AUGUST 2017

M.Sc. in Food Engineering

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UNIVERSITY OF GAZİANTEP

GRADUATE SCHOOL OF

NATURAL & APPLIED SCIENCES

STABILITY OF APIGENINIDIN IN MAIZE PORRIDGE

M.Sc. THESIS

IN

FOOD ENGINEERING

BY

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in

Food Engineering

University of Gaziantep

Supervisor

Asst. Prof. Dr. Derya KOÇAK YANIK

Co-supervisor

Asst. Prof. Dr. Anita LINNEMANN

by

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August 2017

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UNIVERSITY OF GAZİANTEP
GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES
FOOD ENGINEERING DEPARTMENT

Name of the thesis: Stability of Apigeninidin in Maize Porridge

Name of the student: Filiz HAZAL

Exam date: 11.08.2017

Approval of the Graduate School of Natural and Applied Sciences

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Filiz HAZAL

ABSTRACT

STABILITY OF APIGENINIDIN IN MAIZE PORRIDGE

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M.Sc. in Food Engineering
Supervisor(s): Asst. Prof. Dr. Derya KOÇAK YANIK
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August 2017
59 pages

The objective of this study is to investigate the stability of apigeninidin in maize porridge. For this purpose; extract of leaf sheaths of sorghum which includes apigeninidin as a predominant 3-deoxyanthocyanidin was used in maize porridge preparation. The stability of apigeninidin in maize porridge (MP) and maize porridge with sorghum (MPS) was studied at different pH values from 4 to 9. The apigeninidin content, antioxidant activity and colour stability were examined within this scope. Apigeninidin content and antioxidant activity of prepared maize porridges were investigated using HPLC and Quencher approach, respectively. Although the highest apigeninidin content was found at pH 9, the antioxidant activity was the lowest at pH 9. The highest antioxidant activity was observed at pH 6 with 19.1 μmol Trolox equivalent/g DM. The colour of MP was yellow at all pH values studied while the colour of MPS was red due to presence of apigeninidin. The strongest red colour was observed at pH 9 followed in order of decreasing redness by pH 4, 5 and 6.

The study showed that apigeninidin was stable to some extent in maize porridge in slightly acidic, neutral and slightly alkaline conditions. Therefore, sorghum might be an important source for natural colorants to bio-colour heat-treated foods, as well as a potential natural dye replacing to synthetic dyes for food industries.

Key words: Apigeninidin, sorghum, colourant, antioxidant capacity, stability, 3-deoxyanthocyanidins, maize porridge

ÖZET

MISIR LAPASINDA APİGENİNİDİNİN KARARLILIĞI

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Ağustos 2017
59 sayfa

Bu çalışmanın amacı, mısır lapasında apigeninidinin kararlılığını araştırmaktır. Bu amaçla mısır lapası hazırlanırken içerisinde yüksek miktarda apigeninidin (3-deoksiantosiyanin) bulunduran sorgum yaprak kılıfları ekstraktı kullanılmıştır. Apigeninidinin kararlılığı, sorgum yaprak kılıfları ekstraktı kullanılarak ve kullanılmadan (kontrol) hazırlanan mısır lapasında pH 4 ile pH 9 arasında farklı pH değerlerinde incelenmiştir. Bu kapsamda apigeninidin içeriği, antioksidan aktivitesi ve renk kararlılığı araştırılmıştır. Hazırlanan mısır lapalarının apigeninidin içeriği ve antioksidan aktivitesi, sırasıyla HPLC ve Quencher yaklaşımı kullanılarak incelenmiştir. Apigeninidin içeriği en yüksek pH 9'da bulunmasına rağmen, antioksidan aktivitesi pH 9'da en düşüktür. En yüksek antioksidan aktivitesi ise 19.1 µmol Trolox eşdeğeri/g kuru madde ile pH 6'da gözlenmiştir. Çalışılan tüm pH değerlerinde kontrol mısır lapalarının rengi sarıyken sorgum yaprak kılıfları ekstraktı kullanılarak hazırlanan mısır lapasının rengi apigeninidin varlığından dolayı kırmızı olarak gözlemlenmiştir. En kuvvetli kırmızı renk pH 9'da, ardından azalan sırayla pH 4, 5 ve 6'da görülmüştür.

Bu çalışma, apigeninidinin mısır lapasında hafif asidik, nötr ve kısmen bazik ortamlarda bir noktaya kadar kararlı olduğunu gösterdi. Bu nedenle, sorgum yaprak kılıfı ekstraktı ısıl işlem görmüş gıdaların renklendirilmesinde kullanılan doğal renklendiricilere önemli bir alternatif olabilirken gıda endüstrileri için de sentetik boyaların yerine kullanabilecek bir kaynak olarak düşünülebilir.

Kelimeler: Apigeninidin, sorgum, renklendirici, antioksidan kapasitesi, stabilite, 3-deoksiantosiyanidins, mısır lapası

To My Parents...

ACKNOWLEDGEMENTS

I would firstly like to thank PhD candidate Folachode Akogou for all his input, patience, advice, and encouragement throughout my research at Wageningen University. I would also like to express my gratitude to my co-supervisor at Wageningen University, Asst.Prof. Dr. Anita Linnemann for her useful comments, remarks, and engagement.

I am very grateful to have this exchange program provided by Gaziantep University, and those at Gaziantep University who gave me this opportunity to have such an experience abroad.

I would also like to express my deepest thanks and gratitude to my supervisor Asst. Prof. Dr. Derya Koçak Yanık, and I am gratefully indebted to her very valuable comments on this thesis. The door to Asst. Prof. Dr. Derya Koçak Yanık office was always open whenever I ran into a trouble spot or had a question about my research or writing. She steered me in the right direction whenever she thought I needed.

I would like to acknowledge Prof. Dr. Fahrettin Göğüş who supported me from the beginning of this thesis.

A very special goes to Wageningen University & Research Centre, Netherlands for the financial, administrative, and technical support throughout this research. It was fantastic to have the opportunity to work majority of my research in your facilities.

Finally, I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

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LIST OF SYMBOLS / ABBREVATIONS

E-120	Cochenille
E-162	Beetroot red
E-163	Anthocyanins
LUT	Luteolinidin
AP	Apigeninidin
DPPH	2, 2-diphenyl-1-picrylhydrazyl
TROLOX	6 hydroxy 2, 5, 7, 8 tetramethylchroman 2-carboxylic acid
THF	Tetrahydrofuran
MP	Maize porridge
MPS	Maize porridge with sorghum colourant
MC	Moisture content
*L**	Lightness
*a**	Redness-greenness
*b**	Yellowness-blueness

CHAPTER I

INTRODUCTION

Recently, the increasing concern in artificial colorants has remarkably influenced the use of natural food colorants due to causing toxicity and carcinogenicity in human body (Jie et al., 2013; Zhang et al., 2011). Therefore, food industry is constantly seeking for natural colorants to replace the synthetic petroleum-based dyes commonly used in foods. Some of these natural colorants are cochenille (E-120), beetroot red (E-162), and anthocyanins (E-163) (Kayodé et al., 2011).

Anthocyanins are becoming gradually important as food colorants with their antioxidant properties, as well as beneficial effects in reducing capillary permeability and fragility, attenuating the proliferation of hepatic stellate cells, and improving visual acuity (Jie et al., 2013; Awika et al., 2004). Much research is directed towards the identification and characterisation of anthocyanin pigments from plant sources such as vegetables, fruits, and flowers. Anthocyanins are also able to polymerize over thermal processing, which can enhance the colour stability (Yang et al., 2014). Besides the advantages of anthocyanins, the stability of anthocyanin pigments can be influenced under various conditions such as pH, temperature, light, oxygen, enzymes, and presence of sugars, ascorbic acids, copigments, and ions. Thus, the use of natural colorants in food applications can be increased through improving these factors (Awika et al., 2004; Kayodé et al., 2011; Arslan, 2015).

Sorghum is one of the important sources of 3-deoxyanthocyanidins (luteolinidin and apigeninidin) which are rarely found in higher plants (Kayodé et al., 2011). Moreover the sorghum leaf sheath contains higher amount of apigenidinin (39,900 µg/g dry matter) than that of other parts (grains, leaves and stalk) of sorghum plant (Benson et al., 2013). Sorghum cereal grown in Africa is used for substantial benefits by the rural woman, especially in Benin, for instance, bio-colouring some foods like

local cheese, porridge, and more. Also, sorghum has been used by the local people in Africa due to its beneficial impact on treating anaemia and menstrual disorders (Awika et al., 2005).

Many studies were carried out with an objective of improving stability of anthocyanins by some additives such as acids, sugars, salts, hydrocolloids, and different phenolic compounds (Rein&Heinonen, 2004; Brenes et al., 2005; Patras et al., 2010). Apigeninidin, which is one of the principal pigments found in sorghum, has a noticeable antioxidant activity due to its lack of oxygen at the C-3 position that is believed to improve its stability (Awika et al., 2004). Among many factors, heat treatment and pH of food matrix have a big influence on the colour and stability of 3-deoxyanthocyanidins (Yang et al., 2014). However, there are limited researches in determining stability of 3-deoxyanthocyanidins in food systems. The purpose of this study is therefore to investigate the effect of pH on stability of apigeninidin in maize porridge using the watery extract of the leaf sheaths from sorghum.

CHAPTER II

LITERATURE REVIEW

2.1 Sorghum

Sorghum (*Sorghum bicolour L.)* is one of the most important cereals in the world after maize, wheat, rice, and barley (Ademiluyi et al., 2014; Ayala-Soto et al., 2015). Sorghum is grown particularly on low-potential, shallow soils with a high clay content, which are not suitable for maize production in Africa as well as across America and Asia. It is efficient to produce these kinds of grains and corn, because of the characteristics of the climate in these specific areas, which are too hot and dry for maize. Therefore, the most appropriate season of the production of the dye sorghum is between May and December (Kayodé et al., 2011; Benson et al., 2013). Besides sorghum has an essential interest in human diet in Africa, and other parts of the world along with producing alcoholic drinks such as beer in Africa and liquor in China (Rhodes et al., 2014).

Figure 2.1 Sorghum in the field

In many parts of the world sorghum has been used traditionally in food products; porridge, unleavened bread, cookies, cakes, couscous, and malted beverages are made from this versatile grain. Especially in Benin, the leaf sheaths of dye sorghum mostly common for the use of foods due to its pigments in the leaf sheaths (Kayodé et al., 2011).

The kernel colour sorghum has an essential role for the importance of the product colour quality. Sorghum has diversity of colour such as red, lemon, yellow, brown or white. White or light sorghums are mostly favoured to make porridge in Africa. Red coloured sorghums are commonly used for brewing traditional beer. However, white sorghums are commercially preferred contrary to red sorghums for the production of beer in the industry. These white sorghums do not include coloured pigments which are partially pale or fully white. The existence of polyphenols provide black, purple, red and yellow colour in sorghum grains, glumes, sheaths, stems and leaves (Awika et al 2011; Hikeezi, 2010).

Figure 2.2 Some colours of sorghum cultivars (white, red, brown, black) (Njongmeta, 2009)

According to Dicko et al., (2006) red and white sorghum grains have considerable differences in proanthocyanidin concentrations. Red sorghums with 9400 mg/kg appeared almost 7 times higher than 1300 mg/kg of white sorghums (Bröhan et al., 2011).

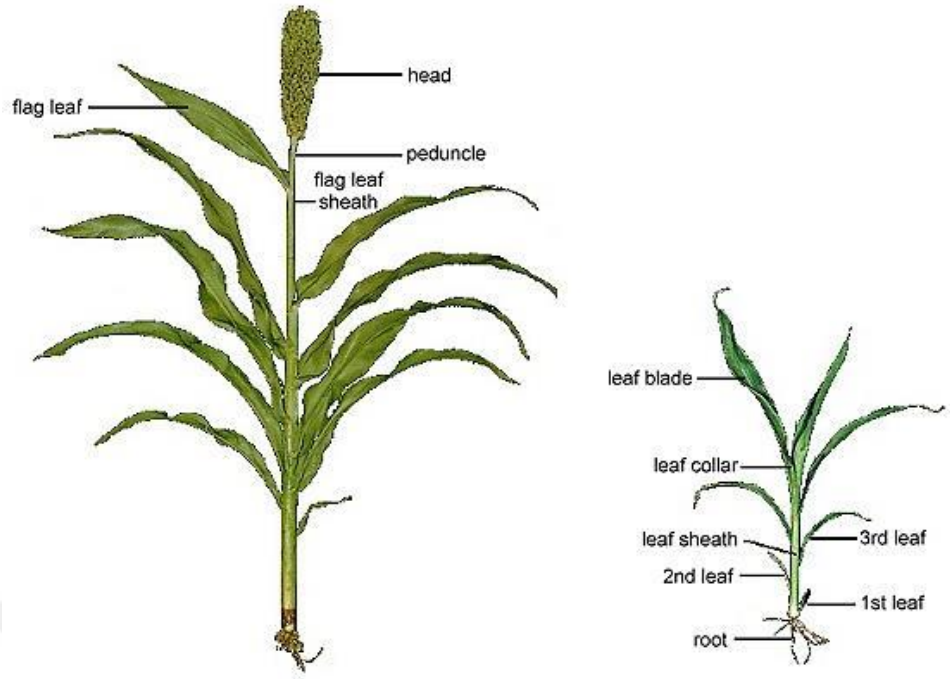


Figure 2.3 Basic parts of sorghum plant (Vanderlip, 1979)

Sorghum species are known to have a high content of antioxidants, including simple phenolic acids, as well as polyphenols, particularly 3-deoxyanthocyanins such as luteolinidin and apigeninidin, which are commonly identified in higher plants (Benson et al., 2013; Kouda-Bonafos et al., 1994). The significant quantities of these antioxidant compounds are present in sorghum-based beers and provide the inhibition of lipid peroxidation during mashing and boiling (Dykes et al., 2006; Benson et al., 2013).

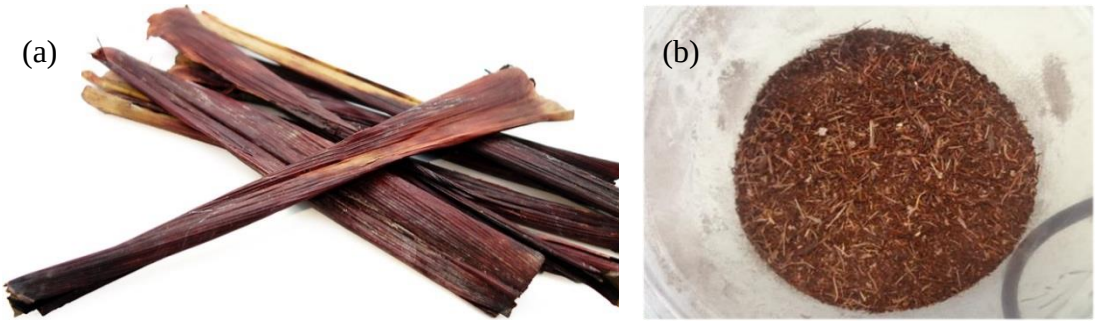


Figure 2.4 (a) The leaf sheaths of sorghum and (b) sorghum leaf sheaths after grinding

2.2 Phenolic Compounds in Sorghum

Phenolic compounds are concerned as nutraceutical impact for healthy food due to their antioxidant activity (Dicko et al., 2005). A great diversity of phenolic compounds with large amounts exist in sorghum plants. Some of their derivatives appear in sorghum such cinnamic acid, monomeric, and oligoremic flavonoids (Mueller-Harvey and Reed, 1992; Njongmeta, 2009; Hithamani et al., 2014). Hepatoprotective impact of the sorghum leaf sheaths might be contributed because of its constituent phytochemicals such as anthocyanins and other phenolic compounds. Sorghum includes a large class of phytochemicals such as anthocyanins, tannins, phenolic acids, phytosterols, and policosanols known as a significant influence on human health (Ademiluyi et al., 2014; Sereme et al., 1993; Kamath et al., 2004).

Polyphenols are determined from the Shikimate and acetatemalonate pathways. Shikimate pathways continues with the production of phenylalanine, which is subsequently deaminated by the enzyme phenylalanine lyase into cinnamate derivatives (Dicko et al 2006). Moreover, acetatemalonate pathways provide flavonoid biosynthesis which continues with the transformation of acetyl CoA to malonyl CoA by acetyl CoA carboxylase (ACC) (Figure 2.5). Phenolic compounds present in entire sorghum types compared to other cerals. Phenolic compounds generally are present in nature companied with a sugar moiety (glycosides) which are particularly water-soluble. The presence of phenolic compounds in sorgum give a particular property among in the cereals. Phenolic compounds can scavende free radicals by their some properties such electron-donating which contribures a stable phenoxyl radical or nonradical varieties. Besides their resistance to neurological diseases, some of them have anticarcinogenic, antimutagenic, and cardioprotective properties with the scavenging of free radicals (Dicko et al 2006; Mueller-Harvey and Reed, 1992; Dicko et al., 2005).

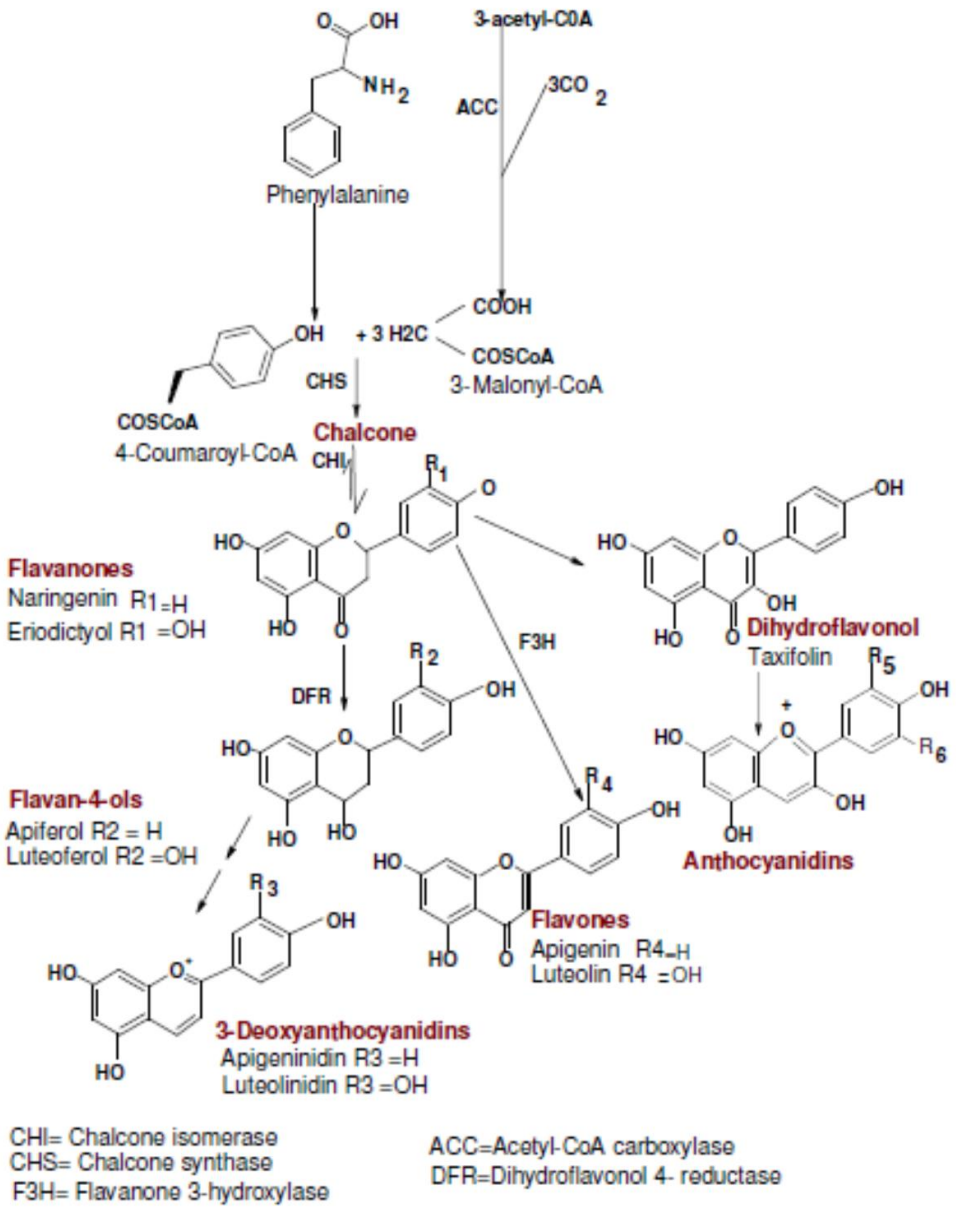


Figure 2.5 The biosynthetic pathway of major sorghum flavonoids. Note how the biosynthetic route of 3-deoxyanthocyanidins and anthocyanindins are proposed to diverge from one another after the synthesis of naringenin. Modified from Wharton & Nicholson, (2000); Boddu et al., (2005).

Phenolic compounds are found in both free and bound form as esters. Free phenolic compounds are proanthocyanidins or flavonoids, while the bound phenolic compounds are ester-linked to cell-wall polymers with ferulic acid and its dehydrodimer derivatives being the major bound phenolic compounds present (Bonoli et al., 2004; Dykes et al., 2005).

Table 2.1 Polyphenol contents in sorghum leaf sheath (Ademiluyi et al., 2014)

Polyphenol compounds	Composition mg/g	LOD µg/mL	LOQ µg/mL
Gallic acid	5.83±0.01	0,04	0,12
Caffeic acid	70.95±0.01	0,01	0,04
Ferulic acid	61.39±0.02	0,02	0,08
Rutin	21.06±0.01	0,02	0,06
Kaempferol	24.52±0.03	0,01	0,05

Phenolic compounds identified in sorghum belong to three major groups: phenolic acids, polymeric flavonoids and simple flavonoids (Awika and Rooney, 2004; Dykes and Rooney, 2006).

2.2.1 Phenolic acids

Phenolic acids in sorghum are classified into two categories: hydroxybenzoic acid which include gallic, protocatechuic, gentistic, *p*-Hydroxybenzoic, salicylic, vanillic, and syringic; and hydroxycinnamic acids which are coumaric, caffeic, ferulic, and sinapic acids (Hahn et al., 1983; Njongmeta, 2009). The benzoic acids derivatives are more common than the cinnamic acid derivatives in plants.

Awika et al. (2004); Waniska, (1989), found free or bound phenolic acids as esters in sorghum, although the bound forms were predominant. Ferulic acid is the abundant (24-47%) of the phenolic acids followed by *p*-coumaric acid (100-200 mg/g dry weight). Gallic acid is found in bound form, whereas cinnamic acid exists in the free form in sorghum (Dicko et al., 2006).

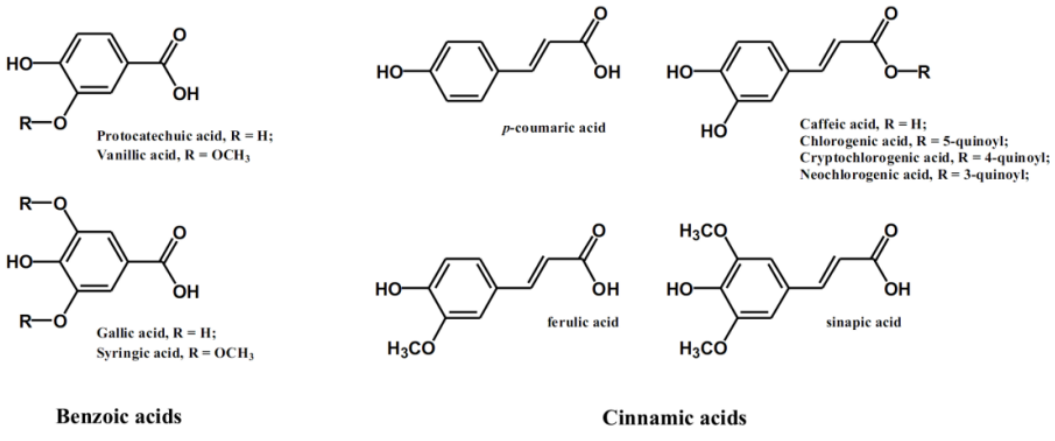


Figure 2.6 Structure of phenolic acids and benzoic acids in sorghum (Tsao, 2010)

These compounds are undesirable in food products since they can cause some health problems with their carcinogenic, hepatotoxic, and goitrogenic effects. However, they can be removed by some food processing such as heating (Dicko et al., 2006).

2.2.2 Tannins (Polymeric Flavonoids)

Tannins are high molecular weight polymeric flavonoids with molecular weights between 500 and 3000. Tannins are divided into hydrolysable or condensed tannins. Hydrolysable and condensed tannins can easily be discriminated by their structure and reactivity against hydrolytic agents (Waniska et al., 1989).

Hydrolyzable tannins (e.g, tannic acid) contain a central core of polyhydric alcohol such as glucose and hydroxyl groups which are fully or partially esterified by gallic acid or hexahydroxydiphenic acid. Hydrolyzable tannins change into sugars and a phenolic acid (gallic or ellagic) when processed with acid, alkali or some hydrolytic enzymes (Njongmeta, 2009). Sorghum does not contain tannic acid and hydrolysable tannins (Awika and Rooney, 2004; Waniska et al., 1989).

Sorghum with a pigmented testa containing condensed tannins, mainly polymerized products of flavan-3-ols and/or flavan 3, 4-diol subunits accumulating in the pigmented testa layer of sorghum kernels (Beta et al., 2000). Only sorghum cultivars with a pigmented testa (*B1_B2*_genes), produce condensed tannins or proanthocyanidins (Waniska et al., 1989; Hahn et al., 1984). According to Hahn and Rooney, (1986), tannins appear only in the pericarp and pigmented testa layers yet Ring, (1984) stated that tannins also exist in the glumes and leaves of sorghum. Besides their structure, they have antioxidant properties *in vitro* antioxidant (Hagerman et al., 1998).

2.2.3 Simple Flavonoids in Sorghum

Flavonoids are polyphenolic compounds dominantly present in the plant. Flavonoids contain some natural pigments which have important biochemical and antioxidant properties. Some of the flavonoids are found in sorghum such as flavanols (flavan-3-ols, flavan-4-ols, etc.), flavanones, flavones, anthocyanins and 3-deoxyanthocyanins (Figure 2.7).

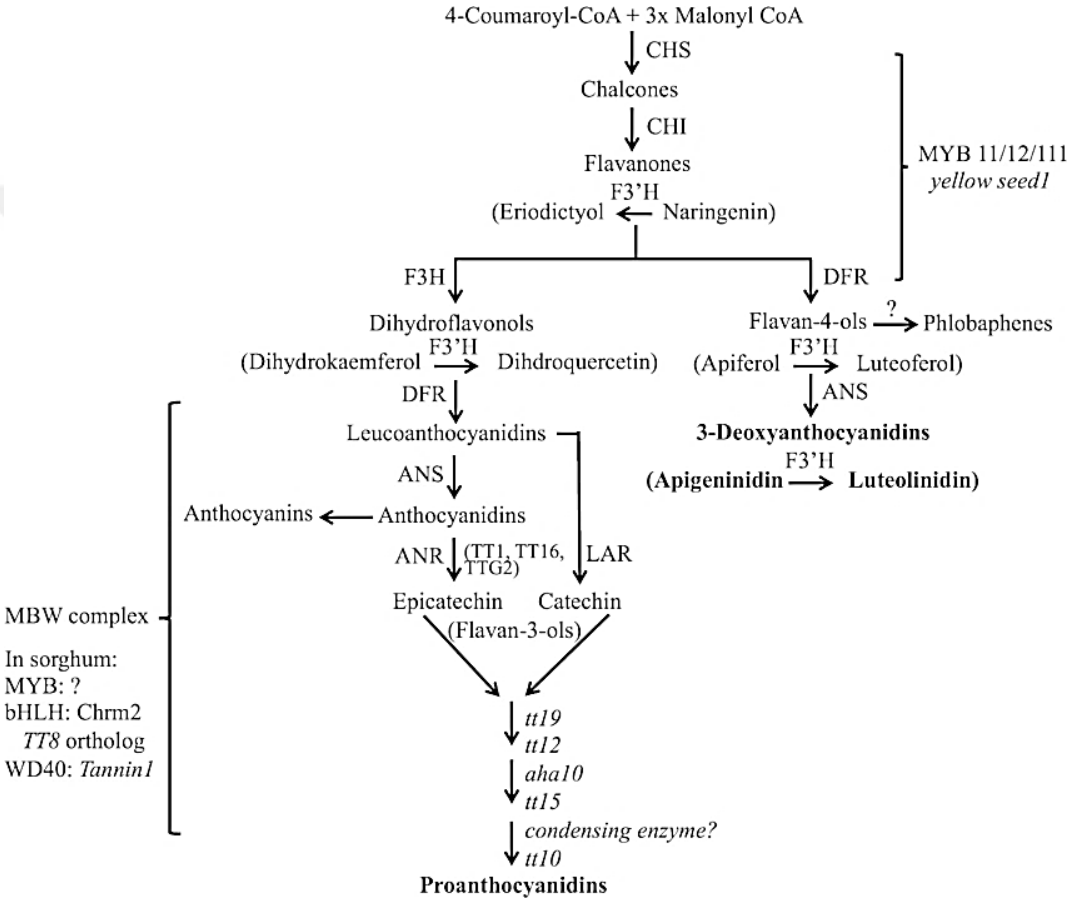


Figure 2.7 Scheme of flavonoid biosynthetic pathway in sorghum grain with candidate genes. Enzyme abbreviations are in uppercase letters, while gene abbreviations are in italics. Question marks depict unknown steps. Chalcone synthase (CHS), chalcone-flavanone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavanone 3′-hydroxylase (F3′H), dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS), anthocyanidin reductase (ANR), leucoanthocyanidin reductase (LAR); MYB-bHLH-WD40 (MBW) (Rhodes et al., 2014)

Table 2.2 Flavonoids in sorghum plant components (Njongmeta, 2009)

Sorghum Varieties	Flavonoids [a]	Leaves	Sheaths	Glumes	Grains
Tx2911 *Red	3-Deoxyanthocyanins	744.00±4.21	3629.10±28.08	8737.90±25.51	75.00±3.89
	Flavones	244.37±12.19	344.58±9.60	1276.03±89.63	21.91±0.72
	Flavanones	ND	6.84±0.57	1348.98±20.11	318.03±13.55
SC748-5 Purple	3-Deoxyanthocyanins	94.70±4.60	1661.50±9.45	1502.00±8.45	17.7±0.56
	Flavones	265.53±6.03	731.99±6.27	147.75±2.03	54.81±0.58
	Flavanones	173.92±1.04	265.74±12.78	159.85±5.19	911.25±35.83
R-07007 Red	3-Deoxyanthocyanins	60.70±3.35	660.00±6.56	8766.90±24.9	15.70±1.16
	Flavones	815.45±2.33	681.39±13.92	754.25±42.33	21.68±0.68
	Flavanones	1260.30±7.17	377.2±5.19	923.70±39.11	1375.06±38.84
Tx430 Black Purple	3-Deoxyanthocyanins	849.60±8.52	9738.40±54.6	5439.90±31.41	877.40±10.21
	Flavones	379.42±10.45	319.74±41.62	857.34±45.79	69.80±0.37
	Flavanones	ND	ND	ND	47.95±0.67
Tx430 Black x Sumac Red	3-Deoxyanthocyanins	1810.60±20.11	7094.40±61.81	4431.70±34.82	156.50±4.10
	Flavones	1448.50±8.31	708.99±7.46	408.38±15.67	19.41±2.43
	Flavanones	ND	ND	ND	ND
ATx631xRTx436 Tan	3-Deoxyanthocyanins	12.60±4.72	5.50±0.24	226.90±3.87	1.50±0.23
	Flavones	255.46±10.93	236.00±7.94	775.89±6.51	79.43±2.59
	Flavanones	ND	ND	ND	ND
Sweet Sorghum (Collier)	3-Deoxyanthocyanins	6198.51±24.45	196.24±7.73	ND	ND
	Flavones	ND	ND	NA	NA
	Flavanones	ND	395.30±1.95	NA	NA

*ND =Not detected. NA= Not analysed. * Secondary plant colour.*

The structure of flavonoid is composed of the flavan nucleus, which contain 15 carbon atoms derived from two phenyl groups (A and B rings), united by a three-carbon bridge (C ring) in order to construct a C6-C3-C6 skeleton (Njongmeta, 2009).

Table 2.3 Content and properties of flavones in the West African sorghum leaf sheaths (Benson et al., 2013)

	µg/g	Rings	Phenolic class	Solubility in water	Solubility in ethanol
Naringenin	130	3	Flavanone	Almost insoluble	Yes
Apigeninidin	39900	3	3-deoxyanthocyanidin		
Luteolinidin	450	3	3-deoxyanthocyanidin		
Apigenin	6910	3	Flavone		
Luteolin	570	3	Flavone	Almost insoluble	Yes

Flavonoids in plants can only synthesised due to their biosynthetic abilities. In the plants flavanoids appear in the variety of forms such as aglycones, glycosides or hydroxyl, methyl and methoxyl derivatives. Regarding the molecular structure, polyphenols are classified by their level of oxidation and substitution pattern on the C ring, while individual compounds can vary in the substitution pattern on the A and B rings.

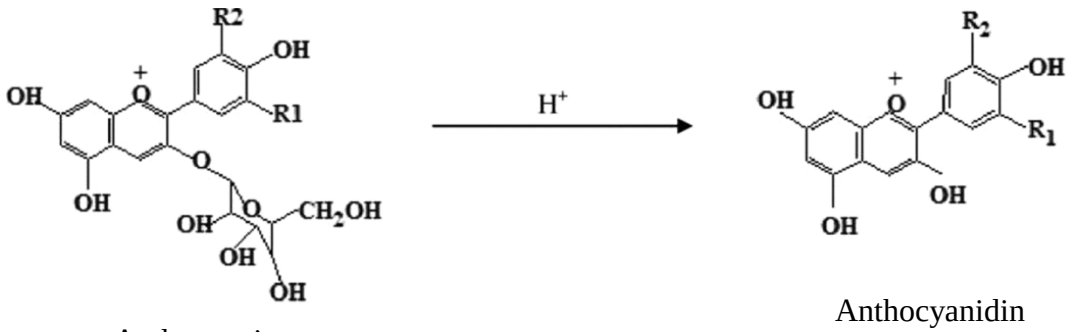
Most of the flavonoids have been isolated and identified in sorghum leaf sheaths (Table 2.3) with flavanols, flavanones, flavones and anthocyanins (Awika et al., 2004; Dykes, 2008).

2.2.3.1 Anthocyanins

Anthocyanins are part of the plant-derived flavonoid compounds, and are well-known with their colour palette ranges from orange to bright red and deep blue to violet in flowers, fruits, vegetables, and grains (Delgado-Vargas et al., 2010; Deroles et al., 2008; Li et al., 2013). Their benefits of therapeutic and medical properties, such as antioxidant activity, anti-inflammatory, anti-convulsant, and chemo-protectant activities, also increase their interest in healthy foods (Benson et al., 2012).

Consumer demand in attention to buy healthy foods is remarkably increasing (Boo et al., 2012). With consumer preference for natural colorants, food industries have therefore gradually substituted to apply natural dyes instead of using petroleum-based dyes in foods (Yang et al., 2014). Anthocyanins well-known, as natural colorants, obtained from diversified alternatives in nature could be an exceptional

source of antioxidant additives and natural colorants in food applications (Zhang et al., 2011; Delgado-Vargas et al., 2010). However, their sensitivity to pH, temperature, light, oxygen and some enzymes is challenging in food application (Fossen et al., 1998).



Anthocyanin

Anthocyanidin

Figure 2.8 Structures of anthocyanin and anthocyanidin (Kumara et al., 2013)

Anthocyanins have some limitations associated with their narrow variability in the plants. Sorghum is one of the applicable sources that have a high antioxidant activity in its existing colourant pigments such as apigeninidin and luteolinidin. However, there are limited sources regarding the application of the anthocyanins in cereals. Thus, they have never been considered as commercially natural colorants (Benson et al., 2013; Gould et al., 2009).

The six common anthocyanins (Figure 1.9) in nature are cyanindin, delphinidin, malvinidin, pelargonidin, petunidin, and peonidin. However, the 3-deoxyanthocyanins are a rare form of anthocyanins commonly found in ferns and mosses with sorghum being the only known dietary source (Wu et al., 2005).

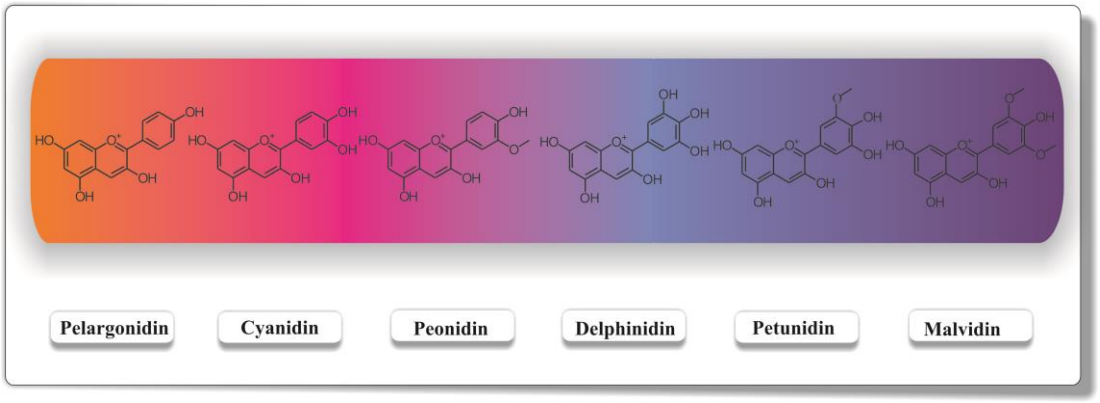


Figure 2.9 The structure of main anthocyanins with a reflection of their colour change (Delores et al.,2008)

2.2.3.2 Stability of anthocyanins in food

There are some factors that considerably influence the stability of anthocyanins such as pH, temperature, light, and enzyme reactions. There have been advanced research investigating to prevent the degradation of anthocyanins due to these factors (Awika et al., 2004). The colour stability of anthocyanins in many fruits and vegetables has increasingly developed in the food industry (Kjell et al., 2005; Chung et al., 2016). Some applications of the anthocyanins in foods are showed under various conditions in Table 2.4 (Delgado-Vargas et al., 2010).

Table 2.4 The stability of anthocyanins applied in food systems (Delgado-Vargas et al., 2010)

Model	Conditions	Effects
Blacberry juice	Different temperatures and addition of aldehydes	Disappearance rate followed a first-order kinetics and was higher in the presence of aldehyde and dependent of temperature
Quick frozen strawberries	Addition of different levels of sucrose	Sugar addition stabilizes the pigment content and produce a reduction in browning reactions. The protective effect was assigned to inhibition of degredative enzymes and to steric interference with condensation reactions
Grape musts	Addition of glutathione	Quinones are involved in anthocyanin degradation because of this is inhibited by glutathione
Barley	Heating (40 - 100°C)	Hordeumin anthocyanin showed an improved stability because of it is constituted by a molecular complex betwee anthocyanins and polyphenols
Marashino cherries	Brined cherries pigmented red radish anthocyanin extracts (RAE)	The coloration imparted by RAE and FD&C Red No. 40 was similar; the kinetics of degradation followed first-order kinetics

Not only colour parameters of natural colorants, but also their antioxidant activity plays an essential role for healthy foods (Boo et al., 2012; Kayodé et al., 2011). However, their sensitive characteristics make them less stable in food processing (Kayodé et al., 2012). Therefore, artificial colorants are commercially used more than natural colorants, even though consumer interest is gradually increasing for healthy foods (Ojwang&Awika, 2010).

Apparently, because of hydroxyl groups, anthocyanins have a distinct relation with the instability (Fossen et al., 1998). Delgado-Vargas et al., (2010) reported that the glycosylation indicates a considerable effect on stability and diglucosides are more stable than monoglucosides.

In Figure 2.10 the transformation of apigeninidin shows that the neutral species A, B, and C do not deprotonote to an considerable extent. AH^+ and A absorb visible light as well as they are in charge of colours (orange, red, and blues) to some extent in flowers and fruits. Reactions 1 are faster than the reactions in 2. The number of the quinoidal bases lessen in case one or two free hydoxyl groups in AH^+ are treated with methyl or glycosyl. Colorless carbinol pseudobase B is yield by exceptional attack at C-2. The rate of equilibrium 3 is low in acidic solutions with regards to the rates of equilibrium 1 and 2. Ring opened chalcone pseudobase C does not consume light in the visible range. Equilibrium 3 that is not dependent to pH is positioned through the value of the tautomeric equilibrium constant(K_T). An acidity constant (K'_c) can be described for the chalcone pseudobase C formation reaction beginning from the flavylium cation AH^+: $K'_c=([C]/[AH^+]a_H= K'_h K_T$. Therefore the stability of the falvylium cation AH^+ in acidic conditions associated with the values of the three constants (K'_a, K'_h, and K'_c). The relation with the stabilities of A, B, and C connected to the associated values of K'_a, K'_h, and K'_c (Mazza and Brouillard, 1987).

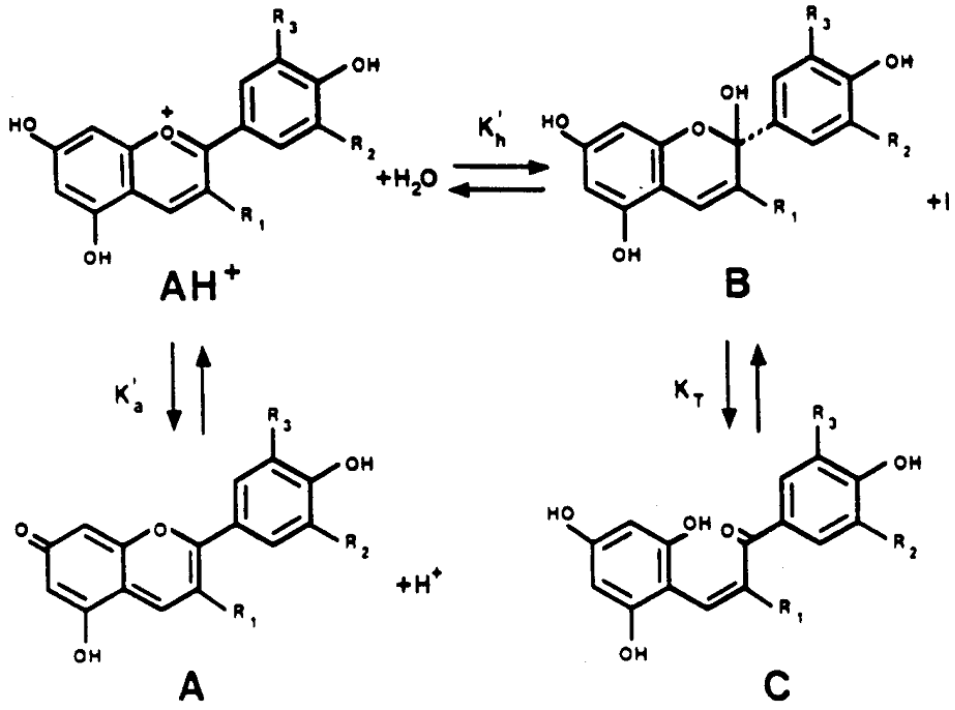


Figure 2.10 Structural transformation of apigeninidin (Mazza and Brouillard, 1987)

In solution, the equilibrium between the coloured cationic form and colourless pseudo base form of anthocyanin molecules is influenced by pH. It is believed that the red colour of anthocyanins in acidic solutions is desired for colouring foods compared to colour change in alkaline conditions (Ojwang et al., 2010). In pH values especially below four, anthocyanins could be used as colorants (Delgado-Vargas et al., 2010).

2.2.3.3 3-deoxyanthocyanins

Luteolinidin (LUT) and apigeninidin (AP), and their glycosylated derivatives known as 3-deoxyanthocyanins and a class of flavonoids are commonly present in sorghum (Fossen et al., 1998; Awika et al., 2005; Dykes et al., 2006). 3-deoxyanthocyanins are produced as phytoalexins in plants as a response to mold invasion or other stress factors in sorghum (Waniska et al., 1989). They have *in vitro* antioxidant activity (Awika et al 2004). The yellow apigeninidin and the bright orange luteolinidin are the two most prominent sorghum 3-deoxyanthocyanins (Nip et al., 1971; Awika et al 2004; Wu and Prior, 2005).

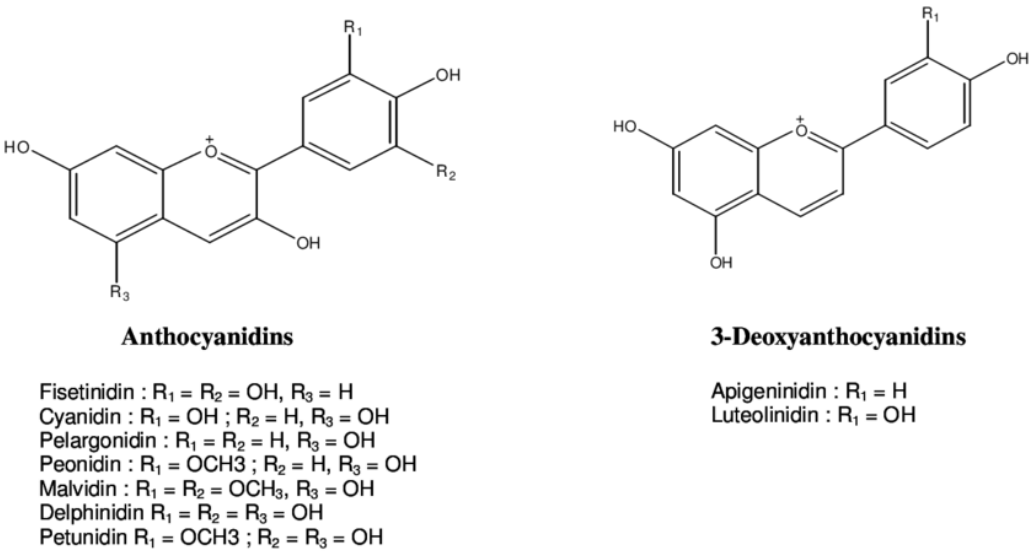


Figure 2.11 Chemical structure of some common anthocyanidins compared to 3-deoxyanthocyanidins (Awika et al., 2005; Dicko et al., 2006)

Sorghum 3- deoxyanthocyanins are similar to the common anthocyanins, however they do not contain the hydroxyl group in the 3-position of the C-ring (Figure 2.11). This unique structural feature gives sorghum 3 deoxyanthocyanins very different 22 chemical and biochemical properties. For example, 3-deoxyanthocyanins are more stable to pH changes and other conditions compared to the common anthocyanins (Awika et al., 2004; Awika and Rooney, 2004; Mazza and Brouillard, 1987; Njongmeta, 2007), giving these compounds a competitive advantage as potential natural food colorants compared to the common anthocyanins.

3-deoxyanthocyanidin compounds are more stable due to the bleaching effect on food additives such as ascorbic acid and bisulphites. Therefore, the stability of these pigments in the bleaching agents might be considered as commercially in food productions (Geera et al., 2012). However, their existence in nature is very limited because of their stability compared to other anthocyanins, which are commonly found in plant pigments (Awika et al., 2003). Furthermore, the extraction of the 3-deoxyanthocyanins is extremely difficult from bran tissue due to the relation with cell wall material. In the recent studies have showed that plant tissues, sheaths, and leaves from sorghum might an important function to use 3-deoxyanthocyanidins in food processing (Ojwang et al., 2010). On the other hand, one of the advantages of

the 3-deoxyanthocyanins could be a source for preventing pathogen attack in the leaf sheaths (Ademuliyu et al., 2014).

3-deoxyanthocyanins found in large quantities in sorghum, are a class of anthocyanins unsubstituted at position C-3. The 3-deoxyanthocyanins in various food processing conditions and with food additives relative to the common anthocyanins have a remarkably increase on the stability (Geera et al., 2012). Additionally, recent developments provide that the 3-deoxyanthocyanins have a higher potential activity against cancer cell proliferation than their anthocyanin analogues. Thus, the 3-deoxyanthocyanin pigments present an opportunity as biologically important natural colorants (Ji et al., 2013).

On the other hand, the 3-deoxyanthocyanins often show different patterns when compared to anhocyanins. In another finding shows that the 3-deoxyanthocyanins with the addition of the sulphite, falvylium cation occurred at C-2 and not C-4 as reported for anthocyanins; however, it still has to be investigated since they do not have a proof (Torskangerpoll et al., 2005). Another study showed that sulphites formed a relatively weak complex with 3-deoxyanthocyanidins, apigeninidin, and luteolinidin, compared to their anthocyanin analogues pelargonidin and cyanidin at pH 1.05 (Ojwang&Awika, 2010; Awika et al., 2004).

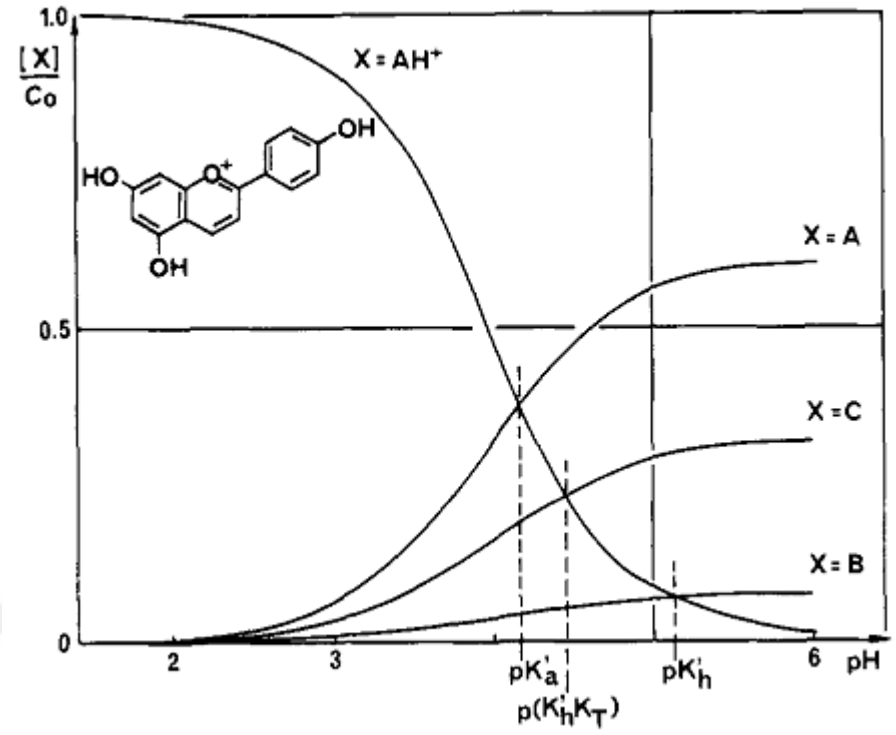


Figure 2.12 Equilibrium distribution of AH^+, A, B, and C for apigeninidin chloride as a function of pH (Brouillard et al., 1982)

Apigeninidin is the most common natural anthocyanins. Figure 2.12 shows the equilibrium distributions of AH^+, A, B, and C for apigeninidin chloride. Appearently, the equilibrium value of the chalcone pseudobase C is diminished the effect of the presence of the 5-OH; K_T 20.6 and 4.4 for 1 and 2, respectively. Additionally, the amount of K'_h has a considerable reduce contrary to the amount of 1. According to the result, the carbinol pseudobase shows a lower stable from 2 to 1. The position of the flavylium cation-quinoidal bases system which are pK'_a is 4.30 of the reaction 1 and 4.20 of the reaction 2 is changed by the SOH. For this reason, the most stable neutral varieties of the acidity constant of the quinoidal bases is K'_a; followed by K'_c, and with a lower value of K'_h. The flavylium cation evolves into more stable form at higher pH values when K'_c is reduced. Although the apigeninidin colour shows a greater rate than the colour of 1, B and C forms which are not coloured contain around 40% of all pigment. The methylated apigeninidin (4',5,7-trimethoxyflavyliucma tion) presents commonly in the chalcone structure due to no quinoidal bases formation and also because of a lower K'_h value than the K'_c value of

the methylated apigeninidin. The apigeninidin shows an increase in the acid-base equilibrium (Brouillard et al., 1982; Baranac and Amic, 1990).

2.3 Aim

The colour and stability of anthocyanins are considerably influenced by mainly pH and temperature, as well as other factors in the food applications. However, there is limited research concerning the effect of the pH on stability of 3-deoxyanthocyanidins. Knowing the degradation mechanisms is another valuable factor to stabilise the 3-deoxyanthocyanidins during processing. In addition, the use of apigeninidin as natural colourant is not well-known since there are not enough sources as to whether it is a potential for food industry. The purpose of this study was therefore to investigate stability of 3-deoxyanthocyanidins at various pH and to evaluate the effect of pH on antioxidant activity in maize porridge.

2.4 Research Questions

In this research mainly two questions were investigated following as:

1. What is the effect of pH on the stability of 3-deoxyanthocyanidins in maize porridge?
2. How does pH influence the antioxidant activity in maize porridge?

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and reagents

2, 2-diphenyl-1-picrylhydrazyl (DPPH), 6 hydroxy 2,5,7,8 tetramethylchroman 2-carboxylic acid (Trolox) was purchased from Sigma Aldrich. Tetrahydrofuran (THF) was obtained from Biosolve. Cellulose from spruce was purchased from Fluka. The batch of leaf sheaths were bought at the local market of Dassa-Zoume in Benin. Methanol and formic acid were obtained from Actu-All Chemicals (The Netherlands). These chemicals were used to analyse the determination of antioxidant activity and the identification and quantification of anthocyanin. All other chemicals and solvents were analytical and chromatographic grade.

3.2 Experimental design

3.2.1 General set up

Figure 3.1 shows the flow diagram of the experimental set up of this research. First of all, two extracts were prepared. One of the extracts (with sorghum and kanwu) was for preparation of coloured maize porridge. Another extract (without sorghum) was for the preparation of control porridge. Then pH of extracts was adjusted to the intended pH values (4, 5, 6, and 9) before making porridge. The extracts of control and sorghum extracts at various pH were mixed with maize flour, and cooked separately at 95˚C for 20 min. Samples of porridges were then taken; a) for measurement of dry matter, pH, and colour; b) to cool using liquid nitrogen. The cooled porridges are then freeze-dried. Consequently, the freeze-dried samples are analysed for total apigeninidin content and antioxidant activity.

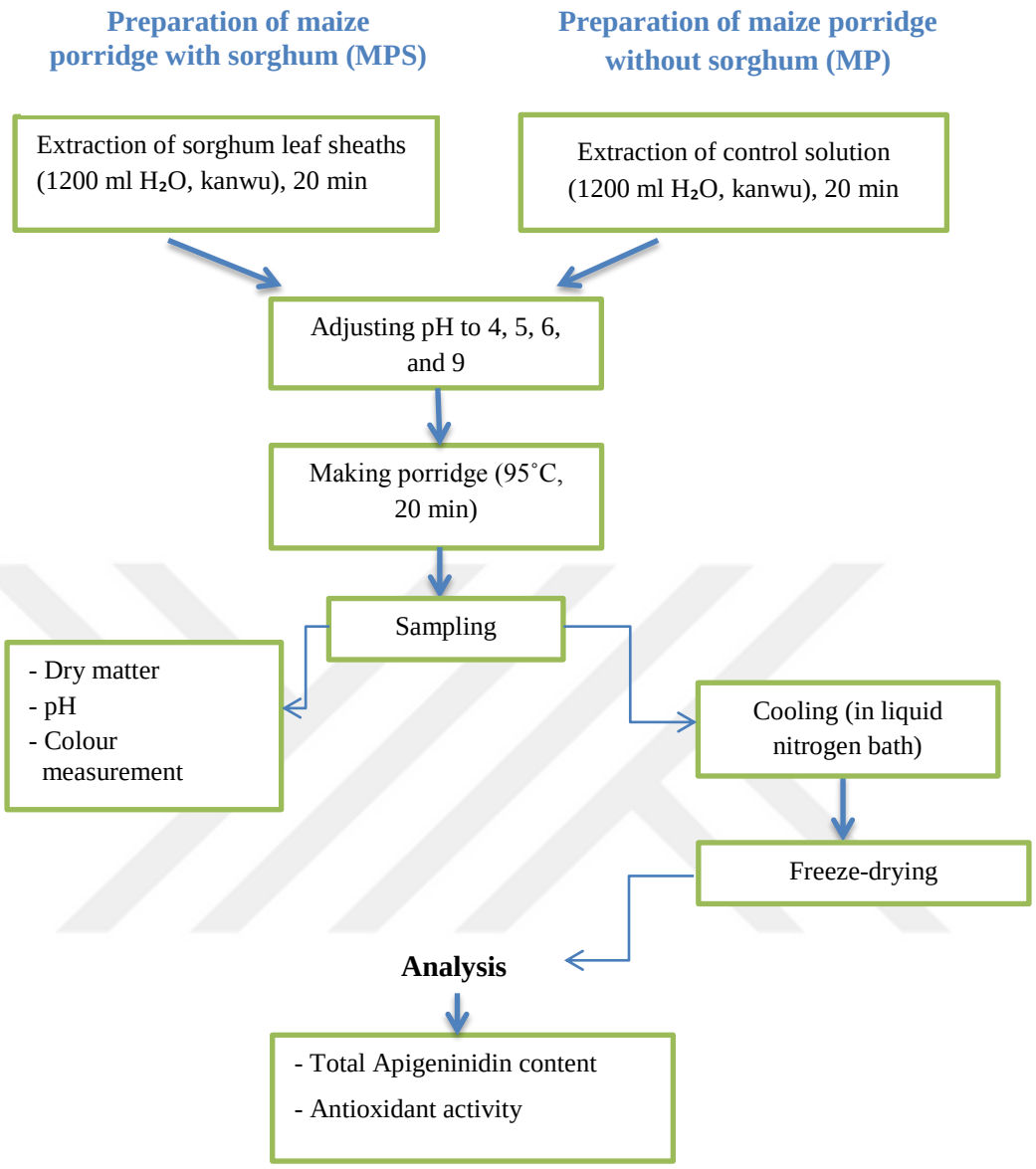


Figure 3.1 Flow diagram for the preparation of MP and MPS

3.3 Methods

3.3.1 Preparation of Extracts

3.3.1.1 Preparation of extract for sorghum bio-colourant

13.3 g of sorghum leaf sheaths and 1.8 g of *kanwu* were weighed. They were mixed and completed with 1200 ml of demi water in glassware and the colourant was extracted at room temperature for 20 min using a magnetic stirrer. In order to remove leaf sheaths from the colourant a 0.3 mm sieve was used. The extract was centrifuged at 3000 rpm at 4˚C for 30 min. The supernatant was collected and the sorghum bio-colourant was stored at 4˚C until next day.

3.3.1.2 Preparation of extract for control sample

1.8 g of *kanwu* was completed with demi water up to 1200 ml to make extraction. The extraction was performed at room temperature for 20 min using a magnetic stirrer. The extract was centrifuged at 3000 rpm at 4˚C during 30 min and the supernatant was collected. The solution of *kanwu* was stored at 4˚C for next day.

3.3.2 Making Porridge

3.3.2.1 Making porridge with sorghum bio-colourant

810 ml of colourant was measured in glass beakers. The pH of the extract was around 8. Therefore, the pH was adjusted to 4, 5, and 6 using citric acid (1 M) and adjusted to 9 using NaOH (1M). 81 g of maize flour was added to 810 ml of the colourant at pH 4, 5, 6, and 9. The flour and the colourant were mixed and cooked at 95˚C 20 min using Thermomix (Vorweck, France). I-buttons were used to measure temperature and cooking time (Figure 3.2).

After cooking, porridge was poured into an aluminium container to cool it down using liquid nitrogen.

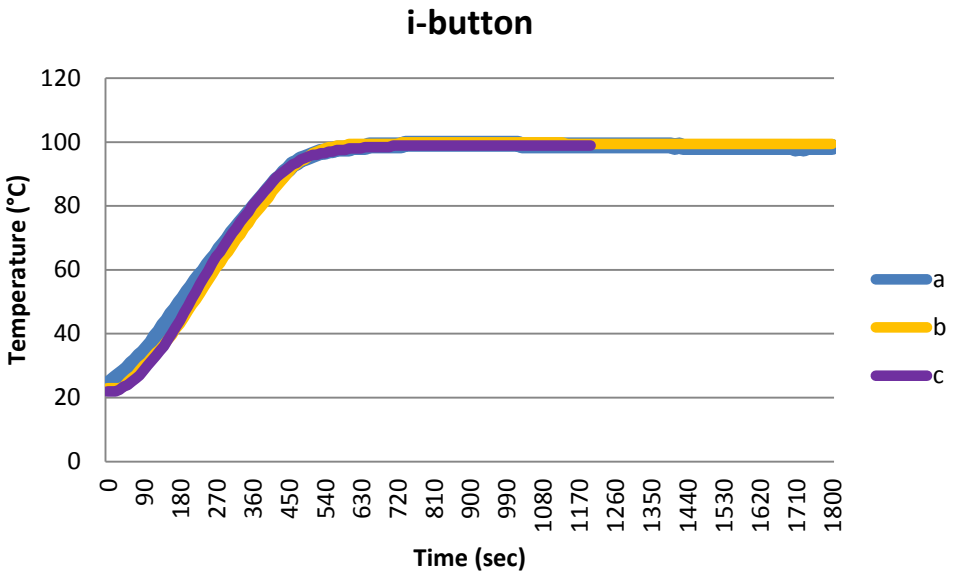


Figure 3.2 Cooking temperature values obtained using i-buttons in different cooking time range of 30 min for (a) i-button_1, and (b) i-button_2, and 20 min for (c) i-button_3

3.3.2.2 Making porridge for control samples

810 ml of extract was measured in glass beakers. The pH of extract was determined around 10. Thus, the pH was adjusted to 4, 5, 6, and 9 using citric acid (1M). 81 g of maize flour was added to 810 ml of the solution of *kanwu* at pH 4, 5, 6, and 9.

The flour and the solution were mixed in a glass beaker and cooked at 95˚C during 20 min using Thermomix (Vorweck, France). The same method was followed to cool down the porridges using liquid nitrogen. After that the frozen porridges were stored in the freezer at -20˚C until freeze drying.

Subsequently the samples were freeze-dried using Christ Alpha 1-4 LD plus freeze-drier and RZ 6 vacuum pump for determination of apigeninidin content and antioxidant activity (Figure 3.3).

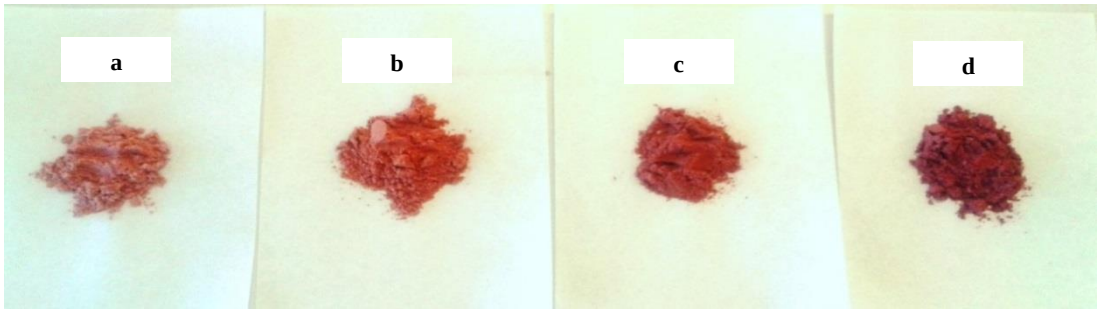


Figure 3.3 An overview of the freeze-dried MPS with various pH values: (a) pH4, (b) pH5, (c) pH6, and (d) pH9

3.3.3 Dry matter and pH

The fresh samples were taken into aluminium containers to measure pH using VWR pHenomenol^{TM} before freeze-drying the porridges. For Dry Matter, the aluminium boxes were dried at 100˚ at least 30 minutes and cooled at room temperature in the exicator for 20 minutes at least. Firstly, the empty aluminium boxes were weighed, and then 1 g of the samples were weighed in the aluminium boxes. The samples were dried in the incubator (VWR Venti-Line Incubator) over night at 100˚C. The samples were then weighed with the aluminium boxes to calculate the dry matter (% dm) content using the equation 3.1 following as:

$$\%\ dm = \frac{(weight\ after\ drying - weight\ box\ before\ drying)}{weight\ of\ sample} \times 100 \qquad (3.1)$$

3.3.4 Colour measurement

Colour of the porridges was measured using HunterLab Colorflex (A60-1010-615 Model Colorimeter, Hunter Lab, Reston, VA) with a standard white and black tile (L_0=93.01, a_0=-1.11 and b_0= 1.30). The Commission Internationale de l'Eclairage (CIE), CIE Lab colour system, (L*; lightness-darkness, a^*; redness-greenness, b^*; yellowness-blueness), was used to express the colour of the maize porridges prepared at different pH. L* scale illustrates lightness or darkness where a low number (0-50) shows dark and a high number (51-100) indicates light. When a* value is positive it means that the colour is red while a negative number showing green colour. A

positive number of b* illustrates yellow colour and a negative value points out blue colour (Fig 3.4).

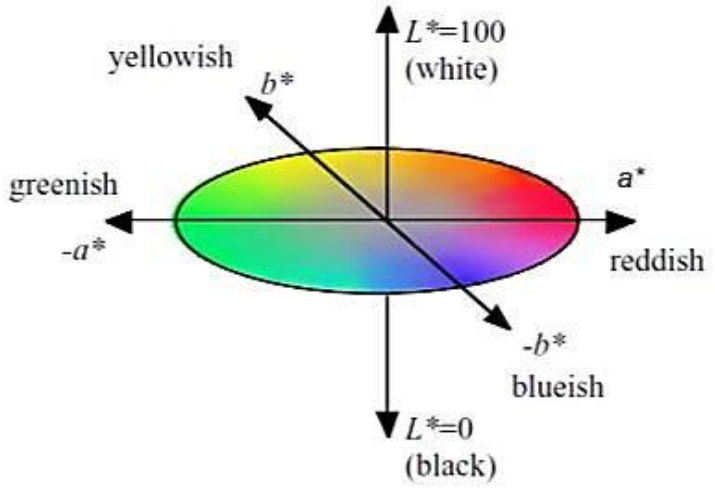


Figure 3.4 CIELAB colour scale (Molino et al., 2013)

3.3.5 Determination of antioxidant activity

The freeze-dried samples were milled using Retsch ball mill (MM400, Germany). Then, they were analysed for their antioxidant activity with Quenching method modified by Serpen et al., (2012). The results were expressed as both Trolox equivalent (mmol TE/ mg porridge) and the half maximal inhibitory concentration (IC_{50}).

3.3.5.1 Preparation of DPPH solution

10 mg/mL stock solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in 100% ethanol was prepared. The solution was sonicated for 5 min using HBM Industriele Ultrasoon Reiniger to dissolve the DPPH. The solution was then diluted to 5 mg/mL with milli Q water and subsequent dilution of 125x by adding 200 µL stock solution to 25 mg/ml 50% ethanol. The solution was prepared one day in advance, covered with aluminium folio, and then stored overnight at room temperature in the dark in order to reach the absorbance >0.5 of the stock solution. Finally, the absorbance of DPPH was measured at 525 nm using Bio – UV – Visible Spectrophometer (CARY 50, Australia).

3.3.5.2 Calibration curve of Trolox

20 mg/mL stock solution of 6 hydroxy 2,5,7,8 tetramethylchroman 2-carboxylic acid (Trolox) in 100% ethanol was prepared and diluted 5.33x/ 8x /16x /40x in 100% ethanol to get 4 point calibration curve of 3.75 / 2.5 / 1.25 / 0.5 mg/ ml (0.15, 0.1, 0.05, 0.02 µmol Trolox). 8 sample tubes were prepared in 5 mL Eppendorf tubes by adding 10 mg of cellulose. Four of them were used for the preparation of calibration curve of Trolox and the rest for preparation of blank. Then, 10 µL of Trolox solutions (5.33x/ 8x /16x /40x) were added separately into test tubes for calibration curve. In order to prepare blanks 10 µL of 100% methanol were added instead of Trolox. 5 ml of DPPH solution was added to the trolox solutions. The mixtures were incubated 100 minutes at room temperature using a mixer (Heidolph Multi Reax, Germany) and then centrifuged at 9000 g for 5 min. Subsequently the absorbance of the samples were measured at 525 nm using CARY 50 Bio-UV-Visible Spectrophotometer. The calibration curve of Trolox was constructed (Figure 3.5). After that the linear regression was applied. The mmol of Trolox of sample using absorption and equation was calculated and subsequently the mmol Trolox / mg were recalculated.

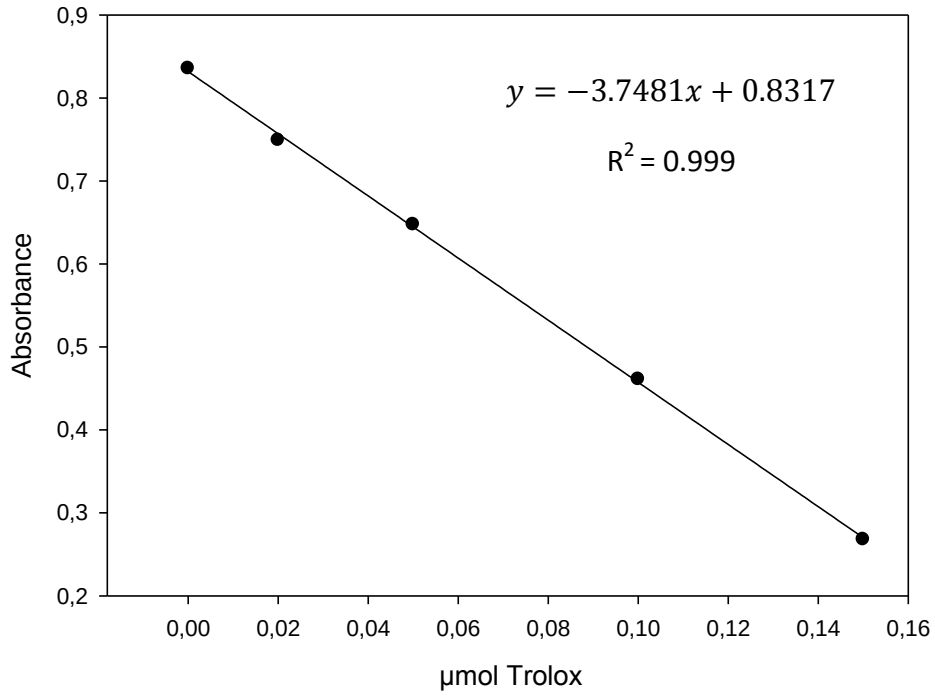


Figure 3.5 The concentration curve of Trolox with the range of 0.05-3.75

3.3.5.3 Preparation of porridges for IC_{50} measurement

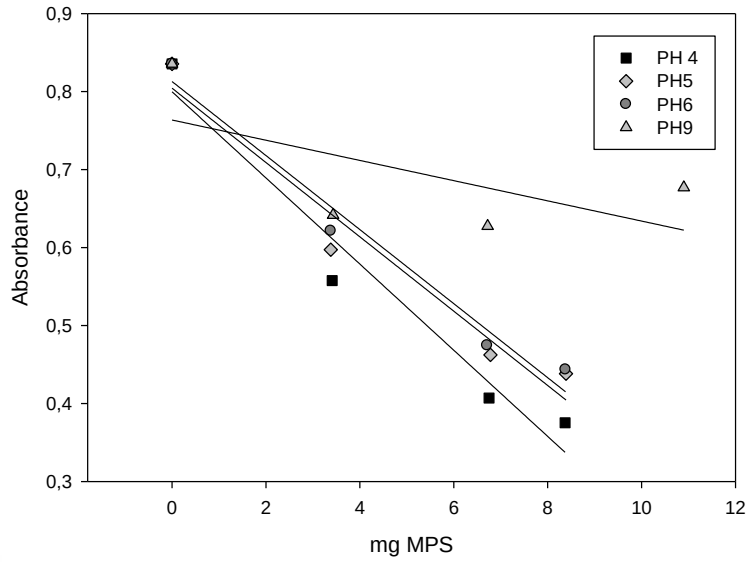
In order to get absorbance between 20 and 80% of inhibition of DPPH different dilutions were tested for each MPS. Firstly, the ratio of 1:1 (100 mg of cellulose: 100 mg of sample) was tried. The 5 mg/ 10 mg/ 15 mg/ 20 mg of samples were taken from this porridge-cellulose mixture in 5 ml Eppendorf tubes. 5 ml of DPPH solution was added to the samples. The samples were incubated 100 minutes at room temperature using a mixer (Heidolph Multi Reax, Germany) and then centrifuged at 9000 g for 5 min. Subsequently the absorbance of the samples were measured at 525 nm using CARY 50 Bio-UV-Visible Spectrophotometer. The ratio of 1:1 did not give absorbance between 20 and 80% of inhibition of DPPH. Therefore the ratio of 3:1 (300 mg cellulose: 100 mg of sample) was tried using the same procedure mentioned above. The 3:1 ratio did not also work properly. Lastly, the ratio of 5:1 (500 mg of cellulose and 100 mg of sample) was also tried. The ratio of 5:1 worked accurately as regards to other pH studied. For the measurements of MP samples any dilution was not applied. Table 3.1 shows the amount of mixture used in the ratio of 5:1 for MPS and the amount of porridges used for MP in the final measurements.

Table 3.1 Diluted samples used for each pH value

	MP (mg)			MPS (mg)		
pH4-1	10	20	40	20	40	50
pH4-2	20	40	50	20	40	50
pH5-1	10	20	40	20	40	50
pH5-2	20	40	50	20	40	50
pH6-1	10	20	40	20	40	50
pH6-2	20	40	50	20	40	50
pH9-1	10	20	40	20	40	65
pH9-2	20	40	65	20	40	65

MP, Maize porridge; MPS, Maize porridge with sorghum

The curves constructed to calculate IC_{50} measurements for pH 4, 5, 6 and 9 was shown in Figure 3.6.



$y = -0.0552x + 0.7996$

R^2=0.9546 (pH4)

$y = -0.0476x + 0.8043$

R^2=0.8043 (pH5)

$y = -0.0475x + 0.8127$

R^2=0.8127 (pH6)

$y = -0.013x + 0.7635$

R^2=0.3974 (pH9)

Figure 3.6 The regression curves for calculation of IC_{50}

3.3.6 HPLC analysis

3.3.6.1 Sample Preparation

100 mg of sample was weighed and 3 mL of HCl/methanol (1% v/v) was added to the sample. The sample was incubated one hour at room temperature using the shaker (Heidolph Multi Reax, Germany). The sample was then centrifuged 10 min at 5000 g and the supernatant was collected. The extraction was repeated using the same method. The supernatants from the first and second extraction were mixed and kept at -20˚C in the freezer until the analysis.

3.3.6.2 HPLC Determination of Anthocyanidins

The apigeninidin content was determined using HPLC system consisting of an Ultimate 3000 RS Diode Array Detector, a Ultimate 3000 RS Pump, a Ultimate 3000 Autosampler, and a Ultimate 3000 RS Column Compartment. Analytical separation of apigeninidin and phenolic acids was carried out using a Varian Polaris 5 C18-A 4.6 x 150 mm, a 5 µm column.

Identification and quantification were done by use of a standard curve (Figure 3.7) obtained by injecting different concentrations of apigeninidin standard (Extrasynthese, France) as follows: 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, 0.97 µg/mL.

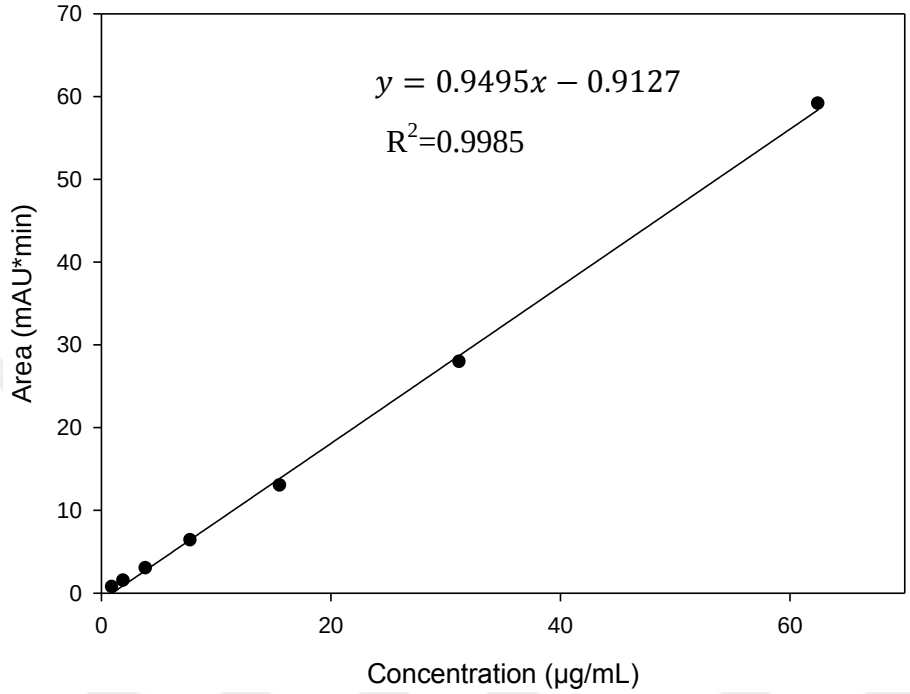


Figure 3.7 Calibration curve of apigeninidin with range of 0.97, 1.95, 3.9, 7.8, 15.6, 31.25, and 62.5 µg/mL

Elution for apigeninidin was executed under gradient conditions with (A) 10% formic acid in water and (B) methanol. The solvent gradient was programmed as follows: 0-20 min, from 5% to 60% B; 20 to 25 min, from 60% to 100% B; 25 to 30 min with 100% B; 30 to 31 min from 100% to 5% B; 31 to 35 min with 5% B. The solvent flow rate was set at 1 mL/min, and the chromatogram was recorded at 480 nm (apigeninidin). Data signals were processed on a personal computer (PC) running Chromeleon 7 Chromatography Data System. Peak areas were used for all calculations.

3.3.7 Statistical analysis

One-way analyses of variance (ANOVA) were conducted to determine;

- the effect of pH on Hunter colour parameters (L*, a*, b*) of maize porridges

- the effect of pH on antioxidant activity of maize porridges
- the differences of apigeninidin content of maize porridges.

The IBM SPSS Statistics for Windows (2013), Version 22.0 (Armonk, NY: IBM Corp.) was used. Each measurement was triplicated. In order to determine which means are significantly different from each other, Duncan multiple range test method was used. Trends were considered significant when means of compared parameters differed at $p < 0.05$ significance level.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Dry matter content of maize porridges

Table 4.1 shows the moisture content of porridges (MP and MPS) before and after the freeze drying. As it was seen after the freeze drying the moisture contents of porridges were lower than 7 % wet basis.

Table 4.1 Moisture content of maize porridges before and after freeze drying

	MC (%) before freeze-drying	MC (%) after freeze-drying	MC (%) before freeze-drying	MC (%) after freeze-drying
MP			**MPS**	
pH4	90.2	4.9	89.5	4.2
pH5	89.9	6.4	90.1	5.5
pH6	90.5	5.3	90.6	7
pH9	90.7	5.4	90.4	4.4

MC, moisture content; MP, Maize porridge; MPS, Maize porridge with sorghum

4.2 pH values of maize porridges after cooking

In this study the effect of pH on apigeninidin stability were evaluated at pH 4, 5, 6, and 9. These pH values have been decided according to two criteria. One of them is that apigeninidin should stay stable. The other one is that it will be possible to prepare maize porridge with appropriate taste. Devi et al., (2012) have reported that sorghum extracts shows high absorbance at pH 1.1, 4, 6, 8.9. Due to these reasons the above pH values have been evaluated in this study.

The pH values of maize porridges (MP and MPS) were adjusted before the cooking during porridge preparation in order to see whether there are any differences on their pH values. They were measured again after cooking. The pH change of MP and the MPS were shown in Figure 4.1.

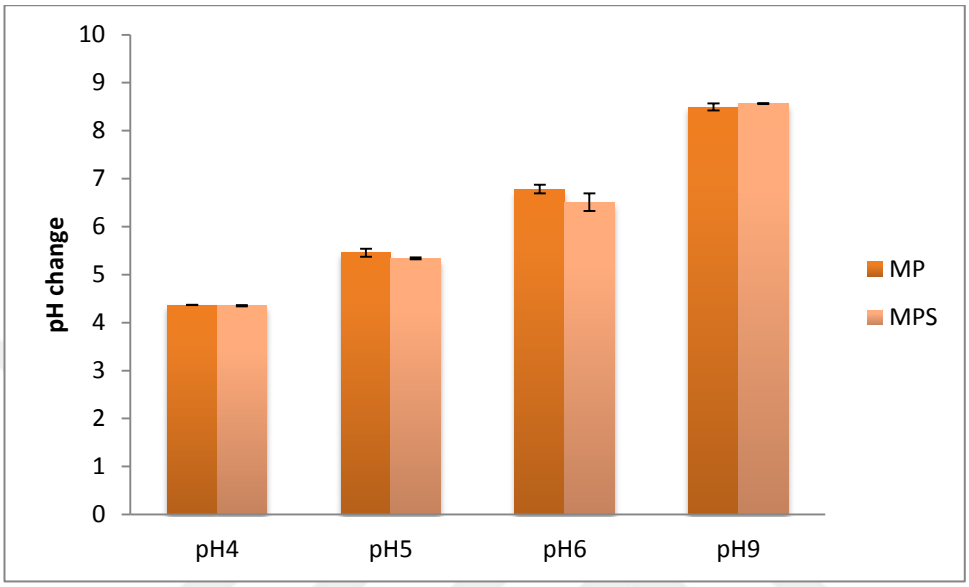


Figure 4.1 The pH change of MP and MPS after making porridge at pH range of 4, 5, 6, and 9

As can be seen in the Fig 4.1 there is an increasing trend on the pH values of both MP and MPS after the cooking at pH 4, 5, 6 and 9. However, decreasing was observed on the pH values of MP and MPS samples at pH 9 after the cooking when the change of pH for MP and MPS were examined separately for each pH (4, 5, 6 and 9). The results show that there are no significant differences ($p>0.05$) on the pH values of MP and MPS samples.

4.3 Antioxidant activity of apigeninidin in maize porridge

4.3.1 Preliminary studies

The antioxidant activities of porridges (MP and MPS) were expressed as both IC_{50} and Trolox equivalent µmol/g. A number of preliminary experiments were performed to obtain the best dilution factors for samples during antioxidant activity determination.

Firstly, in order to obtain the inhibition of DPPH between 20 and 80% for MPS samples the ratio of cellulose to sample were changed from 1:1(100 mg cellulose and 100 mg of sample) to 5:1 (500 mg cellulose and 100 mg of sample). The percentage of DPPH inhibition at various pH for ratio of 1:1 has been given in Appendix A.1. It shows that the inhibition of DPPH for pH 9 was higher than 100% which is not appropriate for the Quencher approach. Therefore the ratio of 3:1 (300 mg cellulose and 100 mg of sample) was tried. The results for the ratio of 3:1 were given in Appendix A.2. The percentage of the absorbance at pH of 4, 5, and 6 was lower than 80% and higher than 20% in the ratio 3:1. It means that the absorbance could work for the method. However, the proportion of pH 9 was not proper also in the ratio of 3:1. Subsequently the ratio of 5:1 worked better than other ratios applied in this experiment (Table 4.2). Despite the lowest point of pH 9 at this ratio, there was still a higher antioxidant activity in comparison with the ratio 1:1 and 3:1. Therefore, the ratio of 5:1 (500 mg cellulose and 100 mg of sample) was used for the analysis of antioxidant activity of all samples.

Table 4.2 The percentage of DPPH at different pH with Trolox equivalents for the ratio of 5:1 (500 mg cellulose / 100 mg coloured porridge)

MPS		**(1)**		**MP**	**(2)**	
		% inhibition of DPPH	**µmol/g**		**% inhibition of DPPH**	**µmol/g**
	0 mg					
	20 mg	65.3	22.7		68.2	20.4
pH4	40 mg	48.4	16.1		49	16.7
	50 mg	44.7	13.3		45.1	14.5
	0 mg					
	20 mg	64.9	22.8		78	14.1
pH5	40 mg	50.6	16.8		60.1	12.9
	50 mg	49.6	14.6		55.3	11.8
	0 mg					
	5 mg	73.1	17.5		56.7	28.3
pH6	10 mg	56.9	14.2		51.4	15.9
	20 mg	54.8	11.9		50.1	13.2
	0 mg					
	5 mg	75.5	15.6		78.2	13.9
pH9	10 mg	73.3	8.71		76.9	7.51
	20 mg	82.8	3.42		79.3	4.16

MP, Maize porridge; MPS, Maize porridge with sorghum

4.3.2 Effect of pH on antioxidant activity

The ratio of 5:1 was used to obtain whether there were any differences on the antioxidant activity of MP and MPS at different pH (Fig 4.2). As it was expected, the antioxidant activity of MPS was higher than that of MP at all pH studied. This is due to high anthocyanin particularly apigeninidin content of sorghum leaf sheath extract which is used to colour maize porridge.

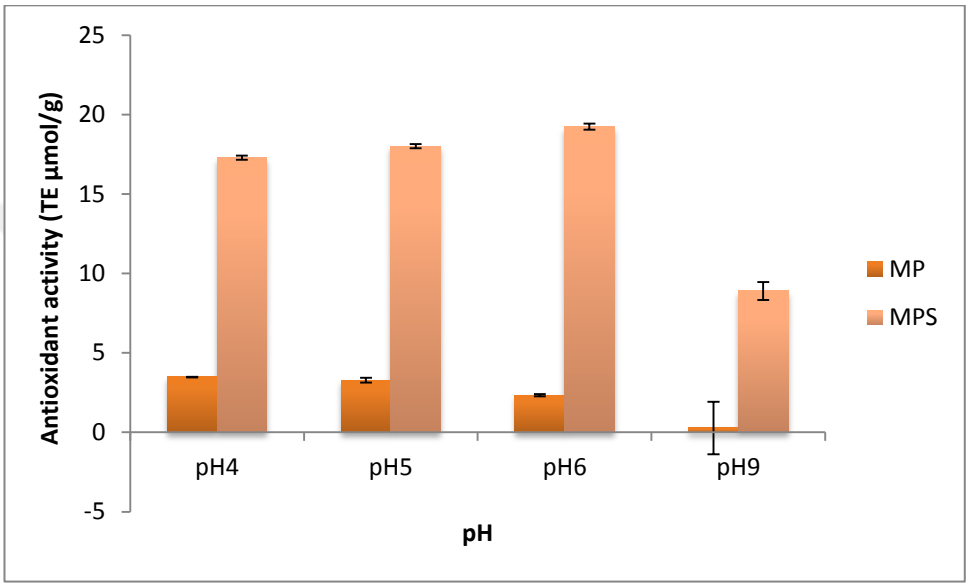


Figure 4.2 The antioxidant activity in MP (control) and MPS (maize porridge coloured with sorghum) with pH range of 4, 5, 6, and 9

The differences in antioxidant activities of MP and MPS samples were found statistically significant ($p<0.05$). Fig 4.2 also shows that the antioxidant activity of MP samples changed due to pH. Therefore, Fig 4.3 was constructed by omitting the antioxidant effect of MP to see just the effect of pH on antioxidant activity of MPS.

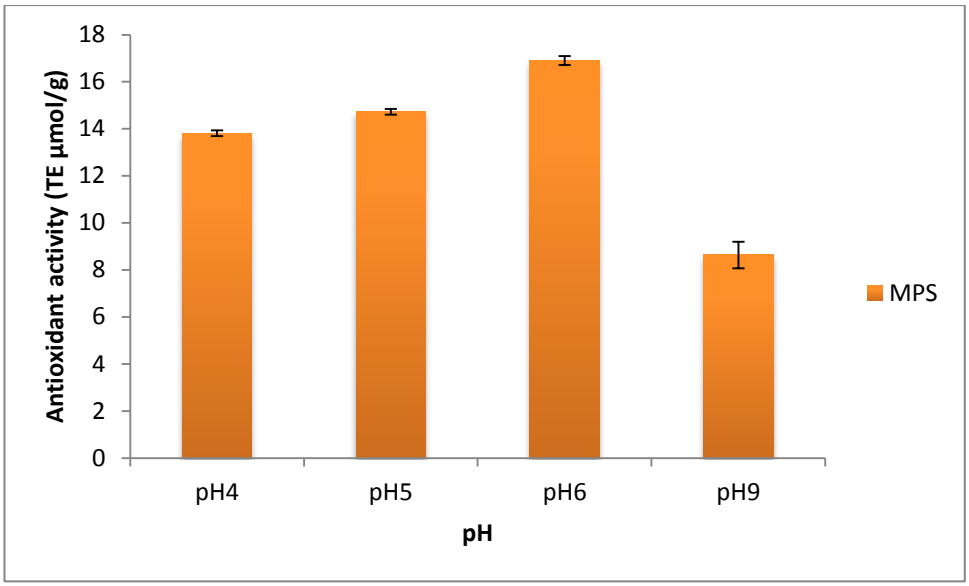


Figure 4.3 The antioxidant activity in MPS (coloured porridge samples) by omitting the antioxidant activity of control samples with pH range of 4, 5, 6 and 9

It was demonstrated that there is no regular effect of pH on the antioxidant activity of MPS (Fig 4.3). According to the statistical results there is significantly difference ($p<0.05$) between the antioxidant activities of MPS with respect to pH (Appendix A.4). Since, the pH-transformed forms of anthocyanins exhibit different antioxidant activity in aqueous solutions. There are very sophisticated reversible structural transformations such as proton transfer between coloured forms and water addition to the pyrylium ring which leads formation of colourless hemiacetals and chalcones (Kähkönen et al., 2003, Lapidot et al., 1999, Dangles et al., 2000; Estevez et al., 2010). As it can be seen from the Figure 4.3 the order of antioxidant activity was as followed; pH 6 > pH 5 > pH 4 > pH9. According to these results while the antioxidant activity in MPS was the highest at pH 6 with 17.29 µmol TE/g, the lowest antioxidant activity (8.89 µmol TE/g) was observed at pH 9. Dangles et al., (2000) reported that even if the pH-transformed forms of the anthocyanins remained effective antioxidants, the quinoidal-base and chalcone forms are powerful hydrogen donors. The findings of this study were in agreement with literature. Because as it was demonstrated in the study of Brouillard et al., (1982) the concentration of quinoidal-base and chalcone forms of apigeninidin was higher at pH 6 than that of other pH. Baranac and Amic (1990) found that the anionic form of A^- and trans-chalcone tC^- forms of apigeninidin were abundant at pH 9. The reactivity of trans-chalcone towards DPPH was considerably lower than the coloured forms (Dangles et

al., 2000). This might be the reason of why pH 9 showed the lowest antioxidant activity in MPS.

The results of antioxidant activity for MPS with respect to IC_{50} were given in Table 4.3. The lowest IC_{50} value represents the higher antioxidant activity. Therefore it can be concluded that IC_{50} results showed completely similar trend with Trolox equivalent values of MPS.

Table 4.3 The antioxidant activity of MPS with respect to IC_{50}

	IC_{50}			
pH	4	5	6	9
MP	18,47	17,90	13,45	2,2
MPS	15,35	13,15	12,7	6,5

MP, Maize porridge; MPS, Maize porridge with sorghum

4.4 Effect of pH on the total apigeninidin content

The HPLC chromatograms in Fig 4.4 a, b, c and d show the results of anthocyanidin analysis of MPS at pH 4, 5, 6 and 9, respectively. The 3-deoxyanthocyanidin, apigeninidin, was only determined in our MPS. The luteolinidin which is another important 3-deoxyanthocyanidin has not been detected in MPS. However, Benson et al., (2013) have reported that sorghum leaf sheath contains 39,900 µg apigenidinin/g and 450 µg luteolinidin/ g dry matter. Although some phenolic acids, such as benzoic acids (801.4 µg/g), *p*-coumaric (681.6 µg/g), and *o*-coumaric acid (67.9 µg/g) were found in the extracts of sorghum leaf sheath (Kayodé et al., 2011), these phenolic acids were not also detected in MPS. The missing of the luteolinidin and phenolic acids in MPS was due to the presence in small quantities when mixed with maize flour. Any apigeninidin content was not observed in MP due to the lack of sorghum leaf sheath extract in it. Dykes and Rooney (2007) have also reported that apigeninidin was not found in maize flour. Therefore the result of apigeninidin content in maize porridge does not affected by maize flour in this study.

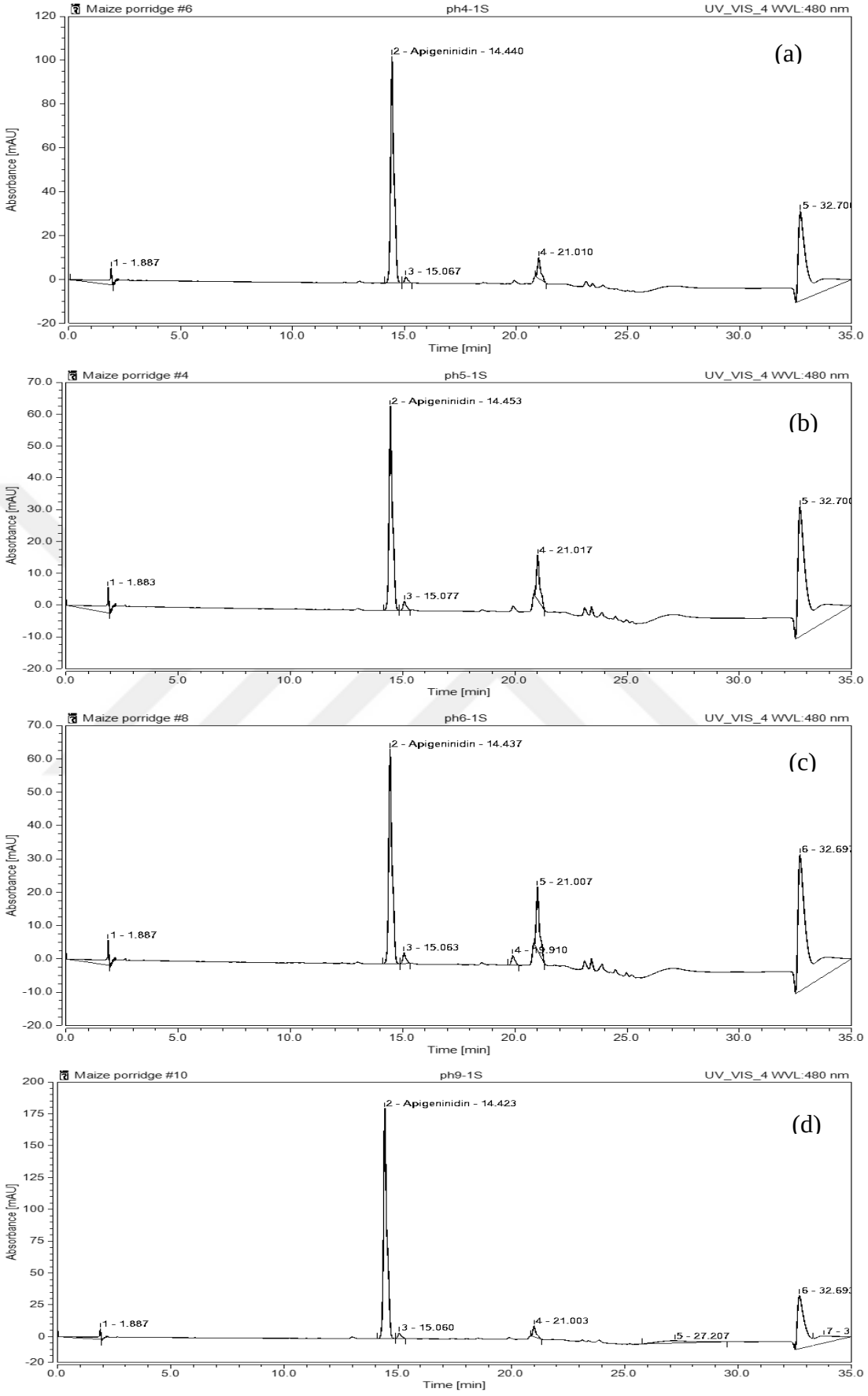


Figure 4.4 The chromatogram of apigeninidin content of maize porridges coloured with sorghum at pH (a) 4, (b) 5, (c) 6, and (d) 9

Figure 4.5 has been created from the results of HPLC analysis in order to see the apigenidinin content of MPS with changing pH. Based on the results, pH had a great influence on the stability of apigeninidin pigment. The highest apigeninidin content was observed at pH 9. While the apigeninidin content was almost same at pH 5 and 6, it was lower than that of pH 4. Devi et al., (2012) were evaluated the stability of methanol extracts of sorghum anthocyanins by measuring absorbance at 480 nm at changing pH from 1.1 to 10.5. They have found almost the same results. They reported also that anthocyanins were more stable at around pH 9 followed by pH 4 and then 6 and 5. These differences on apigeninidin content with changing pH due to pH-transformed forms of anthocyanins resulted from simultaneous degradation of anthocyanins. Since the characteristics absorbance of the pH-transformed forms of anthocyanins shows differences on UV-Vis spectra. Baranac and Amic, (1990) stated that flavylium cation structure (AH^+) of apigeninidin was stable between the pH 2 and 4 and had a strong peak absorption (λ_{max}) at 468 nm whereas the colourless pseudo-base form (B) had λ_{max} at 276 nm. The hydro-base form (A) at 496 nm and the formation of chalcone (C) at 410 nm occurred at pH 4.5. When increased the pH, anionic form A^- of apigeninidin at 530 nm appeared until pH 5.5 as well as the ionised form of cis-chalcone (cC^-) was present in the same pH range. A^- and cC^- maintained their presence in the low alkaline medium. The increase in alkaline medium till pH 11 induced the formation of A^- in the apigeninidin with the ionized trans-chalcone form (tC^-) at 474 nm. In conclusion the results of our study also showed that at pH 4 the concentration of coloured AH^+ and A forms were predominant so the absorbance was higher at 480 nm. Then the concentration of AH^+ reduced by increasing pH (pH 5), while the concentrations of colourless ones, C and B, increased. On the other hand when pH reached to 6 the concentration of coloured A was a little bit higher than that of at pH 5. Therefore the absorbance was higher at pH 6 than that of pH 5. Finally at pH 9, the new coloured forms, A^- and tC^- was observed so the absorbance again increased at pH 9.

The stability of anthocyanins was normally very low within the pH range of 3.0 and 6.0. However 3-deoxyanthocyanidins were more stable than other anthocyanins at these pH ranges (Yang et al., 2014). From that statement, similar results were obtained in present study. Even if the apigeninidin showed poor stability at pH 5 and 6 compared to other pH, they present in some extent (0.82 µg/mg) (Fig 4.5). Thus,

the 3-deoxyanthocyanidins may have high potential for the application in food systems (Awika et al., 2004). Moreover, according to the statistical analysis, the apigeninidin content in MPS were significantly different ($p<0.05$) from each other at pH values studied (Appendix A.5). However the apigeninidin content of MPS at pH 5 and pH 6 were not found significantly different.

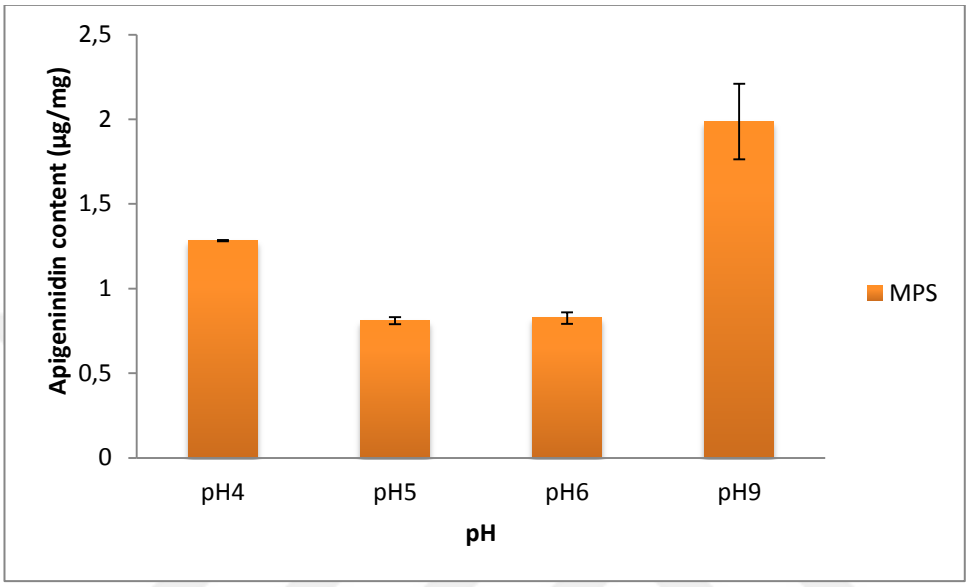


Figure 4.5 Apigeninidin content in maize porridge with sorghum colourant (MPS) at different pH values (4, 5, 6, and 9)

4.5 Effect of pH on colour stability of apigeninidin

The colour stability is an important criterion in food quality. The pH is one of the main factors that influence the colour expression of anthocyanins (Torskangerpoll&Andersen, 2005). The CIE Lab colour parameters (L*; lightness-darkness, a*; redness-greenness, b*; yellowness-blueness) are commonly used in describing visual colour of foods. The colour parameters, L*, a* and b*, of MP and MPS were shown in Figure 4.6 a, b and c, respectively. First of all, results show that MP exhibit a quite bright yellow colour with high L*, b* and low a* values at almost all pH. However, MPS have lower L* values due to their high apigeninidin content than that of MP. Since L* is a measure of lightness and darkness, low L* values means that MPS were dark in colour. Figure 4.6 (b) also shows that there is a loss on the yellowness of MPS with increasing pH. The high a* value for MPS demonstrated that the anthocyanins present in MPS give predominantly red colour at pH studied

(Figure 4.6 (c)). Moreover, the L*, a* and b* values of MP and MPS have been found significantly different ($p<0.05$) from each other (Appendix A.3).

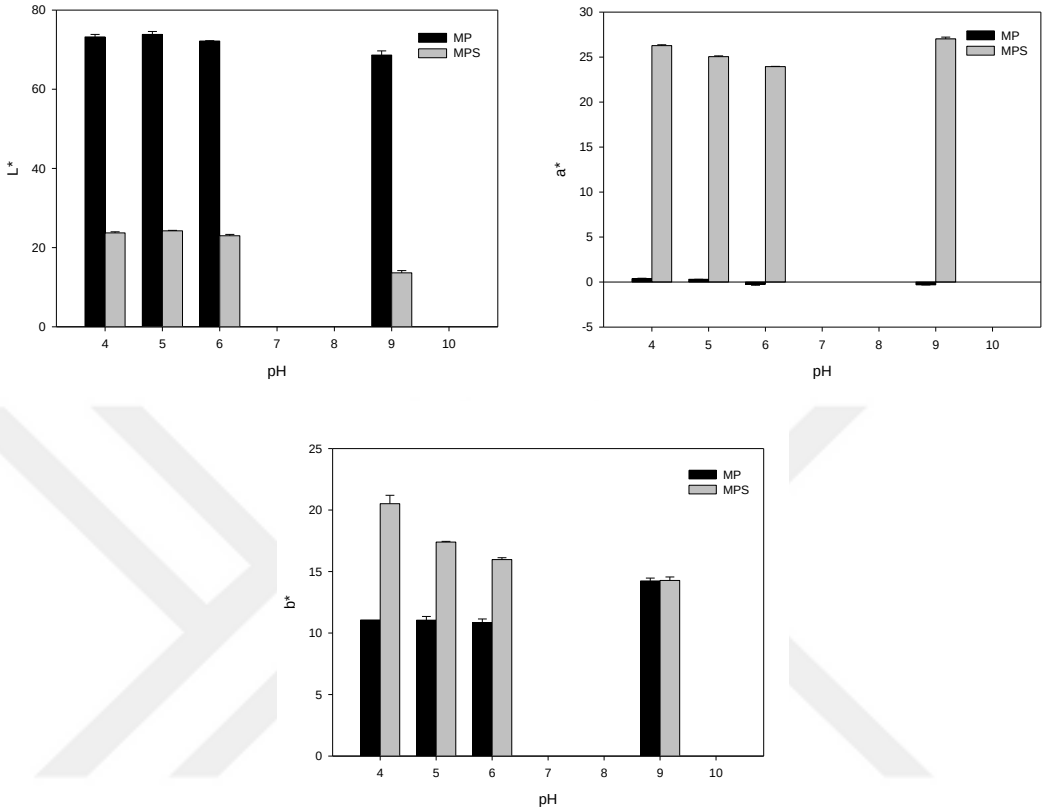


Figure 4.6 L* (a), a* (b), b* (c) for MP (maize porridge) and MPS (maize porridge coloured with sorghum) at pH of 4, 5, 6, and 9

As it is known the anthocyanins show structural transformations in aqueous media with changing pH. So the final colour of anthocyanins depends on the concentration of two coloured forms, flavyluim cation and quinoidal base and two colourless forms, carbinol pseudobase and chalcone at any pH (Brouillard et al., 1982). Mazza and Brouillard, (1987) reported that anthocyanins and associated compounds can present as red and yellow colour in the falvylium cation form, red or blue in the quinoidal base, colourless in the form of the carbonal pseudobase, and chalcone. When the colour parameters (L*, a* and b*) of maize porridges at different pH was compared there are significant differences ($p<0.05$) between them for both MP and MPS. The significant differences unexpected for MP would be related with the variation in colour of its own anthocyanins at different pH. The change on a* and b* values of MPS followed almost the same pattern except pH 9 (Figure 4.6). The

differences on a* values of MP and MPS was striking and the visual perception of the MPS was also red in colour. Therefore the differences on a* values with changing pH was particularly discussed. There is a decreasing on a* value of MPS from pH4 to pH6. This alteration on the redness of MPS would be related with the structural transformation of apigeninidin as the pH changes. In aqueous solutions there is equilibrium between the different forms of apigeninidin and the apigeninidin largely present in the coloured forms (flavylium cation and quinoidal base) at pH 4. Then the chemical equilibrium between them shifts towards increasing the colourless forms by increasing pH (Brouillard et al., 1982). Therefore at pH 5 and pH 6 MPS had lower a* values than that of at pH4. However the highest redness was observed at pH 9. These results show that apigeninidin in maize porridge shows colour stability not only at slightly acidic (pH 4) but also at slightly basic conditions (pH 9). The similar results have been previously reported by Devi et al., (2012). Normally anthocyanins exhibit greater stability under acidic conditions. However 3-deoxyanthocyanins have better stability due to lack of a hydroxyl group at carbon 3 positions than that of regular anthocyanins (Mazza and Brouillard, 1987). Therefore stability of 3-deoxyanthocyanins pigments compared to commercial anthocyanin pigments make them important for the food applications.

The results of colour stability were found almost the same with the results of apigeninidin degradation analysis. For instance the highest apigeninidin content was observed at pH 9 followed by pH 4 and then pH 6 and pH5 according to the HPLC analysis.

CHAPTER V

CONCLUSION

It is known that 3-deoxyanthocyanins are able to have high stability in both acidic and slightly alkaline conditions. Therefore they have been considered as alternative natural food colorants for food industry in recent years. Up to now the stability of 3-deoxyanthocyanins has not been studied in a food matrix. The apigeninidin is one of the abundant 3-deoxyanthocyanins which present in sorghum leaf sheaths. This study is the first attempt to evaluate the stability of apigeninidin in maize porridge in the pH ranged from 4 to 9. In this context the colour stability, apigeninidin content and antioxidant activity have been evaluated successfully. Although pH had a great influence on the stability of apigeninidin in maize porridge, apigeninidin exhibited activity to a certain extent at all pH values studied. Interestingly, pH 9 showed different aspects as regards to antioxidant activity, apigeninidin content and colour stability in maize porridge. Although the apigeninidin content was higher and the stronger the reddish colour was observed at pH 9, the antioxidant activity was the lowest at pH 9 than the other pH values.

As a result, apigeninidin, colourant pigment in sorghum, gave promising results for food applications in both acidic (pH 4) and alkaline (pH 9) conditions, because of high apigeninidin content, good colour and moderate antioxidant activity. Sorghum has been used due to its bright red colour in various applications. In this research, the red colour from apigeninidin was observed apparently different in maize porridges at various pH.

CHAPTER VI

RECOMMENDATION

The thermal stability of 3-deoxyanthocyanins of sorghum should be examined during porridge making as well as cooking time since the temperature and time are other important parameters to keep the stability of anthocyanins. The food matrixes other than the maize porridge should be also used to see the effect of pH on antioxidant activity and other parameters.

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APPENDIX

A.1 The percent of DPPH at different pH with Trolox equivalents for the ratio of 1:1 (100 mg cellulose / 100 mg coloured porridge)

		(1)			(2)		
			mmol			mmol	
MPS		**%**	**Trolox**	**µmol/g**	**%**	**Trolox**	**µmol/g**
pH4	0 mg						
	5 mg	67.6	0.0001	38.9	84.29	2.1E-05	8.21
	10 mg	52.08	0.00017	33.2	79.7	2.8E-05	5,54
	15 mg	45.74	0.0002	27.2	63.23	5.3E-05	6.99
	20 mg	42.61	0.00022	21.3	54.6	6.7E-05	6.63
pH5	0 mg						
	5 mg	68.7	0.00021	77.9	71.58	6E-05	22.63
	10 mg	55.06	0.0003	55.6	58.03	9.2E-05	17.86
	15 mg	54.89	0.0003	40.2	51.21	0.00011	14.23
	20 mg	47.97	0.00035	34.2	43.47	0.00013	12.65
pH6	0 mg						
	5 mg	75.46	5.35E-05	20.9	76.2	8.15E-05	30.78
	10 mg	63.38	7.77E-05	15.2	66.79	0.000114	22.01
	15 mg	51.47	0.000102	13.4	61.54	0.000133	17.14
	20 mg	49.51	0.000106	10.5	61.46	0.000133	12.92
pH9	0 mg						
	5 mg	84.87	5.24E-05	19.1	76	4.16E-05	16.58
	10 mg	101.4	-9.5E-07	-0.18	73.5	4.69E-05	9.35
	15 mg	104	-9.4E-06	-1.25	87.47	1.72E-05	2.29
	20 mg	118.3	-5.5E-05	-5,42	103.9	-1.8E-05	-1.74

MP, Maize porridge; MPS, Maize porridge with sorghum

A.2 The percent of DPPH at different pH with Trolox equivalents for the ratio of 3:1 (300 mg cellulose / 100 mg coloured porridge)

		(1)			(2)		
			mmol			mmol	
MPS		**%**	**Trolox**	**µmol/g**	**%**	**Trolox**	**µmol/g**
pH4	0 mg						
	5 mg	75.7	6.3E-05	45.53	91.62	9E-06	6.42
	10 mg	62.31	0.00013	46.34	73.72	3.7E-05	13.65
	15 mg	55.47	0.00016	41.84	75.54	3.42E-05	9.07
	20 mg	48.26	0.00019	37.52	73.8	3.69E-05	7.27
pH5	0 mg						
	5 mg	78.78	0.00014	112.9	82.13	3.5E-05	28.05
	10 mg	71.1	0.00019	70.71	70.79	6.2E-05	23.36
	15 mg	64.81	0.00024	62.87	67.61	6.9E-05	17.9
	20 mg	59.1	0.00027	52.56	59.41	8.9E-05	17.33
pH6	0 mg						
	5 mg	79.54	4.53E-05	32.89	84.68	5.18E-05	38.19
	10 mg	64.76	7.49E-05	28.76	79.46	7.65E-05	29
	15 mg	61.23	8.2E-05	21.83	70.71	9.26E-05	23.92
	20 mg	50.83	0.000103	20.22	64.18	0.000123	24.57
pH9	0 mg						
	5 mg	85.21	5.13E-05	37.1	76.49	4.05E-05	31.07
	10 mg	85.08	5.17E-05	19.23	73.22	9.37E-05	36.27
	15 mg	87.04	4.54E-05	11.65	69.34	0.000101	26.71
	20 mg	95.91	1.68E-05	3,27	70.44	5.34E-05	10.28

MP, Maize porridge; MPS, Maize porridge with sorghum

A.3 One-way analysis of variance (Anova) results of Hunter colour parameters (L*, a*, b*) for MP and MPS samples at different pH

a (MPS)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16,602	3	5,534	753,799	,000
Within Groups	,059	8	,007		
Total	16,661	11			

a (MPS)	Ph	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	6,00	3	23,9367			
	5,00	3		25,0467		
	4,00	3			26,2700	
	9,00	3				27,0200
	Sig.		1,000	1,000	1,000	1,000

a (MP)	Ph	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	9,00	3	-,3050	
	6,00	3	-,2400	
	5,00	3		,3050
	4,00	3		,3700
	Sig.		,118	,118

a (MP)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1,129	3	,376	182,461	,000
Within Groups	,017	8	,002		
Total	1,145	11			

b (MPS)	Ph	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	9,00	3	14,2800			
	6,00	3		15,9700		
	5,00	3			17,4000	
	4,00	3				20,5100
	Sig.		1,000	1,000	1,000	1,000

b (MPS)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	62,799	3	20,933	289,730	,000
Within Groups	,578	8	,072		
Total	63,377	11			

b (MP)	Ph	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	6,00	3	10,8550	
	5,00	3	11,0450	
	4,00	3	11,0600	
	9,00	3		14,2350
	Sig.		,194	1,000

b (MP)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23,820	3	7,940	275,033	,000
Within Groups	,231	8	,029		
Total	24,051	11			

L (MPS)	Ph	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	9,00	3	13,6250			
	6,00	3		23,0000		
	4,00	3			23,7200	
	5,00	3				24,2300
	Sig.		1,000	1,000	1,000	1,000

L (MPS)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	228,418	3	76,139	1166,327	,000
Within Groups	,522	8	,065		
Total	228,940	11			

L (MP)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	46,947	3	15,649	55,276	,000
Within Groups	2,265	8	,283		
Total	49,212	11			

L (MP)	Ph	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	9,00	3	68,6350		
	6,00	3		72,1800	
	4,00	3			73,2233
	5,00	3			73,6783
	Sig.		1,000	1,000	,326

A.4 Anova results of antioxidant activity for MP and MPS samples at different pH

MP	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8,200	3	2,733	690,712	,000
Within Groups	,032	8	,004		
Total	8,231	11			

MP	ph	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	9,00	3	1,4033			
	6,00	3		2,3300		
	5,00	3			3,2800	
	4,00	3				3,4763
	Sig.		1,000	1,000	1,000	1,000

MPS	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	199,499	3	66,500	1368,129	,000
Within Groups	,389	8	,049		
Total	199,888	11			

MPS	ph	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	9,00	3	8,9000			
	4,00	3		17,2900		
	5,00	3			18,0100	
	6,00	3				19,2350
	Sig.		1,000	1,000	1,000	1,000

A.5 Anova results of apigeninidin content for MPS samples at different pH

Apigeninidin (MPS)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2,751	3	,917	141,966	,000
Within Groups	,052	8	,006		
Total	2,803	11			

Apigeninidin (MPS)	pH	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	5,00	3	,8097		
	6,00	3	,8267		
	4,00	3		1,2853	
	9,00	3			1,9880
	Sig.		,802	1,000	1,000