

Bioaccessibility of phenolic compounds and related antioxidant and health promoting properties of soured sorghum porridges

By

Nomfundo Dlamini

Submitted in partial fulfilment of the requirements for the degree.

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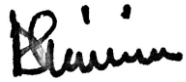
University of Pretoria

South Africa

June 2022

DECLARATIONS

I declare the dissertation which I hereby submit for the degree MSc (Food Science) at the University of Pretoria is my work and has not previously been submitted by me for any other degree at another University or Institution of higher learning.



Nomfundo Dlamini

2022

DEDICATION

This dissertation is dedicated to my late father Mr. Amos M. Dlamini. Thank you, Dad, for giving me a chance in life.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following for their valuable assistance throughout the study:

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The Almighty God for giving me such an opportunity. I bless your name Jehovah.

ABSTRACT

Bioaccessibility of phenolic compounds and related antioxidant and health promoting properties of soured sorghum porridges

By

Nomfundo Dlamini

Degree: MSc Food Science

Supervisor: Prof K.G. Duodu

Co-supervisor: Prof E. Kayitesi

The percentage of deaths due to non-communicable diseases (NCDs) such as diabetes, cancer, obesity and heart disease has been growing steadily in South Africa. Rapid urbanization and increased demand for convenience foods have driven changes in dietary choices towards those based on highly refined grains which has led to an increase in susceptibility to oxidative stress and NCDs among consumers. This brings into focus the importance of sorghum which is a cereal crop consumed in many parts of Africa and is well known as an important source of bioactive phenolic compounds with the potential to protect against diet-related NCDs. Phenolic compounds in sorghum occur mainly in bound forms which limits their extractability, bioaccessibility and subsequently their ability to exert health-promoting effects. Processing methods such as souring could release phenolics in sorghum from their bound forms and therefore enhance their bioaccessibility. This research studied the effect of souring using spontaneous fermentation and citric acid acidification on the bioaccessibility of phenolic compounds and related antioxidant and health promoting properties of sorghum porridges.

Two type I non-tannin sorghums (red and white phenotype) were used. Soured sorghum porridges were prepared using spontaneous fermentation and exogenous acidification with citric acid. For determination of bioaccessibility of phenolic compounds, supernatants were obtained from sorghum porridges digested using a static simulated *in vitro* gastrointestinal digestion procedure.

Total phenolic content (TPC) using the Folin-Ciocalteu assay, phenolic composition using Ultra Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UPLC-QToF-MS), esterase enzyme activity, ABTS and DPPH radical scavenging activity, Oxygen Radical Absorbance Capacity (ORAC), Nitric Oxide (NO) inhibition, Caco-2 cellular antioxidant activity as well as the capacity to protect against plasmid DNA oxidative damage were evaluated for the sorghum flours, porridges and supernatants of digested porridges.

Phenolic compounds identified in the two sorghums were phenolic acids and flavonoids. The major phenolic acids class present were the hydroxycinnamic acids. Flavonoids identified were flavanones (the major identified flavonoid class), flavonols, flavan-3-ols and flavones. Red sorghum flour, porridges and supernatants contained higher levels of total phenolic compounds compared to that for white sorghum.

Souring through spontaneous fermentation led to an increase in TPC and radical scavenging activity against ABTS, DPPH and ORAC values for the sorghum flours and resultant porridges. This increase was attributed to the acidic conditions imposed by the fermentation process combined with the action of esterase enzymes which facilitate ester bond hydrolysis and the release of bound phenolic compounds. Fermented red sorghum slurry exhibited higher esterase enzyme activity compared to that for white sorghum. On the other hand, acidification with citric acid had no effect on the TPC but produced more bioaccessible phenolic compounds compared to souring through fermentation. This souring method also enhanced the antioxidant activity in all the samples except for ABTS radical scavenging. Although souring had no significant effect on NO inhibition, red sorghum flours, porridges and supernatants of digested porridges had significantly higher NO inhibition capacity than those from white sorghum.

Simulated *in vitro* gastrointestinal digestion also enhanced the TPC and the radical scavenging properties of the porridges. This was attributed to the action of the enzymes used during the *in vitro* digestion process such as amylase, pepsin, pancreatin leading to breakdown of the food structure and enhanced extractability of phenolic compounds.

The flours, porridges and supernatants from both sorghum types exerted Caco-2 cellular antioxidant activity and protected the DNA from AAPH-induced oxidative damage. This is an

indicator of the potential health promoting properties of the soured sorghum porridges by offering protection against diet-related NCDs.

This research has shown that souring, either through spontaneous fermentation or through exogenous acidification with citric acid can enhance the bioaccessibility of phenolic compounds and antioxidant properties of sorghum porridges. These soured sorghum porridges can therefore play a vital role in contributing towards efforts to combat and reduce the current rising trends of the occurrence and mortality from diet- and oxidative stress-related NCDs in Africa.

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1 INTRODUCTION

1.1 INTRODUCTION AND PROBLEM STATEMENT

Over 71% of all global deaths can be attributed to non-communicable diseases (NCDs) (Banwo, Asogwa, Ogunremi, Adesulu-Dahunsi & Sanni, 2021), which include cancer, cardiovascular diseases and diabetes. In 2011, NCDs were estimated to account for 29% of all deaths in South Africa (World Health Organization, 2011). By 2014, the proportion had increased to 43% (World Health Organization, 2014) and further up to 51% in 2018 (World Health Organization, 2018). This clearly shows that there has been a steady rise in the percentage of deaths due to NCDs in South Africa over the years since 2011.

NCDs can be triggered by oxidative stress (Lakshmi, Naushad, Rao & Kutala, 2013). Rapid urbanization together with changes in life patterns (Banwo *et al.*, 2021), which includes lack of physical exercise and the demand for convenience foods that are high in saturated fats and also mostly based on highly refined grains has led to a greater susceptibility to oxidative stress and NCDs among consumers. This has a negative impact on the economy as a lot of people will depend on health facilities (Bloom, Cafiero-Fonseca, Candeias, Adashi, Bloom, Gurfein, Jané-Llopis, Lubet, Mitgang & Saxena, 2014).

Sorghum is a cereal crop which can endure semi-arid climates characterized by high temperatures, poor soils and low rainfall (Adeyanju, Kruger, Taylor & Duodu, 2019). Various food products are made from sorghum ranging from beverages, baked products and different types of porridges (Rashwan, Yones, Karim, Taha & Chen, 2021) and are consumed by people living in these regions where sorghum is an important cereal staple. Sorghum is also a well-known source of phenolic compounds (Salazar-López, González-Aguilar, Rouzaud-Sáñez & Robles-Sánchez, 2018a). These phenolic compounds possess bioactive properties with related health benefits, hence the intake of sorghum is thought to be correlated with reduction in the incidence of a number of NCDs (Gabaza, Shumoy, Muchuweti, Vandamme & Raes, 2016).

In most of sub-Saharan Africa, porridges made from sorghum are a significant part of the daily diet and they are obtained from fermented, unfermented or acidified slurries (Rashwan *et al.*, 2021). Fermentation is mostly done spontaneously in Africa (Onyango, Noetzold, Ziems,

Hofmann, Bley & Henle, 2005). However, processes for producing soured sorghum porridges which involve exogenous acidification with food grade acids like citric acid (Onyango *et al.*, 2005) are on the rise in Africa (Adeyanju *et al.*, 2019; Onyango & Wanjala, 2018). The advantage of producing soured porridges using exogenous acidification is that it is a quicker process of souring and produces a consistent product quality compared with the less controlled spontaneous fermentation.

For phenolic compounds in sorghum to exert meaningful bioactive and health promoting effects, they must be bioaccessible and bioavailable (Carbonell-Capella, Buniowska, Barba, Esteve & Frígola, 2014). Phenolic compounds in sorghum occur mostly in bound forms via ester bonds to cell wall components and macromolecules such as proteins (Ravisankar, Dizlek & Awika, 2021) and this limits their bioaccessibility. Processing can be used to improve bioaccessibility of phenolic compounds in sorghum and there are opportunities for achieving that with the souring process used to produce soured sorghum porridges. The acidic conditions created during souring could potentially hydrolyse the ester bonds that promote binding of phenolic compounds. Furthermore, the action of various hydrolytic enzymes activated during spontaneous lactic acid fermentation could enhance the release of bound phenolics (Gabaza *et al.*, 2016).

Information on the effect of souring on the bioaccessibility of phenolic compounds and health promoting properties of sorghum porridges is lacking. Therefore, in this research the effect of souring using spontaneous fermentation and citric acid acidification on the bioaccessibility of phenolic compounds and antioxidant related health promoting properties of sorghum porridges were studied. Given the current rise in diet-related NCDs in sub-Saharan Africa, such soured sorghum porridges with enhanced bioaccessibility of bioactive phenolics and health promoting properties can play a significant role in improving the health of many communities.

2 LITERATURE REVIEW

2.1 SORGHUM AS A CEREAL GRAIN AND ITS IMPORTANCE FOR FOOD

Sorghum (*Sorghum bicolor* (L) Moench) is the fifth major cereal food crop in the world after barley, maize, rice and wheat (Xiong, Zhang, Warner & Fang, 2019; Zhu, Shi, Yao, Hao & Ren, 2017) and it is extensively consumed as a cereal staple food in semi-arid regions including Africa and Asia (Girard & Awika, 2018). In the western hemisphere, sorghum is mainly used as animal feed (Girard & Awika, 2018; Xiong *et al.*, 2019). Compared to other cereals, sorghum is more economical to produce shown by the world's production of 812 000 tonnes of sorghum grains in 2016 (Khoddami, Mohammadrezaei & Roberts, 2017).

Sorghum, like all cereal grains, has three main anatomical parts: endosperm, pericarp and germ, (Xiong *et al.*, 2019), although some varieties (condensed tannin-containing types) have a testa as a fourth anatomical feature (de Morais Cardoso, Pinheiro, Martino & Pinheiro-Sant'Ana, 2017). Sorghum can be categorised into three types: Type I which have no pigmented testa and lack condensed tannins, Type II which have a pigmented testa and condensed tannins that can be extracted only in acidified methanol and Type III that also have a pigmented testa and whose tannins are extracted in non-acidified and acidified methanol (Rao, Santhakumar, Chinkwo, Wu, Johnson & Blanchard, 2018a). The sorghum grain occurs in various phenotypic forms in terms of pericarp colour (black, red, white and lemon-yellow) (Dykes, Peterson, Rooney & Rooney, 2011).

The grain can be milled into flour and processed into various foods such as cookies, snacks, thin and thick porridges (Bvochora, Danner, Miyafuji, Braun & Zvauya, 2005). In South Africa, sorghum-based food products include sorghum meal, known as “mabele”, malted instant porridge products and “umqombothi”, a traditional sorghum beer. Sorghum porridge can be eaten in unfermented or fermented forms and as either thin or thick porridges. *Ting* is one example of a sorghum porridge from South Africa and Botswana that is fermented traditionally (Adebo, Njobeh, Mulaba-Bafubandi, Adebisi, Desobgo & Kayitesi, 2018). Other examples of fermented sorghum porridges within the sub-Saharan African sub-region include *nasha* in Sudan, *uji* from East Africa (Adebo, 2020), *obushera* from Uganda and *ogi* in Nigeria and examples of unfermented thin porridges include *edi* in Uganda and *kunu* in Nigeria (Rashwan *et al.*, 2021). These sorghum products contain nutrients like carbohydrates, minerals and vitamins. Apart from the nutrients,

they also have bioactive compounds with health promoting factors such as phytosterol, policosanols, bioactive lipids and phenolic compounds (Adebo, 2020).

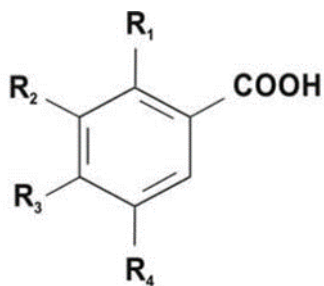
2.2 SORGHUM PHENOLIC COMPOUNDS

Sorghum contains two main classes of phenolic compounds: flavonoids and phenolic acids (de Morais Cardoso *et al.*, 2017). As mentioned earlier, some sorghum types (Type II and Type III) also have condensed tannins which are polymers of flavonoids. The phenolic composition and content vary in sorghums and they are mostly affected by the environment and genetics (Kang, Price, Ashton, Tapsell & Johnson, 2016).

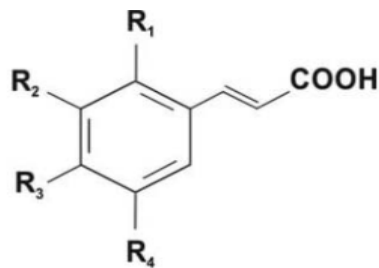
2.2.1 Phenolic acids

These are derivatives of cinnamic acid (hydroxycinnamic acids) and benzoic acid (hydroxybenzoic acids) (Figure 2-1) found in the testa, aleurone layer, pericarp and endosperm in many sorghum varieties (Dykes & Rooney, 2006). Caffeic, chlorogenic, sinapic, *p*-coumaric and ferulic acid are examples of hydroxycinnamic acids (Boz, 2015). Examples of hydroxybenzoic acids include protocatechuic, *p*-hydroxybenzoic and vanillic acid (Van Hung, 2016). Ferulic, protocatechuic, caffeic and vanillic acid are the most reported phenolic acids in sorghum (Xiong *et al.*, 2019). In plants, phenolic acids are associated with a lot of functions such as protection against pathogens, pests and oxidative stress (Awika & Rooney, 2004), enzyme activity, nutrient uptake, photosynthesis, protein synthesis, structural components and allelopathy (Van Hung, 2016).

In terms of health promotion, phenolic acids have been found to confer antimutagenic, antimicrobial, antioxidant, anti-inflammatory, anticancer and antitumor properties (Martins, Mussatto, Martínez-Avila, Montañez-Saenz, Aguilar & Teixeira, 2011). In sorghums, phenolic acids exist in free (unconjugated) and bound forms (Girard & Awika, 2018) but mostly bound forms with ferulic acid as the most abundant bound phenolic acid (100-500 µg/g) (Xiong *et al.*, 2019). Bound phenolic acids are esterified to lignin, cell walls, hemicellulose and pectin while free (unconjugated) phenolic acids are commonly situated in the outer layer of the pericarp (Awika & Rooney, 2004).



a) Benzoic acid



b) Cinnamic acid

Hydroxybenzoic acid derivatives

	R ₁	R ₂	R ₃	R ₄
Gallic	H	OH	H	OH
Gentisic	OH	H	H	OH
Salicylic	OH	H	H	H
<i>p</i> -hydroxybenzoic	H	H	H	OH
Syringic	H	OCH ₃	OH	OCH ₃
Protocatechuic	H	OH	OH	H

Hydroxycinnamic acid derivatives

	R ₁	R ₂	R ₃	R ₄
Caffeic	H	OH	OH	H
Ferulic	H	OCH ₃	OH	H
<i>o</i> -coumaric	OH	H	H	H
<i>p</i> -coumaric	H	H	H	H
Sinapic	H	OCH ₃	OCH ₃	OCH ₃
Vanillic	H	OCH ₃	OCH ₃	H

Figure 2-1: Structures of the main phenolic acids in foods as well as sorghum
 (From de Morais Cardoso *et al.*, (2017))

The levels of phenolic acids range between 135.5 and 479.40 $\mu\text{g/g}$ in sorghum varieties (de Morais Cardoso *et al.*, 2017). Protocatechuic aldehyde, caffeic and protocatechuic acid are some of the phenolic acids identified in sorghum by Svensson, Sekwati-Monang, Lutz, Schieber and Gänzle (2010). Sinapic acid has also been identified as one of the abundant phenolic acid in sorghums by Awika and Rooney (2004). Apea-Bah, Minnaar, Bester and Duodu (2014) identified *p*-coumaric, protocatechuic and caffeic acid in sorghum flour and sorghum porridges. Cinnamic, vanillic, protocatechuic and *p*-coumaric acid were also identified in white and red sorghum varieties by Dykes and Rooney (2006). Gallic acid and gallic acid monohydrate were detected and characterized by Kang *et al.* (2016) in red, brown and white sorghum whole grains.

2.2.2 Flavonoids

Flavonoids are more complex phenolic compounds (compared to phenolic acids), having a benzopyran nucleus (a two-ring system referred to as the A and C rings) and an aromatic ring B attached to the C ring (of the benzopyran nucleus) forming a C6-C3-C6 oxygenated heterocyclic carbon skeleton (Figure 2-2) (Şöhretoğlu & Sari, 2020).

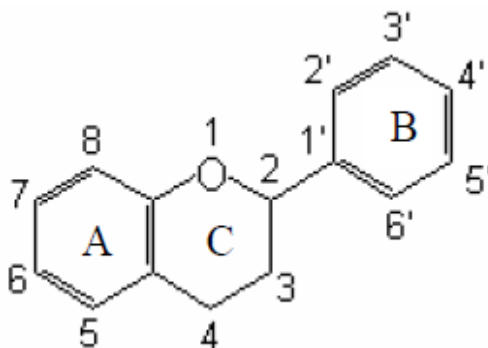


Figure 2-2: The general structure of flavonoids (From Girard and Awika (2018))

Flavonoids can exist as glycosides bound to sugar moieties by ester bonds at their hydroxyl groups or carbon-carbon bonds (Dykes & Rooney, 2006). The major flavonoid subgroups in plants are flavanols, flavones, flavonols, flavanones, anthocyanins and isoflavones (Figure 2-3) (Karakaya, 2004). In sorghum, the main flavonoids are anthocyanins, flavanones and flavones (Dykes, Seitz, Rooney & Rooney, 2009). Anthocyanins in sorghum are mainly the 3-deoxyanthocyanins such as

apigeninidin and luteolinidin (Kumari, Umakanth, Narsaiah & Uma, 2021). Some examples of major flavonoids that have been identified in sorghum grain are given in Table 2-1.

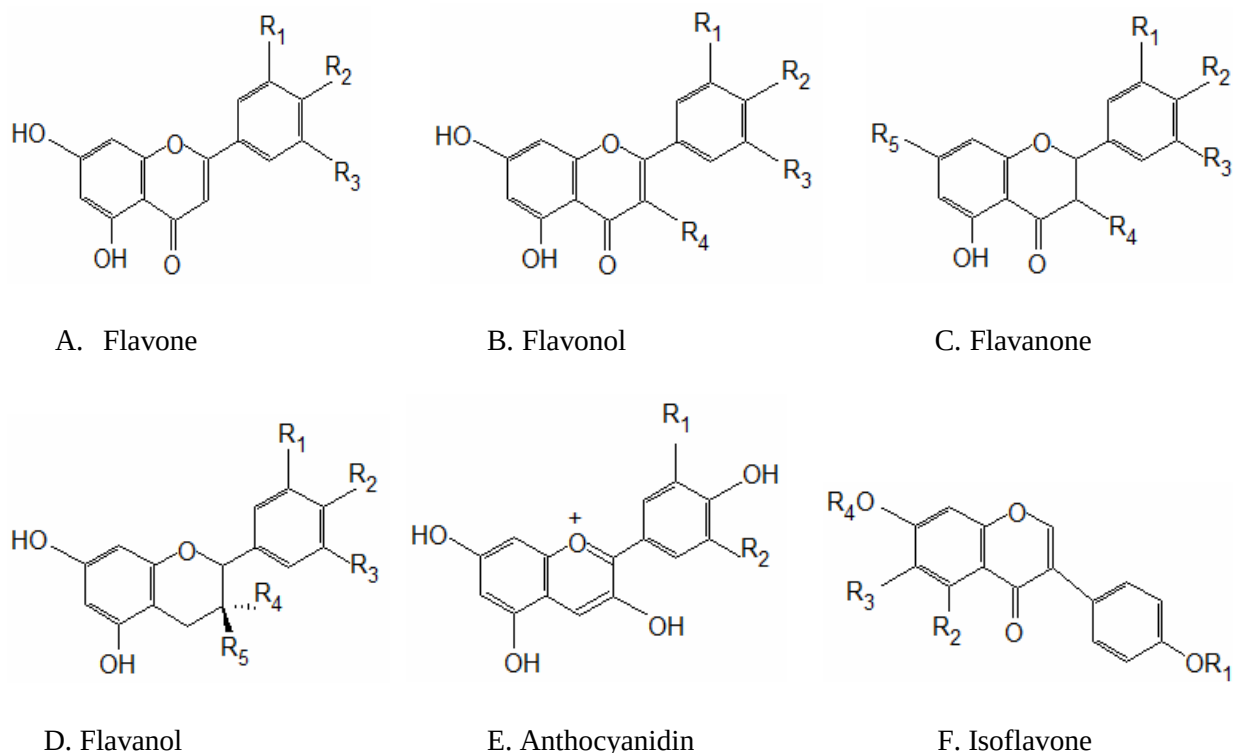


Figure 2-3: Chemical structures of flavonoid subclasses (From Shelembe (2012))

Most flavonoids are situated in the outer layers of the sorghum grain (Dykes *et al.*, 2009) and they are effective against pathogens, ultra violet damage, and pests (Awika, Piironen & Bean, 2011). The profile and concentration of flavonoids is influenced by the presence of the testa, difference in colour and thickness of the pericarp (de Morais Cardoso *et al.*, 2017).

Table 2-1: Some examples of major flavonoids identified in sorghum grain (From Awika and Rooney (2004))

Flavonoid subclass	Compound
3-Deoxyanthocyanins	Apigeninidin-5-glucoside Luteolinidin-5-glucoside
3-Deoxyanthocyanidins	Apigeninidin Luteolinidin 7-O-methyl apigeninidin
Flavones	Luteolin 7-O-methyl luteolin
Flavanones	Naringenin Eriodictoyl Eriodictoyl 5-O- β -glucoside
Flavan-4-ols	Apiforol Luteoforol
Polyflavans	Polyflavan-3-ol Procyanidin B ₁ Proluteolinidin Proapigeninidin Prodelphinidin

Flavanones such as eriodictyol, eriodictyol glucoside and naringenin were identified in red and black sorghum by Dykes *et al.* (2009). The flavones (luteolin and apigenin) and the 3-deoxyanthocyanidins (apigeninidin and luteolinidin) were identified by Kumari *et al.* (2021) in red-brown sorghum and by Girard and Awika (2018) in sorghum. Catechin (a flavan-3-ol), taxifolin a flavanonol, apiforol and luteoforol (flavan-4-ols) and eriodictyol and their glucosides were reported in sorghum grains by Awika and Rooney (2004) (Table 2-1). Taxifolin and ($\pm$)-catechin were also reported in sorghum by Apea-Bah *et al.* (2014). Kang *et al.* (2016) further identified quercetin, naringenin hexoside, luteolin hexoside and eriodictyol in red, brown and white sorghum.

2.3 STRUCTURE-ANTIOXIDANT ACTIVITY RELATIONSHIPS OF PHENOLIC COMPOUNDS

The structure of phenolic compounds influences their antioxidant action (Dueñas, Hernández & Estrella, 2006), in particular their side chain structure and substitutions on the aromatic ring (Natella, Nardini, Di Felice & Scaccini, 1999). An important structural feature for antioxidant action of phenolic acids is the number of hydroxyl groups (Rice-Evans, Miller & Paganga, 1996) whereas in general, more hydroxyl groups result in higher antioxidant activity due to more sites for radical scavenging (Dueñas *et al.*, 2006). Secondly, the presence of a catechol group enhances the antioxidant activity in phenolic acids as these facilitate the donation of hydrogen atoms (Platzer, Kiese, Herfellner, Schweiggert-Weisz, Miesbauer, Eisner & Bilewicz, 2021). Derivatives of cinnamic acid have higher antioxidant activity than benzoic acid derivatives which is attributed to the propenoid side chain in cinnamic acid derivatives with a double bond that is conjugated with double bonds of the benzene ring (Figure 2-4) (Natella *et al.* (1999). This conjugation stabilizes the resonance structures of the resulting phenolic radicals and enhance antioxidant activity (Platzer *et al.*, 2021; Shelembe, 2012). The presence of the carboxylic group in benzoic acid (Figure 2-4) negatively influences the hydrogen donating abilities of the phenolic ring linked with electron withdrawing activity (Rice-Evans *et al.*, 1996). In general, glycosylation reduces the antioxidant activity of phenolic acids (Rice-Evans *et al.*, 1996).

Gallic acid has a structural advantage compared to protocatechuic acid due to the presence of a pyrogallol structure (Rösch, Bergmann, Knorr & Kroh, 2003). However, loss of one hydroxyl group may slightly reduce the antioxidant activity and a further loss reduces the antioxidant activity even more (Fukumoto & Mazza, 2000).

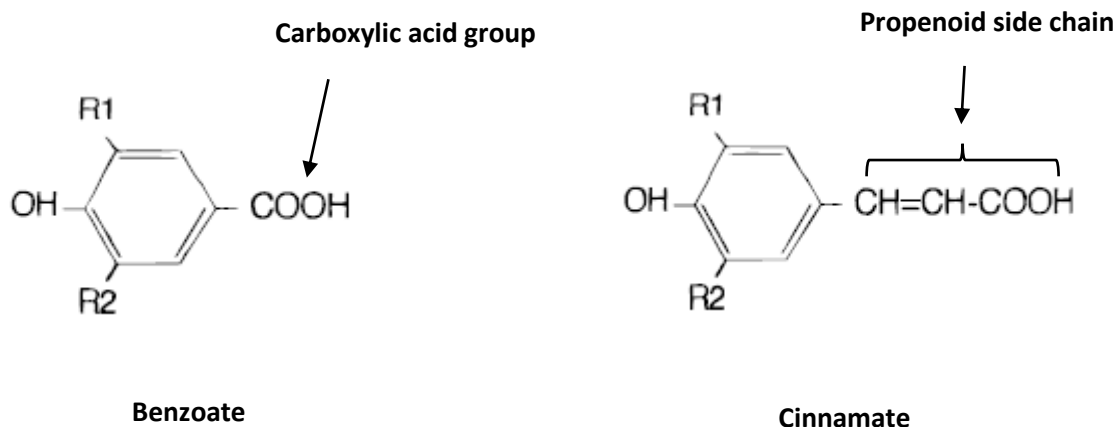


Figure 2-4: Chemical structures of phenolic acids showing the carboxylic acid group (in benzoates) and propenoid side chain with its double bond conjugated with double bond in the benzene ring (in cinnamates) (From Van Hung (2016))

The degree and position of hydroxylation are associated with the antioxidant activity in flavonoids (Platzer *et al.*, 2021). The 2,3-double bond coupled with a 4-oxo function in the C-ring, 3- and 5-hydroxy groups adjacent to the 4-keto structure (Nwachukwu, Sarteshnizi, Udenigwe & Aluko, 2021), and a catechol group in the B-ring are structural requirements for maximum antioxidant activity in flavonoids (Figure 2-5) (Wolfe & Liu, 2008). The catechol group in the B-ring stabilizes the radical formed (Williams, Spencer & Rice-Evans, 2004), while the conjugation of the B-ring to the 4-oxo group through the 2,3-double bond delocalize the electrons from the B-ring (Apea-Bah *et al.*, 2014). The 3- and 5-hydroxyl groups with the 4-oxo group also delocalize electrons from the 4-oxo group (Platzer *et al.*, 2021). The increase in catechol groups substantially increases the antioxidant potential by further stabilizing the phenoxyl radical formed (Natella *et al.*, 1999). Flavonoids also have several free hydroxyl groups found in the A and C rings increasing their antioxidant activity (Rao, Schwarz, Santhakumar, Chinkwo & Blanchard, 2018b). Methoxylation and glycosylation reduces the antioxidant of flavonoids when compared to their aglycones (Rice-Evans *et al.*, 1996).

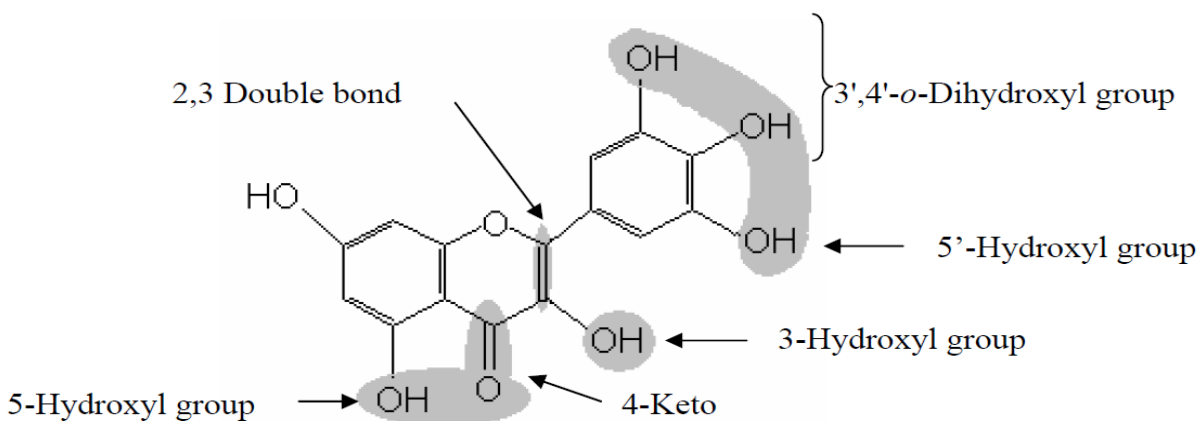


Figure 2-5: The structural groups affecting antioxidant activity in flavonoids (From Shelembe (2012))

As an example, the flavonol quercetin has superior antioxidant potential attributed to the presence of the 3-hydroxy group that forms hydrogen bonds with the 2''/6' positions of the B ring hence facilitates radical formation of the 4'-hydroxy group (Bors & Michel, 2002). The higher antioxidant activity of quercetin compared to kaempferol was also reported by Rösch *et al.* (2003), who attributed this activity to the catechol structure in the B-ring in quercetin which is absent in kaempferol. The flavonol, myricetin was reported as the most efficient flavonoid antioxidant before quercetin by Silva, Santos, Caroço, Rocha, Justino and Mira (2002).

2.4 OXIDATIVE STRESS AND ITS ROLE IN THE DEVELOPMENT OF NCDS

The body's continuous metabolic activities results in the production of reactive species like reactive oxygen and nitrogen species (ROS/RNS) (Asmat, Abad & Ismail, 2016). These reactive species are mostly free radicals like superoxide anion (Burton & Jauniaux, 2011), hydroxyl radical and hydrogen peroxide (Nwachukwu *et al.*, 2021). The human body has a natural antioxidant system for removing these free radicals, but an imbalance between these free radicals and antioxidants results in oxidative stress (Nwachukwu *et al.*, 2021). In simple terms, oxidative stress is a condition where the body's natural defense against free radicals has been overwhelmed (Lakshmi *et al.*, 2013; Van Hung, 2016) due to increased levels of free radicals and weakened antioxidant systems (Bondia-Pons, Ryan & Martinez, 2012).

Oxidative stress is associated with several health conditions such as aging, cardiovascular diseases (Bondia-Pons *et al.*, 2012; Sosa, Moliné, Somoza, Paciucci, Kondoh & Lleónart, 2013; Van Hung, 2016), diabetes (Asmat *et al.*, 2016; Bondia-Pons *et al.*, 2012), insulin resistance, allergies, immune system dysfunctions (Sosa *et al.*, 2013), metabolic syndrome, inflammatory diseases, cancer and neurodegenerative disorders such as Parkinson's diseases (Nwachukwu *et al.*, 2021).

Endogenous or exogenous antioxidants obtained from the diet such as phenolic compounds help maintain the homeostasis of the body by neutralizing free radicals (Asmat *et al.*, 2016). These phenolic compounds also donate hydrogen atoms that scavenge highly reactive oxygen and nitrogen species (Apea-Bah *et al.*, 2014), and therefore potentially offer protection against oxidative stress and associated diet-related non-communicable diseases. Van Hung (2016) further noted that phenolic compounds can combat oxidative stress through maintaining the balance between oxidants and antioxidants.

2.5 HEALTH-PROMOTING PROPERTIES OF PHENOLIC COMPOUNDS IN SORGHUM AND SORGHUM FOODS

2.5.1 Anti-cancer properties

There have been various reports in the literature about anti-cancer properties of sorghum and sorghum foods through various mechanisms that are linked to their phenolic compounds. These include their ability to control phase II enzymes, inhibit the initiation, progression and spread of cancers cells *in vitro* and prevent the formation and growth of tumors (Davidson, Zhu & Fang, 2016). Concerning the control of phase II enzymes like quinone oxidoreductase, the phenolic compounds in sorghum are reported to have the ability to activate cellular signaling pathways which consequently influence the expression of phase II enzymes that convert reactive species (ROS and RNS) to non-toxic metabolites that can be excreted out of the body (Xiong *et al.*, 2019). In this regard, Awika (2011) reported that 3-deoxyanthocyanidins in sorghum induced quinone oxidoreductase in a murine hepatoma cell model *in vitro* suggesting the capability of the 3-deoxyanthocyanidins for cancer chemoprevention.

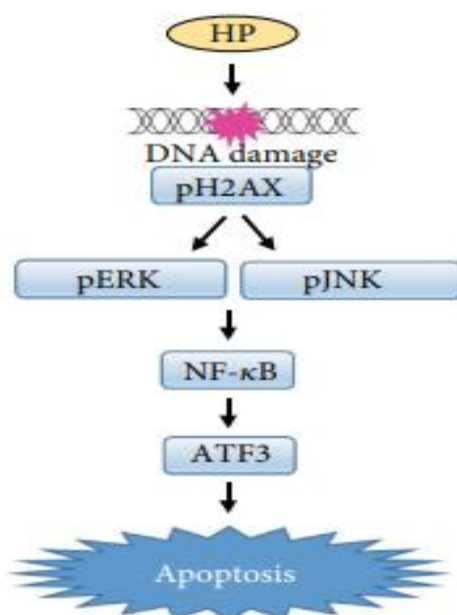


Figure 2-6: A projected method of anticancer activity for phenolic sorghum extracts (From Lee, Lee, Herald, Cox, Noronha, Perumal, Lee & Smolensky (2020))

In general, phenolic compounds are proposed to have the ability to prevent the formation and growth of tumors by inducing apoptosis (Dai & Mumper, 2010) through various growth suppression methods including activation of transcription signaling pathways (Figure 2-6) and organizing the cytochrome which inhibits the progression of tumor growth (Mollakhalili Meybodi, Mortazavian, Bahadori Monfared, Sohrabvandi & Aghaei Meybodi, 2017). It has been reported by de Morais Cardoso *et al.* (2017) and Xiong *et al.* (2019) have reported that sorghum 3-deoxyanthocyanins can prevent growth of cancer cells in the liver, colon and bone marrow by apoptosis. Phenolic acids in sorghum like *p*-coumaric, vanillic and protocatechuic acid have all demonstrated pro-apoptotic and antiproliferative activities particularly in colon cancer cell lines as reported by Vanamala, Massey, Pinnamaneni, Reddivari and Reardon (2018). Recently, Amarakoon, Lou, Lee, Smolensky and Lee (2020) stated that phenolic compounds in sorghum are involved in modulating various cancer pathways such as growth arrest, apoptosis, metastasis and angiogenesis and exert cancer chemoprevention in the process.

Various *in vitro* studies have shown the capacity of phenolic compounds in sorghum to inhibit the progression and spread of cancers cells (Davidson *et al.*, 2016). This has been shown by various

authors such as Simnadis, Tapsell and Beck (2016), who stated that the phenolic compounds in sorghum could prevent the spread of breast and gastrointestinal cancer cells. Furthermore, in a study conducted by Chen, Shen, Xu, Herald, Smolensky, Perumal and Wang (2021), the phenolics in sorghum inhibited cancer cell growth through cell cycle arrest which prompted DNA repair resulting to apoptosis.

2.5.2 Anti-diabetic properties

Phenolic compounds in sorghum inhibit the starch degrading enzymes α -glucosidase and α -amylase and hence reduce the glycemic level (Kumari *et al.*, 2021; Xiong *et al.*, 2019), hinder carbohydrate digestion (Amarakoon *et al.*, 2020) and limit absorption of glucose (Arbex, Moreira, Toledo, de Morais Cardoso, Pinheiro-Sant'ana, Benjamin, Licursi, Carvalho, Queiroz & Martino, 2018). The α -glucosidase and α -amylase inhibition by the phenolic compounds in sorghum reduces starch digestion and glucose absorption (Amarakoon *et al.*, 2020), thus provide an effective mechanism for the control of diabetes (de Morais Cardoso *et al.*, 2017; Kim, Hyun & Kim, 2011).

A previous investigation showed that oral administration of sorghum phenolic extracts to diabetic mice improved insulin sensitivity therefore played a significant role in the cure of diabetes (de Morais Cardoso *et al.* (2017). The improvement in the diabetic mice was related to the phenolic compounds from sorghum that reduced the tumor necrosis factor- α (TNF- α), through the overexpression of peroxisome proliferator-activated receptor- γ (PPAR- γ) and increased adiponectin gene expression (de Morais Cardoso *et al.*, 2017).

The phenolic compounds found in sorghum may also hinder the glycation of proteins by up to 60%. An excess of protein glycation products can result in insulin resistance and formation of ROS (Yamagishi, 2011). Therefore, the ability of sorghum phenolic compounds to hinder protein glycation represents an anti-diabetic effect.

2.5.3 Anti-cardiovascular disease

The monomeric flavonoids and phenolic acids in sorghum have the potential to reduce oxidative stress which can lead to atherosclerosis (a principal cause of cardiovascular disease) (Stefoska-Needham, Beck, Johnson & Tapsell, 2015). Amarakoon *et al.* (2020) observed that phenolic compounds in sorghum may ease the occurrence of cardiovascular diseases by regulating

cholesterol synthesis. Additionally, de Morais Cardoso *et al.* (2017) observed a decrease in Low Density Lipoprotein (LDL), triglycerides and total cholesterol in diabetic rats symbolizing the anti-cardiovascular effect of the phenolic compounds in sorghum. Similarly, an increase in high-density lipoprotein (HDL) followed by a decrease in plasma cholesterol were observed in rats fed with the phenolic extracts of whole grain sorghum by Chung, Kim, Yeo, Kim, Seo and Moon (2011).

Monomeric 3-deoxyflavonoids in sorghum inhibit the prevalence of cardiovascular disease attributed to their high anti-inflammatory and antioxidant action (Awika, 2011). The phenolic compounds in sorghum are also reported to inhibit thrombus formation (which can trigger cardiovascular diseases) by reducing platelet microparticle release and platelet aggregation (Amarakoon *et al.*, 2020). Phenolic extracts from black sorghum regulated gene expression for the producers of ROS associated with endothelial dysfunction which is one of the characteristics of cardiovascular diseases (Nidhish, Shiwangini, Christopher & Abishek, 2019).

2.5.4 Chronic inflammation

Chronic inflammation could contribute to numerous diseases like cancer, obesity and cardiovascular diseases (Awika *et al.*, 2011; Zhang, Li, Gao, Wang, Tongcheng, Gao & Yu, 2019). During inflammation, several pro-inflammatory compounds are produced such as prostaglandin E2 (PG-E2), tumor necrosis factor (TNF)- α and interleukin (IL). Several phenolic compounds in sorghum have been reported to inhibit cyclooxygenase (COX- 2) enzyme which is involved in these inflammatory pathways, hence prevents the formation of the pro-inflammatory TNF- α (Xiong *et al.*, 2019). Phenolic compounds in sorghum bran can inhibit inflammatory response by reducing inducible COX-2 and nitric oxide synthase (iNOS) (Amarakoon *et al.* (2020). Phenolic compounds may also alter the activity of lipoxygenases (LOX) (whose overexpression can lead to inflammation) both *in vivo* and *in vitro* (Dai & Mumper, 2010).

Awika *et al.* (2011) noted that the bran from white, black and red sorghum pericarp inhibited the enzyme hyaluronidase which contributes to chronic inflammation. Similar observations were stated by Xiong *et al.* (2019) and de Morais Cardoso *et al.* (2017), who highlighted that the 3-deoxyanthocyanidins found in sorghum can inhibit hyaluronidase.

2.6 FACTORS AFFECTING BIOACCESSIBILITY OF PHENOLIC COMPOUNDS IN CEREAL-BASED FOODS

Phenolic compounds in sorghum are mainly found in conjugated and in bound forms esterified to various components such as proteins, carbohydrates and cell walls (Awika & Rooney, 2004). For phenolic compounds to optimally exert their bioactive action, they have to be released from the food matrix during digestion and converted into an absorbable form (bioaccessibility), absorbed and then transported to the bloodstream (bioavailability) (Carbonell-Capella *et al.*, 2014). Bioaccessibility of phenolic compounds can be influenced by several factors like food matrix, food processing and molecular structure of the phenolic compound (Manach, Scalbert, Morand, Rémésy & Jiménez, 2004). The presence of digestive enzymes, physicochemical characteristics of the gastro-intestinal fluids (Helal, Tagliazucchi, Verzelloni & Conte, 2014), chemical interactions with other phenolic compounds and bio-molecules present in the food (Chitindingu, Benhura & Muchuweti, 2015) also affects the bioaccessibility. Two of these factors namely food processing and structure of the phenolic compounds will be briefly discussed.

The processing of food can induce physical or chemical changes leading to the release and absorption of phenolic compounds especially during digestion (Ribas-Agustí, Martín-Belloso, Soliva-Fortuny & Elez-Martínez, 2018). Several studies have shown that application of processes like extrusion, wet heating (Salazar-López *et al.*, 2018a), grinding, acid hydrolysis and fermentation can promote the release of phenolic compounds (especially bound forms), hence improve their bioaccessibility and bioavailability (Tagliazucchi, Verzelloni & Conte, 2012).

The molecular structure of the phenolic compound, especially the molecular size, degree of glycosylation and the class of the phenolic compound also affects the bioaccessibility of phenolic compounds (Manach *et al.*, 2004). In this regard, the potential role of digestive enzymes in enhancing bioaccessibility of phenolic compounds is brought into focus with the observation that glycosylated, and polymerized phenolic compounds are converted into aglycones by β -glucosidase and lactase phlorizin hydrolyse enzymes before they can be absorbed through a diffusion mechanism (Erick Paul, Dulce Libna, Nayely, Ramón Ignacio & José Basilio, 2016; Ribas-Agustí *et al.*, 2018).

Ribas-Agustí *et al.* (2018) mentioned that highly unstable anthocyanins can only be absorbed in their glycosylated form. Manach *et al.* (2004) and Awika (2011) noted that in sorghum, the aglycones of flavanones and flavones are more rapidly absorbed in the small intestines compared to anthocyanins which have a relatively low bioavailability due to the anthocyanin's structure and nature of the sugar aglycone. Manach, Williamson, Morand, Scalbert and Rémésy (2005) agreed with this, stating that anthocyanins and proanthocyanidins are the least absorbed phenolic compounds. Interestingly, epicatechin gallate (flavan-3-ol) can be absorbed without deconjugation or hydrolysis (Erick Paul *et al.*, 2016). Most phenolic acids are readily absorbed because of their small molecular sizes (Awika & Rooney, 2004).

2.7 PREPARATION OF SOURED CEREAL PRODUCTS

Various soured cereal products especially lactic-acid fermented foods are common in sub-Saharan Africa (Adeyanju *et al.*, 2019). In general, soured cereal products in Africa are prepared using two main methods: spontaneous lactic acid fermentation or by exogenous acidification using mainly citrus fruit acids (Gabaza *et al.*, 2016). A variety of traditionally fermented foods like thin porridges form a significant part of the daily diet for most African communities (Chelule, Mokoena & Gqaleni, 2010). Cereals including sorghum and maize can be fermented and further processed after fermentation to produce several foods (Galati, Oguntinyinbo, Moschetti, Crescimanno & Settanni, 2014) like biscuits, bread and porridges. Fermentation has several significant roles such as enhancing digestibility and phenolic content, formation of acidity and flavour, enhanced nutritional quality and extended shelf life (Chelule *et al.*, 2010). Exogenous acidification is also widely practiced where organic acids like citric acid are used to produce soured products (Onyango & Wanjala, 2018). A major advantage of using exogenous acidification is that soured products can be prepared within a short time (Adeyanju *et al.*, 2019).

2.7.1 Spontaneous lactic acid fermentation

Typically, the process for spontaneous fermentation of cereal grains involves cleaning, milling and soaking the grain in water for some days at ambient temperature, during which naturally occurring microorganisms, dominated by lactic acid bacteria (LAB) build up in the medium (Blandino, Al-Aseeri, Pandiella, Cantero & Webb, 2003). Fermentable sugars are produced in such fermentations by endogenous enzymes like amylase that in turn provide energy for the LAB (Nout, 2009). This

process can last for about 36 hrs at ambient temperatures, after which the fermented slurries can be cooked (Gabaza *et al.*, 2016) in the case of thin porridge preparation.

The most dominant fermenting bacteria include species of *Streptococcus*, *Leuconostoc*, *Lactobacillus* and *Lactococcus lactis*. *subsp. Lactis* is mostly associated with fermented thin porridges (Blandino *et al.*, 2003). In terms of how they metabolize hexose sugars, LAB are categorized into two main groups: Homofermentative and heterofermentative bacteria. Homofermenters produce lactic acid as the only final product after glucose fermentation. They include *Lactococcus* and some *Lactobacilli*. Heterofermenters like *Leuconostoc*, *Weisella* and some *Lactobacilli* produce lactate, ethanol and carbon dioxide from glucose fermentation (Nout, 2009).

2.7.2 Exogenous acidification

As an alternative to lactic acid fermentation, exogenous acidification is also used in preparation of soured cereal food products which contributes to increased shelf life, enhancement of food safety and an improvement in sensory properties (Adeyanju *et al.*, 2019). In some communities around Africa, lemon, orange, or tamarind juice are added to sorghum or maize porridges to produce souring. Commercially, in the food industry, food grade acids (e.g. citric and lactic acid) are used (Onyango & Wanjala, 2018). These acids can exert hydrolysing effects on ester bonds resulting in the release of bound phenolic compounds (Shelembe, Cromarty, Bester, Minnaar & Duodu, 2014). Soured sorghum porridges prepared using exogenous acidification could therefore contain more extractable phenolic compounds than unsoured porridges leading to the development of a soured sorghum porridge with enhanced health promoting properties.

2.8 EFFECTS OF LACTIC ACID FERMENTATION AND EXOGENOUS ACIDIFICATION ON PHENOLIC COMPOUNDS IN SOURED CEREAL FOODS

Processes such as lactic acid fermentation can increase soluble phenolic compounds in cereals and reduce bound ones (Gabaza *et al.*, 2016). This is because lactic acid fermentation can activate microbial enzymes such as esterases and hydrolases (Shumoy, Gabaza, Vandeveld & Raes, 2017), which in turn cause structural breakdown of the cereal matrix. This disruption of the cereal matrix results in the release of insoluble bound phenolics since they are covalently bonded to macromolecules like proteins and non-starch polysaccharides. Therefore breaking down of the

ester linkages between the macromolecules and phenolic compounds liberates the phenolic compound from their bound form (Shahidi & Yeo, 2016).

In particular, feruloyl esterase releases phenolic compounds bound to arabinoxylans by hydrolysing the ester linkages between hydroxycinnamates and arabinose particularly in cereal grains (Salazar-López *et al.*, 2018a). The enzyme tannase which is also an esterase, breaks down hydroxybenzoic esters such as the ester bonds of protocatechuic and gallic acids and the resulting phenolic acids are then decarboxylated (Esteban-Torres, Landete, Reverón, Santamaría, de las Rivas & Muñoz, 2015). Specific glycosyl hydrolases also convert flavonoid glycosides to their aglycones (Svensson *et al.*, 2010).

In a study by Salazar-López *et al.* (2018a), lactic fermentation significantly increased the contents of polyphenols including phenolic compounds in red sorghum which was related to the presence of enzymes like glucosidase and phenolic acid reductase which metabolized the phenolic acid esters and flavonoid glucosides. Contrary to this, fermenting *mahewu* (a sorghum beverage) for about 36 hours led to a 63% decrease in proanthocyanidins in sorghum in a study conducted by Bvochora, Reed, Read and Zvauya (1999). Towo, Matuschek and Svanberg (2006) also noted a significant decrease in total phenols after fermenting sorghum gruels. The decrease in phenolics during fermentation was attributed to the rearrangement of phenolic structures and abstraction of hydride ions due to the acidic fermenting conditions. Another reason for the reduction was self-polymerization of phenolic compounds resulting in interactions between phenolic compounds and macromolecules like proteins, which reduced the extractability of phenolic compounds (Towo *et al.*, 2006).

The metabolism of phenolic acids in lactic acid fermentations is facilitated by decarboxylases and reductases (Gänzle, 2014) of which several phenolic acids are converted by strain-specific decarboxylases and reductases such as cinnamic acid reductase (Svensson *et al.*, 2010). Decarboxylases convert hydroxycinnamates and hydroxybenzoates to vinyl or phenol derivatives and the reductases further metabolize the hydroxycinnamates and their vinyl derivatives by hydrogenating the double bond (Gänzle, 2014).

Svensson *et al.* (2010) noted that fermenting sorghum doughs showed activity of phenolic acid decarboxylase, glucosidase, esterase (tannase) and phenolic acid reductase which are used in various pathways for flavonoid and phenolic acid metabolism.

Acidification with organic acids like citric acid is also a growing trend particularly in Africa (Onyango & Wanjala, 2018). The acidic conditions can hydrolyse ester linkages between phenolic compounds and various macromolecules and cell wall components and release phenolic acids from their insoluble esterified forms (Liyana-Pathirana & Shahidi, 2005) and flavone and flavanone aglycones from their glycoside forms (Tarko, Duda-Chodak & Zajac, 2013). Acidification can also break down cell walls, hence enhance the solubilization and diffusion of phenols from plant material like sorghum (Shelembe, 2012), leading to an increased phenolic content.

2.9 CONCLUSION AND GAPS IN KNOWLEDGE

Sorghum, is a cereal that can withstand drought, hence valuable in the semi-arid regions of Africa compared to other cereals like wheat (Taylor & Dewar, 2001). It is a source of phenolic compounds like flavonoids and phenolic acids (Salazar-López *et al.*, 2018a). These phenolic compounds exert their antioxidant effects by their capability to donate hydrogen atoms that scavenge highly reactive oxygen and nitrogen species (Apea-Bah *et al.*, 2014) and therefore potentially offer protection against oxidative stress. Oxidative stress is associated with several health conditions, NCDs in particular like cancer and diabetes (Bondia-Pons *et al.*, 2012). However, in order for the phenolic compounds in sorghum to exercise their biological activity, they have to be bioaccessible and bioavailable (Carbonell-Capella *et al.*, 2014). Processes like acid hydrolyses and fermentation promote the extraction of phenolic compounds hence improve their bioaccessibility (Tagliazucchi *et al.*, 2012). Even so, information of the effect of spontaneous fermentation and citric acid acidification of sorghum porridges on the bioaccessibility of phenolic compounds and antioxidant properties is lacking. Therefore, in this research the effect of spontaneous fermentation and citric acid acidification on the bioaccessibility of phenolic compounds and antioxidant properties of soured sorghum porridges is studied, with the aim of producing a soured sorghum porridge with improved health promoting properties.

2.10 HYPOTHESES AND OBJECTIVES

2.10.1 Hypotheses

Hypothesis 1

Soured (citric acid acidification and spontaneous fermentation) sorghum porridges will have more bioaccessible phenolic acids such as protocatechuic, vanillic, gallic and sinapic acids and flavonoid aglycones from the flavone and flavanone subclasses compared to the unsoured sorghum porridges. This is because the acidic conditions in the soured porridges will hydrolyse ester linkages and release these phenolic acids from their soluble esterified forms (Liyana-Pathirana & Shahidi, 2005) and flavone and flavanone aglycones from their glycoside forms (Tarko *et al.*, 2013), thus making them more bioaccessible.

Hypothesis 2

Sorghum porridges soured either through spontaneous fermentation or citric acid acidification will have greater antioxidant-related health promoting properties compared to the unsoured sorghum porridges. This is because the soured sorghum porridges will have more bioaccessible phenolic compounds leading to a higher antioxidant activity. The acidic conditions together with the presence of cell wall degrading enzymes in the soured porridges can bring about structural breakdown of the cereal matrix leading to the liberation of insoluble bound phenolics (Shahidi & Yeo, 2016) and enhanced health promoting properties of the soured porridges.

2.10.2 Objectives

Objective 1

To determine the effect of souring (citric acid acidification and spontaneous fermentation) on the bioaccessibility of phenolic compounds and antioxidant properties of sorghum porridges in order to develop a soured sorghum porridge with enhanced health promoting properties.

Objective 2

To determine the effect of souring (citric acid acidification and spontaneous fermentation) on the antioxidant-related health promoting properties of sorghum porridges with the aim of developing a soured sorghum porridge with enhanced health promoting properties.

2.11 EXPERIMENTAL DESIGN

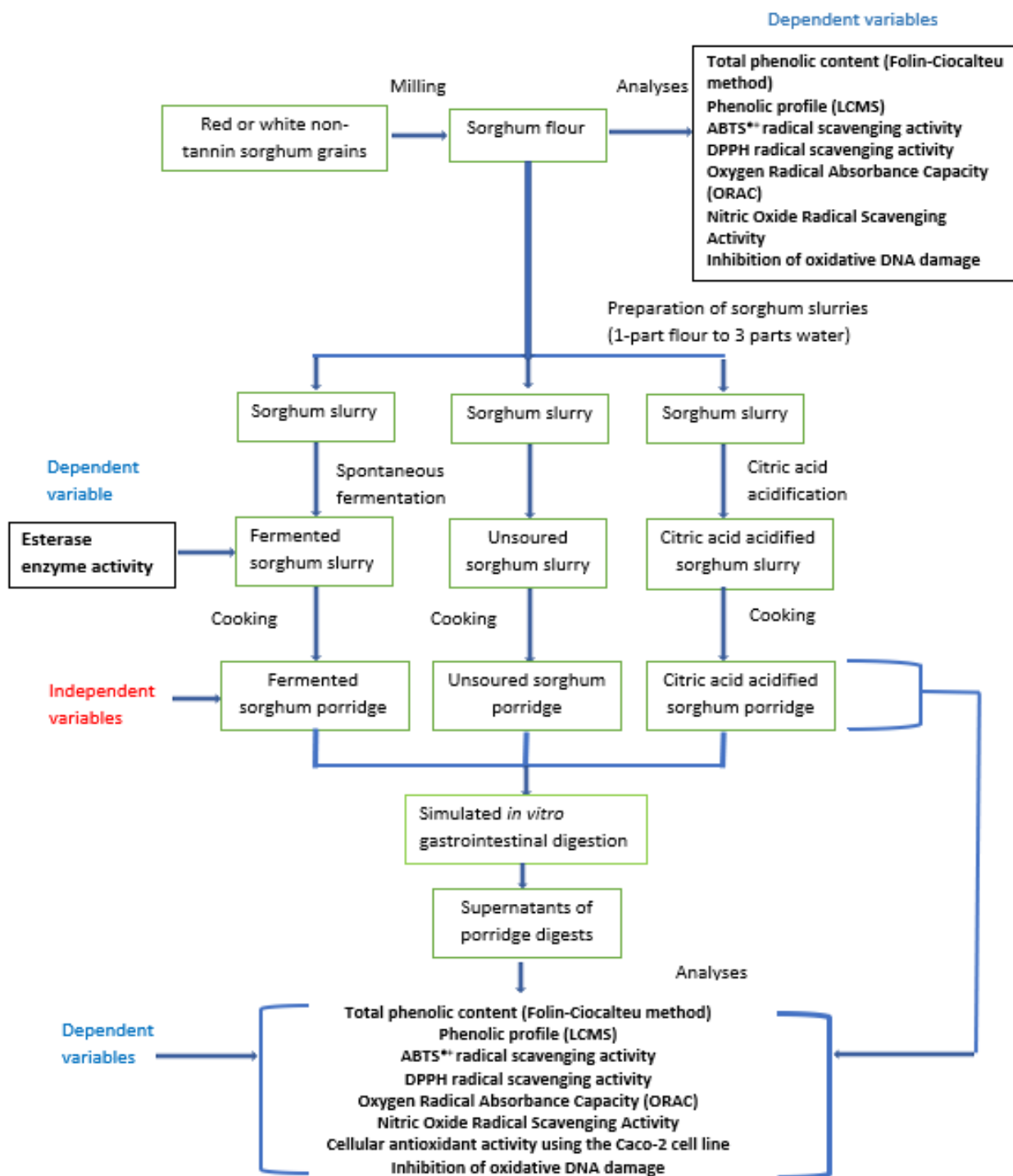


Figure 2-7: Experimental design used to determine the effect of souring and simulated *in vitro* gastrointestinal digestion on phenolic compounds and antioxidant related health promoting properties of sorghum flours, porridges and supernatants

3 RESEARCH

3.1 EFFECTS OF SOURING AND SIMULATED *IN VITRO* GASTROINTESTINAL DIGESTION ON CONTENT AND BIOACCESSIBILITY OF PHENOLIC COMPOUNDS IN RED AND WHITE SORGHUM FLOURS AND PORRIDGES

ABSTRACT

The effects of souring through spontaneous fermentation and exogenous acidification using citric acid on phenolic content and bioaccessibility in red and white non-tannin sorghum porridges were evaluated. Total phenolic content (TPC) was determined using the Folin-Ciocalteu method. Esterase enzyme activity of fermented sorghum slurries was determined with a spectrophotometric method using *p*-nitrophenol butyrate as a substrate. Liquid Chromatography-Mass Spectrometry (LC-MS) was used to characterise phenolic compounds and their bioaccessibility in soured sorghum flours, their porridges and supernatants obtained from simulated *in vitro* gastrointestinal digestion of the porridges. Souring through fermentation led to a significant increase in TPC values of sorghum flours and resultant porridges while no significant difference were observed in the TPC values after acidification with citric acid. Fermented sorghum slurries exhibited esterase enzyme activity which was higher in fermented red sorghum slurries compared to white sorghum. Souring through exogenous acidification led to more bioaccessible phenolic compounds compared to souring through fermentation. Soured sorghum porridges generally have enhanced bioaccessibility of bioactive phenolic compounds and therefore have the potential to prevent diet-related non-communicable diseases.

3.1.1 Introduction

Sorghum is an important staple cereal grain for many countries in the semi-arid tropics of Africa and Asia (Li, Zhao, Zhang & Liu, 2021) and millions of people living in these countries depend on sorghum as a major food source (Adeyanju *et al.*, 2019). It contains macronutrients like lipids, proteins and starch and micronutrients such as vitamins and minerals (Lee, Lee, Herald, Cox, Noronha, Perumal, Lee & Smolensky, 2020). Sorghum has a very wide range of food uses from porridges, baked foods to several alcoholic and non-alcoholic beverages in many countries in Africa (Adeyanju *et al.*, 2019).

In 2014, the proportion of deaths due to NCDs had increased to 43% (World Health Organization, 2014), and further up to 51% in 2018 in South Africa (World Health Organization, 2018). It is clear that NCDs are on the increase in Africa. Rapid urbanization and rising incomes in Africa as well as a demand for convenience have caused a shift in people's dietary choices to highly refined foods that are high in energy and saturated fat. As a result, there is increased susceptibility to oxidative stress which leads to an increase in occurrence of NCDs in Africa (Banwo *et al.*, 2021). There is increasing focus on bioactive dietary phenolic compounds that are hypothesised to have the capacity to offer protection against oxidative damage through scavenging of highly reactive radicals that are produced during oxidative stress (Apea-Bah *et al.*, 2014). In this regard, phenolic compounds in sorghum can play a significant role in offering health promoting properties (Luo, Hou, Dong, Huang, Zhang & Su, 2020).

During food digestion, the portion of the food that is released and is available for absorption is considered to be bioaccessible (Ribas-Agustí *et al.*, 2018). In order to exert bioactive and health-promoting properties, it would be expected that phenolic compounds contained in any given food such as sorghum must be bioaccessible (Garzón, Van de Velde & Drago, 2020). However, a high proportion of the phenolic compounds in sorghum are bound to indigestible polysaccharides and other components which restricts their bioaccessibility and ultimately, their ability to exert health-promoting effects (Salazar-López, González-Aguilar, Rouzaud-Sáñez & Robles-Sánchez, 2018b).

Soured sorghum porridges are part of the regular diet of people in many communities in Africa. These soured porridges are prepared using mainly spontaneous lactic acid fermentation processes. However, there is an increasing trend of using exogenous acidification using either fruit acids or food grade acids to produce soured sorghum products (Adeyanju *et al.*, 2019; Onyango & Wanjala, 2018). There are several indications from the literature that processes that involve souring such as acidification (Tagliazucchi *et al.*, 2012) and fermentation (Garzón *et al.*, 2020) can enhance phenolic content and antioxidant properties of sorghum foods (Zaroug, Orhan, Senol & Yagi, 2014) due to improvement in bioaccessibility of phenolic compounds (Blandino *et al.*, 2003; Ravisankar *et al.*, 2021).

Therefore, this study seeks to evaluate the effects of souring through spontaneous fermentation and citric acid acidification on the content and bioaccessibility of phenolic compounds in sorghum porridge with the aim of producing soured sorghum porridges with enhanced health-promoting properties.

3.1.2 Materials and methods

3.1.2.1 Materials

Two Type 1 non-tannin sorghums (a red and a white phenotype) were obtained from the Ukulima Farm, Mpumalanga, South Africa and used in this research. Folin-Ciocalteu reagent, citric acid monohydrate, porcine pancreatin (Cat. No. P1760-100G), porcine pepsin A (Cat. No. P7000-100G), porcine bile extract (Cat. No. B8631-100G), *p*-nitrophenyl butyrate, Triton X100, naringin, naringenin, hesperetin, quercetin, kaempferol, rutin, myricetin, catechin, epicatechin, apigenin, luteolin, syringic acid, *p*-coumaric acid, vanillic acid, caffeic acid, 4-hydroxybenzoic acid, ferulic acid, sinapic acid, protocatechuic acid, gallic acid and chlorogenic acid were obtained from Sigma-Aldrich. Propanol and acetonitrile were purchased from Merck.

3.1.2.2 Preparation of sorghum flours

The white and red sorghum grains were cleaned and milled to produce flour using a laboratory hammer mill fitted with a 500 µm screen opening (Figure 3-1). The flours were stored in an air-tight zip-lock polyethylene bags at 4-6°C before analyses.



Figure 3-1: Red (A) and white (B) sorghum flour used in this research

3.1.2.3 Preparation of unsoured, fermented, and acidified sorghum flour slurries and porridges

Three separate sorghum flour slurries from both the red and white non-tannin sorghum were made by mixing 200 g sorghum flour with 600 ml distilled water (1:3) followed by stirring for uniform mixing. One of the sorghum slurries was fermented naturally in an incubator (Analis Namur Soc. Anonyme) at 30°C, while monitoring the pH until a pH of 3.5 was attained.

Another mixture was acidified exogenously by titrating with citric acid monohydrate (1.0 mol/ L) until a pH of 3.5 was attained. The third sorghum slurry was used as a control sample (unsoured). This was done for both sorghum types.

The control, fermented and citric acidified slurries were then cooked into porridges by boiling on an electric hot plate for about 12 min with continuous stirring at 3 min intervals under low heat to prevent the formation of lumps. The cooked porridges were cooled between 18-22°C before subjection to simulated *in vitro* gastrointestinal digestion as described below.

3.1.2.4 Determination of esterase enzyme activity of fermented sorghum slurries

A modified version of the esterase enzyme activity method of Pliego, Mateos, Rodriguez, Valero, Baeza, Femat, Camacho, Sandoval and Herrera-López (2015) which uses *p*-nitrophenol butyrate as a substrate was used in this research. Fermented sorghum slurries were centrifuged at 6500 rpm for 20 min at 4°C using a centrifuge (Rotana 460 Hettiech, South Africa). The supernatants which represent the esterase enzyme preparation were recovered and used for the esterase enzyme activity determination. The substrate solution was prepared by dissolving 2.1 ml *p*-nitrophenyl butyrate in a 1:4 (v/v) acetonitrile: isopropanol solution. About 1 ml of this solution was dissolved in 18 ml phosphate buffer (50 mM at pH 7) followed by the addition of 95 µl (0.5%) Triton X100. Thirty microlitres of the enzyme preparation (i.e. supernatants of the fermented sorghum slurries) was reacted with 170 µl of the substrate solution in a well of a 96-well microplate. The release of the *p*-nitrophenolate anion as a result of the action of the esterase enzyme on the *p*-nitrophenol butyrate substrate was monitored using a microplate reader (FLUOstar Omega, BMG LABTECH, Software version 5.50 R4). The following conditions were used: 30°C incubation temperature and measurement of absorbance of the reaction mixture at 30 s intervals for 9 min using a wavelength of 415 nm. The method was performed in duplicate and the enzyme activity was described as the amount of enzyme that releases 1 µmol of *p*-nitrophenol per minute. Esterase activity was expressed as (U/mL) using the molar extinction coefficient for the *p*-nitrophenol of 11700 M⁻¹cm⁻¹ (Furukawa, Kawakami, Tomizawa & Miyamoto, 2019).

$$\frac{U}{mL} = \frac{\Delta A - \Delta A_y}{\epsilon} \chi \frac{\alpha}{\beta}$$

Where: ΔA = slope of the sample curve (Abs/min), ϵ = molar extinction coefficient (L/ μ mol), α = reaction volume (L), β = sample volume (ml) and ΔA_{γ} = slope of the blank curve (without enzyme) (Abs/min).

3.1.2.5 Simulated *in vitro* gastrointestinal digestion of sorghum porridges

The method described by Adarkwah-Yiadom and Duodu (2017) for simulated *in vitro* gastrointestinal digestion was used with modifications. For each sorghum type, 90 ml of oral digestive juice (consisting of 2.38 g sodium hydrogen phosphate, 0.19 g potassium dihydrogen phosphate and 8 g sodium chloride dissolved in 1 L of distilled water) was added to 30 g of the porridge in a 250 ml conical flask. The pH was modified to 6.75 using conc. HCL followed by incubating the mixture in an orbital shaking water bath for 10 min at 37°C. For the gastric digestion phase, 90 ml gastric juice (consisting of 0.32% (w/v) porcine pepsin A prepared in 0.03 M aqueous NaCl) was added to the digestion mixture, pH was modified to 2.0 using conc. HCl followed by incubation again at 37°C for 2 h. For the duodenal digestion phase, 90 ml duodenal juice (consisting of 0.14% (w/v) porcine pancreatin and 0.86% (w/v) porcine bile extract in 0.1 M aqueous NaHCO₃) was added to the digestion mixture, pH was modified to 7.0 using conc. HCl then incubated again for 2 h at 37°C. The digests were heated at 75°C for 10 min to inactivate the enzyme activity. Control samples were prepared using the same procedure with the addition of 90 ml distilled water instead of the digestive juices followed by heating at 75°C for 10 min. The digests and controls samples were centrifuged (Hettich Mikro 120, Refrigerated Centrifuge Z 366 K, Germany) at 2000 x g for 35 min at 4°C and the supernatants and residues were packed separately into plastic tubes, labelled, and stored at -20°C. The supernatants were freeze-dried, milled into a fine powder packed in an air-tight zip-lock polyethylene bag and stored between 4-6 °C.

3.1.2.6 Preparation of phenolic extracts

The method described by Apea-Bah *et al.* (2014) for extraction was used with modifications. Freeze-dried sample (1 g) was extracted in duplicates using 10 ml acidified methanol (1% (v/v) conc. HCl in methanol) through magnetic stirring for 2 h. This was followed by centrifuging at 3500 rpm for 10 min at 10°C and the supernatant was collected. The residue was re-extracted twice with 10 ml acidified methanol for 30 min followed by centrifugation as described above. The supernatants were pooled together, labelled, and kept at -20°C before analysis.

Phenolic extracts for Liquid Chromatography-Mass Spectrometry (LC-MS) analysis were also prepared by extracting 1 g of each sample with 5 ml acidified methanol. The extracts were filtered through 0.45 μm Arcrodisc PSF syringe filters (Pall Life Sciences, Ann Arbor, MI, USA) and the filtrates were stored in 2 mL amber glass vials (12*32 mm, Flat Base) at -20°C before analysis. All samples were extracted in duplicate.

3.1.2.7 Determination of total phenolic content (TPC)

The Folin-Ciocalteu method modified for the use of a 96-well microplate as described by Ainsworth and Gillespie (2007) was used. In each well of a 96-well microplate, 100 μL of sample extract, catechin standard solution (0-0.45 mM) or blank (1% conc. HCl in methanol) was reacted with 200 μL of 10% (v/v) Folin-Ciocalteu reagent (diluted with distilled water). This was followed by adding 800 μL of 700 mM Na_2CO_3 and thorough mixing by vortex. The reaction mixture was incubated in the dark for 2 h and the absorbance read at 765 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Software version 5.50 R4). Total phenolic content was calculated with the aid of a catechin standard calibration curve and reported as milligrams catechin equivalents per gram (mg CE/g) dry weight basis.

3.1.2.8 Identification and quantification of phenolic compounds using liquid chromatography-mass spectrometry (LC-MS)

The LC-MS method for identification and quantification of phenolic compounds described by Apea-Bah *et al.* (2014) was used. A Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system coupled with a binary pump (Waters, Milford, MA, USA) and a Waters Synapt G2 system comprising a Quadrupole-Time of Flight Mass Spectrometer (QToF-MS) (Waters, Milford, MA, USA) using an electrospray ionization (ESI) source was used. An incorporated photodiode array (PDA) detector (Waters, Milford, MA, USA) monitored the phenolic compounds at the wavelength range of 230-500 nm. A waters BEH C18 (100 $\times$ 2.1 mm, 1.7 μm) reverse phase column was used for separation. The mobile phase contained 0.1% (v/v) aqueous formic acid (solvent A) and 0.1% (v/v) aqueous formic acid in acetonitrile (solvent B). Gradient elution of phenolic compounds was conducted as follows: 100% A (0-22 min); 72% A (22-22.5 min); 60% A (22.5-23 min); 0% A (23-24.5); 100% A (24.5-26). The injection volume was 3 μL and the flow rate was 0.3 ml/min. Ionization was in the negative mode with a capillary voltage of 2.5 kV and a cone voltage of 25 V. Identification was carried out by comparing the retention times, mass and

UV-visible spectral data of the peaks observed in this study with those of pure phenolic compound as standards and what has been reported in literature. Integrated peak areas of phenolic compounds in sample extracts were compared with those of standards to quantify the phenolic compounds. Leucine enkephalin (molecular weight 555 Da) was used as lock mass. Data collection was done using MassLynx v. 4.1 software (Waters, Milford, MA, USA).

3.1.2.9 Determination of bioaccessibility of phenolic compounds in sorghum porridges

The bioaccessibility of phenolic compounds in the sorghum porridges was determined by expressing the total concentration of any particular group of phenolic compounds (phenolic acids, flavonoids or their sum) in the supernatants of digested porridges as a percentage of the total concentration of that group of phenolic compounds in the undigested porridges as follows:

$$\text{Bioaccessibility (\%)} = \left(\frac{A}{B} \right) \times 100$$

Where A represents the total concentration of any group of phenolic compounds in the supernatants of digested porridges, and B represents the total concentration of that group of phenolic compounds in the undigested porridges.

3.1.2.10 Statistical Analysis

Each experiment was done in duplicate and multifactor analysis of variance was used to determine the effects of sample treatments on dependent variables. Fisher's least significant difference were used for mean separation and significant differences indicated at ($p < 0.05$). Statistica 8 (StatSoft Inc., Tulsa, OK, USA) was used for statistical analysis.

3.1.3 Results and discussion

3.1.3.1 Esterase enzyme activity of fermented sorghum slurries

Figure 3-2 shows the esterase enzyme activity of the fermented red and white sorghum slurries. During the reaction, the esterase enzyme in the fermented slurries hydrolyses the ester bond in *p*-nitrophenyl butyrate and releases *p*-nitrophenol and this is indicated by a decline in the absorbance with time as seen in the graphs in Figure 3-2. The fermented red sorghum had higher esterase activity (3.52×10^{-4} U/mL) than the fermented white sorghum (2.21×10^{-8} U/mL). This could have an effect on the extent to which phenolic compounds can be released during fermentation in the two sorghum phenotypes and hence their bioaccessibility.

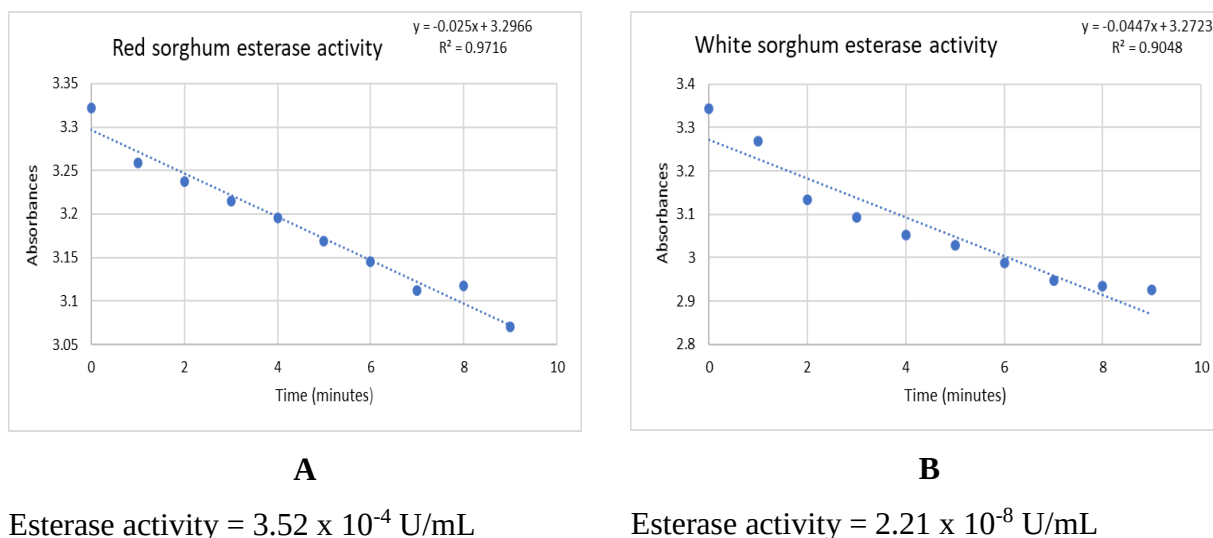


Figure 3-2: Esterase enzyme activity of fermented red and white sorghum slurries. A: fermented red sorghum slurry; B: fermented white sorghum slurry

A wide range of enzymes including esterases have been demonstrated to be active during sorghum fermentation (Svensson *et al.* (2010)). These include phenolic acid decarboxylase, glucosidase, tannase and phenolic acid reductase. These enzymes could also be involved in hydrolysis reactions to release bound phenolic compounds during sorghum fermentation.

3.1.3.2 Total phenolic content (TPC) of extracts from flour, porridges and digested porridges (supernatants)

Table 3-1: Effects of sorghum type, souring method, cooking and *in vitro* digestion on the TPC of extracts from flour, porridges and supernatant in sorghum

Treatments	TPC mg CE/g dry basis						Overall effect of souring method
	Uncooked flours		Undigested porridges		Supernatants of digested porridges		
	White sorghum	Red sorghum	White sorghum	Red sorghum	White sorghum	Red sorghum	
Raw flour (unsoured)	1.60 ^a ± 0.08	2.86 ^e ± 0.07	2.14 ^{bc} ± 0.08	3.01 ^{ef} ± 0.12	2.83 ^e ± 0.04	3.30 ^g ± 0.72	2.62 ^a
Spontaneously fermented	2.13 ^{bc} ± 0.03	3.62 ^{ij} ± 0.06	2.25 ^{cd} ± 0.15	3.75 ^j ± 0.11	3.30 ^{gh} ± 0.05	3.49 ^{hi} ± 0.09	3.09 ^b
Citric acid acidified	1.67 ^a ± 0.05	2.86 ^e ± 0.13	2.06 ^b ± 0.05	3.08 ^f ± 0.06	2.40 ^d ± 0.02	2.96 ^{ef} ± 0.04	2.51 ^a
Effect of sorghum Type	1.80 ^a	3.11 ^{de}	2.15 ^b	3.28 ^e	2.84 ^c	3.25 ^e	
Overall effect of treatment	2.46 ^a		2.72 ^b		3.05 ^c		

¹Mean±standard deviation of two independent experiments. Figures in a row and column with different superscripts differ significantly from each other ($p \leq 0.05$).

Table 3-1 shows that in terms of the overall effects, souring through fermentation and citric acid acidification had different effects on the TPC. While fermentation led to a significant overall increase in the TPC ($p < 0.05$), exogenous acidification had no effect on TPC of the sorghum samples. The process of fermentation enhances the depolymerization of high molecular weight phenolic compounds and simple phenolic conversion which enables the production of more phenolic compounds (Nazarni, Purnama, Umar & Eni, 2016). The activity of enzymes such as esterase (Ravisankar *et al.*, 2021) during fermentation may also enhance the extractability of phenolic compounds leading to the observed increased TPC upon souring by fermentation. This is demonstrated in this research by the observation that the fermented sorghum slurries had esterase activity (Figure 3-2). Based on the observation that the fermented red sorghum slurries had higher esterase enzyme activity than the fermented white sorghum slurries, it would have been expected that the percentage increase in total phenolic content upon fermentation of red sorghum would be greater than for white sorghum. However, the results in Table 3-1 indicate that the percentage increase in total phenolic content after fermenting the red sorghum flour (27%) was lower than for the white sorghum flour (33%). It is worth noting that a constraint in using the Folin-Ciocalteu technique is the fact that it is not restricted to phenolic compounds hence not specific. This can potentially confound any expected trends and correlations between esterase enzyme activity and associated percentage increases in total phenolic content.

Table 3-1 shows that flours, cooked and digested porridge samples from red sorghum had higher TPC than corresponding samples from white sorghum which is a well-known trend regarding how pericarp colour influences phenolic content of sorghum (Punia, Tokas, Malik & Sangwan, 2021; Rashwan *et al.*, 2021; Salawu, Bester & Duodu, 2014; Salazar-López *et al.*, 2018a). Pigmented sorghums contain higher levels and a wider variety of phenolic compounds compared to white sorghums and this accounts for the observed higher total phenolic content of samples from red sorghum compared to white sorghum.

Cooking flours into porridges increased the overall TPC in both sorghum types. Similar effects of cooking on TPC have been reported (Li *et al.*, 2021; N'Dri, Mazzeo, Zaupa, Ferracane, Fogliano & Pellegrini, 2013; Zaroug *et al.*, 2014) in sorghum. The increase in TPC has been attributed to enhanced extractability of phenolic compounds in sorghum through cell wall disruption and solubilization during thermal treatment (Apea-Bah *et al.*, 2014; Zaroug *et al.*, 2014).

Simulated *in vitro* gastrointestinal digestion of porridges produced a significant overall increase in TPC as has been reported by other workers (Apea-Bah, Minnaar, Bester & Duodu, 2016; Chandrasekara & Shahidi, 2012; Luzardo-Ocampo, Ramírez-Jiménez, Cabrera-Ramírez, Rodríguez-Castillo, Campos-Vega, Loarca-Piña & Gaytán-Martínez, 2020). The hydrolytic action of the enzymes used during the *in vitro* digestion process (amylase, pepsin, pancreatin) leads to breakdown of the food structure and matrix and can enhance extractability of phenolic compounds (Gawlik-Dziki, Dziki, Baraniak & Lin, 2009).

3.1.3.3 Phenolic compounds found in soured red and white non-tannin sorghum flour porridge and digested extracts

Table 3-2 shows the phenolic acids and flavonoids identified in flours, porridges and digested porridges (supernatants) of soured red and white sorghum. The retention times, UV-visible mass spectral characteristics and absorption maxima of the compounds and their identified fragments were used to identify the phenolic compounds compared with standards and literature.

Table 3-2: Retention time (Rt), UV-visible absorption maxima ($\lambda_{\max}$) and mass spectral characteristics of phenolic compounds identified in the sorghum flours and porridges

Peak No	Rt (min)	$\lambda_{\max}$ (nm)	[M-H] ⁻ (m/z)	MS/MS Fragments	Proposed Compound
<i>Benzoic acid derivatives</i>					
1	7.23	229, 254	137	109 (27), 93 (4)	4-hydroxybenzoic acid
2	8.44	229	153	109 (100)	Protocatechuic acid
3	15.75	229, 253	169	125 (17)	Gallic acid
<i>Hydroxycinnamic derivates</i>					
4	20.84	229, 319	193	161 (31), 133 (21)	Ferulic acid
5	11.63	229, 324	179	161 (2), 135 (35)	Caffeic acid
6	16.19	229, 273	167	123 (1)	Vanillic acid
7	24.63	229, 254	223	179 (3)	Sinapic acid
8	25.69	229	353	309 (31)	Chlorogenic acid
9	11.91	280	181	137 (21)	Dihydro caffeic acid
10	18.45	280	195	151 (100)	Dihydro ferulic acid
11	13.54	319	253	179 (17), 161 (63), 135 (39)	1-O-Caffeoyl-glycerol
12	15.81	279	237	145 (100), 119 (74)	1-O-coumaroyl-glycerol
13	23.92	230, 281	415	253 (66), 179 (26), 161 (48)	1,3-dicaffeoyl-glycerol
<i>Flavanones</i>					
14	24.34	229	271	151 (33), 119 (20)	Naringenin
15	14.50	287	287	151 (27), 135 (4)	Eriodictyol
16	17.25	286, 229	433	271 (100), 151 (40)	Naringenin-7-O-glucoside
17	14.90	319	449	287 (22)	Eriodictyol 7-O-glucoside
18	20.12	286	721	559 (29), 433 (100)	5,7,3',4'-Tetrahydroxy-flavan-5-O- β -glucosyl-4-8-eriodictyol
<i>Flavonols</i>					
19	21.67	229	301	193 (100), 179 (18)	Quercetin
20	12.29	253	625	301 (7)	Quercetin diglucoside
<i>Flavan-3-ols and Flavones</i>					
21	11.71	229	289	245 (27), 137 (14)	(+)-Catechin
22	13.48	229	289	137 (34)	Epicatechin
23	12.32	229, 253	451	451 (100), 289 (1)	Catechin glycoside
24	15.80	229, 303	269	119 (21)	Apigenin

Phenolic acids

The compound labelled peak 1 with retention time 7.23 min, molecular ion at m/z 137 was identified as 4-hydroxybenzoic acid (Figure 3-3). It showed a fragment ion at m/z 109 attributed to the loss of a CO group (-28 amu) (Luo *et al.*, 2020) and another at m/z 93 based on the loss of CO₂ (-44 amu) (Kang *et al.*, 2016). Compound 2 with retention time 8.37 min, molecular ion at m/z 153 was identified as protocatechuic acid (Figure 3-3). It produced a fragment with m/z 109 based on the loss of CO₂ (-44 amu) (Cheiran, Raimundo, Manfroi, Anzanello, Kahmann, Rodrigues & Frazzon, 2019). Compound 3 with retention time 15.75 min, molecular ion at m/z 169 was identified as gallic acid (Figure 3-3). It produced a fragment with m/z 125 based on the loss of CO₂ (-44 amu) (Liu, Guo, Ye, Lin, Sun, Xu & Guo, 2007).

The compound labelled peak 4 with retention time 20.84 min, molecular ion at m/z 193 was identified as ferulic acid (Figure 3-3). It produced fragments with m/z 161 which could be due to loss of OCH₃ (-31 amu) and a hydride from the ferulate anion and m/z 133 due to loss of a methyl group (CH₃; -15 amu), CO₂ (-44 amu) and a hydride ion (Kang *et al.*, 2016). The compound labelled peak 5 with retention time 11.63 min, molecular ion at m/z 179 was identified as caffeic acid (Figure 1). It produced a fragment m/z 135 based on the loss of CO₂ (-44 amu) and 161 due to loss of an OH group and a hydride (-18 amu) (Wu, Johnson, Bornman, Bennett, Clarke, Singh & Fang, 2016). The compound labelled peak 6 with retention time 16.19 min, molecular ion at m/z 167 was identified as vanillic acid (Figure 3-3). It produced a fragment at m/z 123 via the loss of CO₂ (-44 amu) (Liu *et al.*, 2007). The compound labelled peak 7 with retention time 24.63 min and molecular ion at m/z 223 was identified as sinapic acid (Figure 3-3). The compound produced a fragment at m/z 179 due to the loss of CO₂ (-44 amu) group from the carboxylic acid functional group (Liu *et al.*, 2007; Wu *et al.*, 2016).

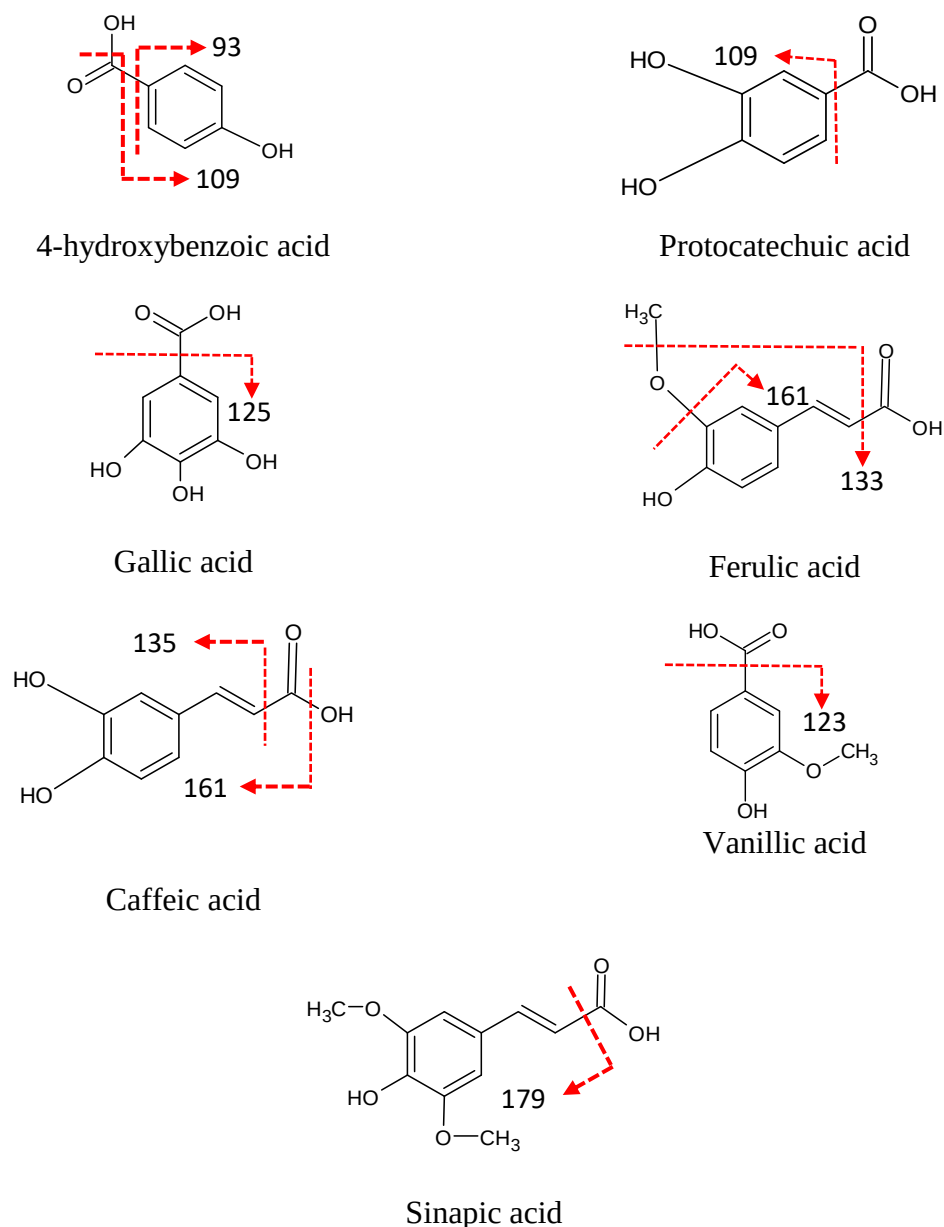


Figure 3-3: Proposed fragmentation patterns for phenolic acids identified

The compound labelled peak 8 with retention time 25.688 min and molecular ion at m/z 353 was identified as chlorogenic acid (Figure 3-4). The compound produced a fragment at m/z 309 due to the loss of CO_2 group from the carboxylic acid functional group (Wu *et al.*, 2016). The compound labelled peak 9 with retention time 11.91 was identified as dihydrocaffeic acid. The compound produced a fragment at m/z 137 due to the loss of CO_2 group from the carboxylic acid functional group, and the compound labelled peak 10 with retention time 18.45 was identified as dihydroferulic acid. This compound produced a fragment at m/z 151 also due to the loss of a CO_2 group in the carboxylic acid functional group (Wu *et al.*, 2016) (Table 3-2).

The compound labelled peak 11 with retention time 13.54 min, molecular ion at m/z 253 was identified as 1-*O*-caffeoyl-glycerol (Figure 3-4). It produced fragments at m/z 179, 161 and 135. The fragment ion at m/z 179 was obtained due to loss of a $-C_3H_6O_2$ unit from the glycerol residue (-74 amu) (Wu *et al.*, 2016), the one at m/z 161 was due to loss of a glycerol unit (-92 amu) (Pontieri, Pepe, Campiglia, Merciai, Basilicata, Smolensky, Calcagnile, Troisi, Romano & Del Giudice, 2021) and that at m/z 135 was due to loss of a glycerol and C=O unit (-118 amu). The compound labelled peak 12 with retention time 15.81 min, molecular ion at m/z 237 was identified as 1-*O*-coumaroyl-glycerol (Figure 3-4). It produced a fragment with m/z 145 due to the loss of a glycerol unit (-92 amu) and at m/z 119 due to loss of a glycerol and C=O unit (-118 amu). The compound labelled peak 13 with retention time 23.916 min, molecular ion at m/z 415 was identified as 1,3-*O*-dicafeoyl-glycerol (Figure 3-4). It produced fragments at m/z 253, 179 and 161. The fragment at m/z 253 was due to loss of a caffeoyl unit (-162 amu) (Pontieri *et al.*, 2021), that at m/z 179 was due to loss of a caffeoyl and a $-C_3H_6O_2$ unit from the glycerol (-236 amu) and that at m/z 161 was due to loss of a caffeoyl and glycerol unit (-254 amu).

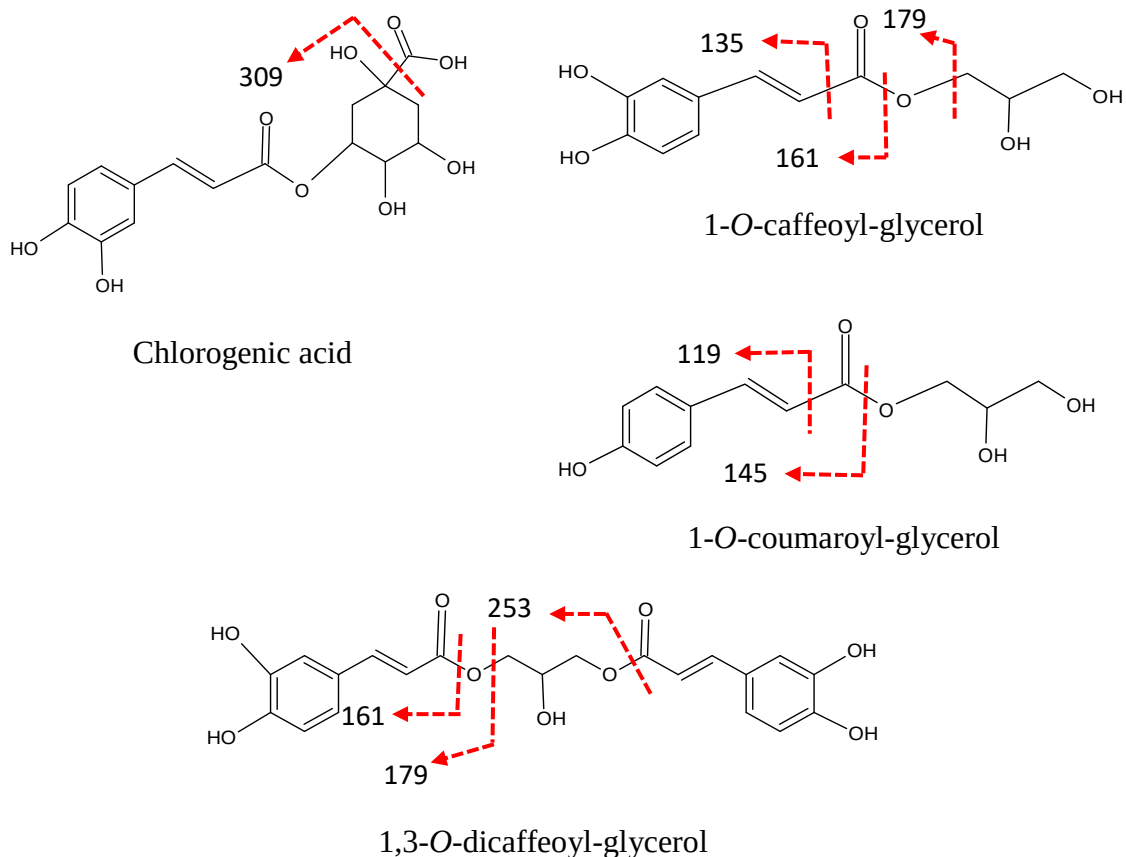
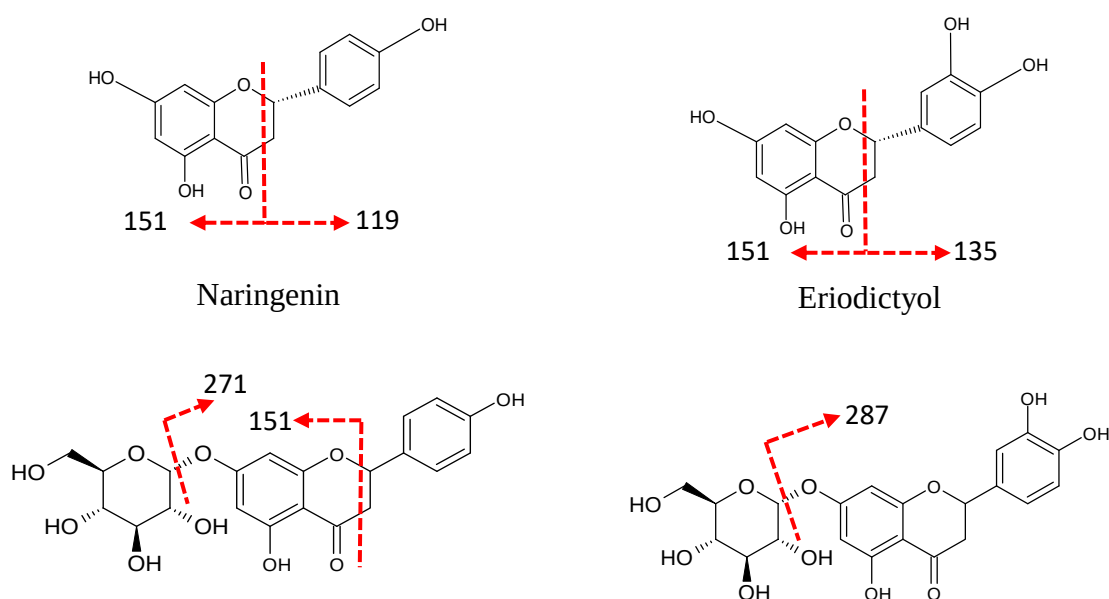


Figure 3-4: Proposed fragmentation patterns for derivatives of phenolic acids identified

Flavanones

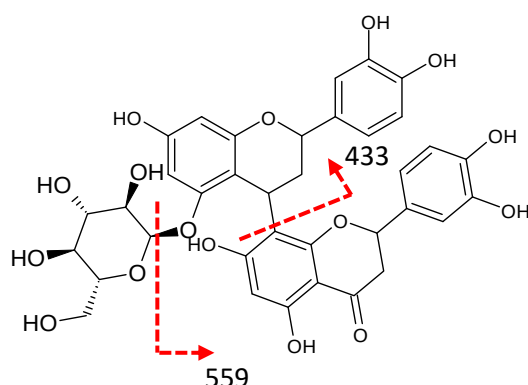
The compound labelled peak 14 with retention time 24.35 min, molecular ion at m/z 271 was identified as naringenin (Figure 3-5). It produced fragment ions at m/z 151 and 119. These were produced from cleavage of the C-ring at positions 1 and 3 to produce $^{1,3}A^-$ (m/z 151) and $^{1,3}B^-$ (m/z 119) fragments (Fabre, Rustan, de Hoffmann & Quetin-Leclercq, 2001). The compound labelled peak 15 with retention time 14.496 min, molecular ion at m/z 287 was identified as eriodictyol (Figure 3-5). It produced fragment ions at m/z 151 and 135. These were produced from cleavage of the C-ring at positions 1 and 3 to produce $^{1,3}A^-$ (m/z 151) and $^{1,3}B^-$ (m/z 135) fragments (Fabre *et al.*, 2001).

The compound labelled peak 16 with retention time 17.25 min, molecular ion at m/z 433 (Figure 3-5) was identified as naringenin-7-*O*-glucoside. It produced a fragment at m/z 271 which represents the naringenin aglycone after loss of the glucose (-162 amu). The ion at m/z 151 represents the $^{1,3}A^-$ fragment after cleavage of the C-ring at positions 1 and 3 of the naringenin aglycone (Fabre *et al.*, 2001). The compound labelled peak 17 with retention time 15.13 min was identified as eriodictyol-7-*O*-glucoside (Figure 3-5). It produced a fragment at m/z 287 identified as eriodictyol aglycone after the loss of glucose (-162 amu) (Wu *et al.*, 2016). The compound labelled peak 18 with retention time 19.41 min, molecular ion at m/z 721 (Figure 3-5) was identified as 5, 7, 3', 4',-tetrahydroxy-flavan-5-*O*- β -glucosyl-4-8-eriodictyol (Apea-Bah *et al.*, 2014). It produced fragments at m/z 559 due to loss of a glucose unit (-162 amu) and at m/z 433 due to the loss of an eriodictyol group (-288 amu).



Naringenin-7-*O*-glucoside

Eriodictyol-7-*O*-glucoside

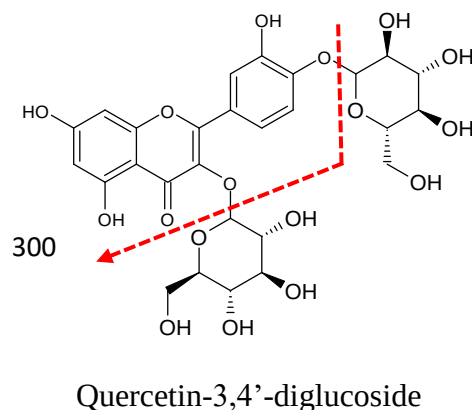
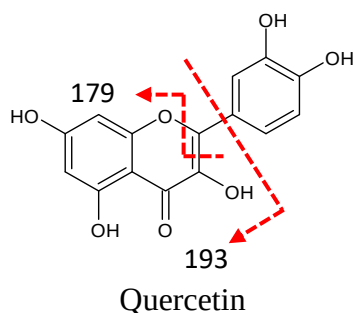


5,7,3',4'-tetrahydroxy-flavan-5-*O*- β -glucosyl-4-8-eriodictyol

Figure 3-5: Proposed fragmentation patterns for flavanones identified

Flavonols

The compound labelled peak 19 with retention time 19.54 min, molecular ion at m/z 301 (Figure 3-6) was identified as quercetin. It produced an ion at m/z 179 which represents the $^{1,2}A^-$ fragment after fragmentation of the C ring of quercetin at positions 1 and 2. It also produced a fragment at m/z 193 resulting from cleavage of the B-ring. The compound labelled peak 20 with retention time 12.286 min, molecular ion at m/z 625 (Figure 3-6) was identified as a diglucoside of quercetin (e.g., quercetin-3,4'-diglucoside). It produced fragment at m/z 301 which represent the quercetin aglycone after loss of the two glucose molecules (Cheiran *et al.*, 2019).



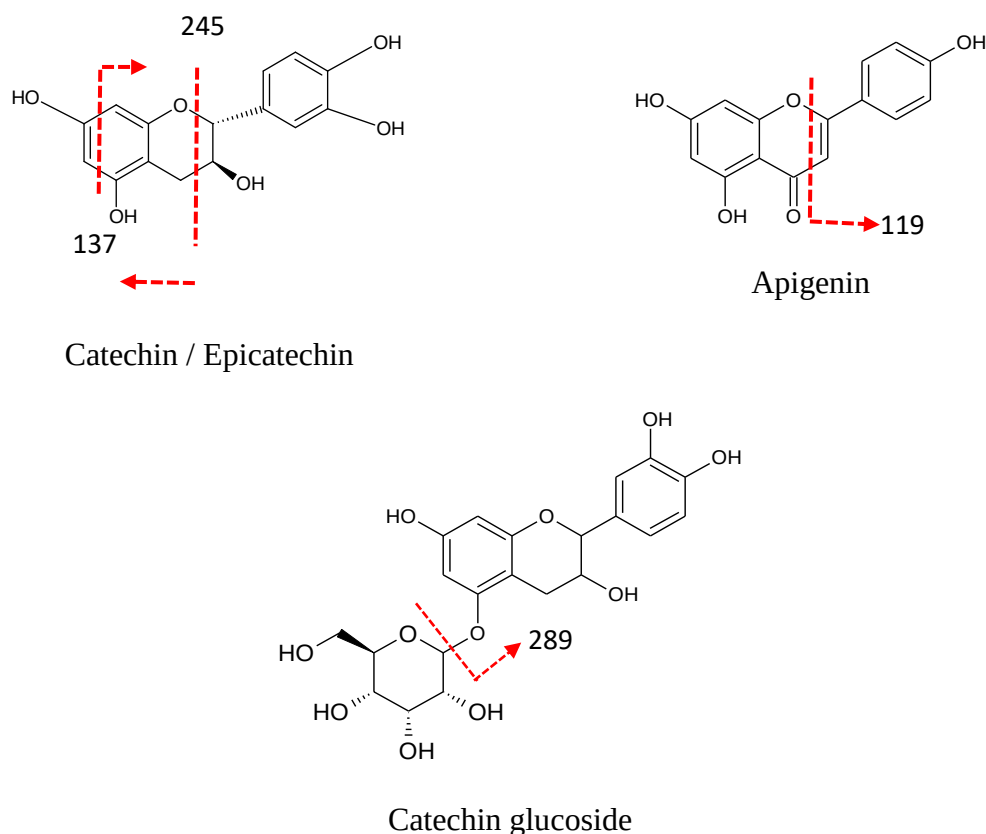


Figure 3-6: Proposed fragmentation patterns for flavonols, flavan-3-ols and flavones identified

Flavan-3-ols and flavones

The compounds labelled peaks 21 and 22 with retention times 11.71 min and 13.84 min were identified as catechin and epicatechin respectively (Figure 3-6). Both compounds had a molecular ion at m/z 289. Catechin produced fragments with m/z 245 and 137, while epicatechin produced a fragment at m/z 137. The fragment at m/z 245 might be due to the loss of a CH=C-OH group (– 42 amu) (Pérez-Magariño, Revilla, González-SanJosé & Beltrán, 1999) together with H₂ molecule (– 2 amu) most likely from the A-ring. The ions at m/z 137 might be due to fragmentation of the C ring at positions 1 and 3 resulting in ^{1,3}A⁻(m/z 137) (Fabre *et al.*, 2001).

The compound labelled peak 23 with retention time 8.95 min, molecular ion at m/z 451 (Figure 17) was identified as catechin glucoside (Figure 3-6). It produced a fragment at m/z 289 after loss of the glucose moiety (– 162 amu) (Zerbib, Cazals, Enjalbal & Saucier, 2018). The compound labelled peak 24 with retention time 15.89 min, molecular ion at m/z 269 was identified as apigenin (Figure 3-6). It produced a fragment at m/z 119 which could represent the ^{1,3}B⁻ ion obtained from cleavage of the C ring at positions 1 and 3 (Fabre *et al.*, 2001).

Table 3-3: Effect of souring on concentration ($\mu\text{g/g}$) of phenolic compounds in sorghum flour

	Red sorghum				White sorghum		
	Unsoured	Fermented	Acidified		Unsoured	Fermented	Acidified
Phenolic acids							
Protocatechuic acid	$6.51^c \pm 0.10$	$13.6^e \pm 0.01$	$7.60^d \pm 0.02$		$4.57^a \pm 0.02$	$7.69^d \pm 0.00$	$6.24^b \pm 0.02$
Caffeic acid	$71.3^c \pm 2.35$	$9.38^a \pm 0.64$	$310^d \pm 9.48$		$35.4^b \pm 0.77$	$6.71^a \pm 0.02$	$36.2^b \pm 3.89$
Ferulic acid	$21.1^c \pm 0.27$	$2.35^a \pm 0.35$	$48.1^d \pm 4.21$		$9.32^b \pm 0.47$	$1.92^a \pm 0.06$	$9.29^b \pm 0.51$
P-hydroxybenzoic	$36.4^d \pm 1.92$	$30.7^c \pm 3.23$	$70.8^e \pm 1.16$		$13.7^b \pm 0.82$	$0.80^a \pm 0.01$	$15.6^b \pm 1.73$
Syringic acid	$1.13^b \pm 0.11$	$6.77^d \pm 0.45$	$2.42^c \pm 0.02$		$0.26^a \pm 0.02$	$1.16^b \pm 0.15$	$1.13^b \pm 0.01$
Vanillic acid	$0.02^a \pm 0.00$	$1.35^c \pm 0.03$	$0.11^{ab} \pm 0.01$		$0.02^a \pm 0.00$	$0.65^b \pm 0.04$	$0.06^a \pm 0.01$
Gallic acid	$2.88^d \pm 0.05$	$1.06^c \pm 0.06$	$0.42^b \pm 0.02$		$0.02^a \pm 0.00$	$0.08^a \pm 0.00$	$0.35^b \pm 0.01$
Sinapic acid	$0.72^b \pm 0.00$	-	-		$0.42^a \pm 0.04$	-	-
Chlorogenic acid	$16.7^d \pm 0.06$	$35.4^e \pm 0.75$	$10.2^c \pm 0.20$		$5.08^a \pm 0.71$	$4.75^a \pm 0.36$	$7.29^b \pm 0.31$
Dicaffeoyl-glycerol	$589^e \pm 6.82$	$45.4^b \pm 1.29$	$663^f \pm 13.7$		$467^d \pm 15.2$	$7.19^a \pm 0.33$	$94.4^c \pm 8.15$
Caffeoyl-glycerol	$441^d \pm 23.9$	$77.2^{ab} \pm 0.12$	$398^d \pm 50.4$		$309^c \pm 3.93$	$44.1^a \pm 3.10$	$113^b \pm 6.29$
Dihydro ferulic acid	$0.14^a \pm 0.00$	$114^c \pm 1.17$	$2.27^a \pm 0.02$		-	$70.8^b \pm 5.37$	-
Dihydro caffeic acid	-	$8.32^b \pm 0.48$	$18.8^c \pm 0.62$		-	$0.63^a \pm 0.03$	-
1-coumaroyl glycerol	$1372^f \pm 10.0$	$191^d \pm 8.62$	$82.33^c \pm 6.89$		$1016^e \pm 7.58$	$15.0^a \pm 0.67$	$52.3^b \pm 0.14$
Total phenolic acids	2559	537	1614		1861	162	336

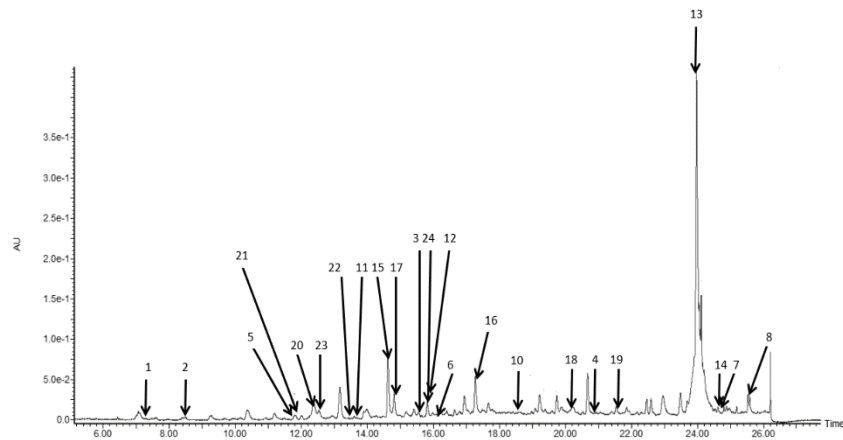
	Red sorghum				White sorghum		
	Unsoured	Fermented	Acidified		Unsoured	Fermented	Acidified
Flavonoids							
Naringenin	15.2 ^c ± 1.14	33.6 ^e ± 2.92	21.6 ^d ± 0.94		3.21 ^a ± 0.31	5.02 ^a ± 0.19	8.91 ^b ± 0.02
Catechin	0.41 ^e ± 0.00	0.68 ^f ± 0.02	0.33 ^d ± 0.00		0.12 ^c ± 0.01	0.05 ^a ± 0.00	0.09 ^b ± 0.00
Epicatechin	0.30 ^b ± 0.00	0.83 ^c ± 0.00	1.08 ^d ± 0.00		0.11 ^a ± 0.02	0.36 ^b ± 0.01	0.10 ^a ± 0.00
Kaempferol	10.8 ^b ± 0.53	13.4 ^c ± 0.42	17.2 ^d ± 1.98		4.65 ^a ± 0.36	9.53 ^b ± 0.07	4.99 ^a ± 0.18
Apigenin	11.9 ^c ± 0.14	11.9 ^c ± 0.23	10.2 ^b ± 0.75		2.96 ^a ± 0.09	10.4 ^b ± 0.31	2.89 ^a ± 0.12
Eriodictyol	36.0 ^b ± 0.70	137 ^d ± 2.02	57.6 ^c ± 0.04		0.47 ^a ± 0.03	1.11 ^a ± 0.00	0.60 ^a ± 0.00
Quercetin	1.86 ^b ± 0.10	6.53 ^c ± 0.36	6.38 ^c ± 0.04		1.62 ^b ± 0.10	0.38 ^a ± 0.05	0.77 ^a ± 0.00
Luteolin	6.80 ^b ± 0.02	21.1 ^e ± 1.16	8.88 ^c ± 0.05		4.05 ^a ± 0.13	17.0 ^d ± 0.92	4.03 ^a ± 0.07
Naringenin-7-glucoside	0.04 ^b ± 0.00	0.06 ^c ± 0.00	0.12 ^d ± 0.01		0.03 ^b ± 0.00	0.01 ^a ± 0.00	0.01 ^a ± 0.00
Eriodictyol- glucoside	37.3 ^c ± 0.35	1.72 ^b ± 0.00	44.9 ^d ± 0.01		1.07 ^a ± 0.08	-	-
Quercetin-diglucoside	18.5 ^c ± 0.47	-	1.83 ^a ± 0.08		11.9 ^b ± 0.13	-	-
Catechin glycoside	0.23 ± 0.01	-	-		-	-	-
5,7,3,4 tetrahydroxyflavan-5-O-β-glucosyl-4-8-eriodictyol	111 ^b ± 1.01	117 ^c ± 0.97	34.9 ^a ± 1.25		-	-	-
Total flavonoids	250	344	205		30	44	23
Total phenolic compounds	2809	881	1819		1891	206	359

Key: - = not identified, ¹Mean ± standard deviation from two independent experiments. Figures in a row with different superscripts differ significantly from each other ($p \leq 0.05$). NB: Dicafeoyl-glycerol, Caffeoyl-glycerol and Dihydro caffeic acid were quantified as caffeic acid equivalents, Dihydro ferulic acid was quantified as ferulic acid equivalents, 1-coumaroyl glycerol was quantified as *p*-coumaric acid equivalents, Eriodictyol, Eriodictyol-glucoside and 5,7,3,4 tetrahydroxyflavan-5-O-β-glucosyl-4-8-eriodictyol was quantified as naringin equivalents, Naringenin-7-glucoside was quantified as naringenin equivalents, Quercetin-diglucoside quantification was quantified as quercetin equivalents and Catechin glycoside quantification was quantified as catechin equivalents.

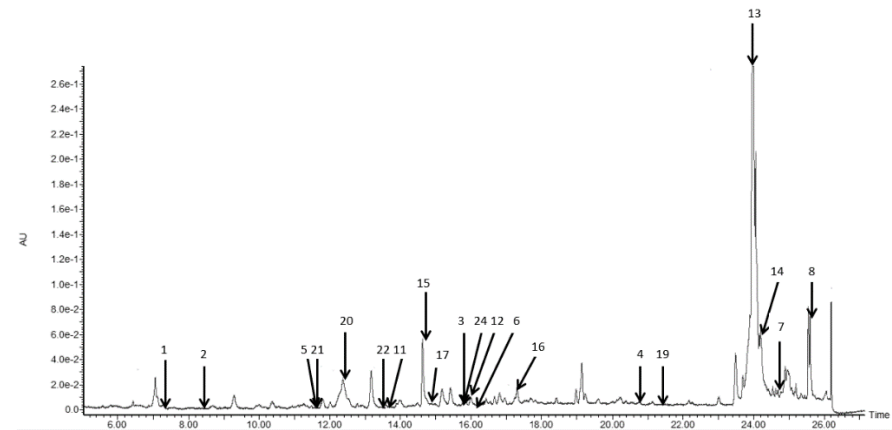
Table 3-3 shows the effect of souring on the levels of phenolic compounds (phenolic acids and flavonoids) in the red and white sorghum flours. Almost all the phenolic acids identified in this study such as protocatechuic acid, caffeic acid, ferulic acid, syringic acid, vanillic acid, gallic acid and sinapic acid have been reported in sorghum (Ironi, Adegoke, Effion, Oyewo, Alamu & Boligon, 2019; Kang *et al.*, 2016; Ofosu, Elahi, Daliri, Yeon, Ham, Kim, Han & Oh, 2020; Rao *et al.*, 2018a; Wu *et al.*, 2016). Flavonoids such as quercetin, apigenin, luteolin, naringenin, kaempferol and catechin have been reported in sorghum by various authors (Apea-Bah *et al.*, 2014; Ironi *et al.*, 2019; Kang *et al.*, 2016; Ofosu *et al.*, 2020).

According to de Morais Cardoso *et al.* (2017), the levels of phenolic acids range between 135.5 and 479.40 $\mu\text{g/g}$ in sorghum varieties (de Morais Cardoso *et al.*, 2017). Zhang *et al.* (2019) have reported that sorghum phenolic acids concentration ranges between 445 and 2.850 $\mu\text{g/g}$. The levels of all the phenolic acids quantified in this study fell within these ranges. Flavones and flavanones form part of the three most dominant classes of flavonoids in sorghum (de Morais Cardoso *et al.*, 2017). Sorghum flavone concentration ranges of up to 386 $\mu\text{g/g}$ and flavanone concentration ranges of up to 2000 $\mu\text{g/g}$ (Zhang *et al.*, 2019) have been reported. Flavan-3-ols are also important flavonoids in sorghum and levels of up to 33,000 have been reported (Girard & Awika, 2018). Quercetin (flavonol) at levels of up to 614 $\mu\text{g/g}$ have also been found in sorghum by Li *et al.* (2021). The levels of flavone (apigenin), flavanones (naringenin, eriodictyol, eriodictyol-glucoside and 5,7,3,4 tetrahydroxyflavan-5-O- β -glucosyl-4-8-eriodictyol, naringenin-7-glucoside quantification), flavan-3-ols (catechin, epicatechin and catechin glycoside) and flavonols (quercetin and quercetin-diglucoside) quantified in this study fell within these ranges.

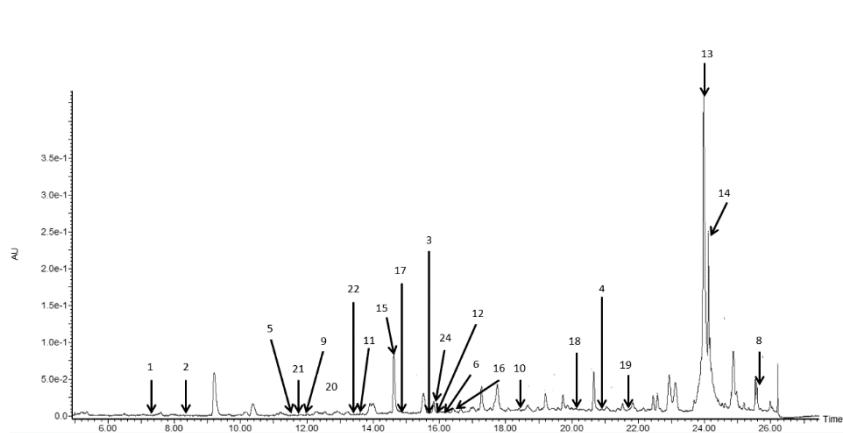
Table 3-3 also shows that the more pigmented red sorghum flour contained more phenolic compounds than the less pigmented white sorghum, an observation which has been widely reported by various authors (Buitimea-Cantúa, Torres-Chávez, Ledesma-Osuna, Ramírez-Wong, Robles-Sánchez & Serna-Saldívar, 2013; Rashwan *et al.*, 2021; Salazar-López *et al.*, 2018a). This is further demonstrated by the chromatograms in Figure 3-7 from the extracts of red sorghum flour (A, C and E) compared to the white sorghum flour extracts (B, D and F). The chromatograms for extracts from red sorghum flour contained more peaks with higher peak areas in many instances compared to extracts from white sorghum flour.



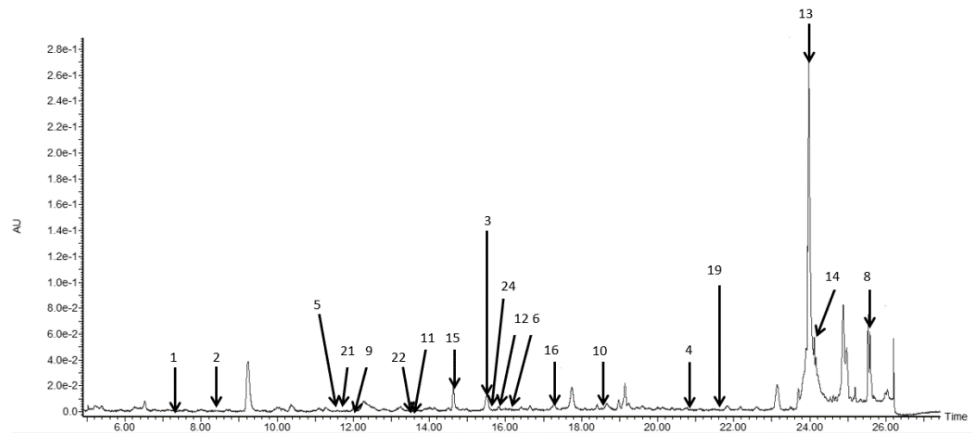
A



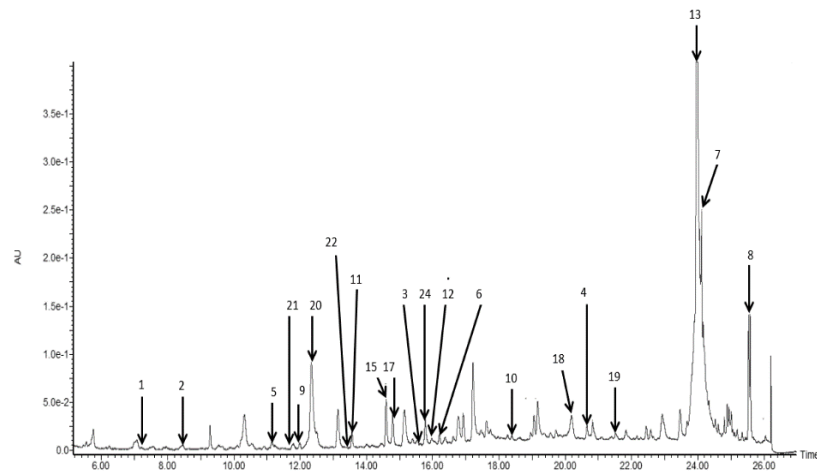
B



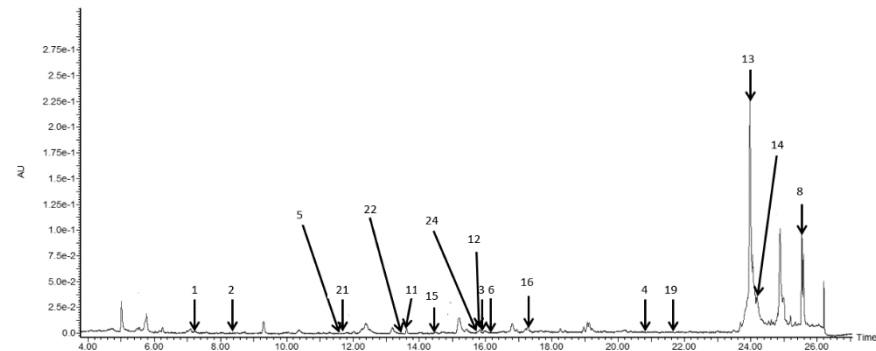
C



D



E



F

Figure 3-7: UV diode array chromatograms (extracted at 280 nm) of extracts from unsoured (A), fermented (C) and citric acid acidified (E) red sorghum and unsoured (B), fermented (D) and citric acid acidified (F) white sorghum flours. Numbers shown by the arrows represent peak numbers as shown in Table 3-2.

Effect of souring on phenolic compounds in sorghum

Table 3-3 shows that the effects of souring whether by fermentation or by citric acid acidification on phenolic compounds in sorghum were varied. There were increases in the levels of some phenolic compounds while some decreased after souring in both sorghum varieties. Given the complexity of the sorghum grain system in terms of how phenolic compounds occur in the grain (free and bound forms), the observed increases and decreases in phenolics due to souring could be expected and are generally in line with literature. Increases in phenolic concentrations after souring could be related to the acidic conditions which facilitate ester bond hydrolysis and the release of bound phenolic compounds (Tarko *et al.*, 2013) and also the presence of esterase enzymes (Shumoy *et al.*, 2017), especially in the fermented sorghum. On the other hand, decreases in phenolic concentrations after souring can be related to phenolic compounds that self-polymerize and interact with macromolecules such as starch and proteins induced by the acidic environments. Other mechanisms include the degradation of phenolic compounds by the fermenting microflora during fermentation and possible polyphenol oxidase activity might also lead to the observed decrease (Taylor & Duodu, 2015).

Table 3-3 shows that fermentation led to increases in the concentrations of some phenolic acids (protocatechuic, syringic, vanillic, chlorogenic, dihydroferulic and dihydrocaffeic acid in both red and white sorghum flour) including gallic acid for white sorghum flour. Fermentation also led to increases in the concentrations of most of the flavonoids with the exception of apigenin and eriodictyol glucoside for red sorghum flour and catechin, quercetin and naringenin-7-glucoside for white sorghum flour. As mentioned earlier, such increases in phenolic compounds due to fermentation can be related to the activity of esterase enzymes which agrees with the observation in this research that there was esterase enzyme activity in both sorghum types during fermentation. The proportion of total concentration of phenolic compounds in fermented red sorghum flour (881 µg/g) relative to unsoured red sorghum flour (2809 µg/g) was 31% which was greater than the corresponding proportion for white sorghum flour which was 11%. This is an indication that fermentation increased extractability of phenolic compounds to a greater extent in red sorghum flour in comparison with white sorghum flour which can be linked to the observation in this research that esterase activity during fermentation was greater in red sorghum flour compared to white sorghum flour.

Table 3-4: Effect of souring on concentration ($\mu\text{g/g}$) of phenolic compounds in sorghum porridges

	Red sorghum				White sorghum		
	Unsour	Fermented	Acidified		Unsour	Fermented	Acidified
Phenolic acids							
Caffeic acid	97.8 ^c $\pm$ 0.77	-	112 ^c $\pm$ 13.2		67.9 ^b $\pm$ 3.55	-	29.2 ^a $\pm$ 1.07
Ferulic acid	73.4 ^{cd} $\pm$ 2.24	5.59 ^a $\pm$ 0.00	86.3 ^d $\pm$ 1.97		21.1 ^b $\pm$ 0.03	4.14 ^a $\pm$ 0.00	23.15 ^b $\pm$ 0.01
P-hydroxybenzoic	-	3.88 $\pm$ 0.25	-		-	-	-
Vanillic acid	-	23.67 $\pm$ 0.01	-		-	-	-
Gallic acid	43.9 ^d $\pm$ 0.99	42.1 ^d $\pm$ 0.80	33.3 ^c $\pm$ 0.12		26.7 ^b $\pm$ 0.14	35.8 ^c $\pm$ 1.68	13.5 ^a $\pm$ 0.88
Chlorogenic acid	649 ^f $\pm$ 14.7	472 ^e $\pm$ 0.01	259 ^c $\pm$ 1.02		128 ^a $\pm$ 1.11	340 ^d $\pm$ 1.83	177 ^b $\pm$ 1.08
Dicaffeoyl-glycerol	631 ^e $\pm$ 0.14	66.4 ^b $\pm$ 1.40	349 ^d $\pm$ 0.74		20.4 ^a $\pm$ 0.04	22.1 ^a $\pm$ 0.03	262 ^c $\pm$ 0.03
Caffeoyl-glycerol	523 ^e $\pm$ 0.01	126 ^b $\pm$ 1.76	478 ^d $\pm$ 1.10		500 ^d $\pm$ 4.83	87.1a $\pm$ 1.19	345 ^c $\pm$ 4.17
Dihydro ferulic acid	-	134 ^b $\pm$ 1.23			-	61.1 ^a $\pm$ 0.18	-
Dihydro caffeic acid	-	962 ^b $\pm$ 16.3			-	30.6 ^a $\pm$ 2.00	-
1-coumaroyl glycerol	651 ^c $\pm$ 39.8	-	475 ^b $\pm$ 17.0		514 ^{bc} $\pm$ 16.7	-	316 ^a $\pm$ 27.3
Total phenolic acids	2669	1836	1793		1278	581	1166
Flavonoids							
Naringenin	32.2 ^a $\pm$ 0.78	48.2 ^b $\pm$ 0.64	30.6 ^a $\pm$ 0.16		-	-	-
Apigenin	-	0.15 ^a $\pm$ 0.01	1.45 ^c $\pm$ 0.17		1.12 ^{bc} $\pm$ 0.01	-	0.72 ^b $\pm$ 0.02
Eriodictyol	32.4 ^c $\pm$ 0.39	49.9 ^d $\pm$ 1.00	9.57 ^b $\pm$ 0.27		-	0.70 ^a $\pm$ 0.01	-
Naringenin-7-glucoside	-	1.55 $\pm$ 0.00	-		-	-	-
5,7,3,4 tetrahydroxyflavan-5-O- β -glucosyl-4-8-eriodictyol	18.3 ^a $\pm$ 0.02	22.2 ^{ab} $\pm$ 0.82	16.8 ^a $\pm$ 1.06				
Total flavonoids	83	122	58		1	1	1
Total phenolic compounds	2752	1958	1851		1279	582	1167

Key: - = not identified, ¹Mean $\pm$ standard deviation from two independent experiments. Figures in a row with different superscripts differ significantly from each other ($p \leq 0.05$). NB: Dicaffeoyl-glycerol, Caffeoyl-glycerol and Dihydro caffeic acid were quantified as caffeic acid equivalents, Dihydro ferulic acid was quantified as ferulic acid equivalents, 1-coumaroyl glycerol was quantified as *p*-coumaric acid equivalents, Eriodictyol and 5,7,3,4 tetrahydroxyflavan-5-O- β -glucosyl-4-8-eriodictyol was quantified as naringin equivalents and Naringenin-7-glucoside was quantified as naringenin equivalents

Table 3-4 shows that after cooking, some of the phenolic acids like sinapic, protocatechuic, syringic acid and flavonoids such as catechin, epicatechin, kaempferol, quercetin, luteolin, eriodictyol-glucoside, quercetin-diglucoside and catechin glycoside identified in the flours (Table 3-3) could not be identified in the porridges, which indicates reduced extractability of those phenolic compounds after cooking. The observed decrease in phenolic compounds after cooking could be related to degradation of phenolic compounds by the heat treatment. In addition, it has been proposed that during thermal processing (such as in wet cooking), phenolic compounds could leach into the endosperm and form complexes with macromolecules like proteins, resulting in a decrease in their extractability (Salazar-López *et al.*, 2018a; Taylor & Duodu, 2015).

Table 3-5: Effect of souring on concentration ($\mu\text{g/g}$) of phenolic compounds in supernatants of digested sorghum porridges

	Red sorghum				White sorghum		
	Unsoured	Fermented	Acidified		Unsoured	Fermented	Acidified
Phenolic acids							
Caffeic acid	316 ^a $\pm$ 18.7	-	363 ^b $\pm$ 7.94		-	-	-
Ferulic acid	6.13 $\pm$ 0.04	-	-		-	-	-
P-hydroxybenzoic	-	4.58 ^a $\pm$ 0.16	50.3 ^b $\pm$ 1.89		-	-	-
Vanillic acid	-	112 $\pm$ 2.67	-		-	-	-
Chlorogenic acid	278 ^b $\pm$ 0.47	-	881 ^c $\pm$ 10.2		-	-	257 ^a $\pm$ 0.36
Dicaffeoyl-glycerol	37.1 ^a $\pm$ 0.23	77.8 ^b $\pm$ 2.48	96.5 ^c $\pm$ 2.45		35.2 ^a $\pm$ 2.24	-	-
Caffeoyl-glycerol	543 ^d $\pm$ 2.19	-	422 ^c $\pm$ 1.51		106 ^a $\pm$ 5.51	-	310 ^b $\pm$ 18.8
Dihydro ferulic acid	-	20.0 $\pm$ 0.00	-		-	-	-
1-coumaroyl glycerol	359 ^c $\pm$ 39.3	-	40.8 ^b $\pm$ 3.73		-	-	26.6 ^a $\pm$ 0.45
Total phenolic acids	1539	214	1854		141	-	594
Flavonoids							
Naringenin	4.87 $\pm$ 0.77	-	-		-	-	-
Eriodictyol	-	8.21 $\pm$ 0.12	-		-	-	-
Naringenin-7-glucoside	8.37 ^a $\pm$ 0.06	-	16.2 ^b $\pm$ 0.19		-	-	-
Eriodictyol- glucoside	6.17 ^a $\pm$ 0.69	-	8.75 ^b $\pm$ 0.37		-	-	-
Quercetin-diglucoside	-	30.2 ^c $\pm$ 0.51	15.8 ^{ab} $\pm$ 0.08		-	23.8 ^b $\pm$ 0.22	10.5 ^a $\pm$ 0.52
5,7,3,4 tetrahydroxyflavan-5-O- β -glucosyl-4-8-eriodictyol	-	-	8.40 $\pm$ 0.78		-	-	-
Total flavonoids	19	38	49		-	24	11
Total phenolic compounds	1558	252	1903		141	24	605

Key: - = not identified, ¹Mean $\pm$ standard deviation from two independent experiments. Figures in a row with different superscripts differ significantly from each other ($p \leq 0.05$). NB: Dicaffeoyl-glycerol and Caffeoyl-glycerol were quantified as caffeic acid equivalents, Dihydro ferulic acid was quantified as ferulic acid equivalents, 1-coumaroyl glycerol was quantified as *p*-coumaric acid equivalents, Eriodictyol, Eriodictyol-glucoside and 5,7,3,4 tetrahydroxyflavan-5-O- β -glucosyl-4-8-eriodictyol was quantified as naringin equivalents, Naringenin-7-glucoside was quantified as naringenin equivalents and Quercetin-diglucoside quantification was quantified as quercetin equivalents

Table 3-6: Effect of souring on percentage bioaccessibility of phenolic acids, flavonoids and total phenolic compounds in red and white sorghum porridges

	Total bioaccessible phenolic acids					
	Unsoured		Fermented		Citric Acid acidified	
	Red sorghum	White sorghum	Red sorghum	White sorghum	Red sorghum	White sorghum
Phenolic acids	58	11	12	0	103	51
Total bioaccessible Flavonoids						
Flavonoids	22	0	31	2400	84	1100
Total bioaccessible phenolic compounds						
Total phenolic compounds	57	11	13	4	103	52

Table 3-5 shows that even more phenolic compounds could not be identified after simulated digestion compared with the flours and undigested porridges. However, it is clear from Table 3-5 that some phenolic compounds could be identified and quantified in the supernatants of the porridge digests which indicates that the phenolic compounds in the red and white sorghum do have a certain measure of bioaccessibility.

Table 3-5 also shows that there were more total phenolic compounds in the supernatants of the exogenously acidified red and white sorghum porridges compared to the fermented and unsoured red and white sorghum porridges. This is an indication that there were more bioaccessible phenolics in the exogenously acidified sorghum porridges compared to the fermented and unsoured porridges. This is reflected in Table 3-6 which shows the percentage bioaccessibility of the phenolic compounds in the porridges. The percentage bioaccessibility was calculated by expressing the concentration of phenolic compounds (phenolic acids, flavonoids and total phenolic compounds) in the supernatants of the digested porridges as a percentage of the concentration of phenolic compounds in the corresponding undigested porridge. Table 3-6 clearly shows that the exogenously acidified red and white sorghum porridges had higher bioaccessibility of phenolic acids, flavonoids and total phenolic compounds compared with the unsoured and fermented sorghum porridges. The only exception was with the fermented white sorghum porridge which had the highest bioaccessibility of flavonoids. However, it is also worth noting that only one phenolic compound (eriodictyol) could be identified in the fermented white sorghum porridge and in the supernatant of its digest (quercetin diglucoside) which is an indication of an overall lower bioaccessibility of phenolic compounds.

In summary, phenolic compounds in red and white sorghum porridges soured by exogenous acidification with citric acid were more bioaccessible than those in porridges soured by fermentation. In the fermented sorghum porridges, there could be enhanced activity of polyphenol oxidase enzymes due to the action of the fermenting microflora. The polyphenol oxidase enzymes oxidize phenolic compounds and convert them to quinones which leads to the formation of large polymeric compounds, which in turn reduces the extractability (and by implication bioaccessibility of phenolic compounds) (Taylor & Duodu, 2015) in the fermented sorghum porridge.

3.1.4 Conclusions

Souring through spontaneous fermentation enhances the content and extractability of phenolic compounds in sorghum. Fermented sorghum slurries exhibit esterase enzyme activity which may contribute to enhanced extractability of phenolics in fermented sorghum. More significantly, exogenous acidification with citric acid produces more bioaccessible phenolic compounds in red and white sorghum porridges compared to fermentation. Souring of sorghum porridges leads to enhanced bioaccessibility of bioactive phenolic compounds and such soured sorghum products therefore have the potential to combat diet related NCDs.

3.2 EFFECTS OF SOURING AND SIMULATED *IN VITRO* GASTROINTESTINAL DIGESTION ON ANTIOXIDANT-RELATED HEALTH-PROMOTING PROPERTIES OF SORGHUM PORRIDGE

ABSTRACT

The effects of souring through spontaneous fermentation, and exogenous acidification by citric acid and simulated *in vitro* gastrointestinal digestion on antioxidant-related health-promoting properties of porridges from red and white sorghum were studied. Samples (red and white flours, porridges, and supernatants of digested porridges) were analysed for radical scavenging activity against ABTS, DPPH and ORAC assays, as well as NO inhibition, Caco-2 cellular antioxidant activity and their capacity to protect plasmid DNA against oxidative damage. In general, both souring methods spontaneous fermentation and citric acid acidification enhanced the antioxidant activity for all samples analysed except for ABTS where souring through citric acid acidification led to a significant decrease in radical scavenging activity and there was no observed effect with NO inhibition after souring. Overall, spontaneous fermentation increased ABTS, DPPH and ORAC values, while souring through citric acid acidification had varied effects on the antioxidant property. The radical scavenging activity of the porridges were also enhanced after simulated *in vitro* gastrointestinal digestion. Flours, porridges, and supernatants of digested porridges from both sorghum types exhibited protective effects against AAPH-induced oxidative DNA damage and Caco-2 cellular damage. Flours, porridges, and supernatants of digested porridges from red sorghum demonstrated higher radical scavenging activities compared to white sorghum, possibly due to the higher phenolic content of the red sorghum extracts. Results obtained from this study show the potential of soured sorghum porridges to offer protection against oxidative stress-induced non-communicable diseases.

3.2.1 Introduction

Oxidative stress is identified as a leading cause of the occurrence of many non-communicable diseases (NCDs) (Apea-Bah *et al.*, 2016). The body is said to be in an oxidative stress state when it is overwhelmed with highly reactive components such as reactive oxygen and nitrogen species which can trigger damage to various body cells and lead to the occurrence of NCDs (Nwachukwu *et al.*, 2021). This has increased the interest in bioactive antioxidant components from dietary sources that are suggested to have the ability to scavenge these reactive oxygen and nitrogen species (Apea-Bah *et al.*, 2014), and by so doing, prevent oxidative stress and protect against NCDs. Sorghum is an important cereal staple food for approximately 7% of the world's population (Khoddami *et al.*, 2017). It is known as an important source of bioactive phenolic compounds with potential to protect against oxidative stress and related NCDs (Gabaza *et al.*, 2016).

Soured sorghum products are popular among many communities in sub-Saharan Africa and in particular, souring through spontaneous fermentation has been used in Africa for ages as a method to produce fermented sorghum porridge (Onyango & Wanjala, 2018). A growing trend in Africa is the production of soured cereal products such as porridges (mostly ready-to-consume), using exogenous acidification with acidulants like citric acid (Adeyanju *et al.*, 2019). From the perspective of potential health-promoting properties of bioactive phenolics in sorghum, such soured products are important due to the potential of the souring process to improve extractability of phenolic compounds that are found excessively in bound forms and enhance antioxidant activity (Tagliazucchi *et al.*, 2012).

This research seeks to study the effects of souring through spontaneous fermentation and citric acid acidification on the antioxidant-related health promoting properties of sorghum porridges.

3.2.2 Materials and Methods

3.2.2.1 Materials

Red and white sorghum (non-tannin type) was used. ABTS 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt), DPPH (2,2-Diphenyl-1-picrylhydrazyl), Trolox ((±)-6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid), hydrochloric acid (HCl), 2,2-azobis (2-methylpropionamidine) dihydrochloride (AAPH) sodium carbonate (Na₂CO₃), methanol (CH₃OH), disodium hydrogen phosphate (Na₂HPO₄), potassium dihydrogen phosphate (KH₂PO₄), citric acid monohydrate (C₆H₈O₇·H₂O), sodium hydroxide (NaOH) were sourced from Sigma-Aldrich. The pBR 322 vector DNA was purchased from Promega (Madison, WI, USA). Human colon adenocarcinoma cell line (Caco-2) was purchased from the European Collection of Cell Cultures (ECACC) through Sigma-Aldrich (Johannesburg, South Africa). All other chemicals were purchased from Sigma-Aldrich.

3.2.2.2 Sample preparation

Preparation of sorghum flour unsoured and soured porridges and simulated *in vitro* gastrointestinal digestion of porridges were carried out as described in Chapter 3.1.

3.2.2.3 Determination of ABTS radical scavenging activity

ABTS radical scavenging activity of the sorghum extracts was determined using a modified form of the method described by Ofosu *et al.* (2020). The cooked and uncooked sorghum extracts were diluted 5 times with acidified methanol (1% (v/v) conc HCl in methanol), while the digested extracts were diluted 10 times. The ABTS radical stock solution (mother solution) was prepared by mixing equal volumes of 7 mM ABTS radical (obtained by dissolving 6.68 mg ABTS diammonium salt in 1 ml deionized water) and 3 mM potassium persulphate (obtained by dissolving 1.32 mg potassium persulphate in 1 ml deionized water) in an Eppendorf tube for 12-14 h between 18-22°C in the dark. The working solution was prepared by diluting 2 ml of the mother solution with 58 ml phosphate buffer saline (PBS) at pH (7.4). In each well of a 96-well microplate, 10 µl diluted sample extract or Trolox standards (0-600 mM) [prepared by dissolving 6.2825 mg Trolox in 25 ml 1% (v/v) HCl in methanol] were mixed with 190 µl working solution followed by incubation in the dark for 30 min between 18-22°C. The absorbance was read at 734 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Software version 5.50 R4). ABTS radical scavenging activity was calculated with

the aid of a Trolox standard calibration curve and results reported in millimolar Trolox equivalent per gram sample (mM TE/g) dry weight basis.

3.2.2.4 Determination of DPPH radical scavenging activity

DPPH radical scavenging activity of the sorghum extracts was determined using a modified method described by Apea-Bah *et al.* (2016). The uncooked, cooked, and digested sorghum extracts were diluted 15 times with absolute methanol. The mother solution was prepared by dissolving 2.4 mg DPPH radical in 10 ml absolute methanol followed by vortexing. The working solution was prepared by adding 10 ml mother solution to 50 ml absolute methanol. In each well of a 96-well microplate, 10 μ l diluted sample extract or Trolox standards (0-600 mM prepared in absolute methanol) were each mixed with 190 μ l working solution and incubated in the dark for 1 h at room temperature. The absorbance was read at 515 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Software version 5.50 R4). Using a Trolox standard calibration curve, the DPPH radical scavenging activity was calculated and reported in millimolar Trolox equivalents per gram sample (mM TE/g) dry weight basis.

3.2.2.5 Determination of Oxygen Radical Absorbance Capacity (ORAC)

Oxygen radical absorbance capacity was determined using a modified version of the method described by Zhu *et al.* (2017). AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) was used as a peroxy radical generator. About 4 ml AAPH solution was prepared by dissolving 0.0065 g AAPH in 4 ml deionized water. Working solution of 0.19 mM fluorescein salt was prepared using phosphate buffer saline (PBS). Trolox standards (0-1 mM) were prepared by mixing 0.0025 g Trolox with 10 ml 0.1 M PBS. Undigested and digested samples were diluted 50 times with PBS. In a 96-well microplate, 10 μ l extracts or Trolox was reacted with 165 μ l fluorescein salt and 25 μ l AAPH solution. The fluorescence was obtained every minute for 2 h at an excitation wavelength of 485 nm and an emission wavelength of 520 nm using a spectrophotometer (FLUOstar Omega, BMG LABTECH, Software version 5.50 R4). The ORAC was determined using the area under the curves (AUC) and reported in millimolar Trolox equivalents per gram sample (mM TE/ g) dry weight basis.

3.2.2.6 Determination of Nitric Oxide Radical Scavenging Activity

A method adapted from Apea-Bah *et al.* (2014) was used to determine the nitric oxide (NO) radical scavenging activity. A volume of 10 ml PBS (0.1 M) (pH 7.4) was reacted with 0.015 g sodium nitroprusside to obtain a 5 mM solution and left to stand for 1 h in the light between

18-22°C. Sodium nitrite (NaNO₂) standards (0-0.1 mM) were prepared in PBS (0.1 M) pH 7.4. Griess reagent was prepared by mixing 0.2% (w/v) N-(1-naphthyl) ethylenediamine (NED) (0.02 g NED in 10 ml 2.5% phosphoric acid) with a 2% solution w/v of sulphanilamide (0.2 g sulphanilamide in 10 ml 2.5% phosphoric acid). These two solutions were mixed to a final concentration of 1% sulphanilamide and 0.1% NED Griess reagent. 50 µl sample extract was reacted with 50 µl sodium nitroprusside followed by 1 h incubation in the dark between 18-22°C. After that, 100 µl Griess reagent was added followed by another 10 min incubation between 18-22°C. Absorbance was read at 570 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Software version 5.50 R4). The NO scavenged by the sorghum extracts was reported as percentage inhibition (% inhibition).

3.2.2.7 Determination of the effect of sorghum extracts on Caco-2 cellular growth (cytotoxicity)

About 100 µl of Caco-2 cells at a concentration of 4 x 10⁴ cells/ml were plated in the wells of a 96-well plate followed by incubation for 24 h at 37°C in an atmosphere of 5% carbon dioxide. The effect of the porridge extract/digest was determined by adding 10 µl extract/digest to the cells. The control contained 10 µl PBS instead of extract/digest. Then the plates were incubated for 48 h at 37°C in an atmosphere of 5% carbon dioxide. Thereafter, 11 µl 20% formaldehyde was added to each well followed by incubation for 30 min at 37°C in an atmosphere of 5% carbon dioxide to fix the cells. After that, the medium was discarded and 100 µl Crystal Violet (CV) stain was added. The plates were incubated again between 18-22°C for 30 min. Running water was used to rinse the CV stain until the water ran clear. After rinsing, 100 µl 10% acetic acid was added to the remaining CV stain in the well. Absorbance was read at 630 nm using a microplate plate reader (FLUOstar Omega, BMG LABTECH, Software version 5.50 R4). The % growth of cells was calculated as follows:

$$\frac{\text{Sample well absorbance}}{\text{Control well absorbance}} \times 100 = \% \text{ Cell Viability}$$

3.2.2.8 Determination of cellular antioxidant activity using the Caco-2 cell line

Cellular antioxidant activity (CAA) in Caco-2 cells was determined using the DCFH-DA (dichlorofluorescein diacetate) method adapted from McDowell, Thompson, Stark, Ou and Gould (2011). About 100 µl of Caco-2 cells at a concentration of 4 x 10⁴ cells/ml were plated into wells of a 96-well plate. Cells were allowed to attach overnight for 24 h at 37°C in an atmosphere of 5% carbon dioxide. After incubation, 50 µl of 75 µM DCFH-DA working

solution was added to each well of the 96-well plate making a final concentration of 25 μ M DCFH-DA then incubated the plates again for 1 h at 37°C. The medium was removed and cells were rinsed once with 300 μ l PBS. The cells were exposed to 50 μ l of the sample extracts followed by 50 μ l 7.5 mM AAPH solution. The excitation wavelength was 485 nm and the emission wavelength was 520 nm and the change in fluorescence was measured every two minutes for 1 h using a microplate reader (FLUOstar Omega, BMG LABTECH, Software version 5.50 R4). The gradient of the curve was calculated and results reported as % oxidative damage as follows:

$$\% \text{ oxidative damage (OD)} = \frac{\text{Sample} - \text{PBS}}{(\text{AAPH} - \text{PBS}) \times 100}$$

The change in fluorescence for the well containing AAPH without sample extracts was designated as having 100% cellular oxidative damage. Cellular antioxidant effect was indicated by a significant decrease in fluorescence.

3.2.2.9 Determination of inhibition of oxidative DNA damage

A modification of the method by Nderitu, Dykes, Awika, Minnaar and Duodu (2013) which measures protection against oxidative damage of pBR 322 plasmid DNA by 2,2-azobis (2-methylpropionamidine) dihydrochloride (AAPH) in the presence or absence of sorghum flour porridge extracts and digests was used. The pBR 322 plasmid DNA (1 mg/ml) was diluted 20 times using sterile distilled water. The sample extracts were diluted 20 times with sterilized PBS (pH 7.4) and the digests were diluted 10 times with pH 7.4 PBS. The negative control contained 2.5 μ l DNA, 2.5 μ l sterile distilled water and 2.5 μ l PBS (pH 7.4) and the positive control contained 2.5 μ l DNA, 2.5 μ l AAPH and 2.5 μ l PBS (pH 7.4). Reaction mixtures containing 2.5 μ l DNA, 2.5 μ l 10% (v/v) solutions of sample digests or extract and 2.5 μ l 3 mM AAPH were incubated at 37°C for 90 min. After incubation, 2.5 μ l gel loading buffer (consisting of 0.13% bromophenol blue and 40% sucrose) was added into each tube including the negative and positive control and 8 μ l of the mixture was loaded into a 1% agarose gel consisting of 40 mM tris, 20 mM glacial acetic acid, 0.5 M ethylenediaminetetraacetic acid (pH 8.0) and 0.5 μ g/ml ethidium bromide. The gel was then electrophoresed in a horizontal slab gel apparatus in tris acetate–EDTA buffer (pH 8.0) for 1 h (60 V, 500 mA, 150W). DNA bands were then photographed (Gel Doc EZ Imager, Bio-Rad Labs, Hercules, CA, USA) and the bands analysed using Image Lab 5.1 software (Bio-Rad Labs, Hercules, CA, USA).

3.2.2.10 Statistical Analysis

Each experiment was done in duplicate and multifactor analysis of variance was used to determine the effects of sample treatments on dependent variables. Fisher's least significant difference were used for mean separation and significant differences indicated at ($p < 0.05$). Statistica 8 (StatSoft Inc., Tulsa, OK, USA) was used for statistical analysis.

3.2.3 Results and Discussion

3.2.3.1 Radical scavenging activity of extracts from porridges and supernatants

Table 3-7: Effect of souring on radical scavenging activity (ABTS, DPPH, ORAC and NO) of extracts from sorghum flours and porridges and supernatants of digested porridges

ABTS (mM TE/g dry basis)							
	Uncooked flours		Undigested porridges		Supernatants of digested porridges		Overall Effect
	White sorghum	Red sorghum	White sorghum	Red sorghum	White sorghum	Red sorghum	
Raw flour (unsoured)	21.03 ^b ± 0.54	57.09 ⁱ ± 1.85	16.22 ^a ± 1.00	33.52 ^e ± 1.32	67.85 ^j ± 2.57	104.71 ^o ± 2.89	50.07 ^b
Spontaneously fermented	23.94 ^c ± 0.78	47.74 ^h ± 1.34	28.93 ^d ± 2.20	47.56 ^h ± 1.23	79.41 ^l ± 4.77	88.74 ⁿ ± 6.88	52.72 ^c
Citric acid acidified	23.39 ^{bc} ± 0.82	37.89 ^f ± 1.10	25.12 ^c ± 1.72	42.19 ^k ± 0.81	72.19 ^k ± 3.69	83.45 ^m ± 3.77	47.37 ^a
Overall effect of sorghum Type	22.79 ^a	47.57 ^c	23.42 ^a	41.09 ^b	73.15 ^d	92.30 ^c	
Overall effect of treatment	35.18 ^b		32.25 ^a		82.73 ^c		
DPPH (mM TE/g dry basis)							
Raw flour (unsoured)	70.51 ^a ± 3.50	95.34 ^c ± 4.66	94.41 ^c ± 2.71	113.82 ^d ± 4.05	95.13 ^c ± 6.46	122.41 ^{de} ± 8.87	98.60 ^a
Spontaneously fermented	97.85 ^c ± 3.44	110.32 ^d ± 3.84	99.30 ^c ± 3.80	113.75 ^c ± 5.02	90.06 ^{bc} ± 3.89	131.90 ^e ± 7.48	107.20 ^b
Citric acid acidified	82.89 ^b ± 2.36	96.09 ^c ± 4.28	112.38 ^d ± 3.18	132.58 ^e ± 1.44	105.29 ^{cd} ± 7.47	133.08 ^e ± 9.34	110.38 ^c
Overall effect of sorghum Type	83.75 ^a	100.58 ^c	102.03 ^c	120.05 ^d	96.82 ^b	129.13 ^e	
Overall effect of treatment	92.17 ^a		111.04 ^b		112.98 ^b		
ORAC (mM TE/g)							
Raw flour (unsoured)	66.38 ^b ± 3.01	66.38 ^b ± 1.12	113.84 ^d ± 3.60	160.37 ^f ± 0.59	74.15 ^c ± 1.72	167.62 ^f ± 3.76	108.12 ^a
Spontaneously fermented	61.64 ^a ± 1.13	143.04 ^e ± 0.47	115.03 ^d ± 1.25	173.55 ^g ± 1.03	120.67 ^{de} ± 9.60	180.97 ^g ± 7.41	132.48 ^c
Citric acid acidified	77.65 ^c ± 1.69	113.45 ^d ± 0.58	68.28 ^b ± 1.22	200.51 ^h ± 4.22	93.82 ^{cd} ± 8.91	126.32 ^{de} ± 7.62	113.34 ^b
Overall effect of sorghum Type	68.56 ^a	107.62 ^c	99.05 ^b	178.14 ^e	96.21 ^b	158.30 ^d	
Overall effect of treatment	88.09 ^a		138.60 ^c		127.26 ^b		
NO INHIBITION (%)							
Raw flour (unsoured)	36.20 ^{bc} ± 4.53	62.39 ^j ± 1.83	35.40 ^{bc} ± 2.44	60.45 ^{gh} ± 0.83	28.67 ^a ± 1.08	55.12 ^e ± 2.76	46.37 ^{ab}
Spontaneously fermented	37.63 ^c ± 3.30	56.83 ^{ef} ± 1.21	33.70 ^b ± 1.42	58.21 ^{efg} ± 2.35	38.81 ^{cd} ± 0.79	59.32 ^{fgh} ± 2.35	47.41 ^b
Citric acid acidified	27.66 ^a ± 0.97	57.22 ^{efg} ± 1.92	28.99 ^a ± 0.66	56.38 ^{ef} ± 1.86	62.63 ⁱ ± 1.88	41.78 ^{de} ± 1.69	45.77 ^a
Overall effect of sorghum Type	33.83 ^a	58.81 ^c	32.70 ^a	58.35 ^c	36.42 ^b	59.02 ^c	
Overall effect of treatment	46.32 ^{ab}		45.52 ^a		47.72 ^b		

ⁱMean±standard deviation of two independent experiments. Figures in a row and column with different superscripts differ significantly from each other (p ≤ 0.05).

Overall, the results of the effect of citric acid fermentation (Table 3-7) revealed an increase in the radical scavenging activities with the exception of results obtained from NO inhibition and ABTS radical inhibition assays. It was however noted that the effect of souring varied with the sorghum type, the method used for antioxidant activity and the souring method. Repeatedly, after souring whether using spontaneous fermentation or citric acid acidification, there was a significant increase in radical scavenging activities in white sorghum using ABTS and DPPH, while there was a significant decrease in radical scavenging activity in some red sorghum samples after souring using these methods. These could be attributed to the higher phenolic content in red sorghum compared to white, meaning there could be more bound phenolic compounds in the red sorghum compared to the white sorghum hence these souring methods were not effective enough to extract the bound phenolic compounds in some of the samples.

When using ABTS for the porridges and supernatants, there was an increase in radical scavenging activity after spontaneous fermentation compared to the unsoured and citric acid acidified porridges and supernatants. Similar trends were noted after using ORAC except for red undigested porridges. From the overall effects, there was a significant decrease in radical scavenging activity using ABTS after citric acid acidification, while when using DPPH and ORAC, there was a significant increase in radical scavenging activities. The observed difference between the methods used could be attributed to the sensitivity and instability of the ABTS radical (Romulo, 2020) compared to the DPPH radical which is quite stable and reliable (Nwachukwu *et al.*, 2021).

Increases in antioxidant activity after fermentation of sorghum have been reported by other authors (Banwo *et al.*, 2021; Ravisankar *et al.*, 2021; Shumoy *et al.*, 2017; Zaroug *et al.*, 2014). This is a clear indication of the enhancement of antioxidant properties of the sorghum porridges due to souring. The observed increase in antioxidant activity during souring can be related to enhanced extractability of bioactive antioxidant phenolic compounds. During souring in general (by fermentation or citric acid acidification), there could be hydrolysis of ester bonds and release of bound phenolics due to the acidic conditions. In addition, during souring by fermentation, the presence of cell wall degrading enzymes activated by the fermenting microflora can cause structural breakdown of the cereal matrix leading to the liberation of insoluble phenolic compounds that are bound to cell wall material (Shahidi & Yeo, 2016; Shumoy *et al.*, 2017). The production of other metabolites like amino acids during fermentation (D'Este, Alvarado-Morales & Angelidaki, 2018) could also contribute to the increased radical scavenging activities in the spontaneously fermented samples.

Table 3-7 shows that overall, the supernatants of the digested porridges had high radical scavenging activity. The supernatants had higher overall ABTS, DPPH and ORAC radical scavenging activities compared to the flours, and higher ABTS radical scavenging activity and NO inhibition compared to the undigested porridges. The significance of the observed high radical scavenging activity of the supernatants of the digested porridges is that it provides an indication that the sorghum porridges would exert radical scavenging properties in the body even after digestion which is desirable for health promotion. Similar increases in radical scavenging activity of sorghum porridge products after simulated *in vitro* gastrointestinal digestion have been reported by other authors (Adarkwah-Yiadom & Duodu, 2017; Apea-Bah *et al.*, 2016). The *in vitro* gastrointestinal digestion process used in this research involved the use of enzymes such as amylase, pepsin and pancreatin. During the digestion, macromolecules like starch and proteins are broken down due to the action of these enzymes resulting in the breakdown of the food matrix and facilitation of the release of phenolic compounds that have been bound to these macromolecules. Also the low pH particularly in the gastric phase can enhance hydrolysis and release of the phenolic compounds bound to macromolecules (Adarkwah-Yiadom & Duodu, 2017).

Results from Table 3-7 also show that flours, porridges, and supernatants of digested porridges from red non-tannin sorghum had higher radical scavenging activity than corresponding samples from white non-tannin sorghum. The higher antioxidant activity observed for the red sorghum samples can be attributed to the higher phenolic content in red sorghum compared to white sorghum reported earlier in the study.

3.2.3.2 Cytotoxicity of extracts from porridges and supernatants

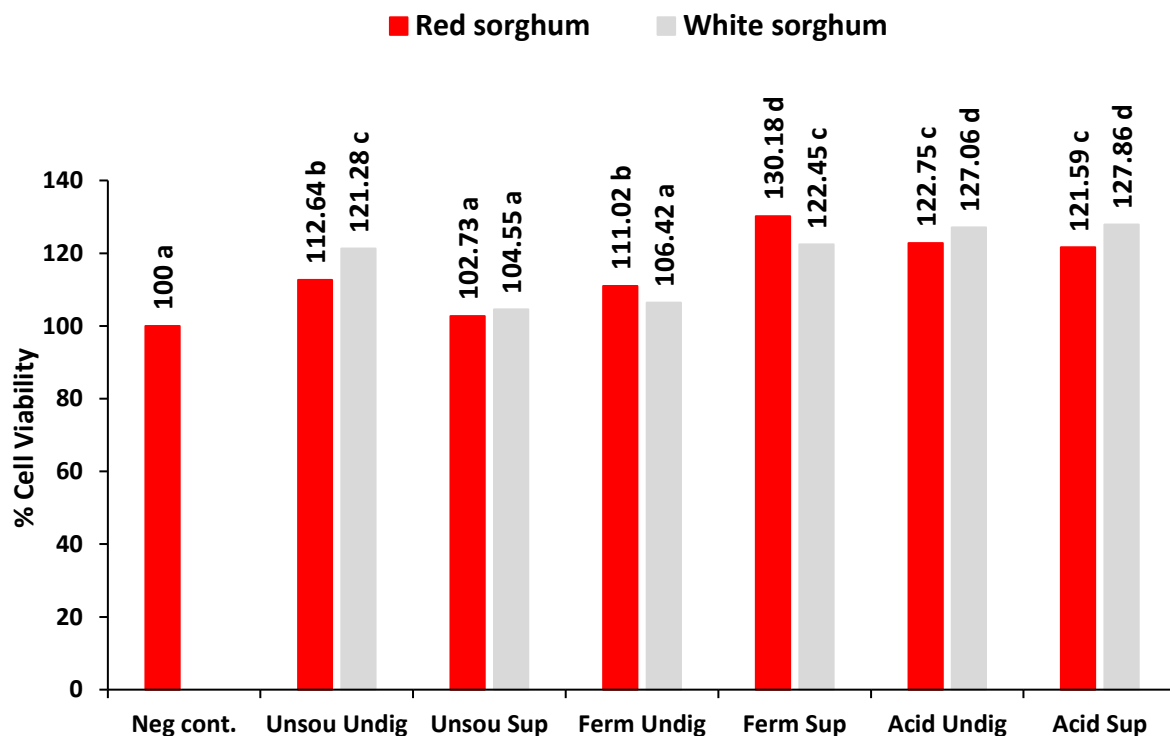


Figure 3-8: Effects of sorghum porridge extracts and supernatants on the viability of Caco-2 cells after 48 h of incubation

Key: Unsou Undig- unsoured undigested porridge extracts, Unsou Sup- unsoured porridge supernatants, Ferm Undig- fermented undigested porridge extracts, Ferm Sup- fermented porridge supernatants, Acid Undig- citric acid acidified undigested porridge extracts, Acid Sup- citric acid acidified porridge supernatants. For each sorghum type (red or white), columns (representing mean values) with different letters are significantly different, ($p < 0.05$).

Figure 3-8 shows the effect of the red and white non-tannin sorghum porridge extracts and supernatants on the viability of Caco-2 cells after 48 h of incubation. The negative control consisted of Caco-2 cells with no sample extract or supernatant added. All the Caco-2 cells incubated with extract or supernatant had slightly greater than 100% cell viability. This indicates that the extracts and supernatants exerted no toxic effects on the Caco-2 cells and so could be reliably used to study cellular antioxidant effects.

3.2.3.3 Cellular antioxidant activity of extracts from porridges and supernatants

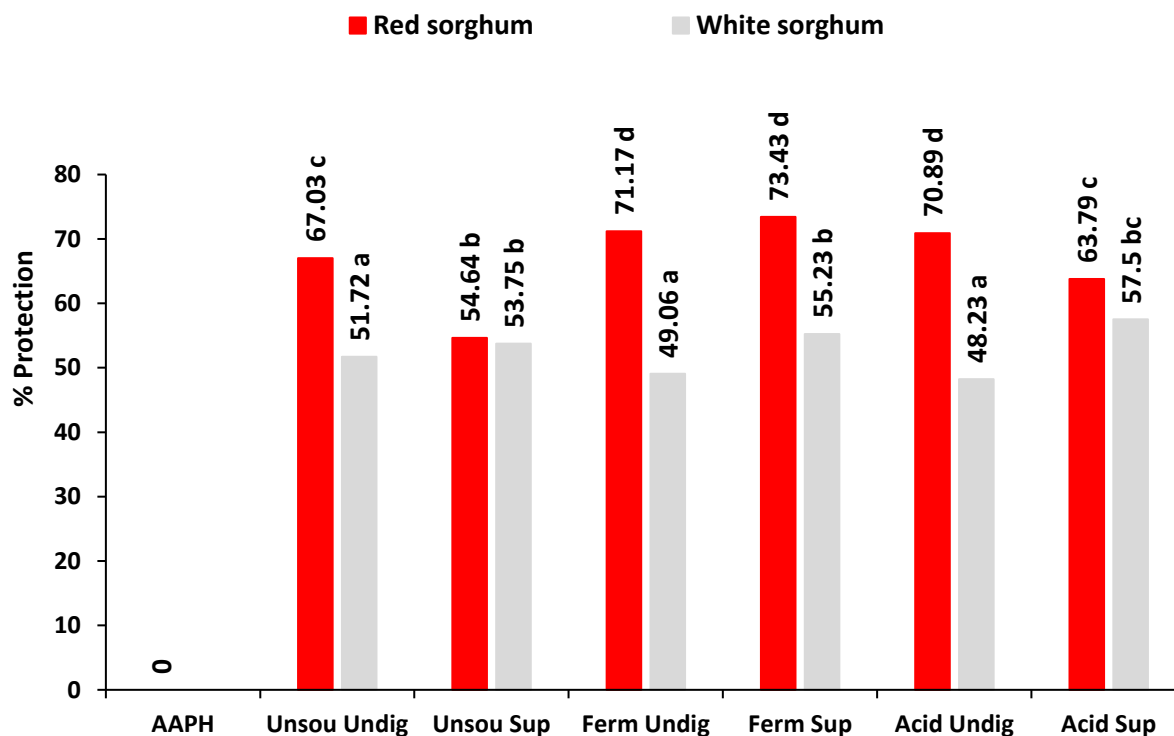


Figure 3-9: Protective effects of sorghum porridge extracts and supernatants against AAPH radical-induced oxidation in Caco-2 cells

Key: Unsou Undig- unsoured undigested porridge extracts, Unsou Sup- unsoured porridge supernatants, Ferm Undig- fermented undigested porridge extracts, Ferm Sup- fermented porridge supernatants, Acid Undig- citric acid acidified undigested porridge extracts, Acid Sup- citric acid acidified porridge supernatants. For each sorghum type (red or white), columns (representing mean values) with different letters are significantly different, ($p < 0.05$).

Figure 3-9 shows the ability of the porridge extracts and digest supernatants to protect the Caco-2 cells from oxidative damage induced by the AAPH radical. There was no protection of the Caco-2 cells against oxidative damage when they were treated with the AAPH radical without sample extract or supernatant. All sample extracts and supernatants from the red and white non-tannin sorghum showed protection of the Caco-2 cells against oxidative damage.

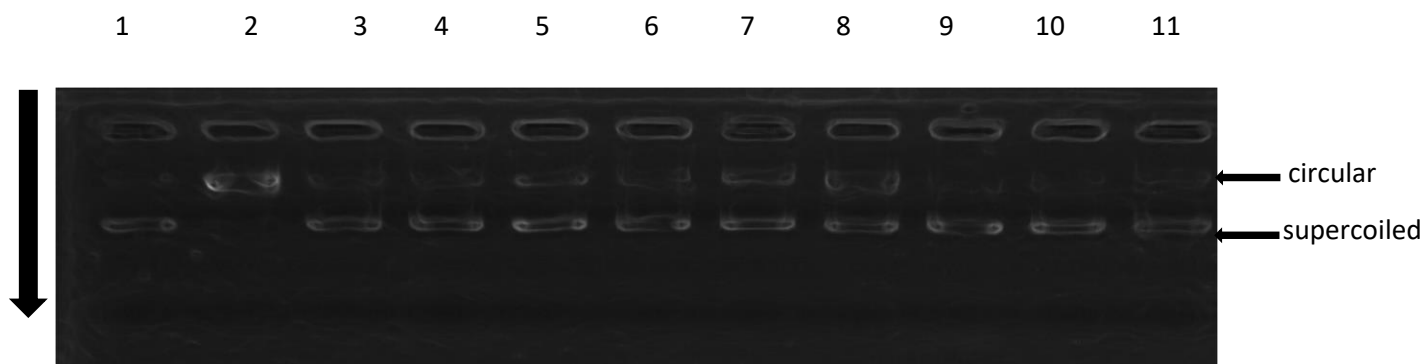
The observed protection of the Caco-2 cells from oxidative damage induced by the AAPH radical demonstrated by the extracts from porridges and supernatants of red and white non-tannin sorghum can be attributed to the phenolic compounds in sorghum. These phenolic

compounds donate hydrogen atoms that scavenge highly reactive species (Apea-Bah *et al.*, 2014). The protection of the cells from oxidative damage exerted by the samples demonstrates the potential of the soured sorghum porridges to offer protection against NCDs that are brought on by oxidative stress.

Extracts and supernatants from red sorghum had higher average protection of the Caco-2 cells against oxidative damage (66.83%) compared with extracts and supernatants from white sorghum (52.58%). This can be attributed to the higher phenolic content in red sorghum observed earlier in the study.

3.2.3.4 Protective effects of extracts and supernatants against oxidative DNA damage

a) White non-tannin sorghum



b) Red non-tannin sorghum

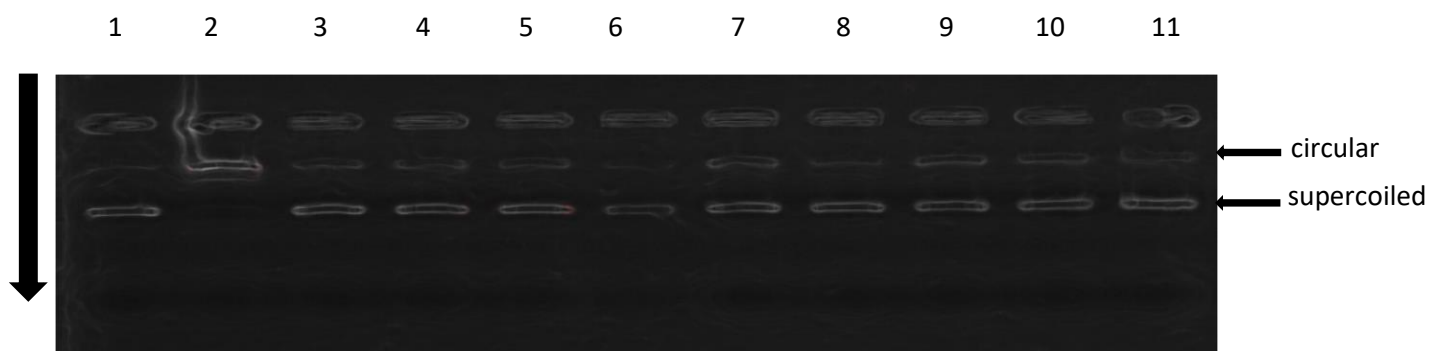


Figure 3-10: Agarose gel electrophoretogram and pBR 322 plasmid DNA band intensity showing the effects of flour extracts, cooked porridge extracts and digest supernatants of white and red non-tannin sorghum (unsoured, fermented and exogenously citric acid acidified) on pBR322 vector DNA treated with AAPH)

Allocation of wells for white and red sorghum: 1 = negative control (DNA + H₂O), 2 = positive control (DNA + AAPH), 3= DNA + AAPH + unsoured uncooked flour extract, 4= DNA + AAPH + fermented uncooked flour extract, 5= DNA + AAPH + acidified uncooked flour extract, 6= DNA + AAPH + unsoured porridge extract, 7= DNA + AAPH + fermented porridge extract, 8= DNA + AAPH + acidified porridge extract, 9= DNA + AAPH + unsoured porridge supernatants, 10= DNA + AAPH + fermented porridge supernatants, 11= DNA + AAPH + citric acidified porridge supernatants.

Direction of electrophoretic migration is shown by the downward arrow.

The principle of the oxidative DNA damage assay is that the AAPH radical attacks the plasmid DNA which results in strand breaks and oxidative damage (Wei, Zhou, Cai, Yang and Liu (2006). The oxidative damage is indicated by the change of the supercoiled plasmid DNA to open-circular or linear forms (Apea-Bah *et al.*, 2016) which have different mobilities during electrophoresis.

The plasmid DNA was mostly in the supercoiled form in the absence of AAPH as shown by Figure 3-10 lane 1 (DNA + H₂O; negative control). When AAPH was added (DNA + AAPH; positive control), the plasmid DNA was present only in circular form (Figure 3-10 lane 2) which indicates oxidative damage to the plasmid DNA due to the AAPH generated radicals. When DNA was exposed to the AAPH radical in the presence of all the extracts and supernatants from red and white sorghum, they showed some protection of the DNA against oxidative damage as seen by the presence of a significant portion of the DNA in the supercoiled form. It is worth noting that some of the DNA were still in circular form (Figure 3-10 (a) lanes 5-8 and Figure 3-10 (b) lanes 3-5 and 7-9) which indicates that some oxidative DNA damage did occur despite the presence of the extracts and supernatants. However, the bands due to supercoiled DNA were significantly more intense than the bands due to DNA in the circular form. This is an indication that the protection against DNA damage exerted by extracts and supernatants was extensive. Similar observations of protection against DNA damage by extracts from sorghum have been reported (Apea-Bah *et al.*, 2016; Devi, Kumar & Das, 2012). The protection of DNA from oxidative damage can be attributed to the phenolic compounds that are able to scavenge the AAPH generated radicals (Nderitu *et al.*, 2013). Damage to DNA is one of the factors that can lead to NCDs such as cancer. Therefore, the protection of DNA from oxidative damage exerted by the extracts and supernatants from the red and white non-tannin sorghum porridges provides an indication of the potential of sorghum to contribute to protection against NCDs.

3.2.4 Conclusions

Souring through fermentation and citric acid acidification generally enhances the radical scavenging properties of sorghum porridges. This can be attributed to the acidic medium created during souring which facilitates the hydrolysis and release of bound phenolic compounds. The action of hydrolytic enzymes which enhance release of bound phenolic compounds also plays a role. Simulated *in vitro* gastrointestinal digestion also enhances radical scavenging properties of soured sorghum porridges which is an indication of the potential of the porridges to exert antioxidant health-promoting effects after ingestion and digestion. Red non-tannin sorghum flours, porridges and supernatants have higher antioxidant properties than white non-tannin sorghum likely due to the higher phenolic content of red sorghum. Although the porridges from both red and white sorghum exhibit antioxidant properties, the red sorghum

porridges have a higher potential to contribute to the alleviation of oxidative stress, hence reduce the incidence of non-communicable diseases compared to white sorghum porridge.

4 GENERAL DISCUSSION

The first part of the general discussion focuses on the methods used in this study, their advantages and disadvantages including some considerations about how they can be improved upon when utilized in future research. The second part focuses on a more in-depth discussion of the main findings of the study. This includes the proposal of some models to explain some of the effects observed in this study.

4.1 EXPERIMENTAL APPROACH

Two Type I non-tannin sorghums (red and white pericarp) were used in this study. Red and white sorghums are consumed extensively in Africa and sorghum is an essential food source for many people in this region (Garzón & Drago, 2018). As also observed in this research, it is well known that red sorghum contains more phenolic compounds than white sorghum (Rashwan *et al.*, 2021; Salazar-López *et al.*, 2018a). It was therefore of interest in this research to determine whether the pericarp colour phenotypic trait of sorghum would have an effect on bioaccessibility of phenolic compounds in soured sorghum porridges.

Acidified methanol was used to extract phenolic compounds in this study. Methanol has been used extensively as an extraction solvent for phenolic compounds not only in sorghum but also for other food matrices (Khoddami, Wilkes & Roberts, 2013) due to its polar-organic nature which is suitable for extracting polar compounds which are also organic in nature such as phenolic compounds. Organic solvents used in the extraction of phenolic compounds are usually acidified (Khoddami *et al.*, 2013) as was done in this research where concentrated hydrochloric acid (HCl) was used as a solvent acidification agent. Phenolic compounds in sorghum mostly occur in bound form mainly through ester bonds (Ravisankar *et al.*, 2021). The acid promotes hydrolysis of these ester bonds which results in disruption of cell walls as well as solubilisation and release of bound phenolic compounds (Apea-Bah *et al.*, 2014; Ju & Howard, 2003). In effect, the acidified methanol extraction solvent enhances the extraction of both free and bound phenolic compounds.

Two souring methods were used in the study. These were spontaneous fermentation and exogenous acidification using citric acid. Souring through spontaneous fermentation to produce lactic acid fermented products is common practice in most sub-Saharan African countries (Adeyanju *et al.*, 2019). However, soured products prepared using exogenous acidification are also extremely popular and citric acid being a common food grade acidulant

is used to produce these products. Therefore, citric acid was used as the acidification agent for the porridge in this study in an effort to study a soured product as close in character as possible to what is available commercially.

The simulated *in vitro* gastrointestinal digestion method used in this study is classified as a static *in vitro* digestion method (Santos, Saraiva, Vicente & Moldão-Martins, 2019). This method can imitate the physiological release of phenolic compounds and other food components during digestion (Chandrasekara & Shahidi, 2012), hence provide an indication of the behaviour of food during digestion. However, it may have been better to use a dynamic *in vitro* digestion method which incorporates dynamic digestion processes such as churning of food in the stomach and peristaltic movements in the intestine involving the application of shear forces (Santos *et al.*, 2019). The dynamic *in vitro* digestion method was not used in this research simply due to the unavailability of the required equipment but could be considered in future research. Nevertheless, the static *in vitro* digestion method used has some advantages in being affordable and easy to execute (Schaich, Tian & Xie, 2015). In general, static *in vitro* digestion methods are used extensively and they correlate with what happens *in vivo* during digestion (Hur, Lim, Decker & McClements, 2011), but it may have been useful to use an *in vivo* method to get a true reflection of what happens in the entire gastrointestinal tract.

Esterase enzyme activity was evaluated using *p*-nitrophenol butyrate as a substrate. In this method when *p*-nitrophenol butyrate is hydrolysed, it releases *p*-nitrophenolate (a chromophore) which can be evaluated spectrophotometrically at various time intervals at a wavelength of 415 nm (Figure 4-1) (Pliego *et al.*, 2015). A limitation in the experimental design for this study was that the esterase activity was only determined for the fermented sorghum slurries. It would have been useful to also evaluate the esterase activity for the unsoured sorghum slurry and the citric acid acidified sorghum slurry in order to establish if there was any endogenous esterase activity in these samples. This can be done in future research. Various disadvantages have been associated with this method such as its relatively lower sensitivity and inflexibility which can alter the absorbance of *p*-nitrophenol (Gilham & Lehner, 2005). However, its efficiency (Pliego *et al.*, 2015), convenience and low cost (Gilham & Lehner, 2005) are some advantages which make this method popular.

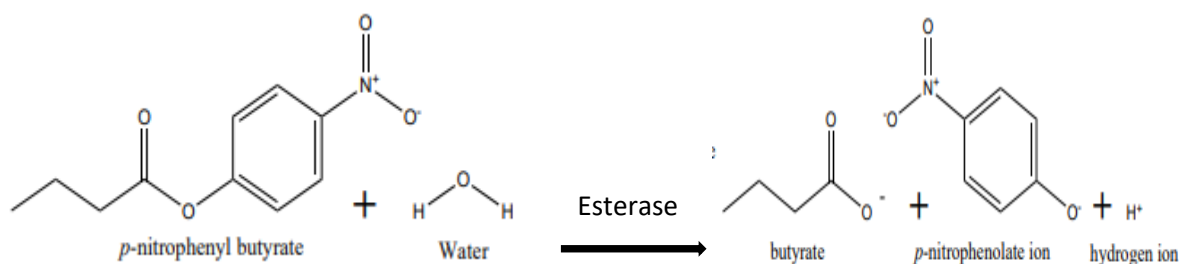


Figure 4-1: Enzymatic hydrolysis reaction of p-nitrophenyl butyrate (From Pliego *et al.*, (2015)).

The Folin-Ciocalteu assay was used to determine total phenolic content while the ABTS and DPPH radical scavenging assays were two of the methods used to determine antioxidant activity. These three methods are all based on reduction-oxidation reactions involving electron transfer. In the Folin-Ciocalteu assay, there is electron transfer from the antioxidant (phenolic compounds) to the Folin-Ciocalteu reagent (Romulo, 2020) while the ABTS and DPPH radical scavenging assays involve electron transfer to the ABTS^{•+} radical cation and DPPH radical respectively (Nwachukwu *et al.*, 2021; Romulo, 2020; Shahidi & Zhong, 2015). These reactions result either in formation of chromophores or decolourisation which allows for the measurement of absorbance which is then related to either the total phenolic content or antioxidant activity. These methods are generally easy to conduct (Shahidi & Zhong, 2015) and reproducible. However, they are not specific for phenolic compounds as they essentially measure all reducing substances (Romulo, 2020). They are also considered to be biologically irrelevant (Schaich *et al.*, 2015) as the radicals (ABTS^{•+} and DPPH) are not found in biological systems thus the antioxidant activity obtained is not a true representation of physiological reactions (Prior, Wu & Schaich, 2005). Despite these disadvantages, these methods are still popular and they are used extensively to screen samples for gross phenolic content and antioxidant activity.

The biological irrelevance of the methods discussed above has led to the interest in using biological relevant assays like ORAC and NO inhibition (Shahidi & Zhong, 2015) to evaluate antioxidant activity. This is because peroxy radicals which are of importance for both assays are found in biological systems especially during lipid oxidation under physiological conditions (Shahidi & Zhong, 2015). In the ORAC assay, fluorescein is used as a fluorescent probe, AAPH generates radicals and the decay of fluorescence due to the radicals is demonstrated by the area under the curve which is equal to the antioxidant activity of the evaluated sample (Blasa, Angelino, Gennari & Ninfali, 2011). In the NO inhibition assay,

antioxidants compete with oxygen to bind to NO• which prevents it from oxidizing into nitrite and nitrates (Alam, Bristi & Rafiquzzaman, 2013). Apart from the biological relevance of these methods, NO is important for organ and cellular functioning but when in excess, it can result in cytotoxicity and inflammatory events like septic shock (Nguyen, Zhao, Lee, Kim, Min & Woo, 2015).

Due to the non-specificity of the Folin-Ciocalteu assay for phenolic compounds, Liquid Chromatography Mass Spectrophotometry (LCMS) was used to identify and quantify the phenolic compounds present in the sorghum samples. Mass spectrometry is a powerful technique for characterising phenolic compounds and in addition, it allows for detection of trace amounts and the structural identification of unfamiliar compounds (Liu *et al.*, 2007). LCMS has now become the preferred method for effective identification of phenolic compounds in food matrices (Kang *et al.*, 2016).

The dichlorodihydrofluorescein diacetate (DCFH-DA) method was used to evaluate the capability of the sorghum samples to prevent oxidative damage in Caco-2 cells. In this method, esterase enzymes present in the Caco-2 cells diacetylate the non-fluorescent DCFH-DA probe and convert it into non-fluorescent DCFH (Nwachukwu *et al.*, 2021; Shahidi & Zhong, 2015), which is further oxidized by AAPH-generated radicals to form fluorescent dichlorofluorescein (DCF) (Figure 4-2). This oxidation process is indicative of oxidative stress. Phenolic compounds in the sorghum extracts can scavenge the AAPH generated radicals and inhibit the oxidation of DCFH to DCF (Blasa *et al.*, 2011) which produces a decrease in fluorescence which is measured. This method has been suggested for its biological relevance (Blasa *et al.*, 2011) as it gives a good indication of oxidative stress events in human cells and the potential protective effects of antioxidants.

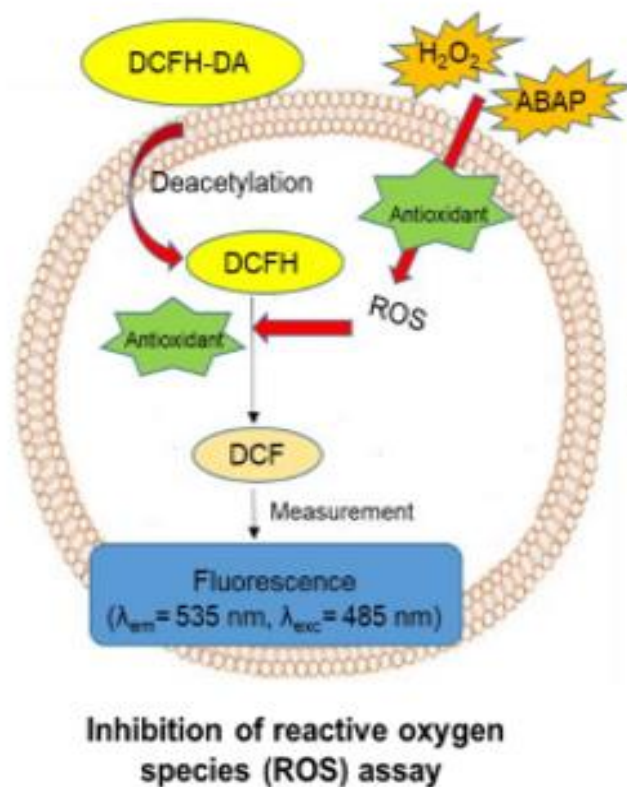


Figure 4-2: An illustration of AAPH induced cellular oxidative damage (From Nwachukwu *et al.*, 2021)

Inhibition of DNA damage was another method used to evaluate antioxidant activity. When DNA gets oxidised, it results in its supercoiled form being converted to open-circular and linear forms which is indicative of DNA damage (Apea-Bah *et al.*, 2016; Fernández, Montes-Bayón, González, Sierra, Sanz-Medel & Bettmer, 2011; Nderitu *et al.*, 2013). Oxidative DNA damage may lead to various diet-related chronic diseases such as cancer (Chen *et al.* (2021). The oxidation of the DNA can be induced by highly reactive free radicals (e.g. peroxy and hydroxyl radicals) which attack the DNA and induce strand breakage. Phenolic compounds can scavenge these radicals and thus prevent the strand breaks which results in inhibition of the oxidative DNA damage. One of the advantages of this method is that the radical generating rate of AAPH can be easily measured and controlled (Wei *et al.*, 2006).

4.2 KEY RESEARCH FINDINGS

4.2.1 Effect of souring on extractability of phenolic compounds in sorghum porridge

This study provided evidence that souring, whether through spontaneous fermentation or through exogenous acidification enhanced the total extractable phenolic content, phenolic concentration, antioxidant activity and bioaccessibility of phenolic compounds. The enhanced extractability of phenolic compounds can be attributed to the release of bound phenolic compounds within the sorghum porridge matrix by the souring process. Figure 4-3 presents a model to illustrate the mechanism by which the fermentation process and exogenous acidification can enhance extractability of phenolic compounds in sorghum. Within the sorghum grain, phenolic compounds occur mainly in bound forms, bound to primarily cell wall structures through ester bonds. During fermentation, various hydrolytic enzymes including esterases (as shown in this study) are activated. The esterase enzymes in particular can break ester linkages and release the bound phenolic compounds (Shumoy *et al.*, 2017) and enhance the extractability of phenolic compounds in fermented sorghum.

The observed enhanced phenolic extractability after exogenous acidification with citric acid can be attributed to the acidic conditions (Svensson *et al.*, 2010), which can also hydrolyse ester bonds leading to the release of bound phenolic compounds (Liyana-Pathirana & Shahidi, 2005). In effect, the enhanced extractability of phenolic compounds in sorghum during fermentation is due to the action of esterase enzymes and the presence of acidic conditions while that during exogenous acidification is primarily due to the acidic conditions (Figure 4-3). In addition, both fermentation and exogenous acidification may also lead to the breakdown of cell wall structures which may facilitate the release of bound phenolic compounds.

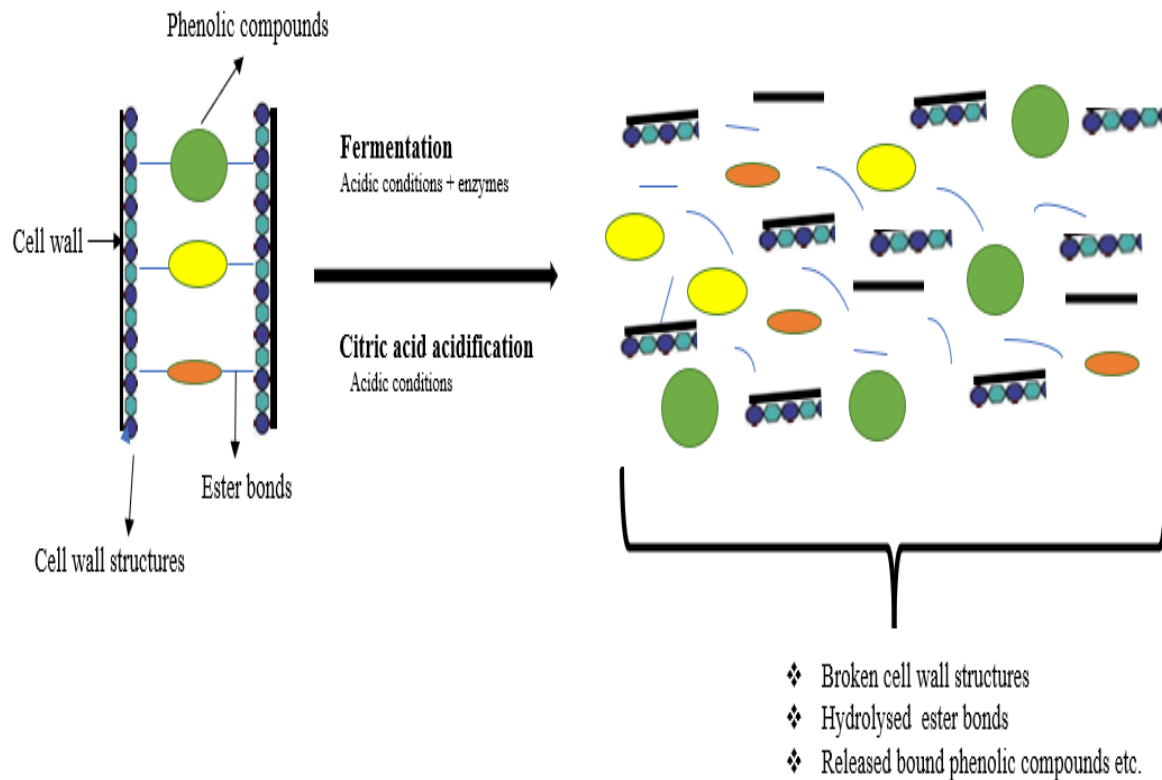


Figure 4-3: A proposed model to illustrate the mechanism by which fermentation and exogenous acidification can enhance extractability of phenolic compounds in sorghum

There were some instances where fermentation seemed to lead to reduced extractability of phenolic compounds. There could be a few reasons for this. It is possible that some of the phenolic compounds released by the fermentation process could bind to macromolecules like starch and proteins, especially during cooking, thus reducing their extractability (Nkhata, Ayua, Kamau & Shingiro, 2018). Some phenolic compounds could also be degraded during fermentation (Adebo *et al.*, 2018) or oxidised through the action of polyphenol oxidase enzyme (Sun, Wang, Zhang, Ajlouni & Fang, 2020). It has been noted that degradation of phenolic compounds under acidic conditions can occur through abstraction of hydride ions and rearrangement of the structure of the phenolic compound (Taylor & Duodu, 2015). Despite the observed decrease in extractability of phenolic compounds during souring in certain instances, it is likely that the mechanisms discussed above which led to increased extractability of phenolic compounds were more predominant. This would account for the overall observed enhanced extractability of phenolic compounds in sorghum after souring.

4.2.2 Effect of simulated *in vitro* gastrointestinal digestion on extractability and bioaccessibility of phenolic compounds

In this study, the supernatants of the digested sorghum porridges (which represent the bioaccessible fraction of the porridges) had significantly higher total phenolic content and exhibited higher radical scavenging activity than the undigested porridges. Importantly, the supernatants of the digested porridges did contain some bioaccessible phenolic compounds. Exogenously acidified sorghum porridges had more bioaccessible phenolic compounds compared to the fermented and unsoured porridges. This indicates that these sorghum porridges (especially the exogenously acidified porridge) can still potentially exert health beneficial effects even after digestion and ingestion.

Figure 4-4 presents a model to illustrate the mechanism by which simulated *in vitro* gastrointestinal digestion of the sorghum porridges can lead to phenolic compounds present in a bioaccessible fraction. During the digestion process, the hydrolytic action of enzymes such as amylase, pepsin and pancreatin leads to the breakdown of the food structure by hydrolysis of macromolecular components such as gelatinised starch and proteins (Figure 4-4). The broken down or ruptured food matrix will then facilitate the release of phenolic compounds, which could be found in the supernatants. In addition, the hydrothermal conditions during digestion can also contribute to liberation of some of the phenolic compounds from the food matrix (Figure 4-4) (Gawlik-Dziki *et al.*, 2009).

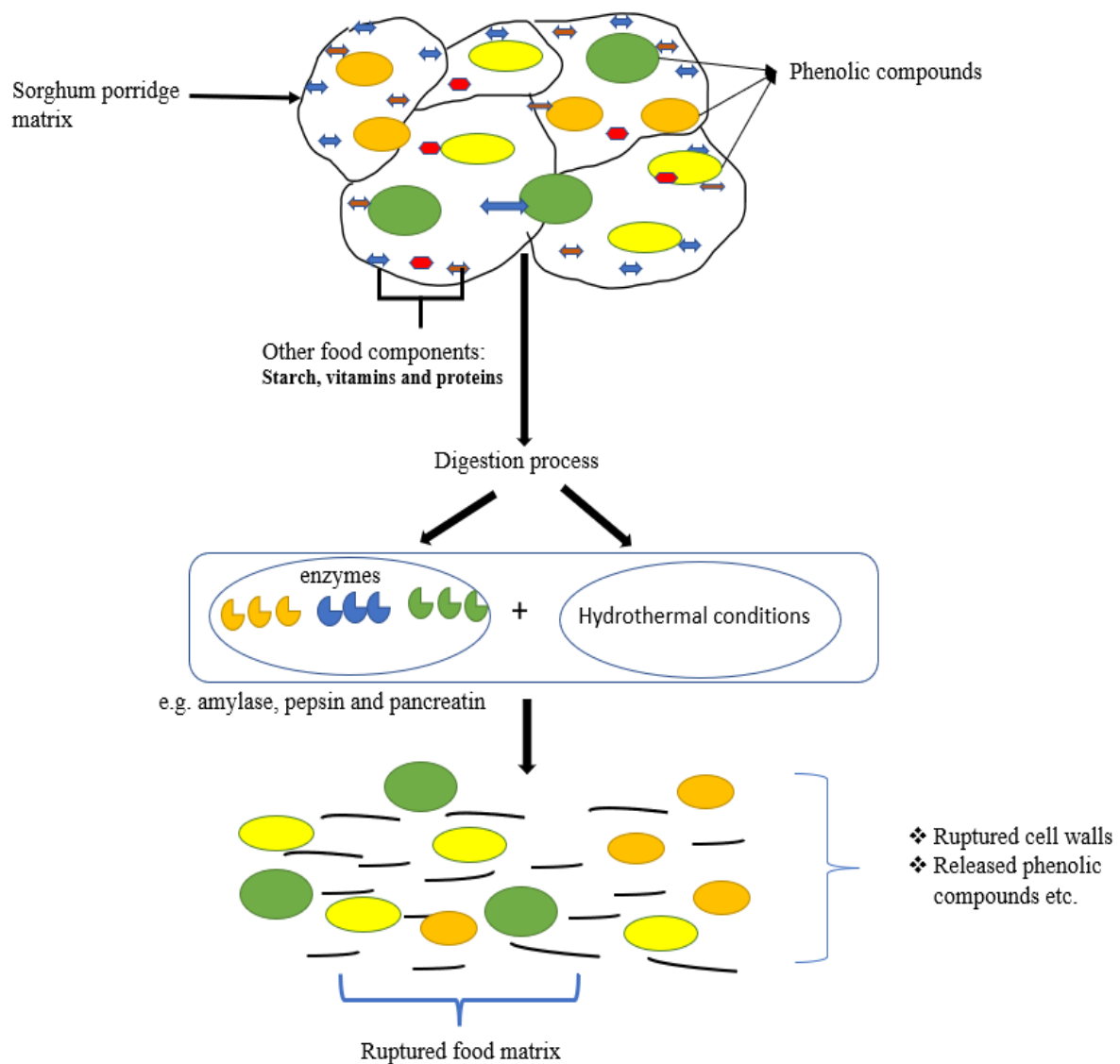


Figure 4-4: A proposed model to illustrate the effect of *in vitro* gastrointestinal digestion on phenolic compounds in sorghum porridges

It is notable that despite the presence of some phenolic compounds in the bioaccessible fraction of the porridges, majority of the phenolic compounds which were found in the extracts of the porridges were not found in the supernatants of the digested porridges (especially the unsoured and fermented porridges). This may be attributed to degradation of some phenolic compounds during digestion or the limited ability of the digestive enzymes to release them from cell wall structures (Apea-Bah *et al.*, 2016). It is also proposed that some phenolic compounds can be transformed into non-phenolic forms during digestion (Chandrasekara & Shahidi, 2012). Another reason could be that some of the phenolic compounds, phenolic acids in particular

could be retained in the residual sample because of their small molecular sizes hence they are readily absorbed (Awika & Rooney, 2004).

4.2.3 Health promoting properties of soured sorghum porridge

An important result from this study was the observation that the extracts from the soured porridges as well as the supernatants of the digested soured porridges protected against oxidative DNA damage and exerted antioxidant activity in Caco-2 cells. This can be attributed to the antioxidative action of phenolic compounds in the extracts and supernatants of the digested porridges which indicates the potential health-promoting properties of the soured porridges in offering protection against diet-related NCDs.

A diagrammatic representation of how the bioactive phenolic compounds in the soured sorghum porridges could potentially act to exert health benefits is shown in Figure 4-5. Highly reactive molecules such as reactive oxygen species or reactive nitrogen species can be present in the body. These may come from exogenous sources (e.g. drugs, radiation) or from endogenous sources (metabolic reactions in the body) (Figure 4-5) (Asmat *et al.*, 2016). The accumulation of these radicals in the body results in oxidative stress (Nwachukwu *et al.*, 2021) and damage to biological molecules like DNA, which leads to chronic inflammation and NCDs such as cancer and diabetes (Asmat *et al.*, 2016; Bondia-Pons *et al.*, 2012; Van Hung, 2016).

Although the body has antioxidant defense systems (e.g. Phase I and Phase II enzyme systems) that work to remove excess free radicals from the body (de Morais Cardoso *et al.*, 2017; Xiong *et al.*, 2019) these systems can get overwhelmed and dietary sources of antioxidants can assist. As shown in Figure 4-5, phenolic antioxidants (ferulic acid as an example) in the soured sorghum porridges can donate hydrogens and scavenge the reactive oxygen or reactive nitrogen species. In this way, the soured sorghum porridges can potentially protect against oxidative stress and associated NCDs. In this regard, sorghum can be considered as an important cereal with potential health-promoting properties due to the fact that it is a rich source of bioactive antioxidant phenolic compounds (Simnadis *et al.*, 2016).

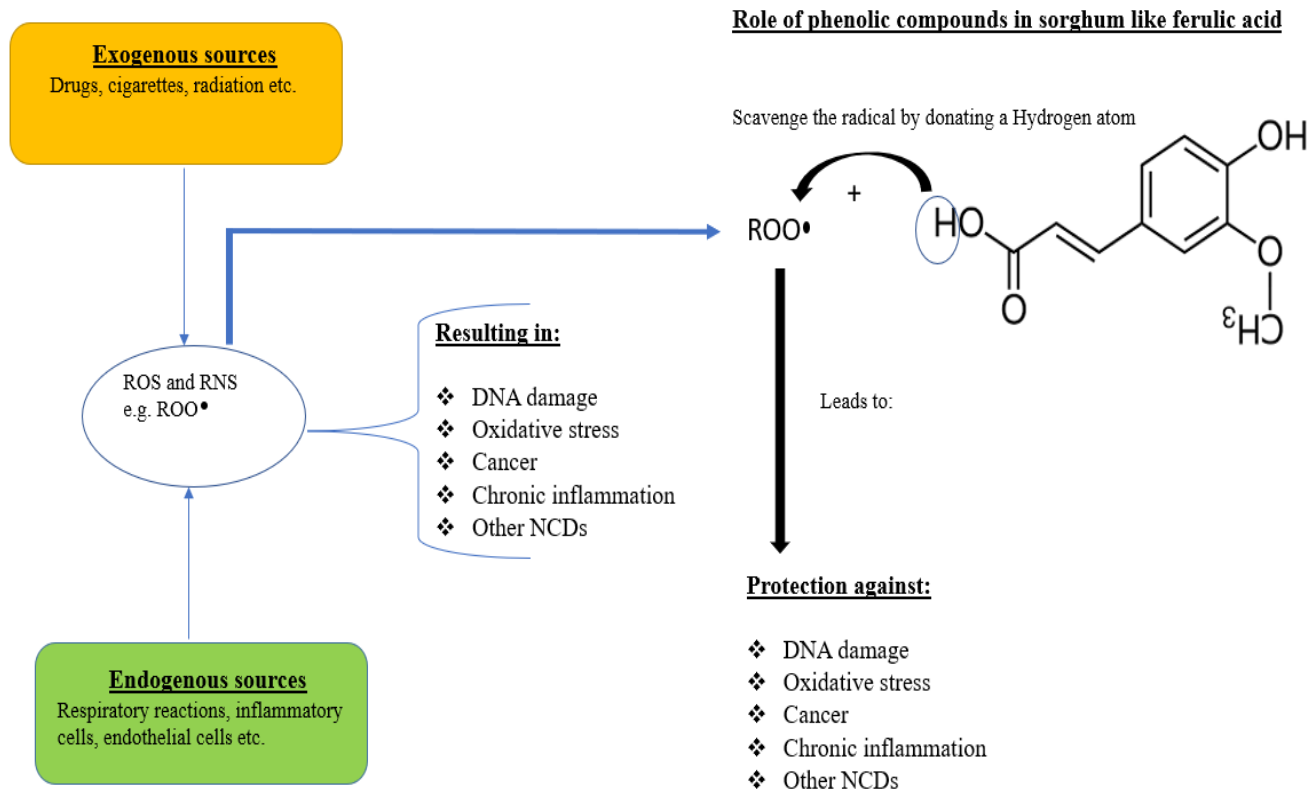


Figure 4-5: An illustration of how phenolic compounds in sorghum (e.g. ferulic acid) could offer protection against various NCDs

5 CONCLUSIONS AND RECOMMENDATIONS

This study has shown that the phenolic composition of red and white sorghum consists of phenolic acids and flavonoids. The phenolic acids identified are composed of hydroxycinnamic acids and hydroxybenzoic acids with hydroxycinnamic acids constituting the greater proportion. The flavonoid classes identified are flavanones (the major class of flavonoids identified), flavonols, flavan-3-ols and flavones. The red sorghum (flour, porridge and supernatants) contains higher levels of total phenolic compounds compared to white sorghum, suggesting its greater potential in contributing to the health promoting properties by reducing the incidence of NCDs.

Souring of sorghum through spontaneous fermentation enhances the extractability of phenolic compounds which contributes to increased total phenolic content and radical scavenging activity. This enhancement is attributed to the acidic conditions imposed by the fermentation process combined with the action of esterase enzymes (as demonstrated in this study), which enables the release of bound phenolic compounds by hydrolysing ester bonds.

Souring of sorghum through exogenous acidification with citric acid produces more bioaccessible phenolic compounds compared to souring through spontaneous fermentation in both red and white sorghum porridges. Acidic conditions in acidified sorghum porridge facilitate the release of bound phenolic compounds by hydrolysing ester bonds. Despite the acidic conditions and esterase enzyme activity in fermented sorghum porridge, the potential action of oxidative enzymes such as polyphenol oxidase can lead to oxidation and polymerisation of phenolic compounds which reduces their extractability and bioaccessibility. Therefore, sorghum porridges soured by exogenous acidification show promise in contributing to the health promoting properties due to their enhanced content of bioaccessible phenolics.

Generally, souring of red and white sorghum through spontaneous fermentation and citric acid acidification enhances the radical scavenging properties (ABTS, DPPH and ORAC) which is an indicator of the potential of the soured sorghum porridges to contributing to the antioxidant health-promoting effects. However, souring appears to have no significant effect on NO inhibition, although the red sorghum flours, porridges and supernatants of digested porridges have significantly higher NO inhibition capacity than those from white sorghum. NO is a biological indicator of oxidative stress, hence the ability of the red sorghum samples to scavenge the NO shows their potential in alleviating oxidative stress in biological systems.

Simulated *in vitro* gastrointestinal digestion enhances phenolic content and radical scavenging properties of soured sorghum porridges. The presence of some identifiable and quantifiable phenolic compounds in the supernatants of digested soured porridges indicates a measure of bioaccessibility of these phenolic compounds after the sorghum porridge has been digested. This shows that the soured sorghum porridges can still offer antioxidant health-promoting effects even after digestion.

The soured red and white sorghum porridges and supernatants of digested porridges exert protective effects against oxidation in Caco-2 cells (an indication of ability to protect against oxidative stress) and protect plasmid DNA from oxidative damage (an indication of ability to protect against cancer). Therefore, these observations provide an indication of their potential to contribute to protection against NCDs.

For future studies, it is recommended that the esterase enzyme activity is also evaluated on the unsoured and citric acidified sorghum slurries in order to establish if there was any endogenous esterase activity in these slurries contributing to the extractability of phenolic compounds particularly in the exogenously acidified samples.

The data collected in this study for protection of plasmid DNA against AAPH induced oxidative damage was qualitative. The drawback with this qualitative data is that it was not possible to determine the effects of souring and *in vitro* gastrointestinal digestion on protection against oxidative DNA damage. For future research, it will be useful to obtain measurements of the intensities of the DNA bands which can be captured as quantitative data in order to draw comparison between treatments. In such a study, the plasmid DNA could be treated with the extracts at different concentrations in order to obtain information about any possible dose-response effects.

A static *in vitro* gastrointestinal digestion method was used in this study to determine bioaccessibility. For future studies, a dynamic *in vitro* gastrointestinal method which incorporates dynamic digestion processes such as churning of food in the stomach and peristaltic movements in the intestine involving the application of shear forces could be used as this provides the best simulation of the digestion process. *In vivo* methods using available animal models can also be recommended for the study of bioaccessibility of phenolic compounds in sorghum in future studies since these provide the best indication of what happens in the gastrointestinal tract.

For this research, a commercial food grade acidulant (citric acid) was used for souring. For future studies, natural acidulants which are mostly used in African communities such as lemons and other citrus fruits could also be explored to determine their effects on bioaccessibility of phenolic compounds in sorghum porridges. The focus of this research was on non-tannin Type I sorghum. However, tannin-containing Types II and III sorghums are also consumed in many parts of Africa. These tannin-containing sorghum types could also be similarly studied in the future to expand the knowledge base about how sorghum type and souring affect phenolic compounds and health promoting properties of sorghum porridges.

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7 APPENDICES

APPENDIX A

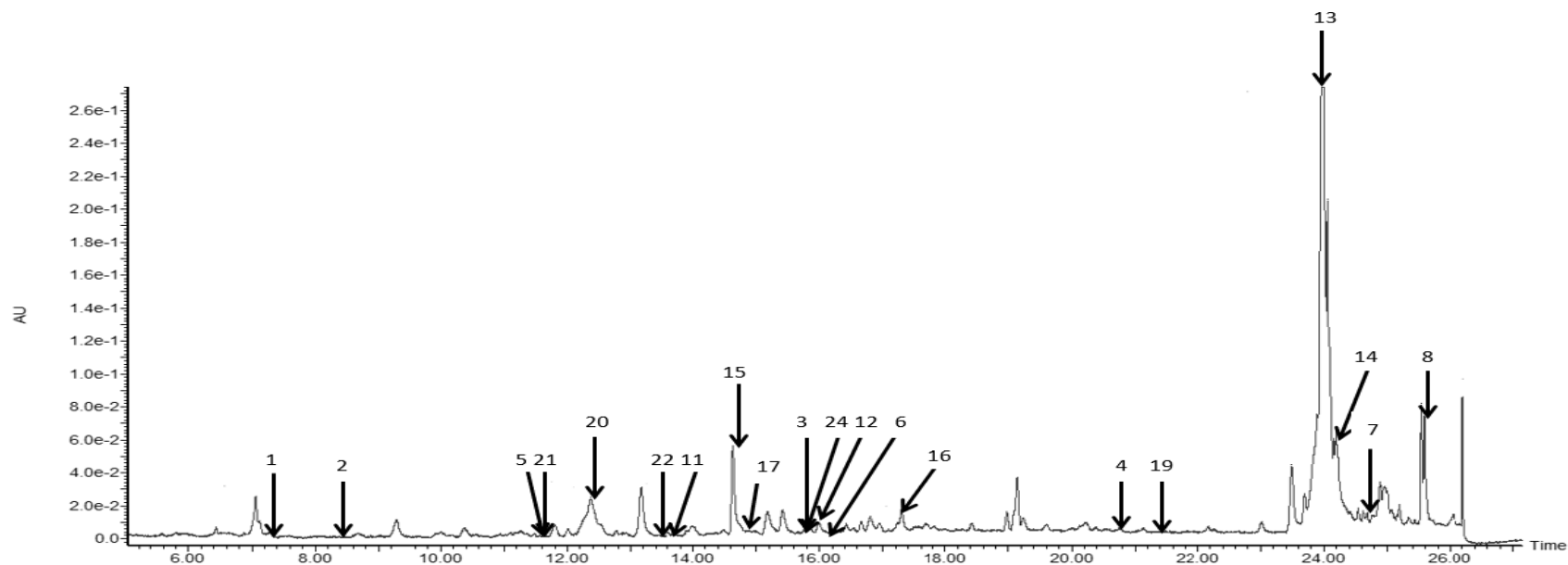


Figure 7-1: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from unsoured white sorghum flour. 1= 4-hydroxybenzoic acid, 2= Protocatechuic acid, 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 6= Vanillic acid, 7=Sinapic acid, 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 15= Eriodictyol, 16= Naringenin-7-O-glucoside, 17= Eriodictyol 7-O-glucoside, 19= Quercetin, 20= Quercetin diglucoside, 23= Catechin glycoside and 24= Apigenin

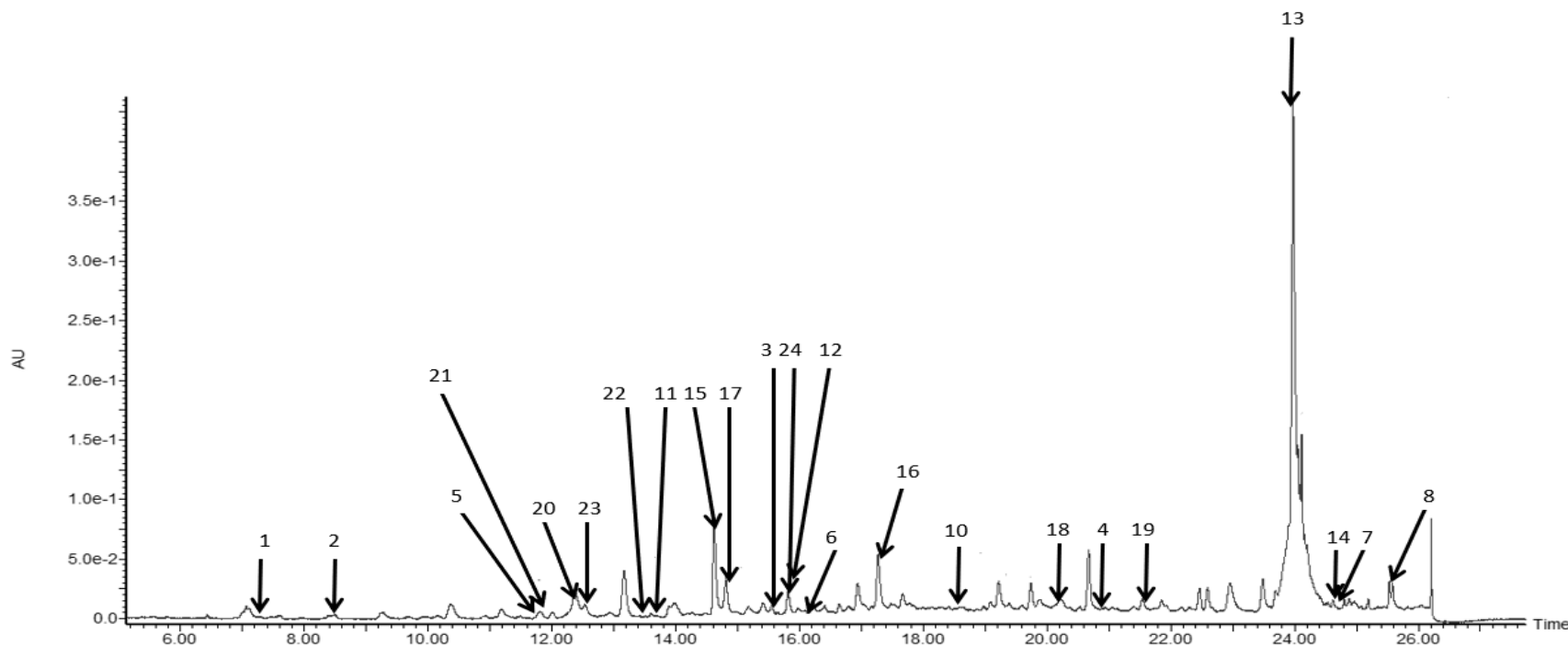


Figure 7-2: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from unsoured red sorghum flour. 1= 4-hydroxybenzoic acid, 2= Protocatechuic acid, 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 6= Vanillic acid, 7=Sinapic acid, 8= Chlorogenic acid, 10= Dihydro ferulic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 15= Eriodictyol, 16= Naringenin-7-O-glucoside, 17= Eriodictyol 7-O-glucoside, 18= 5,7,3',4', -Tetrahydroxy-flavan-5-O- β -glucosyl-4-8-eriodictyol, 19= Quercetin, 20= Quercetin diglucoside, 21= (+)-Catechin, 22= Epicatechin, 23= Catechin glycoside and 24= Apigenin

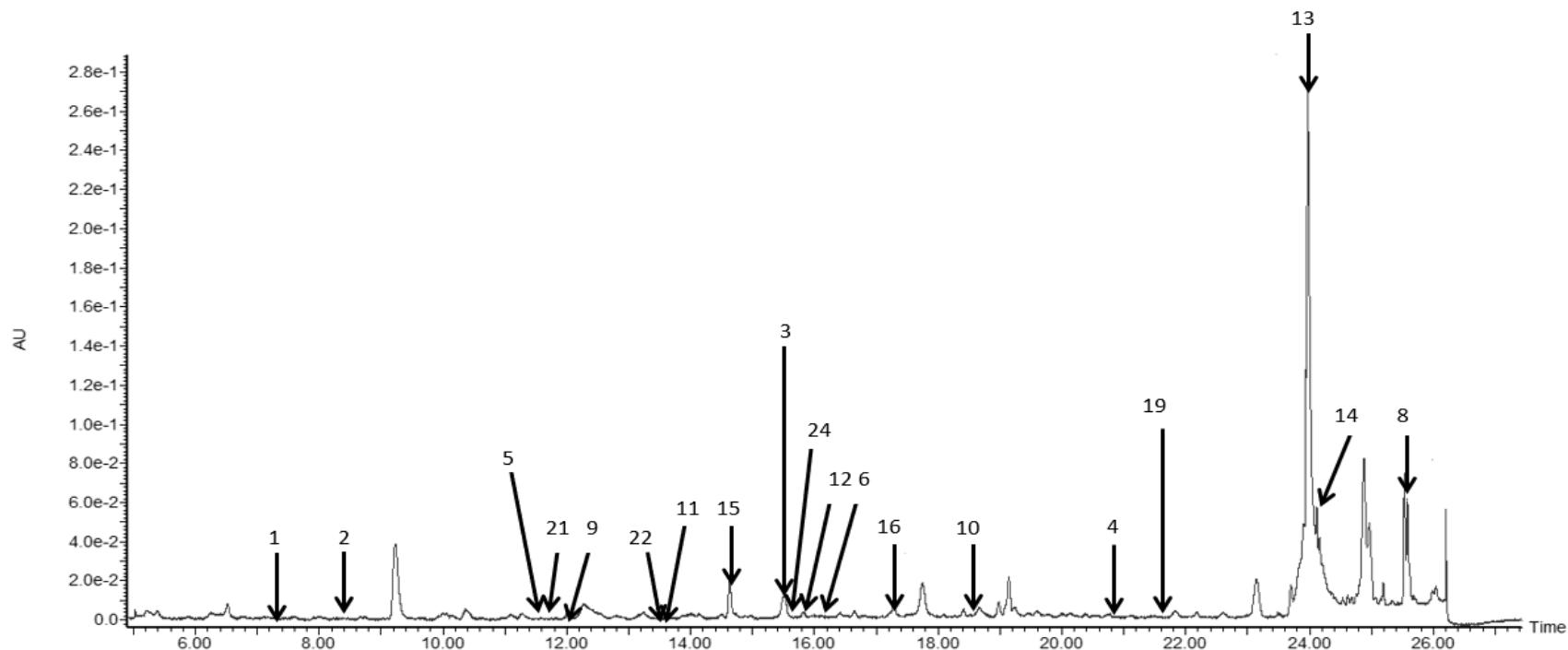


Figure 7-3: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from fermented white sorghum flour. 1= 4-hydroxybenzoic acid, 2= Protocatechuic acid, 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 6= Vanillic acid, 8= Chlorogenic acid, 9= Dihydro caffeic acid, 10= Dihydro ferulic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 15= Eriodictyol, 16= Naringenin-7-O-glucoside, 19= Quercetin, 21= (+)-Catechin, 22= Epicatechin and 24= Apigenin

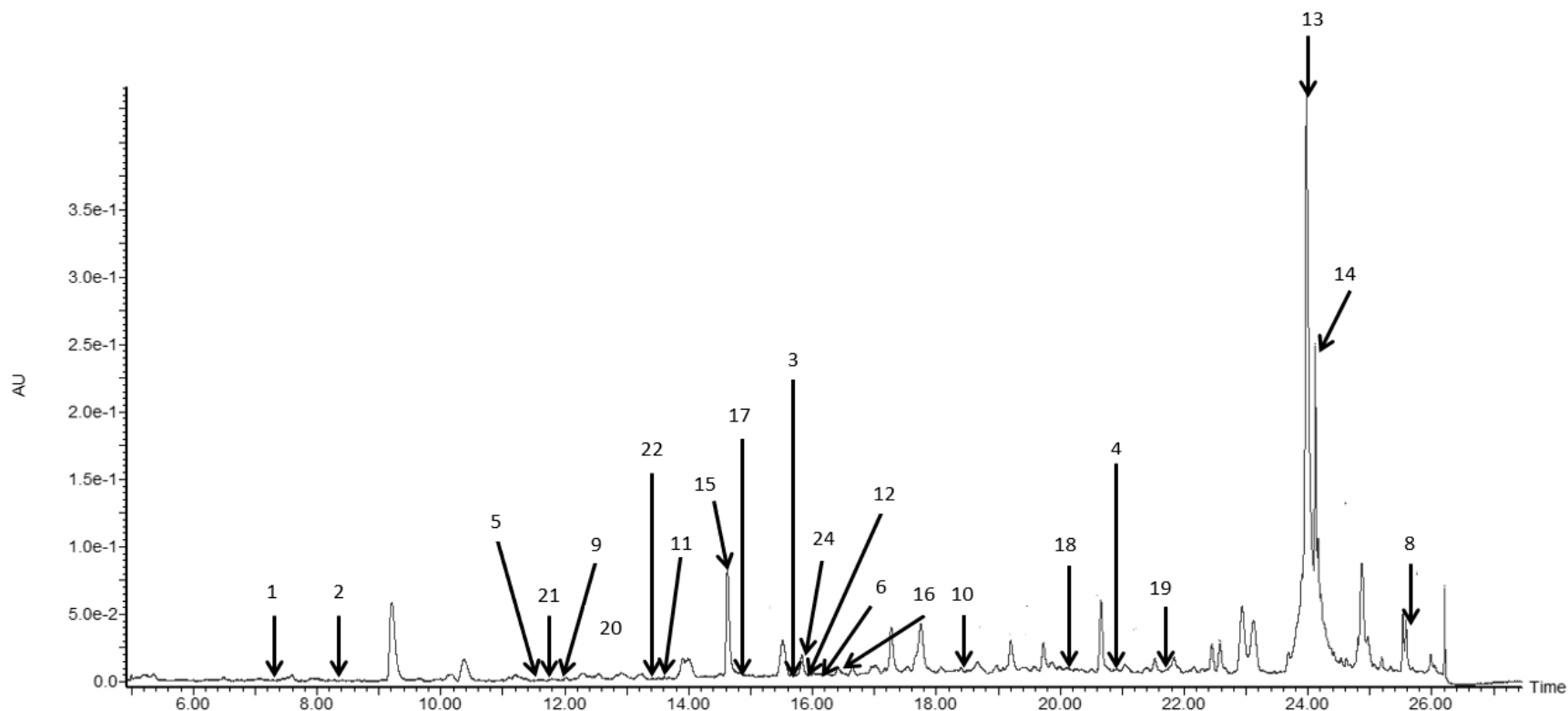


Figure 7-4: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from fermented red sorghum flour. 1= 4-hydroxybenzoic acid, 2= Protocatechuic acid, 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 6= Vanillic acid, 8= Chlorogenic acid, 9= Dihydro caffeic acid, 10= Dihydro ferulic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 15= Eriodictyol, 16= Naringenin-7-O-glucoside, 17= Eriodictyol 7-O-glucoside, 18= 5,7,3',4', -Tetrahydroxy-flavan-5-O- β -glucosyl-4-8-eriodictyol, 19= Quercetin, 20= Quercetin diglucoside, 21= (+)-Catechin, 22= Epicatechin and 24= Apigenin

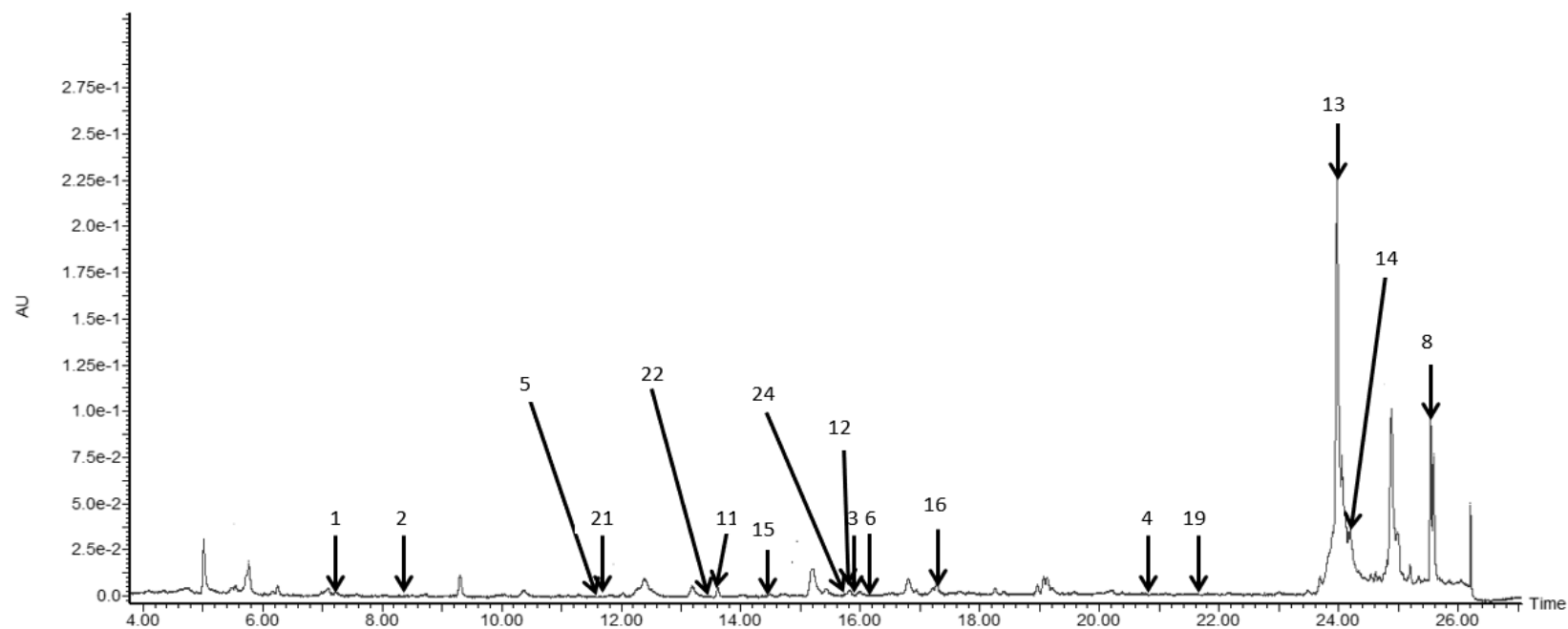


Figure 7-5: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from citric acidified white sorghum flour. 1= 4-hydroxybenzoic acid, 2= Protocatechuic acid, 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 6= Vanillic acid 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 15= Eriodictyol, 16= Naringenin-7-O-glucoside, 19= Quercetin, 21= (+)-Catechin, 22= Epicatechin and 24= Apigenin

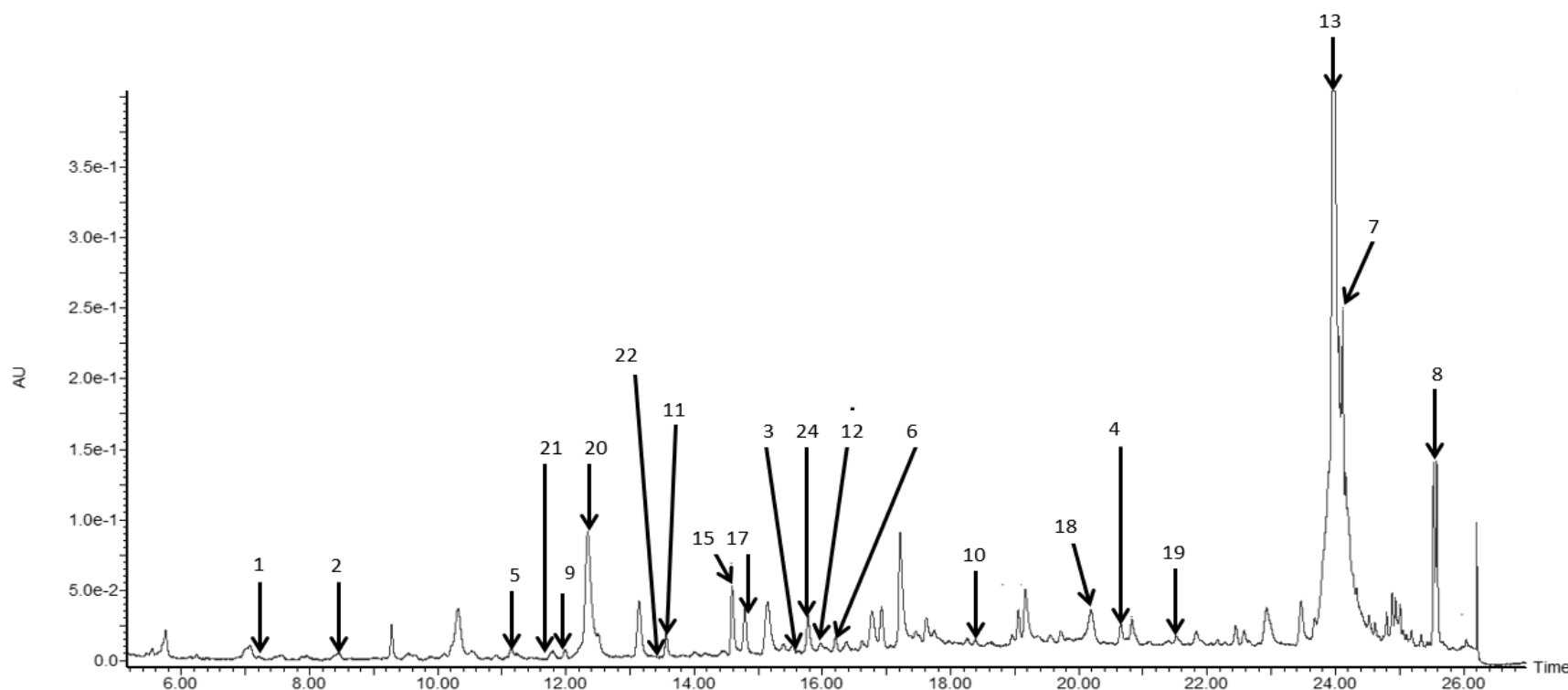


Figure 7-6: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from citric acidified red sorghum flour. 1= 4-hydroxybenzoic acid, 2= Protocatechuic acid, 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 6= Vanillic acid, 7=Sinapic acid, 8= Chlorogenic acid, 9= Dihydro caffeic acid, 10= Dihydro ferulic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 15= Eriodictyol, 17= Eriodictyol 7-O-glucoside, 18= 5,7,3',4', -Tetrahydroxy-flavan-5-O- β -glucosyl-4-8-eriodictyol, 19= Quercetin, 20= Quercetin diglucoside, 21= (+)-Catechin, 22= Epicatechin and 24= Apigenin

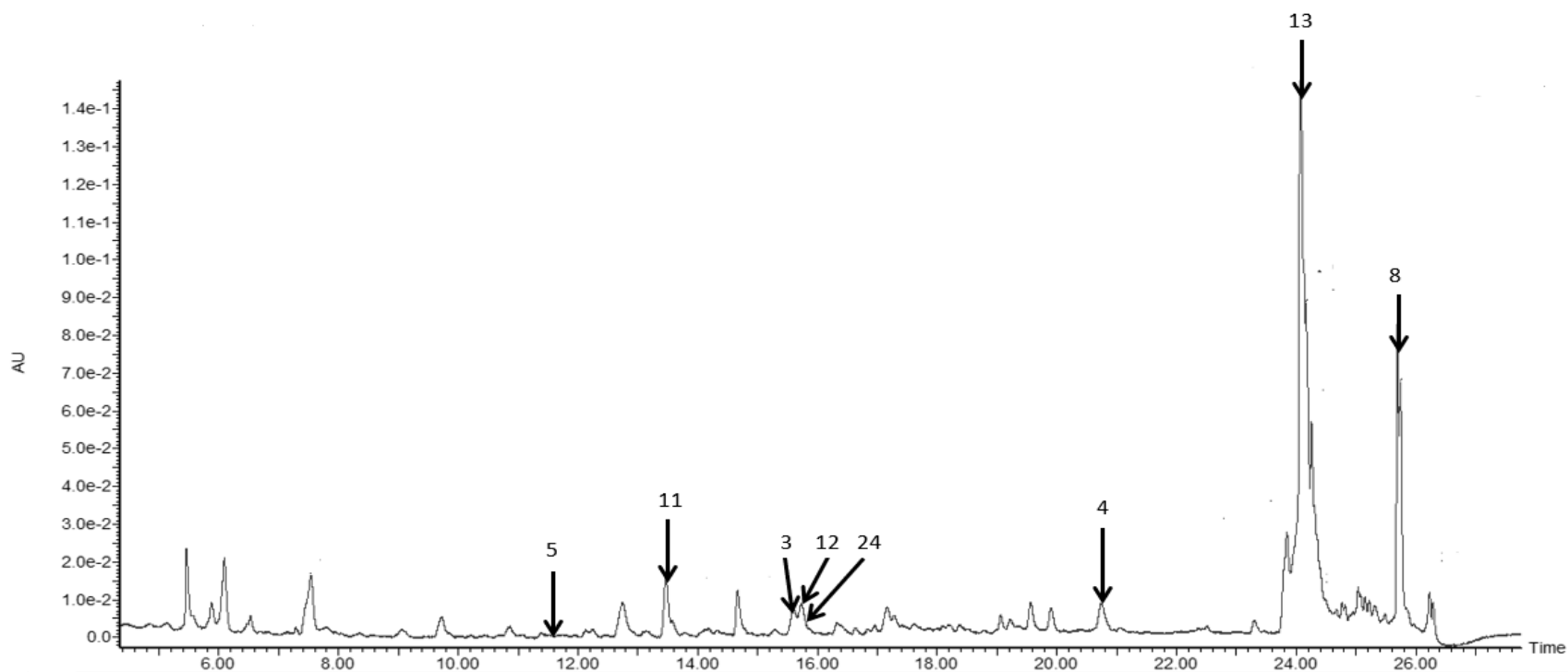


Figure 7-7: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from unsoured white sorghum porridge. 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol and 24= Apigenin

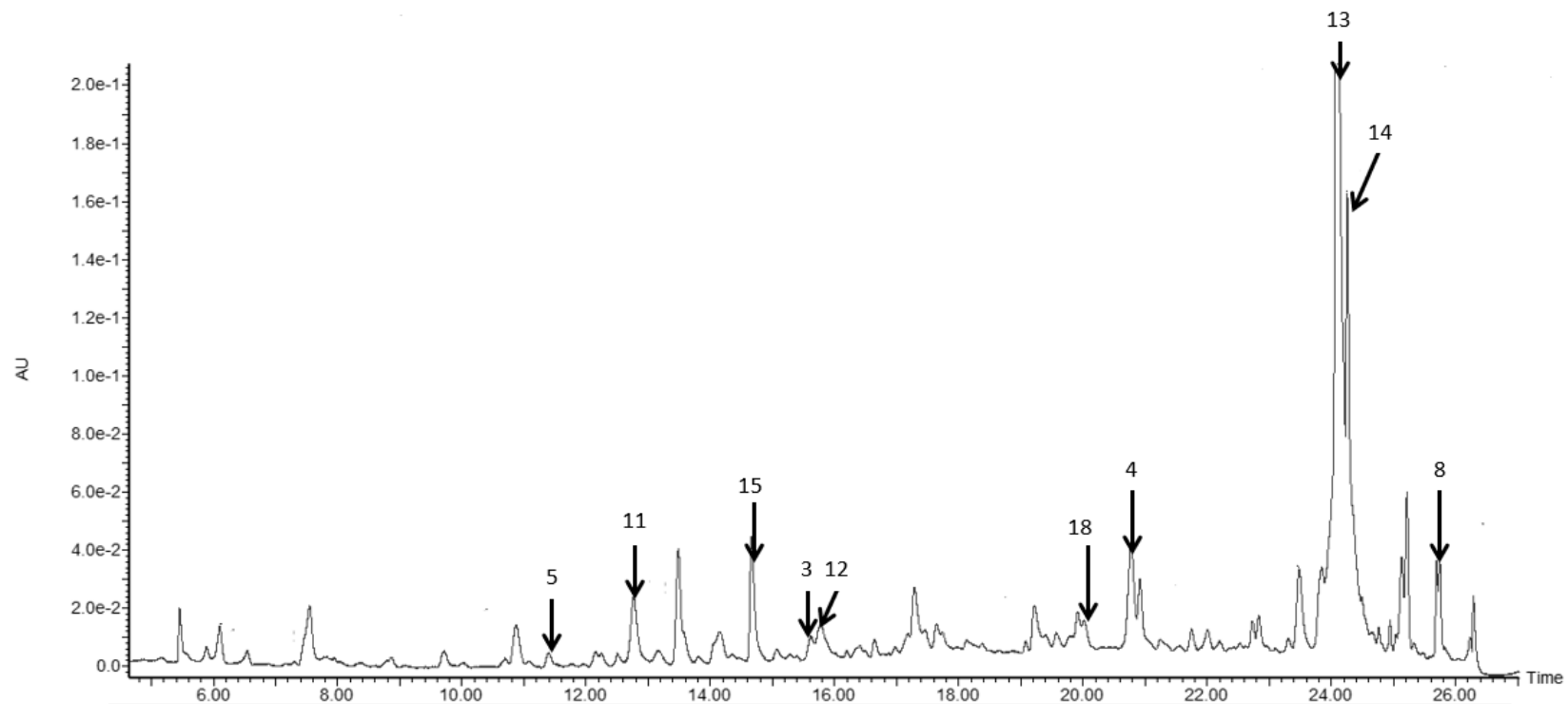


Figure 7-8: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from unsoured red sorghum porridge. 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 15= Eriodictyol and 18= 5,7,3',4', -Tetrahydroxy-flavan-5-O- β -glucosyl-4-8-eriodictyol

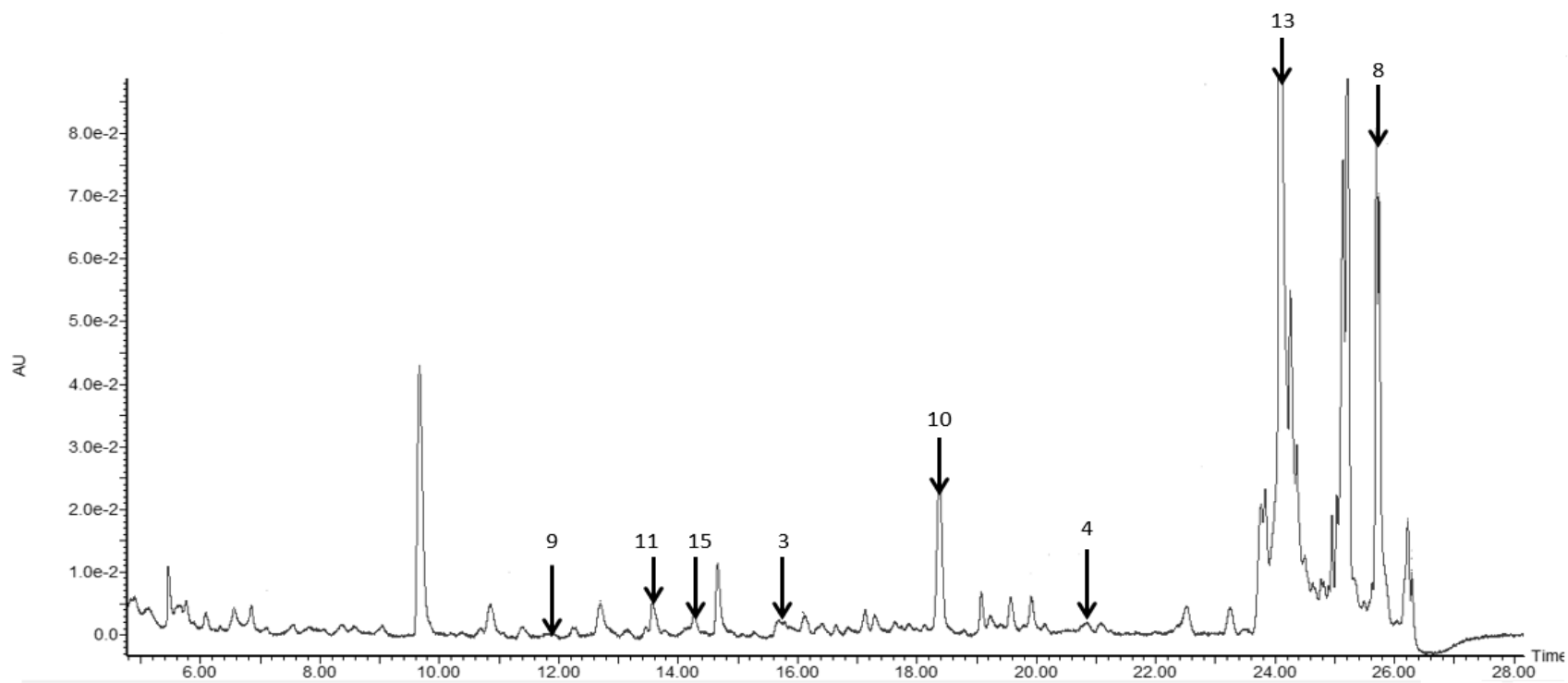


Figure 7-9: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from fermented white sorghum porridge. 3= Gallic acid, 4= Ferulic acid, 8= Chlorogenic acid, 9= Dihydro caffeic acid, 10= Dihydro ferulic acid, 11= 1-O-Caffeoyl-glycerol, 13= 1,3-dicaffeoyl-glycerol and 15= Eriodictyol

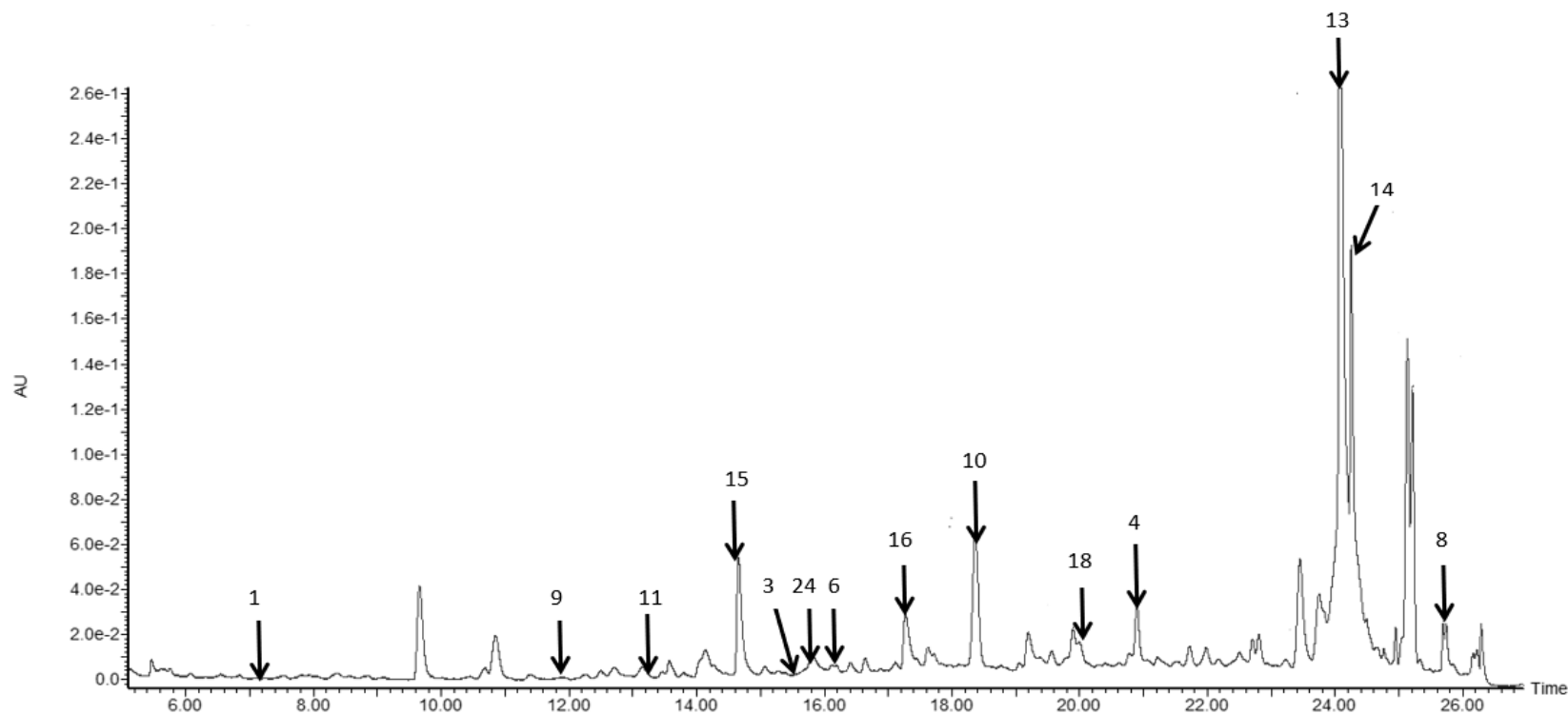


Figure 7-10: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from fermented red sorghum porridge. 1= 4-hydroxybenzoic acid, 3= Gallic acid, 4= Ferulic acid, 6= Vanillic acid, 8= Chlorogenic acid, 9= Dihydro caffeic acid, 10= Dihydro ferulic acid, 11= 1-O-Caffeoyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 15= Eriodictyol, 16= Naringenin-7-O-glucoside, 18= 5,7,3',4', -Tetrahydroxy-flavan-5-O- β -glucosyl-4-8-eriodictyol and 24= Apigenin

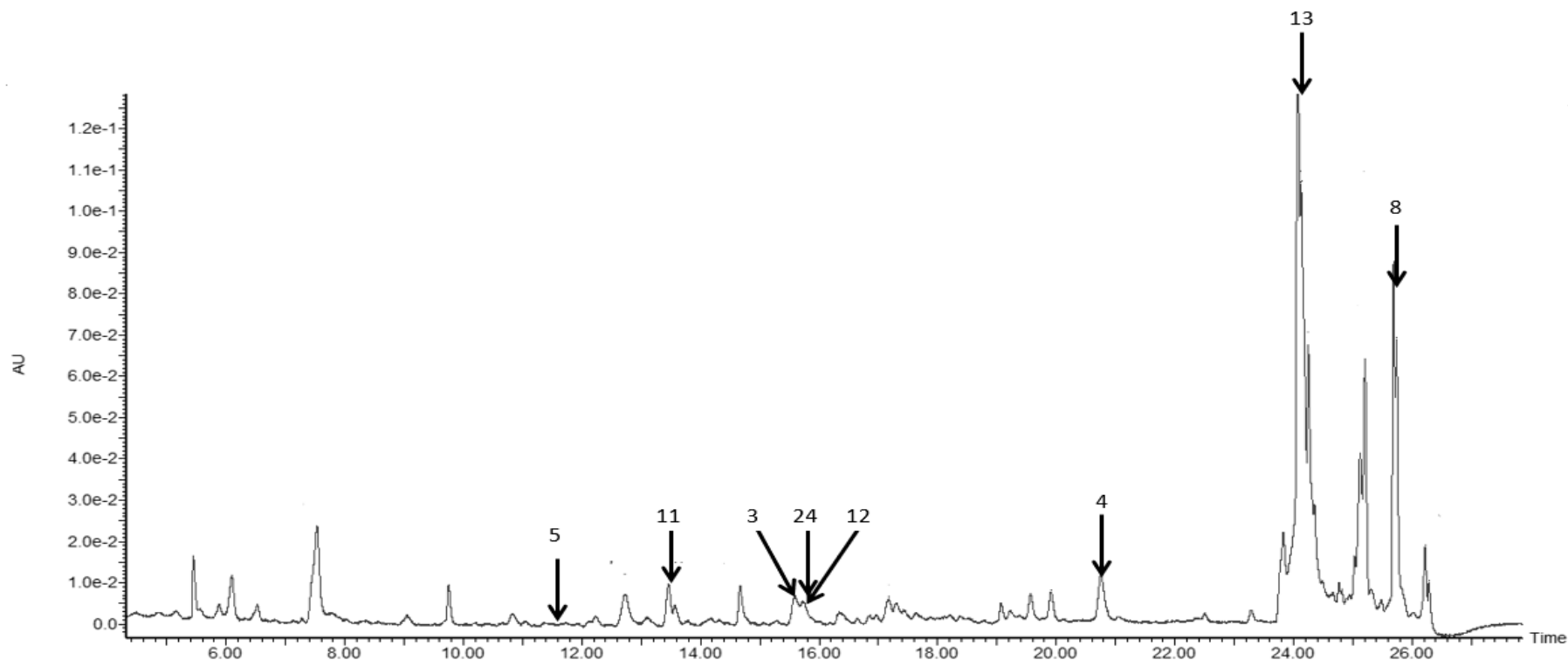


Figure 7-11: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from citric acidified white sorghum porridge. 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 6= Vanillic acid, 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol and 24= Apigenin

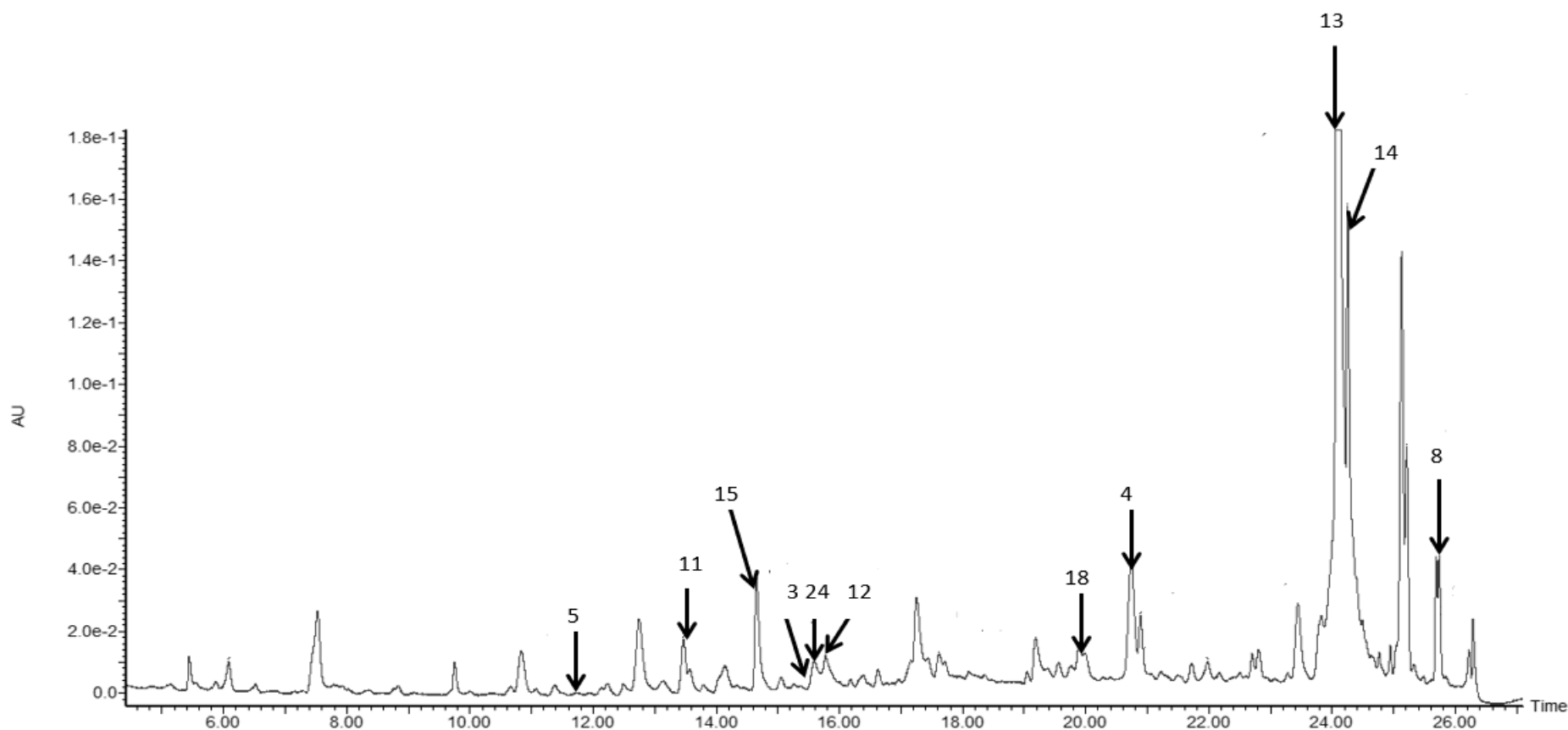


Figure 7-12: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from citric acidified red sorghum porridge. 3= Gallic acid, 4= Ferulic acid, 5= Caffeic acid, 6= Vanillic acid, 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 15= Eriodictyol, 18= 5,7,3',4', -Tetrahydroxy-flavan-5-O- β -glucosyl-4-8-eriodictyol and 24= Apigenin

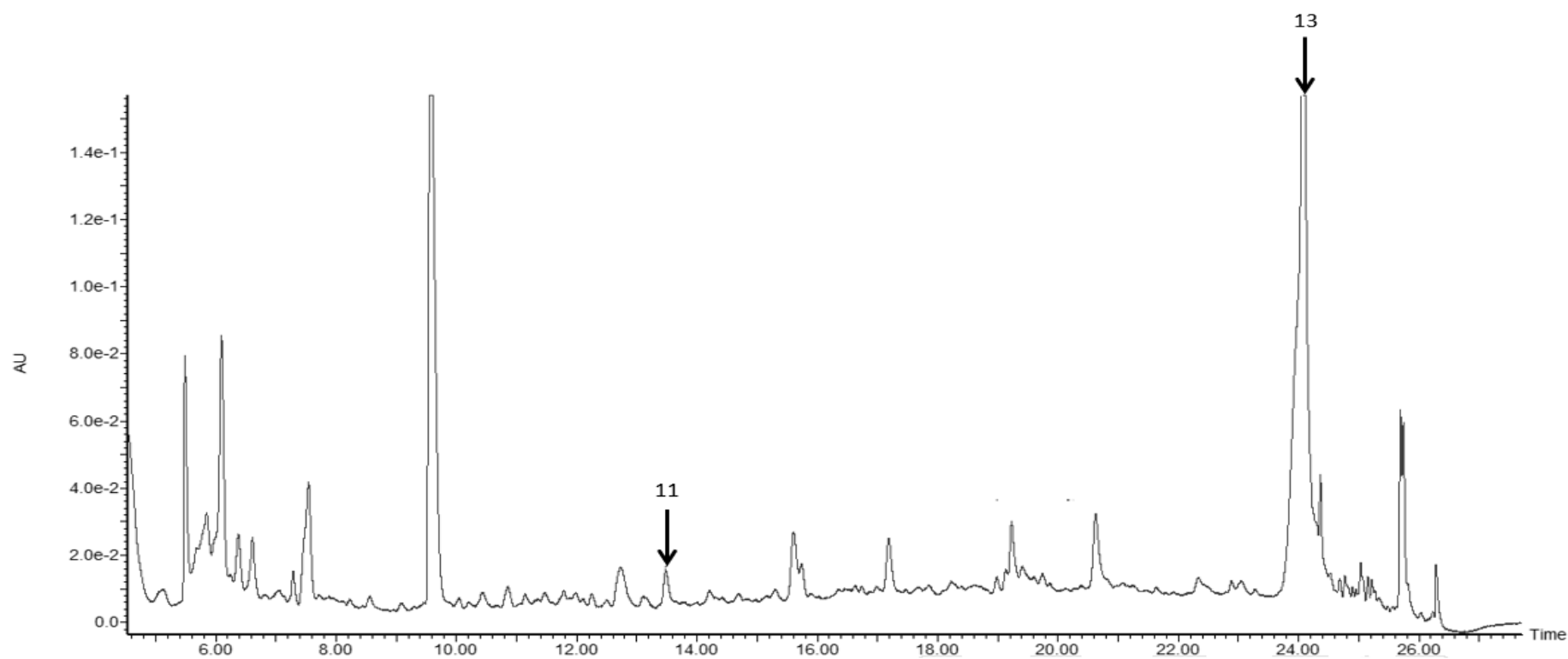


Figure 7-13: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from unsoured white sorghum digest (supernatant). 11= 1-O-Caffeoyl-glycerol and 13= 1,3-dicaffeoyl-glycerol

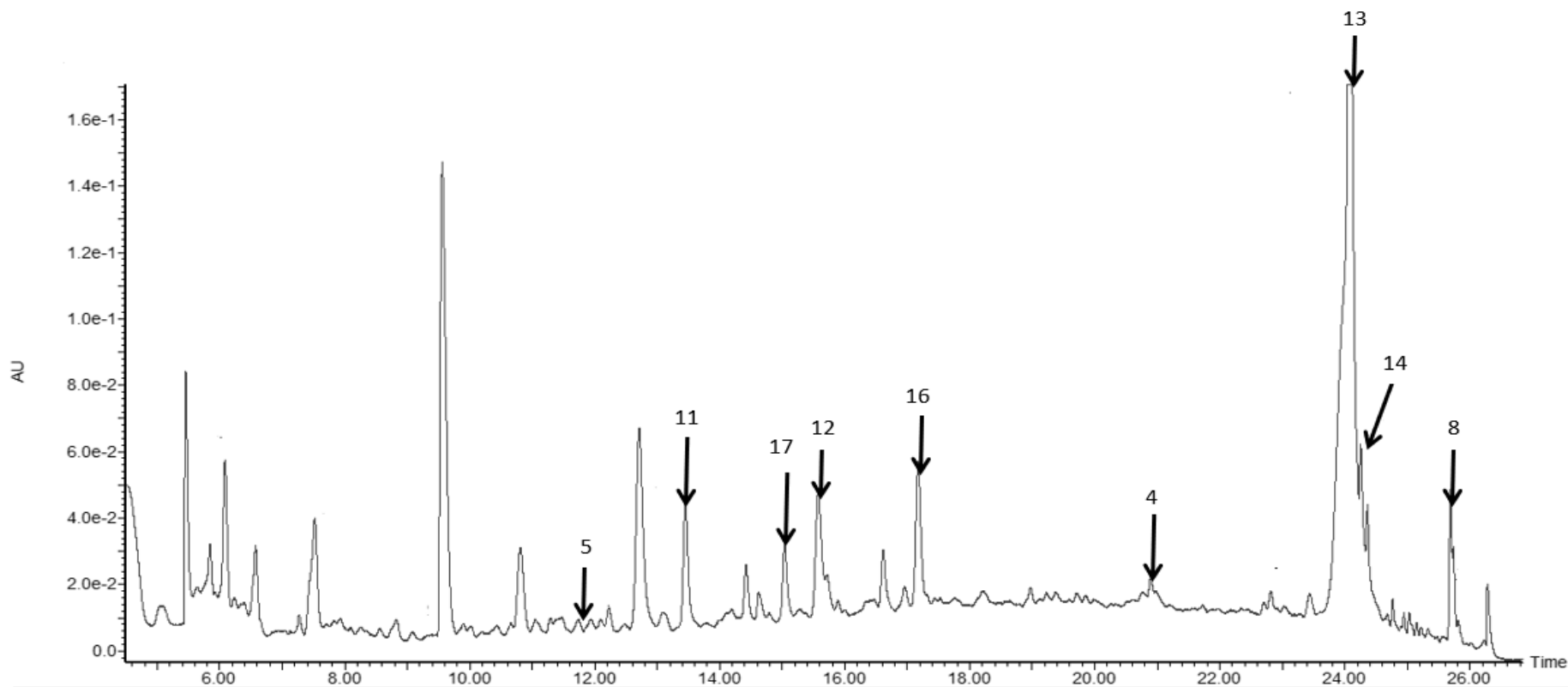


Figure 7-14: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from unsoured red sorghum digest (supernatant). 4= Ferulic acid, 5= Caffeic acid, 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 14= naringenin, 16= Naringenin-7-O-glucoside and 17= Eriodictyol 7-O-glucoside

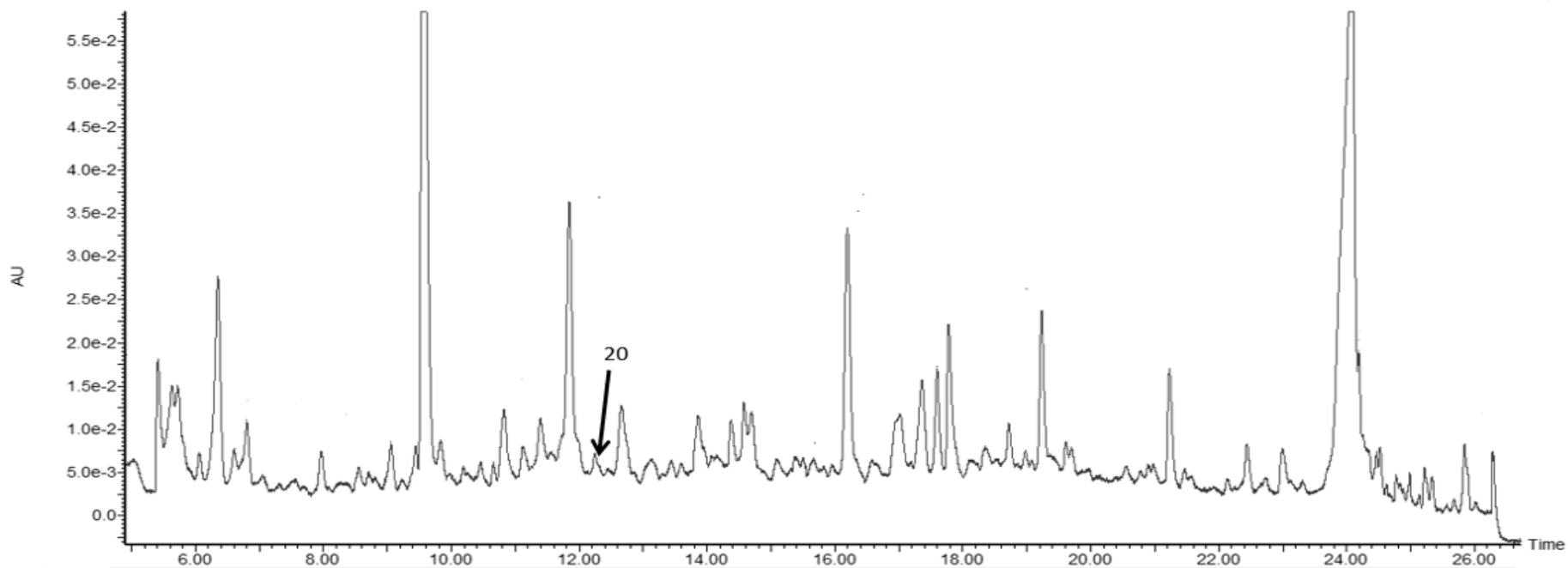


Figure 7-15: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from fermented white sorghum digest (supernatant). 20= Quercetin diglucoside

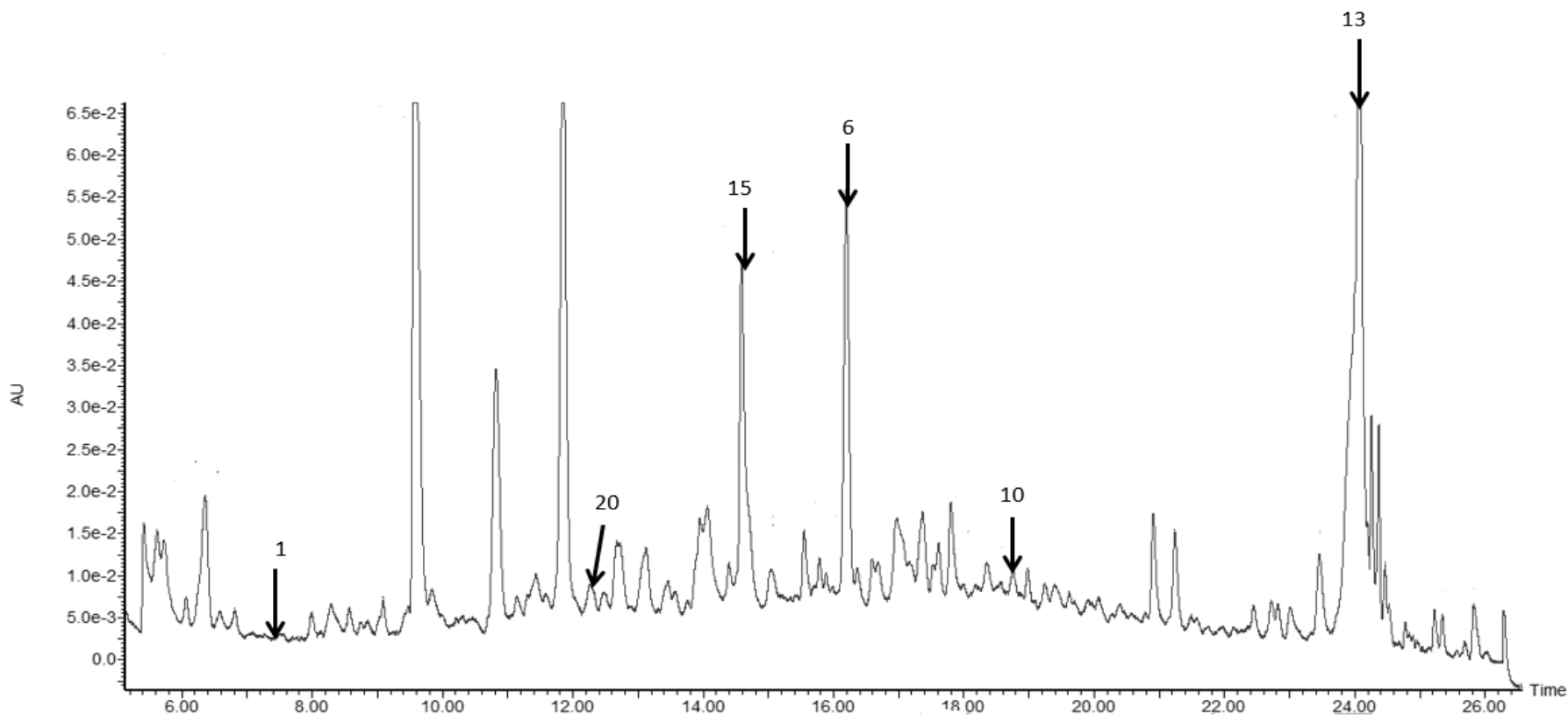


Figure 7-16: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from fermented red sorghum digest (supernatant). 1= 4-hydroxybenzoic acid, 6= Vanillic acid, 10= Dihydro ferulic acid, 13= 1,3-dicaffeoyl-glycerol, 15= Eriodictyol and 20= Quercetin diglucoside

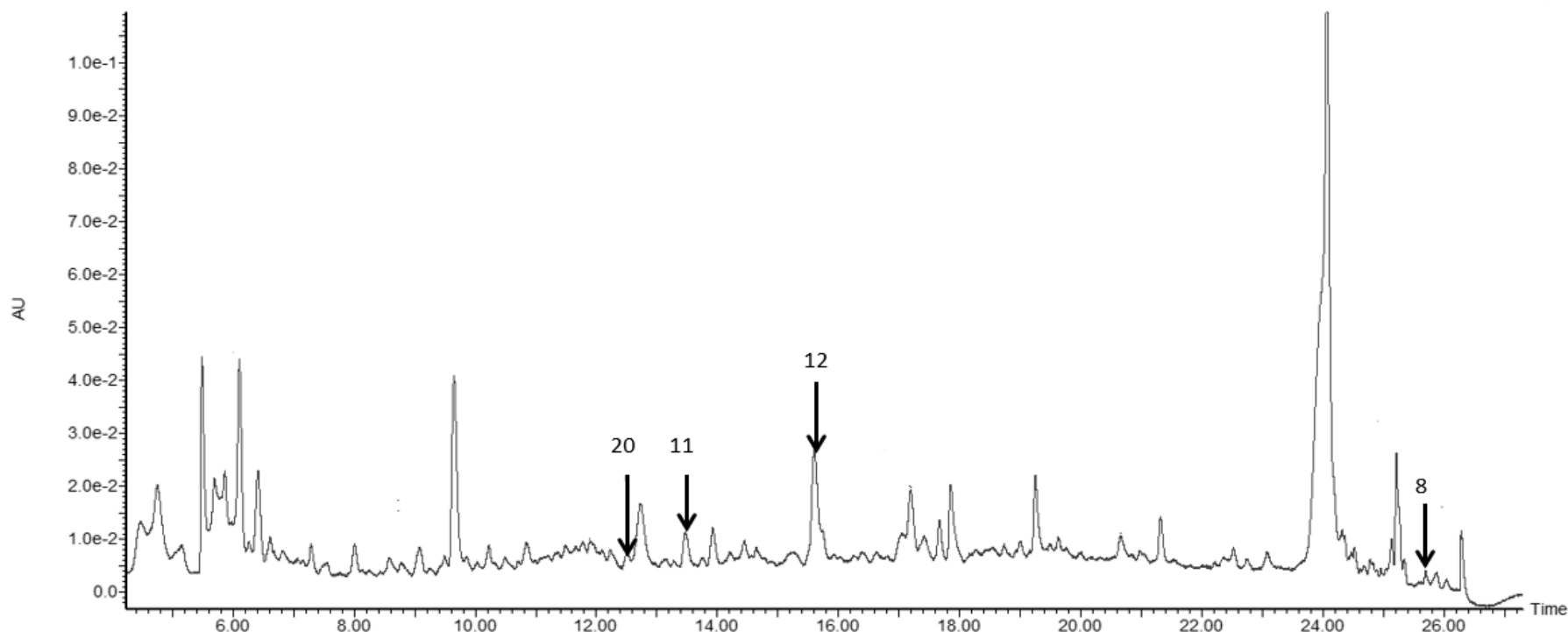


Figure 7-17: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from citric acidified white sorghum digest (supernatant). 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol and 20= Quercetin diglucoside

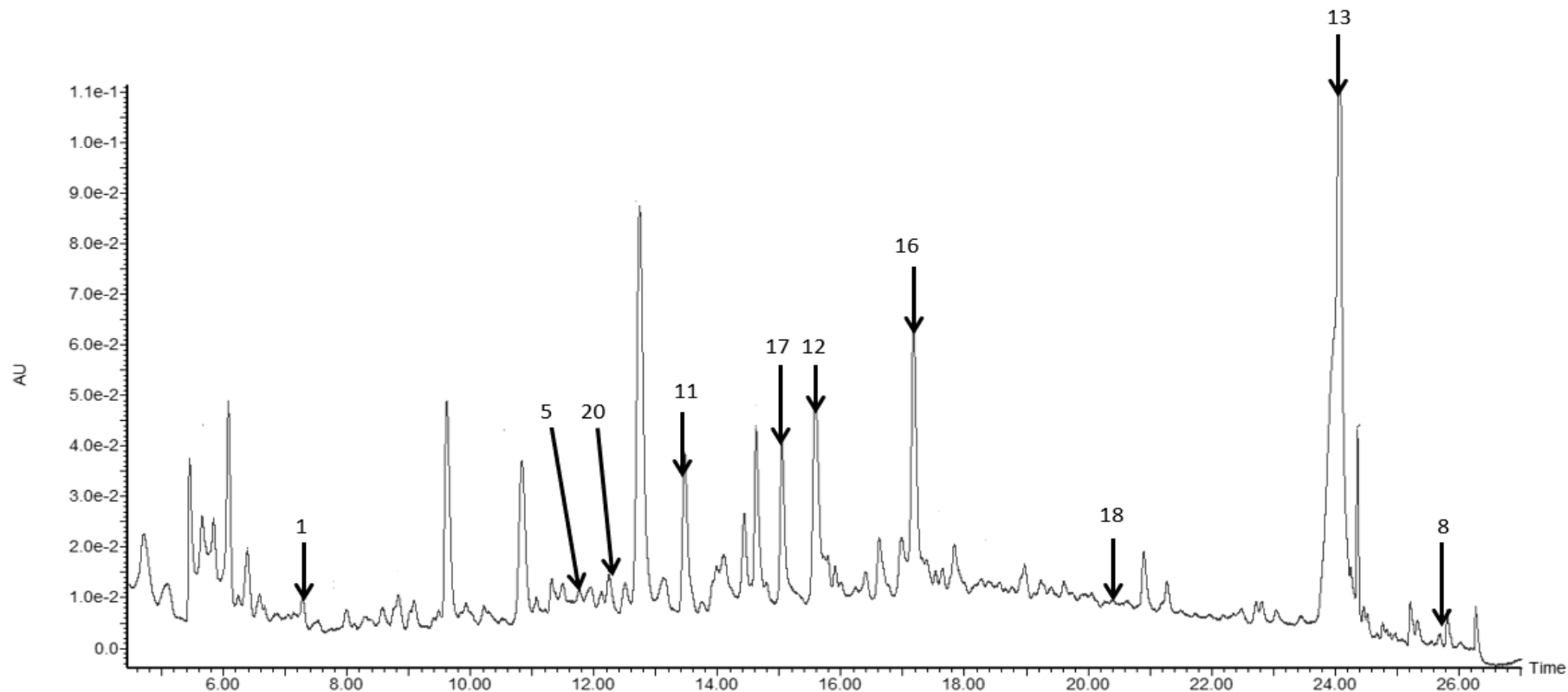


Figure 7-18: Chromatogram ($\lambda_{\text{max}} = 280\text{nm}$) of extracts from citric acidified red sorghum digest (supernatant). 1= 4-hydroxybenzoic acid, 5= Caffeic acid, 6= Vanillic acid, 7=Sinapic acid, 8= Chlorogenic acid, 11= 1-O-Caffeoyl-glycerol, 12= 1-O-coumaroyl-glycerol, 13= 1,3-dicaffeoyl-glycerol, 16= Naringenin-7-O-glucoside, 17= Eriodictyol 7-O-glucoside, 18= 5,7,3',4', -Tetrahydroxy-flavan-5-O- β -glucosyl-4-8-eriodictyol and 20= Quercetin diglucoside

APPENDIX B

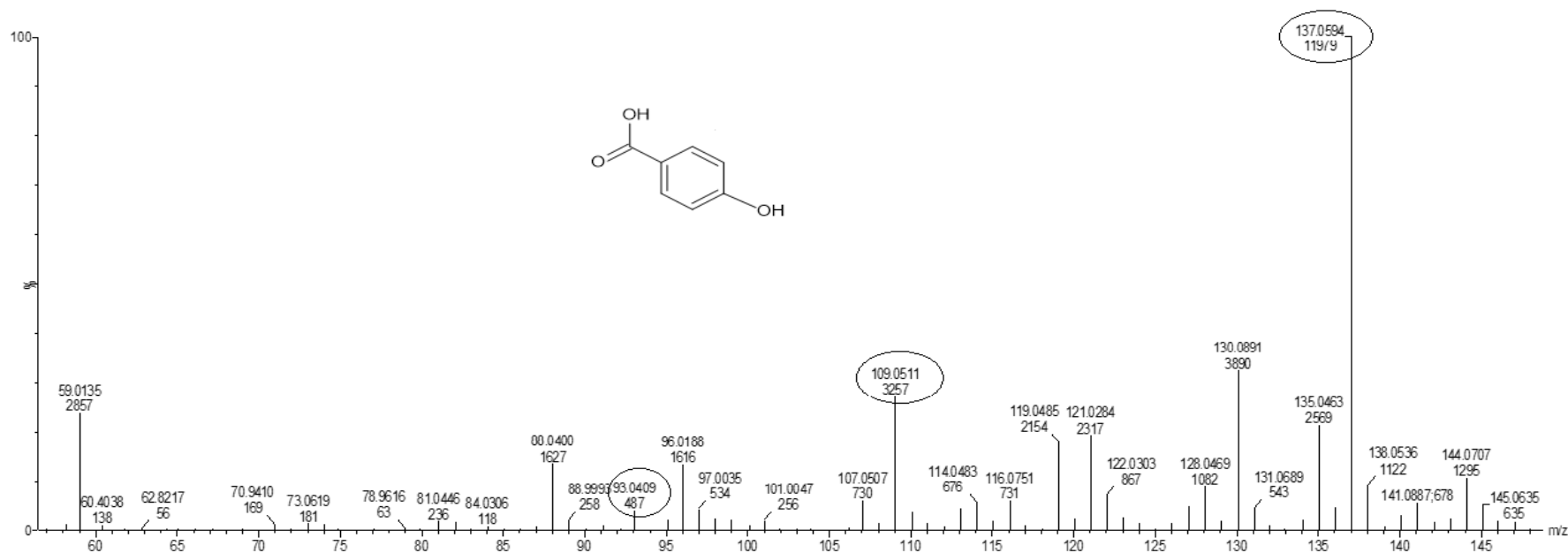


Figure 7-19: Mass spectrum of 4-hydroxybenzoic acid (m/z 137) identified in extracts of sorghum

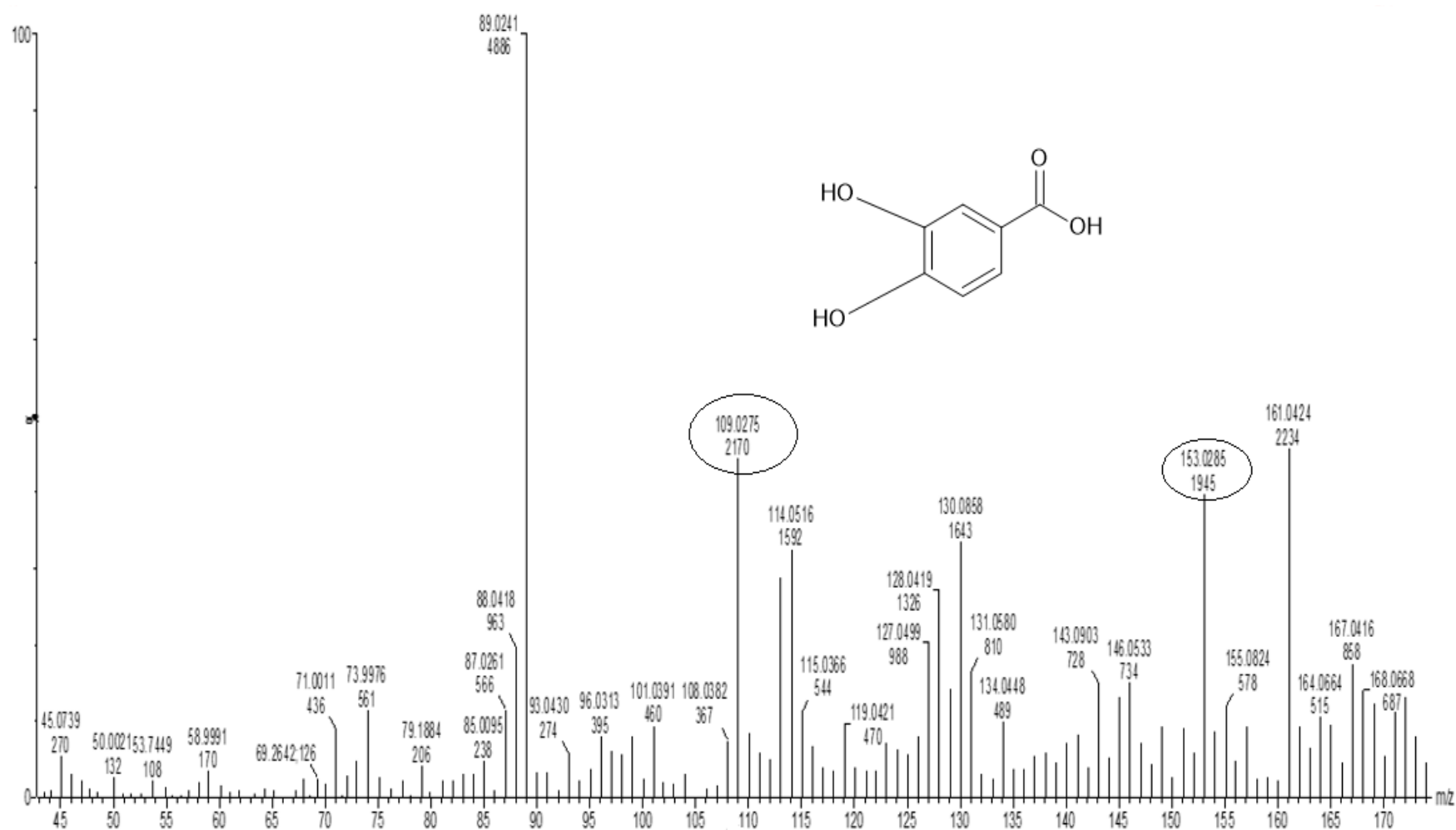


Figure 7-20: Mass spectrum of protocatechuic acid (m/z 153) identified in extracts of sorghum

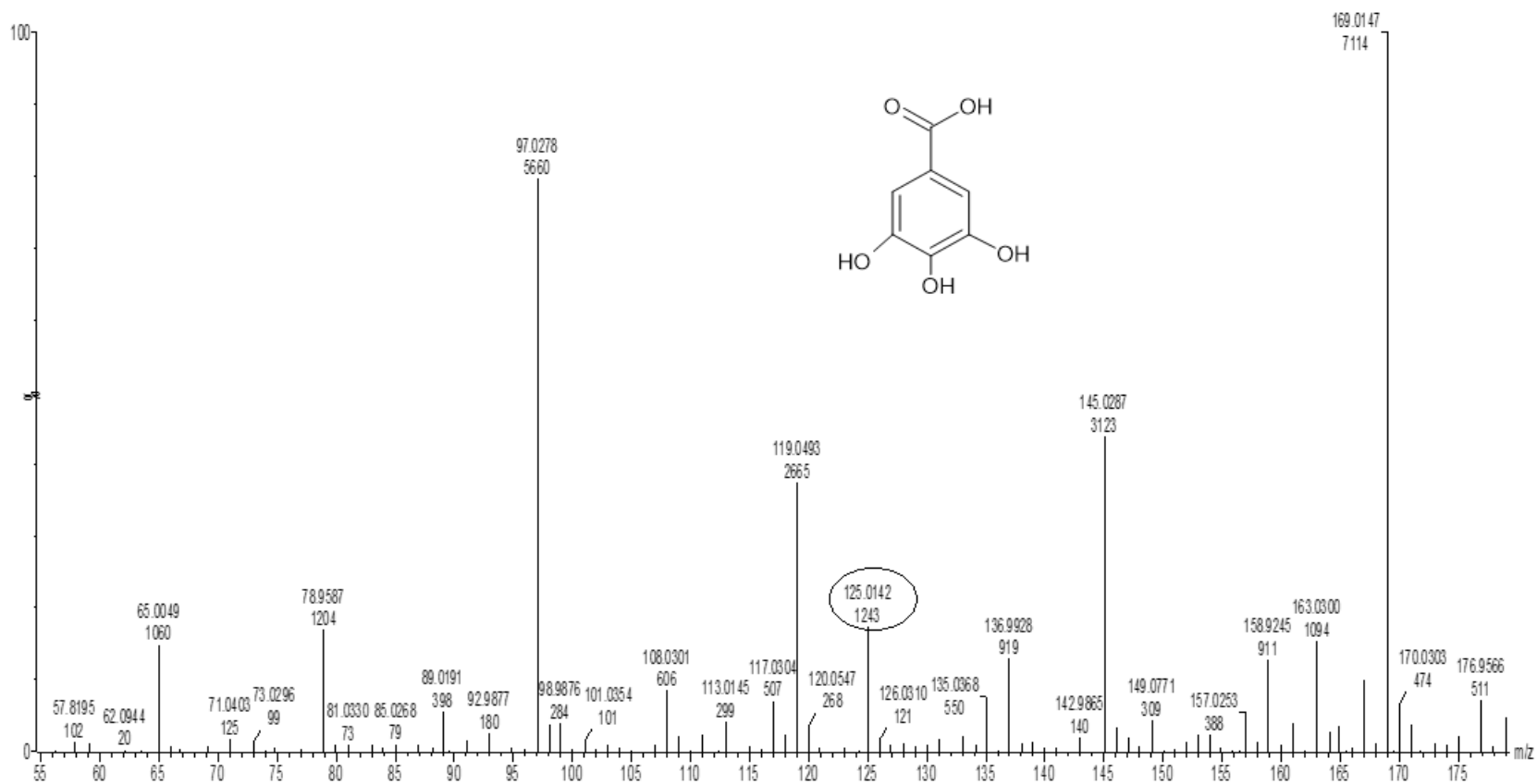


Figure 7-21: Mass spectrum of gallic acid (m/z 169) identified in extracts of sorghum

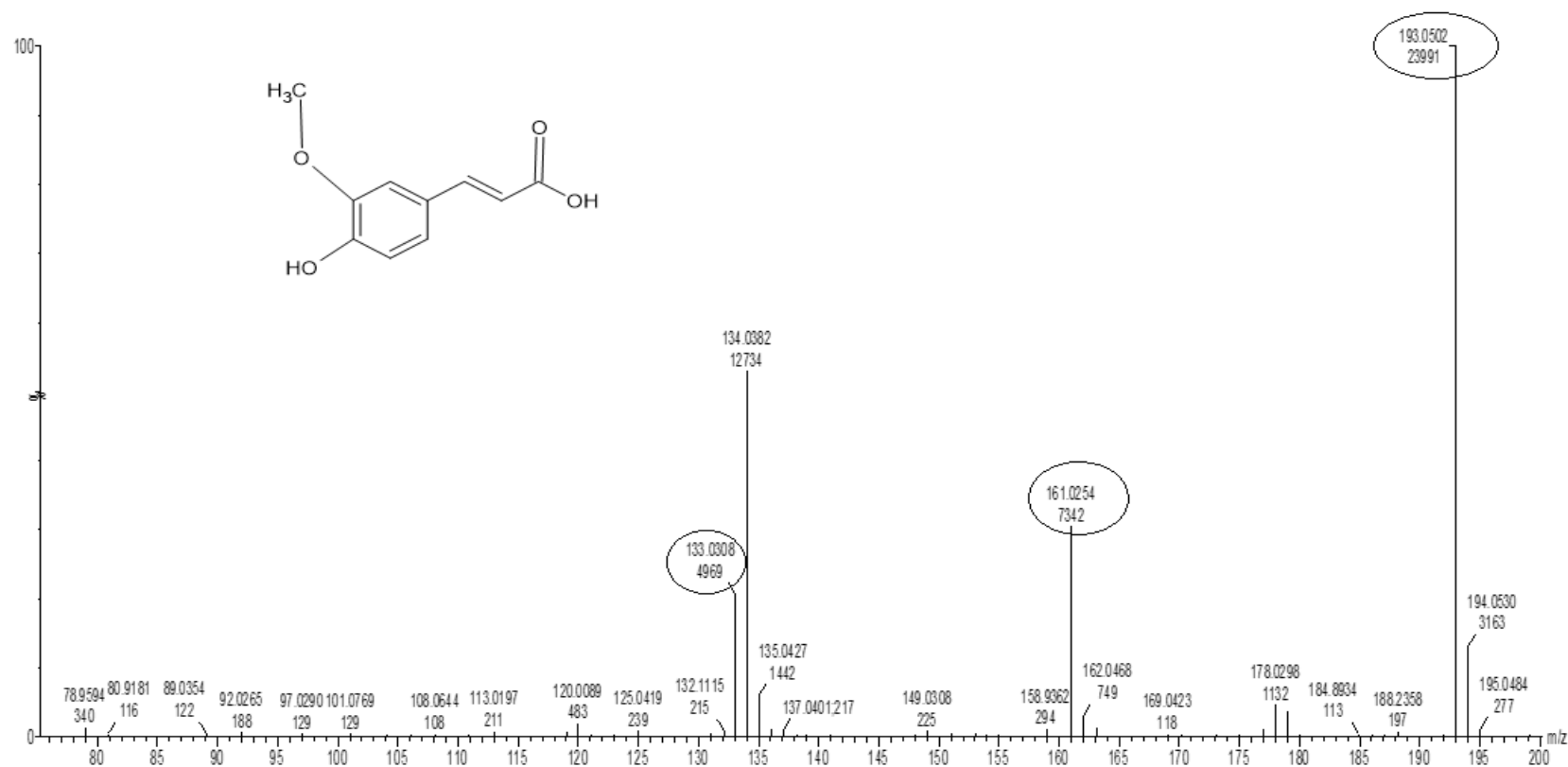


Figure 7-22: Mass spectrum of ferulic acid (m/z 193) identified in extracts of sorghum

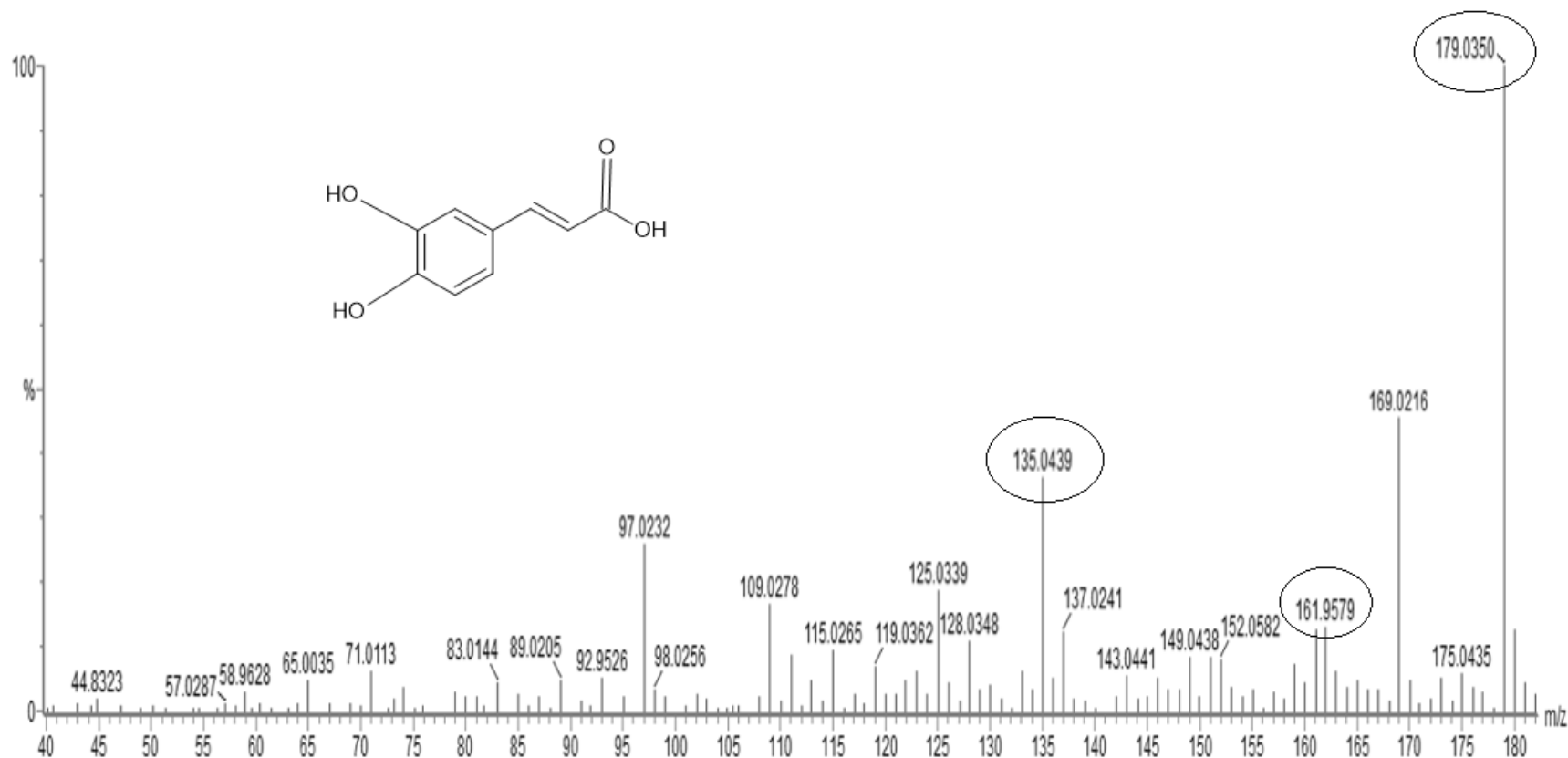


Figure 7-23: Mass spectrum of caffeic acid (m/z 179) identified in extracts of sorghum

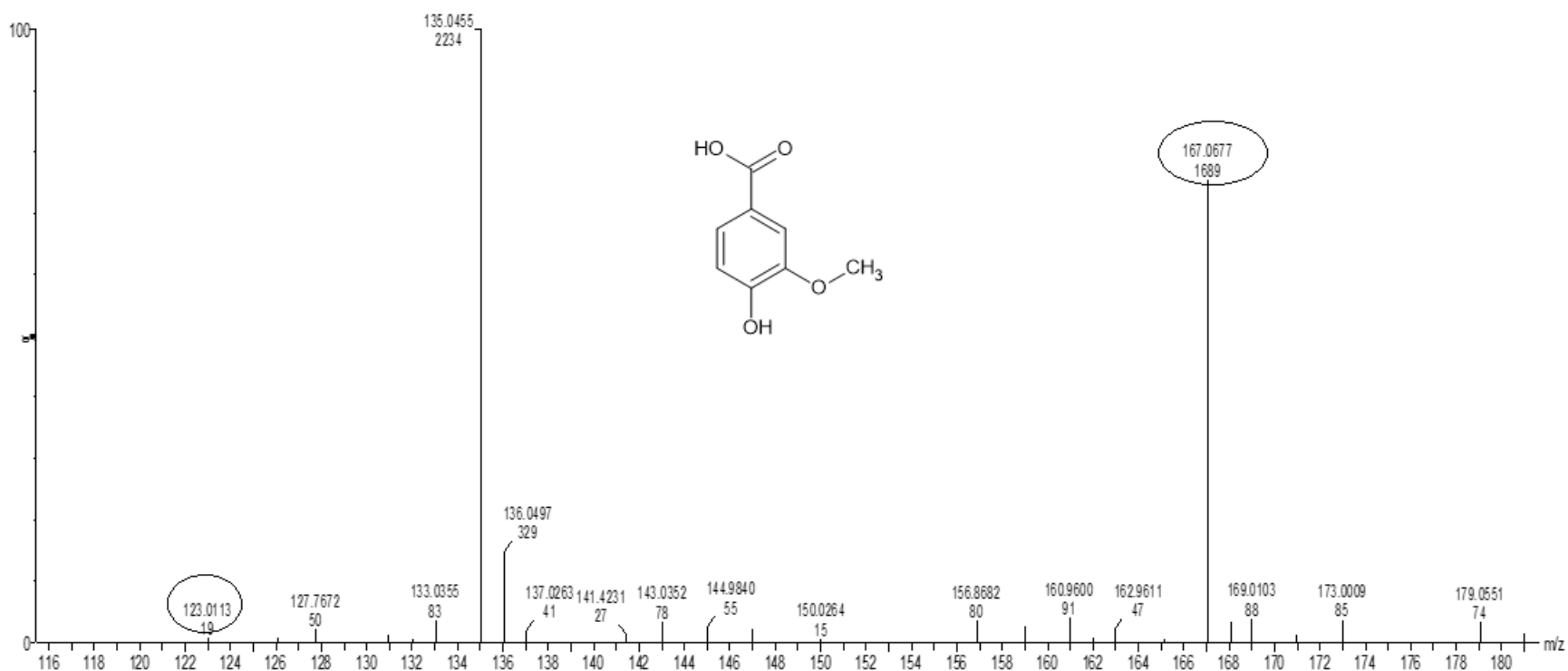


Figure 7-24: Mass spectrum of vanillic acid (m/z 167) identified in extracts of sorghum

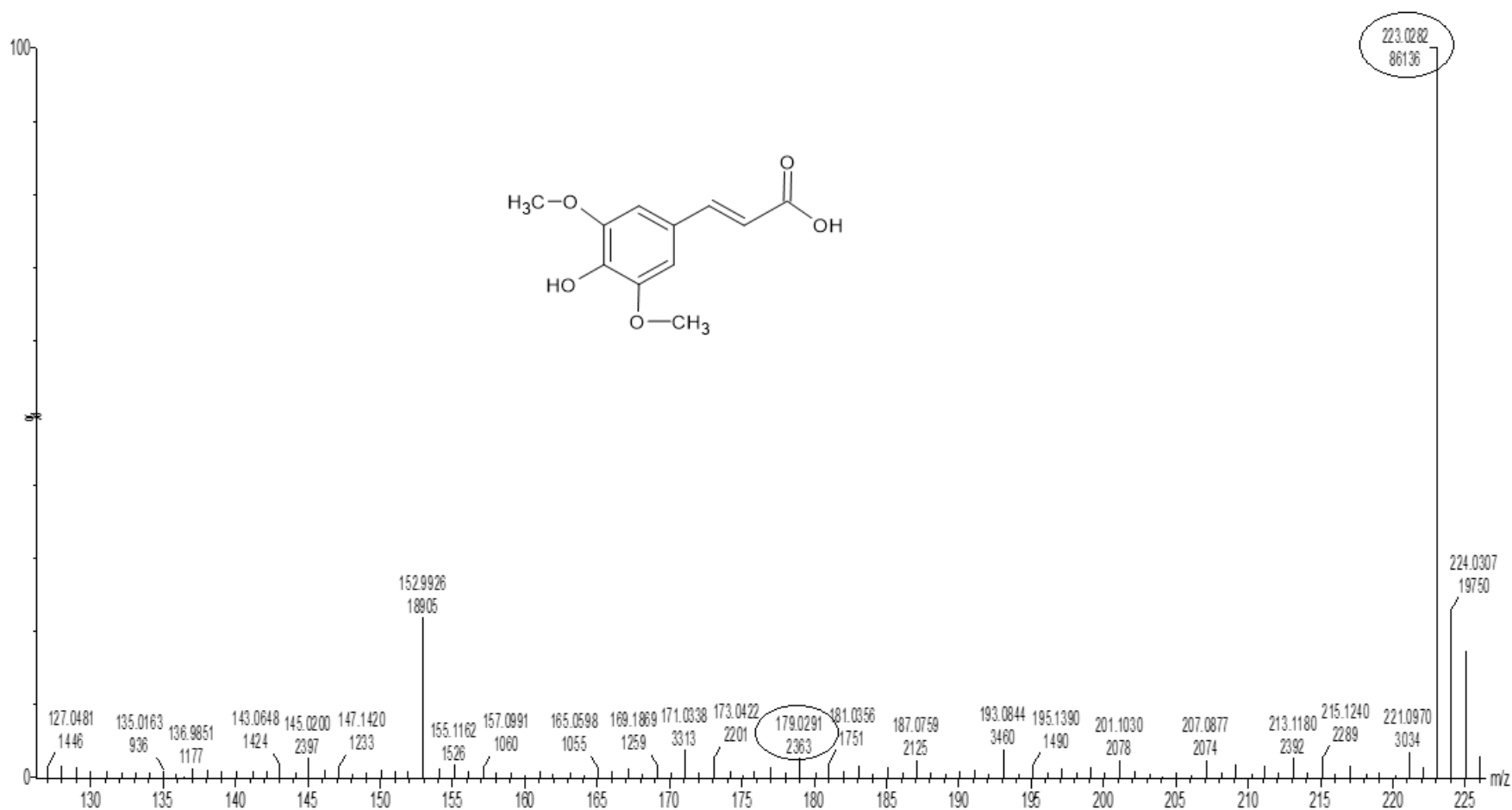


Figure 7-25: Mass spectrum of Sinapic acid (m/z 223) identified in extracts of sorghum

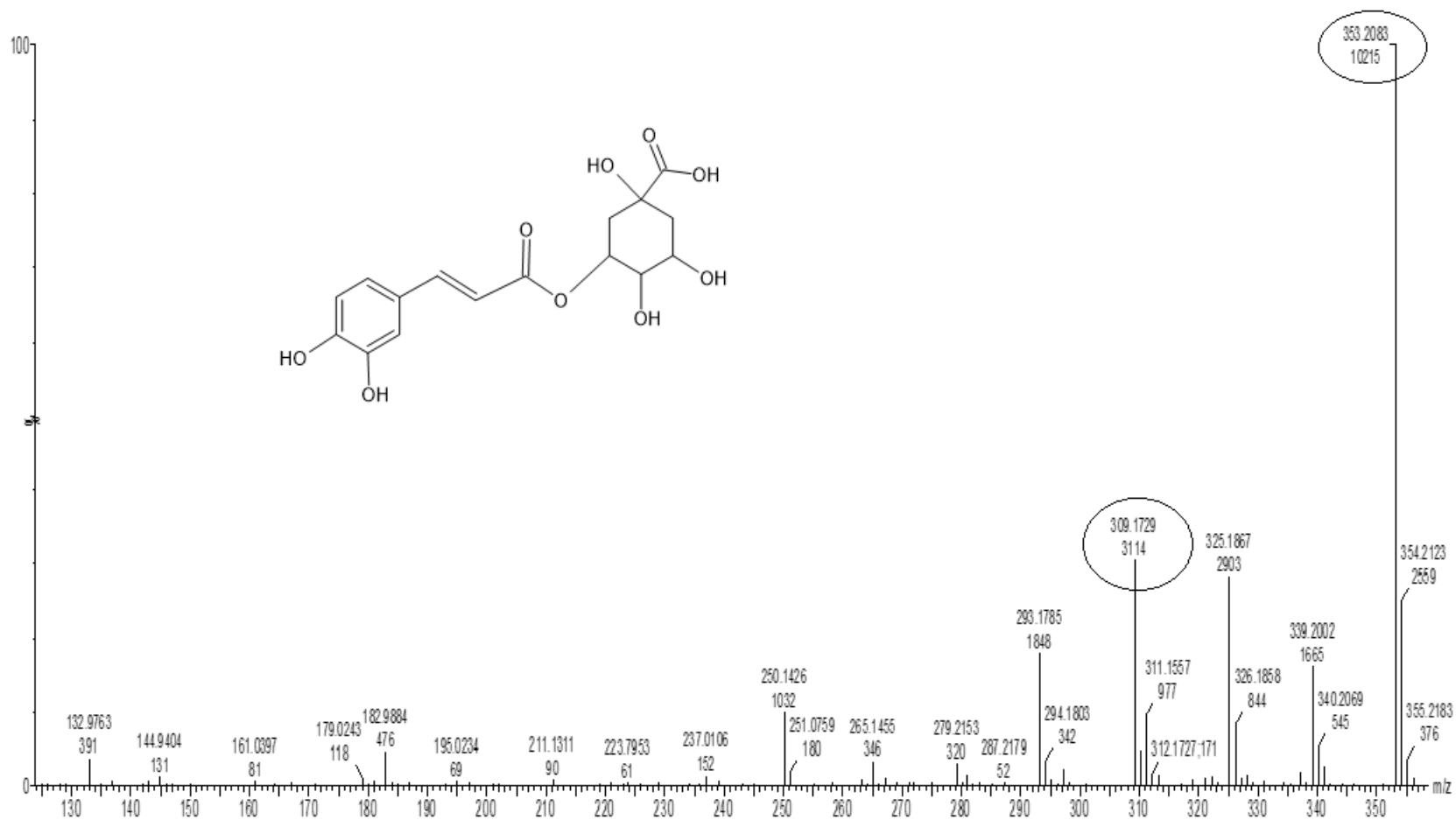


Figure 7-26: Mass spectrum of Chlorogenic acid (m/z 353) identified in extracts of sorghum

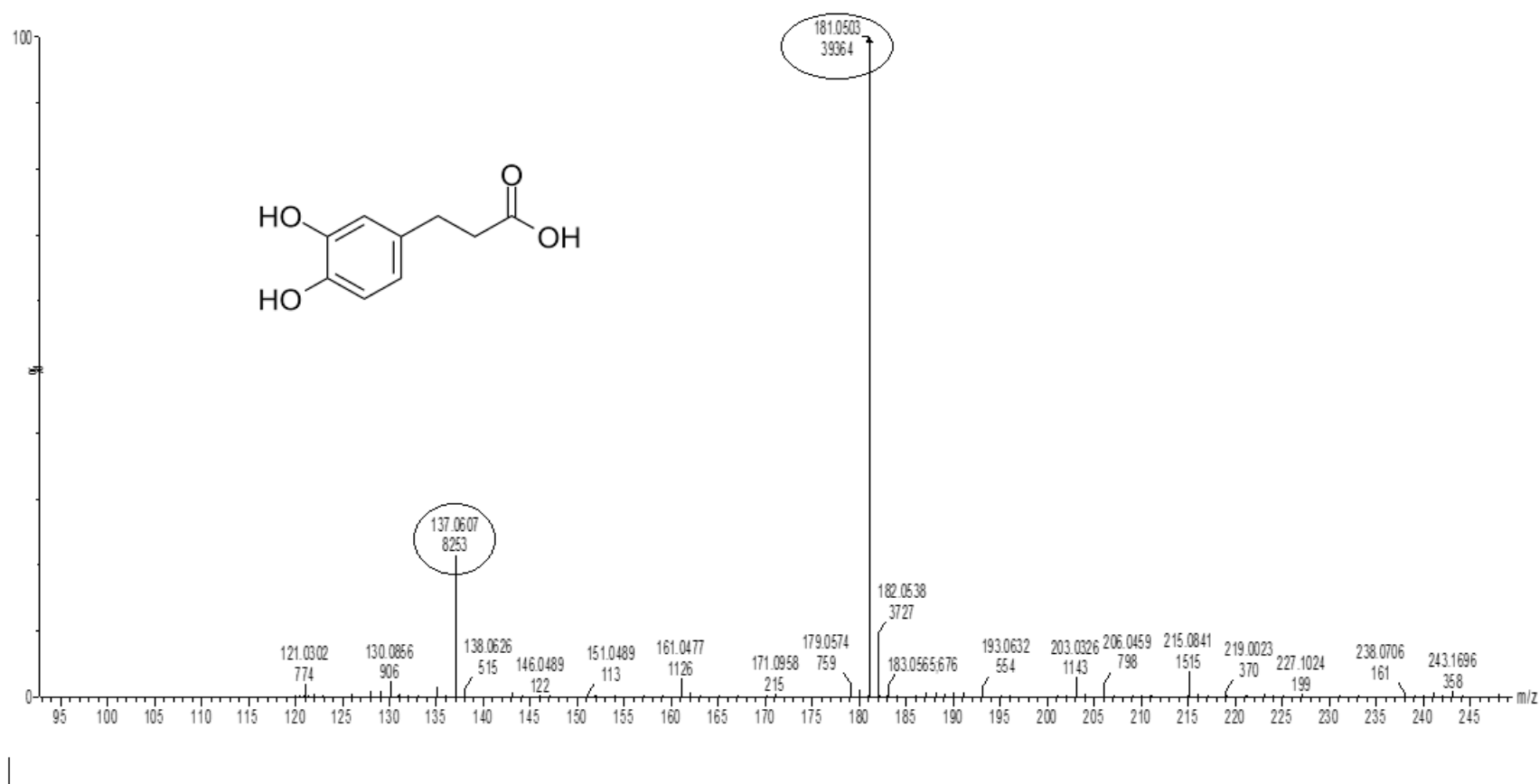


Figure 7-27: Mass spectrum of Dihydro caffeic acid (m/z 181) identified in extracts of sorghum

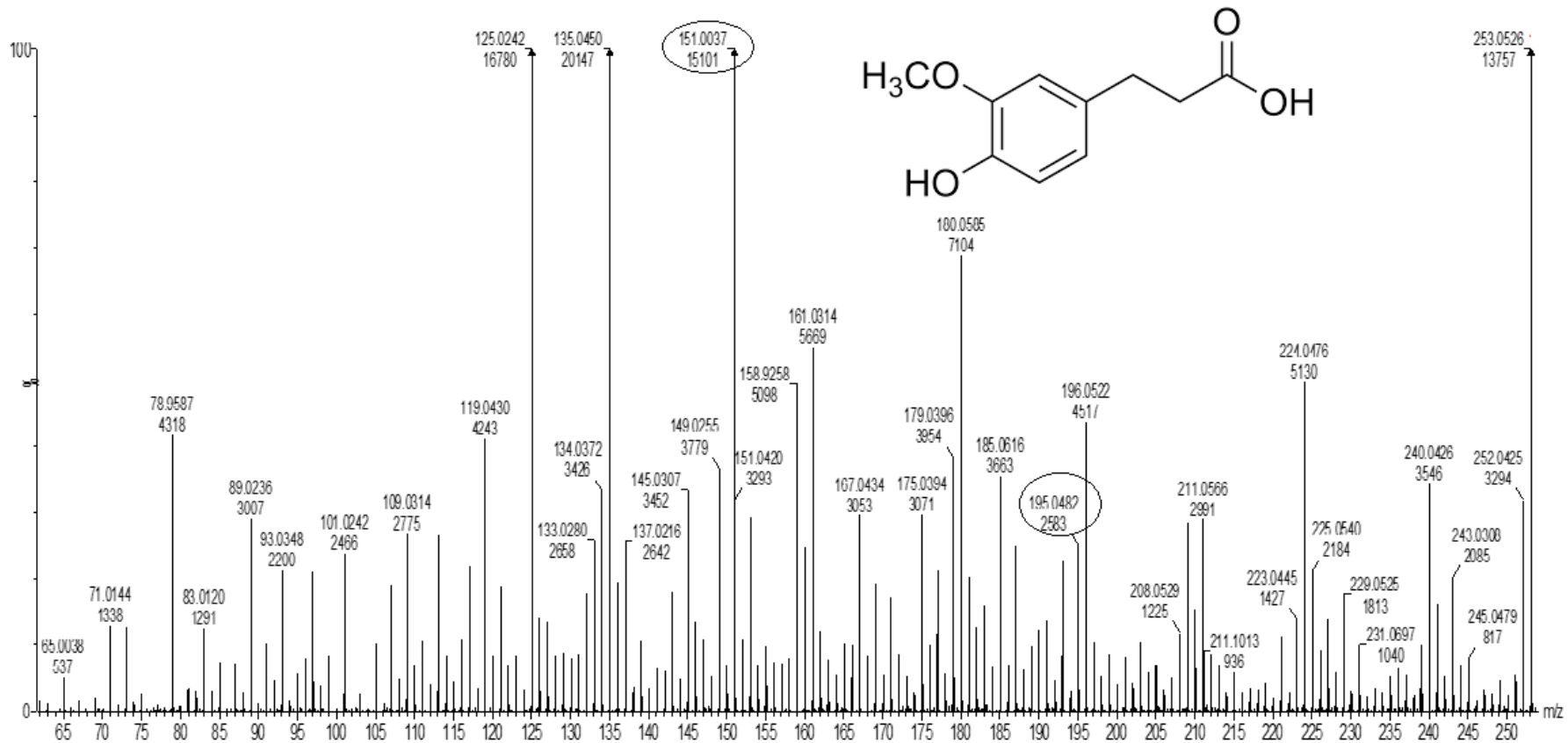


Figure 7-28: Mass spectrum of Dihydro ferulic acid (m/z 195) identified in extracts of sorghum

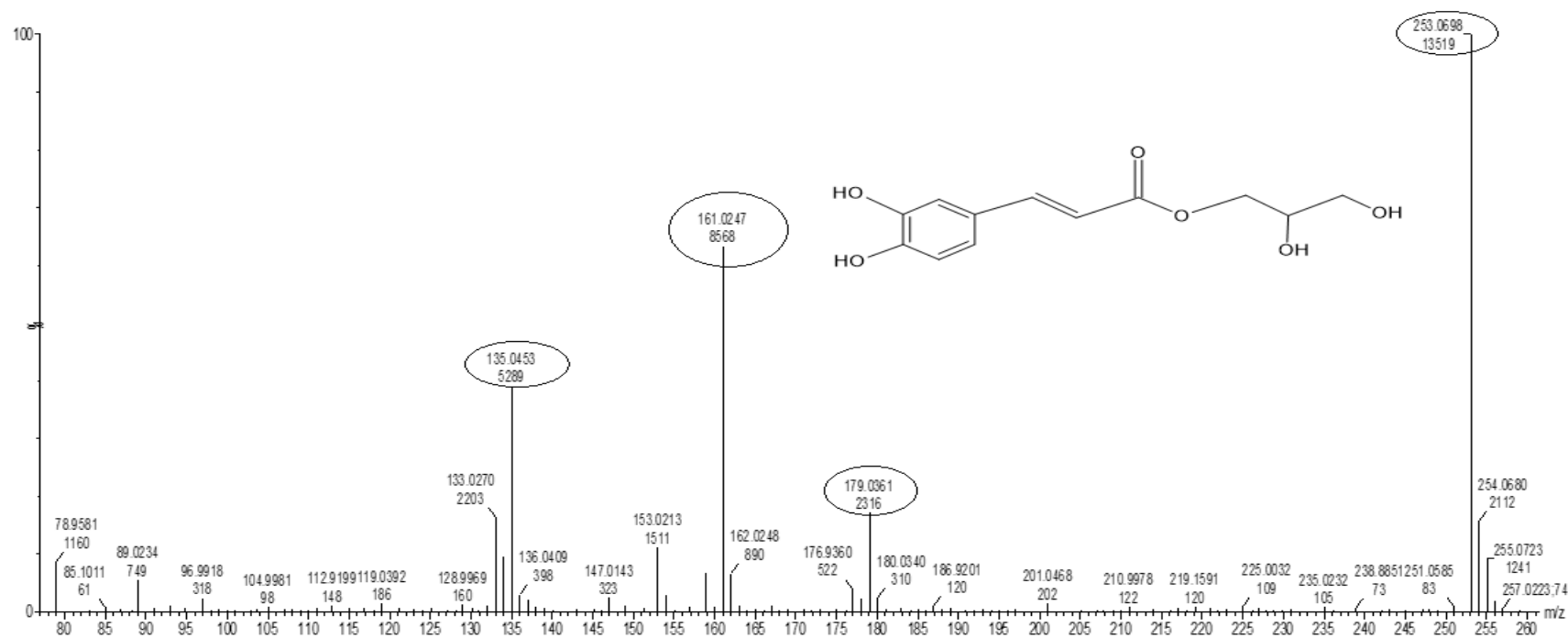


Figure 7-29: Mass spectrum of 1-O-Caffeoyl-glycerol (m/z 253) identified in extracts of sorghum

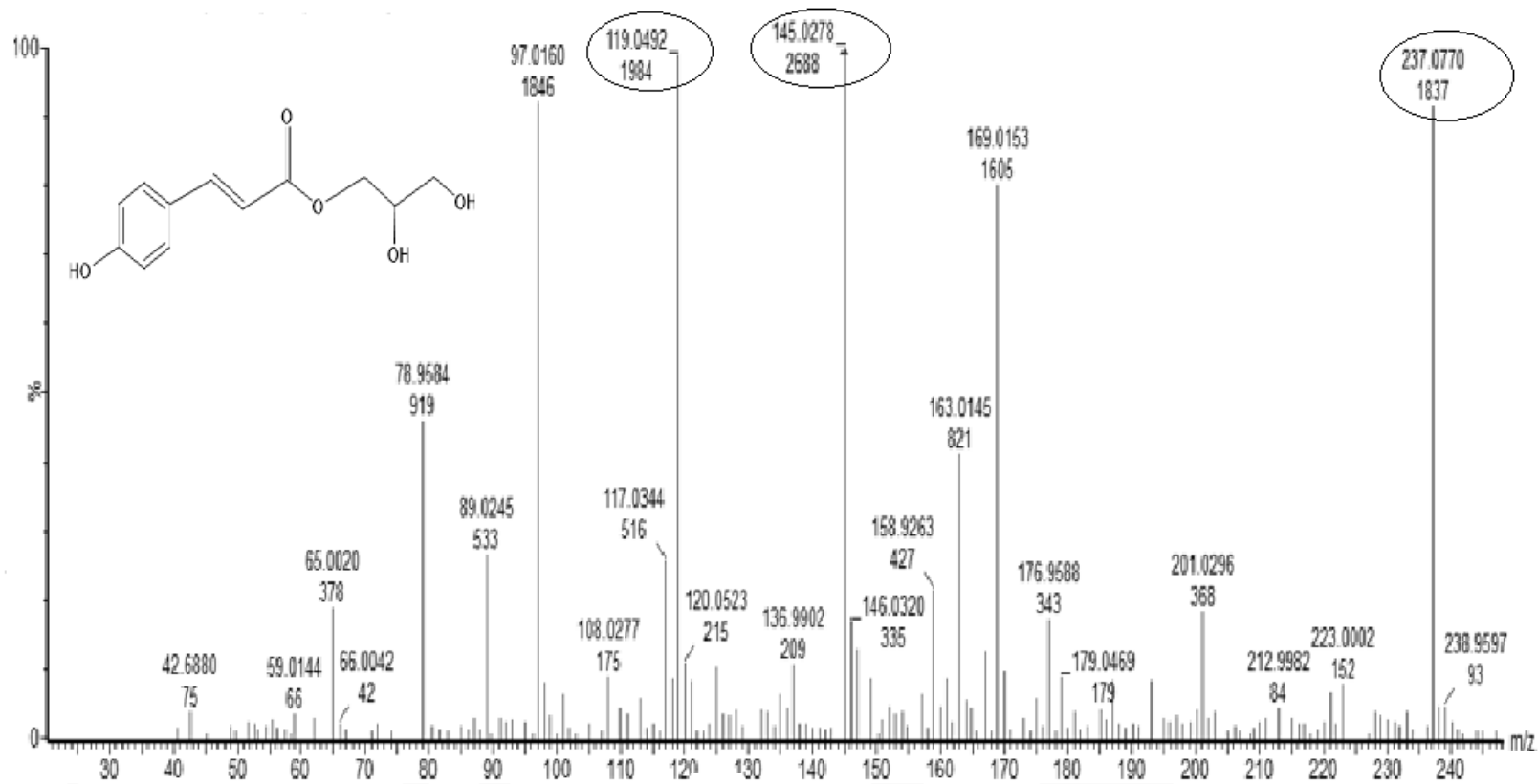


Figure 7-30: Mass spectrum of 1-O-coumaroyl-glycerol (m/z 237) identified in extracts of sorghum

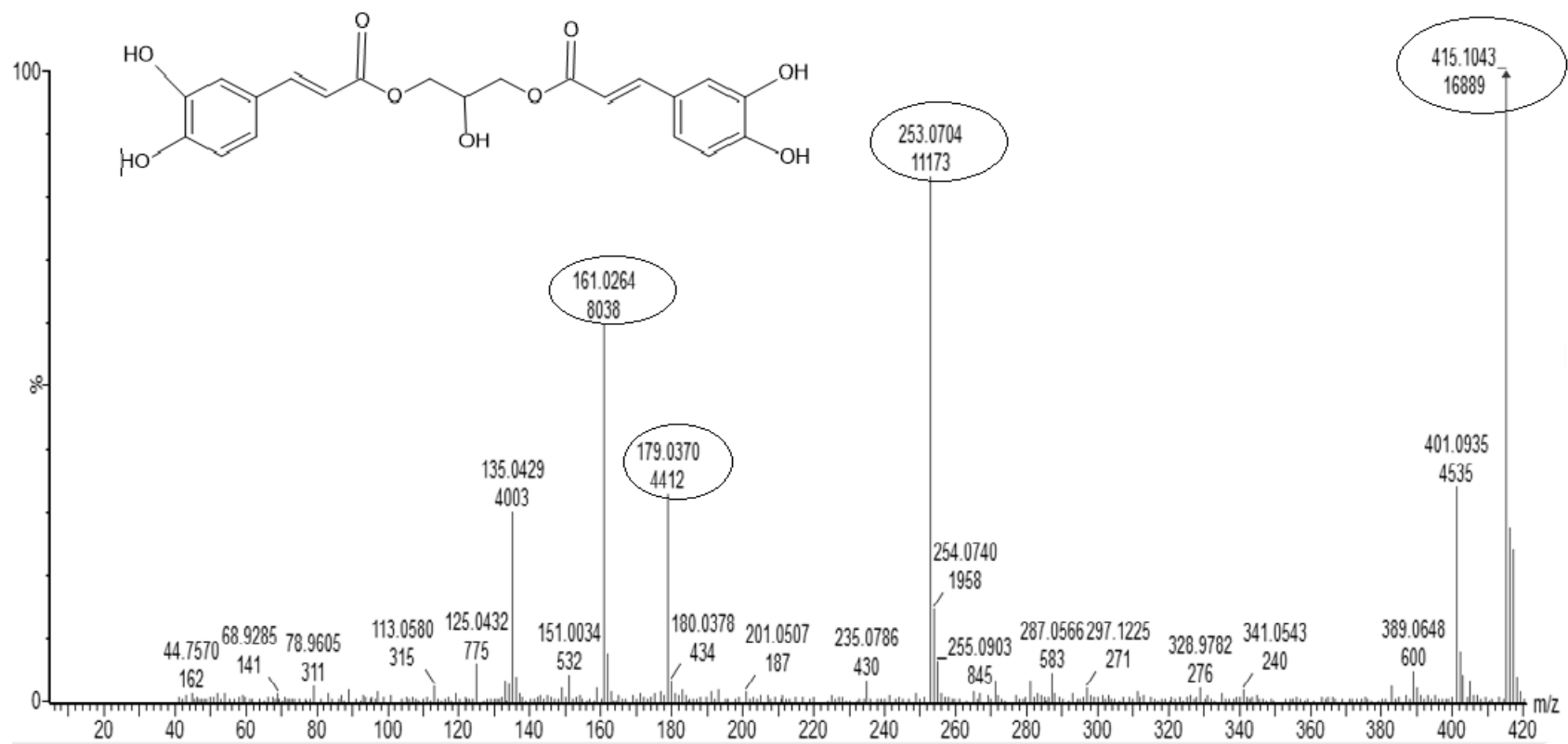


Figure 7-31: Mass spectrum of 1,3-dicaffeoyl-glycerol (m/z 415) identified in extracts of sorghum

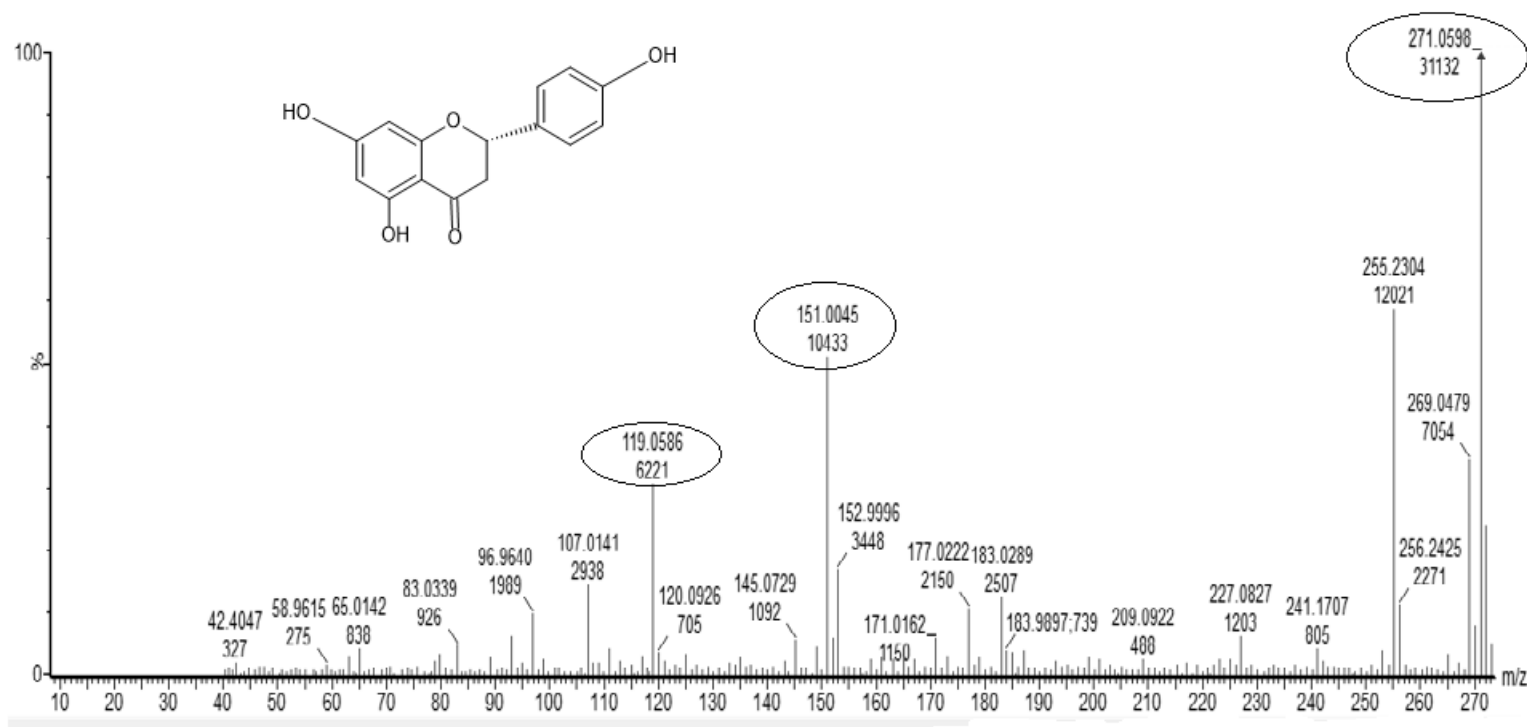


Figure 7-32: Mass spectrum of Naringenin (m/z 271) identified in extracts of sorghum

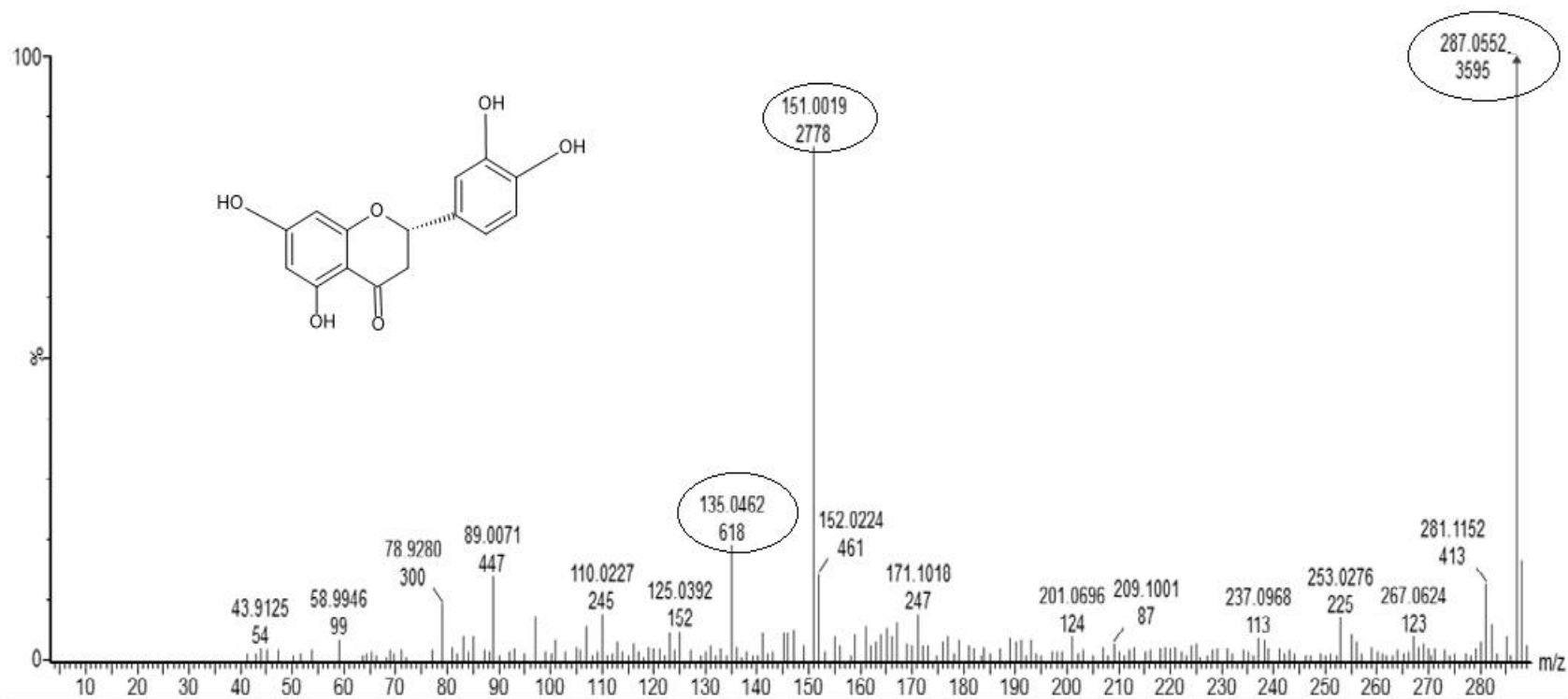


Figure 7-33: Mass spectrum of Eriodictyol (m/z 287) identified in extracts of sorghum

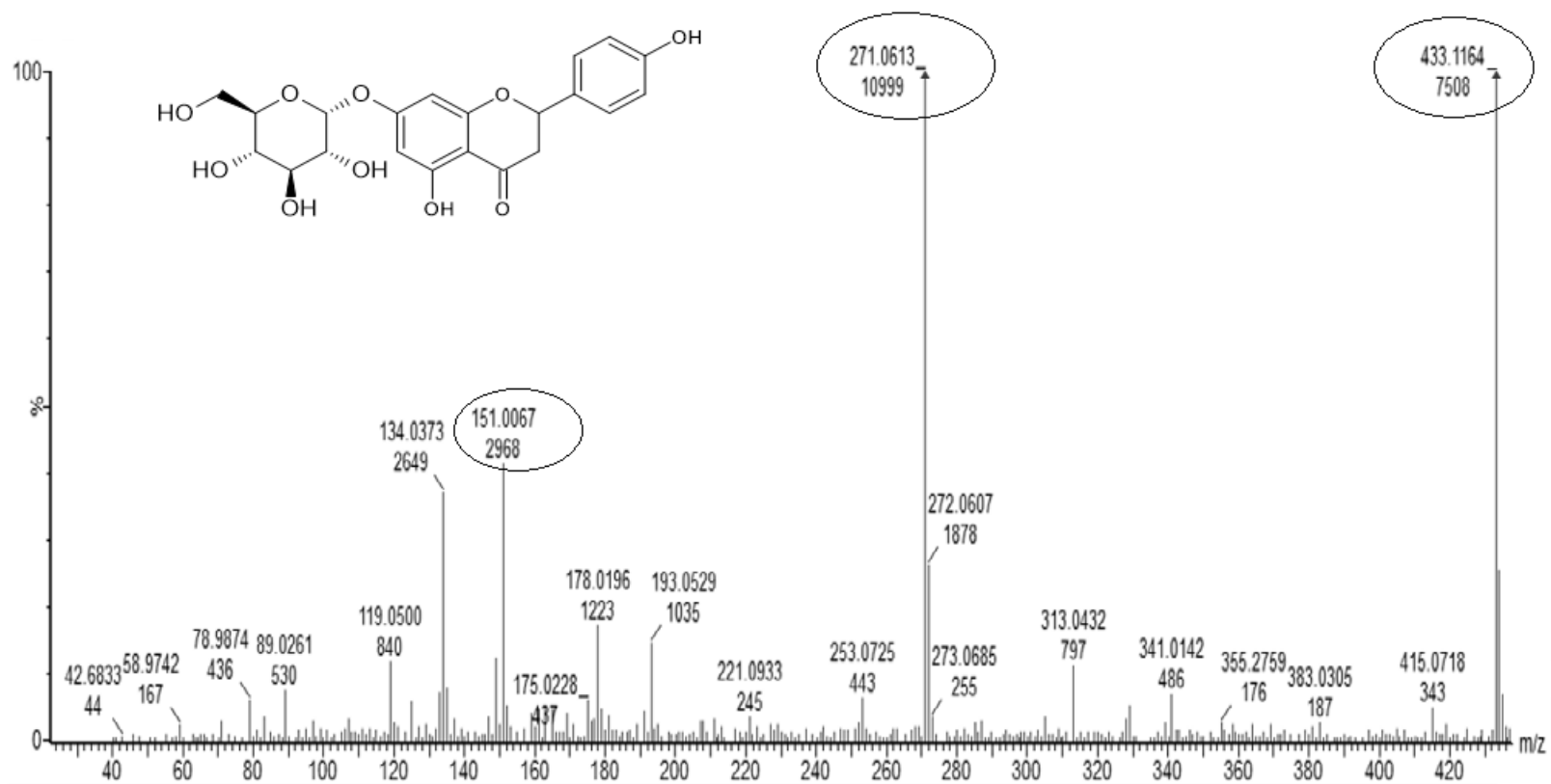


Figure 7-34: Mass spectrum of Naringenin-7-O-glucoside (m/z 433) identified in extracts of sorghum

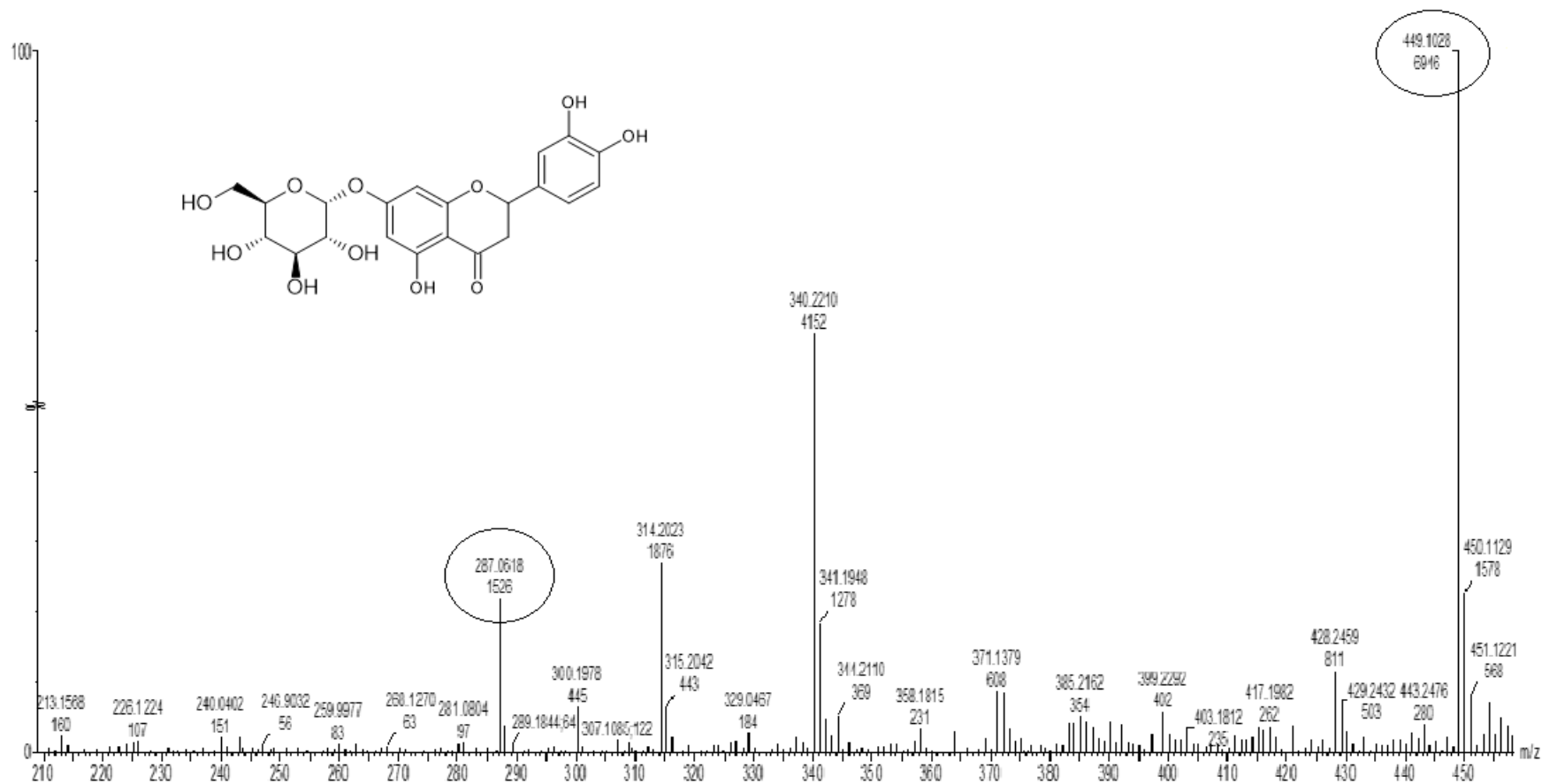


Figure 7-35: Mass spectrum of Eriodictyol 7-O-glucoside (m/z 449) identified in extracts of sorghum

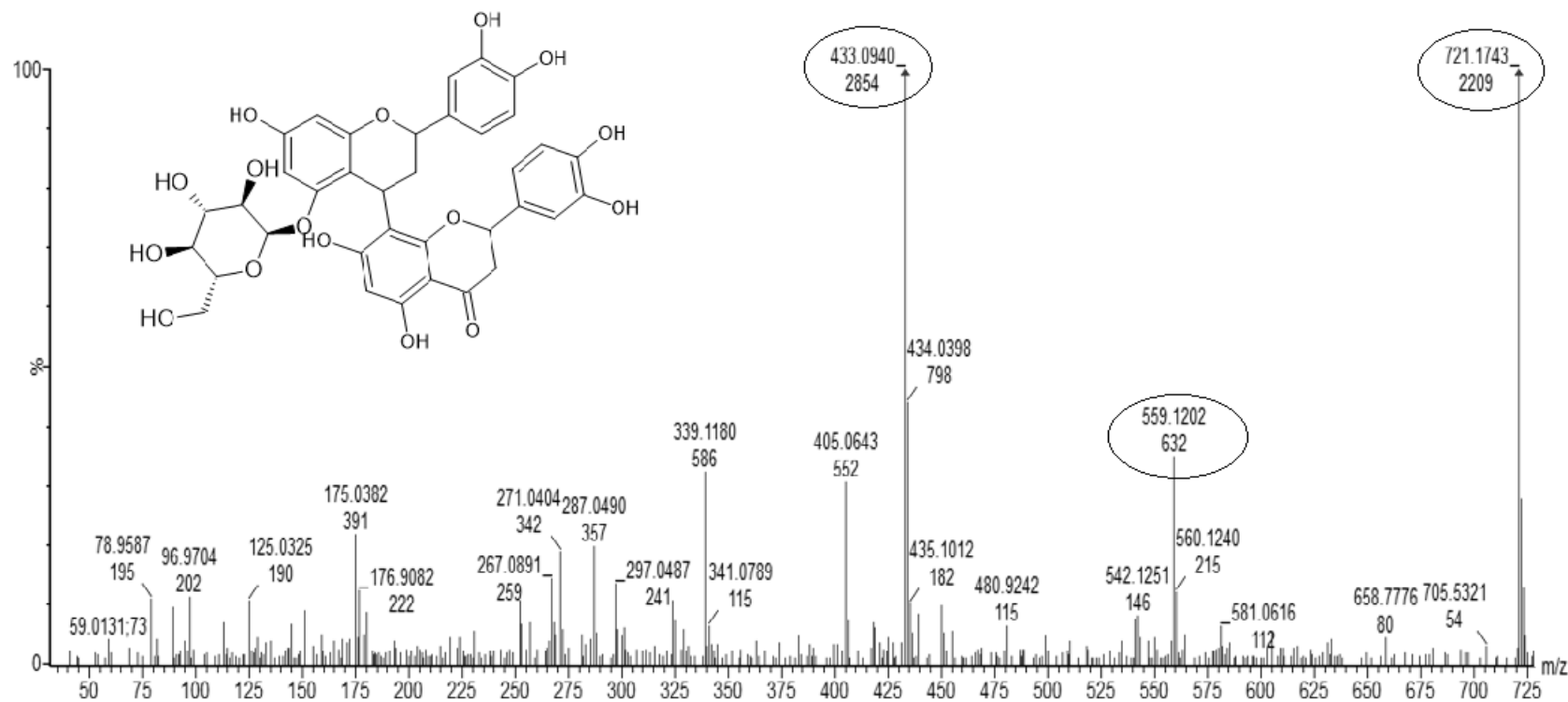


Figure 7-36: Mass spectrum of 5,7,3',4',-Tetrahydroxy-flavan-5-O-β-glucosyl-4-8-eriodictyol (m/z 721) identified in extracts of sorghum

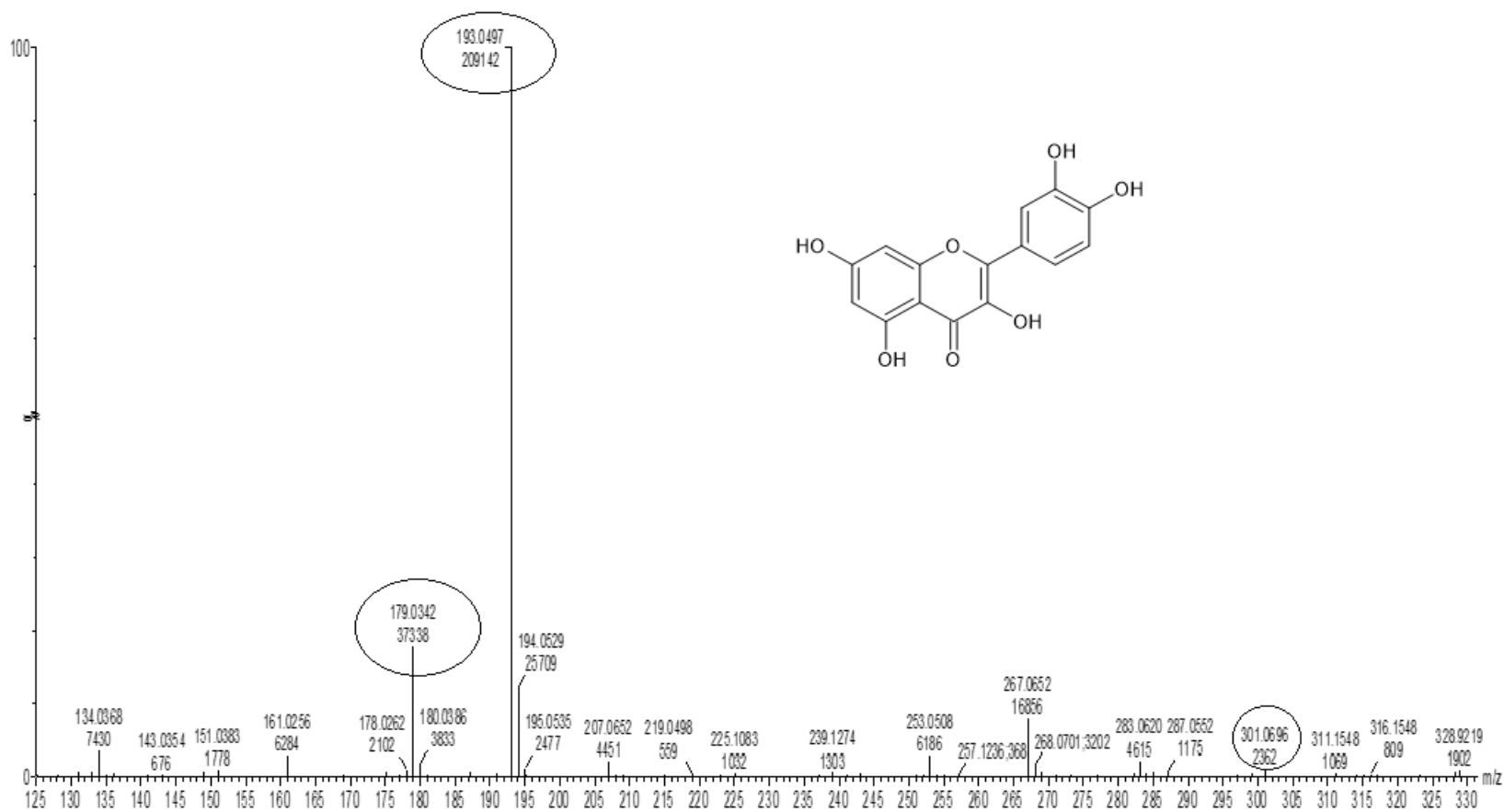


Figure 7-37: Mass spectrum of Quercetin (m/z 301) identified in extracts of sorghum

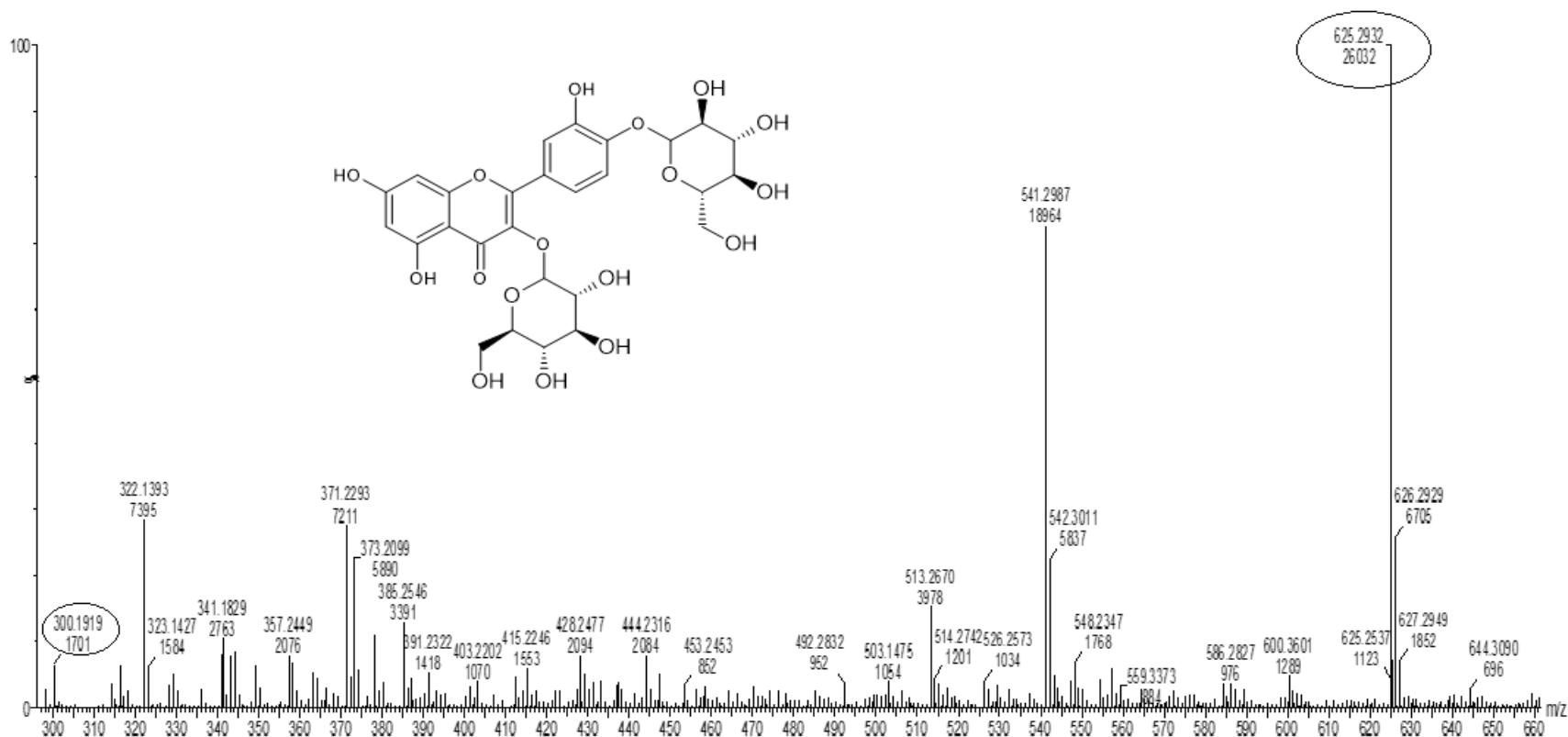


Figure 7-38: Mass spectrum of Quercetin diglucoside (m/z 625) identified in extracts of sorghum

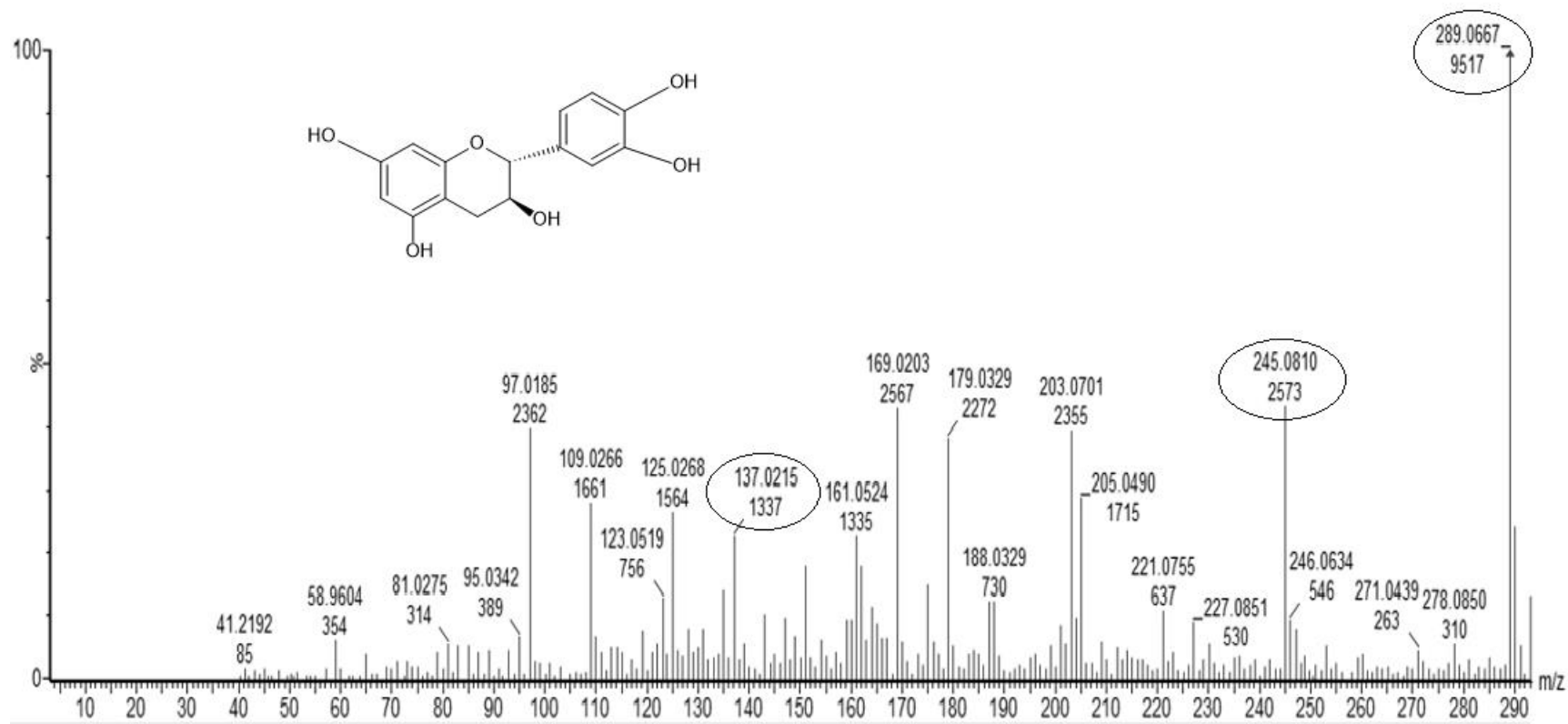


Figure 7-39: Mass spectrum of (+)-Catechin/ Epicatechin (m/z 289) identified in extracts of sorghum

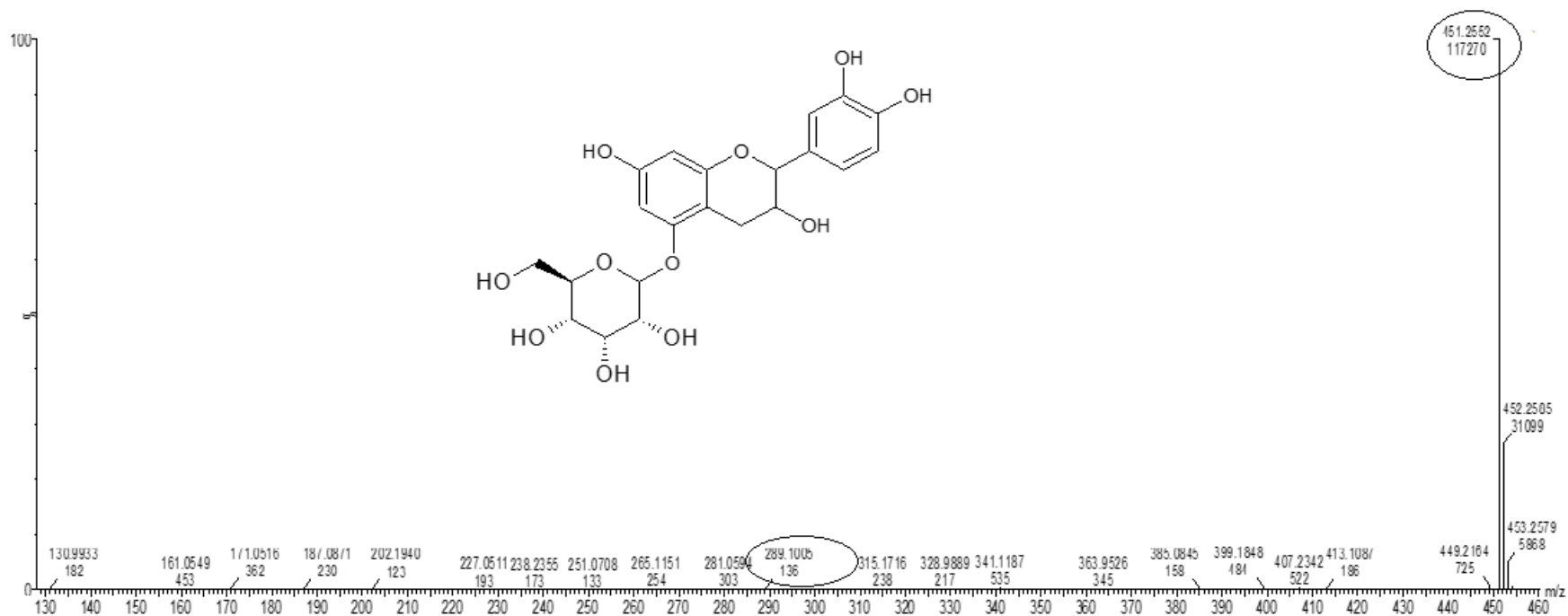


Figure 7-40: Mass spectrum of Catechin glycoside (m/z 451) identified in extracts of sorghum

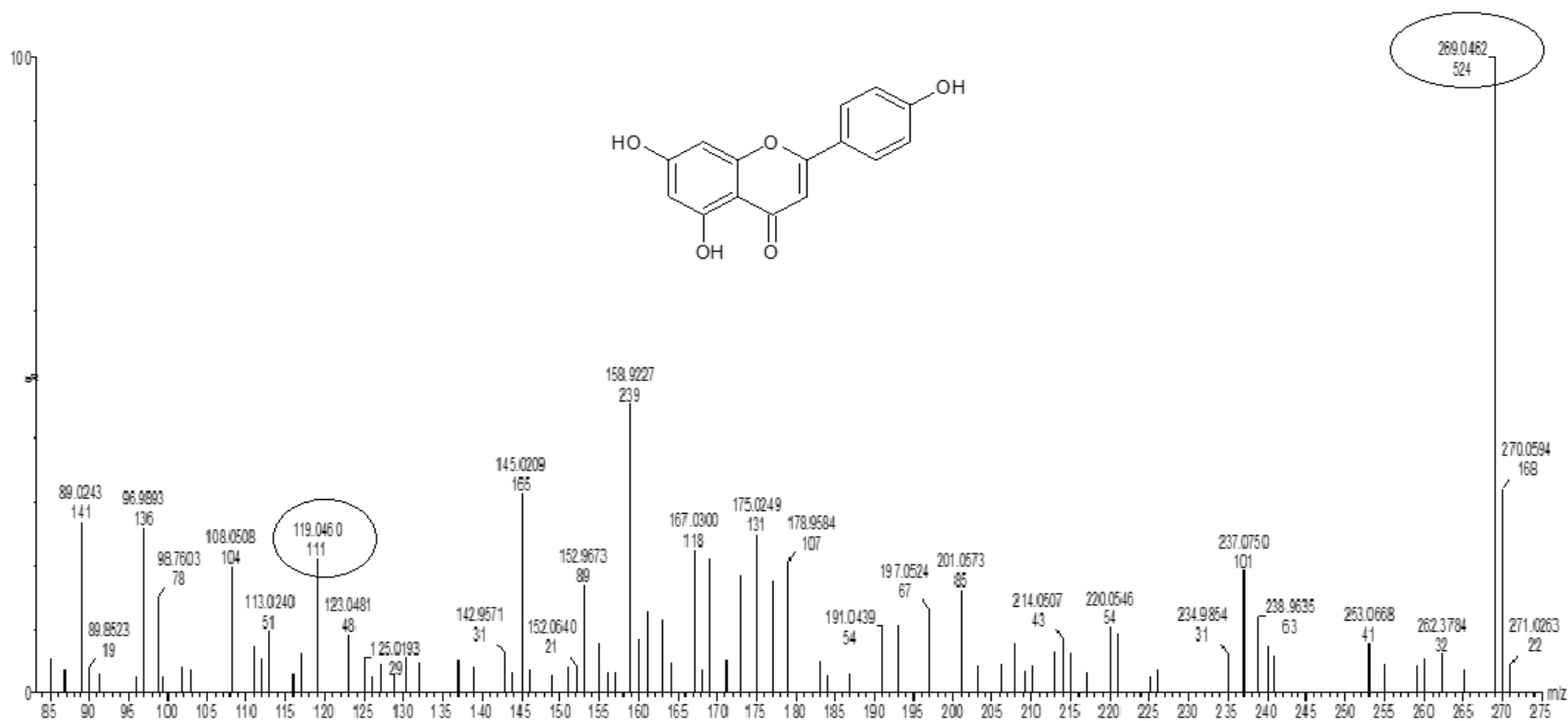


Figure 7-41: Mass spectrum of Apigenin (m/z 269) identified in extracts of sorghum