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Development of biscuits using purple rice flour, defatted green-lipped mussel powder and spices

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Abstract

Development of biscuits using purple rice flour, defatted green-lipped mussel powder and spices

by

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Biscuits are the most popular bakery food consumed worldwide. The nutritional values of biscuits can be fortified by adjusting their formulations. Wheat flour is one of the main ingredients in a biscuit mix and biscuits can be fortified by using alternative flours containing a high nutritional value, such as Thai purple rice (*Oryza sativa* L.) flour. However, this has seldom been used in bakery products, i.e. bread or biscuits. Defatted green-lipped mussel powder (*Perna canaliculus*) is a by-product, which is a good source of protein to add to biscuits. Spices tend to give the biscuits a nice smell and taste, particularly in biscuits where defatted green-lipped mussel powder *(P. canaliculus)* has been added to the recipe to increase the protein content. Wheat-purple rice biscuits (50:50) supplemented with defatted mussel powder at 0-20%, together with spices, were evaluated by measuring their proximate compositions, physical characteristics and antioxidant contents along with their liking scores as derived from a standard tasting trial. This study was carried out in 5 stages: 1) substituting a portion of wheat flour with Thai purple rice flour, 2) producing biscuits from different flour mixtures, 3) incorporating defatted mussel powder to the biscuits containing purple rice flour, 4) using spices in the formulation of biscuits with defatted mussel powder, 5) studying changes in the quality of biscuits containing defatted mussel powder and spices during storage for four months.

An increase in substitution levels of purple rice flour increased the level of dietary fibre from 2.3 to 5.6% and protein digestibility from 24.8 to 66.46%, and decreased the predicted glycaemic index (pGI) from 63.1 to 48.6 compared to refined wheat flour. Lower amounts of rapidly digested starch with higher slowly digested starch contents were found in whole flour made from purple rice with a 75% substitution of purple rice flour for wheat flour. In addition, antioxidant compounds (total phenolic compounds, anthocyanin and total flavonoid) and

antioxidant activity significantly increased ($P < 0.05$) in the substituted flour compared to the refined wheat flour.

The wheat-purple rice biscuits contained high fibre contents (4.1%) with small only changes in physical properties. The biscuits also showed positive characteristics using *in vitro* digestibility methods. The lowest pGI was found in the 100% purple rice flour biscuits. The change in colour of the biscuits was due to the antioxidant compounds from the purple rice flour. The total phenolics, anthocyanin and flavonoid contents of the fortified biscuits ranged from 0.8-3.0 mg gallic acid equivalents/100 g dry weight (DW), anthocyanins 9.4-51.5 mg/kg DW and flavonoids 0.6-1.3 mg catechin equivalents/100 g DW, respectively. Sensory evaluation revealed that panellists liked the 50% substitution level of purple rice flour for wheat flour in the biscuit mix compared to the 75% and 100% substitution levels.

The highest crude protein level (11.3%) with the highest protein digestibility (83.5%) was found in the 20% defatted mussel powder biscuits mixed with ginger and galangal compared to biscuits enriched with 10% and 15% defatted mussel powder. The antioxidant compounds and antioxidant activity also significantly increased ($P < 0.05$), and the total starch contents decreased, when increased amounts of protein from defatted mussel powder were incorporated into the biscuit mix. Overall, the supplementation of 10% defatted mussel powder was accepted by all the ethnic groups with a higher score given than for the 20% supplemented biscuits. Pacific Islanders appreciated the mussel-supplemented biscuits more than the other three ethnic groups, Caucasian, Chinese and Thai.

The fortified biscuits enriched with defatted mussel powder and spices contained higher protein (26.4%), fibre (52.9%) and ash (6.0%) contents with a lower fat (5.6%) content compared to the wheat-purple rice biscuits (50:50). The *in vitro* starch digestibility and pGI decreased in the fortified biscuits by 19% and 6.2%, respectively, while the protein digestibility increased by 3.7%, corresponding to the increased levels of defatted mussel powder present. The inclusion of defatted mussel powder at 15% showed no significant differences in liking scores in terms of colour and overall appearance; whereas, the flavour and overall acceptability scores were significantly lower than the control biscuits from 7.0 to 4.6 and from 7.4 to 5.3, respectively.

Storage of the 15% defatted mussel biscuits at $21.6 \pm 0.4°C$ and $34.2 \pm 0.1°C$ in two different packaging types (polyethylene terephthalate, PET and aluminium foil laminate, AL) for 12 weeks showed small reductions in antioxidant compounds and antioxidant activity ($P < 0.05$), but no changes were observed in the colour and physical parameters.

Overall, substitution of purple rice flour can be used as an alternative flour in terms of functional properties to produce a good texture in biscuits with a higher antioxidant potential and lower predicted glycaemic index. Spices can be successfully incorporated into mussel-containing biscuits to improve the overall taste of the biscuits. A combination of lower temperature (21.6 ± 0.4°C) and AL pouches proved to be the best storage condition for biscuits giving, overall, acceptable rates of free fatty acid and peroxide values even after storage for 12 weeks.

Keywords: Biscuits, purple rice flour, wheat flour, oil extracted green-lipped mussel powder, *in vitro* digestibility of protein, starch, antioxidants, flour functional properties, ethnic groups, sensory evaluation, fortification, protein enrichment

Acknowledgements

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Chapter 1

Introduction

1.1 Research background

Bakery products are the most popular processed food items around the world (Caleja, Barros, Antonio, Oliveira, & Ferreira, 2017). Of these, biscuits represent the largest category of snack foods among bakery products because they are made from simple, cheap and easily available raw materials. They are widely consumed because they have a very acceptable taste and their low water activity allows a long shelf-life (Chauhan, Saxena, & Singh, 2015). Unfortunately, biscuits, because they are generally made from wheat flour and fat, are easily digestible high-energy foods. This can negatively impact on health if they are consumed regularly, particularly in excess (Caleja *et al*., 2017). Marketing forecasts suggest that global biscuit sales will grow significantly by 2020, and healthy biscuits are expected to perform well in this sector (Čukelj *et al.,* 2017). Fortification of food products plays an important role in increasing health-promoting functional components in bakery products to provide additional benefits to meet consumers' demands (Świeca, Gawlik-Dziki, Dziki, & Baraniak, 2017). There is also a good possibility to improve the overall nutritional contribution of the biscuits by reducing the content of wheat flour. Researchers have started to evaluate the possibility of using locally-available products, such as soybean, plantain, coconut and amaranth flours as substitutes for wheat flour. These flours provide a useful means of improving the nutritional values of the protein and bioactive compounds of the biscuits through the incorporation of less expensive non-wheat flours. Many researchers have used alternative locally-grown crops in place of wheat flour in baked goods in order to decrease the cost associated with using imported wheat (Raihan & Saini, 2017). Many different cereals have been added to wheat flour biscuits, but the addition of rice flour has not yet been fully investigated.

Refined wheat flour contains relatively low levels of antioxidants and it is well known that these are largely destroyed during baking. However, herbs and spices also contain a wide range of antioxidants (Sui, Zhang, & Zhou, 2016) and these are frequently added to all kinds of baked products to add flavour (Uhl, 2000). Antioxidants also preserve food

products and extend their shelf-life (Sui *et al.,* 2016). Herbs and spices also increase the nutritional value of biscuits (Świeca *et al.,* 2017).

Tasty and healthy foods depend on the addition of quality ingredients, such as herbs and spices (Vitali, Dragojević, & Šebečić, 2009), and biscuits can easily be fortified to manufacture healthier products. However, the addition of these products has not been fully studied because of the complexity of their chemical nature and the enormous range of products that can be incorporated to produce a new healthy product.

The food industry is facing the challenge of developing new food products with special health-enhancing characteristics. Proteins are the main structural components of several natural foods that can enhance the consistency, textural properties and stability of food products (Shahidi & Ambigaipalan, 2015). Whey, soy, rice, and lupin proteins have been widely used in the final products to improve their overall qualities using different functions (Sharif *et al.,* 2003). Therefore, the protein and digestible protein content of biscuits can be improved by investigating the addition of new and high protein sources. The novel developments and innovations of by-products from food processing have led to a number of new products that contain increased amounts of novel bioactive compounds and proteins. Fish meal by-products contain high quality proteins and some interesting bioactive compounds (Nørgaard, Petersen, Tørring, Jørgensen, & Lærke, 2015) but these by-products present many challenges when incorporating them into baked products.

Determining the physical and chemical properties of a new product is important in assisting the development process. The basic determination of moisture, protein, fat, ash and carbohydrates give an indication of the nutritive value of a product but the values of these change depending on the mixture of raw materials used to make the product (Owusu-Apenten, 2005). Physical and textural characteristics of biscuits, especially hardness, are important characteristics for consumer acceptance (Owusu-Apenten, 2005). Changes in nutritional composition and the textural properties of bakery products using fortified flour as a replacement for wheat flour has been studied (Oladale & Aina, 2007). Different materials and methods lead to important physicochemical changes in the final product. Serna-Saldivar (2012) showed that replacing refined wheat flour with rice flour changed the chemical composition, physical characteristics, functional properties and the sensory evaluation of the final product.

One of the biggest challenges in creating a new food product is predicting how it will be accepted by consumers. The physicochemical changes of new products are also important factors for consumer acceptance as a new product can have improved nutrition (Vitali *et al.,* 2009; Čukelj *et al.*, 2017). The colour of a food is a very important quality attribute that is evaluated before a food is even tasted (Markovic, Ilic, Markovic, Simonovic, & Kosanic, 2013). Food colour is related to many physicochemical changes that occur during food processing. Food processors use colour measurements of food products as an indirect measurement of other quality attributes, such as antioxidant contents, and colour correlates well with other physicochemical properties.

The development of high protein containing biscuits is a worthwhile development provided the product is accepted by consumers, but the long-term outcome of these developments is very difficult to predict. The inclusion of new ingredients is not often undertaken as the overall acceptability of consumers is difficult to predict (Čukelj *et al.,* 2017). There are a number of factors that determine consumers' acceptance, including convenience, price, nutritional value, and packaging; however, the key factor that deserves significant evaluation is the sensory experience of tasting the new product. Jantathai, Sungsri-in, Mukprasirt, & Duerrschmid (2014) reported that colour and texture characteristics had a significant influence on the evaluation of new food products by consumers. Sensory evaluation is a method to determine the overall tastes of food products using human senses to identify the properties of food products, such as taste, flavour and texture. Consumer acceptance tests need to be used as a tool in the development of the new products. Untrained consumer panels may be used to determine whether there are differences between products or to evaluate the preference for a new product (Yeh *et al.,* 1998).

Every ethnic group has their own traditional foods with unique and distinctive flavours and tastes. Therefore, understanding taste perception between different ethnic groups is critical in understanding the food choices of consumers and the possible design of new food products. It is important to note that individuals within different ethnic groups may also have a wide range of taste preferences. Currently, interest in traditional tastes is growing (Jantathai *et al*., 2014). When the development of a new biscuit is being considered, it is important to use locally-sourced ingredients and spices whose tastes are appreciated by each ethnic group the products are intended for. To achieve success in the

marketplace, a new product needs to meet the preferences of several different ethnic groups. Therefore, the study of consumer acceptance by different ethnic groups is a fundamental task in developing a new functional food (Yeh *et al.,* 1998).

Shelf-life is also a very important factor for a new food product, as export logistics, profitability and efficiency are all improved when a produce can be stored for a longer time (Masson, Delarue, & Blumenthal, 2017). Therefore, a long shelf-life of fortified biscuits, with an acceptable taste, enables their large-scale production and widespread distribution. The storage of a food product is important and crucial for food processing and depends on the chemical composition of the products and how they have been processed, packed, and distributed (Sharif *et al.,* 2003). Storage temperatures and types of packaging materials used for the products are important factors affecting the food qualities of products (Vanhanen & Savage, 2006). Consumers will not accept food products that have unacceptable characteristics. Therefore, investigation of the storage characteristics under normal and elevated temperatures is important aspect of the development of a new product.

In order to deepen understanding about the fortification of biscuits using substituted flour mixtures, new high protein sources from by-products combined with spices, this research programme will incorporate rice flour into wheat flour at different levels. The new products will be investigated by measuring their proximate compositions, antioxidant status and physical characteristics. The development of new fortified biscuits, which could have a significant improvement in nutritional value and antioxidant contents, could reduce the risk of health problems and would be highly desirable for the market. Since limited information is available, this research is critical to determining the physicochemical properties of fortified biscuits enriched with natural sources, as well as identifying the antioxidant compounds and carrying out taste testing using different ethnic groups. This would give a clear understanding of the benefits of fortified biscuits, their correlations and, hence, reduce the costs of production.

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Chapter 2
Literature review

2.1 Biscuits

Although biscuits vary in their shapes, sizes and composition, the three main ingredients are always flour, sugar and fat (butter or vegetable shortenings) (Caponio, Summo, Delcuratolo, & Pasqualone, 2006). Commercial biscuits normally constitute 50% of calories from fat and carbohydrates, with over 400 calories per 100 g in plain biscuits (Protonotariou, Batzaki, Yanniotis, & Mandala, 2016). Biscuits broadly form into two groups, hard and soft biscuits, depending on their ingredient mix (Sudha, Vetrimani, & Leelavathi, 2007). Commercial biscuits are prepared from refined wheat flour that consists mainly of carbohydrate as starch (70–75%), water (13-15%), protein (10-12%), fat 1-2%, together with some fibre, ash and minerals (Goesaert *et al.,* 2005). The protein in wheat flour is mainly gluten, which is made up of two major protein fractions - monomeric gliadins and polymeric glutenins. Gluten proteins are an important element in bakery products and are responsible for the elasticity, cohesiveness and viscosity characteristics of the dough. The percentage of protein in wheat flour affects the flour strength (Goesaert *et al.,* 2005). Therefore, replacing wheat flour with another type of flour will change the amount of gluten protein, which will then affect dough formation (Wrigley & Beitz, 1998).

Sugar is commonly used as a sweetener, but it also serves various functional properties in food processing, such as the flavour, colour and texture of the dough (Gallagher, O'Brien, Scannell, & Arendt, 2003). A complex series of browning reactions involving sugar occur above 160°C that cause the baked products to develop a brown crust. This phenomenon is known as a Maillard reaction (Gallagher *et al.,* 2003). In baked biscuits, sugar binding with water molecules prevents the growth of microorganisms and improves the shelf-life (Gallagher *et al*., 2003). Fats are the principal ingredients that contribute to the general appearance of the product and raise the quality of the biscuits, the mouthfeel and the texture by interacting with other ingredients (Pareyt *et al*., 2009; Mamat & Hill, 2014). Fats reduce the abrasive effect of flour and sugar during mixing and processing. In addition, fats also enhance aeration giving an increased volume and open texture to

create a softer mouthfeel (Mamat & Hill, 2014). The secondary function of fats in biscuits is to create the flavour of the finished product. Bakery fats (particularly butter), which contain about 80% fat, are ingredients in the dough that have the most dominant effect on the final flavour. In addition, fats can extend the shelf-life of products by masking or slowing the staling process as fats in the crust slow moisture migration into the biscuit (Pareyt *et al*., 2009).

2.1.1 Biscuit production

The four main processes to make biscuits are mixing, cutting, baking and packing (Okpalan & Egwu, 2015). Biscuit production needs precise preparation to make a successful product (Uchenna & Omolayo, 2017). The ingredients are mixed to form a dough using mixers that are either operated manually or using a pre-set mixing programme (Whitely, 1971). As the dough is mixed, the protein molecules form long strands of gluten resulting in an elastic web, which, essentially, controls the quality of wheat flour-based products (Wrigley & Beitz, 1998). Once the dough is mixed, it is then made into different shape and sizes. This process leads to an increase in the stress on the gluten structure.

Baking is a very important process to achieve good quality biscuits. This process transforms the physical and chemical characteristics of the dough when baked in an oven (Khater & Bahnasawy, 2014), where the temperature and time will be accurately controlled. The oven temperature affects the moisture loss during baking, which plays an important role in achieving a good texture and the structure of the biscuits (Whitely, 1971) since gluten needs a large amount of water to form and develop a gluten web as well as hydrate the starch granules (Mamat & Hill, 2014). The structure of the biscuits is formed, and free water evaporated when gluten and starch have been sufficiently hydrated. The evaporation starts from the dough surface, achieving about a 2-5% moisture content in the final products (Khater & Bahnasawy, 2014). Baking also alters the colour of the biscuit surface; namely, the browning process. There are three main browning processes: caramelisation of sugars, dextrinization of starch and the Maillard reaction in reducing sugars and amino acids, associated with the biscuit production. These processes occur when the biscuit surface is already dry and the temperature is high (above 100°C). Colouring of the biscuits takes place in the final stage of the baking process (Whitely, 1971). Packaging is used as a barrier to protect the biscuits and prevent the deterioration

following absorption of moisture from the air. Moreover, biscuits can be protected from cracking and being broken by choosing the right packaging materials. Selecting and handling the materials used to pack the biscuits are crucial for the life and qualities of the final products (Whitely, 1971).

Biscuit production changes in both the chemical and physical characteristics, which result in the structure, texture and colour changes in the biscuits (Sudha *et al*., 2007). Overall, the characteristics of high quality biscuits also depend on the type and proportions of the ingredients.

2.2 Fortification of biscuits

Biscuits can hardly be regarded as a healthy snack because they usually contain high levels of rapidly digested carbohydrate, high fat, generally low levels of fibre and only modest amounts of protein (Chinma, Igbabul, & Omotayo, 2012; Park, Choi, & Kim, 2015). Several studies have used blended flours or composite flours to produce biscuits (Oladale & Aina, 2007; Khouryieha & Aramouni, 2012; Okpala, Okoli, & Udensi, 2013; Chauhan, Saxena, & Singh, 2015; Okpalan & Egwu, 2015; Park *et al*., 2015; Akesowan, 2016; Mir, Bosco, Shah, Santhalakshmy, & Mir, 2017; Raihan & Saini, 2017; Uchenna & Omolayo, 2017; Adeola & Ohizua, 2018; Gbenga-Fabusiwa, Oladele, Oboh, Adefegha, & Oshodi, 2018). There have also been a number of attempts to improve their nutritional characteristics by partially replacing the wheat flour with non-wheat ingredients in the production of biscuits. Replacing wheat flour with different types of flour mostly affects their nutritional values and physical characteristics, such as hardness and spread ratio as well as colour parameters, which can be observed in Table 2.1. Brown rice flour has been used to improve the nutritive values of biscuits together and increase the overall acceptability of the fortified biscuit (More, Ghodke, & Chavan, 2013).

In consideration of the development of a new product, it is important to use locally sourced ingredients, as the tastes would then be appreciated by the ethnic groups the products were intended for (Yeh *et al.,* 1998). The texture, flavour and appearance of biscuits are major attributes that affect biscuit acceptability (Torbica, Hadnađev, & Hadnađev, 2012). Moreover, many studies have investigated the properties of gluten-free biscuits using different types of rice flours, such as white rice flour with buckwheat flour (Torbica *et al.,* 2012), waxy rice flour (Giuberti, Marti, Fortunati, & Gallo, 2017), brown

rice flour (More *et al.*, 2013; Chung, Cho, & Lim, 2014; Mir *et al.*, 2017) and a composite rice flour, together with green gram flour and potato flour (Chandra, Singh, & Kumari, 2015), or broken rice mixed with cocoyam flour (Okpalan & Egwu, 2015). Unfortunately, rice protein cannot generate a viscoelastic network like gluten in wheat, which retains carbon dioxide during biscuit dough fermentation. Therefore, the addition of rice to a biscuit mix can have a significant effect on the textural qualities of the cooked biscuit.

Table 2.1   Effects of replacing wheat flour on physicochemical characteristics and antioxidant properties of wheat-based biscuits.

Types of flour	Replacing wheat flour (%)	Outcome	References
Barley	10, 20, 30 and 40	-decreased protein content -increased darkness -decreased spread ratio	Gupta, Bawa, & Abu-Ghannam (2011)
	25, 50 and 75	-increased darkness -decreased spread ratio -increased antioxidant activity	Sharma and Gujral (2014)
Beniseed and unripe plantain	10, 20, 30 and 40 of beniseed with 10% unripe plantain flour	-increased spread ratio -increased protein content	Agu and Okoli (2014)
Chestnut	20, 40 and 60	-increased fibre content -increased hardness -increased darkness -decreased spread ratio	Šoronja-simović *et al.* (2017)
Chia seeds	5, 10, 15 and 20	-increased darkness -increased polyphenols -no change in hardness	Mesías, Holgado, Marquez-Ruiz, & Morales (2016)
Chick pea	5, 10 and 15	-decreased spread ratio -decreased water absorption	Hegazy and Faheid (1990)
	20, 40, 60, and 80	-increased darkness -no change in spread ratio -increased hardness	Yamsaengsung, Berghofer, & Schoenlechner (2012)
Defatted groundnut	5, 10, 15, 20, 25 and 30	-improved protein content	Dauda, Abiodun, Arise, & Oyeyinka (2018)
Defatted maize germ	5, 10, 15, 20 and 25	-decreased spread ratio -increased protein content -increased darkness	Nasir *et al.* (2010)
Defatted soy	28.6, 50 and 71.4	-increased protein and fibre contents -increased lysine content	Serrem, de Kock, & Taylor (2011)

Types of flour	Replacing wheat flour (%)	Outcome	References
Flaxseed	6, 12 and 18	-increased darkness -decreased height	Khouryieha and Aramouni (2012)
	20 with multigrain flour	-increased bioactive compounds -increased spread ratio	Čukelj *et al.* (2017)
	5, 10, 15, 20, 25 and 30	-increased protein content -increased antioxidant activity -increased darkness -increased spread ratio	Kaur, Singh, & Kaur (2017)
Fluted pumpkin seeds	5, 10, 15, 20 and 25	-increased protein and moisture content -decreased spread ratio -decreased hardness	Giami, Achinewhu, & Ibaakee (2005)
Green gram flour	10, 20, 30, 40 and 50	-increased protein content with decreasing fat content -increased darkness -decreased spread ratio	Rajiv, Lobo, Lakshmi, & Rao (2012)
Jering seeds	5, 10, 15 and 20	-increased protein and fibre content -decreased fat content -decreased spread ratio -increased hardness	Cheng and Bha (2016)
Lupin	5, 10 and 15	-decreased spread ratio -decreased water absorption	Hegazy and Faheid (1990)
	10, 20, 30, 40 and 50	-increased protein and moisture content -decreased spread ratio -increased hardness -increased darkness	Jayasena and Nasar-Abbas (2011)
Lupin (germinated)	10, 20, 30, 40 and 50	-increased protein and fibre contents -decreased spread ratio	Obeidat, Abdul-hussain, & AL Omari (2013)
Mahaleb cherry seeds	1, 2, 3 and 4	-increased darkness -increased hardness -increased moisture content -increased antioxidant capacity	Herken, Simsek, Ohm, & Yurdunuseven (2017)
Navy bean	10, 20 and 30	-increased protein content	Dreher and Patek (1984)
Oak fruit	15, 30 and 45	-decreased moisture content -increased antioxidant activity	Parsaei, Goli, & Abbasi (2018)
Papaya pulp	15, 30 and 50	-increased crude fibre content -increased antioxidant capacity -increased hardness	Varastegani, Zzaman, & Yang (2015)

Types of flour	Replacing wheat flour (%)	Outcome	References
Pigeon pea	25, 50 and 75	-decreased sugar content -slow the hydrolysis of starch	Gbenga-Fabusiwa *et al*. (2018)
Potato	10, 20 and 30	-decreased spread ratio -decreased carbohydrate	Seevaratnam, Banumathi, Premalatha, Sundaram, & Arumugam (2012)
Purple rice	25, 50 and 75	-increased darkness -increased antioxidant properties -increased spread ratio	Klunklin and Savage (2018d)
Quinoa	15, 30, 45, 60 and 90	-increased darkness -no changes in texture properties	Wang, Opassathavorn, & Zhu (2015)
Sorghum	5, 10, 15 and 20	-not significantly changed in physical characteristics (thickness, width and spread ratio) -decreased moisture content	Adebowale, Adegoke, Sanni, Adegunwa, & Fetuga (2012)
	30, 50 and 70	-increased spread ratio	Emmanuel, Omolara, & Michael (2012)
Soy bean	5, 10 and 15	-decreased spread ratio -increased water absorption	Hegazy and Faheid (1990)
	10, 15 and 20	-decreased moisture content -increased protein and fibre contents	Farzana and Mohajan (2015)
Taro	51% and 100	-lowed spread ratio -soften the texture of the cookies	Nip, Whitaker, & Varg (1994)
	5, 10, 15, 20, 25 and 30	-increased protein -increased elasticity	Himeda *et al.* (2014)
Unripe banana	15, 30 and 50	-increased fibre content -increased slowly digested starch	Agama-Acevedo, Islas-Hernández, Pacheco-Vargas, Osorio-Díaz, & Bello-Pérez (2012)
Water chestnut	60, 70, 80 and 90	-increased water absorption -decreased spread ratio	Singh *et al.* (2011)

Recently, products with high protein and fibre contents are more commonly chosen by consumers to reduce the risk of diabetes and obesity. The protein enrichment of biscuits can be achieved by adding different kinds of ingredients, such as Nile tilapia fish bones

(Abdel-Moemin, 2015), soya protein (Marco & Rosell, 2008; Sarabhai *et al.,* 2015) and defatted green-lipped mussel powder (Klunklin & Savage, 2018b; Klunklin & Savage, 2018c). The development of a high protein containing biscuit is a worthwhile challenge when the overall nutritional status of underprivileged sections of the population is considered. Dietary fibre in the bran and germ of cereal grains has been added to biscuit formulations that originally contained almost no dietary fibre in the original recipe (Mir *et al.,* 2017; Varastegani *et al.,* 2015; Cheng & Bha, 2016). Substitution of purple rice flour for wheat flour will not only increase the dietary fibre and bioactive contents, but it is also a cheaper raw material (Klunklin & Savage, 2018a).

Consumers are always concerned about the fat content of biscuits. In recent years, an enormous amount of research work has been carried out to study the possibility of adding healthy fats to biscuits without changing the flavour and texture of the final products. Fat and sugar replacement using dietary fibre, such as inulin, β-glucan, potato fibre, mango peel, rice bran, wheat bran, arabinoxylan and complex oligosaccharides, have been added to increase the nutritional quality of biscuits (Sozer, Cicerelli, Heiniö, & Poutanen, 2014; Giarnetti, Paradiso, Caponio, Summo, & Pasqualone, 2015; Vujić, Vital, & Vedrina-Dragojević, 2015). Garden cress seed oil has been used to enrich the α-linolenic acid content of biscuits. It was added to produce ω-3 fatty acid-rich biscuits in order to increase antioxidant properties of the product (Umesha, Manohar, Indiramma, Akshitha, & Naidu, 2015). Grape marc extracts have been added to biscuits and this has increased their antioxidant activity and phenolic contents compared to control biscuits made using wheat flour (Pasqualone *et al.*, 2014). The overall flavour and cross-sectional structure and appearances of the biscuits were improved by these additions.

Since biscuits are easy to consume as a snack or dessert with long shelf-life when efficiently packed, many food manufacturers are interested in developing new and nutritional biscuits. Commercial bakery products generally contain high levels of carbohydrates and fats with small amounts of protein and fibre. Modifying the composition of biscuits is a way to increase the nutritional value of the product, such as by adding rice to increase protein and fibre content of final product.

2.3 Purple rice

Rice (*Oryza sativa* L.) is one of the most widely eaten cereal grains in Asia (Butsat & Siriamornpun, 2010). In Thailand, red, purple and black rice is widely grown in different locations (Yodmanee, Karrila, & Pakdeechanuan, 2011), which results in many different pigmentation phenotypes being observed throughout the country (Boonsit, Karladee, & Phongpiachan, 2006). Many of the pigmented rice cultivars are reported to contain higher levels of antioxidants than white rice (Yawadio, Tanimori, & Morita, 2007; More *et al.,* 2013; Klunklin & Savage, 2018a; Klunklin & Savage, 2018d). This can be classified depending on anthocyanin and proanthocyanidin compounds in the aleurone layer of rice grain (Thitipramote *et al*., 2016).

Purple rice, also known as forbidden rice, has been grown in China since the Tang and Sung Dynasties (Kushwaha, 2016). Purple rice was served only to Chinese royalty but the emperors would share purple rice with warriors who were about to go into battle due to its perceived medicinal properties (Yawadio *et al*., 2007). As well as China, this rice has a long history in South Asia, including Indonesia, India and Thailand. Purple rice is a glutinous, whole grain containing a high amylopectin content (Boonsit *et al*., 2006; Tang, Cai, & Xu, 2016), which can be grown in many Asian countries, such as China, Indonesia, Japan, Korea, Laos, Malaysia, Philippines and Thailand (Kushwaha, 2016). Purple rice (var. Sanpatong) is widely grown in Northern Thailand and is becoming a popular crop because of its improved taste compared to white rice (Jang & Xu, 2009; Klunklin & Savage, 2018a; Klunklin & Savage, 2018d). However, the price of purple rice is five times higher than normal white rice due to its lower productivity. Nowadays, the demand for pigmented rice consumption is increasing rapidly throughout European countries and also in the USA because of its high nutritive value and antioxidant properties (Boonsit *et al*., 2006; Yawadio *et al*., 2007; Kaur *et al.,* 2017; Umesha *et al*., 2015).

2.3.1 Bioactive components in purple rice

Purple rice has several nutritional advantages over white rice, such as higher protein, vitamin and mineral contents; for example, Fe, Zn, Mn and P (Yawadio *et al.,* 2007). The bran layer of this rice has a high level of antioxidant compounds, mainly phenolic acids, flavonoids, anthocyanins and oryzanols (Shao, Feifei, Xiao, Jinsong, & Trust, 2014;

Sumczynski, Kotásková, Družbíková, & Mlček, 2016; Kushwaha, 2016; Shao *et al*., 2018). Purple rice has been shown to have significantly more anthocyanin contents than pigmented corn, wheat and barley (Yodmanee *et al.,* 2011; Kushwaha, 2016; Klunklin & Savage, 2018a; Klunklin & Savage, 2018d). The study of the anthocyanin profile by spectroscopy assay showed that extracted purple rice has four active components; cyanidin-3-O-glucoside (C3G), peonidin-3-O-glucoside, cyanidin-3,5-diglucoside, and cyanidin-3-glucoside (Yawadio *et al*., 2007; Yodmanee *et al.*, 2011). The predominant anthocyanins are C3G (around 92% of total anthocyanins) and peonidin-3-O-glucoside (Yawadio *et al*., 2007; Kushwaha, 2016). Many researchers have reported that C3G contributed the highest antioxidant activity of raw and purple rice bran (Jang & Xu, 2009; Sompong, Siebenhandl-Ehn, Linsberger-Martin, & Berghofer, 2011; Yodmanee *et al*., 2011; Chatthongpisut, Schwartz, & Yongsawatdigul, 2015; Kushwaha, 2016; Thitipramote *et al.*, 2016; Jiamyangyuen, Nuengchamnong, & Ngamdee, 2017; Klunklin & Savage, 2018a; Shao *et al*., 2018). However, only a few researchers have studied the phytochemical and antioxidant activities of different cultivars of purple rice flour, as shown in Table 2.2.

Table 2.2 Antioxidant compounds (total phenolic compounds, TPC and cyanidin-3-O-glucoside, C3G) and antioxidant activity (DPPH and ABTS assays) of raw purple rice.

Cultivars	Origins	TPC (mg GAE/100 g DW)	C3G (kg/g DW)	DPPH (mmol TEAC/100 g DW)	ABTS (mmol TEAC/100 g DW)	References
Unknown	China	492.0	-	22.7	-	Tang *et al.* (2016)
	China	709.3	-	23.6	3.1	Sumczynski *et al.* (2016)
	Italy	282.2	-	10.0	2.2	Sumczynski *et al.* (2016)
	Japan	252.6	-	10.7	1.8	Sumczynski *et al.* (2016)
	Laos	504.6	-	16.8	3.1	Sumczynski *et al.* (2016)
	Thailand	458.3	-	1.8	2.6	Sumczynski *et al.* (2016)
D Youzinuo 161	China	41.5	12.0	1.6	3.2	Shao *et al.* (2018)
Heimi No. 1	China	38.6	83.3	1.3	2.7	Shao *et al.* (2018)
Heixiangnuo No. 3	China	51.4	226.7	2.01	4.4	Shao *et al.* (2018)
Heinuomi	China	62.6	683.7	2.7	5.8	Shao *et al.* (2018)
Heimi 2420	China	54.8	1106.0	1.6	4.7	Shao *et al.* (2018)
Heiyounian	China	643.0	0.3	-	-	Ti *et al.* (2015)
Sintoheugmi	Korea	356.3	0.018	-	-	Surh and Koh (2014)
Sinnongheugchal	Korea	179.5	0.0009	-	-	Surh and Koh (2014)
Luem Pua	Thailand	75.5	17.8	7.2	8.5	Thitipramote *et al.* (2016)
Mali Nil Surin No. 6	Thailand	492.0	49.0	-	2.8	Chatthongpisut *et al.* (2015)
Niew Dum	Thailand	336.7	137.4	5.0	-	Sompong *et al.* (2011)
Riceberry	Thailand	116.0	37	130.0	-	Jiamyangyuen *et al.* (2017)
Sanpatong	Thailand	318.3	492.6	10.3	4.7	Klunklin and Savage (2018a)

GAE, gallic acid equivalents; TEAC, trolox equivalent antioxidant capacity; DPPH, 2,2-diphenyl-2-picrylhydrazyl radical scavenging activity; ABTS, [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging activity.

2.3.2 Analytical methods

It is important to determine the antioxidant contents of food products, to identify their effectiveness for preventing or delaying lipid oxidation in the cooked product. Ghani, Barril, Bedgood Jr., & Prenzler (2017) and Tan and Lim (2015) have reviewed many different antioxidant assays to evaluate the antioxidant compounds in foods, such as total phenolic compounds, anthocyanin and flavonoids (Tan & Lim, 2015). Anthocyanin and total phenolic compounds (TPC) of purple rice have been shown to have high antioxidant capacities using the oxygen radical absorbing capacity method (Yodmanee *et al.,* 2011; Hou, Qin, & Ren, 2010). However, the recommended assay for determination of antioxidant capacity was to use 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), since they were considered to be simple, accurate and economic assays (Pasqualone *et al.,* 2015).

2.3.3 Use of purple rice in the food products

The development of rice production to enhance human health benefits is very attractive because rice is economically a very important crop for Asian countries. Apart from daily consumption as cooked rice grains, purple rice is also used to produce desserts and snacks in China and Thailand, such as coconut black rice pudding (Kushwaha, 2016; Kraithong, Lee, & Rawdkuen, 2018). Food manufacturers are beginning to develop high quality good-tasting food using the unpolished rice grain, which includes the germ, bran, and endosperm (Kushwaha, 2016). The whole grain contains phenolic acids (ferulic, coumaric and caffeic acids) and free phenolic compounds (i.e. gallic acid). Kim, Kikuchi, Kim, & Park (2010) confirmed that purple rice powders were able to enhance the anthocyanin and overall antioxidant qualities of the traditional Korean rice wine (takju). These phenolic acids and anthocyanin compounds are thought to reduce the risk of colon cancer when eaten in moderate amounts (Sumczynski *et al.,* 2016). Pigments extracted from purple rice bran can also be used as alternative of artificial colourants for foods and beverages (Shao & Bao, 2015). These pigments have been used to increase the functional components of various snacks, cakes and breakfast cereals (Kushwaha, 2016).

2.3.4 Application of rice and purple rice flours

Rice flour

Rice flour is gluten free and useful as alternative to wheat flour, which contains gluten that can cause celiac disease in susceptible individuals (Cho *et al.,* 2014). Rice flour is not widely used in bakery products since it has no protein structure to hold the carbon dioxide in the product during baking (Noomhorm, Bandola, & Kongseree, 1994). Other proteins are added as foaming agents in bakery products to improve the elasticity of the products, enhance the taste and help develop a gelatinisation structure (Nammakuna, Barringer, & Ratanatriwong, 2015; Sirichokworrakita, Phetkhuta, & Khommoon, 2015). Therefore, rice flour has been used, together with proteins, as ingredients to make gluten-free crackers (Nammakuna *et al.,* 2015), brown rice flour is used to make traditional Korean rice cakes (Cho *et al*., 2014) and riceberry flour can also be used to replace wheat flour in noodles (Sirichokworrakita *et al.,* 2015). Nevertheless, this is not so important in biscuit-type products as they are not required to rise in the same way as bread. Broken rice blend flour is also used to produce biscuits that are baked in a microwave (Gonzalez-galan, Wang, Sgarbieri, & Moraes, 1991). Rice flour with its small particle size also needs to absorb more water during food processing compared to wheat flour (Sirichokworrakita *et al*., 2015); this means the production of soft and smooth products, such as rice noodles, fermented condiments (miso and mirin), congee need more care taken during their production (Surh & Koh, 2014).

Purple rice flour

Finely milled and graded purple rice flour has been used in many traditional desserts made in Thailand, Indonesia and China. It is also used as a gelling or thickening agent in many cakes and breads due to its high viscosity (Kushwaha, 2016; Itthivadhanapong & Sangnark, 2016). The nutritional properties of purple rice flours in various cultivars have been investigated (Table 2.3). Recently, Klunklin and Savage (2018a) reported that refined wheat flour contained more starch and protein contents than purple rice flour which, in contrast, contained higher total fibre contents. Purple rice flour was used to develop cake properties, such as firmness, gumminess and chewiness, without changing sensory quality characteristics (Itthivadhanapong & Sangnark, 2016). Jung, Lee, & Eun (2002) reported that the supplementation of 20% purple rice flour for wheat flour did not show any significant differences in the sensory characteristics of the bread. Purple rice

flour has also been used as a base ingredient for making pasta (Laishram & Das, 2017) or partially used to replace wheat flour to make biscuits. Their functional properties, such as foaming, bulk density, swelling power and lower gelation concentration of the flour, affect the texture characteristic of the biscuits and, generally, without changing the overall taste (Klunklin & Savage, 2018a; Klunklin & Savage, 2018d).

Table 2.3   **Nutritional compositions of Thai purple rice flour compared with rice berry flour, white rice flour and refined wheat flour.**

Nutritional properties (% DW basis)	Purple rice flour			Riceberry flour[3] (var. Khao Dawk Mali 105)	White rice flour[1] (var. Unknown)	Refined wheat flour[2]
	(var. Sanpatong)[2]	(var. Niew Dum)[4]	(var. Kam Doi Saket)[5]			
Moisture content	11.6	12.0	<14%	9.6	5.5	12.5
Ash	1.1	1.48	1.3	1.1	1.0	0.9
Total fat	2.8	3.7	1.8	3.6	8.1	2.0
Crude protein	13.0	10.9	6.6	7.5	13.2	15.4
Total dietary fibre	5.6	3.4	2.5	1.66	-	2.3
Total carbohydrate	71.5	71.9	87.8	77.1	72.2	69.2

[1]More *et al.* (2013); [2]Klunklin and Savage (2018a); [3]Kraithong *et al.* (2018); [4]Sompong *et al.* (2011); [5]Laokuldilok, Surawang, & Klinhom (2013).

2.4 Evaluation of fortified biscuits

One of the most important concerns of pilot scale production is to make a product that can be efficiently made in a full-scale commercial facility. It is important that the final product can be made consistently with the same appearance, texture, flavour, colour and shelf-life as the experimental test products. It is important that a new product containing multiple, processed ingredients has a consistent quality. The quality of foods is difficult to define precisely; however, all characteristics need to be measured as these are significant in the fortification of the quality of new products and to make the food acceptable for consumers (Mir *et al.,* 2017). Replacing wheat flour with several types of flour was shown in the previous section (Table 2.1). The evaluation of sensory characteristics and the changes of physicochemical content are properties that have a critical influence on food quality, processing and the acceptance of fortified biscuits.

2.4.1 Physicochemical analysis

Proximate analysis

One of the major concerns for biscuit manufacturers is to determine how to improve the formulation of biscuits with suitable ingredients, and to increase the nutritional and functional properties of the biscuit to satisfy consumer demands. Proximate analysis can estimate the characteristics of the food products that affect consumers' food choices (Passos *et al.,* 2013). This analysis provides basic information related to the water, protein, fibre, ash, fat and carbohydrate contents of biscuits. Protein is an essential nutrient responsible for multiple functions in biscuits, such as texture and *in vitro* digestion (Chung *et al.,* 2014). The protein contents of different ingredients have been observed even within the same varieties of purple rice (Yodmanee *et al.,* 2011). Dietary fibre is mostly found in cereal grains and influences biscuit quality. The inclusion of dietary fibre into the biscuit mix affects biscuit dough shrinkage and thickness, as well as reducing the force needed to snap fortified biscuits during texture analysis (Chandra *et al*., 2015). In baking, water provides the medium for the physicochemical reactions that convert raw materials into the finished baked foods (Ahmad & Ahmed, 2014). Increasing the water content in biscuit doughs will raise the hardness of finished baked products and this tends to be good for the final product (Ahmad & Ahmed, 2014). Therefore, proximate compositions have an influence on the overall quality of the biscuits. The information from proximate analysis is essential for labelling purposes (Owusu-Apenten, 2005), since

the purpose of the fortification of food products is to improve the nutritional value of the product and change the characteristics of the final product (Owusu-Apenten, 2005). It is important to emphasize that despite the declaration of nutritional compositions, the levels of these contents are useful information for both consumers and health professionals.

Biscuit quality and colour measurement

The most important characteristic of the quality of biscuits produced from any ingredients is its appearance, including shape, size and colour, as consumers can easily be influenced by the appearance of food products. An understanding of the combination of the ingredients, together with baking process, has an important effect on the quality and colour of the final product (Sudha *et al.,* 2007). Andresen, Dissing, & Loje (2013) suggested that the main drivers determining consumer preferences of biscuits are closely related to the major ingredients, the spreadability of the biscuits and appearance (i.e. width and thickness) because these aspects can make biscuits look delicious. Rice flour is used to make biscuits more spreadable; however, rice flour is known to have significantly lower protein contents and viscosity in comparison to refined wheat flour (Thomson, 1976).

The colour and appearance are the first parameters that a consumer uses in their decision to purchase a product (Sozer *et al.,* 2014). A colorimeter calibrated to measure the CIE *L*a*b** colour scale is an important tool during manufacturing to maintain a consistent colour during commercial production. The development of biscuits needs to maintain or improve the appearance of biscuits in order to give the consistency consumers expect. Since, the measurement of colour, which has also been used as quality parameters in food industries, is an important consideration when foods are fortified by various coloured ingredients (Jha, 2010).

Texture analysis

The texture, appearance and flavour of food products are the important key factors for sampling, buying and repurchasing by consumers. Texture analysis is also important to predict the acceptability of the finished product, since it can predict the force needed to bite through a biscuit (Owusu-Apenten, 2005). Multiple component biscuits are characterised by different mechanical properties within a single bite. Although, the product has an acceptable flavour and colour, an undesirable texture in a food product can easily be a factor that influences whether a customer will continue to purchase the

product or not. Therefore, the development of biscuits must be carefully designed in order to meet the required physicochemical properties among various environmental conditions, such as processing and storage. Changing the formulation of any products may alter their texture and consistency. To improve consumer acceptance of new products more research needs to be undertaken to improve the nutritional contents and the appearance of the products. In biscuits, hardness is influenced by particle size distribution, and the fat and protein contents. Consumers tend to like soft biscuits (Chugh, Singh, & Kumbhar, 2013); however, consumer perceptions vary in different countries and between ethnic groups (Williams, Bartoshuk, Fillingim, & Dotson, 2016). The optimisation of the grinding process needs to be controlled as well, the ingredients can change the texture of the biscuits (Rolle *et al*., 2012). Therefore, new products need to assessed using a wide range of properties, such as hardness, springiness, fracturability, cohesiveness, etc. (Owusu-Apenten, 2005). Texture analysis instruments are a way of giving numbers to these physical properties (Rolle *et al.,* 2012). Consequently, analytical techniques are needed to test foods to ensure that they have the appropriate physicochemical properties that meet consumer demands (Owusu-Apenten, 2005).

2.4.2 Sensory evaluation

Following the development of a new product, it is necessary to measure both physicochemical characteristics along with sensory evaluation using the ethnic groups the product is intended for (Williams *et al.,* 2016). Determining how new food products affect consumers' acceptance is one of the main goals that food scientists need to develop healthier recipes. Whereas some attributes of a food, such as nutritional quality, can be measured by chemical analysis, food acceptability is not easy to measure since it is very subjective. One of the biggest challenges in creating a new food product is predicting how it will be accepted by consumers. Sensory evaluation is a scientific method that uses human senses, such as vision, taste, smell and touch, to evaluate the characteristics of foods (Tuorila, 2015).

There are three different methods that use different kinds of information and methods; discrimination (paired comparison, duo–trio, triangle, etc.), descriptive (quantitative descriptive analysis, etc.) and affective (acceptance, preference, 9-point hedonic, labelled magnitude (LAM) scale). A hedonic scale is used as a simple and effective method to

apply in many areas, such as, the inspection of raw materials, correlation between the target products and the prototypes (instrumental analysis), determination of food choices, product development and improvement, quality control, cost reduction and storage studies, together with critical information about individuals' likes and dislikes of food products (Stone, Bleibaum, & Thomas, 2012). The 9-point hedonic scale is most often used to test foods, beverages and consumer products (Tuorila, 2015). This category-type scale uses a series of nine verbal categories ranging from "1 = dislike extremely" to "9 = like extremely." A neutral midpoint (5 = neither like nor dislike) is included. Consumers or panellists rate the product on the scale based on their response. The development of new food products, in terms of marketing, the guidelines require a sensory score above 7 to proceed to production (Stone *et al.,* 2012).

Therefore, the hedonic scale is a suitable method to predict the acceptability of a new innovative product or to improve a product so that it will be accepted by consumers. This test can be conducted using untrained consumer panels; however, descriptive analysis techniques require trained panellists (Stone *et al*., 2012). Typically, a hedonic test needs between 75–150 consumers and each test needs to be carried out using standardised equipment and protocols (Tuorila, 2015).

Many researchers have compared consumer taste tests of new bakery products using instrumental analysis (Sozer *et al.,* 2014; Tuorila, 2015; Sarabhai *et al.,* 2015; Vujić *et al.*, 2015; Sharma, Saxena, & Riar, 2016). Williams *et al.* (2016) showed that there was a correlation between the analytical results obtained from human-based sensory analysis (such as crunchiness, softness, or hardness) and results obtained using mechanical instrumentation. Unfortunately, many people do not have sufficient experience to evaluate taste sensations, such as sweetness, saltiness or sourness. In addition, different ethnic groups perceive the taste of foods in quite different ways. Comparisons of eating, food choices and the nutrition of different cultures have received more attention in recent studies (Williams *et al.,* 2016). Recently, there were several studies that have reported a correlation in food choices and taste preferences among individuals, groups or cultures. Reed, Tanaka, & McDaniel (2006) reported that different ethnic groups showed significantly different responses in taste intensity due to genetic variations. In addition, human behaviour toward food consumption choices can be affected in various ways due to the complex nature of human motives and emotions. Prescott, Young, O'neill, Yau, &

Stevens (2002) studied the influence of consumers' eating habits in many European and Asian countries and found that health, weight control, convenience, mood, natural content, sensory appeal, price, and familiarity are important factors influencing consumers' food choices. Studies on human preferences across various cultures provide significant data and increase our understanding of ethnic influences on individual preferences (Prescott *et al.,* 2002). When attempting to modify the nutritional value of biscuits, different ethnicities need to be involved since these individuals may have distinct preferences.

2.5 Lipid oxidation in biscuits

One of most important non-microbial degradations that can occur in biscuits is oxidation, which results in a change of colour, aroma and flavour and loss in nutritional value (Guyon, Meynier, & Lamballerie, 2016). Lipids, which are one of the main components of biscuits, can be oxidized by non-enzymatic and enzymatic reactions related to many mechanisms such as photooxidation, enzymatic oxidation and autoxidation. Autoxidation is the most important mechanism of lipid oxidation during storage of biscuits induced by a continuous free-radical chain reaction. It also involves with three sequences: initial oxidation, propagation and termination (Kozłowska, Zbikowska, Gruczyńska, Zontała, & Półtorak, 2014). Lipid oxidation leads to the formation of a fattty acid hydroperoxide (primary oxidation products at propagation stage) which are tasteless, odourless and very unstable and decompose to form secondary oxidation products such as aldehydes, ketones and carboniles that adversly affect to food flavour quality (St. Angelo, 1996). Lipid oxidation is known to cause rancidity in many types of foods, including low moisture food (water activity lower than 0.5) (Kumar, Manohar, Indiramma, & Krishna, 2014) and with small amounts of lipids (less than 1%) (Barden, 2014). The effects of lipid oxidation from triacylglycerols and phospholipids in butter are changes in functional and sensory characteristics and decreased shelf-life (Barden, 2014; Cheng & Bha, 2016). Lipids containing high amount of polyunsaturated fatty acids are also highly susceptible to the oxidation (Kozłowska *et al.,* 2014). Free fatty acid (FFA) content is one of quality indicators and acted as a pro-oxidant. The FFA is a hydrolytic rancidity product, which is an important value to determine the quality parameter of fat-containing foods. Since, FFA can generated by microbial activity that leads to strong flavours and odours at comparatively very low levels. The determination of FFA is important factor for rancidity

shelf-life testing of foods. On the other hand, oxidised products can be assessed by measuring the formation of compounds, such as peroxide values (PV) and TBA reactive substances (TBARS), which are most commonly evaluated in food products (Cheng & Bha, 2016). PV is a useful indicator of the extent of the primary oxidation of rancidification (the concentration of hydroperoxides decomposes during storage) from oils and fats, which depends on temperature, time, light, content of unsaturated fatty acids and processing. Aldehydes are key compounds of the secondary lipid oxidation products, which also affect the modifications of nutritional properties. Although PV is the most used parameter for estimating the overall oxidation status for lipids, it is not possible to use only this value to judge the rancidity of oils in foods (Guyon *et al.,* 2016).

2.6 Natural antioxidants from spices can inhibit oxidation in biscuits

In recent years, interest in the use of natural antioxidants has risen in improving the oxidative stability of foods. The effectiveness of antioxidant compounds results from their free radical-scavenging activity, the presence of regenerating and/or synergistic antioxidants also have important positive effects (Barden, 2014). Numerous studies have indicated that antioxidants are useful compounds to reduce lipid oxidation (Juntachote, Berghofer, Siebenhandl, & Bauer, 2007). Many types of plants that naturally contain antioxidants (polyphenols), such as grape seeds, are used as a source of antioxidant incorporated in food products (Selani *et al.,* 2011), as well as ginger (Ramanathan & Das, 1993), rosemary (Pereira *et al*., 2017), cinnamon (Hu, Wang, Xiao, & Bi, 2015), nutmeg (Velasco & Williams, 2011; Gupta, Bansal, Babu, & Maithil, 2013; Šojić *et al.,* 2015), ginger (Stoilova, Krastanov, Stoyanova, Denev, & Gargova, 2007) and galangal (Buaniaw, Siripongvutikorn, & Thongraung, 2010). The antioxidant activity of spices has been studied as potential and additional sources of natural antioxidants. Spices contain large amounts of bioactive compounds consisting of phenolic compounds, flavonoids, tannins and vitamins that show different antioxidant activities depending on the type of spice (Yashin, Yashin, Xia, & Nemzer, 2017). Buaniaw *et al.* (2010) studied the effect of an ethanol galangal extract on minced sea bass and observed it inhibited lipid oxidation. Moreover, curry spice, rosemary and thyme when incorporated in the biscuit mix had good sensorial acceptability with a potential to improve oxidative stability of fat in the biscuits (Emmanuel *et al.,* 2012; Kozłowska *et al.,* 2014).

2.7 Summary

Biscuits are popular snack foods made from flour, sugar and fat. Many researchers have tried to develop new nutritious products by incorporating new bioactive compounds and different proteins into biscuits. Substitution of various types of flour for wheat flour was becoming popular because of the nutritional advantages it provided. Purple rice, widely grown in Northern Thailand, was a good choice to replace wheat flour because it contained high fibre contents and a range of antioxidants. In terms of nutritional values, purple rice flour has several nutritional advantages over refined wheat flour, such as higher protein, vitamin and mineral contents. Nevertheless, the consumer acceptability of healthier purple rice flour biscuit should also be considered, on the one hand, since the partial replacement of purple rice flour for wheat flour changes both the chemical and physical characteristics (e.g. hardness). On the other hand, lipids were a major cause for both desirable and undesirable flavours that occurred in biscuits. Several studies have indicated that natural antioxidants were useful compounds to include in biscuits to reduce lipid oxidation during storage. However, at the present, there were very few publications that reported the use of purple rice flour to make biscuits; therefore, further study was essential.

2.8 Research objectives

Experimental research was carried out in the laboratory, with the following objectives:

1) To investigate the functional properties of flour mixtures made from purple rice with wheat flour substitutions at different levels to give improved functional properties.

2) To study whether different proportions of flour mixtures in a standard biscuit mix affect biscuits characteristics and taste as tested by panellists.

3) To identify the optimum amounts of defatted mussel powder and Thai spices that can be added to prepare a balanced and nutritious product acceptable to Caucasian, Chinese, Pacific Island and Thai ethnic groups.

4) To measure the effect of adding spice mixtures on the antioxidant content of the biscuits and acceptability of added defatted mussel powder.

5) To determine the storage characteristics of the optimum biscuit mixtures using two different types of packaging at two different temperatures.

2.9 Research outline

This research was carried out in five major stages. This is summarised in the diagram in Appendix A. The numbers on the left hand side of each main heading are the chapter numbers in this thesis.

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Chapter 3

Physicochemical, antioxidant properties and *in vitro* digestibility of wheat-purple rice flour mixtures

Abstract

The physicochemical characteristics, antioxidant properties and *in vitro* digestibility of high-antioxidant content flours made from different combinations of Thai purple rice flour and refined wheat flour from 25, 50 to 75% (w/w) were investigated these were compared to whole flour from purple rice and refined wheat flour. The increase in substitution levels of purple rice flour affected all the functional properties of flours, at the same time the levels of dietary fibre, protein digestibility and antioxidant compositions were also changed. The purple rice flour exerted a particularly strong effect on starch digestibility as the purple rice increased to 50% in the mixture. Moreover, purple rice flour showed lower amounts of rapidly digested starch, whereas slowly digested starch of whole flour from purple rice and 75% substitution purple rice flour were found to the highest for all samples. The *in vitro* starch digestibility of all samples also showed a positive correlation between dietary fibre and antioxidant compounds. Overall, the addition of purple rice flour improved the final nutritional properties, notably a lower predicted glycaemic index, and a higher antioxidant potential, which are two important nutritional properties for human health.

3.1 Introduction

Thai purple rice (*Oryza sativa* L.) is grown in Northern Thailand and it has been widely recognised as a potential cereal grain that contains high amounts of bioactive compounds, which are as usual located in the bran layer (Jang & Xu, 2009; Hosseinian, Li, & Beta, 2008). The two major anthocyanins found in the purple rice pericarp and aleurone layers, are cyanidin-3-O-glucoside and peonidin-3-O-glucoside. Moreover, phenolic acids are also found in the outer layers of purple rice grains (Jang & Xu, 2009). The bioactive compounds of purple rice exhibit high antioxidant activities due to the presence of antioxidant compounds (Jang & Xu, 2009; Yawadio, Tanimori, & Morita, 2007). Several reports have focused on the extraction and identification of phenolic compounds and

antioxidant activity in different pigmented rice cultivars (Yawadio *et al*., 2007; Loypimai, Moongngarm, & Chottanom, 2016; Shao *et al*., 2018).

Rice flour is one of the alternative flours recommended to produce gluten-free products because it contains no gluten (Chung, Cho, & Lim, 2014). Many commercial gluten-free products are made from white rice because it is a cheap raw material. However, the characteristics and use of flours to produce acceptable sensory characteristics products depends on the functional properties of the flours (Oladale & Aina, 2007).

Because they have high glycaemic indexes, rice and rice-based products are concern as they can increase insulin resistance for individuals who consume large amounts of these products (An, Bae, Han, Lee, & Lee, 2016). The glycaemic index is commonly used to classify dietary carbohydrates and this method is based on the blood glucose response, which can be measured using the area under curve when test subjects are fed test amounts of carbohydrate foods, compared to a reference food or a 50 g sample of glucose (Willett, Manson, & Liu, 2002). The predicted glycaemic index (pGI) uses an *in vitro* method based on a simulated gastric digestion method, together with a small intestine digestion step, to predict the glycaemic index that would be otherwise determined using human subjects (Monro, Mishra, & Venn, 2010). With the concern about the increasing number of people with diabetes worldwide, there is an important need to produce rice-based products with a low glycaemic indexes (low starch digestibility) using pigmented rice flour. Purple rice also contains high dietary fibre in the bran and germ fractions, which delays the starch granules' accessibility to digestive enzymes in the human digestive system (Jang & Xu, 2009; An *et al*., 2016). Therefore, understanding the digestibility of flour mixtures is important in order to achieve or increase product quality.

Colour is an important factor for initial acceptability by consumers who select food products based on their emotional expectation. Making the flours' colour similar to consumers' expectations can affect the food acceptance and liking scores (Sozer, Cicerelli, Heiniö, & Poutanen, 2014). The colour of wheat flour can be a reliable indicator of the quality of the flour. The pigmentation of purple rice is mainly from anthocyanins that accumulate in a single layer of cells (Jang & Xu, 2009). Moreover, Shao *et al*. (2018) reported that there are correlation between *L**, *a**, *b** colours and antioxidant compounds of pigmented rice.

The effect of wheat-purple rice protein on the rate of starch digestion, together with *in vitro* protein digestibility of raw flours, has not been undertaken so far. Moreover, few studies have reported the effects of wheat-purple rice flour mixtures on the nutritional properties, *in vitro* digestibility and antioxidant compounds, such as phenolic acids and anthocyanins. Substitution of purple rice flour might lead to an overall decrease in starch digestibility of the flour mixtures. Therefore, this study investigated the physicochemical characteristics, protein and starch digestibility, antioxidant compounds and antioxidant activities of purple rice blend flours by adding wheat flour at different levels in order to produce a series of biscuits for further study.

3.2 Materials and Methods

3.2.1 Raw materials

Blended flours were prepared with refined wheat flour (Pams Products Ltd., Auckland, NZ) and whole grain purple rice (*Oryza sativa* L. var. Sanpatong) (Big T. supermarket, Upper Riccarton, Christchurch, NZ). The whole rice grains were imported from Thailand in 5 kg bags. Whole purple rice grains were milled into a fine flour using a Whisper Mill (Grote Molen Inc., USA). Flours were then prepared by the substitution of purple rice flour into the wheat flour at levels of 25, 50 and 75% (w/w) of total flour sample weights by comparing with wheat flour and whole flour from purple rice.

3.2.2 Functional properties of flours

Water and oil absorption capacity

Beuchat (1977)'s method was used to determine the water and oil absorption capacity. Distilled water or soybean oil (Goodman Fielder, Auckland, NZ) (10 mL) was added to 1.0 g of flour sample in a centrifuge tube. The suspension was stirred using a vortex mixer. The suspension was then centrifuged at 2200 *g* for 25 min. The separated water and oil was then removed with a pipette and weighed. The water or oil absorption capacities were expressed as g of water or oil absorbed per g of the sample.

Bulk density

The bulk density was determined using the method of Okaka and Potter (1977). 50 g of each flour sample was weighed into a 100 mL graduated cylinder and then tapped

continuously 20-30 times. The bulk density (g/mL) was calculated as weight per unit volume of sample after the flour had settled.

Swelling power

Swelling power was determined by the method of Oladele and Aina (2007). 1 g of each flour sample was mixed with 10 mL of distilled water in a test tube. The suspension was heated in a shaking water bath at 80°C for 30 min. The tube was cooled to room temperature and then centrifuged at 870 g for 15 min. The paste was weighed to determine the swelling power.

Foaming capacity and stability

The foaming capacity and stability were measured using the method of Coffman and Garcia (1977). Sample (2 g) was added to 50 mL distilled water in 100 mL graduated cylinder. The suspension was shaken vigorously for 5 min to form a foam. The volume of the foam (mL) after shaking for 30 s was expressed as the foam capacity. The volume after 1 h was used as an indicator of foam stability and this was expressed as a percentage of the initial foam volume.

Least gelation concentration

The least gelation concentration was determined by the method of Coffman and Garcia (1977). Flour sample was mixed with 5 mL distilled water in test tubes to make flour dispersions at concentrations of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20% (w/v). The suspensions were then heated at 90°C for 1 h in a water bath, then cooled rapidly under running tap water (12°C) and further cooled for 2 h at 4°C. The least gelation concentration was regarded as the concentration at which the inverted sample did not slip down the side of the test tube. All functional properties of the flour mixtures were carried out in triplicate.

3.2.3   Proximate analysis

The moisture content was measured using the oven drying method at 105°C for 18 h (AOAC, 2000). Total nitrogen was determined using the Rapid Max N exceed® method of Dumas (Elementar, Hanau, Germany) and the factor 6.25 was used to calculate crude protein. Fat and ash were evaluated using AOAC methods 920.176 and 900.029, respectively (AOAC, 2000). Total starch content was determined using the Megazyme starch assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland-approved

method 76-13) (Reed, Ai, Leutcher, & Jane, 2013). Total dietary fibre was determined using a total dietary fibre assay kit (Sigma-Aldrich, MO, USA). All proximate composition measurements were carried out in triplicate.

3.2.4 Colour

The colour of samples was determined using a Minolta Chroma Meter CR-410 (Konica Minolta, Osaka, Japan) using the CIE $L*a*b*$ system (Ruangchakpet & Sajjaanantakul, 2007). The $L*$, $a*$, and $b*$ parameters represent lightness-darkness, redness-greenness, and yellowness-blueness of flour mixtures, respectively. Six replicates were performed for each analysis.

3.2.5 *In vitro* digestibility

Protein digestibility

Protein digestibility of the flour mixtures was investigated using the method of Akeson and Stahmann (1964). This method uses a two-stage *in vitro* digestion using pepsin from porcine gastric mucosa (Sigma Aldrich, USA, 66 units/mg protein) followed by pancreatin from porcine pancreas (AppliChem Chemica Synthesis, Germany, 30.315 units/mg protein). The stimulated solutions were centrifuged at 1600 g for 10 min. The supernatants were analysed for their digested protein contents using an Elementar (Hanau, Germany) Vario TOC cube instrument fitted with a chemi-luminescence detector for determining total bound nitrogen (TNb). The analyses were performed in triplicate.

Starch digestibility

Flours were tested *in vitro* to determine a simulated gastric digestion, and a small intestine digestion, using the method from Goni, Garcia-Alonso, & Saura-Calixto (1997) and Rosin, Lajolo, & Menezes (2002). Samples (0.5 g) were digested in 60 mL specimen pots on a multi magnetic stirrer (IKA-Werke RT 15 Power, Staufen/Germany) at the steady speed at 37°C 30 mL of water and 0.8 mL of 1 M HCl were added to adjust the pH of the samples. Then 1 mL of 10% pepsin (Sigma, P 7000; 800–2,500 U/mL) solution in 0.05 M HCl was added immediately. The solutions were digested for 30 min to accomplish gastric digestion. The sample was neutralised the gastric HCl with 2 mL of 1 M NaHCO$_3$ and 5 mL of 0.1 M sodium maleate buffer pH 6. Starch digestion was commenced adding 5 mL of 2.5% pancreatin (EC: 232-468-9, CAS: 8049-47-6, activity: 42362 FIP-U/g, Applichem GmbH, Darmstadt, Germany) in 0.1 M sodium maleate buffer pH 6 and the

pots were immediately filled up to 53 mL with distilled water. Triplicate of 1.0 mL samples were added to 4 mL ethanol, before (T=0), and at 20, 60, 120 and 180 min after adding the pancreatin solution. The digested samples were centrifuged at 180 *g* for 5 min. A 0.05 mL aliquot of ethanolic sample, standard glucose (5 and 10 mg/mL glucose) and reagent blank (distilled water) were added with 0.25 mL of enzyme solution A (1% enzyme invertase and 1% amyloglucosidase (Megazyme, E-AMGDF; 3260 U/mL) in acetate buffer pH 5.2) and incubated at room temperature for 20 min. Reducing sugars were determined by adding 0.75 mL dinitrosalicylic mixture (0.5 mg/mL glucose, 4 M NaOH and DNS reagent were mixed in ratio 1:1:5) and heated at 100°C for 15 min. The solution were cooled before 4 mL distilled water was added and read at 530 nm using a UV-Vis spectrophotometer (V-1200 spectrophotometer, Global Science, Auckland, New Zealand). The measured glucose released from the hydrolysed samples was expressed as the area under curves (AUCs) of digested starch of each sample for up to 180 min (Goni *et al.,* 1997). The AUCs were calculated for each sample from the area under the glucose release curve. The levels of rapidly digestible starch (RDS) and slowly digestible starch (SDS) were measured at 20 and after intestinal digestion between 20 and 180 min, respectively (Rosin *et al*., 2002).

3.2.6 Predicted glycaemic index (pGI)

The hydrolysis index (HI) was the AUC of each sample from the *in vitro* starch digestibility divided by the AUC of a standard (50 g white bread). The predicted glycaemic index (pGI) was calculated based on the HI values using the equation of Goni *et al.* (1997). Predicted glycaemic index = 39.71 + 0.549 × HI.

3.2.7 Extraction and determination of antioxidant compounds and antioxidant activities

The samples were extracted using a method of Jang and Xu (2009). The extracted solution was kept at -20°C until analysis. The antioxidant determinations were carried out in triplicate. The total phenolic contents were determined according to the method of Kaneda, Kubo, & Sakurai (2006). Total flavonoid contents were determined using the method of Zhishen, Mengcheng, & Jianming (1999). Hosseinian *et al*. (2008)'s method was used to analyse the anthocyanin contents. The anthocyanin concentration (mg/L) of

samples were calculated according to the following formula and expressed as Cyanidin-3-O-glucoside (C3G) equivalents:

$$\text{C3G (mg/L)} = \frac{[(A_{\lambda 700-\lambda 520})_{pH\,1.0}] - [(A_{\lambda 700-\lambda 520})_{pH\,4.5}] \times MW\ of\ Cy-3-glu \times DF}{\varepsilon \times 1} \times 1000$$

Where MW = the molecular weight of C3G = 449.2 g/mol, DF = the dilution factor and ε = the extinction coefficient (L$\times$ cm^{-1} $\times$mol^{-1}) = 26,900 for C3G.

The ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] assay was performed using the method of Re *et al*. (1999). The samples (0.1 mL) were mixed with 2.9 mL of ABTS solution and incubated for 6 min before reading at 734 nm. A standard curve of trolox was set up in the range of 0 to 100 mg/100 mL. The results were expressed as trolox equivalents (µmol trolox/g DM of sample). The DPPH (2,2-diphenyl-2-picrylhydrazyl) contents of the sample extracts were analysed according to the method of Mahakunakorn, Tohda, Murakami, Matsumoto, & Watanabe (2004). The extracts were mixed with DPPH (2,2-diphenyl-2-picrylhydrazyl) solution (1:1, v/v) and then incubated for 30 min at room temperature. The absorbance of the resulting yellow colour was measured at 520 nm with a spectrophotometer (UV-Vis). MeOH (4 mL of 80%) was added as a control. All determinations were conducted in triplicate.

3.2.8 Statistical analysis

All results generated in this work were statistically analysed by one-way ANOVA using a complete randomised design. The mean values were analysed by Duncan's multiple range test for a multi-comparison of means. The level of significance was assigned at $P < 0.05$. Pearson correlation coefficients were also performed. All statistical analyses were undertaken using the SPSS Statistics (v. 22.0, SPSS Inc., Chicago, IL, USA).

3.3 Results and discussion

3.3.1 Functional properties of flours

Water absorption capacity (WAC) and oil absorption capacity (OAC)
The WAC of the flour played an important role in the functional and sensory properties during food preparation. Moreover, WAC represents the ability of a product to mix with water under conditions where water is limited (Du, Jiang, Yu, & Jane, 2014). The WACs

of the flours ranged from 98.10 to 135.81%, where the WAC of refined wheat flour was the highest and the whole flour from purple rice was the lowest (Table 3.1). The difference in WAC of flour mixtures is due to the difference in fibre and carbohydrate contents. Moreover, the amylopectin/amylose ratio can cause a lower solubility of the flours (Chandra, Singh, & Kumari, 2015). It is reported that low WAC corresponds to high amylopectin contents of rice (Ihegwuagu, Omojola, Emeje, & Kunle, 2009). The OAC of flours was another important factor for improving the mouthfeel and maintaining the flavour of food products. The OAC of the refined wheat flour was significantly ($P < 0.05$) higher than those of the other flours. The carbohydrate content in the mixtures is also responsible for the elevated OAC. The differences in OAC have been attributed to the variations in the amylose/amylopectin ratio as well as their chain length distribution (Chandra *et al*., 2015). Hydrophobic proteins play an important role in water and oil absorption, and this is important for the development of a stable product (Du *et al.,* 2014).

Bulk density

Bulk density plays an important role in food preparation. Significant differences ($P < 0.05$) were observed among the bulk densities of the different flours (Table 3.1). The bulk density of the flours varied from 0.52 to 0.79 g/mL, where the lowest and the highest values were obtained from the purple rice flour and wheat flour, respectively. It was clear that the proportion of wheat flour increased the bulk density of 50% purple rice flour substitution compared to whole flour from purple rice. Joshi, Liu, & Sathe (2015) reported that the bulk density of different flours ranged from 0.59 g/mL for rice grains to 0.80 g/mL for refined wheat flour; these were comparable to the results in this study. The bulk density had a direct influence on the protein content, preparation, treatment and storage of the sample.

Swelling power

The swelling power represented the degree of starch granules in the flour that can absorb water. Swelling is, essentially, a property of the whole amylopectin molecule, rather than parts of it, and amylose alone appeared to be a diluent, while lipids (as complexes with amylose) strongly inhibited swelling. Table 3.1 shows that the swelling power of the flours ranged from 4.42 to 11.56 g/g. Purple rice flour had high swelling power due to its higher amylopectin content compared to wheat flour (Chan, Bhat, & Karim, 2009).

Foaming properties (foam capacity and foam stability)

The foam properties of flour mostly depend on proteins and the interfacial protein film trapping air bubbles in liquid or solid suspensions and decelerating the rate of accumulation of bubbles in flours (Chandra *et al.,* 2015). The highest foam capacity was observed for refined wheat flour (17.33%), the 50% and 75% purple rice flour substitutions (10.67%), and the lowest foam capacity was the whole flour from purple rice (6.67%). A lower value of foaming capacity for white rice flour (3.52%) had previously been reported by Chandra and Singh (2013) who stated that this was due to differences in protein concentrations in the flours. Foam stability represents the capacity of the foam to retain its structure over time, and can be described as the ability of the proteins to form strong cohesive films around air vacuoles that then resist air diffusion from the vacuoles (Chandra *et al.,* 2015). The foam stability decreased as increasing amounts of purple rice flour were added to the wheat flour mixture. The low foam capacity and stability were desirable attributes for flours intended for the production of a variety of baked products, such as biscuits and crackers (Chandra *et al.,* 2015).

Least gelation concentration

The least gelation concentration measured the lowest concentration of proteins to maintain a gel that formed in the inverted tube, and was defined as an index of gelation capacity. This depended on the relative ratios of their proximate contents, such as protein, carbohydrates, and lipids (Chandra *et al.,* 2015). Purple rice flour quickly formed a gel at the highest concentration (11.33%) while wheat flour formed a gel at a very low concentration (8.67%). The increasing substitution levels of purple rice flour in the flour mix reduced interactions among the binding forces which, in turn, decreased the gelling ability of the flour. It can be implied that whole flour from purple rice needed a higher quantity of flour substituted in order to form a gel; however, refined wheat flour was better in terms of gelling ability.

Table 3.1   **Functional properties of the five different flour mixtures.**

Functional properties	Wheat flour	Purple rice flour substitution (%)			Whole flour from purple rice
		25	50	75	
Water absorption capacity (%)	135.81 ± 1.55^a	120.93 ± 1.69^b	116.25 ± 1.59^c	106.58 ± 0.60^d	98.10 ± 0.59^e
Oil absorption capacity (%)	159.97 ± 1.07^a	149.05 ± 1.27^b	145.28 ± 0.28^c	132.68 ± 0.30^d	119.13 ± 0.53^e
Bulk density (g/mL)	0.79 ± 0.01^a	0.73 ± 0.01^b	0.68 ± 0.01^c	0.58 ± 0.01^d	0.52 ± 0.01^e
Swelling power (g/g)	4.42 ± 0.06^e	5.38 ± 0.01^d	7.17 ± 0.04^c	9.44 ± 0.15^b	11.56 ± 0.16^a
Foaming capacity (%)	17.33 ± 1.76^a	14.00 ± 1.15^b	10.67 ± 0.67^b	10.67 ± 1.33^b	6.67 ± 0.67^c
Foaming stability (%)	1.82 ± 0.01^a	1.76 ± 0.07^a	1.60 ± 0.06^a	1.39 ± 0.06^b	0.96 ± 0.02^c
Least gelation concentration (%)	8.67 ± 0.67^b	9.33 ± 0.67^{ab}	10.00 ± 0.00^{ab}	10.67 ± 0.34^{ab}	11.33 ± 0.67^a

Values represent mean ± standard error. In each row, sample means not having the same letter are significantly different (Duncan's multiple range test, $P < 0.05$).

3.3.2 Proximate composition of flours

The proximate compositions of the different levels of purple rice flour are critical factors in the development of new baked products and were used to adjust the formulations are shown in Table 3.2 (Varastegani, Zzaman, & Yang, 2015). The moisture content of the refined wheat flour (12.53%) was slightly higher compared with the substituted flour samples (11.98%) and the whole flour from purple rice samples (11.57%). The whole flour from purple rice had the highest total dietary fibre contents compared to the other flours. The fibre and ash contents of the refined wheat flour were related to the amounts of bran in the wheat flour (Varastegani *et al.,* 2015). The refined wheat flour contained higher starch and protein contents than the whole flour from purple rice. Increasing purple rice flour substitution significantly reduced ($P < 0.05$) the protein content and at the same time increased the total dietary fibre content, these affected the functional properties such as foaming and least gelation concentration of the flour mixtures (Table 3.1). The starch and protein contents were the major compositions in foods, affecting the physicochemical characteristics of food products and plays an important role in the final quality of bakery products (Varastegani *et al.,* 2015).

Table 3.2 shows the *L*a*b** colour parameters of the raw flours. The lightness (*L**) showing that all flours had significant colour differences among them (see Appendix C). The factor affecting the *L** value of the flour mixtures was wheat protein, which showed a positive relationship with the *b** values (Beuchat, 1977). Okaka and Potter (1977) stated that the brightness of the flours tended to decrease with the increasing protein content; however, the lightness of these flour mixtures decreased due to the dark colour of the purple rice flour. The addition of purple rice flour, at all levels, increased the redness (*a**) and lowered the yellowness (*b**) in the mixture due to the predominance of phenolic acids in rice hulls and bran layers (Jang & Xu, 2009). The colour of purple rice flour is derived from the accumulation of anthocyanins and proanthocyanidins in the outer surface of the sheath (Jang & Xu, 2009).

Table 3.2   Proximate composition and *L**a*b* colour values of the five different flour mixtures.

| Nutritional properties | Wheat flour | Purple rice flour substitution (%) | | | Whole flour from |
		25	50	75	purple rice
Moisture content (%)	12.53 ± 0.23^a	12.29 ± 0.04^a	11.88 ± 0.04^b	11.78 ± 0.06^b	11.57 ± 0.14^b
Crude protein (%)	15.41 ± 0.06^a	14.25 ± 0.03^b	12.93 ± 0.19^c	12.88 ± 0.04^c	12.97 ± 0.12^c
Protein digestibility (%)	24.80 ± 0.50^e	33.33 ± 0.20^d	50.48 ± 0.14^c	60.33 ± 0.20^b	66.46 ± 0.86^a
Total dietary fibre (%)	2.31 ± 0.15^e	2.82 ± 0.01^d	3.32 ± 0.09^c	4.54 ± 0.17^b	5.56 ± 0.10^a
Ash (%)	0.88 ± 0.02^d	1.03 ± 0.01^c	1.06 ± 0.01^{bc}	1.09 ± 0.01^{ab}	1.10 ± 0.02^a
Total fat (%)	1.97 ± 0.05^c	1.97 ± 0.01^c	2.09 ± 0.01^c	2.51 ± 0.12^b	2.77 ± 0.07^a
Colour values *L**	99.72 ± 0.65^a	86.06 ± 0.25^b	78.55 ± 0.66^c	71.87 ± 0.28^d	66.65 ± 0.84^e
a*	-1.42 ± 0.02^e	0.96 ± 0.01^d	2.13 ± 0.03^c	3.14 ± 0.01^b	3.91 ± 0.07^a
b*	13.08 ± 0.16^a	10.83 ± 0.14^b	3.96 ± 0.02^c	3.51 ± 0.02^d	2.16 ± 0.01^e

Values represent mean ± standard error. In each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$).

3.3.3 *In vitro* starch digestibility

Although all flours had similar starch contents, the starch digestion rates were significantly different ($P < 0.05$) (Table 3.3). The total starch contents of all flours were not significantly different from each other ($P > 0.05$). The area under the starch hydrolysis curve (AUC) was evaluated after the digestion of each sample to explain the glycaemic response of the selected foods compared to a reference sample of white bread (An *et al.,* 2016). As described, the delay in starch hydrolysis observed in the whole flour from purple rice led to a decrease in the rapidly digestible starch (RDS) and an increase in the slow digestible starch (SDS) fraction compared to the wheat flour (473.21 mg min dL^{-1}). The measurement of reducing sugar released using the *in vitro* method can be divided into RDS (glucose released in the first 20 min of digestion) and SDS (the amount of glucose released from 20 to 180 min of digestion) based on the amount of glucose released. RDS was the predominant fraction in flour mixtures that were measured as glucose at 20 min of digestion, to reflect the rate of absorption in the small intestine. From Table 3.3, it can be seen that the substitution of purple rice flour in the mixture has significantly lower RDS than wheat flour, whereas the substituted purple rice flour also had a higher SDS compare to wheat flour by 25%. Flours with higher amounts of SDS are required as they are able to reduce the prevalence of health problems such as heart disease and diabetes (An *et al.*, 2016). Raw starch is generally not very digestible since many intrinsic and extrinsic factors can affect the digestibility of starch. Purple rice starches typically contain high levels of amylopectin, which may be retrograded during the hydrolysis to make the flour mixtures less sensitive to the digestion (An *et al.*, 2016). This may be the case with purple rice flour, which shows minimal enzyme digestibility over 120 min of enzyme incubation. The same results were also reported by Sumczynski, Kotásková, Družbíková, & Mlček (2016). Before the purple rice can be used in products, it has to be milled. This processing may modify the amylose content of the purple rice starch, which in turn will affect the digestibility. In addition, it is possible that starch digestibility is related to granule size. Larger granules tend to have a lower digestibility (Reed *et al*., 2013). The correlation between the antioxidant compounds and *in vitro* starch digestibility are also reported in this study.

Glucose release curves in Fig. 3.1 show the rate of reducing sugars released from starch in a digestive enzymatic method. Among all the flour mixtures, the digestion rates of the

whole flour from purple rice samples were the lowest, indicating that purple rice flour had a stronger resistance to digestion than wheat flour after 120 min of enzymatic hydrolysis, which resulted in slower and lower increases of glucose in the *in vitro* method. An *et al.* (2016) showed that different cultivars may have different nature of starch, protein and dietary fibre contents and changes in these may affect the digestibility of starch. Protein can restrict the rate of starch granules swelling and gelatinisation, which was partially responsible for the low digestibility. Moreover, the purple rice flour, which was rich in dietary fibre and protein, showed a significant decreased in starch digestibility. Based on the results of An *et al.* (2016), the starch digestibility of black rice flour substituted in wheat flour is related to the phenolic content rather than dietary fibre with decreasing rate of glucose released in the *in vitro* digestion method. Thompson, Yoon, Jenkins, Wolever, & Jenkins (1984) and Ramdath, Padhi, Hawke, Sivaramalingam, & Tsao (2014) found that anthocyanin-rich extracts from potatoes showed signicant *in vitro* inhibitory activity towards α-glucosidase, the main enzyme which hydrolyses disaccharides into glucose. All samples digested rapidly within the first 20 min of the hydrolysis, and the digestion rate from 20 to 180 min increased slowly thereafter (Fig. 3.1).
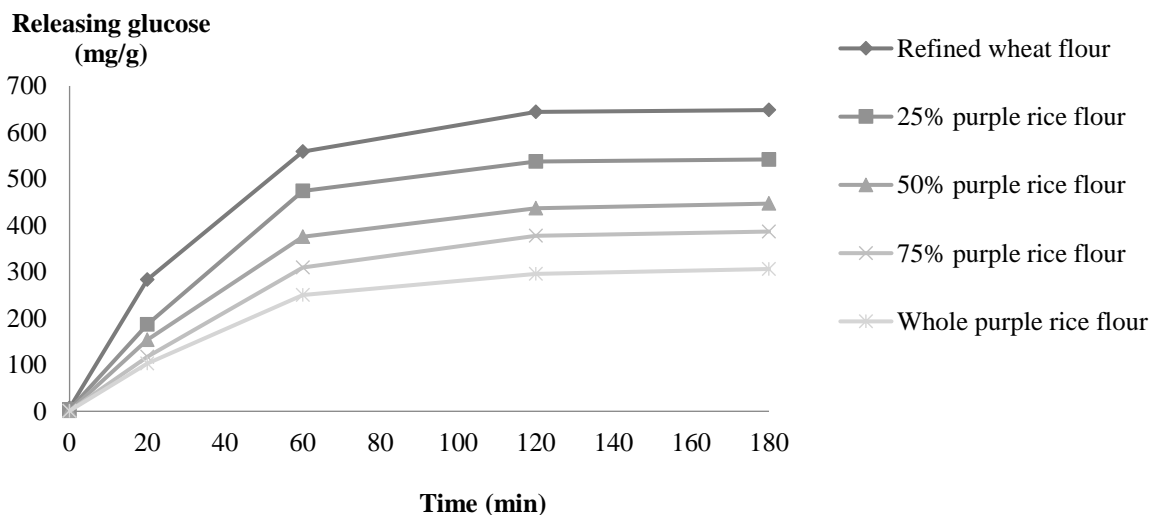
Figure 3.1   Amount of glucose released (mg/g of starch) of each flour mixture during *in vitro* starch digestion using different enzymatic hydrolysis for up to 180 min.

The pGI values of the flours were calculated based on HI derived from the starch digestibility of each flour. As shown in Table 3.3, the calculated pGI values significantly

decreased ($P < 0.05$) with increasing purple rice flour substitution levels. Thus, whole flour from purple rice had the lowest amount of digested starch released using the *in vitro* method. The suppression effects of the substituted purple rice flour in refined wheat flour on starch digestibility were based on the phenolic contents and dietary fibre (An *et al.,* 2016). Moreover, the variation in the proportions of rapidly digestible starch, slowly digestible starch, as well as the content of other macronutrients, such as fat, fibre and protein, had a distinct impact on the pGI of food products (An *et al.,* 2016). As stated previously, purple rice flour, which was rich in dietary fibre, had lower amounts of glucose released over time compared to wheat flour. A similar result was reported by An *et al*. (2016) who stated that the pGI values of black rice flour were significantly lower than wheat flour during an *in vitro* starch digestion. Another study reported that high amylose rice varieties also showed higher pGI values compared to purple rice, which has a lower amylose content (Frei, Siddhuraju, & Becker, 2003). Brand-Miller (1994) showed that brown rice with a low glycaemic index reduced the insulin resistance and adjusted the blood glucose levels in both Type 1 and Type 2 diabetic patients. Moreover, Therefore, the substituted purple rice flour can be said to have a low glycaemic index (GI < 60), which is good for both diabetics and healthy people (Brand-Miller, 1994). Moreover, the control flour (refined wheat flour) can be regarded as an intermediate GI (60-85) ingredient (Brand-Miller, 1994).

Table 3.3 *In vitro* **starch digestion profile and amylolytic digested starch fractions (rapidly digestible starch, RDS and slowly digestible starch, SDS) of flour mixtures.**

Nutritional properties	Wheat flour	Purple rice flour substitution (%)			Whole flour from purple rice
		25	50	75	
Total starch (%)	74.50 ± 0.71^{NS}	73.60 ± 0.38	72.30 ± 0.12	73.23 ± 0.35	73.00 ± 1.04
RDS (mg/g sample)	42.70 ± 0.07^a	34.12 ± 0.17^b	33.74 ± 0.11^b	30.08 ± 0.03^c	22.66 ± 0.28^d
SDS (mg/g sample)	13.74 ± 0.14^c	12.55 ± 0.07^d	15.92 ± 0.31^b	20.05 ± 0.34^a	20.35 ± 0.41^a
AUC of digested starch (mg min dL^{-1})	473.21 ± 0.10^a	389.26 ± 0.31^b	266.32 ± 0.17^c	235.64 ± 0.98^d	178.90 ± 0.28^e
pGI	63.11 ± 0.01^a	58.96 ± 0.03^b	55.29 ± 0.03^c	52.88 ± 0.01^d	48.56 ± 0.02^e

Values represent mean ± standard error; NS = not significant difference. In each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$). AUC = area under the curve; pGI = predicted glycaemic index.

3.3.4 Antioxidant compounds and antioxidant activities of different flour mixtures

The antioxidant characteristics and antioxidant activities of all flour samples are shown in Table 3.4. The whole flour from purple rice obviously had higher total phenolic, anthocyanin and flavonoid contents than the other flours. Large differences in the total phenolics were identified; the total phenolics ranged from 28.66 mg GAE/100 g DW in refined wheat flour to 318.28 mg GAE/100 g DW in whole flour from purple rice. The total phenolic contents of the whole flour from purple rice in this study were similar to the results of Shao *et al*. (2018). In contrast, the results of the whole flour from purple rice in this study were higher than reported by Rocchetti *et al.* (2017). These results may be due to the differences in the extraction methods used by Rocchetti *et al*. (2017). The major anthocyanin of purple rice analysed in this study was cyanidin-3-O-glucoside (C3G). The whole flour from purple rice was the richest in phenolic compounds and these were mainly ascribed to anthocyanins (Shao *et al*., 2018). Shao *et al*. (2018) also stated that black rice from different cultivars had C3G, which ranged from 12.03 to 1106.00 mg/kg DW; these values were similar to the values for whole flour from purple rice in this study. Therefore, it could be expected that purple rice flour had higher flavonoid contents than wheat flour. The total flavonoid contents of the refined wheat flour (29.67 mg catechin (CE)/100 g) were significantly lower ($P < 0.05$) than those of the whole flour from purple rice (149.83 mg CE/100 g). These findings were similar to report from Kim and Kim (2017). The variations of the antioxidant compounds were due to the different levels of purple rice flour added to the wheat flour. Whole flour from purple rice had the highest ABTS and DPPH radical scavenging activity compared to substituted flour and refined wheat flour. The effectiveness in scavenging ability on ABTS and DPPH radicals were in a range of the results reported by Sumczynski *et al.* (2016) that varied from 10.0 to 23.6 mmol trolox/kg and ranged from 7.2 to 12.5 mmol trolox/kg in black rices, respectively. In addition, Sumczynski *et al.* (2016) also stated that results from ABTS scavenging method were consistent with the DPPH scavenging method.

Table 3.4   Antioxidant compounds and antioxidant activities (DPPH and ABTS assays) of the five different flour mixtures.

| Antioxidant properties	Wheat flour	Purple rice flour substitution (%)			Whole flour from purple rice
		25	50	75	
Total phenolics (mg GAE/100 g DW)	28.66 ± 0.09^e	76.46 ± 0.55^d	153.57 ± 0.46^c	211.84 ± 2.54^b	318.28 ± 1.51^a
Anthocyanins (C3G, mg/kg DW)	0.22 ± 0.05^e	118.49 ± 0.61^d	249.69 ± 0.35^c	388.73 ± 1.23^b	492.59 ± 0.95^a
Flavonoid contents (mg CE/100 g DW)	29.67 ± 1.23^c	58.74 ± 0.66^d	95.92 ± 5.55^c	119.92 ± 1.30^b	149.83 ± 2.17^a
DPPH (µmol trolox/g DW)	3.72 ± 0.14^e	16.97 ± 0.46^d	35.46 ± 0.17^c	41.00 ± 0.04^b	46.75 ± 0.08^a
ABTS (µmol trolox/g DW)	6.73 ± 0.21^d	8.28 ± 0.09^d	10.48 ± 0.19^c	77.84 ± 0.38^b	102.77 ± 1.11^a

Values represent mean ± standard error. In each row, sample means not having the same letter attached to them are significantly different from each other (Duncan's multiple range test, $P < 0.05$). GAE, gallic acid equivalents; C3G, cyanidin-3-glucoside; CE, catechin equivalents; DPPH, 2,2-diphenyl-2-picrylhydrazyl radical scavenging activity; ABTS, [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging activity.

3.3.5 Correlation among selected proximate composition, *a** colour parameter, antioxidants and *in vitro* starch digestibility

Pearson's correlations (*r*) among selected proximate composition, *a** colour parameter, antioxidant compounds and antioxidant capacity are shown in Table 3.5. Among all flour accessions, the colour parameter *a** value had significantly positive correlation with anthocyanins ($r = 0.975$ at $P < 0.01$). However, negative correlation of whole rice grain *a** with antioxidant capacity has been reported by Shen, Jin, Xiao, Lu, & Bao (2009). Regarding the correlation among colour parameter *a** value, dietary fibre and protein content, significant correlations were observed. It was also found that dietary fibre and total phenolics significantly correlate with pGI. It can be suggested that the higher the fibre content in flour mixtures the lower the digestibility values could be observed (Table 3.3). *In vivo* study also reported by Jenkins, Lees, Gassell, Cocket, & Alberti (1977) that dietary fibre increase the viscosity of intestinal contents in the intestinal tract and reduce the absorption of carbohydrates. The significant correlation between antioxidant compounds and antioxidant activity has also been observed. From an analytical point of view, more purple rice content in the flour mixtures gives a higher content of antioxidant compounds. Although the data obtained from ABTS and DPPH scavenging activities (Table 3.4) were different, both ABTS and DPPH had highly positive correlations with antioxidant compounds among all flour mixtures. Nearly all other studies found similar results in the correlations between antioxidant compounds and antioxidant activity in rice grains (Sumczynski *et al.,* 2016; Shao *et al.*, 2018).

Table 3.5   Pearson correlation among selected proximate composition, *a* **colour value, antioxidant compounds, antioxidant activities and *in vitro* starch digestibility.**

	Colour *a**	pGI	DPPH assay	ABTS assay
Dietary fibre	0.916**	0.196*	0.901**	0.958**
Protein content	-0.931**	0.190	-0.956**	-0.622**
Total starch	-0.414	0.007	-0.418	-0.070
Total phenolics	0.946**	0.241*	0.944**	0.917**
Flavonoids	0.946**	0.241	0.944**	0.880**
Anthocyanins	0.975**	0.211	0.977**	0.917**

*, ** indicate significant at $P < 0.05$ and $P < 0.01$, respectively.

3.4 Conclusions

The purple rice flour with high antioxidant contents can be used as an effective flour to substitute wheat flour to produce healthy bakery products to meet consumers' demand. This study is the first report on the substitution of purple rice flour for wheat flour where the functional properties, antioxidant characteristics of the flours and their correlations have been measured. The functional properties of flour mixtures were changed after substituted purple rice flour into the mixture, which will affect the quality of the products. The substitution of purple rice flour varied the nutritional properties, *in vitro* digestibility, with only small changes in the *L*a*b** colour values. Inclusion of increasing purple rice flour in the mixture had a significant effect on increasing the *in vitro* digestibility of protein, which can slow the starch digestion rate on human metabolism. The whole flour from purple rice showed a stronger resistance to starch digestion and a higher SDS fraction compared to wheat flour. *In vitro* starch digestion of whole flour from purple rice has a significantly positive correlation with dietary fibre and antioxidant compounds. This study revealed that the purple rice flour could be an effective alternative flour with a low starch digestibility, low pGI and high antioxidant contents which needs further study to understand behaviour of these flour mixtures in a real food product.

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Chapter 4

Effect of substituting purple rice flour for wheat flour on physicochemical characteristics, *in vitro* digestibility and sensory evaluation of biscuits

Abstract

Purple rice flour contains high levels of a number of antioxidant compounds; however, it has seldom been used as an ingredient in bakery products. The aim was to increase the nutritional value of biscuits by adding purple rice flour to a basic wheat flour biscuit. The substitution of purple rice flour in place of wheat flour modified the characteristics of the biscuits in terms of increasing the nutritional values. They contained high fibre, antioxidants and antioxidant activities, while slightly changing the physical properties such as hardness and spread ratio. The study showed that the protein digestibility increased as the content of the purple rice flour was increased in the biscuit mix. The 100% purple rice flour biscuits had the lowest predicted glycaemic index (pGI) of all the biscuits. Sensory analysis showed that the overall acceptability of the blended flour biscuits at 25 and 50% substitution levels were slightly less than the control biscuits with only 9%; however, the scores were over 5 which means the panellists still liked the biscuits a lot. Overall, the inclusion of purple rice flour in biscuits mix increased crude protein content, protein digestibility and bioactive compounds with acceptable sensory scores from consumers up to 50% substitution of purple rice.

4.1 Introduction

Purple rice (*Oryza sativa* L. var. Sanpatong) is a popular cultivar grown widely in Northern Thailand. It has become well known because of its interesting colour, good taste, and lower commodity price compared to wheat flour (Jang & Xu, 2009). Purple rice has also become popular because it contains high amounts of bioactive compounds such as phenolic acids and anthocyanins. It also contains higher levels of dietary fibre than wheat flour (Yawadio, Tanimori, & Morita, 2007). Cyanidin-3-O-glucoside and peonidin-3-O-glucoside are present in the pericarp and aleurone layers, and high levels of phenolic acids are found in the outer layers of the grain (Jang & Xu, 2009). All of these bioactive

compounds give the grain high antioxidant activities (Jang & Xu, 2009). Red rice, black rice, and purple wheat are all valued for their natural antioxidant content (Pasqualone *et al.,* 2015; Yawadio *et al.,* 2007). Antioxidants play important roles in lowering low-density lipoprotein cholesterol (LDL), improving lipid profiles, and reducing inflammation due to free radical inhibition (Hosseinian, Li, & Beta, 2008). In addition, purple rice contains high amounts of dietary fibre (Jang & Xu, 2009).

Biscuits made from wheat flour are one of the most widely eaten consumer products around the world. They are an affordable product, and they have a good taste and a long shelf-life (Park, Choi, & Kim, 2015). Unfortunately, biscuits usually contain high levels of easily digested starch, sugar, and butter, and low levels of dietary fibre which nutritionists suggest make them a rather unhealthy constituent of our diet. Bakers are well aware of these issues, and they have shown some interest in developing biscuits, which can be regarded as functional foods containing less butter (Pasqualone *et al.,* 2015; Sharma, Saxena, & Riar, 2016; Giarnetti, Paradiso, Caponio, Summo, & Pasqualone, 2015). So far, bakery research has attempted to make more healthy products by incorporating new ingredients into biscuit mixes in order to increase their nutritional and textural qualities (Park *et al.,* 2015). The texture, flavour, and appearance of the biscuits are major attributes that affect biscuit acceptability (Torbica, Hadnađev, & Hadnađev, 2012). Unfortunately, rice protein cannot generate a viscoelastic network like gluten in wheat, which retains carbon dioxide during biscuit dough fermentation. So the addition of rice to a biscuit mix can have a significant effect on the textural qualities of the cooked biscuit. However, biscuits require a lower specific volume compared to the familiar structure of bread (Sharma *et al.,* 2016). In attempts to reduce cost, many studies have investigated the properties of biscuits using different types of rice flours such as white rice flour (Torbica *et al.,* 2012), waxy rice flour (Giuberti, Marti, Fortunati, & Gallo, 2017), brown rice flour (Chung, Cho, & Lim, 2014), and a composite rice flour together with green gram flour and potato flour (Chandra, Singh, & Kumari, 2015). Recently, products with high protein and fibre contents are more commonly chosen by consumers in order to combat diseases such as diabetes and obesity. Therefore, dietary fibre in bran and the germ of cereal grains has been added to biscuit formulations, which originally contained almost no dietary fibre in the original recipe (Torbica *et al.,* 2012). Substitution of purple rice flour for wheat flour will definitely reduce the wheat gluten content, but purple rice flour has potential as an inexpensive raw material for biscuits as it has good

textual qualities with high contents of bioactive compounds and dietary fibre. However, the development of new healthy products has to have acceptable sensory attributes, as the product will become a marketing failure. Therefore, the objective of this study was to evaluate the physicochemical characteristics and antioxidant properties of raw flours and biscuits substituted with different levels of purple rice flour. Finally, the preferences for the biscuits made from different levels of purple rice flour were evaluated using consumer taste tests.

4.2 Materials and Methods

4.2.1 Raw materials

The biscuits were prepared by the substitution of purple rice flour for refined wheat flour (Pams Products Ltd., Auckland, NZ), purple rice (*Oryza sativa* L. var. Sanpatong) (Big T. supermarket, Riccarton, Christchurch, NZ) purchased in 5 kg bags, baking powder (Edmonds Limited, Goodman Fielder Ltd., Auckland, NZ), xanthan gum (Lotus Foods Pty Ltd., Cheltenham, Victoria, Australia), butter (Dairyworks Ltd., Hornby, Christchurch, NZ), castor sugar (Pams Products Ltd., Auckland, NZ), salt (Pams Products Ltd., Auckland, NZ), whole egg (Pams Products Ltd., Auckland, NZ), and vanilla extract (Pams Products Ltd., Auckland, NZ). All ingredients were of food grade. The experimental biscuits were made by substituting purple rice flour for wheat flour at levels of 25, 50, 75, and 100% (w/w) of the total flour weight. The control biscuits were made from refined wheat flour.

4.2.2 Biscuits preparation

The control biscuits were prepared from blended wheat flour, and the whole purple rice grains were milled using a Whisper Mill (Grote Molen Inc., USA) as purple rice flour was not available in New Zealand. All ingredients are shown in Table 4.1. The dough was rolled out to a thickness of 4 mm using an adjustable rolling pin (Joseph Joseph 20085, Joseph Joseph Ltd., London, UK) and was then cut out with a 5 cm diameter biscuit cutter. The dough was then baked in the oven (Bakbar Turbofan 32 Max, Ali Group company, Milan, Italy) at 170°C for 9 min and cooled on a wire grid at room temperature for 15 min. Physical analysis and sensory evaluations were carried out on the freshly baked biscuits, whereas chemical analysis such as antioxidants and *in vitro* digestibility was carried out on the ground samples.

Table 4.1   Formulation of biscuits supplemented with purple rice flour.

Ingredients (g)	Control	Purple rice flour substitution (%)			
		25	50	75	100
Refined wheat flour	100	75	50	25	-
Purple rice flour	-	25	50	75	100
Butter	75	75	75	75	75
Sugar	62.5	62.5	62.5	62.5	62.5
Egg	112.5	112.5	112.5	112.5	112.5
Salt	0.75	0.75	0.75	0.75	0.75
Sodium bicarbonate	4.5	4.5	4.5	4.5	4.5
Xanthan gum	1.25	1.25	1.25	1.25	1.25

4.2.3 Proximate analysis

Moisture content of the biscuits was measured by gravimetric method using the oven at 105°C for 18 h (AOAC, 2000). Total nitrogen was determined using the Dumas method; the factor 6.25 was used to calculate the crude protein content of the biscuits (AOAC, 2000). Fat and ash contents of the biscuits were also determined according to the methods of the Association of Official Analytical Chemists (AOAC, 2000). The carbohydrate was calculated by the difference according to the AOAC method (AOAC, 2000). Total starch content was determined using the full instruction and reagents from Megazyme starch assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) (Approved Method 76-13) (Reed, Ai, Leutcher, & Jane, 2013). Total dietary fibre content of biscuits was evaluated using the total dietary fibre assay kit (Sigma-Aldrich, MO, USA). All proximate compositions were analysed in triplicate.

4.2.4 *In vitro* digestibility

Protein digestibility

The *in vitro* protein digestibility was carried out by multienzyme solution according to Akeson and Stahmann (1964) method. The two stages of *in vitro* digestion of pepsin from porcine gastric mucosa (Sigma-Aldrich, USA, 66 units/mg protein) followed by pancreatin from porcine pancreas (AppliChem Chemica Synthesis, Germany, 30.315 units/mg protein) were used to stimulate the gastric and intestinal digestion in humans. The simulated protein solution of each sample was centrifuged at 1600 *g* for 10 min. The supernatants were determined for nitrogen contents using an Elementar (Hanau, Germany) Vario TOC cube instrument fitted with a chemiluminescence detector for determining total bound nitrogen (TNb). The analysis was carried out in triplicate.

Starch *in vitro* digestion

The *in vitro* digestion of biscuits was determined using a simulated gastric digestion followed by pancreatin enzymes (AppliChem Chemica Synthesis, Germany, 30.315 units/mg protein) (Monro, Mishra, & Venn, 2010). The hydrolyzed glucose contents were measured using the glucose released method at following times: 0, 20, 60, 90, 120, and 180 min. The glucose released was measured using a glucose oxidase-peroxidase kit (K-GLOX, Megazyme Bray, Co., Wicklow, Ireland). The digested starch fractions were centrifuged at 180 *g* for 5 min, and then, the supernatants were read at 530 nm (V-1200 spectrophotometer, Global Science, Auckland, New Zealand).

4.2.5 Predicted glycaemic index (pGI)

The hydrolysis index (HI) was derived from the area under the curve of glucose released from each sample (0–180 min) divided by the area under the curve from a reference food (50 g white bread). The predicted glycaemic index (pGI) was calculated using the equation of Goni, Garcia-Alonso, & Saura-Calixto (1997): Predicted glycaemic index = 8.198 + 0.549*HI.

4.2.6 Physical and texture characteristics

The hardness (N) of freshly baked biscuits was measured using a texture analyser (TA-XT2i, Stable Micro System, Godalming, UK) equipped with 30 kg load cell. A small three-point bending test rig with sharp-blade cutting probe was used (Park *et al.,* 2015). The first peak of each penetration was recorded as the hardness force. Fifteen minutes after baking, six freshly baked biscuits were weighted, and the width (W), thickness (T), and biscuit spread ratio (W /T) were determined (AACC method 10-50.05) (AACC, 2000).

4.2.7 Colour determination

Surface CIE colour values (*L*a*b**) were taken at the centre of each biscuit using a Minolta Chroma Meter CR-410 (Konica Minolta, Chiyoda, Tokyo, Japan) (Sozer, Cicerelli, Heiniö, & Poutanen, 2014). The browning index was calculated using following equation:

$$Browning\ index = 100\ (x - 0.31)]/0.17$$

Where, x is $(a* + 1.75L*)/(5.645L* + a* - 0.3012b*)$ according to Ruangchakpet and Sajjaanantakul (2007). Six replicates were performed for each sample in physical analysis.

4.2.8 Extraction and determination of antioxidant compounds and antioxidant activities

All solutions were extracted according to Jang and Xu (2009) and kept at $-20°C$ until further analysis. Samples (1 g) were added to 10mL methanol acidified with 1.2M HCl (50:50, v/v) before vortexing for 30 s. Then, they were incubated in a water bath at 60°C for 1.5 h. The extracts were centrifuged at 4500 g for 10 min. The total phenolic concentration of both the free and bound fractions was analysed using the Folin–Ciocalteu assay as described in Kaneda, Kubo, & Sakurai (2006). Total anthocyanin concentration was determined using the method previously described by Hosseinian *et al.* (2008). Total flavonoid concentration in both the free and bound fractions was determined using the protocol described by Ruangchakpet and Sajjaanantakul (2007). The determinations were carried out in triplicate.

For ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], radical scavenging activity of the free and bound fractions was determined according to Re *et al.* (1999). The DPPH radical scavenging capacity (DPPH, 2,2-diphenyl-2-picrylhydrazyl) was determined according to the method of Mahakunakorn, Tohda, Murakami, Matsumoto, & Watanabe (2004). All determinations were carried out in triplicate.

4.2.9 Consumer acceptance test

Ninety panellists participated in this study. The panellists (19–60 years old) were students and staff members from the University campus. The study was approved by the Lincoln University Human Ethics Committee (number 2017-24). The panellists were asked to sign a standard consent form before evaluating the samples. The participants quantified their preferences using a seven point hedonic scale (1 = dislike very much, 4 = neither like nor dislike, 7 = like very much) (Chandra *et al.,* 2015). The samples were served one day after baking. The evaluations were carried out under standard conditions.

4.2.10 Statistical analysis

Experimental data in this work were statistically analysed by one-way ANOVA using a complete randomised design for all physicochemical experiments and a randomised complete block design for the sensory evaluation data. The mean values were analysed by Duncan's multiple range test for a multicomparison of means. The level of significance was assigned at $P < 0.05$. All statistical analyses were performed using the SPSS Statistics (v. 22.0, SPSS Inc., Chicago, IL, USA).

4.3   Results and Discussion

4.3.1   Basic characteristics of biscuits

The nutritional compositions of biscuits are shown in Table 4.2. The moisture content of biscuits increased linearly from 3.57 to 7.36% ($P < 0.05$) with the increased concentration of purple rice flour in the biscuit mix. Therefore, biscuits prepared from 100% purple rice flour had the highest moisture content due to the high fibre content and swelling power of purple rice flour. Water content in food products affects the texture and consumers' acceptability of the final quality of the biscuits (Sharma *et al.,* 2016). The level of moisture was within the range of 10% moisture contents for baked products (cake, biscuits, and bread) to prevent spoilage and extend shelf-life (Okaka & Potter, 1977).

Crude protein contents significantly ranged from 5.82 (100% purple rice biscuits) to 7.35% (the control biscuits). Torbica *et al.* (2012) also observed a decrease in the protein contents with corresponding increase in the proportion of rice flour substituted for wheat flour in the biscuits. Protein and fat contents differed due to the rice cultivars and biscuit formulations (Torbica *et al.,* 2012; Uthumporn, Woo, Tajul, & Fazilah, 2015). Starch and protein contents are the major compositions in food products, affecting physicochemical characteristics, and play an important role in the final quality of the biscuits (Sharma *et al.,* 2016). As far as only few studies focus on the *in vitro* protein digestibility of purple rice, no literature is also available on the *in vitro* digestibility of purple rice flour biscuits. However, this study found that the protein digestibility of the biscuits showed a significantly increased trend for biscuits made from wheat-purple rice flour blends compared to the control biscuit. The *in vitro* protein digestibility of the control biscuit was 41.13%, while that of the wheat-purple rice flour blend biscuits ranged from 60.15 to 79.65% (Table 4.2). Although substituting purple rice flour in the biscuits mix showed

a decrease of crude protein content by 26.29%, the protein digestibility increased 53.64% when compared to the control biscuits. The suppression effect of protein digestibility of gluten proteins after baking is a consequence of protein denaturation, structure, conformation, and degree of hydrolysis (Zhang, Liu, Ying, Sanguansri, & Augustin, 2017). The fibre contents of the biscuits depend on the flour composition. Purple rice flour substitutions significantly increased fibre ($P < 0.05$) compared to the control biscuit with no significant difference in fat contents. Yawadio *et al.* (2007) also reported that dietary fibres in pigmented rice are much more overwhelming than those in white rice. Fat content is another principal ingredient of biscuits which has an influence on texture characteristics (Giarnetti *et al.,* 2015). All biscuits in this study contained a high fat content (mean 28.7%) from butter, which is a natural fat to use as it gives a good flavour after baking (Giarnetti *et al.,* 2015). However, it is rich in saturated fatty acids. Currently, consumers' awareness of low-fat products is increasing. It is possible to replace, at least partly, butter with healthier lipids such as extra virgin olive oil (Giarnetti *et al.,* 2015).

The starch content of purple rice blend flour biscuits ranged from 30.89 (75% purple rice flour substitution) to 32.35% (25% purple rice flour substitution) which was found to be statistically significant ($P < 0.05$). The control biscuit was reported 33.91% which is higher than blend flour biscuits (Table 4.2). The effects of purple rice flour on the *in vitro* starch digestion in biscuits were investigated by measuring the released reducing sugar contents degraded from starch by a digestive enzyme hydrolysis for 180 min. Area under the curves (AUCs) of glucose released from starch digestion were determined after each sample digestion because it easily explains the glycaemic response of the tested foods compared to the standard amount of white bread (An, Bae, Han, Lee, & Lee, 2016). The control biscuits had the highest AUC content, followed by substitution with 25% purple rice flour, 50% purple rice flour, 75% purple rice flour, and 100% purple rice flour, respectively, due to the fibres and proteins in purple rice acting as a barrier towards starch digestibility which can cause slower and lower increase of the glucose in the *in vitro* method (Sozer *et al.,* 2014). The 100% purple rice biscuit showed the lowest starch digestions according to the low starch hydrolysis determined. A 57.99% reduction was observed in starch digestibility when 100% purple rice flour was replaced with the wheat flour in the biscuit mixtures. Similar to the observation of An *et al.* (2016), the low starch digestibility is influenced by the protein bodies that can restrict the starch granule swelling and starch gelatinisation.

The pGI values of the biscuits were calculated based on the correlation equations between HI and estimated GI derived from the starch digestion of each biscuit. The pGI values of the biscuits were investigated depending on the level of purple rice flour substitutions (Table 4.2). The calculated pGI values of the biscuits decreased from 72.8 units down to 60.64 as the level of purple rice flour was increased in the biscuit mix (Table 4.2). The trend of pGI of wheat-black rice blend flours was similar to the study of An *et al.* (2016). The biscuit compositions have been changed due to the purple rice flour which might influence the moisture retention during baking. Thus, 100% purple rice flour gives the lowest digested starch released using the *in vitro* starch digestibility. The reduction effects on starch digestibility of substituted purple rice flour in wheat biscuits are based on the different starch fractions as well as the other nutrient contents such as fat, fibre, protein, and the phenolic acids (An *et al.,* 2016). As stated previously, the purple rice flour biscuits which are rich in dietary fibre and amylopectin content (An *et al.,* 2016) had lower amounts of glucose released over time compared to wheat biscuits (Frei, Siddhuraju, & Becker, 2003). The similar result was reported by An *et al.* (2016) that pGI values of black rice flour were significantly lower than those of wheat flour in *in vitro* starch digestion. In contrast, the biscuits with low moisture content had lower pGI since the gelatinisation would be restricted if there is not enough water during heating regardless of the amylose content (Frei *et al.,* 2003). Therefore, substituted biscuits with purple rice flour can be estimated as an intermediate GI product (60.64–69.76), which is lower than wheat biscuits (GI = 72.78) (Miller, 1994).

Table 4.2   Basic characteristics of the biscuits containing different levels of purple rice flours.

Nutritional properties	Control wheat biscuit	Purple rice flour substitution (%)			
		25	50	75	100
Moisture content (%)	3.57 ± 0.06^e	4.60 ± 0.12^d	5.64 ± 0.08^c	6.33 ± 0.14^b	7.36 ± 0.13^a
Crude protein (%)	7.35 ± 0.04^a	6.62 ± 0.03^b	6.61 ± 0.02^b	6.13 ± 0.04^c	5.82 ± 0.01^d
Protein digestibility (%)	41.13 ± 0.53^e	60.15 ± 0.29^d	65.36 ± 0.26^c	74.05 ± 0.04^b	79.65 ± 1.01^a
Total dietary fibre (%)	2.01 ± 0.09^d	3.06 ± 0.10^c	3.26 ± 0.04^c	4.40 ± 0.05^b	5.53 ± 0.02^a
Total fat (%)	28.43 ± 0.14^{NS}	28.59 ± 0.07	28.36 ± 0.20	28.69 ± 0.62	29.22 ± 0.46
Ash (%)	1.69 ± 0.02^d	1.86 ± 0.01^c	2.16 ± 0.02^b	2.26 ± 0.01^a	2.32 ± 0.04^a
Total carbohydrate (%)	59.22 ± 0.14^a	58.83 ± 0.12^{ab}	57.86 ± 0.29^b	56.11 ± 0.54^c	54.38 ± 0.47^d
Total starch (%)	33.91 ± 0.15^a	32.35 ± 0.11^b	31.62 ± 0.34^{bc}	30.89 ± 0.19^c	30.36 ± 0.83^c
AUC of digested starch (mg min dL^{-1})	668.69 ± 0.95^a	607.66 ± 0.56^b	559.38 ± 0.43^c	487.23 ± 1.32^d	423.25 ± 1.43^e
pGI	72.78 ± 0.05^a	69.76 ± 0.03^b	67.38 ± 0.02^c	63.81 ± 0.07^d	60.64 ± 0.07^e

Values represent mean ± standard error; In each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$); NS = not significant difference. AUC = area under the curve; pGI = predicted glycaemic index.

4.3.2 Physical properties

The mean values of physical properties of control biscuits and blend flour biscuits are shown in Table 4.3. The textural characteristic of the biscuits is the important factor contributing to the quality of biscuits. Hardness is one of the key factors, which determine the texture properties of biscuits, and is measured as the peak force needed to penetrate the biscuits. It was significantly decreased ($P < 0.05$) when different levels of purple rice flour were incorporated in the biscuit mix. Purple rice flour biscuits required significantly lower force to snap than the control biscuits. However, the hardness of biscuits was found within the range of the commercial hardness of a biscuit substituted with rice flour (Chung *et al.,* 2014). The hardness of blended biscuits is normally lower than the hardness of wheat flour biscuit due to the degradation of macromolecules and low bulk density of flour blends (Sharma *et al.,* 2016). Moreover, high dietary fibre in the wheat-purple rice blended flour increases the viscosity of biscuits with delaying the accessibility of starch granules to digestive enzymes in the human digestive system.

The addition of purple rice flour in the biscuit formulation changed the width, thickness, and spread ratio due to the protein contents (see Appendix C). The thickness of the blend flour biscuits decreased as the protein content decreased in the formula. The 100% purple rice flour biscuit had the highest spread ratio (greater width and thinner), which is considered to be a positive characteristic of biscuits because the spread ratio is one of the physical parameters that influence the consumer liking scores (Sharma *et al.,* 2016). A higher spread ratio is thought to improve the acceptability of biscuits. In general, the highest spread ratio was obtained when no gluten was added to the biscuit recipe (Sharma *et al.,* 2016). Giuberti *et al.* (2017) reported that a higher level of fibre in the biscuit formulation made from modified sticky rice flour absorbed more water; therefore, the biscuit mix has less water left in the mixture which leads to less spread ratio. Chung *et al.* (2014) also reported that biscuits made from different types of rice flour could alter the hardness of the biscuits.

Table 4.3   Physical and textural characteristics of the biscuits containing different levels of purple rice flours.

Physical characteristics	Control wheat biscuit	Purple rice flour substitution (%)			
		25	50	75	100
Hardness (N)	71.21 ± 0.47^a	52.81 ± 0.69^b	28.52 ± 0.18^c	22.18 ± 0.10^d	15.69 ± 0.14^e
Width (W, mm)	55.17 ± 0.31^d	57.67 ± 0.21^c	59.67 ± 0.21^b	60.50 ± 0.22^{ab}	61.17 ± 0.48^a
Thickness (T, mm)	12.00 ± 0.26^a	10.50 ± 0.22^b	8.67 ± 0.21^c	8.00 ± 0.37^{cd}	7.50 ± 0.22^d
Spread ratio (W/T)	4.61 ± 0.12^d	5.50 ± 0.12^c	6.91 ± 0.19^b	7.64 ± 0.35^a	8.20 ± 0.30^a

Values represent mean $\pm$ standard error. For each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$).

4.3.3 Colour characteristics

Colour is one of the most important factors for the consumer's selection and purchase of baked products (Chandra *et al.,* 2015) including biscuits. The change in colour value of the biscuits is presented in terms of $L*$, $a*$, and $b*$ in Table 4.4. The colour of biscuits develops further during the later stage of baking with increasing temperature and baking time (Sharma *et al.,* 2016). The control biscuit showed the highest $L*$ value (74.53) compared to the mean $L*$ value of the substituted biscuits (46.38). The substitutions of purple rice flour in the formulation darkened the surface of the biscuits compared to the control biscuits which had much lower browning index values. The dark colour of biscuits generates from the caramelisation of the sugar in the recipe or a Maillard reaction during baking at high temperature (Sharma *et al.,* 2016). Thus, the control biscuits contained the highest protein content (Table 4.2), the colour values revealed a contrast with Ruangchakpet and Sajjaanantakul (2007) who reported that more protein incorporation resulted in a darker surface of biscuits. In purple rice flour substitution biscuits, besides the protein level, indications also suggested that purple rice flour is deep purple in colour since the naturally high antioxidant contents (Yawadio *et al.,* 2007) can contribute to the colour changes of the biscuits.

The redness ($a*$) also differed among the biscuits since purple rice biscuits are characterized by much darker colour with lower protein content when compared to the control biscuits. Redness (positive $a*$ value) of the biscuits shows an increasing trend from the lowest value (3.84) observed in the control biscuits to the highest value (6.08) found in

100% purple rice flour biscuits. The increase in redness of purple rice flour containing in the biscuits could be attributed to the presence of purple pigments in husk of purple rice. According to Shao *et al.* (2018), phenolic acids and anthocyanins exhibited the antioxidant activities in both DPPH and ABTS assays. The studies on the antioxidants of the biscuits are assured to investigate the radical scavenging ability, which might provide further details in this study. The *b** values indicated the yellowness of all biscuits which were significantly different ($P < 0.05$) with increasing level of purple rice flour substitutions. The control biscuit has greater *b** value than the substituted biscuits. The yellowness of purple rice flour substitution biscuits decreases due to the degradation of unstable yellow compounds of purple rice flour during baking. Moreover, *a** decrease in the *b** value of the substituted biscuits can also be attributed to the lower *b** value (2.16) of 100% purple rice flour than 100% wheat flour (13.08) (Klunklin & Savage, 2018). Hence, the reduction of yellowness of purple rice flour substitution biscuits occurs. The browning index of biscuits is also caused by Maillard reactions and caramelisation of sugar during baking process between reducing sugar and amino acids that can increase the antioxidant properties of the baked products (Chandra *et al.,* 2015). The lightness of biscuits is related not only to the Maillard reactions during baking, but also to the colour of the flour used which can affect the browning index of the biscuits. Wheat flour contains more protein than substituted purple rice flour, and the amount of the melanoidins formed in the blend flour biscuits was significantly greater than those in the control biscuits (Chandra *et al.,* 2015).

Table 4.4   *L*a*b* colour values of the biscuits containing different levels of purple rice flours.*

Physical characteristics	Control wheat biscuit	Purple rice flour substitution (%)			
		25	50	75	100
Colour values *L**	74.53 ± 0.57^a	60.45 ± 0.10^b	45.33 ± 0.06^c	40.85 ± 0.15^d	38.88 ± 0.37^e
a*	3.84 ± 0.08^d	3.98 ± 0.03^{bc}	4.07 ± 0.02^{bc}	4.39 ± 0.04^b	6.08 ± 0.36^a
b*	38.65 ± 0.32^a	12.92 ± 0.14^b	9.15 ± 0.24^c	5.33 ± 0.03^d	3.94 ± 0.10^e
Browning index	76.36 ± 1.02^a	52.18 ± 0.27^b	28.38 ± 0.79^c	20.97 ± 0.14^d	17.87 ± 0.31^e

Values represent mean ± standard error. For each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$).

4.3.4 Antioxidant compounds and antioxidant activities

The overall results of the antioxidant compounds and antioxidant activities of the biscuits obtained from different levels of purple rice flour substitution are shown in Table 4.5. The blend flour biscuits significantly increased ($P < 0.05$) all antioxidant compounds (total phenolics, anthocyanins, and flavonoid content) compared to the control biscuits. Total phenolic acids (including free, soluble conjugated, and insoluble bound forms) are found in high level existed in the whole pigmented cereal grains which have gallic acid (GAE) as a major phenolic acid (Loypimai, Moongngarm, & Chottanom, 2016). Jang and Xu (2009) and Loypimai *et al.* (2009) suggested that purple rice is a potential source of phenolic compounds due to the dark-purple pigment of the anthocyanins. The major anthocyanins of purple rice analysed in this study were cyanidin-3-O-glucoside (C3G), which was reported to have an antidiabetic effect in type 2 diabetes of mice (Hosseinian *et al*., 2008). In case of purple rice flour, it contains high levels of C3G. The 100% purple rice biscuits had significantly higher ($P < 0.05$) anthocyanin and flavonoid contents, followed by 75%, 50%, and 25% purple rice substitution biscuits, respectively. Thus, purple rice flour was found to suppress the glucose released from starch digestion (Table 4.2) based on the inhibition of starch digestive enzymes by anthocyanins such as C3G. Shao *et al.* (2018) also reported that black rice from different cultivars had C3G which ranged from 12.03 to 1106.00 mg/kg DW. The total flavonoid contents of the control biscuit (0.15 mg catechin equivalence (CE)/100 g) were significantly lower ($P < 0.05$) than those of the substituted biscuits (1.32 mg CE/100 g). Even though the anthocyanin and flavonoid stability of purple rice is affected because of thermal processing, the retention rate of antioxidant compounds is still stable in the biscuits. This study suggested that the substitution of purple rice flour for the wheat flour could be another option to retain bioactive compounds in bakery products such as biscuits, with slightly altered texture characteristics.

The accepted methods used for analysing antioxidant activities are the ABTS and DPPH radicals based on measuring colour degradation (Loypimai *et al.*, 2009). The DPPH assay is normally analysed aqueous or organic extracts having hydrophilic and lipophilic compounds. On the contrary, the ABTS method commonly evaluates the antioxidant activity of hydrophilic compounds in the extract (Loypimai *et al.*, 2009). Table 4.5 shows

increases in antioxidant activities of the biscuits with increasing substitution in wheat flour. The DPPH radical scavenging activities and radical cation ABTS scavenging activity ranged from 1.07 to 49.19 µ mol trolox/g DW and from 5.49 to 96.95 µ mol trolox/g DW, respectively. Compared to the control biscuits, the incorporation of purple rice flour showed a significant ($P < 0.05$) increase in the antioxidant activities (DPPH and ABTS assays) of purple rice substitution biscuits (even at the lowest substitution level, 25% purple rice flour substitution). These results were similar to the report of Shao *et al.* (2018). Chung *et al.* (2014) reported that increase in antioxidant activities might be attributed to the formation of browning colour due to the Maillard reaction during baking process which is in contrast with this study since the results of antioxidant activities are also linked with the concentration of phenolic compounds, anthocyanin, and flavonoids that have antioxidant properties (Loypimai *et al.*, 2016). Many studies also reported a high correlation between DPPH radical scavenging activities and antioxidant contents (Loypimai *et al.*, 2016; Klunklin & Savage, 2018; Shao *et al.*, 2018).

Table 4.5   Bioactive compounds contents of the biscuits containing different levels of purple rice flours.

Bioactive compounds	Control wheat biscuit	Purple rice flour substitution (%)			
		25	50	75	100
Total phenolics (μg GAE/100 g DW)	6.51 ± 0.04^e	812.51 ± 0.60^d	2053.81 ± 21.60^c	2319.92 ± 2.89^b	3012.27 ± 33.73^a
Anthocyanins (C3G, mg/kg DW)	0.28 ± 0.05^e	9.35 ± 0.19^d	15.92 ± 0.49^c	37.44 ± 1.43^b	51.49 ± 0.73^a
Flavonoid contents (mg CE/100 g DW)	0.15 ± 0.01^e	0.57 ± 0.02^d	0.92 ± 0.01^c	1.13 ± 0.01^b	1.32 ± 0.01^a
DPPH (μmol trolox/g DW)	1.07 ± 0.05^e	15.63 ± 0.14^d	33.55 ± 0.12^c	39.61 ± 0.12^b	49.19 ± 0.08^a
ABTS (μmol trolox/g DW)	5.49 ± 0.01^e	14.00 ± 0.02^d	36.73 ± 0.01^c	60.45 ± 0.28^b	96.95 ± 0.22^a

Each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$); Values represent mean ± standard error. GAE, gallic acid equivalents; C3G, cyanidin-3-O-glucoside; CE, catechin equivalents; DPPH, 2,2-diphenyl-2-picrylhydrazyl radical scavenging activity; ABTS, [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging activity.

4.3.5 Sensory attributes

The mean liking scores for all attributes of the biscuits for the different levels of purple rice flour substitution in wheat flour are shown in Table 4.6; these scores were neutral or moderately acceptable (scores 4–6). The overall acceptability of blended flour biscuits was slightly different ($P < 0.05$) compared to the control biscuits. Colour, oiliness, and sweetness of the substituted flour biscuits up to 75% showed nonsignificant variation ($P > 0.05$) compared to the control biscuits, which consumers liked a lot. The preferred biscuits were enriched with purple rice flour up to 50% which had significantly different colour parameters (Table 4.4) from the control biscuit. However, overall consumers appear to prefer lighter coloured biscuits (Chandra *et al.,* 2015). In the present study, the term crunchiness was used to describe the consumer preference, which refers to the hardness characteristic of the biscuits. The mean scores of texture and crunchiness of 25% and 50% purple rice flour substitutions decreased from the scores of the control biscuits around 8%. The relationship of physical characteristics and sensory evaluation was presented in this study. As the substitution levels increased from 25% to 100%, the biscuits tended to be much softer. These findings are in agreement with the results indicated in Table 4.3, wherein the hardness of the biscuits showed a decrease with increase in the purple rice flour incorporation levels. The flavour scores of blended flour biscuits significantly decreased ($P < 0.05$) along with increase in the purple rice flour substitution levels. Few studies have observed the influence of rice flour substitution on the sensory acceptance of biscuits. Mancebo, Rodriguez, & Gomez (2016) prepared biscuits from different ratios of white rice flour mixed with protein and showed good overall acceptability scores. The purple rice flour had a dark colour, due to its high antioxidant content, which might cause the reduction of sensory liking scores shown for colour, overall appearance, and texture of the substituted biscuits (Sharma *et al.,* 2016). The mean score of overall acceptability decreased gradually from 5.54 to 3.90 as the purple rice flour incorporation levels were increased beyond 50% in the biscuit mixes. This indicates that at substitution levels of more than 50%, the preference on the biscuits in terms of overall acceptability was significantly reduced. In the case of all sensory attributes of 25% and 50% purple rice substitution biscuits, they scored similar values to the control biscuits with higher antioxidants and gave the best overall responses to the physical changes. However, the liking score values for the 75% and 100% purple rice biscuits showed significantly decreasing values for all attributes when compared to the control wheat biscuits, but the biscuits contained higher levels of antioxidants.

Table 4.6   Mean liking scores of sensory evaluation of the biscuits containing different levels of purple rice flours.

Attributes	Control wheat biscuit	Purple rice flour substitution (%)			
		25	50	75	100
Colour	6.09 ± 0.07^a	6.00 ± 0.07^a	5.83 ± 0.08^{ab}	5.71 ± 0.09^b	5.02 ± 0.13^c
Overall appearance	6.26 ± 0.07^a	5.86 ± 0.07^b	5.84 ± 0.08^b	5.78 ± 0.07^b	5.67 ± 0.08^b
Touch/Texture	6.09 ± 0.07^a	5.68 ± 0.08^b	5.58 ± 0.06^b	4.98 ± 0.11^c	4.28 ± 0.12^d
Touch/Oiliness	5.87 ± 0.07^a	5.83 ± 0.08^a	5.67 ± 0.08^{ab}	5.69 ± 0.08^{ab}	5.56 ± 0.08^b
Sweetness	6.26 ± 0.06^a	6.13 ± 0.06^a	6.26 ± 0.07^a	6.12 ± 0.07^a	5.90 ± 0.08^b
Crunchiness	5.73 ± 0.08^a	5.40 ± 0.08^b	5.24 ± 0.07^b	4.62 ± 0.08^c	4.29 ± 0.08^d
Flavour	6.41 ± 0.06^a	6.10 ± 0.09^b	5.74 ± 0.08^c	5.17 ± 0.08^d	4.71 ± 0.11^e
Overall acceptability	6.14 ± 0.07^a	5.67 ± 0.08^b	5.54 ± 0.08^b	4.47 ± 0.11^c	3.90 ± 0.12^d

Each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$); Data are expressed as mean $\pm$ standard error.

4.4 Conclusions

This study revealed that the purple rice flour could be an acceptable partial wheat substitute to produce biscuits with a low starch digestibility, low pGI with high protein digestibility, and natural antioxidant contents. Inclusion of increasing purple rice flour led to a 54% increase in the digestible protein while at the same time reducing the rate of starch digestion by 58%. Considering that biscuits made with wheat flour have low dietary fibre and protein contents, it seems that maximizing protein digestibility and starch digestibility from the purple rice flour substitution is desirable to increase the nutritional properties of the biscuits. Further research would be needed to improve the formulation regarding the fat content in order to produce a lower fat containing biscuit. Although the physical characteristics of the biscuits were significantly changed, only small negative effects were observed during sensory evaluation up to 50% of purple rice flour substitution compared to the control biscuits. Thus, the present finding suggests that value-added biscuits could be produced by supplementing purple rice flour up to 50%. This could be recommended as suitable food for people with diabetes who need to consume a lower digestibility starch-containing food, which assumed that a lower fat content would be achieved.

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Chapter 5

Physicochemical properties and sensory evaluation of wheat-purple rice biscuits enriched with defatted green-lipped mussel powder (*Perna canaluculus*) and spices

Abstract

Biscuits are one of the most consumed bakery products eaten by everyone. Purple rice contains much higher levels of antioxidants, vitamins and minerals such as iron and zinc compared to wheat. The aim of this work was to produce a protein-rich biscuit made from purple rice flour and defatted green-lipped mussel powder (*Perna canaliculus*) (0-20%) blended with ginger and galangal spices at 4% for each spice. The objective was to produce an inexpensive, balanced, healthy snack product containing increased levels of protein and antioxidants from the defatted mussel powder and to investigate the consumer preferences of these biscuits using four different ethnic groups (Caucasian, Chinese, Pacific Islanders and Thai) living in New Zealand. The addition of the defatted mussel powder increased the crude protein content by 43% and the protein digestibility by 21% at the highest level of inclusion. The addition of defatted mussel powder significantly ($P < 0.05$) increased the hardness of biscuits while making small increases in the browning index of the cooked biscuit. The phenolic contents and antioxidant activities (DPPH and ABTS) were significantly ($P < 0.05$) increased as additional amounts of defatted mussel powder were incorporated into the biscuit mix, resulting in a reduction in the total starch contents. The addition 10% defatted mussel powder to the control biscuit mix was accepted by all the ethnic groups. Overall, the Pacific Islanders showed a higher appreciation for all the attributes tested.

5.1   Introduction

Biscuits are a ubiquitous snack food that many people are unable to resist eating because they are readily available, are bite-sized, affordable, and have a long shelf-life. As a result, biscuits are highly favoured bakery items (Caleja, Barros, Antonio, Oliveira, & Ferreira, 2017). They cannot, however, be regarded as a healthy snack food because they usually contain high levels of easily digested carbohydrates and fats, generally low levels of fibre and only modest levels of protein as they are usually made from flour, butter and sugar (Park, Choi, & Kim, 2015).

Recent trends suggest that people are aware of the food they consume and they are also aware of benefits of consuming nutritious biscuits (Yeh *et al*., 1998).

The development of a high protein containing biscuit is a worthwhile challenge when considering the overall nutritional status of underprivileged sections of the population. In considering the development of a new product, it is important to use locally-sourced ingredients and spices whose tastes are appreciated by each ethnic group the products are intended for (Yeh *et al*., 1998). Commercial green-lipped mussels (*Perna canaliculus*) are popular as raw materials and dietary supplements because they contain high protein levels, omega-3 fatty acids, iodine and carbohydrates (Grienke, Silke, & Tasdemir, 2014). In addition, mussels contain proteins, peptides and amino acids which are bioactive compounds. These have different bioactivities such as antimicrobial mussel peptides or anti-inflammatory, anti-arthritic and anti-cancer agents (Grienke *et al.,* 2014; Cobb & Ernst, 2006). There has been little research on the use of mussel powders in food products. The manufacture of gluten free pasta mixed with different levels of green-lipped mussel powder has been studied by Vijaykrishnaraj, Kumar, & Prabhasankar (2015) and, later by Vijaykrishnaraj, Roopa, & Prabhasankar (2016) who made gluten-free bread enriched with different levels of green mussel protein hydrolysates.

Defatted green-lipped mussel powder is a by-product following the extraction of most of the oil fraction. It still contains some fat, but much less than in the original powder. However, the addition of mussel powder to various food products has a number of problems associated with the processing and shelf-life of the final products (Zhang *et al.,* 2013).

Researchers have recently become interested in functional foods containing antioxidants due to the health-promoting activities of these compounds (Pasqualone *et al*., 2015). Antioxidants are mostly used to preserve food products by extending their shelf-life (Caleja *et al.,* 2017). Purple rice (*Oryza sativa* L.) is reported to contain higher levels of antioxidants than white rice (Yawadio, Tanimori, & Morita, 2007). Purple rice is an economically important rice species so it is preferred for the preparation of snacks and desserts. In addition, it has many nutritional benefits as it contains more protein, vitamins and minerals than white rice (Yawadio *et al.,* 2007).

Spices have been used in Asian cooking for thousands of years. Traditionally, spices, such as ginger, garlic, clove and turmeric, were added to foods to reduce the level of microorganisms but it is now becoming clear that these spices also have important antioxidant properties and can slow down colour degradation in cooked products. Spices can extend the shelf-life by slowing lipid oxidation (Uhl, 2000). Ginger (*Zingiber officinale* Roscoe) and its derivatives

contain major pungent and active compounds that have a high antioxidant activity (DPPH assay) (Stoilova, Krastanov, Stoyanova, Denev, & Gargova, 2007). Galangal (*Alpinia galanga*), a member of the ginger family, is an aromatic spice that is widely used in South East Asia (Uhl, 2000). One of the most interesting properties of galangal is its effect on lipid oxidation in fish muscle systems (Buaniaw, Siripongvutikorn, & Thongraung, 2010). The benefits from biscuits on human health depends on their ingredients (Vitali, Dragojević, & Šebečić, 2009). In spite of the benefits from spices, the impact on health of people consuming high amounts of wheat-purple rice flour mixed with defatted mussel powder remains unstudied and requires clarification.

The aim of this study was to investigate formulations for a series of biscuits using novel ingredients, including purple rice flour, defatted mussel powder, and spices (ginger and galangal). The aim was to produce a balanced, low-cost, and healthy snack product. Considering the potential health benefits of purple rice and defatted mussel powder and the increasing consumption of healthy food, the objective of the present study was to prepare nutritious biscuits to deliver a nutritious and healthy product. It is important, however, to understand the response of different ethnic groups to this new product.

5.2 Materials and Methods

5.2.1 Raw materials

The ingredients used were: plain wheat flour (Pams Products Ltd., NZ), purple rice distributed by the Big T. supermarket in New Zealand, butter (Dairyworks, NZ), castor sugar (Pams Products Ltd., NZ), baking powder (Edmonds Limited, NZ), salt (Pams Products Ltd., NZ), whole egg (Pams Products Ltd., NZ), vanilla extract (Pams Products Ltd., NZ), xanthan gum (Lotus foods Pty Ltd., Australia), honey (Airborne honey limited, NZ), ginger (*Zingiber officinale*) and galangal (*Alpinia galanga*) powders, imported by Sunson gifts and the Asian food market, and defatted green-lipped mussel powder (*Perna canaliculus*) (containing 61.50% protein, 16.70% carbohydrate, 8.89% fat and 8.46% ash) was provided by Aroma New Zealand Limited, Christchurch, NZ. The experimental biscuits were prepared by the addition of defatted green-lipped mussel powder at levels of 0%, 10%, 15% and 20% of total flour weight.

5.2.2 Preparation of the biscuits

The control biscuits were made from a 50:50 blends of wheat flour and purple rice flour milled using a Whisper Mill (Grote Molen Inc., USA). The ingredients are shown in Table 5.1.

Defatted mussel powder was blended with mixed flour at 0-20% based on the dry weight of the flour. The biscuit dough was rolled out to a thickness of 10 mm, using a rolling pin, and then cut into rounds using a 5 cm diameter biscuit cutter. The cut biscuits were baked at 170°C for 8 min and cooled at room temperature for 20 min. The biscuits were stored in sealed plastic bags at -20°C until further analysis could commence. Physical analysis was carried out on the freshly baked biscuits while chemical analysis was carried out on the ground samples.

Table 5.1   Formulation of control and fortified biscuits.

Ingredients (g)	Control	Fortified biscuit
Refined wheat flour	100	100
Purple rice flour	100	100
Defatted mussel powder	-	Varied from 10-20%
Butter	150	150
Sugar	125	125
Egg	225	225
Salt	1.5	1.5
Galangal	-	8
Ginger	-	8
sodium bicarbonate	9	9
Xanthan gum	2.5	2.5

5.2.3 Proximate analysis

Proximate analysis, including crude protein (%N x 6.25) using the Dumas method by rapid Max N exceed® (Elementar, Hanau, Germany), crude fat and ash, were determined using AOAC methods 920.177, 920.176 and 900.029, respectively (AOAC, 2000). The moisture content was determined using a drying oven at 105°C for 18 h. The total starch content was determined using the Megazyme starch assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) approved method 76-13 (Reed, Ai, Leutcher, & Jane, 2013). Total dietary fibre (TDF) was determined using a total dietary fibre assay kit (Sigma-Aldrich, MO, USA). All proximate composition measurements were conducted in triplicate.

5.2.4 *In vitro* protein digestibility

In vitro protein digestibility of the biscuits was assessed according to the method of Akeson and Stahmann (1964). The protein digestion method used a two-step hydrolysis to stimulate continuous gastric and pancreatic digestion in humans using pepsin from porcine gastric mucosa (Sigma Aldrich, USA, 66 units/mg protein) and pancreatin from a porcine pancreas (AppliChem Chemica Synthesis, Germany, 30.315 units/mg protein). The solutions were then centrifuged at 1600 g for 10 min. The supernatants were collected to analyse for total nitrogen

using an Elementar Vario TOC cube instrument (Hanau, Germany) fitted with a chemiluminescence detector for determining total bound nitrogen (TNb). The crude protein contents were calculated (%N x 6.25). The analyses were performed in triplicate.

5.2.5 *In vitro* starch digestibility

The digestion involved a simulated gastric digestion followed by a small intestine digestion using the method from Monro, Mishra, & Venn (2010). Briefly, a 10% pepsin (porcine, Sigma, P 7000; 800–2,500 U/mL) solution in 0.05 M HCl was added to the sample solutions to digest for 30 min to accomplish gastric digestion. Starch digestion was then commenced with the addition of 5 mL of 2.5% pancreatin (EC: 232-468-9, activity: 30315 FIP-U/g, Applichem GmbH, Darmstadt, Germany) in 0.1 M sodium maleate buffer (pH 6). Triplicate samples were taken for each time interval (0, 20, 60 and 120 min) after adding the pancreatin solution. The digested samples were then centrifuged at 180 g for 5 min. The absorbance of the supernatant was read at 530 nm (V-1200 spectrophotometer, Global Science, Auckland, New Zealand).

5.2.6 Physical characteristics

The textural properties of the biscuits were measured using a texture analyser (TA-XT2i, Stable Micro System, Godalming, UK) equipped with 30 kg load cell. The hardness of the freshly baked biscuits was measured by the cutting force, using a small three-point bending test rig with sharp-blade cutting probe (Park *et al.,* 2015). The first peak force was recorded as the hardness force in N. All measurements were repeated six times.

Biscuit quality was assessed by measuring the width, thickness and biscuit spread ratio (width/thickness) (AACC method 10-50.05) (AACC, 1967). This method is also useful in assessing the quality of the flour and other ingredients that can affect the biscuit shape (AACC method 10-50D) (AACC, 2000). All measurements were repeated six times.

A Minolta Chroma Meter CR-410 (Konica Minolta, Japan) was used to measure the colour of the rice flour samples. The colour was expressed using the *L*a*b** CIE system (Sozer, Cicerelli, Heiniö, & Poutanen, 2014). According to Ruangchakpet and Sajjaanantakul (2007), the browning index was calculated using the following equation:

$$\text{Browning index} = [100 (x - 0.31)]/0.17$$

Where, x is $(a* + 1.75L*)/(5.645L* + a* - 0.3012b*)$. All measurements were repeated six times.

5.2.7 Extraction and determination of samples for antioxidant analysis

Sample extraction was performed using a method adapted from Jang and Xu (2009). One gram of each sample was transferred into a test tube before adding 10 mL methanol acidified with 1.2 M HCl (50:50, v/v) then vortexing for 30 s. The tubes were capped and placed in a water bath at 60°C for 1.5 h. The test tubes were mixed by vortexing twice during the incubation then centrifuged at 4500 x g for 10 min. The extracts were kept at -20°C until further analysis could commence. The total phenolic content was analysed by a Folin-Ciocalteu assay adapted from Kaneda, Kubo, & Sakurai (2006). The method of Zhishen, Mengcheng, & Jianming (1999) was used to analyse total flavonoids contents. The anthocyanin content analysis was determined using method of Hosseinian, Li, & Beta (2008). The determinations were carried out in triplicate.

5.2.8 Antioxidant activities (DPPH and ABTS assay)

The DPPH (2,2-diphenyl-2-picrylhydrazyl) radical scavenging activity of the samples was tested according to the method of Mahakunakorn, Tohda, Murakami, Matsumoto, & Watanabe (2004). The ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging activity was performed using Re et al.'s (1999) method. All determinations were conducted in triplicate.

5.2.9 Sensory analysis

Students and staff members from the university campus were invited to participate in the full taste test. The sensory evaluation protocol was approved by the Lincoln University Human Ethics Committee (2017-24). Consumer focus groups used people from the four ethnic groups: Caucasian, Chinese, Pacific Island and Thai. There were between 36 and 37 semi-trained people in each ethnic group (in total, there were 60 males and 85 females ranging from 19 to 50 years).

The participants were asked to use a seven-point hedonic scale to rate their degree of liking (1 = dislike extremely; 4 = neither like nor dislike; 7 = like extremely) (Aminah & Tan, 2001). The samples were presented at room temperature in clear plastic cups labelled with three-digit random numbers. The order of the presentation of the samples was randomised. The samples were served one day after baking.

5.2.10 Statistical analysis

The data collected from all experimental sections were statistically analysed by one- or two-way ANOVA using a complete randomised design and using a randomised complete block design for sensory evaluation. The data means were analysed using Duncan's multiple range test for a multi comparison of means. A bivariate correlation matrix of data was analysed using Pearson's correlation coefficient. The statistical significance was declared at $P < 0.05$. Each of the experimental sections was carried out in triplicate. All statistical analyses were carried out by using the SPSS Statistics (v. 22.0; SPSS Inc., Chicago, IL, USA). The least significant differences (LSDs) were calculated to compare the means at the 95% level ($P < 0.05$) using Minitab (v. 17; Minitab Inc., State College, PA, USA).

5.3 Results and Discussion

5.3.1 Basic composition of the biscuits

Table 5.2 shows the proximate composition of the fortified biscuits containing different amounts of defatted mussel powder. The enriched biscuits contained increased qualities of protein and total dietary fibre (TDF) when compared to the control biscuits. The moisture contents of the biscuits ranged from 5.3–6.5%; 10% is suggested as the upper limit needed for the biscuits to prevent spoilage by microorganisms and to increase the shelf-life (Kure, Bahago, & Daniel, 1998). The moisture contents of all biscuits were below this level. The water released from the biscuit matrix was altered by the defatted mussel powder and spice supplementations due to their higher contents of saccharides in fortified biscuits than the control biscuits. Increasing the fibre contents resulted in higher water absorption capacities of the fortified biscuits (Cauvain & Young, 2009). Therefore, the final moisture content of the 20% defatted mussel powder biscuit had a significantly higher moisture content compared to the other biscuits. The acceptable moisture content in freshly baked biscuits is normally less than 5% (Cauvain & Young, 2009); however, the acceptance of the experimental biscuits was tested by different ethnic groups using a taste test.

The fortified biscuit with 20% defatted mussel powder had the highest crude protein (11.29%) content compared to the control (7.9%). The green-lipped mussel powder has a quantity of protein content around 49.5% (Vijaykrishnaraj *et al.,* 2015; Oliveira, Sykes, Hachero-Cruzado, Azeiteiro, & Esteves, 2015). Normally, biscuits have low protein content, in the region of 4.0% (Boobier, Baker, & Davies, 2006). Sozer *et al.* (2014) reported that wheat flour contains gluten protein, which contains approximately 14% protein. Moreover, Kaneda *et al.* (2006) stated that

protein contents of purple rice flour ranged between 9 and 13%, and this depends on the cultivars evaluated. Such an increase comes from the fact that wheat-purple rice flour that was used for baking as a control also contains some protein contents. Furthermore, the incorporation of mussel powder increased the protein content of these products to more than that found in most commercial products (Vijaykrishnaraj *et al.,* 2015).

The biscuits enriched with defatted mussel powder contained significantly higher amounts of digestible protein (80.9%) in comparison to the control sample (69.1%). The data obtained by Abdel-Aal (2008) and Vitali *et al.* (2009) showed that *in vitro* protein digestibility of wheat-based biscuits ranged from 44.3% to 68.9%, which was lower than that in this study. Vitali *et al.* (2009) stated that the baking process did not cause any rise in protein digestibility since there was no significant difference in the available protein between the dough and biscuit samples. In contrast, Sumczynski, Kotásková, Družbíková, & Mlček (2016) reported that the baking process could increase the protein digestibility by inactivating proteinase inhibitors. Lima *et al.* (2017) stated that pigmented rice such as purple rice has contributed to increased protein digestibility (75.33%). In addition, ginger and galangal have been recognized as functional ingredients, which can improve digestive capacity (Uhl, 2000). Biscuits containing mussel powder showed a direct increase in protein digestibility following increased additions of defatted mussel powder. Nørgaard, Petersen, Tørring, Jørgensena, & Lærke (2015) confirmed that the addition of mussel protein in the diet of pigs increased the overall digestibility of protein in the diet.

The fortified defatted mussel powder biscuits showed significantly ($P < 0.05$) higher fat and ash contents than the control biscuits. Short-dough biscuits commonly sold in shops normally contain high fat contents in the excess of 20.0% (Boobier *et al.,* 2006). Vijaykrishnaraj *et al.* (2015) reported that green-lipped mussel powder contained 2.7% ash. Ginger and galangal also contain 2.6% ash. The addition of defatted mussel powder and spices in the biscuits increased the ash content compared to the control biscuits. Following the incorporation of mussel powder, total starch content of the defatted mussel powder biscuits significantly decreased ($P < 0.05$) compared to the control biscuits.

Biscuit products primarily consist of starch from wheat-purple rice flour and some mussel polysaccharides; therefore, the determination of functionality of the starch is very important to understand. The predicted glycaemic response of each biscuit can be measured by determining the area under the curve (AUC) for the digested starch at 2 h (Table 5.2). The AUC of the control biscuit gave the highest response, while the biscuit enriched with 20% defatted mussel

powder gave the lowest value. An obvious reduction in the AUC of the fortified biscuits compared to the control indicated the lower release of carbohydrates in the enhanced mussel powder biscuits. The lower digestibility of biscuits incorporated with increasing levels of defatted mussel powder together with the inclusion of spices might be attributed to their higher dietary fibre (DF) and resistant starch contents because the DF can reduce the digestibility of starch granules (Ng, Robert, Ahmad, & Ishak, 2017). Moreover, polyphenols have been shown to be inhibitors of α-amylase as they reduced both the activity of the digestive enzymes and the *in vitro* starch digestibility (Thompson & Yoon, 1984).

The total dietary fibre (TDF) content of various varieties of purple rice and wheat varies between 3.0 and 15.5% and 2.0 and 14.0%, respectively (Sompong, Siebenhandl-Ehn, Linsberger-Martin, & Berghofer, 2011). Ginger and galangal also contain up to 11% TDF. Therefore, protein contents from wheat, purple rice, and spices are responsible for the increased level of TDF of the fortified biscuits. Foschia, Peressini, Sensidoni, & Brennan (2013) reported that the consumption of the cereal dietary fibre might reduce the risk of cardiovascular disease, intestinal regulation, high intestinal glucose level, and forms of colorectal cancer. Basically, increasing the level of the defatted mussel powder from 10 to 20% resulted in a significant ($P < 0.05$) increase in the level of protein, ash, and TDF, with the 20% defatted mussel powder biscuit having the highest nutritional values with low carbohydrate levels. Mussel adhesive proteins consisted of rod-like collagenous fibrils, which are of type 1 collagen that may affect the TDF content (Vijaykrishnaraj *et al.*, 2015). Therefore, all biscuits in this study containing TDF of 12.96–28.59% can be claimed to be "high fibre," according to Regulation (EC) no. 1924/2006, which requires "a claim that a food is high in fibre, and any claim likely to have the same meaning for the consumer may only be made where the product contains at least 6.0%" (Foschia *et al.,* 2013). Foschia *et al.* (2013) also reported that a dietary fibre intake among adults is around 18 g per day. The fibre contents of these biscuits showed that defatted mussel powder and spices could be effectively used to enrich their TDF content without exceeding the recommended daily intake of fibre for consumers.

Table 5.2   Proximate analysis, protein digestibility and AUC of digested starch of the fortified biscuits prepared from wheat-purple rice flour blends mixed with defatted mussel powder and spices.

Products	Moisture content (%)	Crude protein (%)	Protein digestibility (%)	Total fat (%)	Ash (%)	Total starch (%)	AUC of digested starch (mg min dL^{-1})	Total dietary fibre (%)
Control	5.31 ± 0.13^b	7.91 ± 0.02^d	69.10 ± 0.94^c	20.68 ± 0.16^b	2.51 ± 0.01^d	31.28 ± 0.25^a	588.05 ± 1.24^a	12.96 ± 0.18^d
10% mussel powder	5.75 ± 0.21^b	9.32 ± 0.03^c	78.19 ± 1.27^b	22.37 ± 0.06^a	2.85 ± 0.01^c	26.95 ± 1.09^b	542.09 ± 1.48^b	22.51 ± 0.22^c
15% mussel powder	5.70 ± 0.23^b	10.22 ± 0.01^b	81.12 ± 0.58^a	22.20 ± 0.13^a	3.15 ± 0.00^b	26.08 ± 0.71^b	507.16 ± 0.74^c	25.85 ± 0.51^b
20% mussel powder	6.50 ± 0.13^a	11.29 ± 0.09^a	83.48 ± 0.33^a	22.43 ± 0.05^a	3.31 ± 0.01^a	27.52 ± 0.30^b	488.24 ± 0.93^d	28.59 ± 0.31^a
LSD	0.51	0.11	2.33	0.25	0.08	1.55	3.20	1.90

Different superscripts (a,b,c,d) in each column represent significant difference (Duncan's multiple range test, $P < 0.05$); Values represent mean ± standard error. AUC = area under the curve; LSD = least significant difference.

5.3.2 Physical characteristics of the biscuits

Data on the texture and geometry of the biscuits are presented in Table 5.3. Biscuits with the addition of defatted mussel powder were significantly ($P < 0.05$) harder than the control biscuits. Therefore, the amount of protein addition seemed to be important to the texture (Krystyjan, Gumul, Ziobro, & Korus, 2015). As the content of defatted mussel powder was increased, the total dietary fibre in the biscuits also increases. It is interesting to note that increasing the mussel powder content also increased the hardness of the biscuits (Table 5.3). The increase in hardness of the 20% mussel powder biscuits was significantly higher than that of the control, at 42.4%. The biscuit matrix consisted of starch and sugar in a glassy state and fat globules that were bound to starch and protein in the matrix (Sozer *et al.,* 2014).

The substitution of defatted mussel powder led to a decrease in the width and spread ratio of the biscuits with a significant increase in their thickness. The highest addition of mussel powder in the biscuits (20%) had the lowest width and spread ratio and the highest thickness compared to the other three biscuit types. Larger diameter and lower thickness values were observed in the control biscuits. Biscuits with a high spread ratio are more desirable for consumers according to Sarabhai and Prabhasankar (2015). The spread ratios of the fortified biscuits were lower than those in the controls, where spread ratio values of 6.0 have been reported in purple wheat biscuits (Pasqualone *et al.,* 2015). Many studies have reported that the width and spread ratio of biscuits decreased with increasing fibre substitution and rice flour incorporation (Sudha, Vetrimani, & Leelavathi, 2007; Sharma & Gujra, 2014). The addition of defatted mussel powder at different levels changed the quality of the product in terms of the texture and colour parameters (Vijaykrishnaraj *et al.,* 2015).

Colour parameters are essential for consumers, who either purchase or select to consume food products, including biscuits, on the basis of their colour (Krystyjan *et al*., 2015). The colour developed during the baking stage can be used to determine the final stage of the baking process. When analysing the colour parameters of the biscuits (Table 5.3), it was observed that the addition of mussel powder to the biscuits resulted in significant differences ($P < 0.05$) in the redness (a^*) of the biscuit surfaces (see Appendix C). This is unsurprising since defatted mussel powder is characterized by a definite green colour when compared to the blended flour used. There were no changes observed in the lightness of the biscuits, regardless of the amount of mussel powder added.

Defatted green-lipped mussel powder contained more protein than the substituted wheat-purple rice-blended flour, and the carbohydrates in the biscuit dough came from the plain wheat flour, purple rice flour, and caster sugar. Therefore, the control showed a significantly lower browning index than the other biscuits. This finding is similar to many other studies (Sozer *et al.,* 2014; Sharma & Gujra, 2014). The Maillard reaction between the carbohydrates and amino acids was the main factor that changed the colour of biscuits in response to the baking temperature and time (Krystyjan *et al*., 2015). Moreover, high browning index values have also been found in bakery products enriched with spices (Przygodzka, ZieliNski, Ciesarová, Kukurová, & Lamparski, 2015).

Table 5.3 **Physical characteristics of fortified biscuits with different levels of defatted mussel powder.**

Products	Hardness (N)	Biscuit quality			L*a*b* Surface colour			Browning index
		Width (W, mm)	Thickness (T, mm)	Spread ratio (W/T)	L*	a*	b*	
Control	11.61 ± 0.30^c	67.75 ± 0.05^a	10.61 ± 0.12^d	6.39 ± 0.10^a	79.13 ± 0.22^{NS}	-3.09 ± 0.12^a	24.85 ± 0.67^{NS}	31.66 ± 1.60^b
10% mussel powder	14.36 ± 0.57^b	64.32 ± 0.04^b	12.75 ± 0.02^c	5.05 ± 0.07^b	79.15 ± 0.27	-3.17 ± 0.30^a	25.74 ± 0.34	34.12 ± 0.82^a
15% mussel powder	14.16 ± 0.38^b	62.33 ± 0.03^c	13.53 ± 0.08^b	4.61 ± 0.04^c	78.44 ± 0.43	-4.14 ± 0.36^{ab}	25.97 ± 0.09	35.91 ± 0.46^a
20% mussel powder	16.53 ± 0.56^a	60.83 ± 0.03^d	14.92 ± 0.17^a	4.08 ± 0.04^d	78.57 ± 0.19	-5.02 ± 0.50^b	25.94 ± 0.16	35.87 ± 0.31^a
LSD	1.69	1.48	0.59	0.27	1.69	0.89	2.25	2.45

Different superscripts (a,b,c,d) in each column represent significant difference (Duncan's multiple range test, $P < 0.05$); Values represent mean $\pm$ standard error; Not significant (NS) = no significant differences were found ($P > 0.05$). L* is measure of brightness from 0 = black to 100 = white; a* indicates red/green colour, with positive a* value redness and negative a* value greenness; b* describes yellowness when the values are positives and blueness when the values are negative. Browning index define brown colour. LSD, least significant difference.

5.3.3 Antioxidant compounds and antioxidant activities

Purple rice is a rich source of phenolic compounds and anthocyanins, including cyanidin-3-O-glucoside, which is the predominant anthocyanin in pigmented rice (Chatthongpisut, Schwartz, & Yongsawatdigul, 2015). The levels of bioactive compounds, such as total phenols, anthocyanin, and flavonoid contents, significantly increased ($P < 0.05$) in the mussel powder biscuits (Table 5.4) with the incorporation of increasing levels of powder. A marked difference in total phenolic compounds between the mussel biscuits and the control biscuits was found in this study. In comparison with the control biscuit, the total phenolic content among the fortified biscuits enriched with 10–20% defatted mussel powder was significantly higher by about 45%. These results could be the effect of the combination of antioxidant compounds derived from defatted mussel powder and spices. Hence, Uhl (2000) reported that the ginger and galangal extracts act as an effective antioxidant supplement. The highest values were observed in total phenolic compounds in the 20% mussel powder biscuits. The total phenolic compounds of the black mussel extract reported by Gorinstein *et al.* (2003) ranged between 3.8 and 6.5 mg/kg DW using the same extraction process as in this study, while the total phenolic compounds of purple rice ranged between 492 and 2013 μg of gallic acid/g DW (Jang & Xu, 2009). Therefore, the increase of total phenolic content among the biscuits enriched with 10–20% defatted mussel powder was observed due to the total phenolic compounds derived from the defatted mussel powder.

The increase of anthocyanin was significantly higher ($P < 0.05$) in the fortified biscuits prepared with the addition of defatted mussel powder compared to the control biscuits, in which antioxidants from purple rice and spices were similarly incorporated. The level of defatted mussel powder addition had no influence on the anthocyanin contents of the biscuits. In general, anthocyanins are found in high amounts in purple rice (Jang & Xu, 2009), but there has been no research on the anthocyanin content in defatted mussel powder. The use of more than 10% mussel powder biscuit formulation impacted on the flavonoid content. The levels were 248.6% higher in the 20% mussel-containing biscuits when compared to those in the control biscuits containing no defatted mussel powder. Flavonoids can be isolated from the ginger and galangal rhizomes (Uhl, 2000), while the major flavonoids in purple rice grains are anthocyanins (Jang & Xu, 2009). Therefore, the fortification of the biscuit ingredients tended to increase the antioxidant compounds from the inclusion of defatted mussel powder, spices, and purple rice flour into the biscuit mix compared to the control biscuits.

Due to the higher levels of bioactive compounds in the mussel biscuits, these biscuits showed ($P < 0.05$) a stronger antioxidant capacity, as measured by the ABTS assays, than the control biscuits (Table 5.4); but they were relatively less active when the DPPH assay was used. Stoilova *et al.* (2007) stated that ginger extracts have a stronger DPPH activity compared to ABTS. The addition of defatted mussel powder and baking seemed to have had a positive effect on antioxidant activities due to an increase in the Maillard reaction products, which are known for their antioxidant effects (Pasqualone *et al.,* 2015). The highest values of polyphenols from the black mussel were extracted with the combination of 50% methanol, 50% water, and 1.2 M HCl (Moncheva *et al.,* 2004), which is a similar extraction used in this study. Many researchers have clearly indicated that ginger and galangal extracts exhibited a strong scavenging effect on DPPH radicals (Uhl, 2000; Stoilova *et al.,* 2007; Buaniaw *et al.,* 2010; Caleja *et al.,* 2017). The ABTS scavenging activity of the biscuit extracts may, therefore, be related to the phenolic compounds present.

Table 5.4 **Bioactive compounds contents of fortified biscuits with defatted mussel powder and spices.**

Products	Total phenolic (µg GAE /g DW)	Anthocyanin (mg C3G /g DW)	Flavonoid contents (µg Catechin /g DW)	DPPH (µmol trolox /g DW)	ABTS (µmol rolox /g DW)
Control	1899.90 ± 6.84^d	12.41 ± 0.72^b	761.94 ± 1.91^c	37.35 ± 0.40^b	10.50 ± 0.26^d
10% mussel powder	2588.70 ± 1.23^c	18.26 ± 0.53^a	840.53 ± 16.86^c	38.69 ± 0.81^{ab}	53.69 ± 1.35^c
15% mussel powder	2623.62 ± 5.41^b	19.04 ± 0.35^a	1341.66 ± 27.00^b	40.11 ± 0.43^{ab}	65.93 ± 0.62^b
20% mussel powder	3081.50 ± 4.50^a	19.59 ± 0.48^a	2656.15 ± 70.45^a	40.59 ± 0.49^a	73.06 ± 0.98^a
LSD	13.97	1.51	109.20	1.58	2.55

Different superscripts (a,b,c,d) in each column represent significant difference (Duncan's multiple range test, $P < 0.05$); Values represent mean ± standard error. LSD, least significant difference. GAE, gallic acid equivalents; C3G, cyanidin-3-O-glucoside; DPPH, 2,2-diphenyl-2-picrylhydrazyl radical scavenging activity; ABTS, [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging activity.

5.3.4 Sensory preference attributes

Comparisons of the mean liking scores for the sensory attributes of the biscuits, as tested by the four ethnic groups, are presented in Table 5.5. The results showed that Pacific Islanders' perceived liking scores were higher than the other three ethnic groups' scores for all attributes tested. An explanation for this finding might be that the preferences towards the food were based on consumers' previous experience (Charlton *et al.,* 2016). Pacific Islanders traditionally eat diets high in fish and shellfish products (Charlton *et al.,* 2016; Polet & Bocherens, 2016). The familiarity of the consumers with ingredients or types of the product consumed might be the essential factor that affected consumers' attitudes towards these biscuits (Polet & Bocherens, 2016). Colour, texture, and flavour are often considered to be the most important attributes of bakery products, and these also received high scores from the Pacific Islands group. Jantathai, Sungsri-in, Mukprasirt, & Duerrschmid (2014) also stated that colour had a significant influence on the food satisfaction of consumers. Subsequently, Thai consumers gave the mussel biscuits higher liking scores in colour, overall appearance, and flavour than consumers from China and Europe, probably resulting from their daily familiarity with purple rice. Biscuits are not usually a preferred choice for Thai consumers; however, the texture of the products was more familiar to them than for the Caucasian consumers. When consumers eat food, they normally choose well-known products rather than less-known ones (Jantathai *et al.,* 2014). The Chinese people living in foreign countries still maintain their Chinese food habits (Ma, 2015). Therefore, this study can assume that the fortified biscuits were unfamiliar for the Chinese people.

The focus of the analysis of the mussel biscuits was on the colour and overall appearance of the biscuits, and the biscuits with 10% defatted mussel powder did not significantly have different liking scores compared to the control biscuits. This result confirmed that all consumers slightly liked the colour, overall acceptance, texture, oiliness, sweetness, and crunchiness of the 10% mussel powder biscuit. The sensory results showed that more than 10% defatted mussel powder addition significantly decreased the acceptable scores because of the flavour of the mussel powder. The liking score of overall acceptability of the biscuits had a linear relationship with the addition of defatted mussel powder.

The interaction of biscuits with increasing levels of defatted mussel powder among the four ethnic groups also showed that Pacific Islanders moderately liked the 10% mussel powder biscuit more than the other ethnic groups, who neither liked nor disliked these biscuits. Caucasians also gave higher scores for the 10% mussel powder biscuits than the other biscuits

for colour, overall appearance, texture, and oiliness attributes, when compared to the other ethnic groups. However, Thais preferred the overall appearance and oiliness of the 15% mussel powder biscuit more than the other biscuits, and they gave a higher score than the consumers from China, Europe, and the Pacific Islands. Vijaykrishnaraj *et al.* (2015) reported that the optimal level of green mussel powder addition in pasta was 5%, while the lowest mussel flavour was found in gluten-free bread fortified with 10% green mussel protein hydrolysates using quantitative sensory descriptive analysis (Vijaykrishnaraj *et al.*, 2016). The finding of this research clearly showed that the 10% mussel powder biscuit was accepted by all consumers.

Pearson's correlation (*r*) between physicochemical properties and sensory evaluation of the biscuits was also undertaken (Table A.1). The texture analysis and moisture content of the biscuits were positively correlated to crunchiness and oiliness preference scores from the sensory evaluation ($r = 0.58$, $P < 0.01$ and $r = 0.71$, $P < 0.05$, respectively). Moreover, the texture liking score appeared to be negatively correlated to the width of the biscuits ($r = -0.41$, $P < 0.05$). There were no significant relationships between the other characteristics and sensory evaluation tests used in this study.

Table 5.5   Average value of sensory liking scores of four different ethnic groups (Caucasian, Chinese, Pacific Island and Thai) for the four different fortified biscuits.

Attributes	Products	Ethnic groups				P-value[a]			LSD E (5%)	LSD P (5%)	LSD E*P (5%)
		Thai	Chinese	Caucasian	Pacific Islanders	E	P	E*P			
Colour	Control	4.50	3.94	4.92	5.78						
	10% mussel powder	5.19	4.78	5.71	5.50	<0.01	<0.01	<0.01	0.46	0.23	0.20
	15% mussel powder	4.50	4.83	4.61	5.03						
	20% mussel powder	4.67	4.64	5.16	5.03						
Overall appearance	Control	4.75	4.47	5.08	5.94						
	10% mussel powder	5.25	4.53	5.26	5.19	<0.01	0.226	0.01	0.44	0.22	0.20
	15% mussel powder	5.03	4.53	4.75	4.97						
	20% mussel powder	4.83	4.86	4.84	5.39						
Touch/Texture	Control	4.56	4.89	5.28	6.08						
	10% mussel powder	4.50	4.86	5.42	5.39	<0.01	<0.01	<0.01	0.42	0.21	0.19
	15% mussel powder	4.33	4.10	5.08	5.08						
	20% mussel powder	4.28	4.58	4.37	5.06						
Touch/Oiliness	Control	5.28	4.61	5.31	6.11						
	10% mussel powder	5.06	4.83	5.11	5.11	<0.01	<0.01	<0.01	0.41	0.20	0.18
	15% mussel powder	5.14	4.39	5.03	4.69						
	20% mussel powder	5.22	5.03	4.37	4.89						
Sweetness	Control	5.22	5.28	5.81	6.19						
	10% mussel powder	4.75	4.83	4.24	5.36	<0.01	<0.01	<0.01	0.47	0.23	0.21
	15% mussel powder	5.19	3.75	3.00	4.86						
	20% mussel powder	4.86	3.78	3.47	4.97						
Crunchiness	Control	3.67	4.86	5.25	6.28						
	10% mussel powder	3.61	4.25	3.79	5.92	<0.01	<0.01	<0.01	0.45	0.22	0.20
	15% mussel powder	3.67	3.83	3.42	5.06						
	20% mussel powder	3.22	4.25	3.61	5.11						
Flavour	Control	5.56	5.50	5.58	6.17						
	10% mussel powder	3.42	3.97	2.66	5.28	<0.01	<0.01	<0.01	0.50	0.25	0.22
	15% mussel powder	3.83	3.03	2.53	4.33						
	20% mussel powder	3.39	3.39	2.21	4.14						
Overall acceptability	Control	5.28	5.14	5.78	6.06						
	10% mussel powder	4.25	3.92	3.55	5.39	<0.01	<0.01	<0.01	0.43	0.21	0.19
	15% mussel powder	4.31	3.44	2.92	5.14						
	20% mussel powder	3.83	3.31	2.24	4.86						

Evaluated on seven-hedonic scales varying from 1 = dislike extremely to 7 = like extremely.

[a]Probability values due to sensory evaluation of four ethnic groups (E), products (P), and interaction effects (E*P) obtained by two-way ANOVA ($P < 0.05$). LSD, least significant difference.

5.4 Conclusions

Defatted green-lipped mussel powder clearly provided increased benefits from the antioxidant compounds and antioxidant activities added to the fortified biscuits, without any significant changes to the colour of the biscuits. The inclusion of defatted mussel powder from 10 to 20% in the biscuit mix also affects the antioxidant compounds, particularly total phenolics and flavonoids. However, the addition of defatted mussel powder in wheat-purple rice biscuits significantly ($P < 0.05$) altered their physical characteristics such as the hardness, spread ratio, and browning index resulting from higher fibre contents compared to the control biscuits. Increasing the purple rice and spice contents results in higher nutritional values, also antioxidant properties, and texture of the fortified biscuits compared to the control biscuits. Since protein accounted for the major part of defatted mussel powder, significant differences in flavour between the defatted mussel powder biscuits and the control biscuits were noticeable. This study concluded that defatted mussel powder could be incorporated as the main protein into wheat-purple rice biscuits up to a 10% level, according to its acceptable product quality as rated by the four different ethnic groups used to evaluate these products.

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Chapter 6

Addition of defatted green-lipped mussel powder and mixed spices to wheat-purple rice biscuits: Physicochemical, *in vitro* digestibility and sensory evaluation

Abstract

Biscuits were formulated using a 50/50 wheat and purple rice flour mix containing mixed spices, galangal, and defatted green-lipped mussel powder (*Perna canaliculus*) added in the range of 5-15% of the total biscuit weight. The fortified biscuits had higher protein (26.36%), fibre (52.90%) and ash (6.00%) contents and a lower total fat (5.64%) content compared to the control biscuits. The *in vitro* starch digestibility and predicted glycaemic index (pGI) decreased in the fortified biscuits at 18.95% and 6.18%, respectively while the *in vitro* protein digestibility increased by 3.73%, corresponding to the increased levels of defatted mussel powder present. The spread ratio and hardness of the fortified biscuits also increased significantly. The colour values of the fortified biscuits after the incorporation of different levels of defatted mussel powder showed significant changes, with a darkening of the biscuit surface and a lowered browning index compared to the control biscuits. Results of the sensory quality evaluation showed that incorporation defatted mussel powder into the biscuit mix of up to 15% showed no significant differences in liking scores in terms of colour, overall appearance; whereas, the flavour and overall acceptability scores, were significantly lower than the control biscuits. Overall, defatted mussel powder can be successfully incorporated into biscuit mixes to enrich the protein, fibre and antioxidants of the biscuits.

6.1 Introduction

Many agricultural crops, such as flaxseeds and garden cress seeds, have been used as natural sources of bioactive compounds that meet the demands of health conscious consumers (Kaur, Singh, & Kaur, 2017; Umesha, Manohar, Indiramma, Akshitha, &

Naidu, 2015). *Oryza sativa* L. var. Sanpatong, purple rice, widely grown in Northern Thailand, is becoming a popular crop because of its improved taste, lower price, and high levels of antioxidants (i.e., anthocyanins and phenolic acids) compared to wheat flour (Jang & Xu, 2009). Anthocyanins (cyanidin-3-O-glucoside and peonidin-3-O-glucoside) are present in the aleurone layers and pericarp of the rice, while phenolic acids are in the outer layers of the rice grain and these have potential antioxidant activities (Jang & Xu, 2009). In addition, purple rice also contains very high dietary fibre levels (Das, Goud, & Das, 2017; Jang & Xu, 2009; Yawadio, Tanimori, & Morita, 2007). Earlier studies by Klunklin and Savage (2018) have reported that purple rice flour is a good ingredient for making biscuits and also contains high levels of antioxidants.

Improvement in the protein contents of biscuits has been undertaken previously by the incorporation of whey protein, rice flour protein and soy protein (Mancebo, Rodriguez, & Gomez, 2016; Šaponjac *et al.,* 2016; Wani, Gull, Allaie, & Safapuri, 2015). Green-lipped mussel (*Perna canaliculus*) powder is widely known as a dietary supplement and contains bioactive components that are rich in polyunsaturated fatty acids. However, there is a limit to the use of mussel powder in food products due to problems with lipid oxidation resulting in the development of undesirable odours and taste (Umesha *et al.,* 2015). Defatted mussel powder has had the oil fraction removed to produce mussel products for the human supplement market, but still contains some residual fatty acids (Vijaykrishnaraj, Kumar, & Prabhasankar, 2015; Vijaykrishnaraj, Roopa, & Prabhasankar, 2016). The defatted mussel powder can be used to enrich the protein in food products (Vijaykrishnaraj *et al.,* 2016). A few studies have evaluated the addition of different levels of green-lipped mussel powder in pasta and bread (Vijaykrishnaraj *et al.*, 2015; 2016). Addition of green-lipped mussel is the cause of high off-flavours in the final product due to lipid oxidation (Vijaykrishnaraj *et al.,* 2015).

Natural antioxidants are important alternative ingredients that can be used to protect the oil from oxidation during thermal processing (Zhang *et al.*, 2013); they are also able to protect the free radicals present (Pasqualone *et al.*, 2015). Natural antioxidant compounds can be found in fruits, vegetables and spices, and also in various types of pigmented cereals, such as red, purple, and black rice or wheat grains (Pasqualone *et al.*, 2015; Yawadio *et al.*,

2007). Since ancient times, spices have been used as basic ingredients in Southeast Asian cuisine, and throughout the world, to develop the flavour of foods (Su *et al*., 2007). Many studies have researched the oxidative stability and antioxidant contents of culinary spices in food products due to their growing use as natural dietary sources (Juntachote, Berghofer, Siebenhandl, & Bauer, 2007; Misan *et al*., 2011; Ramanathan & Das, 1993; Ruangchakpet & Sajjaanantakul, 2007; Su *et al*., 2007; Umesha *et al*., 2015). These studies reported that culinary spices contain antioxidant compounds with effective antioxidant activities. Nutmeg and cinnamon are widely used in food products as flavouring ingredients with the benefits of reducing lipid oxidation and assisting human health by controlling diabetes, as well as having antimicrobial and anti-inflammatory effects (Su *et al*., 2007).

In the baking industry, the application of functional foods is an important developing market. Much bakery research has attempted to make healthy products by incorporating new ingredients into biscuit mixes in order to increase the nutritional qualities of the final product (Giarnetti, Paradiso, Caponio, Summo, & Pasqualone, 2015; Misan *et al.,* 2011; Park, Choi, & Kim, 2015; Sozer, Cicerelli, Heiniö, & Poutanen, 2014; Umesha *et al.*, 2015). Biscuits are largely consumed around the world due to their affordable price, good taste, and long shelf-life (Park *et al*., 2015). Biscuits generally contain high carbohydrate, sugar and fat contents. From a previous study, it was shown that purple rice flour could be substituted for some of the refined wheat flour to make bakery products, such as biscuits. (Klunklin & Savage, 2018). These biscuits had low starch digestibility, low pGI, and high protein digestibility, with only small changes in the physical characteristics of the biscuits. A 50% purple rice flour substitution was accepted by most of the panellists who tasted the experimental biscuits (Klunklin & Savage, 2018). In a previous study (Klunklin & Savage, 2018), the mussel biscuits were fortified by adding ginger and galangal to cover up the slight fishy smell and increase the antioxidant content at the same time. For this study, biscuits were supplemented using wheat–purple rice blended flour containing mixed spices, such as cinnamon, nutmeg, and galangal in order to increase the liking scores of the fortified biscuits tested by untrained consumers. The biscuits were then enriched with different levels of defatted mussel powder that improved the physicochemical, antioxidant properties and sensory evaluation of the fortified biscuits.

The research objective was to evaluate the physicochemical characteristics, antioxidant properties and consumer preferences of fortified biscuits enriched with different levels of defatted green-lipped mussel powder, ranging from 5% to 15% of the total biscuit mix with the added mixed spices.

6.2 Materials and Methods

6.2.1 Raw materials

Biscuits were prepared using refined wheat flour (Pams Products Ltd., Auckland, New Zealand), whole purple rice grains (*Oryza sativa* L. var. Sanpatong) (Big T. supermarket, Riccarton, Christchurch, New Zealand) imported in 5-kg bags, baking powder (Edmonds Limited, Goodman Fielder Ltd., Auckland, New Zealand), xanthan gum (Lotus foods Pty Ltd., Cheltenham, Victoria, Australia), butter (Dairyworks Ltd., Hornby, Christchurch, New Zealand), castor sugar (Pams Products Ltd.), salt (Pams Products Ltd., Auckland, New Zealand), whole egg (Pams Products Ltd.), vanilla extract (Pams Products Ltd.), mixed spices nutmeg and cinnamon in a 2:1:1 ratio (Cerebos Gregg's Limited, Christchurch, New Zealand), galangal powder (Sunson gifts and the Asian food market, Riccarton), and defatted green-lipped mussel powder (*Perna canaliculus*) provided by Aroma New Zealand Limited, Christchurch, New Zealand. All ingredients were food grade and all chemicals were analytical grade. The experimental biscuits were prepared by the addition of defatted mussel powders at levels of 5, 10, and 15% (w/w) of the total flour weight.

6.2.2 Biscuits preparation

The experimental biscuits were prepared using a 50/50 mix of refined wheat flour and whole purple rice flour. The purple rice grains were milled using a Whisper Mill (Grote Molen Inc., USA). All ingredients are shown in Table 6.1. The biscuit dough was rolled to a thickness of 4 mm using an adjustable rolling pin (Joseph Joseph 20085; Joseph Joseph Ltd., London, UK). The dough was cut to round shapes using a 5-cm-diameter biscuit cutter and then baked at 170°C for 9 min in the oven (Bakbar Turbofan 32 Max; Ali group

company, Milan, Italy). The biscuits were cooled at room temperature for 15 min before they were packed in sealed aluminum bags.

Table 6.1   Formulation of different biscuits supplemented with defatted mussel powder.

Ingredients (g)	Control	Fortified biscuit
Refined wheat flour	50	50
Purple rice flour	50	50
Defatted mussel powder	-	Varied from 5-15%
Butter	75	75
Sugar	62.5	62.5
Egg	112.5	112.5
Salt	0.75	0.75
Mixed spices	-	2
Cinnamon	-	1
Nutmeg	-	1
Galangal	-	4
Sodium bicarbonate	4.5	4.5
Xanthan gum	1.25	1.25

6.2.3 Proximate analysis

All biscuit samples were analysed in triplicate. The moisture content was determined using the oven drying method at 105°C for 18 hr (AOAC, 2000). The total nitrogen was evaluated using the Dumas method by rapid Max N Exceed® (Elementar, Hanau, Germany) and a factor of 6.25 was used to estimate the protein contents. The fat and ash contents of the biscuits were determined using standard methods 920.176 and 900.029 respectively (AOAC, 2000). Total carbohydrate was calculated by difference (AOAC, 2000) and total dietary fibre (TDF) was measured using a total dietary fibre assay kit (Sigma-Aldrich, MO, USA). The total starch content was determined using a Megazyme starch assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland), using approved method 76–13 (Reed, Ai, Leutcher, & Jane, 2013).

6.2.4 *In vitro* digestibility

Protein digestibility

The *in vitro* protein digestibility was determined according to the method of Akeson and Stahmann (1964). The enzymes used were pepsin from porcine gastric mucosa (Sigma Aldrich, USA, 66 units/mg protein) followed by pancreatin from porcine pancreas (AppliChem Chemica Synthesis, Germany, 30.315 units/mg protein). All simulated solutions from the digestibility were centrifuged at 1600 g for 10 min. The supernatants were measured using an Elementar Vario TOC cube instrument (Hanau, Germany) fitted with a chemiluminescence detector to determine total bound nitrogen (TNb) to determine the digested protein. The analysis was carried out in triplicate.

Starch digestibility

The available starch contents were assessed following the multienzymatic protocol of Monro, Mishra, & Venn (2010) using porcine pepsin (Sigma Aldrich), porcine pancreatic alpha-amylase (No. 7545, Sigma–Aldrich, St. Louis, MO), and amyloglucosidase (No. 9913, Sigma–Aldrich). The enzyme solutions were made fresh before each analysis. The digested solutions were centrifuged at 180 g for 5 min. The supernatants were read at 530 nm (V-1200 spectrophotometer, Global Science, Auckland, New Zealand).

6.2.5 Predicted glycaemic index (pGI)

The hydrolysis index (HI) was calculated from the area under the curve (AUC) of each biscuit using the *in vitro* starch digestibility results divided by the AUC of a standard (50 g white bread). The predicted glycaemic index (pGI) was calculated according to the method of Goñi, García, & Saura-Calixto (1997). Predicted glycaemic index = 39.71 + 0.549 × HI.

6.2.6 Physical characteristics

All physical analyses of biscuits were measured 15 min after baking. A texture analyser (TA-XT2i, Stable Micro System, Godalming, UK) equipped with a small 3-point bending test rig with a sharp-blade cutting probe and with a 30-kg load cell (Park *et al.,* 2015).

Hardness measurements, expressed as the first peak force of each penetration, were carried out. Six biscuits were weighed and their width (W), thickness (T), and biscuit spread ratio (W/T) measured using AACC method 10-50.05 (AACC, 1967; AACC, 2000).

6.2.7 Colour determination

Surface CIE colour values ($L*a*b*$) were determined using a Minolta Chroma Meter CR-410 (Konica Minolta, Chiyoda, Tokyo, Japan) and the results were presented in the CIE $L*$ $a*$ $b*$ system in which $L*$ represented lightness, $a*$ represented redness, while $b*$ represented yellowness (Sozer *et al.,* 2014). The browning indexes of the biscuits were calculated as reported by Ruangchakpet and Sajjaanantakul (2007). All analyses were performed in triplicate.

6.2.8 Antioxidant compounds and antioxidant activity determinations

Biscuits were extracted following the protocol of Jang and Xu (2009) to obtain both lipophilic and hydrophilic antioxidants. The extracted solutions were kept at −20°C until further analysis. The total phenolic contents in the extracted solutions were measured according to the method of Kaneda, Kubo, & Sakurai (2006). The total flavonoid content was determined according to the procedure used by Zhishen, Mengcheng, & Jianming (1999). The anthocyanin content was determined according to Hosseinian, Li, & Beta (2008). The DPPH scavenging capacity was undertaken according to the method of Mahakunakorn, Tohda, Murakami, Matsumoto, & Watanabe (2004). The ABTS assay was evaluated using the method of Re *et al.* (1999). All analyses were determined in triplicate.

6.2.9 Sensory evaluation

One hundred and seven panellists, aged between 19 and 60 years, evaluated the biscuits in terms of overall appearance, touch/oiliness, crunchiness, flavour, and overall acceptability. The panellists were untrained students and staff members from the Lincoln University. The sensory evaluation was approved by the Lincoln University Human Ethics Committee No. 2017-24. All panellists signed the consent form prior to being served the samples. A 9-point hedonic scale (1 = dislike extremely, 9 = like extremely) was used to assess the liking

and disliking scores of the panellists (Aminah & Tan, 2001). Before consuming the biscuits, panellists were asked to evaluate the overall appearance and touch/oiliness using a 9-point hedonic scale ranging from "like extremely" to "dislike extremely". After scoring the overall appearance, the panellists were then allowed to evaluate the crunchiness, flavour, and overall acceptability of the biscuits. The evaluation of samples was completely randomised and performed 1 day after baking with. The sensory analysis was carried out in a standardized sensory analysis room.

6.2.10 Statistical analysis

The experimental data were statistically analysed by one-way ANOVA using a complete randomised design for all physicochemical experiments, and a randomised complete block design for sensory analysis followed by Duncan's multiple range test using SPSS Statistics (v. 22.0, SPSS Inc., Chicago, IL, USA) at $P < 0.05$ to determine the significant differences. The least significant differences (LSD) were analysed with Minitab (v.17, Minitab Inc., State College, PA, USA).

6.3 Results and Discussion

6.3.1 Nutritional compositions

The chemical compositions of the biscuits prepared using 5%, 10%, and 15% of defatted green-lipped mussel powder are shown in Table 6.2. The moisture content in the biscuits decreased significantly ($P < 0.05$) with increased defatted mussel powder incorporation, from 5% to 15%. In general, biscuits were considered to be very low moisture content products. The moisture content of the biscuits normally ranged from 1% to 5%, which prevents microbiological spoilage and extends the shelf-life of products (Chung, Cho, & Lim, 2014). Chung *et al*. (2014) reported that the fortification of biscuits with increasing fibre and protein contents affected the moisture content of the biscuits. Therefore, the biscuits prepared with the addition of defatted mussel powder had a low water retention capacity in the biscuit matrix compared to the control biscuits. This might have contributed to a change in moisture content during baking. Increasing the defatted mussel powder content in the biscuit mix (Table 6.2) had the greatest effect on the crude protein content.

All biscuits supplemented with defatted mussel powder were found to be nutritious snacks. The consumption of 100 g of defatted mussel biscuits would provide more than half of the recommended daily protein requirements for children and young people aged between 5 and 19 years (25–30 g/day) according to the FAO/WHO (1973). This suggests that these biscuits enriched with defatted mussel powder would be useful as protein-supplemented snacks in developing countries and could be used as a supplement for people who are unable to consume meat protein.

The addition of 15% defatted mussel powder in the biscuits resulted in a significant rise in total fibre content. From a nutritional point of view, to claim that a food is a "source of dietary fibre," it needs to contain at least 3 g/100 g, whereas "high in dietary fibre" foods should contain at least 6 g/100 g according to regulation (EC) 1924/2006 (Official Journal of the European Union, 2006). Therefore, all biscuits in this study can be considered to be high dietary fibre food products. The biscuits enriched with defatted mussel powder contained less fat compared to the control biscuits. A similar trend was reported by Arshad, Anjum, & Zahoor (2007), that the addition of the defatted wheat germ decreased the fat content when the total dietary fibre content of the biscuits was increased. Fat content is one of the basic components of biscuits which has positive effects on texture characteristics such as mouthfeel. Similar fat contents (Table 6.2) have also been observed by Baltsavias, Jurgens, & van Vliet (1999) who reported that fat can vary from 20% to 60% based on percentage by weight of flour used to make the biscuits. However, butter contains high content of saturated fatty acids (Baltsavias *et al*., 1999; Giarnetti *et al*., 2015). A further study would investigate the possibility of reducing the total fat content and also the possibility of using a fat with a healthier fatty acid profile (Giarnetti *et al*., 2015). There were no significant differences ($P > 0.05$) in total starch contents of all the biscuits.

The overall reduction in starch digestibility was of great interest as starch is a major ingredient in these biscuits. The area under the starch hydrolysis curve (AUC) was evaluated, after the digestion of each biscuit, to determine the glycaemic response of the selected foods compared to a reference sample of white bread (An, Bae, Han, Lee, & Lee, 2016). The AUC of the biscuits enriched with defatted mussel powder was significantly lower ($P < 0.05$), by 26.4%, than the control biscuits. This is the first study of the starch

digestibility of defatted mussel powder incorporated within a food product. Lower starch digestibility following the additions of defatted mussel powder resulted from protein–starch interactions, which formed a matrix surrounding the starch granules, which then acted as a physical barrier to limit starch availability (Singh, Dartois, & Kaur, 2010). This result showed that defatted mussel powder can be considered as an important ingredient that was able to reduce a starch digestibility when incorporated into food products.

The pGI was calculated according to Goñi *et al*. (1997) using the starch hydrolysis index (HI) of each biscuit containing added defatted mussel powder. As expected, including defatted mussel powder as an enriched ingredient caused a reduction in the pGI of the biscuits (Table 6.2). Brand-Miller (1994) showed that a diet containing low glycaemic index foods reduced insulin resistance and adjusted the blood glucose levels in both Type 1 and Type 2 diabetic patients. These fortified biscuits have a low glycaemic index (GI < 60), and would be a good snack food for diabetics. The control biscuits can be regarded as an intermediate GI (60–85) snack.

Table 6.2 **Basic characteristics of the fortified biscuits prepared from wheat-purple rice flour blends mixed with mussel powder and mixed spices.**

Parameters (%DM basis)	% defatted green-lipped mussel powder				LSD
	0 (Control)	5	10	15	(5%)
Moisture content (%)	5.06 ± 0.03^a	4.99 ± 0.05^b	4.73 ± 0.01^c	4.69 ± 0.03^d	0.05
Crude protein (%)	7.31 ± 0.13^d	8.50 ± 0.04^c	9.33 ± 0.17^b	9.88 ± 0.07^a	0.14
Protein digestibility (%)	82.59 ± 1.33^{NS}	85.17 ± 0.06	85.73 ± 0.85	86.12 ± 0.70	3.54
Total dietary fibre (%)	15.76 ± 0.11^d	20.61 ± 0.30^c	23.76 ± 0.09^b	27.45 ± 0.07^a	0.28
Ash (%)	2.33 ± 0.03^c	2.27 ± 0.03^{bc}	2.47 ± 0.03^b	2.67 ± 0.07^a	0.07
Total fat (%)	25.30 ± 0.50^a	23.37 ± 0.10^b	24.05 ± 0.27^{ab}	24.43 ± 0.20^{ab}	0.50
Total starch (%)	33.70 ± 1.79^{NS}	36.91 ± 0.37	35.42 ± 1.01	35.05 ± 1.43	3.26
AUC of digested starch (mg min dL^{-1})	462.01 ± 0.43^a	422.17 ± 0.26^b	391.67 ± 0.28^c	351.35 ± 0.75^d	1.26
pGI	62.56 ± 0.02^a	60.59 ± 0.01^b	59.08 ± 0.01^c	57.09 ± 0.04^d	0.07

Values represent mean ± standard error. In each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$); AUC = area under the curve; pGI = predicted glycaemic index; NS = no significant difference.

6.3.2 Biscuit quality

The addition of purple rice flour and defatted mussel powder to a basic biscuit mix influenced the physical characteristics (Table 6.3). The hardness of the biscuits significantly increased ($P < 0.05$), from 5% to 15%, with the increased amounts of defatted mussel powder, compared to the control biscuits. This could be attributed to the lower levels of wheat gluten available to bind with water as the flour content was decreased by adding more defatted mussel powder into the biscuit mix. Moreover, the moisture content of the biscuits inversely affected the hardness of the fortified biscuits (Mancebo *et al.*, 2016). All these factors gave the biscuits a higher hardness and lower spread ratio. However, the hardness of these experimental biscuits was in the same range as biscuits containing rice flour (Chung *et al.,* 2014).

The spread ratio is an important measurement of biscuit quality, higher values are considered to be more desirable. The spread ratio of the fortified biscuits showed a significant decrease ($P < 0.05$) with the increased defatted mussel powder addition levels compared to the control biscuits (Table 6.3). This was the first time that the addition of defatted mussel powder in a biscuit mix has been investigated. Moreover, Vijaykrishnaraj *et al*. (2016) found that adding green-lipped mussel hydrolysate to gluten-free bread affected the physical characteristics, such as the volume and crumb formation. In this study, the width decreased, and the thickness increased after the incorporation of defatted green-lipped mussel powder into the biscuit mix. The increased level of fibre and protein content from the defatted mussel powder (Table 6.2) absorbed more water. The water in the biscuit formulation was limited, as a result the biscuits remained harder so, consequently, they had a smaller spread ratio (Chung *et al.,* 2014). Lower dough expansion during baking was also observed by Park *et al*. (2015) and Pasqualone *et al*. (2015) in biscuits formulated from alternative flours that contained increased protein content.

Consumers considered buying food products based on their colour, and this was an important factor in the acceptance of a new food. The fortified biscuits showed a significant reduction in all *L*a*b** colour parameters (Table 6.3). The control biscuits were relatively dark, as they were made from 50% purple rice flour. Colour darkening of biscuits during

cooking normally occurred from sugar caramelisation and/or the Maillard reactions between sugars and amino acids in the mixture (Chung *et al*., 2014). The addition of protein can also reduce the $L*$ values of the biscuits. Other authors found similar results when they incorporated additional protein into biscuits (Park *et al*., 2015; Sozer *et al*., 2014). As the incorporation of protein decreased the $a*$ and $b*$ values of the biscuits, the additional green colour of the biscuits resulted from the addition of defatted mussel powder (see Appendix C). There were increased negative $a*$ values (greenness) in the fortified biscuits, whereas the $b*$ values of the biscuits decreased, the colour moved from yellow to a blue hue due to the presence of the defatted mussel powder (higher in the control biscuits than in the fortified biscuits). Similar results were also reported by Vijaykrishnaraj *et al.* (2015, 2016) who observed that the addition of green-lipped mussel powder altered the colour values of food products.

The browning index is commonly used to determine the end of the baking process as it was the last step of both the Maillard reaction and caramelisation (Torbica, Hadnađev, & Hadnađev, 2012). In this study, the rate of Maillard reaction reduced with the increased incorporation of the defatted mussel powder in biscuit mixes due to reductions in the browning index. The rate at which the sugars present dissolved, depended on the amount of water present in the biscuit dough (Torbica *et al.,* 2012); hence, the water content of the fortified biscuits was lower than in the control biscuits (Table 6.2). Vijaykrishnaraj *et al*. (2016) also confirmed that the small molecules produced by the thermal degradation of starch and proteins in the mussel powder had not induced pronounced Maillard reactions during the baking process.

Table 6.3 **Physical, textural and *L*a*b** colour characteristics of the fortified biscuits prepared from wheat-purple rice flour blends mixed with defatted mussel powder and mixed spices.**

Physical parameters	% defatted green-lipped mussel powder				LSD
	0 (Control)	5	10	15	(5%)
Hardness (N)	19.05 ± 0.07^d	19.43 ± 0.02^c	19.95 ± 0.11^b	20.49 ± 0.06^a	0.17
Width (W, mm)	62.67 ± 0.21^a	61.17 ± 0.48^b	61.00 ± 0.37^b	60.67 ± 0.33^b	0.75
Thickness (T, mm)	6.50 ± 0.22^c	7.17 ± 0.75^{bc}	7.50 ± 0.22^b	8.33 ± 0.82^a	0.68
Spread ratio (W/T)	9.70 ± 0.33^a	8.60 ± 0.40^b	8.20 ± 0.30^{bc}	7.35 ± 0.32^c	0.81
Colour values					
L*	78.46 ± 0.13^a	77.31 ± 0.08^{bc}	77.62 ± 0.07^b	77.06 ± 0.20^c	0.27
a*	-3.22 ± 0.08^a	-4.49 ± 0.11^b	-4.93 ± 0.05^c	-5.07 ± 0.05^c	0.15
b*	25.40 ± 0.08^a	24.04 ± 0.11^c	24.34 ± 0.12^b	23.87 ± 0.09^c	0.21
Browning index	34.82 ± 0.23^a	31.67 ± 0.21^b	31.60 ± 0.24^b	30.87 ± 0.20^c	0.47

Values represent mean ± standard error. For each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$).

6.3.3 Antioxidant compounds and antioxidant activities

The total phenolic contents of the biscuits significantly increased ($P < 0.05$) as the percentage of defatted mussel powder increased from 5% to 15% in the biscuit mix (Table 6.4). Many researchers have studied wheat biscuits enriched with polyphenols from different ingredients, such as ginger, gooseberry, flaxseed flour, and purple wheat extract (Abdel-Samie, Wan, Huang, Chung, & Xu, 2010; Kaur *et al*., 2017; Pasqualone *et al*., 2015; Ruangchakpet & Sajjaanantakul, 2007). The contents of phenolic compounds in baked cereal products were normally affected by the moisture content on baking, while the total phenolic compounds of purple rice ranged between 492 and 2013 μg of gallic acid/g DW using the same extraction process as in the study by Jang and Xu (2009). The main anthocyanins of defatted mussel powder biscuits were analysed in this study. Cyanidin-3-O-glucoside (C3G) significantly increased ($P < 0.05$) with the increased proportion of defatted mussel powder in the biscuit mix. Anthocyanins have been found in high amounts in purple rice (Jang & Xu, 2009) but there has been no research on the anthocyanin content of defatted mussel powder. In addition to the total flavonoids, the phenolic acids had a high positive correlation with the content of total phenolic compounds. Thus, the flavonoid levels were 89.29% higher in the 15% defatted mussel powder biscuits compared to the control biscuits. The antioxidant compounds present depended on the breakdown of

phenolics by heat in the food matrix, which was affected by the extraction rates of the phenolics (Jang & Xu, 2009).

There have not been any reports about the antioxidant content of defatted green-lipped mussel powder. The protein isolated from blue mussel (*Mytilus edulis*) and green-lipped mussel powder (*Perna canaliculus*) also showed significant radical scavenging activity and acted as inhibitors of auto-oxidation in the linoleic acid model system (Vijaykrishnaraj *et al.,* 2015; Vijaykrishnaraj *et al.,* 2016).

The antioxidant activities of the biscuits were determined by two different methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) assays, as shown in Table 6.4. The highest DPPH activity was exhibited by biscuits containing 15% defatted mussel powder. In general, biscuits made from composite flours had decreased the DPPH activity after the baking process (Kaur *et al.,* 2017). Results by Vijaykrishnaraj *et al*. (2015) showed that the DPPH activity for raw green-lipped mussel powder was 36%; however, there was no significant difference in total antioxidant activity between fresh and cooked products. When included at a high level in pasta, antioxidants showed a loss activity (Vijaykrishnaraj *et al*., 2015). Table 6.4 also shows the antioxidant activities of the biscuits enriched with different levels of defatted mussel powder determined using the ABTS assay. The 15% defatted mussel powder biscuits also exhibited the strongest ABTS capacity followed by the 10% and 5% defatted mussel powder biscuits respectively.

A study by Vijaykrishnaraj *et al*. (2015) confirmed that mussel powder could be incorporated in gluten-free pasta to achieve antioxidant efficacy. According to the results of Abdel-Samie *et al.* (2010), antioxidant compounds and the antioxidant activity of biscuits enriched with 5% cumin and ginger showed higher antioxidant effects than the control biscuits. From an antioxidant point of view, the present study indicated that biscuits containing 15% defatted mussel powder contained significant amounts of antioxidant compounds and, consequently, antioxidant activities.

Table 6.4   Bioactive compounds contents of the fortified biscuits prepared from wheat-purple rice flour blends mixed with defatted mussel powder and mixed spices.

Bioactive compounds	% defatted green-lipped mussel powder				LSD (5%)
	0 (Control)	5	10	15	
Total phenolics (mg GAE/100 g DW)	2664.72 ± 8.68^d	3120.98 ± 0.78^c	3311.62 ± 13.77^b	3522.96 ± 10.87^a	10.88
Anthocyanin (C3G, mg/kg DW)	19.20 ± 0.10^d	24.38 ± 0.25^c	25.88 ± 0.10^b	27.55 ± 0.25^a	0.31
Flavonoid contents (mg CE/100 g DW)	1.12 ± 0.07^d	1.52 ± 0.07^c	1.77 ± 0.10^b	2.12 ± 0.10^a	0.01
DPPH (µmol trolox/g DW)	47.52 ± 0.11^d	48.06 ± 0.01^c	48.62 ± 0.06^b	48.96 ± 0.07^a	0.12
ABTS (µmol trolox/g DW)	11.95 ± 0.18^d	16.39 ± 0.24^c	30.60 ± 0.19^b	41.07 ± 0.03^a	0.29

Each row, sample means not having the same letter attached to them are significantly different (Duncan's multiple range test, $P < 0.05$); Values represent mean ± standard error. GAE, gallic acid equivalents; C3G, cyanidin-3-O-glucoside; CE, catechin equivalents; DPPH, 2,2-diphenyl-2-picrylhydrazyl radical scavenging activity; ABTS, [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging activity.

6.3.4 Sensory attributes for preference

A 9-point hedonic scale method is widely used to evaluate consumers' acceptance of food products by food scientists and technologists. It is effective, used less time than other methods, and can be carried out by either trained or untrained panellists (Sharma, Saxena, & Riar, 2016). The sensory evaluation of biscuits prepared from wheat–purple rice flour that included different levels of defatted mussel powder in the biscuit mix are presented in Table 6.5. The sensory panellists rated the control biscuits with the highest score (over 7) for oiliness, crunchiness, flavour, and overall acceptability. These attributes were significantly decreased in the biscuits which incorporated different levels of defatted mussel powder. The enrichment with defatted mussel powder up to 15% resulted in a significant increase in hardness (Table 6.3) compared to the control biscuits. The texture of gluten-free bread and the acceptance scores improved following the addition of green mussel powder into breads (Vijaykrishnaraj *et al*., 2016). On the other hand, there were no significant differences ($P > 0.05$) in liking scores for colour and overall acceptance among the fortified biscuits and the control biscuits. The defatted mussel powder enrichment significantly decreased the mussel flavour scores of the biscuits; however, the biscuits were still liked slightly by the panellists. The low moisture content in fat-containing food (biscuits contain 23–24% fat) can accelerate lipid oxidation reactions since the substrates and reactants become more concentrated (Chung *et al*., 2014). The highest overall acceptability score was for biscuits containing 5% defatted mussel powder and, after this level of substitution, a decrease in acceptability scores was observed; however, there were no significant differences ($P > 0.05$) among the fortified biscuits for all attributes. Vijaykrishnaraj *et al.* (2016) reported that the sensory evaluation scores were acceptable when 5% green-lipped mussel powder was added to gluten-free bread. In this study, biscuits enriched with defatted mussel powder of up to 15% were accepted by panellists. The defatted mussel biscuits developed were nutritionally enriched and would be acceptable by consumers suffering from diabetes.

Table 6.5 **Mean liking scores of the fortified biscuits prepared from wheat-purple rice flour blends mixed with defatted mussel powder and mixed spices.**

Attributes	% defatted green-lipped mussel powder				LSD
	0 (Control)	5	10	15	(5%)
Colour	6.42 ± 0.26^{NS}	6.19 ± 0.20	6.38 ± 0.24	6.27 ± 0.27	1.86
Overall appearance	6.69 ± 0.23^{NS}	5.92 ± 0.29	6.42 ± 0.27	6.46 ± 0.30	1.97
Touch/oiliness	7.27 ± 0.22^{a}	6.54 ± 0.26^{ab}	6.35 ± 0.25^{b}	6.38 ± 0.30^{b}	0.87
Crunchiness	7.62 ± 0.20^{a}	6.58 ± 0.27^{b}	6.46 ± 0.27^{b}	6.35 ± 0.27^{b}	1.02
Flavour	7.04 ± 0.36^{a}	5.46 ± 0.45^{b}	5.27 ± 0.36^{b}	4.62 ± 0.40^{b}	1.52
Overall acceptability	7.42 ± 0.25^{a}	5.65 ± 0.40^{b}	5.38 ± 0.30^{b}	5.31 ± 0.18^{b}	1.67

***The mean difference is significant at $P < 0.001$; NS = not significant; Data are expressed as mean ± standard error.

6.4 Conclusions

Developing novel biscuits by supplementing refined wheat flour with purple rice flour enriched with defatted mussel powder has been investigated by Klunklin and Savage (2018). The biscuits hold high promise with regard to antioxidant compounds and antioxidant activities. Moreover, the water content and hardness were important factors when evaluating biscuit quality without causing adverse effects after adding the defatted mussel powder. Mixed spices and galangal were used to enhance the flavour of the biscuits enriched with the defatted mussel powder and this was successful, based on the liking scores of the flavour attributes measured by the panellists. Therefore, defatted green-lipped mussel powder was a nutritious, bioactive compound that can be incorporated in biscuit mixes up to the 15% level. The sensory evaluation proved that the addition of defatted mussel powder in the biscuits led to acceptable liking scores at the highest incorporation level. Natural protein from defatted green-lipped mussel powder can be used as an alternative protein to improve the nutritional values (higher protein, fibre and ash content with lower fat content) of food products and achieved acceptable scores by panellists.

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Chapter 7

Effect of storage at different temperatures and packaging on quality properties of wheat-purple rice biscuits enriched with defatted green-lipped mussel powder and spices

Abstract

The quality of biscuits during storage is profoundly influenced by their ingredients. Biscuits were prepared using a 50:50 mix of wheat and purple rice flour. Another batch of biscuits was prepared using the same base mix with the addition of defatted mussel powder and spices. This research aimed to study the different conditions of control and supplemented biscuits stored at two different temperatures (21.6 ± 0.4°C and 34.2 ± 0.1°C) in two different type of packaging (polyethylene terephthalate (PET) and aluminium foil laminate (AL)) for 12 weeks. The moisture content of the biscuits in both types of packaging slowly increased during storage ($r = -0.561$, $P < 0.01$). At the sixth week of storage, antioxidants, free fatty acids (FFA) and peroxide value (PV) showed small changes; however, no differences ($P > 0.05$) were detected for CIE L^*, a^* and b^* values in the control and supplemented biscuits under different storage conditions. At the lower temperature (21.6 ± 0.4°C), the antioxidant compounds were reduced in the supplemented biscuits stored in PET and AL pouches, by 37% and 30%, respectively. However, under all storage conditions the oxidation rate of FFAs and PV was within acceptable limits for this type of product.

7.1 Introduction

In order to produce a new food product, different aspects such as the selection of ingredients, selection of a stable formulation and shelf-life, are important considerations to meet consumer demands (Boobier, Baker, & Davies, 2006). Among bakery products, biscuits are convenient cereal products with a wide popularity across the world due to their low cost and variety of tastes compared to other foods. Several studies have considered fortifying biscuits by enhancing their protein, fibre, and antioxidant contents because of their long shelf-life and popularity (Rebellato, Pacheco, Prado, & Pallone, 2015; Kumar *et al.,* 2016; Klunklin & Savage, 2018a).

Recently, natural products have been used more frequently as food supplements. Antioxidants are substances that can prevent the oxidation of other compounds. Natural antioxidant compounds can be found in many types of foods, such as spices, fruits and vegetables, and also in various types of pigmented cereal grains (Yawadio, Tanimori, & Morita, 2007). Purple rice (*Oryza sativa* L.) is a whole-grain glutinous rice containing high amylopectin contents (Jang & Xu, 2009). It has naturally high antioxidant activity because it contains high levels of anthocyanins and phenolic acids. The predominant anthocyanin in purple rice is cyanidin-3-O-glucoside (C3G) contained 92% of the total quantified anthocyanin, as reported by several researchers (Abdel-Aal, Young, & Rabalski, 2006; Jang & Xu, 2009; Sompong, Siebenhandl-Ehn, Linsberger-Martin, & Berghofer, 2011). Many researchers also reported that C3G was found in high amounts in purple rice bran and this contributed to its high potential antioxidant activity (Jang & Xu, 2009; Yodmanee, Karrila, & Pakdeechanuan, 2011). In addition, purple rice contains more protein, vitamins and minerals than white rice (Jang & Xu, 2009). Among bakery products, biscuits can easily be fortified with antioxidant compounds and that has been a common approach used to extend their shelf-life (Kumar *et al.,* 2016). Spices have been used effectively in culinary and medicinal applications for many years. Asian countries produce many kinds of spices, such as pepper, nutmeg, cinnamon, ginger and galangal (Buaniaw, Siripongvutikorn, & Thongraung, 2010). The fortification of food products with spices can enhance flavouring effects and antioxidant compounds, which can extend their shelf-life (Buaniaw *et al.,* 2010; Przygodzka, ZieliNski, Ciesarová, Kukurová, & Lamparski, 2015).

Defatted green-lipped mussel powder (*Perna canaliculus*) is a good protein source, is processed to remove mussel oil and provides products for human consumption (Klunklin & Savage, 2018a). The supplementation of biscuits with antioxidants and defatted mussel protein during storage has remained almost unexplored. Few researchers have been working on the inclusion of protein from meat, such as green-lipped mussels (Vijaykrishnaraj, Kumar, & Prabhasankar, 2015; Vijaykrishnaraj, Roopa, & Prabhasankar, 2016; Klunklin & Savage, 2018a), chicken meat (Kumar *et al.,* 2016) and fish bones (Abdel-Moemin, 2015) into cereal-based foods, such as bread, pasta and biscuits.

The food storage and shelf-life of foods are important properties for any foods and the nutritional, functional and sensory characteristics of products is of interest to food producers and consumers (Žilić, Kocadağl, Vančetović, & Gökmen, 2016). The moisture content of food products is a key factor that influences shelf-life (Romani *et al.,* 2015). The resulting

physicochemical changes in food during storage lead to a deterioration in the quality of food products, such as off-flavours, loss of crispiness and reductions in antioxidant compounds by oxidative rancidity (Žilić *et al.,* 2016). The majority of food products depend on their particular packaging and storage conditions to achieve the expected shelf-life. The deterioration of packaged foods may be caused by permeation and reactions between the foods and packaging components during storage. Furthermore, lipid oxidation is one of the most important chemical reactions occurring in foods that bring rancid flavours and aromas to make unpalatable and unacceptable products (Romani *et al.,* 2015). However, it is still unclear how these combinations of ingredients influence lipid oxidation and, thus, the shelf-life of biscuits, as different effects from the raw materials under different storage conditions were reported in these studies.

The objective was to determine the effect of storage conditions on the supplemented biscuits, after storing them under different temperatures and packaging conditions, on their antioxidant compounds, antioxidant capacities, colour, texture and lipid oxidation of the biscuits made from wheat-purple rice flour supplemented with defatted mussel powder and spices. The aim was to produce a balanced and healthy snack product with a long shelf-life. Therefore, the primary goal was to enhance the antioxidant compounds from purple rice flour and spices in the supplemented biscuits to assess their antioxidant usefulness as an ingredient in bakery foods.

7.2 Materials and Methods

7.2.1 Materials

Purple rice was purchased from the Big T supermarket in New Zealand. Refined wheat flour (Pams Products Ltd., Auckland, NZ), butter (Dairyworks, Christchurch, NZ), castor sugar (Pams Products Ltd., Auckland, NZ), baking powder (Edmonds Limited, Auckland, NZ), salt (Pams Products Ltd., Auckland, NZ), whole egg (Pams Products Ltd., Auckland, NZ), vanilla extract (Pams Products Ltd., Auckland, NZ), xanthan gum (Lotus foods Pty Ltd., Melbourne, Australia), and mixed spices - nutmeg (*Myristica fragrans* Houtt) and cinnamon (*Cinnamomum verum*) - were purchased from local markets in Christchurch, NZ. Galangal (*Alpinia galanga*) powder was imported by Sunson Asian supermarket, Riccarton, Christchurch, NZ. Defatted green-lipped mussel powder (*Perna canaliculus*) (containing 61.50% protein, 16.70% carbohydrate, 8.89% fat and 8.46% ash) was obtained by Aroma New Zealand Limited, Christchurch, NZ.

7.2.2 Biscuit preparation

The purple rice was milled using a Whisper Mill (Grote Molen Inc., Reno, USA) and the mixed flour made from a 50:50 blend of refined wheat flour and purple rice flour that had been passed through sieve of 60 mesh was used for biscuit preparation. The biscuits were prepared by Klunklin and Savage's (2018a) method. All ingredients are shown in Table 7.1. Control biscuits were prepared using refined wheat flour mixed with purple rice flour. The ingredients were mixed in a professional standard mixer (BBEK1092, Brabantia Branding B.V., Valkenswaard, Netherlands) to get a uniform mixture. According to the study of Klunklin and Savage (2018a), defatted green-lipped mussel powder was added to a standard biscuit formulation at 15% (on a flour basis) together with mixture of spices (mixed spices, nutmeg, cinnamon and galangal) at 8%. The supplemented biscuits were analysed for physicochemical properties and by sensory evaluation (Klunklin & Savage, 2018a). The biscuit dough was kneaded and sheeted to a uniform thickness of 10 mm using a rolling pin (Joseph Joseph 20085; Joseph Joseph Ltd., London, UK) then cut into circular shape using a 5 cm diameter biscuit cutter. Baking was carried out at 180°C for 9 min in an oven (Bakbar Turbofan 32 Max; Ali group company, Milan, Italy).

Table 7.1   Composition of the control and supplemented biscuits.

Ingredients (g)	Control	Supplemented biscuits
Refined wheat flour	100	100
Purple rice flour	100	100
Defatted mussel powder	-	30
Butter	150	150
Sugar	125	125
Egg	225	225
Salt	1.5	1.5
Mixed spices	-	4
Cinnamon	-	2
Nutmeg	-	2
Galangal	-	8
Sodium bicarbonate	9	9
Xanthan gum	2.5	2.5

7.2.3 Packaging and storage conditions

Biscuits (around 40 g or 3 pieces) were sealed into 120-micron polyethylene terephthalate (PET) pouches (140 x 100 mm, Pac Food PTY, LTD., NSW, Australia) and 110-micron aluminium foil laminate (AL) pouches (140 x 100 mm, Contour International, Bay of Plenty,

NZ) using a MEC Impulse Hand Sealer (Accolade Packaging Ltd., Auckland, NZ) (see Appendix C). Temperatures and relative humidity (RH) were recorded in a shelf-life determination incubator (Contherm Scienctific Ltd., Upper Hutt, NZ) using a Tinytag Ultra 2 (Gemini Data Loggers Ltd., Chichester, UK). Readings were taken every 5 min. The weekly averages of daily temperatures and RH inside the incubator were calculated for the 12 weeks of storage (Table B.2). The biscuits were evaluated at two-week intervals during 12 weeks of storage at 21.6 ± 0.4°C with a RH value of 50.3 ± 2.1% and 34.2 ± 0.1°C at 26.8 ± 1.4% RH using conventional methods. A pack was then removed from the incubator and analysed for various parameters, such as moisture content, hardness, colour, antioxidant compounds, antioxidant activity, free fatty acids and peroxide value. All analysis was carried out in triplicate.

7.2.4 Physical and colour analysis

The hardness of the biscuits was determined using a texture analyser (TA-XT2i, Stable Micro System, Godalming, UK) with a 30 kg load cell. The breaking strength of the baked biscuits was determined by performing a small three-point bending test rig with a sharp-blade cutting probe (Park, Choi, & Kim, 2015). The first peak force in Newtons (N) was recorded as the hardness. All measurements were repeated six times.

The colour parameters of biscuits were measured using a Minolta Chroma Meter CR-410 (Konica Minolta, Japan) and expressed using the CIE *L*a*b** scale (Sozer, Cicerelli, Heiniö, & Poutanen, 2014). All determinations were repeated six times.

7.2.5 Chemical analysis

The moisture contents (%) of the biscuits were measured in an oven at 105°C for 18 h (AACC, 2000). The nitrogen contents were determined using the Dumas method by rapid Max N exceed[®] (Elementar, Hanau, Germany) and multiplied by 6.25 to estimate the protein content according to AOAC method 920.177 (AOAC, 2000). Crude fat and ash were determined using AOAC methods 920.176 and 900.029, respectively (AOAC, 2000). The Megazyme starch assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland), approved method 76-13, was used to analyse the total starch content (Reed, Ai, Leutcher, & Jane, 2013). A total dietary fibre assay kit (Sigma-Aldrich, MO, USA) was used to determine total dietary fibre (TDF) of the samples. All measurements were carried out in triplicate.

7.2.6 Extraction and analysis of antioxidant compounds

Biscuit extractions were prepared according to the method reported by Jang and Xu (2009). To obtain the extracts, one gram of each sample was added to 10 mL of methanol acidified with 1.2 M HCl (50:50, v/v) and incubated in a water bath at 60°C for 3 h. The solutions were then centrifuged at 4500 g for 10 min. The extracts were kept at -20°C until further analyses were undertaken. The Folin-Ciocalteu method was used to quantify total phenolic compounds in the samples (Kaneda, Kubo, & Sakurai, 2006). The determination of anthocyanin content was carried out using the method of Hosseinian, Li, & Beta (2008). All determinations were measured in triplicate.

7.2.7 Evaluation of antioxidant capacity

The antioxidant activity of the samples was measured using the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging activity procedure according to the method of Re *et al.* (1999). The determinations were carried out in triplicate.

7.2.8 Fat extraction from the biscuits

Oils from the biscuits were extracted according to the procedure described by AACC (1987) for baked cereal products. These extractions were then used in the free fatty acid and peroxide determinations.

7.2.9 Analysis of free fatty acids (FFA) and peroxide value (PV)

One gram of extracted oil was titrated against 0.1 N potassium hydroxide (KOH) using the method of Cox and Pearson (1962). Peroxide values were determined using the international IDF standard method (74A:1991). This analysis was carried out in triplicate.

7.2.10 Statistical analysis

Data collected from all experiments were analysed with SPSS software (V. 22.0 for Windows, SPSS Inc., Chicago, IL) using a complete randomised design. The data means were analysed by Duncan's multiple range test to verify any significant differences between the means. A bivariate correlation matrix of data was analysed using Pearson's correlation coefficient. Statistical significance was considered to be at $P < 0.05$.

7.3 Results and discussion

7.3.1 Proximate analysis of the biscuits

The nutritional compositions of the biscuits are shown in Table 7.2. The protein content of the supplemented biscuits were significantly ($P < 0.05$) higher than the control biscuits. Dietary fibre increased following the addition of defatted mussel powder and spices to the biscuit mix. The ash content of the supplemented biscuits was found to be significantly higher ($P < 0.05$) than the control biscuit. The higher ash content of the supplemented biscuits compared to wheat-purple rice biscuits were due to the higher mineral contents of the defatted mussel powder and spices. The ash and fibre contents decreased with the increased extraction rate, whereas the higher protein contents and lower fat contents in the biscuits were responsible for the decrease in the water absorption capacity of purple rice flour compared to wheat flour (Klunklin & Savage, 2018b). The increased fat content in the supplemented biscuits could be due to the addition of the defatted mussel powder. However, fat from butter may also have been responsible for the deterioration of biscuits during storage (Boobier *et al.*, 2006). The total starch contents of the supplemented biscuits were significantly higher ($P < 0.05$) than the control biscuits. Similar nutritional compositions of biscuits were reported in our previous paper (Klunklin & Savage, 2018a).

Table 7.2 **Proximate analysis of control and supplemented biscuits.**

Parameters (% DM basis)	Control	Supplemented biscuits	LSD (5%)
Crude protein	6.81 ± 0.09	8.31 ± 0.11	0.31
Total dietary fibre	15.76 ± 0.11	23.60 ± 1.27	2.72
Ash	2.29 ± 0.01	2.63 ± 0.03	0.10
Total fat	26.54 ± 0.36	28.37 ± 0.18	0.85
Total starch	30.48 ± 0.27	28.12 ± 0.24	0.77

The results are expressed as mean $\pm$ standard error; LSD = least significant difference.

7.3.2 Effect of storage conditions on moisture content

Moisture content plays a vital role in influencing the stability of biscuits during storage. The changes in moisture content of the experimental biscuits are shown in Figs 7.1 and 7.2. Initially, the moisture contents of the control and supplemented biscuits were 5.1% and 4.8%, respectively. The changes in the moisture contents of the control biscuits were significantly ($P < 0.05$) higher than the supplemented biscuits under different storage temperatures and

packaging over 12 weeks. After the fourth week of storage, there was a significant increase in the moisture contents for all samples ($P < 0.05$). In particular, there were increases in the moisture content of the experimental biscuits stored in PET at $21.6 \pm 0.4°C$ and $34.2 \pm 0.1°C$. The moisture content in the biscuits stored in PET pouches significantly ($P < 0.05$) increased more than the biscuits stored in the AL pouches at 13%. From Table B.1, temperature, storage time and type of packaging influenced the moisture content during 12 weeks of storage. Similar, to the study of Jan, Saxena, & Singh (2017), the temperature during storage as well as packaging material played important roles that affected the moisture content of the biscuits. Biscuits with 5.2-5.8% moisture content became stale after six weeks of storage (Jan *et al.*, 2017).
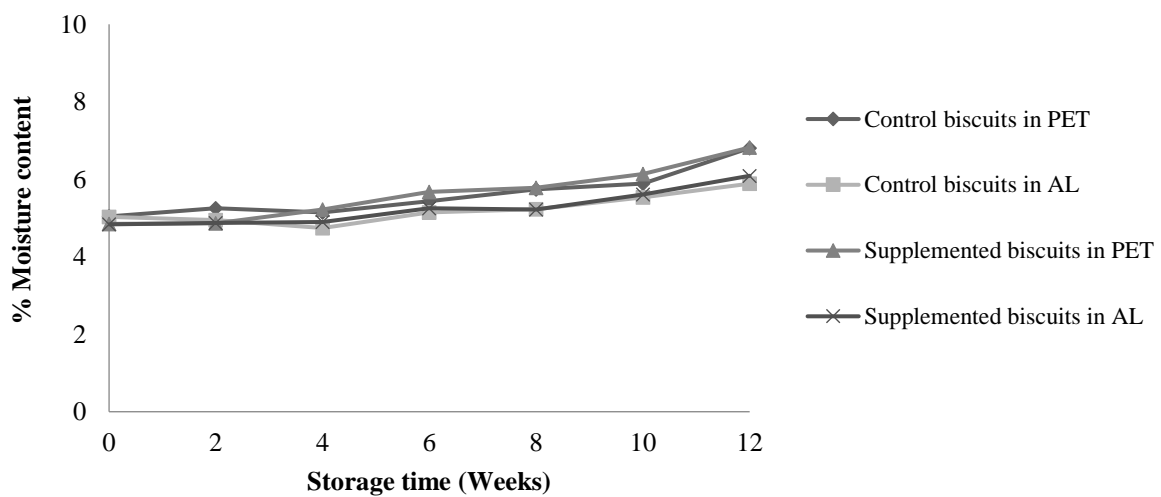
Figure 7.1   Effect of storage on moisture content (%) of biscuits during storage at 21.6 ± 0.4°C, 50.3 ± 2.1% RH for 12 weeks; PET = polyethylene terephthalate pouches; AL = aluminium foil laminate pouches.
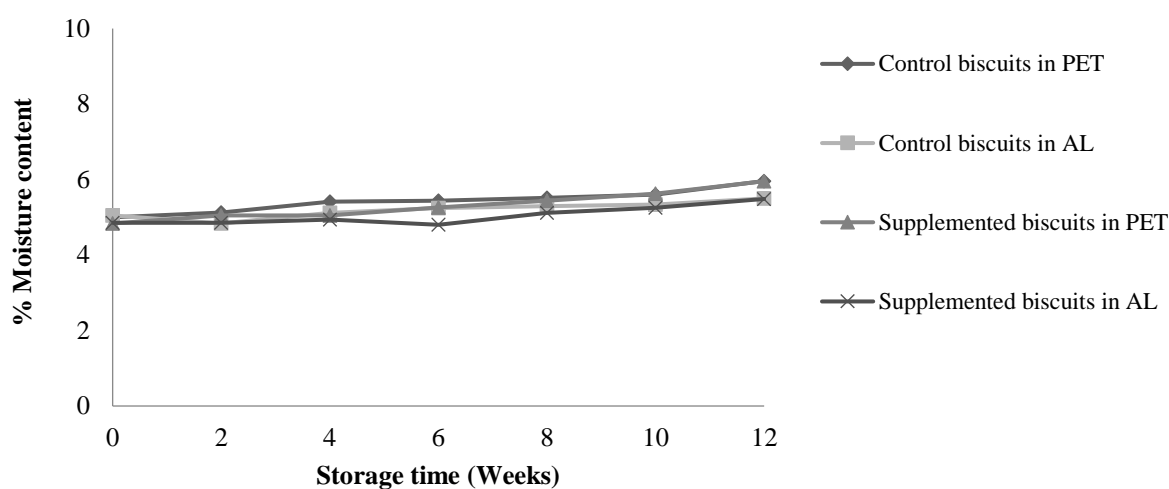
Figure 7.2 **Effect of storage on moisture content (%) of biscuits during storage at 34.2 ± 0.1°C, 26.8 ± 1.4% RH for 12 weeks; PET = polyethylene terephthalate pouches; AL = aluminium foil laminate pouches.**

7.3.3 Effects of temperature and packaging on the colour and physical properties of biscuits during storage

Colour changes during storage can be used as an indicator of the freshness of biscuits (Caleja, Barros, Antonio, Oliveira, & Ferreira, 2017). No significant differences ($P > 0.05$) in the *L*, a*, b** values of the biscuits were shown among the biscuits stored in two different temperatures and packaging (Table 7.3). Similar results were found in the study of Kumar *et al.* (2016) using a storage trial of chicken meat biscuits and Oliveira *et al.* (2017) confirmed that colour from anthocyanins were stable for up to 90 days of storage. The storage time is the only factor that caused significant differences in the colour parameters among the samples (Table B.1). Free radicals generated during lipid oxidation could have reacted with the protein in the biscuits, which could modify the colour parameters of the biscuits (Zamora & Hidalgo, 2005). However, the contribution of lipid oxidation in this study was low due to the addition of spices in the supplemented biscuits.

Hardness is one of the main criterions for determining the overall quality of biscuits (Jan *et al.*, 2017). Significant differences ($P < 0.05$) were observed in the hardness of the biscuits during storage. The effects of temperature and packaging materials on the hardness of the experimental biscuits are shown in Table 7.3. Hardness of the experimental biscuits decreased more in the

PET pouches (21%) compared to the AL pouches (19%) during storage of up to 12 weeks. For temperature, the biscuits stored at 21.6 ± 0.4°C and 34.2 ± 0.1°C softened by 17 and 22%, respectively. These results were similar to the values reported by Romani *et al.* (2015) who reported that the hardness of biscuits gradually decreased with increasing storage time. Biscuits supplemented with defatted mussel powder and spices had a small reduction in hardness compared to the control biscuits. While only a 1 or 2% change of moisture content occurred during storage, the changes in hardness of the biscuits were able to be noticed (Jenkins & Harrington, 1991). The decrease in hardness with increasing moisture contents (Figs 7.1 and 7.2) was due to decreases in the modulus of elasticity of the biscuit matrices and was similar to the results from Jan *et al*. (2017). Controlling the moisture content assures a good hardness quality for the biscuits since the hardness of biscuits is sensitive to moisture changes. Efficient sealing is an important parameter that affects the quality of biscuits since the biscuits might lose crispiness from moisture entering the package (Jan *et al*., 2017).

Table 7.3   Effect of storage at different temperatures and packaging on CIE *L*a*b** colour and hardness of the control and supplemented biscuits.

Physical parameters	Samples	Storage temperatures (°C)	Package types	Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
L*	Control	21.6 ± 0.4	PET	79.2 ± 0.4	79.3 ± 0.2	79.2 ± 0.4	79.4 ±0.6	77.4 ± 0.7	77.5 ± 0.5	77.5 ± 0.5
			AL	79.2 ± 0.3	79.4 ± 0.3	79.2 ± 0.7	79.4 ± 0.4	77.6 ± 0.9	77.5 ± 0.6	77.6 ± 0.5
		34.2 ± 0.1	PET	79.4 ± 0.4	79.5 ± 0.5	79.2 ± 0.4	79.4 ± 0.2	77.4 ± 0.8	77.4 ± 0.9	77.5 ± 0.4
			AL	79.1 ± 0.2	79.6 ± 0.4	79.2 ± 0.7	79.5 ± 0.4	77.5 ± 0.8	77.6 ±0.6	77.6 ± 0.4
	Supplemented biscuits	21.6 ± 0.4	PET	77.3 ± 0.5	77.5 ± 0.2	77.4 ± 0.5	77.3 ± 0.3	79.3 ± 0.6	79.4 ± 0.4	79.6 ± 0.5
			AL	77.4 ± 0.5	77.5 ± 0.4	77.4 ± 0.9	77.4 ± 0.6	79.3 ± 0.3	79.6 ± 0.6	79.1 ± 0.9
		34.2 ± 0.1	PET	77.6 ± 0.3	77.5 ± 0.3	77.5 ±0.7	77.4 ± 0.8	79.4 ± 0.5	79.4 ± 0.3	79.6 ± 0.8
			AL	77.4 ± 0.7	77.5 ± 0.6	77.4 ± 0.2	77.4 ± 0.6	79.5 ± 0.7	79.5 ± 0.1	79.6 ± 0.8
a*	Control	21.6 ± 0.4	PET	-3.2 ± 0.4	-3.2 ± 0.2	-3.3 ± 0.5	-3.2 ± 0.2	-3.2 ± 0.3	-3.2 ± 0.1	-3.2 ± 0.2
			AL	-3.1 ± 0.2	-3.2 ± 0.6	-3.2 ± 0.4	-3.2 ± 0.1	-3.2 ± 0.2	-3.2 ± 0.3	-3.2 ± 0.2
		34.2 ± 0.1	PET	-3.2 ± 0.3	-3.3 ± 0.3	-3.2 ± 0.4	-3.2 ± 0.3	-3.2 ± 0.3	-3.2 ± 0.6	-3.2 ± 0.4
			AL	-2.8 ± 0.4	-3.3 ± 0.5	-3.2 ± 0.3	-3.2 ± 0.4	-3.1 ± 0.1	-3.2 ± 0.4	-3.2 ± 0.3
	Supplemented biscuits	21.6 ± 0.4	PET	-4.3 ± 0.1	-4.4 ± 0.5	-4.3 ± 0.6	-4.3 ± 0.5	-4.3 ± 0.5	-4.3 ± 0.5	-4.3 ± 0.3
			AL	-4.4 ±0.2	-4.4 ± 0.2	-4.3 ± 0.1	-4.5 ± 0.6	-4.3 ± 0.4	-4.3 ± 0.4	-4.3 ± 0.1
		34.2 ± 0.1	PET	-4.3 ± 0.1	-4.2 ± 0.4	-4.2 ± 0.4	-4.3 ± 0.6	-4.2 ± 0.6	-4.3 ± 0.3	-4.3 ± 0.2
			AL	-4.2 ± 0.3	-4.4 ± 0.6	-4.4 ± 0.6	-4.3 ± 0.8	-4.2 ± 0.5	-4.3 ± 0.2	-4.3 ± 0.1
b*	Control	21.6 ± 0.4	PET	25.4 ± 0.5	25.7 ± 0.2	25.7 ± 0.3	25.7 ± 0.2	25.6 ± 0.5	25.4 ± 0.2	25.3 ± 0.2
			AL	25.3 ± 0.1	25.7± 0.3	25.8 ± 0.2	25.5 ± 0.3	25.7 ± 0.3	25.8 ± 0.1	25.2 ± 0.4
		34.2 ± 0.1	PET	25.2 ± 0.1	25.7 ± 0.3	25.5 ± 0.3	25.5 ± 0.2	25.7 ± 0.1	25.8 ± 0.3	25.2 ± 0.1
			AL	25.2 ± 0.2	25.6 ± 0.4	25.5 ± 0.2	25.8 ± 0.3	25.5 ± 0.2	25.6 ± 0.3	25.2 ± 0.3
	Supplemented biscuits	21.6 ± 0.4	PET	25.5 ± 0.3	25.7 ± 0.3	25.3 ± 0.3	25.6 ± 0.2	25.3 ± 0.4	25.3 ± 0.3	25.2 ± 0.3
			AL	25.7 ± 0.1	25.8 ± 0.2	25.6 ± 0.4	25.6 ± 0.1	25.6 ± 0.3	25.6 ± 0.2	25.2 ± 0.2
		34.2 ± 0.1	PET	25.5 ± 0.2	25.7 ± 0.5	25.5 ± 0.5	25.7 ± 0.2	25.6 ± 0.3	25.4 ± 0.2	25.1 ± 0.2
			AL	25.3 ± 0.2	25.7 ± 0.4	25.5 ± 0.2	25.6 ± 0.4	25.3 ± 0.4	25.5 ± 0.1	25.3 ± 0.2
Hardness (N)	Control	21.6 ± 0.4	PET	19.1 ± 0.3	17.3 ± 0.6	17.0 ± 0.3	16.7 ± 0.5	16.6 ± 0.6	16.2 ± 0.5	15.2 ± 0.5
			AL	19.1 ± 0.5	17.2 ± 0.9	16.8 ± 0.2	16.6 ± 0.3	16.4 ± 0.2	16.2 ± 0.3	15.9 ± 0.7
		34.2 ± 0.1	PET	19.1 ± 0.7	17.2 ± 0.6	16.9 ± 0.5	16.6 ± 0.1	16.4 ± 0.3	15.8 ± 0.5	15.3 ± 0.8
			AL	19.1 ± 0.8	17.1 ± 0.1	16.7 ± 0.3	16.5 ± 0.3	16.3 ± 0.3	16.0 ± 0.6	15.6 ± 0.6
	Supplemented biscuits	21.6 ± 0.4	PET	20.5 ± 0.8	19.4 ± 0.8	19.0 ± 0.5	18.9 ± 0.4	18.7 ± 0.2	18.5 ± 0.8	18.1 ± 0.5
			AL	20.5 ± 0.9	19.3 ± 0.2	19.2 ± 0.6	19.0 ± 0.6	18.9 ± 0.2	18.6 ± 0.6	18.4 ± 0.3
		34.2 ± 0.1	PET	20.4 ± 0.3	18.6 ± 0.2	18.2 ± 0.3	18.0 ± 0.8	17.9 ± 0.2	17.3 ± 0.8	16.8 ± 0.5
			AL	20.4 ± 0.5	18.4 ± 0.7	18.0 ± 0.2	17.9 ± 0.5	17.7 ± 0.5	17.5 ± 0.5	17.1 ± 0.4

The results are expressed as mean ± standard error. PET = polyethylene terephthalate; AL = aluminium foil laminate.

7.3.4 Antioxidant stability of biscuits during storage

The antioxidant compounds in methanolic extracts of the samples are shown in Table 7.4. As expected, the control biscuits showed lower levels of antioxidant compounds compared to supplemented biscuits enriched with defatted mussel powder and spices (Klunklin & Savage, 2018a). The total phenolics content (TPC) of the control biscuits packed in PET pouches was significantly ($P < 0.05$) reduced, by 57%, when stored at $21.6 \pm 0.4°C$ for 12 weeks, but the TPC of the biscuits packed in AL pouches was significantly ($P < 0.05$) reduced by 34% when compared to the freshly prepared biscuits. The supplemented biscuits also showed decreasing trends with higher amounts of TPC remaining in the samples compared to the control biscuits (Table 7.4). The highest TPC was recorded in the supplemented biscuits stored at $21.6 \pm 0.4°C$ in AL pouches, followed by biscuits stored at $21.6 \pm 0.4°C$ in PET pouches, then at $34.2 \pm 0.1°C$ in AL pouches and, finally they were stored at $34.2 \pm 0.1°C$ in PET pouches. The results agreed with the TPC, as antioxidant compounds are directly related to temperature, storage time and packaging of the storage conditions (Table B.1).

Anthocyanins are important flavonoids that provide colour to purple rice flour (Klunklin & Savage, 2018b). There was a small reduction in anthocyanin contents during the storage of biscuits kept in both PET and AL pouches at two different temperatures of 72% and 80%, respectively (Table 7.4). This decrease of anthocyanins during storage might be due to the polyphenolic structures, which are prone to oxidation and susceptible to oxidative degradation (Caleja *et al*., 2017). The highest decrease was observed in the control samples packed in PET pouches at $34.2 \pm 0.1°C$ as they allowed moisture to pass through the packages compared to the AL pouches (Galić, Ćurić, & Gabrić, 2009). The temperature and packaging influenced C3G in the experimental biscuits (Table B.1), while anthocyanins were reported to be thermostable compounds (Oliveira *et al*., 2017). However, the temperature and packaging did not show any statistical interactions between each of them for anthocyanin contents (Table B.1). After 12 weeks, the supplemented biscuits still contained higher antioxidant compounds than the study of Uthumporn, Woo, Tajul, & Fazilah (2015) who studied biscuits containing eggplant flour stored for 21 days.

Antioxidant activity is a balance between the loss of antioxidant compounds and the formation of antioxidant products from Maillard reactions that can occur in biscuits (Liang & Were, 2018). Results for the percentages of scavenging or inhibition by ABTS free radical activity

can also be observed in Table 7.4. There was a significant difference ($P < 0.05$) for the antioxidant activities in the biscuits when evaluated every two weeks. The supplemented biscuits stored at $21.6 \pm 0.4°C$ and $34.2 \pm 0.1°C$ in AL pouches also had the highest antioxidant activity for the different weeks among all samples, which agreed with the PV results (Figs 7.5 and 7.6). The antioxidant activity of the control biscuits decreased by 76% while, in the supplemented biscuits, it reduced by only 64% during 12 weeks of storage. Since the antioxidant activity of the supplemented biscuits increased after the incorporation of defatted mussel powder and spices, these biscuits showed significantly higher antioxidant activity than the control biscuits (Klunklin & Savage, 2018b). However, the temperature and packaging showed no significant differences in the samples stored under different conditions. A four-way ANOVA gave no significant results from the interaction between temperature and packaging on antioxidant activity, as assessed by an ABTS assay (Table B.1).

Table 7.4   Effect of storage at different temperatures and packaging on antioxidant compounds and antioxidant activity of control and supplemented biscuits.

Bioactive compounds	Samples	Storage temperatures (°C)	Package types	Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
Total phenolics (g GAE/100 g DW)	Control	21.6 ± 0.4	PET	4.28 ± 0.09	3.84 ± 0.08	2.82 ± 0.06	2.23 ± 0.02	2.22 ± 0.02	2.20 ± 0.01	1.84 ± 0.03
			AL	4.24 ± 0.02	3.89 ± 0.02	3.66 ± 0.08	3.16 ± 0.03	3.07 ± 0.04	2.98 ± 0.01	2.80 ± 0.05
		34.2 ± 0.1	PET	4.31 ± 0.16	3.33 ± 0.09	3.07 ± 0.08	1.84 ± 0.00	1.77 ± 0.02	1.64 ± 0.02	1.51 ± 0.03
			AL	4.25 ± 0.11	4.20 ± 0.21	3.29 ± 0.42	2.23 ± 0.02	2.14 ± 0.01	2.10 ± 0.09	1.67 ± 0.03
	Supplemented biscuits	21.6 ± 0.4	PET	5.49 ± 0.13	4.51 ± 0.13	4.36 ± 0.04	3.73 ± 0.03	3.64 ± 0.02	3.57 ± 0.06	3.48 ± 0.03
			AL	5.38 ± 0.28	4.99 ± 0.12	4.18 ± 0.02	3.84 ± 0.01	3.83 ± 0.02	3.78 ± 0.03	3.78 ± 0.05
		34.2 ± 0.1	PET	5.41 ± 0.10	4.65 ± 0.10	3.80 ± 0.05	2.81 ± 0.04	2.69 ± 0.01	2.47 ± 0.06	2.44 ± 0.09
			AL	5.47 ± 0.01	5.16 ± 0.06	3.87 ± 0.10	3.14 ± 0.09	3.03 ± 0.02	2.96 ± 0.01	2.92 ± 0.03
Anthocyanin (C3G, mg/kg DW)	Control	21.6 ± 0.4	PET	18.5 ± 0.3	15.8 ± 0.6	15.6 ± 0.4	14.8 ± 0.7	12.7 ± 0.5	11.2 ± 0.5	9.7 ± 0.8
			AL	18.6 ± 0.7	17.1 ± 0.3	17.1 ± 0.5	13.5 ± 0.4	12.2 ± 0.4	11.6 ± 0.5	9.8 ± 0.9
		34.2 ± 0.1	PET	18.4 ± 0.2	14.4 ± 0.4	13.8 ± 0.4	9.6 ± 0.1	7.1 ± 0.4	7.1 ± 0.7	4.6 ± 0.8
			AL	18.1 ± 0.3	16.8 ± 0.2	16.9 ± 0.2	11.1 ±0.8	8.4 ± 0.6	8.4 ± 0.3	7.1 ± 0.3
	Supplemented biscuits	21.6 ± 0.4	PET	27.1 ± 0.4	21.4 ± 0.3	20.3 ± 0.4	17.6 ± 0.6	15.3 ± 0.4	15.2 ± 0.4	12.2 ± 0.5
			AL	26.2 ± 0.8	24.4 ± 0.9	23.3 ± 0.5	23.2 ± 0.5	21.4 ± 0.6	16.1 ± 0.6	13.4 ± 0.5
		34.2 ± 0.1	PET	27.0 ± 0.5	20.2 ± 0.6	18.6 ± 0.4	17.4 ± 0.3	15.6 ± 0.4	14.8 ± 0.4	12.8 ± 0.4
			AL	27.1 ± 0.6	22.4 ± 0.9	21.5 ± 0.8	20.9 ± 0.5	14.8 ± 0.7	14.4 ± 0.7	13.1 ± 0.7
ABTS (μmol trolox/g DW)	Control	21.6 ± 0.4	PET	22.1 ± 0.5	18.6 ± 0.5	12.4 ± 0.7	9.8 ± 0.3	9.2 ± 0.4	7.9 ± 0.3	5.3 ± 0.1
			AL	22.1 ± 0.3	19.0 ± 0.1	13.3 ± 0.3	10.0 ± 0.6	9.2 ± 0.3	8.0 ± 0.2	5.3 ± 0.1
		34.2 ± 0.1	PET	22.2 ± 0.7	18.9 ± 0.2	13.0 ± 0.9	9.8 ± 0.8	9.1 ± 0.2	7.9 ± 0.2	5.3 ± 0.1
			AL	22.3 ± 0.6	19.7 ± 0.8	13.9 ± 0.8	9.7 ± 0.5	9.2 ± 0.6	8.0 ± 0.3	5.3 ± 0.1
	Supplemented biscuits	21.6 ± 0.4	PET	66.4 ± 1.3	51.6 ± 1.2	40.5 ± 0.9	38.8 ± 1.2	38.1 ± 1.4	35.4 ± 1.5	23.4 ± 0.8
			AL	66.9 ± 1.2	51.7 ± 1.5	40.7 ± 0.8	40.5 ± 1.5	39.2 ± 1.9	35.7 ± 1.3	23.6 ± 0.9
		34.2 ± 0.1	PET	65.6 ± 1.1	50.0 ± 1.4	40.1± 0.8	38.8 ± 1.6	38.3 ± 1.6	35.2 ± 1.8	23.5 ± 1.6
			AL	65.2 ± 0.9	51.7 ± 0.9	40.5 ± 1.0	39.0 ± 1.9	38.5 ± 1.7	35.2 ± 1.6	23.6 ± 1.5

The results are expressed as mean ± standard error. GAE, gallic acid equivalents; C3G, cyanidin-3-O-glucoside; ABTS, [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging activity; PET = polyethylene terephthalate; AL = aluminium foil laminate.

7.3.5 Free fatty acids in biscuits stored under different temperatures and packaging

The results of the free fatty acid (FFA) analysis for all biscuits are presented in Figs 7.3 and 7.4. Increasing trends were observed in all the stored biscuits. In general, the FFA content of food products do not dramatically change during normal storage periods (Zbikowska & Rutkowska, 2011). The initial values of FFAs in the control biscuits were 0.16 g/100 g oil, whereas there were 0.59 g/100 g oil in the supplemented biscuits. The results indicated that the highest increases in FFA values were noted after six weeks of storage and then the oxidation processes slowed down until week 8. The last week of storage (week 12) had the highest FFA values in the supplemented biscuits stored at $34.2 \pm 0.1°C$ in PET pouches (0.95 g/100 g oil) (Fig 7.4), which were considerably lower than the recommended maximum level of 5% FFAs in products intended for human consumption (Pomeranz, 1992). The supplemented biscuits with added defatted mussel powder contained more lipids than the control biscuits, which might have induced the considerable generation of oxidation products, as shown by the FFA values after 12 weeks of storage. However, the FFA of the control biscuits increased higher than in the supplemented biscuits during storage at $21.6 \pm 0.4°C$ and $34.2 \pm 0.1°C$ in PET pouches, by 70% and 34%, respectively. The experimental biscuits stored in AL pouches also showed a similar trend as Zbikowska and Rutkowska (2011) who stored biscuits under different storage conditions. Among the different packaging, there were little differences between the PET and AL pouches the biscuits were stored in, which is similar to the study of Nagarajaiah and Prakash (2015). The increase in FFA contents during storage generated the hydrolytic deterioration (rancidity) of triacylglycerols in the fat when the moisture content of the biscuits and air in the packaging reacted with the absorbed oil in the biscuits (Agrawal, Shirale, & Syed, 2017).
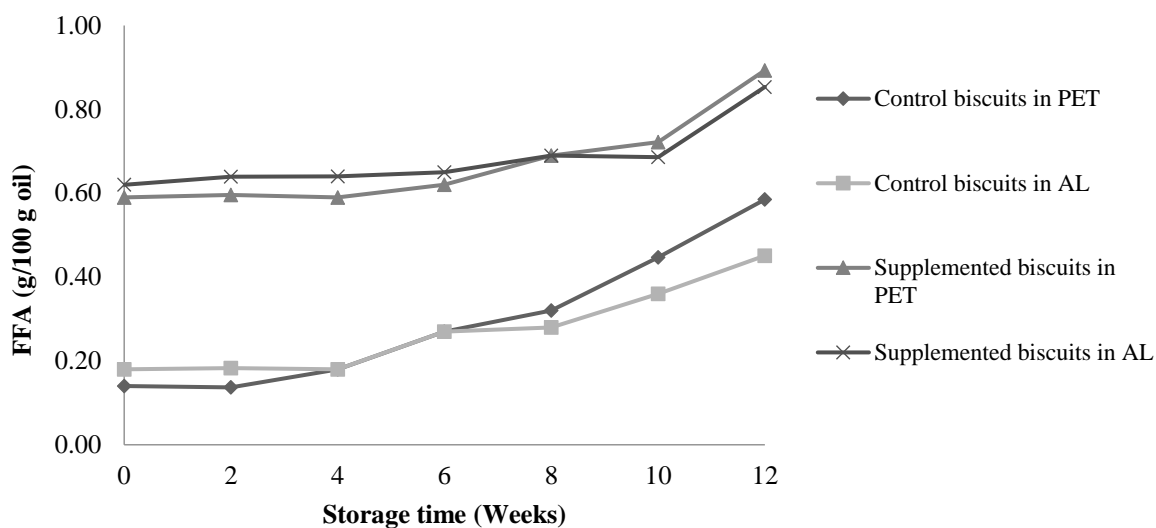
Figure 7.3 **Effect of storage on free fatty acids (FFA, g/100 g oil) in biscuits during storage at 21.6 ± 0.4°C, 50.3 ± 2.1% for 12 weeks; PET = polyethylene terephthalate pouches; AL = aluminium foil laminate pouches.**
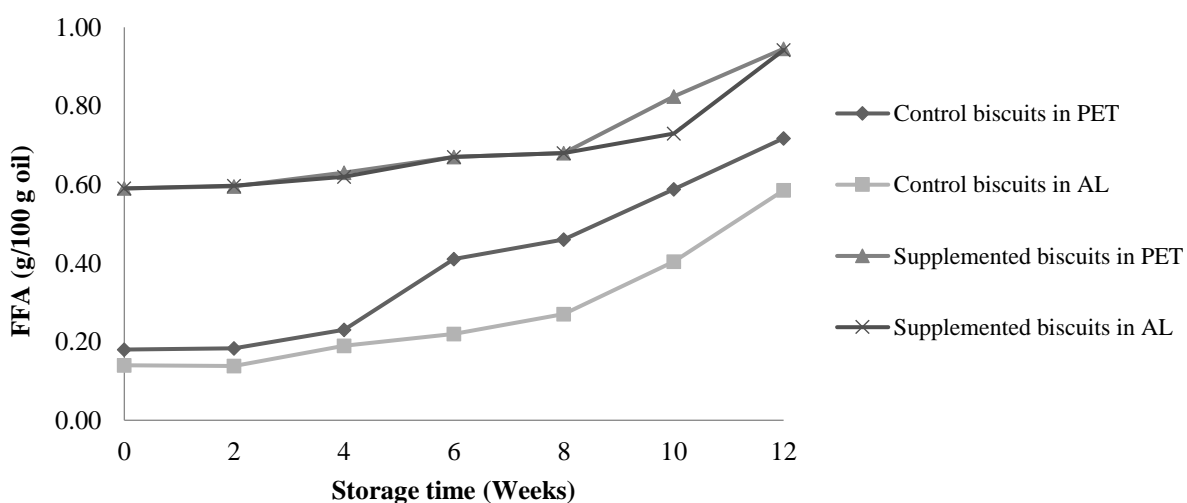
Figure 7.4 **Effect of storage on free fatty acids (FFA, g/100 g oil) in biscuits during storage at 34.2 ± 0.1°C, 26.8 ± 1.4% RH for 12 weeks; PET = polyethylene terephthalate pouches; AL = aluminium foil laminate pouches.**

7.3.6 Peroxide values in biscuits stored in different temperatures and packaging

Supplementation of biscuits with antioxidants has been shown to prolong their shelf-life (Caleja *et al.,* 2017). The lipid fractions extracted from biscuits are presented in Figs 7.5 and 7.6. Initially, the PV in all samples was very low and then significantly increased over each two-week interval ($P < 0.05$). However, a small increase in PV was observed in biscuits stored at $21.6 \pm 0.4°C$ (77%) compared to biscuits stored at $34.2 \pm 0.1°C$ (83%), which produced more PV in the same type of packaging. Hence, biscuits store best at lower temperatures. It is commonly known that PV depends on temperature, time and light during storage (Jan *et al.,* 2017). The supplemented biscuits stored at $21.6 \pm 0.4°C$ in AL pouches had the lowest PV during storage (1.7 meq. O_2/100 g oil), followed by the supplemented biscuits stored at $21.6 \pm 0.4°C$ in PET pouches (1.8 meq. O_2/100 g oil) and then the biscuits stored at $34.2 \pm 0.1°C$ in AL pouches (1.9 meq. O_2/100 g oil). A slow rise in PV was observed in the supplemented biscuits, revealing the effectiveness of the combination of defatted mussel powder, purple rice flour and spices in stabilising the biscuits due to the high levels of antioxidants in the biscuits. This result agrees with several earlier studies (Caleja *et al*., 2017; Nanditha, Jena, & Prabhasankar, 2009; Žilić *et al.,* 2016). However, the effect of the antioxidant compounds on PV depended on many factors, including the structure of the antioxidant, oxidation conditions and the nature of the sample being oxidised (Buaniaw *et al.,* 2010). The packaging material also influences the quality of foods during storage (Romani *et al.,* 2015). Thus, the AL pouches proved to be a better packaging material for biscuits than PET and the biscuits packed in AL pouches could be stored for longer at $21.6 \pm 0.4°C$. The difference in hardness (Table 7.3) among the biscuits stored at different temperatures and packaging materials were also related to the PV of the lipid fraction during storage (Nanditha *et al*., 2009). However, the PVs were within acceptable limits (permissible limit for peroxide value is 10 meq. O_2/kg while meq. O_2/100 g is shown in the following graphs).

Figure 7.5 **Effect of storage on peroxide values (PV, meq. O_2/100 g oil) in biscuits during storage at $21.6 \pm 0.4°C$, $50.3 \pm 2.1\%$ for 12 weeks; PET = polyethylene terephthalate pouches; AL = aluminium foil laminate pouches.**

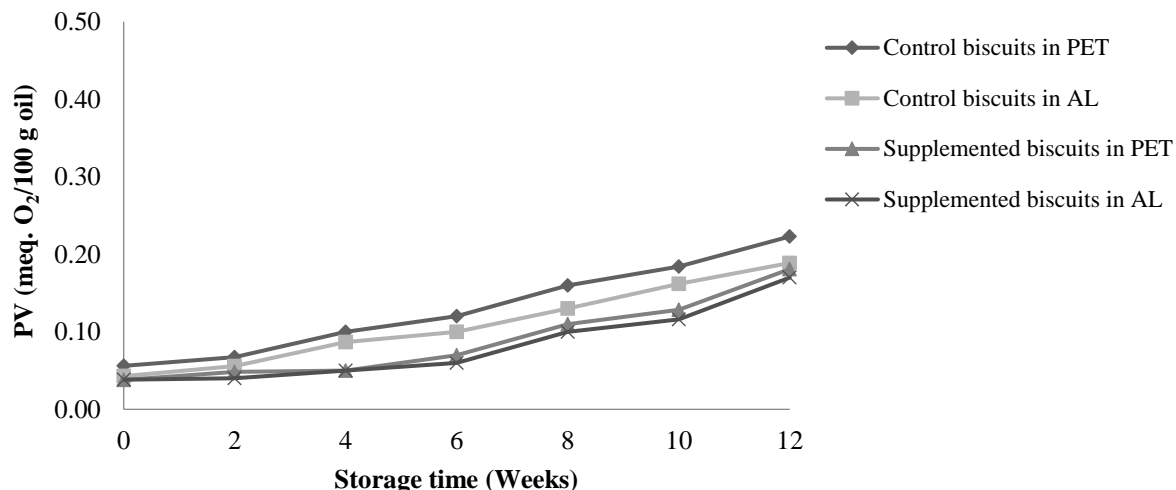**Figure 7.6** **Effect of storage on peroxide values (PV, meq. O_2/100 g oil) in biscuits during storage at $34.2 \pm 0.1°C$, $26.8 \pm 1.4\%$ RH for 12 weeks; PET = polyethylene terephthalate pouches; AL = aluminium foil laminate pouches.**
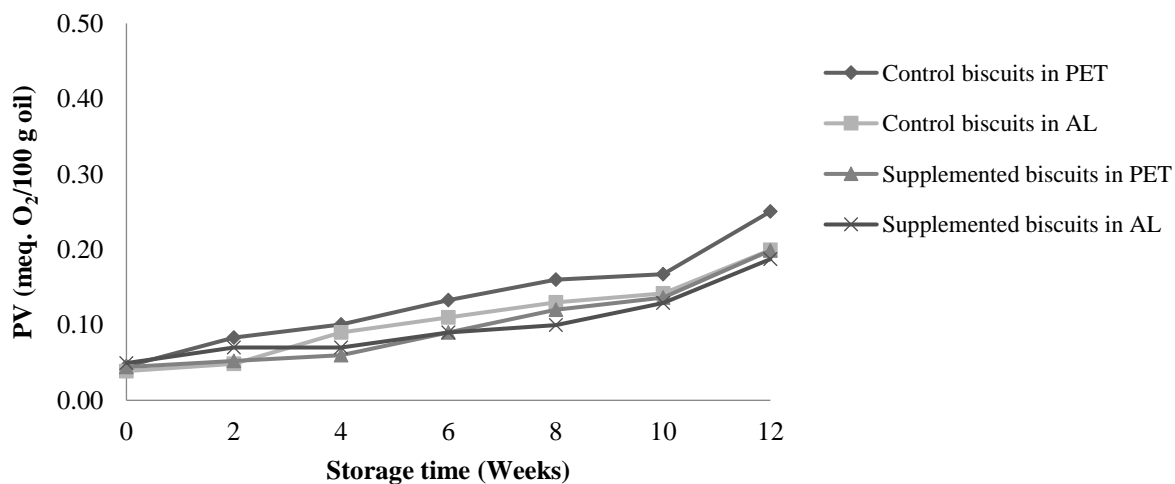
7.3.7 Correlation of temperatures and packaging of the experimental biscuits and all other parameters measured

The Pearson's correlation coefficient (r) among the parameters is shown in Table 7.5. As expected, the moisture content and hardness correlated negatively to each other; the higher moisture content, the softer the biscuits; and this was in agreement with Butt, Nasir, Akhtar, & Sharif (2004). Both parameters are influenced by temperature; however, only the moisture content showed a significant correlation with the packaging ($r = -0.350$, $P < 0.01$). Those parameters (moisture content and hardness) also significantly ($P < 0.01$) correlated with the antioxidant compounds and antioxidant activity (Table 7.5). It can be seen from the results that biscuits showing high antioxidant levels would produce lower PVs due to their negative correlations. There is a direct correlation between antioxidant compounds and antioxidant activity, which is similar to the results presented in a previous study (Klunklin & Savage, 2018a). Temperature showed a negative correlation with antioxidant compounds and no correlation with antioxidant activity. In this case, the decrease in antioxidant compounds of the biscuits was due to the increase in the temperature of the storage conditions, as the moisture content had an inverse relation with the antioxidants in the biscuits. A significant negative correlation was observed between PV and anthocyanins ($r = -0.799$, $P < 0.01$); likewise, the FFA showed a strong correlation with ABTS ($r = -0.454$, $P < 0.01$) without any correlations with temperature and packaging during storage.

Table 7.5   Pearson's correlation coefficients (*r*) of the variables analysed to determine physicochemical, antioxidants, peroxide and free fatty acids of the experimental biscuits stored in two different packaging materials during 12 weeks of storage.

Variables	Hardness	Anthocyanin	ABTS	PV	FFA	Temperature	Packaging
Moisture content	-0.561**	-0.608**	-0.429**	0.817**	0.420**	0.176*	-0.350**
Hardness	1	0.877**	0.880**	-0.743**	0.212**	-0.186	NS
Anthocyanin	0.877**	1	0.854**	-0.799**	NS	-0.157*	NS
Total phenolic	0.885**	0.862**	0.785**	-0.758**	NS	-0.251**	NS
ABTS	0.880**	0.854**	1	-0.613**	-0.454**	NS	NS
PV	-0.743**	-0.799**	-0.613**	1	0.301**	NS	NS
FFA	0.212**	NS	-0.454**	0.301**	1	NS	NS

**Significant at *P* < 0.01 level, *Significant at *P* < 0.05.

7.4 Conclusions

A combination of defatted mussel powder, purple rice flour and spices did not protect the reduction of antioxidant compounds in biscuits during the storage trial. However, the retention rate of antioxidant compounds in the biscuits was still high. Temperature seemed to have a huge impact on the quality of the biscuits during storage. A change in the moisture content during storage had an influence on many parameters, such as hardness, antioxidant compounds, antioxidant activity, PV and FFA due to differences in temperature and packaging material. Nevertheless, the colour parameters did not change during the storage trial. AL pouches proved to be a better packaging material for biscuits than PET and the biscuits packed in it could be successfully stored for six weeks, whereas, for the PET packaging the shelf-life of the biscuits appeared to be approximately four weeks. The keeping quality of biscuits was enhanced at low temperatures when stored in good quality packaging.

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Chapter 8

General discussion and conclusions

8.1   General discussion

The aim of this thesis was to determine the nutritional properties of biscuits where some of the wheat flour had been replaced with finely ground Thai purple rice flour. Further experiments were carried out to enrich the recipe with defatted green-lipped mussel powder and spices commonly used in Thailand. The reformulated, cooked biscuits were then evaluated to measure their proximate analyses, physicochemical properties, antioxidant compounds and antioxidant activities. Standard taste tests were also carried out to determine the responses from four different ethnic groups to the new formulations. In addition, biscuits containing purple rice flour were placed into two different types of packaging and stored at two different environmental temperatures to determine the stability of the nutrients in the fortified biscuits to long-term storage.

Objective 1: To investigate the functional properties of flour mixtures made from purple rice with wheat flour substitutions at different levels to give improved functional properties.

Substitution of wheat flour with purple rice flour can produce a natural and cheaper basic biscuit mixture that has an interesting colour and improved nutritional characteristics. It is important, however, to understand how the new mixture will behave when mixed with other ingredients and cooked. Since increasing the substitution levels of purple rice flour in the biscuit recipe will modify the nutritional composition and functional characteristics of the flour mixtures, it is important that the proximate components, such as fibre, protein, carbohydrate and fat in the flour mixtures, are accurately determined (Chandra, Singh, & Kumari, 2015). The assessment of different substitution levels of purple rice flour for wheat flour is crucial, as the fundamental physicochemical properties that reflect the complex interactions between the composition, structure and physicochemical properties of the biscuits will be altered. Purple rice flour has high swelling index due to its higher amylopectin and fat contents compared to wheat flour. Moreover, the substitution of purple rice flour for wheat flour showed a lower overall foam capacity and stability compared to 100% wheat flour biscuits. Increasing the substitution levels of purple rice flour in the flour mix reduced the interactions among the binding forces which, in turn, decreased the gelling ability of the flour. These were desirable attributes for flours used

to produce biscuits and crackers. Therefore, the functional properties of flour mixtures provide useful information to assist in the formulation of new products that are acceptable to consumers.

Whole (100%) purple rice flour had the highest total dietary fibre contents compared to the other flour mixtures. Increasing the purple rice flour substitution level significantly reduced ($P < 0.05$) the protein content and, at the same time, increased the protein digestibility of the flour mixtures. *In vitro* protein digestibility can predict true protein digestibility with a quick and more convenient process compared to the *in vivo* method (Varastegani, Zzaman, & Yang, 2015). In this study, the increased protein digestibility implied that protein availability in purple rice flour for intestinal absorption after digestion reflected the increased efficiency of protein utilisation in the diet. To the best of our knowledge, this is the first study to measure the protein digestibility of purple rice flour.

Purple rice starch is, typically, not very digestible and contains high levels of amylopectin, which may be retrograded during the hydrolysis will make these flour mixtures less sensitive to digestion (An, Bae, Han, Lee, & Lee, 2016). The substitution of purple rice flour in the flour mixture produced a lower rapidly digestible starch (RDS) with a higher slowly digestible starch (SDS) compared to wheat flour. In addition, purple rice flour had the lowest predicted glycaemic index (pGI) calculated from the hydrolysis index derived from starch digestibility experiments. The variation in the proportions of RDS, SDS and the macronutrient contents in flour (fibre, protein and fat) had a distinct impact on the pGI of the flour mixtures. Overall, the starch digestion rates were significantly reduced after the substitution of purple rice flour in the flour mixture. Based on the results of An *et al.* (2016), the starch digestibility of purple rice flour is related to its phenolic contents, which reduced the rate of glucose released in the *in vitro* digestion method. It is expected that this lowered rate of glucose release would also occur *in vivo*, potentially lowering blood glucose increases following the consumption of these biscuits (Thompson, Yoon, Jenkins, Wolever, & Jenkins, 1984; Ramdath, Padhi, Hawke, Sivaramalingam, & Tsao, 2014).

Anthocyanins (cyanidin-3-O-glucoside, C3G) are found at high levels in whole purple rice flour (492.6 mg/kg DW), which is supported by the studies of Rocchetti *et al.* (2017) and Shao *et al*. (2018). In contrast, refined wheat flour has a much lower anthocyanin content (0.2 mg/kg DW). Moreover, whole purple rice flour is rich in phenolic compounds and these are mainly anthocyanins. Total flavonoids from whole purple rice flour were similar to the report by Kim and Kim (2017). Antioxidants are the predominant compounds accumulated in purple rice flour and they also created a nice colour in the flour mixtures, since, there is a correlation between

the redness and anthocyanins. Both ABTS and DPPH assays also had highly positive correlations with antioxidant compounds among all the flour mixtures.

Objective 2: To study whether different proportions of flour mixtures in a standard biscuit mix affect biscuits characteristics and taste as tested by panellists.

Biscuits were prepared with different substitution levels of purple rice flour. The whole purple rice flour biscuits had the highest moisture contents due to their high fibre contents. Purple rice flour substitutions significantly increased the fibre contents ($P < 0.05$) compared to the control wheat flour biscuits. The total fat contents of the biscuits were not significantly different as the majority of the fat in the final product was supplied by the butter added to the recipe. Yawadio, Tanimori, & Morita (2007) also confirmed that the dietary fibre in pigmented rice is much higher than in white rice. Moreover, the protein content of the purple rice biscuits was lower than in the wheat biscuits. These results were similar to the study by Torbica, Hadnađev, & Hadnađev (2012) who reported a decrease in the protein contents observed in biscuits substituted with rice flour in place of wheat flour. The majority of commercial biscuits contain low amounts of dietary fibre. Thus, increasing dietary fibre following the substitution of purple rice flour could help lower the rate of starch digestion and that provides many health benefits.

When purple rice flour was used to produce biscuits, it can reduce the release of glucose, with its low pGI during *in vitro* digestion due to the fibre and proteins in the purple rice acting as a barrier, thus, lowering the overall starch digestibility. This study confirms the results from a previous study (Objective 1) that purple rice flour had a slower release of glucose compared to wheat flour in a standard biscuit mix. Moreover, the trend of the pGI of biscuits made from wheat-purple rice blend flours was similar to the study by An *et al.* (2016). Only a few studies have focused on the *in vitro* protein digestibility of purple rice so far and no literature is available on the *in vitro* digestibility of purple rice flour biscuits. However, the positive results reported in this study were due to the higher rate of protein digestibility in the wheat-purple rice biscuits compared to the wheat biscuits.

The increase in redness of purple rice flour contained in the fortified biscuits could be attributed to the presence of purple pigments in a bran layer of purple rice from its antioxidant compounds. Although the stability of antioxidant compounds in purple rice can be affected by thermal processing, the retention rates of antioxidant compounds were still high in the biscuits substituted with purple rice flour. This could be an advantage for consuming these fortified biscuits.

The textural quality study of the fortified biscuits revealed that the hardness decreased with the increased addition of purple rice flour, since purple rice flour absorbed more water than wheat flour (Giuberti, Marti, Fortunati, & Gallo, 2017). Biscuits made from whole (100%) purple rice flour had the highest spread ratio (greater width and thinner). This is considered to be a positive characteristic for the overall acceptability of biscuits. However, the overall acceptability of wheat-purple rice flour biscuits was slightly different ($P < 0.05$) compared to the wheat biscuits, as tested by untrained panellists. The purple rice flour had a purple colour because of its high antioxidant content, which led to a reduction in sensory liking scores for the overall appearance and colour of the fortified biscuits. In this case, however, substitution of 25 or 50% purple rice flour showed only slightly lower overall acceptability compared to the refined wheat flour biscuits. Biscuits containing 75% purple rice flour were not readily accepted in taste tests. Overall, biscuits containing 50% purple rice flour were readily accepted because they had only slightly altered textural characteristics from the increased levels of bioactive compounds.

Objective 3: To identify the optimum amounts of defatted mussel powder and Thai spices that can be added to prepare a balanced and nutritious product acceptable to Caucasian, Chinese, Pacific Island and Thai ethnic groups.

The enrichment of purple rice biscuits with defatted green-lipped mussel powder increased the total protein and digestible protein contents, which are positive features to add to a carbohydrate rich food, such as biscuits. Vitali, Dragojević, & Šebečić (2009) and Abdel-Moemin (2015) reported that the protein digestibility of wheat-based biscuits was lower than observed in this study. Nørgaard, Petersen, Tørring, Jørgensen, & Lærke (2015) confirmed that adding mussel protein increased the overall digestibility of the protein. The enrichment of defatted mussel powder in this study was successful in improving the nutritional value, especially protein, in the biscuit mix. Apart from the increasing benefits of the protein contents, the fortified biscuits had lower starch digestibility compared to the wheat-purple rice biscuits, due to the higher fibre content of the defatted mussel powder and spices. This combination contributed to a beneficial improvement in the nutritional value of the biscuits. The addition of defatted mussel powder and spices decreased their width and spread ratio and increased the hardness of the biscuits due to the higher proportion of protein in the biscuit mix.

Increasing levels of defatted mussel powder significantly increased ($P < 0.05$) the levels of bioactive compounds, such as total phenols and total flavonoids, in the biscuits. Gorinstein *et al.* (2003) reported that black mussel powder contained total phenolic compounds that ranged between 3.8 and 6.5 mg/kg DW, and this was lower than found in this study. In contrast, the

incorporation of defatted mussel powder had no effect on C3G in the fortified biscuits. However, there has been no research on the anthocyanin contents of defatted green-lipped mussel powder. The fortified wheat-purple rice biscuits enriched with defatted mussel powder and spices contained high levels of bioactive compounds as they showed strong antioxidant activity in both the DPPH and ABTS assays. This was a very positive feature in the biscuits, which had an excellent antioxidant effect when tested using these *in vitro* methods.

Sensory evaluation plays an important role in quality control during the development of new products. The liking scores from panellists drawn from different ethnic groups showed that Pacific Islanders liked the defatted mussel powder containing biscuits. The familiarity of the consumers with ingredients or types of food products is one of the most important factors affecting consumers' preferences (Polet & Bocherens, 2016). Pearson's correlation (r) of the texture analysis and texture preference scores from the sensory evaluation study was positively related to each other ($r = 0.58$, $P < 0.01$). The fortified biscuits with 10% defatted mussel powder did not show any significant differences ($P > 0.05$) in liking scores for colour and overall appearance compared to the control biscuits. The addition of spices was thought to balance the overall taste of the fortified biscuits and the levels added were higher than found in the other commercial products studied by Vijaykrishnaraj, Kumar, & Prabhasankar (2015) and Vijaykrishnaraj, Roopa, & Prabhasankar (2016).

Objective 4: To measure the effect of adding spice mixtures on the antioxidant content of the biscuits and acceptability of added defatted mussel powder.

Mixed spices, nutmeg, cinnamon and galangal were added to the biscuit mix in this study to improve the taste, antioxidant contents, and to balance the overall smell and taste of the biscuits so the amount of defatted mussel powder could be increased. Defatted mussel powder biscuits mixed with spices contained higher total dietary fibre than mussel biscuits mixed with ginger and galangal (Objective 3). All biscuits in this study can be claimed as high dietary fibre foods (Official Journal of the European Union, 2006). The total dietary fibre levels affected the hardness of the biscuits but the hardness of the fortified biscuits was in the same range of many commercial biscuits (Chung, Cho, & Lim, 2014). The darkness of the fortified biscuits increased compared to the control due to the Maillard reaction, the addition of protein from defatted mussel powder and the purple colour from the purple rice flour. The antioxidant compounds increased compared to the previous experiment (Objective 3). However, it is interesting to note that the anthocyanin content of the fortified biscuits significantly increased ($P < 0.05$) with the increasing levels of defatted mussel powder. The increase in C3G came

from the spices that were incorporated in slightly higher amounts than in the biscuit mixes. Further studies are needed to measure the anthocyanin contents of purple rice flour, spices and defatted mussel powder. The success of this study was to find that there were no significant differences in the overall acceptability between the fortified biscuits as tested by 107 untrained panellists. Therefore, the incorporation levels of defatted mussel powder could be increased from 10 to 15% and still maintain an overall acceptable taste.

Objective 5: To determine the storage characteristics of the optimum biscuit mixtures using two different types of packaging at two different temperatures.

Shelf-life determination is of great importance for the food industry to ensure that consumers will receive a high-quality product for an acceptable time after purchase. Hence, the shelf-life of a packaged food product should be determined after storage at normal room temperatures and at elevated temperatures. The optimum biscuits supplemented with 15% defatted green-lipped mussel powder and 8% of spices from Objective 4 were stored at two different temperatures (21.6 ± 0.4°C and 34.2 ± 0.1°C) and packaging materials (polyethylene terephthalate, PET and aluminium foil laminate, AL) for 12 weeks. Differences in food composition attributed to different recipes may affect the shelf-life (Kumar *et al*., 2016). The temperature and packaging during storage played important roles in the moisture content of the biscuits and also had a negative correlation with the hardness of the control and supplemented biscuits. Nevertheless, the antioxidant compounds and antioxidant activity of the control and supplemented biscuits were affected by the storage conditions. Antioxidants remaining in the biscuits can still inhibit lipid oxidation of other food compounds due to the low contents of free fatty acids and the peroxide values of the stored biscuits. The fortification of defatted mussel powder and spices was successful in prolonging the shelf-life of the biscuits for up to 12 weeks.

8.2 Conclusions

In regard to nutritional problems from eating biscuits, it was necessary to improve their nutritional values; in particular, their protein and dietary fibre contents. The use of traditional ingredients that are easily found in different regions and their by-products are important to provide familiar tastes from the local people's diet. The wheat-purple rice biscuits with increased levels of defatted mussel powder and spices might be acceptable for Thai people as they contained increased levels of digestible protein and reduced rates of starch digestion. The substitution of purple rice flour in the biscuits altered the functional properties of the flour in positive ways that are suitable characteristics for producing biscuits. Wheat-purple rice biscuits

were softer than the refined wheat flour biscuits; however, the fortified biscuits enriched with defatted mussel powder and spices were harder than the wheat-purple rice biscuits, which might be considered to be preferable for most consumers. The fortified biscuits were high in antioxidant compounds obtained from the fortification of the recipe from the incorporation of purple rice flour, defatted mussel powder and spices. The significant increase in antioxidants with high antioxidant activities was a good feature in the experimental biscuits. Changes in the colour of the biscuits associated with antioxidant compounds could affect the liking scores of the panellists. However, the fortified biscuits in this study were acceptable compared with refined wheat flour biscuits with a tendency to have a more prolonged shelf-life after the addition of antioxidants. The use of supplementing purple rice flour with spices may lead to a reduction in the price of the ingredients as purple rice is locally grown in Asian countries where it was readily available as part of Asian diets.

8.3   Recommendations for further studies

This research project started with a plan to replace wheat flour with purple rice flour. The initial idea was to reduce the amount of imported wheat flour in biscuits with a locally-grown rice flour, which would give an interesting purple colour to the final products. In the second stage the enrichment of the biscuits with defatted green-lipped mussel powder, along with typical Thai spices, was investigated. The idea was to develop a product that would be accepted by people in Thailand. The addition of purple rice flour and defatted mussel powder changed the characteristics of the biscuits and improved the overall nutritional characteristics, particularly the antioxidant contents of the biscuits.

The nutritional characteristics of the biscuits could be further improved by reducing the butter and sugar contents. There is some scope to investigate the replacement of the sugar contents in the biscuits with artificial sweeteners, but this can lead to difficulties maintaining a sufficiently sweet taste and acceptable structure in the cooked biscuits. The overall objective of producing a biscuit that contains a greater range of positive nutritive components while retaining a positive texture and a positive consumer acceptance is a complex goal to achieve.

In vitro digestion methods were used in this study to measure the digestibility of the carbohydrate and protein fraction of the biscuits. These studies should be expanded to include *in vivo* experiments using rats to confirm that the new formulations were improved by adding rice flour to the biscuit recipe.

Further studies should investigate the mineral contents of the purple rice containing biscuits as purple rice is known to contain a wider range of minerals and knowledge of this would be a positive promotional feature for a new type of biscuit.

An investigation of different mixtures of spices needs to be carried out and the changes confirmed using consumer appreciation studies with different ethnic groups. The texture, taste and colour of a product are probably more important to a consumer than the improved nutritional qualities.

The concept of adding purple rice flour to wheat flour along with defatted mussel powder and spices could be used in a number of products, such as extruded snacks, breakfast cereal bars or chips. This would encourage the use of more home-grown rice and spices, which could lead to the possibility of reduced production costs.

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Appendix A

Research outline

2 Introduction
Purple rice flour has never been incorporated into biscuits and their resulting characteristics are not known

↓

3 **Flour mixtures**
0, 25, 50, 75 and 100% purple rice flour

Purple rice flour has a low pGI with a high protein

↓

4 Biscuits were made from different flour mixtures 0, 25, 50, 75 and 100% purple rice flour

Panellists preferred 50% purple rice flour biscuits

↓

Addition of defatted mussel powder and spices into biscuit mixes

↓

5 Adding ginger and galangal to purple rice - wheat flour mixture containing 10, 15 and 20% mussel powder

6 Adding mixed spices, cinnamon, nutmeg and galangal to purple rice - wheat flour mixture containing 10, 15 and 20% mussel powder

Pacific Islanders preferred biscuits containing 10% mussel powder

Panellists preferred biscuits containing 15% mussel

↓

7 Control biscuits (50% purple rice flour mixture) and fortified biscuits (15% mussel powder mixed with 8% spices) were stored at different temperatures (21.6 and 34.2°C) and in two different packages (PET and AL) for 4 months

Fortified biscuits containing 8% spices keep better than the control

↓

8 Conclusion
The fortified biscuits have a high nutritional value and good texture with an acceptable taste

Appendix B

Supplemented data for chapter 5

Table A.1 Pearson's correlation coefficients (*r*) of variable analysed to determine physicochemical and sensory characteristics of the fortified biscuits prepared from wheat-purple rice flour blends enriched with defatted mussel powder and spices.

Parameters	Proximate analysis				L*	Colour		Hardness	Moisture content	Biscuit qualities		Sensory evaluation						
	Crude protein	Crude fibre	Total fat	Total starch		a*	b*			Width	Thickness	Appearance	Colour	Texture	Oiliness	Crunchiness	Flavour	Overall acceptability
Crude protein	1.00																	
Crude fibre	0.97*	1.00																
Total fat	0.82*	0.91*	1.00															
Total starch	-0.66*	-0.76**	-0.78**	1.00														
L*	-0.50	-0.45**	-0.25	0.38	1.00													
a*	0.81*	0.82**	0.67	-0.70*	-0.39	1.00												
b*	0.563	0.63*	0.62*	-0.60*	0.08	0.85**	1.00											
Hardness	0.72**	0.70*	0.58*	-0.54	-0.33	0.58*	0.46	1.00										
Moisture content	0.80**	0.73**	0.62*	-0.31	-0.25	0.36	0.21	0.57	1.00									
Width	-0.64*	-0.64*	-0.44	0.43	0.41	-0.79**	-0.62**	-0.77**	-0.22	1.00								
Thickness	0.92**	0.83**	0.61*	-0.49	-0.51	0.81**	0.54	0.83**	0.65*	-0.92**	1.00							
Appearance	-0.03	-0.07	-0.00	0.28	0.69	0.05	0.42	0.11	0.11	-0.16	0.17	1.00						
Colour	-0.29	-0.30	-0.16	0.29	0.68	-0.18	0.23	0.07	-0.10	-0.01	0.05	0.54	1.00					
Texture	0.26	0.23	0.12	-0.11	-0.33	0.16	0.05	0.60	0.14	-0.41*	0.36	0.39	0.39	1.00				
Oiliness	0.47	0.41	0.29	0.02	-0.28	0.13	0.08	0.22	0.71*	-0.05	0.11	0.31	0.30	0.39	1.00			
Crunchiness	0.17	0.14	0.19	-0.08	0.16	0.07	0.16	0.58**	0.10	-0.37	0.33	0.21	0.22	0.43	0.27	1.00		
Flavour	0.45	0.46	0.38	-0.42	-0.12	0.32	0.31	0.03	0.45	0.19	-0.15	0.12	0.06	0.26	0.27	0.45	1.00	
Overall acceptability	0.18	0.25	0.34	-0.19	-0.19	0.10	0.18	0.11	0.06	-0.05	-0.00	0.25	0.22	0.38	0.38	0.53	0.74	1.00

**Significant at $P < 0.01$ level, *Significant at $P < 0.05$.

Appendix C

Supplemented data for chapter 7

C.1 Statistical results of all data

Table B.1 Summary of ANOVA of experimental biscuits stored at different temperature for different length of time in two packaging materials.

Dependent variables	df	Moisture content	Hardness	L*	a*	b*	Total phenolic content	Anthocyanin	ABTS	PV	FFA
S	1	**	**	**	**	**	**	**	**	**	**
T	1	**	**	0.45	0.132	0.112	**	**	0.217	*	**
t	6	**	**	**	**	**	**	**	**	**	**
P	1	**	**	0.313	0.948	0.354	**	**	0.361	**	**
S*t	6	**	**	**	0.264	**	**	**	**	**	**
t*T	6	**	**	0.733	0.250	0.213	**	**	0.972	**	*
t*P	6	**	**	0.207	0.340	0.594	**	**	0.421	**	0.112
S*T	1	**	**	0.879	0.474	0.982	**	**	*	**	0.129
S*P	1	**	**	0.461	*	0.406	**	**	0.505	**	**
T*P	1	**	**	0.101	0.794	*	**	0.487	0.926	0.365	*
t*S*T	6	**	**	0.847	0.365	0.498	**	**	0.578	0.106	0.948
t*S*P	6	**	**	0.693	0.338	0.914	**	**	0.789	0.232	0.344
t*T*P	6	**	**	0.573	0.109	*	**	*	0.697	0.957	0.651
S*T*P	1	0.146	0.092	0.840	0.427	0.282	**	**	0.981	0.217	*
t*S*T*P	6	0.436	**	0.990	0.754	0.354	**	**	0.471	**	0.694
Error	112	0.006	0.004	0.023	0.014	0.031	8492.81	0.365	0.910	0.001	0.004

**Significant at $P < 0.01$ level, *Significant at $P < 0.05$, df = degrees of freedom, S = sample, T = temperature, t = storage time, P = packaging, PV = peroxide vale, FFA = free fatty acid.

C.2 Recorded temperatures during storage for 12 weeks

Table B.2 Calculated weekly temperatures set at 22°C and 34°C with relative humidity (%RH) in the incubators.

Weeks	Temperatures (°C)	%RH	Temperatures (°C)	%RH
1	22.5	45	34.1	27
2	23.1	44	34.2	30
3	23.5	61	34.3	40
4	22.4	49	34.2	27
5	21.3	48	34.1	25
6	21.2	53	34.1	27
7	21.5	41	34.2	22
8	21.2	59	34.1	27
9	18.5	56	34.1	27
10	20.1	60	34.5	23
11	23.2	40	34.5	22
12	21.1	47	34.5	25
	21.6 ± 0.4^a	50.3 ± 2.1^a	34.2 ± 0.1^a	26.8 ± 1.4^a

[a]Mean ± standard error.

Appendix D

Pictures of biscuit experiments
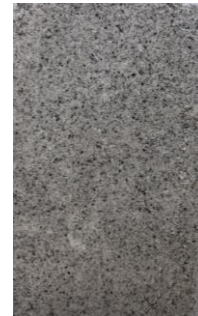
D.1 Flour mixtures

| Refined wheat flour | 25% purple rice flour | 50% purple rice flour | 75% purple rice flour | Whole purple rice flour |

D.2 Biscuits made from different flour mixtures

D.3 Defatted mussel biscuits mixed with ginger and galangal

| Control | 10% mussel powder | 15% mussel powder | 20% mussel powder |

D.4 Defatted mussel biscuits mixed with spice mixtures

D.5 Biscuits packed in different packaging

D.6 Biscuits stored in an incubator

Appendix E
List of publications

E.1   Refereed Published Papers

Klunklin, W., & Savage, G. (2017). Effect on quality characteristics of tomatoes grown under well-watered and drought stress conditions. *Foods, 6*(8), 1-10.

Klunklin, W., & Savage, G. (2018). Physicochemical, antioxidant properties and *in vitro* digestibility of wheat-purple rice flour mixtures. *International Journal of Food Science and Technology,* 1-10. https://doi.org/10.1111/ijfs.13785

Klunklin, W., & Savage, G. (2018). Effect of substituting purple rice flour for wheat flour on physicochemical characteristics, *in vitro* digestibility and sensory evaluation of biscuits. *Journal of Food Quality,* 1-9. https://doi.org/10.1155/2018/8052847

Klunklin, W., & Savage, G. (2018). Physicochemical properties and sensory evaluation of wheat-purple rice biscuits enriched with green-lipped mussel powder (*Perna canaluculus*) and spices. *Journal of Food Quality,*1-9. https://doi.org/10.1155/2018/7697903

Klunklin, W., & Savage, G. (2018). Addition of defatted green-lipped mussel powder and mixed spices to wheat-purple rice flour biscuits: physicochemical, *in vitro* digestibility and sensory evaluation. *Food Science and Nutrition, 00*, 1-9. https://doi.org/10.1002/fsn3.675

Klunklin, W., & Savage, G. (2018). Biscuits: A supplementation of wheat flour with purple rice flour. *Advances in Food Processing and Engineering, 2*(3), 81-97.

E.2   Submitted Refereed Papers

Klunklin, W., & Savage, G. (2018). Effect of storage at different temperatures and packaging on quality properties of wheat-purple rice biscuits enriched with defatted green-lipped mussel powder and spices. *Journal of Food Processing and Preservation.*

E.3   Refereed Conference Podium Presentations

Klunklin, W., & Savage, G. (2016). "*Quality characteristics and sensory evaluation of tomatoes* (Lycopersicon esculentum *Mill) grown under drought stress in a greenhouse*"

proceeding of Annual Scientific Meeting of the New Zealand Nutrition Society at a DoubleTree by Hilton hotel, Christchurch, New Zealand, on 8-9 December 2016.

Klunklin, W., & Savage, G. (2017). "*Effect of the addition of defatted green-lipped mussel powder (*Perna canaliculus*) in wheat-purple rice biscuits*" proceeding of 67th Australasian Grain Science Conference at Rydges Hotel, Christchurch, New Zealand, on 20-22 September 2017.

Klunklin, W., & Savage, G. (2018). "*Physicochemical, antioxidant properties and in vitro digestibility of wheat-purple rice flour Mixtures*" proceeding of The Food and Applied Bioscience International Conference at The Empress Hotel, Chiang Mai, Thailand, on 1-2 February 2018.

E.4   Contribution of authors

Assoc. Prof. Geoffrey Savage supervised the project from the beginning, checked all analysed data, revised the manuscripts and provided precious comments to improve the quality of those study mentioned in the above publications. Warinporn Klunklin conducted research, interpreted all results from data analysis and drafted the papers and presentations.