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Iron bioavailability, nutritional and health-promoting properties of extruded sorghum porridge fortified with Baobab fruit, moringa leaves and Bambara groundnut

by

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Submitted in partial fulfilment of the requirements for the degree

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DECLARATION

I, John Lubaale, hereby declare that this thesis submitted to the University of Pretoria for the award of the degree PhD Food Science is my work and has not been previously submitted by me for a degree at this or any other university or institution of higher education.

John Lubaale (U16209070):

Date: 16th July 2023

DEDICATION

This thesis is dedicated to my mother, Rose Margaret Najjuma Mukiibi-Lubaale, for your love, prayers and encouragement; you are indeed the wind beneath my wings.

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ABSTRACT

Iron bioavailability, nutritional and health-promoting properties of extruded sorghum porridge fortified with Baobab fruit and moringa leaves

By

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Iron deficiency, protein energy malnutrition (PEM) and the rise in diet-related non-communicable diseases (NCDs) are major public health concerns in developing countries. Food-to-food fortification (FtFF) is an emerging strategy that can be used to manage malnutrition. Moringa leaf powder (MLF) (rich in iron) and baobab fruit pulp (BFP) (rich in mineral bioaccessibility enhancers, such as ascorbic and citric acids), can be used in FtFF of starchy foods like sorghum to help reduce iron deficiency. They are also rich in bioactive polyphenols, which have been shown to have health-promoting properties in terms of offering protection against NCDs. Compositing cereals with legumes such as Bambara groundnut can improve the protein quality of cereal-based foods and thus help to address PEM. Extrusion cooking is used to produce convenient food products such as instant porridges, which are popular, particularly among urban communities in sub-Saharan Africa. It has been shown to reduce antinutrients in foodstuffs, thus improving the bioavailability of nutrients. There is, therefore, an opportunity to use FtFF and extrusion cooking technology to enhance nutritional quality (micro- and macronutrients) and health-promoting properties of sorghum-based foods. This research investigates the impact of FtFF (with BFP, MLP, and Bambara groundnut) and extrusion cooking on the iron bioaccessibility, health-promoting properties and macronutrient (protein and starch) quality of sorghum-based porridges.

Formulations of non-tannin sorghum-based flours (sorghum alone or composited with Bambara groundnut flour) were prepared by FtFF with BFP and MLP (either alone or in combination). Flours were cooked into porridges by conventional cooking or instantized using extrusion cooking at a feed moisture level of 3 L/h, barrel temperature zone profile of 60/70/80/140/140ºC and a screw speed of 250 rpm. The iron bioaccessibility (measured as *in vitro* dialyzability and ferritin formation by Caco-2 cells) of the plain sorghum-based porridges FtFF with BFP and MLP, phytate content, and total phenolic content (TPC) (Folin-Ciocalteu method were determined. Antioxidant properties of the porridges were determined using [(2,2-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS) and nitric oxide (NO) radical scavenging and oxygen radical absorbance capacity (ORAC)], while phenolic profiles of the foodstuffs used for fortification and the porridges were determined using liquid chromatography-mass spectrometry. Cellular antioxidant protection in human carcinoma (Caco-2) cells using the dichlorofluorescein diacetate (DCFH-DA) assay, NO scavenging activity in RAW264.7 macrophages, inhibition of advanced glycation end products (AGEs) and prevention and treatment of lipid droplet accumulation in 3T3-L1 cells were determined. The FtF-fortified composite (with Bambara groundnut) porridges were analysed for functional properties (water absorption and solubility index, nitrogen solubility index and flow properties), *in-vitro* starch digestibility (IVSD), soluble and insoluble dietary fibre content and *in-vitro* protein digestibility (IVPD).

Sorgum-based porridges fortified with BFP had higher iron bioaccessibility (*in vitro* iron dialysability) compared with porridges fortified with MLP. This indicates that the type of plant foodstuff used for FtFF had an effect on the resultant iron bioaccessibility. BFP had high levels of organic acids (citric and organic acids) that are well-known mineral bioaccessibility enhancers and could account for the enhanced iron bioaccessibility of porridges fortified with BFP. MLP was high in mineral bioaccessibility inhibitors (polyphenols, calcium and phytate). Polyphenols and phytate could form insoluble complexes with iron, and stable, insoluble complexes could be formed between iron, phytate and calcium, which reduces bioaccessible iron.

Extrusion-cooked instant sorghum-based porridges had increased ferritin formation by Caco-2 cells compared to conventionally wet-cooked porridges, which indicates an enhancing effect of extrusion cooking on iron bioaccessibility. This could be due to the ability of extrusion cooking to reduce the contents of antinutrients such as phytate (probably by dephosphorylation) and polyphenols (probably by degradation). Instant sorghum porridges fortified with BFP

produced higher ferritin formation in Caco-2 cells than porridges where MLP was used for FtFF. This was a further indication of the importance of the role of the type of plant foodstuff used for FtFF in mineral bioaccessibility.

FtFF of wholegrain sorghum-based porridges with BFP and MLP enhanced health-promoting properties of sorghum-based porridges in terms of radical scavenging activity (ABTS and ORAC) (protection against oxidative stress), cellular nitric oxide (NO) inhibition (anti-inflammatory properties) and inhibition of advanced glycation end products (AGEs) formation (antidiabetic properties). The observed enhanced health-promoting properties could be related to the enhanced levels of various bioactive phenolics (phenolic acids and their esters, flavonoids and flavonoid glycosides) in the sorghum-based porridges after FtFF. Phenolic extracts from the sorghum-based porridges showed protection against AAPH radical-induced oxidation in Caco-2 cells, an indication of their potential ability to protect against radical-induced oxidative stress.

Extracts from all the sorghum-based porridges reduced *in vitro* chemical formation of NO, an indication of their potential to contribute to alleviating radical-induced inflammation. FtFF significantly improved the inhibition of cellular NO production in RAW264.7 macrophages, possibly due to the enhancement of the phenolic profile of sorghum-based porridges following FtFF with baobab and moringa. Extrusion-cooked instant porridges exhibited decreased inhibition of NO formation in RAW264.7 macrophages, possibly due to their reduced phenolic content as a result of the extrusion cooking process.

Extracts from all the sorghum-based porridges showed prevention and treatment of accumulated adipocytes in 3T3-L1 cells, indicating their potential application in managing obesity. The porridges exhibited antidiabetic properties by reducing the formation of AGEs. The FtFF porridges, in particular, significantly reduced the formation of AGEs, possibly due to the increase in phenolic content and higher antioxidant activity following FtFF with BFP and MLP.

Sorghum-Bambara groundnut composite (SBC) porridges FtF-fortified with BFP and MLP showed a marked reduction in starch digestibility {decreased rapidly digestible starch (RDS) increased slowly digestible starch (SDS) and resistant starch (RS)} and estimated glycaemic index (GI) compared to the unfortified composite. The high levels of anti-nutritional compounds - polyphenolics, phytate, and soluble and insoluble dietary fibre (SDF and IDF) in the fortificants (BFP and MLP) could account for the reduced starch hydrolysis. Dietary fibre

could entrap starch molecules and reduce their accessibility to digestive enzymes, while polyphenols could form indigestible complexes with starch and could also bind enzymes responsible for the digestion of starch.

Extrusion-cooked instant SBC porridges had higher RS, RDS, and protein digestibility (IVPD) with lower SDS in comparison with conventionally cooked porridges. The dextrinisation of starch and reduction in anti-nutritional compounds (that bind both starch and proteins) because of the high temperature, shear and pressure conditions during extrusion cooking could make the starch and protein molecules more susceptible to enzymatic hydrolysis and lead to increased RDS, IVPD, and lower SDS. Extrusion-cooked SBC porridges had higher SDF and lower IDF compared to conventionally wet-cooked porridges, possibly due to the hydrolysis of glycosidic bonds in IDF during extrusion cooking, solubilising it into SDF. This increase in SDF could account for the increase in RS and SDS as the gelatinised and disrupted starch molecules could be entrapped in the SDF, making them less accessible for enzymatic hydrolysis (resistant starch type 1). Retrogradation of starch in the extruded porridges during storage could also produce enzyme-resistant starch (resistant starch type III). A third possible occurrence during extrusion is the formation of amylose-lipid complexes resistant to enzymatic hydrolysis (resistant starch type V). The high RS content of these instant sorghum-based porridges suggests they could be useful in managing type 2 diabetes.

Extrusion-cooked SBC porridges had lower pasting viscosities, probably due to the dextrinisation of starch (the primary biopolymer responsible for pasting) during the high temperature, shear and pressure conditions of extrusion cooking. This provides a shear-thinning porridge, which could increase nutrient intake for infants who have difficulty orally processing thick foods and thus preventing the prevalence of PEM.

In conclusion, extrusion cooking can be used to produce instant porridges from FtF-fortified and composited non-tannin sorghum with improved bioaccessibility of iron, protein and starch digestibility and health-promoting properties. Thus, extrusion cooking and FtFF of non-tannin sorghum can be employed as strategies to improve the nutritional and health status of at-risk communities.

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CHAPTER 1 INTRODUCTION

Malnutrition can be classified into three related but distinctly different problems: energy deficiencies, nutrient deficiencies, and excessive net energy intake, all of which result in diet-related non-communicable diseases (NCDs), and collectively they may be referred to as "the triple burden of malnutrition" (Davis, Oaks & Engle-Stone, 2020). Macro- and micronutrient (specifically iron deficiency) deficiency, as well as chronic non-communicable diseases (NCDs), are major public health concerns in developing countries (UNICEF/WHO/WBG, 2021). Iron deficiency is one of the micronutrient deficiencies, accounting for 30-50% of anaemia in children and women, with those in underdeveloped countries being the most affected (Rosli, Norhayati & Ismail, 2021). Of the nearly 150 million stunted children globally, 41% are in Africa, and 27% of the nearly 46 million wasted children globally are in Africa (UNICEF/WHO/WBG, 2021). Reports show a high prevalence of protein-energy malnutrition (PEM) and iron deficiency in Africa among children that manifests in the form of stunting, wasting, and increased disease burden, which often leads to mortality (Micha, Mannar, Afshin, Allemandi, Baker, Battersby, Bhutta, Chen, Corvalan & Di Cesare, 2020). Diet-related non-communicable diseases such as diabetes, obesity and cardiovascular diseases are on the rise in Africa, where the prevalence is expected to be about 5.2% by 2045 (Sun, Saeedi, Karuranga, Pinkepank, Ogurtsova, Duncan, Stein, Basit, Chan & Mbanya, 2022).

The leading cause of these forms of malnutrition is diet-related. A high proportion of the population of sub-Saharan Africa, especially the lower socioeconomic group, rely on monotonous starch-based diets (Gibson, Raboy & King, 2018). Starchy foods are often low in bioavailable minerals, and cereals specifically are high in mineral bioavailability inhibitors, notably phytate and polyphenols. The protein quality of cereals is low due to these inhibitors and also due to the fact that they are limiting in essential amino acids, particularly lysine (Hossain, Muthusamy, Zunjare & Gupta, 2019). Rapid urbanisation has driven demand for convenience foods among consumers, which influences dietary choices with a shift towards more energy-dense refined starch-based food products, high in total fats and especially saturated fat, leading to increased incidence of NCDs, in-part a result of oxidative stress (Haggblade, Duodu, Kabasa, Minnaar, Ojijo & Taylor, 2016; Nnyepi, Gwisai, Lekgoa & Seru, 2015; Vorster, Kruger & Margetts, 2011).

Cereals are a major staple and source of nutrition in sub-Saharan Africa. Sorghum is a major food crop across the semi-arid tropics of Africa because of its tolerance to high temperatures

and low rainfall (Taylor, 2019). In Africa, sorghum is cultivated majorly for consumption as soft and stiff porridges as well as alcoholic and non-alcoholic beverages (Taylor & Duodu, 2019).

Food-based approaches, such as dietary diversification and food-to-food fortification (FtFF) using micronutrient-rich foods, have been emphasised as sustainable ways to reduce the prevalence of micronutrient deficiencies in low socioeconomic groups (Olney, Rawat & Ruel, 2011). FtFF is a strategy whereby nutrient-rich food combinations are used to promote the bioavailability of essential micronutrients by increasing the content of micronutrients and /or enhancing their absorption and decreasing the levels of inhibitors of micronutrient bioavailability (Kruger, Taylor, Ferruzzi & Debelo, 2020). FtFF is not only about tackling micronutrient malnutrition. Compositing cereals with legumes for enhanced protein quality can also be regarded as a form of FtFF. Consumption of diets rich in phenolic compounds, such as whole grain-based diets, appears to be associated with the reduction or prevention of NCDs through their antioxidant activity (Duodu & Awika, 2019), thereby promoting health.

Extrusion cooking is a food processing technology that is applied to produce a variety of convenience-type products from a large diversity of raw materials (Guy, 2001). Extrusion cooking is a continuous food manufacturing process that applies high heat, pressure, and friction to transform raw foodstuffs into a cooked and pre-gelatinised form (Fellows, 2009). It can also destroy anti-nutritional compounds and enhances the digestibility of plant macronutrients (El-Hady & Habiba, 2003). Due to increasing urbanisation, there is a growing trend of consumer demand for instant products for the convenience they provide (Brennan, Brennan, Derbyshire & Tiwari, 2011). Extrusion cooking is, therefore, applied to produce convenience-type instant foods from grains in Africa, such as sorghum.

Thus, the purpose of this research was to study the effects of FtFF in combination with extrusion cooking on the nutritional and health-promoting attributes of sorghum-based porridge.

CHAPTER 2 : LITERATURE REVIEW

In this review, the principles of extrusion cooking and food-to-food fortification in the enhancement of the nutritional profile of cereal-based products will be discussed. The nutritional and health-promoting properties of the different foodstuffs used in this study will also be reviewed. The effects of extrusion cooking and FtFF on iron bioaccessibility, protein and starch quality, as well as the health-promoting properties of cereal-based products, will also be briefly discussed. Some of the key methods of analysis employed in this research study will also be discussed.

2.1 EXTRUSION TECHNOLOGY IN PREPARATION OF INSTANT PORRIDGES

2.1.1 Principles of Extrusion cooking

Extrusion processing provides the conditions of high shear, high temperature, and high pressure for a short time to cook food ingredients normally at a low moisture content (Kowalski, Hause, Joyner & Ganjyal, 2018; Guy, 2001). Feed materials typically change their phase into a melt form due to high shear and high temperature under high-pressure conditions. This melt is finally pushed through a die. As it exits the die, it is exposed to atmospheric conditions. At this point, the melt expands because of the immediate pressure drop and vaporisation of the blowing agents (i.e., water) (Moraru & Kokini, 2003). In addition to the steam flash-off, the die-swell characteristics of the materials also play a role in the expansion process. The different extrusion technologies operate on this same principle but have developed over many years to serve many additional functions in food processing. These functions include conveying, mixing, shearing, separation, heating or cooling, co-extrusion, venting volatiles and moisture, flavour generation, encapsulation, and sterilisation (Guy, 2001). Depending on the desired end product, extruders are manufactured and applied to make use of specific functions. Extrusion cooking, also referred to as High-Temperature Short Time (HTST) (temperature conditions above 100°C) extrusion, applies shear as well as pressure to continuously and rapidly process starchy and protein-rich raw materials into pre-cooked foods. Extrusion cooking is applied in the production of ready-to-eat cereals (Singh et al., 2007). Mixing, cooking, kneading, shearing, shaping, and forming are the operations combined in extrusion cooking to continuously break down raw foods into a cooked and pre-digested form (Fellows, 2009). What makes extrusion cooking unique compared to other HTST processes are the shear forces applied, which break covalent bonds in biopolymers (Carvalho & Mitchell, 2000).

Many reactions, such as gelatinisation of starch, denaturation of proteins, and Maillard reactions, also occur during extrusion cooking (Mościcki & van Zuilichem, 2011). All these reactions change the physical and chemical properties of the processed materials, thereby affecting the product's functional and nutritional properties. The outcome of extrusion cooking is ready-to-eat and easily packaged products (Kazemzadeh, 2012). Extrusion cooking also reduces the number of microorganisms and inactivates endogenous enzymes (Fellows, 2009). The low moisture content in the end products of extrusion cooking (7-10%), often achieved by drying the extruded products, is, however, the primary method of preservation (Mościcki, 2011). The benefits of extrusion cooking include versatility in end products, low processing costs with high productivity, and the production of almost no waste products, such as effluents (Guy, 2001).

There are many critical control points throughout the extrusion cooking process which affect the end product quality. To understand the role of raw materials in product quality, an understanding of extrusion cookers and the extrusion cooking process is necessary. Among many options to choose from in extrusion cooking is the application of either one or two screws in the extruder barrel, referred to as single or twin-screw extrusion cookers (Riaz, 2001). The main difference between the two extruders is in the mode of operation (Mościcki & van Zuilichem, 2011). In a single-screw extruder, the screw plays the function of conveying, compressing, melting and plasticising the material and finally forcing it under pressure through small die holes at the end of the barrel. Shear is generated due to the rubbing of the material against the barrel and the screw surfaces and between particles and particles of the raw material. However, in twin screw extruders, the barrel lining is generally smooth; thus, most of the shear is generated due to the rubbing of the particles against each other while the material is being conveyed along the two screws (Mościcki & van Zuilichem, 2011). The opportunities for the shear generation are tremendous in a twin-screw extruder. In short, twin screw extruders have greater flexibility regarding raw material size and nature (viscous, oily, wet, or sticky) and have greater control of parameters to achieve the desired end product (Fellows, 2009). Single screw extruders show low efficiency when a multi-component mixture is used as a raw ingredient (Mościcki & van Zuilichem, 2011). The only disadvantages of twin screw extrusion cookers are that they are of a more complicated design and have higher initial costs (Mościcki & van Zuilichem, 2011).

Cooking in the extrusion cooker starts mainly in the compression section and intensifies in the plasticising section. The change in physical structure and chemical components of the material

related to cooking is due to three sources of energy: conductive energy through heating of the barrel, convective energy within the barrel from incorporated moisture reaching high temperatures and pressures and conversion energy when the movement of the screws in the confined space of the barrel causes shear and produces pressure (Kazemzadeh, 2012). Exposure of the foodmaterial to these energies brings the food material to its melting or plasticising point (Mościcki & van Zuilichem, 2011), after which it is forced through one or more die openings. As the food material is forced out under pressure through the die, it expands to the final shape (due to the rapid evaporative cooling as the product is being pushed through the die) and cools rapidly to retain its structure as moisture is flashed off as steam (Fellows, 2009). Table 2-1 summarises the key variables that are vital during extrusion.

Table 2-1: Extrusion cooking process variables and their function in the process (Yacu, 2011; Chessari & Sellahewa, 2001)

Process variable	Effect
Screw speed	Affects the residence time of the product in the barrel, the amount of shear and frictional energy generated, and barrel fill which affects melt viscosity.
In-barrel moisture content	Controls the frictional energy generated by affecting the melt viscosity.
Feed rate	Controls the amount of feed in the barrel, which in turn affects residence time and pressure in the barrel.
Barrel temperature	Determines product temperature, affecting the degree of cook and melt viscosity.

2.1.2 Effects of extrusion cooking on mineral bioavailability, antinutrients and macronutrient quality of raw materials used

The effects of extrusion cooking on the various nutrients and antinutrients explored in this research study will be briefly discussed.

2.1.2.1 Mineral bioavailability/bioaccessibility

Bioavailability is defined as the amount of an ingested active compound that is absorbed and is available for physiological functions (Price & Patel, 2021). Due to the complexity and time-

consuming nature of assays for bioavailability, *in vitro* approaches are used to estimate bioavailability and referred to as bioaccessibility, which is defined as the amount of an element that has the potential to be absorbed and utilised for physiological functions (Etcheverry, Grusak & Fleige, 2012). Mineral bioavailability in plant-based foods is influenced by two main factors, that is, the chemical state of the element (e.g., mineral, free ion, oxidation state) and its association with flour components such as phytic acid, dietary fibre, and proteins. Extrusion cooking can affect either or both factors positively or negatively and thus influence the bioaccessibility of minerals.

Researchers have reported differing findings on the effect of extrusion on the bioaccessibility of different mineral elements. Several authors have reported a significant improvement in Fe bioaccessibility upon extrusion of maise (Hazell & Johnson, 1989), sorghum (Vilakati, Taylor, MacIntyre & Kruger, 2016), legumes (Ummadi, Chenoweth & Uebersax, 1995), and dry beans (Gulati & Rose, 2018), while others have reported no change in iron and zinc bioaccessibility upon extrusion of a wheat bran-flour mixture and dry beans (Drago, Velasco-González, Torres, González & Valencia, 2007; Fairweather-Tait, Portwood, Symss, Eagles & Minski, 1989). Gulati and Rose (2018) and Alonso, Rubio, Muzquiz and Marzo (2001) observed significant increments in magnesium and phosphorus bioaccessibility in dry bean flours upon extrusion, Kivistö, Andersson, Cederblad, Sandberg, and Sandström (1986) reported significant decreases in Mg and P absorption when a high fibre cereal-based product was extruded. The contradictory results reported by different researchers for various mineral elements could be due to different techniques used for measuring bioaccessibility or due to different raw materials used for extrusion. For example, variable Fe bioaccessibility upon extrusion of five Italian legumes was reported, with some showing improvement, others no change, and still others showing reduced bioaccessibility (Lombardi-Boccia, Lullo & Carnovale, 1991). Extrusion influences mineral bioavailability mainly due to its effect on mineral-binding components present in cereals, such as phytic acid, phenolic compounds, and dietary fibre.

2.1.2.2 Anti-nutritional factors

While vital for health promotion due to their potential to prevent non-communicable diseases (Echeverria & Valenzuela, 2022), phenolic compounds are considered antinutrients due to their ability to bind nutrients and digestive enzymes (Duodu, 2011). Extrusion cooking has been reported to have different effects on phenolic compounds. Extrusion cooking of wholegrain red sorghum was found to cause a significant decrease in assayable phenolic compounds (by 14 to 19%) (Llopart, Drago, De Greef, Torres & González, 2014). In single screw extrusion cooking of sorghum at an in-barrel moisture of 15%, phenolic compounds were more extractable than at 18%, presumably due to their depolymerisation (Dlamini, Taylor & Rooney, 2007a). However, extrusion cooking at 18% moisture rendered them less extractable, probably due to the polymerisation of phenolics. Llopart *et al.* (2014) proposed that greater interaction between protein and phenolic compounds occurs at lower moisture extrusion cooking conditions.

Concerning phytate, an observed reduction in phytate in red sorghum during extrusion cooking was found to be dependent on temperature increase rather than on the in-barrel moisture content (Llopart *et al.*, 2014). Increasing in-barrel moisture content when extrusion cooking cereal brans (18 to 22% moisture) (Kaur, Sharma, Singh & Dar, 2015) and various legumes (14 to 20% moisture) (El-Hady & Habiba, 2003) was, however, found to cause a reduction in phytate. Decreases in phytate during extrusion cooking were found to be due to the hydrolysis of inositol hexakisphosphates into lower phosphates (Alonso *et al.*, 2001) or due to the formation of insoluble complexes between phytate and other charged components (Kaur *et al.*, 2015).

Extrusion cooking has been reported to increase total dietary fibre content through the formation of resistant starch and to change the ratio of soluble dietary fibre to insoluble dietary fibre (Østergård, Björck & Vainionpää, 1989). Extrusion cooking was found to cause fibre depolymerisation and, as a result, increased the proportion of soluble dietary fibre (Oladiran & Emmambux, 2017). Increasing the in-barrel temperature has also been reported to increase the total dietary fibre in wheat due to the formation of lignin-like substances (Theander & Westerlund, 1987). Shear stress in the barrel generates strain on the insoluble fibre macromolecular structure and results in chemical bond breakage and the creation of smaller soluble compounds (Ralet, Saulnier & Thibault, 1993). However, high pressure in the barrel may have a greater effect on fibre solubility than the shear rate generated during high screw speed (Gualberto, Bergman, Kazemzadeh & Weber, 1997). Reduction in insoluble dietary fibre, however, reduces the quality of extrudates by decreasing the radial expansion, hence

increasing the bulk density and giving harder texture and less crispy products (Lue, Hsieh & Huff, 1991).

2.1.2.3 Starch

Considering the majority of the food products that are made using extrusion, the major ingredients used in these products are often cereal grain flours and/ or components of the flours, mainly starches. In many extrusion cooking applications, including sorghum-based systems, starch is the structure-forming material (Guy, 2001). The gelatinisation of the starch (Pelembe, Erasmus & Taylor, 2002) and the viscosity of the resulting starch melt in the extruder barrel (Mahasukhonthachat, Sopade & Gidley, 2010) have implications on the physical and functional properties of the resulting product. The fate of starch in extrusion cooking is thought to take place through a unique mechanism when compared to starch gelatinisation through hot pasting. The mechanism of extrusion relies on granule mechanical disruption due to high friction and shear, compared to the swelling of starch granules due to an excess of water in pasting (De Muelenaere, 1989). The extrusion mechanism largely involves starch gelatinisation and melting (involving destruction of the starch granules as a result of cleavage of the intermolecular hydrogen and covalent bonds) and fragmentation into dextrinised starch and oligosaccharides (Lai & Kokini, 1991; Gomez & Aguilera, 1984). The digestibility of starch in extruded products is high (Péronnet, Meynier, Sauvinet, Normand, Bourdon, Mignault, St-Pierre, Laville, Rabasa-Lhoret & Vinoy, 2015). As a result, extrusion-cooked starchy foods often have a high glycaemic index (GI) (Camire, 2001), which may contribute to insulin resistance and the risk of type 2 diabetes (Ceriello & Colagiuri, 2008). This has informed several studies focusing on the reduction of the rate and extent of starch digestion in food products. The effect of extrusion on starch digestibility is dependent on the different types of starch, whether normal, waxy or high-amylose (Robin, Heindel, Pineau, Srichuwong & Lehmann, 2016).

Partial dextrinisation of starch during extrusion cooking increases its molecular mobility resulting in a greater probability of retrogradation (Zhang, Liu, Liu, Luo, Li, Liu, Wu & Zuo, 2014). Due to the detrimental effect of retrogradation on the sensory quality of starchy foods, research has been focused on reducing or retarding retrogradation (Wang, Li, Copeland, Niu & Wang, 2015). However, many studies have also shown that enzyme-resistant starch (RS) can be formed by retrogradation following extrusion (Kim, Tanhehco & Ng, 2006; Faraj, Vasanthan & Hoover, 2004; Huth, Dongowski, Gebhardt & Flamme, 2000; Unlu & Faller, 1998). RS is particularly important due to its role in reducing the GI of foods. The rate of

digestion of RS-containing foods in the small intestine is lower than rapidly digestible starch-rich foods leading to a sustained and lower level of glucose release (lower glycaemic load). Faraj *et al.* (2004) and Huth *et al.* (2000) have reported increments in retrograded starch and reduction in starch digestibility following storage of undried extrudate. RS formation during extrusion cooking can also result from complexation between starch and particular lipids (Panyoo & Emmambux, 2017). This type of RS is referred to as RS V. When considered as dietary fibre, RS V can be categorised depending on whether it is amorphous or semicrystalline as type I or type II (Panyoo & Emmambux, 2017). These starch-lipid complexes can be applied in the food industry for purposes such as enhancement of freeze-thaw stability, retardation of staling in bread and biscuits, and prevention of stickiness in starchy foods (Gulati, Brahma & Rose, 2020). Due to its slow degradation by bacteria in the lower intestine, RS is associated with health benefits due to a slower release of glucose into the bloodstream, which can result in lower postprandial glycaemic and insulin responses (Birt, Boylston, Hendrich, Jane, Hollis, Li, McClelland, Moore, Phillips & Rowling, 2013).

2.1.2.4 Proteins

The nutritional value of protein is dependent on the quantity, digestibility and availability of essential amino acids (Singh, Gamlath & Wakeling, 2007). Extrusion cooking variables greatly influence protein digestibility (Ek & Ganjyal, 2020). The presence of the anti-nutritional factors phytate, phenolics (tannins), protease inhibitors, and dietary fibre can also reduce the amount of protein available for intestinal absorption (Camire, 2001). Studies have shown that high extrusion temperature along with shear can significantly reduce the anti-nutritional factors and promote protein digestibility (Arun Kumar, Mani, Pramod, Samuel, Jha, Sahoo, Sinha & Kar, 2018; de Morais Cardoso, Pinheiro, Martino & Pinheiro-Sant'Ana, 2017) Proteins can also unfold as a result of thermal and mechanical denaturation during extrusion rendering them more accessible to proteolytic enzymes (Gulati *et al.*, 2020). Fapojuwo, Maga and Jansen (1987) reported improved protein digestibility of sorghum flour when extruded compared to a 20% loss in digestibility when conventionally cooked. Hamaker, Kirleis, Butler, Axtell and Mertz (1987) reported a similar increment in protein digestibility following the extrusion of sorghum. This is primarily due to the breaking of disulphide linkages of sorghum prolamin proteins at high screw speeds that otherwise cause lower protein digestibility of sorghum flour.

2.2 PLANT FOODSTUFFS USED IN THIS STUDY

2.2.1 Sorghum

Sorghum (*Sorghum bicolor* (L.) Moench) is an important food crop in many parts of Africa, Asia and the semi-arid tropics worldwide (Taylor, 2019). Sorghum is a drought-tolerant crop which requires little husbandry during growth and can withstand periods of water logging. In this regard, sorghum has a distinct advantage over other major cereals. World sorghum production was estimated at 61.4 million tonnes in 2021, with Africa contributing over 40% of this production (FAO, 2022). Sorghum is the dietary staple of more than 500 million people in more than 30 countries (Reddy, Ashok Kumar & Sanjana Reddy, 2010).

The nutrient composition of sorghum indicates that it is a good source of energy, protein, carbohydrate, vitamins and minerals, including trace elements (particularly zinc and iron), like most cereals (Serna-Saldivar & Espinosa-Ramirez, 2019). As with most cereals, starch and protein are the major components of sorghum, accounting for 56-76% and 6-20%, respectively (Bean, Smith, Wilson, OIoerger & Tilley, 2019). The starch composition of sorghum is similar to most starches and is majorly (98-99%) composed of the α-glucans, amylose and amylopectin (Bean *et al.*, 2019). While albumins and globulins (accounting for about 10-30%) and glutelins (accounting for about 4-35%) have been reported in sorghum, the primary storage proteins in sorghum are prolamins (accounting for 50-70%) (Taylor, Schussler & Van der Walt, 1984). A major concern of sorghum protein quality is it is limited in amino acid lysine while rich in sulphur-containing amino acids (Bean *et al.*, 2019).

Sorghum's nutritional quality is dictated mainly by its chemical composition and the presence of anti-nutritional factors such as phytate and phenolic compounds. Phytate (phytic acid) is the principal storage form of phosphate in grains and is ubiquitously distributed in plants. The effects of phytate in human and animal nutrition are related to the interaction of phytic acid with proteins, vitamins and several minerals, thereby restricting their bioavailability (Elkhalil, El Tinay, Mohamed & Elsheikh, 2001). In a study of 45 sorghum lines, the phytate content varied between 0.47 and 3.53 g/100 g, depending on genetic and environmental factors (Kayodé, Linnemann, Hounhouigan, Nout & van Boekel, 2006).

Phenolic compounds are the largest group of secondary metabolites found in plant foods (Kroll, Rawel & Rohn, 2003). Although phenolic compounds are seen as health-promoting, they also function as antinutrients due to their ability to bind nutrients, rendering them less available for

utilisation in the body. They can be divided into three major categories: phenolic acids, flavonoids and tannins (Serna-Saldivar & Espinosa-Ramirez, 2019). Dlamini *et al.* (2007a) reported a total phenolics content of 0.27-0.53 g catechin equivalents (CE)/100 g in sorghum.

Due to the high phytate and phenolic content of sorghum, the bioaccessibility of minerals such as iron is reduced. Iron bioaccessibility is negatively influenced by various inherent factors such as phytate, phenolic compounds, calcium and insoluble dietary fibre, calcium and fibre (both soluble and insoluble) (Hemalatha, Platel & Srinivasan, 2007).

2.2.2 Bambara groundnuts

Bambara groundnut (*Vigna subterranea* L. Verdc) is an underutilised annual legume native to Africa (Esan, Oke & Ogunbode, 2023; Khan, Rafii, Ramlee, Jusoh & Al Mamun, 2022; Tan, Azam-Ali, Goh, Mustafa, Chai, Ho, Mayes, Mabhaudhi, Azam-Ali & Massawe, 2020a). It has gained traction lately due to its nutrient density and its tolerance to drought (Esan *et al.*, 2023). Global production is 0.4 million metric tonnes, with nearly all production coming from Africa and scanty production in Thailand, Malaysia, and Indonesia (FAO, 2022). The main chemical component of Bambara groundnuts is also starch (50-65%) (Nwadi, Uchegbu & Oyeyinka, 2020), but it has a larger proportion of amylose (37%) (Ashogbon, 2014) than sorghum starch. The amylose content of Bambara groundnut starch is in line with the starch amylose contents of other pulses, which are generally higher (35-46%) than cereals (Rao, 1976). Bambara groundnuts are also higher in protein (17-25%) compared to sorghum, with major protein fractions being albumins and globulins (Poulter, 1981). The protein content of Bambara groundnuts is similar to that of other legumes such as chickpeas, cowpeas, lentils and green peas (Iqbal, Khalil, Ateeq & Khan, 2006).

The protein quality of Bambara groundnut, as with other legumes, is limited by its deficiency in sulphur-containing essential amino acids, particularly methionine (Tan, Azam-Ali, Goh, Mustafa, Chai, Ho, Mayes, Mabhaudhi, Azam-Ali & Massawe, 2020b; Adeleke, Adiamo, Fawale & Olamiti, 2017). This is coupled with high phenolic content, particularly tannins (0.2-1.8%) and trypsin inhibitors (0.06-73 units/mg of protein) (Tan *et al.*, 2020b; Halimi *et al.*, 2019). Bambara groundnut also contains high contents of phytic acid (0.1-1.5%) (Yao, Kouassi, Erba, Scazzina, Pellegrini & Casiraghi, 2015; Mazahib, Nuha, Salawa & Babiker, 2013). Another critical factor limiting the utilisation of legumes in general and Bambara groundnut, in particular, is the presence of oligosaccharides of the raffinose sugar family

(stachyose being the most abundant, 0.75-1 g/100 g (Apata, 2008). Due to the absence of α-galactosidase in humans, oligosaccharides cannot be digested in the upper part of the gastrointestinal tract leaving them to be fermented in the colon by bacteria causing abdominal discomfort (Halimi, Barkla, Mayes & King, 2019).

Due to Bambara groundnuts' high protein and mineral contents (iron being the mineral of interest, Bambara groundnut is reported to have 11-150 mg/100 g of iron), it is used in composites with cereals and even tubers to improve the nutritional qualities of traditional meals (Tan *et al.*, 2020b; Halimi *et al.*, 2019). Cereals and tubers that are composited with Bambara groundnuts include maize for traditional African maise-based foods such as 'ogi', a starchy gruel (Mbata, Ikenebomeh & Ezeibe, 2009), pearl millet for the formulation of 'agidi', a stiff gel, (Zakari, Hassan & Abbo, 2010); malted sorghum and fermented sweet potato for porridge (Nnam, 2001) and fermented cassava for the production of 'fufu' (Oluwole & Olapade, 2011). Bambara groundnuts have also been investigated in composites in products such as biscuits (Abu-Salem & Abou-Arab, 2011) and bread (Alozie, Iyam, Lawal, Udofia & Ani, 2009). Extrusion cooking has been applied to produce white yam and Bambara groundnut expanded extrudates (Oluwole & Olapade, 2011), sorghum malt and Bambara groundnut-based extrudates (Jiddere & Filli, 2015) and pearl millet and Bambara groundnut based fura (Filli, Nkama & Jideani, 2013).

2.2.3 Moringa

Moringa oleifera Lam. is a tree belonging to the monogeneric genus Moringa of the Moringaceae family. It is native to the Indian subcontinent, but due to its ability to grow in humid and hot dry lands and survive in less fertile soils chronically affected by drought, it is grown in many tropical and subtropical areas around the world (Anwar, Latif, Ashraf & Gilani, 2007). It has been defined as a multi-purpose tree, as all parts can be utilised for different purposes. The leaves are the most used part of the plant. In particular, they are used for human and animal nutrition and in traditional medicine to treat many ailments (Popoola & Obembe, 2013). Several *in vitro* and *in vivo* studies have ascribed numerous pharmacological properties to *M. oleifera* leaves, although the scientific evidence on their health effects in humans is still limited (Leone, Spada, Battezzati, Schiraldi, Aristil & Bertoli, 2015b). On a dry basis, moringa leaves are rich in dietary fibre, proteins, calcium, iron, potassium, vitamins (particularly C and E), and β-carotene (Moyo, Masika, Hugo & Muchenje, 2011; Yaméogo, Bengaly, Savadogo,

Nikiema & Traore, 2011; Jongrungruangchok, Bunrathep & Songsak, 2010; Sánchez-Machado, Núñez-Gastélum, Reyes-Moreno, Ramírez-Wong & López-Cervantes, 2010; Owusu, Ellis & Oduro, 2008; Aslam, Anwar, Nadeem, Rashid, Kazi & Nadeem, 2005), and in antioxidant and bioactive compounds, such as flavonoids, phenolic acids, glucosinolates and isothiocyanates, tannins and saponins (Popoola & Obembe, 2013; Anwar *et al.*, 2007). Although considered antinutrients, phenolic compounds have been proposed to confer health-promoting properties (Awika & Rooney, 2004). Moringa leaves contain approximately 2970 mg total phenolics/100 g (dry basis) (Leone, Fiorillo, Criscuoli, Ravasenghi, Santagostini, Fico, Spadafranca, Battezzati, Schiraldi, Pozzi, Di Lello, Filippini and Bertoli (2015a). These reduce the mineral availability of the moringa. The phytate content of moringa leaves is considerably high, 2-3 g/100 g (dry basis) (Adetola, Kruger, White & Taylor, 2019; Leone *et al.*, 2015a; Stevens, Ugese, Otitoju & Baiyeri, 2015).

2.2.4 Baobab

Baobab (*Adansonia digitata* L.) is a fruit tree endemic to the Savannah drylands of sub-Saharan Africa. Local communities mainly utilise the leaves, pulp, and seeds of baobab as a source of food and for income generation (Muthai, Karori, Muchugi, Indieka, Dembele, Mng'omba & Jamnadass, 2017). Ripe baobab fruits are large, egg-shaped, 15–20 cm long, with a hard woody outer shell covered with yellowish-brown hairs and are filled with a dry white powdery pulp that covers brownish bean-like seeds (Tembo, Holmes & Marshall, 2017; Coe, Clegg, Armengol & Ryan, 2013; Besco, Braccioli, Vertuani, Ziosi, Brazzo, Bruni, Sacchetti & Manfredini, 2007; Shahat, 2006). The pulp, which is usually eaten fresh, is acidic (pH 3.2). Several studies have shown that baobab fruit pulp is very rich in vitamin C with a content of up to 540 mg/100 g on a fresh weight basis. The seeds contain high levels of polyphenols (epicatechin and procyanidin), provitamin A carotenoids and fatty acids (linoleic and oleic acids) and show high antioxidant activity (Del Rio, Rodriguez-Mateos, Spencer, Tognolini, Borges & Crozier, 2013; Vermaak, Kamatou, Komane-Mofokeng, Viljoen & Beckett, 2011). However, utilisation is limited due to insufficient knowledge about the effects of processing conditions for quality control (Tembo *et al.*, 2017). The high vitamin C content (ascorbic acid + dehydro-ascorbic acid) of baobab pulp is completely lost in the final juice due to excessive heating (Tembo *et al.*, 2017).

The fruit pulp is mainly used in beverages and food preparation. It is an excellent source of carbohydrates (85.0%, dry basis) but low in protein and fat (9.2% and 0.3%, dry basis, respectively). The fruit pulp is high in potassium, sodium, calcium and magnesium (approximately 1384, 31, 329, and 100 mg/100 g, respectively) but low in iron, zinc and copper (approximately 10.4, 2.0, and 1.8 mg/100 g, dry basis, respectively). Nhukarume, Chikwambi, Muchuweti and Chipurura (2010) and Braca, Sinisgalli, De Leo, Muscatello, Cioni, Milella, Ostuni, Giani and Sanogo (2018) reported total phenolic contents of between 12-58 mg gallic acid equivalent/100 g and 120-161 mg gallic acid /g in baobab fruit pulp, respectively and phytic acid was reported to be approximately 0.2% on dry basis (Adetola, Kruger, White & Taylor, 2019; Osman, 2004).

2.3 MECHANISM OF FERRITIN FORMATION IN CELLS AS AN INDICATOR OF IRON UPTAKE

Dietary iron can exist as either inorganic iron or haem iron, with inorganic iron being the most prevalent in diets specifically of plant origin (Anderson, Frazer, McKie, Vulpe & Smith, 2005). While the absorption of haem-iron is less well understood, it is far more efficient than non-haem iron, apparently by endocytosis of the intact iron–protoporphyrin complex at the enterocyte brush border (Anderson *et al.*, 2005) Figure 2-1.

From a nutritional perspective, not only is the content of iron in foods of importance but also its bioaccessibility or bioavailability. The bioaccessibility of iron is determined by first its digestion and release from the diet, active absorption into the enterocytes, which starts with its reduction from the prevalent Fe^{3+} to Fe^{2+} (by stomach acid and duodenal cytochrome b (dCtyB) ferric reductase in the lumen, ascorbic acid would, in this case, contribute to the reduction process), and transport from the enterocytes to the circulation (Ferruzzi, Kruger, Mohamedshah, Debelo & Taylor, 2020). Absorption of iron in the intestine is mediated by the divalent metal transporter-1 (DMT1) (Sharp & Srai, 2007) (Figure 2-1). In the cytoplasmic matrix, the iron enters a labile iron pool where it is incorporated in ferritin for storage using poly rC-binding protein (PCBP1). For this reason, this study used the ferritin formed in a Caco-2 cell model following treatment with the digests from the different porridges as a measure of iron uptake and hence, its bioaccessibility.

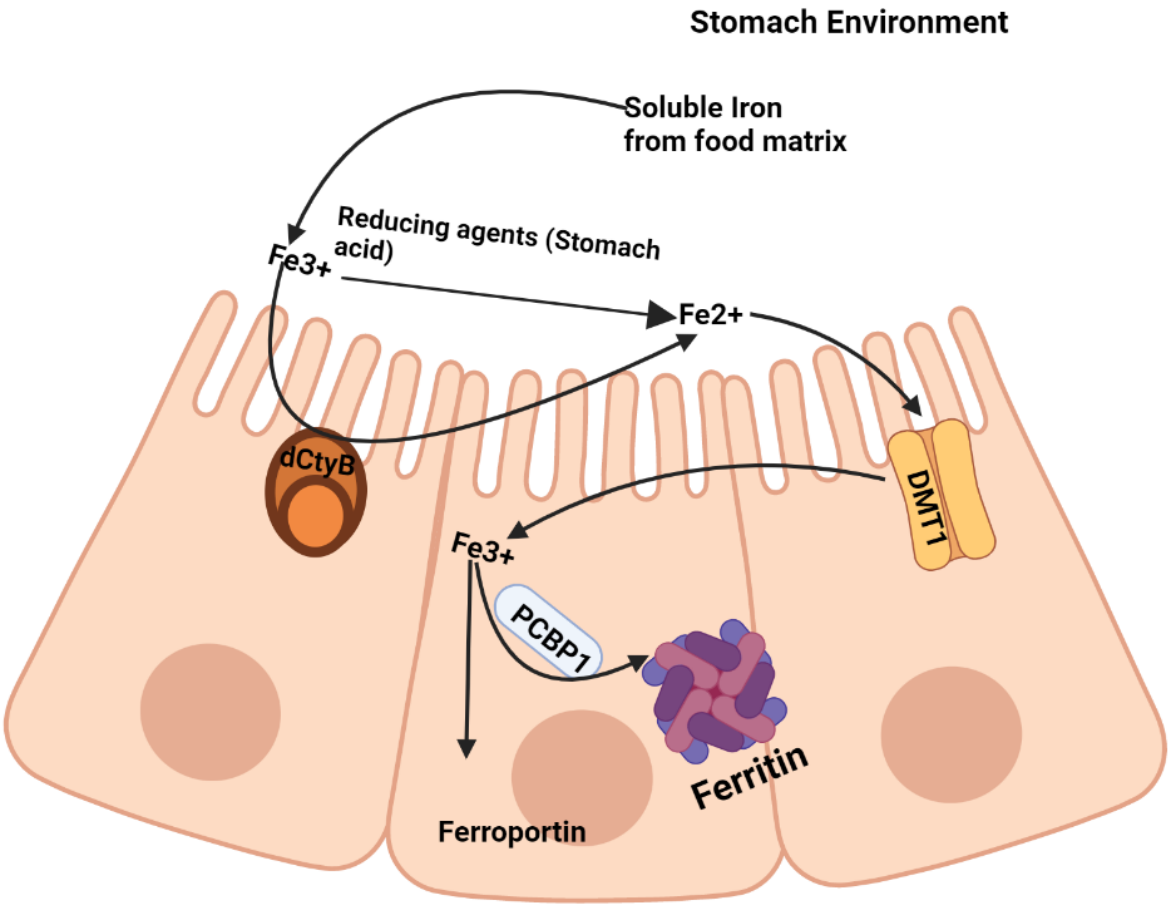


Figure 2-1: Schematic representation of the mechanism of iron uptake by enterocytes (adopted from (Dasa & Abera, 2018). dCtyB- duodenal cytochrome b ferric reductase, DMT1- divalent metal transporter, PCBP1- poly rC-binding protein

2.3.2 Factors affecting iron bioaccessibility and bioavailability

2.3.2.1 phytate

During digestion, the phytate molecule (inositol hexakisphosphate, IP6) is negatively charged, indicating a potential for binding positively charged metal ions like iron (Kumar, Sinha, Makkar & Becker, 2010; Harland & Oberleas, 1987), (Figure 2-2). It can form stable complexes with divalent or trivalent mineral ions like iron, zinc, calcium, and magnesium through electrostatic interactions. Complexation between phytic acid and minerals can occur in the digestive system and is dependent on the atomic mass and electronegativity of the minerals. Since humans are monogastric, complexes of mineral ions with phytates are not easily digestible due to the lack of endogenous phytase enzymes (Hurrell & Egli, 2010), thus impairing their bioavailability. The higher inositol-phosphates (i.e., IP6 and IP5) have a greater

capacity to bind minerals and reduce their bioavailability compared with lower inositol-phosphates (i.e., IP4, IP3, IP2 and IP1) which also generally form relatively more soluble complexes (Sandberg, Carlsson & Svanberg, 1989).

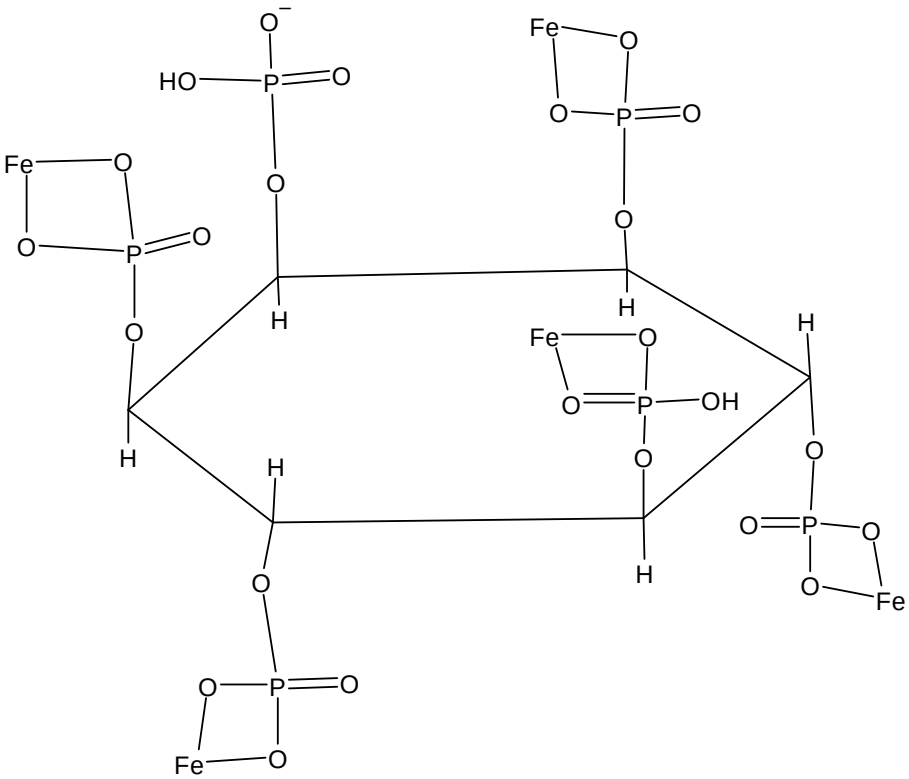


Figure 2-2: Structure of the phytate-iron complex

2.3.2.2 Calcium

Calcium inhibits the absorption of both haem and non-haem iron in a similar way (Dasa & Abera, 2018), and thus, this inhibition by calcium likely occurs after the haem iron is freed from the porphyrin ring (Hallberg, Rossander-Hulthèn, Brune & Gleerup, 1993). Moreover, complexes between iron, phytate and calcium (Figure 2-3) are stable and insoluble and thus render iron less bioavailable (Rousseau, Kyomugasho, Celus, Hendrickx & Grauwet, 2020). While the actual mechanism of iron inhibition by calcium is not fully understood, Hallberg, Rossander-Hulten, Brune and Gleerup (1992) proposed that inhibition of iron absorption occurs during the intestinal membrane transfer process rather than during iron's initial uptake into the enterocyte.

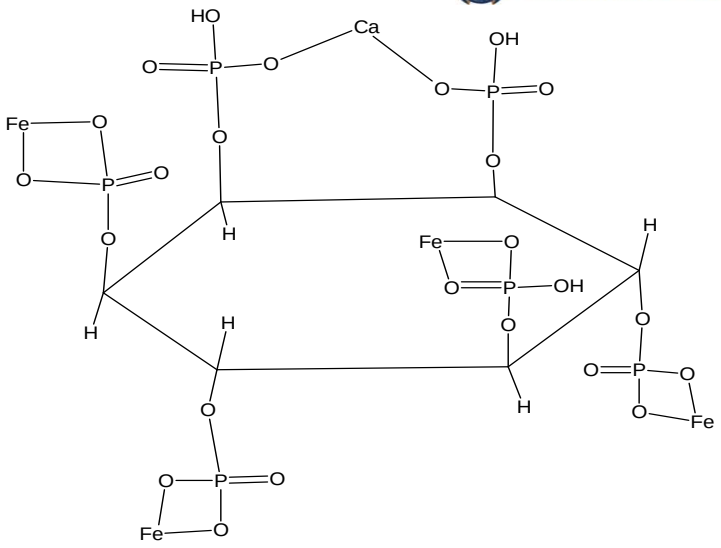


Figure 2-3: Structure of the phytate-iron-calcium complex

2.3.2.3 Phenolic compounds

During digestion, phenolic compounds in the food can be released, forming complexes with iron in the intestinal lumen, making it unavailable for absorption (Hart, Tako, Kochian & Glahn, 2015). However, it appears that phenolic compounds differ in their ability to form complexes with iron and their capacity to reduce its bioavailability (Dasa & Abera, 2018; Andjelković, Van Camp, De Meulenaer, Depaemelaere, Socaciu, Verloo & Verhe, 2006). Phenolics with galloyl and catechol groups can form insoluble complexes with iron, rendering the mineral unavailable for absorption (Figure 2-4). It has been suggested that the amount of iron-binding phenolic galloyl groups in foods roughly corresponds to the degree of inhibition of iron absorption (Brune, Hallberg & Skanberg, 1991). The inhibitory effect of phenolics on iron absorption is likely due to a condensation reaction with iron, which results in the formation of insoluble complexes (Siegenberg, Baynes, Bothwell, Macfarlane, Lamparelli, Car, MacPhail, Schmidt, Tal & Mayet, 1991), (Figure 2-4).

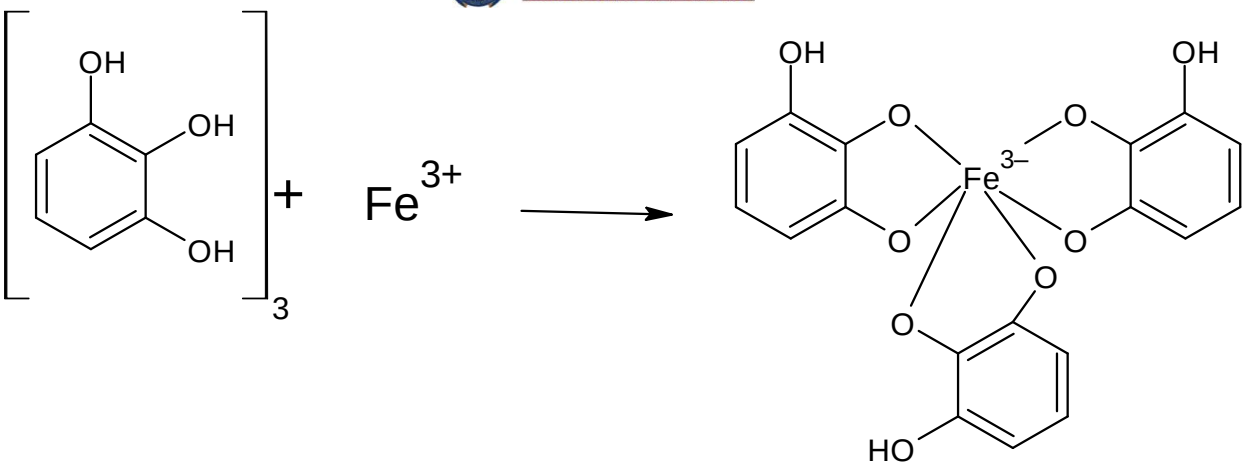


Figure 2-4: Binding of iron with the catechol group of phenolic compounds

2.3.2.4 Organic acids

Citric acid and ascorbic acid appear to be the organic acids that are most potent in enhancing iron absorption, particularly of non-haem iron (Teucher, Olivares & Cori, 2004; Salovaara, Sandberg & Andlid, 2003, 2002). It is proposed that organic acids enhance iron absorption by lowering the pH of the food and by chelating the iron and keeping it in a soluble form, thereby preventing the formation of insoluble and bound iron compounds (Salovaara *et al.*, 2003, 2002). Organic acids, particularly ascorbic acid, also act to reduce the oxidation state of iron from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state, the form of iron absorbed at the enterocytes.

As explained, iron is absorbed in the divalent (Fe^{2+}) state as it is more soluble at the relatively high pH of the duodenum and small intestine (Lopez, Leenhardt, Coudray & Remesy, 2002). Thankachan, Walczyk, Muthayya, Kurpad and Hurrell (2008) showed that when ascorbic acid was added to rice meal at a molar ratio of 2:1 (ascorbic acid: iron), iron absorption was significantly increased (by over 200%). Ascorbic acid has been found to promote the absorption of non-haem iron from the diet to the extent that it counteracts the adverse effects of dietary phytate, tannins and other phenolics (Siegenberg *et al.*, 1991), calcium and the milk protein, casein (Stekel, Olivares, Pizarro, Chadud, Lopez & Amar, 1986) on iron absorption.

2.3.2.5 Dietary fibre

Péneau, Dauchet, Vergnaud, Estaquio, Kesse-Guyot, Bertrais, Latino-Martel, Hercberg and Galan (2008), using fibre-rich fruit and vegetable juices, found that fibre only influenced non-haem iron absorption in human participants whose non-haem iron absorption was high due to

low iron stores. However, fibre did not affect non-haem iron absorption in participants with high iron stores. Conflicting results have been found concerning the effect of fibre on iron bioavailability. In several animal studies, dietary fibre was shown not to significantly affect iron uptake (haemoglobin being used as an indicator), in rats (Zhang, Yung & KongYeung, 2021; Carvalho, Brait, Vaz, Lollo, Morato, Oesterreich, Raposo Jr & Freitas, 2017; Laparra, Díez-Municio, Herrero & Moreno, 2014; Kobayashi, Ohbuchi, Fukuda, Wakasugi, Yasui, Hamada, Yokoyama, Kuwahata & Kido, 2011), in broiler chickens (Gomes, Kolba, Agarwal, Kim, Eshel, Koren & Tako, 2021; Carboni, Reed, Kolba, Eshel, Koren & Tako, 2020; Pereira da Silva, Kolba, Stampini Duarte Martino, Hart & Tako, 2019; Pacifici, Song, Zhang, Wang, Glahn, Kolba & Tako, 2017; Tako & Glahn, 2012), in pigs (Samolinska, Grela & Kiczorowska, 2019) and in fish (Tiengtam, Khempaka, Paengkoum & Boonanuntanasarn, 2015). Cade, Moreton, O'Hara, Greenwood, Moor, Burley, Kukalizch, Bishop and Worwood (2005) observed no effect of fibre on serum ferritin in women, and conflicting results were found in elderly individuals (Fleming, Tucker, Jacques, Dallal, Wilson & Wood, 2002) where consumption of fruits rich in fibre seemed to improve iron uptake. The authors attributed this to the high ascorbic acid content in the fruits, which enhanced iron uptake.

Cook, Noble, Morck, Lynch and Petersburg (1983) found that only wheat bran had a statistically significant inhibition of iron bioavailability from wheat muffins prepared with added bran, cellulose, or pectin. They demonstrated that inhibition of iron absorption is not a universal property of all fibre sources. Moreover, the modest effect of maximally altering the natural fibre levels of a meal suggests that dietary fibre per se is not a major determinant of food iron bioavailability in humans. Rather it is the high phytate content of some crude forms of fibre, such as wheat bran, which inhibits non-haem iron absorption (Cook, Reddy, Burri, Juillerat & Hurrell, 1997). Furthermore, high-fibre-containing foods with reduced phytate levels showed increased mineral absorption to a degree similar to that of low-fibre-containing foods (Nävert, Sandström & Ake, 1985). Hence, Lönnerdal (2000) concluded that dietary fibre on its own has little or no effect on mineral absorption.

2.3.3 Food-to-food fortification as a strategy for improving iron quality.

The nutrient content of cereal-based foods can be greatly improved by combining them with locally available nutrient-rich plant foodstuffs (Onofiok & Nnanyelugo, 1998). While several authors have explored the use of iron-rich food crops to improve the iron content of cereal-based foods, emphasis should be placed on the bioaccessibility of iron in the food. Icard-

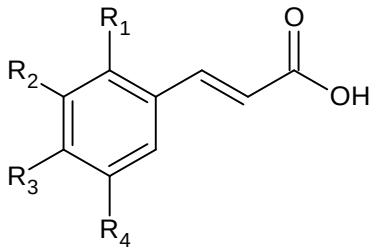
Vernière, Olive, Picq and Mouquet-Rivier (2015), Van der Merwe, Kruger, Ferruzzi, Duodu and Taylor (2019), Adetola *et al.* (2019) and Adetola, Kruger, Ferruzzi, Hamaker and Taylor (2021) have reported increments in the iron contents of sauces, pearl millet porridges, and maize respectively following food-to-food fortification with iron-rich foodstuffs. However, while moringa increased the amount of iron, the amount of bioaccessible iron measured using the dialyzability assay was reduced by 24% following the addition of moringa to pearl millet (Adetola *et al.*, 2019). This was attributed to the high content of phytate and calcium in moringa that forms stable complexes rendering the iron less bioaccessible (also see Section 3.1 of this thesis). This was the same trend reported by other authors regarding FtFF with moringa, such as Van der Merwe *et al.* (2019) with pearl millet-based porridges and Adetola *et al.* (2021) with maize-based porridges. In contrast, the addition of baobab fruit pulp reduced the iron content of pearl millet-based porridge by 4%, and there was an increase in the amount of bioaccessible iron (by 36%) Adetola *et al.* (2019). This increase was attributed to the high content of organic acids (citric and ascorbic acids) in baobab, which are known to enhance mineral bioaccessibility (Iyengar, Pullakhandam & Nair, 2010; Lönnerdal, 2000). Similar increments in iron bioaccessibility following FtFF of pearl millet-based porridges with baobab were reported by Van der Merwe *et al.* (2019) and maize-based porridges by Adetola *et al.* (2021). In the same study, Van der Merwe *et al.* (2019) noted that the addition of moringa, roselle and baobab to pearl millet improved its iron content by 2.5, 2.1 and 2.3 times. The authors, however, reported that the improvement in iron bioaccessibility by the addition of moringa was related to the percentage used, as beyond a threshold of 30% of moringa in the formulation, iron bioaccessibility was significantly reduced. The authors proposed that this was a result of the high calcium content contributed by moringa (Van der Merwe *et al.*, 2019). Gautam, Platel and Srinivasan (2010) reported that the addition of carrot to rice and sorghum resulted in a 14–86% increase in bioavailable iron, while amaranth leaves yielded 11–193% increases. In apparent contradiction, Cercamondi, Icard-Verniere, Egli, Vernay, Hama, Brouwer, Zeder, Berger, Hurrell and Mouquet-Rivier (2014) observed that accompanying a maize paste-type porridge ‘tô’ with sauces made with amaranth or jute leaves did not provide additional bioavailable iron, which the authors attributed to the high phenolic levels of the leaves.

2.4 PHENOLIC COMPOUNDS IN FOODSTUFFS STUDIED

Phenolic compounds are substances that have a minimum of one benzene ring in their structure with one or more hydroxyl groups attached to them. They are produced by plants as secondary metabolites in times of stress to defend against pathogens (phytoalexins), pests and diseases and oxidative stress (Awika & Rooney, 2004). They possess antioxidant, anticarcinogenic, anti-inflammatory, immunomodulatory and antimicrobial properties *in vitro* (WCRF/AICR, 2007; Rao, 2003). They also contribute sensory functions such as the aroma, astringency and colour of some plant parts. The majority of these phenolic compounds (95%) in plants exist in a bound form (Gabaza, Shumoy, Muchuweti, Vandamme & Raes, 2016) although free forms are also present in minor fractions due to their apparent toxicity that is reduced or eliminated by conjugation. They are primarily conjugated (glycosylated) with sugars such as glucose. Other common interactions include association with proteins, carboxylic and organic acids, amines and lipids (Morales-González, 2013). Phenolic compounds present in plants can be broadly categorised as phenolic acids, flavonoids and tannins.

2.4.1 Phenolic acids

Phenolic acids can be categorised as either benzoic acid derivatives, having a C6-C1 structure (Figure 2-6), or cinnamic acid derivatives, with a C6-C3 structure (Figure 2-5) (Dykes & Rooney, 2006). In grains like sorghum, they are located in the pericarp, testa, aleurone layer and endosperm (Dykes & Rooney, 2006). In sorghum, phenolic acids can exist in either free form or bound to various components, such as hemicelluloses (Apea-Bah, Minnaar, Bester & Duodu, 2016, 2014). Free phenolic acids are located in the outer layer of the pericarp and are extracted using organic solvents (Mattila, Pihlava & Hellström, 2005; Subba Rao & Muralikrishna, 2002). Bound phenolic acids are esterified to cell walls; acid or base hydrolysis is required to release these bound compounds from the cell matrix (Chiremba, Taylor, Rooney & Beta, 2012). Some phenolic acids, such as ρ-coumarates and ρ-hydroxybenzoates, are found in association with lignin (Wallace, Chesson, Lomax & Jarvis, 1991).



p-Coumaric acid (R_1, R_2, R_4-H, R_3-OH)

o-Coumaric acid (R_2, R_3, R_4-H, R_1-OH)

m-Coumaric acid (R_1, R_3, R_4-H, R_2-OH)

Caffeic acid (R_1, R_2-H, R_3, R_4-OH)

Ferulic acid (R_1, R_2-H, R_3-OH, R_4-OCH_3)

Sinapic acid (R_1-H, R_3-OH, R_2, R_4-OCH_3)

Figure 2-5 Cinnamic acid derivatives

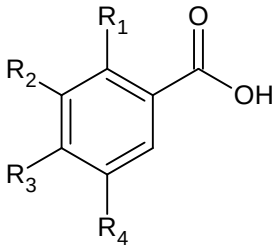


Figure 2-6: Benzoic acid derivatives

p-Hydroxybenzoic acid (R_1, R_2, R_4-H, R_3-OH)

Vanillic acid (R_1, R_4-H, R2-OCH_3, R_3-OH)

Gallic acid (R_1-H, R_2, R_3, R_4-OH)

Syringic acid (R_1-H, R_2, R_4-OCH_3, R_3-OH)

Protocatechuic acid (R_1,R_4-H, R_2, R_3-OH)

2.4.2 Flavonoids

These are compounds comprising a 3-ring system with rings A and C forming a benzopyran nucleus and an aromatic ring B attached to the C ring, forming a C6-C3-C6 oxygenated heterocyclic carbon skeleton (Figure 2-7) (Dykes & Rooney, 2007; Waterman & Mole, 1994). They are phenylalanine derivative products of a combination of acetic acid and shikimic pathways (Aherne & O'Brien, 2002). Anthocyanins form an important group or class of flavonoids that are water-soluble pigments which contribute to the blues, purples, and reds in plant foods (Dykes & Rooney, 2007). Sorghums contain unique anthocyanins called 3-deoxyanthocyanins, which lack the hydroxyl group at position 3 of the C-ring (Dykes & Rooney, 2007). This feature is believed to increase their stability at high pH compared to common anthocyanins (Awika, Rooney & Waniska, 2004).

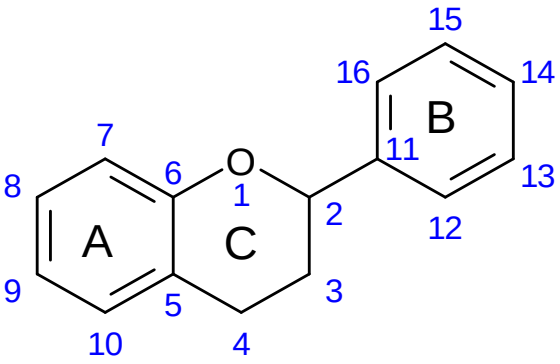
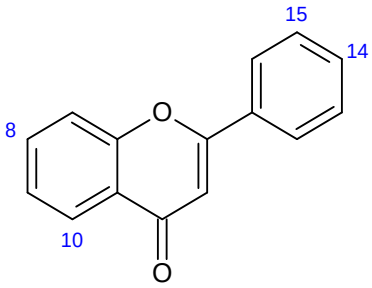


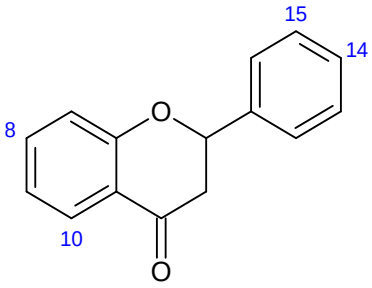
Figure 2-7: General flavonoid structure

Although aglycones (unconjugated or free flavonoid molecules) do occur, the majority of flavonoids exist as glycosides (bound to a sugar moiety). The extent of oxidation and pattern of substitution of the C ring largely determine the difference within classes of flavonoids, while differences between individual compounds are dependent on the substitution of the A and B rings, as indicated in Figure 2-8 below (Pietta, Simonetti, Gardana & Mauri, 2000).



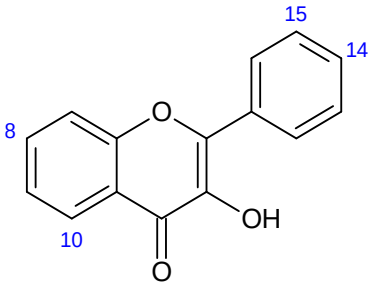
Flavones

	10	8	15	14
luteolin	OH	OH	OH	OH
apigenin	OH	OH		OH



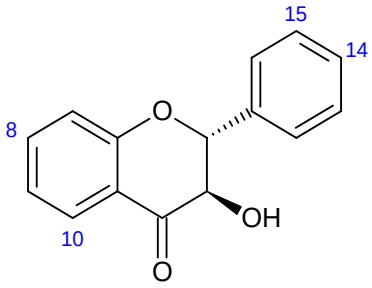
Flavanones

	10	8	15	14
hesperetin	OH	OH	OH	OCH_3
naringenin	OH	OH		OH



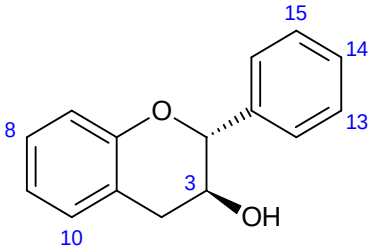
Flavonols

	10	8	15	14
quercetin	OH	OH	OH	OH
kaempferol	OH	OH		OH



Flavanol

	10	8	15	14
taxifolin	OH	OH	OH	OH



Flavan-3-ols

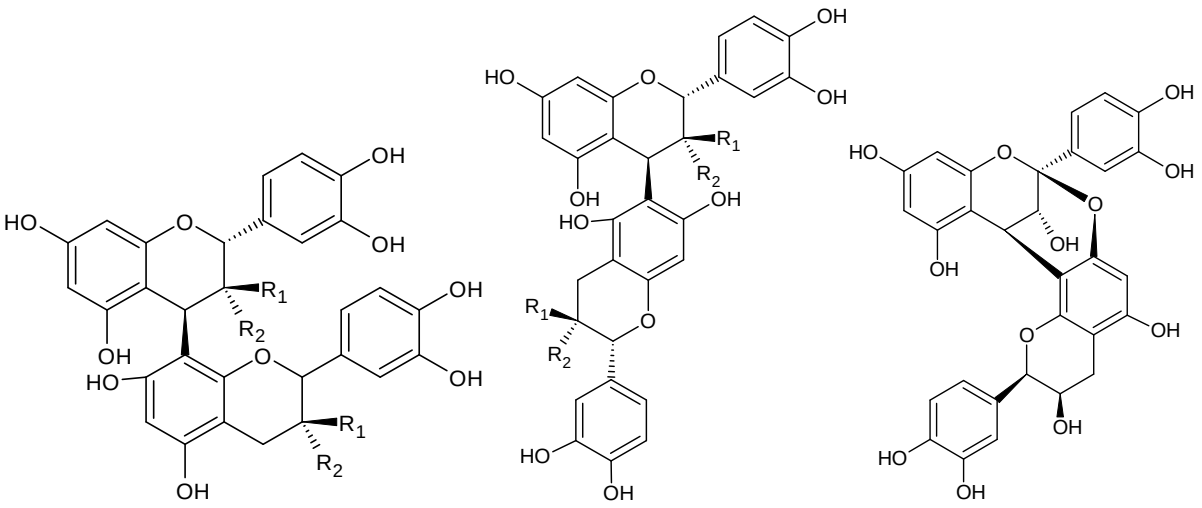
	3	10	8	15	14	13
(+)-catechin	β-OH	OH	OH	OH	OH	
(-)-epicatechin	α-OH	OH	OH	OH	OH	
(-)-epigallocatechin	α-OH	OH	OH	OH	OH	OH

Figure 2-8: Structures of common plant flavonoids

2.4.3 Tannins

Generally, tannins can be categorised as hydrolysable and condensed tannins (Hagerman, Riedl, Jones, Sovik, Ritchard, Hartzfeld & Riechel, 1998; Strumeyer & Malin, 1975). Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid (gallotannins) or with hexahydroxydiphenic acid (ellagitannins) and are only found in Angiospermae or dicotyledonous plants. Condensed tannins, also termed proanthocyanidins or procyanidins, are the most prevalent types of tannin in cereals such as sorghum. They are essentially oligomeric or polymeric flavanol compounds and may be referred to as procyanidins, propelargonidins or prodelphinidins, depending on the monomeric flavanol unit involved. The most prevalent proanthocyanidins in food are procyanidins and prodelphinidins (Cheynier, Dueñas-Paton, Salas, Maury, Souquet, Sarni-Manchado & Fulcrand, 2006).

Procyanidins consist exclusively of flavan-3-ol units, (+)-catechin and/or (-)-epicatechin (Xu & Chang, 2007; Awika, Dykes, Gu, Rooney & Prior, 2003a). The simplest procyanidins are dimeric with either C4-C8 (Figure 2-9a) or C4-C6 (Figure 2-9b)-linked monomeric units (Da Silva, Rigaud, Cheynier, Cheminat & Moutounet, 1991). The units can also be double-linked through an additional ether bond between C2 and O7 (Figure 2-9c). Propelargonidins are made up of afzelechin and/or epiafzelechin subunits, while prodelphinidins are made up of gallocatechin and/or epigallocatechin subunits (Awika *et al.*, 2003a).



a: Procyanidin (4β->8)-dimer b: Procyanidin (4β->6)-dimers c: Procyanidin (4β->8;2β->O->7)-dimers

Figure 2-9: Proanthocyanidin chemical structures showing the different linkages (Prior, Lazarus, Cao, Muccitelli & Hammerstone, 2001)

Table 2-2: Phenolic compounds identified in the different plants used in this study

Food Stuff	Phenolic Compound	Reference
Sorghum	Caffeic acid, cinnamic acid, ferulic acid, gallic acid, sinapic acid, vanillic acid, and ρ-coumaric acid are the main phenolic acids, while luteolin, catechin, epicatechin, apigenin, eriodictyol, and naringenin are the main flavonoids reported in sorghum.	(Xiong, Teixeira, Zhang, Warner, Shen & Fang, 2021; Xiong, Zhang, Warner, Shen, Johnson & Fang, 2020; Luo, Cui, Zhang & Duan, 2018; Rao, Santhakumar, Chinkwo, Wu, Johnson & Blanchard, 2018; Apea-Bah *et al.*, 2014)
Baobab fruit pulp	Phenolic acids chlorogenic acid, caffeic acid, ρ-hydroxycinnamic acid, protocatechuic acid, ρ-hydroxybenzoic acid and ellagic acid, flavonoids catechin, epicatechin, kaempferol, quercetin, apigenin, and myricetin along with Procyanidins and proanthocyanins have been previously reported in baobab fruit pulp.	(Ismail, Guo, Pu, Çavuş, Ayub, Watharkar, Ding, Chen & Liu, 2021a; Ismail, Liu, Pu, He & Guo, 2021b; Ismail, Guo, Pu, Wang, Ye & Liu, 2019a; Ismail, Pu, Fan, Dandago, Guo & Liu, 2019b; Ismail, Pu, Guo, Ma & Liu, 2019c; Sokeng, Sobolev, Di Lorenzo, Xiao, Mannina, Capitani & Daglia, 2019)
Bambara groundnut	Ferulic acid, ρ-hydroxycinnamic acid, syringic acid, caffeic acid, protocatechuic acid, gallic acid, ellagic acid, and quinic acid (phenolic acids), catechin, epicatechin, myricetin, kaempferol, luteolin, quercetin, and naringenin (flavonoids) and procyanidins have been identified in Bambara groundnut.	(Chinnapun & Sakorn, 2022; Okafor, Jideani, Meyer & Le Roes-Hill, 2022; Adedayo, Anyasi, Taylor, Rautenbauch, Le Roes-Hill & Jideani, 2021; Adebiyi, Njobeh & Kayitesi, 2019; Mubaiwa, Fogliano, Chidewe & Linnemann, 2019; Nyau, Prakash, Rodrigues & Farrant, 2017, 2015)

Moringa leaves	Phenolic acids chlorogenic acid, caffeic acid, coumaric acid, sinapic acid, ferulic acid, gallic acid, vanillic, ρ-hydroxybenzoic acid, and ellagic acid and flavonoids kaempferol, quercetin, myricetin, catechin, apigenin, and epigallocatechin gallate are the major phenolic compounds in moringa leaves.	(Hassan, Xu, Tian, Zhong, Ali, Yang & Lu, 2021; Mumtaz, Kausar, Hassan, Javaid & Malik, 2021; Wu, Li, Chen, Wang & Lin, 2020; Coz-Bolaños, Campos-Vega, Reynoso-Camacho, Ramos-Gómez, Loarca-Piña & Guzmán-Maldonado, 2018; Rodríguez-Pérez, Quirantes-Piné, Fernández-Gutiérrez & Segura-Carretero, 2015)

2.5 SORGHUM PROTEIN CONTENT AND DIGESTIBILITY

The quality of protein in any food is determined by how digestible the protein is as well as its amino acid composition (WHO/FAO/UNU, 2007). Therefore, even though a food source might have a high protein content, the amino acid composition (particularly essential (indispensable) amino acids) and digestibility of the proteins present would dictate whether they are of high quality to the consumer. Sorghum is relatively high in protein, but the digestibility of sorghum proteins is generally low, especially when wet-cooked (Duodu, Taylor, Belton & Hamaker, 2003). The low IVPD has been attributed chiefly to the disulphide crosslinking of sorghum prolamin proteins, making them resistant to enzyme attack (Duodu *et al.*, 2003).

Protein digestibility indicates the proportion of a food protein ingested, which is digested and absorbed in the gastrointestinal tract (WHO/FAO/UNU, 2007). It is essentially a measure of the extent and ease of hydrolysis of amino acids after proteolysis (Hsu, Vavak, Satterlee & Miller, 1977). The *in vitro* protein digestibility (IVPD) of uncooked decorticated sorghum is reported to be between 72 and 86% (Hamaker, Mertz & Axtell, 1994; Axtell, Kirleis, Hassen, D'Croz Mason, Mertz & Munck, 1981). The IVPD of sorghum has, however, been shown to decrease by 16-41% (Oria, Hamaker & Shull, 1995; Hamaker, Kirleis, Mertz & Axtell, 1986; Chibber, Mertz & Axtell, 1978) after processing of sorghum flour by wet cooking. This decrease in IVPD after cooking appears to be unique to sorghum grain (Hamaker *et al.*, 1986) and does not occur to any significant extent in other cereals such as wheat and maize (Mertz, Hassen, Cairns-Whittern, Kirleis, Tu & Axtell, 1984). Duodu *et al.* (2003) reviewed the factors which can affect sorghum protein digestibility. The major cause of the decreased IVPD of sorghum upon wet cooking is regarded to be due to cross-linking of its prolamin protein (kafirin). It has been proposed that disulphide bonds form between cysteine-rich β- and γ-type kafirins at the periphery of sorghum protein bodies (Hamaker *et al.*, 1994), the organelles of kafirin storage, possibly leading to a rigid β-sheet conformational structure (Emmambux & Taylor, 2009). It has been further proposed that disulphide bonding of protein at the protein body surface inhibits proteolytic attack of the more digestible α-kafirins located in the centre of protein bodies, therefore hindering the digestibility of the major kafirin sub-group, the α-kafirins (Oria *et al.*, 1995).

2.5.2 Compositing as a measure of improving the protein quality of sorghum.

While cereals are limited in the essential amino acid lysine, legumes, with the exception of soybean, are limited in sulphur-containing amino acids (Temba, Njobeh, Adebo, Olugbile & Kayitesi, 2016). A combination of cereals with legumes improves the protein content and quality of the subsequent food products (Feyera, 2020; Temba *et al.*, 2016). Numerous authors have reported improvement in the protein quality of sorghum-based products following the incorporation of various legumes such as cowpea (Vilakati, MacIntyre, Oelofse & Taylor, 2015; Dovi, 2013; Okpala, Okoli & Udensi, 2013; Pelembe *et al.*, 2002), marama bean (Kayitesi, de Kock, Minnaar & Duodu, 2012), chickpea (Rani, Kumar & Sabikhi, 2016), soy (Bolarinwa, Olaniyan, Adebayo & Ademola, 2015), and sugar bean (Jackson, Weatherspoon, Nnyepi, Malete, Mokgatlhe, Lyoka & Bennink, 2013).

2.6 METHODS OF ANALYSIS

In this section, some of the methods for determining the nutritional, functional and health-promoting properties of foods used in this research are briefly discussed.

2.6.1 *In vitro* iron dialysability determination as a measure of iron bioaccessibility

The *in vitro* iron dialysability assay is widely used for the determination of *in vitro* bioaccessibility of iron. It is based on a modification of the *in vitro* digestion model by Miller, Schricker, Rasmussen and Van Campen (1981). The original method was designed to determine the gastric and upper intestinal digestive release and solubility (bioaccessibility) of iron using dialyzability as a predictor of iron availability for absorption (Ferruzzi *et al.*, 2020). The modified method simulates gastric and small intestinal digestion coupled with a dialysis step using dialysis tubing with an approx. 10 kDa molecular weight cut off, enabling discrimination between low molecular weight soluble mineral complexes, assumed to be bioaccessible, and high molecular weight ones that are assumed not to be bioaccessible (Luten, Crews, Flynn, Van Dael, Kastenmayer, Hurrell, Deelstra, Shen, Fairweather-Tait & Hickson, 1996). The major drawbacks of the *in vitro* iron dialysability assay are that its measure of bioaccessibility is limited as it cannot assess the rate of absorption, absorption, or transport kinetics. It can also not measure nutrient or food competition at the site of absorption (Etcheverry *et al.*, 2012). Another limitation is the precipitation of a significant amount of iron that has diffused into the dialysis bag due to the higher pH of the dialysate, which may significantly affect the results (Van Campen & Glahn, 1999).

2.6.2 Estimation of *in vitro* bioaccessibility using Caco-2 cells

Caco-2 cells (human colon adenocarcinoma cell line) demonstrate numerous morphological and biochemical characteristics that are similar to those of enterocytes (Au & Reddy, 2000). These cells can be used to assess bioaccessibility through the determination of nutrient uptake, transport, or both (Etcheverry *et al.*, 2012). Iron uptake can be estimated by the Caco-2 human epithelial cell line via ferritin formation, via atomic absorption spectroscopy or via radioisotopic forms of the mineral. The *in vitro* Caco-2 absorption model has been suggested as the recommended bioaccessibility method for iron (Etcheverry *et al.*, 2012). This assay can provide more information than *in vitro* dialysability bioaccessibility studies alone, such as the impact of food or nutrient components on the absorption rate and efficiency and possible competition at the absorption site (Glahn, Wortley, South & Miller, 2002). The Caco-2 absorption model has been validated against human iron absorption results, with a significant correlation found (Au & Reddy, 2000).

2.6.3 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity determination

A main advantage of the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) assay over other assays in the chemical determination of antioxidant activity is the fact that the $ABTS^{\bullet+}$ radical is soluble in both aqueous and organic solvents and therefore, the $ABTS^{\bullet+}$ assay can be used to determine the antioxidant capacity of both hydrophilic and lipophilic compounds in samples (Arnao, Cano & Acosta, 2001). However, the $ABTS^{\bullet+}$ assay is long and time-consuming because the $ABTS^{\bullet+}$ radical needs to be prepared first in a reaction that takes at least 12 hours, in comparison with another antioxidant assay such as the oxygen radical absorbance capacity (ORAC) using 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) where the radical is available commercially as an already-made radical. Another drawback that applies to the ABTS assay is that the $ABTS^{\bullet+}$ radical does not occur in biological systems, and therefore the antioxidant activities obtained are of little physiological relevance (Prior, Wu & Schaich, 2005). Nonetheless, the assay is useful for routine antioxidant screening exercises and provides meaningful trends in differences in the antioxidant activity of different samples.

2.6.4 Chemical nitric oxide radical scavenging capacity determination

The nitric oxide ($NO^{\bullet}$) free radical is produced by inducible nitric oxide synthase (iNOS) *in vivo* from arginine with oxygen as a substrate (Tsai, Lin-Shiau & Lin, 1999). Scavenging of NO produced by iNOS is crucial because it may help prevent the development of systematic inflammation response or multiple organ dysfunction (Guzik, Korbut & Adamek-Guzik, 2003). The NO antioxidant assay measures the ability of antioxidants to scavenge $NO^{\bullet}$. Nitrite is first treated with a diazotizing reagent, namely sulphanilamide (SA), in acidic media to form a transient diazonium salt (Sun, Zhang, Broderick & Fein, 2003). This intermediate is then allowed to react with a coupling reagent, N-naphthyl-ethylenediamine (NED), to form a stable azo compound which has an intense purple colour. The intense purple colour of the product allows nitrite determination with high sensitivity and can be used to measure nitrite concentration as low as ~0.5 µM level. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample. The amount of nitric oxide after reaction with sample extracts is measured as nitrite equivalents (Gülçin, 2012). It is worth noting that during the reaction, nitrates may also be formed; hence the nitrates need to be reduced to nitrites before determination (Gülçin, 2012). The reaction mechanism of $NO^{\bullet}$ is based on the ability of antioxidants to bind to $NO^{\bullet}$ thereby preventing its oxidation to nitrates or nitrites (Gülçin, 2012). The limitation of the assay is that $NO^{\bullet}$ is not stable and needs to be generated *in situ* for each analysis (Jayachandra, Maheswaran & Murali, 2012). Also, different intermediate products from the oxidation of nitric oxide to nitrite, i.e. NO_2, N_2O_3, N_2O_4 and $ONOO^-$ may interfere with the results due to possible interactions of the extracts and the nitric oxide-related chemical species (Marcocci, Maguire, Droylefaix & Packer, 1994).

2.6.5 Oxygen radical absorbance capacity (ORAC) determination

ORAC is a biologically relevant assay since the peroxyl radical (ROO•) generated from 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) is found in the human system. It measures the inhibition of peroxyl radicals by antioxidants and is reflective of radical chain-breaking antioxidant activity by hydrogen atom transfer (Ou, Hampsch-Woodill & Prior, 2001). In this regard, the ORAC assay differs from the ABTS and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays, which are based on an electron transfer mechanism (Rivero-Pérez, MUNiz & González-Sanjosé, 2007; Huang, Ou & Prior, 2005). The ORAC assay involves the reaction of peroxyl radicals (ROO•) with a fluorescent probe to form a non-fluorescent product which can be quantified by monitoring reduction in fluorescence (Prior *et al.*, 2005). Although

this assay is biologically relevant and combines both inhibition time and degree of inhibition of antioxidants, it requires the use of expensive equipment (Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003b), which limits its availability in most laboratories (MacDonald-Wicks, Wood & Garg, 2006).

2.6.6 Advance glycation end-products (AGEs) determination

AGEs are formed by spontaneous post-translational modification of proteins or amino acids by reducing sugars in Maillard-type reactions, also known as nonenzymatic glycation (Yeh, Hsia, Lee & Wu, 2017). The Maillard reaction is essential in food processing for sensory enhancement; however, excessive formation of AGEs during food storage and processing which end up being ingested, could result in numerous disorders along with associated complications such as diabetes mellitus and kidney complications and also play a role in tumour development and malignancy, Alzheimer's disease, atherosclerosis, and chronic heart failure (Sadowska-Bartosz & Bartosz, 2016; Uribarri, del Castillo, de la Maza, Filip, Gugliucci, Luevano-Contreras, Macías-Cervantes, Markowicz Bastos, Medrano & Menini, 2015).

The major AGEs are classified into 3 groups: (1) fluorescent crosslinking AGEs, such as carboxymethyl lysine and pentosidine; (2) nonfluorescent crosslinking AGEs, such as imidazolium dilysine crosslinks; and (3) nonfluorescent non-crosslinking AGEs, such as N 3-carboxyethyl lysine (CEL) and N 3 -carboxymethyl-lysine (CML) (Nowotny, Jung, Höhn, Weber & Grune, 2015; Yan, Ramasamy & Schmidt, 2008). Except for pyrraline and pentosidine, the production of AGEs is irreversible. Several methods have been proposed for the measurement of AGEs, as reviewed by Corica, Pepe, Currò, Aversa, Tropeano, Ientile and Wasniewska (2021). Siddiqui, Rasheed, Saquib, Al-Khedhairy, Al-Said, Musarrat and Choudhary (2016) describe a fluorescence spectroscopy (at an emission of 330 nm and excitation of 420 nm) method to quantify AGEs using methylglyoxal and bovine serum albumin as precursors for AGEs formation. The technique is relatively simple but cannot detect non-fluorescent compounds, and it is not specific for fluorescent AGEs; hence non-AGE fluorophore scans interfere with measurement (Corica *et al.*, 2021).

2.6.7 Cellular antioxidant activity assay (CAA)using Caco-2 cells

Cellular antioxidant assays can simulate physiological conditions. Samples are tested for their ability to protect human adenocarcinoma cells (Caco-2 cells) against oxidation by AAPH-induced peroxyl radicals using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay.

For cellular antioxidant assays of this kind, it is essential to determine if the extracts or samples under study are cytotoxic, and this is usually done using the crystal violet assay. The crystal violet assay can be used for indirect quantification of cell death and to determine differences in proliferation upon stimulation with death-inducing agents such as polyphenols (Kanduc, Mittelman, Serpico, Sinigaglia, Sinha, Natale, Santacroce, Di Corcia, Lucchese & Dini, 2002). The CAA assay can differentiate between live and dead cells and is free from interference from other death-inducing agents (Bruggisser, von Daeniken, Jundt, Schaffner & Tullberg-Reinert, 2002).

The cellular antioxidant assay itself uses DCFH-DA, which is a cell-permeable dye. Once within the Caco-2 cells, DCFH-DA is deacetylated by cellular esterases forming dichloro-dihydro-fluorescein (DCFH), which is non-fluorescent (Blasa, Angelino, Gennari & Ninfali, 2011), (Figure 2-10). In the presence of radicals such as ONOO-, NO• and peroxyl radicals, DCFH oxidises to a fluorescent derivative dichlorofluorescein (DCF), and on the addition of a sample containing an antioxidant, the antioxidant quenches the radicals and blocks the conversion of DCFH to DCF in the Caco-2 cells (Blasa *et al.*, 2011; Wolfe & Liu, 2007). Thus, the decrease in cellular fluorescence when compared to the control cells is indicative of the cellular antioxidant capacity of the antioxidant compounds. A general limitation of the DCFH-DA *in vitro* antioxidant properties assay is that they may not measure the protective effect of antioxidant compounds under normal physiological conditions in humans (Wolfe & Liu, 2007).

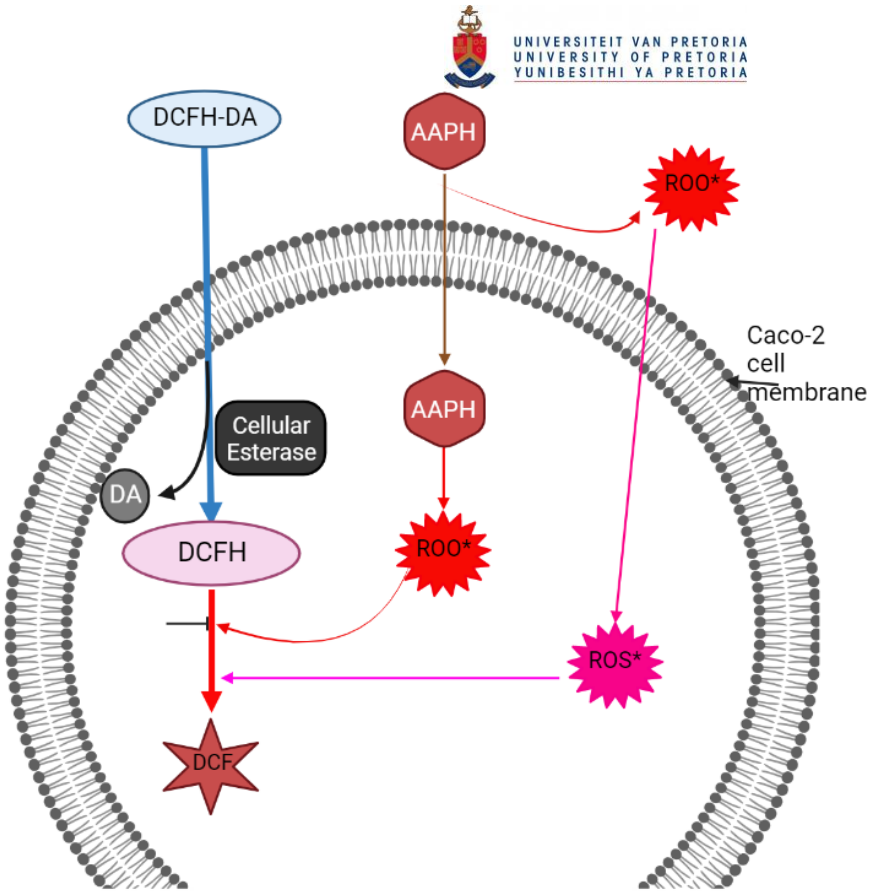


Figure 2-10: Mechanism of measuring antioxidant activity using DCFH-DA in Caco-2 cells (Kellett, Greenspan & Pegg, 2018).

2.6.8 Nitric oxide (NO) scavenging activity in RAW264.7 cells

The RAW264.7 murine cell line is an essential tool for *in vitro* study of inflammation (Murakami, Kawata, Suzuki & Fujisawa, 2020). As innate cells, macrophages are recruited to inflammatory sites, activated, and release cascades of inflammatory molecules, including NO under the stimulation of lipopolysaccharide (LPS), a well-known endotoxin from Gram-negative bacteria (Hong, Pangloli, Perumal, Cox, Noronha, Dia & Smolensky, 2020). The ability of the extract or sample under study to suppress the production of NO in the RAW264.7 macrophages is measured and used as an indication of anti-inflammatory effects.

LPS can be recognized by toll-like receptor 4 (TLR4) and induce acute or chronic inflammatory responses (Raetz & Whitfield, 2002). It then activates the nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) pathway and MAPK (mitogen-activated protein kinases) pathways such as Janus kinase 2-JAK2, leading to the production of NO, and some other inflammatory factors, (Figure 2-11) (Beutler, 2004; Johnson & Lapadat, 2002). The NF-κB is activated by phosphorylation and subsequent degradation of its inhibitor nuclear factor of

kappa light polypeptide gene-enhancer in B-cells inhibitor (IκB), which is present in three different isoforms, IκBα, IκBβ, and IκBε (Kanarek, London, Schueler-Furman & Ben-Neriah, 2010). Jagetia and Baliga (2004) described a method for determining cellular NO production in RAW264.7 macrophages. The reaction involves inducing cellular NO production in RAW264.7 macrophages using LPS before subjecting the cell extracts to a chemical NO assay.

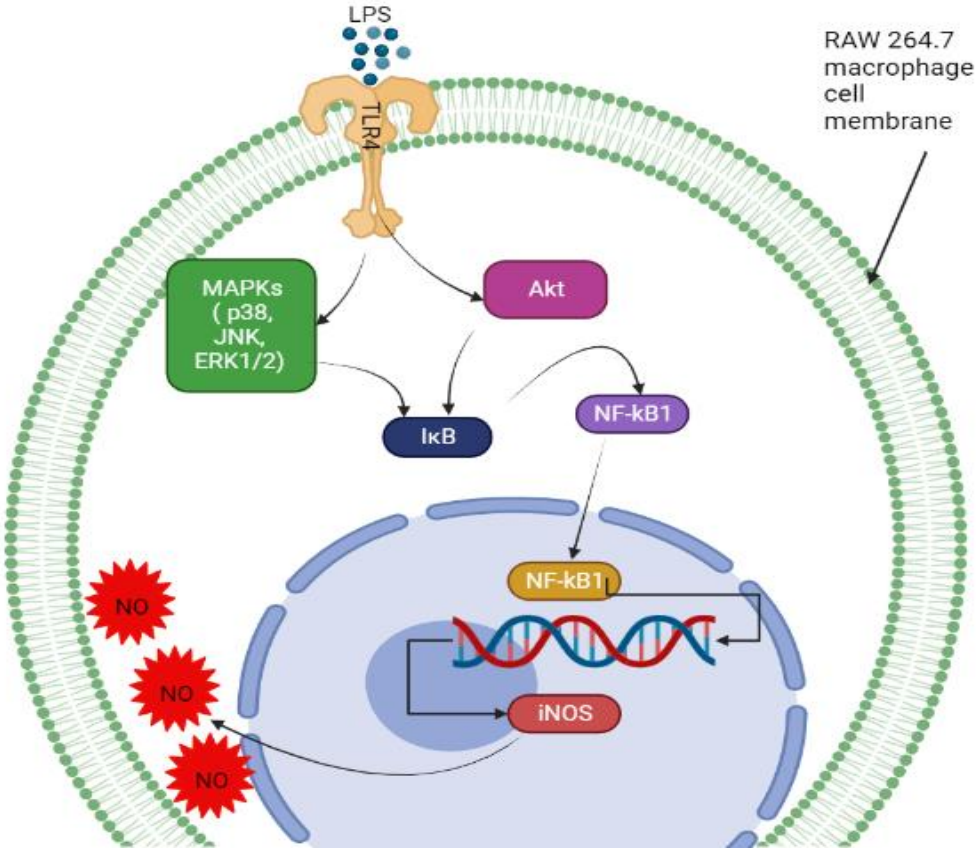


Figure 2-11: Mechanism of LPS-induced NO production in RAW264.7 macrophages (Serreli, Melis, Corona & Deiana, 2019).

2.6.9 Cellular lipid droplet reduction in 3T3-L1 cells

At the cellular level, obesity is defined by an increase in adipose tissue mass, which is the result of an enlargement in fat cells and/or an increase in their number (Chae, Seo, Yang, Yu, Suk, Jung, Ji, Kwon, Lee & Lee, 2015). Adipogenesis is the process of preadipocyte differentiation into adipocytes (Ghaben & Scherer, 2019; Ali, Hochfeld, Myburgh & Pepper, 2013). Due to the ability of 3T3-L1 cells to differentiate from fibroblast into adipocytes (Zebisch, Voigt, Wabitsch & Brandsch, 2012), 3T3-L1 preadipocyte fibroblast clonal cell line can mature into fat cells (Jakab, Miškić, Mikšić, Juranić, Ćosić, Schwarz & Včev, 2021). Adipogenesis has

been well-characterized using 3T3- L1 preadipocytes and can be generally divided into early phase differentiation, which includes growth arrest and mitotic clonal expansion (MCE), and intermediate and late phase differentiation with chronological changes in gene expression. Adipogenesis *ex vivo* can further be divided into four steps: growth arrest, mitotic clonal expansion (MCE), early differentiation, and terminal differentiation (Gregoire, Smas & Sul, 1998) (Figure 2-12). After contact inhibition and growth arrest of post-confluent 3T3-L1 preadipocytes, the differentiation is induced by hormonal stimulation with insulin, dexamethasone, and 1-methyl-3-isobutyl-xanthine (IBMX) (Rubin & OM, 1978). In this stage, transient high expression of cytosine-cytosine-adenosine-adenosine-thymidine/enhancer-binding proteins (C/EBP), C/EBPδ, and C/EBPβ occur. In the intermediate differentiation, C/EBPβ/δ stimulate C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ). PPARγ and C/EBPα promote the induction of several adipocyte-specific genes, including lipoprotein lipase (LPL), adipocyte protein 2 (aP2), fatty acid synthase (FAS), and perilipin in the terminal stage of differentiation (Figure 2-12) (Rosen, Walkey, Puigserver & Spiegelman, 2000). 3T3-L1 cells can be stained by oil Red-O dye (Kwan, Wu, Su, Chao, Liu, Fu, Chan, Lau, Tse & Han, 2017), and absorbance can be measured at a wavelength of 520 nm following extraction of the dye from the cells with 60% isopropanol. Polyphenol-rich diets have directly or indirectly resulted in the downward regulation of adipose tissues (preadipocytes, adipose stem cells and immune cells) (Wang, Moustaid-Moussa, Chen, Mo, Shastri, Su, Bapat, Kwun & Shen, 2014).

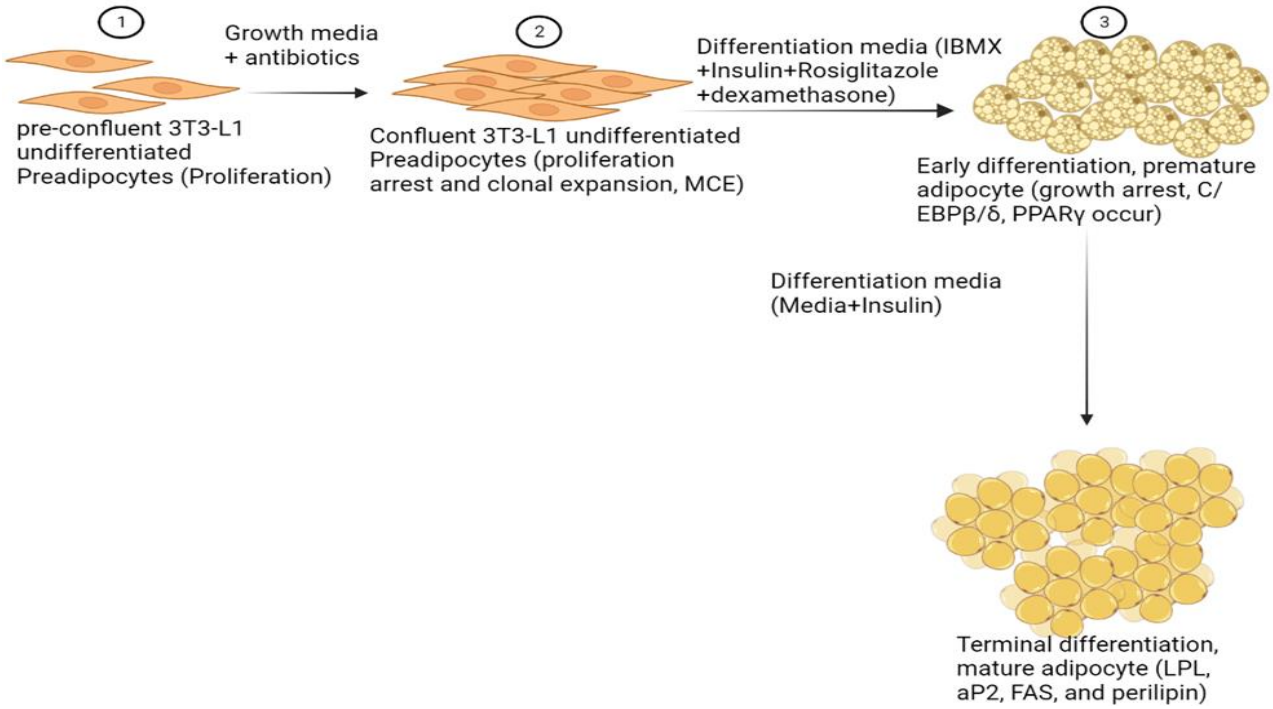


Figure 2-12: Stages of differentiation of 3T3-L1 cells *ex vivo* (Murata, Yamaguchi, Kohno, Takahashi, Risa, Hatori, Hikita & Kaneda, 2020)

2.6.10 Nitrogen solubility index

The nitrogen solubility index (NSI) assays the soluble nitrogenous compounds in the extruded products. The reduction in nitrogen solubility observed upon extrusion cooking may be attributed to the formation of disulphide and hydrophobic linkages due to high temperature in the extrusion cooker (Prudencio-Ferreirar & Areas, 1993). The major storage proteins in legumes are globulins, and they are soluble in salt solutions (Kiosseoglou, Paraskevopoulou & Poojary, 2021). Samples are dispersed in water at 30 ºC and stirred at a low speed of 100 rpm for 30 min, after which they are centrifuged twice with NaCl solution to extract all solubilized proteins. The filtrate is then freeze-dried to concentrate soluble protein. This is done because preliminary studies on liquid extracts showed wide variations in the results obtained, possibly due to the dilution of soluble protein, which puts the nitrogen content below the detection limit of the Dumatherm instrument (DT, Gerhardt Konigswinter, Germany). The detection limit of the Dumatherm is 0.01 mg of Nitrogen. Freeze drying helps improve the detection level as it concentrates the nitrogenous compounds and ensures the repeatability of the results.

2.6.11 Pepsin *In vitro* protein digestibility (IVPD) assay

Protein digestibility may be used as an indicator of protein quality as it is a measure of the susceptibility of a protein to proteolysis during the digestion of proteins (Schaafsma, 2012, 2000). As discussed previously, a protein with high digestibility is the potential of better nutritional value than one of low digestibility because it would provide more amino acids for absorption after proteolysis. The IVPD assay relies on the solubilisation of proteins following digestion with the pepsin enzyme (Hamaker *et al.*, 1986). Once digested with pepsin, the food sample is centrifuged, the residue dried, and its protein content determined. The digestibility of the food sample is obtained as a percentage difference between the solubilized protein and the protein initially present in the sample. It has been used extensively to determine the protein digestibility of sorghum foods and is considered to have a good correlation with *in vivo* human protein digestibility studies (Mertz *et al.*, 1984; Maclean Jr, RomaÑa, Placko & Graham, 1981).

2.6.12 Starch digestibility

Starch is hydrolysed by amylolytic enzymes before being absorbed as glucose in the small intestine (Peyrot des Gachons & Breslin, 2016). There are several methods used for the determination of starch digestibility *in vitro*, but the most common are the Englyst, Kingman and Cummings (1992) and Goñi, Garcia-Alonso and Saura-Calixto (1997) methods. The Goñi *et al.* (1997) method is the most widely applied technique. This method involves the use of α-amylase and amyloglucosidase to digest starch, while protein is digested using pepsin. The use of α-amylase and amyloglucosidase in conjunction closely stimulates the process of starch digestion in the small intestine, where a larger percentage of starch is digested (Hasjim, Lee, Hendrich, Setiawan, Ai & Jane, 2010). A drawback is that the Goñi *et al.* (1997) method excludes the oral digestion phase, where the food is made into a bolus by mixing with saliva, and the salivary α-amylase begins the process of starch breakdown. In this method, the sample is mechanically disrupted using glass beads due to the exclusion of the chewing phase of digestion. Certain food and human physiological factors such as digesta viscosity, gastric emptying rate and transit time through the gastrointestinal tract that affect starch digestion are not considered in the Goñi *et al.* (1997) method. Several *in-vivo* studies have shown that these aforementioned factors influence the rate of starch digestion (Turnbull, Baxter & Johnson, 2005) and as such, their omission are likely to be another limitation of the Goñi *et al.* (1997) method.

2.7 CONCLUDING REMARKS

The consumption of foods low in minerals and dense in energy should be addressed to help prevent the triple malnutrition in sub-Saharan Africa. Foodstuffs such as sorghum, moringa and baobab could be used as fortificants to improve the iron quality of sorghum and thus play a significant role in helping to address iron deficiency in sub-Saharan Africa. Legumes are a source of proteins since they are high in lysine and have been used in managing protein malnutrition in communities consuming cereal-based diets. Bambara groundnut is a legume that is underutilised and drought-resilient and could be used to improve the protein quality of sorghum. Twin screw extrusion cooking is a high throughput and continuous food manufacturing process that can process sorghum to produce nutritious, convenience-type foods for the rapidly urbanising communities in Africa. However, there has been little research on starch-based systems concerning the effect of food-to-food fortification and the effects of extrusion cooking on macro- and micronutrient quality and health-promoting properties of sorghum-based foods. This research aims to establish the effect of food-to-food fortification and extrusion cooking on the macro- and micronutrient quality of sorghum-based porridges.

2.10 HYPOTHESES AND OBJECTIVES

2.10.1 Hypotheses

1. Extrusion-cooked sorghum-based porridges will have a higher iron bioaccessibility when compared to conventionally wet-cooked porridges. Due to the high shear and temperatures involved in extrusion cooking (Cheftel, 1986) when compared to conventional cooking, there is the hydrolysis of insoluble dietary fibre (Rashid, Rakha, Anjum, Ahmed & Sohail, 2015) and dephosphorylation of phytate (Alonso *et al.*, 2001) both of which bind minerals (Dust, Gajda, Flickinger, Burkhalter, Merchen & Fahey, 2004; Ljøkjel, Sørensen, Storebakken & Skrede, 2004; Murray, Flickinger, Patil, Merchen, Brent Jr & Fahey Jr, 2001), reducing their bioaccessibility. Their hydrolysis during extrusion cooking will improve iron bioaccessibility compared to conventional cooking.

   Food-to-food fortification (FtFF) of sorghum with baobab fruit pulp will result in higher iron bioaccessibility compared to FtFF with moringa leaf powder. Baobab fruit pulp has a higher content of ascorbic and citric acids compared to moringa leaf, which can enhance mineral bioaccessibility (Iyengar *et al.*, 2010; Lönnerdal, 2000). While moringa leaves have a higher content of phenolics and phytate (Leone *et al.*, 2015a) but are low in organic acids. Phenolic compounds and phytate bind iron (Brune, Rossander-Hultén, Hallberg, Gleerup & Sandberg, 1992; Brune, Hallberg & Skanberg, 1991). The high content of phenolics and phytate will lead to reduced iron bioaccessibility of sorghum-based porridges fortified with moringa leaf powder.

2. Conventionally wet-cooked sorghum-based porridges will have higher phenolic content and health-promoting properties (radical scavenging properties, inhibition of advanced glycation end products and antilipogenic properties) when compared to extrusion-cooked sorghum-based instant porridges. The high temperatures coupled with high shear and high pressure used in extrusion cooking will lead to the destruction of phenolic compounds, and the combination of high shear, pressure and moisture could cause the polymerisation of phenolic compounds, both of which would reduce the extractability of phenolic compounds and reduce antioxidant activity (Sharma, Gujral & Singh, 2012; Brennan *et al.*, 2011). FtFF of sorghum with baobab and moringa will

enhance the phenolic content and health-promoting properties of the sorghum-based porridges due to the high phenolic content of baobab and moringa. Baobab fruit pulp and moringa leaf have been reported to be high in phenolic compounds (Kashyap, Kumar, Riar, Jindal, Baniwal, Guiné, Correia, Mehra & Kumar, 2022; Ismail, Guo, Pu, Çavuş, Ayub, Watharkar, Ding, Chen & Liu, 2021; Adetola, Kruger, White & Taylor, 2019; Ismail, Pu, Guo, Ma & Liu, 2019; Sokeng, Sobolev, Di Lorenzo, Xiao, Mannina, Capitani & Daglia, 2019; Tembo, Holmes & Marshall, 2017; Leone, Fiorillo, Criscuoli, Ravasenghi, Santagostini, Fico, Spadafranca, Battezzati, Schiraldi, Pozzi, Di Lello, Filippini & Bertoli, 2015; Stevens, Ugese, Otitoju & Baiyeri, 2015; Moyo, Masika, Hugo & Muchenje, 2011). Their addition to sorghum will result in higher phenolic content of the sorghum-based porridges. Since phenolic compounds have been proposed to confer antioxidant properties (Duodu & Awika, 2019; Awika & Rooney, 2004), the antioxidant properties (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), Oxygen radical absorbance capacity, cellular antioxidant activity), nitric oxide scavenging capacity, advanced glycation end products inhibition and antilipogenic properties of sorghum-based porridges FtF fortified with moringa and baobab will be higher than those of plain sorghum.

3. Extrusion-cooked sorghum-based porridges will have higher starch and protein digestibility than conventionally wet-cooked porridges. The higher temperature and shear of extrusion cooking will lead to depolymerization of starch, thereby making the starch more readily accessible for enzymatic hydrolysis (Alonso, Aguirre & Marzo, 2000) and also disrupting the protein bodies of sorghum, exposing more of the α-kafirins to proteolysis (Hamaker *et al.*, 1994).

   The starch and protein digestibilities of extrusion-cooked and conventionally cooked sorghum-based porridges fortified with baobab and moringa will be lower than those of unfortified porridges. This is due to the high content of antinutritional compounds, including phytate, phenolics and dietary fibre, in these FfF fortificants. Antinutritional compounds bind to starch and proteins as well as enzymes responsible for hydrolysing them, resulting in lower starch and protein digestibility (Kumar, Basu, Goswami, Devi, Shivhare & Vishwakarma, 2021; Thakur, Sharma & Thakur, 2019). The pasting profile, starch and protein digestibilities of extrusion-cooked and conventionally cooked sorghum-based porridges fortified with baobab and moringa will be lower than those

of unfortified porridges. This is due to the high content of antinutritional compounds, including phytate, phenolics and dietary fibre, in these FfF fortificants. Antinutritional compounds bind to starch and proteins as well as enzymes responsible for hydrolysing them, resulting in lower starch and protein digestibility (Kumar, Basu, Goswami, Devi, Shivhare & Vishwakarma, 2021; Thakur, Sharma & Thakur, 2019). Furthermore, the main contributor to the viscosity of foods is starch, and a reduction in starch would directly be reflected in higher viscosity properties.

2.10.2 Objectives

1. To determine the effects of FtFF of whole-grain sorghum with baobab fruit pulp powder and moringa leaf powder, as well as extrusion cooking to produce instant porridges on porridge iron bioaccessibility, with the aim of addressing micro-nutrient deficiencies in sub-Saharan Africa.

2. To determine the effects of FtFF of whole-grain sorghum with baobab fruit pulp powder and moringa leaf powder, as well as extrusion cooking to produce instant porridges on porridge phenolic compounds and health-promoting properties, with the aim of addressing the rising incidence of NCDs in sub-Saharan Africa.

3. To determine the effects of FtFF of whole-grain sorghum with baobab fruit pulp powder, moringa leaf powder, and Bambara groundnuts, as well as extrusion cooking, to produce instant porridges on porridge starch and protein digestibilities, with the aim of addressing diabetes and protein-energy malnutrition in sub-Saharan Africa.

2.11 Experimental Design 1 (Chapter 3, Sections 3.1 and 3.2)

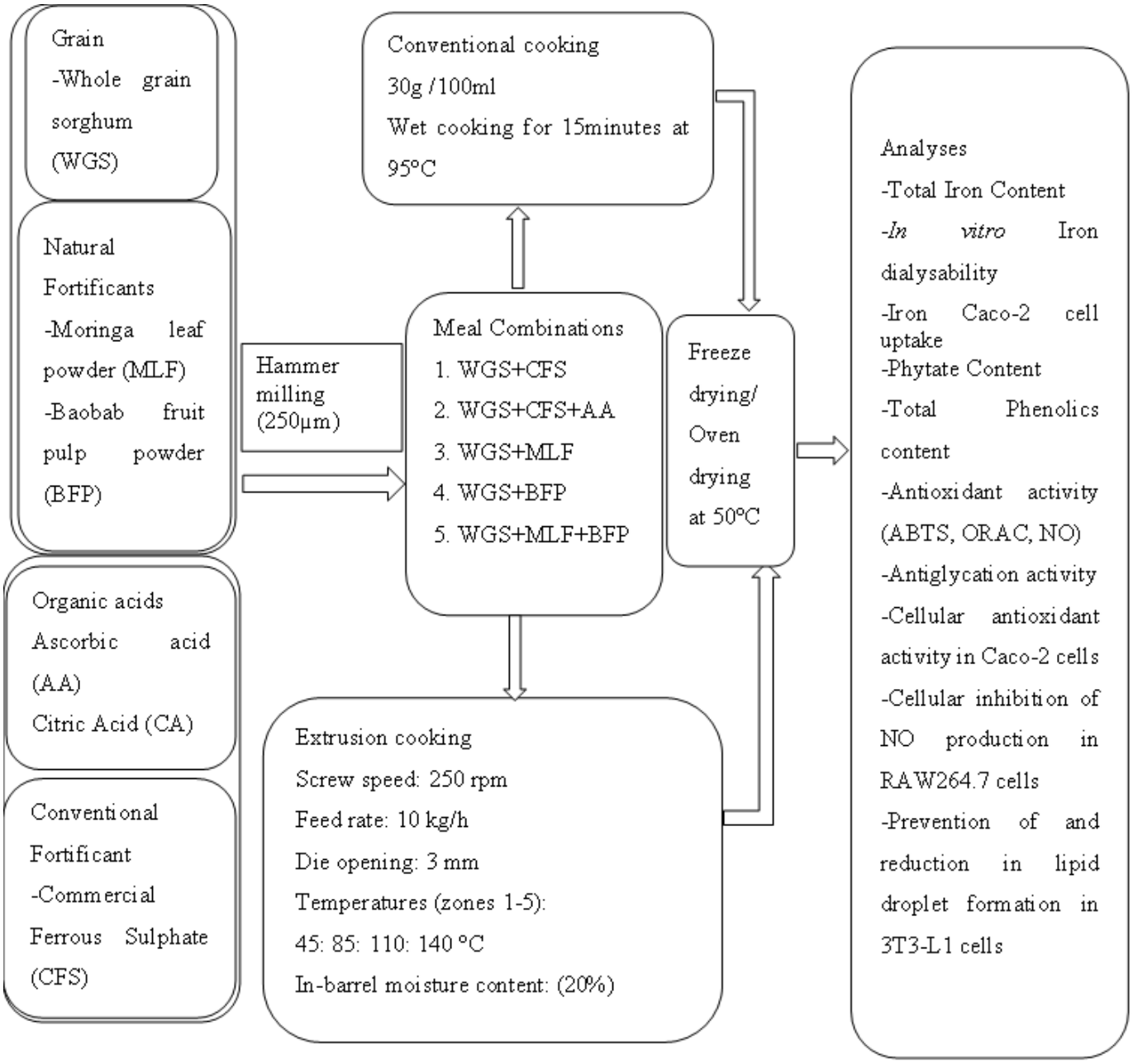


Figure 2-13: Flow diagram of experimental design from raw material preparation, compositing, extrusion cooking conditions and drying to analyses for sections 3.1 and 3.2

2.12 Experimental Design 2 (Chapter 3, Section 3.3)

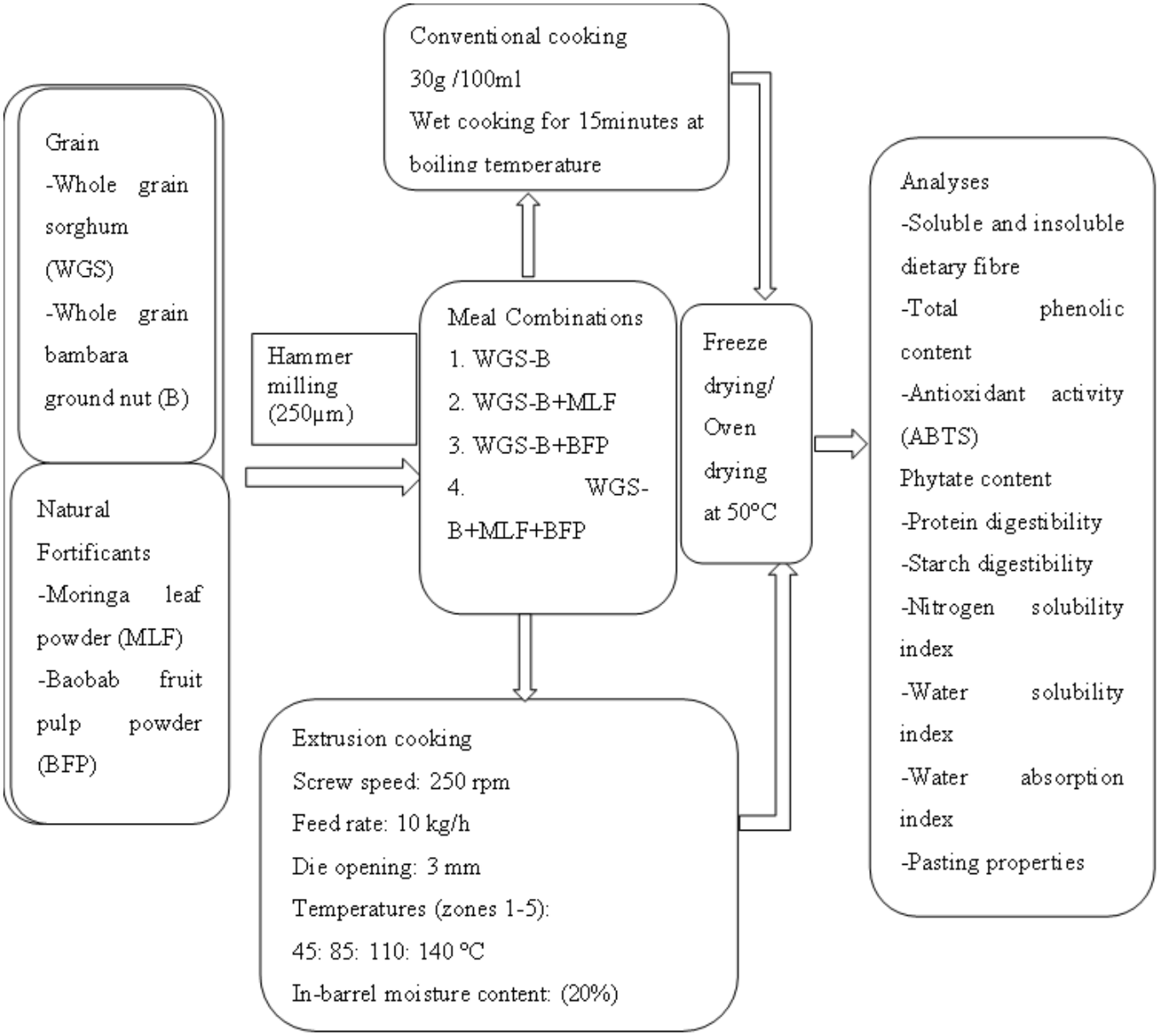


Figure 2-14: Flow diagram of experimental design from raw material preparation, compositing, extrusion cooking conditions and drying to analyses for sections 3.3

CHAPTER 3 : RESEARCH

This research chapter consists of three sections. The first section (3.1) deals with the effects of extrusion cooking of sorghum-based porridges food-to-food (FtF) fortified with baobab fruit powder and moringa leaf powder on iron bioaccessibility. The second section (3.2) concerns the bioactive phenolics and antioxidant properties of the FtF-fortified extruded porridges measured by both *in vitro* chemical and cellular assays. Section three (3.3) covers the *in vitro* digestibility (starch and protein) as well as pasting properties of extruded and FtF-fortified sorghum-Bambara groundnut composite porridges.

3.1 EXTRUSION COOKING OF FOOD-TO-FOOD FORTIFIED WHOLEGRAIN SORGHUM-BASED PORRIDGES ENHANCES CACO-2 FERRITIN FORMATION

Abstract

Iron deficiency is still a major public health concern in sub-Saharan Africa, and this is in part due to a monotonous diet of cereals often low in bioavailable minerals and high in mineral bioavailability inhibitors, notably phytate and polyphenols. Sorghum is a major food crop across the semi-arid tropics of Africa because of its tolerance to high temperatures and low rainfall. Extrusion cooking is a process that applies high heat, pressure, and shear to raw food materials to produce ready-to-eat products and can destroy anti-nutrients in plant foods and hence enhance the digestibility of their macronutrients. Food-to-food fortification (FtFF) is a strategy where micronutrient-rich food combinations are used to promote the bioavailability of essential micronutrients by increasing the content of micronutrients and enhancers of their absorption and decreasing the levels of inhibitors of micronutrient bioavailability. The objective of this study was to determine the effects of extrusion cooking of sorghum-based porridges FtF fortified with baobab fruit powder and moringa leaf powder on iron bioaccessibility, as measured by both dialyzability and Caco-2 cell assay.

Effects of extrusion cooking of FtFF wholegrain sorghum-based porridges using iron-rich foodstuffs (moringa) or high in iron absorption-enhancers (baobab) on iron bioaccessibility were determined. Although extrusion reduced bioaccessible iron content (BIC) and percentage bioaccessible iron (PBI), it enhanced ferritin-formation in Caco-2 cells (by 38%) compared to conventional cooking, most probably because extrusion reduced contents of phenolics and phytate, hence freeing more iron. Fortification with baobab increased PBI by 14-34%, whether extruded or conventionally cooked, probably due to its organic acids. Fortification with moringa reduced BIC and PBI (by 30% and 71%, respectively), whether extruded or conventionally cooked, probably due to its high calcium and phytate contents.

Extrusion cooking with baobab has the potential to help alleviate iron deficiency in sorghum-based foods because it reduces the content of anti-nutrients and could play a role in alleviating iron deficiency. This study highlights the potential of extrusion cooking coupled with tropical foodstuff high in organic acids to improve iron bioavailability in wholegrain-based starchy staple foods.

Keywords: Extrusion, sorghum, wholegrain, food-to-food fortification, iron, bioaccessibility, ferritin-formation

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Lubaale, J., Taylor, J.R.N., Emmambux, M.N. and Duodu, K.G. (2023) Extrusion cooking of food-to-food fortified wholegrain sorghum-based porridges enhances Caco-2 ferritin formation. Cereal Chemistry 100: 371-383.

3.1.2 Introduction

Extrusion cooking is a food processing technology that can be applied to produce a variety of convenience-type products from diverse plant foods (Guy, 2001). It is a continuous cooking process that applies high heat, pressure and shear to raw food materials to produce ready-to-eat products (Fellows, 2009). Due to rapid urbanisation in Africa, there is increasing demand for ready-to-eat foods (Tiuganji, Nehme, Marqueze, Isherwood, Martins, Vasconcelos, Cipolla-Neto, Lowden, Skene & Moreno, 2020), especially in families with working mothers. Another advantage of extrusion cooking is that it can destroy anti-nutrients in plant foods and hence enhance the digestibility of their macronutrients (Nikmaram, Leong, Koubaa, Zhu, Barba, Greiner, Oey & Roohinejad, 2017). For example, it has been reported to improve iron availability in peas and kidney beans (Alonso *et al.*, 2001) and in rice and maize-based protein-enriched snacks (Wani & Kumar, 2016).

Iron deficiency is still a major public health concern in sub-Saharan Africa (Lemoine & Tounian, 2020; Bouis, Saltzman, Low, Ball & Covic, 2017). The deficiency of micronutrients such as iron, while seldom causing death, adversely affects health. Iron deficiency accounts for 30-50% of anaemia reported in children and women (Pasricha, Tye-Din, Muckenthaler & Swinkels, 2020). These deficiencies are in part due to a monotonous diet of cereals (Gibson *et al.*, 2018), roots and tubers (Gregory & Wojciechowski, 2020). These starchy foods are often low in bioavailable minerals, and cereals are specifically high in mineral bioavailability inhibitors, notably phytate and polyphenols (Gibson *et al.*, 2018). Notwithstanding these nutritional drawbacks, sorghum is a major food crop across the semi-arid tropics of Africa because of its tolerance to high temperatures and low rainfall (Taylor, 2019).

To help prevent mineral deficiencies, at-risk communities are encouraged to diversify their diets by including vegetables rich in essential minerals and fruits rich in promoters of mineral bioavailability (WHO, 2017). FtFF is a strategy where micronutrient-rich food combinations are used to promote the bioavailability of essential micronutrients by increasing the content of micronutrients and enhancers of their absorption and decreasing the levels of inhibitors of micronutrient bioavailability (Kruger, 2020). FtFF with moringa leaves and baobab fruit pulp has been shown to improve iron and zinc bioaccessibility in pearl millet (Adetola *et al.*, 2019) and maize (Adetola *et al.*, 2021).

Hence, the objective of this study was to determine the effects of extrusion cooking of sorghum-based porridges FtF fortified with baobab fruit powder and moringa leaf powder on iron bioaccessibility, as measured by both dialyzability and Caco-2 cell assay.

3.1.3 Materials and methods

3.1.3.1 Materials

Red non-tannin sorghum was procured from Mpumalanga Province, South Africa. The grain was milled using a hammer mill fitted with a 500 µm mesh size screen. The wholegrain flour was stored at 4°C in sealed plastic buckets. Baobab fruit powder was from Nautica Organic Trading, Durban, South Africa. Dried moringa leaf powder was from Supa Nutri, Cape Town, South Africa.

Digestive enzymes and bile salts used were pepsin (P-7000, CAS Number: 9001-75-6), pancreatin (P-1750, CAS Number: 8049-47-6), and bile extract (B-8631, CAS Number: 8008-63-7) (Sigma-Aldrich, Johannesburg, South Africa). Dialysis tubing Spectra/Por 7 (Ø=20.4 mm) with a molecular weight cut-off of 10 kDa was used (G.I.C. Scientific, Johannesburg, South Africa).

3.1.3.2 Porridge Formulations

The following formulations of sorghum-based flours with fortificants were prepared (corn starch was included as a filler to maintain a constant final percentage weight for all formulations, figures in brackets represent the ratios of the ingredients):

A. Wholegrain sorghum flour+corn starch (85:15)
B. Wholegrain sorghum flour+ferrous sulphate+corn starch (85:0.02:14.98) as a conventional iron fortification standard
C. Wholegrain sorghum flour+ferrous sulphate+corn starch+ascorbic acid+citric acid (85:0.02:14.35:0.01:0.62) as a conventional iron fortification gold standard, with organic acids being added before processing. The organic acids added were based on the amount present in the baobab fruit pulp and moringa leaf powders.
D. Wholegrain sorghum flour+ferrous sulphate+corn starch+ascorbic acid+citric acid (85:0.02:14.35:0.01:0.62) as a conventional iron fortification gold standard, with organic acids being added after processing.
E. Wholegrain sorghum flour+baobab fruit pulp powder+corn starch (85:6:9), with baobab being added before processing.
F. Wholegrain sorghum flour+baobab fruit pulp powder+corn starch (85:6:9), with baobab being added after processing.
G. Wholegrain sorghum+moringa leaf powder+corn starch fortified (85:6:9).

H. Wholegrain sorghum+moringa leaf powder+baobab fruit pulp powder+corn starch (85:6:6:3), with baobab being, was added before processing.
I. Wholegrain sorghum+moringa leaf powder+baobab fruit pulp powder+corn starch (85:6:6:3), with baobab being, was added after processing.

All formulations were made to meet approximately 25% of the recommended dietary intake for iron at low bioavailability of an adult woman (32.4 mg) in the total formulation (Saunders, Craig, Baines & Posen, 2013), except the formulations containing only baobab fruit pulp powder. This is because the iron content of the baobab was low.

From the *in vitro* iron dialyzability results, porridge formulations A, E, G, and H were used for the ferritin ELISA assay with Caco-2 cells in order to study the effect of extrusion cooking compared to conventional cooking as well as the effect of fortification with moringa and baobab on ferritin formation:

3.1.3.3 Conventional Wet Cooking

Deionised water was added to each wholegrain sorghum-based flour in a ratio of 3:10, flour: water (w/w). The slurry was heated to boiling temperature (95ºC) and maintained with constant stirring for 25 minutes. The slurry was left to cool at ambient temperature, after which it was placed in plastic containers and frozen to -20ºC and freeze-dried in an Instruvac freeze-dryer model RFR 3878 (Air and Vacuum Technologies, Johannesburg, South Africa. Freeze-dried porridge flour was crushed to a particle size that passed through a 500 µm opening screen before further analysis. The pre-cooked porridge flour was stored at 4ºC in double-sealed, airtight plastic bags.

3.1.3.4 Extrusion cooking

A co-rotating twin-screw extrusion cooker model TX 32 (CFAM Technologies, Potchefstroom, South Africa) (L/D = 21.5:1) was used. Porridge formulations prepared as above were extruded separately. The barrel comprised of five heating zones towards the die was set at 60/70/80/140/140ºC, respectively. Water was fed into the system at a dosing rate of 3 l/h (to obtain a final moisture content of 20% calculated based on the moisture content of the flours), and the feed rate was 10 kg/h. A die opening of 3 mm was used, and the screw speed was maintained at 250 rpm. Extrudates were collected three times after every 30 min interval to produce triplicates. They were dried immediately in a force draught oven at 50ºC for 5 min to a moisture content below 10%. The cooled extrudates were milled using an air-cooled analytical

mill to a maximum particle size of 500 µm. The milled extrudates were stored at 4ºC in double-sealed, airtight plastic bags.

3.1.3 Analyses

3.1.3.5 Determination of phytate content

Phytate was determined using the extraction and indirect quantitative assay of Fruhbeck, Alonso, Marzo and Santidrián (1995). The method is based on the spectrophotometric determination of organic phosphate present in the sample that has been acid extracted and purified to remove the inorganic phosphate. Dowex1-anion-exchange resin-AG 1 x 4 (4% Cross-linkage, chloride form, 100–200 mesh, 74–149 µm) in glass barrel Econo-columns, 7 × 5 mm was used for purification of the extracts. The standard, sodium phytate (P-8810, Sigma-Aldrich, Johannesburg, South Africa) and purified extracts were reacted with Wade reagent, after which absorbance was measured at 500 nm.

3.1.3.6 Determination of Total Phenolic Content

The total phenolic content of the extracts was determined as described by Apea-Bah *et al.* (2016). In each well of a 96-well microplate, 18.2 µl volume of the sample extract or catechin standard solution (0 - 0.5 mg/ml) was reacted with 36.4 µl 10% Folin-Ciocalteu reagent (diluted with distilled water) and 145.4 µl of 700 mM sodium carbonate. The reaction mixture was incubated for 2 hours in the dark, after which absorbance was read at 750 nm using an Omega FluoSTAR microplate reader (BMG Labtechnologies, Ortenberg, Germany). Total phenolic content was calculated with the aid of the catechin standard calibration curve and expressed as milligrams of catechin equivalents per gram (mg CE/g) dry weight basis.

3.1.3.7 Determination of Organic acids

The extraction and quantification of organic acids were by reversed phase-HPLC according to Tembo *et al.* (2017) with modifications as per Adetola *et al.* (2019).

3.1.3.8 Determination of iron content

Acid digestion of the plant foods was performed using conc. nitric acid plus hydrogen peroxide according to EPA method 3051A (U.S.EPA, 2007). Iron contents of the digested flour samples were analysed by EPA method 200.7 (U.S.EPA, 1996) using inductively coupled plasma atomic emission spectrometry (ICP-AES) (iCAP 6000 series, Thermo Fisher Scientific, Waltham, USA). Iron was analysed at 239.5 nm. To ensure accuracy, samples were analysed against National Institute for Standards and Technology traceable standards and independent

quality control solutions. A calibration acceptance criterion of $R^2 > 0.9995$ was used, and an internal standard technique was used to check result accuracy.

3.1.3.9 Determination of iron bioaccessibility using the *in vitro* dialyzability assay

The *in vitro* dialyzability method of Miller *et al.* (1981) was used. Digestive enzymes and bile salts used were pepsin (P-7000), pancreatin (P-1750) and bile extract (B-8631). Dialysis tubing Spectra/Por 7 (Ø 20.4 mm) with a molecular weight cut-off of 10 kDa was used (G.I.C. Scientific, Johannesburg, South Africa). Mineral contents of the dialysates were measured using ICP-AES as described. Iron bioaccessibility was calculated as the percentage of the mineral in the dialysate relative to the total mineral content in the porridge sample.

3.1.3.10 *In Vitro* Digestion for Caco-2 cells: Solubility Method

The *in vitro* digestion method of Glahn, Lee, Yeung, Goldman and Miller (1998) with modifications according to Perales, Barberá, Lagarda and Farré (2005) was used and comprised sequential gastric and intestinal stages. The modifications mainly involved demineralizing the pepsin and pancreatin-bile salt solutions. In addition, instead of using dialysis tubing, aliquots of 20 g sample were transferred to polypropylene centrifuge tubes (50 mL) and centrifuged at 3500 g for 1 h at 4ºC and supernatants were used to determine the bioaccessible iron content. The bioaccessible fraction (soluble fraction) was used in the Caco-2 cell ferritin assay.

3.1.3.11 Culturing of Caco-2 cells and determination of ferritin synthesis by Caco-2 cells

Cell culturing and determination of ferritin were done as described by Viadel, Perales, Barberá, Lagarda and Farré (2007) with modifications. Caco-2 cells were obtained from Separation Scientific, Johannesburg-South Africa (Cellonex Cell Line) and were used between passage numbers 8 and 20. The cells were sub-cultured and maintained at 37ºC in an incubator (under a 5% CO_2/95% air atmosphere at constant humidity). Growth medium of Dulbecco's minimum essential media (Sigma-Aldrich) with Earle's salts, L-glutamine, sodium bicarbonate and sodium pyruvate supplemented with 10% v/v foetal bovine serum, 1% v/v non-essential amino acids (Sigma-Aldrich), and 1% v/v antibiotic-antimycotic solution (Sigma-Aldrich) was used. At 80% confluence, cells were seeded at a density of $5x10^4$ cells/ cm^2 in 6-well plates and maintained at 37ºC in an incubator under a 5% CO_2/95% air atmosphere at constant humidity. The seeded cells were grown under low iron conditions, minimum essential medium (Sigma-Aldrich) supplemented with 10% demineralized FBS and 1% v/v antibiotic-antimycotic solution (Gibco). The culture medium was changed every 2 days. The iron uptake assays were performed with differentiated cells 14–16 days after seeding. At the end of each assay, the cell

monolayers were washed three times with buffer solution, and the cells were detached with a cell scrapper.

Cell monolayers were collected with 2 ml deionized water at 4ºC and sonicated at 30 kHz for 30 seconds at 4ºC. Ten microliter aliquots of the sonicated Caco-2 monolayer were used in ferritin measurement (Human Ferritin ELISA Kit for serum, plasma, cell culture supernatant and urine, Sigma-Aldrich). The cell protein content was determined using a TaKaRa Bradford Protein Assay Kit Bicinchoninic Acid (Separations, Johannesburg, South Africa). Ferritin contents were expressed as ng ferritin/mg protein.

3.1.3.12 Statistical Analyses

Each experiment was performed thrice (except for dialyzability and ferritin formation, which were repeated 6 times), and a multiple analysis of variance was used to determine the differences between treatments. Fisher's LSD test at a 0.05 level of significance was applied. Statistica 10 (StatSoft Inc., Tulsa, OK, USA) was used.

3.1.4 Results and discussion

3.1.4.1 Antinutritional components (phytate, total phenolics and dietary fibre) and organic acids (ascorbic acid and citric acid)

3.1.4.1.1 Mineral Absorption Enhancers (Organic acids - ascorbic acid and citric acid)

Due to their role as enhancers of mineral bioavailability in foods (Iyengar *et al.*, 2010), the content of organic acids (ascorbic acid and citric acid) in wholegrain sorghum-based porridges was of interest. Baobab had higher contents of ascorbic and citric acids (50-times more than moringa with none in sorghum for ascorbic acid, and 1.3 times more than moringa and 112 times more than sorghum for citric acid) (Table 3-1). Similar trends in the organic acid contents of baobab compared to moringa have been reported previously (Adetola *et al.*, 2021; Adetola *et al.*, 2019).

Table 3-1: Organic acids, phytate, total phenolic content (TPC), insoluble dietary fibre (IDF), soluble dietary fibre (SDF) and iron contents of wholegrain sorghum, baobab fruit pulp powder and moringa leaf powder determined on a dry basis

Food material	Citric acid (mg/100 g)	Ascorbic acid (mg/100 g)	Phytate (mg/100 g)	TPC (mg CE/100 g)	IDF (g/100 g)	SDF (g/100 g)	Iron Content (mg/100 g)
Wholegrain Sorghum	284±15[1a]	ND[2]	1078±136[b]	404±19[a]	8.66±0.51[a]	1.51±0.48[a]	4.84±0.80[b]
Baobab Fruit Pulp Powder	3506±148[c]	164±27[b]	418±7[a]	4119±7[c]	12.95±0.77[b]	42.61±1.43[c]	2.32±0.44[a]
Moringa Leaf Powder	2722±358[b]	2±0[a]	1721±61[c]	3852±0[b]	38.17±0.85[c]	4.32±1.07[b]	100.93±7.52[c]

[1]Values are the means±SD of at least two samples of each plant food analysed independently in triplicate (n=6)
[2]Not detected
[3]Means of with different superscript uppercase letters in a column differ significantly ($p<0.05$)

3.1.4.1.2 Phytate and total phenolics

Moringa leaf powder had the highest phytate content, 1.6 times higher than wholegrain sorghum and 4.1 times higher than baobab fruit pulp powder (Table 3-1). The values reported here are similar to those reported by Kruger, Oelofse and Taylor (2014) for wholegrain sorghum, baobab fruit pulp (Adetola *et al.*, 2021), and moringa leaf powder Leone *et al.* (2015a).

The wholegrain sorghum-based porridges prepared by extrusion cooking consistently had a lower phytate content than their conventionally wet-cooked counterparts (on average, 16% lower) (Table 3-2). This lower phytate content was probably due to the thermal dephosphorylation of the phytate into lower inositol phosphates (Watson, Smernik & Doolette, 2019) by extrusion cooking. In fact, it has been observed that with extrusion cooking of kidney beans and peas, there was an increase in inositol-penta/tetra/tri-phosphates accompanied by a corresponding decrease in inositol hexaphosphate content. Reduction in phytate content by extrusion cooking has also been reported in wholegrain sorghum-based composite flours by Tadesse, Beri and Abera (2019) Tadesse, Beri, and Abera (2019) and Arun Kumar *et al.* (2018). Concerning the effects of fortification, generally, none of the various fortification treatments reduced phytate content.

Table 3-1 shows that baobab fruit pulp powder had the highest TPC, 6% more than moringa leaf powder and 90% more than wholegrain sorghum. Braca *et al.* (2018) similarly reported high values of TPC in baobab. Similarly, Leone *et al.* (2015a) reported high TPC values of moringa leaf. The TPC values for wholegrain sorghum (Table 3-1) are also within the range reported by Kruger, Taylor and Oelofse (2012). Table 3-2 shows that the wholegrain sorghum porridges prepared by extrusion cooking had lower TPC than the conventionally cooked porridges. The combination of high temperatures, shear and pressure during extrusion cooking lead to the degradation of phenolic compounds or alteration of their chemical activity (Sharma *et al.*, 2012), which might alter their ability to chelate iron. Fortification with baobab fruit pulp and moringa leaf powder alone or in combination resulted in increased TPC (15-64% higher), due to their high TPC contents. A principal component analysis (PCA) plot projecting the dependent variables and treatments on a two-dimensional factor plane (Figure 3-1 A&B) showed that the extruded treatments, except those containing moringa, were in different quadrants to total phenolic content and phytate content. This illustrates clearly the effect of extrusion cooking of reducing phytate and phenolics content.

Table 3-2: Effects of processing (conventional and extrusion cooking) and addition of organic acids, baobab fruit pulp and moringa leaf powder to wholegrain sorghum porridge on phytate and total phenolic content (TPC) (dry basis) of sorghum-based porridge formulations

Formulation	Phytate (mg/100 g)			TPC (mg CE/100 g)		
	Conventionally cooked	Extrusion cooked	Average effect of porridge formulation[4,8]	Conventionally cooked	Extrusion cooked	Average effect of porridge formulation
A. Wholegrain Sorghum	$808^{bBC}\pm133^{1,2,3}$	$679^{aB}\pm18$	$744^{BC}\pm76$	$340^{bB}\pm46$	$250^{aC}\pm16$	$295^{B}\pm31$
B. Wholegrain Sorghum+$FeSO_4$	$955^{bCD}\pm67$	$672^{aB}\pm15$ (-31%)[6]	$813^{CD}\pm186$	$394^{bBC}\pm8$	$204^{aB}\pm3$ (-18%)	$299^{B}\pm104$
C. Wholegrain Sorghum+$FeSO_4$+Ascorbic Acid+Citric Acid (added before processing)	$783^{bAB}\pm11$(-3%)	$699^{aB}\pm7$ (-28%)	$714^{BC}\pm104$	$303^{bA}\pm10$(-12%)	$131^{aA}\pm14$ (-48%)	$217^{A}\pm95$(-26%)
D. Wholegrain Sorghum+$FeSO_4$+Ascorbic Acid+Citric Acid (added after processing)	$846^{bBC}\pm23$	$670^{aAB}\pm68$ (-31%)	$758^{BC}\pm104$	$342^{bAB}\pm43$	$142^{aA}\pm5$ (-43%)	$242^{A}\pm113$(-18%)
E. Wholegrain Sorghum+Baobab	$800^{bAB}\pm21$	$705^{aB}\pm39$ (-27%)	$752^{B}\pm57$	$465^{bD}\pm45$(+37%)	$278^{aCD}\pm28$	$372^{C}\pm108$(+26%)

(Baobab added before processing)						
F. Wholegrain Sorghum+Baobab (Baobab added after processing)	765^{bAB}±36(-15%)	603^{aA}±45 (-38%)	684^{AB}±94	401^{bBC}±12	452^{aF}±20 (+81%)	427^{D}±31(+45%)
G. Wholegrain Sorghum+Moringa	960^{bD}±41(+19%)	845^{aC}±12 (-13%)	903^{C}±67	563^{bE}±49(+66%)	316^{aD}±17 (+26%)	439^{D}±139(+49%)
H. Wholegrain Sorghum+Moringa+Baobab (Baobab added before processing)	1058^{bE}±130(+31%)	682^{aB}±101 (-29%)	870^{D}±33(+17%)	664^{bF}±51(+95%)	405^{aE}±50 (+62%)	535^{E}±149(+81%)
I. Wholegrain Sorghum+Moringa+Baobab (Baobab added after processing)	893^{aBC}±22 (-8%)	885^{aC}±45 (+30%)	889^{E}±34(+19%)	547^{aE}±33(+61%)	536^{aG}±29 (+114%)	542^{E}±27(+84%)
Average effect of cooking method[5,9]	904^{b}±47	747^{a}±41 [-17%][7]		454^{b}±47	302^{a}±41 [-25%]	

[1]Values are the means±SD of at least two samples of each formulation analysed independently in triplicate (n=6)
[2]For each dependent variable (phytate, TPC), the means of each treatment (conventionally cooked, extruded) with different superscript lowercase letters in a row differ significantly ($p<0.05$) by pairwise comparison
[3]Means of each treatment (conventionally cooked, extruded) with different superscript uppercase letters in a column differ significantly ($p<0.05$)
[4]For each overall effect of porridge formulation, the means of each formulation with different superscript uppercase letters in a column differ significantly ($p<0.05$)
[5]For each overall effect of processing technology (cooking or extruded), the means of each formulation with different superscript lowercase letters in a row differ significantly ($p<0.05$)
[6]Figures in curved brackets are the average percentage difference of extruded porridges compared to conventionally wet-cooked sorghum porridges where statistically significant
[7]Figures in square brackets are the average percentage difference between extrusion cooked and conventionally cooked where statistically significant
[8]Average effect of porridge formulation refers to the average of each treatment (fortification) across the rows regardless of whether the formulation was conventionally wet-cooked or extruded
[9]Average effect of cooking method refers to the average of each cooking method along the column regardless of the treatment performed

3.1.4.2 Total iron content, iron bioaccessibility and ferritin formation in Caco-2 cells

3.1.4.2.1 Total iron content, percentage iron bioaccessibility and bioaccessible iron (dry basis)

Moringa contained 25 times more iron than sorghum and 44 times more iron than baobab (Table 3-1). While varying data for the iron content of moringa leaf powder have been reported, 19 mg/100 g by Adetola *et al.* (2019) and 58.4 mg/100 g by Van der Merwe *et al.* (2019), it consistently has a higher iron content than sorghum. Adetola *et al.* (2019) reported an iron content of 3.7 mg/100 g in baobab, close to what we reported in this study, and Kayodé *et al.* (2006) reported iron content (3.0-11.3 mg/100 g) in sorghum within range of what was reported in this study.

Regarding the total iron content (TIC), extrusion cooking significantly increased the iron content of the sorghum-based porridges (by 9%) (Table 3). This is probably due to the abrasive action of the plant materials resulting in some iron from the extruder parts, probably in the form of iron oxide (rust), being incorporated into the extrudates (Alonso *et al.*, 2001). Extrusion cooking, when compared to conventional wet cooking, had no significant ($p>0.05$) effect on the bioaccessible iron content (BIC) of the porridges (Table 3-3). However, extruded porridge fortified with moringa and with moringa plus baobab had significantly lower BIC, by 59% and 53%, respectively, compared to the conventionally wet-cooked porridge treatments. The reduction in BIC could result from the iron incorporated in the formulation following extrusion not being bioaccessible.

Fortification with ferrous sulphate increased BIC by an overall 143% but reduced the percentage of bioaccessible iron (PBI) by overall 19% (Table 3-3). Fortification with ferrous sulphate plus ascorbic and citric acids, whether before or after cooking (conventional or extrusion cooking), increased both BIC and PBI (by 540% and 99% before processing by 419% and 64% after, respectively). The increase in both BIC and PBI was due to the enhancing effects of the organic acids. Ascorbic and citric acids are known mineral bioaccessibility enhancers as they chelate minerals and keep them in a soluble and absorbable form (Iyengar *et al.*, 2010; Lönnerdal, 2000). The PCA projecting the BIC and PBI and fortification treatments on a two-dimensional factor plane (Figure 3-1 A&B) showed that all the treatments containing organic acids were associated with PBI and BIC, further supporting the enhancing effect of organic acids on mineral bioaccessibility.

FtFF with baobab before processing significantly increased BIC, while baobab FtFF both before and after processing significantly increased PBI by 23-37%. This was supported by the PCA plot where all treatments containing baobab, observed along factor 1, were associated with PBI (Figure 3-1 A&B), suggesting an enhancing role of baobab in iron bioaccessibility. While the content of ascorbic and citric acids in the baobab was equivalent to the organic acids added in the ferrous sulphate treatment, the effect of the baobab on the bioaccessible iron was far less. This can be attributed to the phenolic compounds in baobab binding the iron. The increase in PBI following FtFF with baobab fruit powder can therefore be attributed to the presence of organic acids (mainly citric acid) in the baobab fruit powder. Similar results for BIC and PBI were reported by (Adetola *et al.*, 2019) baobab fruit powder FtFF of pearl millet porridges.

FtFF with moringa, whether alone or in combination with baobab, increased the iron content of the sorghum-based porridges by approximately 3 times due to its high iron content (Table 3-3). However, moringa caused an overall significant reduction in both the bioaccessible iron content and percentage of bioaccessible iron, by 30% and 71%. This is likely due to its high phytate and calcium contents (Adetola *et al.*, 2019). This is supported by the PCA plot, which revealed that all treatments containing moringa were associated with high phytate and phenolics contents and were in the opposite quadrant and plane to the BIC and PBI when projected on a two-dimensional factor plane (Figure 3-1 A&B). When complexes are formed between phytic acid with calcium and iron, they are more stable and less soluble than complexes of iron with phytic acid making complexes formed in the presence of high calcium content far less available (Rousseau *et al.*, 2020).

Table 3-3: Effects of processing (conventional and extrusion cooking) and addition of organic acids, baobab fruit pulp and moringa leaf powder to wholegrain sorghum porridge on total iron content (TIC), bioaccessible iron content (BIC) and percentage bioaccessible iron (PBI) of sorghum-based porridge formulations

Formulations	TIC (mg/100 g db)			BIC (mg/100 g db)			PBI (%)		
	Conventionally cooked	Extrusion cooked	Average effect of porridge formulation[4,8]	Conventionally cooked	Extrusion cooked	Average effect of porridge formulation	Conventionally cooked	Extrusion cooked	Average effect of porridge formulation
A. Wholegrain Sorghum	$3.7^{aA}\pm0.4^{1,2,3}$	$5.3^{bB}\pm0.1$	$4.5^{B}\pm0.9$	$0.4^{aA}\pm0.1$	$0.4^{aB}\pm0.1$	$0.4^{B}\pm0.1$	$9.2^{aD}\pm2.4$	$7.1^{aCD}\pm1.8$	$8.1^{E}\pm2.4$
B. Wholegrain Sorghum+$FeSO_4$	$12.9^{aD}\pm0.1$(+250%)[6]	$14.2^{bE}\pm0.2$(+170%)	$13.5^{E}\pm0.7$(+202%)	$0.9^{dC}\pm0.0$(+143%)	$0.9^{aD}\pm0.0$(+143%)	$0.9^{D}\pm0.0$(143%)	$6.9^{aC}\pm1.4$(-24%)	$6.0^{aC}\pm1.1$(-15%)	$6.6^{D}\pm1.3$ (-19%)
C. Wholegrain Sorghum+$FeSO_4$+Ascorbic Acid+Citric Acid (added before processing)	$13.4^{aE}\pm0.1$(+264%)	$15.0^{bF}\pm0.9$(+185%)	$14.2^{F}\pm1.1$(+218%)	$2.4^{aE}\pm0.1$(+549%)	$2.4^{aE}\pm0.1$(+535%)	$2.4^{F}\pm0.1$(+540%)	$16.7^{aH}\pm0.7$(+82%)	$15.7^{aG}\pm0.7$(+121%)	$16.2^{H}\pm0.9$(+99%)
D. Wholegrain Sorghum+$FeSO_4$+Ascorbic Acid+Citric Acid (added after processing)	$13.8^{aF}\pm0.2$(+273%)	$15.4^{bF}\pm0.0$(+193%)	$14.6^{G}\pm0.9$(+227%)	$1.8^{fD}\pm0.1$(+395%)	$2.0^{gE}\pm0.3$(+441%)	$1.9^{E}\pm0.2$(+419%)	$13.7^{aG}\pm0.7$(+49%)	$13.0^{aF}\pm1.9$(+84%)	$13.3^{G}\pm1.4$(+64%)
E. Wholegrain Sorghum+Baobab (Baobab added before processing)	$3.6^{aA}\pm0.2$	$4.8^{bA}\pm0.0$(-10%)	$4.2^{AB}\pm0.6$	$0.5^{aB}\pm0.0$ (+22%)	$0.5^{aC}\pm0.1$(+27%)	$0.5^{C}\pm0.1$(+27%)	$12.1^{bF}\pm0.8$(+32%)	$9.7^{aE}\pm1.1$(+37%)	$10.9^{F}\pm1.6$(+34%)
F. Wholegrain Sorghum+Baobab (Baobab added after processing)	$3.6^{aA}\pm0.2$	$5.0^{bAB}\pm0.2$	$4.3^{AB}\pm0.8$	$0.4^{aAB}\pm0.1$	$0.4^{aB}\pm0.1$	$0.4^{B}\pm0.0$	$11.3^{bEF}\pm1.8$(+23%)	$7.3^{aCD}\pm1.2$	$9.3^{E}\pm2.5$
G. Wholegrain Sorghum+Moringa	$10.4^{aB}\pm0.1$(+183%)	$11.2^{bD}\pm0.3$(+116%)	$10.8^{C}\pm0.5$(+142%)	$0.4^{bA}\pm0.0$	$0.2^{aA}\pm0.0$(-59%)	$0.3^{A}\pm0.0$(-30%)	$3.4^{bAB}\pm0.4$(-63%)	$1.3^{aA}\pm0.1$(-81%)	$2.4^{AB}\pm1.1$(-71%)
H. Wholegrain Sorghum+Moringa+Baobab (Baobab added before processing)	$10.8^{aC}\pm0.3$(+194%)	$11.4^{bD}\pm0.2$(+115%)	$11.1^{D}\pm0.4$(+148%)	$0.3^{bA}\pm0.1$	$0.2^{aA}\pm0.0$(-59%)	$0.2^{A}\pm0.1$(-38%)	$3.0^{bA}\pm0.4$(-68%)	$1.3^{aA}\pm0.2$(-81%)	$2.2^{A}\pm0.9$(-74%)

I. Wholegrain Sorghum+Moringa+Baobab (Baobab added after processing)	10.8^{aC}±0.2(+193%)	10.7^{aC}±0.0(+106%)	10.7^{C}±0.2(+140%)	0.5^{aB}±0.0 (+27%)	0.5^{aC}±0.0(+22%)	0.5^{C}±0.1(+24%)	4.4^{aB}±0.2(-52%)	4.3^{aB}±0.8(-40%)	4.3^{C}±0.5 (-47%)
Average effect of cooking methods[5,9]	9.2^{a}±4.2	10.3^{b}±4.1 [+10%][7]		*0.8^{a} ± 0.7*	0.8^{a}±0.8		*9.2^{b}±4.8*	7.5^{a}±4.8[-19%]	

[1]Values are the means±SD of at least two samples of each formulation analysed independently in triplicate (n=6)
[2]For each dependent variable (TIC, BIC and PBI), the means of each treatment (conventionally cooked, extruded) with different superscript lowercase letters in a row differ significantly ($p<0.05$) by pairwise comparison
[3]Means of each treatment (conventionally cooked, extruded) with different superscript uppercase letters in a column differ significantly ($p<0.05$)
[4]For each average effect of porridge formulation, the means of each formulation with different superscript lowercase letters in a column differ significantly ($p<0.05$)
[5]For each average effect of processing technology (cooking or extruded), the means of each formulation with different superscript lowercase letters in a row differ significantly ($p<0.05$)
[6]Figures in curved brackets are the average percentage difference compared to sorghum porridge were statistically significant
[7]Figures in square brackets are the average percentage difference between extrusion cooked and conventionally cooked were statistically significant
[8]Average effect of porridge formulation refers to the average of each treatment (fortification) across the rows regardless of whether the formulation was conventionally wet-cooked or extruded
[9]Average effect of cooking method refers to the average of each cooking method along the column regardless of the treatment performed

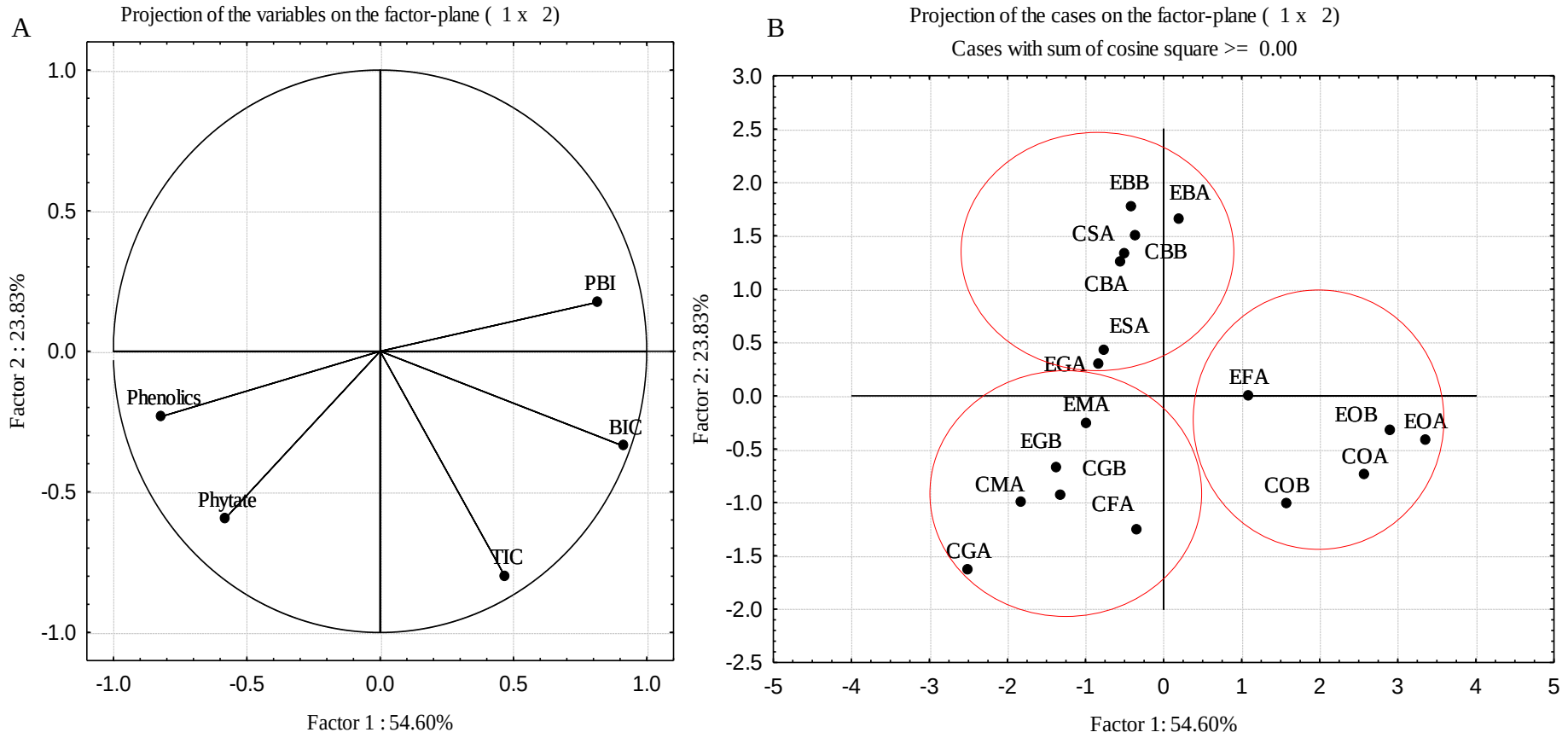


Figure 3-1: Principal component analysis showing 1×2 factor coordinate plots of dependent variables (phytate, phenolics, total iron content-TIC, bioaccessible iron content-BIC and percentage bioaccessible iron- PBI), A, and independent variables (fortification and processing technique-wet-cooking and extrusion), B.

Key: CSA-Cooked Sorghum, CFA- Cooked Sorghum+Fe, COA- Cooked Sorghum+Fe+Organic Acids co-cooked, COB- Cooked Sorghum+Fe+Organic Acids added after, CBA- Cooked Sorghum+Baobab co-cooked, CBB- Cooked Sorghum+Baobab added after, CMA- Cooked Sorghum+Moringa, CGA- Cooked Sorghum+Moringa+Baobab co-cooked, CGB- Cooked Sorghum+Moringa+Baobab added after, ESA- Extruded Sorghum, EFA- Extruded Sorghum+Fe, EOA- Extruded Sorghum+Fe+Organic Acids co-

cooked, EOB- Extruded Sorghum+Fe+Organic Acids added after, EBA- Extruded Sorghum+Baobab co-cooked, EBB- Extruded Sorghum+Baobab added after, EMA- Extruded Sorghum+Moringa, EGA- Extruded Sorghum+Moringa+Baobab co-cooked, EGB- Extruded Sorghum+Moringa+Baobab added after

3.1.4.2.2 Ferritin formation in Caco-2 cells

While *in vitro* dialyzable iron assesses gastric/upper intestinal digestive release and solubility of iron and is an indicator of iron availability for absorption, ferritin formation by Caco-2 cells provides information about how much of the soluble iron may be available for utilization by enterocytes (Ferruzzi *et al.*, 2020). This is because when Caco-2 cells fully differentiate, they express transport and metabolizing systems critical to iron absorption (such as divalent metal ion transporter-1 and ferroportin transporters). As the storage form of cellular iron is ferritin, higher levels of available iron induce greater ferritin formation by Caco-2 cells and hence are an indicator of iron bioavailability (Glahn *et al.*, 1998). In fact, quantitative determination of iron bioaccessibility using the *in vitro* digestion/Caco-2 cell culture model has been well correlated with human data (Mahler, Shuler & Glahn, 2009; Glahn *et al.*, 1998) and, as such, presents a method of predicting iron bioavailability *in vivo.*

Figure 3-2 shows that when wholegrain sorghum was extrusion cooked, there was a 46% increment in ferritin synthesis when compared to conventionally cooked wholegrain sorghum porridge. In this regard, it is notable that extrusion cooking caused a significant decrease in both total phenolics and phytate (Table 3-2). As these compounds decrease iron availability (Gabaza, Shumoy, Muchuweti, Vandamme & Raes, 2018), their reduction by extrusion cooking is the likely cause of the increase in ferritin formation. FtFF of co-extruded sorghum with baobab and moringa and their combination showed a reduction in ferritin synthesis by 18%, 34% and 26%, respectively, when compared to sorghum alone**.** This is apparently somewhat contradictory to the dialyzable iron results, where there was an increase in bioaccessible iron with baobab inclusion (Table 3-3).

The *in vitro* iron dialyzability assay assesses iron bioaccessibility in terms of the amount of iron that is released by simulated digestion processes and soluble to cross a semi-permeable membrane of a particular threshold, in this case, 10 kDa. In contrast, ferritin formation by Caco-2 cells measures how much iron can be taken up by the cells to form ferritin (Ferruzzi *et al.*, 2020). Therefore, while with *in vitro* iron dialyzability analysis of the extrusion cooked and conventionally cooked porridges, there was the same amount of iron crossing the dialysis membrane; more iron was available for ferritin formation in the extruded porridges. This was probably because the contents of the iron-binding phenolics and phytate were reduced by extrusion cooking.

It is also possible that another reason for the observed difference in trends between the *in vitro* iron dialyzability assay and the Caco-2 ferritin formation assay is that the dialyzability assay used in this research involved the utilisation of an older *in vitro* gastrointestinal food digestion instead of more advanced techniques such as INFOGEST 2.0 method described by Brodkorb, Egger, Alminger, Alvito, Assunção, Ballance, Bohn, Bourlieu-Lacanal, Boutrou and Carrière (2019).

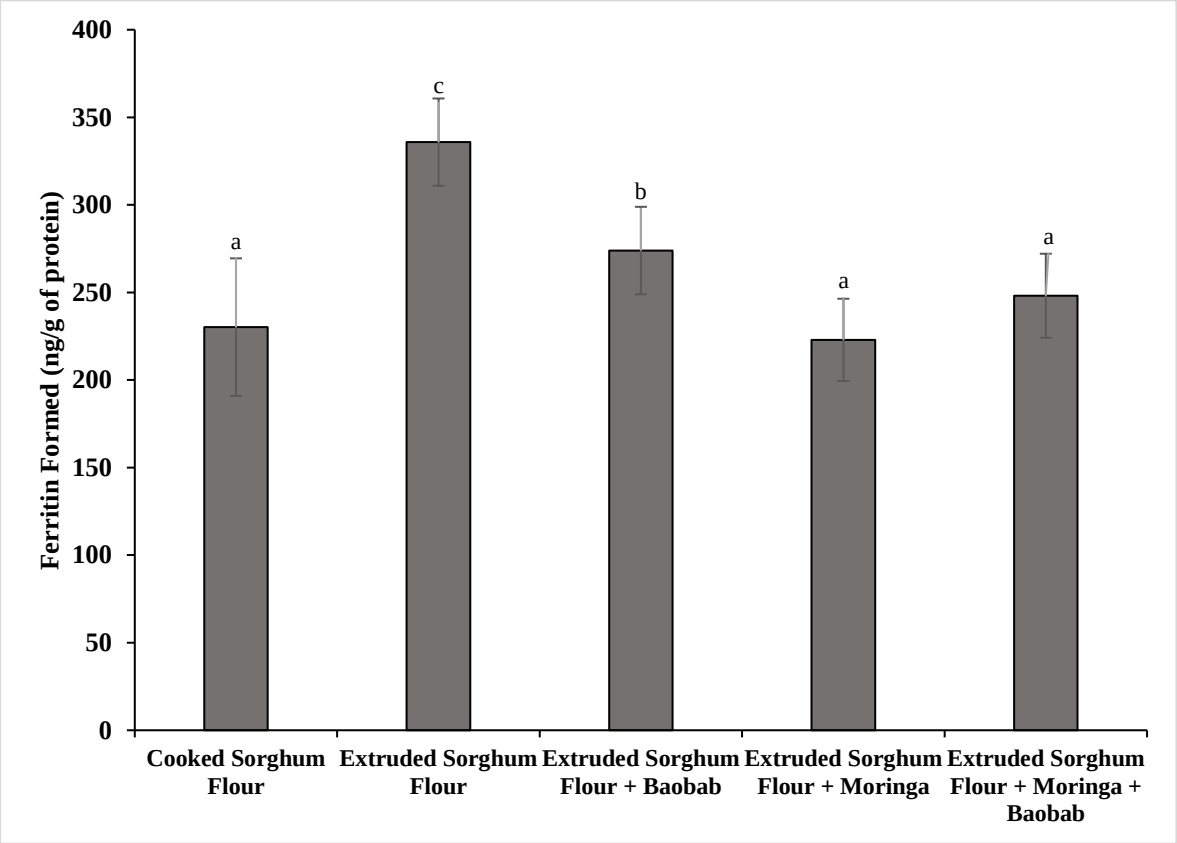


Figure 3-2: Effects of processing (conventional and extrusion cooking), FtFF with baobab fruit pulp and moringa leaf powder (added before processing) to wholegrain sorghum porridge on ferritin formation in Caco-2 cells grown under low iron conditions. Data are the mean±standard deviation of three independent experiments carried out in triplicate (n=9). Different letters indicate significant differences at $p<0.05$. Error bars indicate standard deviation.

3.1.5 Conclusions

Extrusion cooking increases ferritin formation in Caco-2 cells when compared to conventional wet cooking, which is indicative of the enhancing effect of extrusion cooking on iron bioaccessibility. This is largely due to its ability to reduce the content of phytate (probably by dephosphorylation) and phenolic content (probably by degradation). However, extrusion did not affect the amount and percentage of dialyzable iron. The apparent contradiction between

dialyzability and ferritin formation data is probably because they measure different aspects of bioaccessibility. By *in vitro* iron dialyzability analysis of the extrusion-cooked and conventionally cooked porridges, there was the same amount of iron crossing the dialysis membrane. However, more of this iron was available for ferritin formation in the extruded porridges due to the reduction in phytate and phenolics. While an acute iron uptake study in animal or human subjects is required to provide more definitive data regarding the effect on iron bioavailability of wholegrain sorghum fortification with tropical foodstuffs, this study highlights the potential of extrusion cooking to improve iron bioavailability in wholegrain-based starchy staple foods.

3.2 EFFECTS OF EXTRUSION COOKING AND FOOD-TO-FOOD FORTIFICATION ON THE BIOACTIVE PHENOLIC COMPOUNDS AND HEALTH-PROMOTING PROPERTIES OF SORGHUM-BASED PORRIDGES

3.2.1 Abstract

Currently, there is a growing burden of diet-related non-communicable diseases (NCDs) in developing countries, in part due to urbanization that has led to a shift in diet from traditional nutrient-dense diets to highly processed foods that are energy dense and high in saturated fat. Sorghum is a major food crop across the semi-arid tropics of Africa because of its tolerance to high temperatures and low rainfall. Extrusion cooking is a process that applies high heat, pressure, and shear to raw food materials to produce ready-to-eat products and can destroy anti-nutrients in plant foods and hence enhance the digestibility of their macronutrients. Food-to-food fortification (FtFF) is a strategy where nutrient-rich food combinations are used to enhance their nutrient value but has not been explored for potential improvement of health-promoting properties. The objective of this study was to determine the effects of extrusion cooking of sorghum-based porridges FtF fortified with baobab fruit powder and moringa leaf powder on antioxidant, anti-inflammatory, antidiabetic and antilipogenic properties.

Effects of extrusion cooking and food-to-food fortification (FtFF) (with moringa and baobab) of wholegrain sorghum-based porridges on bioactive phenolic content, *in vitro* radical scavenging properties, antiglycation properties (AGEs), cellular antioxidant activity (CAA), cellular anti-inflammatory activity (inhibition of NO) and anti-obesity properties (reduction in lipid droplet formation) were determined. FtFF porridges showed greater phenolic content (phenolic acids and their esters, flavonoids and their glycosides), radical scavenging properties (ABTS radical scavenging and ORAC) and greater reduction in AGEs compared to unfortified porridges. In contrast, extruded instant porridges had lower phenolic content, radical scavenging properties and showed a lesser reduction in AGEs compared to conventionally wet-cooked porridges. All porridges exhibited chemical inhibition of nitric oxide with no apparent effects of fortification or extrusion. While FtFF with moringa alone reduced CAA and extrusion cooking increased it, FtFF with baobab alone or in combination with moringa did not affect CAA. FtFF produced greater higher inhibition of NO formation in RAW264.7 cells while extrusion cooking reduced cellular NO inhibition. Extracts from all porridge samples exhibited prevention and reduction of adipocyte formation in 3T3-L1 cells, indicating anti-

lipogenic effects. In conclusion, FtFF (with moringa and baobab) and extrusion cooking can be used to produce instant porridges from wholegrain sorghums with targeted health-promoting properties to address rising non-communicable diseases in sub-Saharan Africa.

FtFF has the potential to help alleviate antioxidant, anti-inflammatory and antidiabetic properties in ready-to-eat sorghum-based porridges because it enhances their phenolics profile and could play a role in alleviating NCDs in at-risk communities.

This study highlights the potential of FtFF of tropical foodstuff high in phenolic compounds coupled with extrusion cooking to improve health-promoting properties in wholegrain-based starchy staple foods.

Keywords: Extrusion, sorghum, wholegrain, food-to-food fortification, antioxidants, anti-inflammatory, antilipogenic

3.2.1 Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is adjudged as the fifth most important cereal crop in the world after wheat, rice, maize and barley (Taylor, 2019). Over 35% of sorghum is grown directly for human consumption (Taylor & Duodu, 2019). As a drought-resistant crop, sorghum represents an important cereal for food use because of the increase in world population and decreasing water supplies due to climate change (Taylor, 2019). Sorghum is, therefore, an essential and strategic cereal food crop, especially in the dry, semi-arid regions of the world, which include much of sub-Saharan Africa, where sorghum is already primarily used for human food within certain countries. Currently, there is a growing burden of diet-related non-communicable diseases (NCDs) in developing countries. NCDs were responsible for nearly 74% of deaths globally, accounting for more than 52% of the deaths in Africa, with rising numbers in low-income countries (WHO, 2021). NCDs stem primarily from reactive oxygen and nitrogen species as biomarkers of oxidative stress and inflammation (Seyedsadjadi & Grant, 2020). Sorghum is particularly rich in phenolic compounds that have gained popularity for their potential to combat NCDs (Duodu & Awika, 2019).

Food-based strategies such as food-to-food fortification (FtFF) to address micronutrient deficiencies are being applied using micronutrient-rich foodstuffs (such as moringa) and foods rich in mineral bioaccessibility enhancers (such as baobab fruit pulp) (Adetola *et al.*, 2021; Kruger, 2020; Adetola *et al.*, 2019; Van der Merwe *et al.*, 2019). While these foodstuffs are rich in micronutrients and mineral enhancers, they also contain high contents of phenolic compounds, with baobab being rich in flavonoids and tannins (Ismail *et al.*, 2019) and moringa being rich in phenolic acids and flavonoids (Kashyap *et al.*, 2022).

Extrusion cooking is a food processing technology which can be applied to produce a variety of convenience-type products from a large diversity of raw materials (Camire, 2001). An example of such convenience-type products is instant porridge which is popular among consumers in urban and pre-urban communities in sub-Saharan Africa. Extrusion cooking is a continuous manufacturing process which applies high heat, pressure and friction to break down raw foods into a cooked and pre-gelatinised form (Fellows, 2009). While extrusion cooking has been reported to destroy phenolic compounds under certain conditions (Gu, Bk, Wu, Lu, Nawaz, Barrow, Dunshea & Suleria, 2022), it has also been reported to improve phenolic bioaccessibility through depolymerization of polymeric phenolic compounds (Adarkwah-Yiadom & Duodu, 2017).

The main aim of this research was to study the effects of FtFF of sorghum with moringa leaf and baobab fruit pulp, as well as the effect of extrusion cooking on bioactive phenolics and antioxidant properties of sorghum-based porridges. The information gained from this research could contribute to a broader application of FtFF in enhancing the health-promoting properties of cereal-based foods.

3.2.2 Materials and methods

3.2.2.1 Materials

Whole grain red non-tannin sorghum was procured from a local farmer in Mpumalanga, South Africa. Clean sorghum grains were milled into flour using a Drotsky S1 Hammer mill (Alberton, South Africa) fitted with a 500 µm mesh size screen to ensure a particle size of 500 µm or smaller. Prepared flours were stored at 4°C. Naturally dehydrated organic baobab fruit powder from the endocarp was obtained from Nautica Organic Trading, Durban, South Africa. Dried moringa leaf powder was obtained from Supa Nutri (Pty) Ltd, Cape Town, South Africa.

The human colon adenocarcinoma (Caco-2) cell line, adult murine macrophage RAW 264.7 cells and murine fibroblast cell line (3T3-L1) were obtained from CELLONEX through Separations, Johannesburg, South Africa (SA). All cell lines were maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin/fungizone formulation (Highveld Biological Company, Johannesburg, South Africa) and maintained at 37°C 5% CO_2. The Caco-2 cell line was used between passages 9-18, the 3T3-L1 was used between passages 39-45, and the RAW 264.7 cell line was used between passages 8-15. All chemicals were obtained from Sigma- Aldrich Company, Atlasville, SA.

3.2.2.2 Porridge formulations

The following formulations of sorghum-based flours with fortificants were prepared:

A. Wholegrain sorghum flour+corn starch (85:15)
B. Wholegrain sorghum flour+baobab fruit pulp powder+corn starch (85:6:9), with baobab being added before processing.
C. Wholegrain sorghum flour+baobab fruit pulp powder+corn starch (85:6:9), with baobab being added after processing.
D. Wholegrain sorghum+moringa leaf powder+corn starch fortified (85:6:9).
E. Wholegrain sorghum+moringa leaf powder+baobab fruit pulp powder+corn starch (85:6:6:3), with baobab being added before processing.
F. Wholegrain sorghum+moringa leaf powder+baobab fruit pulp powder+corn starch (85:6:6:3), with baobab being, was added after processing.

3.2.2.3 Conventional wet cooking

Distilled water was added to each whole sorghum-based flour in a ratio of 3:10, flour: water (w/w). The slurry was heated to boiling temperature and maintained with constant stirring for

25 minutes. The slurry was left to cool at ambient temperature, after which it was placed in plastic containers and frozen to -20˚C and freeze-dried. Freeze-dried porridge flour was crushed to a particle size that passed through a 500 µm opening screen before further analyses. The pre-cooked porridge flour was stored at 4°C in double-sealed, airtight plastic bags.

3.2.2.4 Extrusion cooking of sorghum

A co-rotating twin-screw extrusion cooker model TX 32 (CFAM Technologies, Potchefstroom-South Africa) (L/D = 21.5:1) was used. Porridge formulations prepared as above were extruded separately. The barrel comprising five heating zones towards the die was set at 60/70/80/140/140ºC, respectively. Water was fed into the system at a dosing rate of 3 l/h (to obtain a final moisture content of 20% calculated based on the moisture content of the flours), and the feed rate was 10 kg/h. A die opening of 3 mm was used, and the screw speed was maintained at 250 rpm. Extrudates were collected three times after every 30 min interval to produce triplicates. They were dried immediately in a forced draught oven at 50ºC for 5 min to a moisture content below 10%. The cooled extrudates were milled using an air-cooled analytical mill to a maximum particle size of 500 µm. The milled extrudates were stored at 4ºC in double-sealed, airtight plastic bags.

3.2.2.5 Preparation of phenolic extracts

Phenolic extracts for TPC and ABTS were prepared from the plant materials according to the procedure previously described by Apea-Bah *et al.* (2014). Approximately 1 g of each dry sorghum-based porridge sample was extracted in duplicate using 10 ml acidified methanol (1% (v/v) conc HCl in methanol) by magnetic stirring for 2 hours. The suspension was centrifuged at 1650 g for 10 min at 4ºC, and the supernatant was collected. The residue was similarly re-extracted twice, each with 10 ml acidified methanol for 30 minutes. The supernatants were then pooled together and stored at -20ºC in the dark before analysis.

Phenolic extracts for liquid chromatography-mass spectrometry (LC-MS) analysis were prepared using a modification of a method described by Nderitu, Dykes, Awika, Minnaar and Duodu (2013). Approximately 5 g of each sorghum-based porridge flour was extracted using 10 ml 1% (v/v) hydrochloric acid in methanol for 2 hours with vortexing after every 5 minutes. The suspension was centrifuged at 1650 g for 10 minutes at 4ºC, and the supernatant was collected and filtered through 0.45 µm Arcrodisc PSF syringe filters (Pall Life Sciences, Ann Arbor, MI, USA) into 1.5 ml amber vials ahead of chromatographic analysis.

3.2.3 Analyses

3.2.3.1 Determination of total phenolic content (TPC)

The total phenolic content of the extracts was determined as described by Apea-Bah *et al.* (2016). In each well of a 96-well microplate, 18.2 µl volume of the sample extract or catechin standard solution (0 - 0.5 mg/ml) was reacted with 36.4 µl 10% Folin-Ciocalteu reagent (diluted with distilled water) and 145.4 µl of 700 mM sodium carbonate. The reaction mixture was incubated for 2 hours in the dark, after which absorbance was read at 750 nm using an Omega FluoSTAR microplate reader (BMG Labtechnologies, Ortenberg, Germany). Total phenolic content was calculated with the aid of the catechin standard calibration curve and expressed as milligrams of catechin equivalents per gram (mg CE/g) dry weight basis.

3.2.3.2 Determination of ABTS radical scavenging capacity

ABTS radical scavenging capacity of the sample extracts was determined using the method described by Apea-Bah *et al.* (2016). The extracts were diluted depending on their concentration with acidified methanol (1% (v/v) conc HCl in methanol). The ABTS radical cation stock solution was prepared by reacting equal volumes of 7 mM ABTS salt with 2.54 mM potassium persulphate in distilled water for 12-16 hours at room temperature in the dark. A working solution was prepared by diluting the ABTS mother solution with 0.2 M phosphate-buffered saline at pH (7.4) in the ratio of 1:29. In each well of a 96-well microplate, 10 µl of the diluted sample extract extracts or trolox standards (0 - 600 mM prepared in acidified methanol) were mixed with 190 ul of the working solution and incubated in the dark for 30 minutes at room temperature (20^{o}C). The absorbance was read at 750 nm using the Omega FluoSTAR microplate reader (BMG Labtechnologies, Ortenberg, Germany). With the aid of a Trolox standard calibration curve, the ABTS radical scavenging capacity was calculated and expressed as micromole Trolox equivalent per gram sample (µmol TE/g) dry weight basis.

3.2.3.3 Nitric oxide radical scavenging capacity

The nitric oxide (NO) radical scavenging capacity of the samples was determined using a modification of the method of Jagetia and Baliga (2004). All extracts were diluted 3 times with 0.1 M PBS. The sodium nitroprusside (5 mM in 0.1 M PBS) was left on the working bench (25-27 C) for 90 minutes before use. The reaction mixture comprising 20 µl extract and 80 µl sodium nitroprusside, was incubated in the dark for 60 minutes at 37^{o}C. A 100 µl volume of 1% (w/v) sulphanilamide and 0.1% (w/v) naphthyl ethylenediamine dihydrochloride in 2.5%

(v/v) aqueous H_3PO_4 was then added. The absorbance of the reaction mixture was measured at 546 nm using the Omega FluoSTAR microplate reader (BMG Labtechnologies, Ortenberg, Germany). NO scavenged by the extracts was expressed as molar $NaNO_2$ equivalents, dry weight basis.

3.2.3.4 Oxygen radical absorbance capacity (ORAC)

The assay was performed using the method described by Ou *et al.* (2001) with modifications described by Serem and Bester (2012). The extracts were diluted 10 times with 0.1 M phosphate-buffered saline (PBS), pH 7.4. A 165 µl volume of 8.8 nM disodium fluorescein working solution and 25 µl of 0.24 M aqueous 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) were added to 10 µl of each diluted extract in a 96-microplate well. The reaction mixtures were shaken to mix well and incubated at 37ºC while measuring their fluorescence decay every minute for 2 hours at 485 nm excitation and 520 nm emission wavelengths, using an Omega FluoSTAR microplate reader (BMG Labtech, Ortenberg, Germany). The ORAC values of the samples were calculated using the net area under the fluorescence decay curves and expressed as mmol TE/g flour, dry weight basis.

3.2.3.5 Anti-glycation assay

The anti-glycation assay was performed according to the method of Siddiqui *et al.* (2016) under a sterile environment. A 50 µl volume of diluted 1% conc. hydrochloric acid-methanolic extracts of sorghum-based porridges (diluted in PBS to yield 500 µg/ml in the well) were transferred into a 96-well opaque fluorescence plate, followed by the addition of 50 µl of bovine serum albumin (BSA, 40 mg/ml, final concentration 10 mg/ml) and 50 µl methylglyoxal (MGO, 56 mM, final concentration 14 mM). Thereafter, 50 µl of 0.1 M PBS pH 7.4 was added to the plate and incubated at 37°C for 7 days. After incubation, fluorescence was measured at an emission of 330 nm and excitation of 420 nm. The positive control contained 50 µl BSA, 50 µl MGO and 100 µl buffer, while the negative control consisted of 50 µl BSA and 150 µl buffer. Sample controls consisted of 50 µl sample, 50 µl MGO, and 100 µl buffer (no BSA). The % advanced glycation end-product (AGE) formation relative to the 100% AGE formation by BSA and MGO alone was calculated.

3.2.3.6 Cellular antioxidant activity assay

The dichlorofluorescein diacetate (DCFH-DA) assay was used to determine cellular antioxidant activity (CAA) using a modified method of Blasa *et al.* (2011). Caco-2 cells were plated in a 96-well plate at a concentration of 10×10^4 cells/ ml (1×10^4 cells/100 µl) and

incubated at 37℃ for 24 hours. Following incubation, 50 µl of 75 µM DCFH-DA was added to each well to a final concentration of 25 µM. After an incubation period of 1 hour at 37℃, the medium was removed, and the cells were gently rinsed once with PBS. Immediately, a 50 µl volume of diluted 1% conc. hydrochloric acid-methanolic extracts of sorghum-based porridges (diluted in PBS to yield 50 µg/ml in the well) were added; thereafter, 50 µl of 4.9 µM AAPH was added. The blank control was Caco-2 cells exposed to DCFH-DA and PBS, and the positive control was cells exposed to DCFH-DA and AAPH.

The %CAA was calculated as: $\frac{\text{(Gradient sample - Gradient sample blank)} \times 100}{\text{(Gradient control - Gradient control blank)}}$

The change in fluorescence was measured every 2 min for 60 min, at excitation/ emission of 485 nm and 520 nm, respectively.

3.2.3.7 Cellular anti-inflammatory activity- lipopolysaccharide assay

Nitric oxide scavenging activity in RAW 264.7 cells was done using a modified method of Malan, Serem, Bester, Neitz and Gaspar (2016). Cells were grown until confluent at 37℃ and 5% CO_2 in DMEM supplemented with 10% FBS and 1% antibiotics. When confluent, cells were serum starved for 24 hours. After 24 hours, 80 µl cells (final concentration 1×10^6 cells/mL) were combined with 10 µl of diluted 1% conc. hydrochloric acid-methanolic extracts of sorghum-based porridges (diluted in PBS to yield 50 µg/ml in the well) and 10 µl lipopolysaccharide (LPS, final concentration 100 ng/ml) and further incubated for 24 hours. After 24 hours, 50 µl of the cell supernatant was assayed for nitric oxide (NO) production using 50 µl Griess reagent (0.1% N-1-naphthyl ethylenediamine di-hydrochloride, 1% sulphanilamide in 2.5% phosphoric acid) and the absorbance was read at 570 nm. Results were reported at % NO production, compared with RAW 264.7 cells exposed to only LPS (100% NO produced). Cell viability was determined with the crystal violet assay.

3.2.3.8 Cellular lipid droplet reduction in 3T3-L1 cells

Lipid droplet reduction and inhibition assays were performed in a similar manner as described by Ibrahim, Serem, Bester, Neitz and Gaspar (2020). Briefly, 3T3- L1 pre-adipocyte cells were maintained in DMEM containing 10% FBS and 1% antibiotic solution (DMEM/FBS). Confluent cells were plated at a concentration of 1×10^3/100 µl in a 96-well plate and grown for 3 days until confluent. For the lipid droplet reduction assay, cells were differentiated for 14 days with differentiation medium (DM) 1 (DMEM/FCS containing final concentrations of 10 µg/ ml insulin, 25 mM IBMX, 50 µM dexamethasone, and 100 µM rosiglitazone) changed on

days 4 and 7, and then with DM 2 (DMEM/FCS + final concentration of 10 µg/ml insulin) on day 10. On day 14, cells were replenished with 90 µl DMEM/FCS only and exposed to 10 µl of the sample.

3.2.3.9 Oil red O (ORO) staining assay.

To determine lipid content, the cultures, after differentiation and exposure to sorghum-based porridge extracts, were fixed with 2% formaldehyde for 30 minutes at 37°C. The formaldehyde was removed, the plates were dried, and 100 µl ORO solution (5% w/v in 60% isopropanol, then further diluted 1.7× in distilled-deionised water) was added for 1 hour. The plate was then rinsed with water and left to dry. Phase contrast images were taken before dye extraction with 100 µl of a 60% isopropanol solution for 5 min. Dye absorbance of the ORO taken up by cells was measured at 405 nm, and the data was reported as % lipid present relative to unexposed differentiated 3T3-L1 cells (100% lipid formation).

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

Phenolic compound characterization by LC-MS was performed using the method described by Apea-Bah et al. (2014). A Waters Acquity Ultra-Performance Liquid Chromatograph (UPLC), equipped with a binary pump system (Waters, Milford, MA, USA) and coupled to 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) was set to monitor phenolic compounds at the wavelength range of 230-500 nm. Separation was done on a Waters BEH C18 (100 × 2.1 mm, 1.7 µm) reverse phase column. The mobile phase consisted of 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 of phenolic compounds in the sample extracts was done by comparing the retention times, mass and UV-visible spectral data of the peaks which were observed in this study with those of pure authentic phenolic compound standards and with what has been reported in literature. Integrated peak areas of phenolic compounds in 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).

3.2.4 Statistical Analyses

Each experiment was performed twice, and a multiple analysis of variance was used to determine the differences in sample parameters. Fisher's LSD test at a 0.05 level of significance was applied. Statistica 10 (StatSoft Inc., Tulsa, OK, USA) was used.

3.2.5 Results and Discussion

3.2.5.1 Effect of FtFF and of extrusion cooking on the total phenolic content (TPC), antioxidant activity against 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate) radical (ABTS), oxygen radical absorbance capacity (ORAC), *in vitro* chemical nitric scavenging capacity (NO) and advanced glycation end products (AGES) of sorghum-based porridges.

Table 3-4: Effects of extrusion cooking and food-to-food fortification of sorghum with baobab fruit pulp and moringa leaf powder on total phenolic content (TPC), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate) radical (ABTS) radical scavenging capacity and Oxygen Radical Absorbance Capacity (ORAC) of sorghum-based porridges on dry basis

Treatment	Conventionally Cooked			Extruded		
	TPC (mgCE/100 g)	ABTS (µMTE/100 g)	ORAC (µMTE/100 g)	TPC (mgCE/100 g)	ABTS (µMTE/100 g)	ORAC (µMTE/100 g)
Plain Sorghum	$404^{d} \pm 19$	$2538^{b} \pm 50$	$90^{cd} \pm 6$	$250^{a} \pm 16$	$2177^{a} \pm 18$	$62^{ab} \pm 11$
Baobab Fruit Pulp Powder	$4792^{i} \pm 102$	$28648^{k} \pm 512$	$385^{h} \pm 14$	N/A	N/A	N/A
Moringa Leaf Powder	$4300^{h} \pm 167$	$37506^{l} \pm 365$	$463^{g} \pm 17$	N/A	N/A	N/A
Sorghum + Baobab (Baobab added before processing)	$465^{e} \pm 45$	$5271^{h} \pm 416$	$132^{e} \pm 15$	$278^{ab} \pm 28$	$3044^{c} \pm 40$	$88^{de} \pm 14$
Sorghum + Baobab (Baobab added after processing)	$452^{e} \pm 20$	$4221^{e} \pm 8$	$146^{e} \pm 12$	$401^{d} \pm 12$	$4851^{fg} \pm 198$	$153^{e} \pm 17$
Sorghum + Moringa	$563^{f} \pm 49$	$3334^{d} \pm 201$	$152^{e} \pm 16$	$316^{c} \pm 17$	$3301^{cd} \pm 393$	$79^{bc} \pm 16$

Sorghum + Moringa + Baobab (Baobab added before processing)	$664^{g} \pm 51$	$8151^{j} \pm 805$	$214^{g} \pm 23$	$405^{de} \pm 50$	$5071^{g} \pm 134$	$132^{e} \pm 45$
Sorghum + Moringa + Baobab (Baobab added after processing)	$547^{f} \pm 33$	$6155^{i} \pm 34$	$200^{fg} \pm 14$	$536^{f} \pm 29$	$6030^{i} \pm 211$	$179^{f} \pm 12$

[1] Values are the means ± 1 Standard deviation of at least three samples of each plant food analysed independently in triplicate (n = 9).
[2]Means with different superscript letters in a column differ significantly ($p \leq 0.05$).
[3]N/A- Not applicable

The TPC of sorghum (404 mg CE/100 g) was within the range of 1 - 13 mg CE/g (that is, 100 – 1300 mg CE/100 g) reported by Kruger *et al.* (2014). The TPC of baobab fruit pulp powder (4792 mg CE/100 g) was higher than both moringa leaf powder and sorghum. Braca *et al.* (2018) reported TPC in gallic acid equivalents at 120 – 148 mg GAE/g. Moringa leaf powder had a TPC of 4300 mg CE/100 g (43 mg CE/g) which was in the range of what was reported by Leone *et al.* (2015b) (29 – 53 mg CE/g). Moringa leaf powder had the highest ABTS radical scavenging activity, 1.3 times higher than baobab fruit pulp and 14.8 times higher than sorghum.

Fortification of the sorghum flour with baobab, moringa and a combination of the two increased TPC (by 12% - 64%), ABTS radical scavenging (by 31% - 177%) and ORAC (by 27% - 189%) compared to sorghum flour (Table 3-4). The most significant increases in TPC and radical scavenging properties were observed in sorghum-based porridges fortified with a combination of baobab fruit pulp and moringa leaf powder. This suggests potential synergistic effects of the different phenolic compounds in the baobab fruit pulp and moringa leaf powder in exerting antioxidant properties.

Instant porridges prepared by extrusion cooking had lower TPC (by 11% - 44%), ABTS radical scavenging activity (by 14% - 42%) and ORAC (by 11% - 48%) compared to conventionally wet cooked porridges (Table 3-4). The high temperature coupled with shear and pressure used in extrusion cooking could lead to decarboxylation of phenolic compounds, and high moisture could cause the polymerisation of phenolic compounds, both of which would reduce the extractability of phenolic compounds and reduce antioxidant activity (Sharma *et al.*, 2012; Brennan *et al.*, 2011). Furthermore, phenolic compounds such as flavonoids can bind to proteins, reducing their extractability (Arts, Haenen, Wilms, Beetstra, Heijnen, Voss & Bast, 2002) and, in effect, reducing the TPC and antioxidant activity.

A principal component analysis (PCA) plot projecting the dependent variables and treatments on a two-dimensional factor plane (Figure 3-3a,b) showed that samples FtF-fortified with moringa and baobab whether alone or in combination were correlated with TPC and antioxidant radical scavenging capacity measured as ORAC and ABTS compared to the unfortified controls. This indicates the positive effect of FtFF on the phenolic profile and antioxidant properties of wholegrain sorghum. The plot also revealed a greater correlation between conventionally wet cooked samples with TPC, ORAC and ABTS compared to their extruded counterparts, clearly illustrating the negative impact of extrusion cooking on these properties

of wholegrain sorghum. Notwithstanding this effect of extrusion cooking, FtF-fortified wholegrain sorghum-based porridges depicted a greater correlation with these properties compared to the unfortified porridges, whether conventionally wet-cooked or extruded. This highlights the positive impact of FtFF on the antioxidant properties of wholegrain sorghum-based porridges.

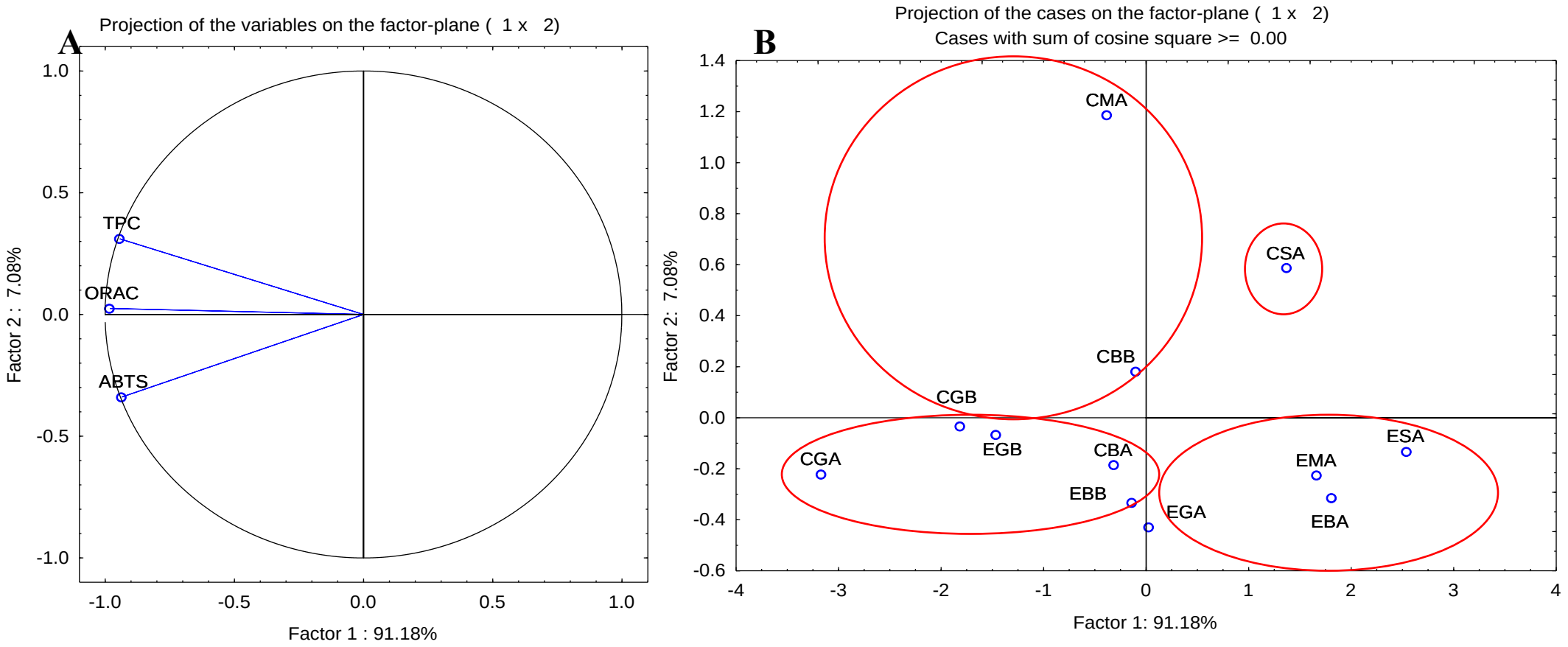


Figure 3-3: Principal component analysis showing 1×2 factor coordinate plots of dependent variables (total phenolic content-TPC, 2,2′ -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-ABTS radical scavenging activity, oxygen radical absorbance capacity-ORAC), A, and independent variables (fortification and processing technique-wet-cooking and extrusion), B.

Key: CSA-Cooked Sorghum, CBA- Cooked Sorghum+Baobab co-cooked, CBB- Cooked Sorghum+Baobab added after, CMA- Cooked Sorghum+Moringa, CGA- Cooked Sorghum+Moringa+Baobab co-cooked, CGB- Cooked Sorghum+Moringa+Baobab added after, ESA- Extruded Sorghum, EBA- Extruded Sorghum+Baobab co-cooked, EBB- Extruded Sorghum+Baobab added after, EMA- Extruded Sorghum+Moringa, EGA- Extruded Sorghum+Moringa+Baobab co-cooked, EGB- Extruded Sorghum+Moringa+Baobab added after

3.2.5.1.1 *In vitro* nitric oxide scavenging

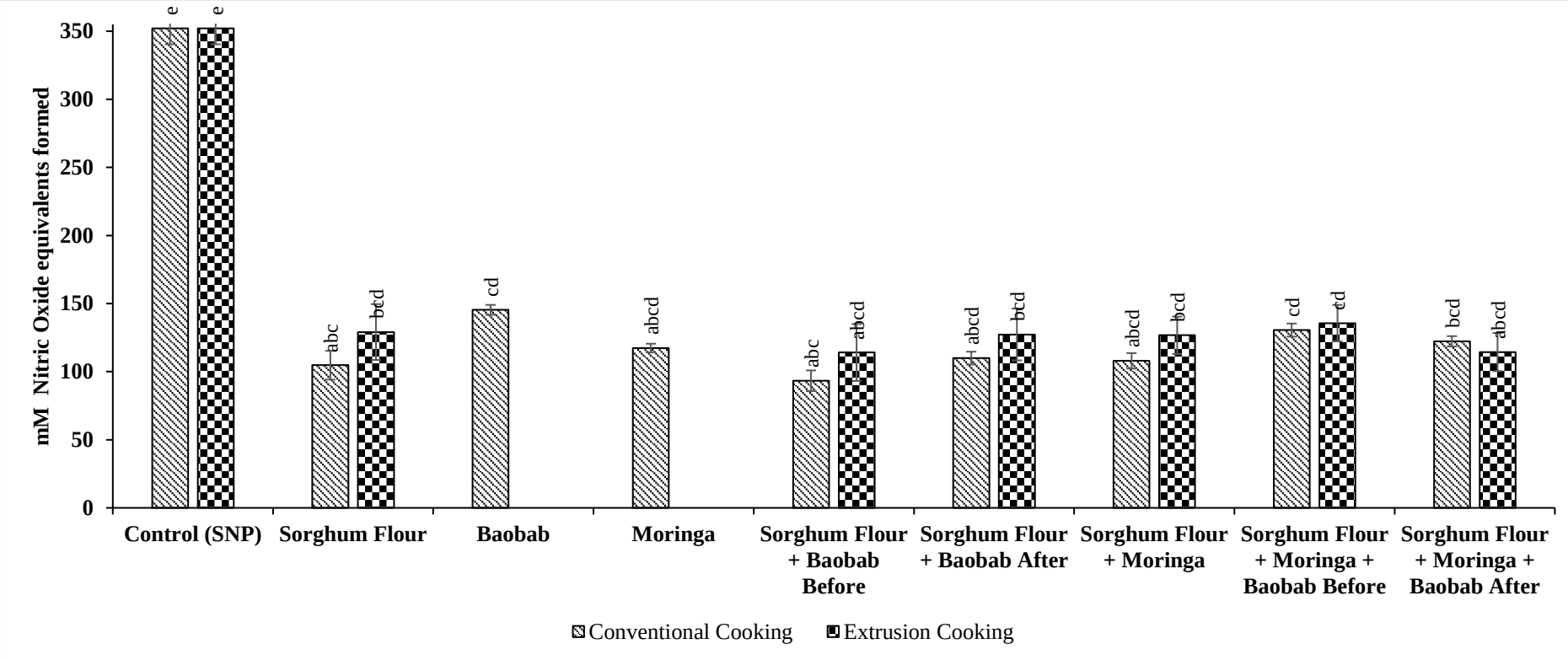


Figure 3-4: The inhibitory effects of phenolic extracts from sorghum-based porridges fortified with baobab and moringa (at a concentration of 50 µg/ml) against nitric oxide radical formation. Data are presented as the mean ± standard deviation (n = 3). Columns marked by different small letters indicate significant differences at $p \leq 0.05$.

[1]Baobab before: Denotes addition of baobab before cooking.
[2]Baobab after: Denotes addition of baobab after cooking.

Regarding the NO radical scavenging capacity of the phenolic extracts, the amounts of NO radical formed ranged between 92-130 mM NO equivalents and 114-135 mM NO equivalents for conventionally wet cooked and extruded sorghum-based porridges respectively, Figure 3-4. Unlike TPC, ABTS and ORAC, NO scavenging showed no significant trends following either fortification with baobab fruit pulp and moringa leaf powders or extrusion. However, all phenolic extracts significantly ($p \leq 0.05$) reduced the amount of NO radicals formed compared to the control. The NO scavenging capacity of the sorghum-based porridges demonstrates their potential in contributing to alleviating radical-induced inflammation. This difference in trends may reflect the different mechanisms of antioxidant action between the methods of analysis. ABTS radical scavenging depends on electron-transfer potential, while ORAC depends on hydrogen atom transfer of the antioxidant (Huang *et al.*, 2005). NO radical scavenging capacity, on the other hand, relies upon the binding of NO radical by the antioxidant and therefore preventing its oxidation into nitrites and nitrates, which are detected by the Griess reaction (Jagetia & Baliga, 2004).

3.2.5.1.2 Advanced glycation endproducts (AGEs)/ Antiglycation

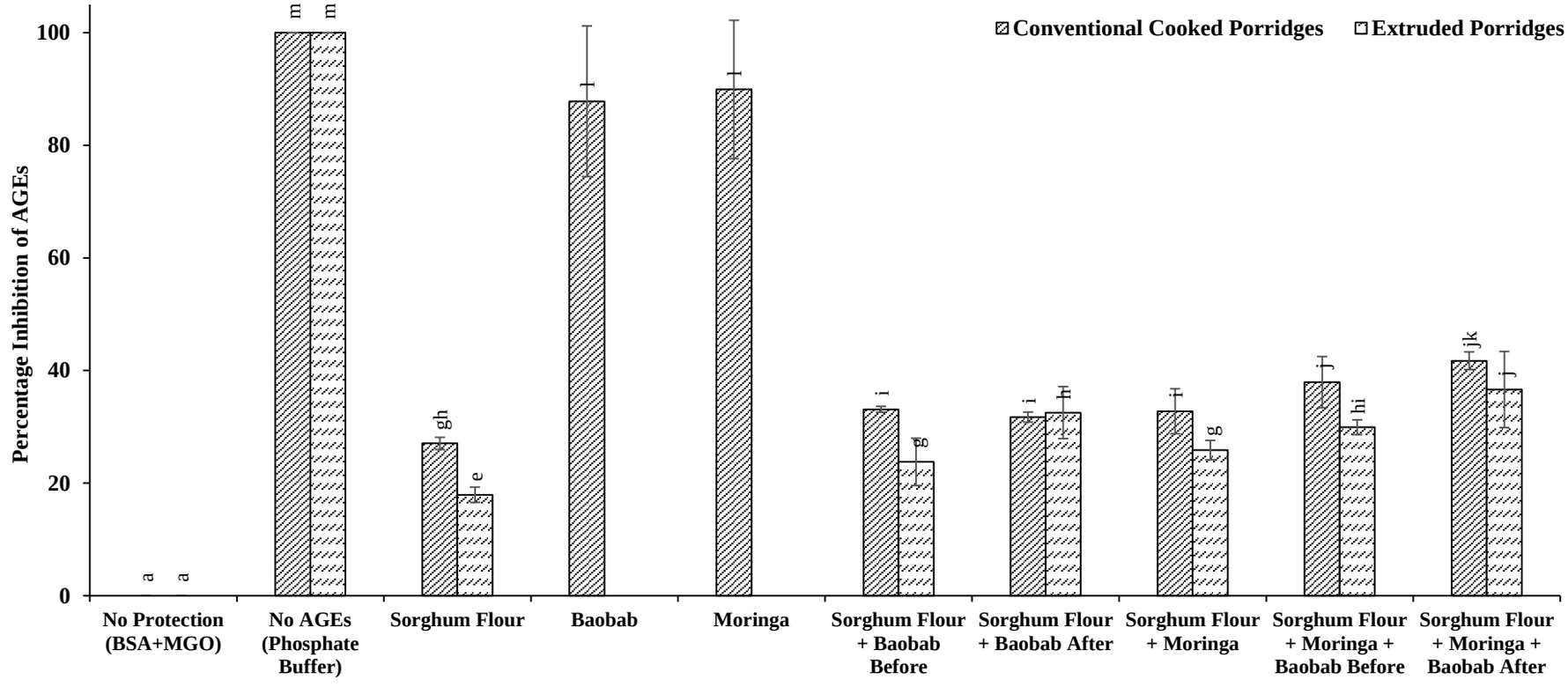


Figure 3-5: The inhibitory effects of phenolic extracts from sorghum-based porridges fortified with baobab and moringa (at a concentration of 500 µg/ml) against advanced glycation end-products (AGEs) formation as measured in bovine serum albumin-methylglyoxal model system. Data are presented as the mean ± standard deviation (n = 3). Columns marked by different small letters indicate significant differences at $p \leq 0.05$.

[1]Baobab before: Denotes addition of baobab before cooking.
[2]Baobab after: Denotes addition of baobab after cooking.

Protein glycation by methylglyoxal is a nonenzymatic modification whereby arginine and lysine side chains of proteins participate in forming a heterogeneous group of advanced glycation end-products (AGEs) (Wu & Monnier, 2003). Long-term hyperglycaemic conditions in diabetic patients cause the formation of AGEs through the protein glycation reaction. Therefore, inhibition of the formation of AGEs could be monitored as an indicator of anti-diabetic properties.

Extracts from sorghum flour as well as from the conventionally cooked and extruded instant porridges significantly inhibited the formation of AGEs. Extracts from sorghum porridges FtFF with baobab fruit pulp and moringa leaf powder exhibited relatively higher inhibition of AGE formation by 5-7% and 6-8%, respectively, compared to extracts from sorghum porridge alone. FtFF fortification with a combination of BFP and MLP resulted in a significantly higher reduction when compared to either BFP or MLP alone (lower inhibition of AGEs by 11-19% was observed when compared to sorghum alone). This could be attributed to the increase in phenolic and higher antioxidant activity following FtFF with BFP and MLP. Compounds with antioxidant activity have been reported to be useful in preventing diabetic complications through the reduction of AGEs formation by preventing oxidation of Amadori products and metal-catalysed glucoxidation (Dil, Ranjkesh & Goodarzi, 2019).

Concerning extrusion cooking, extruded porridges consistently showed significantly lower inhibition of AGEs when compared to the conventionally cooked counterparts (by 5-9%). These results are consistent with this study's TPC and antioxidant data.

3.2.5.1.3 Correlations between phenol content, tannin content and antioxidant activity of tannin sorghum

TPC exhibited a positive correlation with ABTS ($r = 0.87$, $p \leq 0.05$), AGEs ($r = 0.84$, $p \leq 0.05$) and ORAC ($r = 0.65$, $p \leq 0.05$), Table 3-5. Adarkwah-Yiadom and Duodu (2017) reported a similar correlation between TPC and ABTS for sorghum. These suggest that phenolic compounds contribute to the observed radical antioxidant activities; this is supported by the strong between TPC and total quantified phenolics ($r = 0.96$, $p \leq 0.05$). Radical scavenging activity (ABTS and ORAC) also correlated strongly with each other ($r = 0.79$, $p \leq 0.05$), Table 3-5. Awika *et al.* (2003b) reported a similarly high correlation ($r = 0.98$) between ABTS radical scavenging activities for sorghum and sorghum products, while (Adarkwah-Yiadom & Duodu, 2017) reported a high correlation of the same. This suggests that both methods could be equally useful for assessing antioxidant activities. TPC in this study exhibited a high negative

correlation with NO (r = -0.70, p≤0.05), weak negative correlation with CAA (r = -0.29, p≤0.05), Cellular NO inhibition (r = -0.50, p≤0.05) inhibition of lipid accumulation (r = 0.43, p≤0.05), Table 3-5. This could be attributed to the differences in principles of action by the different assays. ABTS showed a strong negative correlation with cellular NO inhibition (r = -0.94, p≤0.05), while weak correlations were observed with CAA (r = 0.32, p≤0.05) and inhibition of lipid accumulation (r = 0.35, p≤0.05), Table 3-5. ORAC had a strong correlation with CAA (r = 0.79, p≤0.05) and moderate but negative correlations with Cellular NO inhibition (r = -0.42, p≤0.05) and inhibition of lipid accumulation (r = -0.57, p≤0.05) which could suggest that ORAC could be a good indicator of CAA. *In vitro* chemical, NO inhibition had a strong correlation with cellular NO inhibition (r = 0.93, p≤0.05) and weak but negative correlations with CAA (r = -0.44, p≤0.05) and inhibition of lipid accumulation (r = -0.24, p≤0.05), Table 3-5, suggesting *in vitro* NO inhibition could be used to indicate cellular anti-inflammatory properties.

Table 3-5: Pearson's correlation coefficient of total phenolic content, antioxidant activities (ABTS, ORAC, and cellular antioxidant activity), anti-inflammatory properties (chemical and cellular NO production), antidiabetic properties (AGEs) and antilipogenic properties (3T3-L1)

	TPC	ABTS	ORAC	CAA	mM NO	RAW264.7	3T3-L1	AGEs
TPC	1	0.87**	0.65**	-0.29**	-0.70**	-0.50**	0.43**	0.84**
ABTS		1	0.79**	0.32**	-0.70**	-0.94**	0.34**	0.89**
ORAC			1	0.79**	-0.64**	-0.42**	0.57**	0.89**
CAA				1	-0.44**	-0.08**	-0.38**	0.63**
mM NO					1	0.93**	0.24**	-0.63**
RAW264.7						1	-0.35**	-0.66**
3T3-L1							1	0.33**
AGEs								1

[1]** Correlation is significant at the 0.05 level (2-tailed)
[2]TPC- total phenolic content, ABTS-2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), ORAC- Oxygen Radical Absorbance Capacity, CAA- cellular antioxidant protection of 2′,7′-Dichlorofluorescein diacetate, mM NO- chemical inhibition of nitric oxide production, RAW264.7- inhibition of nitric oxide production in LPS stress RAW26.7 macrophages, 3T3-L1- prevention and treatment of lipid droplets, AGEs- Advanced glycation endproducts.

3.2.5.2 Liquid chromatography-mass spectrometry (LCMS)

3.2.5.2.1 Compound Identification

Table 3-6: Retention time, UV-visible absorption maxima and mass spectral characteristics of phenolic compounds found in extracts of cooked sorghum (sorgh), Baobab fruit pulp (Bao), moringa (mor), extruded sorghum (ESA) extruded sorghum fortified with baobab (EBA), extruded sorghum fortified with moringa (EMA) and extruded sorghum fortified with moringa and baobab (EGA)

t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS fragments (Intensity, %)	Proposed compounds	Peak	Sorg	Bao	Mor	ESA	EBA	EMA	EGA
Hydroxybenzoic acid derivatives												
10.85	254, 253	137	137 (67), 93 (100)	4-Hydroxy benzoic acid	6	+	+	+	+	+	+	+
8.08	294, 259, 230	153	153(42), 109(100)	Protocatechuic acid	2	+	+	+	+	+	+	+
15.27	297, 263	167	167 (17), 123 (1), 108 (100)	Vanillic acid	20	+	+	+	+	+	+	+
6.48	272, 230	169	169 (72), 125 (100), 107 (3)	Gallic acid	1	+	+	+	+	+	+	+
19.56	230	197	197 (77), 182 (35), 179 (7), 153 (45), 138 (22), 123 (100), 121 (70)	Syringic acid	32	+	+	+	+	+	+	+
Hydroxycinnamic acid derivatives												

15.29	310, 230	163	163 (25), 147 (10) 145 (53), 119 (76)	P-Coumaric acid	21	+	-	+	-	-	+	+
10.16	272, 230	179	179 (42), 164 (1), 161 (1), 135 (100)	Caffeic acid	5	+	-	+	+	+	+	+
18.48	324, 244	193	133 (100), 161 (15)	Ferulic acid	28	+	-	+	+	+	+	+
17.33	328, 325	223	223 (38), 208 (21), 179 (9), 164 (40)	Sinapic acid	27	+	+	-	-	+	+	+
Phenolic esters												
15.51	230	237	237 (10), 163 (12), 147 (5), 145 (100), 119 (52), 117 (91)	p-Coumaroyl glycerol	23	+	-	-	+	+	+	+
14.25	288,230	253	253 (40), 179 (11), 164 (3), 161 (100), 135 (61)	Caffeoylglycerol	15	+	-	+	+	+	+	+
24.86	230	255	255 (100), 179 (7), 164 (2), 161 (8), 135 (3)	Dihydrocaffeoylglycerol	46	+	+	+	+	+	+	+
11.07	309, 230	337	337 (26), 191 (29), 173 (13), 163 (100), 119 (46)	3-p-coumaroylquinic acid	7	-	-	+	-	-	+	+
13.84	309, 230	337	337 (20), 191 (9), 173 (100), 163 (12), 119 (19)	4-p-coumaroylquinic acid	14	-	-	+	-	-	+	+

9.43	325, 230	353	353 (54), 191 (100), 179 (53), 173 (4), 161 (3), 135 (46)	3-Caffeoyl-quinic acid	3	+	+	+	+	+	+	+
11.91	325, 230	353	353 (70), 191 (45), 179 (63), 173 (100), 161 (2), 135 (38)	4-Caffeoyl-quinic acid	10	+	+	+	+	+	+	+
12.32	325, 230	367	367 (26), 193 (100), 191 (24), 178 (2), 173 (20), 149 (17), 135 (20), 134 (78)	3-Feruloylquinic acid	11	-	-	+	-	-	+	+
14.91	325, 230	367	367 (24), 193 (17), 191 (6), 178 (1), 173 (100), 149 (9), 134 (14)	4-Feruloylquinic acid	19	-	-	+	-	-	+	+
9.73	230	399	399 (31), 253 (2), 191 (100), 179 (50), 135 (81)	Coumaroyl-caffeoyl-glycerol	4	-	-	+	-	-	-	-
18.77	230	415	415 (13), 253 (40), 179 (11), 161 (55), 135 (100)	Dicaffeoylglycerol	30	+	-	-	+	+	+	+
14.72	230	468	468 (14), 332 (16), 306 (27), 179 (14), 161 (73), 151 (45), 135 (100), 133 (44)	Dicaffeoyl spermidine	17	+	-	-	+	+	+	+
Flavonols													

19.78	365, 266, 234	285	285 (100), 257 (2), 243 (1), 241 (1), 213 (2), 197 (1), 151 (5)	Kaempferol	33	+	+	+	+	+	+	+
23.90	363, 230	301	301 (100), 273 (7), 257 (1), 229 (1), 179 (22), 151 (69), 121 (15), 107 (18)	Quercetin	41	+	+	+	+	+	+	+
20.41	350, 265, 230	317	317 (100), 289 (4), 179 (55), 151 (11), 107 (19)	Myricetin	36	+	+	-	+	+	+	+
	Flavonol glycosides												
19.53	350, 265, 230	447	447 (100), 285 (27), 257 (4), 243 (1), 229 (1), 227 (27), 199 (1), 151 (2)	Kaempferol glycoside	31	+	+	+	+	+	+	+
14.67	230	449	449 (10), 285 (65), 257 (14), 243 (16), 241 (5), 229 (9), 199 (2), 151 (100)	Dihydrokaempferol glycoside	16	+	-	+	+	+	+	+
22.85	230, 251	463	463 (100), 301 (41), 273 (4), 257 (1), 229 (3), 179 (3), 151 (6), 121 (1), 107 (1)	Quercetin glycoside	38	+	+	+	+	+	+	+

20.04	352, 230	477	477 (60), 301 (75), 273 (5), 257 (2), 229 (7), 179 (11), 151 (16), 121 (4), 107 (2)	Quercetin glucuronide	35	-	-	+	-	-	+	+
17.31	354, 255	609	609 (100), 301 (75), 229 (2), 179 (4), 151 (10), 121 (9), 107 (2)	Rutin	26	+	+	+	+	+	+	+
Flavan-3-ols												
11.36	274, 230	289	289 (100), 245 (36), 203 (46), 179 (14), 135 (5), 125 (32), 109 (40)	Catechin	9	+	+	-	+	+	+	+
13.48	274, 230	289	289 (100), 245 (36), 203 (46), 179 (13), 135 (100), 125 (23),109 (48)	Epicatechin	13	+	+	-	+	+	+	+
Flavan-3-ol glycosides												
15.43	272	451	451 (6), 407 (26), 289 (43), 245 (5), 203 (10), 179 (9), 125 (100), 109 (7)	Epicatechin glycoside	22	-	+	+	-	+	+	+
Flavanones												
24.19	288, 234	271	271 (100), 227 (2), 177 (10), 151 (47), 119 (59), 107 (23)	Naringenin	42	+	-	-	+	+	+	+

23.05	289, 230	287	287 (2), 272 (7), 269 (34), 245 (5), 243 (7), 151 (29), 135 (100), 133 (25), 125 (5), 107 (8), 93 (8)	Eriodictyol	39	+	-	-	+	+	+	+
24.27	288, 234	301	301 (100), 286 (22), 272 (12), 243 (3), 151 (67), 135 (4), 125 (6), 107 (25)	Hesperetin	44	+	+	+	+	+	+	+
Flavanone glycosides												
17.04	324, 229	433	433 (33), 271 (100), 313 (4), 227 (4), 177 (19), 151 (92), 119 (35), 107 (18)	Naringenin glycoside	25	+	+	-	-	-	-	-
19.98	230	449	449 (15), 287 (34), 151 (100), 135 (67), 125 (16), 107 (10)	Eriodictoyl glycoside	34	+	-	-	+	+	+	+
21.72	283, 230	579	579 (100), 459 (11), 271 (51), 227 (2), 177 (4), 151 (54), 119 (15), 107 (24)	Naringin	37	+	+	+	+	+	+	+
Flavones												
24.24	346, 334, 266, 230	269	269 (100), 241 (0.1), 227 (0.2), 225 (4), 201 (2), 183 (3), 181 (2), 159 (2), 151 (11), 117 (21), 107 (5)	Apigenin	43	+	-	-	+	+	+	+

23.64	340, 266, 230	285	285 (100), 267 (1), 257 (1), 243 (2), 241 (1), 229 (1), 217 (3), 213 (1), 199 (4), 197 (1), 151 (1), 133 (24)	Luteolin	40	+	+	+	+	+	+	+
Flavone glycosides												
24.41	230, 646	431	431 (78), 341 (12), 311 (11), 269 (4), 243 (1), 241 (3), 225 (3), 201 (1), 183 (3), 181 (8), 151 (11), 117 (6), 107 (3)	Vitexin	45	+	+	+	+	+	+	+
18.60	350, 265	447	447 (100), 285 (100), 257 (4), 243 (2), 241 (1), 151 (1)	Luteolin glycoside	29	+	+	+	+	+	+	+
16.04	232, 530	563	563 (100), 506 (5), 473 (9), 443 (29), 383 (31), 353 (69),269 (38), 241 (11), 225 (10), 201 (2), 183 (2), 181 (4), 159 (31), 151 (8), 117 (61), 107 (3)	Glucosyl-arabinosyl apigenin	24	+	+	+	+	+	+	+
Proanthocyanidins												
11.33	279, 233	577	577 (26), 451 (45), 425 (20), 407 (45), 289 (100), 245 (39), 203 (23), 125 (91), 109 (19)	Procyanidin dimer	8	-	+	-	-	-	-	-

12.50	279, 234, 230	865	865 (18), 577 (29), 451 (9), 425 (21), 407 (46), 289 (100), 245 (38), 203 (22), 109 (17)	Procyanidin trimer	12	-	+	-	-	-	-	-
14.81	274, 233	1154	1154 (9), 865 (19), 577 (28), 425 (10), 407 (44), 289 (100), 245 (45), 203 (22), 109 (9)	Procyanidin tetramer	18	-	+	-	-	-	-	-

t_R- retention time, λ_{max}- Wavelength of maximum absorption, $[M-H]^-$(*m/z*)- negatively charged atomic mass unit

The compound labelled peak 1 (t_R = 6.48 min, λ_{max} = 230, 272 nm) with $[M-H]^-$ at *m/z* 169 (Table 3-6) was identified as gallic acid. It produced a fragment at *m/z* 125 due to the loss of CO_2 (-44 amu) (Table 3-6 and Figure 7-8, appendix) (Pérez-Magariño, Revilla, González-SanJosé & Beltrán, 1999). The fragment at *m/z* 107 is due to the loss of H_2O from the *m/z* 125 fragment.

The compound labelled peak 2 (t_R 8.08 min, λ_{max} = 294, 259, 230 nm) had $[M-H]^-$ at *m/z* 153 (Table 3-6 and Figure 7-9, appendix) and was identified as protocatechuic acid. It produced a fragment at *m/z* 109 due to loss of CO_2 (-44 amu) (Pérez-Magariño *et al.*, 1999).

The compound labelled peak 6 (t_R 10.85 min, λ_{max} = 254, 253 nm) had $[M-H]^-$ at *m/z* 137 (Table 3-6) and was identified as 4-hydroxy benzoic acid. It produced a fragment at *m/z* 97 (Table 3-6 and Figure 7-13, appendix) due to the loss of CO_2 (-44 amu).

The compound labelled peak 20 (t_R 15.27 min, λ_{max} = 297, 263 nm) had $[M-H]^-$ at *m/z* 167 (Table 3-6 and Figure 7-25, appendix) and was identified as vanillic acid. It had a fragment at *m/z* 123 due to the loss of CO_2 (-44 amu) (Pérez-Magariño *et al.*, 1999) and at m/z 108 due to possible loss of a methyl group (-15 amu) from the *m/z* 123 fragment.

The compound labelled peak 32 (t_R 19.83 min, λ_{max} = 230 nm) had $[M-H]^-$ at *m/z* 197 (Table 3-6 and Figure 7-37, appendix) and produced a major fragment at *m/z* 153 corresponding to the loss of a CO_2 moiety (-44 amu) (Sun *et al.*, 2007). The compound was identified as syringic acid

The compound labelled peak 5 (t_R 10.16 min, λ_{max} = 230, 272 nm) had $[M-H]^-$ at *m/z* 179 (Table 3-6) and was identified as caffeic acid. Fragmentation produced ions at *m/z* 164, 161, and 135 (Table 3-6 and Figure 7-12, appendix). The fragment at *m/z* 164 results from demethylation (-CH_3 group) of caffeic acid (-15 amu), while dehydration of caffeic acid could account for the *m/z* 161 (-18 amu). The loss of CO_2 (-44 amu) from the caffeic acid accounts for the *m/z* 135 fragment (Pérez-Magariño *et al.*, 1999).

The compound labelled peak 21 (t_R 15.29 min, λ_{max} = 310, 230 nm) had $[M-H]^-$ at *m/z* 163 (Table 3-6 and Figure 7-26, appendix) and was identified as ρ-coumaric acid. It had a fragment at *m/z* 147 indicative of loss of O_2 (-16 amu) (Pérez-Magariño *et al.*, 1999), at *m/z* 145 corresponding to loss of an H_2O group from the parent ion (-18 amu), and at *m/z* 119 as a result of loss of a CO_2 moiety (-44 amu) from the parent ion.

The compound labelled peak 27 (t_R 17.33 min, λ_{max} = 328, 325 nm) had $[M-H]^-$ at *m/z* 223 (Table 3-6) and was identified as sinapic acid. Fragmentation produced ions at *m/z* 208, 179, and 164 (Table 3-6 and Figure 7-32, appendix). The fragments at *m/z* 208 and 179 correspond to demethylation (-CH_3 group, -15 amu) and decarboxylation (-CO_2, -44 amu), respectively, of sinapic acid (Sinosaki, Tonin, Ribeiro, Poliseli, Roberto, Silveira, Visentainer, Santos & Meurer, 2020). The fragment at *m/z* 164 is due to the elimination of CO_2 (-44 amu) from the fragment at *m/z* 208.

Peak 28 (t_R 18.48 min, λ_{max} = 324, 244 nm) with $[M-H]^-$ at *m/z* 193 (Table 3-6) was identified as ferulic acid. It produced fragments at *m/z* 133 and 161 (Table 3-6 and Figure 7-33, appendix). The *m/z* 133 fragment could be due to the loss of a methyl, CH_3 (-15 amu) from the methoxy group, the loss of CO_2 (-44 amu) from the carboxylate moiety on the ethylene short chain of the ferulic acid molecule and the loss of a hydride ion (H, 1 amu) ($[M-H]^-$ (193) – 15 – 44 – 1) (Svensson, Sekwati-Monang, Lutz, Schieber & Gänzle, 2010). The fragment at *m/z* 161 corresponds to the loss of O_2 (-32 amu) from the ferulate anion ($[M-H]^-$ (193) – 32).

The compound labelled peak 3 (t_R 9.43 min, λ_{max} = 325, 230 nm) had $[M-H]^-$ at *m/z* 353 (Table 3-6) and produced fragments at *m/z* 191, 179, 173, 164, 161, 135 and 127 (Table 3-6 and Figure 7-10, appendix). The corresponding to the loss of a CO_2 moiety (-44 amu). The compound was identified as 3-Caffeoylquinic acid based on its fragmentation pattern. The fragments at *m/z* 191 and *m/z* 179 correspond to quinic acid and caffeic acid, respectively, from the cleavage of the compound. The fragment at *m/z* 173 corresponds to dehydration of quinic acid (-18 amu) (Clifford, Johnston, Knight & Kuhnert, 2003). The fragment at *m/z* 164 could correspond to the demethylation (-CH_3 group) of the caffeic acid (-15 amu), while dehydration of caffeic acid could account for the *m/z* 161 (-18 amu). The loss of CO_2 (-44 amu) from the caffeic acid accounts for the *m/z* 135 fragment (Pérez-Magariño *et al.*, 1999).

The compound labelled peak 4 (t_R 9.73 min, λ_{max} = 230 nm) had $[M-H]^-$ at *m/z* 399 (Table 3-6) and produced fragments at *m/z* 253, 179, 163 and 135 (Table 3-6 and Figure 7-11, appendix). The fragment at *m/z* 253 could be accounted for as caffeoyl glycerol, *m/z* 179 being the caffeic acid moiety, *m/z* 163 being the coumaric acid moiety, and the *m/z* 135 resulting from the loss of a CO_2 (-44 amu) from the caffeic acid. The compound was identified as coumaroyl caffeoyl glycerol based on its fragmentation pattern in literature (Kang, Price, Ashton, Tapsell & Johnson, 2016). It was present only in the moringa sample chromatograms at peak 4 (Figure 3-4, appendix). Its absence in sorghum-based porridges fortified with Moringa could be

attributed to the breakdown of the compound due to high temperature, shear and pressure following extrusion.

The compound labelled peak 7 (t_R 11.07 min, λ_{max} = 309, 230 nm) had $[M-H]^-$ at *m/z* 337 (Table 3-6) and produced fragments at *m/z* 191, 173, 163, and 119 (Table 3-6 and Figure 7-14, appendix) and was identified as 3-ρ-coumaroyl quinic acid based on its fragmentation in literature (Clifford *et al.*, 2003). The fragment at *m/z* 191 could be accounted for as quinic acid, *m/z* 173 could be a result of dehydration of the quinic acid (-18 amu), *m/z* 163 being the caffeic acid moiety, *m/z* 163 being the coumaric acid moiety, and *m/z* 119 could be from the loss of a CO_2 (-44 amu) from coumaric acid.

The compound labelled peak 10 (t_R 9.43 min, λ_{max} = 325, 230 nm) had $[M-H]^-$ at *m/z* 353 (Table 3-6) and produced fragments at *m/z* 191, 179, 173, 164, 161, 135 and 127 (Table 3-6 and Figure 7-17, appendix). The corresponding to the loss of a CO_2 moiety (-44 amu). The compound was identified as 4-Caffeoylquinic acid based on its fragmentation pattern in literature. The fragments at *m/z* 191 and 179 correspond to quinic acid and caffeic acid, respectively, from the cleavage of the compound. The fragment at *m/z* 173 corresponds to the dehydration of quinic acid (-18 amu) (Clifford *et al.*, 2003). The fragment at *m/z* 164 could correspond to the demethylation (-CH_3 group) of the caffeic acid (-15 amu), while dehydration of caffeic acid could account for the *m/z* 161 (-18 amu). The loss of CO_2 (-44 amu) from the caffeic acid accounts for the *m/z* 135 fragment (Pérez-Magariño *et al.*, 1999). According to Clifford *et al.* (2003), isomers substituted at position 4 produce a characteristic major fragment at *m/z* 173. Thus, the conclusion is that the compound at peak 10 is a position 4 substituted isomer of the compound at peak 3.

The compound labelled peak 11 (t_R 12.32 min, λ_{max} = 325, 230 nm) had $[M-H]^-$ at *m/z* 367 (Table 3-6) and produced fragments at *m/z* 193, 191, 178, 173, 149, and 134 (Table 3-6 and Figure 7-18, appendix). The compound was identified as 3-feruloyl quinic acid based on its fragmentation pattern in literature. The fragments at *m/z* 193 and 191 correspond to ferulic and quinic acids, respectively, from the cleavage of the compound. The fragment at *m/z* 178 could be a result of the demethylation of ferulic acid (-15 amu). The fragment at *m/z* 173 corresponds to dehydration of quinic acid (-18 amu) (Clifford *et al.*, 2003). The fragment at *m/z* 149 could be a result of the decarboxylation of the ferulic acid moiety (-44 amu). The loss of CO_2 (-44 amu) from the fragment at *m/z* 178 accounts for the *m/z* 134 fragment.

The compound labelled peak 14 (t_R 13.84 min, λ_{max} = 309, 230 nm) had $[M-H]^-$ at *m/z* 337 (Table 3-6) and produced fragments at *m/z* 191, 173, 163, and 119 (Table 3-6 and

Figure 7-52, appendix) and was identified as 4-ρ-coumaroyl quinic acid based on its fragmentation in literature (Clifford *et al.*, 2003). The fragment at *m/z* 191 could be accounted for as quinic acid, *m/z* 173 could be a result of dehydration of the quinic acid (-18 amu), *m/z* 163 being the caffeic acid moiety, *m/z* 163 being the coumaric acid moiety, and *m/z* 119 could be from the loss of a CO_2 (-44 amu) from coumaric acid. Due to its major fragment at *m/z* 173, it was differentiated from the 4-p-coumaroyl quinic acid, according to Clifford *et al.* (2003).

The compound labelled as peak 15 (t_R 14.25 min, λ_{max} = 230 nm) had $[M-H]^-$ at *m/z* 253 (Table 3-6) and was identified as caffeoyl glycerol based on its fragmentation pattern in literature (Kang *et al.*, 2016). The fragments at *m/z* 179 correspond to the loss of the glycerol molecule (-74 amu) to form caffeic acid (*m/z* 179) (Table 3-6 and Figure 7-12, appendix). The fragment at *m/z* 164 could correspond to the demethylation (-CH_3 group) of the caffeic acid (-15 amu), while dehydration of caffeic acid could account for the *m/z* 161 (-18 amu). The loss of CO_2 (-44 amu) from the caffeic acid accounts for the *m/z* 135 fragment (Pérez-Magariño *et al.*, 1999).

The compound labelled peak 17 (t_R 14.72 min, λ_{max} = 230 nm) had $[M-H]^-$ at *m/z* 468 (Table 3-6) and was identified as dicaffeoyl spermidine based on the fragmentation pattern in literature. It produced fragments at *m/z* 332, 306, 179, 161, 151 and 135 (Table 3-6 and Figure 7-23, appendix). The fragment at *m/z* 179 is the caffeic acid moiety, *m/z* 161 corresponds to dehydration of the caffeic acid molecule, and the *m/z* 135 results from the loss of CO_2 (-44 amu) from the caffeic acid. Kang *et al.* (2016) reported dicaffeoyl spermidine in sorghum before.

The compound labelled peak 19 (tR 14.91 min, λmax = 325, 230 nm) had [M-H]- at *m/z* 367 (Table 3-6) and produced fragments at m/z 193, 191, 178, 173, 149, and 134 (Table 3-6 and Figure 7-18, appendix). The compound was identified as 3-feruloyl quinic acid based on its fragmentation pattern in literature. The fragments at *m/z* 193 and 191 correspond to ferulic and quinic acids, respectively, from the cleavage of the compound. The fragment at *m/z* 178 could be a result of the demethylation of ferulic acid (-15 amu). The fragment at *m/z* 173 corresponds to dehydration of quinic acid (-18 amu) (Clifford *et al.*, 2003). The fragment at m/z 149 could be a result of the decarboxylation of the ferulic acid moiety (-44 amu). The loss of CO_2 (-44 amu) from the fragment at *m/z* 178 accounts for the *m/z* 134 fragment. Due to its major fragment

at *m/z* 173, it was differentiated from the 3-feruloyl quinic acid, according to Clifford *et al.* (2003).

The compound labelled as peak 23 (t_R 15.51 min, λ_{max} = 230 nm) had $[M-H]^-$ at *m/z* 237 (Table 3-6) and was identified as ρ-coumaroyl-glycerol based on its fragmentation pattern in literature (Kang *et al.*, 2016). The fragments at *m/z* 163 correspond to the loss of the glycerol molecule (-74 amu) to form p-coumaric acid (*m/z* 163) (Table 3-6 and Figure 7-26, appendix). The fragment at m/z 147 is indicative of loss of O_2 (-16 amu) (Pérez-Magariño *et al.*, 1999), at m/z 145 corresponding to possible loss of an H_2O group from the parent ion (-18 amu), and at m/z 119 probably as a result of loss of a CO_2 moiety (-44 amu).

The compound labelled as peak 30 (t_R 18.77 min, λ_{max} = 230 nm) had $[M-H]^-$ at *m/z* 415 (Table 3-6 and Figure 7-35, appendix) and was identified as dicaffeoyl glycerol based on its fragmentation pattern in literature (Kang *et al.*, 2016). The fragments at *m/z* 179 correspond to the liberation of caffeic acid (*m/z* 179) (Table 3-6 and Figure 7-12, appendix). The dehydration of caffeic acid could account for the *m/z* 161 (-18 amu). The loss of CO_2 (-44 amu) from the caffeic acid account for the *m/z* 135 fragment (Pérez-Magariño *et al.*, 1999).

The compound labelled as peak 46 (t_R 24.56 min, λ_{max} = 230 nm) had $[M-H]^-$ at *m/z* 255 (Table 3-6 and Figure 7-49, appendix) and was identified as dihydrocaffeoyl glycerol based on its fragmentation pattern in literature (Kang *et al.*, 2016). It produced ionic fragments at m/z 179, 164, 161, and 135. The fragments at *m/z* 179 correspond to the liberation of caffeic acid (*m/z* 179) (Table 3-6 and Figure 7-12, appendix). The demethylation and dehydration of caffeic acid could account for the fragments at *m/z* 164 (-15 amu) and 161 (-18 amu), respectively. The loss of CO_2 (-44 amu) from the caffeic acid accounts for the *m/z* 135 fragment (Pérez-Magariño *et al.*, 1999).

The compound at peak 33 (t_R = 19.78 min and λ_{max} = 365, 266, 234 nm) (Table 3-6) had a molecular ion *m/z* at 285 and was identified as kaempferol. It produced anionic fragments at *m/z* 257, 243, 241, 229, and 151 (Table 3-6 and Figure 7-38, appendix). The loss of CO (-28 amu), C_2H_2O (-42 amu) and CO_2 (-44 amu) each from kaempferol produce the fragments at *m/z* 257 (Fabre, Rustan, de Hoffmann & Quetin-Leclercq, 2001), 243 and 241 respectively. The loss of two CO molecules (-56 amu) accounts for the ion at *m/z* 229. Loss of a C_2H_2O molecule (42 amu) and a CO_2 molecule (-44 amu) together from kaempferol accounts for the *m/z* 199 fragment. Retrocyclization cleavage of kaempferol at bond positions 1 and 3 of the C ring produces a $^{1,3}A^-$ fragment at *m/z* 151 (Figure 3-7) (Fabre *et al.*, 2001).

The compound labelled peak 36 with molecular ion at *m/z* 317 (t_R = 20.41 min and λ_{max} = 350, 265, 230 nm) (Table 3-6) was identified as myricetin. Fragmentation produced ionic fragments at *m/z* 289, 179, 151 and 107 (Table 3-6 and Figure 7-41, appendix). The fragment at *m/z* 289 could be a result of the loss of a CO molecule from the myricetin aglycone (-28 amu). The ionic fragments at *m/z* 179 and 121 could result from retrocyclization cleavage of the myricetin molecule involving bonds 1 and 2 of the C ring to produce the $^{1,2}A$ and $^{1,2}B^-$ fragments, respectively (Figure 3-6). The fragment at *m/z* 107 is indicative of consecutive loss of a CO moiety (-28 amu) and a CO_2 moiety from the $^{1,2}A^-$ fragment (-44 amu).

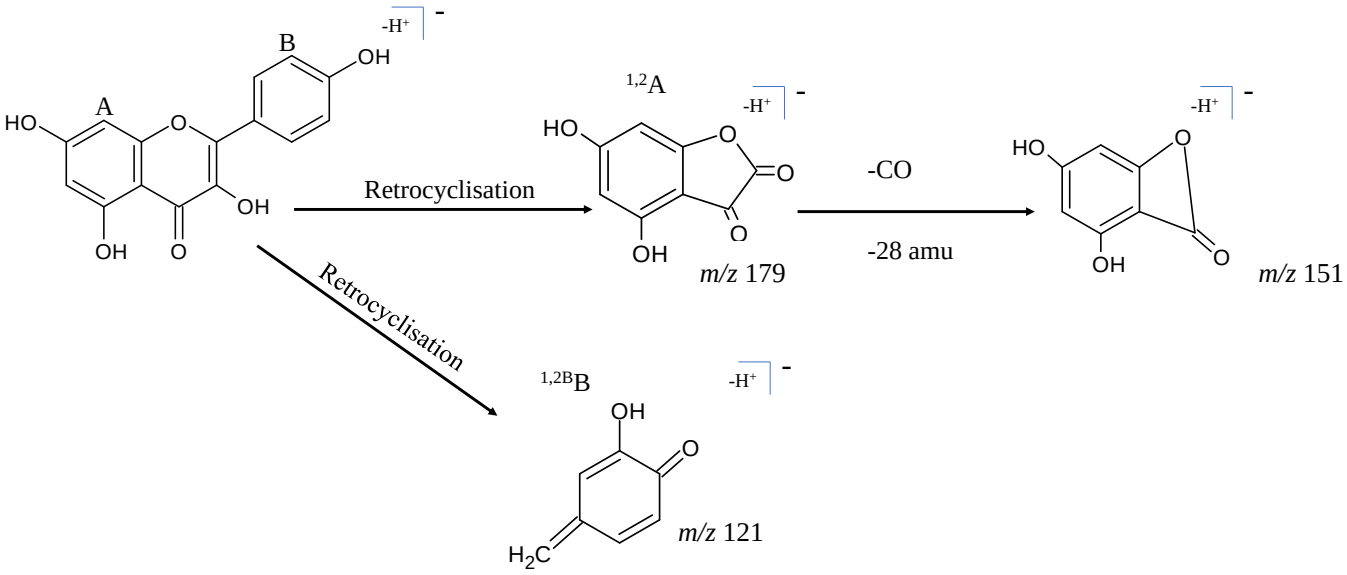


Figure 3-6: Proposed fragmentation scheme for flavonols (e.g., quercetin and myricetin)

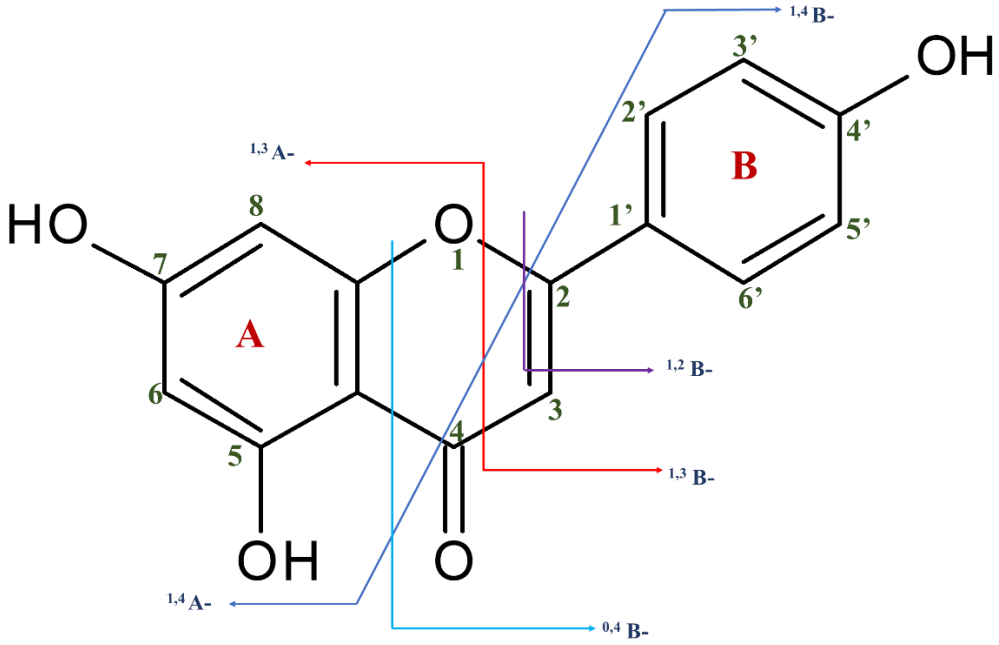


Figure 3-7: Proposed general fragmentation scheme for flavonoids

The compound at Peak 41 had a molecular ion at *m/z* 301 (t_R = 23.90 min and λ_{max} = 363, 230 nm) (Table 3-6) and was identified as quercetin. It produced fragments at *m/z* 273, 229, 179, 151, 121, and 107 (Table 3-6 and Figure 7-46, appendix). Loss of a CO molecule (-28 amu) and a C_2H_2O molecule (-42 amu) from the quercetin aglycone produces the fragments at *m/z* 273 and 257, respectively (Fabre *et al.*, 2001). The loss of both a CO molecule and a CO_2 molecule from the quercetin aglycone results in the fragment at m/z 229. Retrocyclization cleavage of the quercetin molecule at bond positions 1 and 2 of the C ring produces a $^{1,2}A^-$ fragment at *m/z* 179 and a $^{1,2}B^-$ fragment at m/z 121 (Figure 3-6 and Figure 3-7) (Fabre *et al.*, 2001). The loss of a CO molecule (-28 amu) from the $^{1,2}A^-$ fragment yields the fragment at *m/z* 151 (Figure 3-6). The loss of both a CO molecule (-28 amu) and a CO_2 molecule (-44 amu) from the $^{1,2}A^-$ fragment produces the fragment at *m/z* 107.

The compound at peak 16 (t_R = 14.67 min and λ_{max} = 230 nm) had a molecular ion at *m/z* 449 (Table 3-6) and was identified as dihydrokaempferol glycoside. It produced fragments at *m/z* 285, 257, 243, 241, 229, 199 and 151 (Table 3-6 and Figure 7-22, appendix). The fragment at *m/z* 285 is the kaempferol aglycone produced from the loss of the hexose unit along with 2 hydrogen units (-164 amu). The loss of a CO molecule (-28 amu), a C_2H_2O molecule (-42 amu) and a CO_2 molecule (-44 amu) each from the kaempferol aglycone moiety produces the fragments at *m/z* 257, 243 and 241, respectively (Fabre *et al.*, 2001). The loss of two CO molecules (-56 amu) from the kaempferol aglycone fragment produces the ion at *m/z* 229, while the loss of a C_2H_2O molecule (-42 amu) together with a CO_2 molecule (-44 amu) from the kaempferol aglycone fragment produces the fragment at *m/z* 199. The ionic fragment at *m/z* 151 is the $^{1,3}A^-$ fragment produced from retrocyclization cleavage of kaempferol at bond positions 1 and 3 of the C ring (Figure 3-7) (Fabre *et al.*, 2001).

The compound at peak 26 (t_R = 17.04 min and λ_{max} = 324, 229 nm) (Table 3-6) had a molecular ion at *m/z* 609 and was identified as rutin. It produced fragments at *m/z* 301, 227, 179, 151, 121, and 107 (Table 3-6 and Figure 7-31, appendix). The fragment at *m/z* 301 is the quercetin aglycone produced from the loss of the disaccharide from the rutin molecule. The fragment at *m/z* 179 is the $^{1,2}A^-$ fragment produced from retrocyclization cleavage of the quercetin molecule at bond positions 1 and 2 of the C ring (Figure 3-6 and Figure 3-7). The loss of a CO molecule (-28 amu) from the $^{1,2}A^-$ fragment yields the fragment at *m/z* 151 (Figure 3-6). The fragment at

m/z 121 is the $^{1,2}B^-$ fragment produced from retrocyclization cleavage of the C ring of quercetin aglycone at bond positions 1 and 2 (Figure 3-6 and Figure 3-7).

The compound at peak 31 (t_R = 19.53 min and λ_{max} = 350, 265, 230 nm) (Table 3-6) had a molecular ion at *m/z* 447 and was identified as kaempferol glycoside. It produced fragments at *m/z* 285, 257, 243, 229, 199, and 151 (Table 3-6 and Figure 7-36, appendix). The fragment at *m/z* 285 is the kaempferol aglycone after the loss of the glycoside unit (-162 amu). The loss of a CO molecule (-28 amu) and a C_2H_2O molecule (-42 amu) each from the kaempferol aglycone produces the fragments at *m/z* 257 and *m/z* 243 respectively (Fabre *et al.*, 2001). The loss of two CO molecules (-56 amu) from the kaempferol aglycone produces the fragment at *m/z* 229, while the loss of a C_2H_2O molecule (-42 amu) together with a CO_2 molecule (-44 amu) from the kaempferol aglycone produces the fragment at *m/z* 199. The fragment at *m/z* 151 is the $^{1,3}A^-$ fragment produced from retrocyclization cleavage of the kaempferol aglycone at bond positions 1 and 3 of the C ring (Figure 3-7) (Fabre *et al.*, 2001).

The compound at Peak 35 (t_R = 20.04 min and λ_{max} = 352, 230 nm) (Table 3-6) had a molecular ion *m/z* at 477 and was identified as quercetin glucuronide. It produced fragments at *m/z* 301, 273, 229, 179, 151, 121, and 107 (Table 3-6 and Figure 7-40, appendix). The fragment at *m/z* 301 is the quercetin aglycone after the loss of the glucuronide moiety. Loss of a CO molecule (-28 amu) and a C_2H_2O molecule (-42 amu) each from the quercetin aglycone produces the fragments at *m/z* 273 and 257, respectively (Fabre *et al.*, 2001). The fragments at *m/z* 179 and 121 are, respectively, the $^{1,2}A^-$ and $^{1,2}B$ fragments produced from retrocyclization cleavage of the quercetin aglycone at bond positions 1 and 2 of the C ring (Figure 3-6 and Figure 3-7) (Fabre *et al.*, 2001). The loss of a CO molecule (-28 amu) from the $^{1,2}A^-$ fragment produces the fragment at *m/z* 151 (Figure 3-6). The loss of a CO molecule (-28 amu) together with a CO_2 molecule (-44 amu) from the $^{1,2}A^-$ fragment produces the fragment at *m/z* 107.

The compound at peak 38 (t_R = 20.04 min and λ_{max} = 352, 230 nm) (Table 3-6) had a molecular ion *m/z* at 463 and was identified as quercetin glycoside. It produced fragments at *m/z* 301, 273, 229, 179, 151, 121, and 107 (Table 3-6 and Figure 7-43, appendix). The fragment at *m/z* 301 is the quercetin aglycone after the loss of the glucose moiety. Loss of a CO molecule (-28 amu) and a C_2H_2O molecule (-42 amu) each from the quercetin aglycone produce the fragments at *m/z* 273 and 257, respectively (Fabre *et al.*, 2001). The loss of a CO molecule (-28 amu) together with a CO_2 molecule (-44 amu) produces the fragment at *m/z* 229. The fragments at *m/z* 179 and 121 are, respectively, the $^{1,2}A^-$ and $^{1,2}B^-$ fragments produced from retrocyclization

cleavage of the quercetin aglycone at bond positions 1 and 2 of the C ring (Figure 3-6 and Figure 3-7) (Fabre *et al.*, 2001). The loss of a CO molecule (-28 amu) from the $^{1,2}A^-$ fragment yields the fragment at *m/z* 151 (Figure 3-6). The loss of a CO molecule (-28 amu) together with a CO_2 molecule (-44 amu) from the $^{1,2}A^-$ fragment produces the fragment at *m/z* 107.

The compound labelled peak 9 (t_R 11.36 min, λ_{max} = 274, 230 nm) had a molecular ion at *m/z* 289 (Table 3-6) and was identified as catechin. Its fragmentation (Table 3-6 and Figure 7-16, appendix) produced an ion at *m/z* 245, which could be due to the loss of a CO_2 molecule (-44 amu) (Sandhu & Gu, 2010; Pérez-Magariño *et al.*, 1999). The fragment at *m/z* 203 could result from the loss of a CO_2 (-44 amu) together with a C_2H_2O molecule (-42 amu) (Stöggl, Huck & Bonn, 2004). The fragment at *m/z* 179 corresponds to the loss of dihydroxybenzene moiety (-110 amu), which was also detected as an anionic fragment at *m/z* 109 (Ben Said, Hamed, Mahalel, Al-Ayed, Kowalczyk, Moldoch, Oleszek & Stochmal, 2017).

The compound labelled peak 13 (t_R 13.48 min, λ_{max} = 274, 230 nm) had a molecular ion at *m/z* 289 (Table 3-6 and Figure 7-20) and was tentatively identified as epicatechin. Its fragmentation pattern was identical to that of the catechin described above. Although catechin and epicatechin have similar molecular ions at *m/z* 289 and similar fragmentation patterns, epicatechin is regarded to elute after catechin because, stereogeometrically, catechin is more polar than epicatechin (Dou, Lee, Tzen & Lee, 2007).

The compound labelled peak 22 (t_R *15.43* min, λ_{max} = 272 nm) had molecular ion at *m/z* 451 (Table 3-6) and was proposed to be epicatechin glycoside. Its fragmentation (Table 3-6 and Figure 7-27, appendix) produced an ion at *m/z* 407, which could be attributed to the loss of a CO_2 molecule (-44 amu). The anion at *m/z* 289 is the epicatechin aglycone produced after the loss of a hexose unit (-162 amu) (Apea-Bah *et al.*, 2014; Nderitu *et al.*, 2013). Loss of a CO_2 molecule (-44 amu) from the epicatechin aglycone produces the fragment at *m/z* 245 (Sandhu & Gu, 2010; Pérez-Magariño *et al.*, 1999). The fragment at *m/z* 203 could result from the loss of a CO_2 (-44 amu) together with a C_2H_2O molecule (-42 amu) from the epicatechin aglycone (Stöggl *et al.*, 2004). The fragment at *m/z* 179 corresponds to the loss of dihydroxybenzene moiety (-110 amu), which was also detected as an anionic fragment at *m/z* 109 (Ben Said *et al.*, 2017).

The compound labelled peak 39 (t_R 23.05 min, λ_{max} = 289, 230 nm) had a molecular ion at *m/z* 287 (Table 3-6) and was identified as eriodictoyl (Fabre *et al.*, 2001). It produced fragments at *m/z* 272, 269, 245, 243, 151, 135, 133, 125, and 107 (Table 3-6 and Figure 7-44, appendix).

The fragments at *m/z* 272 and 269 could be attributed to the demethylation (-CH_3, -15 amu) and dehydration (-H_2O, -18 amu) of the eriodictoyl moiety, respectively. The fragments at *m/z* 245 and 243 could be due to the loss of a C_2H_2O (-42 amu) molecule and a CO_2 (-44 amu) molecule, respectively. The fragments at *m/z* 151 and 135 are, respectively, the $^{1,3}A^-$ and $^{1,3}B^-$ fragments produced from retrocyclization cleavage of eriodictyol at bond positions 1 and 3 of the C ring (Figure 3-7) (Fabre *et al.*, 2001). The fragment at *m/z* 125 is the $^{1,4}A^-$ produced from retrocyclization cleavage of eriodictyol at bond positions 1 and 4 of the C ring (Figure 3-7). Dehydration (-H_2O, -18 amu) of the moiety at *m/z* 151 could yield the fragment at *m/z* 133. The loss of a CO molecule from the fragment at *m/z* 125 (-28 amu) could account for the fragment at *m/z* 107.

The compound at Peak 42 (t_R = 21.72 min and λ_{max} = 288, 234 nm) (Table 3-6) had a molecular ion at *m/z* at 271 and was identified as naringenin. It produced fragments at *m/z* 227, 177, 151, 119 and 107 (Table 3-6 and

Figure 7-51, appendix). The fragment at *m/z* 227 is produced from the loss of CO_2 (-44 amu) from the naringenin moiety. The fragment at *m/z* 177 results from the loss of the B-ring via position 2 and position 1' of naringenin, Figure 3-7. The ionic fragment at *m/z* 151 and 119, respectively, are the $^{1,3}A^-$ and $^{1,3}B$ fragments resulting from retrocyclization cleavage of naringenin (Figure 3-7) (Fabre *et al.*, 2001) while the fragment at *m/z* 107 results from loss of a CO_2 moiety from the $^{1,3}A^-$ fragment (-44 amu). Thus, the compound at *m/z* 271 was identified as naringin.

The compound labelled peak 44 (t_R 23.05 min, λ_{max} = 289, 230 nm) had a molecular ion at *m/z* 301 (Table 3-6) and was identified as hesperetin (Fabre *et al.*, 2001). It produced fragments *m/z* 286, 272, 243, 151, 135, 133, 125, 107 and 93 (Table 3-6 and Figure 7-48, appendix). The fragments at *m/z* 286 could be attributed to the demethylation (-CH_3, -15 amu) of the hesperetin molecule. The fragments at *m/z* 272 could be attributed to the demethylation (-CH_3, -15 amu) of the fragment at m/z 286. The fragments at *m/z* 243 could be due to the loss of a CO_2 molecule (-44 amu). The fragments at *m/z* 151, 135 and 125 are, respectively, the $^{1,3}A^-$, $^{1,3}B^-$ and $^{1,4}A^-$ fragments produced from retrocyclization cleavage of hesperetin. Dehydration (-H_2O, -18 amu) of the moiety at *m/z* 151 could yield the fragment at *m/z* 133. The loss of a CO molecule from the fragment at *m/z* 125 (-28 amu) could account for the fragment at *m/z* 107).

The compound at Peak 25 (t_R = 17.04 min and λ_{max} = 324, 229 nm) (Table 3-6) had a molecular ion at *m/z* 433 and was identified as naringenin glycoside (Apea-Bah *et al.*, 2014; Svensson *et*

al., 2010). It produced a fragment at *m/z* 271 (Table 3-6 and Figure 7-30, appendix), which is the naringenin aglycone after the loss of one hexose unit (-162 amu). The fragment at *m/z* 227 results from the loss of CO_2 (-44 amu) from the naringenin moiety, while the fragment at *m/z* 177 results from loss of the B-ring via position 2 and position 1' of naringenin, Figure 3-7. The ionic fragment at *m/z* 151 and 119, respectively, are the $^{1,3}A^-$ and $^{1,3}B$ fragments resulting from retrocyclization cleavage of naringenin (Figure 3-7) (Fabre *et al.*, 2001) while the fragment at *m/z* 107 results from loss of a CO_2 moiety from the $^{1,3}A^-$ fragment (-44 amu). Based on this fragmentation pattern, the compound was identified as naringenin glycoside.

The compound labelled peak 34 (t_R 19.98 min, λ_{max} = 230 nm) had a molecular ion at *m/z* 449 (Table 3-6) and was identified as eriodictyol glycoside (Apea-Bah *et al.*, 2014). It produced fragments at *m/z* 287, 151, 135, 125, and 107 (Table 3-6 and Figure 7-39, appendix). The fragment at *m/z* 287 (eriodictyol aglycone) is due to the loss of one hexose unit (-162 amu). The fragments at *m/z* 151, 135 and 125 are, respectively, the $^{1,3}A^-$, $^{1,3}B^-$ and $^{1,4}A^-$ fragments produced from retrocyclization cleavage of eriodictyol, (Figure 3-7) (Fabre *et al.*, 2001). The loss of a CO molecule from the fragment at *m/z* 125 (-28 amu) could account for the fragment at *m/z* 107.

The compound at Peak 37 (t_R = 21.72 min and λ_{max} = 283, 230 nm) (Table 3-6) had a molecular ion at *m/z* at 573 and was identified as naringin. It produced fragments at *m/z* 459, 313, 271, 227, 177, 151, 119 and 107 (Table 3-6 and Figure 7-42, appendix). The fragment at *m/z* 459 could be a $^{1,3}B$ fragment resulting from retrocyclization cleavage of naringenin aglycone in naringin (Figure 3-7) (Zeng, Su, Zheng, Liu, Li, Zhang, Liang, Bai, Peng & Yao, 2018). The fragment ion at *m/z* 313 could be because of the loss of the rhamnose moiety from the moiety at *m/z* 459. The ion at *m/z* 271 is naringenin, formed by the successive loss of rhamnose and a glucose moiety from naringin. The fragment at *m/z* 227 corresponds to the loss of CO_2 (-44 amu) from the carboxylate moiety, while the fragment at *m/z* 177 is a result of loss of the B-ring via position 2 and position 1' of naringenin, Figure 3-7. The ionic fragment at *m/z* 151 and 119, respectively, are the $^{1,3}A^-$ and $^{1,3}B$ fragments resulting from retrocyclization cleavage of naringenin (Figure 3-7) (Fabre *et al.*, 2001), while the fragment at *m/z* 107 is indicative loss of a CO_2 moiety from the $^{1,3}A^-$ fragment (-44 amu). This cleavage of the naringin molecule could also account for the fragment at m/z 459.

The compound at peak 40 (t_R = 23.64 min and λ_{max} = 340, 266, 230 nm) (*Table 3-6*) with molecular ion at *m/z* 285 was identified as luteolin. It produced fragments at *m/z* 267, 257, 243,

217, 213, 199, 151 and 133 (Table 3-6 and Figure 7-45, appendix). The fragment at *m/z* 267 results from dehydration (-18 amu) of the luteolin aglycone (Fabre *et al.*, 2001). The loss of a CO molecule (-28 amu) and a C_2H_2O molecule (-42 amu) each from the luteolin aglycone produce the fragments at *m/z* 257 and 243, respectively. The fragment at *m/z* 217 is consistent with the loss of a carbon suboxide molecule (C_3O_2, -68 amu) ((Fabre *et al.*, 2001), while the fragment at *m/z* 213 is consistent with the loss of a CO_2 molecule (-44 amu) together with a CO group (- 28 amu) from the luteolin aglycone. The loss of a C_2H_2O molecule (-42 amu) together with a CO_2 molecule (-44 amu) from the luteolin aglycone produces the fragment at *m/z* 199. The fragments at *m/z* 151 and 133 are, respectively, the ^{1,3}A- and ^{1,3}B- fragments resulting from the retrocyclization cleavage of luteolin at bond positions 1 and 3 of the C ring (Figure 3-7).

The compound labelled peak 43 (t_R 24.24 min, λ_{max} = 346, 334, 266, 230 nm) had molecular ion at *m/z* 269 (Table 3-6) and was identified as apigenin. It produced fragments at *m/z* 241, 227, 225, 201, 183, 181, 159, 151, 149, and 117 (Table 3-6 and Figure 7-47, appendix). The loss of CO (-28 amu), C_2H_2O (- 42), CO_2 (-44 amu) and C_2O_3 (-68 amu) moieties each from the apigenin aglycone produces the fragments at *m/z* 241, 225, 227, and 201 respectively. The loss of a C_2H_2O molecule (-42 amu) together with a CO_2 molecule (-44 amu) from the apigenin aglycone produces the fragment at *m/z* 183, while the loss of two CO_2 molecules (-88 amu) from the apigenin aglycone accounts for the fragment at *m/z* 181. The loss of a C_2O_3 molecule (-42 amu) together with a C_2H_2O molecule (-44 amu) from the apigenin aglycone accounts for the fragment at *m/z* 183. Ionic fragments at *m/z* 151 and 117, respectively, are the $^{1,3}A^-$ and $^{1,3}B^-$ fragments from the retrocyclization cleavage of apigenin at bond positions 1 and 3 of the C ring (Figure 3-7) (Fabre *et al.*, 2001). The loss of a CO_2 molecule (-44 amu) from the $^{1,3}A^-$ fragment (*m/z* 151) could account for the fragment at *m/z* 107.

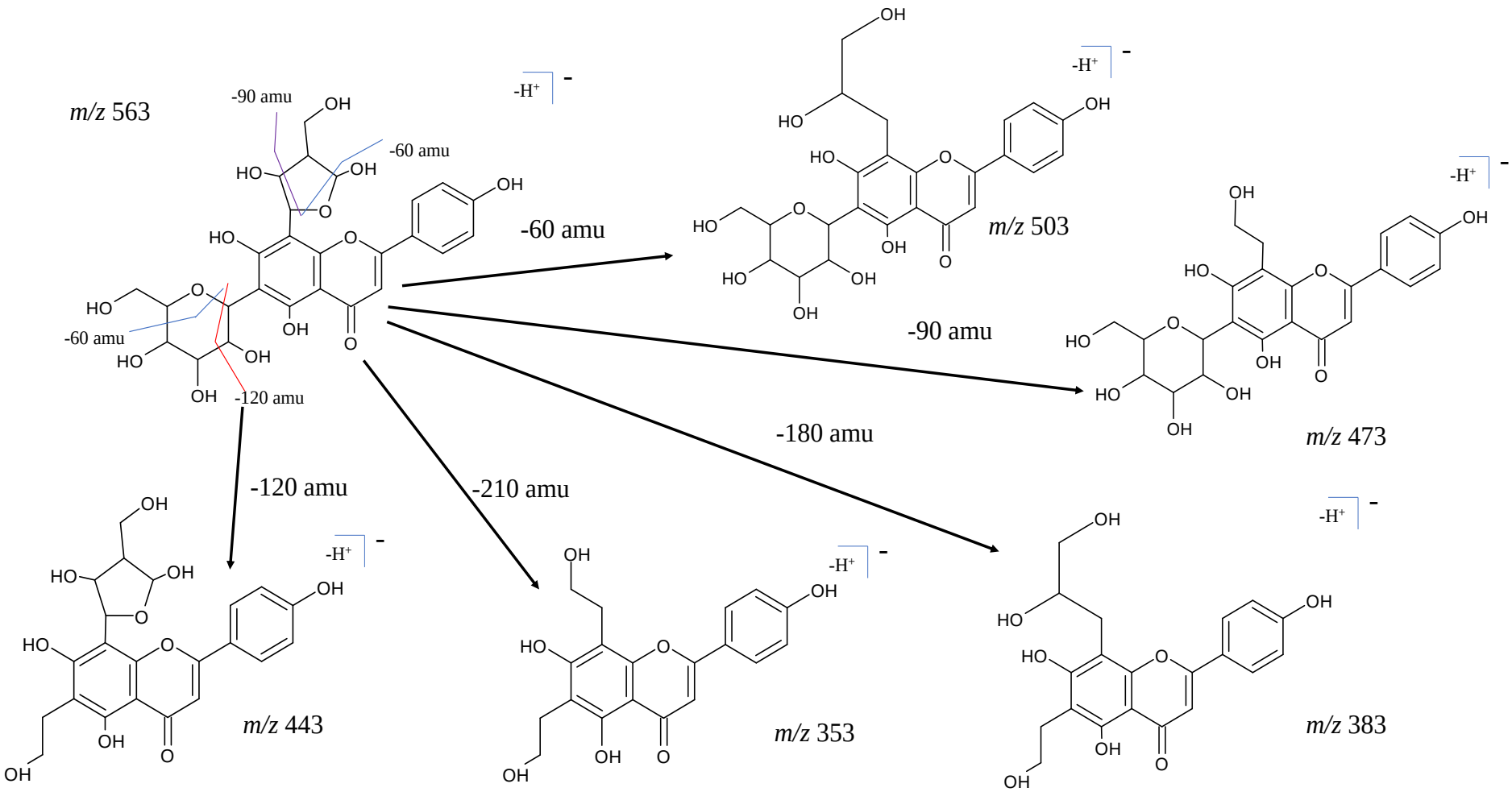


Figure 3-8: Proposed fragmentation scheme for apigenin glycoside compounds showing potential modes of cleavage of sugar moieties

The compound labelled peak 24 with molecular ion at *m/z* 563 (Table 3-6) was identified as glucosyl-arabinosyl apigenin. It produced fragments at *m/z* 506, 473, 383, 353, 269, 241, 225, 201, 183, 181, 159, 151, 117, and 107 (Table 3-6 and Figure 7-29, appendix). These fragments arise from various modes of fragmentation of the sugar moieties (Figure 3-8) and the apigenin aglycone (Figure 3-7). The fragment at *m/z* 506 corresponds to the loss of a 60 amu moiety (CH_2O_3 molecule), while the fragment at *m/z* 473 occurs as a result of cleavage and the loss of a 90 amu fragment ($C_3H_6O_3$ molecule) (Figure 3-8) (Benayad, Gómez-Cordovés & Es-Safi, 2014; Tahir, Shaari, Abas, Parveez, Ishak & Ramli, 2012). The fragment at *m/z* 383 corresponds to cleavage of 120 amu ($C_4H_8O_4$ molecule) and 60 amu fragments (-180 amu). The fragment at *m/z* 353 corresponds to cleavage and the loss of 90 amu ($C_3H_6O_3$ molecule) and 120 amu ($C_4H_8O_4$ molecule) fragments (-210 amu). The fragment at *m/z* 269 (identified as apigenin aglycone) could be due to the loss of the glucose and arabinose units. The apigenin aglycone could further undergo loss of CO (-28 amu), CO_2 (-44 amu), and C_2H_2O (-42), yielding fragments at *m/z* 241, 225, and 227, respectively. Ionic fragments at *m/z* 151 and 117, respectively, are the $^{1,3}A^-$ and $^{1,3}B^-$ fragments due to the retrocyclization cleavage of apigenin aglycone at bond positions 1 and 3 of the C ring (Figure 3-7) (Fabre *et al.*, 2001). The loss of a CO_2 molecule from the $^{1,3}A^-$ fragment (*m/z* 151) could account for the fragment at *m/z* 107.

The compound labelled peak 29 (t_R = 18.60 min and λ_{max} = 350, 265 nm) (Table 3-6) had a molecular ion *m/z* at 447 and was identified as luteolin glycoside. It produced fragments at *m/z* 285, 257, 243, 241, 229, 199 and 151 (Table 3-6 and Figure 7-34, appendix). The fragment at *m/z* 285 is the luteolin aglycone after the loss of the glucose unit (-162 amu). The loss of a CO molecule (-28 amu) and a C_2H_2O molecule (-42 amu) each from the luteolin aglycone produces the *m/z* 257 and 243 fragments (Fabre *et al.*, 2001). The fragment at *m/z* 151 is the $^{1,3}A^-$ fragment resulting from retrocyclization cleavage of luteolin at bond positions 1 and 3 of the C ring (Figure 3-7).

The compound labelled peak 45 with molecular ion at *m/z* 431 (Table 3-6) was identified as vitexin (apigenin-8-C-glycoside) based on its fragmentation pattern in literature. It had fragments at *m/z* 341, 311, 269, 241, 225, 201, 183, 181, 159, 151, 117, and 107 (Table 3-6 and Figure 7-50, appendix). The fragments at *m/z* 341 and *m/z* 311 correspond to the loss of 90 amu ($C_3H_6O_3$ molecule) and 120 amu, Figure 3-8, respectively, from the molecular ion via different modes of cleavage of the sugar moiety as illustrated in Figure 3-8 (Krasteva & Nikolov, 2008). The fragment at *m/z* 269 (identified as apigenin aglycone) is produced after the loss of the

glucose unit (-162 amu). Loss of CO (-28 amu), CO_2 (-44 amu) and C_2H_2O (-42 amu) units each from the apigenin aglycone yields the fragments at *m/z* 241, 225, and 227, respectively. The fragments at *m/z* 151 and 117 are, respectively, the $^{1,3}A^-$ and $^{1,3}B^-$ fragments as a result of the retrocyclization cleavage of apigenin at bond positions 1 and 3 of the C ring (Figure 3-7) (Fabre *et al.*, 2001). The loss of a CO_2 (-44 amu) molecule from the $^{1,3}A^-$ fragment (*m/z* 151) produces the fragment at *m/z* 107.

The compound labelled peak 8 (tR 11.33 min, λmax = 279, 233 nm) had a molecular ion at *m/z* 577 (Table 3-6) and was identified as a procyanidin dimer. It had fragments at *m/z* 451, 425, 407, 289, 245, 203, 125 and 109 (Table 3-6 and Figure 7-15 appendix). The fragment ion at *m/z* 451 results from the loss of a phloroglucinol molecule (A-ring) (–126 amu) by a heterocyclic ring fission (HRF) reaction (Figure 3-9) (Hayasaka, Waters, Cheynier, Herderich & Vidal, 2003),. The fragment ion at *m/z* 425 is proposed to be a retro-Diels-Alder (RDA) fragment produced via cleavage of the C-ring of one of the monomeric flavan-3-ol components of the procyanidin dimer, as illustrated in Figure 3-9. The fragment at *m/z* 407 results from the loss of a water molecule (–18 amu) from the *m/z* 425 fragment, most likely from the 3-OH (Figure 3-9) (Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz & Prior, 2003). The fragment at *m/z* 289 is a monomeric flavan-3-ol subunit (epicatechin or catechin) produced from the fragmentation of the procyanidin dimer by the quinone methide (QM) reaction. Either of the monomeric flavan-3-ol subunits can lose a CO_2 molecule (-44 amu) to produce the fragment at *m/z* 245 (Figure 3-9). The fragment ion at *m/z* 125 is proposed to be an RDA fragment produced via cleavage of the C-ring of one of the monomeric flavan-3-ol components of the procyanidin dimer, as illustrated in Figure 3-9.

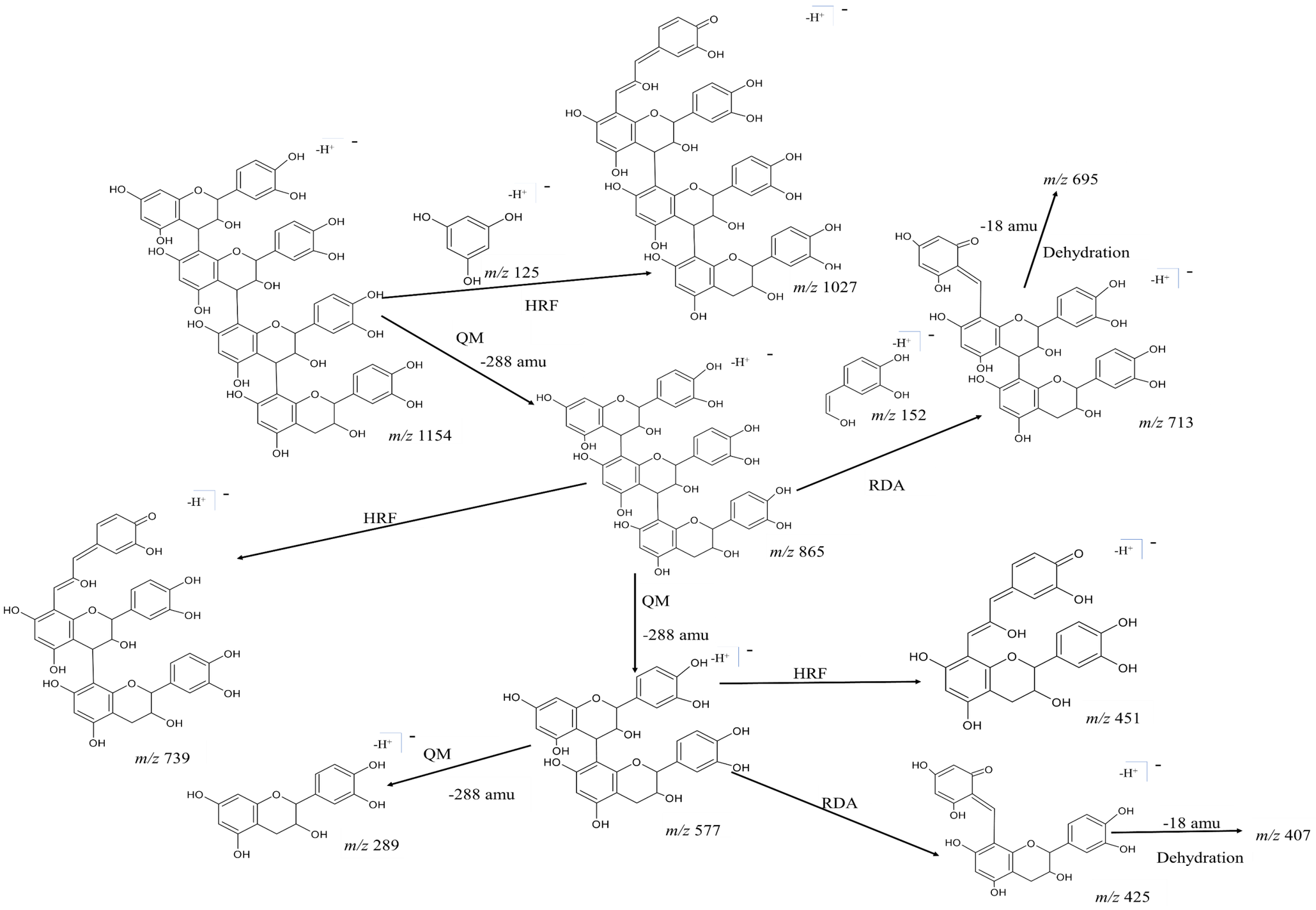


Figure 3-9 The structures of procyanidin dimer, trimer and tetramer showing quinone-methide (QM), retro-Diels-Alder (RDA) and heterocyclic ring fission (HRF) modes of cleavage

The compound labelled peak 12 (tR 12.50 min, λmax = 279, 234, 230 nm) had a molecular ion at *m/z* 865 (Table 3-6) and was identified as a procyanidin trimer. It had fragments at *m/z* 577, 451, 425, 407, 289, 245, 203, 125 and 109 (Table 3-6 and Figure 7-19, appendix). The fragment ion at *m/z* 451 corresponds to the loss of a phloroglucinol molecule (A-ring) (–126 amu) (Hayasaka *et al.*, 2003) via the HRF reaction (Figure 3-9). The fragment at *m/z* 577 represents a dimeric procyanidin specie which results from splitting the procyanidin trimer via a QM reaction (Figure 3-9). The rest of the fragments are produced via similar fragmentation patterns described above for procyanidin dimer.

The compound labelled peak 18 (tR 14.81 min, λmax = 274, 233 nm) had a molecular ion at *m/z* 1154 (Table 3-6) and was identified as procyanidin tetramer. It had fragments at *m/z 865*, 577, 451, 425, 407, 289, 245, 203, 125 and 109 (Table 3-6 and Figure 7-24, appendix). The molecular fragments at m/z 865 and 577 are, respectively, the procyanidin trimer and dimer fragments produced from the splitting of the procyanidin tetramer via a QM reaction (Figure 3-9). The rest of the fragments are produced via similar fragmentation patterns described above for procyanidin trimer and dimer.

3.2.5.2.2 Effect of food-to-food fortification (FtFF) and extrusion on the concentration of phenolic compounds in wholegrain sorghum-based porridges

Table 3-7: Effect of FtFF and extrusion on the concentration of phenolic compounds (µg/g) in wholegrain sorghum-based porridges

Compound	**Cooked Sorghum**	**Baobab**	**Moringa**	**Extruded Sorghum**	**Extruded sorghum + Baobab**	**Extruded sorghum + Moringa**	**Extruded sorghum + Moringa + Baobab**
Phenolic acids							
p-Hydroxybenzoic acid	617^{c}±66	86^{a}±21	371^{b}±12	340^{b}±29	405^{b}±20	341^{b}±44	371^{b}±39
Protocatechuic acid	121^{c}±13	599^{e}±9	271^{d}±11	28^{a}±5	50^{b}±3	72^{b}±4	86^{bc}±35
Vanillic acid	12^{d}±0	26^{e}±2	5^{a}±2	4^{a}±0	10^{cd}±1	6^{ab}±0	9^{bc}±1
Gallic acid	7^{a}±1	42^{c}±2	110^{d}±7	2^{a}±0	13^{ab}±11	5^{a}±1	20^{b}±0
Syringic acid	21^{b}±0	33^{c}±4	51^{d}±6	1^{a}±0	6^{a}±0	4^{a}±2	22^{b}±3
p-Coumaric acid	181^{c}±20	ND	919^{d}±34	ND	ND	42^{a}±3	101^{b}±3
Caffeic acid	941^{d}±49	ND	647^{c}±7	263^{b}±25	285^{b}±5	256^{b}±13	305^{b}±8
Ferulic acid	92^{e}±0	ND	2^{a}±0	42^{b}±0	58^{d}±3	50^{c}±3	55^{d}±3
Sinapic acid	4^{a}±0	3^{a}±0	ND	ND	24^{b}±3	4^{a}±0	20^{b}±0
Total Phenolic acids	**1996**	**789**	**2376**	**680**	**851**	**780**	**989**
Phenolic acid esters							
p-Coumaroyl glycerol	1837^{c}±82	ND	ND	1491^{b}±59	1220^{a}±8	1186^{a}±34	1091^{a}±84
Caffeoyl glycerol	3104^{e}±79	ND	57^{a}±4	1743^{d}±97	1603^{cd}±27	1277^{b}±29	1470^{c}±6

Dihydrocaffeoyl glycerol	501^{a}±62	3008^{b}±91	2636^{b}±184	169^{a}+8	617^{a}±45	235^{a}±8	418^{a}±48
3-Coumaroyl quinic acid	ND	ND	5309^{b}±53	ND	ND	36^{a}±3	42^{a}±6
4-Coumaroyl quinic acid	ND	ND	2882^{c}±139	ND	ND	14^{a}±2	22^{a}±2
3-Caffeoyl quinic acid	57^{a}±19	93^{a}±4	22695^{b}±1350	12^{a}±1	9^{a}±1	64^{a}±4	136^{a}±12
4-Caffeoyl quinic acid	1594^{a}±155	2819^{a}±69	464493^{b}±4985	523^{a}±91	277^{a}±22	1708^{a}±78	1730^{a}±130
3-Feruloyl quinic acid	ND	ND	390^{b}±8	ND	ND	239^{a}±10	406^{c}±4
4-Feruloyl quinic acid	ND	ND	265^{d}±3	ND	ND	247^{c}±14	319^{e}±9
Coumaroyl caffeoyl glycerol	ND	ND	41751^{a}±2279	ND	ND	ND	ND
Dicaffeoyl glycerol	3506^{c}±82	ND	ND	2253^{a}±41	2459^{b}±92	2292^{a}±20	2408^{b}±17
Dicaffeoyl spermidine	663^{c}±58	ND	16^{a}±5	728^{c}±68	446^{b}±4	492^{b}±75	524^{b}±31
Total Phenolic acid esters	**11262**	**5920**	**540494**	**6919**	**6631**	**7790**	**8566**
Flavonoid aglycones							
Kaempferol	115^{f}±5	16^{a}±0	61^{d}±0	20^{a}±0	40^{b}±6	46^{bc}±4	53^{e}±1
Quercetin	15^{b}±0	17^{b}±0	83^{d}±1	11^{a}±0	11^{a}±0	17^{b}±1	36^{c}±0
Myricetin	5^{d}±0	9^{f}±0	ND	1^{a}±0	6^{e}±	3^{b}±0	6^{e}±0
Catechin	7^{b}±1	309^{d}±6	ND	2^{a}±0	25^{c}±0	1^{a}±0	19^{c}±1
Epicatechin	145^{a}±8	3563^{b}±226	ND	20^{a}±4	133^{a}±17	3^{a}±1	128^{a}±13
Naringenin	71^{d}±6	ND	ND	33^{bc}±8	41^{c}±6	27^{b}±1	38^{bc}±6
Apigenin	29^{d}±1	ND	ND	11^{b}±1	19^{c}±2	10^{b}±0	27^{d}±5
Eriodictoyl	119^{e}±3	ND	ND	52^{c}±4	82^{d}±5	6^{a}±0	28^{b}±0
Hesperitin	10^{b}±0	12^{c}±0	78^{g}±0	4^{a}±0	17^{d}±0	19^{e}±2	32^{f}±0

Luteolin	99^{e}±4	5^{a}±0	29^{b}±0	32^{b}±2	36^{bc}±6	41^{cd}±4	45^{d}±1
Total flavonoid aglycones	**610**	**3931**	**251**	**186**	**410**	**173**	**406**
Flavonoid glycosides							
Kaempferol glycoside	8^{a}±1	23^{a}±1	1425^{c}±42	5^{a}±1	7^{a}±0	213^{b}±11	244^{b}±17
Dihydrokaempferol glycoside	34^{f}±0	ND	8^{b}±1	15^{e}±2	11^{cd}±0	9^{bc}±0	13de±2
Quercetin glucoside	24^{a}±4	23^{a}±0	1687^{d}±37	8^{a}±1	9^{a}±2	281^{b}±14	360^{c}±21
Rutin	60^{ab}±2	100^{c}±1	5604^{e}±12	22^{a}±1	29^{ab}±1	681^{c}±57	861^{d}±40
Epicatechin glucoside	ND	ND	293^{e}±3	ND	ND	5^{b}±1	9^{c}±0
Eriodictoyl glucoside	96^{b}±4	ND	ND	36^{a}±2	36^{a}±2	35^{a}±2	38^{a}±3
Naringenin glucoside	53^{e}±2	22^{b}±2	ND	27^{bc}±2	30^{cd}±0	27^{bc}±2	36^{d}±6
Naringin	7^{a}±0	8^{a}±1	577^{b}±49	10^{a}±1	9^{a}±0	11^{a}±1	5^{a}±0
Vitexin	7^{c}±0	1^{a}±0	40^{d}±1	4^{b}±0	3^{b}±0	4^{b}±0	6^{c}±0
Luteolin glucoside	41^{a}±1	24^{a}±0	1261^{c}±23	41^{a}±0	36^{a}±2	190^{b}±9	29^{a}±0
Glucosyl-arabinosyl apigenin	23^{e}±1	1^{a}±0	10^{d}±0	6^{bc}±1	8^{cd}±0	5^{b}±0	6^{bc}±0
Total flavonoid glycosides	**353**	**202**	**10865**	**174**	**151**	**1461**	**1607**
Proanthocyanidins							
Procyanidin dimer	ND	896^{a}±47	ND	ND	ND	ND	ND
Procyanidin trimer	ND	947^{a}±17	ND	ND	ND	ND	ND
Procyanidin tetramer	ND	2.02^{a}±0.09	ND	ND	ND	ND	ND
Total proanthocyanidins		**1845.02**					

[1]Values are the means±SD of at least two samples of each formulation analysed independently.
[2]Values within the same column followed by different letters are significantly different ($p<0.05$).

[3]ND = not detected

Sorghum and moringa both contained 2 times more total phenolic acids than baobab fruit pulp. The predominant phenolic acids were caffeic acid in sorghum, p-coumaric acid in moringa leaf powder (MLP) and protocatechuic acid in baobab fruit pulp (BFP) (Table 3-7). Food-to-food fortification (FtFF) with BFP alone, whether alone or in combination with MLP, had no effect on the phenolic acids quantified in this study except for protocatechuic acid (increased by 79% and 207 respectively), vanillic acid (increased by 150% and 125% respectively), caffeic acid (increased by 8% and 16% respectively), ferulic acid (increased by 38% and 31% respectively) and sinapic acid (increased by 100% and 100% respectively). FtFF with MLP had no effect on phenolic acids quantified except protocatechuic acid (increased by 157%), ferulic acid (increased by 19%) and sinapic acid (increased by 100%). A combination of MLF and BFP increased the gallic and syringic acid contents of sorghum-based porridges (by 900% and 210%, respectively). Concerning phenolic acid esters, MLP had the highest content of esters, with caffeoylquinic acid being the predominant ester. Caffeoyl glycerol was the predominant ester in sorghum, while dihydrocaffeoyl glycerol was the predominant ester in baobab fruit pulp powder. Caffeoyl glycerol and diacaffeoyl spermidine were undetected in BFP, while feruloyl quinic acids were absent in sorghum and BFP. FtFF with BFP had no effect on the caffeoyl glycerol and 3-caffeoyl quinic acid contents of sorghum but increased the dihydrocaffeoyl glycerol content (by 265%) and diacaffeoyl spermidine (by 39%). FtFF with MLP alone decreased the caffeoyl glycerol and diacaffeoyl spermidine contents (by 27% and 39%, respectively), increased the contents of dihydrocaffeoyl glycerol and 3-caffeoylquinic acid (by 39% and 433% respectively) and introduced 3- and 4- feruloyl quinic acids that were absent in sorghum. FtFF with a combination of MLP and BFP decreased the caffeoyl glycerol and diacaffeoyl spermidine contents (by 16% and 28%, respectively), increased the contents of dihydrocaffeoyl glycerol and 3-caffeoylquinic acid (by 147 and 433% respectively) and introduced 3- and 4- feruloyl quinic acids that were absent in sorghum.

Concerning flavonoids, quercetin was the main flavonoid in MLP while epicatechin was the principal flavonoid in sorghum and BFP. FtFF with BFP, whether alone or in combination with MLP, caused a significant increase in the contents of kaempferol (by 100% and 165%, respectively), myricetin (by 500% and 500% respectively), catechin (by 1150% and 850% respectively), epicatechin (by 565% and 540% respectively), apigenin (by 73% and 145% respectively), and hesperetin (by 325% and 700% respectively) (Table 3-7). FtFF with BFT alone did not affect the quercetin, naringenin, or luteolin content. FtFF with MLP alone caused a significant increase in the contents of kaempferol (by 130%), quercetin (by 54%), myricetin

(by 200%), and hesperetin (by 325%) while significantly reducing the content of epicatechin (by 85%). FtFF with MLP had no effect on catechin, naringenin, apigenin and Luteolin content. FtFF, with a combination of BFT and MLP, significantly increased the quercetin (by 227%) and luteolin (by 41%) contents but did not affect the naringenin content.

Regarding flavonoid glycosides, overall, MLP had the highest content of flavonoid glycosides (about 42 times more than sorghum and 53 times more than BFP). Rutin was the predominant glycoside in all the plant samples. Except for dihydrokaempferol glycoside, which was significantly reduced (by 27%), FtFF with BFP had no effect on the flavonoid glycoside content of sorghum. FtFF with MLP alone significantly increased kaempferol glycoside (by 4160%), quercetin glucoside (by 3413%), and luteolin glycoside (by 363%), while incorporating epicatechin glucoside, which was initially absent in the sorghum and decreasing the dihydrokaempfeol glycoside content (by 40%). FtFF with a combination of BFP and MLP significantly increased kaempferol glycoside (by 4780%), quercetin glucoside (by 4400%) and vitexin (by 50%) while incorporating epicatechin glucoside, which was initially absent in the sorghum. Proanthocyanidins were only identified in BFP but were absent in all samples fortified with BFP, possibly due to their breakdown following extrusion cooking.

Concerning extrusion, extrusion cooking consistently reduced the concentration of phenolic compounds (total phenolic acids, total phenolic acid esters, total flavonoid aglycones, and total flavonoid glycoside), which can be clearly shown when concentrations of total phenolic compounds in conventionally cooked sorghum porridge are compared to extruded sorghum porridge, Table 3-7. Various workers have also reported a reduction in phenolic extrusion cooking treatment of sorghum (Awika *et al.* (2003b); (Adarkwah‐Yiadom & Duodu, 2017); Dlamini *et al.* (2007b)), faba bean and kidney bean (Alonso *et al.* (2000)). The decrease in total phenolic content during extrusion cooking may be ascribed to the binding of phenolic compounds with protein (Emmambux & Taylor, 2003) and other cell wall macromolecules thereby reducing their extractability (Taylor & Duodu, 2015; Awika *et al.*, 2003a). High-temperature conditions during extrusion cooking may denature protein and cause it to assume a more open structure with exposed sites, which promotes phenolic-protein interaction (Dlamini *et al.*, 2007b).

3.2.5.3 Assays involving the use of cell tissue cultures.

For all cell tissue culture assays (Caco-2, 3T3-L1 and RAW264.7), the 1% Conc. hydrochloric acid-methanolic phenolic extracts of sorghum-based porridges were tested for cytotoxicity at the concentrations used (dilutions with PBS to yield 50 µg/ml in the well for all cell lines). For all concentrations of the extracts used, the cells showed a viability of at least 80% for 72 hours, indicating minimal cytotoxicity. The concentration that showed tissue culture activity with minimal cytotoxicity after 72 hours was a final concentration of 50 µg/ml in the tissue culturing well.

3.2.5.3.1 Cellular antioxidant activity (DCFH-DA assay)

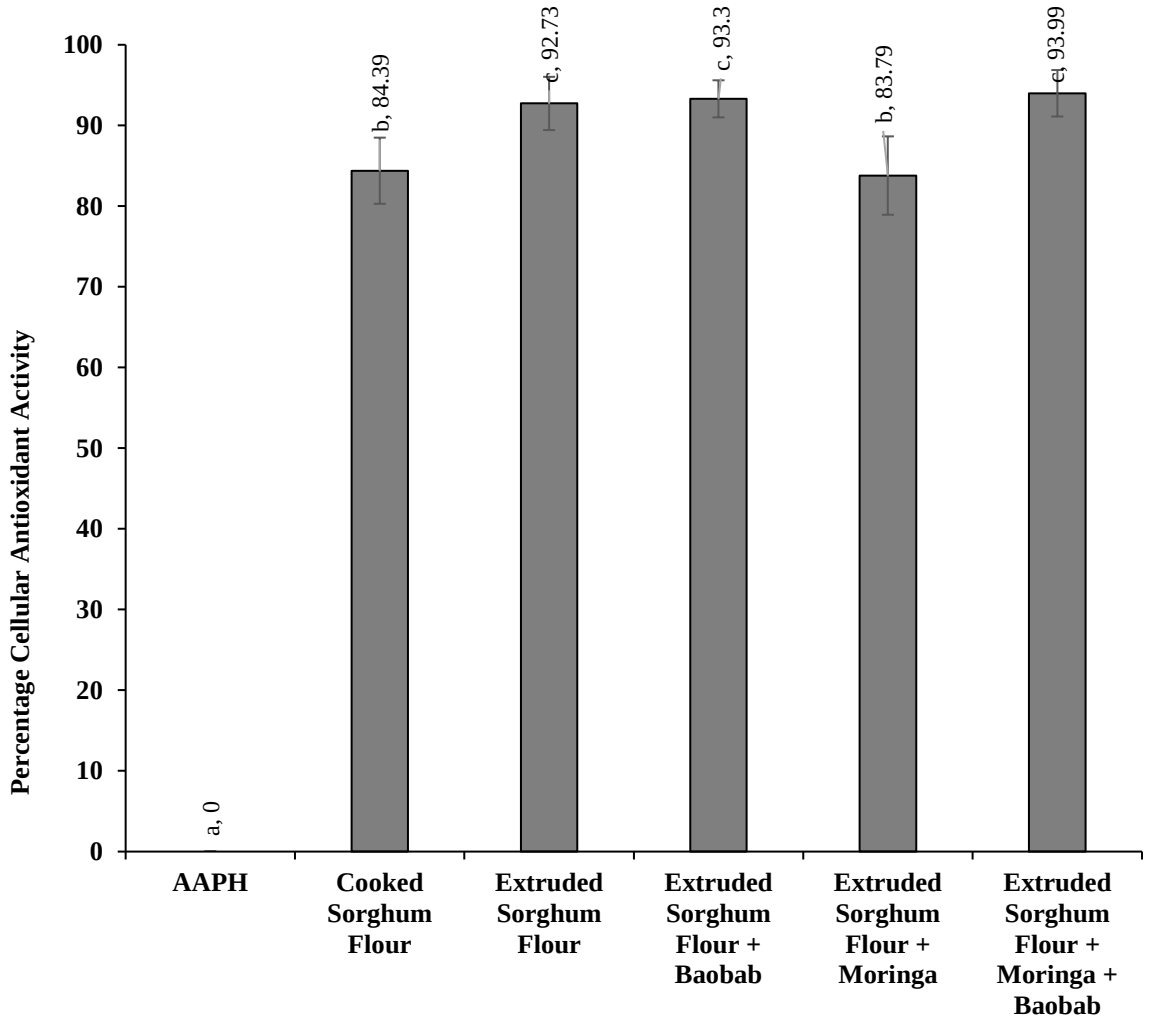


Figure 3-10: Cellular protection (mean ± SEM) against AAPH-induced oxidative damage for sorghum-based porridge extracts (diluted in PBS to yield 50 µg/ml in the well) in the Caco-2 cell line, measured using the DCFH-DA assay. Bars with different letters are significantly different, $p \leq 0.05$.

The cellular antioxidant activities (CAA) based on the Caco-2 cell line model of the sorghum-based sample extracts were determined. A positive control consisting of Caco-2 cells treated with phosphate-buffered saline without the sorghum extracts in the presence of AAPH was used to indicate maximum oxidative damage. Extracts from extruded sorghum FtFF with BFP alone or in combination with MLP exerted similar CAA to extracts from extruded sorghum alone. Extracts from extruded sorghum FtFF with MLP exerted lower CAA (by 10%) compared to extracts from extruded sorghum alone.

Apart from the extract from extruded sorghum FtFF with MLP extracts from all the other extruded samples had higher CAA (by up to 11.4%) than the extract from cooked (unextruded) sorghum flour. The improvement of CAA by extrusion is probably because extrusion has been reported to increase free phenolic compounds while reducing bound phenolic compounds. Approximately 80% of sorghum phenolic compounds are linked to arabinoxylans by ester bonds, which are capable of resisting the digestion process in the upper gastrointestinal tract, compromising their bioaccessibility (Salazar Lopez, Loarca-Piña, Campos-Vega, Gaytán Martínez, Morales Sánchez, Esquerra-Brauer, Gonzalez-Aguilar & Robles Sánchez, 2016). Extrusion cooking has been reported to increase the proportion of free phenolic compounds and thus increase their bioaccessibility (Herrera-Cazares, Luzardo-Ocampo, Ramírez-Jiménez, Gutiérrez-Uribe, Campos-Vega & Gaytán-Martínez, 2021). Regarding the effect of MLP, this could be attributed to the high content of soluble and insoluble dietary fibre. Zhao, Zhang, Dong, Huang, Liu, Deng, Ma, Zhang, Wei and Xiao (2018) reported that insoluble dietary fibre decreased free phenolic compounds and increased their bound form in rice.

Concerning extrusion, CAA indicates significantly higher values in extruded sorghum when compared to conventionally cooked sorghum (by 10%). The cell type effect is a function of the oxidative status of different cellular compartments (cytoplasm, mitochondria and nucleus) (Koren, Zverev, Ginsburg & Kohen, 2008), as well as the rate and degree of absorption, metabolism, conjugation and secretion. Literature has documentation of various phenolic compounds identified in this study and their role in CAA, including caffeic acid (Fan, Liu, Gao, Zhang & Yi, 2018), gallic acid, ferulic acid, luteolin, quercetin, quercetin glycoside, myricetin, kaempferol, catechin and epicatechin (Wan, Liu, Yu, Sun & Li, 2015). The antioxidant activity ranking of tested compounds in the CAA assay differed from the results in the ORAC assay *in vitro* but was more in agreement with the ranking in the ORAC assay *in vitro*, demonstrating the good biological relevance of the CAA assay developed in this study. Thus, the CAA values could be used to predict the antioxidant activity of antioxidants *in vivo*.

3.2.5.3.2 Lipid accumulation in 3T3-L1 cells

Figure 3-11 shows the effect of sorghum-based porridge phenolic extracts on the formation of lipid droplets (prevention) and reduction of lipid droplet size and/or density (treatment). Prevention and treatment of lipid droplet formation by phenolic extracts ranged from 25-29% and 13-22%, respectively, with no significant differences amongst different treatments compared to the control. This was confirmed by microscopic images (Figure 3-12 and Figure 3-13) that showed a reduction in the density of lipid droplets.

Phenolic compounds identified in the sorghum-based porridges from this study have been reported to play a vital role in lipid accumulation at different stages (Aranaz, Navarro-Herrera, Zabala, Miguéliz, Romo-Hualde, López-Yoldi, Martínez, Vizmanos, Milagro & González-Navarro, 2019). The authors indicated that quercetin reduced lipid accumulation along the entire incubation period while apigenin and myricetin were active during the onset and completion of differentiation. Phenolic acids, including ferulic, gallic and vanillic acids, were active in downward lipid accumulation during the onset of differentiation, with only p-coumaric acid showing downward regulation of lipid accumulation throughout the whole differentiation (Aranaz *et al.*, 2019). Aranaz *et al.* (2019) also reported that the anti-adipogenic effect was accompanied by the downregulation of Stearoyl-CoA desaturase-1 and Lipoprotein lipase, both relevant in adipogenesis.

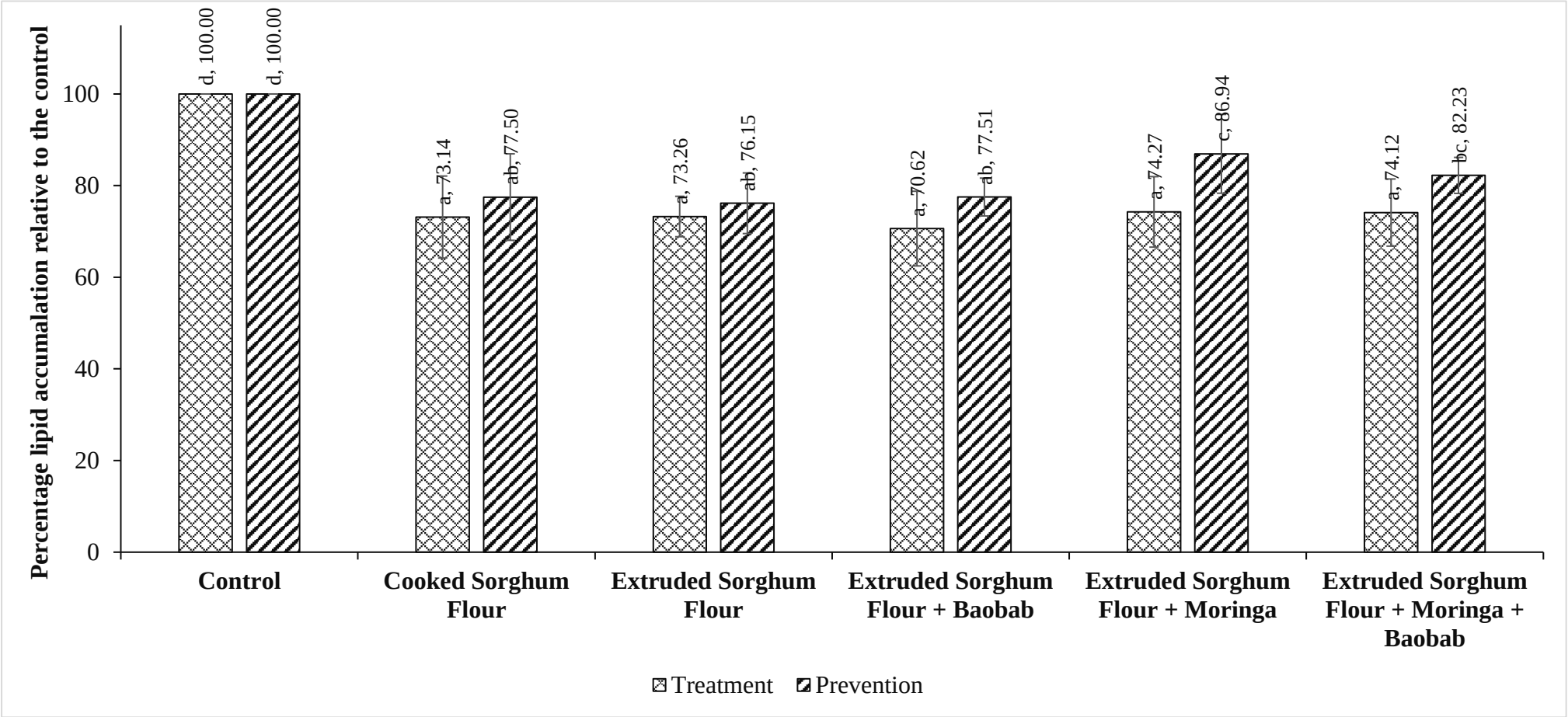


Figure 3-11: Effect of sorghum-based porridge phenolic extracts (diluted in PBS to yield 50 µg/ml in the well) on percentage lipid accumulation, compared with differentiated controls. Data are the mean±standard deviation of three independent experiments carried out in triplicate (n=9). Different letters indicate significant differences at $p<0.05$. Error bars indicate standard deviation.

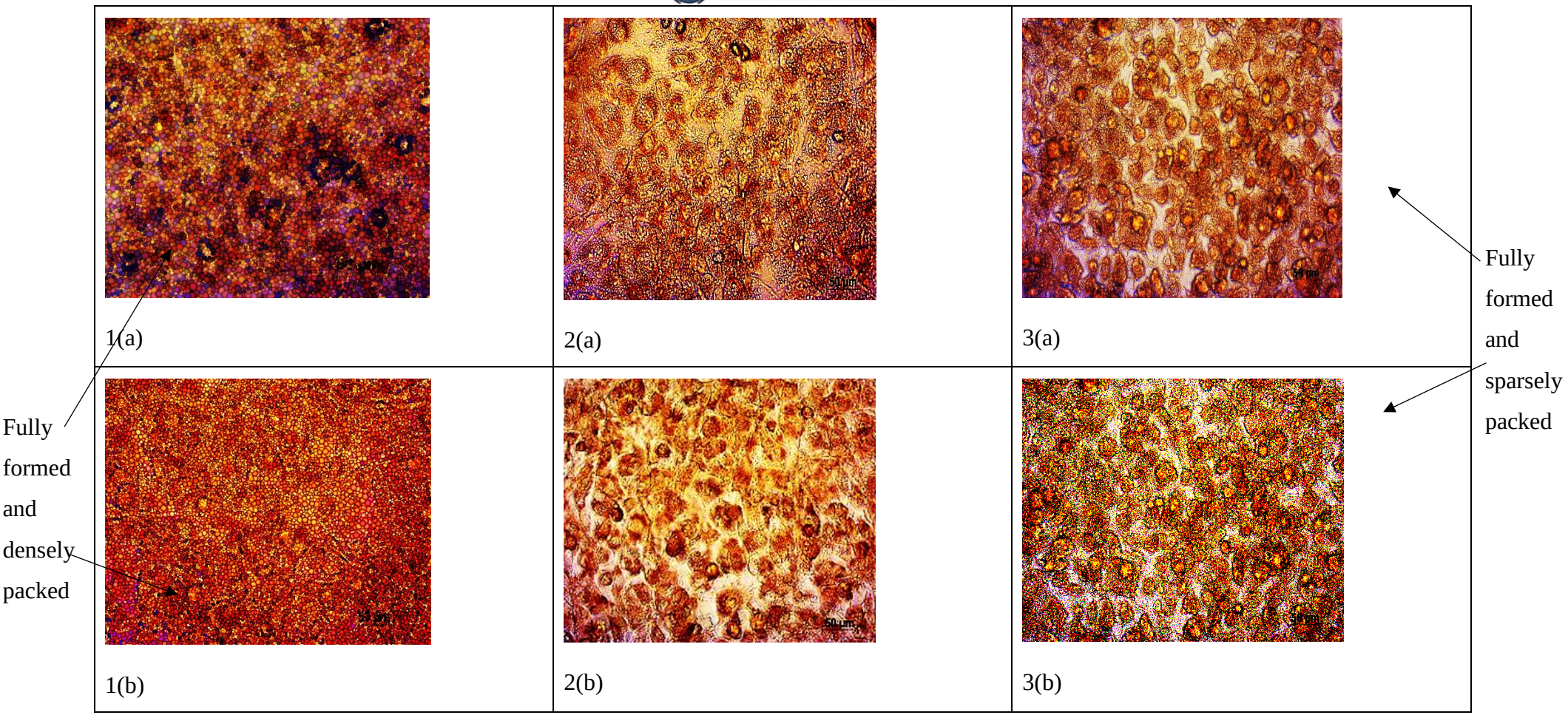


Figure 3-12: Effect of sorghum-based porridge phenolic extracts (diluted in PBS to yield 50 µg/ml in the well) on 3T3-L1 adipocyte differentiation process, compared with differentiated controls. Phase contrast micrographs of Oil Red O stained differentiated murine (3T3-L1) adipocytes at 40×. Bar = 50 µm. 1-Control, 2-Cooked sorghum, 3- Extruded sorghum: a- Prevention of lipid droplet accumulation, b- Treatment of accumulated lipid droplets

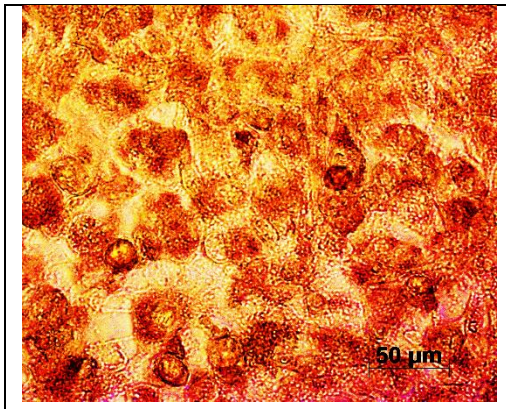
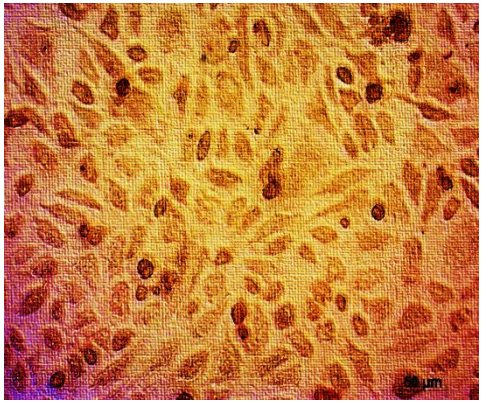
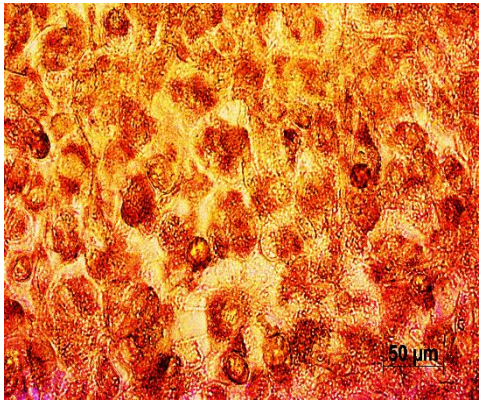
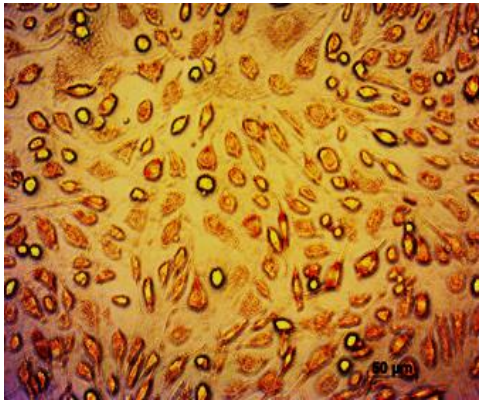
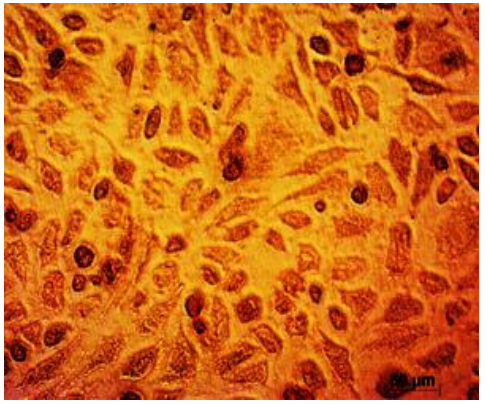
 4 (a)	 5(a)	 6(a)
 4(b)	 5(b)	 6(b)

Figure 3-13: Effect of sorghum-based porridge phenolic extracts (diluted in PBS to yield 50 µg/ml in the well) on 3T3-L1 adipocyte differentiation process, compared with differentiated controls. Phase contrast micrographs of Oil Red O stained differentiated murine (3T3-L1) adipocytes at 40×.

Bar = 50 μm. 4- Extruded sorghum fortified with BFP, 5- Extruded sorghum fortified with MLP, 6- Extruded sorghum fortified with BFP and MLP: a- Prevention of lipid droplet accumulation, b- Treatment of accumulated lipid droplets

3.2.5.3.3 Nitric oxide (NO) scavenging activity in RAW264.7 cells.

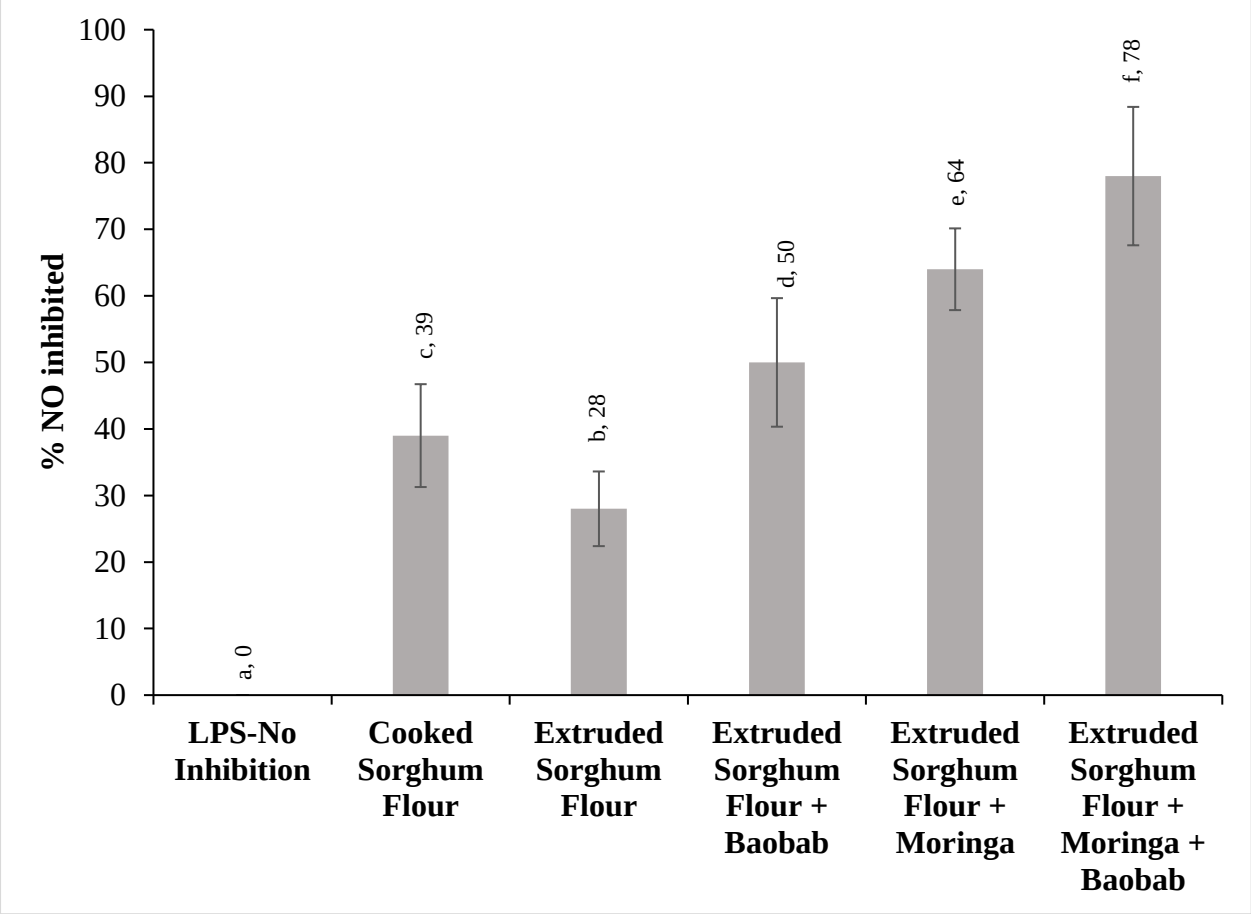


Figure 3-14: Percentage inhibition of LPS-induced nitric oxide production in RAW264.7 macrophages of sorghum-based phenolic extracts (diluted in PBS to yield 50 µg/ml in the well). The bars represent means from three independent experiments ± SEM. Different letters indicate significant differences at $p<0.05$. Error bars indicate standard deviation.

All extracts showed a significant reduction in NO formed by the cells *(*Figure 3-14). Extracts from extruded sorghum FtFF with BFP reduced NO formation in RAW 264.7 cells by 50% relative to the control MLP FtFF extracts resulting in a significantly higher reduction in NO than BFP (64%). A combination of MLP and BFP caused the most significant reduction in NO formed (78%). Choo, Lee, Nguyen, Lee, Woo, Min and Lee (2015) and Nguyen, Zhao, Lee, Kim, Min and Woo (2015) reported that phenolic acid esters (caffeoyl glycerols) reduced NO production in RAW 264.7. They further concluded that the suppression of NO production (anti-inflammatory activity) is predominantly exerted by methyl ester of phenolic acid derivatives and not simple methylation or O-methyl substitution of OH group in free phenolic acid derivatives *(*Nguyen et al., 2015). This is consistent with the findings in this study which showed that while sorghum and BFP contained phenolic acid esters, MLP had the highest

content of phenolic acid esters, thus the high NO inhibition when sorghum was fortified with MLP. The higher inhibition observed when a combination of MLP and BFP is consistent with the higher content of phenolic acid esters that increased with a combination of the two.

Concerning extrusion cooking, extruded sorghum showed a lower inhibition of NO when compared to conventionally wet-cooked sorghum. This is consistent with the reduction in phenolic acid esters following extrusion.

3.2.6 Conclusion

FtFF of wholegrain sorghum with baobab and moringa, either alone or in combination, enhances the TPC, radical scavenging activity (ABTS and ORAC), cellular NO inhibition and AGEs but does affect the chemical NO inhibition and adipogenesis in 3T3-L1 cells. Fortification with baobab shows no marked significance on CAA, while moringa reduces CAA marginally. This could be attributed to the different phenolic compositions of baobab and moringa. Extrusion of wholegrain sorghum reduced the TPC, radical scavenging activity (ABTS and ORAC), cellular NO inhibition and AGEs but does not affect the chemical NO inhibition and adipogenesis in 3T3-L1 cells, while it increases CAA. This may be an indication that the prevailing conditions during extrusion cooking, such as high temperature in the presence of moisture and intense shear, may break down phenolic compounds, making them more active. The results from this study demonstrate the potential for health-promoting properties of extruded wholegrain FtFF sorghum in terms of protection against oxidative stress and related non-communicable diseases.

3.3 EFFECTS OF EXTRUSION COOKING AND FOOD-TO-FOOD FORTIFICATION ON NUTRITIONAL (*IN VITRO* STARCH AND PROTEIN DIGESTIBILITY), PASTING AND FUNCTIONAL PROPERTIES OF SORGHUM-BAMBARA GROUNDNUT-BASED PORRIDGE

3.3.1 Abstract

Protein-energy malnutrition (PEM) and micronutrient deficiencies remain burdens among children in Africa due to monotonous cereal-based diets. Compositing with local pulses such as Bambara groundnuts can be a solution. Food-to-food fortification (FtFF) of cereals with micronutrient-rich food products has become pivotal in addressing micronutrient deficiency. Rapidly urbanising communities in Africa, however, demand convenience-type products. To address these needs, the nutritional, physical and functional properties of extrudates of composited whole sorghum and whole grain Bambara groundnut flours fortified with baobab and moringa leaf powder using a twin screw extrusion cooker were investigated. Moringa leaf powder and baobab were used as fortificants either individually or in combination at rates of 10% of the sorghum-Bambara groundnut composite. This research aimed to establish the effects of food-to-food fortification of sorghum-Bambara groundnut composite and extrusion moisture on product nutritional, physical and functional properties. This would indicate which FtFF porridge could yield nutritionally beneficial products for specific at-risk groups and how they could be applied as convenience-type nutritious foods.

Compositing sorghum with Bambara groundnut increased *in vitro* protein digestibility (IVPD) while reducing the extent of starch hydrolysis, estimated glycaemic index (EGI) and the estimated glycaemic load (EGL). Fortification of the composite with moringa leaf powder (MLP) and baobab fruit pulp (BFP), whether alone or in combinations, significantly increased the total dietary fibre (TDF), resistant starch (RS), and slowly digestible starch (SDS) while considerably decreasing rapidly digestible starch (RDS), EGI, and EGL. This was attributed to the high antinutrients (phenolic compounds, phytate and dietary fibre) present in the fortificants. Extrusion cooking of the composites significantly increased the TDF, IVPD, RDS, RS, EGI and EGL while decreasing the SDS. This was attributed to the conditions of high temperature, shear and pressure during extrusion cooking that leads to the degradation of starch and proteins, making them more susceptible to enzymatic hydrolysis as well as the reduction of antinutrients in the foodstuffs. All composites exhibited a shear-thinning behaviour.

FtFF and extrusion cooking of wholegrain sorghum-Bambara groundnut composite porridges has the potential to reduce PEM through boosting the protein content and digestibility of sorghum (by addition of Bambara groundnut) as well as diet-related noncommunicable diseases such as diabetes (through FtFF with BFP and MLP).

This study highlights the potential of FtFF and extrusion to enhance protein quality and starch digestibility of wholegrain-based starchy staple foods.

Keywords: Food-to-food fortification, Protein, Extrusion, Pasting, Starch, Fibre

3.3.1 Introduction

The Covid-19 pandemic is fuelling the global nutrition crisis and highlighting the importance of good nutrition for human health. Sub-Saharan Africa remains the region with the highest prevalence of undernutrition (UNICEF/WHO/WBG, 2021). Of the nearly 150 million stunted children globally, 41% are in Africa, and 27% of the nearly 46 million children who are severely wasted globally are in Africa. Reports show a high prevalence of protein-energy malnutrition (PEM) and iron deficiency in Africa among children and manifest in the forms of stunting, wasting, and increased disease burden, which often leads to mortality (Micha *et al.*, 2020). A predominance of poor diets and infectious diseases in developing communities go hand-in-hand as causes of these mortalities (Müller & Krawinkel, 2005). Moreover, diet-related noncommunicable diseases such as diabetes are on the rise, with Africa expected to have a prevalence of about 5.2% by 2045 (Sun *et al.*, 2022). Diet plays a crucial role in managing both type 1 and type 2 diabetes, with low GI foods being essential in handling it and the presence of fibre and resistant starch can reduce the glycaemic load of a food (Zafar, Mills, Zheng, Regmi, Hu, Gou & Chen, 2019). The glycaemic load reduction is attributed mainly to the diluting effect of fibre and resistant starch on the rapidly digestible starch (RDS), thus reducing the amount of RDS (Pugh, Cai, Altieri & Gary Fros, 2023).

Starchy cereals, roots and tubers are the staple foods of people in sub-Saharan Africa (Gregory & Wojciechowski, 2020; Gibson & Hotz, 2001). The monotony of starch possibly explains the lack of protein and micronutrients in the diets of children, leading to malnutrition. Sorghum is quantitatively a major cereal in sub-Saharan Africa (Taylor, 2019). One of the key values of sorghum is that it can grow in poor soils and drought-stricken areas where subsistence farmers do not have irrigation systems in place. Nutritionally, however, sorghum is especially poor in the essential (indispensable) amino acid lysine (Serna-Saldivar & Espinosa-Ramirez, 2019). Furthermore, sorghum protein digestibility is largely reduced on wet cooking to prepare traditional foods (Duodu *et al.*, 2003). The low IVPD has been attributed chiefly to the disulphide crosslinking of sorghum prolamin proteins, making them resistant to enzyme attack (Duodu *et al.*, 2003).

Food-based strategies, such as dietary diversification, have successfully addressed malnutrition (Duodu, Lubaale & Kayitesi, 2021). As animal proteins are costly and scarce in developing communities (Ryckman, Beal, Nordhagen, Chimanya & Matji, 2021), incorporating these excellent protein sources into children's diets to prevent PEM is sometimes not feasible.

Developing cereal and legume composite foods is an excellent dietary diversification strategy to improve the protein quality of traditional starchy foods. Compositing has proven to be successful in the production of protein-rich foods where sorghum has been composited with various legumes (Jadhavar, Jaiswal & Bornare, 2021). Food-to-food fortification is where micronutrient-rich food combinations are used to promote the bioavailability of essential micronutrients by increasing the nutrient levels and the levels of enhancers and decreasing the levels of inhibitors of nutrient bioavailability (Thompson, 2007). Food-to-food fortification (as an intervention) with iron-rich products such as moringa has the potential to alleviate iron nutritional deficiencies but also increase fibre content and help manage diabetes. Fortification with baobab, which is high in tannins, could increase resistant starch and reduce rapidly digestible starch (Coe *et al.*, 2013). Moreover, tannins can bind enzymes to starch hydrolysing enzymes, thus decreasing the hydrolysis of starch (Giuberti, Rocchetti & Lucini, 2020)

Bambara groundnut is a drought-tolerant and nutritious indigenous African grain legume (pulse) with high protein content but is underutilized (Majola, Gerrano & Shimelis, 2021). Due to the Bambara groundnut plants' nitrogen fixation abilities, it is applied in intercropping and crop rotation with other grains such as sorghum (Majola *et al.*, 2021). Being tolerant to low soil fertility and low rainfall while rich in high-quality protein makes Bambara groundnuts a potential solution to address poor diets, recurring drought and soil degradation in sub-Saharan Africa. However, Bambara groundnuts remain utilised mainly by subsistence farmers (Pasipanodya, Horn, Achigan-Dako, Musango & Sibiya, 2022).

The presence of anti-nutritional compounds in sorghum and Bambara groundnuts present limitations to the nutritional attributes of these grains. The presence of antinutritional factors such as saponins, tannins, phytic acid, gossypol, lectins, protease inhibitors, amylase inhibitor, and goitrogens compounds in plant foods inhibits the absorption of nutrients in plant-based diets (Suhag, Dhiman, Deswal, Thakur, Sharanagat, Kumar & Kumar, 2021). The rapidly urbanising communities of Africa (Blekking, Giroux, Waldman, Battersby, Tuholske, Robeson & Siame, 2022) lead to fast-paced lifestyles where long food preparation times are burdensome.

Extrusion cooking is a food processing technology which can be applied to produce a variety of convenience-type products from a large diversity of raw materials (Camire, 2001). Extrusion cooking is a continuous manufacturing process which applies high heat, pressure and friction to break down raw foods into a cooked and pre-gelatinised form (Fellows, 2009). It also effectively destroys anti-nutritional compounds and enhances the digestibility of plant proteins

(Gu *et al.*, 2022). Extrusion cooking could, therefore, be applied to produce convenience-type foods from African grains such as sorghum and Bambara groundnuts. Compositing sorghum with Bambara groundnuts could help alleviate protein deficiency in the starch-based diets of children. Extrusion of composites could also hold the benefits of improved protein quality.

The nutritional, physical and functional properties of food-to-food fortified sorghum-Bambara groundnut composites fortified with moringa leaf powder and baobab fruit pulp are investigated in the current study. The aim is to establish the effects of food-to-food fortification of sorghum-Bambara groundnut composite and extrusion moisture on product nutritional, physical and functional properties. This will indicate which FtFF porridge could yield nutritionally beneficial products for specific at-risk groups and how they could be applied as convenience-type nutritious foods.

3.3.2 Materials and methods

3.3.2.1 Materials

Red non-tannin sorghum was procured from Mpumalanga Province, South Africa. The grain was milled using a hammer mill fitted with a 500 µm mesh size screen. The wholegrain flour was stored at 4°C in sealed plastic buckets. Baobab fruit powder was from Nautica Organic Trading (Durban, South Africa). Dried moringa leaf powder was from Supa Nutri, Cape Town, South Africa. Bambara groundnut was procured from Limpopo Province, South Africa.

3.3.2.2 Porridge formulations

The following formulations of sorghum-based flours with fortificants were prepared (figures in brackets represent the ratios of the ingredients):

a. Wholegrain sorghum flour (WSF)+wholegrain Bambara groundnut flour (WBF) (70:30)
b. Wholegrain sorghum flour-Bambara groundnut flour (WSB)+Moringa leaf powder (MLF) (90+10)
c. Wholegrain sorghum flour-Bambara groundnut flour (WSB)+Baobab fruit pulp (90+10)
d. Wholegrain sorghum flour-Bambara groundnut flour+Moringa+Baobab fruit pulp (90+5+5)

3.3.2.3 Conventional Wet Cooking

Deionised water was added to each wholegrain flour along with the flour formulations in a ratio of 3:10, flour: water (w/w). The slurry was heated to boiling temperature (95ºC in Pretoria with an altitude of 1,339 metres above sea level) and maintained with constant stirring for 25 min. The slurry was left to cool at ambient temperature, after which it was placed in plastic containers and frozen to -20ºC and freeze-dried. Freeze-dried porridge flour was crushed to a particle size that passed through a 500 µm opening screen before further analysis. The pre-cooked porridge flour was stored at 4ºC in double-sealed, airtight plastic bags.

3.3.2.4 Extrusion cooking

A co-rotating twin-screw extrusion cooker model TX 32 (CFAM Technologies, Potchefstroom, South Africa) (L/D = 21.5:1) was used to produce instant porridge based on the stated formulations. The barrel comprised of five heating zones towards the die was set at 60/70/80/140/140ºC, respectively. Water was fed into the system at a dosing rate of 3 l/h (to obtain a final barrel moisture content of 20% calculated based on the moisture content of the flours), and the feed rate was 10 kg/h. A die opening of 3 mm was used, and the screw speed was maintained at 250 rpm. The extrudates were dried immediately in a force draught oven at 90ºC for 5 min to a moisture content below 10%. The cooled extrudates were milled using an air-cooled analytical mill to a maximum particle size of 500 µm. The milled extrudates were stored at 4ºC in double-sealed, airtight plastic bags.

3.3.3 Analyses

3.3.3.1 Water absorption capacity and Water solubility index

The procedure described by (Gujral & Pathak, 2002) was used to determine the water absorption and solubility index. Extrudate (2.5 g) was dispersed in 30 mL of distilled water at 30°C for 30 minutes in a shaking water bath, and the mixture was vortexed every 5 min interval. The sample solution was centrifuged at 2490 x *g* for 15 min, and the supernatant was decanted into an aluminiumpan of known weight. Water absorption capacity was recorded as the pellet (g) weight obtained per gram of dry ground sample. The amount of dry solids recovered after evaporating the supernatantin an oven at 100°C overnight was expressed as a percentage of dry solids in the sample and defined as the water solubility index.

3.3.3.2 Nitrogen solubility index

The nitrogen solubility index was determined according to the AACC Method 46-23 (AACC, 2000) with modification. About 1 g flour of each sample was dispersed in 20 mL of 0.1M NaCl solution at pH7 and stirred continuously for 1 hour at 30°C. The suspension was centrifuged (9154.3 x *g*, 15 min, and 4°C), and the supernatant was filtered through a Whatman No. 1 filter paper. The residue was re-washed twice in 10 mL of 0.1M NaCl solution at pH 7. The filtrate was frozen (-18°C) overnightand freeze-dried (13KL, Instruvac Lyophilizer, Midrand, South Africa) for 4 days. The nitrogen content of the freeze-dried sample was determined using a Dumatherm (DT, Gerhardt Konigswinter, Germany). The nitrogen solubility index was expressed as a percentage of the total nitrogen content of the freeze-dried sample divided by the total nitrogen content in the flour sample on a dry basis.

3.3.3.3 Determination of Total Phenolic Content

Total phenolic contents were extracted, and content was determined as described by Apea-Bah *et al.* (2016). Approximately 1 g of each dry sorghum-based porridge sample was extracted in duplicate using 10 ml acidified methanol (1% (v/v) conc HCl in methanol) by magnetic stirring for 2 h. The suspension was centrifuged (Hermle refrigerated centrifuge Z 366 K, New Jersey, USA) at 1650 g for 10 min at 4^{o}C and the supernatant was collected. The residue was similarly re-extracted twice, each with 10 ml acidified methanol for 30 min. The supernatants were then pooled together and stored at -20^{o}C in the dark before analysis. In each well of a 96-well microplate, 18.2 µl volume of the sample extract or catechin standard solution (0-0.5 mg/ml) was reacted with 36.4 µl 10% Folin-Ciocalteu reagent (diluted with distilled water) and 145.4 µl of 700 mM sodium carbonate. The reaction mixture was incubated for 2 h in the dark, after which absorbance was read at 750 nm using a microplate reader (FLUOstar Omega Filter-based multi-mode microplate reader, Ortenberg, Germany). Total phenolic content was calculated with the aid of the catechin standard calibration curve and expressed as milligrams of catechin equivalents per gram (mg CE/g) dry weight basis.

3.3.3.4 Determination of ABTS radical scavenging capacity

ABTS radical scavenging capacity of the sorghum-Bambara-based phenolic extracts was determined using a modification of the method described by (Apea-Bah *et al.*, 2016). The extracts were diluted 10 times with acidified methanol (1% (v/v) conc HCl in methanol). The ABTS radical cation stock solution was prepared by reacting equal volumes of 7 mM ABTS

salt with 2.54 mM potassium persulphate in distilled water for 12-16 hours at room temperature in the dark. A working solution was prepared by diluting the ABTS mother solution with 0.2 M phosphate-buffered saline at pH (7.4) in the ratio of 1:29. In each well of a 96-well microplate, 10 µl of the diluted sample extract extracts or trolox standards (0 - 600 mM prepared in acidified methanol) were mixed with 190 ul of the working solution and incubated in the dark for 30 min at room temperature (20^{o}C). The absorbance was read at 750 nm using a microplate reader (FLUOstar Omega Filter-based multi-mode microplate reader, Ortenberg, Germany). With the aid of a Trolox standard calibration curve, the ABTS radical scavenging capacity was calculated and expressed as micromole Trolox equivalent per gram sample (µmol TE/g) dry weight basis.

3.3.3.5 *In vitro* Protein digestibility

In vitro protein digestibility (IVPD) was determined for the different whole grain, soured flours and gruels by the pepsin digestibility method described by Hamaker *et al.* (1986) modified by Da Silva, Taylor and Taylor (2011). In brief, the method involved incubating approximately 200 mg of the flour for 2 h at 37^{o}C in pepsin suspension (CAS Number 9001-75-6, P7000 Sigma-Aldrich Pepsin from porcine gastric mucosa, activity ≥250 units/mg solid) prepared by dissolving 105 g of the enzyme in 100 ml of pH 2 0.1 M sodium citrate buffer. Protein digestibility is defined as the percentage of nitrogen solubilized under the conditions of the assay relative to flour's total nitrogen. This was measured in terms of insoluble residual total protein content (N x 6.25) determined by Dumas combustion using a Dumatherm (DT N64+, Gerhardt Königswinter, Germany) nitrogen analyser. Protein digestibility was calculated by the difference between the total protein and the residual protein after pepsin digestion expressed as a percentage of the total protein.

3.3.3.6 *In vitro* kinetics of starch digestibility (IVSD)

The method, according to (Goñi *et al.*, 1997), was used with slight modification. A sample (conventionally wet cooked and extruded) containing 50 mg starch was used per assay, and 1 mL of boiling water was added to each sample for easy dispersion before 10 mL of HCl-KCl buffer (pH 1.5) and 0.2 mL of a solution containing 1 mg of pepsin (Sigma Aldrich P7000-100G) were added followed by incubation at 40°C for 60 min with constant agitation. Ten (10 mL) of tris-maleate buffer (pH 6.9) was added, and pH was adjusted with 1M NaOH. The volume was made up to 25 mL with tris-maleate buffer, and the 0-minute aliquot of 0.1 mL

was taken before the addition of 5 mL tris-maleate buffer (pH 6.9) containing 2.6IU of pancreatic α-amylase with an activity of 19.6 units/mg (Sigma-Aldrich A-3176) followed by incubation at 37°C with constant shaking. Aliquots of 0.1 mL were taken at 5 min and then at intervals of 30 min until 3 h. The tubes containing the aliquots taken were placed in boilingwater for 15 min to inactivate α-amylase. Then, 1 mL of 0.4M sodium-acetate buffer (pH 4.75) and 90 µL of amyloglucosidase with an activity of 64.7 U/mg (Megazyme E-AMGDF) was added and incubated at 60°C for 45 min. Glucose concentration was measured using a glucose oxidase-peroxidase kit, and the rate of *in vitro* starch digestion was expressed as the percentage of the total starch digested at time intervals (0, 5, 30, 60, 90, 120 and 180 min).

The first-order equation proposed by Goñi *et al.* (1997) was used to describe the kinetics of starchhydrolysis:

$$C = C_\infty (1 - e^{-kt})$$

Where *C* is the concentration at time *t*, C_∞ is the percentage of starch hydrolyzed after 180 min, *k* is kinetic constant (min^{-1}), and *t* is time (min). The parameters *K* and C∞ were estimated for each treatment based on the data obtained from the *in vitro* hydrolysis procedure. The equation by (Jaisut, Prachayawarakorn, Varanyanond, Tungtrakul & Soponronnarit, 2008) was used to calculate the area under the curve (AUC):

$$AUC = (C_\infty (t_f - t_0) - C_\infty/K) (1 - \exp(-K(t_f - t_0)))$$

Where t_f is the final time (180 min), t_0 is the initial time (time 0). The hydrolysis index (HI) was defined as the area under the hydrolysis curve of the sample divided by the corresponding area of white bread. The estimated glycaemic index was then estimated using the equation according to (Goñi *et al.*, 1997):

$$EGI = 39.71 + 0.549HI$$

Estimated glycaemic load (EGL) was calculated using the following:

$$EGL = \frac{EGI}{100} \times \text{Available Carbohydrate}$$

The enzymatic hydrolysis method of (Goñi *et al.*, 1997) was used to obtain the rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) fractions. The RDS was defined as the percentage of starch digested at 30 min, the SDS as the percentage of starch digestedat 120 min, and the RS was defined as the sum of RDS and SDS subtracted from the total starch.

3.3.3.9 Soluble and insoluble dietary fibre determination

This was estimated according to AOAC 991.43 (1995) method using the total dietary fibre megazyme kit (K-TDFR). Approximately 1 g of the sample was dissolved in 40 mL of mes-tris (pH 8.2) buffer solution, and thermostable α-amylase with an activity of 3,000 U/ml (E-AMGDF) wasadded to hydrolyze starch to dextrins at 100 °C. Protease with an activity of 350 tyrosine U/ml (E-BSPRT) was used to solubilize protein. Amyloglucosidase with an activity of 3,300 U/ml (E- BLAAM) was used to hydrolyze starch fragments to glucose. The sample and enzyme mixture was filtered, and the residue was washed with ethanol and acetone to obtain the insoluble dietaryfibre (IDF) portion. Four volumes of ethanol heated to 60 °C were added to the filtrate to precipitatethe SDF and were left to stand for 1 hour, after which it was filtered. The soluble dietary fibre (SDF) residues were washed with 78%, 95% (v/v) ethanol and acetone. The IDF and SDF residues were dried overnight at 100 °C. The SDF and IDF residues were corrected for protein and ash for the final calculation of SDF and IDF values.

3.3.3.10 Pasting Properties

The pasting properties of all raw and extruded flours were measured using a rheometer equipped with a starch pasting cell with Rheoplus software®, (Anton Paar, Ostfilderm, Germany). Flour and distilled water suspensions were prepared at 10% solids. During the pasting procedure, suspensions were equilibrated at 50 ˚C for 5 minutes, after which heating to 90 ˚C at a uniform rate of 5°C per min with constant stirring at 160 rpm was applied. The heated slurry was held at 91°C for 5 minutes, then cooled to 50°C at 5°C per min. It was then held at 50°C for 5 minutes.

3.3.4 Statistical Analysis

Each experiment was performed thrice, and multiple analysis of variance was used to determine the differences between treatments. Fisher's LSD test at a 0.05 level of significance was applied. Statistica 10 (StatSoft Inc., Tulsa, OK, USA) was used. Fisher's LSD posthoc test was also applied for pair-wise comparison between the control formulation and the treatments.

3.3.4 Results and Discussion

3.3.4.1 Nutritional properties

Wholegrain Bambara groundnut porridge was significantly higher in protein (61% higher), fat (21% higher) and ash (minerals) (225% higher) than wholegrain sorghum (Table 3-8). Wholegrain sorghum had significantly higher starch (45% higher), insoluble dietary fibre (IDF) (30% higher) and total dietary fibre (22% higher). The composition of sorghum is in the ranges reported by Bean *et al.* (2019), while the Bambara groundnut compositions are in the range of what was reported by Anhwange & Atoo (2015).

Sorghum, like most cereals, is dense in starch but limited in the essential amino acid lysine. Bambara groundnut, like all legumes, is rich in protein and amino acid-rich but limited in sulphur-containing amino acids (Temba *et al.*, 2016). Complementing sorghum with Bambara groundnut improves the protein quality of the subsequent food products. Extrusion, due to its high temperature, pressure and shear, plays a great role in reducing the antinutrients that are rich in grains (Duodu, 2014).

As expected, compositing sorghum with Bambara groundnut significantly lowered the starch content of the composite. Whether alone or in combination, fortification with moringa and baobab also resulted in a significant reduction in the starch content (8-21%), probably because of the dilution effect of the moringa or baobab replacing the composite flours. Bambara groundnuts had higher protein content than sorghum which resulted in a substantial ($p \leq 0.05$) overall increase in protein (12%) content when compared with wholegrain sorghum (Table 3-8). Fortification with moringa leaf powder, on the other hand, resulted in a significant increase in the protein content of the composite, probably due to the high protein content of moringa (29.2%). As expected, extrusion cooking did not cause a significant difference ($p \leq 0.05$) in protein contents. Proper Adequate nutrition within the first 1000 days of life (0-23 months) is critical for optimal physical and cognitive development, with malnutrition leading to impaired growth and mortality (Akombi, Agho, Merom, Renzaho & Hall, 2017). The WHO, FAO, and UNU (2007) suggested that the average protein requirement for children aged 2 years and between 3-5 years be 0.79 and 0.70 g protein/kg body weight per day, respectively. A serving size of 100 g dry base composite porridges could contribute approximately 50-100% of this protein requirement.

Sorghum IDF values were in close range with those reported by Bader Ul Ain, Saeed, Khan, Niaz, Khan, Anjum, Tufail and Hussain (2019), while Bambara groundnut values were far

below those reported by Tanya, Mbofung and Keshinro (1997) (Table 3-8). Regarding SDF, Tanya *et al.* (1997) reported higher levels of SDF in Bambara groundnut than those found in this study (3.5 g/100 g), while sorghum had SDF values close to those reported by Bader Ul Ain *et al.* (2019). As expected, fortification with either moringa or baobab significantly increased both the IDF and SDF of the composite porridges (by 22-58% and 81-183%, respectively). This was because the IDF and SDF content of baobab (12.95 g/100 g and 42 g/100 g, respectively) and moringa (38.17 g/100 g and 4.32g/100 g) were significantly higher than that of composite porridge. Concerning extrusion cooking, there was a significant decrease in the IDF (overall by 16%) content coupled with a corresponding significant increase in the SDF (overall by 46%). This can be attributed to the formation of smaller and more soluble molecules due to hydrolysis of the glycosidic linkages and disruption of noncovalent bonds in polysaccharides by extrusion cooking (Oladiran & Emmambux, 2018, 2017; Spotti & Campanella, 2017; Rashid *et al.*, 2015).

Polyphenolic compounds are considered antinutrients due to their ability to form complexes with dietary proteins, enzymes and minerals (Duodu, 2011). In this study, total phenolic content (TPC as mg catechin equivalents/g) and antioxidant properties (radical scavenging activity against ABTS radical, micromolar Trolox equivalents/g) for individual porridges and composites were determined. Sorghum had 1.3 times more phenolics than Bambara groundnut, and its antioxidant properties measured as ABTS was lower than that of Bambara groundnut (Table 3-8). Phenolic compounds possess antioxidant properties (Duodu, 2011; Awika & Rooney, 2004; Rice-Evans, Miller & Paganga, 1997), and as such, the antioxidant properties of sorghum and Bambara groundnut are related to the TPC. Compositing Bambara groundnut with sorghum resulted in a 26% reduction in the TPC with a corresponding 8% reduction in ABTS due to the lower phenolic content of Bambara groundnut. Fortification with baobab and moringa, whether alone or in combination, resulted in a significant increase in the TPC (by 69%, 92% and 117%, respectively) and the ABTS (by 56%, 78% and 94%). The high temperature coupled with shear and pressure used in extrusion cooking could lead to decarboxylation of phenolic compounds, and high moisture could cause polymerization of phenolic compounds, both of which would reduce the extractability of phenolic compounds and reduce antioxidant activity (Sharma *et al.*, 2012; Brennan *et al.*, 2011). Furthermore, phenolic compounds such as flavonoids can bind to proteins which would reduce their extractability (Arts *et al.*, 2002) and, in effect, reduce the TPC and antioxidant activity.

A projection of principal components on a principal component bi-plot projecting the dependent variables and treatments on a two-dimensional factor plane (Figure 3-15a,b) showed that samples FtF-fortified with moringa and baobab whether alone or in combination, were correlated with TPC, ABTS, IDF, SDF, TDF and crude ash compared to the unfortified controls whether they were extrusion-cooked or conventionally wet cooked. This confirmed the findings discussed in Table 3-8, indicating the augmenting effect of FtFF on these properties of wholegrain sorghum-Bambara groundnut-based porridges. The biplot revealed that FtFF samples were negatively correlated with starch content which reinforced the findings presented in Table 3-8 that alluded to a lower starch content in FtF-fortified porridges as a result of the diluting effect of the fortificant on the starch content. The plot also revealed a greater correlation between conventionally wet cooked porridges with TPC, ABTS, IDF, and total starch, compared to their extruded counterparts which were correlated with SDF and TDF, confirming findings presented in Table 3-8. This illustrated the effect of extrusion on dietary fibre and starch and the potential for modifying properties of wholegrain sorghum-based porridges essential for managing several chronic NCDs such as type 2 diabetes.

Table 3-8: Effects of compositing, extrusion cooking and food-to-food fortification (with baobab fruit pulp and moringa leaf powder) on the proximate composition of wholegrain sorghum-Bambara groundnut composite

Treatment	Formulation	Total Starch (%)	Crude Protein (%)	Crude fat (%)	Crude Ash (%)	IDF (%)	SDF (%)	TDF (%)	TPC (mg/g)	ABTS (mmolTE/g)	Moisture Content
Cooked Samples	Wholegrain Sorghum-Bambara	60.01^{gh}±2.46	13.79^{b}±0.01	2.31^{bc}±0.11	2.62^{b}±0.04	9.46^{bc}±1.61	1.99^{ab}±0.49	11.45^{bc}±1.05	2.99^{b}±0.25	30.13^{d}±0.11	5.27^{d}±0.08
	Wholegrain Sorghum-Bambara+Baobab	55.91^{f}±2.15	13.13^{b}±0.18	2.48^{bc}±0.20	2.62^{bcd}±0.04	12.93^{e}±0.01	5.01^{d}±0.02	17.94^{d}±0.03	5.48^{f}±0.33	49.53±0.97	3.60^{c}±0.18
	Wholegrain Sorghum-Bambara+Moringa	52.08^{ef}±2.23	15.06^{d}±0.20	3.16^{c}±1.55	2.90^{cd}±0.06	14.03^{f}±0.52	3.61^{c}±1.87	17.64^{d}±1.20	6.14^{g}±0.45	54.11±4.19	2.32^{b}±0.24
	Wholegrain Sorghum-Bambara+Baobab+Moringa	52.91^{ef}±3.32	14.12^{c}±0.38	1.54^{ab}±0.37	2.85^{cd}±0.48	14.90^{f}±0.64	5.63^{de}±0.48	20.53^{e}±1.12	7.11^{h}±0.16	61.58±1.26	1.75^{a}±0.15
Extruded samples	Wholegrain Sorghum-Bambara	60.16^{g}±2.51	13.64^{b}±0.19	1.63^{ab}±0.72	2.32^{b}±0.04	8.87^{b}±1.31	3.16^{c}±0.02	12.03^{c}±1.51	2.38^{a}±0.09	14.85^{a}±1.11	8.23^{g}±0.05
	Wholegrain Sorghum-Bambara+Baobab	55.79^{f}±1.89	14.15^{bc}±0.94	1.25^{a}±0.49	2.53^{bc}±0.15	10.57^{c}±0.02	7.01^{f}±0.73	17.58^{d}±0.75	3.63^{c}±0.18	20.78^{b}±1.89	7.76f±0.16
	Wholegrain Sorghum-Bambara+Moringa	52.63^{ef}±3.44	15.66^{d}±.04	1.74^{ab}±0.82	2.95^{d}±0.17	11.51^{d}±0.33	5.78^{e}±0.03	17.29^{d}±0.36	4.18^{d}±0.16	25.95^{c}±2.46	7.14^{e}±0.04
	Wholegrain Sorghum-Bambara+Baobab+Moringa	42.60^{cde}±7.93	14.49^{cd}±0.16	1.92^{ab}±0.70	2.76^{cd}±0.24	12.24^{e}±0.41	7.74^{f}±0.01	19.98^{e}±0.42	4.57^{e}±0.31	25.62^{c}±3.55	7.07^{e}±0.10

[1]Values are the means±SD of at least two samples of each formulation analyzed independently in triplicate (n=6). Values within the same column followed by different letters are significantly different ($p<0.05$).
[2]For each dependent variable (phytate, TPC), the means of each treatment (conventionally cooked, extruded) with different superscript lowercase letters in a row differ significantly ($p<0.05$) by pairwise comparison
[3]Means of each treatment (conventionally cooked, extruded) with different superscript uppercase letters in a column differ significantly ($p<0.05$)

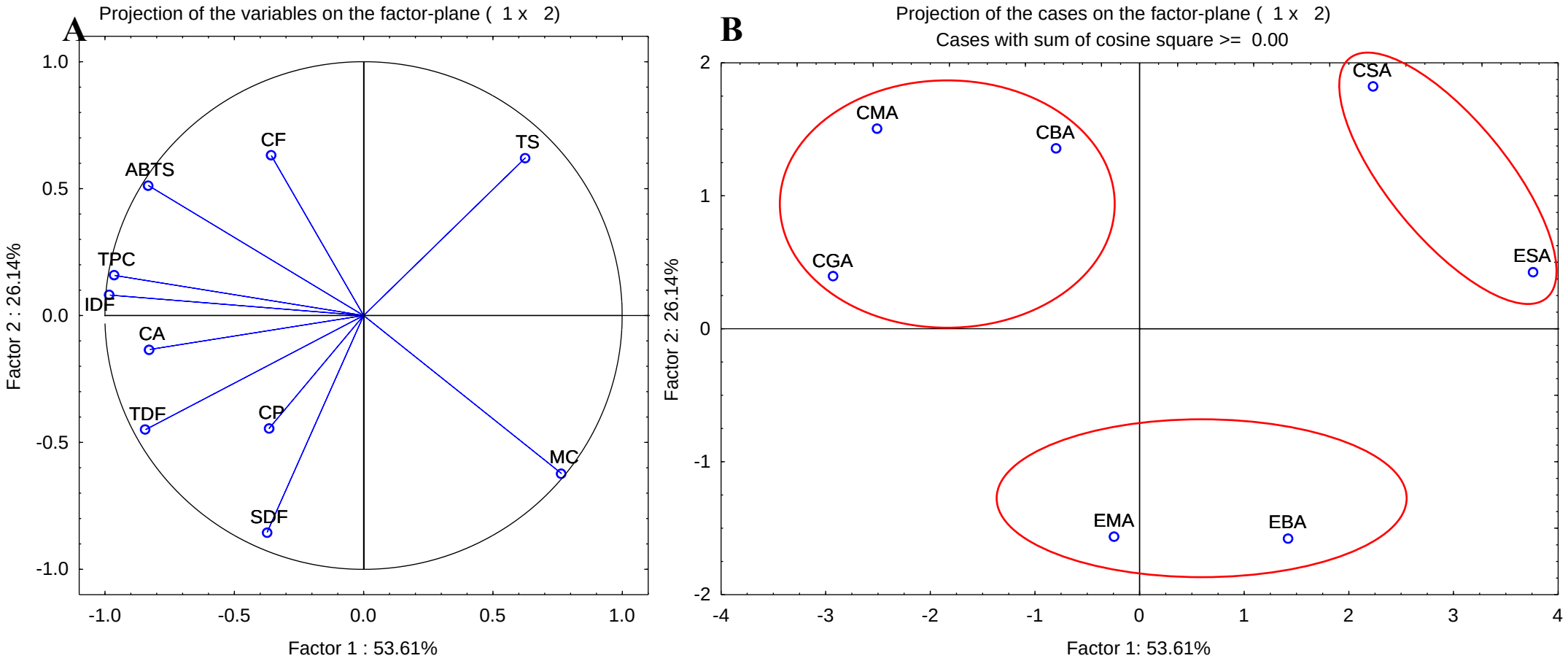


Figure 3-15: Principal component analysis showing 1×2 factor coordinate plots of dependent variables (total phenolic content-TPC, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)-ABTS radical scavenging activity, crude protein-CP, crude fat-CF, crude ash-CA, total starch-TS, insoluble dietary fibre-IDF, soluble dietary fibre-SDF, total dietary fibre-TDF, moisture content-MC), A, and independent variables (fortification and processing technique-wet-cooking and extrusion), B.

Key: CSA-Cooked Sorghum-Bambara, CBA- Cooked Sorghum-Bambara+Baobab co-cooked, CMA- Cooked Sorghum-Bambara+Moringa, CGA- Cooked Sorghum-Bambara+Moringa+Baobab co-cooked, ESA- Extruded Sorghum-Bamabara, EBA- Extruded Sorghum-Bambara+Baobab co-cooked, EMA- Extruded Sorghum-Bambara+Moringa, EGA- Extruded Sorghum-Bambara+Moringa+Baobab co-cooked

3.3.4.3 *In vitro* Starch kinetics and protein digestibility

The effects of fortification on starch digestibility before and after extrusion cooking on sorghum-Bambara groundnut composite porridges are shown in Figure 3-16 and the derived parameters, C_∞, HI, K, RDS, SDS, RS, EGI and EGL, in Table 3-9. The *in vitro* starch digestibility parameters, C_∞, HI, K, and RDS with lower SDS (Table 3-9), were generally higher within extruded porridges than in the conventionally wet cooked porridges. This is more likely due to the dextrinization and depolymerization of starch, which occurred during the extrusion, making starch more readily accessible for enzymatic hydrolysis (Alonso *et al.*, 2000). Extrusion-cooked porridges also had significantly higher RS than conventionally wet-cooked porridges. The slightly higher content of RS in extruded porridges could be attributed to the retrogradation of starch during the storage of the porridges during storage (resistant starch type III) (Alsaffar, 2011; Chanvrier, Uthayakumaran, Appelqvist, Gidley, Gilbert & López-Rubio, 2007). Another possible cause could be that the increased solubilization of fibre (section 3.3.4.1) could result in the entrapment of the starch molecules in the SDF matrix rendering it inaccessible for enzymatic degradation (resistant starch I) (Brennan, Blake, Ellis and Schofield, 1996; Oladiran and Emmambux, 2017; Oladiran and Emmambux, 2018). Another possible explanation could be that the process of extrusion resulted in the formation of amylo-lipid complexes (resistant starch V) that are resistant to enzymatic breakdown (Panyoo & Emmambux, 2017) owing to the relatively high-fat content of Bambara groundnut.

The total starch digested for the sorghum-Bambara groundnut composite was significantly ($p \leq 0.05$) lower than that of sorghum flour for both extruded and conventionally cooked porridge. This was expected since Bambara groundnut showed lower digested total starch when compared to sorghum. The compositing of sorghum with Bambara groundnut significantly ($p \leq 0.05$) lowered the C_∞, hydrolysis index (HI), K, estimated glycaemic index (EGI) and estimated glycaemic load (EGL) of the composite porridge (by 8%, 15%, 26%, 6% and 15% respectively). The reduction in these starch digestion parameters could be due to the interaction between proteins in Bambara groundnut with the starch in sorghum. Proteins can enfold themselves around starch and act as a barrier to the enzymatic hydrolysis of starch (Yang, Zhong, Goff & Li, 2019). These findings were supported by starch digestion fractions data that showed a decrease in rapidly digestible starch (RDS) (by 19%) and an increase in resistant starch (RS) (by 9%).

Fortification with baobab and moringa alone and in combination significantly ($p<0.05$) decreased the IVSD whether alone or in combination (Figure 3-16) in both conventionally wet cooked and extruded porridges as shown by a lower C_∞, HI, K, EGI and EGL (Table 3-9) whether conventionally wet cooked or extruded with meals fortified with a combination of baobab and moringa showing the lowest of these parameters. These effects following fortification with baobab and moringa, either alone or in combination, could be attributed to the high phenolics and fibre content of both as well as the high phytate content in moringa (Section 3.1). While baobab had the highest phenolic and total dietary fibre (the majority of it being soluble) contents, moringa had the highest phytate content (Section 3.1). In studying the glycaemic index and α-amylase activity of different rice cultivars, Kumar, Sahu, Panda, Biswal, Sah, Lal, Baig, Swain, Behera and Chattopadhyay (2020) reported decreased glycaemic index and α-amylase activity in varieties with high phytic acid content. Thompson and Yoon (1984) reported reduced wheat starch hydrolysis when phytic acid was incorporated. The mechanism of impediment of starch digestion could be directly by binding starch in the cell wall matrix or binding starch hydrolysing enzymes (Selle, Cowieson, Cowieson & Ravindran, 2012). Deshpande and Salunkhe (1982) reported that phenolic compounds like tannic acid and catechin interacted with the starch of legumes and potatoes as well as amylose and amylopectin, resulting in a reduction in the *in vitro* starch digestibility. Thompson and Yoon (1984) reported similar effects of tannic acid and catechin on wheat starch hydrolysis. Coe *et al.* (2013) confirmed these findings when *in vivo*, incorporating baobab in white bread reduced the glycaemic response and rapidly digestible starch. Brennan, Merts, Monro, Woolnough and Brennan (2008) reported a reduction of starch digestibility when gaur gum and wheat brain were incorporated in wheat bread, results that were consistent with Jenkins, Wolever, Thorne, Jenkins, Wong, Josse and Csima (1984)'s findings. Yağcı and Göğüş (2009) also reported a reduction in starch digestibility when various food by-products were added to rice grits and durum flour. Brennan *et al.* (2008) suggested that fibre may directly hinder digestion by limiting contact between starch and digestive enzymes.

Similarly, Brennan, Blake, Ellis and Schofield (1996), Oladiran and Emmambux (2017), and Oladiran and Emmambux (2018) suggested that gelatinized and disrupted starch granules could be within soluble fibre matrix, and this would reduce starch digestibility due to limited access by starch degrading enzymes to the substrate. Other mechanisms of reduction of starch hydrolysis have been suggested to be starch-phenolic complexes formed through hydrogen and hydrophobic interactions that make the starch inaccessible to the enzymes (Sun & Miao, 2020;

Amoako & Awika, 2016; Zhu, 2015). Other mechanisms have been the interaction of phenolic compounds with starch hydrolysis enzyme at the active site inhibition (Lv, Zhang, Li, He, Hao & Dai, 2019; Sun, Gidley & Warren, 2018; Liu, Hu, Zhang, Zhang, Wang, Qian & Qi, 2017)

The results of *in vitro* protein digestibility (IVPD) are presented in Table 3-9. Compositing sorghum with Bambara groundnut significantly increased the IVPD (by 44%). These findings are consistent with Patil, Rudra, Varghese and Kaur (2016), who reported that the incorporation of legumes in wheat showed a significant increase in IVPD. The high IVPD of Bambara groundnuts is probably due to its more soluble globulin-type proteins (Yagoub & Abdalla, 2007) as opposed to the less soluble kafirins predominant in sorghum (Duodu *et al.*, 2003). In conventionally wet-cooked composite porridges, fortification with either moringa or baobab had no significant effect on the IVPD. However, on extrusion, fortification with baobab, whether alone or in combination with moringa, increased the IVPD (by 12-16%). Fortification with moringa alone still did not affect the IVPD when extruded. Extrusion cooking led to an overall 34% higher IVPD and amount of digestible protein when compared to conventionally wet-cooked composite porridges. The reduction of phenolic content and phytate content that are known to bind proteins could also explain the increase in IVPD in extruded porridge, as extruded porridges showed reduction in phenolic and phytate content. Previous research has indicated that the presence of antinutritional compounds can decrease the protein digestibility (Park, Kim & Baik, 2010). Patil *et al.* (2016) observed a double increment in IVPD when wheat-based flour was extruded. Moreover, the heat and shear of extrusion cooking are thought to disrupt the protein bodies of sorghum, exposing the α-kafirins to proteolytic attack (Hamaker *et al.*, 1994). The α-kaffirins are thought to be more digestible than the β- and γ- kaffirins and is possibly related to the higher digestibility of α-kaffirins, especially after extrusion cooking.

For greater visibility of the effects of the dependent variables on the independent variables, a projection of dependent variables and treatments on a principal component bi-plot was prepared (Figure 3-17a,b). The plot revealed that samples FtF-fortified with moringa and baobab, whether alone or in combination, were correlated with reduced IVSD indices (RS and SDS). In contrast, the unfortified controls were correlated with higher starch digestibility indices (C_∞, HI, K, EGI and EGL) further affirming the findings presented in Table 3-9, thus showing a negative effect of FtFF on the IVSD. On close inspection of the biplot, it was observed that extrusion cooked porridges were correlated with IVPD and the indices of high starch digestibility. In contrast, conventionally wet-cooked samples were correlated with indices of reduced IVSD. An agreement with the role of extrusion in improving the IVPD and

IVSD in Table 3-9. Notably, while generally, extruded porridges correlated with indices of high IVSD, the FtFF porridges showed lesser correlation than the unfortified controls. These porridges could be essential in managing type 2 diabetes.

Table 3-9: Effects of compositing, extrusion cooking and food-to-food fortification (with baobab fruit pulp and moringa leaf powder) on the starch kinetics, starch fractions, *in vitro* protein and starch digestibilities of wholegrain sorghum-Bambara groundnut composite

Formulations	C∞ (%)		HI (%)		K (min^{-1})		RDS (%)		SDS (%)	
	Conventionally cooked	Extrusion cooked	Conventionally cooked	Extrusion cooked	Conventionally cooked	Extrusion cooked	Conventionally cooked	Extrusion cooked	Conventionally cooked	Extrusion cooked
Wholegrain Sorghum-Bambara	50.54±0.49$^{f\,A}$	61.19±1.50^{hB}	45.35±0.49^{fA}	49.24±0.16^{gB}	0.13±0.01^{cA}	0.29±0.01^{eB}	38.89±1.25^{gA}	43.16±0.50^{iB}	7.21±1.60^{cB}	1.37±0.87^{aA}
Wholegrain Sorghum-Bambara+Baobab	40.07±0.80^{cA}	49.04±1.05^{fB}	31.18±0.87^{cA}	38.10±1.22^{eB}	0.01±0.00^{aA}	0.02±0.00^{bB}	9.45±1.34^{abA}	22.19±1.05^{deB}	29.77±2.27^{gB}	13.24±1.85^{dA}
Wholegrain Sorghum-Bambara+Moringa	39.23±0.57^{cA}	44.52±0.86^{eB}	30.13±0.53^{cA}	35.54±0.25^{dB}	0.01±0.00^{aA}	0.02±0.00^{bB}	11.79±2.59^{bA}	20.84±1.01^{dB}	26.94±2.16^{fgB}	15.27±0.14^{dA}
Wholegrain Sorghum-Bambara+Baobab+Moringa	32.89±1.40^{bA}	40.09±0.70^{cB}	22.68±0.45^{aA}	30.47±0.12^{cB}	0.01±0.00^{aA}	0.02±0.00^{bB}	7.93±0.28^{aA}	15.19±1.41^{cB}	24.17±0.56^{fB}	14.44±1.41^{dA}

Formulations	RS (%)		EGI (%)		EGL (%)		IVPD (%)-Amount of digestible protein per 100 g sample	
Wholegrain Sorghum-Bambara	53.90±0.34^{bA}	55.48±0.38^{cB}	64.61±0.27^{eA}	66.74±0.10^{fB}	39.28±0.08^{ghA}	42.90±2.89^{hA}	44.59±1.08^{bA}	57.58±1.22^{dB}
Wholegrain Sorghum-Bambara+Baobab	60.78±0.94^{dA}	64.57±0.79^{eB}	56.83±0.48^{bA}	60.63±0.67^{dB}	33.34±0.08^{eA}	33.92±0.17^{eA}	45.96±2.36^{bcA}	66.02±3.38^{eB}
Wholegrain Sorghum-Bambara+Moringa	61.27±0.43^{dA}	63.88±0.14^{eB}	56.25±0.29^{bA}	59.22±0.14^{cB}	31.06±0.11^{deA}	33.42±0.17^{eA}	49.08±4.39^{cA}	55.61±2.90^{dB}
Wholegrain Sorghum-Bambara+Baobab+Moringa	67.90±0.28^{gA}	70.62±0.35^{hB}	52.16±0.23^{aA}	56.44±0.10^{bB}	29.53±0.15^{cA}	28.34±0.92^{bcA}	45.01±1.39^{bA}	64.57±1.03^{eB}

[1]Values are means ± standard deviations of 3 independent experiments.
[2]Values within the same column followed by different letters are significantly different ($p<0.01$)
[3]For each dependent variable (C∞, HI, K, RDS, SDS, RS, EGI, EGL, IVDP), the means of each treatment (conventionally cooked, extruded) with different superscript lowercase letters in a row differ significantly ($p<0.05$) by pairwise comparison
[4]Means of each treatment (conventionally cooked, extruded) with different superscript uppercase letters in a column for each dependent variable differ significantly ($p<0.05$)
[5]C∞ = % starch digested after 180 min, RDS- rapidly digestible starch, SDS- slowly digestible starch, RS- resistant starch, HI- hydrolysis index, EGI-estimated glycaemic index, EGL- estimated glycaemic load, IVPD- *in vitro* protein digestibility
[6]HI, k and GI were calculated from the equation: $AUC = (C\infty\,(tf - t0) - C\infty/K)\,(1 - \exp(-K(tf - t0))$ EGL per g solids was estimated as EGI*S/100 where S is starch content (g/100g solids) White bread was used as the reference for calculating EGI

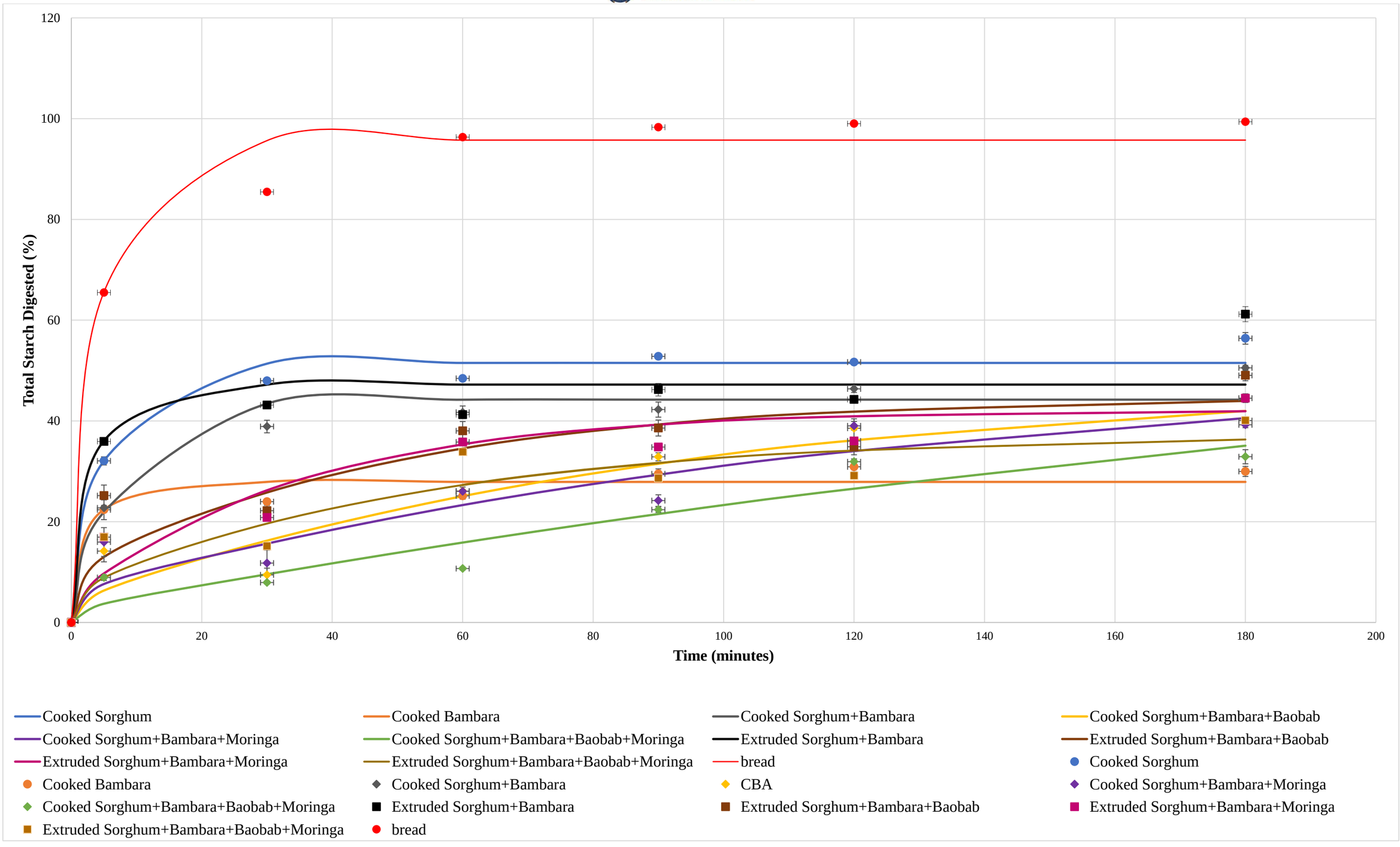


Figure 3-16: Effects of compositing, extrusion cooking and food-to-food fortification on the kinetics of starch digestion of wholegrain sorghum-Bambara composite

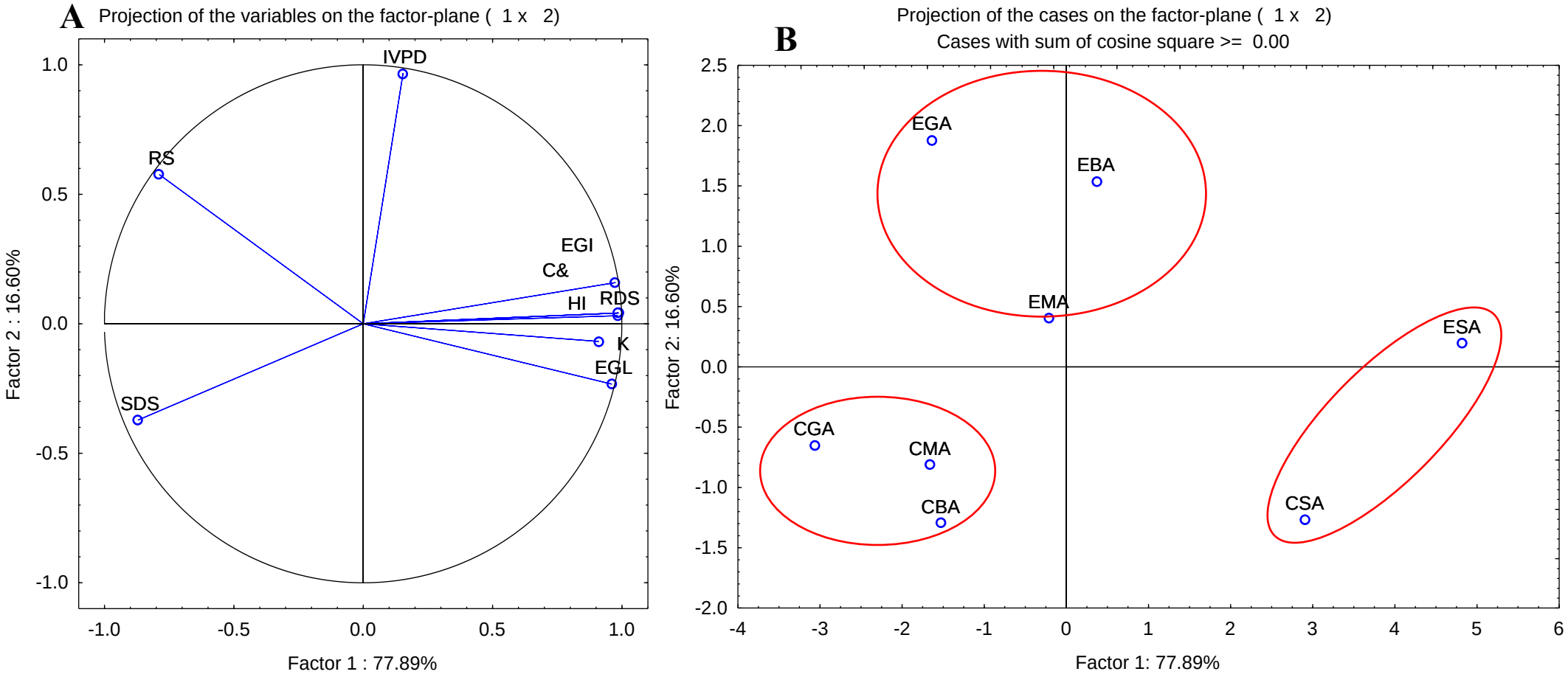


Figure 3-17: Principal component analysis showing 1×2 factor coordinate plots of dependent variables (% starch digested after 180 min- C&, rapidly digestible starch- RDS, slowly digestible starch-SDS, resistant starch-RS, hydrolysis index-HI, estimated glycaemic index-EGI, estimated glycaemic load-EGL, in vitro protein digestibility-IVPD), A, and independent variables (fortification and processing technique-wet-cooking and extrusion), B.

Key: CSA-Cooked Sorghum-Bambara, CBA- Cooked Sorghum-Bambara+Baobab co-cooked, CMA- Cooked Sorghum-Bambara+Moringa, CGA- Cooked Sorghum-Bambara+Moringa+Baobab co-cooked, ESA- Extruded Sorghum-Bamabara, EBA- Extruded Sorghum-Bambara+Baobab co-cooked, EMA- Extruded Sorghum-Bambara+Moringa, EGA- Extruded Sorghum-Bambara+Moringa+Baobab co-cooked

3.3.4.4 Pasting and functional properties

Pasting viscosities of wholegrain sorghum-based composite porridges are shown in Figure 3-18, Figure 3-19 and Table 3-10. All porridges indicated shear-thinning behaviour indicating their ability to be used as weaning foods. Foods that are thick and pasty are difficult for infants to process orally, and thus their nutrient intake is limited with several weaning foods available in Africa, resulting in PEM (Makame, De Kock & Emmambux, 2020). Overall, including Bambara groundnut in sorghum caused a significant decrease ($p \leq 0.05$) in maximum viscosity, trough viscosity and final viscosity for both conventional and extrusion-cooked porridge. The pasting viscosity reduction corresponds with previous studies when Bambara groundnuts were added to sorghum (Muller, 2017). This was due to the decrease in the starch content of the sorghum when Bambara groundnut was included, and starch is the main food biopolymer that contributes to pasting viscosity (Sarker, Elgadir, Ferdosh, Akanda, Aditiawati & Noda, 2013).

Fortification with either moringa or baobab, alone or in combination, significantly reduced the maximum viscosity and trough viscosity during heating for conventional and extrusion-cooked porridges. This reduction in maximum and trough viscosity could be a function of the high content of insoluble dietary fibre in the moringa and baobab and, thus, a lower starch content. The apparent viscosity of non-starch polysaccharides depends on ionically charged groups, molecular weight, the concentration of dietary fibre, surrounding structures (Caprita & Caprita, 2011) and pH (Guillon & Champ, 2000).

Notably, the final viscosity of conventionally cooked porridge for all fortified flours with moringa and baobab was significantly higher than that of composite flour alone. The final pasting viscosity after cooling to 50 ºC is essentially a three-dimensional network of intertwined amylose molecules incorporating dispersed swollen and ruptured starch granules (Langton & Hermansson, 1989). In the current system (Table 3-10 and Figure 3-18, Figure 3-19), the final viscosity is a combination of cooked starch from sorghum and Bambara groundnut, denatured protein from sorghum and Bambara groundnut, and the soluble/insoluble dietary fibres from moringa and Baobab containing some minerals and vitamins depending on the treatment. The higher final viscosity of conventionally cooked samples with moringa and baobab (both high in fibre) could suggest that the three-dimensional network is strengthened by the presence of the soluble dietary fibre in moringa and baobab.

Extrusion cooking also caused a decrease in maximum, trough, and final paste viscosities. This was previously found in the extrusion cooking of sorghum (Mahasukhonthachat *et al.*, 2010).

The decrease in pasting viscosity during extrusion was due to starch dextrinization and depolymerisation (Lai & Kokini, 1991) and depolymerization of fibre (Brennan *et al.*, 2008). The depolymerized polysaccharides may lower final viscosity during cooling because of lower molecular weight/short-chain polymers contributing to lower hydrodynamic volumes. The ability to increase the viscosity of solutions depends on the hydrodynamic volume of the polysaccharide. A large hydrodynamic volume results in increased viscosity at low concentrations (Lovegrove, Edwards, De Noni, Patel, El, Grassby, Zielke, Ulmius, Nilsson & Butterworth, 2017). Relatively lower chain length molecules, due to depolymerization, have lower hydrodynamic volume.

Fortification with either moringa or baobab did not significantly affect the NSI; however, extrusion cooking significantly decreased the NSI (by 78%) Table 3-10. Hot, moist conditions in extrusion cooking probably caused exposure of hydrophobic protein cores (Camire, 1991) and polymerization (via disulphide bonds) after unfolding of the protein matrix during protein denaturation (Chen, Wei & Zhang, 2011), which could reduce protein solubility.

Fortification with baobab caused no significant changes in the WAI or WSI of conventionally wet cooked sorghum-Bambara groundnut porridge but significantly decreased the WAI (by 30%) and increased the WSI of extruded sorghum porridge and (by 52%) (Table 3-10). Fortification with moringa caused a significant reduction in the WAI of the composite porridge that was conventionally wet cooked (by 39%) but did not affect the WSI. The reduction could be attributed to the high content of insoluble dietary fibre in moringa.

The significantly lower ($p \leq 0.05$) WAI and higher WSI following fortification with baobab could be explained by the dextrinization/depolymerisation of starch polymers due to the severe cooking conditions at low extrusion moisture caused by high friction, pressure and heat (Pham & Del Rosario, 1984). Dextrinised starch is more soluble than conventionally cooked starch (Altan, McCarthy & Maskan, 2008).

Extruded composite porridges fortified with moringa had a significantly higher WAI when compared to the conventionally wet-cooked porridge compared to other extruded composites with baobab. This can be attributed to an increase in the partially depolymerised dietary fibre component of moringa. An increase in WAI can be due to modification of the insoluble dietary fibre. Extrusion cooking under high mechanical energy can also defibrillate and reduce the size of dietary fibre, exposing more hydroxyl groups to bind water (Menegassi, Pilosof & Arêas, 2011).

A presentation of the results from Table 3-10 in a projection of dependent variables and treatments on a principal component bi-plot (Figure 3-20a,b) revealed that samples extruded porridges FtF-fortified with moringa and conventionally wet cooked porridges FtF-fortified with baobab were correlated with WAI, NSI, initial viscosity, and trough viscosity. The plot also revealed that extruded porridges FtF-fortified with baobab alone or with moringa and conventionally wet-cooked porridges fortified with moringa alone and with baobab correlated with WSI, NSI, maximum viscosity, and final viscosity which confirmed the findings presented in Table 3-10. This demonstrates the effect of FtFF on increasing WSI, NSI, maximum viscosity, trough viscosity, and final viscosity and, thus, potential application in producing porridges with prolonged gastric emptying. Concerning the cooking method, the biplot showed that extrusion cooked porridges correlated with WAI, WSI and initial viscosity. In contrast, conventionally wet-cooked porridges correlated with NSI, maximum viscosity, trough viscosity and final viscosity, further confirming the findings discussed in Table 3-10. The PCA lays bare the negative effect of extrusion cooking on viscosity, WSI and WAI and its potential application in delivering nutrient-dense yet less viscous foods.

Table 3-10: Effects of compositing, extrusion cooking and food-to-food fortification (with baobab fruit pulp and moringa leaf powder) on some functional and pasting properties of wholegrain sorghum-Bambara groundnut composite

Formulations	Initial Viscosity (mPa·s)		Maximum Viscosity (mPa·s)		Trough Viscosity (mPa·s)		Final Viscosity		WAI (g/100g)	
	Conventionally cooked	Extrusion cooked	Conventionally cooked	Extrusion cooked	Conventionally cooked	Extrusion cooked	Conventionally cooked	Extrusion cooked	Conventionally cooked	Extrusion cooked
Wholegrain Sorghum-Bambara	8.25±0.22^{aA}	105.37±4.53^{eB}	1203.00±5.00^{hB}	167.00±1.00^{dA}	776.20±7.20^{hB}	96.23±1.20^{dA}	588.73±6.14^{dB}	96.32±2.33^{cA}	4.21±0.53^{dA}	4.18±0.44^{dA}
Wholegrain Sorghum-Bambara+Baobab	12.32±4.26^{aA}	93.43±4.66^{dB}	1133.00±63.00^{gB}	85.00±1.00^{bA}	711.80±20.44^{gB}	39.69±0.30^{bA}	1133.33±63.52^{fB}	63.58±0.55^{bA}	3.48±0.92^{cdA}	2.94±0.08^{bcA}
Wholegrain Sorghum-Bambara+Moringa	10.45±1.12^{aA}	51.00±3.69^{bB}	1168.00±13.00^{ghB}	112.00±3.00^{cA}	603.97±19.35^{fB}	60.75±2.82^{cA}	959.17±8.81^{eB}	93.32±2.33^{cA}	2.56±0.33^{abA}	3.94±0.37^{cB}
Wholegrain Sorghum-Bambara+Baobab+Moringa	9.26±0.40^{aA}	84.86±3.54^{cB}	1058.00±39.00^{fB}	63.00±2.00^{aA}	560.73±20.43^{eB}	33.39±0.35^{aA}	560.73±20.43^{dB}	51.23±0.58^{aA}	2.52±0.09^{aA}	2.79±0.05^{abA}

Formulations	WSI (g/100g)		NSI (%)	
Wholegrain Sorghum-Bambara	9.15±1.19^{bcA}	16.01±0.76^{fB}	15.77±1.34^{deB}	3.46±0.0.25^{abA}
Wholegrain Sorghum-Bambara+Baobab	10.33±0.57^{cA}	24.35±1.61^{hB}	17.41±1.6^{eB}	3.37±0.24^{abA}
Wholegrain Sorghum-Bambara+Moringa	10.78±0.72^{cdA}	14.05±0.34^{eB}	12.42±2.15^{cdB}	2.91±0.50^{aA}
Wholegrain Sorghum-Bambara+Baobab+Moringa	11.84±0.45^{dA}	23.59±1.03^{gB}	14.80±0.73^{dB}	2.88±0.15^{aA}

[1]Values are means ± standard deviations of 3 independent experiments.
[2]Values within the same column followed by different letters are significantly different ($p<0.01$)
[3]For each dependent variable (Initial, peak and trough viscosities, WAI-Water absorption index, WSI-Water solubility index, NSI, Nitrogen solubility index), means of each treatment (conventionally cooked, extruded) with different superscript lowercase letters in a row differ significantly ($p<0.05$) by pairwise comparison
[4]Means of each treatment (conventionally cooked, extruded) with different superscript uppercase letters in a column for each dependent variable differ significantly ($p<0.05$)

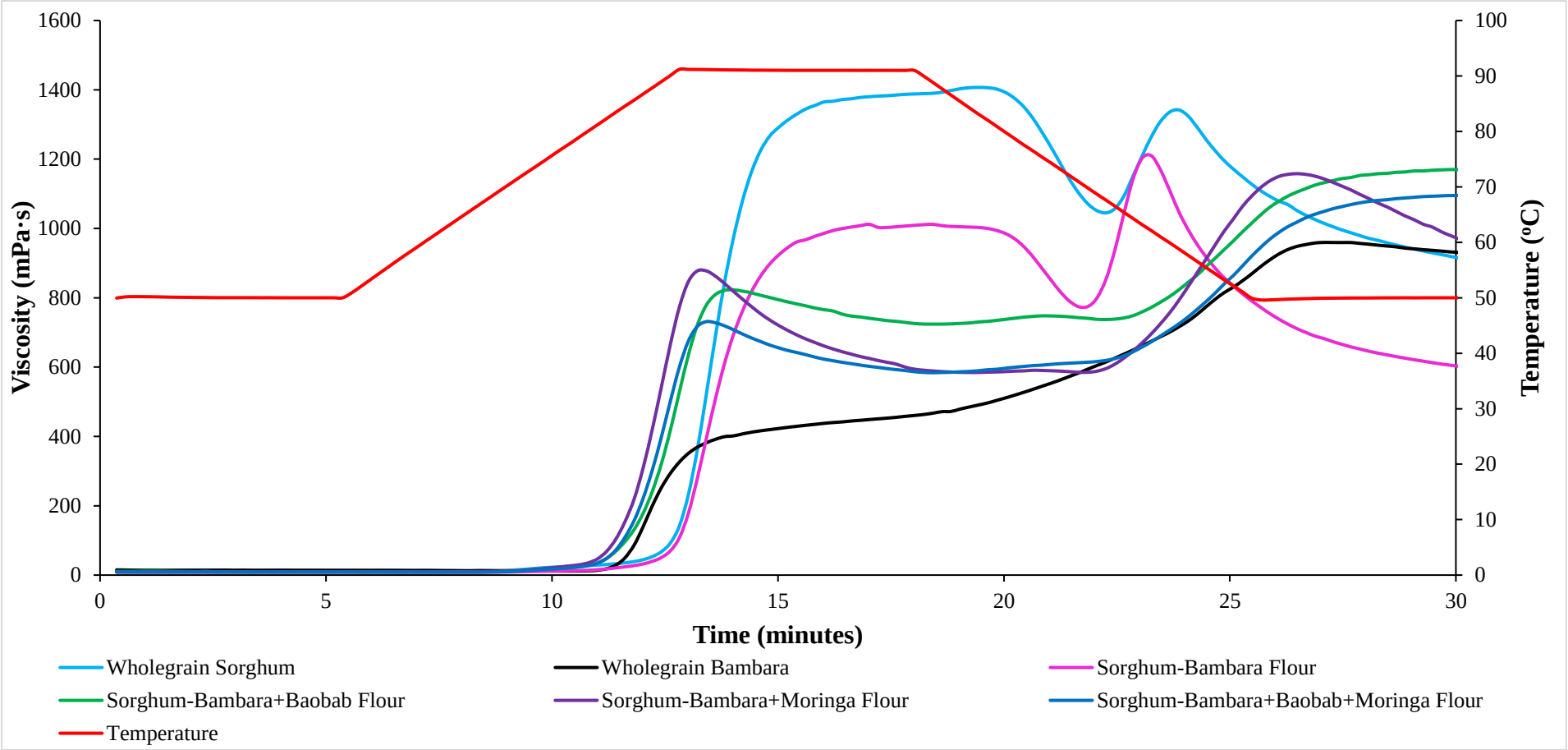


Figure 3-18: Effects of compositing, extrusion cooking and food-to-food fortification (with baobab fruit pulp and moringa leaf powder) on the pasting curves of wholegrain sorghum-Bambara groundnut flours

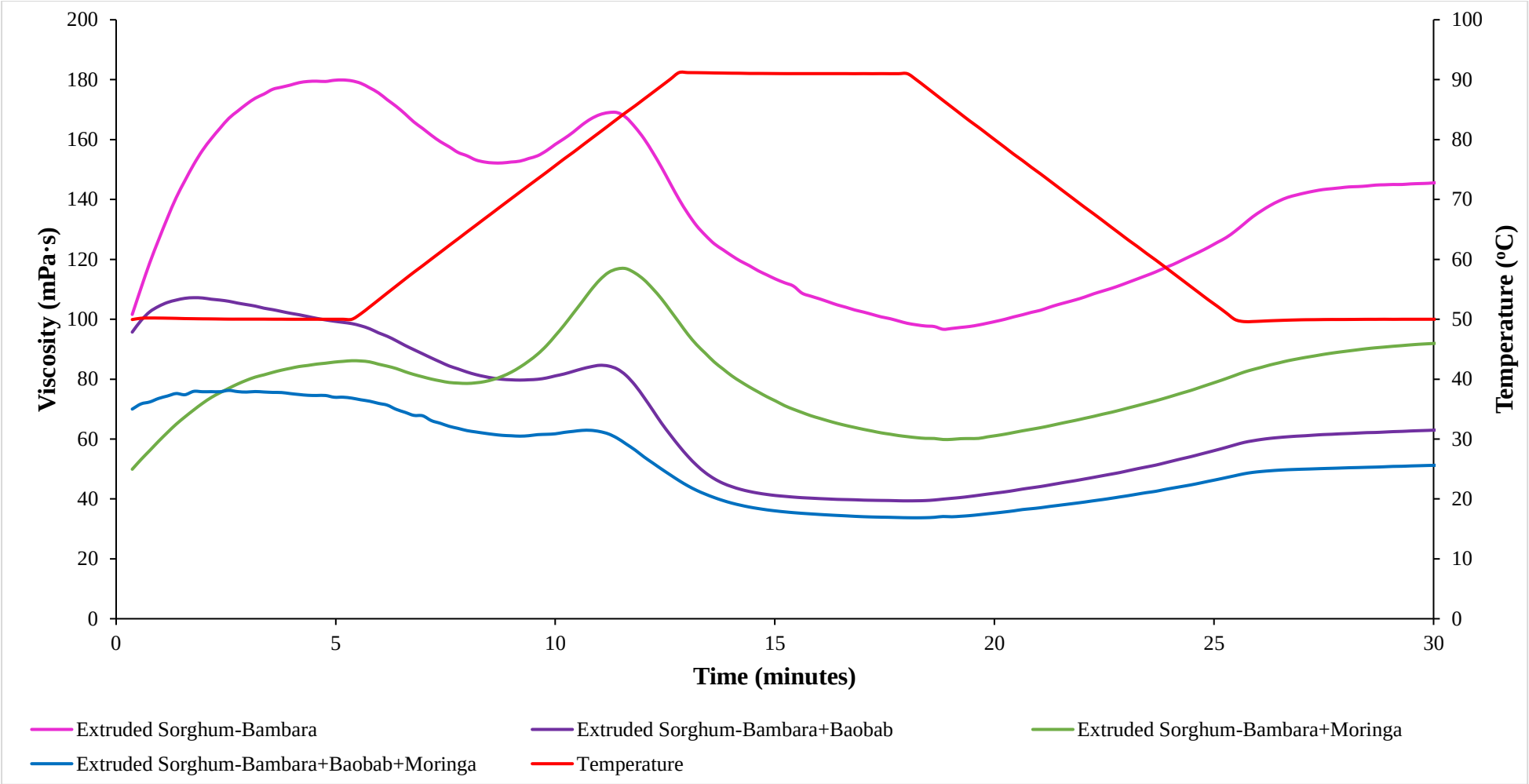


Figure 3-19: Effects of compositing, extrusion cooking and food-to-food fortification (with baobab fruit pulp and moringa leaf powder) on the pasting curves of wholegrain sorghum-Bambara groundnut flours

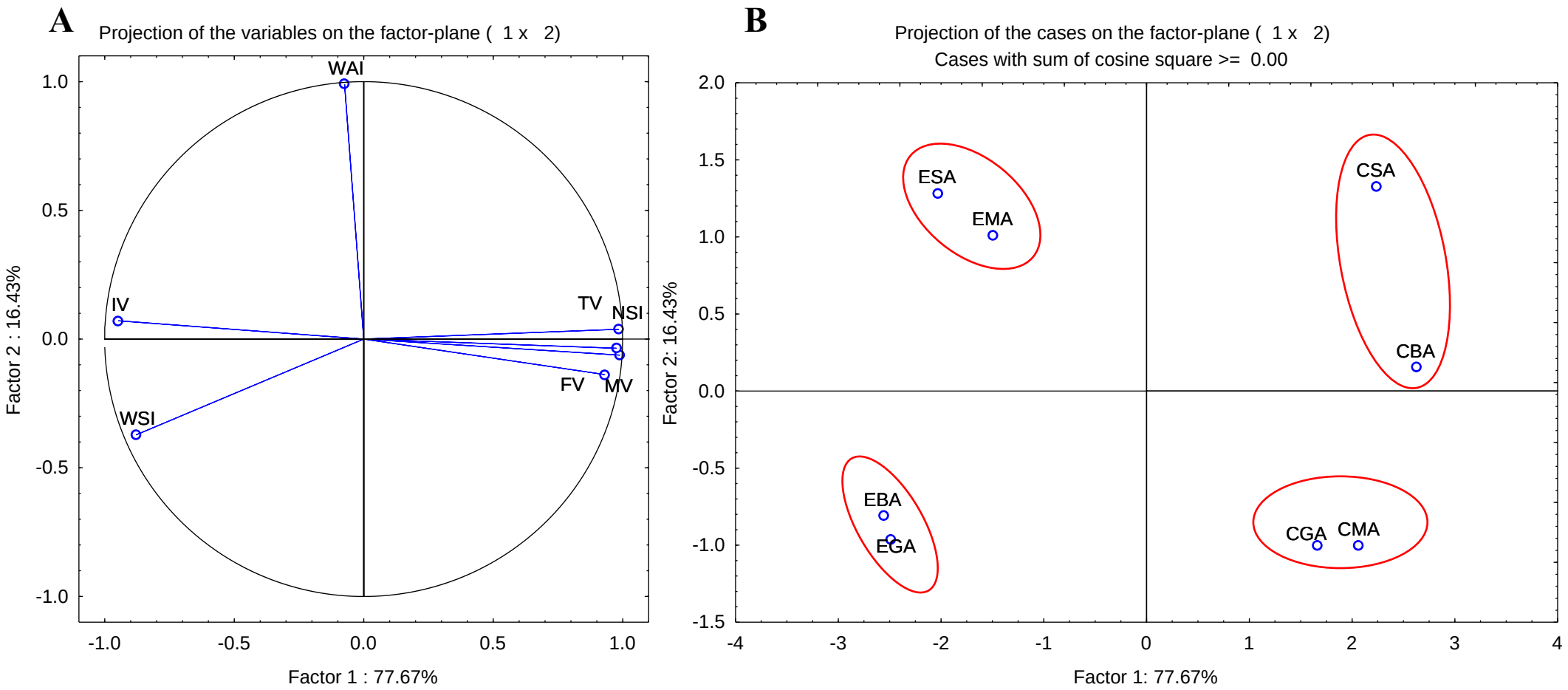


Figure 3-20: Principal component analysis showing 1×2 factor coordinate plots of dependent variables (initial viscosity-IV, tough viscosity-TV, maximum viscosity-MV, Final viscosity-FV, water absorption-WAI, water solubility index-WSI, nitrogen solubility index-NSI), A, and independent variables (fortification and processing technique-wet-cooking and extrusion), B.

Key: CSA-Cooked Sorghum-Bambara, CBA- Cooked Sorghum-Bambara+Baobab co-cooked, CMA- Cooked Sorghum-Bambara+Moringa, CGA- Cooked Sorghum-Bambara+Moringa+Baobab co-cooked, ESA- Extruded Sorghum-Bamabara, EBA- Extruded Sorghum-Bambara+Baobab co-cooked, EMA- Extruded Sorghum-Bambara+Moringa, EGA- Extruded Sorghum-Bambara+Moringa+Baobab co-cooked

3.3.5 Conclusions

Including wholegrain, Bambara groundnut flour with wholegrain sorghum flour increases the protein content and improves IVPD. A serving size of 100 g dry base composite porridges could contribute approximately 50-100% of the daily protein requirements of children between 2-5 years, alluding to possible application in the mitigation of PEM. Extrusion-cooked porridges showed lower polyphenols and phytate contents, both of which are antinutrients translating into improved IVPD and IVSD, thus strengthening the potential for implementation in managing PEM. Extrusion and Bambara groundnut inclusion reduce extrudate peak, trough and final pasting viscosities, which could increase nutrient density per gram of composite porridge when the flours are applied as porridges. Sorghum-Bambara groundnut composite flour extruded at low extrusion moisture could yield nutrient-dense instant porridge flour. The positive nutritional improvement and promising physical and functional qualities of composite flours extruded at low extrusion moisture produce extrudates which are potentially suitable as convenience-type products. The reduction observed in the starch digestibility and EGI of sorghum-Bambara groundnut composite fortified with baobab fruit pulp and moringa leaf powder is a result of changes in functional and rheological properties of fortificants during extrusion cooking. This study, therefore, demonstrates that food-to-food fortification with moringa and baobab could be added to locally available food crops to produce food products suitable for the management of nutrition-related diseases such as type 2 diabetes.

Chapter 4 : GENERAL DISCUSSION

The general discussion is divided into two sections. The first section is a critical review of the experimental design and methodologies used in this study, focusing on the challenges faced and the strengths and weaknesses of the experimental approach used in the research, leading to recommendations for future research. The second section discusses the significant findings in this research, particularly concerning the effect of food-to-food fortification (with baobab and moringa) and extrusion cooking on iron bioaccessibility, phenolics and antioxidant properties, *in vitro* digestibility (protein and starch digestibility) as well as pasting properties of sorghum-based porridges.

4.1 METHODOLOGY

4.1.1 Selection of raw materials

This study used whole-grain sorghum, moringa leaf powder, baobab fruit pulp powder, and whole-grain Bambara groundnut. These are all locally available food crops, some of which are indigenous and drought tolerant. Therefore, they are crucial for food security, particularly in sub-Saharan Africa, which informed the choice to study them in this research. The sorghum used was a red non-tannin type (type 1), which was preferred because of the negative effect of tannins on nutritional quality (Sharma, Kumar, Kaur, Tanwar, Goyal, Sharma, Gat & Kumar, 2021). Moringa leaves are high in micronutrients, including iron (Moyo *et al.*, 2011) and therefore could be explored for the potential to tackle iron-deficiency anaemia. Baobab fruit pulp is high in organic acids such as citric and ascorbic acids (Adetola *et al.*, 2019) that are well-known mineral bioaccessibility enhancers (Iyengar *et al.*, 2010; Lönnerdal, 2000). All these foods are also rich in phenolic compounds that can potentially prevent certain NCDs (Duodu & Awika, 2019).

Fortification of the sorghum-based porridges in this research at a rate of 6 g/100 g was informed by previous studies that showed that the addition of mineral-rich foodstuffs such as moringa beyond a certain threshold (30%) resulted in no significant effect on mineral bioaccessibility (Van der Merwe *et al.*, 2019).

4.1.2 Analytical Methods

4.1.2.1 Phytate Determination

In this research, the method used to determine phytate was the one defined by Fruhbeck *et al.* (1995). Phytates are extracted from the sample using 0.066 M HCl, which dissociates the phytate from the plant matrix. Anion-exchange purification is used to remove the non-phytate phosphate. This is necessary as the spectrophotometric determination measures the total amount of phosphate in the sample. The phosphate complexes with ferric chloride and sulphosalicylic acid exhibiting maximum spectrophotometric absorbance at a wavelength of 500 nm. The major drawback of this method is the possibility of inaccuracy due to large and varying degrees of iron (III) adsorption onto the phytate complex (Wu, Tian, Walker & Wang, 2009), leading to possible over or underestimation. This can be addressed by using chromatography techniques. These allow for direct measurement of the phytates in the sample rather than the estimation using indirect methods like the one employed. However, chromatography techniques are expensive and time-consuming, and this method offers a rapid way of estimating the phytate content of plants.

4.1.2.3 Total phenolic and antioxidant activity determination

The Folin-Ciocalteu assay of Singleton and Rossi (1965) was used to determine the total phenolic content and radical scavenging against $ABTS^{\bullet+}$ [2,2´-azinobis (3-ethyl-benzothiazole-6-sulphonic acid)] was used to determine the antioxidant properties of the sorghum-based porridges. The methods are both based on reduction-oxidation reactions involving electron transfer. The Folin-Ciocalteau assay involves oxidation of the phenolate ion under alkaline conditions (Romulo, 2020; Shahidi & Zhong, 2015; Waterman & Mole, 1994), and the ABTS radical scavenging assay involves electron transfer to the $ABTS^{\bullet+}$ radical cation (Nwachukwu, Sarteshnizi, Udenigwe & Aluko, 2021; Shahidi & Zhong, 2015). While the assays are simple, convenient and reproducible, they lack specificity (measuring all reducing compounds) and are of relatively weak physiological relevance as radicals formed in ABTS do not exist in physiological systems (Nwachukwu *et al.*, 2021; Shahidi & Zhong, 2015). A possible way of accounting for potential interferences from non-phenolic reducing substances (such as ascorbic acid present in the plant materials) is by determining their contribution to total phenolic content and subtracting the values obtained from the gross total phenolic content. However, this could be a rather long and cumbersome approach. Perhaps a more realistic

approach to adopt in phenolics research would be to combine the Folin-Ciocalteu assay with other more specific methods for the determination of phenolic compounds, such as HPLC or LCMS, as was done in this research. To mimic more physiologically relevant radicals, the ORAC and NO assays were also performed.

Nitric oxide ($NO^{\bullet}$) and ORAC are physiologically relevant as the reactive nitrogen species (produced in NO in the NO scavenging assay) and the peroxyl radical (ROO•) (produced in ORAC) occur in physiological systems (Nwachukwu *et al.*, 2021). The mechanism of the $NO^{\bullet}$ assay is based on the competitive binding of $NO^{\bullet}$ by antioxidants preventing its oxidation to nitrates or nitrites (Gülçin, 2012), while ORAC involves a reaction of peroxyl radicals (ROO•) with a fluorescent probe to form a non-fluorescent product quantified by monitoring loss in fluorescence (Prior *et al.*, 2005). The limitations of the $NO^{\bullet}$ assay are that $NO^{\bullet}$ is unstable and needs to be generated in situ for each analysis (Jayachandra *et al.*, 2012) and the possibility of different intermediate products from the oxidation of NO to nitrite, i.e. NO_2, N_2O_3, N_2O_4 and $ONOO^-$ interfering with the results by interactions with phenolic extracts (Marcocci *et al.*, 1994). The requirement of sophisticated and expensive equipment (Awika *et al.*, 2003b) in ORAC assay is its only major limitation.

In this research, samples were tested for their ability to protect human adenocarcinoma cells (Caco-2 cells) against oxidation by AAPH-induced peroxyl radicals using the DCFH-DA assay. The assay employs dichlorofluorescein diacetate (DCFH-DA), a cell-permeable dye, which in the presence of cellular esterases, is deacetylated, forming non-fluorescent DCFH (Blasa *et al.*, 2011). In the presence of radicals such as ONOO-, NO• and peroxyl radicals, the DCFH oxidises to a fluorescent derivative DCF, and on the addition of a sample containing antioxidants, the antioxidants quench the radicals and block the conversion of DCFH to DCF in the Caco-2 cells (Blasa *et al.*, 2011; Wolfe & Liu, 2007). The decrease in cellular fluorescence when compared to the control cells is indicative of the cellular antioxidant capacity of the antioxidant compounds. The limitation of this assay is that exposure of DCFH-loaded cells to light in the presence of oxygen causes photo-reduction of DCFH, causing continuous oxidation as free radicals can be generated continuously contribute (Wolfe & Liu, 2007). In this research, this limitation was overcome by performing the test in the absence of light and using an opaque 96-well plate to contain the DCFH-loaded cells.

The phenolic content of plants has been directly linked to their antioxidant activity (Rice-Evans *et al.*, 1997). While the Folin-Ciocalteu TPC assay provides a rapid and inexpensive means of

estimating the phenolic content of plants, it should be coupled with methods such as LCMS and antioxidant assays such as ABTS, ORAC, and NO inhibition for the certainty of the findings. Notwithstanding this, TPC was shown to be highly correlated with the LCMS data and antioxidant properties in this study (Section 3.2), indicating the reliability of the assay.

4.1.2.4 Determination of organic acids

Two main organic acids (ascorbic acid and citric acid) were of interest in this research, and these were determined using reversed-phase HPLC. For ascorbic acid in particular, an important factor which needed to be taken into consideration, especially during the extraction phase, was its extreme susceptibility to oxidation (Hooper & Ayres, 1950). For this reason, extraction of ascorbic acid from plant tissues for analysis is commonly done with acid solvents, as was done in this research with the use of metaphosphoric acid. It has been shown that metaphosphoric acid preserves ascorbic acid in atmospheric conditions (Musulin & King, 1936).

4.1.2.6 *In vitro* iron dialysability determination as a measure of iron bioaccessibility

As a measure of bioaccessibility, the *in vitro* dialysability assay (conducted as shown in Figure 4-1) is limited as it cannot assess the rate of absorption or transport kinetics of minerals. Furthermore, it cannot measure the actual amount of nutrients at the site of absorption (Etcheverry *et al.*, 2012). In this research, an essential feature of the *in vitro* dialysability assay which needed to be noted is that some of the iron which diffuses into the dialysis bag immediately becomes insoluble due to the higher pH of the dialysate. There is, therefore, the potential for underestimation of the amount of soluble iron (Van Campen & Glahn, 1999). This could likely be an intrinsic weakness of the assay as the high pH of the medium within the dialysis tubing is required because of the requirement to simulate the duodenal conditions of the digestive tract.

Nonetheless, the dialysability assay remains a useful measure of iron (or nutrient) bioaccessibility and has been found to have a reasonable agreement with human absorption studies (Etcheverry *et al.*, 2012). Dialysable iron values from a study performed by Luten *et al.* (1996) had good similarity in terms of ranking and magnitude with non-haem iron absorption values from a human subject study. For future research, more sophisticated gut digestion models, such as the computer-controlled gastrointestinal model (TIM) system

developed by The Netherlands Organization for Applied Scientific Research (TNO), could be considered. This system simulates conditions in the human stomach, duodenum, jejunum and ileum (Minekus, Marteau, Havenaar & Veld, 1995), including dynamic aspects such as the simulation of peristaltic movements. This gastrointestinal model has a much greater *in vivo* predictive value than other *in vitro* systems, such as the mineral dialysability assay (Minekus, 2015).

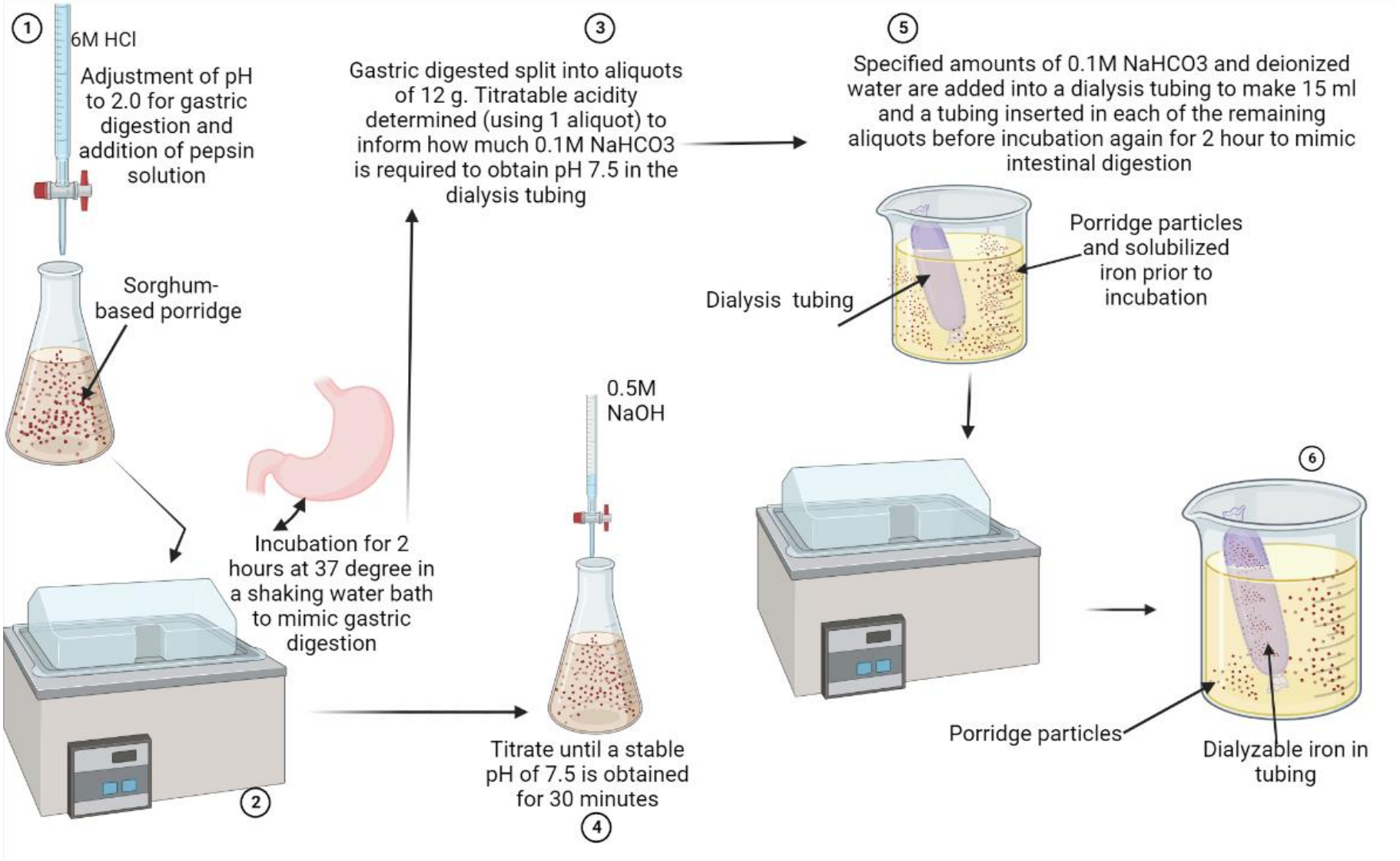


Figure 4-1: Illustration of the *in vitro* dialyzability assay to assess iron bioaccessibility

4.1.2.7 Estimation of *in vitro* iron bioaccessibility using Caco-2 cells

In this research, *in vitro* iron bioaccessibility was also determined by the estimation of iron uptake in terms of ferritin formation by Caco-2 cells. The assay procedure is illustrated in Figure 4-2. This assay can provide more information than bioaccessibility studies alone, such as the impact of food or nutrient components on the absorption rate and efficiency and possible competition at the absorption site (Glahn *et al.*, 2002). The Caco-2 cell assay has been validated against human absorption of iron data and showed a significant correlation found (Au & Reddy, 2000). The Caco-2 cell model is thus a useful technique to assess human iron absorption as well as iron and zinc bioavailability from various food combinations (Garcia, Flowers & Cook, 1996).

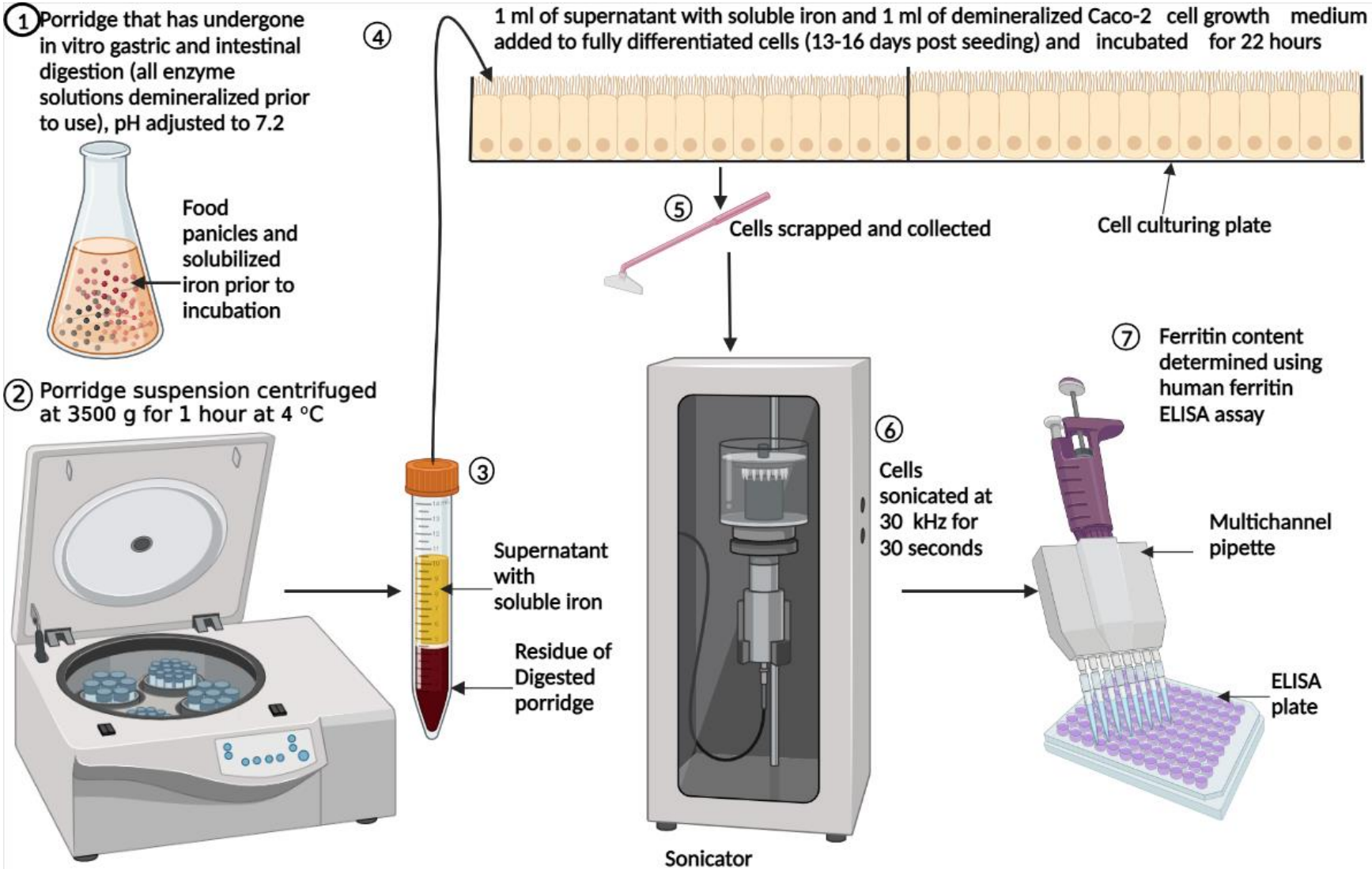


Figure 4-2: Estimation of iron bioaccessibility using Caco-2 cells

4.1.2.8 Advanced glycation end-products (AGEs) determination

AGEs result from spontaneous post-translational modification of proteins or amino acids through reducing sugars via Maillard-type reactions or nonenzymatic glycation (Yeh *et al.*, 2017) (Figure 4-3). Consumption of foods containing excessive levels of AGEs formed during processing and storage has been implicated in several disorders and their associated complications, such as diabetes mellitus, kidney complications, tumour development and malignancy, Alzheimer's disease, atherosclerosis, and chronic heart failure (Sadowska-Bartosz & Bartosz, 2016; Uribarri *et al.*, 2015). In this research, the effect of phenolic extracts on the formation of AGEs was determined using the fluorescence spectroscopy method described by Siddiqui *et al.* (2016), where methylglyoxal and bovine serum albumin act as precursors for the formation of AGEs. The treatments are prepared in sterile centrifuge tubes in a sterile environment to minimize contamination before incubation at 37 ºC for 7 days, after which fluorescence is measured at emission and excitation wavelengths of 330 nm and 420 nm, respectively. Spectrofluorimetric detection is simple and rapid but has a major drawback in that it cannot detect the majority of non-fluorescent AGEs, and in addition, it is not specific for fluorescent AGEs; hence non-AGE fluorescent compounds can interfere with measurement (Corica *et al.*, 2021). More specific analytical chromatography techniques could be useful in identifying fluorescent AGEs to back up the spectrofluorimetric data.

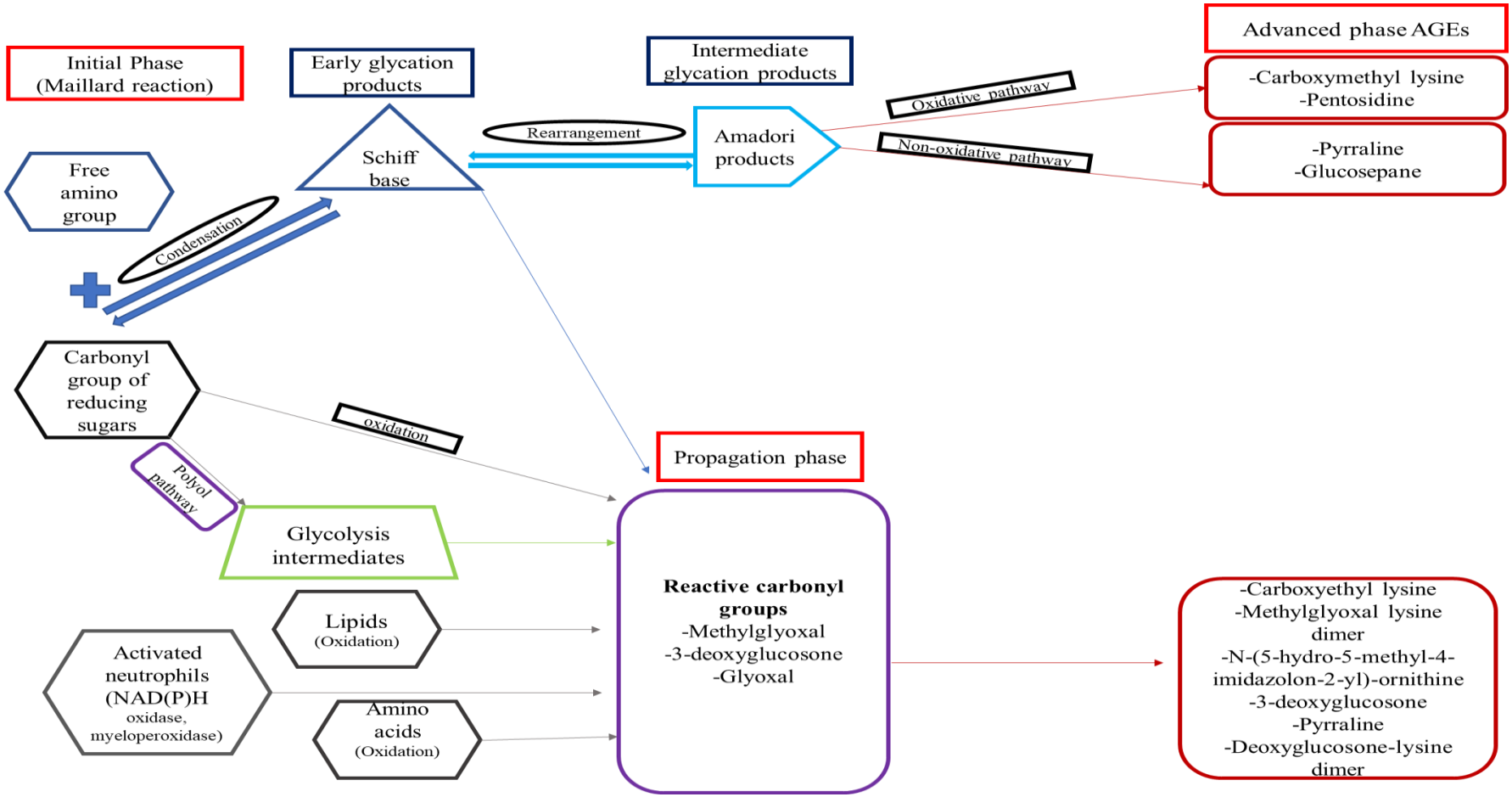


Figure 4-3: Different pathways of advanced glycation end products (AGEs) formation. The Figure schematically depicts the Maillard reaction leading to AGE formation through the initial reaction between reducing sugars and the free amino group of a protein through the stages of Schiff base and Amadori product formation. The Figure also illustrates the many other different pathways that may lead to the formation of AGEs, even in the absence of glucose.

4.1.2.9 Cellular anti-inflammatory activity-lipopolysaccharide assay

The RAW264.7 murine cell line is an important tool for *in vitro* study of inflammation (Murakami *et al.*, 2020), which is conducted by monitoring the production of NO by the cells. In this research, anti-inflammatory effects were studied by determining the ability of phenolic extracts to prevent NO accumulation in lipopolysaccharide- (LPS) induced RAW264.7 cell macrophages. A major challenge encountered while working with RAW264.7 cells was their sensitivity to the enzyme mix used to dislodge them from the tissue culturing plate before subculturing or plating. This was resolved by using a cell scraper rather than the enzyme mix. While these techniques are a cheap and rapid indicator of physiological activity, a weakness in this study was that the extracts used did not undergo *in vitro* digestion prior to analysis. This is mainly because this section did not focus on *in vitro* digestion attributes, but this could be explored in future research.

4.1.2.10 Cellular lipid droplet reduction in 3T3-L1 cells

3T3-L1 cells were used in this study to study the potential antiadipogenic properties of the sorghum-based porridge phenolic extracts. The growth and differentiation of the 3T3-L1 cells were carried out using the method described by Zebisch *et al.* (2012). Owing to their ability to be stained by oil Red-O dye (Kwan *et al.*, 2017), this dye was used to quantify lipids by extracting the dye from the cells with 60% isopropanol, and absorbance was then measured at 520 nm. Laboratory studies indicate that the antiobesity effects of polyphenol-rich diets may be attributed to the ability of polyphenols to interact, directly or indirectly, with adipose tissues (pre-adipocytes, adipose stem cells and immune cells) (Wang *et al.*, 2014). One main drawback found when using the oil Red-O dye in this research was that some undifferentiated cells were also stained. This was because the dye attached to certain components of the cells could lead to an overestimation of the adipocyte content. Exploring alternative types and concentrations of the solvents for the oil Red-O dye could help resolve this. Kinkel, Fernyhough, Helterline, Vierck, Oberg, Vance, Hausman, Hill and Dodson (2004) found that 99% isopropanol was effective in selective staining of 3T3-L1 differentiated cells with oil Red-O dye.

4.2 KEY RESEARCH FINDINGS

One of the significant findings from this study is that extrusion cooking of sorghum-based porridges resulted in an increase in ferritin formation in Caco-2 cells. Specifically, Caco-2 cells treated with extruded instant sorghum porridge exhibited greater ferritin formation (higher iron uptake) than cells treated with conventionally cooked sorghum porridge. This finding suggests that extrusion cooking enhances iron bioavailability in sorghum porridge. This was possibly due to the reduced mineral bioaccessibility inhibitors, phytate and polyphenols by the extrusion cooking process. Several studies suggest that phytate can be degraded by extrusion cooking into lower inositols such as inositol-penta, tetra, tri, di and monophosphate (Watson *et al.*, 2019), as illustrated in Figure 4-4. The mineral binding capacity of phytate is reduced as phosphate groups are removed from the higher inositol phosphates to form lower inositol phosphates (Sandberg, Brune, Carlsson, Hallberg, Skoglund & Rossander-Hulthén, 1999; Persson, Türk, Nyman & Sandberg, 1998; Kaufman & Kleinberg, 1971).

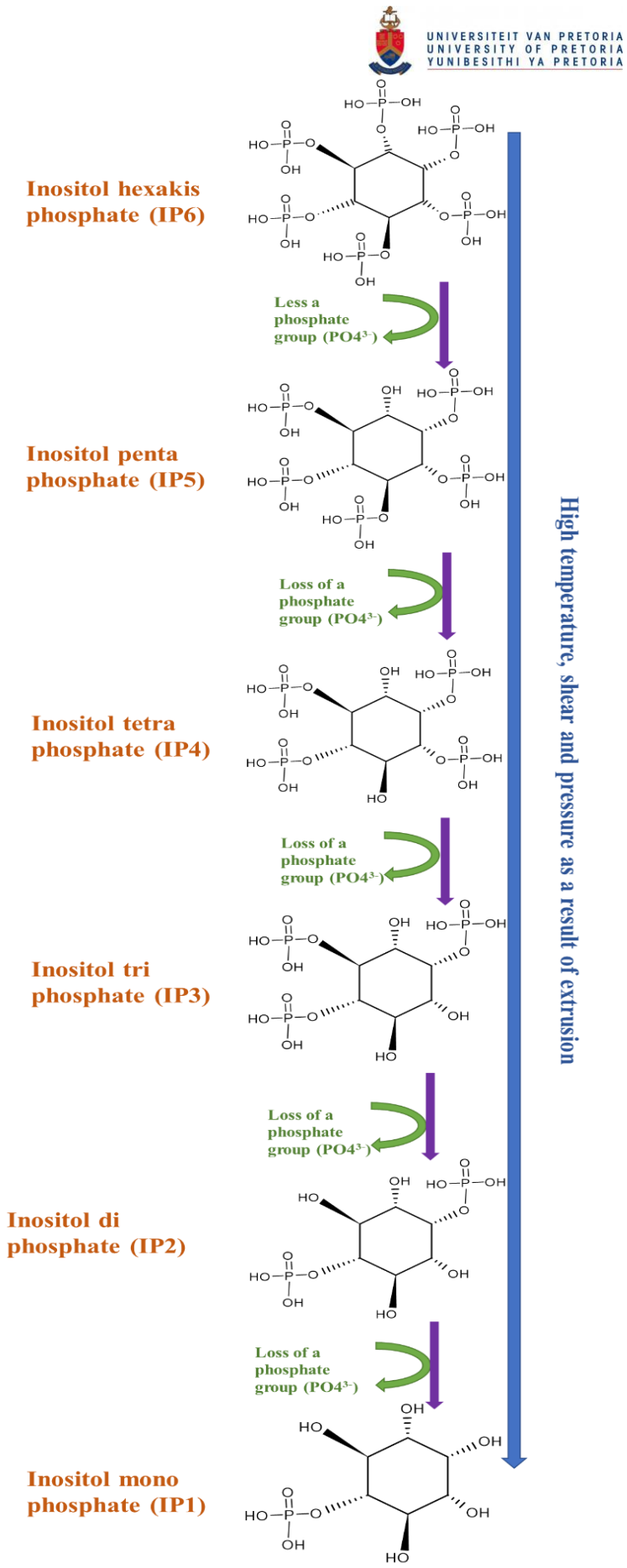


Figure 4-4: Dephosphorylation of phytate during extrusion cooking

This study also showed that FtFF had an effect on the iron bioaccessibility of sorghum-based porridges, with the nature of the effect being dependent upon whether moringa leaves or baobab fruit pulp was used for the fortification. FtFF with moringa, whether alone or in combination with baobab, resulted in major reductions in bioaccessible iron measured by the *in vitro* iron dialysability assay [bioaccessible iron content (BIC) and percentage bioaccessible iron (PBI)]. In contrast, BIC and PBI were significantly increased when baobab was co-processed (extrusion-cooked or conventionally wet-cooked) with whole-grain sorghum. As explained, baobab contains high levels of organic acids, which can chelate iron and keep it in the soluble form required for absorption (Iyengar *et al.*, 2010; Lönnerdal, 2000). As described, ascorbic and citric acid reduces iron from its F^{3+} to its Fe^{2+} form, thereby keeping it soluble and in a bioavailable form (Mackenzie & Garrick, 2005). This difference in effect on iron bioaccessibility depending on whether fortification was done with moringa or baobab was also reflected, to some extent, concerning ferritin formation in the Caco-2 cell assay. Co-extruded sorghum porridges fortified with moringa and baobab showed significantly lower ferritin formation in Caco-2 cells when compared to ferritin formation in Caco-2 cells, with the sorghum porridge alone, with the effect of moringa being significantly greater than baobab alone.

The results from this research have also provided evidence that sorghum-based porridges may have health-promoting properties in terms of offering protection against diet-related NCDs. This is important given the growing concern about NCDs, such as cancer, diabetes and obesity in sub-Saharan Africa (Awika & Rooney, 2004).

All the porridges exhibited the ability to protect against oxidative stress through their antioxidant properties, either by *in vitro* radical scavenging effects or by their display of antioxidant activity in Caco-2 cells. FtFF enhanced the radical scavenging properties of the porridges, which is an indicator that compared to the unfortified sorghum porridge, the FtFF porridges may provide better protection against oxidative stress.

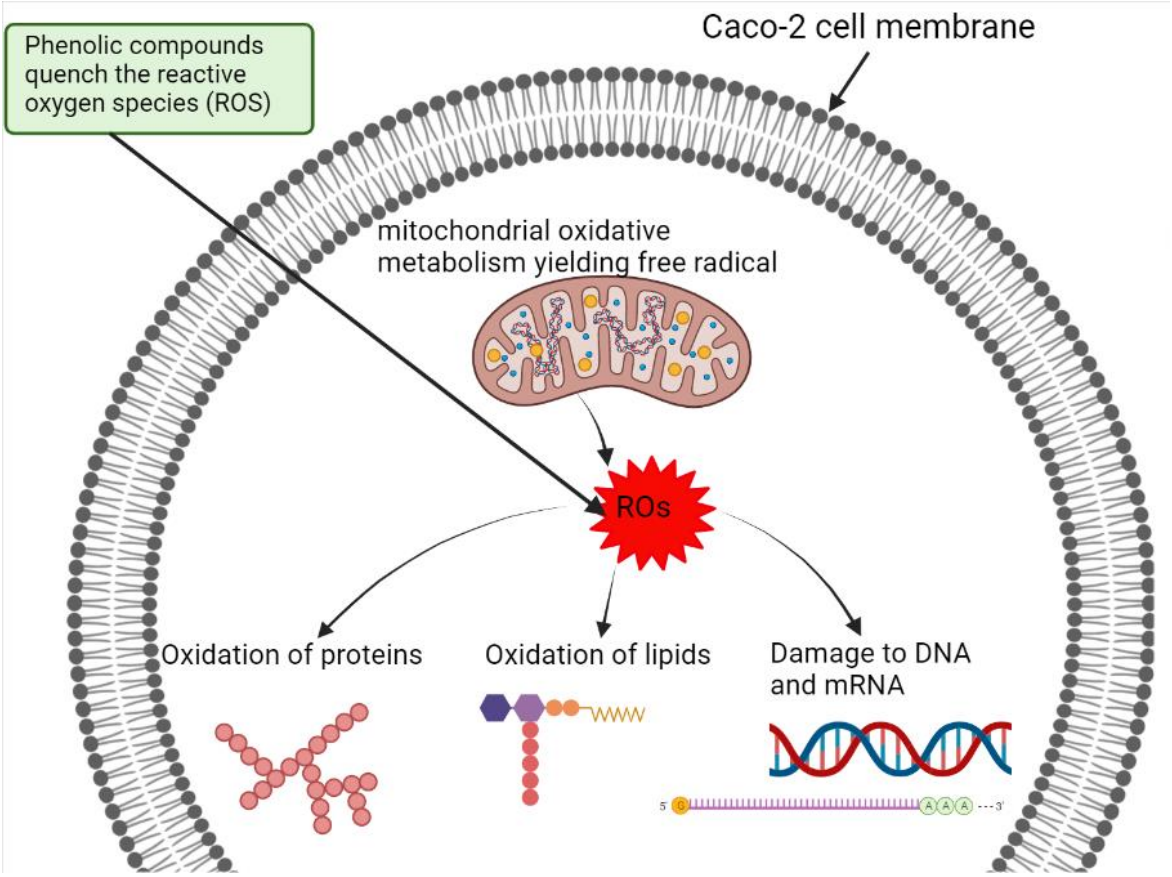


Figure 4-5: Summary of the role of phenolic compounds in protecting against oxidative stress in Caco-2 cells *ex vivo* (Furger, 2021).

In exhibiting antioxidant properties and hence the potential ability to protect against oxidative stress, the results indicated that there could be some additive effects. The antioxidant capacity (ABTS, ORAC and cellular antioxidant activity in Caco-2 cells, (Figure 4-5)) observed when baobab and moringa were combined was significantly higher than those when the individual foods were used as fortificants, whether extrusion- or conventionally wet-cooked. This could be attributed to a possible synergistic effect of the phenolic compounds present in each. Hajimehdipoor, Shahrestani and Shekarchi (2014) studied the effects of different combinations of phenolic compounds and reported higher antioxidant activities when certain combinations of flavonoids and phenolic acids were made. In this study, moringa was particularly high in phenolic acids and their esters, while baobab was high in flavonoids (section 3.2.5.2.2).

The degree of hydroxylation in phenolic acids is important with respect to antioxidant activity. Generally, the greater the number of hydroxyl groups in the phenolic structure, the higher the radical scavenging capacity (Marinova & Yanishlieva, 2003; Van den Berg, Haenen, Van den Berg, Van der Vijgh & Bast, 2000). Moringa and baobab were particularly high in the phenolic

acids protocatechuic acid and gallic acid. More hydroxyl groups on a phenolic compound provide more sites for radical scavenging.

Concerning flavonoids, moringa was rich in quercetin, which has a unique structure comprising a 2,3 double bond in combination with a 3-hydroxy group on the C ring, Figure 4-6. These structures have higher antioxidant activity because the resonance stabilization for electron delocalization across the flavonoid molecule is increased (Rice-Evans, Miller & Paganga, 1996). These unique structures could account for the higher antioxidant activities of porridges fortified with moringa and baobab, whether alone or in combination.

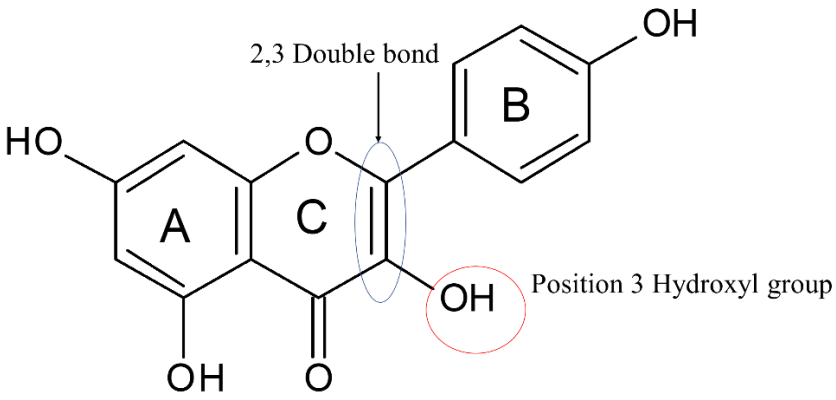
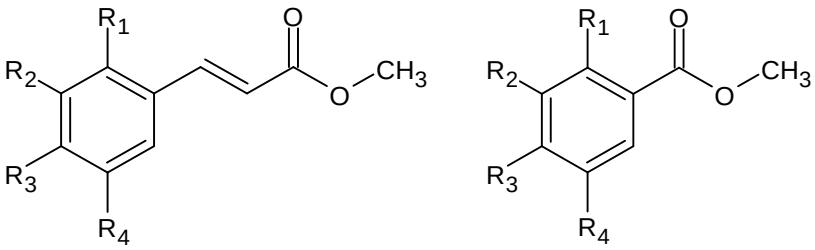


Figure 4-6: Flavonoid structure showing some of the relevant structures for antioxidant activity (hydroxylation at the meta-position on the C-ring, an ortho group at the para-position of the C-ring and a double bond between position 2 and 3 of the C-ring)

Sorghum porridges fortified with baobab and moringa, whether alone or in combination, consistently showed greater inhibition of NO production in LPS activated RAW264.7 macrophages, an indication of enhanced anti-inflammatory activity of the fortified sorghum porridges compared to the unfortified porridges. In reviews of the role of phenolics in the prevention of inflammation, Conforti and Menichini (2011) and Shahidi and Yeo (2018) summed up the role of phenolic compounds as interrupting the pathways involved in the activation of inducible nitric oxide synthase (iNOS), an enzyme that catalyses the production of NO, thus preventing the activation of nuclear factor kappa-B (NF-κB), a protein transcription factor that increases the production of inflammatory cytokines. Phenolic compounds can also inhibit the iNOS enzyme itself (Figure 4-8). The phenolic compounds identified in this study have been shown to suppress inflammation through this mechanism, such as caffeic acid (Chao, Mong, Chan & Yin, 2010; Da Cunha, Duma, Assreuy, Buzzi, Niero, Campos & Calixto, 2004), naringin (Chtourou, Aouey, Aroui, Kebieche & Fetoui, 2016), rutin (Kamel, Abd El-Raouf, Metwally, Abd El-Latif & El-sayed, 2014), quercetin (Hämäläinen, Nieminen, Vuorela,

Heinonen & Moilanen, 2007; Kim, Cheon, Kim, Kim & Kim, 1999), apigenin (Ju, Kang, Bae, Pae, Lyu & Jeon, 2015; Kim *et al.*, 1999), kaempferol (Devi, Malar, Nabavi, Sureda, Xiao, Nabavi & Daglia, 2015; M Calderon-Montano, Burgos-Morón, Pérez-Guerrero & López-Lázaro, 2011; Hämäläinen *et al.*, 2007), naringenin (Hämäläinen *et al.*, 2007), and luteolin (Kim *et al.*, 1999).

Moringa was exceptionally high in 3- and 4-coumaroyl quinic acids and 3- and 4-feruloyl quinic acids, which were absent in sorghum. In a study of the anti-inflammatory properties of roots and rhizomes, Wu, Wei, Yang, Zhang, Xu, Yang, Zhong, Liu and Yang (2017) reported enhanced inhibition of NO production in LPS-activated RAW264.7 macrophage when a feruloyl group was present in phenolic acid esters than when *p*-hydroxyl or cinnamoyl groups were present. Suppression of NO production (anti-inflammatory activity) is predominantly exerted by methyl esters of phenolic acid derivatives and not simple methylation or *O*-methyl substitution of the OH group in free phenolic acid derivatives (Nguyen *et al.*, 2015) (Figure *4-7*a and b). On the other hand, Baobab contained significantly higher amounts of quercetin and apigenin, which may account for its higher inhibition of NO production. There is a 2,3 double bond in both apigenin and quercetin and a 3-hydroxyl group in quercetin, Figure *4-6*.



(a) (b)

Figure 4-7: Core structures of the methyl esters of coumaric acid (a) and benzoic acid derivatives (b)

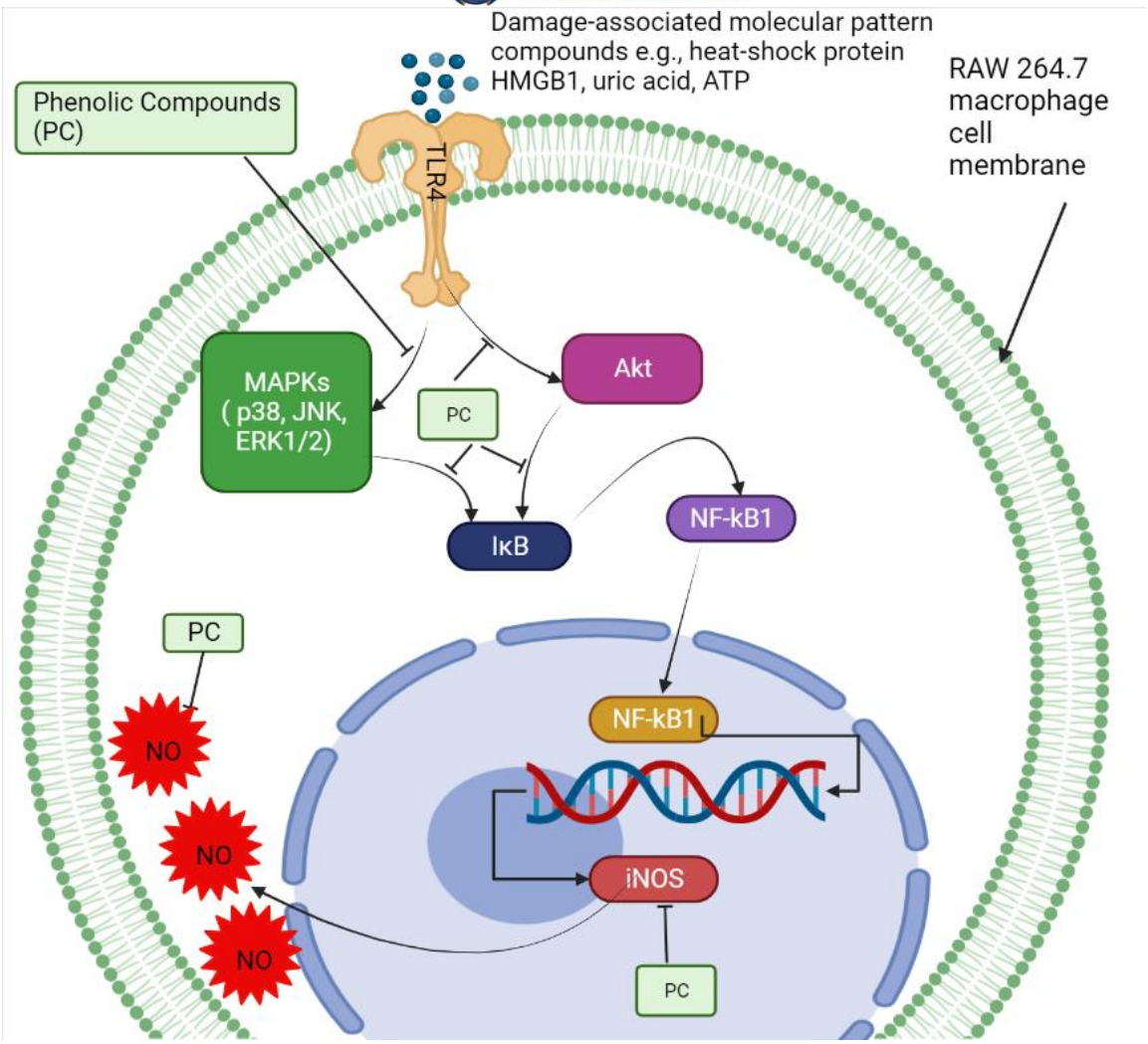


Figure 4-8: Summary of the role of phenolic compounds in the inhibition of cellular NO production in RAW264.7 macrophages (Serreli *et al.*, 2019).

This research used inhibition of AGEs, reduction in starch digestibility and reduction in the glycaemic index as indicators of anti-diabetic activities. With all these indicators, FtFF produced enhanced the anti-diabetic activities of the porridges.

An increase in the inhibition of AGEs was observed with FtFF of whole-grain sorghum using baobab and moringa, whether alone or in combination, which may be related to the observed increase in the concentration of phenolic compounds following FtFF with moringa and baobab. Figure 4-9 illustrates how phenolic compounds could inhibit the formation of AGEs in the human body and thus exert anti-diabetic properties. Several different phenolic compounds have been demonstrated to prevent AGEs formation through various mechanisms. These include the trapping of reactive carbonyl species such as methylglyoxal (Chen, Huang, Hwang, Ho, Li & Lo, 2014; Gutierrez, 2012; Wu, Huang, Lin & Yen, 2011; Peng, Cheng, Ma, Chen, Ho, Lo,

Chen & Wang, 2008) by the phenolic compound, (Figure 4-9). According to Shao, Bai, He, Ho, Yang and Sang (2008), this function is highly dependent on the hydroxyl group at position 2 of the A ring (for flavonoids) as they reported slower trapping of methylglyoxal and glyoxal when phloretin (a dihydrochalcone) was glycosylated at position 2 to form phloridzin. Moreover, both compounds were more reactive than lysine and arginine in trapping reactive carbonyl groups indicating a competitive action in the prevention of AGEs by flavonoids with the A-ring (Shao *et al.*, 2008).

Other mechanisms by which phenolic compounds inhibit the formation of AGEs are inhibition of the formation of Amadori products (Chompoo, Upadhyay, Kishimoto, Makise & Tawata, 2011; McIntyre, Harris, Saleem, Beaulieu, Ta, Haddad & Arnason, 2009; Yoshikawa, Pongpiriyadacha, Kishi, Kageura, Wang, Morikawa & Matsuda, 2003), trapping already formed α-dicarbonyl compounds, glycation of amino groups (Mesías, Navarro, Gökmen & Morales, 2013; Wang & Ho, 2012; Chompoo *et al.*, 2011; McIntyre *et al.*, 2009; Hsieh, Lin, Yen & Chen, 2007; Sang, Shao, Bai, Lo, Yang & Ho, 2007; Lo, Li, Tan, Pan, Sang & Ho, 2006), exhibiting antioxidant activity against free radicals formed during glycation (McIntyre *et al.*, 2009; Ardestani & Yazdanparast, 2007) and reduction of protein carbonyl and thiol oxidation (Ardestani & Yazdanparast, 2007), (Figure 4-9). The phenolic compounds identified in this study, such as chlorogenic acid, gallic acid, quercetin, rutin, catechin, and coumaric acid, among others, have been implicated in playing a role in the inhibition of AGEs (Anwar, Khan, Almatroudi, Khan, Alsahli, Almatroodi & Rahmani, 2021; Khan, Liu, Wang & Sun, 2020; Khangholi, Majid, Berwary, Ahmad & Abd Aziz, 2016). As mentioned earlier, moringa was exceptionally high in phenolic acid esters, coumaric acid, gallic acid and rutin, while baobab was high in rutin and catechin.

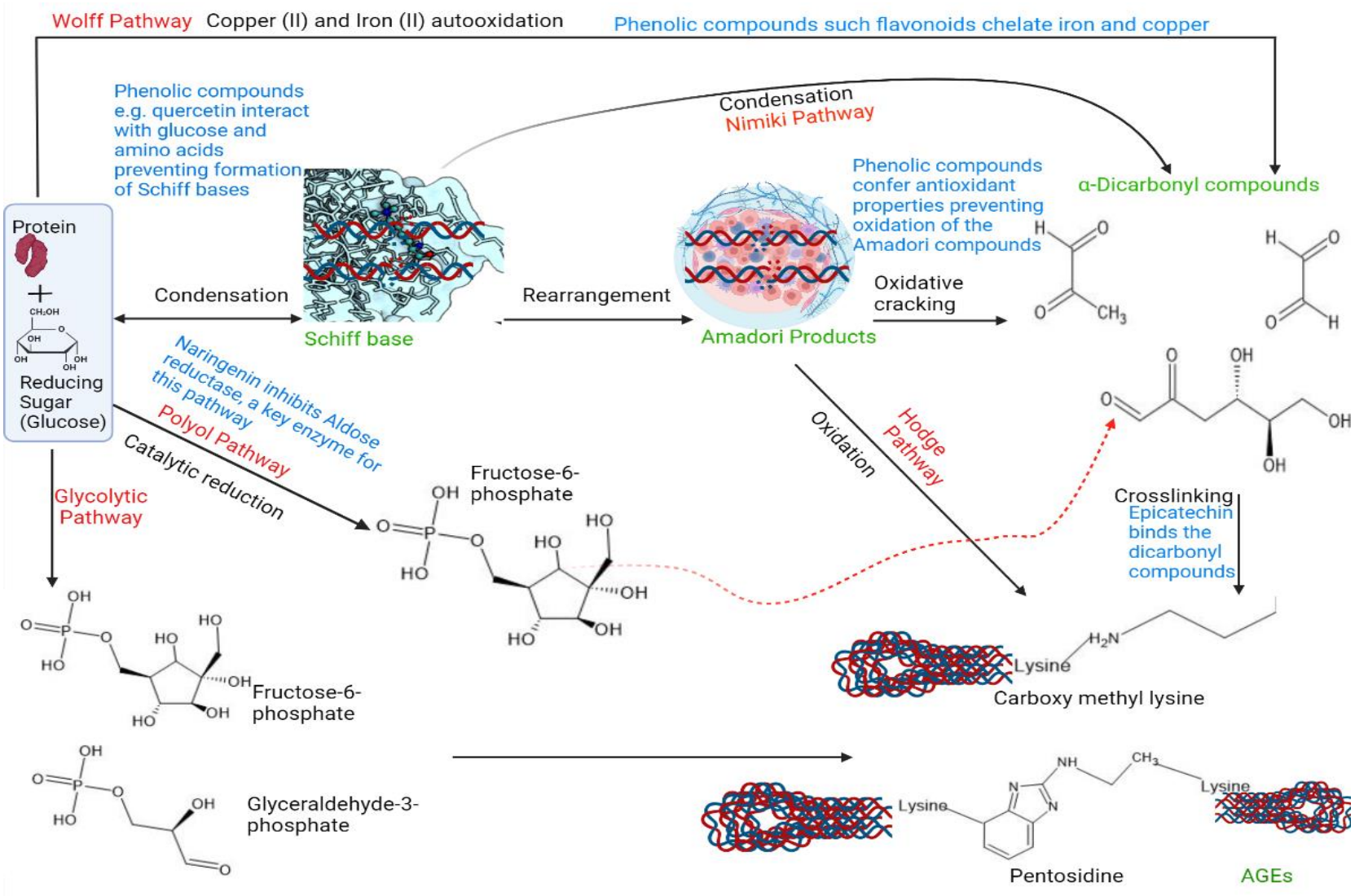


Figure 4-9: Summary of the role of phenolic compounds in the inhibition of advanced glycation end products (AGEs) in the human body

FtFF with baobab and moringa alone and, in combination, decreased the *in vitro* starch digestibility and the estimated glycaemic index of both conventionally wet cooked and extruded porridges. These effects following fortification with baobab and moringa, either alone or in combination, could be attributed to both the high phenolics and fibre content of both and the high phytate content in moringa (Section 3.1). Tannins and flavonoids interact with starch, reducing *in vitro* starch digestibility (Coe *et al.*, 2013; Thompson & Yoon, 1984; Deshpande & Salunkhe, 1982). Coe *et al.* (2013) reported similar findings in *in vivo* research where incorporating baobab fruit pulp in white bread reduced the glycaemic response and rapidly digestible starch in human participants. Soluble dietary fibre has been reported to reduce starch digestibility (Yağcı & Göğüş, 2009; Brennan *et al.*, 2008; Brennan *et al.*, 1996; Jenkins *et al.*, 1984). Brennan *et al.* (2008) suggested that fibre may directly hinder digestion by limiting contact between starch and digestive enzymes. Previously, Brennan *et al.* (1996) suggested that gelatinized and disrupted starch granules could be entrapped within the soluble fibre matrix, and this would reduce starch digestibility due to limited access by amylase enzymes. Furthermore, starch-phenolic complexes formed through hydrogen and hydrophobic interactions can make the starch inaccessible to the enzymes (Sun & Miao, 2020; Amoako & Awika, 2016; Zhu, 2015). Lastly, the interaction of phenolic compounds with amylase enzymes at their active sites could also be a cause for inhibition (Lv *et al.*, 2019; Sun *et al.*, 2018; Liu *et al.*, 2017)

The porridges showed the ability to prevent and reduce adipocyte formation, indicating their potential anti-obesity activities. Extracts from all the porridges showed effective prevention and treatment of adipocytes, with no evidence of effects of fortification or extrusion cooking. This prevention and treatment of adipocytes are indicative of the different roles played by the different phenolic compounds in the lipid droplet formation and treatment process. According to Aranaz *et al.* (2019), different types of phenolic compounds identified in the sorghum-based porridges in this study have differing effects at various stages of the lipogenesis process, which could account for the lack of significant differences between the different treatments (FtFF and extrusion). Phenolic compounds have been reported to exert antilipogenic properties through various mechanisms. Apigenin is believed to activate 5' adenosine monophosphate-activated protein kinase (AMPK), resulting in reduced lipolytic and adipogenic gene expression (Ono & Fujimori, 2011). Catechin and kaempferol act by enhancing adiponectin expression, which enhances glucose uptake *ex vivo* (Lee, Rao, Chen, Lee & Tzeng, 2009; Cho, Park, Shin, Kim, Shin, Shin, Lee, Lee, Baik & Lee, 2007). Myricetin and quercetin reduce the accumulation of

triglycerides, and rutin down-regulates the adipogenic transcription factors PPARγ and C/EBPα (Chang, Tzeng, Liou, Chang & Liu, 2012; Yang, Della-Fera, Rayalam, Ambati, Hartzell, Park & Baile, 2008; Choi, Park, Choi & Lee, 2006). While cellular lipid studies can be used as an indicator of human physiological activity, studies that show a correlation between these two are lacking, presenting a limitation.

Figure 4-10, Figure 4-11, and Figure 4-12 highlight the key findings of this research concerning the overall effects of FtFF with moringa leaf powder, whether alone or in combination with baobab fruit pulp and extrusion cooking on the nutritional and health-promoting potential of whole-grain sorghum-based porridges and their potential applications. While moringa could be used as a fortificant to increase iron content, it leads to reduced iron bioavailability due to its high levels of anti-nutritional factors. Fortification with moringa, however, produces porridges with enhanced potential health benefits in terms of possibly providing protection against diet-related NCD due to the high phenolic content of moringa. Fortification with baobab, on the other hand, produces porridges with enhanced iron bioaccessibility (probably as a consequence of its high content of organic acids) and potentially enhanced health benefits. Based on these findings, it is useful to investigate the extent to which both moringa and baobab could be used together optimally to achieve enhanced iron bioavailability and health benefits regarding protection against NCDs. Extrusion cooking as a processing technique could be used to produce instant cereal-based porridges with improved iron bioavailability and health benefits.

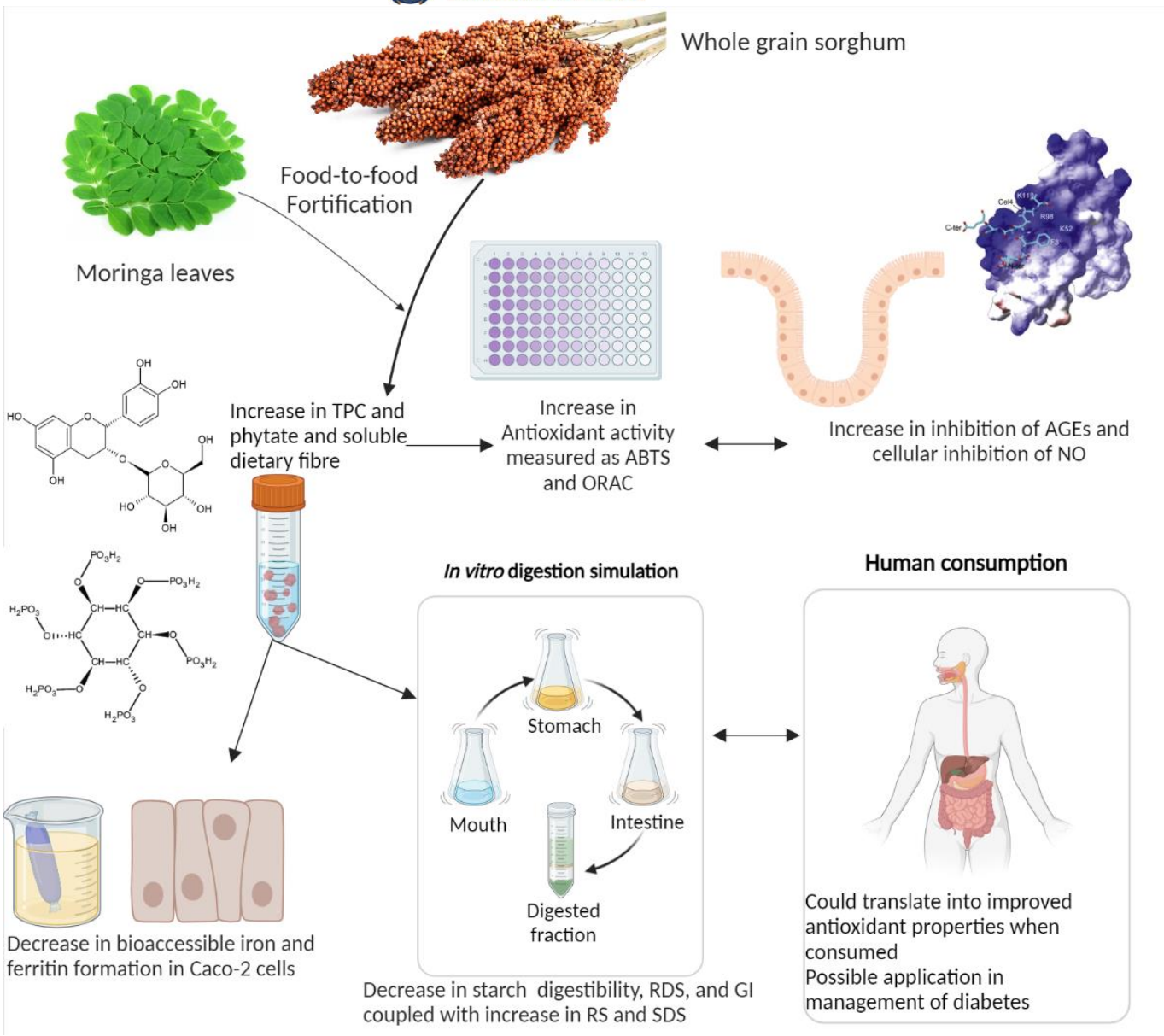


Figure 4-10: Schematic showing major findings concerning FtFF of sorghum-based porridges with moringa leaf powder. RS-resistant starch, RDS- Rapidly digestible starch, GI-estimated glycaemic index, AGEs-Advanced glycation end-products, TPC-total phenolic content, ABTS-2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ORAC-Oxygen Radical Absorbance Capacity, NO-Nitric oxide

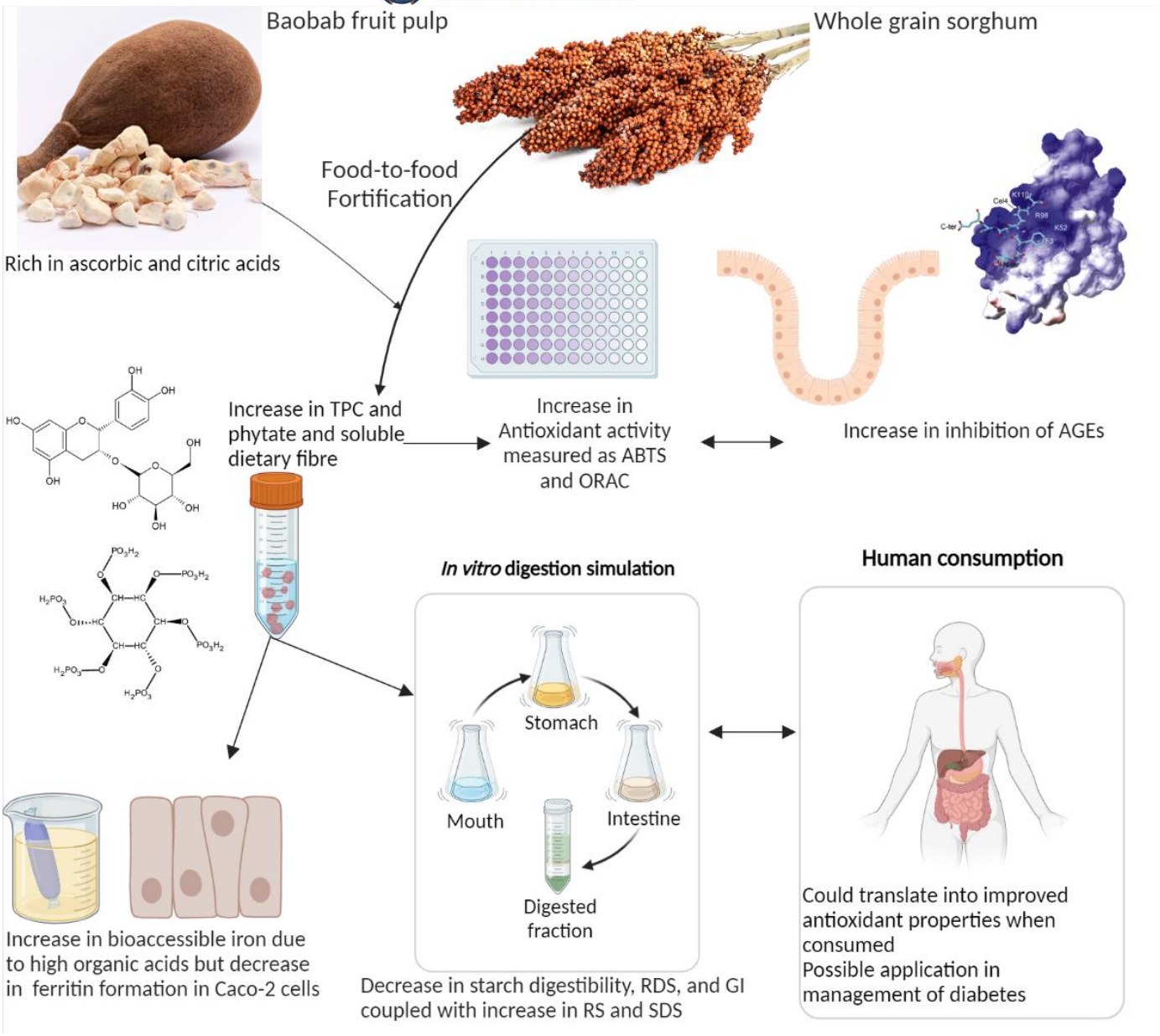


Figure 4-11: Schematic showing major findings following FtFF of sorghum-based porridges with baobab fruit pulp powder. RS-resistant starch, RDS- Rapidly digestible starch, GI-estimated glycaemic index, AGEs- Advanced glycation end-products, TPC-total phenolic content, ABTS-2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ORAC-Oxygen Radical Absorbance Capacity

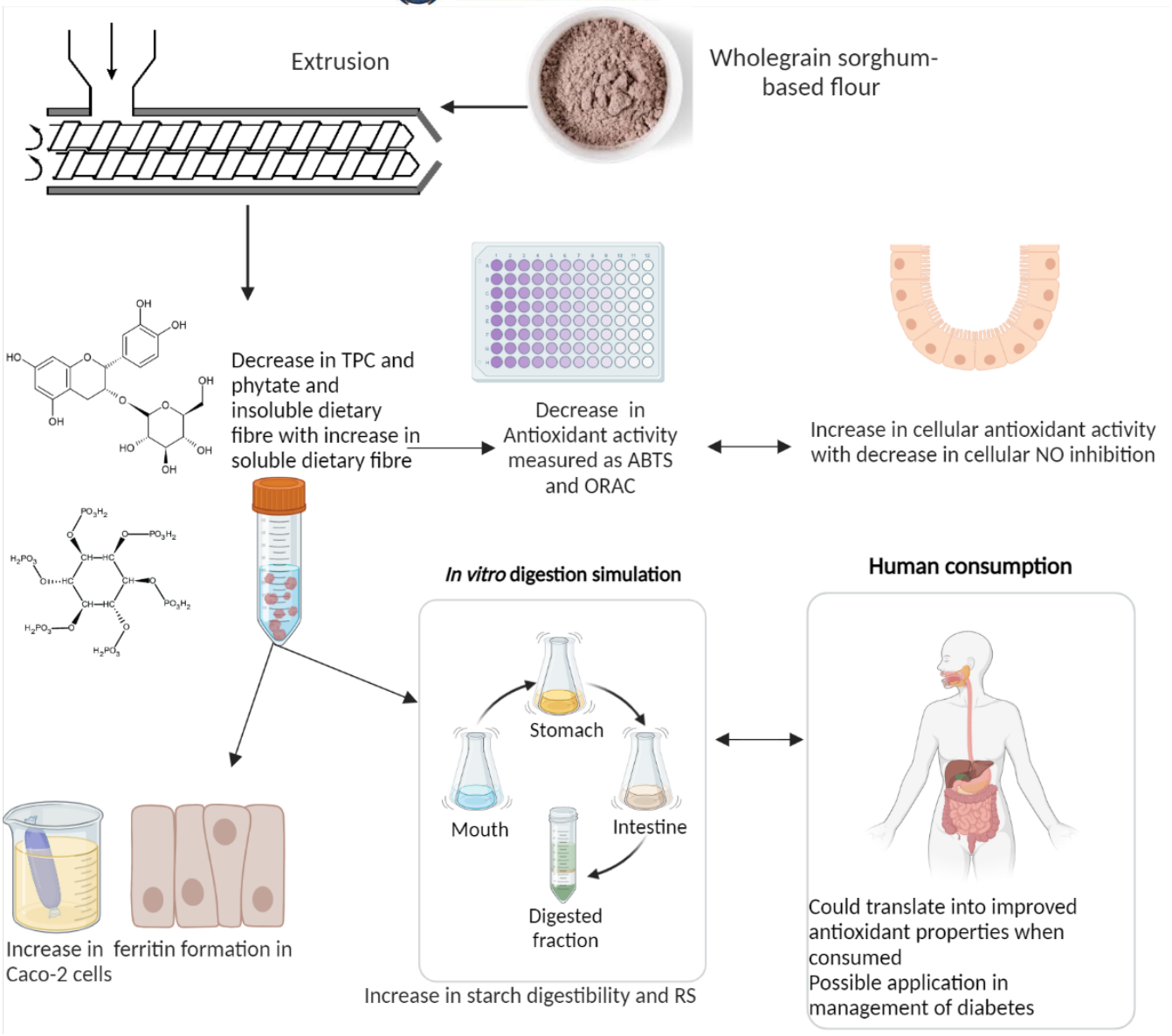


Figure 4-12: Schematic showing major findings following extrusion cooking of FtF-fortified sorghum. RS-resistant starch, TPC-total phenolic content, ABTS-2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ORAC-Oxygen Radical Absorbance Capacity, NO-Nitric oxide

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

Sorghum-based porridges FtF-fortified baobab fruit pulp (BFP) had higher iron bioaccessibility measured as percentage bioaccessible iron (PBI). However, fortification with moringa leaf powder (MLP) significantly reduced the PBI and bioaccessible iron content (BIC). This indicates that the type of plant foodstuff used for FtFF has an effect on the resultant iron bioaccessibility obtained. The increase in PBI following fortification with BFP could be attributed to the high content of organic acids (citric and organic acids) in the BFP that are well-known mineral bioaccessibility enhancers. BFP is also low in phytate, a mineral bioaccessibility inhibitor that could account for the improvement in PBI. On the other hand, MLP is high in mineral bioaccessibility inhibitors, namely, polyphenols, calcium and phytate. Polyphenols and phytate form insoluble complexes with iron, and stable, insoluble complexes can be formed between iron, phytate and calcium, which has the overall effect of reducing bioaccessible iron. These findings confirmed the first hypothesis of this study and highlight FtFF with baobab fruit pulp, an essential investigation in this study, as a novel technique for improving iron bioaccessibility. The application of FtFF presents iron deficiency at-risk communities within sub-Saharan Africa (where sorghum and baobab are prevalent) with a sustainable strategy for improving iron bioaccessibility, an important aim of this research study.

Extrusion-cooked instant sorghum-based porridges had increased ferritin formation by Caco-2 cells when compared to conventionally wet-cooked porridges, which is indicative of an enhancing effect of extrusion cooking on iron bioaccessibility and uptake. This is mainly due to the ability of extrusion cooking to reduce the contents of the mineral bioaccessibility inhibitors phytate (probably by dephosphorylation) and polyphenols (probably by degradation). Instant sorghum porridges fortified with BFP produced higher ferritin formation in Caco-2 cells than porridges where MLP is used for FtFF. This is a further indication of the importance of the role of the type of plant foodstuff used for FtFF in mineral bioaccessibility. This was critical because it further confirmed the first hypothesis of this study, place extrusion cooking and instant products at the centre of addressing iron deficiency through potentially improving iron bioaccessibility. This study provides a novel application for measuring the effect of extrusion cooking on iron bioaccessibility using a physiological system, a combination of *in vitro* iron dialysability and ferritin formation by Caco-2 cells. This is the first research

undertaken to show the augmenting effect of extrusion cooking on ferritin formed by Caco-2 cells treated with digested wholegrain sorghum porridge extracts.

FtFF of wholegrain sorghum-based porridges with BFP and MLP enhanced health-promoting properties of sorghum-based porridges in terms of radical scavenging activity (ABTS and ORAC) (protection against oxidative stress), cellular nitric oxide (NO) inhibition (anti-inflammatory properties) and inhibition of advanced glycation end products (AGEs) formation (anti-diabetic properties). The observed enhanced health-promoting properties can be related to the enhanced levels of various bioactive phenolics in the sorghum-based porridges after FtFF. These bioactive phenolics range from phenolic acids and phenolic acid esters to flavonoids and flavonoid glycosides. Phenolic extracts from the sorghum-based porridges show protection against AAPH radical-induced oxidation in Caco-2 cells, an indication of their potential ability to protect against radical-induced oxidative stress. These findings confirm the second hypothesis of this study, presenting a novel angle of exploring FtFF with baobab and moringa aimed at augmenting the phenolic profile and health-promoting profile of wholegrain sorghum-based porridges in communities at-risk of contracting chronic diet-related NCDs. Studies exploring *in vivo* and human methods should be further explored to drive the potential application in curbing chronic diet-related NCDs.

Extracts from all the sorghum-based porridges reduced *in vitro* chemical formation of NO, an indication of their potential to contribute to alleviating radical-induced inflammation. Cellular inhibition of NO production in RAW264.7 macrophages revealed that FtFF significantly improved the inhibition of cellular NO production in RAW264.7 macrophages, whilst extrusion resulted in a reduction. The improvement in cellular NO inhibition in RAW264.7 macrophages may be attributed to the enhancement of the phenolic profile of sorghum-based porridges following FtFF with baobab and moringa. However, extrusion-cooked instant porridges exhibit decreased inhibition of NO formation in RAW264.7 macrophages, possibly due to their reduced phenolic content as a result of the extrusion cooking process. These findings also confirm the second hypothesis of this study and, as previously discussed, present a potential for FtFF as a technique to improve the phenolic profile and antioxidant properties of wholegrain sorghum-based porridges prepared through FtFF.

Extracts from all the sorghum-based porridges showed prevention and treatment of accumulated adipocytes in 3T3-L1 cells, indicating their potential application in the management of obesity. The porridges also exhibited anti-diabetic properties through their

ability to reduce the formation of AGEs. The FtFF porridges, in particular, significantly reduced the formation of AGEs, possibly due to the increase in phenolic content and higher antioxidant activity following FtFF with BFP and MLP.

Sorghum-Bambara groundnut composite (SBC) porridges (whether extrusion- or conventionally wet-cooked) FtF-fortified with BFP and MLP (whether alone or in combination) showed a marked reduction in starch digestibility (decreased rapidly digestible starch, RDS, coupled with increased slowly digestible starch, SDS and resistant starch, RS) and estimated glycaemic index (GI) compared to the unfortified composite. This decrease in starch digestibility and estimated GI suggest a potential for the application of these porridges in the management of type 2 diabetes. The reduction in the RDS, GI and increase in SDS following FtFF with BFP and MLP could be attributed to the high levels of antinutritional compounds - polyphenolics, phytate, and soluble and insoluble dietary fibre (SDF and IDF) in the fortificants that reduce starch hydrolysis. These antinutritional compounds, such as dietary fibre (which entraps starch molecules), polyphenols (which complex with starch and starch hydrolysing enzymes) and starch reducing digestibility and could also bind enzymes responsible for the digestion of the starch. This is in line with the third hypothesis of this study and should further be investigated with systems that would better predict potential applications in managing type 2 diabetes, such as animal trials and human intervention studies.

Extrusion-cooked instant SBC porridges had higher resistant starch (RS), RDS, and protein digestibility (IVPD) with lower SDS in comparison with conventionally cooked porridges. The higher RDS, IVPD and lower SDS could be due to the dextrinization of starch and the reduction in antinutritional compounds (that bind both starch and proteins) that occur because of the high temperature, shear and pressure, making the starch and protein molecules more susceptible to enzymatic hydrolysis and thus accounting for the increased RDS, IVPD, and lower SDS. Extrusion-cooked SBC porridges in this study had higher SDF and lower IDF than conventionally wet-cooked porridges, which is attributed to the hydrolysis of the glycosidic bonds in the IDF as a result of extrusion cooking solubilizing it into SDF. This increase in SDF could account for the increase in SDS as the gelatinized, and disrupted starch molecules could be entrapped in the SDF, making them less accessible for enzymatic hydrolysis. Another possible cause could be the retrogradation of the starch in the extruded porridges during storage forming enzyme-resistant starch. The high RS content of these instant sorghum-based porridges makes them possibly relevant in managing type 2 diabetes. This underpins the third

hypothesis of this study, and while further research is needed before application in managing type 2 diabetes, these results are proof of concept.

Extrusion-cooked SBC porridges had lower pasting viscosities, probably due to the dextrinization of starch (the primary biopolymer responsible for pasting) during high temperature, shear and pressure conditions during extrusion cooking. This provides a shear-thinning porridge, which could increase nutrient intake for infants who have difficulty orally processing thick foods and thus preventing the prevalence of PEM.

While further studies that incorporate *in vivo* methods of analysis and, where possible, human studies should be explored before the techniques applied in this study can be conclusive, this study is a proof of concept that FtFF can be used to alter particular properties of cereal staples to address the double burden of malnutrition and diet-related NCDs. At the centre of this approach is extrusion cooking as a technique to reduce the antinutritional properties of the final product and deliver healthy and nutritious products to at-risk communities.

It is recommended that studies on the bioavailability of iron and dietary phenolics from these fortified porridges, using models such as *ex vivo* inverted rat intestine and *in vivo* animal models, be studied to better understand their contribution to alleviating iron deficiency and oxidative stress in physiological systems.

Future studies on the consumer sensory acceptability, satiety-promoting potential and oral processing properties of the fortified porridges are needed to provide valuable information about the commercial potential of the instant sorghum-based porridges fortified with baobab and moringa.

CHAPTER 6 REFERENCES

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CHAPTER 7 APPENDIX

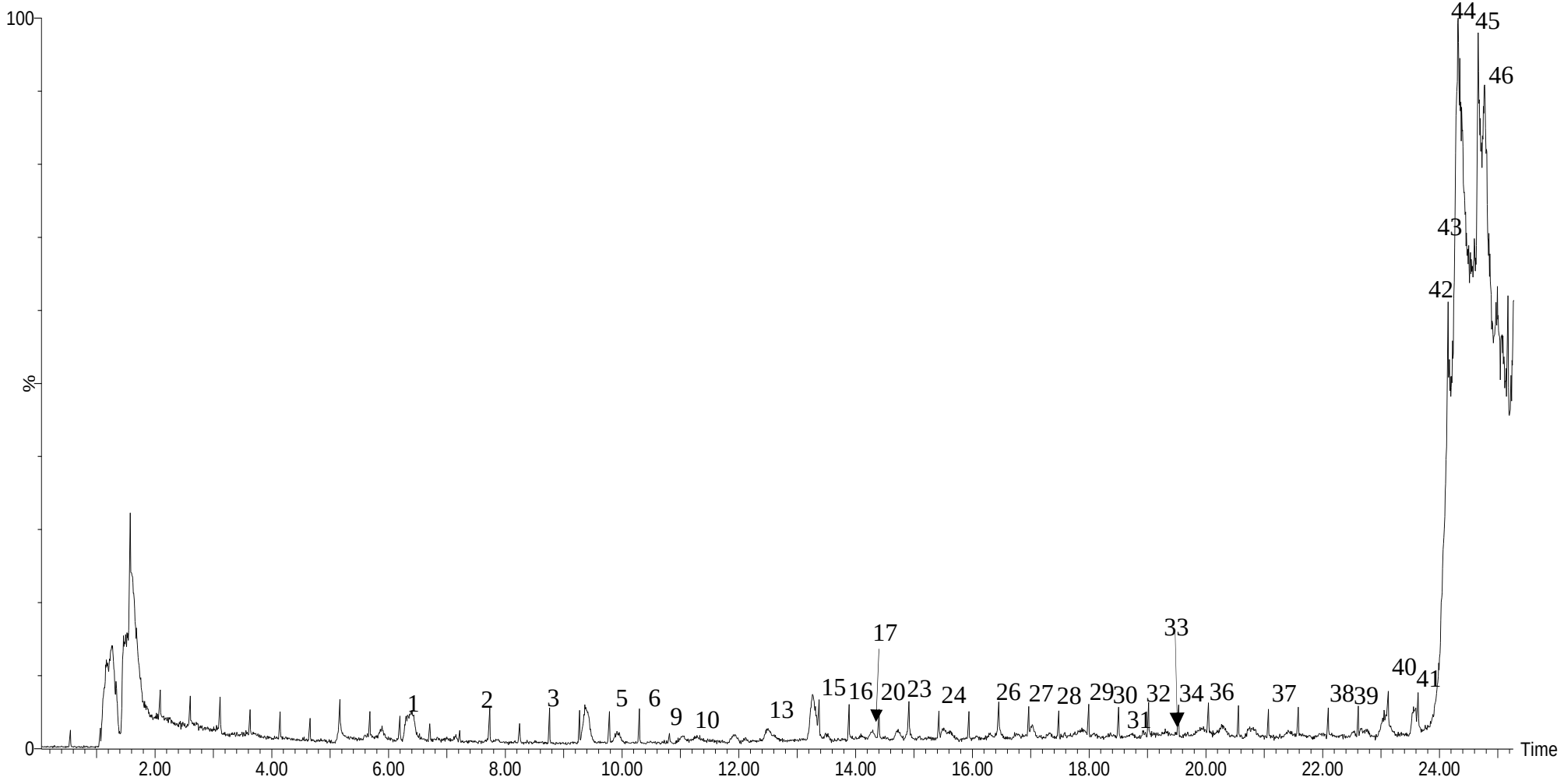

Figure 7-1: Total ion chromatogram of 1% (v/v) HCl in methanol extract from cooked wholegrain sorghum flour

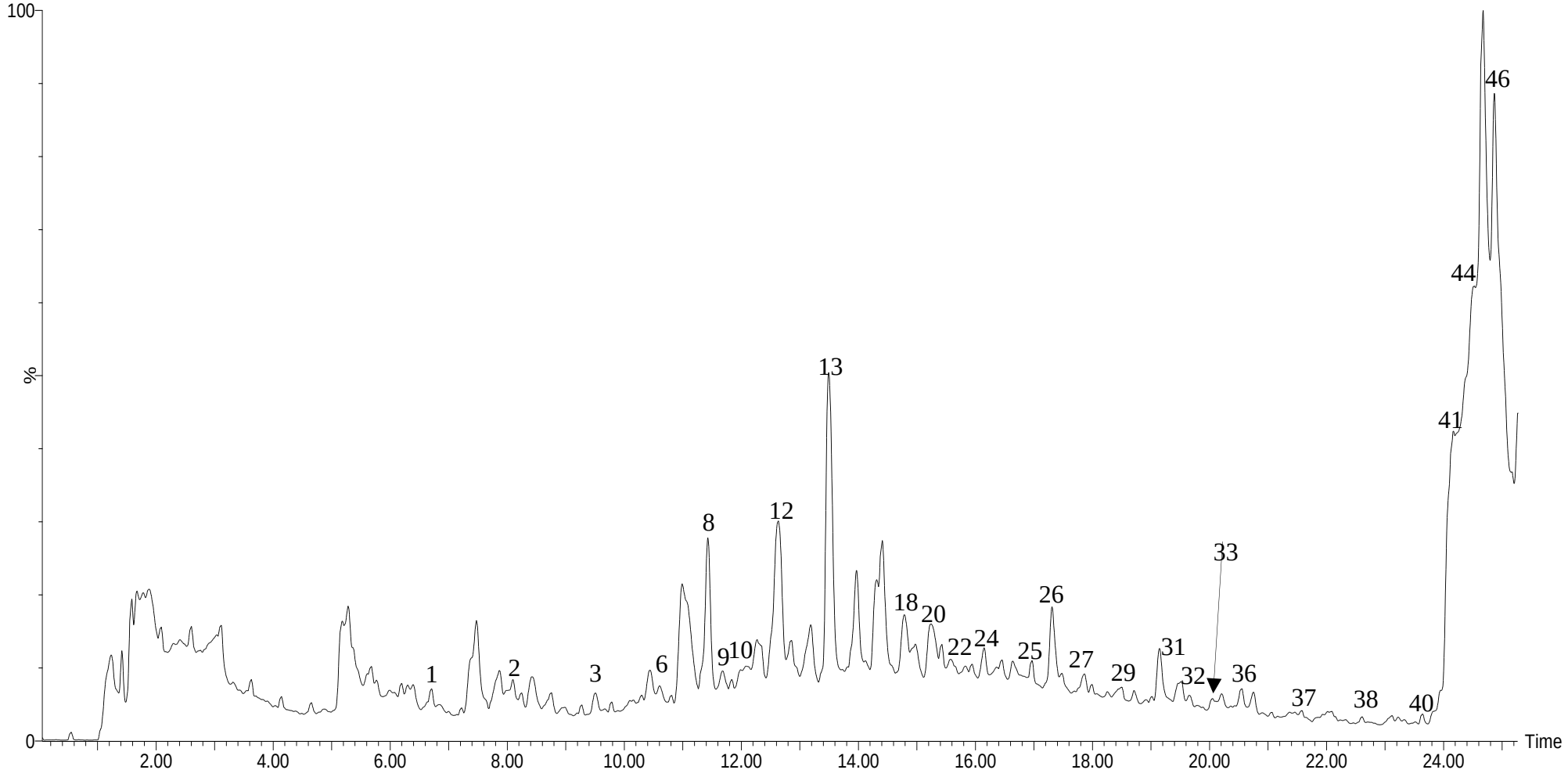

Figure 7-2: Total ion chromatogram of 1% (v/v) HCl in methanol extract from baobab fruit pulp powder

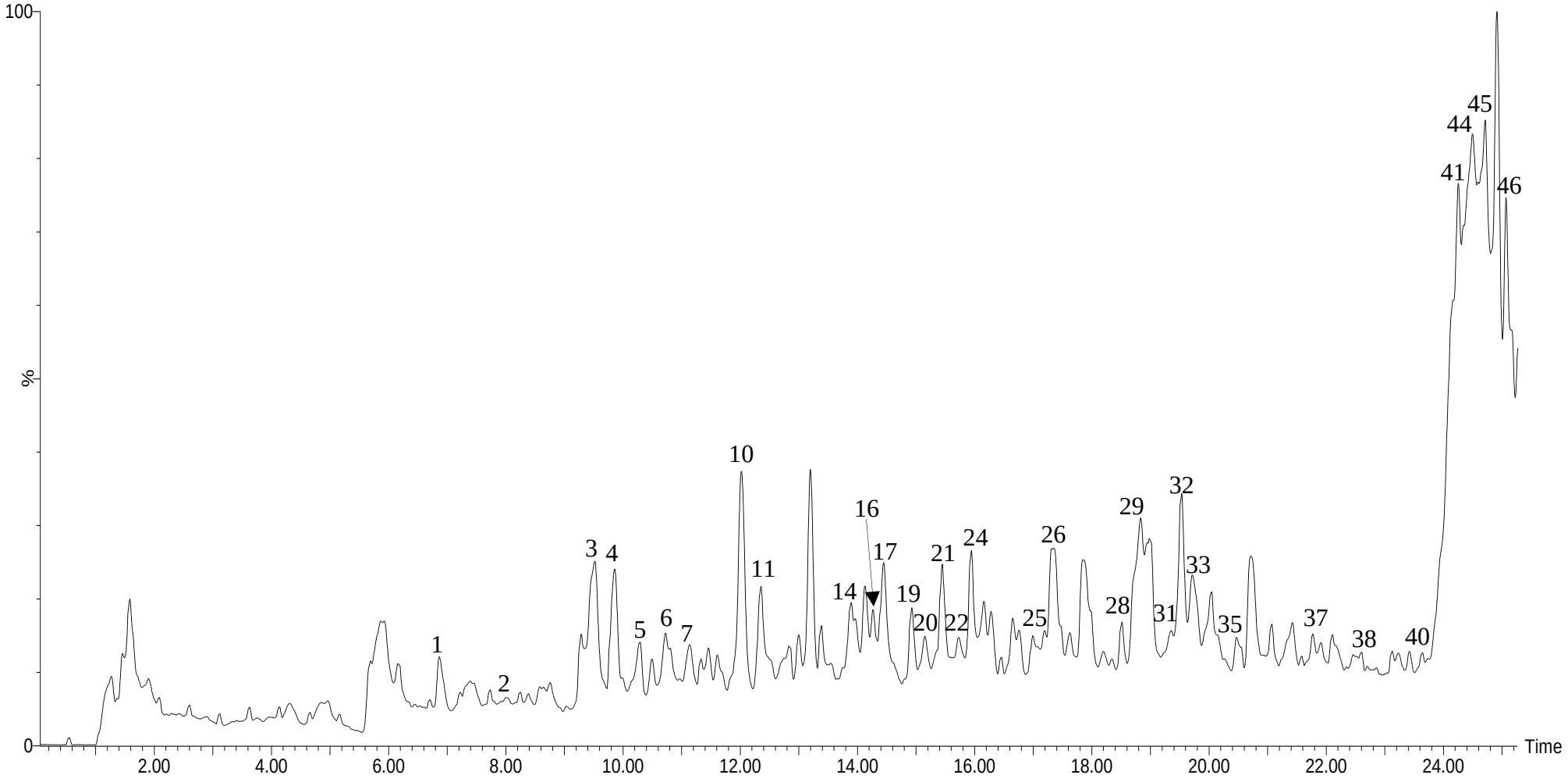

Figure 7-3: Total ion chromatogram of 1% (v/v) HCl in methanol extract from moringa leaf powder

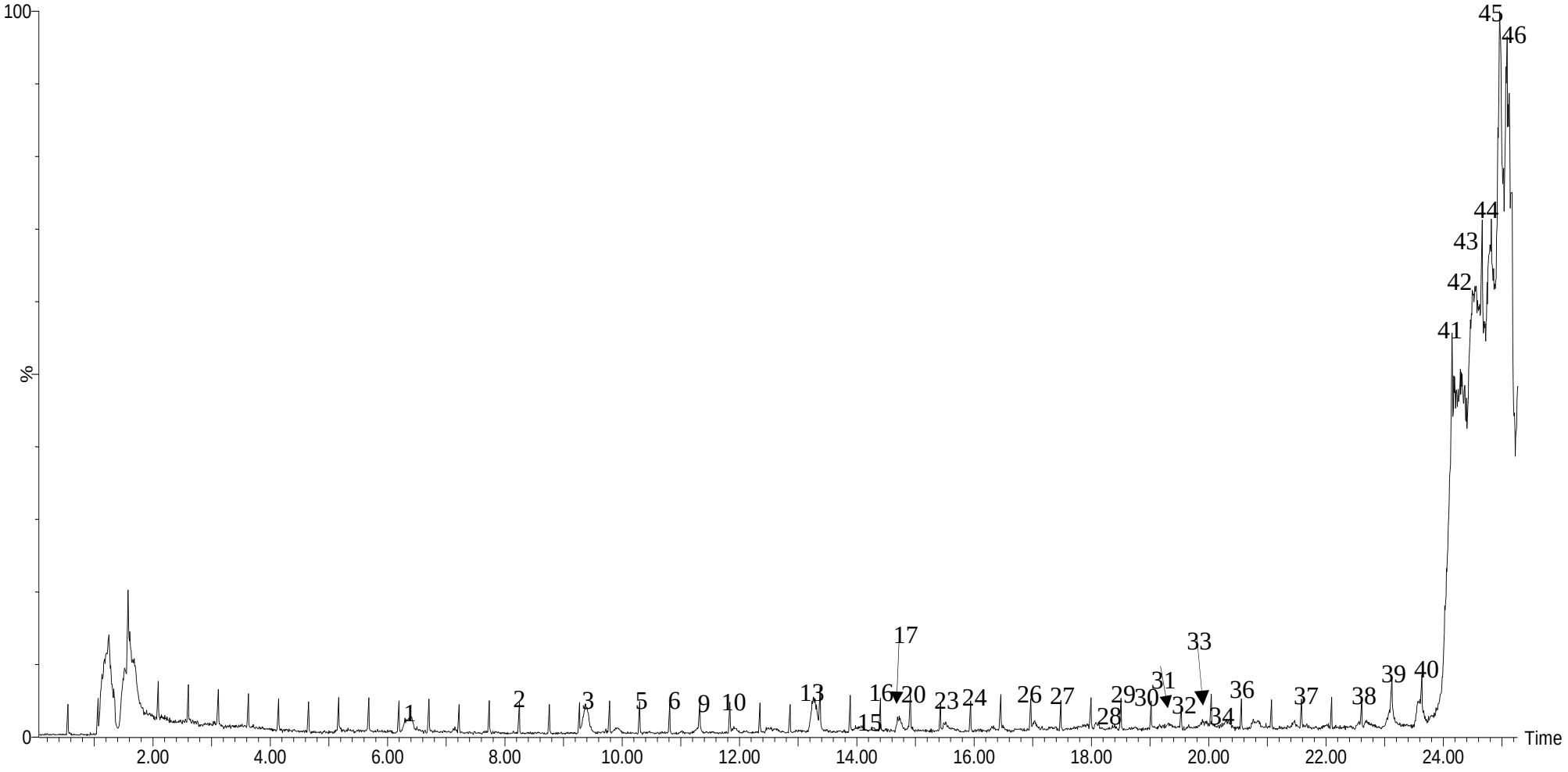

Figure 7-4: Total ion chromatogram of 1% (v/v) HCl in methanol extract from extruded wholegrain sorghum flour

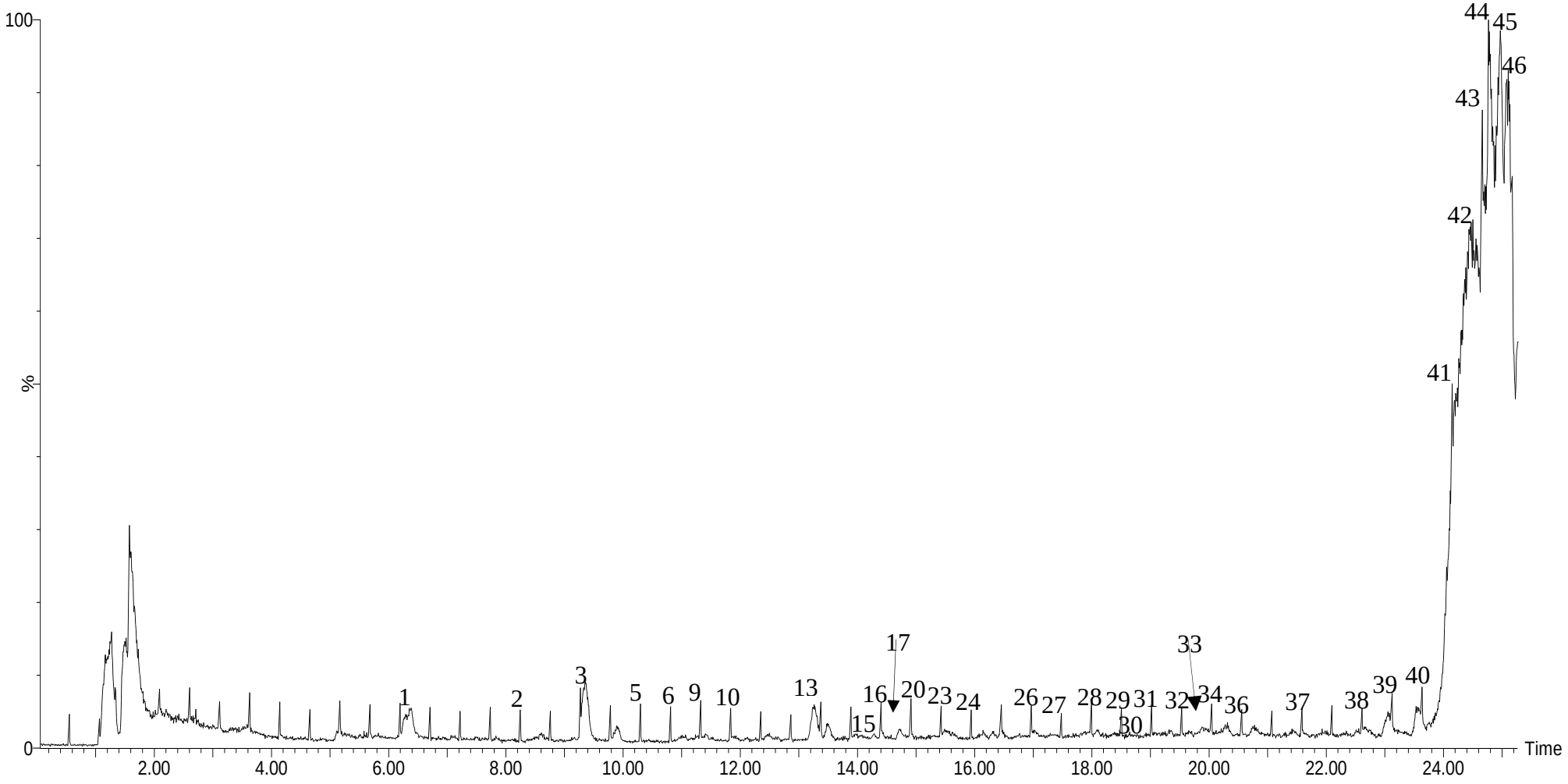

Figure 7-5: Total ion chromatogram of 1% (v/v) HCl in methanol extract from wholegrain sorghum flour co-extruded with baobab fruit pulp powder

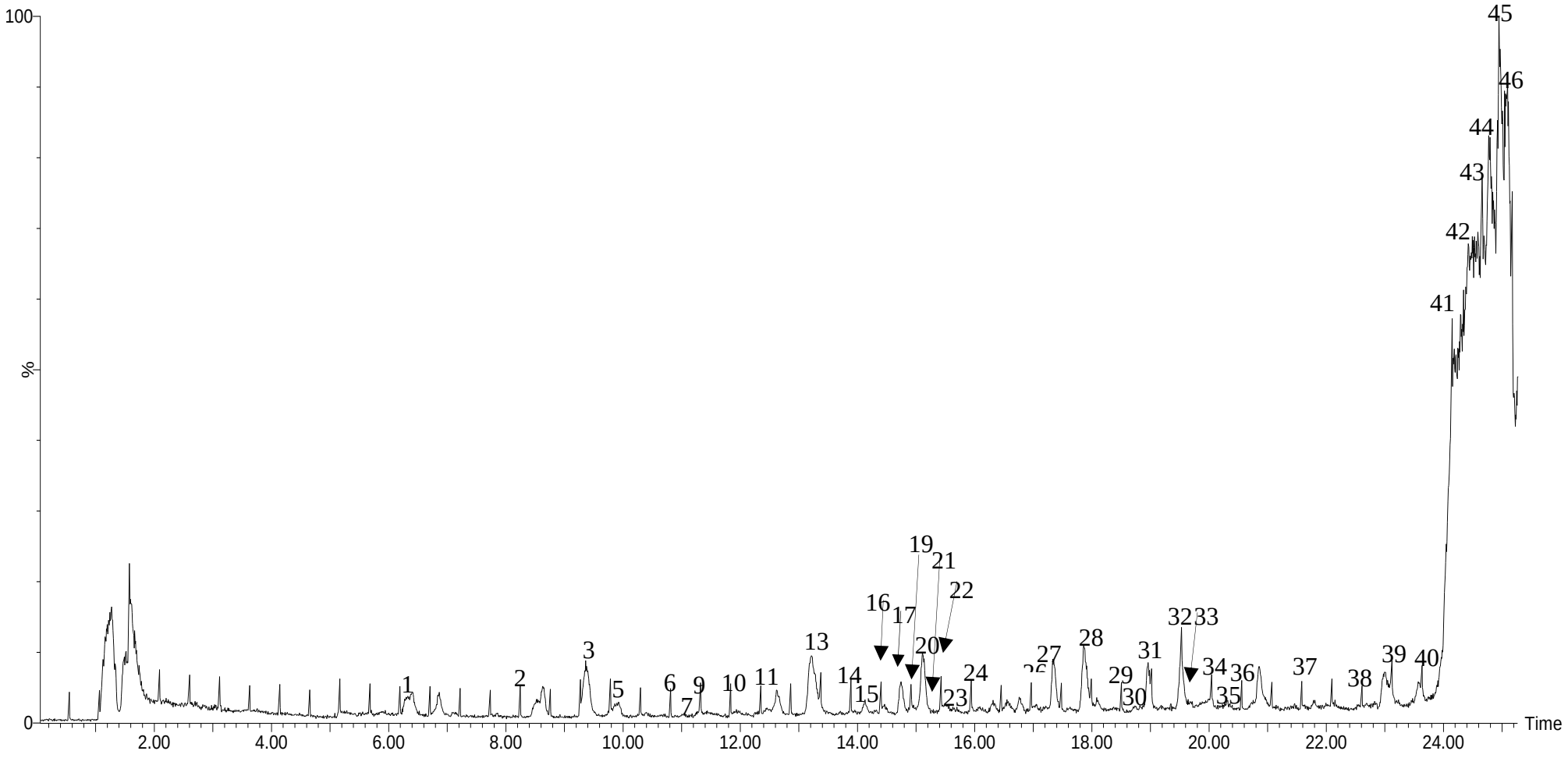

Figure 7-6: Total ion chromatogram of 1% (v/v) HCl in methanol extract from wholegrain sorghum flour co-extruded with moringa leaf powder

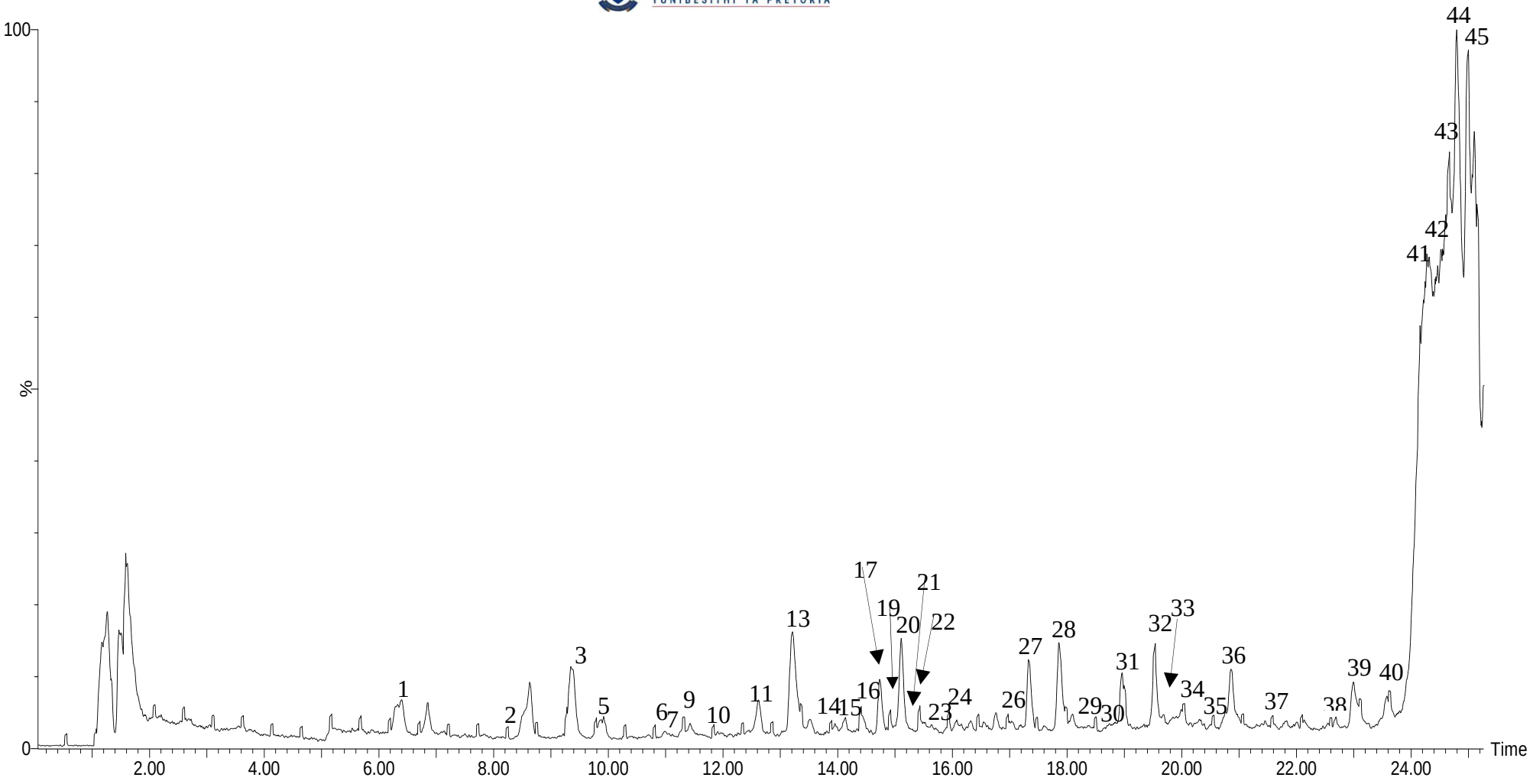

Figure 7-7: Total ion chromatogram of 1% (v/v) HCl in methanol extract from wholegrain sorghum flour co-extruded with baobab fruit pulp and moringa leaf powders

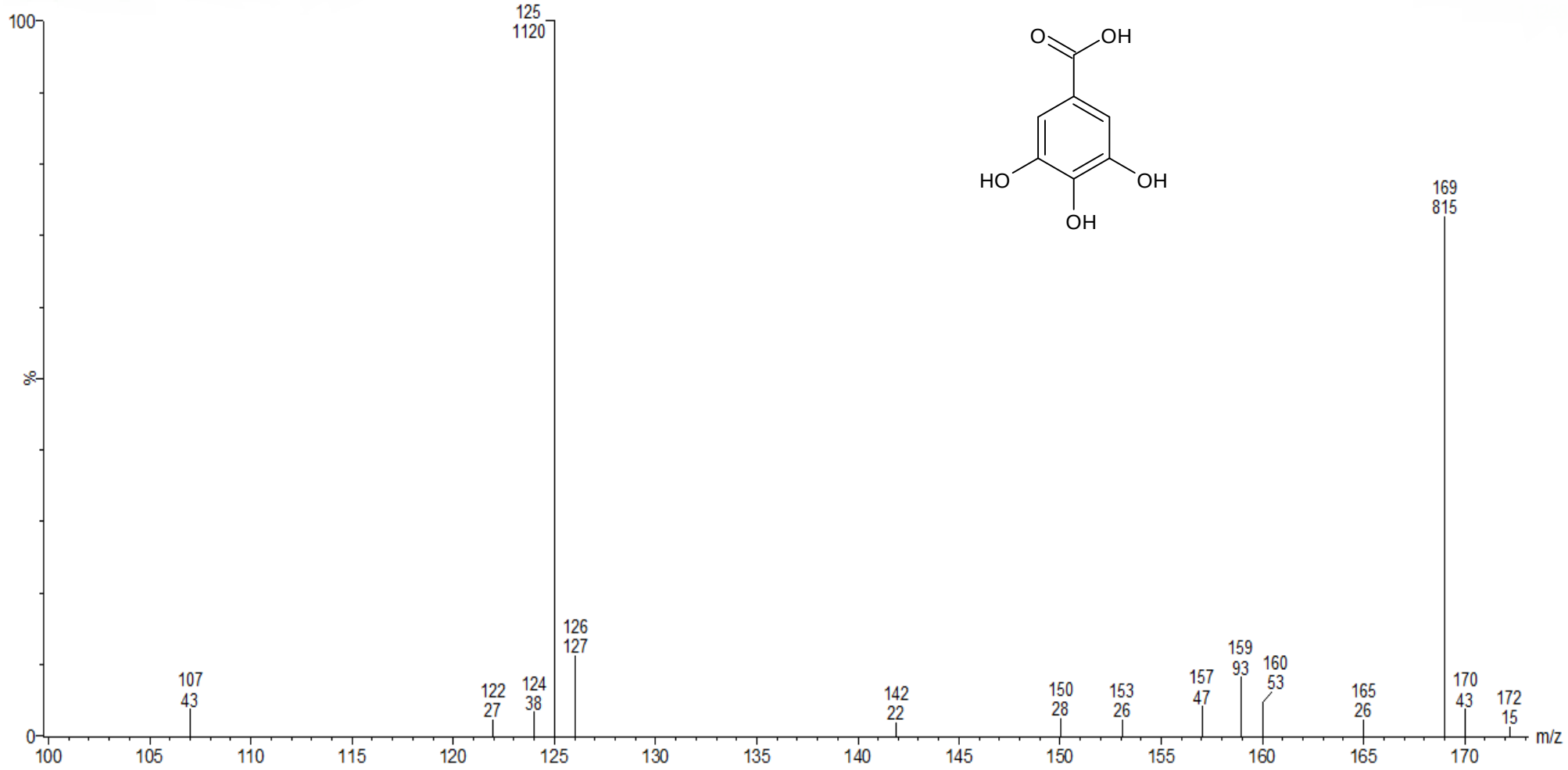

Figure 7-8: Mass spectrum of gallic acid (m/z at 169) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

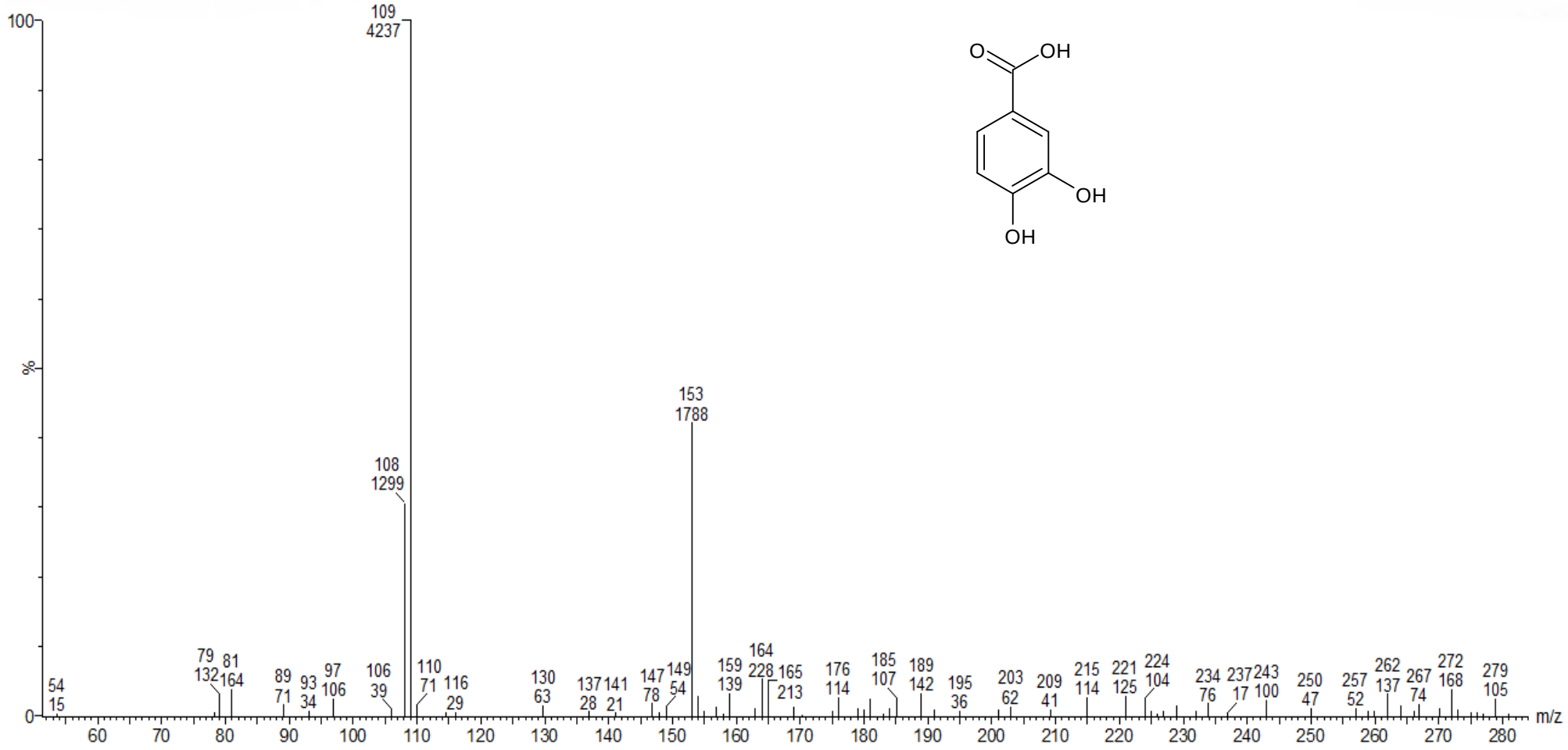

Figure 7-9: Mass spectrum of protocatechuic acid (m/z at 153) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

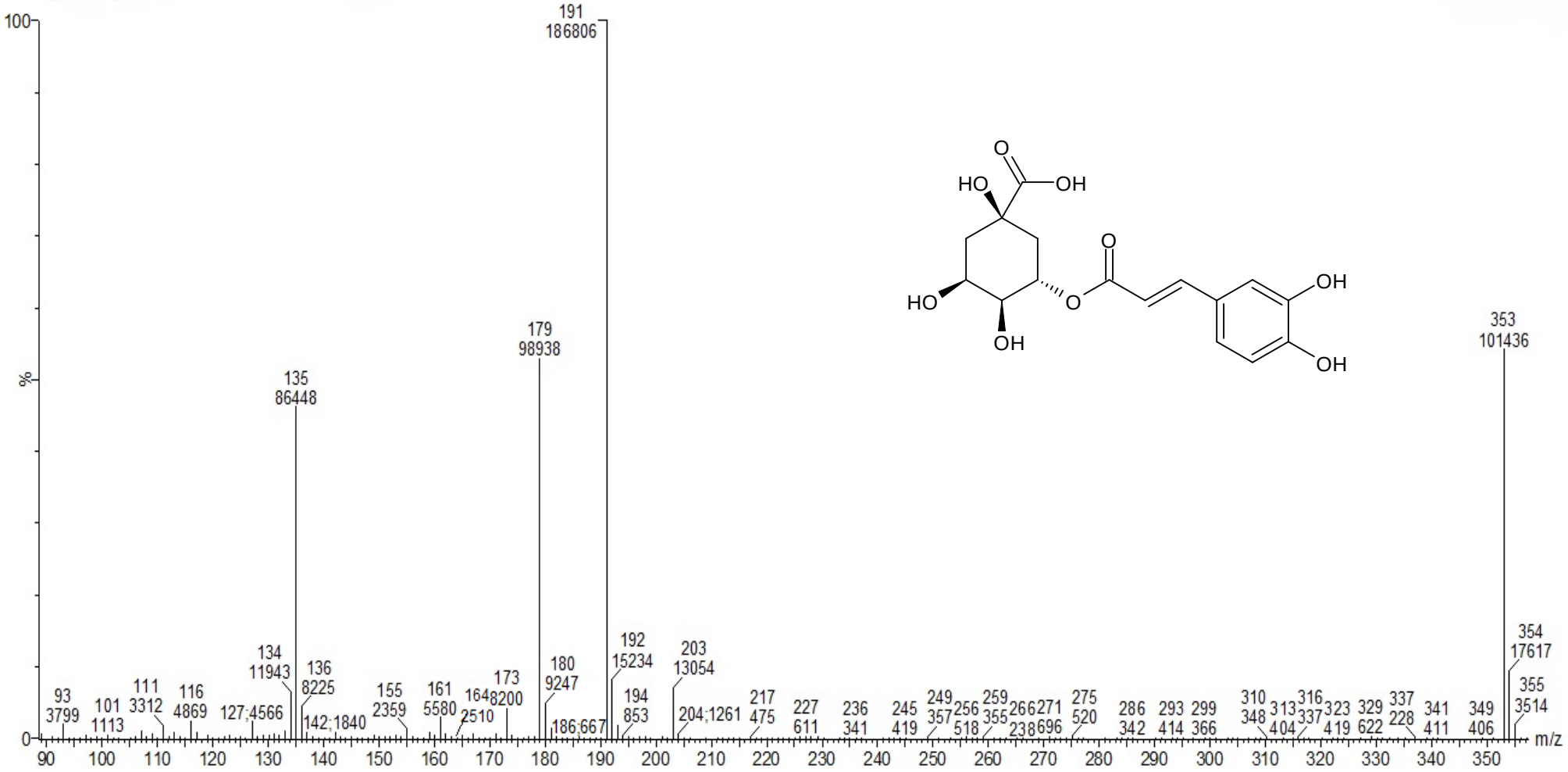

Figure 7-10: Mass spectrum of 3-Caffeoyl-quinic acid (m/z at 353) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

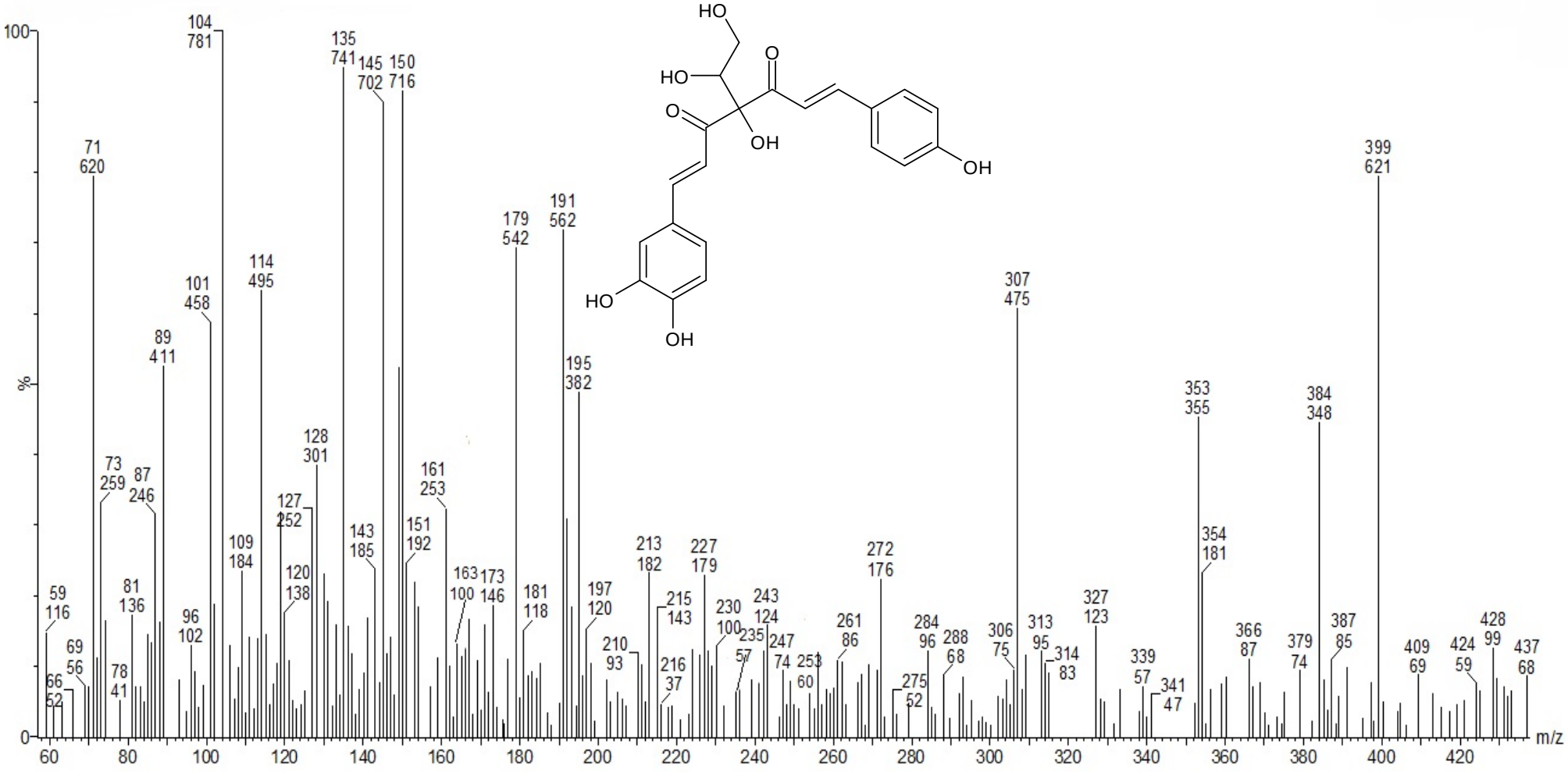

Figure 7-11: Mass spectrum of Coumaroyl-caffeoyl-glycerol (m/z at 399) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

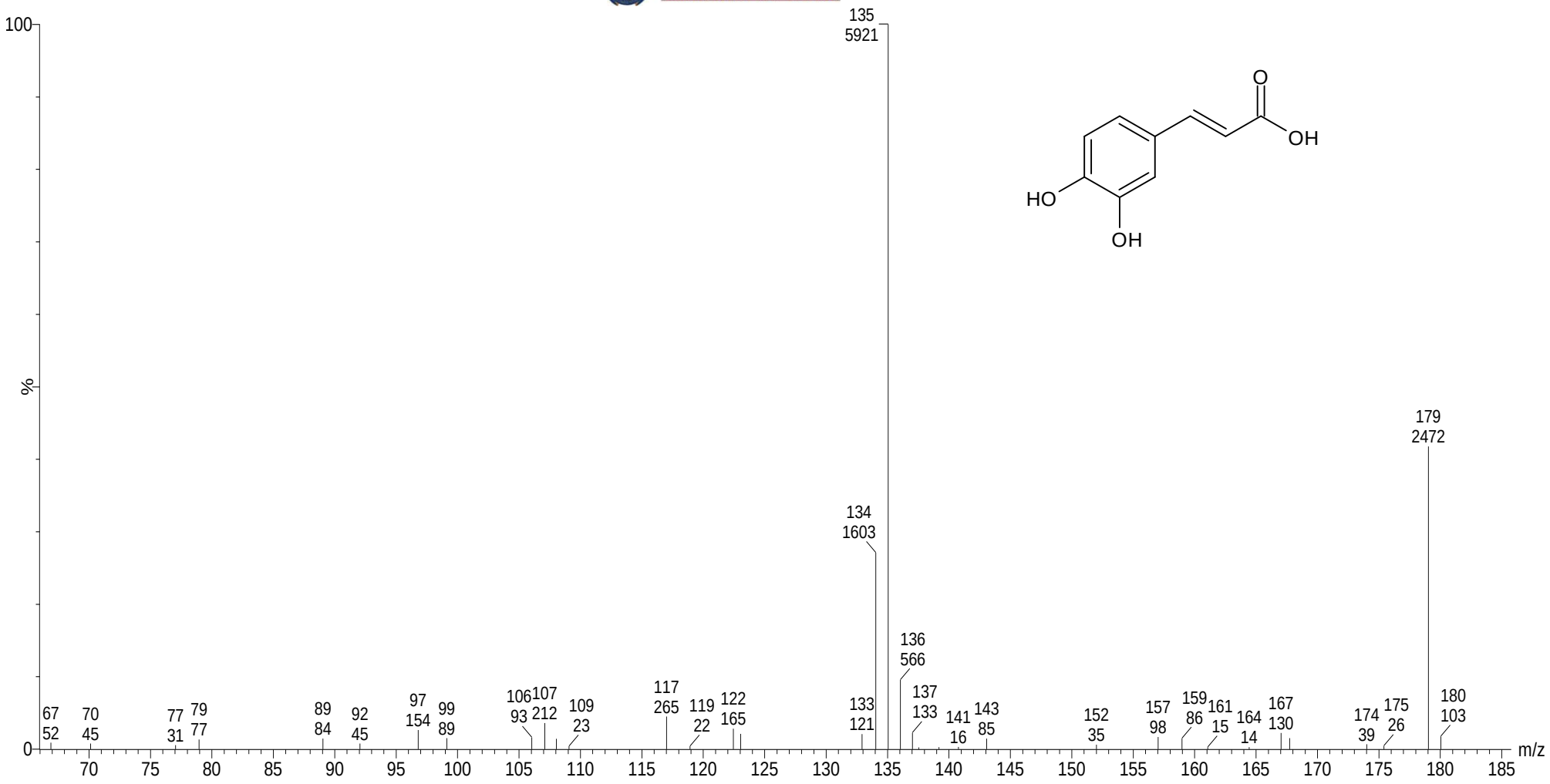

Figure 7-12: Mass spectrum of caffeic acid (m/z at 179) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

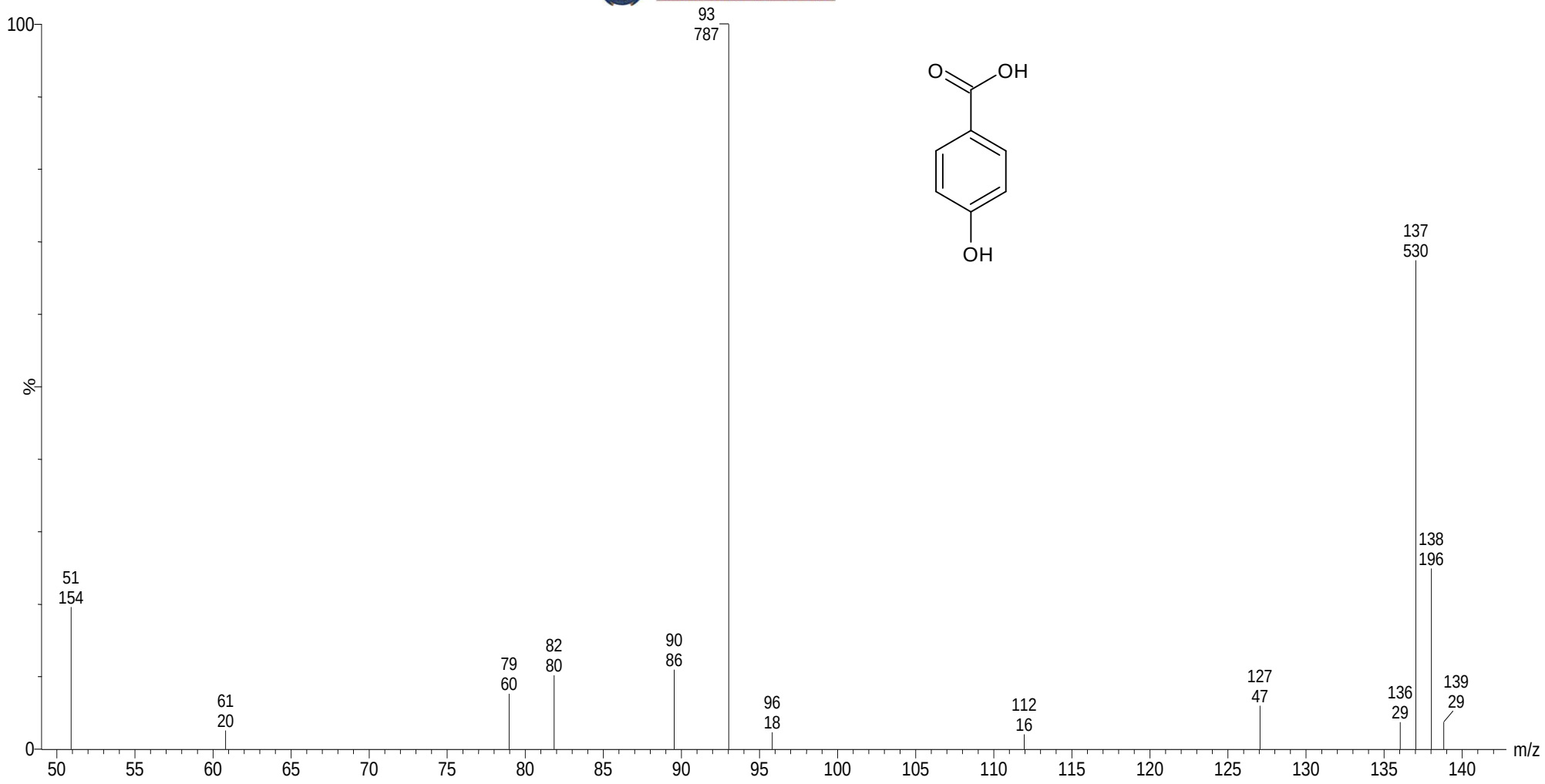

Figure 7-13: Mass spectrum of 4-hydroxy benzoic acid (m/z at 137) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

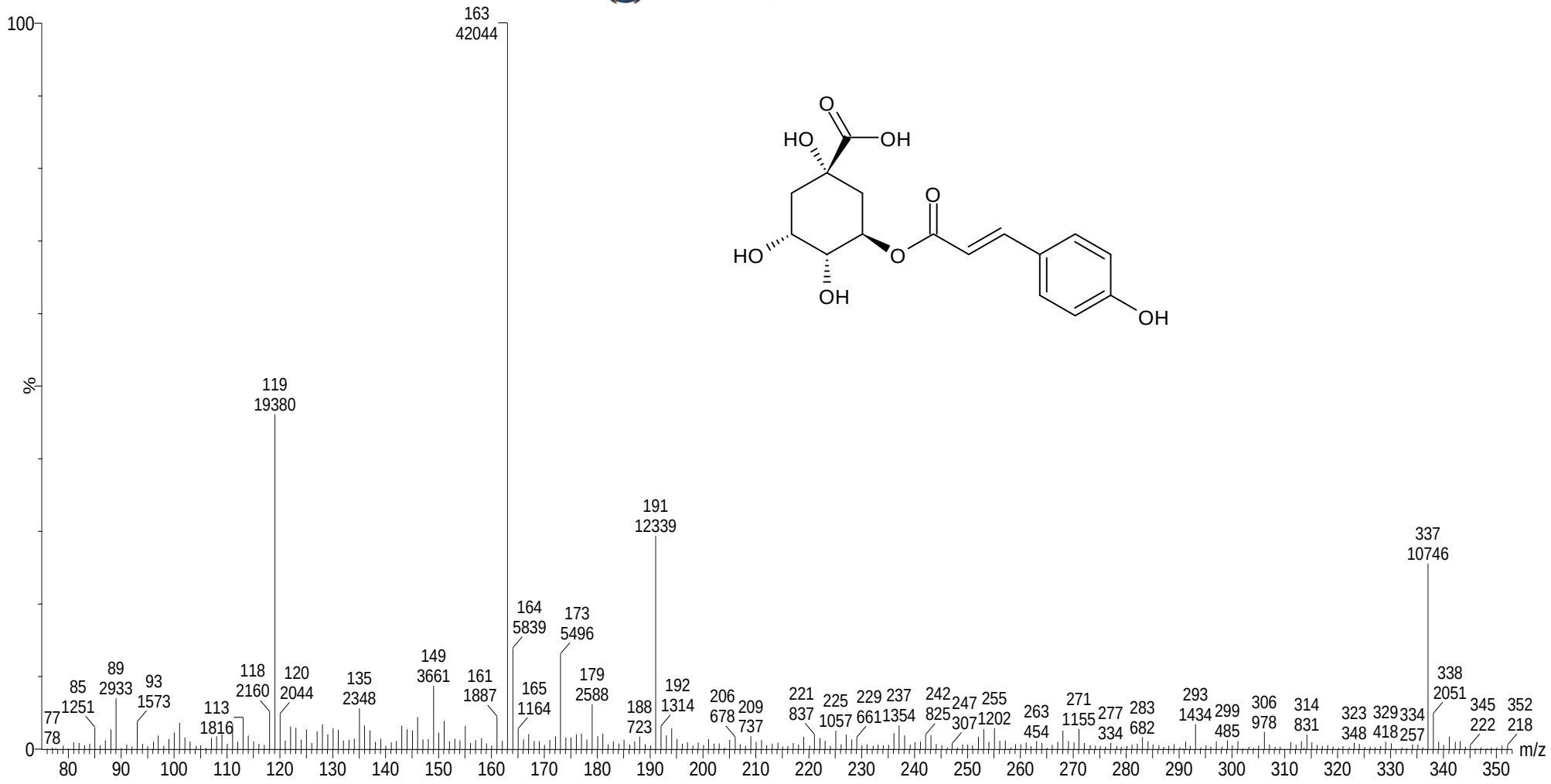

Figure 7-14: Mass spectrum of 3-ρ-coumaroylquinic acid *(m/z* at 337) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

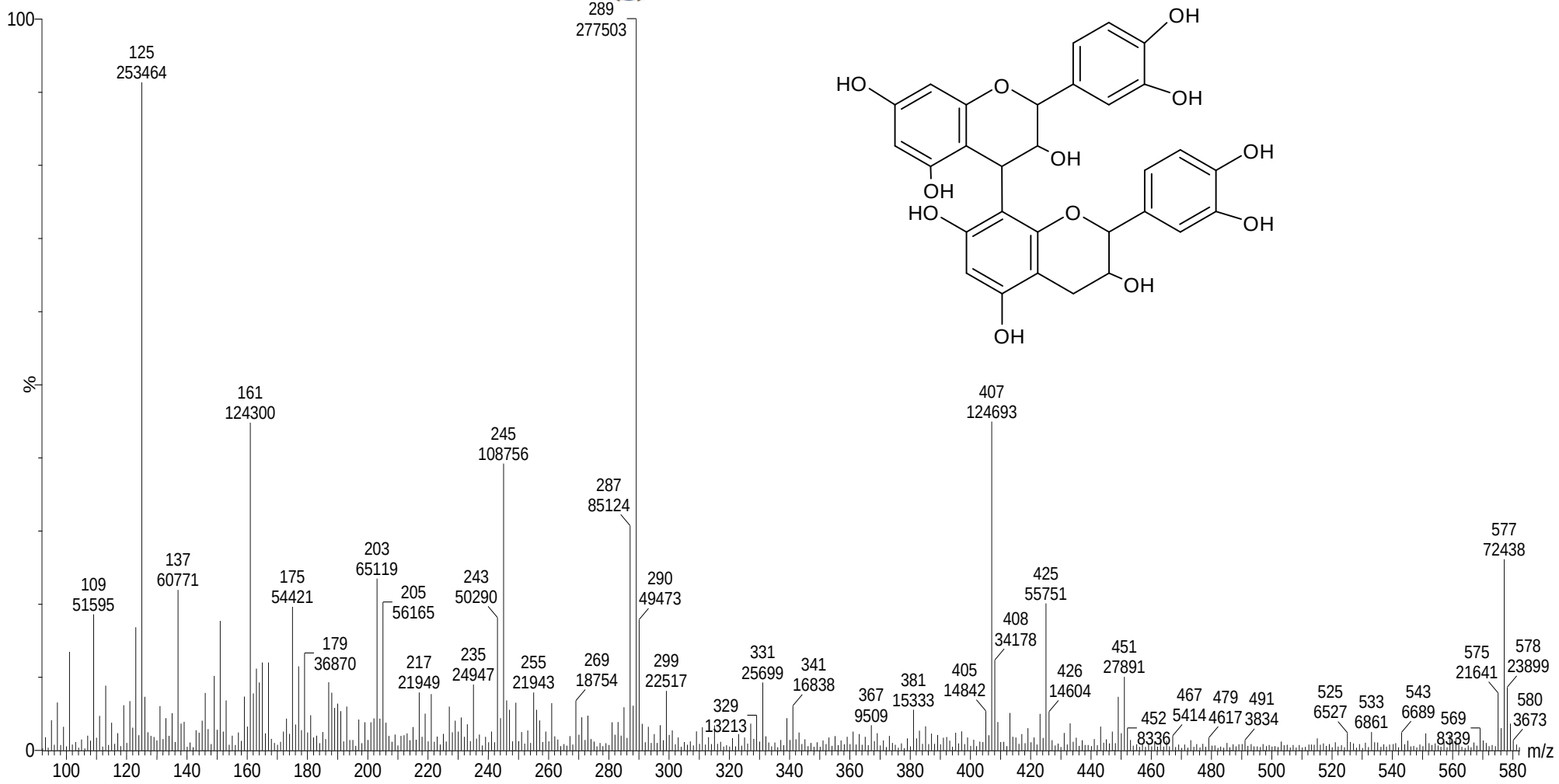

Figure 7-15: Mass spectrum of procyanidin dimer (m/z at 353) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

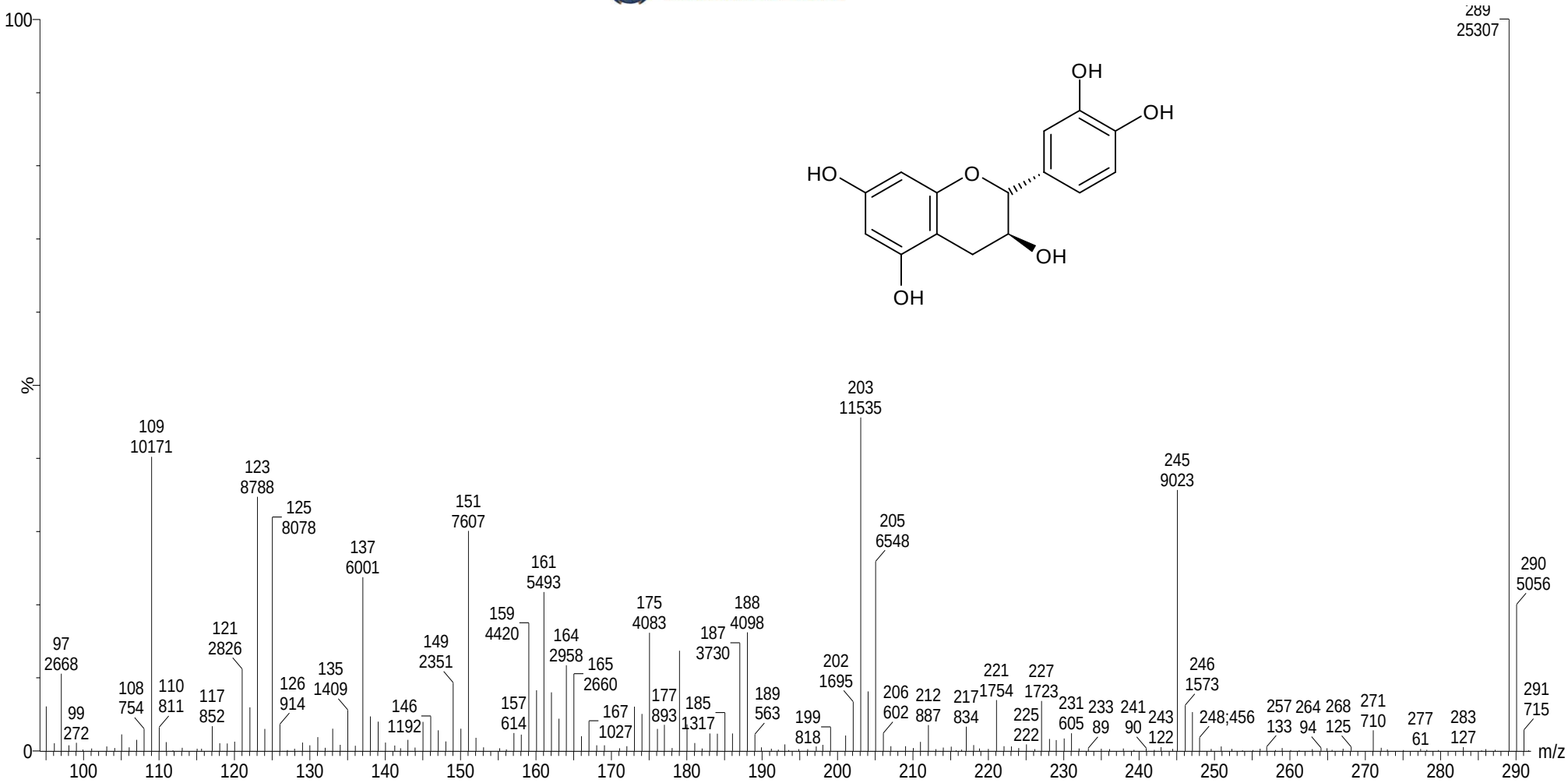

Figure 7-16: Mass spectrum of catechin *(m/z* at 289) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

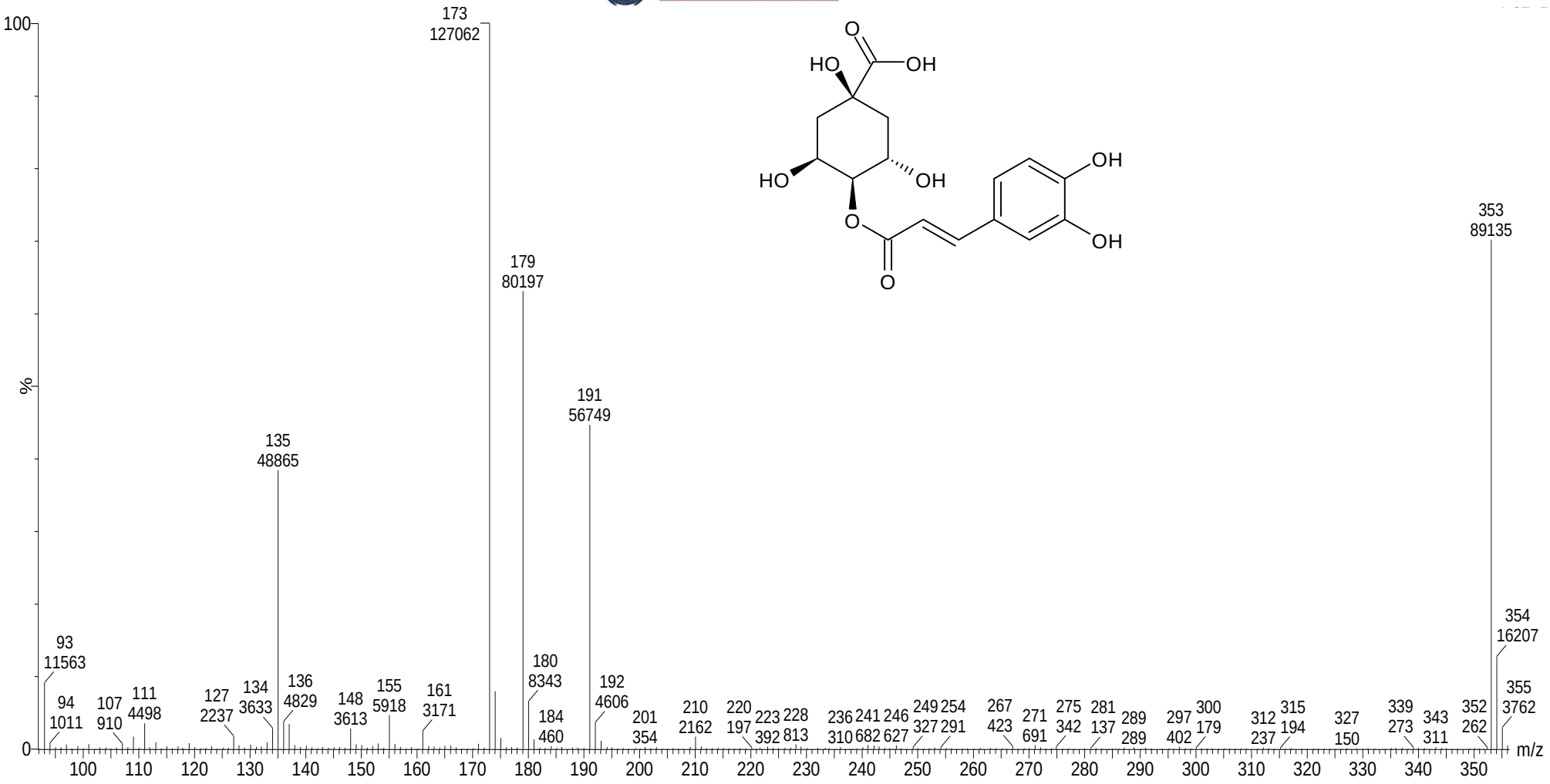

Figure 7-17: Mass spectrum of 4-caffeoyl-quinic acid (m/z at 353) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

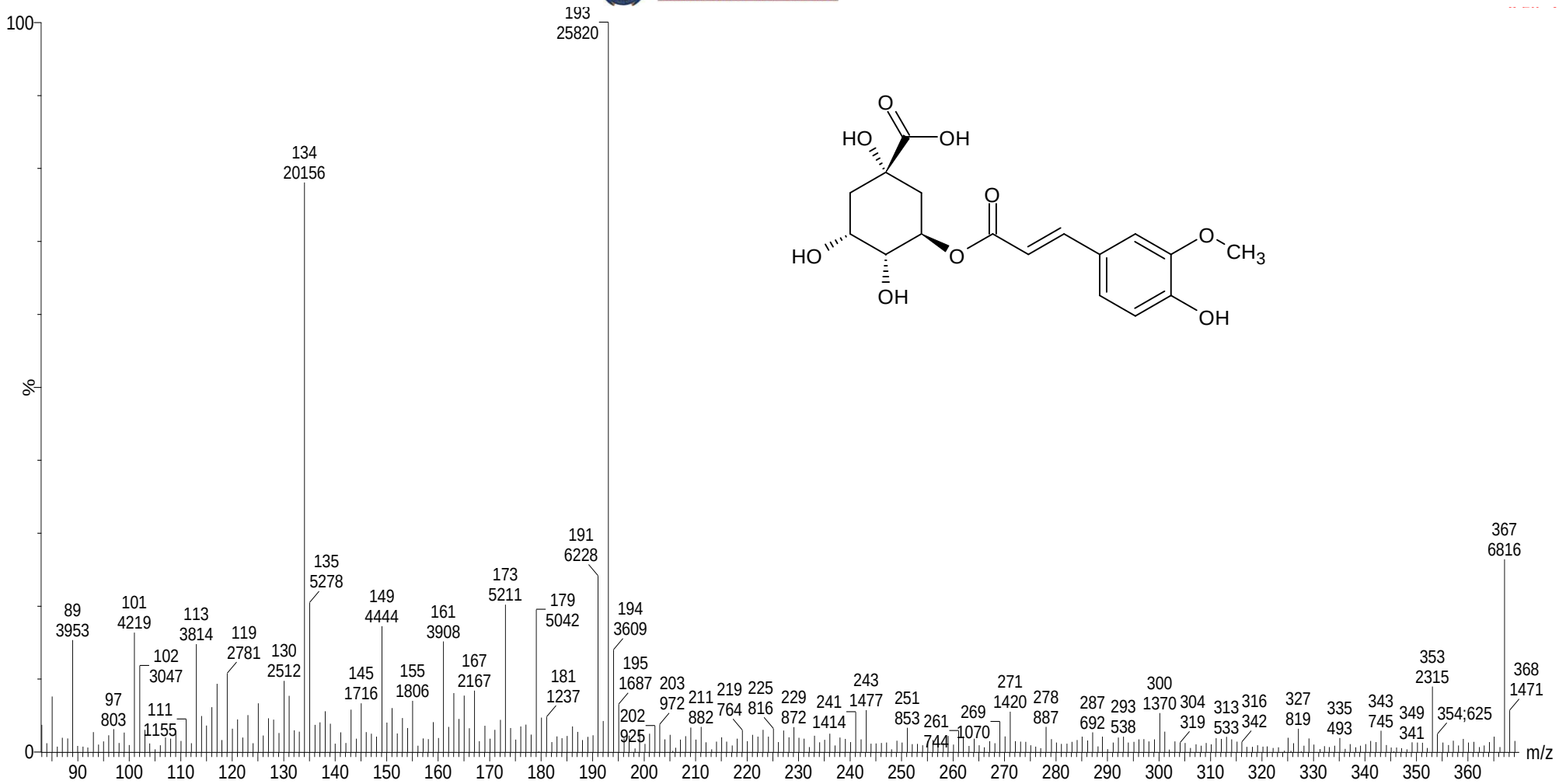

Figure 7-18: Mass spectrum of 3-feruloyl quinic acid (m/z at 367) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

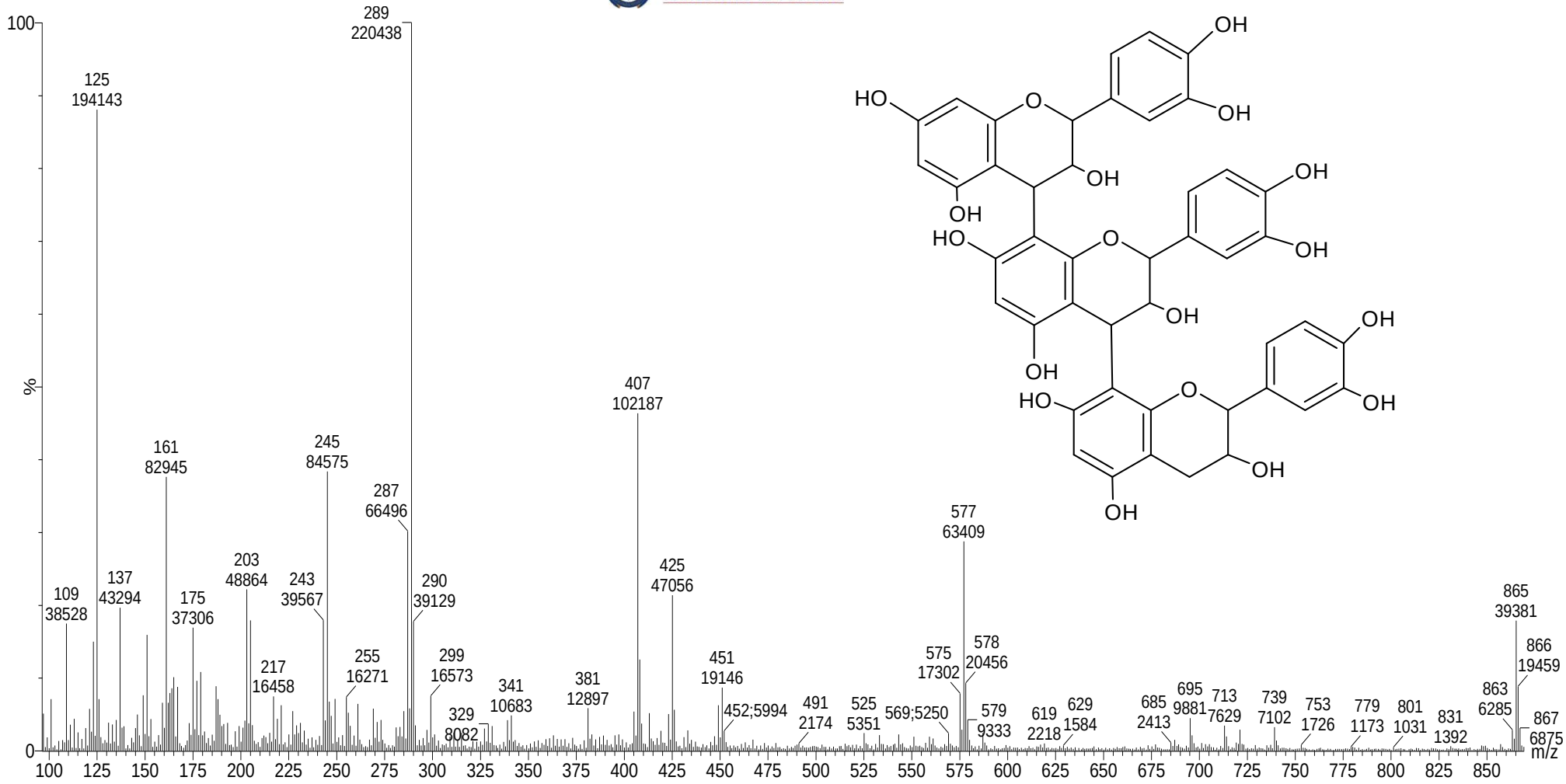

Figure 7-19: Mass spectrum of procyanidin trimer (m/z at 865) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

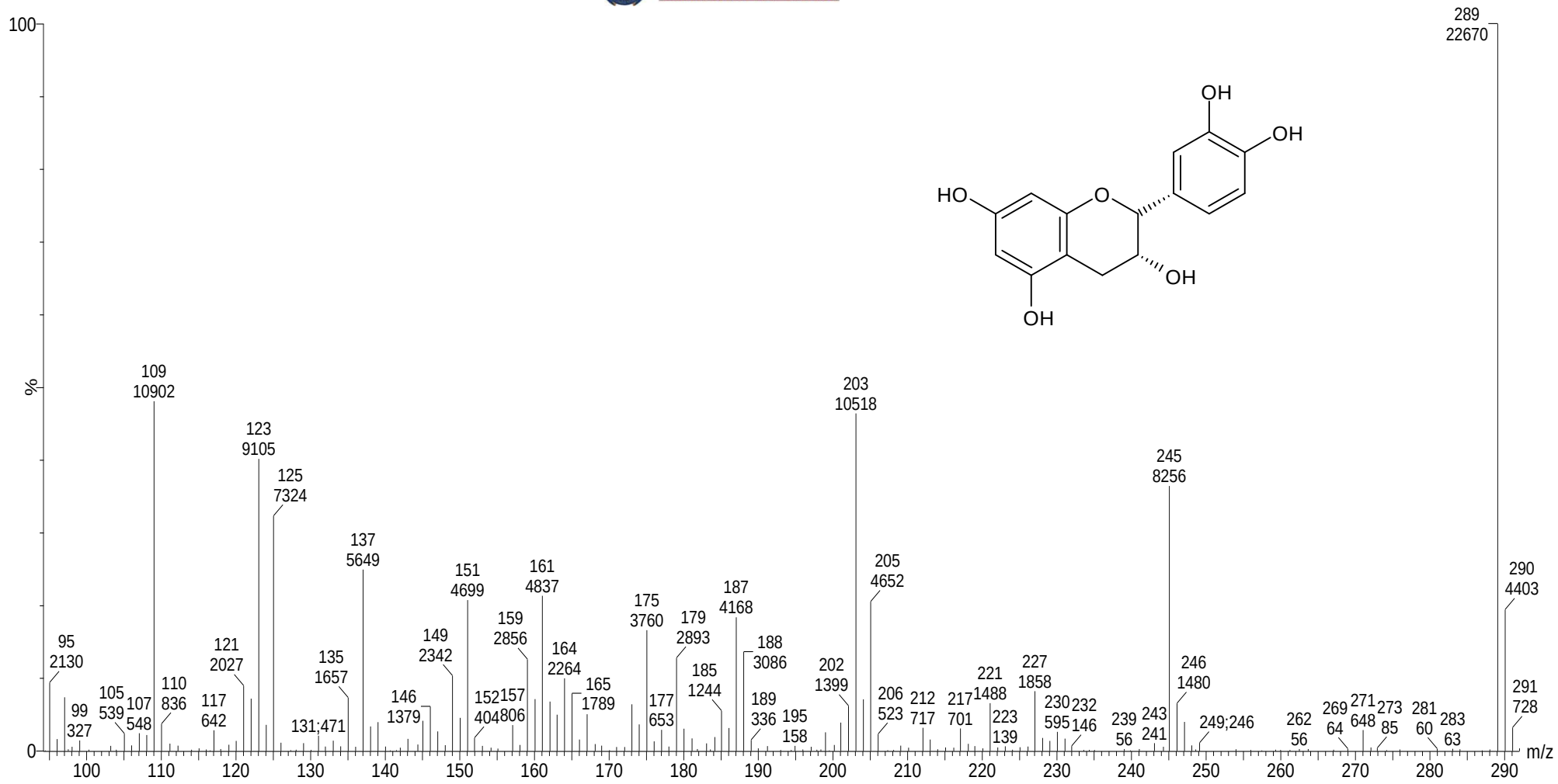

Figure 7-20: Mass spectrum of epicatechin (m/z at 289) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

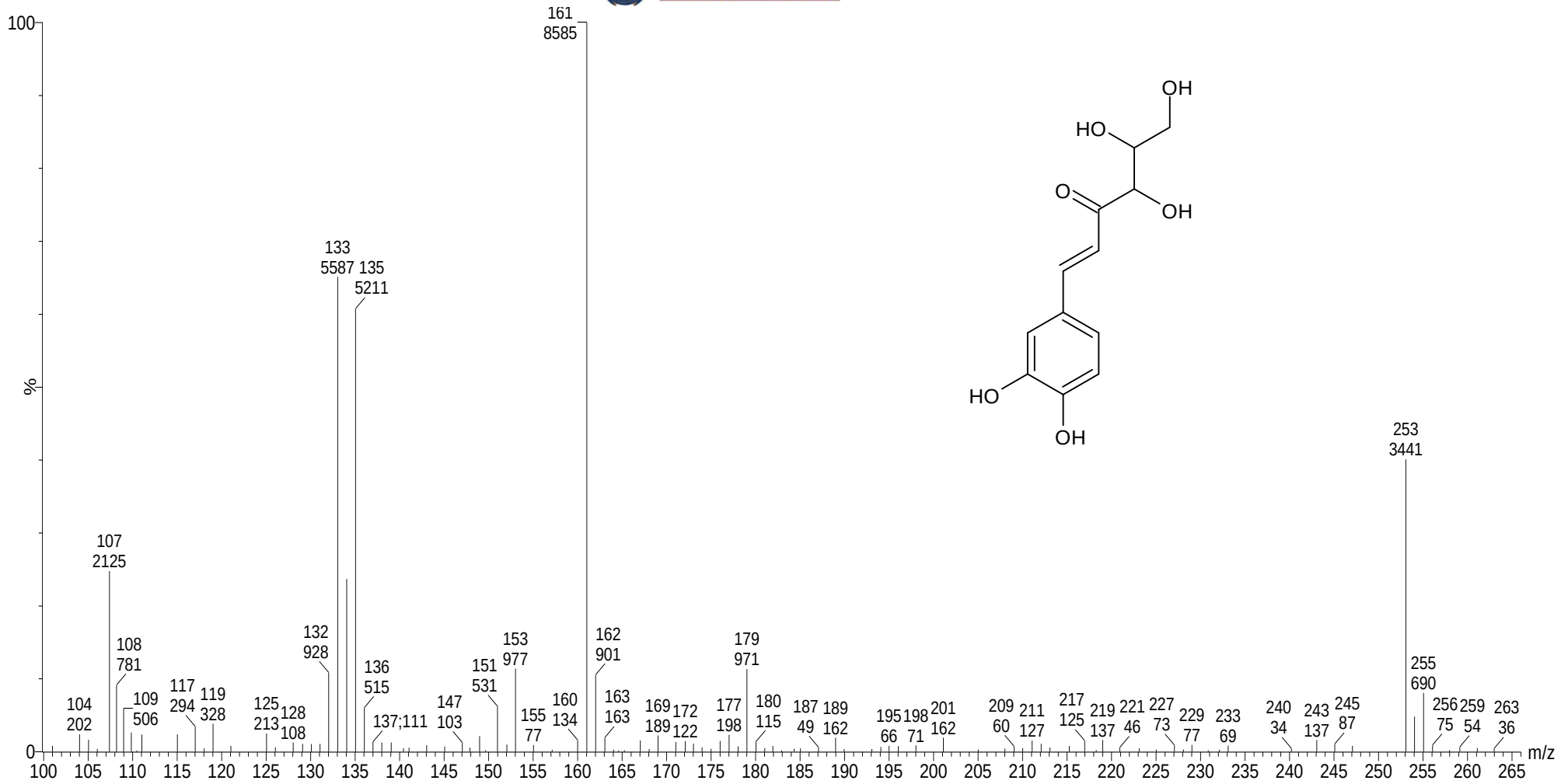

Figure 7-21: Mass spectrum of Caffeoylglcerol (*m/z* at 253) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

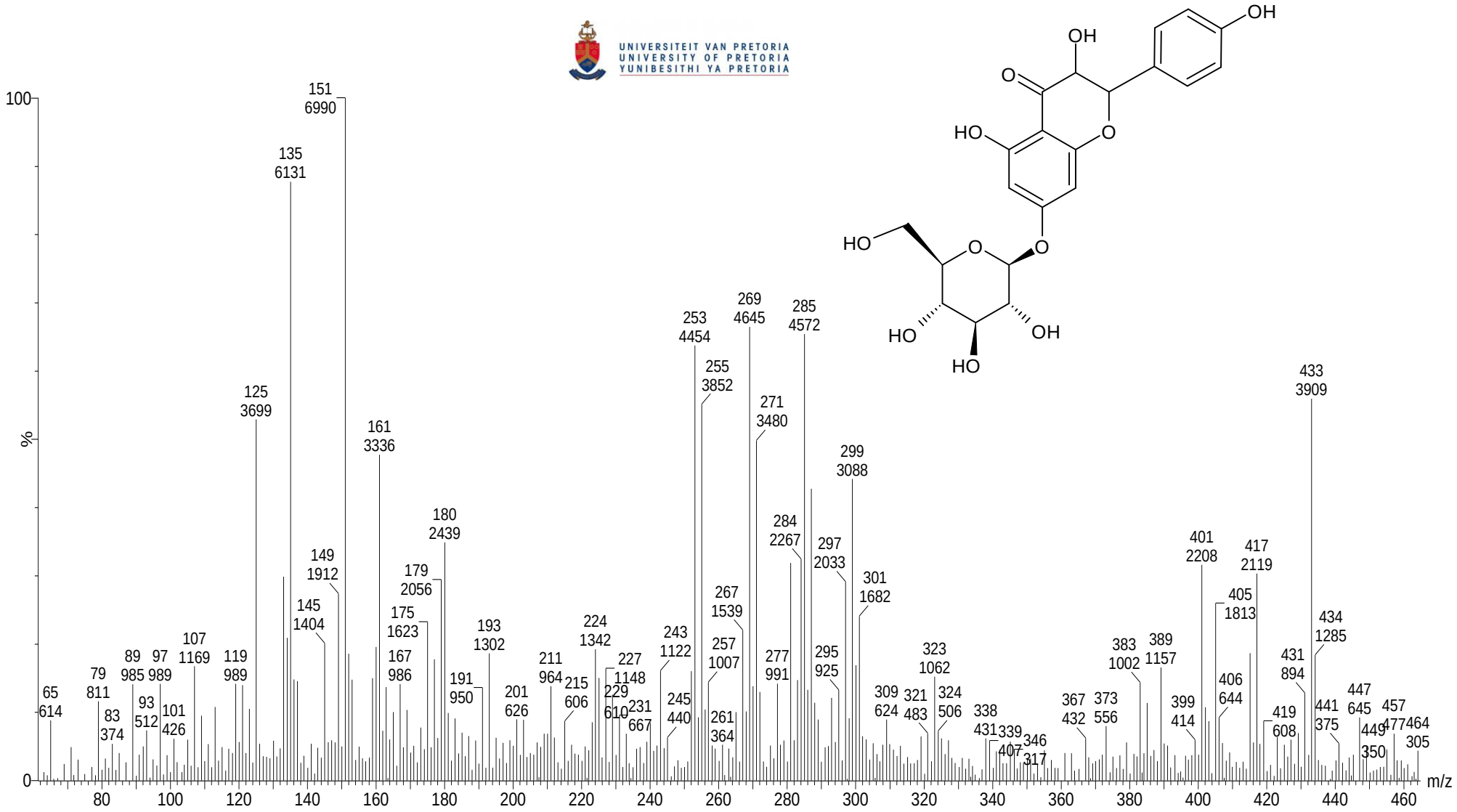

Figure 7-22: Mass spectrum of dihydrokaempferol glycoside (m/z at 449) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

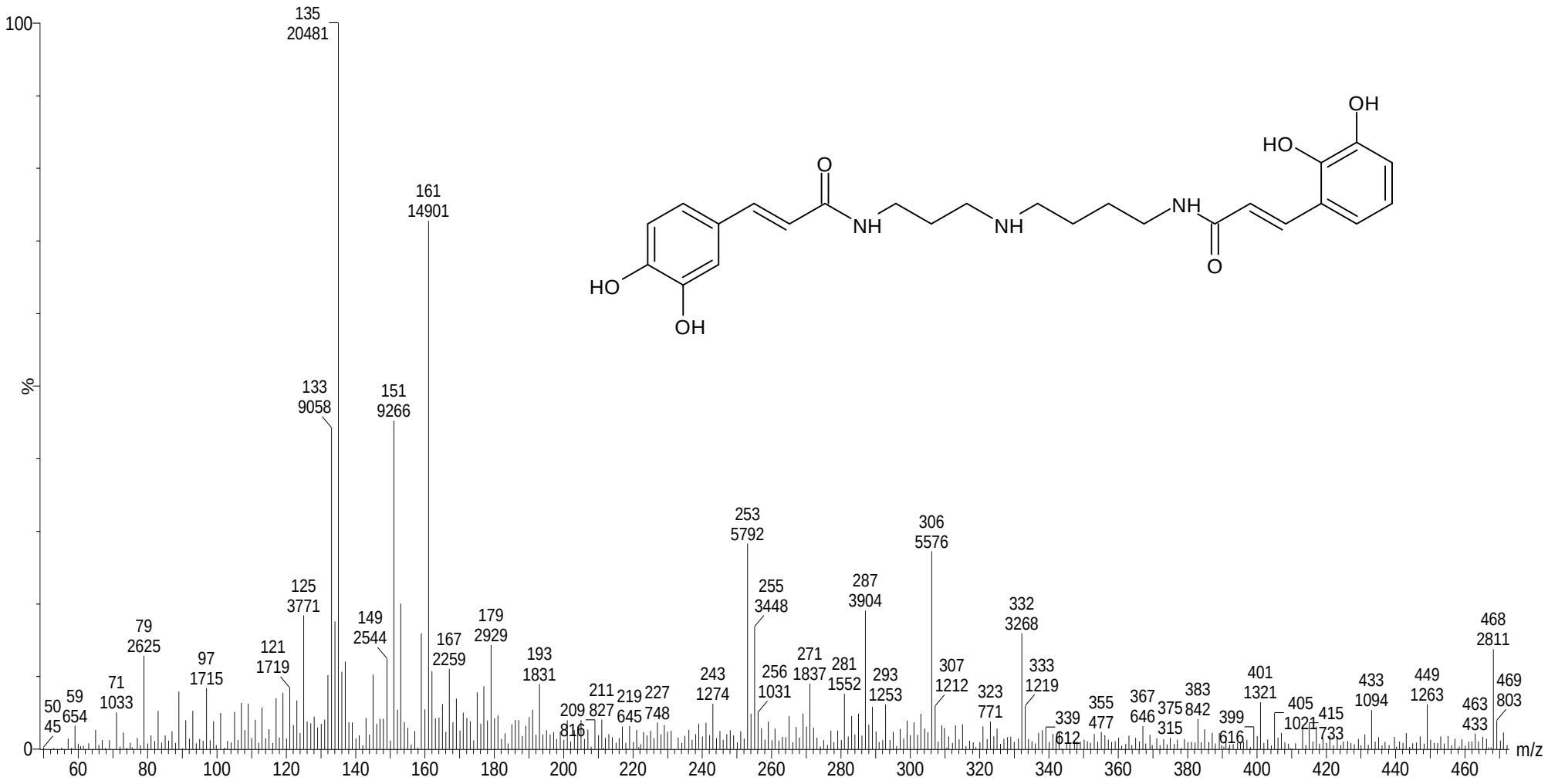

Figure 7-23: Mass spectrum of dicaffeoyl spermidine (m/z at 468) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

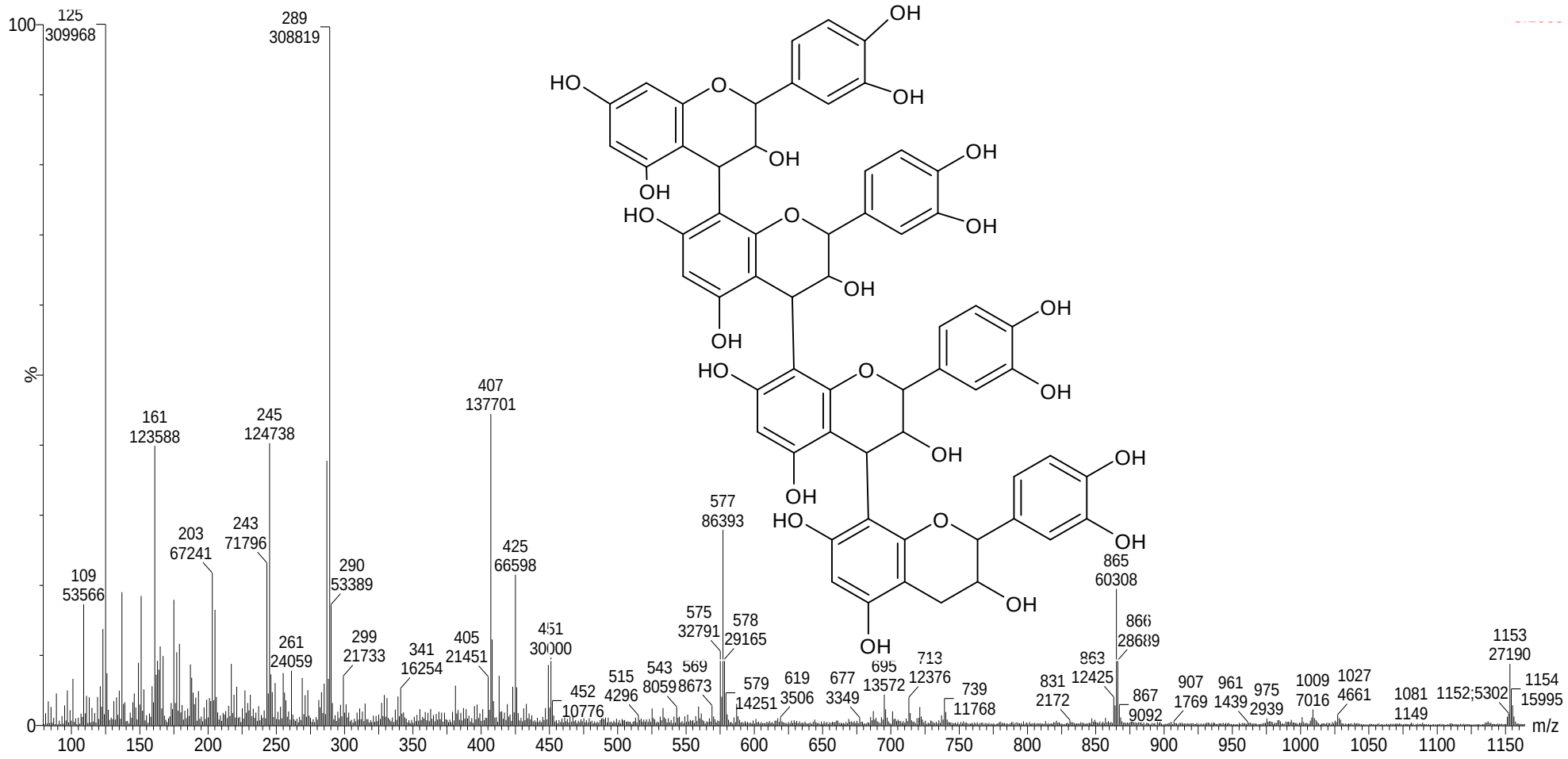

Figure 7-24: Mass spectrum of procyanidin tetramer (*m/z* at 1154) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

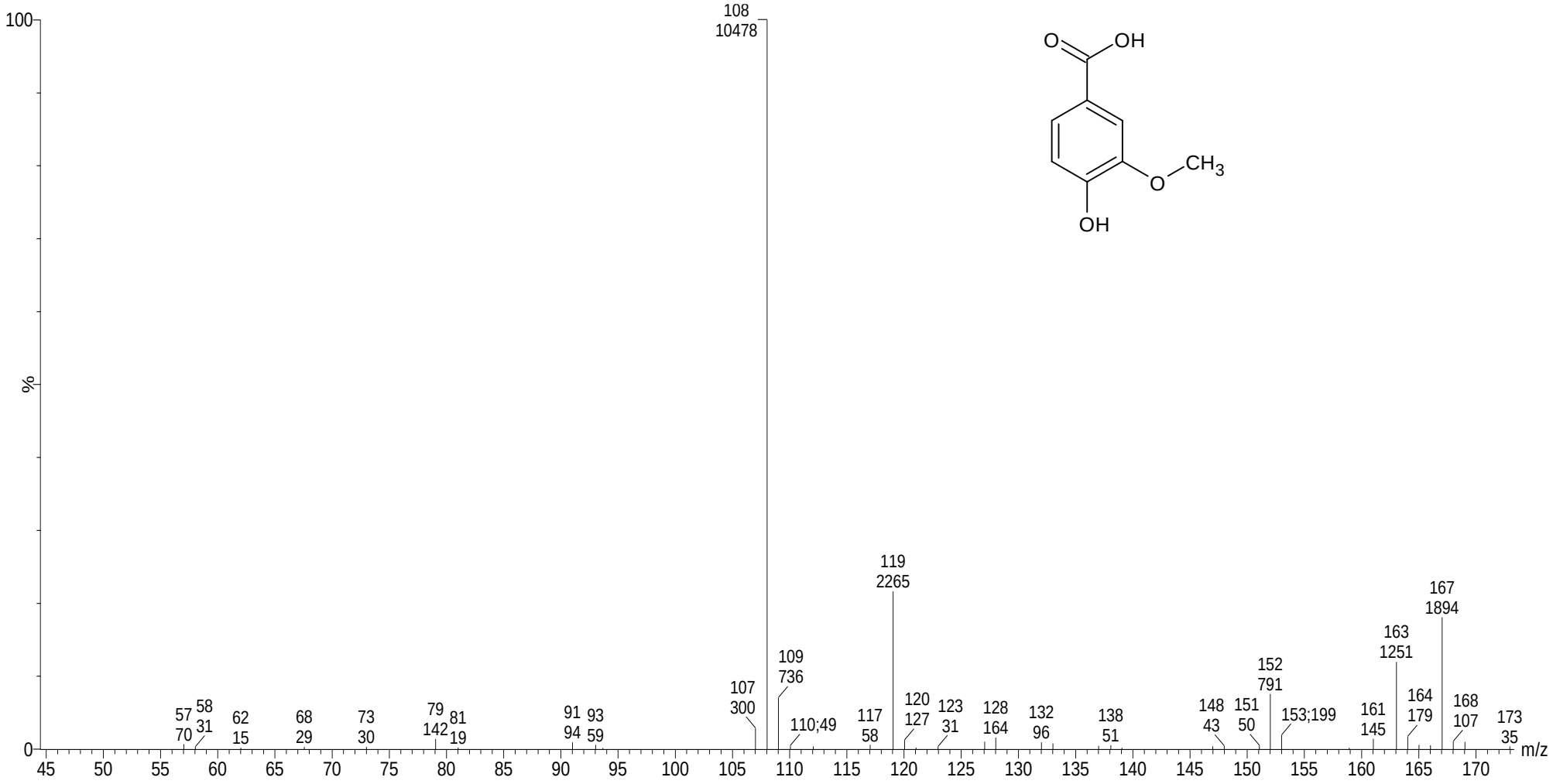

Figure 7-25: Mass spectrum of vanillic acid (*m/z* at 167) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

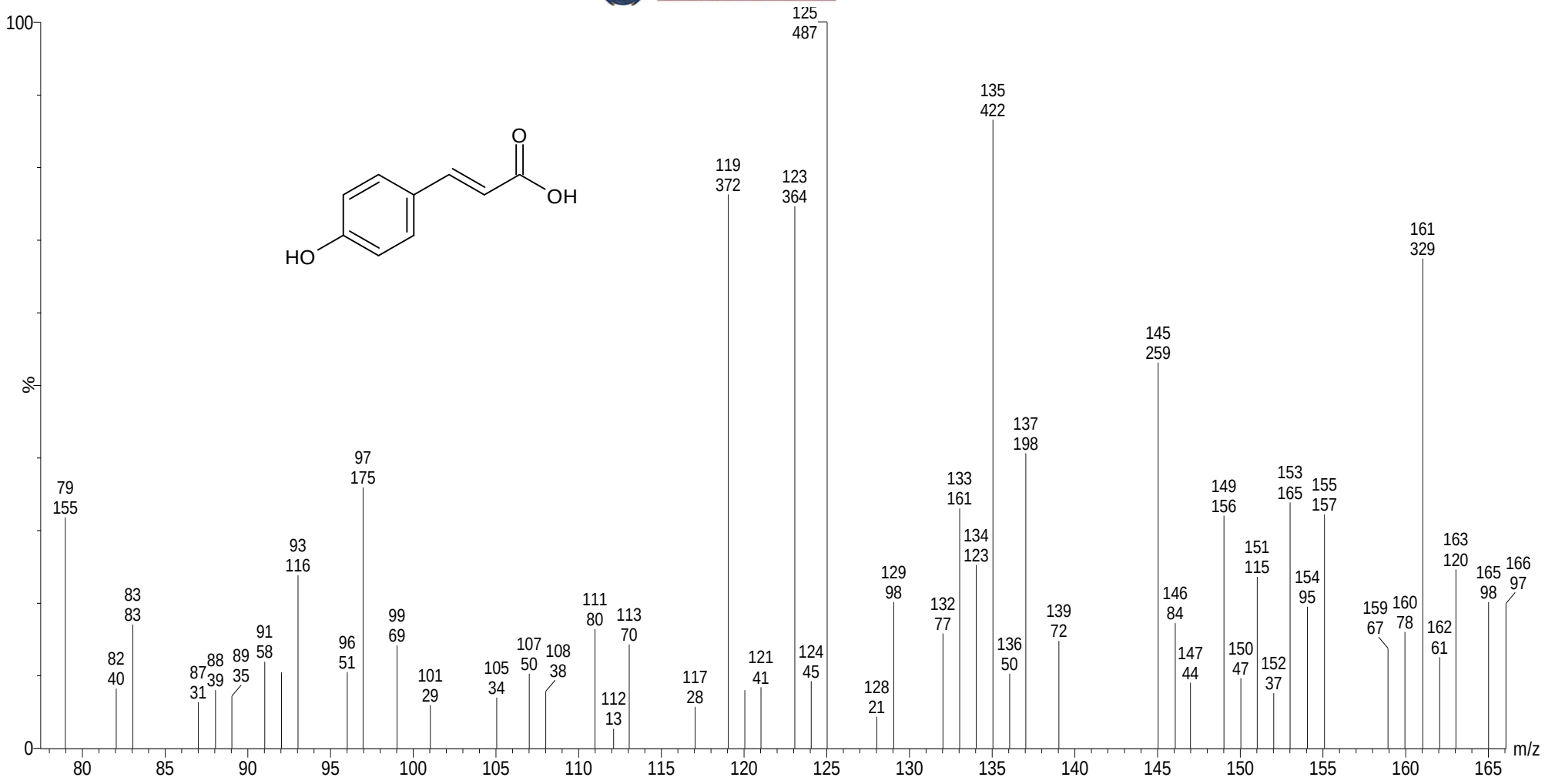

Figure 7-26: Mass spectrum of ρ-coumaric acid (*m/z* at 163) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

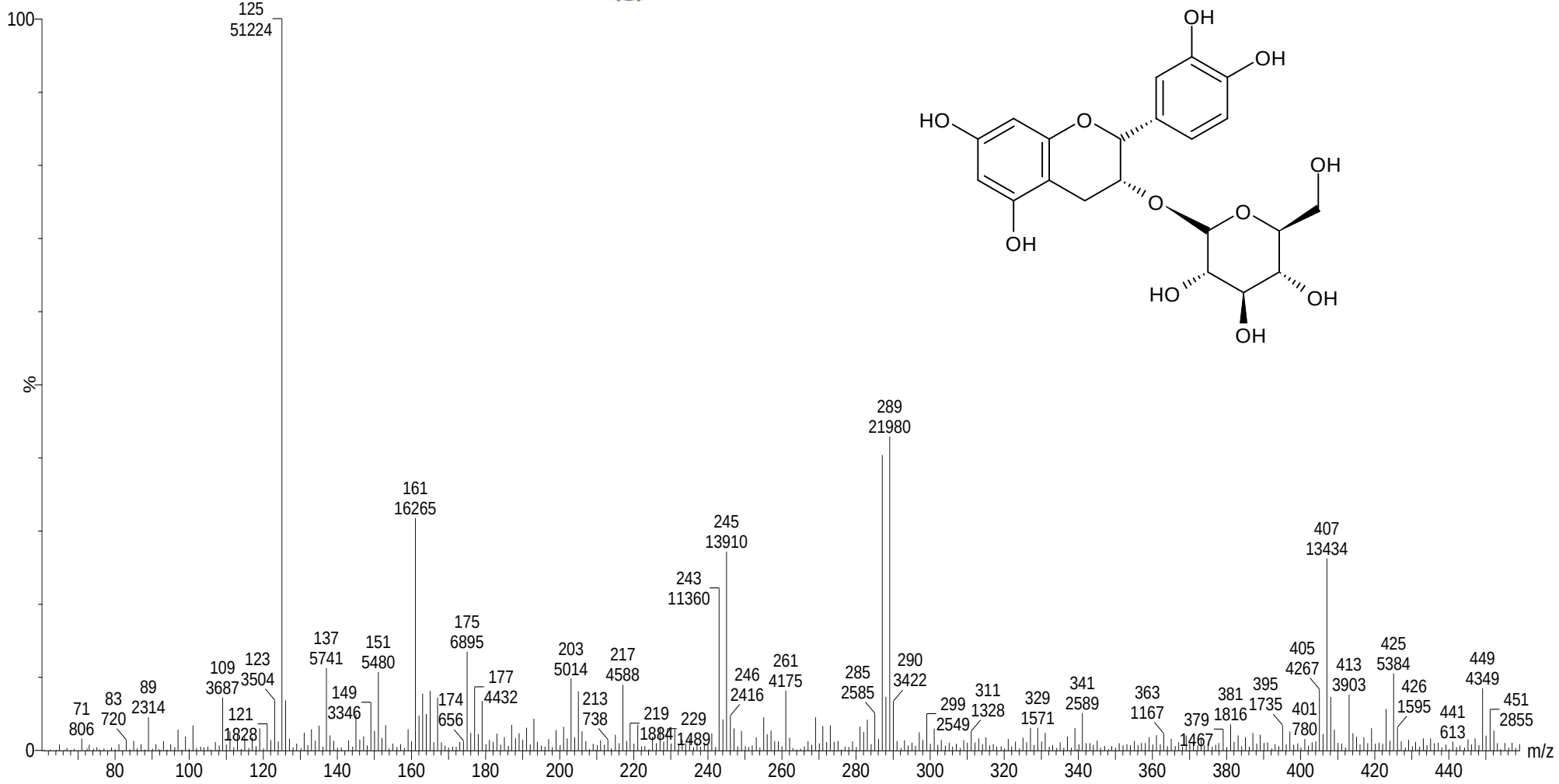

Figure 7-27: Mass spectrum of epicatechin glycoside (m/z at 451) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

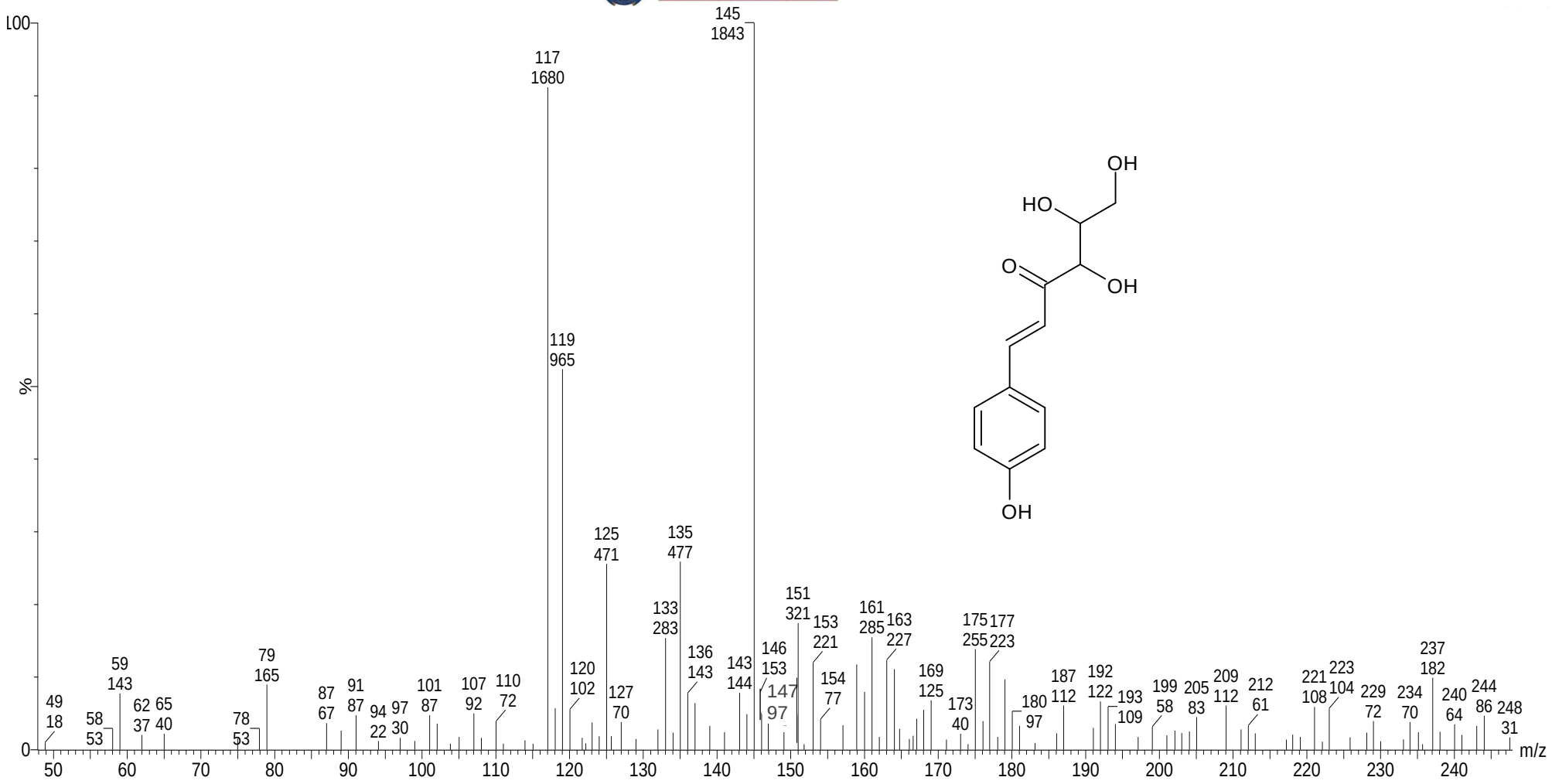

Figure 7-28: Mass spectrum of ρ-coumaroyl glycerol (m/z at 237) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

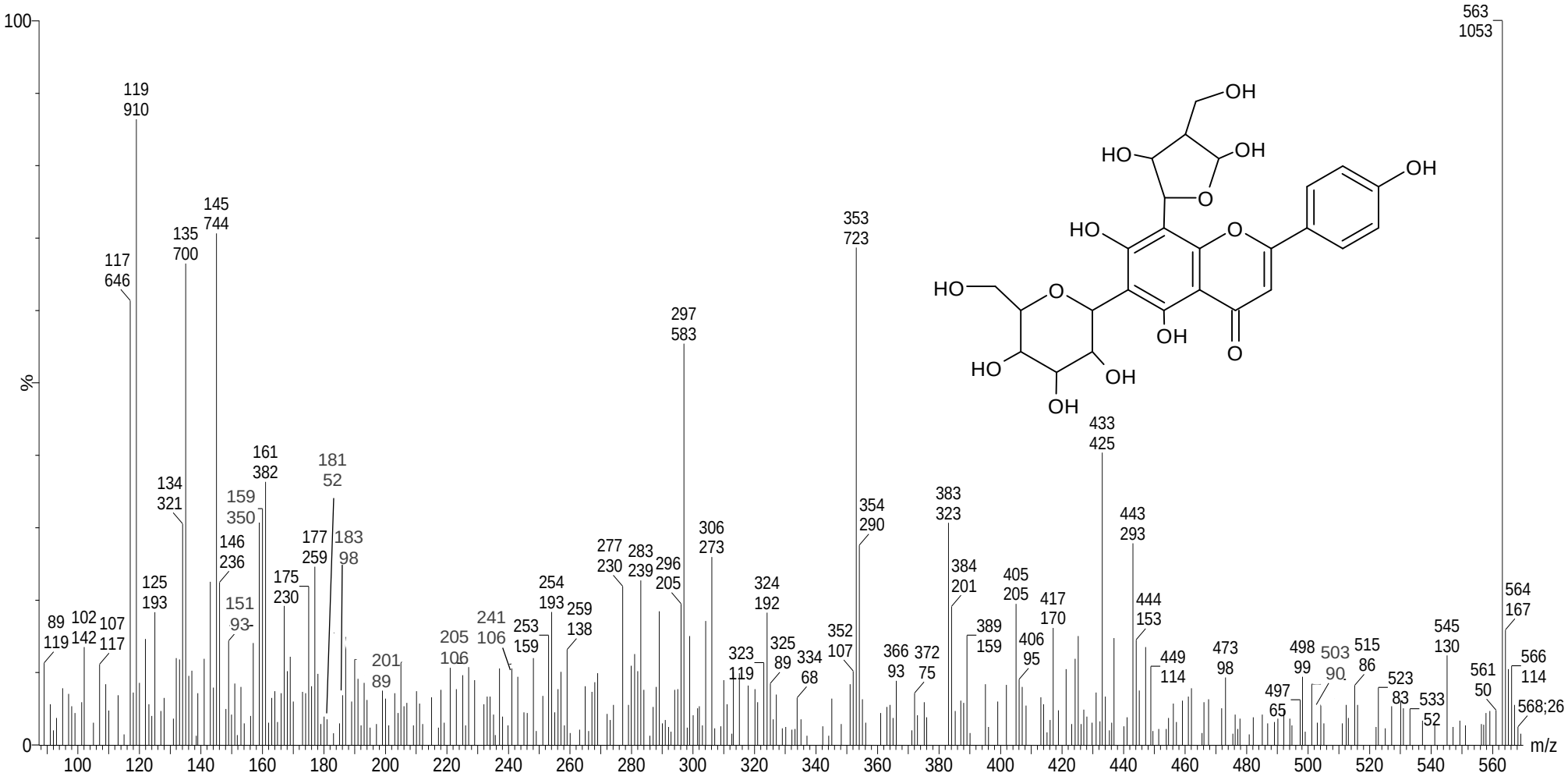

Figure 7-29: Mass spectrum of glucosyl-arabinosyl apigenin (m/z at 563) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

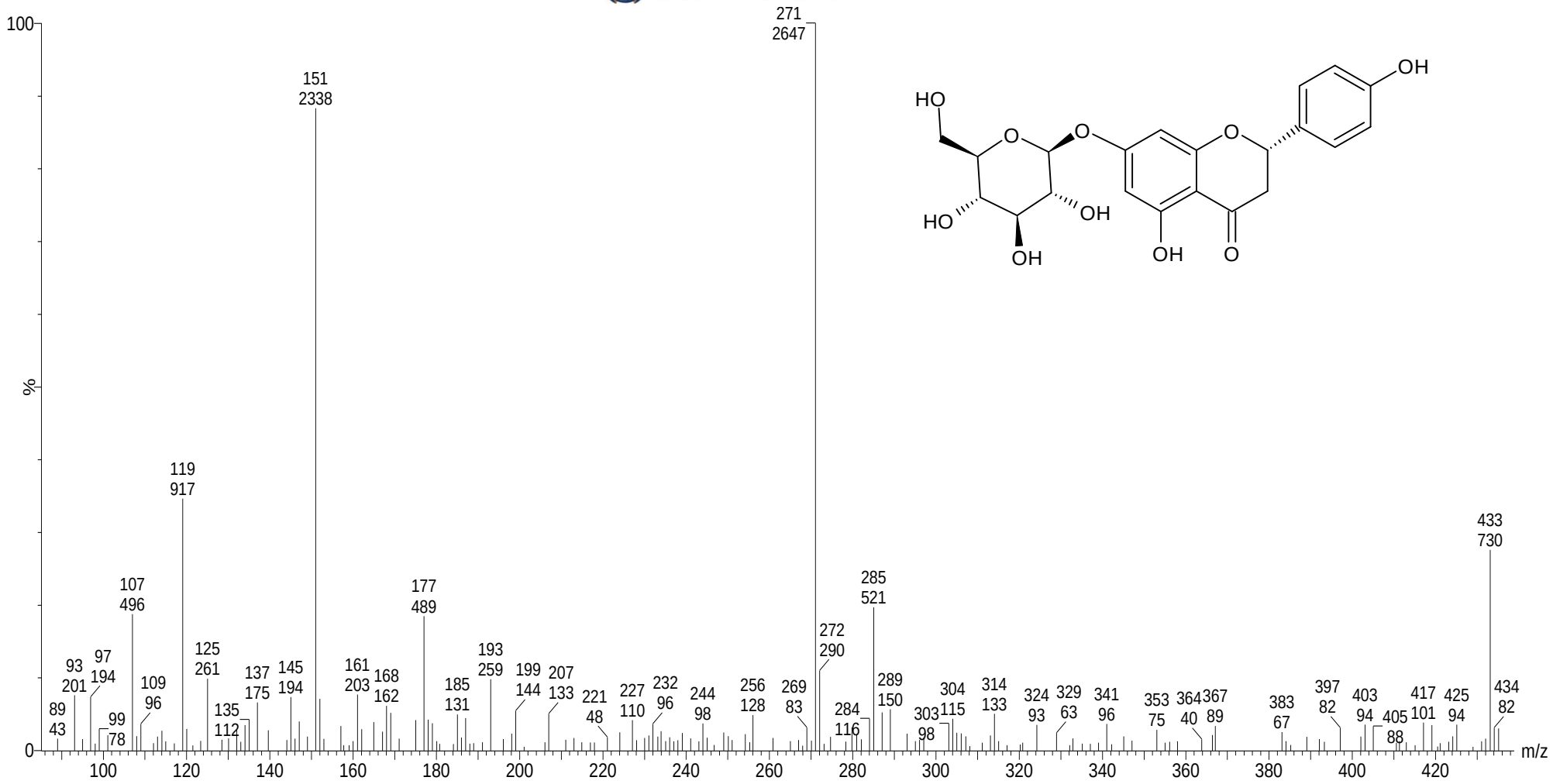

Figure 7-30: Mass spectrum of naringenin glycoside (m/z at 433) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

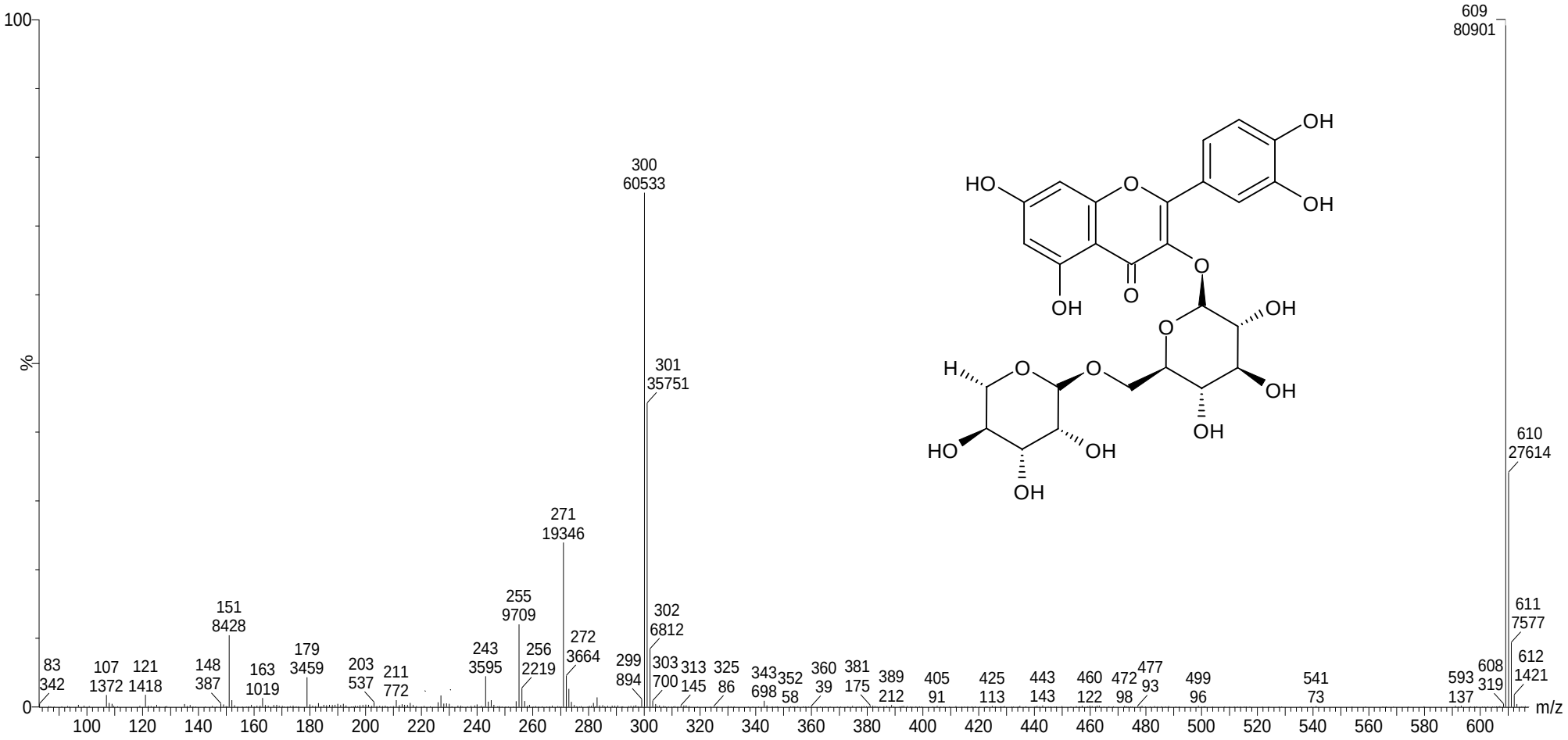

Figure 7-31: Mass spectrum of rutin (m/z at 609) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

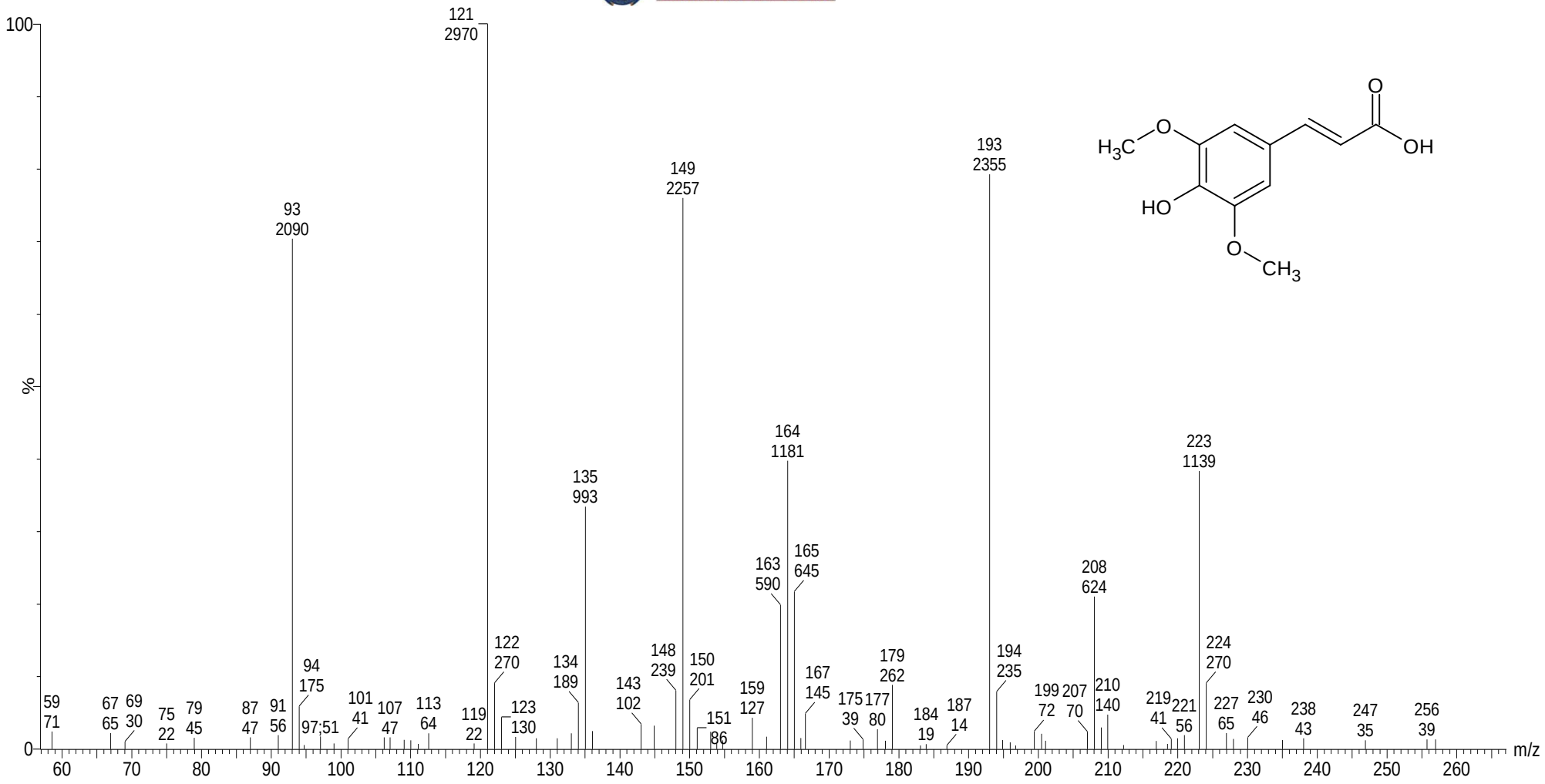

Figure 7-32: Mass spectrum of sinapic acid (m/z at 223) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

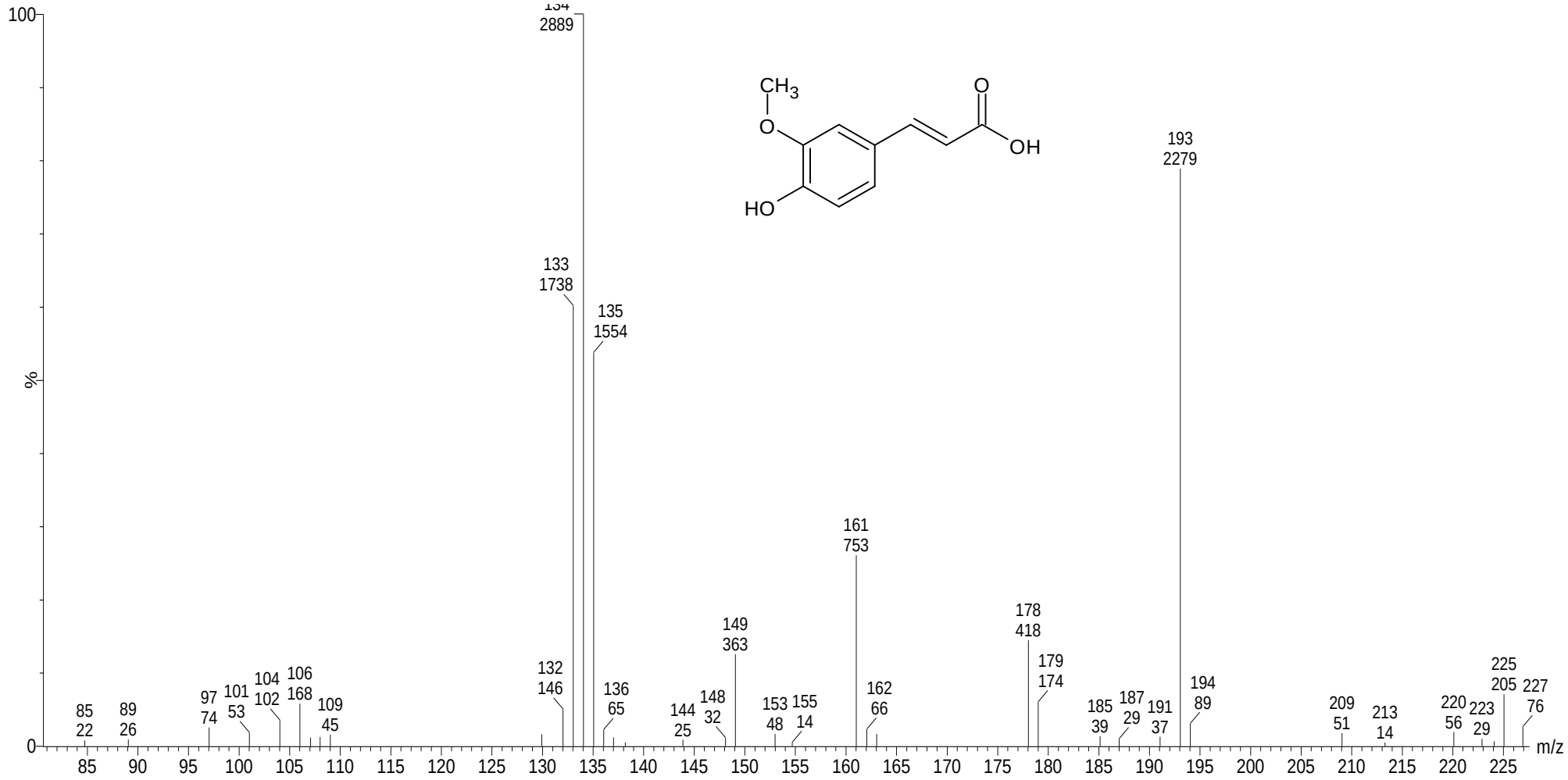

Figure 7-33: Mass spectrum of ferulic acid (m/z at 193) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

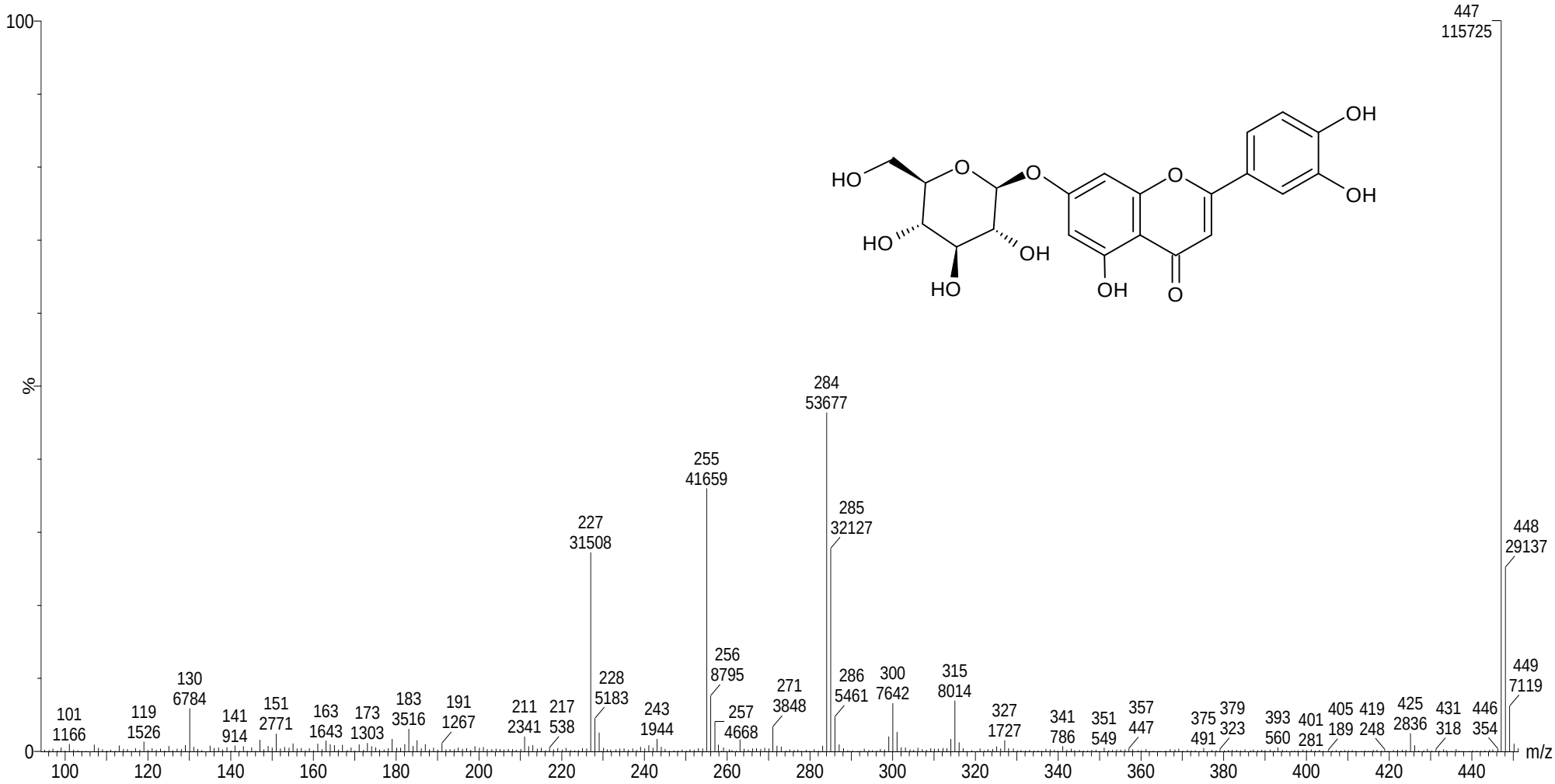

Figure 7-34: Mass spectrum of luteolin glycoside (m/z at 447) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

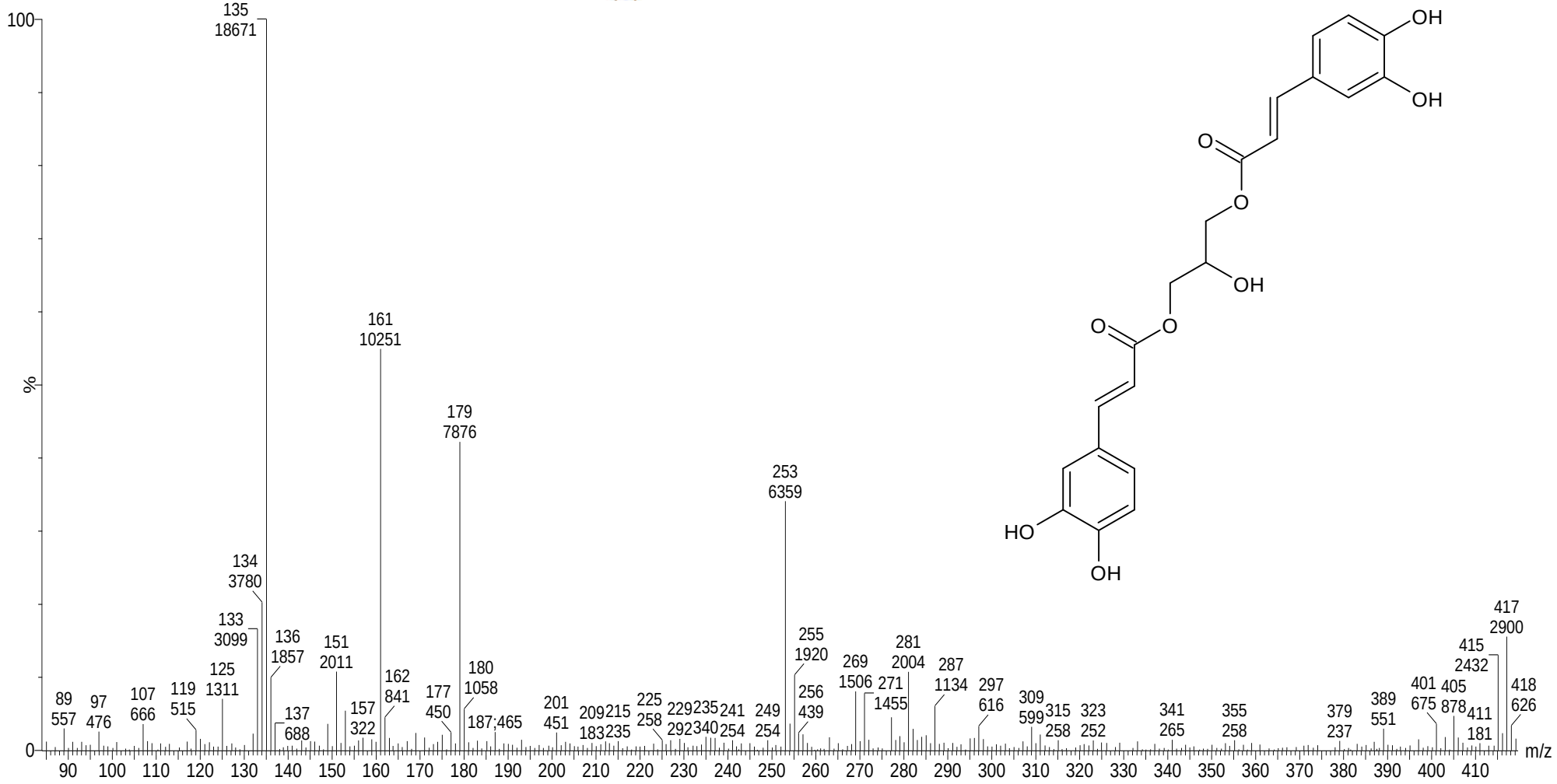

Figure 7-35: Mass spectrum of dicaffeoyl glycerol (m/z at 415) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

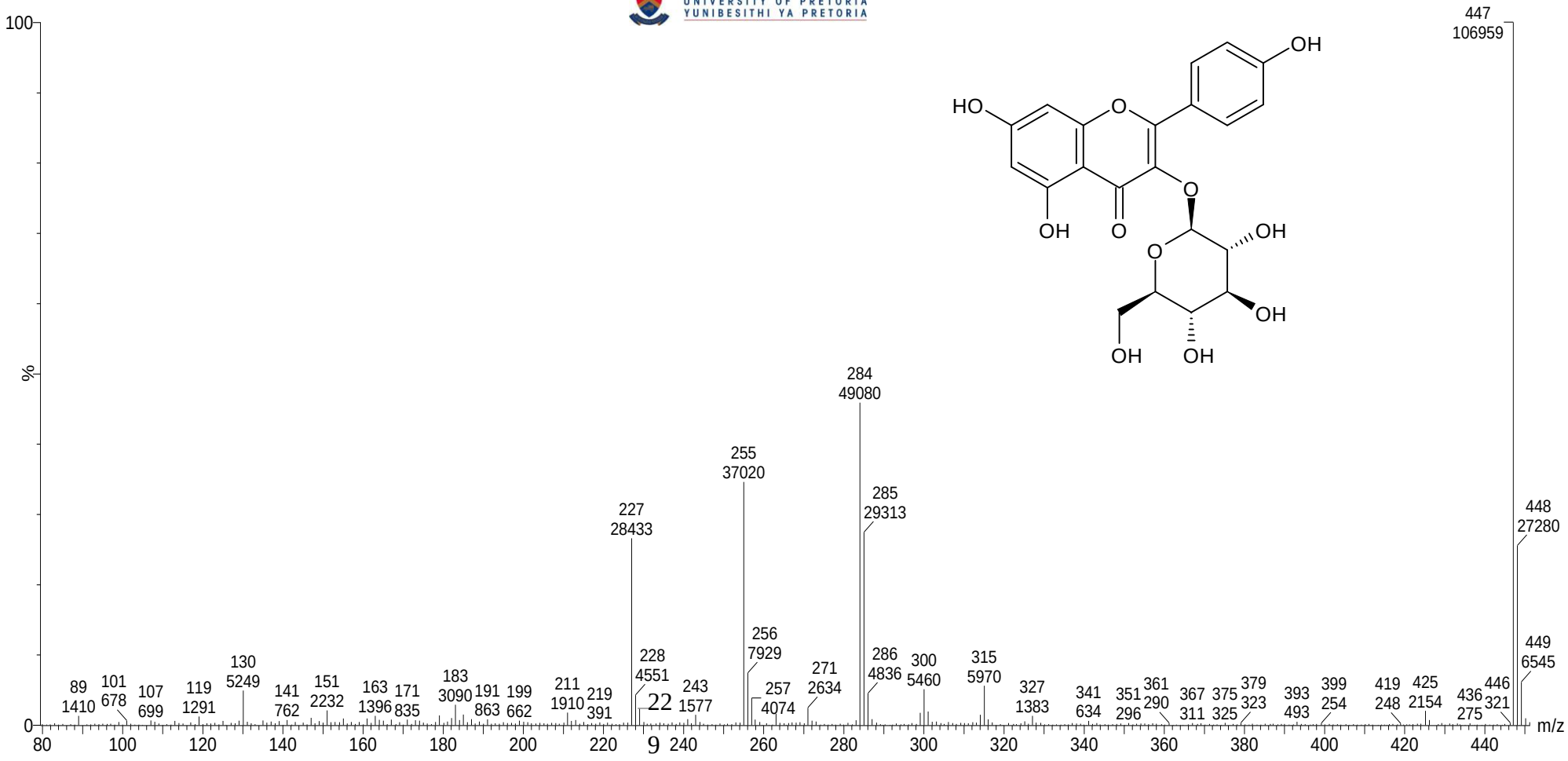

Figure 7-36: Mass spectrum of kaempferol glycoside (m/z at 447) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

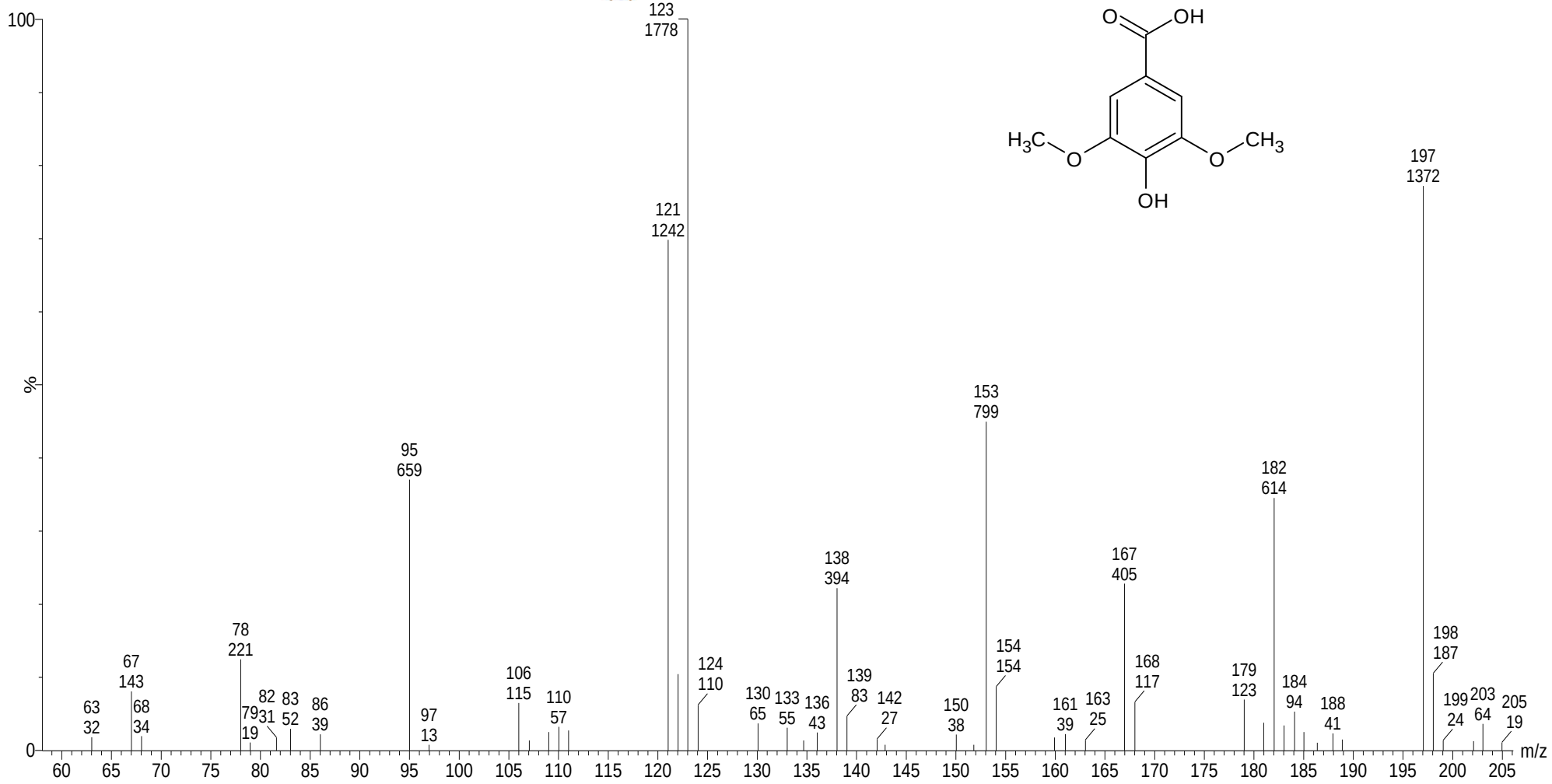

Figure 7-37: Mass spectrum of syringic acid (m/z at 197) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

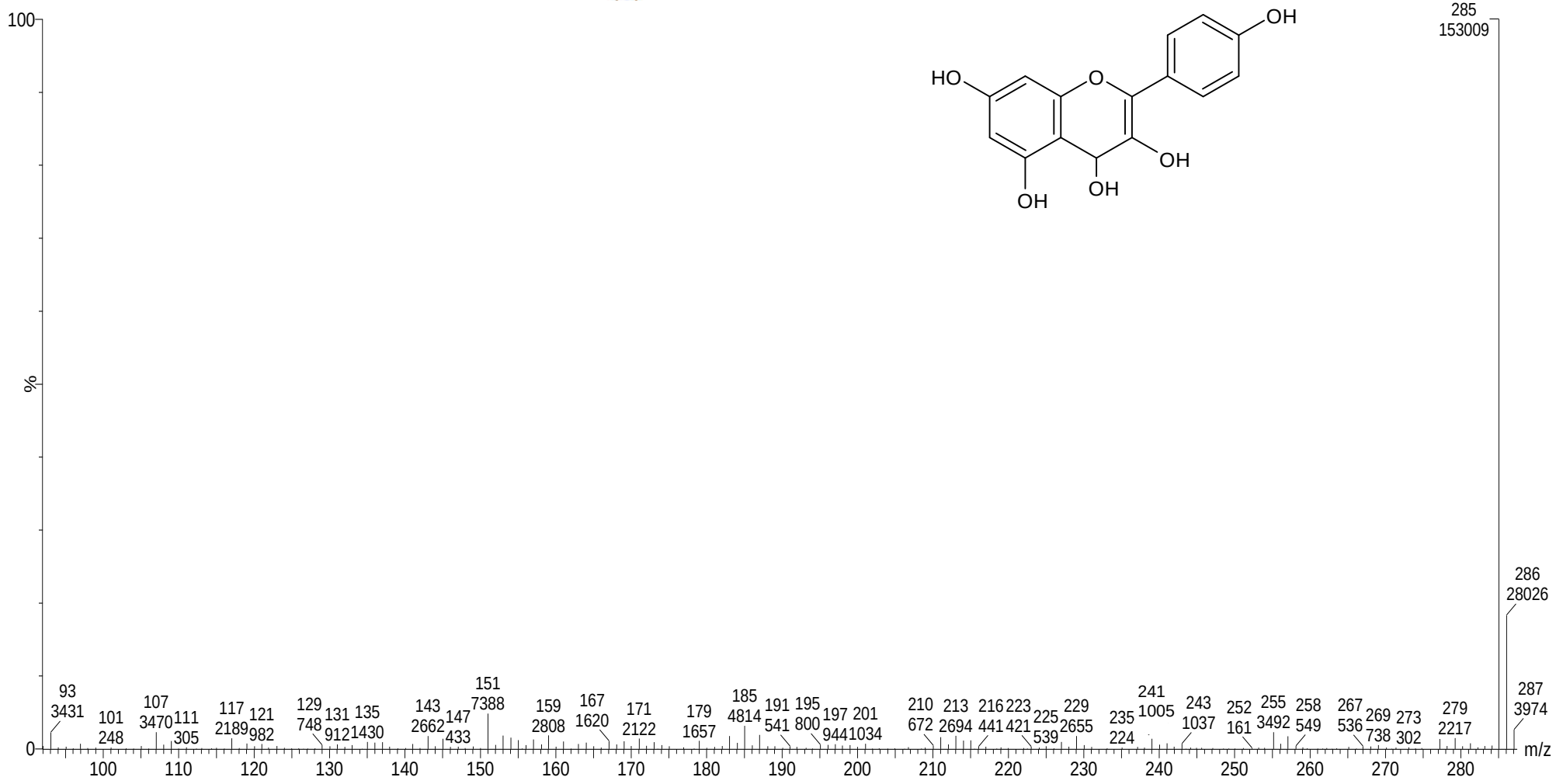

Figure 7-38: Mass spectrum of kaempferol (m/z at 285) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

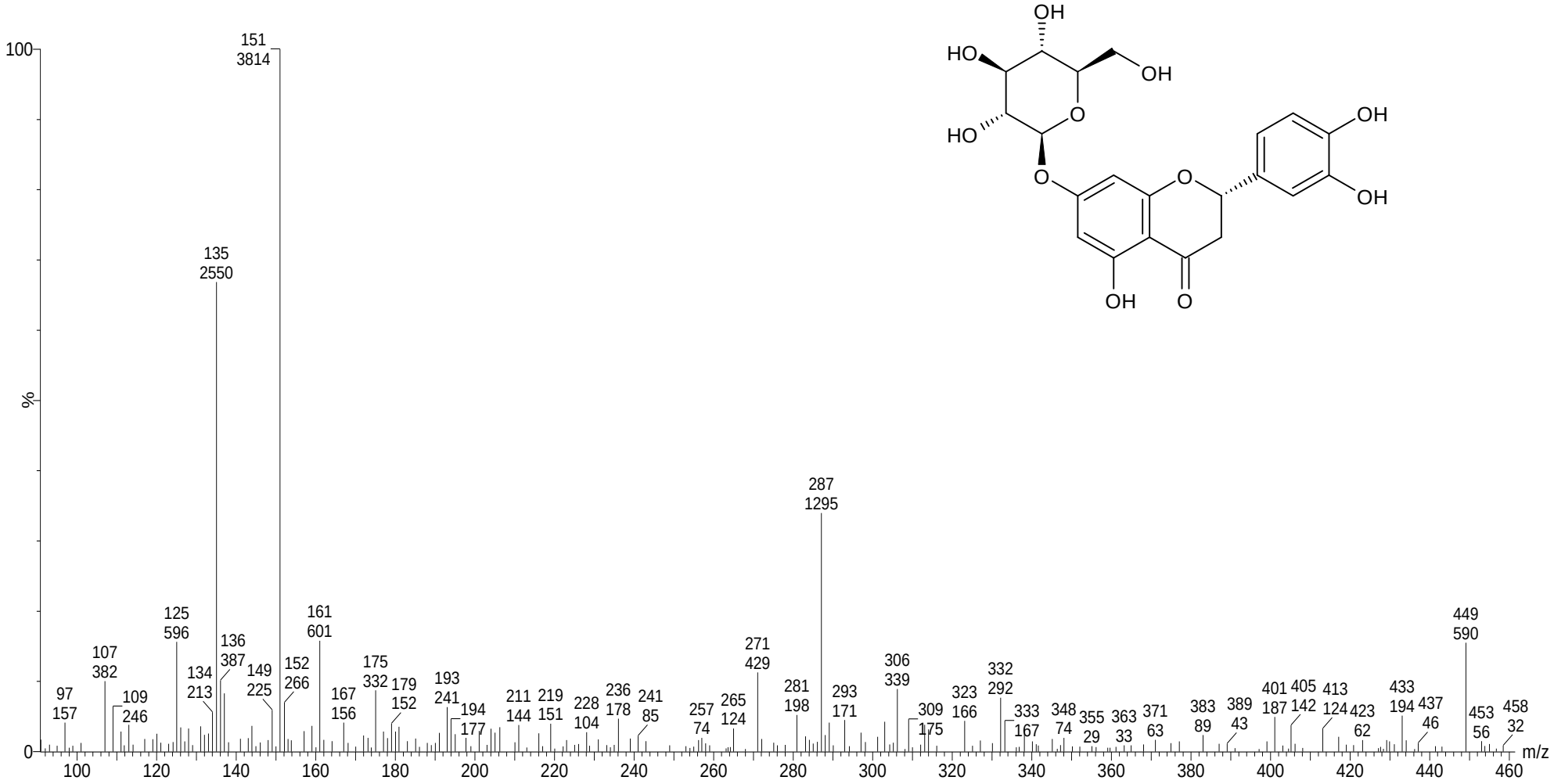

Figure 7-39: Mass spectrum of eriodctoyl glycoside (m/z at 449) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

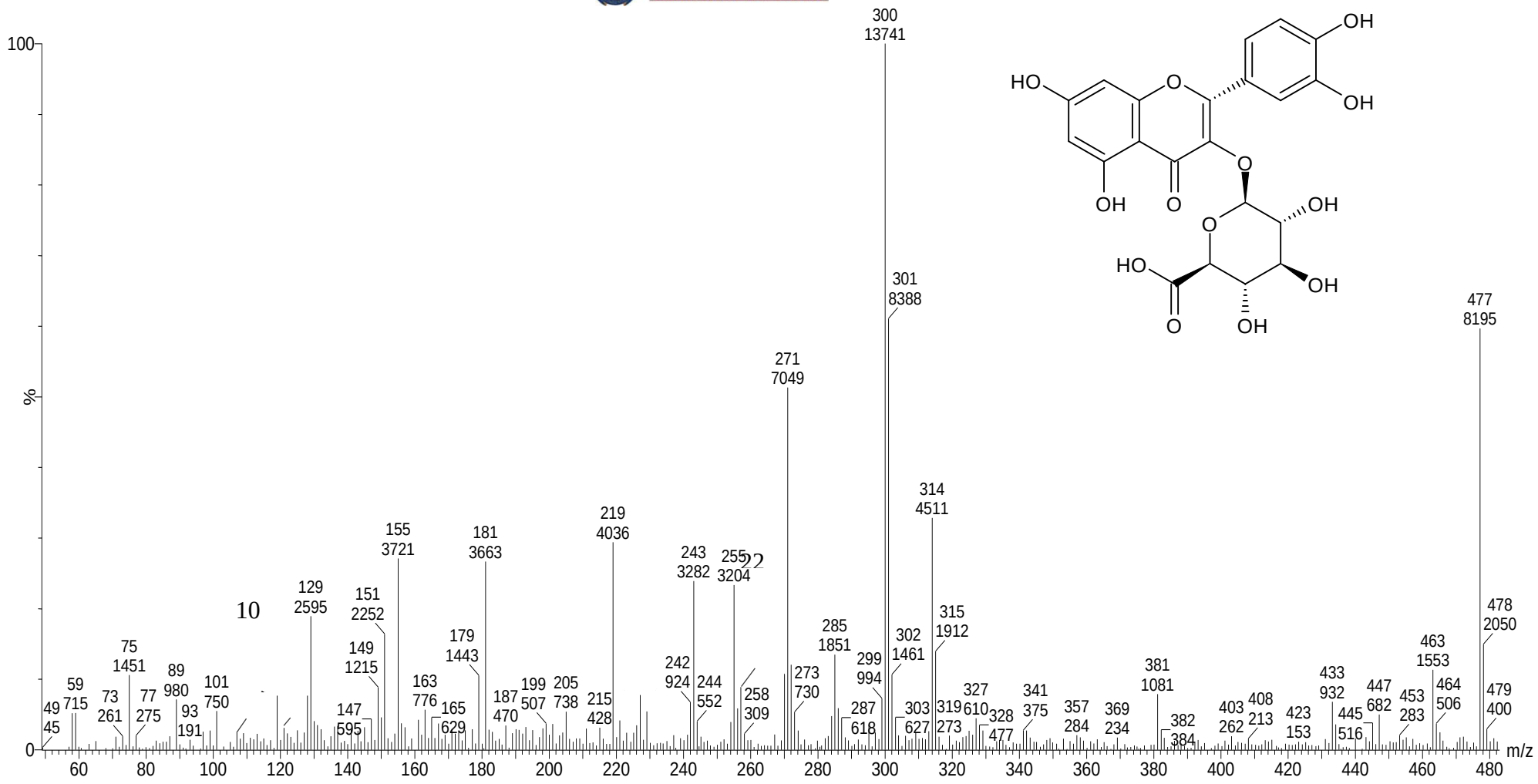

Figure 7-40: Mass spectrum of quercetin glucuronide (m/z at 477) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

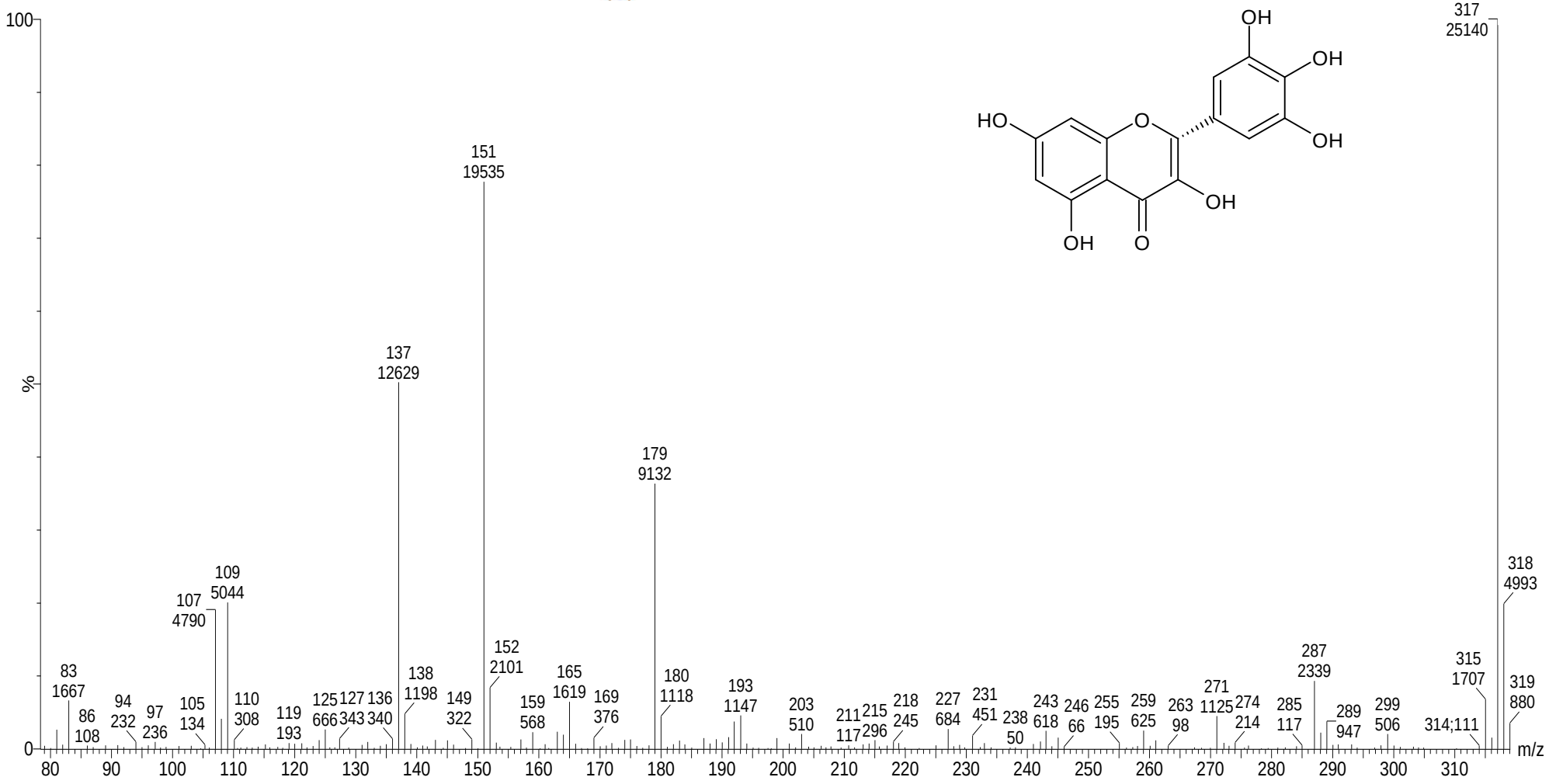

Figure 7-41: Mass spectrum of myricetin (m/z at 449) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

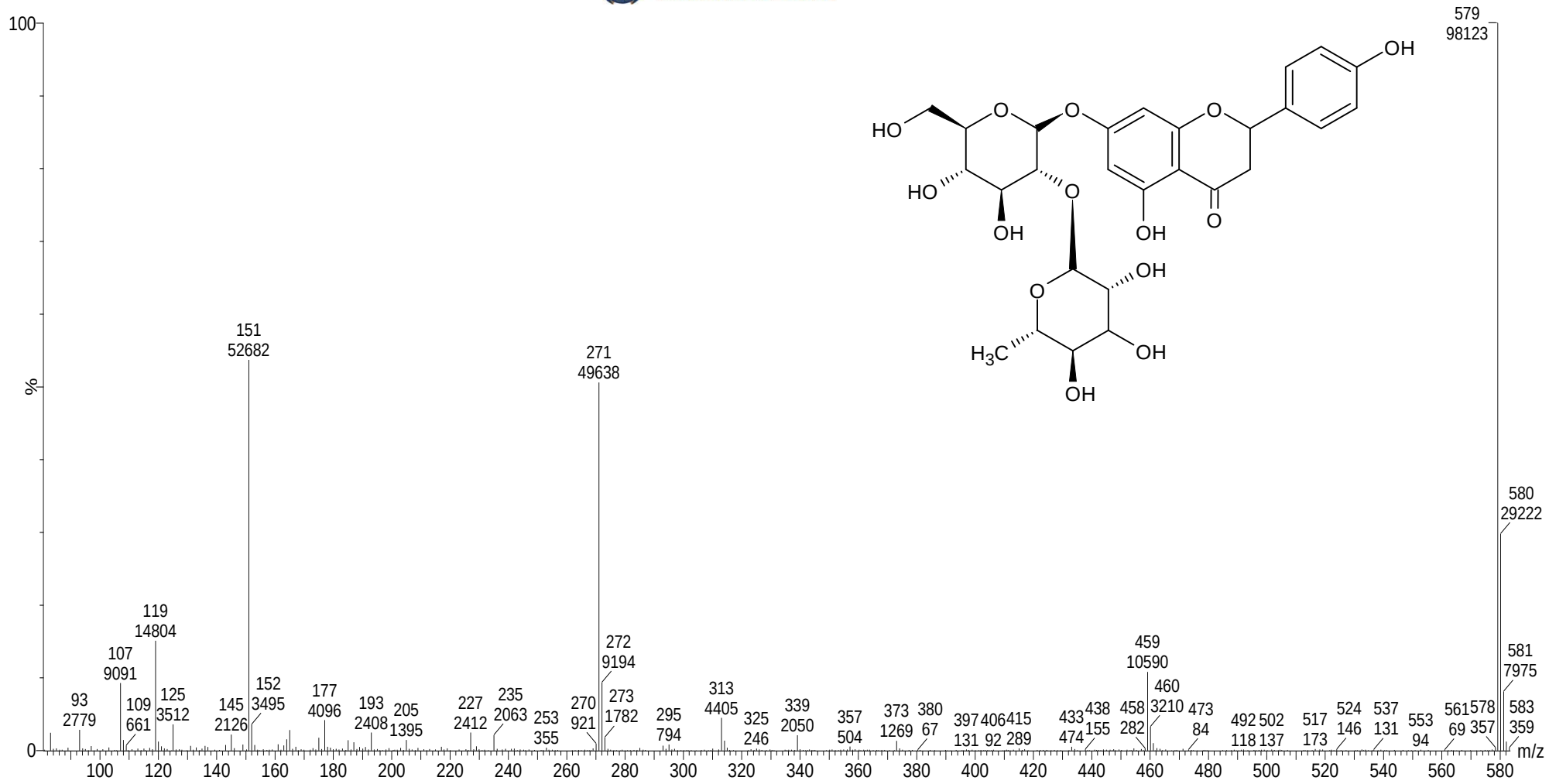

Figure 7-42: Mass spectrum of naringin (m/z at 579) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

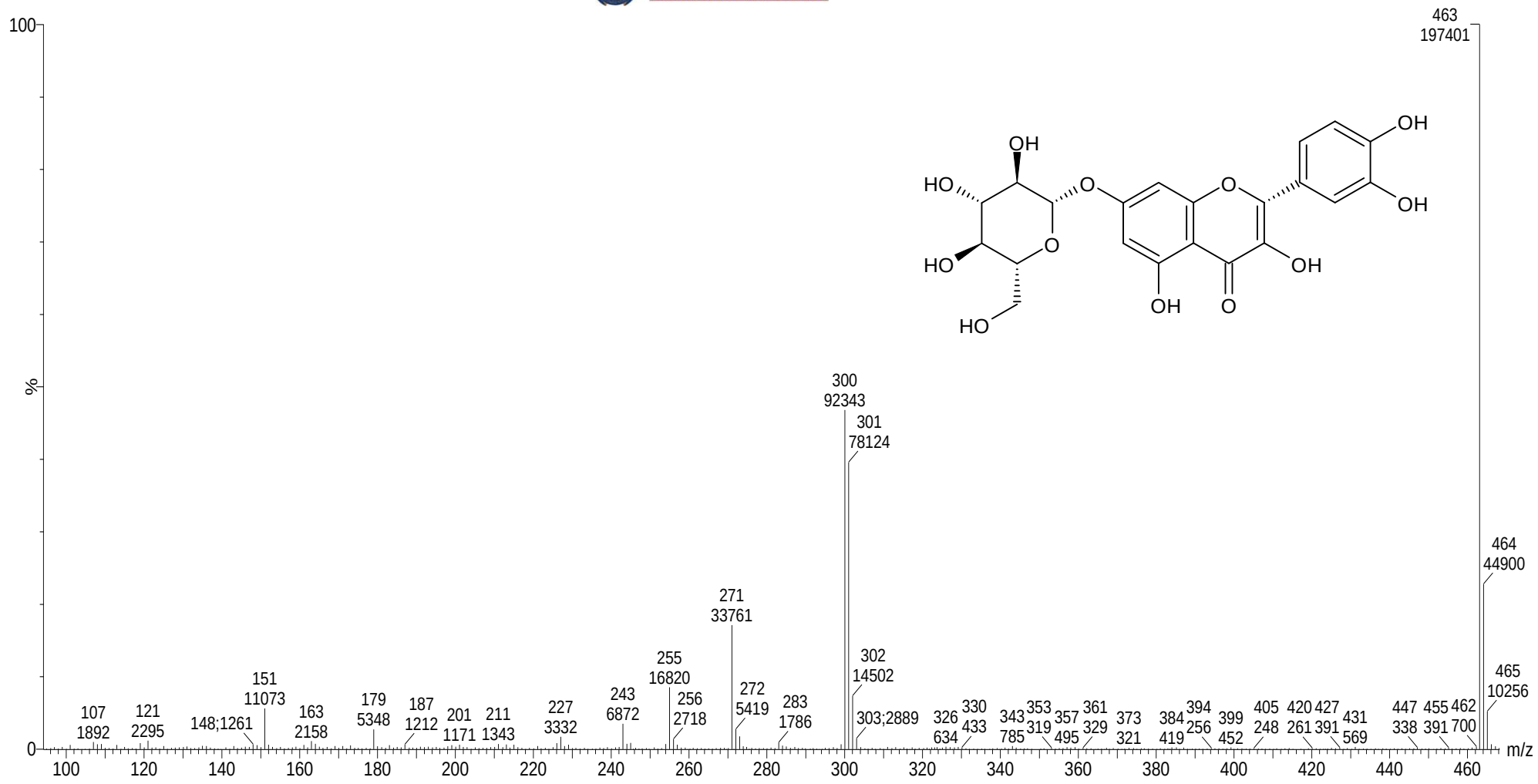

Figure 7-43: Mass spectrum of quercetin glycoside (m/z at 463) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

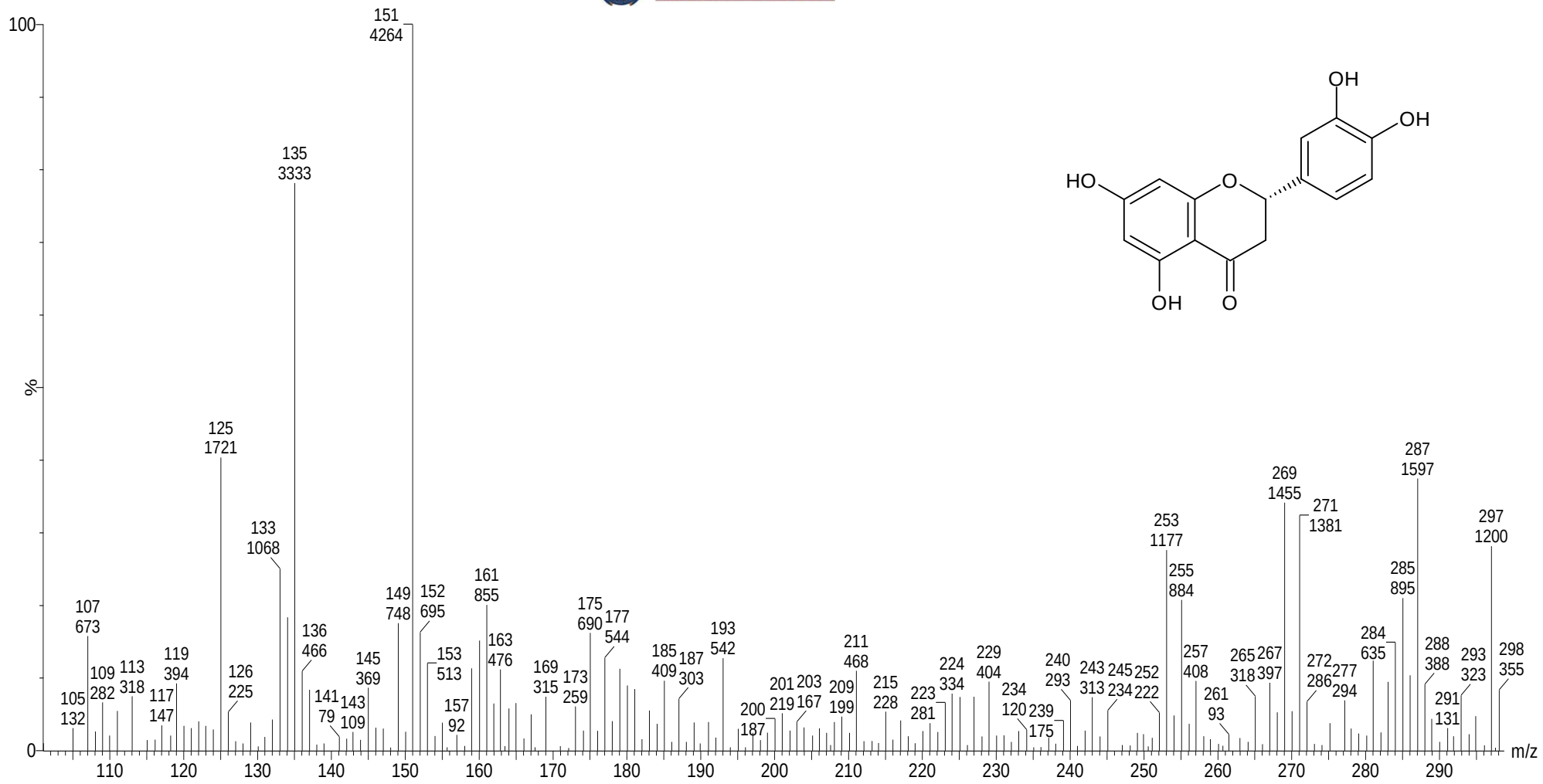

Figure 7-44: Mass spectrum of eriodctoyl (m/z at 287) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

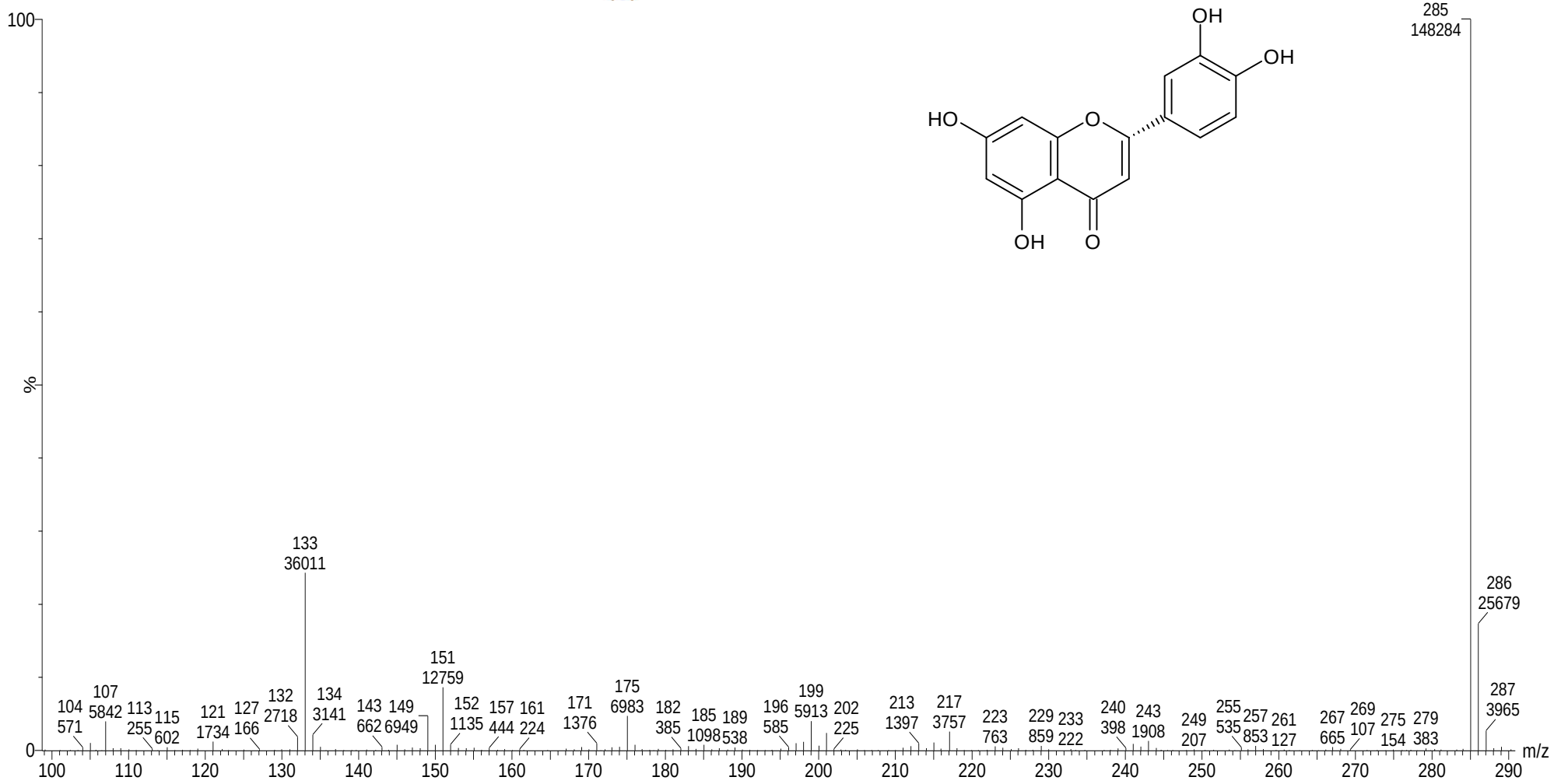

Figure 7-45: Mass spectrum of luteolin (m/z at 285) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

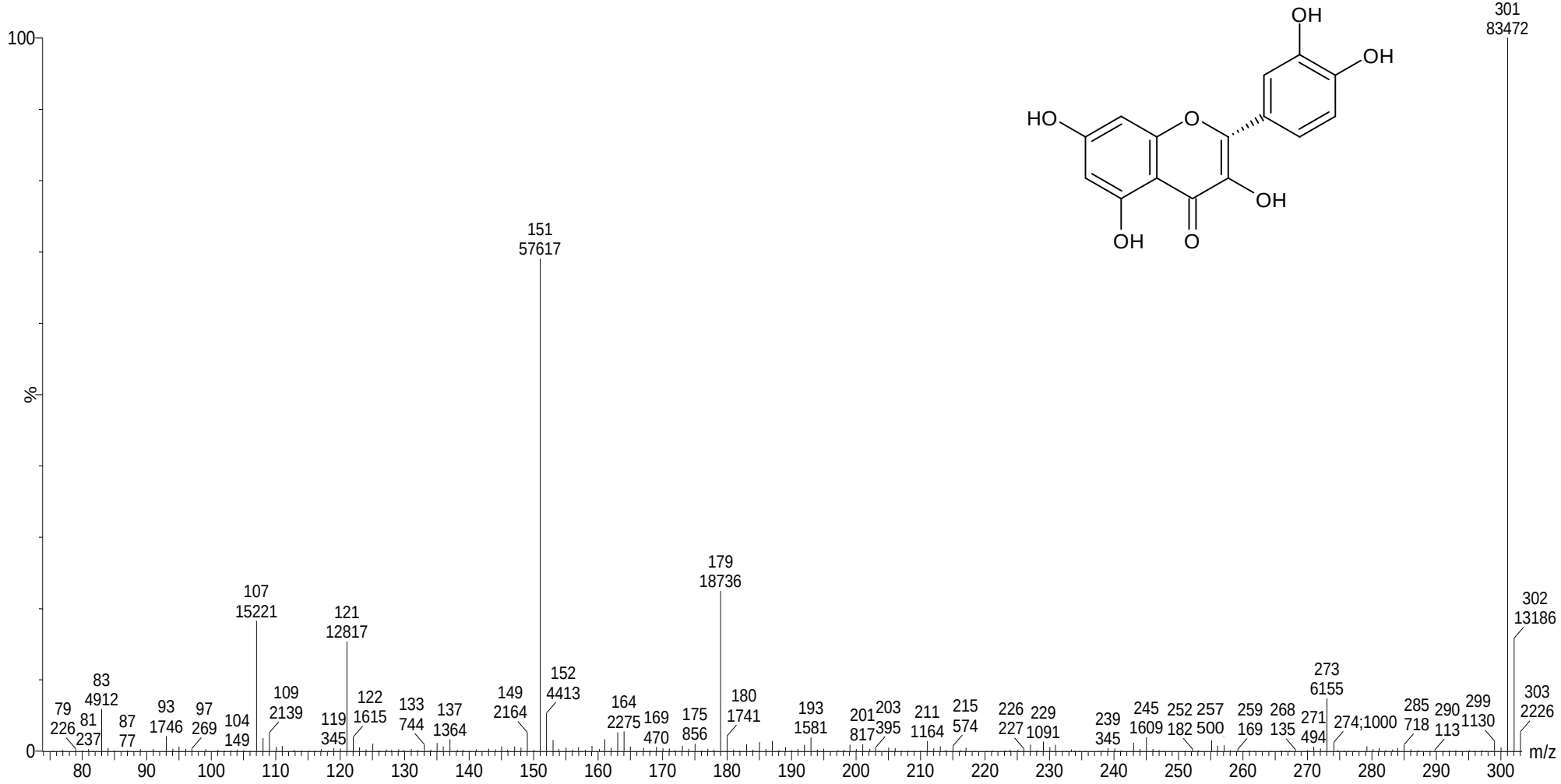

Figure 7-46: Mass spectrum of quercetin (m/z at 301) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

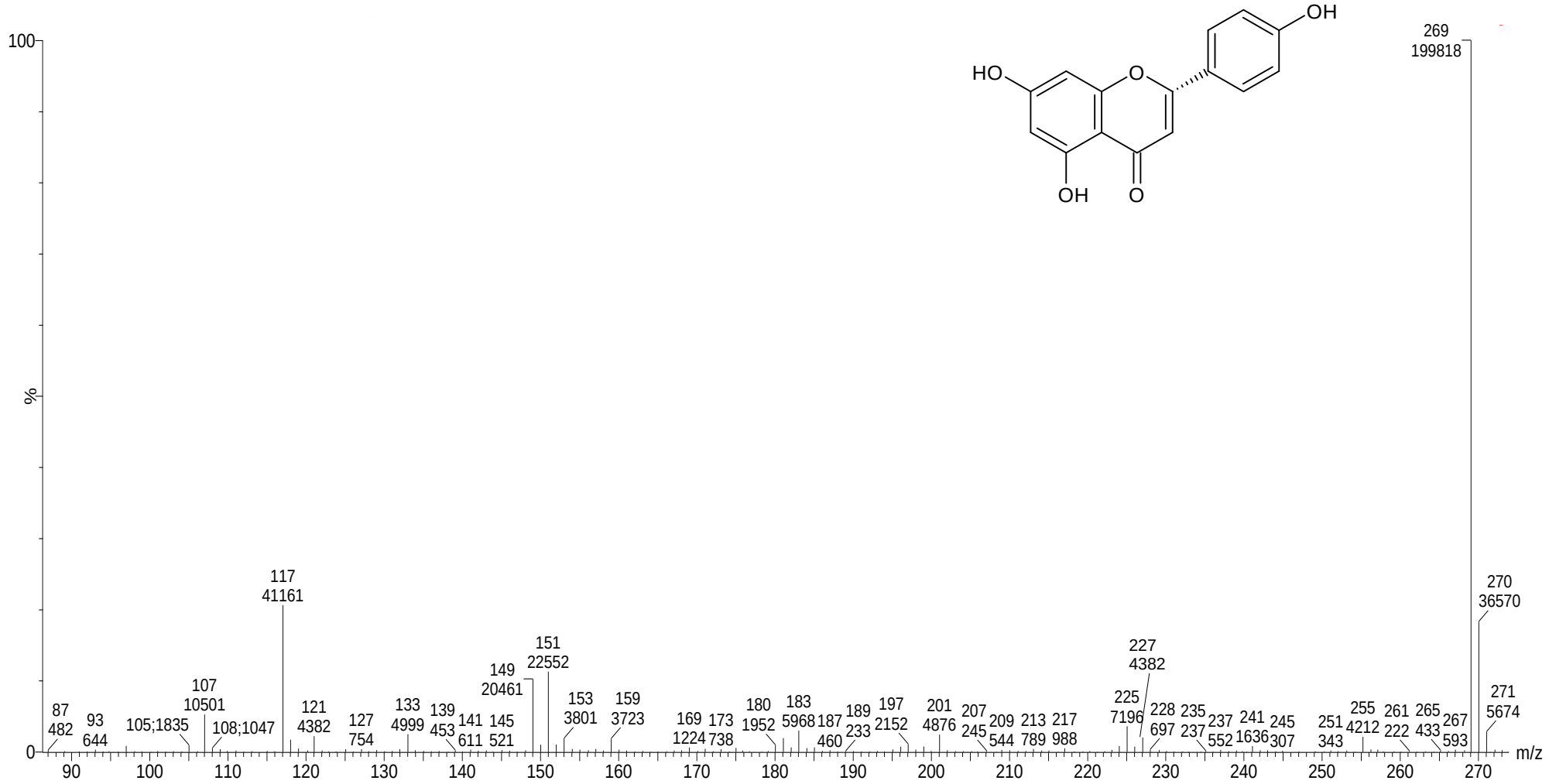

Figure 7-47: Mass spectrum of apigenin (m/z at 269) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

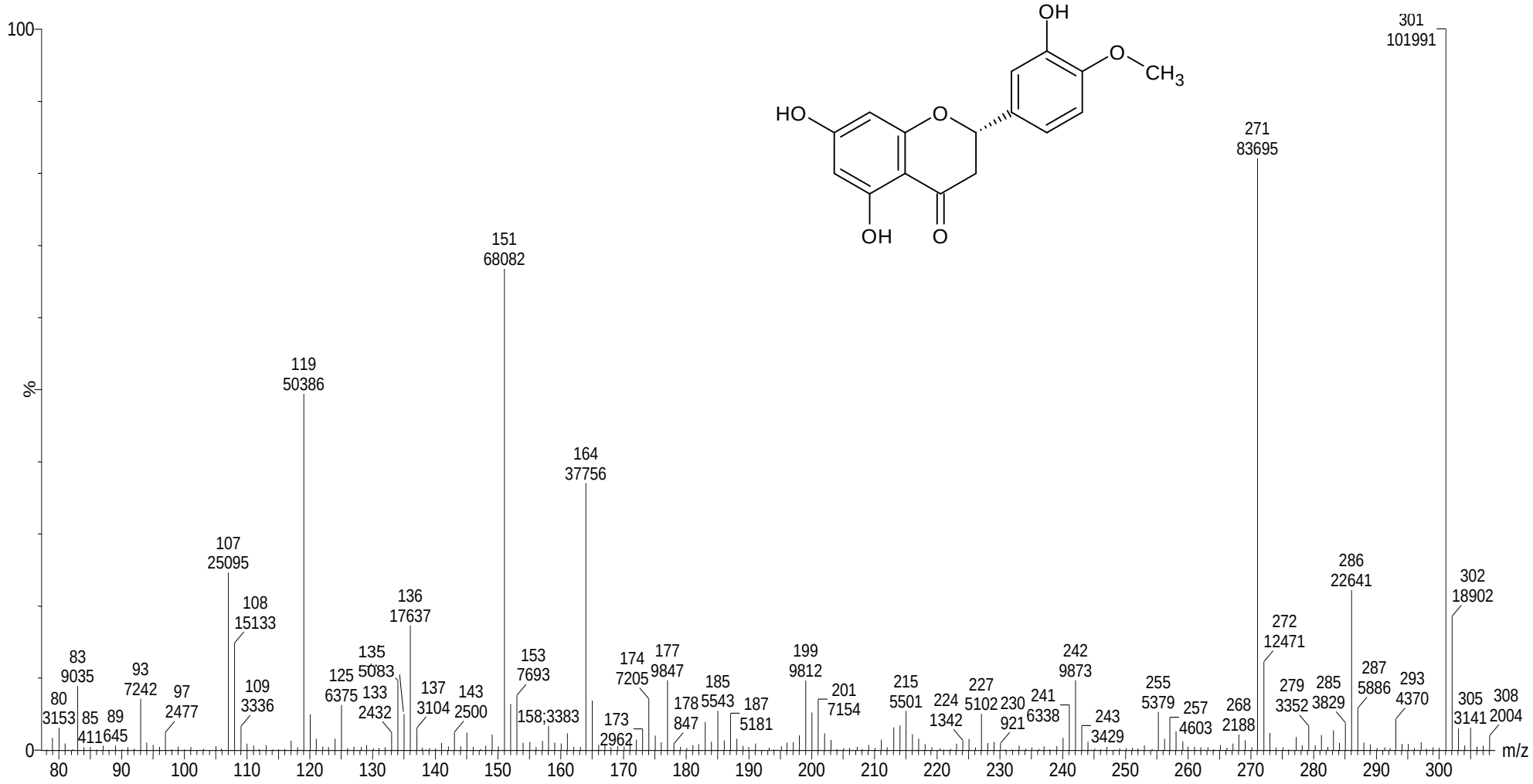

Figure 7-48: Mass spectrum of hesperetin (m/z at 301) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

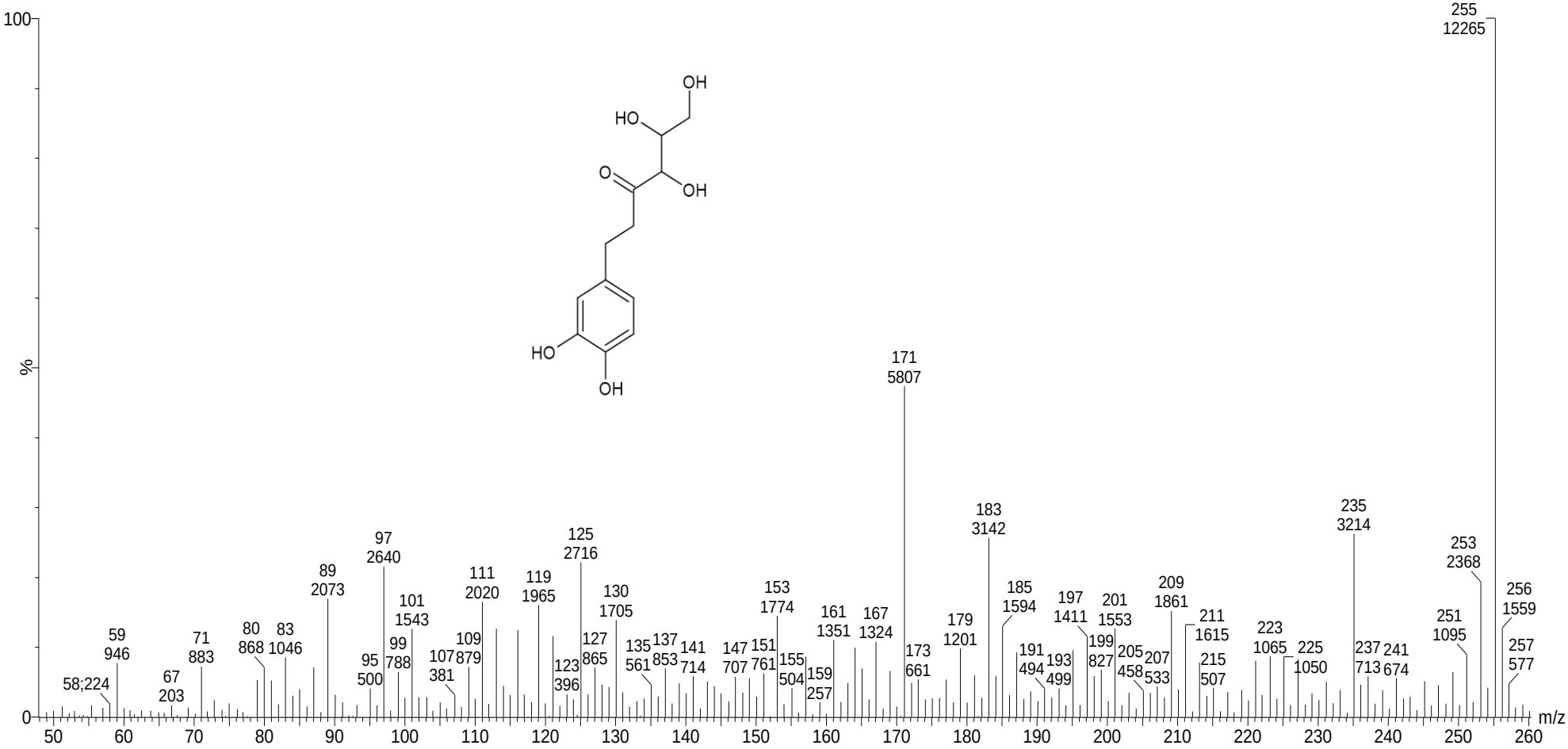

Figure 7-49: Mass spectrum of dihydrocaffeoyl glycerol (m/z at 255) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

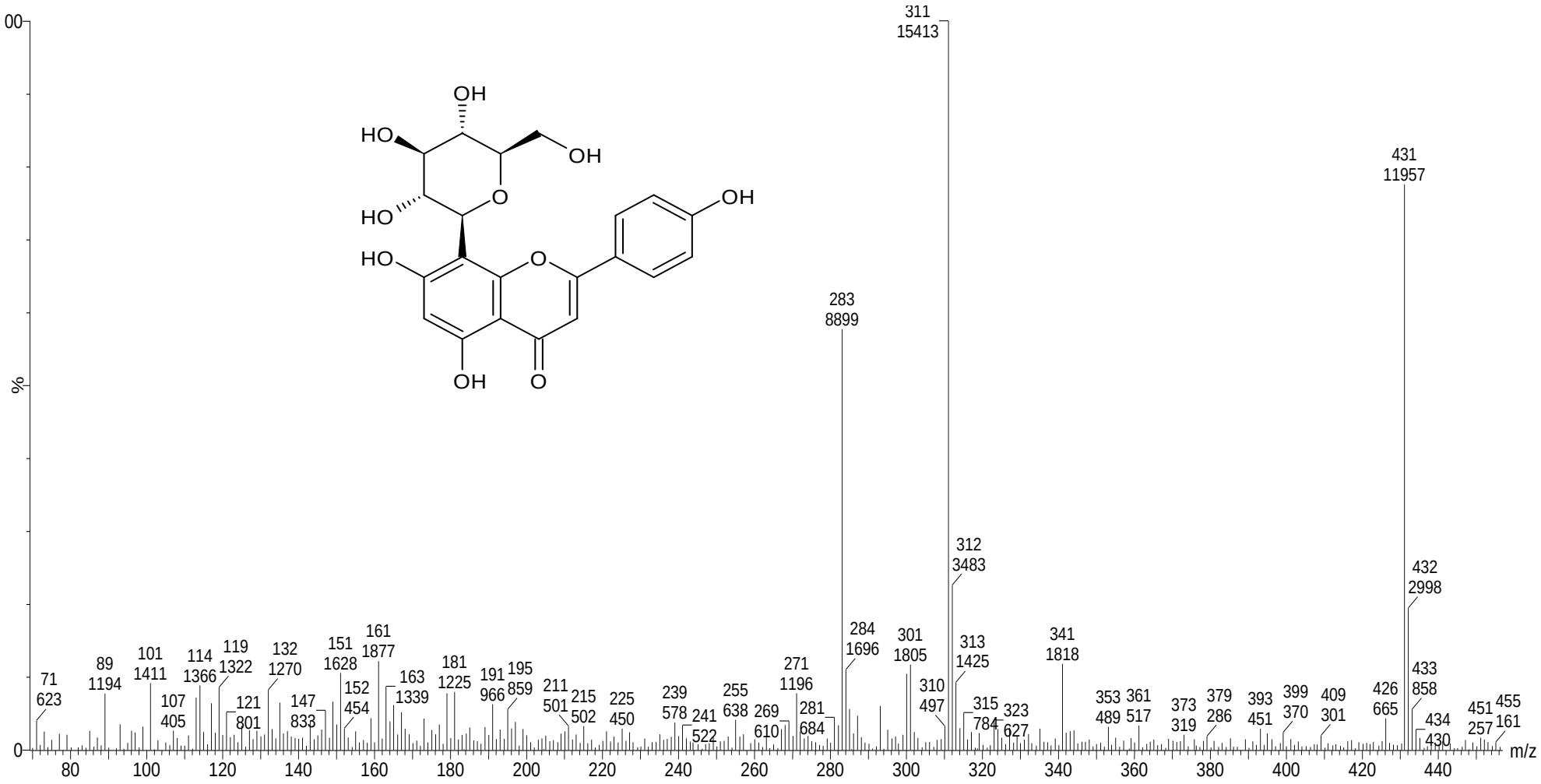

Figure 7-50: Mass spectrum of vitexin (m/z at 431) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

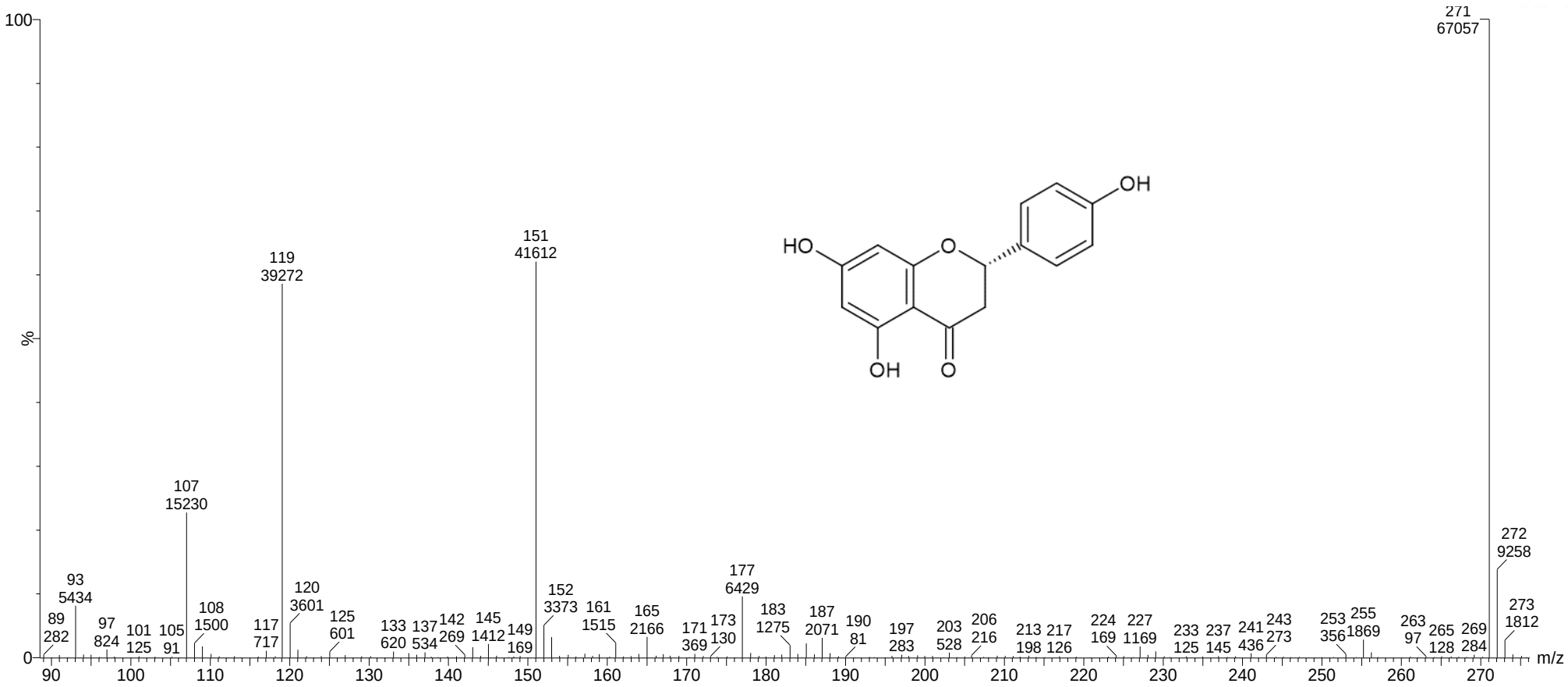

Figure 7-51: Mass spectrum of naringenin (m/z at 271) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study

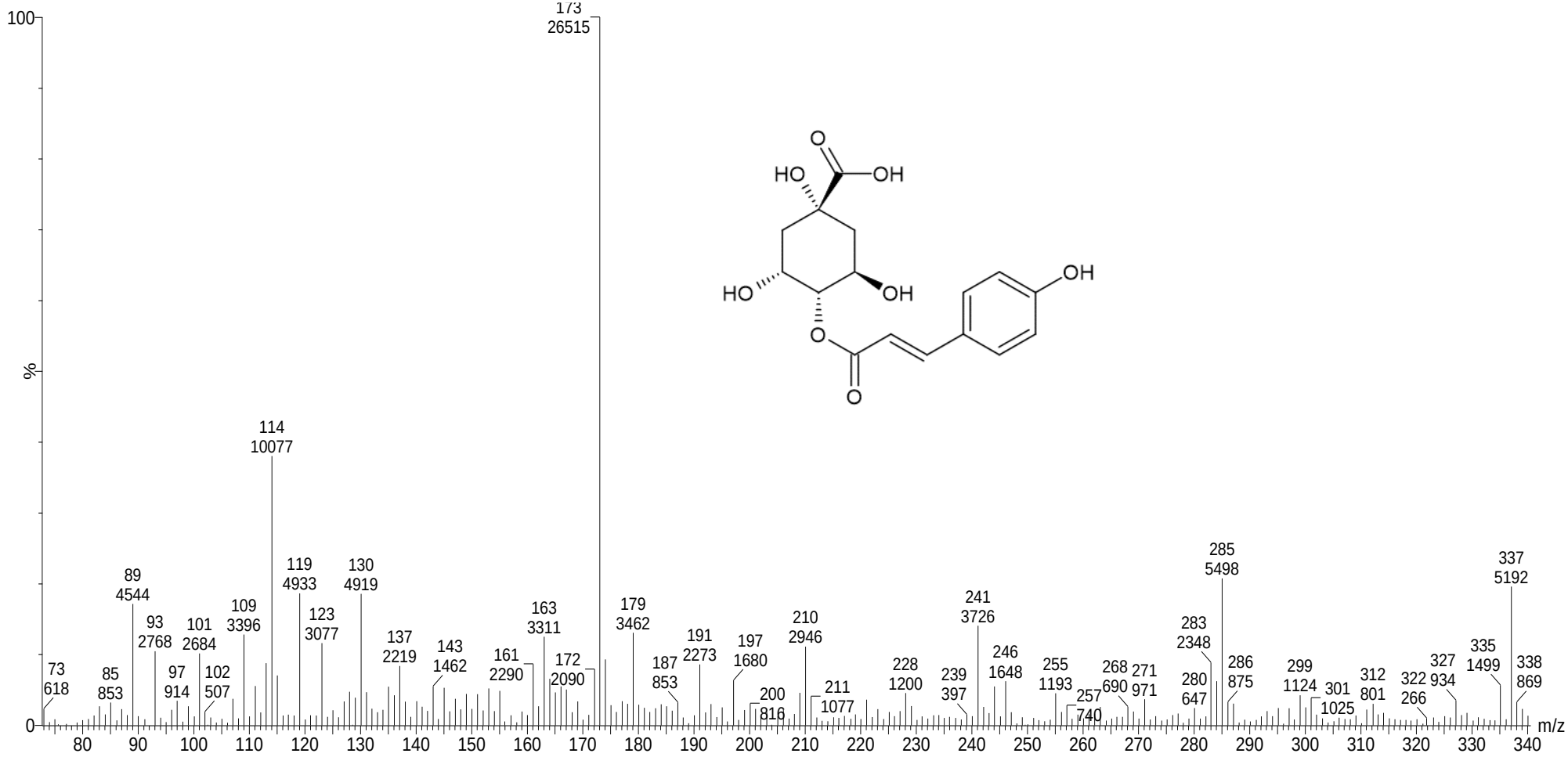

Figure 7-52: Mass spectrum of 4-ρ-coumaroyl quinic acid (m/z at 271) in 1% (v/v) hydrochloric acid in methanol extracts from the foodstuffs used in this study