

**Characterisation, health-promoting properties and food
applications of anthocyanin-rich pigments of flowers from the
Geraniaceae and Lamiaceae plant families**

by

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DECLARATION

I, Anton Venter, declare that the thesis, which I hereby submit for the degree PhD in Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

Date: 06/09/2022

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ABSTRACT

Characterisation, health-promoting properties and food applications of anthocyanin-rich pigments of flowers from the Geraniaceae and Lamiaceae plant families

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Colour is an important characteristic of food and plays a significant role in enhancing the visual appeal of food products. The colourants mostly used in the food industry are synthetic azo-dyes due to their brightness and stability. However, scientific evidence has shown that these compounds may pose adverse health effects. This has created a demand for more natural food colourants by consumers who have become more health conscious. Plant species from the Geraniaceae (*Pelargonium*) and Lamiaceae (*Salvia* (sage) and *Plectranthus* (spurflower)) families produce flowers with a range of different colours. The flowers are non-toxic and could be a source of novel stable pigments for potential application as natural colourants in foods with added health benefits.

Plant species from the Geraniaceae (*Pelargonium grandiflorum* Willd., *P. × hortorum* L.H. Bailey and *Pelargonium zonale* (L.) L'Hér.) and Lamiaceae (*Salvia aurea* × *dolomitica*, *Salvia dolomitica* Codd and *Plectranthus zuluensis* T. Cooke) families were used in this research. The petals of their flowers were collected, air-dried at 30°C for 24 hours and ground into a powder. Pigmented extracts were prepared by extracting the flower powder with formic acid-acidified water for 24 hours.

The pigmented flower extracts were evaluated for total phenolic content (Folin-Ciocalteu assay), anthocyanin and phenolic profiles and concentrations of phenolic compounds (Ultra Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry). Degradation kinetics experiments were conducted to determine the thermal and oxidative stability of the pigmented flower extracts. Health-promoting properties of the pigmented flower

extracts were determined using chemical radical scavenging assays (2,2-diphenyl-1-picrylhydrazyl [DPPH] radical scavenging, oxygen radical absorption capacity [ORAC] and nitric oxide [NO] radical scavenging), cellular assays (Caco-2 cellular antioxidant activity, NO radical scavenging in RAW 264.7 macrophages and anti-adipogenic activity in 3T3-L1 adipocytes) and anti-glycation activity (Advanced Glycation End Products assay). The pigmented flower extracts were applied as natural colourants in yogurt, fondant and gelatine candies while the flower petal powder was applied directly as colourant in yogurt. The food products with the natural colourants were evaluated for their colour stability (chromaticity analysis) during storage.

Anthocyanins in the Geraniaceae pigments were mainly 3,5-diglucoside of delphinidin, petunidin, pelargonidin, peonidin and malvidin and acetyl-acylated malvidin, delphinidin and petunidin. Anthocyanins in the Lamiaceae pigments were rutinosides of pelargonidin, glucosides of petunidin, pelargonidin and *p*-coumaric acid- and malonyl-acylated delphinidin and malvidin. Geraniaceae pigments also contained rutinosides of kaempferol and quercetin, quercetin-3-*O*-glucoside (isoquercetin), luteolin-7-*O*-glucoside and hydrolysable tannins (β -glucogallin, penta and tetra-galloyl glucose). Lamiaceae pigments contained naringin and hesperidin. The only phenolic acid in the Geraniaceae pigments was *p*-coumaric acid and the Lamiaceae pigments contained rosmarinic acid and its derivatives such as sagerinic acid and salvianolic acid B.

Lamiaceae pigments were more thermally and oxidatively stable (lower degradation rate constants and longer half-life) than Geraniaceae pigments. The greater stability of the Lamiaceae pigments could be due to hydrophobic intramolecular self-association interactions of their aromatic acylated anthocyanins in combination with stabilising interactions of these aromatic acylated anthocyanins with rosmarinic acid and its derivatives.

The pigmented flower extracts from the Geraniaceae had higher total phenolic content and DPPH radical scavenging activity than the Lamiaceae. All pigmented flower extracts exhibited Caco-2 cellular antioxidant activity (reduction of oxidative stress), inhibition of LPS-induced NO production in RAW 267.4 macrophage cells (anti-inflammatory effects) and ability to reduce lipid accumulation in 3T3-L1 adipocytes after 14 days treatment (anti-obesity effects). Despite their lower total phenolic content and DPPH radical scavenging activity, Lamiaceae pigments showed similar Caco-2 cellular antioxidant activity and inhibition of NO production in RAW 267.4 macrophages to Geraniaceae pigments. This may be related to the observed

greater thermal and oxidative stability of the Lamiaceae pigments. *P. grandiflorum* and *P. × hortorum* pigmented flower extracts showed significant anti-glycation activity (anti-diabetic effects) which was notably similar to that of the anti-diabetic drug aminoguanidine.

The pigmented flower extracts were successfully applied as natural colourants in yogurt (low pH food), fondant (sugar-based food system) and gelatine (protein-based food system) in comparison with the commercial E163 anthocyanin colourant. The colour of the food products was stable over a 15-day storage period with only some indications of slight decreases in redness and total colour. Compared to the commercial E163 colourant which only gave a purple colour, the pigmented flower extracts imparted a greater range of colours to the food products (red, purple, pink and orange) and in different shades of intensity and brightness. The application of the powdered flower samples directly into yogurt as colourant led to development of brown colouration in the yogurt possibly due to residual activity of browning enzymes in the flowers. This indicates that direct incorporation of the powdered flower samples as colourants in foods may not always be desirable.

This research shows the potential of flowers from the Geraniaceae and Lamiaceae plant families for application as novel natural food colourants. It represents important innovations in natural food colourants and provides novelty in terms of the stability of the pigments, the variety of colours they confer to food systems and their health-promoting properties. The findings of this study open up new opportunities for production of natural colourants for application in the food industry and also meets consumer demands.

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Chapter 1: Introduction and problem statement

Over the last 20 years, consumers worldwide have become increasingly health conscious and more aware of ingredients in food products (Downham and Collins, 2000) such as food colourants. Synthetic food colourants are used extensively in the food industry however, there is concern about their potential negative health effects. Some negative health effects associated with synthetic colourants such as azo dyes include increased hyperactivity in children (McCann, Barrett, Cooper, Crumpler, Dalen, Grimshaw, Smith, Lok, Porteous, Prince, Sonuga-barke, Warner and Stevenson, 2007) and potential carcinogenic properties upon gut bacterial transformation (McCann, Choi, Yamasaki and Ames, 1975). This has created a demand among consumers for more natural and presumably healthier sources of food colourants and the food industry has been on a quest for such colourants in order to satisfy consumer demand.

Colour pigments are widespread throughout nature from sources such as in fruits, vegetables and flowers. Many natural food colourants consist of compounds such as anthocyanins and other phenolic compounds, carotenoids and betalains. Some of the permitted natural food colourants used in the food industry include curcumin from turmeric, natural carotenes derived from algae, lycopene from tomatoes, anthocyanins from grape skin and red carrot among many others (Downham and Collins, 2000; Díaz-García, Castellar, Obón, Obón, Alcaraz and Rivera, 2015).

Concerning natural anthocyanin pigments, a potential limitation to their application in foods is the stability of the anthocyanins. Anthocyanin stability is affected by factors such as light, temperature, pH and presence of other compounds (Rein, 2005). However, anthocyanins are also known for their health-promoting properties due to their bioactive properties (Kong, Chia, Goh, Chia and Brouillard, 2003) and could provide potential health benefits if applied as food colourants.

Flowers from plants occur in a wide range of colours and this makes them ideal sources for the exploration and production of different natural colourants. In this regard, plant species from the Lamiaceae (*Salvia* and *Plectranthus* species) and Geraniaceae (*Pelargonium*) families are worth exploring as they produce flowers with different colours. Plants from these two families have a long history of use as herbs in culinary practices. The medicinal uses of plants from the

Geraniaceae and Lamiaceae form a major part of traditional medicines in South Africa and its use date back 20 000 years to the San people (Scott *et al.* 2004; Deacon, 1983).

They are also most commonly used as a source of essential oils (Zielińska and Matkowski, 2014; Bedigian, 2005) and most research on Lamiaceae and Geraniaceae explore the health benefits of these essential oils and the compounds responsible for the health benefits. Research on these plants has also focused on the plant as a whole or only on aerial roots or leaves. The very limited research on South African plant species from these two families have only focused on some health-promoting properties of the leaf extracts (Adebayo, Ondua, Shai and Lebelo, 2019).

Research on flowers aimed at the production of natural colourants include investigation into the use of extracts from rose petals (*Rosa damascena* Mill.) as a potential food colourant and source of antioxidant properties (Schieber, Mihalev, Berardini, Mollov and Carle, 2005). The use of encapsulated *Hibiscus sabdariffa* L. pigmented extract and the effect of pH and temperature on colour stability in the production of strawberry jams and hard candies has been investigated (Selim, Khalil, Abdel-Bary and Abdel-Azeim, 2008). Studies have also been carried out on the potential of *Thymus moroderi* (Lamiaceae) flower extracts as a natural pink colourant and its application in yogurt (Díaz-García, Castellar, Obón, Obón, Alcaraz and Rivera, 2015).

Flowers of plant species from the Lamiaceae and Geraniaceae families have not been sufficiently explored and studied as potential sources of natural food colorants. This presents an opportunity for natural plant pigment prospecting for application in foods. Therefore, this research aims to evaluate pigments from flowers of plants from the Lamiaceae and Geraniaceae families as natural colourants by studying their stability, health-promoting properties and application in different food systems. This research will open up new avenues and provide more options for plant sources of natural food colourants.

Chapter 2: Literature review

2.1 Importance of colour in food

Colour is one of the most important characteristics of food. It is the first intrinsic sensory information that the consumer receives about the freshness and overall quality of food and may also influence a consumer's perception of the taste and flavour. The brightness of the colour can also impact the perceived intensity of these sensory characteristics by consumers (Spence, 2015). All these characteristics, in turn, contribute to consumer choice and acceptance of food and therefore highlights the importance of colour in this regard. Colourants are thus an important aspect of the food ingredients industry.

2.2 Synthetic colourants used in foods and associated issues

A wide range of colourants are used in the food industry worldwide, most of which are synthetic in nature. The European Union (EU) has approved 43 colourants as food additives, of which 17 are synthetic (Downham and Collins, 2000). South Africa currently permits the use of 44 colourants, of which 18 are synthetic (South African Department of Health (DOH), 1999). The synthetic food colourants which are permitted for use in food by the US Food and Drug Administration (FDA) are classified into three distinct groups namely, azo dyes, triphenylmethane dyes and xanthene dyes (Shamina, Shiva and Parthasarathy, 2007).

Research over the last 20 years has shown that these synthetic dyes could cause negative health effects in humans. Azo dyes have been shown to cause increased levels of hyperactivity in children and are potential carcinogens (McCann et al., 1975; McCann et al., 2007). Erythrosine (a xanthene dye) at high doses of 4% in the diet has been shown to influence circulating thyroid hormones in rats which may lead to thyroid tumours (Chequer, Venâncio, Bianchi and Antunes, 2012). Chequer et al (2012) confirmed the genotoxicity and mutagenicity of erythrosine in HepG2 cells due to DNA damage. These potential negative health effects of synthetic colourants have therefore ignited an interest in natural sources of colourants which are perceived to be healthier.

2.3 Lamiaceae and Geraniaceae plant families as potential sources of natural colourants

The Lamiaceae (mint) family of plants are identified by short-stalked epidermal glands containing intangible oils which consist of terpenoid compounds such as mono, di and sesquiterpenes (Hutchings & Scott, 1996). This plant family consists of 236 genera and more than 6000 species (Carović-Stanko, Petek, Grdiša, Pintar, Bedeković, Čustić and Satovic, 2016). Some plants from the Lamiaceae family emit a strong mint smell (Pingale, Pokharkar and Phadtare, 2013; Ascensão, Marques and Pais, 1997), and most would be considered aromatic. The members of this plant family are used extensively as culinary herbs such as basil (*Ocimum* spp.), mint (*Mentha × piperita* L), rosemary (*Rosmarinus officinalis*) and sage (*Satureja hortensis* L.). Many of the Lamiaceae family of plants have been used as traditional medicine for the treatment of a variety of diseases (Carović-Stanko et al., 2016).

The Geraniaceae plant family consist of two main genera, *Geranium* and *Pelargonium*, which are generally confused by the public as being the same (Lis-Balchin, 2002). The flowers of *Pelargonium* species have an umbel-like arrangement. The species are often identified by their aromatic oils which are usually only produced at night (Miller, 2002). The essential oils in *Pelargonium* species are extracted from the leaves and have been used in aromatherapy practices for centuries and have spasmolytic effects on intestinal smooth muscle (Williams and Harborne, 2002). *Pelargonium* species have been used for medicinal purposes as water-soluble teas or ethanol extracts. Most research has focused on the pharmacological activities of the essential oil although, research on methanolic extracts from the roots and leaves of several *Pelargonium* plant species has found that the alkaloids they contain possess spasmolytic activity (Lis-Balchin and Hart, 1997).

These two plant families produce vivid pigmented flowers with a variety of different colours. The pigments of these flowers are rich in anthocyanin compounds which could be used as potential natural colourants.

2.5 Anthocyanins as plant pigments

Anthocyanins are a group of pigmented flavonoids produced by plants and bacteria (Shamina., 2007). Anthocyanins give colours in nature such as red, purple, blue and orange and have been consumed for millennia by humans and animals in fruits and vegetables with no toxic effect. For this reason, anthocyanins from natural sources have been considered as a viable replacement for synthetic colourants (Markakis, 1982). The EU permits the use of anthocyanin extracts as natural food colourants from sources such as black currants, strawberries, elderberries, and red cabbage. The most common natural anthocyanin colourant (E163) is derived from grape skins, a by-product of wine-making (Bueno, Sáez-Plaza, RamosEscudero, Jiménez, Fett and Asuero, 2012). Anthocyanins are mainly used in the beverage industry as natural colourants and they are increasingly being used in other food products such as dry mixes and yoghurt products (He Jian and Giusti, 2010). Anthocyanins extracted from natural sources such as grape skins or red carrots have also been authorized for use in Japan, USA, and other countries around the world (Elder, 2000). South Africa permits the use of an anthocyanin colourant extracted from various fruits such as grape skin. (Department of Health, 1999).

2.5.1 Chemistry of anthocyanins

Anthocyanins are part of a larger group of phenolic compounds known as flavonoids (He Jian and Giusti, 2010). The anthocyanin aglycone consists of a C-6 (A ring), C-3 (C ring) and C-6 (B ring) (Harborne, 1998) (Figure 2.5.1) backbone chemically known as 2-phenylbenzopyrylium. Differences between individual anthocyanins depend on the number of hydroxyl groups, type and number and position of sugar molecules attached as well as the number and type of aliphatic or aromatic acids attached to the sugar component. Anthocyanins are known for their wide range of colours in plants and they act as attractants for animals to aid in seed dispersal (Kong et al., 2003). Due to the strong absorption of light by anthocyanins, they also play an essential role in the protection of plants against UV damage (Mazza, 1993). Anthocyanins are rarely found in the aglycone form and occur mostly bonded to other constituents by hydroxyl groups which confer stability to the compounds. Glycosylation of anthocyanins confers stability via intramolecular hydrogen bonding within the anthocyanin molecule (Borkowski, Szymusiak, Gliszczyńska-Świąło and Tyrakowska, 2005). The wide range of colours observed from anthocyanins in the acidic environment is mainly due to the

presence of eight conjugated double bonds and a positive charge on the oxygen. The colour of anthocyanins is also primarily influenced by pH (Kong et al., 2003).

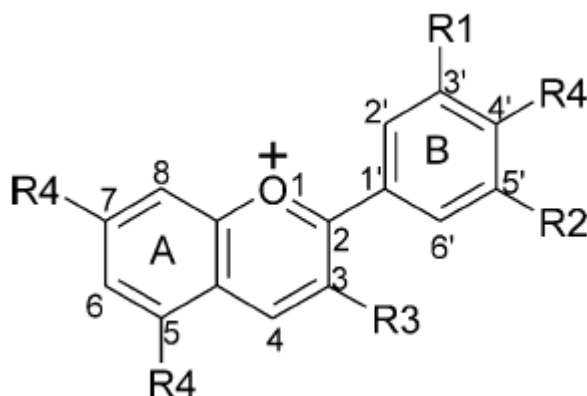


Figure 2.5.1: Structure of the anthocyanin aglycone backbone (Kong et al., 2003)

2.5.2 Influence of pH on anthocyanin structure

As shown in Figure 2.5.2, in acidic medium ($\text{pH} < 2$), the anthocyanin exists in its flavylium cation form (AH^+) (red). With the deprotonation of the AH^+ as the pH changes to neutral and slightly acidic, the flavylium cation converts to the quinonoidal base (A) (purple to violet). Further deprotonation above a pH of 7 results in a blue anionic quinoidal base form (Anderson & Jordheim, 2010). The flavylium cation also converts to the carbinol base and chalcone form (pH 3-6) (B) (colourless or yellow) upon hydration (Brouillard and Dubois, 1977; He Jian and Giusti, 2010; Ananga, Georgiev, Ochieng, Phills and Tsolova, 2013). The pH-induced colour changes of anthocyanins has recently led to them being used as a non-toxic pH indicator in the application of agar/potato starch intelligent packaging films for the detection of product quality changes during bacterial spoilage (Choi, Lee, Lacroix and Han, 2017).

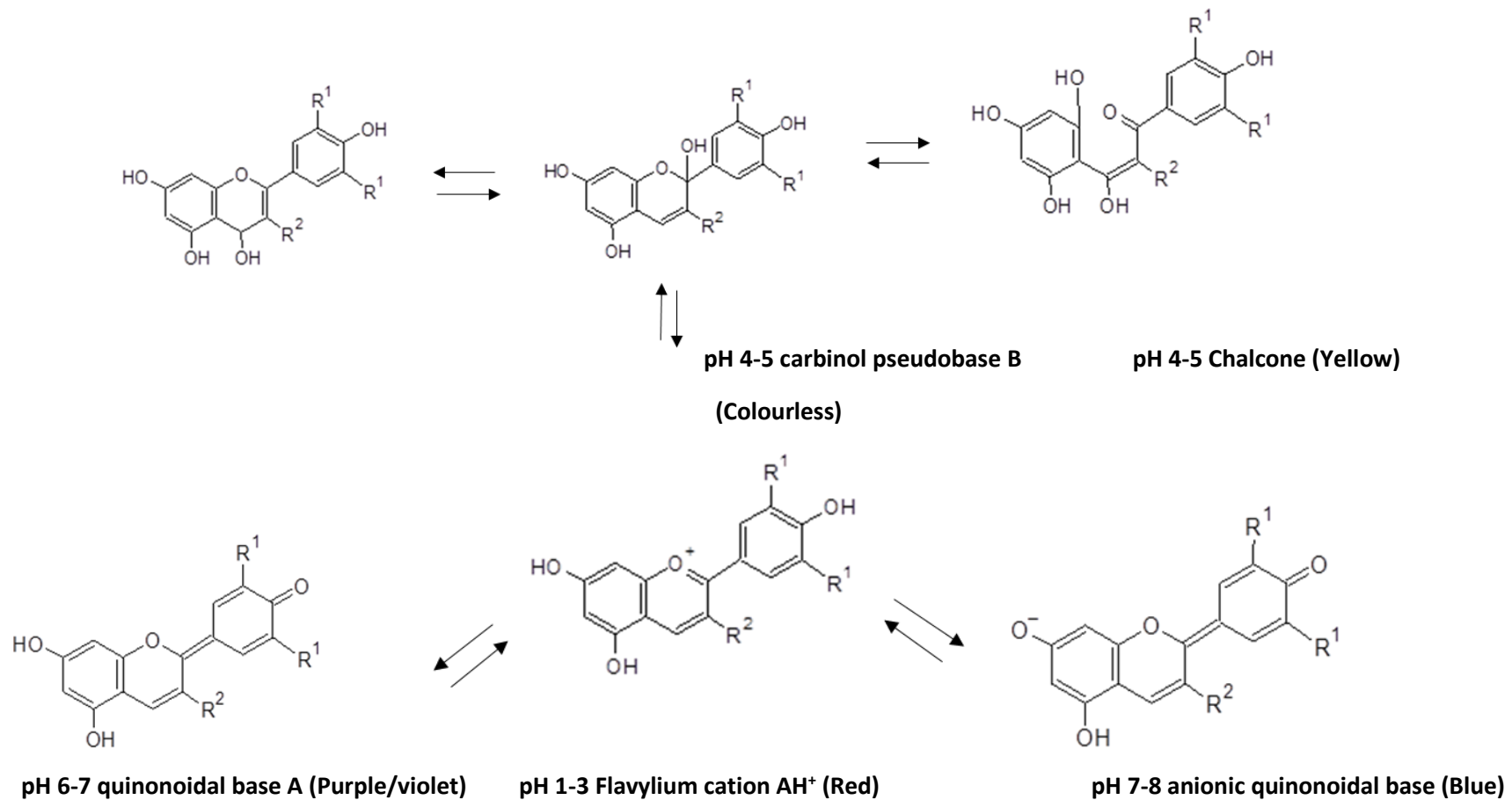


Figure 2.5.2: Effect of pH on the structure of anthocyanins

2.5.3 Influence of temperature on anthocyanin structure

Thermal treatment of anthocyanins can cause degradation of the pigment depending on the severity and type of heating. Thermal degradation of anthocyanins includes enhanced oxidation and cleavage of covalent bonds leading to several chemical species (Patras, Brunton, O'Donnell and Tiwari, 2010). Sadilova, Stintzing and Carle (2006) studied thermal degradation of acylated and non-acylated anthocyanins of elderberry, strawberry and black carrots at 95°C at pH of 1. They found that anthocyanin glycosides such as pelargonidin and cyanidin initially undergo loss of the sugar moiety through deglycosylation followed by cleavage into reaction products such as phloroglucinaldehyde, 4-hydroxybenzoic acid (from pelargonidin) and protocatechuic acid (from cyanidin) (Figure 2.5.3). Other possible mechanisms of thermal degradation have been described in a review article by Xu, Zhang, Fang and Wang (2020). The degradation starts with hydrolysis of the C-3 glucoside followed by isomerization into the chalcone and α -diketone. The other degradation mechanism allows for the opening of the anthocyanin structure to form the chalcone form.

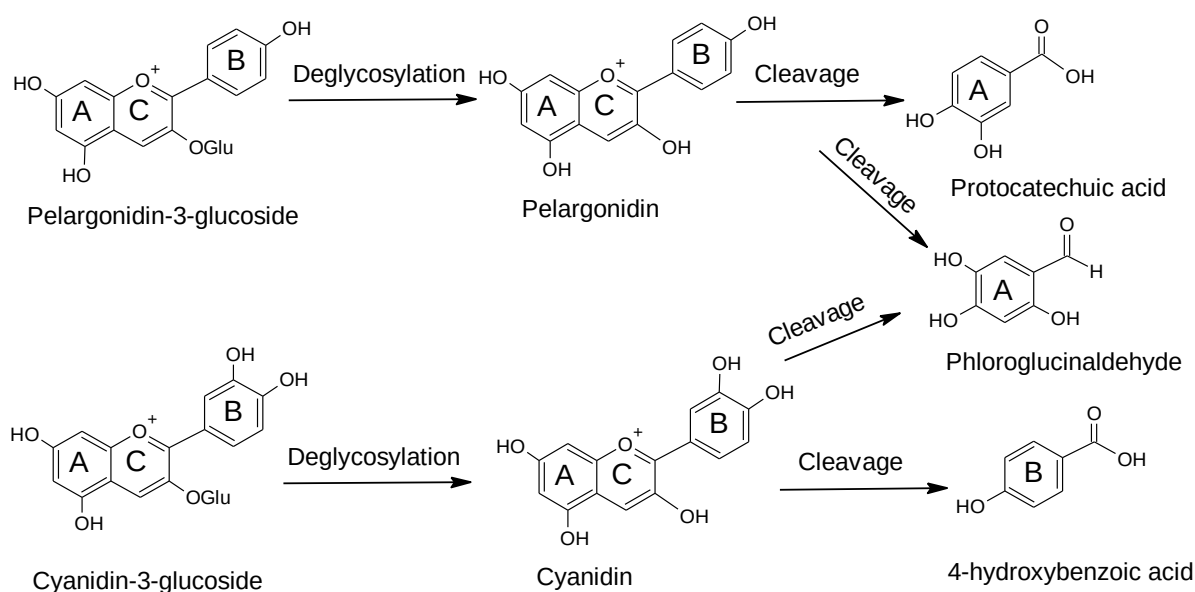


Figure 2.5.3: Thermal degradation pathways for pelargonidin-3-glucoside and cyanidin-3-glucoside. Adapted from Patras, Brunton, O'Donnell and Tiwari (2010)

2.5.4 Influence of oxidation on anthocyanin structure

Anthocyanins can undergo degradation through different oxidation mechanisms such as enzymatic oxidation or oxidation through radicals generated by transition metals. Research by Kader, Nicolas and Metche (1999) showed the important role of polyphenol oxidase (PPO) enzyme and a phenolic acid such as chlorogenic acid in the oxidation of anthocyanins such as pelargonidin-3-glucoside. They found that blueberry PPO oxidizes chlorogenic acid to the chloroquinone form followed by reaction of chloroquinone with pelargonidin-3-glucoside which leads to the formation of brown polymers through condensation reactions (Kader, Nicolas and Metche, 1999; Kader, Irmouli, Nicolas and Metche, 2002).

Radical generated oxidation reactions of anthocyanins are very complex and result in the production of different products depending on the position of nucleophilic attack by radicals. Satake and Yanase (2018) studied different modes of oxidation of anthocyanins by hydrogen peroxide and hydroxyl radicals. They found that oxidative degradation with hydrogen peroxide and hydroxyl radicals produce the same reaction products and degraded anthocyanins by the addition of hydrogen peroxide at different positions of the C ring, leading to ring-opening and loss of aromaticity as well as the release of protocatechuic acid (compound 3) (Figure 2.5.4).

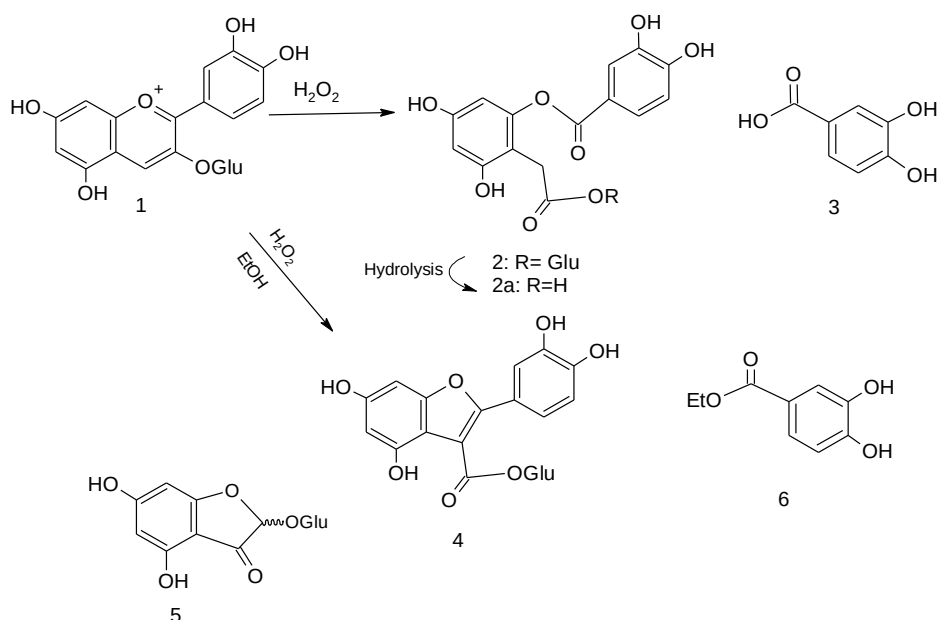


Figure 2.5.4: Hydrogen peroxide oxidation pathway of anthocyanin. Adapted from Satake and Yanase (2018)

Dangles and Fenger (2018) proposed a mechanism for the reaction of anthocyanins with oxygen and transition metal-assisted hydrogen radical. The hydrogen radical is transferred to the oxygen, followed by the addition of hydroperoxide radicals at different positions of the anthocyanin molecules. This leads to a cascade of reactions involving the addition and removal of water (H_2O) leading to C ring-opening and cleavage of the C-2, 3 bonds (Figure 2.5.5).

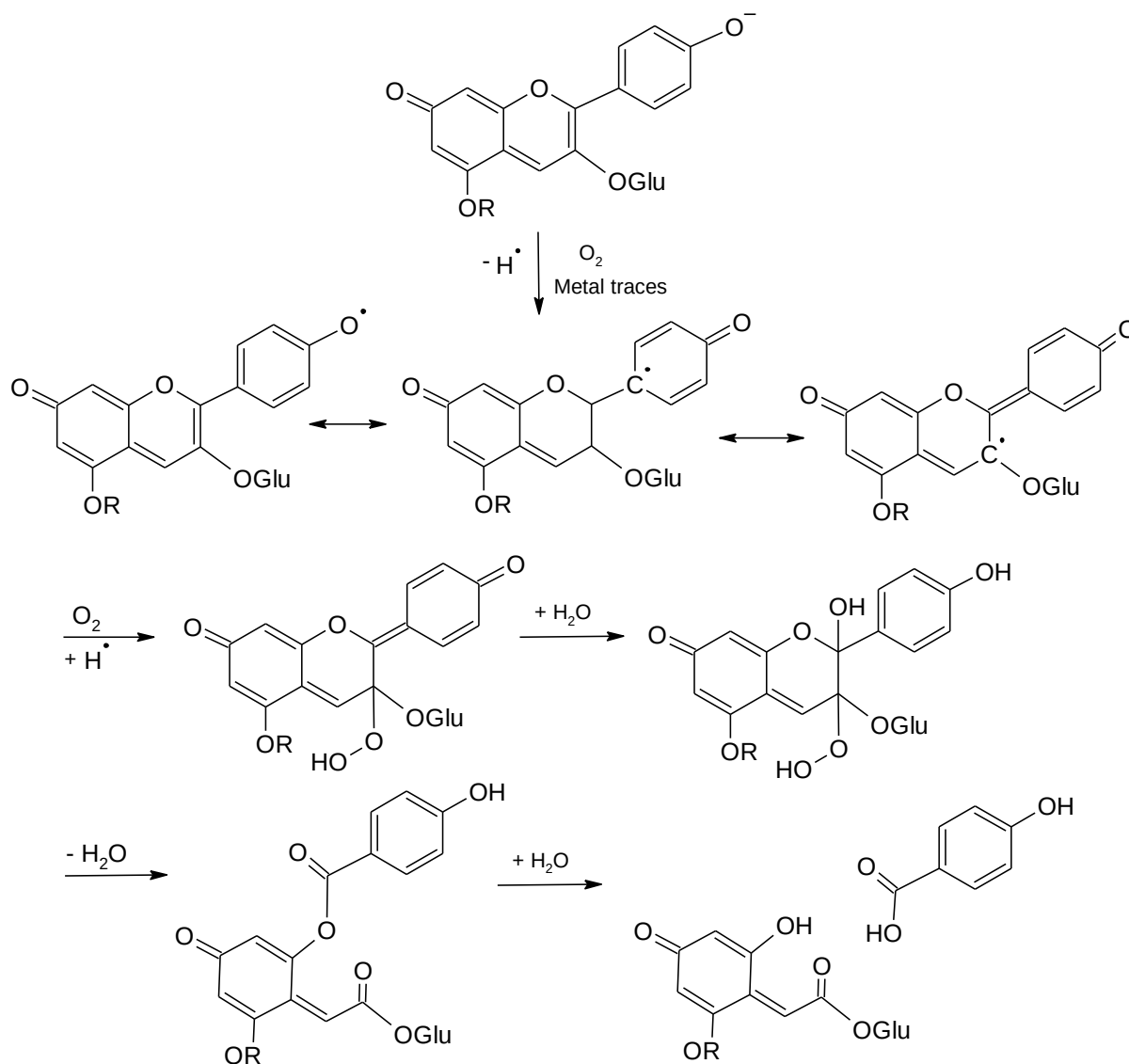


Figure 2.5.5: Possible mechanism of anthocyanin oxidation through reactive hydroperoxide radicals. Adapted from Dangles and Fenger (2018)

2.5.5 Influence of UV radiation on anthocyanin structure

The exposure of *Hibiscus acetosella* anthocyanins to UV radiation under different pH conditions has been extensively studied by Março, Poppi, Scarminio and Tauler (2011). They found that the degradation of anthocyanin pigments at different pH levels occurred at a faster rate with UV radiation. These authors also found that degradation species increased with UV radiation from seven species to nine species. UV radiation seemed to degrade anthocyanin pigment (AH^+) to different chemical species such as the formation of a B_4 species which is similar to the *cis*-chalcone (C_c) form (Março et al., 2011) (Figure 2.5.6). UV radiation causes ionization or deprotonation of anthocyanins leading to different species such as the ionized quinoidal species (A^-), di-ionized quinoidal species (A^{2-}) and ionized *trans*-chalone or quinoidal (C_T/A^{*-} tautomers (Figure 2.5.6) (Março et al., 2011) at different pH levels. Março et al. (2011) found that the quinoidal anidrobases (A) formed from the flavylium cation (AH^+) at an alkaline pH (6.5-8) is however unaffected by UV radiation. Anthocyanin structures can also transition between *cis*/*trans* forms through light excitation, leading to different structural tautomers (Maestri, Pina, Roque and Passaniti, 2000).

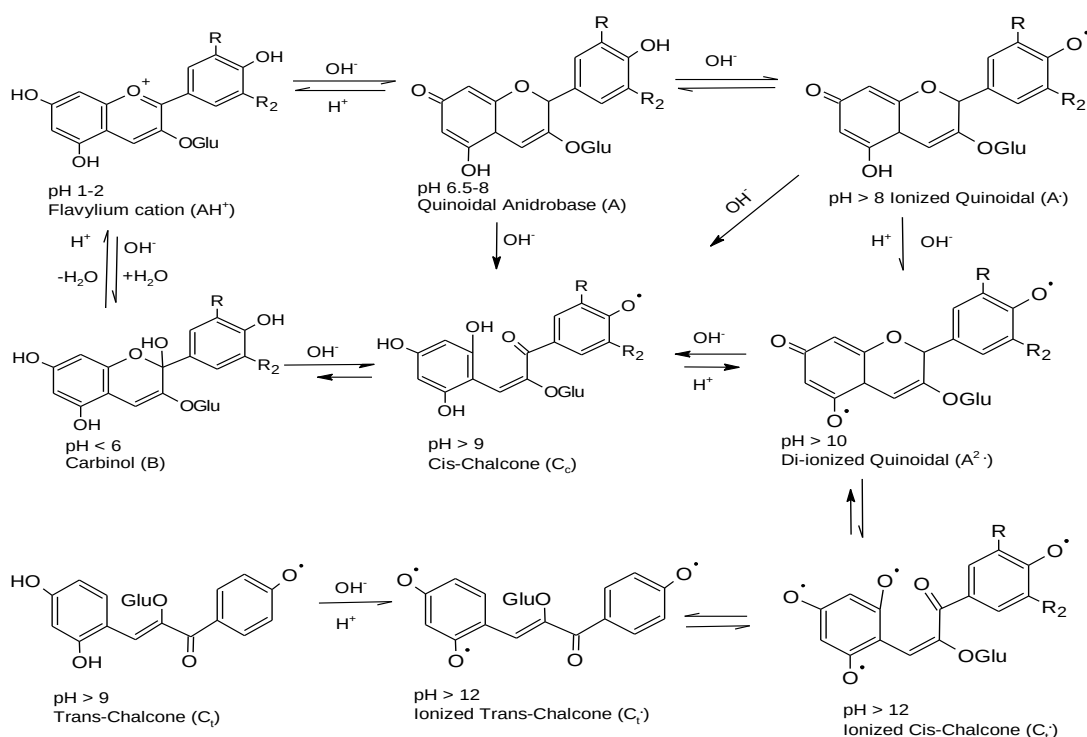


Figure 2.5.6 Influence of pH and UV radiation on anthocyanin structure. Adapted from (Março et al., 2011)

2.6 Mechanisms for improving the stability of anthocyanins

As discussed in the previous sections, anthocyanins are susceptible to degradation under various conditions such as light, pH, temperature, enzymes and other substances present. The stability of natural food colourants, such as anthocyanins, is a crucial part of their success for use in food application (Rein, 2005). Co-pigmentation can protect the anthocyanin molecule from nucleophilic attack by water or protect against pH change, thereby conferring stability (Renis, Calandra, Scifo, Tomasello, Cardile, Vanella, Bei, La Fauci and Galvano, 2008). Several mechanisms have been proposed for anthocyanin stability through co-pigmentation.

2.6.1 Intermolecular co-pigmentation

Intermolecular co-pigmentation involves the molecular interactions between coloured anthocyanins and colourless co-pigment molecules such as other flavonoids and phenolic acids (Brouillard, 1983). Flavylium cation and quinoidal forms of anthocyanins can both be involved in co-pigmentation as both are planar, have delocalized π -electrons and have the same structural features as co-pigments such as other flavonoids (Rein, 2005). The co-pigmentation could also be facilitated by hydrogen bonding and hydrophobic interactions between anthocyanin and the co-pigments (Figure 2.6.1 B) (Asen, 1976). This configuration could also allow for π - π bond overlap of the anthocyanin and flavonoid molecules, as well as hydrogen bonding between the anthocyanin and co-pigment thereby contributing to stabilization (Figure 2.6.1 A) (Dangles and Brouillard, 1992).

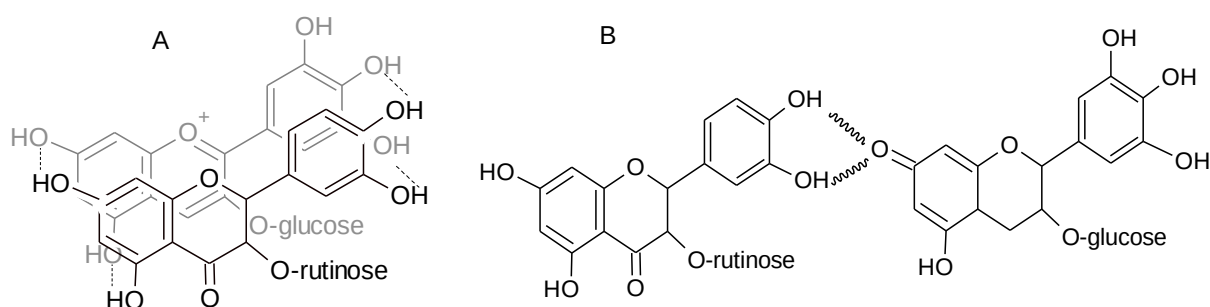


Figure 2.6.1 Mechanisms of intermolecular co-pigmentation. A) Interlocking complex and B) parallel complex between delphinidin-3-glucoside and rutin. Adapted from Williams and Hrazdina (1979); Osawa (1982)

2.6.2 Intramolecular co-pigmentation

The key to intramolecular co-pigmentation is the acylated anthocyanin molecules which confer stability to the co-pigmentation (Francis, 1989, Dangles et al., 1993). The stability is due to a folded conformation initiated by acyl group on the sugar residue of the anthocyanin bringing together the anthocyanins with the phenolic acid residues as described by Dangles et al (1993) such as the cinnamic acids. It has been proposed that diacylated anthocyanins resort to a sandwich-like stacking mechanism with the phenolic acids such that the cinnamic acids reside on both sides of the anthocyanin molecule (Figure 2.6.2 A). Dangles et al (1993) pointed out that phenolic acid residues not only produce a stable anthocyanin complex but also shift the maximum absorption in the visible range and thus allows for a production of a diverse range of colours. The authors attribute the shift to the decrease in polarity of the anthocyanin due to the interaction with hydrophobic caffeoyl residue. The stability of flowers in nature is accredited to the presence of glycosidic acylated anthocyanins. Acylated anthocyanins have an important role in photoprotection. The charge and energy from UV light are rapidly transferred from acyl residues to the anthocyanin molecule (Figure 2.6.2 B) (Dangles et al., 1993). Acylated residues can also protect anthocyanins from oxidative degradation by hydrogen peroxide through impediment of its addition (Fenger et al., 2019).

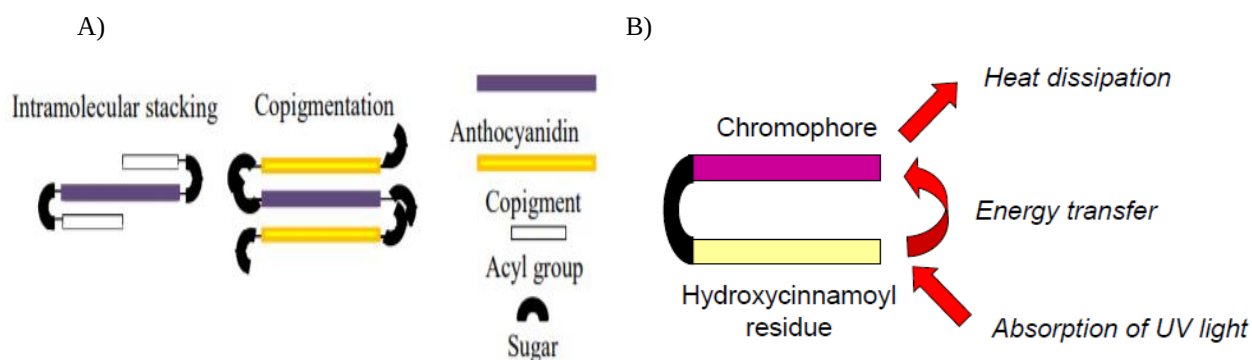


Figure 2.6.2 A) Mechanisms of intramolecular co-pigmentation of diacylated anthocyanins and B) protection of acylated residues from UV light (Yoshida et al., 2012; Dangles et al., 2018)

2.6.3 Metal complexation

Metal complexation has been identified as an anthocyanin stability mechanism and development of the blue colour in blue flowers. Goto and Kondo (1991) among many other researchers have described the formation of so called metalloanthocyanin complexes, which involves the association of anthocyanins, flavones and metal ions such as Fe^{3+} , Mg^{2+} and Al^{3+} (Brouillard, Chassaing, Isorez, Kueny-Stotz and Figueiredo, 2010) in a stoichiometric ratio of 6:6:2 respectively (Figure 2.6.3). Metalloanthocyanin complexes are very rare and usually only occur in blue flowers as colour stability mechanism (Tuominen, 2017).

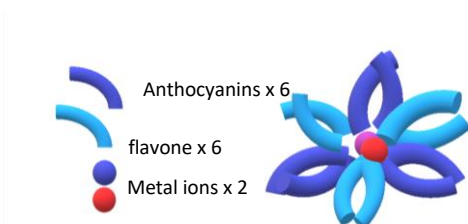


Figure 2.6.3 Metalloanthocyanin complex formation. Adapted from Yoshida, Mori and Kondo, (2009)

2.7 Preparation of colourants from plants

Many research efforts have focused on improving the extraction methods of anthocyanins from biological material such as flowers. The most common method of extraction of anthocyanins utilizes polar solvents such as methanol with small quantities of hydrochloric or formic acid. The acids aid in extraction of bound phenolic compounds and anthocyanins but could cause degradation if the extract is concentrated by evaporation (Kong et al., 2003). Acetone has also been used as an extraction solvent for anthocyanins and shown to produce greater quantitative yields of anthocyanins (Garcia-Viguera, Zafrilla and Tomás-Barberán, 1998).

Da Porto and Natolino (2018) evaluated solid-liquid, ultrasound-assisted extraction and microwave-assisted extraction methods of anthocyanins and total phenolic compounds from saffron (*Crocus sativus*) flowers using 59% v/v ethanol as extraction solvent. They identified the solid-to-liquid ratio to be an essential factor for extraction efficiency. The researchers also found the ultrasound-assisted extraction method to have shorter extraction times and higher extraction yields as compared to solid-liquid and microwave-assisted extraction.

Segura-Carretero, Puertas-Mejía, Cortacero-Ramírez, Beltrán, Alonso-Villaverde, Joven, Dinelli and Fernández-Gutiérrez (2008) used solid-phase extraction-capillary electrophoresis-mass spectrometry (time-of-flight/ion trap) as a technique to selectively extract and identify anthocyanins from *Hibiscus sabdariffa* L. flowers. Capillary electrophoresis proved to be a very efficient method for extraction and separation of anthocyanins (Segura-Carretero et al., 2008). One of the extraction methods used included extraction of dried flower calyces with 15 % v/v acetic acid and filtered through Amberlite XAD-2 silica particles packed in a glass column and washed through the column with distilled water. The anthocyanin adsorbed onto the Amberlite XAD-2 column was then eluted using a 70% v/v ethanol-1% acetic acid solution. After subsequent procedures of drying and re-dissolving, the extract was then analysed by capillary electrophoresis-electrospray-ionization-mass spectrometry (CE-ESI-IT-MS) and capillary electrophoresis-electrospray-ionization-time-of-flight-mass spectrometry (CE-ESI-TOF-MS). The authors concluded that capillary electrophoresis mass spectrometry is a rapid and sensitive method for the extraction and identification of anthocyanins in *Hibiscus* flower extracts.

Jiang, Ding, Wang, Deng and Zhao (2020) utilized a radio frequency-assisted enzymatic extraction method to optimize extraction of anthocyanins from purple *Akebia trifoliata* (Thunb.) Koidz flowers for the pharmaceutical and food industry. The optimal conditions obtained for extraction of anthocyanins were 0.1% equal masses of cellulase and pectinase enzymes (1:1), pH of 4, 50% ethanol extraction solvent with a solid-to-liquid ratio of 20 ml/g and radio frequency electrode gap of 5 cm for 10 minutes at 40°C. The optimized method resulted in the highest crude solids yield of 26.55% with an anthocyanin content of 50.87 mg cyanidin-3-O-glucoside equivalents/100 g compared with conventional extraction methods.

The application of radio frequency as an extraction aid did not result in any detectable degradation of anthocyanins and was proven to be more efficient and rapid than conventional extraction methods such as the use of hot water and acidified ethanol as extraction solvents as well as enzymatic extraction (without radio frequency assistance). Although the greater efficiency of the radio frequency-assisted procedure was attributed to the action of the enzymes that release the anthocyanins from the cell matrix thereby increasing the anthocyanin yield (Bei, Chen, Lu, Wu and Wu, 2018), the radio frequency also aided in acceleration of the extraction process.

2.8 Application of pigments from flowers as natural colourants in foods

Pigments from flowers are generally applied in foods as an aqueous- or ethanol-based tincture. Several research attempts have been made to incorporate flower anthocyanin extracts in different types of food systems. In most applications, anthocyanin-rich pigmented extracts from flowers have been prepared in acidified solvents and applied to low pH foods and this generates the stable red/orange to pink coloured flavylum cation form of the anthocyanin pigment. The low pH of the extracts also allows it to be incorporated in most food systems as acidic foods are regarded as foods with a pH of ≤ 4.5 . Amr and Al-Tamimi (2007) successfully incorporated Persian buttercup flower (*Ranunculus asiaticus*) ethanolic extracts in various food systems including carbonated beverages, gelatine jelly and yogurt. The extracts proved to retain most of their colour after 6-month storage and developed a consumer acceptable purple colour. The alcoholic extracts proved to be advantageous in their application in gelatine as the lower boiling point allowed for concentration of pigment with minimal to no heat damage.

Pigmented extracts from Roselle calyces (*Hibiscus sabdariffa*) were encapsulated in maltodextrin and gum Arabic and applied in hard candy and strawberry jam (Selim et al., 2008). The maltodextrin-encapsulation increased the stability of extracts during storage for up to 93.94 days at different water activities of up to 0.75. The extracts were successfully applied in strawberry jam and hard candy and proved to be sensorially acceptable as compared to the control.

Díaz-García et al (2015) applied extracts prepared from *Thymus moroderi* (Lamiaceae) flowers in a yogurt food system. For incorporation into the yogurt, a solution of 1% citric acid in water (pH 2) was used as solvent. Various amounts of extracts were used for the application and compared with commercial E-163 colourants. The yogurt containing extracts of *T. moroderi* showed no visual difference as compared to the commercial colourants. However, they showed the most significant change in colour as calculated with the total colour difference (ΔE^*_{ab}) and therefore was considered the least stable.

A recent study by Pires, Inês Dias, Barros, Barreira, Santos-Buelga and Ferreira (2018) applied flower petal extracts from Dahlia (*Dahlia mignon*), rose (*R. damascene*) 'Alexandria' and *R. gallica* 'Francesa' and cornflower (*Centaurea cyanus*) L in yogurt and compared their stability to commercial E-163 colourant. The rose and dahlia-coloured yogurts showed no significant difference in a^* (red/green) parameter compared to the artificial E-163 colourant after 7 days

of storage and was considered stable enough as a colourant to potentially replace commercial E-163 colourants.

2.9 Health-promoting properties of anthocyanins

2.9.1 Anti-cancer properties

Several mechanisms have been proposed for the anti-cancer properties of anthocyanins. These include inhibition of oxidative DNA damage (Sarma and Sharma, 1999), induction of phase II detoxifying enzymes, inhibition of phase I detoxifying enzymes (Srivastava, Akoh, Fischer and Krewer, 2007, Shih, Yeh and Yen, 2007), cell cycle arrest and inhibition of cyclooxygenase-2 (COX-2) enzymes (Renis et al., 2008, Seeram, Momin, Nair and Bourquin, 2001).

Kamei, Kojima, Hasegawa, Koide, Umeda, Yukawa and Terabe (1995) reported that anthocyanin extracts from flower petals were more effective in cell growth inhibition of human malignant intestinal carcinoma-derived HCT-15 cell line than non-anthocyanin flavonoids. Tsai, Huang, Chang, Wang and Chang (2017) found that anthocyanin extracts from *Hibiscus sabdariffa* L. flowers caused induction of the G2/M phase (a cell cycle phase checkpoint that initiates mitosis) arrest resulting in cell death in leukaemia HL-60 cancer cells. Anthocyanin-rich rose (*Rosa centifolia*) flower petal extracts exhibited anti-mutagenic effects on *E. coli* cells with anthocyanin and peonidin-3-glucoside contributing most of the observed anti-mutagenesis (Kumar, Gautam and Sharma, 2013).

2.9.3 Anti-inflammatory properties

The process of inflammation involves the activation and expression of several cytokines and enzymes such as tumor necrosis factor (TNF) α , interleukin (IL)1 β , inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein (MCP)1, and cyclooxygenase (COX) 2 among several others. These cascades of reactions produce reactive oxygen species (ROS) which are associated with a variety of chronic diseases (Lee et al., 2020).

Phenolic compounds such as anthocyanins are known to inhibit enzymes involved in the inflammation pathway and prostaglandin synthesis such as cyclooxygenase (COX) (Seeram et al., 2001) and the production of nitric oxide (NO) involved in inflammation. Ternatin anthocyanin and quercetin glycoside fractions of butterfly pea extracts (*Clitoria ternatea*) showed inhibition of different factors involved in lipopolysaccharide (LPS)-induced

inflammation through the toll-like receptor (TLR4) signalling pathway in RAW 264.7 macrophages.

Flavonol fractions (F3) showed powerful cyclooxygenase (COX-2) inhibitory activity and partial reactive oxygen species (ROS) suppression in RAW 264.7 macrophage cells while the ternatin anthocyanin fractions (F4) showed non-ROS-inhibition of NO production through inhibition of nuclear NF- κ B translocation and suppression of inducible nitric oxide synthase (iNOS) expression through inhibition of signalling factors (Nair, Bang, Schreckinger, Andarwulan & Cisneros-Zevallos, 2015).

Lee, Nam, Lee, Shin, Han, Lee, Lee and Lim (2018) investigated the use of extracts from rose flower petals for the prevention of skin inflammation. They found that anthocyanins and other phenolic compounds in rose (*Rosa gallica*) flower extracts inhibited the Mitogen-Activated Protein Kinase (MAPK) signalling pathway activation probably via the antioxidant activity of reactive oxygen species (ROS) thereby preventing COX-2 production and eventually inflammation.

Red clover (*Trifolium pratense*) extract (RC) and anthocyanin extract (RCA) fractions were reported in a recent study by Lee, Brownmiller, Lee and Kang (2020), to effect inflammatory actions in RAW264.7 microphages by suppressing the LPS-induced expression of several genes in the inflammation pathways. Genes for cytokines and enzymes such as tumor necrosis factor (TNF) α , interleukin (IL)1 β , inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein (MCP)1, and cyclooxygenase (COX)2 were suppressed, and LPS-induced ROS production was also inhibited by both RC and RCA fractions. NADPH oxidase 1 (NOX-1) gene expression and its phosphorylation were suppressed by RCA fractions. ROS production was induced by LPS through the upregulation of the NOX-1 gene which encodes for an important ROS producer. The upregulation by LPS was completely inhibited by 5 μ g/ml RCA.

2.9.4 Anti-obesity properties

Flower extracts from *Hibiscus sabdariffa* L. have been shown to prevent obesity through several factors during adipocyte cell differentiation. Some mechanisms include inhibition of expression of transcription factors involved in adipogenesis such as C/EBP α and PPAR γ through the PI3-K and mitogen-activated protein kinase (MAPK) pathway (Kim, So, Youn, Kim, Kim, Park, Kim, Ha, Chai, Kim, Kim and Park, 2008).

Park and Lee (2019) and Guo, Kang, Kang, Je, Lee, Park and Choi (2020) showed that the anthocyanins delphinidin-3-O- β -glucoside and pelargonidin suppressed the expression of transcription factor proteins PPAR γ and sterol regulatory element-binding transcription factor 1 (SREBP1), CCAAT/enhancer-binding protein alpha (C/EBP α) in 3T3-L1 cells. Additionally, pelargonidin was also reported to inhibit the transcription of PPAR γ target genes thereby reducing the expression of several enzymes and binding proteins involved in adipogenesis such as lipoprotein lipase (LPL), adipocyte fatty acid binding protein (A-FABP) and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), thus reducing lipid droplet accumulation significantly.

Anti-obesity can also be controlled at gene expression level as shown by several studies. A study by Kim et al. (2003) showed that anthocyanin-rich extracts of *Hibiscus sabdariffa* significantly inhibited the expression of PPAR γ and C/EBP α transcription factors in 3T3-L1 cells which in turn caused a reduction in lipid accumulation after 4 days of differentiation.

Blue butterfly pea (*Clitoria ternatea*) extracts (CT) have shown potential as an anti-obesity agent through a recent study by Chayaratanasin, Caobi, Suparpprom, Saenset, Pasukamonset, Suanpairintr, Barbieri and Adisakwattana (2019). They showed that the delphinidin-based ternatins, anthocyanin glucosides and other phenolic compounds in the CT extracts inhibited anti-adipogenic activity by delaying cell distribution in G0/G1 phase and decreasing G2/M phase cycles in 3T3-L1 cells. The CT extracts also showed increase in suppression of protein kinase B (Akt1) and ERK1/2 phosphorylation at concentrations of 750-1000 μ g/ml.

The CT extracts also reduced lipid accumulation in 3T3-L1 cells by increasing lipolysis significantly at CT concentrations of 500-1000 μ g/ml, with a decrease in lipid and triglyceride content and an increase in released glycerol. The CT extracts also had a significant effect on the expression of adipogenic transcription factors and lipogenic enzymes. The CT extracts exhibited a dose-dependent reduction in expression of peroxisome proliferator-activated

receptor (PPAR γ) and CCAAT element binding protein (C/EBP α) as well as fatty acid synthase (FAS), acetylCoA-carboxylase (ACC)/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzymes.

2.9.5 Anti-diabetic properties

Haemoglobin glycation can be used as a target for the measurement of glycaemic metabolism and can thereby identify diabetic conditions (Nathan, Kuenen, Borg, Zheng, Schoenfeld & Heine, 2008). Haemoglobin glycation occurs when excess glucose or fructose in blood serum bind to haemoglobin proteins via the aldehyde and ketone carbonyl group and amino groups of proteins. The Schiff base formed through this reaction undergoes rearrangement to form more stable Amadori compounds. Amadori products can further undergo cross-linking reactions with dicarbonyl intermediates (glyoxal and methylglyoxal) (Figure 2.9.6) to form advanced glycation end-products (AGE's) (Ahmed, 2005). Autoxidative glycation can also lead to the formation of AGE's through the reaction of radical oxygen with metal-catalysed radical enediol (monosaccharide equilibrium structure) (Wolff & Dean, 1987). The resulting dicarbonyl ketoaldehydes (Figure 2.9.6) could react with amino groups of proteins to form ketoimines (Ahmed, 2005).

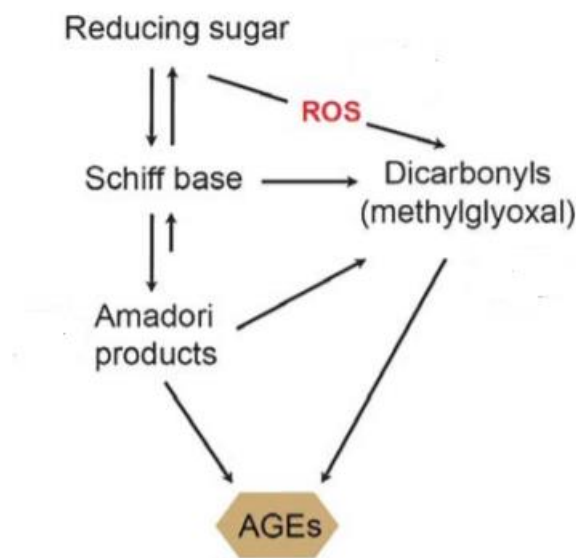


Figure 2.9.6 Formation of advanced glycation end-products (AGE's). Adapted from Wu et al. (2011)

A study by Chayaratanasin et al. (2015) investigated the potential of blue butterfly pea (*Clitoria ternatea*) (CT) petal extracts to inhibit bovine serum albumin (BSA) protein glycation with fructose. CT petal extracts exhibited a dose-dependent reduction in protein carbonyl content and increase in protein thiol groups. The CT extracts also showed dose-dependent reduction in fructosamine and advanced glycation end-products (AGE's). The prevention of protein glycation was attributed to the strong radical scavenging and ferric-reducing ability of anthocyanins, ternatin anthocyanins, phenolic acids and flavonoids identified in CT extracts. Other mechanisms of glycation prevention could be blocking of receptors for AGE's, breaking the cross-links between AGE's and chelation or reduction of transition metals that catalyses free radical formation (Wu, Huang, Lin & Yen, 2011).

Another pathway of anti-diabetic activity was shown by the use of *Hibiscus sabdariffa* L. flower extracts on rat and pig models by Preuss (2007). The author reported that the glucose level in rats after administration of starch or sucrose, and the *Hibiscus* flower extract was decreased significantly. The *Hibiscus* flower extract also induced lower glucose levels in the blood of pigs after they were administered with human dosages of starch and sucrose (Preuss, 2007). This result indicated that *Hibiscus* flower extracts inhibit enzymes such as sucrase and α -amylase, which thus prevents their breakdown into glucose units.

Blue Butterfly pea (*Clitoria ternatea*) flower extract has also shown potential in reduction of *in vitro* starch digestibility with its incorporation in bread. The release of maltose from potato, rice, glutinous rice, wheat, corn and casava flours in combination with different concentrations of butterfly pea flower extract upon treatment with pancreatic α -amylase was significantly decreased with increase in butterfly pea flower extract concentration in all flour types. Similar trends were shown for glucose release with different concentrations (5, 10 and 20 %) of butterfly pea flower extracts incorporated in wheat bread. These results suggest that anthocyanins in *Clitoria ternatea* flower extracts including ternatins as well as other phenolic compounds slow down the hydrolysis of starch by inhibition of the starch digestion enzymes such as α -amylase (Charoonsri, Chusak, Christiani, Praew, Varanya, Sirichai, 2018).

2.9.5 Anti-hypertensive activity

Hypertension is a cardiovascular disease associated with high blood pressure among many other symptoms. Angiotensin converting enzyme (ACE) plays an important role in controlling blood pressure by catalysing the conversion of ACE I to ACE II, a powerful vasoconstrictor. Another role of ACE is to catalyse the degradation of a vasodilator, bradykinin (Murray & Fitzgerald, 2007).

Several studies have highlighted the potential of anthocyanins from different sources as anti-hypertensive compounds. Anthocyanin-rich crude lyophilised extracts of blue butterfly pea flowers (*Clitoria ternatea*) (Escher et al., 2020) and *Centaurea cyanus* flower extracts (Escher et al., 2018) exhibited anti-hypertensive activity through inhibition of ACE *in vitro*. An *in vivo* study on spontaneously hypertensive rats showed that oral administration of *Hibiscus sabdariffa* calyces extracts in distilled water for 10 weeks resulted in reduced systolic and diastolic blood pressure and increased myocardial capillary length density and surface area up to 77% and 86% respectively without affecting the heart rate (Beegam, Inuwa, Ali, Blunden, Ziada & Al-Lawati, 2012). Rats treated with water extracts of *Hibiscus sabdariffa* calyces also had notably reduced myocyte nuclear volume. All these affects were attributed to the inhibition of ACE, largely by the anthocyanins and other phenolic compounds according to the results from previous reports by Ojeda, Jiménez-Ferrer, Zamilpa, Herrera-Arellano, Tortoriello and Alvarez (2012).

2.10 Conclusions and gaps in knowledge

Most natural colourants are extracted from fruits or vegetables, but little research has focused on flower components of plants as the source for colourants. Research on plants has looked mainly at the essential oils and their health-benefits as has been used in traditional medicine for centuries. Studies on flower colourants usually only focus on a single colour such as the red from *Hibiscus sabdariffa*, the pink from rose petals (*Rosa damascena*) and *Thymus moroderi* (Lamiaceae), or blue from *Clitoria ternatea* L. flowers.

Thermal, light and pH stabilities of flower anthocyanins from *Hibiscus sabdariffa* and *Clitoria ternatea* L. have been reported however, very limited studies have been done to establish the oxidative stability of anthocyanins from flowers and have only been reported on fruit juices (sour cherry, pomegranate and strawberry).

Limited research exists on the identification of anthocyanins of flower species from the Geraniaceae and Lamiaceae plant families. Some of the most recent research has focused on health-promoting properties of essential oils from leaves of some plant species from the Geraniaceae and Lamiaceae families. The older research on Geraniaceae families have focused on the characterisation of colour compounds in their pigments. *Pelargonium* flower pigment chemistry is relatively well documented while only few species of *Salvia* and a *Plectranthus* specie have been explored.

A careful examination of the literature indicates that no research into identification, stability, cellular health-promoting properties and potential commercial culinary application of anthocyanins extracted from flower species from the Geraniaceae and Lamiaceae plant families has been done and this represents a significant gap in knowledge. This research aims to evaluate the potential of flower species from Lamiaceae and Geraniaceae families as sources of novel natural colourants through studies on chemical characterisation, stability and health-promoting properties of their pigments.

Chapter 3: Hypotheses and Objectives

3.1 Hypotheses and objectives

3.1.1 Hypothesis 1

Pigmented flower extracts of plant species from the Lamiaceae family (*Salvia aurea* × *dolomitica*, *Salvia dolomitica* Codd and *Plectranthus zuluensis* T. Cooke) will contain conjugated anthocyanins consisting of aromatic acylated residues whereas pigmented flower extracts of plant species from the Geraniaceae family (*Pelargonium grandiflorum* Willd., *P. × hortorum* L.H. Bailey and *Pelargonium zonale* (L.) L'Hér.) will mainly consist of 3, 5-diglucosides acylated with acetates (Saito & Harborne, 1992; Mitchell et al., 1997; Jordheim et al., 2016). The aromatic acylated nature of the anthocyanins of flowers from the Lamiaceae will confer greater thermal and oxidative stability to their pigmented extracts compared to the acetyl acylated anthocyanins from pigmented extracts of flowers from the Geraniaceae family. The aromatic residues of anthocyanins can form hydrophobic intramolecular self-association, intermolecular interactions with other flavonoids and also form metalloanthocyanin complexes which could aid in stabilization of the flower anthocyanin compounds (Brouillard, 1981; Yoshida et al., 2009) and contribute to greater thermal and oxidative stability of the Lamiaceae flower pigments.

3.1.2 Hypothesis 2

Pigmented flower extracts of plant species from the Lamiaceae family (*Salvia aurea* × *dolomitica*, *Salvia dolomitica* Codd and *Plectranthus zuluensis* T. Cooke) will exhibit greater health-promoting properties (in terms of chemical and cellular radical scavenging activity, anti-glycation activity and anti-lipogenic activity) compared to pigmented flower extracts of plant species from the Geraniaceae family (*Pelargonium grandiflorum* Willd., *P. × hortorum* L.H. Bailey and *Pelargonium zonale* (L.) L'Hér.). In addition to the relatively greater stability of the aromatic acylated anthocyanins in the Lamiaceae flower pigments, these anthocyanins could interact with other flavonoids and/or phenolic acids to exert either additive or synergistic effects in their antioxidant, anti-inflammatory, anti-adipogenic and anti-glycation activities (Hajimehdiipoor, Shahrestani & Shekarchi, 2014). Phenolic compounds exhibit antioxidant activity toward cells through radical scavenging, inhibition or induction of cell signalling

enzymes involved in cell inflammation and lipogenesis or chelation of pro-oxidant metal ions (Rice-Evans et al., 1996; Berdowska et al., 2013; Lee *et al*, 2020; Lingesh *et al*, 2019).

3.1.3 Hypothesis 3

The pigmented flower extracts from the Geraniaceae and Lamiaceae plant families will have greater or comparable colour stability and colour variability when applied as natural colourants in yoghurt, fondant and gelatine candies compared to the commercial E163 anthocyanin colourant. The flower extracts will produce a variety of different colours (red, orange and purple) as compared to the pink colour of the E163 colourant in yogurts and purple colour in fondant and gelatine candies. The colour of flower extracts in the yoghurt, fondant and gelatine candies is determined by the anthocyanin types of the flower extracts. The comparable storage stability of the flower extracts compared to the concentrated commercial E163 could be due to inter and intramolecular interactions between the anthocyanins and phenolic compounds (Brouillard, 1981; Yoshida et al., 2009) thereby conferring stability during storage.

3.1.4 Objective 1

To determine the anthocyanin and phenolic profiles of pigmented flower extracts of plant species from the Geraniaceae (*Pelargonium grandiflorum* Willd., *P. × hortorum* L.H. Bailey and *Pelargonium zonale* (L.) L'Hér.) and Lamiaceae (*Salvia aurea* × *dolomitica*, *Salvia dolomitica* Codd and *Plectranthus zuluensis* T. Cooke) families and the effect of this on their thermal and oxidative stability with the aim of producing pigmented extracts for potential application as natural colourants.

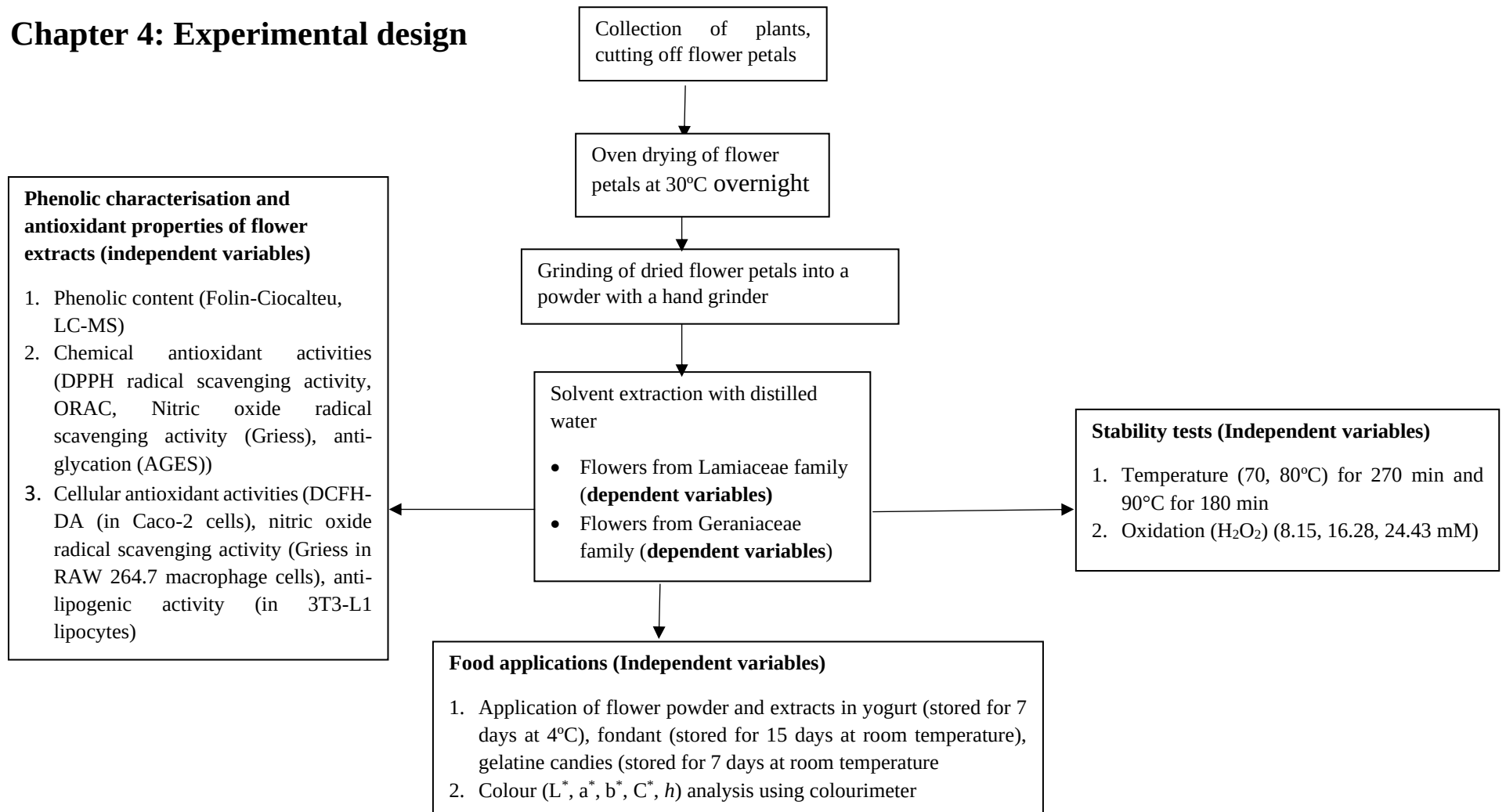
3.1.5 Objective 2

To determine the chemical and cellular radical scavenging, anti-glycation activity and anti-lipogenic activity of pigmented flower extracts of plant species from the Geraniaceae (*Pelargonium grandiflorum* Willd., *P. × hortorum* L.H. Bailey and *Pelargonium zonale* (L.) L'Hér.) and Lamiaceae (*Salvia aurea* × *dolomitica*, *Salvia dolomitica* Codd and *Plectranthus zuluensis* T. Cooke) families with the aim of producing pigmented extracts with enhanced health-promoting properties for application as natural colourants.

3.1.6 Objective 3

To determine the colour storage stability of yogurts, fondant and gelatine candies incorporated with pigmented flower extracts of plant species from the Geraniaceae (*Pelargonium grandiflorum* Willd., *P. × hortorum* L.H. Bailey and *Pelargonium zonale* (L.) and Lamiaceae (*Salvia aurea* × *dolomitica*, *Salvia dolomitica* Codd and *Plectranthus zuluensis* T. Cooke) families as compared to commercial E163 anthocyanin colourant with the aim of evaluating their applicability as natural colourants and storage stability in different food systems.

Chapter 4: Experimental design



Chapter 5, 6 & 7: Research

The research conducted in this thesis is organized in three chapters. Chapter 5 deals with the identification, quantification and temperature and oxidative stability of anthocyanins and phenolic compounds in pigmented extracts from South African flower species of the Geraniaceae and Lamiaceae plant families. Chapter 6 reports on the total phenolic content, DPPH and nitric oxide radical scavenging antioxidant activities and anti-diabetic activity as well as cellular radical scavenging, anti-inflammatory and anti-lipogenic activities of pigmented extracts from South African Geraniaceae and Lamiaceae flower species. Chapter 7 shows the potential of the pigmented flower extracts as potential food colourant in different food systems.

Chapter 5: Characterisation of anthocyanins and other phenolics and stability of pigmented flower extracts of species from the Geraniaceae and Lamiaceae plant families

Abstract

This study determined the profile of anthocyanins and other phenolics of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families found in South Africa and their thermal and oxidative stability as a way of assessing their potential food application. The main anthocyanins in flower pigments from the Geraniaceae plant family were 3,5-diglucosides of delphinidin, petunidin, pelargonidin, peonidin and malvidin and acetyl-acylated malvidin, delphinidin and petunidin. The Lamiaceae plant family mainly contained rutinosides of pelargonidin, glucosides of petunidin, pelargonidin and *p*-coumaric acid- and malonyl-acylated delphinidin and malvidin. The main flavonoids in Geraniaceae pigmented extracts were rutinosides of kaempferol and quercetin, as well as glucosides of quercetin and luteolin while the main flavonoids identified in the Lamiaceae were naringin and neohesperidin. Lamiaceae pigmented extracts contained high amounts of caffeic acid, rosmarinic acid and its derivatives while pigmented extracts from the Geraniaceae contained high amounts of hydrolysable tannins. The pigmented flower extracts from the Lamiaceae plant family were more thermally and oxidatively stable as shown by their lower degradation rate constants and longer half-life. This could be due to aromatic-malonyl-acylated anthocyanin self-association and strong intermolecular interactions with rosmarinic acid and its derivatives which may not be present in the Geraniaceae plant family pigments. Flower species from Lamiaceae and Geraniaceae plant families have potential application as natural food colourants, but temperature and oxidising conditions must be considered particularly for the Geraniaceae pigments.

Keywords: Anthocyanins, Geraniaceae, Lamiaceae, Temperature stability, Oxidative stability

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Venter, A, Fisher, H., Stafford, G.I. and Duodu, K.G. (2022) Pigmented flower extracts of plant species from the Geraniaceae and Lamiaceae families as natural food colourants: anthocyanin composition, thermal and oxidative stability. International Journal of Food Science and Technology 57, 4347-4355.

5.1 Introduction

Although colourants extracted from various natural sources are used extensively in the food industry worldwide (Downham and Collins, 2000), the demand for such nature-derived food colourants remains. Plants from the Geraniaceae and Lamiaceae families have a long history of use as herbal medicine and as aromatic herbs in culinary practice in South Africa (Scott *et al.*, 2004). The flowers from these plant families present with different colours and therefore could have potential as sources of natural food colourants.

Anthocyanins are major components of most colourants used in the food industry. These anthocyanins are also known to be affected by several factors such as pH, temperature, oxidation, and light which ultimately affect stability of the food colourant. Co-pigment molecules can interact with anthocyanin molecules through several bonding mechanisms thereby improving their stability by protecting the anthocyanin nucleus from nucleophilic attack by water and oxidizing agents and could protect the anthocyanin molecules from light (Renis *et al.*, 2008).

Some work on anthocyanin characterisation going back almost three decades indicate that pigmented flowers from the Geraniaceae contain 3,5-diglucoside- and acetyl-acylated anthocyanins while pigmented flowers from the Lamiaceae contain aromatic acylated anthocyanins and malonyl anthocyanins (Saito and Harborne, 1992; Mitchell *et al.*, 1998; Jordheim *et al.*, 2016). However, there seems to be no research on stability of pigmented extracts from these two plant families. There have been some reports on the thermal, hydrogen peroxide (oxidative) and light stability of pigmented extracts from red cabbage and black carrot (Matsufuji *et al.*, 2007; Fenger *et al.*, 2019; Kırca *et al.*, 2007). There have also been some reports on thermal stability of pigmented extracts from *Hibiscus sabdariffa* L. (Maciel *et al.*, 2018), *Tulipa gesneriana* L. (tulip) (Sagdic *et al.*, 2013) and *Clitoria ternatea* L. (butterfly pea) (Escher *et al.*, 2020).

There is increasing demand among consumers for food ingredients from natural sources. This presents an opportunity for innovation in food colourants by studying the flowers of plant species as potential sources of natural food colourants. The objective of this study was therefore to determine the anthocyanin profile of pigmented extracts of a sample of flowers from some species of the Geraniaceae and Lamiaceae plant families found in South Africa and their stability with the aim of evaluating their potential as natural food colourants.

5.2 Materials and methods:

5.2.1 Samples

Flower samples of Geraniaceae family: *Pelargonium grandiflorum* Willd. (large-flowered pelargonium) and *P. × hortorum* L.H. Bailey were procured from Plant Land, Centurion, Gauteng, South Africa. *Pelargonium zonale* (L.) L'Hér. hybrids were procured in Lynwood Ridge, Pretoria, South Africa.

Flower samples of Lamiaceae family: *Salvia aurea* × *dolomitica* and *Salvia dolomitica* Codd were procured from the botanical gardens at the Future Africa campus of the University of Pretoria, South Africa. *Plectranthus zuluensis* T. Cooke was procured from the Hatfield Campus, University of Pretoria, South Africa.

Flower samples were air-dried at 27°C in an air convection oven for 24-48 hours. After drying, the dried flower petals were ground into a fine powder using a Waring hand blender. The flower powder was stored in airtight resealable bags (Ziplock) at 4°C.

5.2.2 Chemicals

Pure standard anthocyanin and other phenolic compounds namely, ideain (cyanidin-3-*O*-galactoside) caffeic acid, (+)-catechin, quercetin, kaempferol and rutin, and other reagents namely, formic acid and hydrogen peroxide were purchased from Merck.

5.2.3 Preparation of pigmented extracts

Approximately 0.5 g of dried flower powders were extracted with 150 ml acidified distilled water (1% (v/v) formic acid in water) using magnetic stirring for 24 hours. After extraction, the extracts were filtered through a Büchner funnel with filter paper under vacuum and freeze-dried. For all degradation kinetics analyses, 10 mg of the freeze-dried extract were re-dissolved in 5 ml distilled water. Yields for each extract were as follows: *P. grandiflorum*: 58.7%, *P. × hortorum*: 44.4%, *P. zonale* hybrid: 54.6 %, *S. aurea* × *dolomitica*: 52.4%, *S. dolomitica*: 48.4% and *P. zuluensis*: 45.6%.

Extracts for UPLC-MS analysis were prepared by extracting 0.5 g of flower powder with 10 ml of 0.5 % formic acid in distilled water for 24 hours at room temperature using magnetic

stirrers. The extracts were filtered through a 0.45 µm PTFE filters into amber vials and stored at -20°C until UPLC-MS analysis.

5.2.4 Characterisation of anthocyanins and other phenolics and stability of pigmented flower extracts

5.2.4.1 UPLC-MS analysis

The chromatographic analysis of anthocyanins was performed as described by Alberts et al. (2012). A Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) incorporating a binary pump, vacuum degasser, autosampler, column oven and Micromass Xevo tandem quadrupole mass spectrometric detector (Manchester, UK) equipped with ESI probe was used. Reversed phase separation was performed using an Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size) at a temperature of 50°C. Mobile phase A was 7.5% formic acid in water and mobile phase B was acetonitrile. The gradient started with 1% B isocratically for 0.5 min followed by a linear increase to 15% at 15 min, 23% at 20 min and 28% at 25 min. Column clean-up at 100% B then followed for 1 min followed by re-equilibration for 4 min to obtain a total run-time of 30 min. The flow-rate was 0.1 mL/min throughout and injection volumes of 10 µL were used. The source conditions were as follows: capillary voltage 2.5 kV, cone voltage 15V, whilst the desolvation temperature was 500°C and the desolvation- and cone gas (both nitrogen) flows were 1000 and 50 L/h, respectively. Ideain (cyanidin-3-O-galactoside) was used as a standard for anthocyanin quantification. The concentration of anthocyanins identified in the pigmented flower extracts were measured based on the individual compound peak area response and calculated using the Ideain standard curve.

The chromatographic analysis of non-anthocyanin phenolic compounds was performed as described by Stander, Van Wyk, Taylor & Long, (2017). A Waters Synapt G2 Quadrupole time-of-flight (QToF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (Waters, Milford, MA, USA) was used for high-resolution UPLC-MS analysis. Electrospray ionization was used in negative mode with a cone voltage of 15 V, desolvation temperature of 275°C, desolvation gas at 650 L/h, and the rest of the MS settings optimized for best resolution and sensitivity. Data were acquired by scanning from m/z 150 to 1500 m/z in resolution mode as well as in MS^E mode. In MS^E mode two channels of MS data were acquired, one at a low collision energy (4 V) and the second using a collision energy ramp (40–100 V) to obtain fragmentation data as well. The instrument was calibrated

with sodium formate. Separation was achieved on a Waters HSS T3, 2.1×100 mm, $1.7 \mu\text{m}$ column. An injection volume of $2 \mu\text{L}$ was used and the mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B.

Gradient elution was done according to the following program: 100% A from 0 to 0.5 min; 74% A and 26% B from 0.5 min to 6 min; 56% A and 44% B from 6 min to 15 min.; 0% A and 100% B from 15 min to 17.5 min. Ionisation was in negative mode with a capillary voltage of 3 kV and cone voltage of 15 V. Identification was done by comparing chromatograms and retention times of phenolic constituents in the extracts with external phenolic acid and flavonoid standards as well as comparison of MS/MS fragmentation data and UV spectra with those of phenolic compounds reported in literature. Quantification was done by comparing integrated peak areas of phenolic compounds in extracts with that of standards and from generated calibration curves.

5.3 Thermal stability

The thermal stability of flower extracts was determined using a temperature degradation kinetics method described by Selim et al. (2008) and Sagdic et al. (2013) with modifications. A 1 ml volume of sample extract was made up to 9 ml with 0.1 M citrate buffer (pH 1), placed in a screw cap test tube and heated in a thermostatically controlled water bath at temperatures of 70, 80°C for 270 min and 90°C for 180 min. Test tubes were cooled down immediately to ambient temperature and the change in absorbance at 520 nm measured every 30 min. The zero-time absorbance values were determined before each experiment. The experiment was done in duplicate.

5.4 Hydrogen peroxide (H_2O_2) oxidative stability

Oxidative stability of flower extracts was determined using an oxidative degradation kinetics method described by Özkan et al. (2002) with modification. Three different concentrations of H_2O_2 (9.31, 18.61 and 27.92 mM) were used. The pre-determined H_2O_2 amounts were rapidly added to 1 ml of the flower extracts (in citrate-phosphate buffer pH 1) to make up 8 ml of 8.15, 16.28, 24.43 mM. The change in absorbance at 520 nm was measured every 15 min for 180 min for each concentration of hydrogen peroxide used. The zero-time absorbance values were determined by preparing the samples with the same amount of distilled water instead of hydrogen peroxide. The experiment was done in duplicates.

5.5 Calculation of degradation kinetics parameters

The degradation rate constant (k) and half-life ($t_{1/2}$) for stability was calculated with the following first order kinetic equations used by Sagdic et al. (2013).

$$\frac{dC}{dt} = -kC \quad (1)$$

Solving equation (1) gives (3)

$$C = \frac{A_t}{A_0} \quad (2)$$

$$\ln(C) = -kt \quad (3)$$

$$\ln\left(\frac{A_t}{A_0}\right) = -kt \quad (4)$$

$$t_{1/2} = \ln \frac{0.5}{K} \quad (5)$$

Where A_t : Absorbance at 520 nm at t min.

A_0 : Absorbance at 520 nm at 0 min.

5.6 Statistical analysis

Experiments were conducted triplicates. One-way analysis of variance (ANOVA) was conducted as required using SPSS software. Significant differences between means were determined at $p \leq 0.05$. Significant means were separated using the least significant difference (LSD), Tukey and Scheffe tests at $p \leq 0.05$. Independent variables were temperatures (70, 80 and 90°C), hydrogen peroxide concentrations (9.31, 18.61 and 27.92 mM), pH values of extract solutions (1, 3, 5, 7 and 9), storage time (5 min and 72 hours) and flower types from Lamiaceae and Geraniaceae families (*P. grandiflorum*, *P. × hortorum*, *P. zonale hybrid*, *S. aurea × dolomitica*, *S. dolomitica* and *P. zuluensis*). Dependent variables were the calculated values for rate constants (k), half-life ($t_{1/2}$), measured absorbance values of extracts at 520 nm and the calculated total anthocyanin and total phenolic acid and flavonoid contents.

5.4 Results and discussion

5.4.1 Identification of anthocyanins in flower extracts of the Geraniaceae and Lamiaceae

Tables 5.4.1 and 5.4.2 show chromatographic and mass spectral data of anthocyanins identified in the pigmented flower extracts. The anthocyanins were all sugar derivatives of delphinidin (Figure 5.4.1), petunidin (Figure 5.4.2), cyanidin (Figure 5.4.3), pelargonidin (Figure 5.4.4), peonidin (Figure 5.4.5) and malvidin (Figure 5.4.6) and they were identified mainly based on their reported mass spectral characteristics and fragmentation patterns (Cuyckens & Claeys, 2004; Alberts *et al.*, 2012). All mass spectra and chromatograms are provided in Appendix A and B respectively.

Delphinidin derivatives

All the nine delphinidin derivatives identified produced a main fragment at m/z 303 which corresponds to the delphinidin aglycone. Peaks 5 and 10 were identified as delphinidin-3-*O*-glucoside and delphinidin-3-*O*-rhamnoside due to loss of glucose (-162 amu) and rhamnose (-146) moieties respectively from the anthocyanin. Peaks 1, 14 and 3 and 21 were identified as delphinidin-3,5-*O*-diglucoside (Figure 5.4.1a), delphinidin-3,7-*O*-diglucoside (Figure 5.4.1e) delphinidin-3-*O*-rutinoside (Figure 5.4.1b) and its isomer due to loss of diglucoside (-324 amu) and rutinoside (-308 amu) respectively from the anthocyanin. Peak 13 was identified as delphinidin-3-*O*-(6-acetyl)-5-*O*-diglucoside (Figure 5.4.1d) based on loss of a glucose moiety (-162 amu) from the anthocyanin to produce the fragment at m/z 507 and loss of an acetyldiglucoside (-336 amu) to produce the delphinidin aglycone (m/z 303). Peak 16 was identified as delphinidin-3-*O*-robinobiosido-5-*O*-rhamnoside based on loss of the trisaccharide unit of robinose and rhamnose (-454 amu) from the anthocyanin to produce the delphinidin aglycone. Peaks 26 and 30 were identified as isomers of delphinidin-3-(6-*p*-coumaroylglucoside)-5-(4,6-dimalonyl) glucoside (Figure 5.4.1f) based on loss of a 642 amu fragment [*p*-coumaroylglucoside (-308 amu) + dimalonylglucoside (-334 amu)] from the anthocyanin to produce the delphinidin aglycone. The 637 amu fragment is produced via loss of the *p*-coumaroylglucoside (-308 amu) moiety.

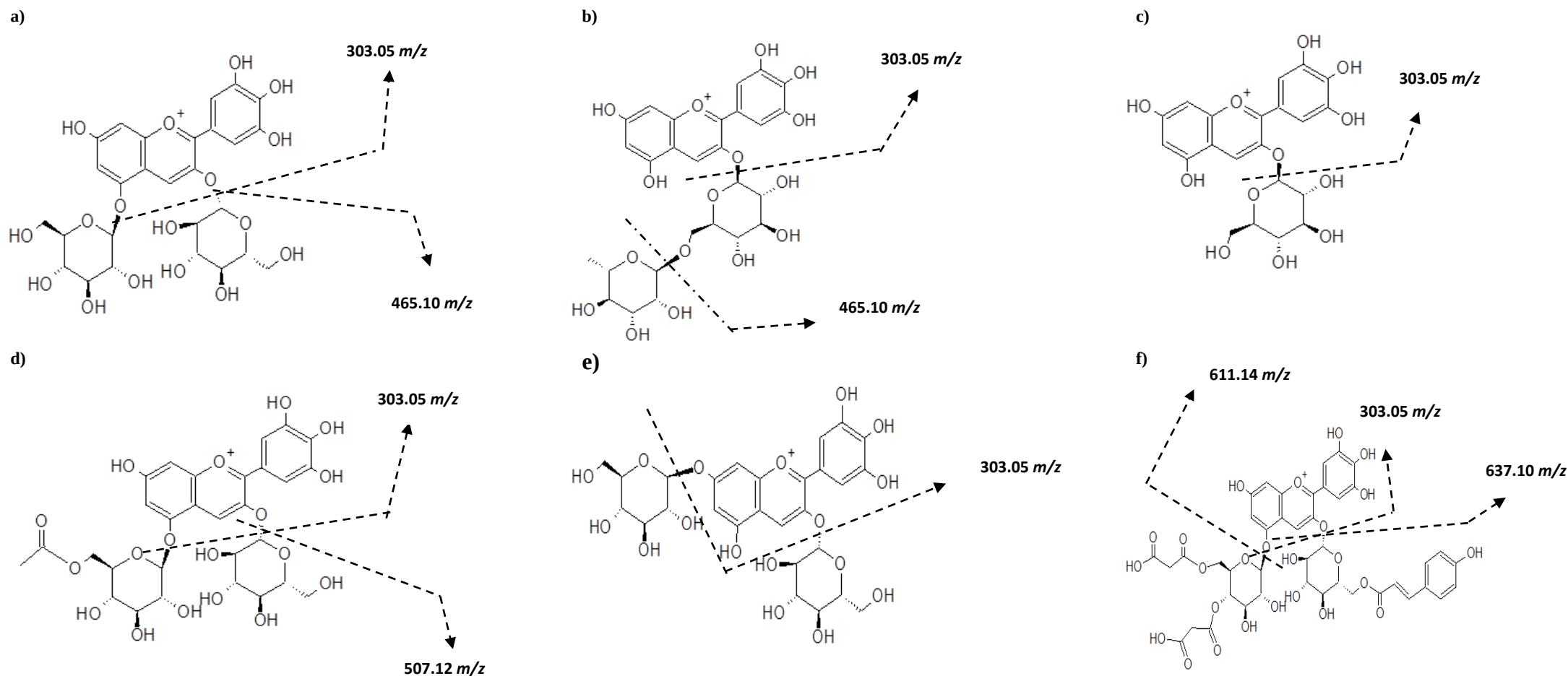


Figure 5.4.1 Proposed fragmentation of some delphinidin derivatives identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families a) delphinidin-3,5-*O*-diglucoside, b) delphinidin-3-*O*-rutinoside, c) delphinidin-3-*O*-glucoside, d) delphinidin-3-*O*-(6-acetyl)-5-*O*-diglucoside, e) delphinidin-3,7-*O*-diglucoside, f) delphinidin-3-(6-*p*-coumaroylglucoside)-5-(4,6-dimalonyl) glucoside

Petunidin derivatives

All three petunidin derivatives identified produced a main fragment at m/z 317.07 corresponding to the aglycone. Peaks 6 and 8 were identified as petunidin-3,5-*O*-diglucoside (Figure 5.4.2a) and petunidin-3-*O*-rutinoside (Figure 5.4.2b) respectively, based on the loss of a diglucoside (-324 amu) and rutinoside (-308 amu) from the petunidin aglycone. Peak 19 was identified as petunidin-3-*O*-(6-acetyl)-5-*O*-diglucoside (Figure 5.4.2c) based on the loss of an acetyldiglucoside (-366 amu) to produce the m/z 317.065, a glucose (-162 amu) moiety to produce a m/z 521.13 fragment and loss of an acetylglucoside (-204 amu) to produce a m/z 479.12 fragment.

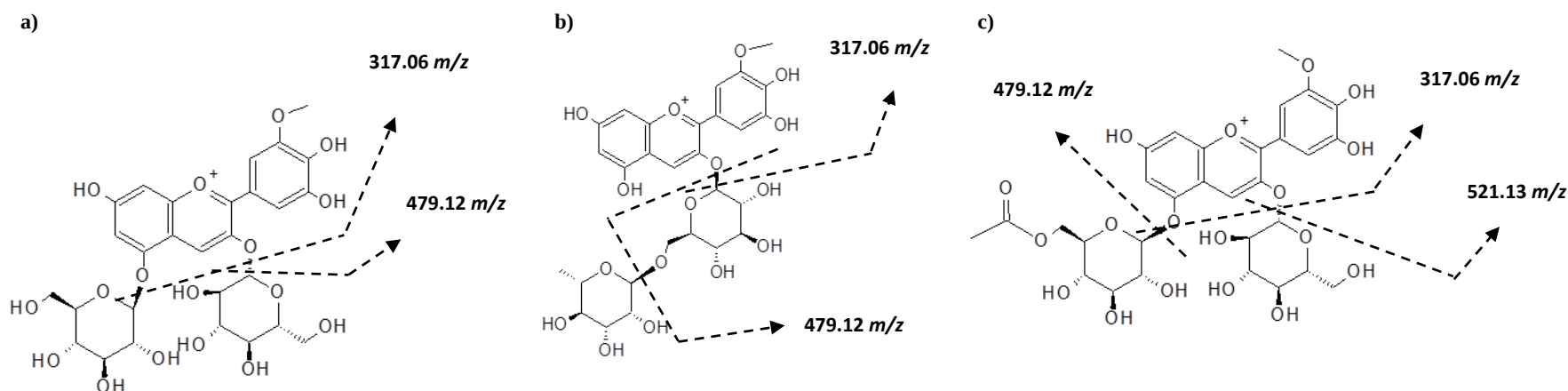


Figure 5.4.2 Proposed fragmentation of petunidin derivatives identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families a) petunidin-3,5-*O*-diglucoside, b) petunidin-3-*O*-rutinoside, c) petunidin-3-*O*-(6-acetyl)-5-*O*-diglucoside

Cyanidin derivatives

All three of the cyanidin derivatives identified produced a main fragment at m/z 287.06 which corresponds to the cyanidin aglycone. Peak 4 was identified as cyanidin-3,5-*O*-diglucoside (Figure 5.4.3a) based on the loss of a diglucoside (-324 amu) to yield the cyanidin aglycone. Peaks 23 and 25 were identified as cyanidin-3-*O*-rutinoside (Figure 5.4.3b) and its isomer, cyanidin-3-(glycosyl) rhamnoside (Figure 5.4.3c) respectively based on the loss of a rutinose (-308 amu) and a rhamnose (-146 amu) moiety resulting in the m/z 449.11 fragment.

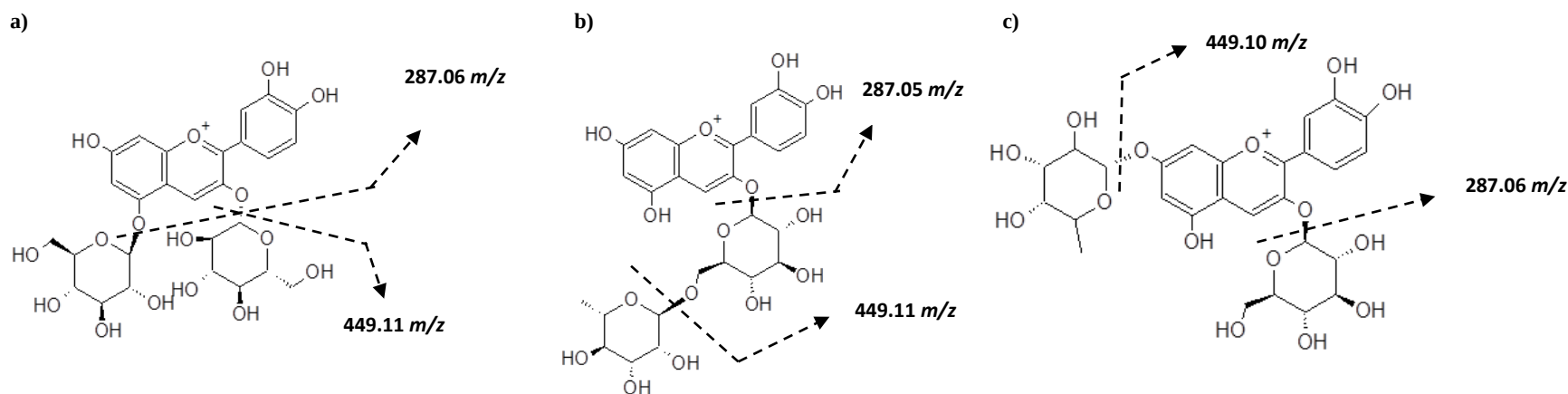


Figure 5.4.3 Proposed fragmentation of cyanidin derivatives identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families a) cyanidin-3,5-*O*-diglucoside, b) cyanidin-3-*O*-rutinoside, c) cyanidin-3-(glycosyl) rhamnoside a) cyanidin-3,5-*O*-diglucoside, b) cyanidin-3-*O*-rutinoside, c) cyanidin-3-(glycosyl) rhamnoside

Pelargonidin derivatives

All four pelargonidin derivatives identified produced a main fragment at m/z 271.06 which corresponds to the pelargonidin aglycone. Peak 11 was identified as pelargonidin-3-*O*-glucoside (Figure 5.4.4a) based on the loss of a glucose (-162 amu) moiety to produce the aglycone fragment (m/z 271.06). Peak 7 was identified as pelargonidin-3,5-*O*-diglucoside (Figure 5.4.4b) based on the loss of a diglucoside (-324 amu) from the anthocyanin to produce the aglycone. Peak 15 was identified as a pelargonidin glucoside derivative based on the loss of a glucose (-162 amu) molecule resulting in the m/z 461.11 fragment. Peak 31 was identified as pelargonidin-3-*O*-rutinoside (Figure 5.4.4c) based on the loss of a rutinose (-308 amu) from the anthocyanin.

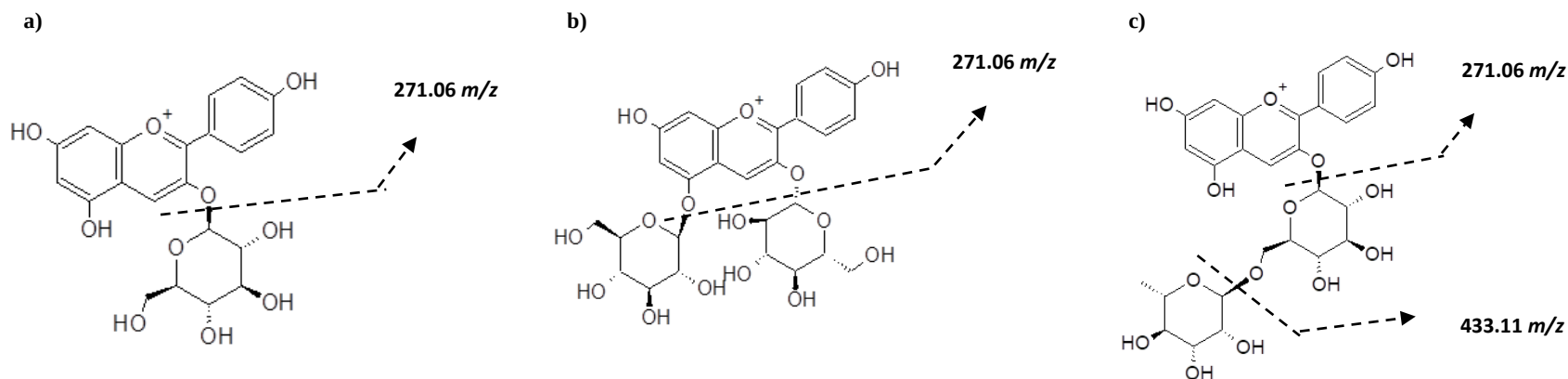


Figure 5.4.4 Proposed fragmentation of some pelargonidin derivatives identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families a) pelargonidin-3-*O*-glucoside, b) pelargonidin-3,5-*O*-diglucoside, c) pelargonidin-3-*O*-rutinoside

Peonidin derivatives

All three peonidin derivatives identified produced a main fragment at m/z 301.07 which corresponds to the peonidin aglycone. Peaks 9 and 17 was identified as peonidin-3,5-*O*-diglucoside (Figure 5.4.5a) and a peonidin glucoside derivative respectively based on the loss of a diglucoside (-324 amu) moiety to produce the m/z 301 fragment (peak 9) and loss of a glucoside (-162 amu) resulting in the m/z 491.11 fragment (peak 17). Peak 20 was identified as peonidin 3-glucoside-5-(6-acetylglucoside) (Figure 5.4.5b) based on the loss of an acetyldiglucoside (-366 amu) to produce the m/z 301.07 fragment.

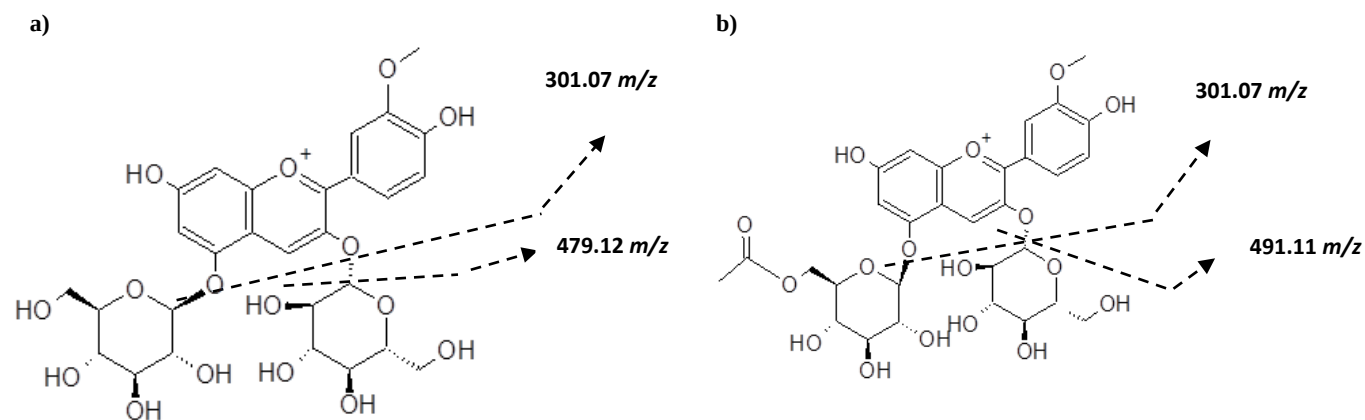


Figure 5.4.5 Proposed fragmentation of some peonidin derivatives identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families a) peonidin-3,5-*O*-diglucoside, b) peonidin 3-glucoside-5-(6-acetylglucoside)

Malvidin derivatives

All five malvidin derivatives identified produced a main fragment at m/z 331.08 corresponding to the malvidin aglycone. Peak 12 was identified as malvidin-3,5-*O*-diglucoside (Figure 5.4.6a) based on the loss of a diglucoside (-324 amu) moiety from the malvidin aglycone. The loss of a glucoside (-162 amu) resulted in the m/z 493.13 fragment. Peak 24 was identified as malvidin-3-*O*-(6-*O*-acetyl)-5-*O*-diglucoside (Figure 5.4.6b) based on loss of an acetyldiglucoside (-366 amu) to produce the m/z 331 fragment. The loss of an acetylglucoside (-204 amu) resulted in a fragment at m/z 493.14 while the m/z 535.15 fragment is due to the loss of a glucoside (-162 amu) moiety. Peaks 27 and 32 were identified as malvidin-3-(6-*p*-coumaroylglucoside)-5-(4-malonylglucoside) (Figure 5.4.6c) and its isomer. For both compounds, loss of a *p*-coumaroyl-malonyldiglucoside (-556 amu) moiety from the malvidin molecule produced the m/z 331.08 fragment and the loss of coumaroylglucoside (-308 amu) resulting in the m/z 579.14 m/z fragment. Peak 28 was identified as malvidin 3-(6-coumaroylglucoside)-5-glucoside (Figure 5.4.6d) based on the loss of a coumaroyldiglucoside (-470 amu) to produce the m/z 331.08 fragment and the loss of a glucose (-162 amu) molecule resulting in the m/z 639.17 fragment.

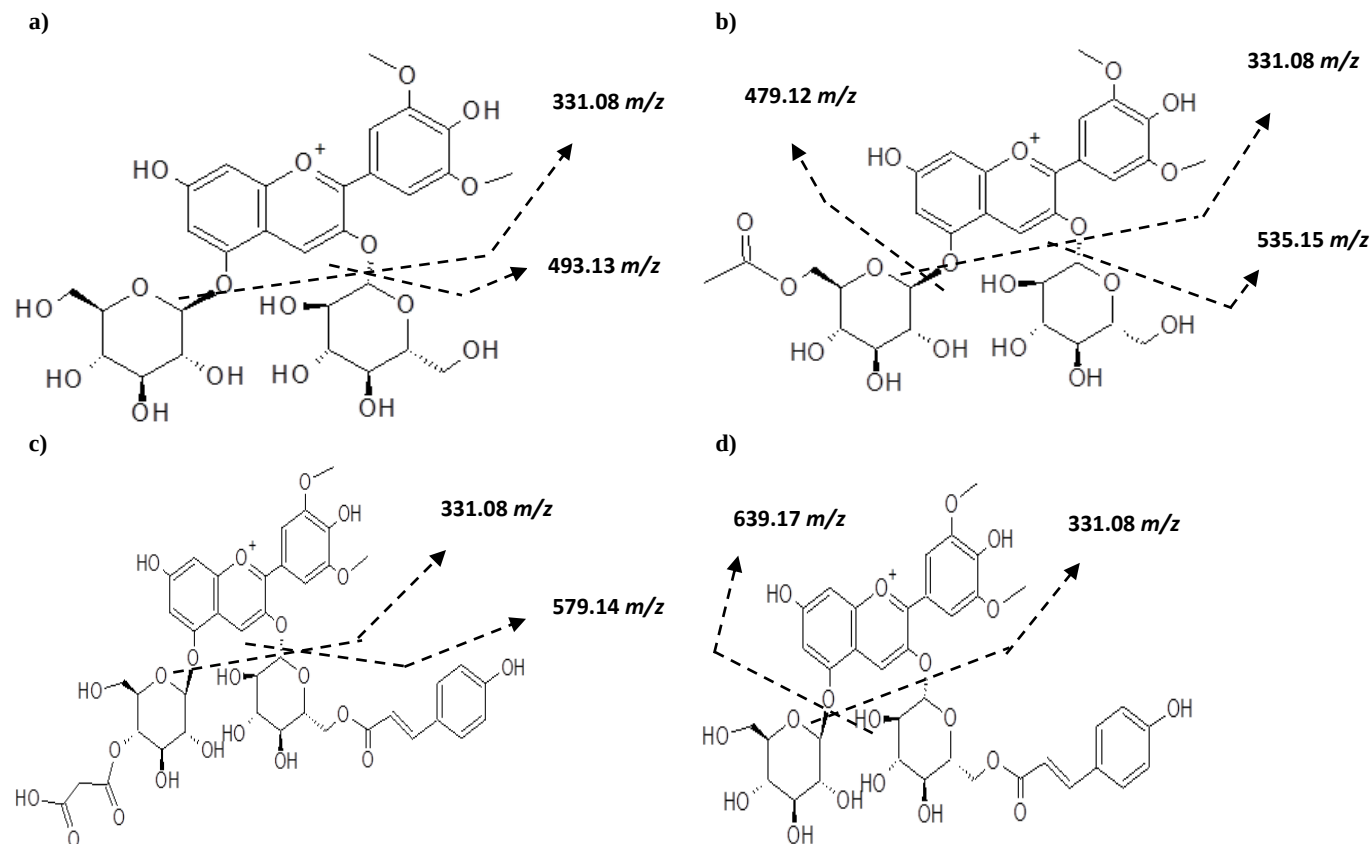


Figure 5.4.6 Proposed fragmentation of some malvidin derivatives identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families a) malvidin-3,5-O-diglucoside, b) malvidin-3-O-(6-O-acetyl)-5-O-diglucoside, c) malvidin-3-(6-p-coumaroylglucoside)-5-(4-malonylglucoside), d) malvidin 3-(6-coumaroylglucoside)-5-glucoside

Table 5.4.1 Retention time (TR), UV-visible absorption maxima ($\lambda_{\max}$) and mass spectral characteristics of anthocyanins found in pigmented flower extracts from the Geraniaceae plant family

T _R (min)	$\lambda_{\max}$ (nm)	Molecular formula [M ⁺]	[M ⁺] (<i>m/z</i>)	MS/MS fragments (% intensity)	Proposed compound	Peak no.	<i>P.</i> <i>grandiflorum</i>	<i>P. × hortorum</i>	<i>P.</i> <i>zonale</i> <i>hybrid</i>
8.33	277, 520	C ₂₇ H ₃₁ O ₁₇	627.15	303.05 (100), 465.10 (12)	Delphinidin-3,5-diglucoside	1	X		
9.16	282, 524	C ₂₆ H ₃₁ O ₁₆	599.16	467.12 (28), 305.065 (56), 437.106 (85)	unknown	2	X		
9.55	278, 518	C ₂₇ H ₃₁ O ₁₆	611.16	303.05 (100), 465.10 (21)	Delphinidin-3- <i>O</i> -rutinoside	3	X		
10.00	276, 516	C ₂₇ H ₃₁ O ₁₆	611.16	287.06 (100), 449.11 (17)	Cyanidin-3,5-diglucoside	4	X	X	
10.10	276, 525	C ₂₁ H ₂₁ O ₁₂	465.10	303.05 (100)	Delphinidin-3- <i>O</i> -glucoside	5	X		
10.99	279, 524	C ₂₈ H ₃₃ O ₁₇	641.17	641.173 (100), 317.066 (93), 479.118 (21)	Petunidin-3,5- <i>O</i> -diglucoside	6	X		
11.37	267, 500	C ₂₇ H ₃₁ O ₁₅	595.17	271.06 (100)	Pelargonidin-3,5- <i>O</i> -diglucoside	7		X	X
12.05	281, 524	C ₂₈ H ₃₃ O ₁₆	625.18	317.066 (100), 479.121 (14)	Petunidin-3- <i>O</i> -rutinoside	8	X		
12.78	284, 525	C ₂₈ H ₃₃ O ₁₆	625.18	301.070 (100), 463.122 (22)	Peonidin-3,5- <i>O</i> -diglucoside	9	X	X	X
13.03	276, 524	C ₂₁ H ₂₁ O ₁₁	449.11	303.049 (100)	Delphinidin-3- <i>O</i> -rhamnoside	10	X		

13.44	242, 500	C ₂₁ H ₂₁ O ₁₀	433.12	271.06 (100)	Pelargonidin-3- <i>O</i> -glucoside	11				X
13.49	274, 524	C ₂₉ H ₃₅ O ₁₇	655.19	331.080 (100), 493.134 (22)	Malvidin-3,5- <i>O</i> -diglucoside	12	X		X	X
13.71	245, 529	C ₂₉ H ₃₃ O ₁₈	669.17	303.049 (100), 507.116 (20)	Delphinidin-3- <i>O</i> -(6-acetyl)-5- <i>O</i> -diglucoside)	13	X			
14.04	271, 525	C ₂₇ H ₃₁ O ₁₇	627.16	319.0453 (100), 303.05 (37)	Delphinidin-3,7- <i>O</i> -diglucoside	14	X			
14.53	244, 500	C ₂₈ H ₃₁ O ₁₆	623.16	271.06 (100), 461.11 (23)	Pelargonidin glucoside derivative	15			X	X
15.17	273, 522	C ₃₃ H ₄₁ O ₂₀	757.22	303.050 (100)	Vincanin (delphinidin-3-robinobiosido-5-rhamnoside)	A 16	X			
15.67	237, 500	C ₂₉ H ₃₃ O ₁₇	653.17	301.07 (100), 491.11 (14)	Peonidin glucoside derivative	17			X	X
16.31	275, 525	-	574.15	163.039 (100)	Unknown	18				
16.39	275, 524	C ₃₀ H ₃₅ O ₁₈	683.18	317.065 (100), 521.127 (25), 479,1232 (5)	Petunidin-3- <i>O</i> -(6-acetyl)-5- <i>O</i> -diglucoside)	19	X		X	
17.04	275, 500	C ₃₀ H ₃₅ O ₁₇	667.19	301.07 (100)	Peonidin-3- <i>O</i> -glucoside-5-(6-acetylglucoside)	20			X	
17.21	267, 351, 514	C ₂₇ H ₃₁ O ₁₆	611.16	303.048 (21), 465.102 (15)	Delphinidin-3- <i>O</i> -rutoside isomer	21	X			

17.63	277, 525	-	434.20	434.202 (100), 317.06 (32)	Unknown	22		
18.83	275, 527	C ₃₁ H ₃₇ O ₁₈	697.20	331.081 (100), 535.146 (23), 493.136 (9)	Malvidin-3- <i>O</i> -(6- <i>O</i> -acetyl)-5- <i>O</i> -diglucoside	24	X	X
19.59	245, 532	C ₂₇ H ₃₁ O ₁₅	595.17	287.054 (100), 449.105 (7)	Cyanidin-3-(glycosyl) rhamnoside	25	X	

X: denotes the presence of the compound

Table 5.4.2 Retention time (TR), UV-visible absorption maxima ($\lambda_{\max}$) and mass spectral characteristics of anthocyanins found in pigmented flower extracts from the Lamiaceae plant family

T_R (min)	$\lambda_{\max}$ (nm)	Molecular formula [M ⁺]	[M ⁺] (m/z)	MS/MS fragments (% intensity)	Proposed compound	Peak no.	<i>S. aurea</i> × <i>dolomitica</i>	<i>S.</i> <i>dolomitica</i>	<i>P.</i> <i>zuluensis</i>
8.33	277, 520	C ₂₇ H ₃₁ O ₁₇	627.15	303.05 (100), 465.10 (12)	Delphinidin-3,5- <i>O</i> -diglucoside	1	X	X	
13.71	245, 529	C ₂₉ H ₃₃ O ₁₈	669.17	303.049 (100), 507.116 (20)	Delphinidin-3- <i>O</i> -(6-acetyl)-5- <i>O</i> -diglucoside)	13	X		
18.14	266, 525	C ₂₇ H ₃₁ O ₁₅	595.16	287.054 (100), 449.108 (8)	Cyanidin-3- <i>O</i> -rutinoside	23	X	X	
19.02	278, 531	C ₄₂ H ₄₁ O ₂₅	945.20	303.05 (100), 637.104 (17)	Dimalonylawobanin (delphinidin-3-(6- <i>p</i> -coumaroylglucoside)-5-(4,6-dimalonylglucoside) isomer	26	X		
19.60	245, 500	C ₄₁ H ₄₃ O ₂₂	887.23	331.08 (100), 579.14 (15)	Malvidin-3-(6- <i>p</i> -coumaroylglucoside)-5-(4-malonylglucoside) isomer	27			X
19.94	245, 500	C ₃₈ H ₄₁ O ₁₉	801.22	331.08 (100), 639.17 (15)	Malvidin 3-(6-coumaroylglucoside) 5-glucoside	28			X
20.07	276, 534	C ₂₇ H ₂₁ O ₁₁	521.11	323.053 (59), 295.06 (41), 163.04 (30)	Unknown	29	X		
20.44	276, 531	C ₄₂ H ₄₁ O ₂₅	945.19	303.049 (100), 637.104 (26), 611.14 (6)	Dimalonylawobanin (delphinidin-3-(6- <i>p</i> -coumaroylglucoside)-5-(4,6-dimalonylglucoside)	30	X		
20.56	268, 531	C ₂₇ H ₃₁ O ₁₄	579.17	271.061 (100), 433.11 (23)	Pelargonidin-3- <i>O</i> -rutinoside	31	X	X	
20.72	245, 500	C ₄₁ H ₄₃ O ₂₂	887.23	331.08 (100), 579.14 (15)	Malvidin-3-(6- <i>p</i> -coumaroylglucoside)-5-(4-malonylglucoside)	32			X

X: denotes the presence of the compound

5.4.2 Quantification of anthocyanins in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

Table 5.4.3 shows the anthocyanin content ($\mu\text{g IE/g}$) of the pigmented extracts from flowers of plants from the two families. The total anthocyanin content was highest in the *P. grandiflorum* (Geraniaceae) ($19745 \mu\text{g IE/g}$) followed by *P. zonale* hybrid (Geraniaceae) ($7606 \mu\text{g IE/g}$). For the Lamiaceae family, pigmented extracts from flowers of the *S. aurea* $\times$ *dolomitica* contained the highest total anthocyanins ($334 \mu\text{g IE/g}$) followed closely by the *P. zuluensis* ($329 \mu\text{g IE/g}$).

The main anthocyanins in flowers from the Geraniaceae were 3,5-diglucosides of delphinidin, petunidin, pelargonidin, peonidin and malvidin and acetyl acylated malvidin, delphinidin and petunidin. For the Lamiaceae, the main anthocyanins identified in their flowers were rutosides of pelargonidin, glucosides of petunidin, pelargonidin and *p*-coumaric acid- and malonyl-acylated delphinidin and malvidin.

Work on the identification of the anthocyanins of the flower species of these two plant families is limited and most research has focused on other phenolic compounds or compounds in the essential oils from the leaves (Kwee and Niemeyer, 2011; Zhu et al., 2014; Marchioni et al., 2020; Jordheim et al., 2016) and other aerial parts (Katanić Stanković et al., 2020) of these plants. The findings from this study on anthocyanin identification generally agree with the limited reports available on anthocyanins in flowers of the Geraniaceae and Lamiaceae.

Mitchell et al. (1998) identified 3, 5-diglucoside and 3-glucoside-5-(6-acetyl) glucoside of delphinidin, malvidin and petunidin in a wide variety of zonal *Pelargonium* flowers. They also reported that the salmon pink *Pelargoniums* consist almost entirely of pelargonidin derivatives which agrees with the salmon pink *P. zonale* hybrid used in this study. The red coloured *P. $\times$ hortorum* mainly contained peonidin, malvidin, pelargonidin and cyanidin derivatives respectively. Mitchell et al. (1998) reported the main pigment in red coloured *Pelargoniums* as pelargonidin and peonidin derivatives.

Our identification of mainly delphinidin glucosides in the blue *Salvia* species studied in this research is in agreement with Saito and Harborne (1992) who reported that blue flower species from the Lamiaceae generally contain delphinidin glucosides as major pigment. They also identified the malonyl acylated anthocyanins and aromatic anthocyanins acylated with *p*-coumaric and caffeic acids in various species of flowers in the Lamiaceae as found in the current study.

The anthocyanins identified in the dark purple *Plectranthus zuluensis* (Lamiaceae) used in this study exclusively contained *p*-coumaroylglucoside and malonylglucoside acylated malvidin derivatives. In contrast, Jordheim et al. (2016) reported high concentrations of coumaroylglucoside, caffeoylglucoside and malonylglucoside of peonidin in the cauline hairs of the leaves of the dark purple *Plectranthus ciliates* E. Mey.

Table 5.4.3 Anthocyanins (µg IE/g) in pigmented flower extracts from Geraniaceae and Lamiaceae plant families

Compound	Geraniaceae			Lamiaceae		
	<i>P. grandiflorum</i>	<i>P. × hortorum</i>	<i>P. zonale hybrid</i>	<i>S. aurea × dolomitica</i>	<i>S. dolomitica</i>	<i>P. zuluensis</i>
Delphinidin-3,5-diglucoside	6693 ± 1003	ND	ND	153 ± 12	75 ± 6	ND
Delphinidin-3,7-diglucoside	479 ± 22	ND	ND	ND	ND	ND
Pelargonidin glucoside derivative	ND	620 ± 62	120 ± 45	ND	ND	ND
Pelargonidin-3-O-glucoside	ND	ND	294 ± 12	ND	ND	ND
Vincanin A (delphinidin-3-robinobiosido-5-rhamnoside)	50 ± 6	ND	ND	ND	ND	ND
Peonidin glucoside derivative	ND	146 ± 0	88 ± 0	ND	ND	ND
Delphinidin-3-O-rutinoside	859 ± 45	ND	ND	ND	ND	ND
Delphinidin 3-neohesperidoside	238 ± 20	ND	ND	ND	ND	ND
Delphinidin-3-O-(6-acetyl)-5-O-diglucoside	2426 ± 465	ND	ND	46 ± 1	ND	ND
Dimalonylawobanin (delphinidin-3-(6-p-coumaroylglucoside)-5-(4,6-dimalonylglucoside)	ND	ND	ND	22 ± 0	ND	ND
Dimalonylawobanin isomer (delphinidin-	ND	ND	ND	67 ± 4	ND	ND

3-(6- <i>p</i> -coumaroylglucoside)-5-(4,6-dimalonylglucoside) Malvidin-3-(6- <i>p</i> -coumaroylglucoside)-5-(4-malonylglucoside)	ND	ND	ND	ND	ND	142 ± 0
Malvidin-3-(6-coumaroylglucoside) 5-glucoside/isomer	ND	ND	ND	ND	ND	187 ± 45
Pelargonidin-3-rutinoside	ND	ND	ND	35 ± 6	56 ± 2	ND
Cyanidin-3,5-diglucoside	107 ± 2	473 ± 0	ND	ND	ND	ND
Delphinidin-3- <i>O</i> -rhamnoside	622 ± 83	ND	ND	ND	ND	ND
Cyanidin-3-rutinoside	192 ± 4	ND	ND	14 ± 2		ND
Petunidin-3,5-diglucoside	2488 ± 110	ND	ND	19 ± 1	ND	ND
Pelargonidin-3,5-diglucoside	ND	ND	6349 ± 0	ND		ND
Petunidin-3- <i>O</i> -rutinoside	120 ± 2	ND	ND	ND	ND	ND
Petunidin-3- <i>O</i> -(6-acetyl)-5- <i>O</i> -diglucoside	2656 ± 150	84 ± 0	ND	ND	ND	ND
Malvidin-3- <i>O</i> -(6- <i>O</i> -acetyl)-5- <i>O</i> -diglucoside	3889 ± 76	730 ± 0	ND	ND	ND	ND
Peonidin-3, 5- <i>O</i> -diglucoside	124 ± 0	3750 ± 0	2061 ± 0	ND	ND	ND
Malvidin-3,5- <i>O</i> -diglucoside	1899 ± 1	1803 ± 0	396 ± 0	ND	ND	ND

Total anthocyanins	19745	7606	8912	334	131	329
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IE: Ideain (cyanidin-3-galactoside) equivalents

ND: Not detected

5.4.3 Identification and quantification of phenolic acids and flavonoids in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

Table 5.4.4 Retention time (T_R), UV-visible absorption maxima (λ_{max}), mass spectral characteristics of phenolic acids and flavonoids in pigmented flower extracts from Geraniaceae and Lamiaceae plant families

T _R (min)	λ _{max} (nm)	Molecular formula [M ⁻]	[M-H] ⁻	MS/MS fragments (% intensity)	Proposed compound	Peak no	Geraniaceae <i>P. grandiflorum</i>	<i>P. × hortorum</i>	<i>P. zonale hybrid</i>	Lamiaceae <i>S. aurea × dolomitica</i>	<i>S. dolomitica</i>	<i>P. zuluensis</i>
Hydroxycinnamic acid derivatives												
10.02	246, 327	C ₁₃ H ₁₁ O ₉	311.04	149.01 (100), 179.03 (78)	Caffeoyltartaric acid	3	X					
11.19	312	C ₉ H ₇ O ₃	163.04	119.05 (34)	<i>p</i> -coumaric acid	4		X	X			X
11.87	230, 315	C ₁₅ H ₁₇ O ₈	325.09	163.04 (8)	<i>p</i> -coumaroylglucose	5		X	X			
12.50	274	C ₁₁ H ₁₁ O ₅	223.06	179.05 (12)	Sinapic acid isomer	6					X	
12.86	320	C ₉ H ₇ O ₄	179.03	135.04 (100)	Caffeic acid	7				X	X	X
Hydroxybenzoic acid derivatives												
6.16	261, 279	C ₁₃ H ₁₅ O ₁₀	331.07	169.02 (6)	β-Glucogallin (Galloyl-β-D-glucoside)	1	X	X	X	X		
8.08	279	C ₉ H ₉ O ₅	197.04	109.03 (2.3), 153.02 (2)	Syringic acid	2				X	X	
16.63	277	C ₃₄ H ₂₇ O ₂₂	787.10	617.08 (27), 465.06 (4), 635.08 (3.7)	Tetra-O-galloyl-glucose	9	X			X		
18.31	279	C ₄₁ H ₃₁ O ₂₆	939.11	169.01 (62), 787.10 (9)	Penta-O-galloyl-glucose	13	X					
Flavonol derivatives												

17.40	260, 350	C ₂₇ H ₂₉ O ₁₆	609.15	463.09 (80), 301.04 (5)	Quercetin-3-O-rutinoside (Rutin)	11	X	X	X
17.82	265, 348	C ₂₁ H ₁₉ O ₁₂	463.09	301.04 (6), 151.00 (2)	Quercetin-3-O-glucoside (Isoquercetin)	12	X	X	X
13.85	265, 340	C ₂₇ H ₂₉ O ₁₆	609.15	285.04 (100)	Kaempferol-3-O-glucosylgalactoside	8		X	X
16.90	265, 340	C ₃₃ H ₃₉ O ₁₉	739.21	285.04 (29)	Robinin (kaempferol 3-O-robinoside-7-O-rhamnoside)	10			X
19.03	265, 346	C ₂₇ H ₂₉ O ₁₅	593.15	285.04 (28)	Kaempferol-3-O-rutinoside	15	X	X	X
Rosmarinic acid derivatives									
19.53	274	C ₃₆ H ₃₁ O ₁₇	735.15	359.08 (100), 717.15 (41)	Rosmarinic acid derivative	18		X	X
20.69	307	C ₃₆ H ₃₁ O ₁₆	719.16	359.08 (96)	Sagerinic acid	20		X	X
20.71	324, 331	C ₁₈ H ₁₅ O ₈	359.08	197.04 (19), 179.03 (1)	Rosmarinic acid	21		X	X
22.43	283	C ₃₆ H ₂₉ O ₁₆	717.14	519.09 (100), 321.04 (37)	Salvianolic acid B	22		X	X
Flavanone derivatives									
19.04	279	C ₂₇ H ₃₁ O ₁₄	579.17	271.06 (66), 151.00 (10)	Naringin	16		X	

20.34	266, 345	C ₂₈ H ₃₃ O ₁₅	609.18	301.07 (100)	Neohesperidin	19						X
<i>Flavone derivatives</i>												
18.47	333	C ₂₁ H ₁₉ O ₁₀	431.10	269.05 (100)	Apigenin-7-O- glucoside	14						X
19.66	266	C ₂₁ H ₁₉ O ₁₁	447.09	285.04 (25)	Luteolin-7-O- glucoside	17	X	X	X			X

X: denotes the presence of the compound

Phenolic acids identified

Table 5.4.4 shows chromatographic and mass spectral data of phenolic acids identified in the pigmented flower extracts. All mass spectra and chromatograms are illustrated in Appendix A and B respectively.

All three gallic acid derivatives identified produced a main fragment at m/z of 169.02 (Figure 5.4.7) which represents gallic acid. The compound labelled peak 1 with retention time of 6.16 min, molecular ion at m/z 331.07, maximum absorption wavelength at 261, 279 nm and molecular formula of $C_{13}H_{15}O_{10}$, was tentatively identified as β -glucogallin (galloyl- β -D-glucoside). The gallic acid fragment at m/z 169.02 is produced after the loss of a glucose molecule (-162 amu) (Table 5.4.3) (Figure 5.4.7a) (Yisimayili, Abdulla, Tian, Wang, Chen, Sun, Li, Liu, Aisa and Huang, 2019). The compound labelled peak 9 with retention time of 16.63 min, molecular ion at m/z 787.10, maximum absorption wavelength at 277 nm and molecular formula of $C_{34}H_{27}O_{22}$, was tentatively identified as tetra-O-galloyl-glucose (Table 5.4.3) (Figure 5.4.7b). Ionic fragments produced were m/z 617.08, 465.06 and 635.08. The m/z 617.08 fragment corresponds to the loss of a gallic acid molecule (-170 amu) while the m/z 635.08 fragment corresponds to the loss of a galloyl moiety (-152 amu). The m/z 465.06 fragment is the result of the loss of a gallic acid molecule (-170 amu) together with the loss of a galloyl moiety (-152 amu) (Berardini, Carle and Schieber, 2004). The compound labelled peak 13 with retention time of 18.31 min, molecular ion at m/z 939.11, maximum absorption wavelength at 279 nm and molecular formula of $C_{41}H_{31}O_{26}$, was tentatively identified as penta-O-galloyl-glucose (Table 5.4.3). Ionic fragments produced were at m/z 169.01 and 787.10. The m/z 169.01 fragment corresponds to the loss of a gallic acid molecule (-770 amu) while the m/z 787.10 fragment corresponds to the tetra-O-galloyl molecule (Berardini et al., 2004).

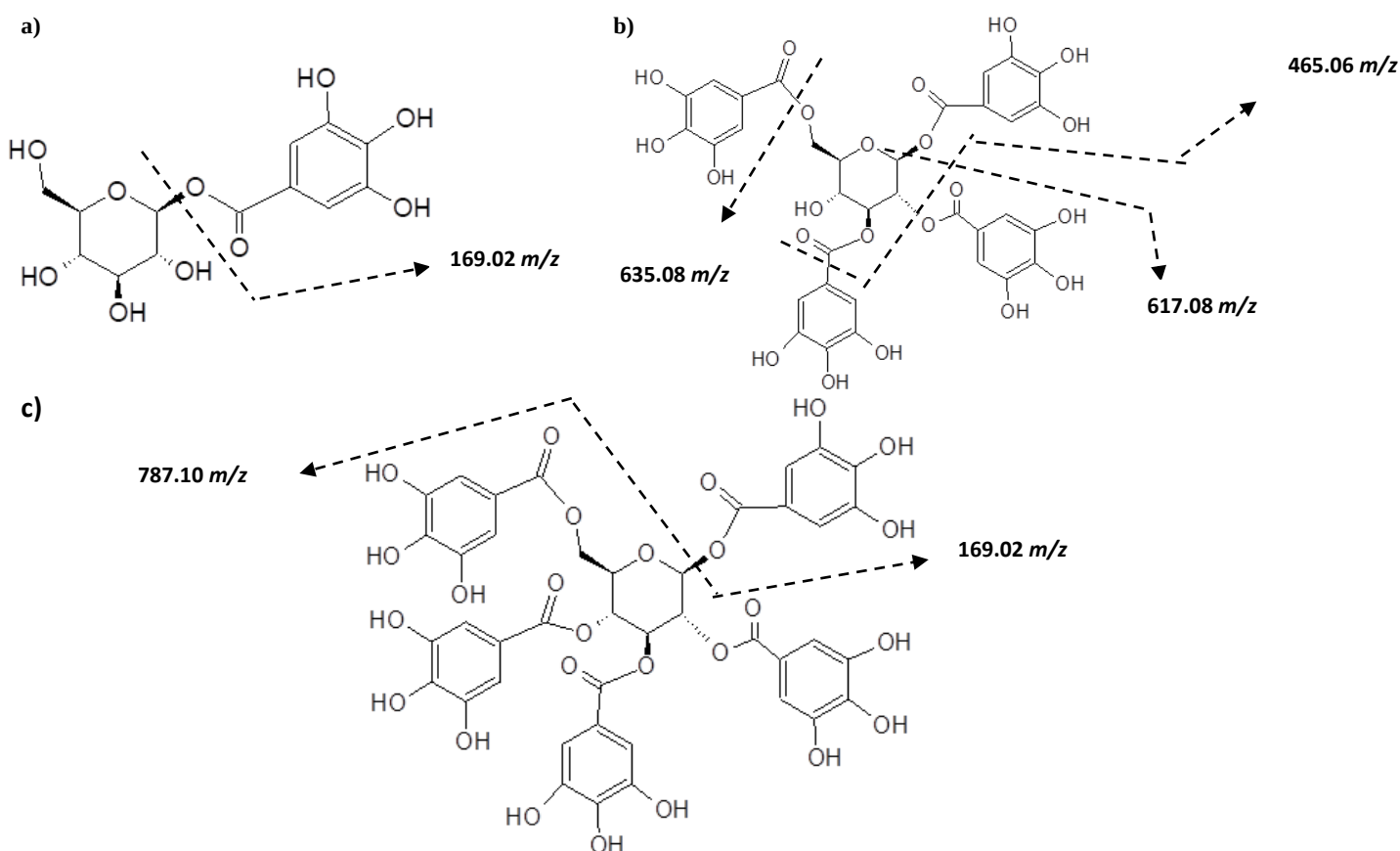


Figure 5.4.7 Proposed fragmentation of some gallic acid derivatives identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families a) β -glucogallin, b) tetra-O-galloyl-glucose

The compound labelled peak 2 with retention time of 8.08 min, molecular ion at m/z 197.04, maximum absorption wavelength at 279 nm and molecular formula of $C_9H_9O_5$, was tentatively identified as syringic acid (Table 5.4.3). Loss of a CO_2 molecule (-44 amu) produces the fragment at m/z 153.02, while loss of a CO_2 (-44 amu) together with CH_3 (-15 amu) produces the m/z 138 fragment (Gruz, Novák and Strnad, 2008) (Figure 5.4.8).

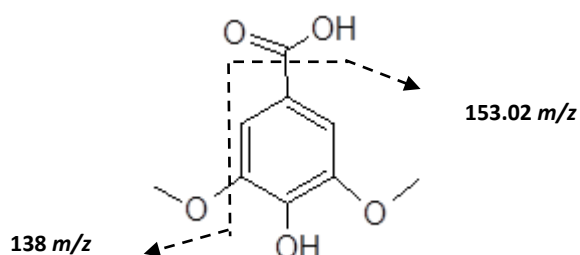


Figure 5.4.8 Proposed fragmentation of syringic acid identified in pigmented flower extracts from the Lamiaceae plant family

The compound labelled peak 6 with retention time of 12.50 min, molecular ion at m/z 223.06, maximum absorption wavelength at 274 nm and molecular formula of $C_{11}H_{11}O_5$, was tentatively identified as sinapic acid isomer (Table 5.4.3). Fragmentation produced an ionic fragment at m/z 179.05 corresponding to loss of a CO_2 (-44 amu).

The compound labelled peak 3 with retention time of 10.02 min, molecular ion at m/z 311.04, maximum absorption wavelength at 246, 327 nm and molecular formula of $C_{13}H_{11}O_9$, was tentatively identified as caffeoyltartaric acid (Table 5.4.3). Fragments produced were at m/z 179.03 and 149.01. The m/z 179.03 fragment corresponds to the loss of caffeic acid molecule while the m/z 149.01 corresponds to a tartarate molecule (Schütz, Kammerer, Carle and Schieber, 2005) (Figure 5.4.9).

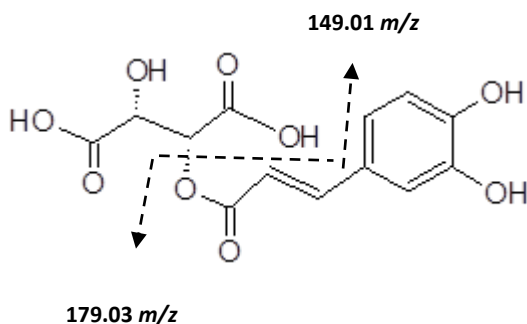


Figure 5.4.9 Proposed fragmentation of caffeoyltartaric acid

The compound labelled as peak 4 with retention time of 11.19 min, molecular ion at m/z 163.04, molecular formula of $C_9H_7O_5$ and maximum UV-vis absorption wavelength of 312 nm was identified as *p*-coumaric acid (Figure 5.4.10a). It produced a fragment at m/z 119.05 corresponding to the loss of a CO_2 molecule (-44 amu) (Yisimayili et al., 2019) (Figure 5.4.10a). The compound labelled peak 5 with retention time of 11.87 min, molecular ion at m/z 325.09, molecular formula of $C_{15}H_{17}O_8$ and maximum UV-vis absorption wavelength of 230, 315 nm was identified as *p*-coumaroylglucose (Figure 5.4.10b). Fragmentation produced a fragment at m/z 163 (*p*-coumaric acid) due to the loss of a glucose moiety (-162 amu) (Cuyckens et al., 2004).

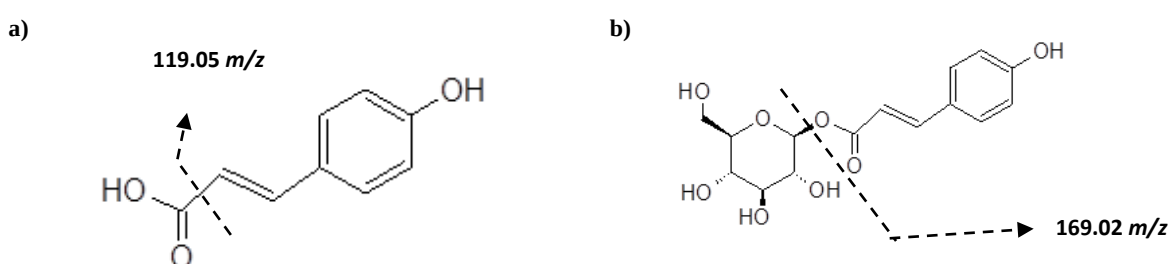


Figure 5.4.10 Proposed fragmentation of coumaric acid and its derivative identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant family a) *p*-coumaric acid and b) *p*-coumaroyl glucose

The compound labelled peak 7 with retention time of 12.86 min, molecular ion at m/z 179.03, maximum absorption wavelength at 320 nm and molecular formula of $C_9H_7O_4$, was identified as caffeic acid and confirmed with an external standard (Table 5.4.3). The fragment at m/z 135.04 corresponds to the loss of a CO_2 molecule (-44 amu) (Schütz et al., 2005). (Figure 5.4.11a).

The compound labelled peak 21 with retention time of 20.71 min, molecular ion at m/z 359.08, maximum absorption wavelength at 324, 331 nm and molecular formula of $C_{18}H_{15}O_8$, was tentatively identified as rosmarinic acid (Table 5.4.3) (Figure 5.4.11b). It produced fragments at m/z 197.04 and 179.03. The m/z 197.04 fragment is the result of the loss of a 2-hydroxy derivative of hydrocaffeic acid (danshensu) while the m/z 179.03 fragment corresponds to the loss of a caffeic acid (-180 amu) (Nuengchamnong et al., 2011). The compound labelled peak 20 with retention time of 20.69 min, molecular ion at m/z 719.16, maximum absorption wavelength at 307 nm and molecular formula of $C_{36}H_{31}O_{16}$, was tentatively identified as sagerinic acid (Table 5.4.3) (Figure 5.4.11c). It produced a major rosmarinic acid ionic fragment at m/z 359.08 due to loss of a rosmarinic acid molecule (-360 amu) (Nuengchamnong, Krittasilp and Ingkaninan, 2011). The compound labelled peak 21 with retention time of 22.43

min, molecular ion at m/z 17.14, maximum absorption wavelength at 324, 331 nm and molecular formula of $C_{18}H_{15}O_8$, was tentatively identified as salvianolic acid B (Table 5.4.3) (Figure 5.4.11d). It produced fragments at m/z 519.09 and 321.04. The m/z 519.09 fragment corresponds to the loss of a 2-hydroxy derivative of hydrocaffeic acid (danshensu) (-198 amu) while the loss of two danshensu molecules (-396 amu) results in the m/z 321.04 fragment (Nuengchamnong et al., 2011).

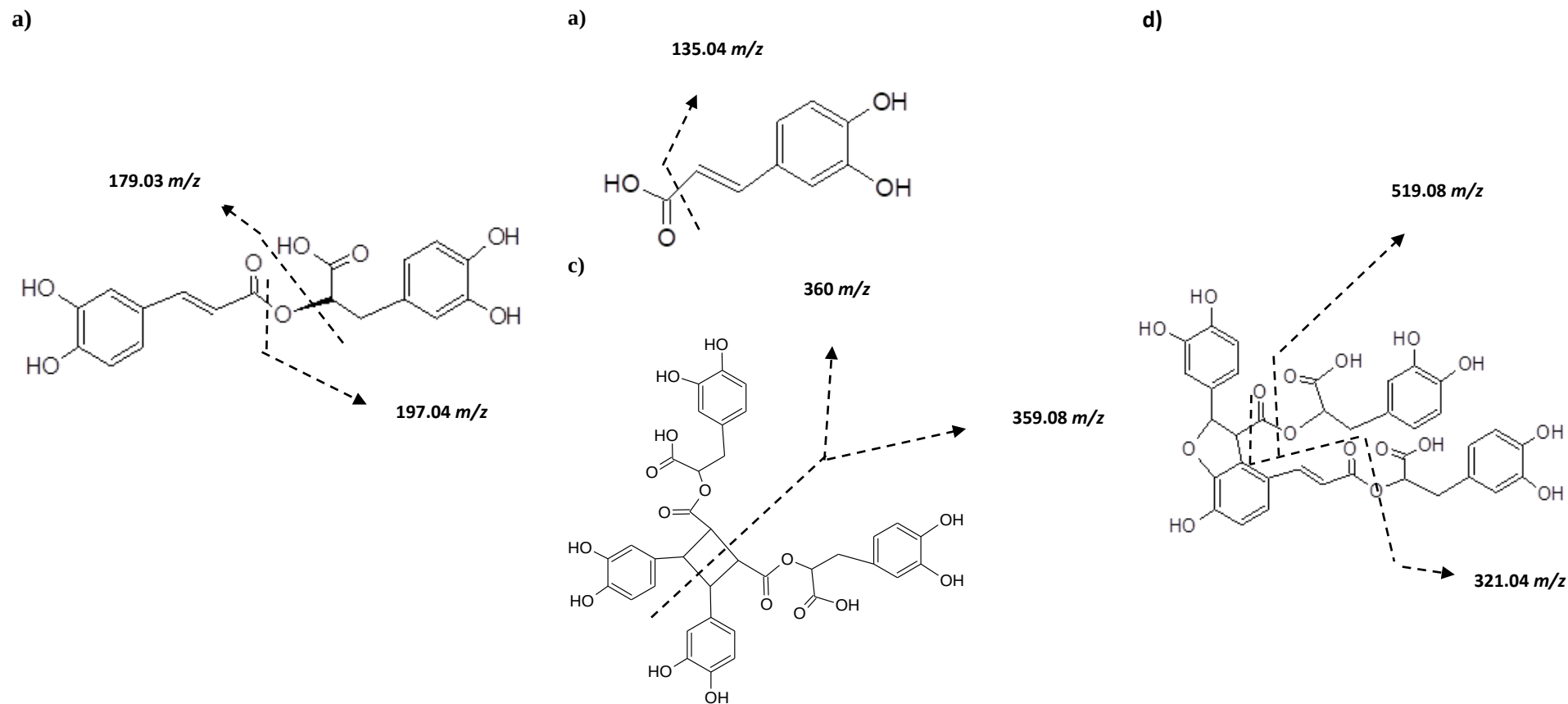


Figure 5.4.11 Proposed fragmentation of caffeic acid derivatives identified in pigmented flower extracts from the Lamiaceae plant family a) caffeic acid, b) rosmarinic acid, c) sagerinic acid, d) salvianolic acid B

The compound labelled peak 6 with retention time of 12.50 min, molecular ion at 223.06 m/z , maximum absorption wavelength at 274 nm and molecular formula of $C_{11}H_{11}O_5$, was tentatively identified as sinapic acid isomer (Table 5.4.3). Fragmentation produced an ionic fragment of m/z 179.05 corresponding to a CO_2 loss (-44 amu).

Flavonoids identified:

Table 5.4.3 shows chromatographic and mass spectral data of flavonoids identified in the pigmented flower extracts. All mass spectra and chromatograms are provided in Appendix A and B respectively.

Flavonol derivatives identified

All three kaempferol derivatives identified produced a main ionic fragment at m/z 285.04 corresponding to the kaempferol aglycone. The compound labelled peak 8 with retention time of 13.85 min, molecular ion at m/z 609.14, maximum absorption wavelength at 265, 340 nm and molecular formula of $C_{27}H_{29}O_{16}$, was tentatively identified as kaempferol-3-*O*-glucosylgalactoside (Table 5.4.3). It produced a main fragment at m/z 285.04 which corresponds to the kaempferol aglycone after the loss of a diglucoside moiety (-324 amu) (Alberts et al., 2012). The compound labelled peak 10 with retention time of 16.90 min, molecular ion at m/z 739.21, molecular formula of $C_{33}H_{39}O_{19}$ and maximum UV-vis absorption wavelength of 265, 340 nm was identified as robinin (kaempferol-3-*O*-robinoside-7-*O*-rhamnoside). It produced a main fragment at m/z 285.04 which corresponds to the kaempferol aglycone (Tsiklauri, An, Ruszaij, Alaniya, Kemertelidze & Morris, 2011) after the loss of a dihexose moiety (-308 amu) and a rhamnoside (-146 amu) (Alberts et al., 2012; Cuyckens et al., 2004) (Figure 5.4.12a).

The compound labelled peak 15 with retention time of 19.03 min, molecular ion at m/z 593.15, maximum absorption wavelength at 265 and 346 nm and molecular formula of $C_{27}H_{19}O_{15}$, was tentatively identified as kaempferol-3-*O*-rutinoside (Table 5.4.3). The fragment at m/z 285.04 is the kaempferol aglycone produced after the loss of a rutinoside unit (-308 amu) (Cuyckens and Claeys, 2004) (Figure 5.4.12b).

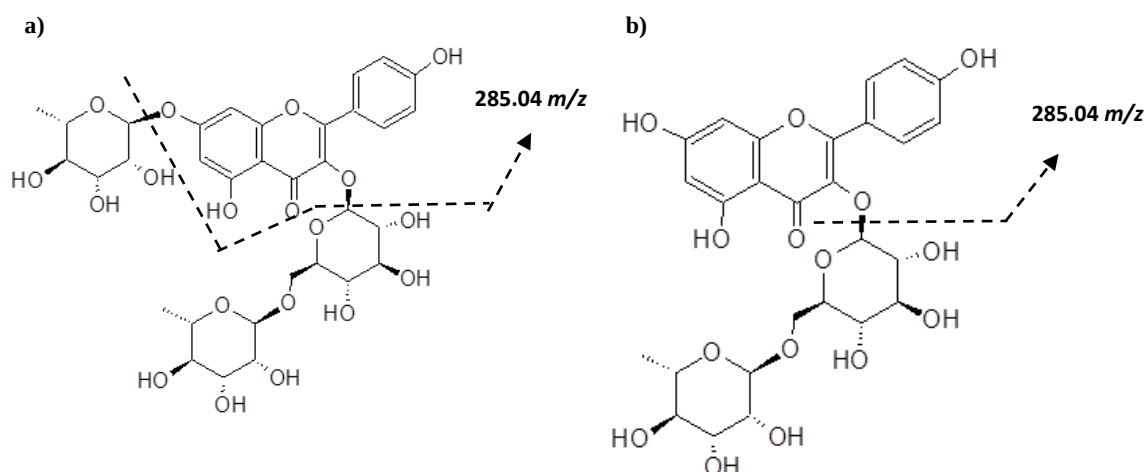


Figure 5.4.12 Proposed fragmentation for some kaempferol derivatives identified in pigmented flower extracts from the Geraniaceae plant family a) Robinin (kaempferol-3-O-robinoside-7-O-rhamnoside), b) kaempferol-3-O-rutinoside

The two quercetin derivatives identified produced an ionic fragment at m/z 301.04 corresponding to the quercetin aglycone. The compound labelled peak 11 with retention time of 17.40 min, molecular ion at m/z 609.15, maximum absorption wavelength at 260, 350 nm and molecular formula of $C_{27}H_{29}O_{16}$, was identified as quercetin-3-O-rutinoside (rutin) (Table 5.4.3). It produced a fragment at m/z 303.03 due to the loss of a rutinoside unit (-308 amu) (Chunhua, Yingyi, Zhouxi and Gang, 2018) and a m/z 463.098 fragment due to loss of a rhamnose unit (-146 mu) (Figure 5.4.13a). The compound labelled peak 12 with retention time of 17.49 min, molecular ion at m/z 463.09, maximum absorption wavelength at 265, 348 nm and molecular formula of $C_{21}H_{19}O_{12}$, was tentatively identified as quercetin-3-O-glucoside (Isoquercetin). It produced ionic fragments at m/z 301.04 and 151.00. The m/z 301.04 fragment is the quercetin aglycone after the loss of a glucose moiety (-162 amu). The fragment at m/z 151.00 is the $^{1,3}A$ fragment produced after Retro-Diels-Alder (RDA) cleavage of the quercetin aglycone at bond positions 1 and 3 of the C ring (Fabre, Rustan, de Hoffmann and Quetin-Leclercq, 2001) (Table 5.4.3) (Figure 5.4.13b).

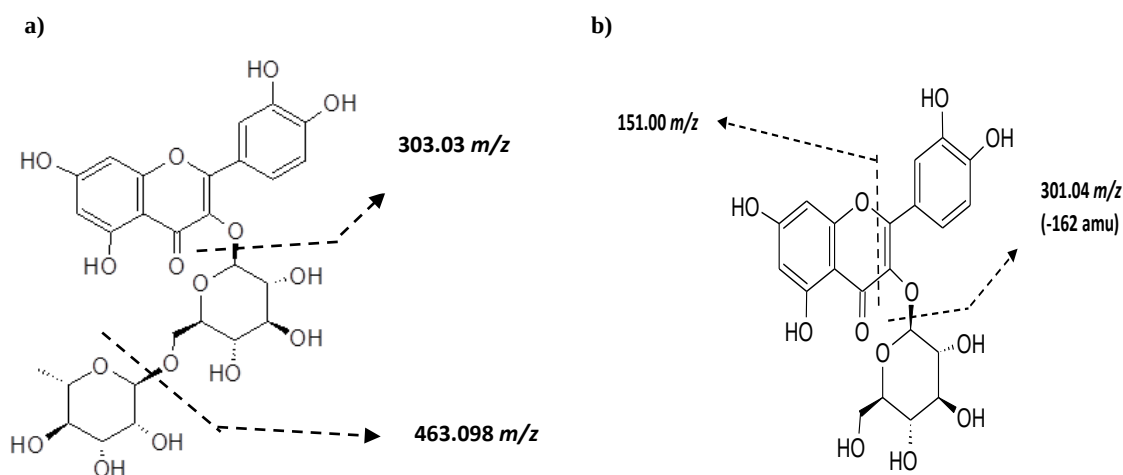


Figure 5.4.13 Proposed fragmentation of quercetin derivatives identified in the pigmented flower extracts from the Geraniaceae plant family a) quercetin-3-O-rutinoside (Rutin), b) quercetin-3-O-glucoside (Isoquercetin)

Flavanone derivatives identified

The compound labelled peak 16 with retention time of 19.04 min, molecular ion at m/z 579.17, maximum absorption wavelength at 279 nm and molecular formula of $C_{27}H_{31}O_{14}$, was identified as naringin (Table 5.4.3). It produced fragments at m/z 271.06 and 151.00. The m/z 271.06 fragment corresponds to the naringenin aglycone after the loss of a neohesperidose moiety (diglucoside) (-308 amu) [glycosyl (162 amu) + rhamnosyl (146 amu)] (Xu, Liu, Zhang, Yang and Tian, 2009). The fragment at m/z 151.00 is the $^{1,3}A$ fragment obtained from RDA cleavage of the naringenin aglycone at bond positions 1 and 3 of the C ring (Fabre et al., 2001) (Figure 5.4.14a).

The compound labelled peak 19 with retention time of 20.43 min, molecular ion at m/z 609.18, maximum absorption wavelength at 266, 345 nm and molecular formula of $C_{28}H_{33}O_{15}$, was identified as neohesperidin as confirmed with an internal standard (Table 5.4.3). It produced a major fragment at m/z 301.07 which is the hesperetin aglycone after the loss of a neohesperidose (diglucoside) unit (-308 amu) (Xu et al., 2009) (Figure 5.4.14b).

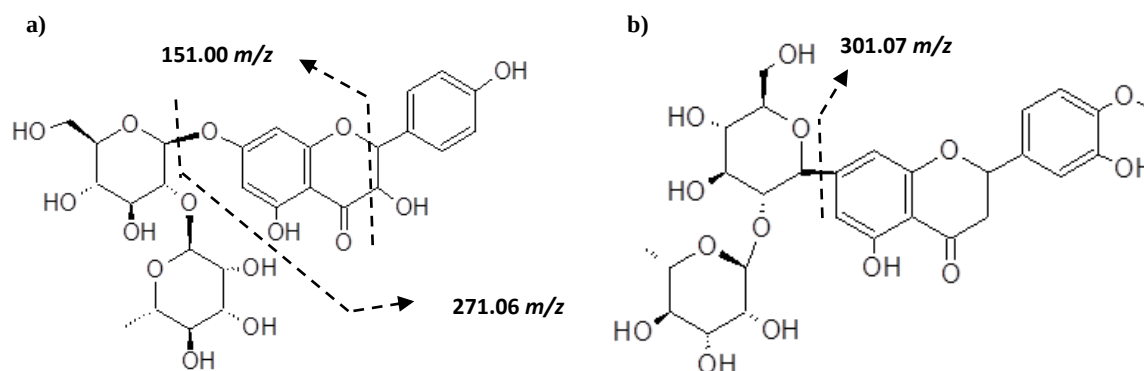


Figure 5.4.14 Proposed fragmentation of flavanones identified in pigmented flower extracts from the Geraniaceae plant family a) naringin, b) Neohesperidin

Flavone derivatives identified

The compound labelled as peak 14 with retention time of 18.47 min, molecular ion at m/z 431.10, molecular formula of $C_{21}H_{19}O_{10}$ and maximum UV-vis absorption wavelength of 333 nm was identified as apigenin-7-*O*-glucoside. It produced a main fragment at m/z 269.05 which is the apigenin aglycone after the loss of a glucose moiety (-162 amu) (Fabre et al., 2001) (Figure 5.4.15a).

The compound labelled peak 18 with retention time of 19.66 min, molecular ion at m/z 447.09, maximum absorption wavelength at 266 nm and molecular formula of $C_{21}H_{19}O_{11}$, was tentatively identified as luteolin-7-*O*-glucoside (Table 5.4.3). It produced a fragment at m/z 285.04 which is the luteolin aglycone after the loss of a glucose unit (-162 amu) (Figure 5.4.15b).

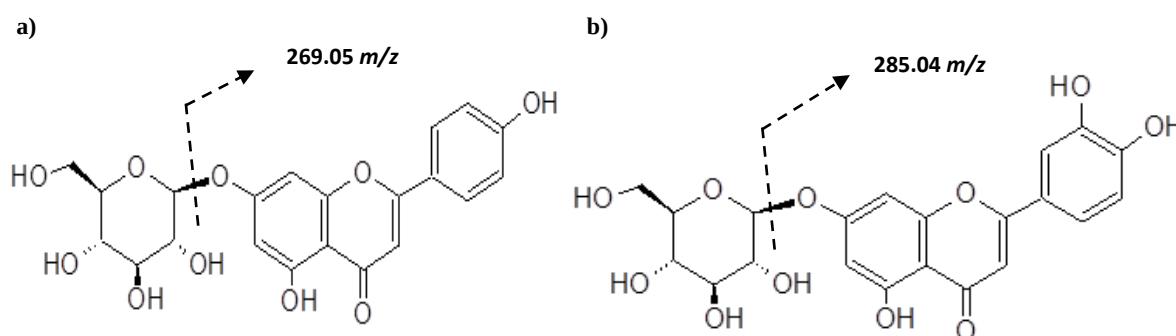


Figure 5.4.15 Proposed fragmentation of flavone derivatives identified in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families a) apigenin-7-*O*-glucoside, b) luteolin-7-*O*-glucoside

5.4.3 Quantification of flavonoids and phenolic acids in pigmented flower extracts from the Geraniaceae and Lamiaceae families

Tables 5.4.5 and 5.4.6 show the flavonoid and phenolic acids contents respectively of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families. For the Geraniaceae, the *P. grandiflorum* extract contained the highest total flavonoids (3361 µg/g) followed by *P. × hortorum* (2458 µg/g) and *P. zonale* hybrid (2182 µg/g) while *S. dolomitica* (607 µg/g) contained the highest total flavonoids for the Lamiaceae plant family followed by *P. zuluensis* (202 µg/g) and *S. aurea × dolomitica* (50 µg/g). *P. × hortorum* contained the highest total phenolic acid content (58 mg/g) in the Geraniaceae plant family followed closely by *P. zonale* hybrid (56 mg/g) and *P. grandiflorum* (21 mg/g) while for the Lamiaceae, *S. aurea × dolomitica* contained the highest total phenolic acid content (125 mg/g) followed by *S. dolomitica* (102 mg/g) and *P. zuluensis* (8 mg/g).

The main flavonoids in the Geraniaceae flower species were rutinoides of kaempferol and quercetin, quercetin-3-*O*-glucoside (isoquercetin) and luteolin-7-*O*-glucoside. The main flavonoids in the Lamiaceae flower species were naringin and neohesperidin. The main phenolic acids in the Geraniaceae species were hydrolyzable tannin types (β-glucogallin, penta and tetra-galloyl glucose) and *p*-coumaric acid and its derivative *p*-coumaroylglucose. The main phenolic acids in the Lamiaceae plant species were caffeic acid, rosmarinic acid and its derivatives such as sagerinic acid and salvianolic acid B.

Table 5.4.5 Flavonoid content of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

	Geraniaceae			Lamiaceae		
	<i>P. grandiflorum</i>	<i>P. × hortorum</i>	<i>P. zonale hybrid</i>	<i>S. aurea</i> <i>× dolomitica</i>	<i>S. dolomitica</i>	<i>P. zuluensis</i>
*Quercetin-3-O-rutinoside (Rutin)	693 ± 51	78 ± 0	47 ± 0	ND	ND	ND
*Quercetin-3-O-glucoside (Isoquercetin)	1043 ± 28	83 ± 8	6 ± 0		ND	ND
*Kaempferol-3-O-rutinoside	1468 ± 79	1533 ± 0	1188 ± 0	ND	ND	ND
*Kaempferol-3-O-glucosylgalactoside	ND	144 ± 0	49 ± 0			
*Robinin (kaempferol-3-O-robinoside-7-O-rhamnoside)	ND	ND	567 ± 0	ND	ND	ND
***Apigenin-7-O-glucoside	ND	ND	ND	ND	ND	191 ± 2
**Naringin	ND	ND	ND	50 ± 9	ND	ND
**Neohesperidin	ND	ND	ND	ND	607 ± 39	ND
***Luteolin-7-O-glucoside	158 ± 8	619 ± 95	326 ± 76	ND	ND	11 ± 0
Total flavonoids	3361	2458	2182	50	607	202

*Flavonols: µg QRE/g: Quercetin-rutinoside equivalents

**Flavanones: µg NE/g: Naringin equivalents

***Flavones: µg AE/g: Apigenin equivalents

ND: not detected

Table 5.4.6 Phenolic acid content of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

	Geraniaceae			Lamiaceae		
	<i>P. grandiflorum</i>	<i>P. × hortorum</i>	<i>P. zonale hybrid</i>	<i>S. aurea × dolomitica</i>	<i>S. dolomitica</i>	<i>P. zuluensis</i>
***β-Glucogallin (Galloyl-β-D-glucoside)	10 ± 0	38 ± 0	28 ± 0	0.12 ± 0	ND	0.05 ± 0
*Syringic acid	ND	ND	ND	2 ± 0	1 ± 1	ND
*Caffeoyltartaric acid	6 ± 1	ND	ND	ND	ND	ND
** <i>p</i> -coumaric acid	ND	14 ± 0	15 ± 0	ND	ND	0.2 ± 0
** <i>p</i> -coumaroylglucose	ND	5 ± 0	13 ± 0			ND
*Caffeic acid	ND	ND	ND	1 ± 0	2 ± 0	3 ± 0
***Tetra- <i>O</i> -galloyl-glucose	4 ± 0	ND	ND	0.1 ± 0	ND	ND
***Penta- <i>O</i> -galloyl-glucose	1 ± 0	ND	ND	ND	ND	ND
*Rosmarinic acid derivative	ND	ND	ND	22 ± 2	12 ± 1	ND
*Sagerinic acid	ND	ND	ND	30 ± 1	33 ± 3	0.1 ± 0
*Rosmarinic acid	ND	ND	ND	44 ± 2	48 ± 4	4 ± 0
*Salvianolic acid B	ND	ND	ND	26 ± 2	6 ± 0	ND
Total phenolic acids	21	58	56	125	102	8

*mg CAE/g: Caffeic acid equivalents

*mg CouE/g: coumaric acid equivalents

***mg GAE/g: Gallic acid equivalents

ND: not detected

5.4.3. Degradation kinetics

5.4.3.1. Thermal and oxidative (hydrogen peroxide) stability of pigmented flower extracts of some plant species from the Geraniaceae and Lamiaceae families

For food applications, thermal and oxidative stability of pigments are important parameters to consider. The thermal and oxidative (hydrogen peroxide concentration) degradation kinetics parameters of the pigmented flower extracts from the Geraniaceae and Lamiaceae are shown in Tables 5.4.3.1 and 5.4.3.2 respectively. The thermal and oxidative degradation of the pigments showed first order reaction kinetics. Figures 5.4.3.1 and 5.4.3.2 show that there was a decrease in absorbance of the pigment extracts with increasing time. More importantly, the slope of the straight lines generally increased with increasing temperature and H₂O₂ concentration. Overall, there was a general trend in which all the pigmented flower extracts showed an increase in degradation rate constant (k) accompanied with a decrease in half-life (t_{1/2}) with increasing temperature (Table 5.4.3.1) and increasing H₂O₂ concentration (Table 5.4.3.2). This is an indication that the pigments became less stable with increasing temperature and increasing H₂O₂ concentration.

Similar trends have been reported from thermal degradation kinetics studies of red Roselle (*Hibiscus sabdariffa*) anthocyanin extracts (Mourtzinou *et al.*, 2008; Maciel *et al.*, 2018) and corn poppy (*Papaver rhoeas* L.), red tulip (*Tulipa* sp.), rose (*Rosa* sp) anthocyanin extracts (Bayram *et al.*, 2015) and various types of tulip (*Tulipa* spp.) (Sagdic *et al.*, 2013) and from oxidative degradation kinetics studies of red radish extracts (Matsufuji *et al.*, 2007). These studies also reported decreases in thermal and oxidative degradation rate constants with increase in temperature and concentration of oxidising agent.

High temperatures may promote breakdown of anthocyanins via hydrolysis of the sugar residue of anthocyanins followed by isomerization and opening of the ring system resulting in the formation of chalcones which could ultimately be cleaved into various degradation products (Patras *et al.*, 2010). Oxidising agents such as hydrogen peroxide and its radicals could degrade anthocyanins by adding to the C ring of anthocyanins resulting in opening of the structure and release of usually phenolic acids as by-products (Satake & Yanase, 2018). These mechanisms could account for the observed decreased stability of the flower pigments with increasing temperature and increasing H₂O₂ concentration.

In this study, the pigmented flower extracts from the plant species from the Lamiaceae had lower thermal degradation rate constant (Mean $k = 1.2 \times 10^3 \text{ min}^{-1}$) and longer half-life (Mean $t_{1/2} = 6.22 \text{ min}$) than the pigmented extracts from the Geraniaceae (Mean $k = 3.86 \times 10^3 \text{ min}^{-1}$; Mean $t_{1/2} = 5.26 \text{ min}$) (Table 5.4.3.1). Thermal degradation rate constants have also been reported for several tulip flower species with mean k range of -3.23 - $4.38 \times 10^3 \text{ min}^{-1}$ at temperatures of 70, 80 and 90°C (Sagdic *et al.*, 2013), poppy flower extract (mean $k = -0.63 \times 10^3 \text{ min}^{-1}$), rose flower extract (mean $k = -4.29 \times 10^3 \text{ min}^{-1}$) and roselle extract (mean $k = -5.21 \times 10^3 \text{ min}^{-1}$) at the same temperature ranges (Bayram *et al.*, 2015).

The oxidative stability kinetics (Table 5.4.3.2) showed a similar trend with the pigmented flower extracts from the Lamiaceae having lower oxidative degradation rate constant (Mean $k = 1.08 \times 10^3 \text{ min}^{-1}$) and longer half-life (Mean $t_{1/2} = 6.24 \text{ min}$) than the pigmented extracts from the Geraniaceae family (Mean $k = 2.47 \times 10^3 \text{ min}^{-1}$; Mean $t_{1/2} = 5.62 \text{ min}$). Özkan *et al.* (2002) reported oxidative degradation rate constants for strawberry anthocyanin extract (mean $k = -2.37$ to $-19.46 \times 10^3 \text{ min}^{-1}$), pomegranate anthocyanin extract (mean $k = -1.80 \times 10^3 \text{ min}^{-1}$ to $-21.19 \times 10^3 \text{ min}^{-1}$) and sour cherry anthocyanin extract (mean $k = -1.54 \times 10^3 \text{ min}^{-1}$ to $-15.25 \times 10^3 \text{ min}^{-1}$) at hydrogen peroxide concentrations of 9.31, 13.96, 23.27 and 27.92 mM and temperatures of 10, 20, and 30°C.

The pigmented flower extracts from plant species from the Lamiaceae studied in the current research were more thermally and oxidatively stable than the pigmented extracts from the Geraniaceae. This might be related to the anthocyanin type (acetyl or aromatic acylated) identified in the pigments and/or the presence of other phenolic compounds such as flavonoids or phenolic acids. The pigmented flower extracts from the Lamiaceae contained *p*-coumaroyl- and malonyl-acylated anthocyanins (aromatic acylated) whereas the pigmented flower extracts from the Geraniaceae contained acetyl-acylated anthocyanins. The aromatic residues from the acylated anthocyanins identified in the pigmented flower extracts from the Lamiaceae could protect the flavylium nucleus from degradation through intra-molecular self-association interactions as described by Goto and Kondo (1991). This could partly explain the relative stability of the pigmented flower extracts from the Lamiaceae.

The pigmented flower extracts of the Lamiaceae in this study contained high amounts of rosmarinic acid (96 mg CAE/g) and its derivatives sagerinic acid (64 mg CAE/g) and salvianolic acid B (32 mg CAE/g) (Table 5.4.6) while these were not detected in pigmented flower extracts from the Geraniaceae. The results of this study suggest that the self-association

interactions of aromatic acylated anthocyanins (Goto & Kondo, 1991) could be accompanied by the strong intermolecular interactions between rosmarinic acid and its derivatives thereby preventing nucleophilic attack by water and hydrogen peroxide (H₂O₂).

Some studies have reported on the role of rosmarinic acid in the stabilisation of anthocyanins. Kean and Reilly (2011) patented a method for stabilisation of red radish anthocyanin extract with rosmarinic acid during storage for use in food beverages and cosmetics where addition of rosmarinic acid resulted in a 36% retention in colour outperforming all other stabilising agents tested. Zhao *et al.* (2021) also reported that rosmarinic acid in an anthocyanin extract consisting of mainly cyanidin-3-*O*- β -glucoside, caused a bathochromic shift in maximum absorption wavelength (λ_{max}) and resulted in an improved stability after 7 days of storage.

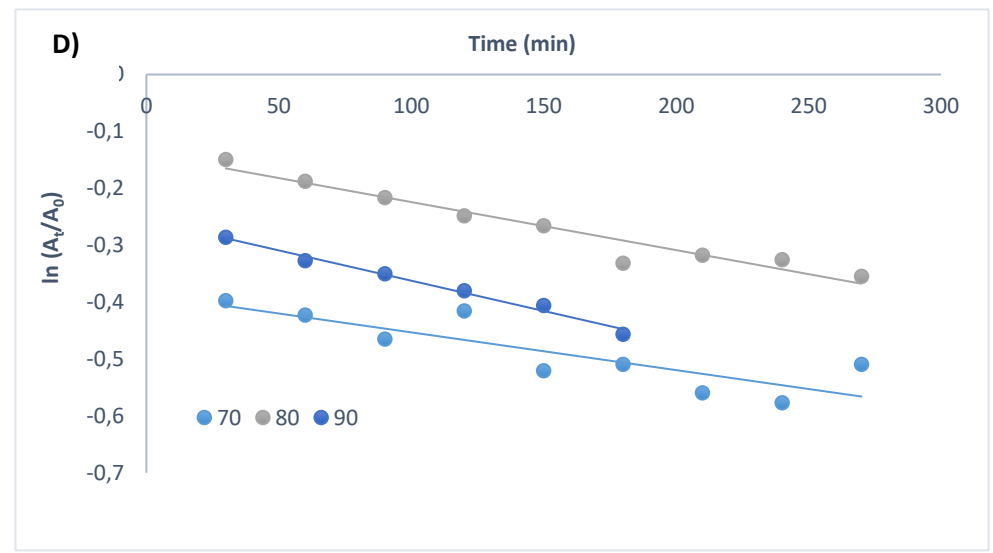
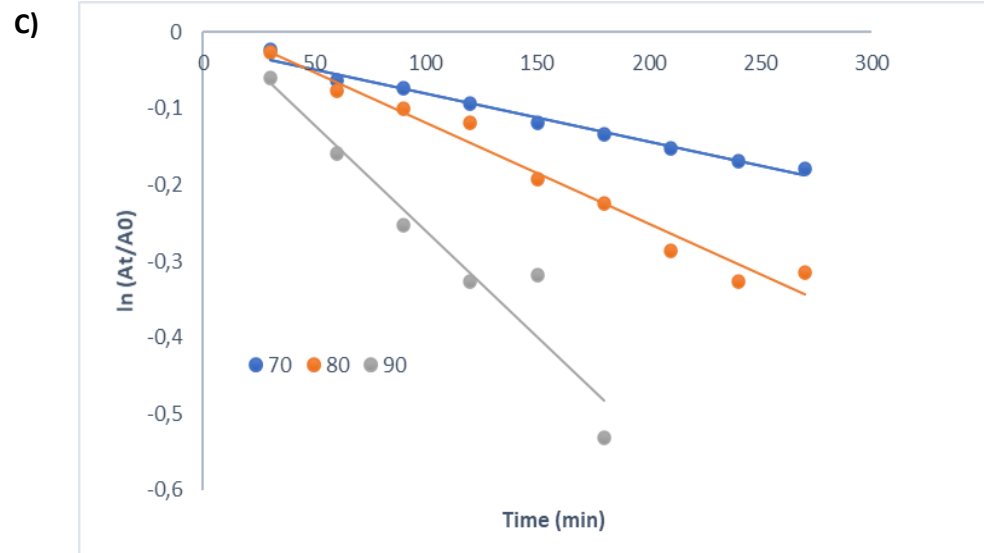
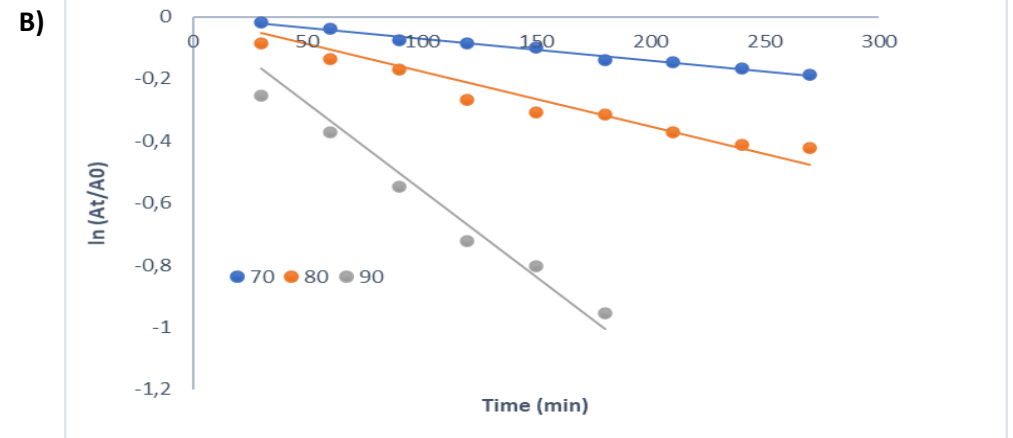
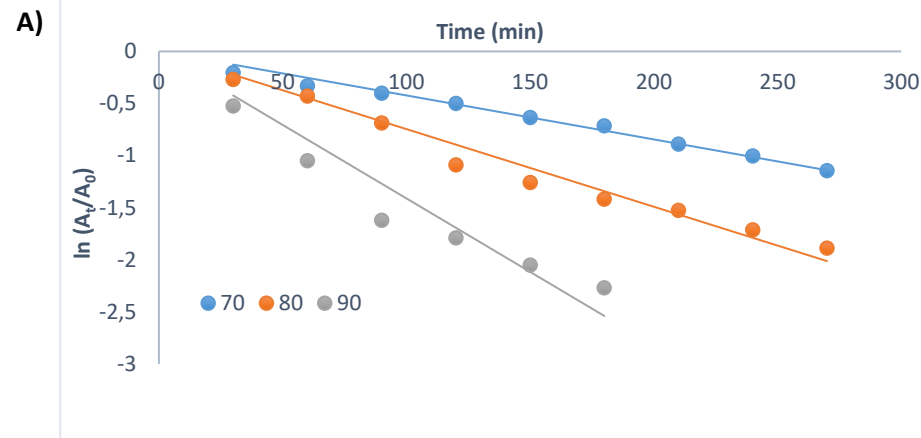
From the results obtained in this study, it is clear that for use of these pigmented extracts in food applications, the thermal and oxidizing conditions must be considered especially for the pigmented flower extracts from the Geraniaceae studied in this research.

Table 5.4.3.1 Thermal degradation kinetics parameters [degradation rate constant (k) and half-life ($t_{1/2}$)] of pigmented flower extracts from some plant species of the Geraniaceae and Lamiaceae

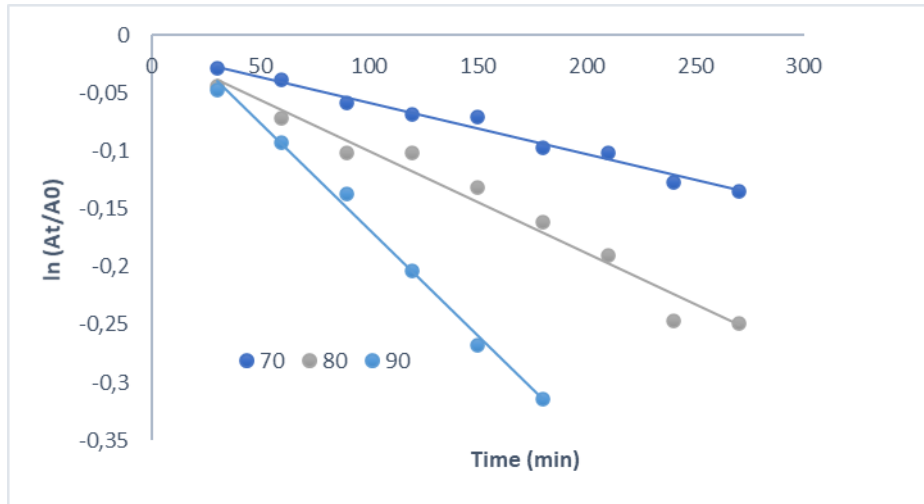
Sample	Temperature (°C)	-k x 10 ³ (min ⁻¹)	$t_{1/2}$ (min)
Geraniaceae			
<i>Pelargonium.</i>	70	3.90 ^o ± 0.30	4.86 ^d ± 0.08
<i>grandiflorum</i>	80	7.00 ^q ± 0.10	4.27 ^b ± 0.01
(purple)	90	11.15 ^t ± 0.25	3.80 ^a ± 0.02
<i>Pelargonium</i> ×	70	0.85 ^{ec} ± 0.20	6.39 ^{no} ± 0.18
<i>hortorum</i>	80	1.50 ^{ji} ± 0.10	5.81 ^{ij} ± 0.07
(scarlet)	90	5.50 ^{pn} ± 0.30	4.51 ^{ce} ± 0.05
<i>Pelargonium</i>	70	0.70 ^{ceg} ± 0.00	6.57 ^{onlq} ± 0.00
<i>zonale</i> hybrid	80	1.30 ^{ijk} ± 0.10	5.96 ^{jihp} ± 0.08
(pink)	90	2.80 ^{npm} ± 0.00	5.18 ^{ecfk} ± 0.00
		Mean = 3.86	Mean = 5.26
Lamiaceae			
<i>Salvia aurea</i> ×	70	0.45 ^{ab} ± 0.05	7.02 ^{rl} ± 0.11
<i>dolomitica</i> (dark	80	0.95 ^{fd} ± 0.15	6.28 ^{mh} ± 0.16
purple)	90	1.75 ^{lh} ± 0.65	5.73 ^{gf} ± 0.39
<i>Salvia</i>	70	0.60 ^{bg} ± 0.20	6.78 ^{qr} ± 0.35
<i>dolomitica</i> (light	80	0.75 ^{dk} ± 0.45	6.73 ^{pm} ± 0.69
purple)	90	1.10 ^{hm} ± 0.30	6.16 ^{kg} ± 0.28
<i>Plectranthus</i>	70	1.05 ^{gec} ± 0.45	6.27 ^{lo} ± 0.46
<i>zuluensis</i> (Dark	80	1.65 ^{kji} ± 0.55	5.77 ^{hj} ± 0.35
purple)	90	2.70 ^{mnp} ± 0.10	5.22 ^{fe} ± 0.04
		Mean = 1.22	Mean = 6.22

Values as mean ± SD

Different letters within the same column indicate significant difference ($p \leq 0.05$)



E)



F)

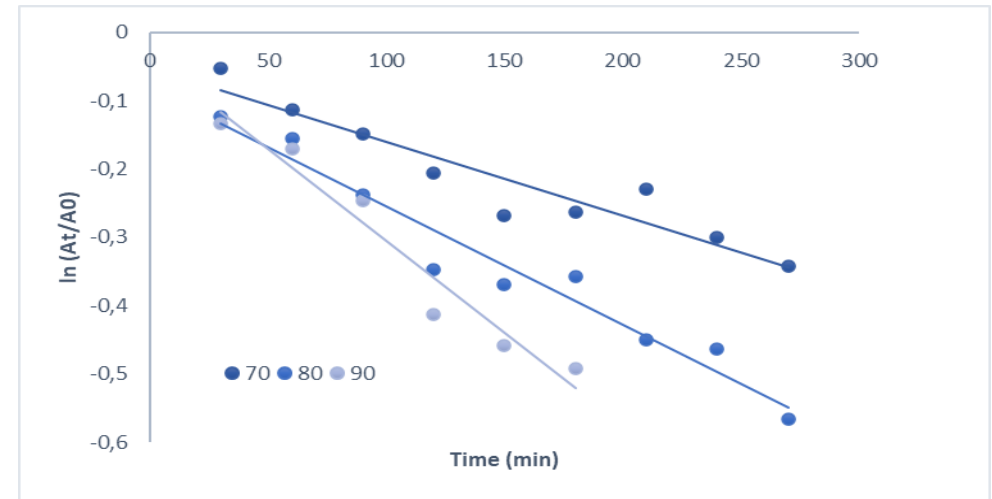


Figure 5.4.3.1. Effect of temperature on degradation of anthocyanins degradation in pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

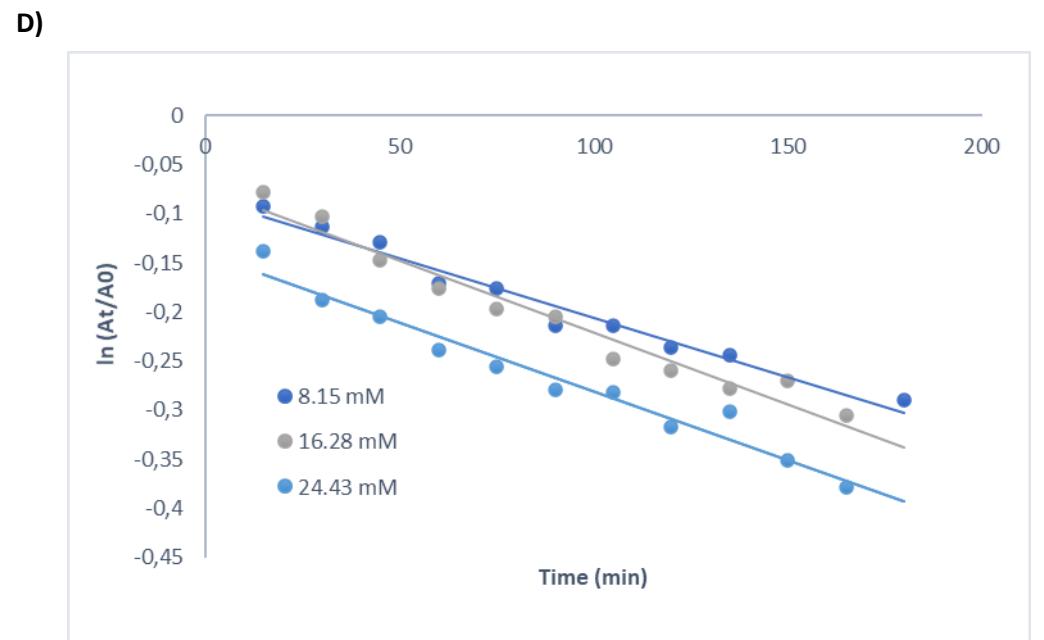
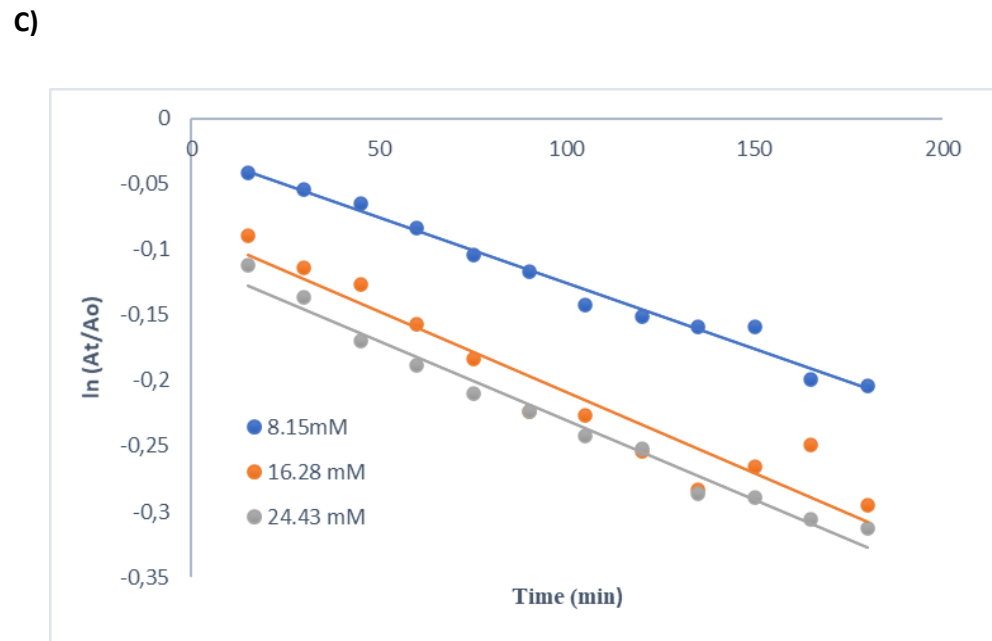
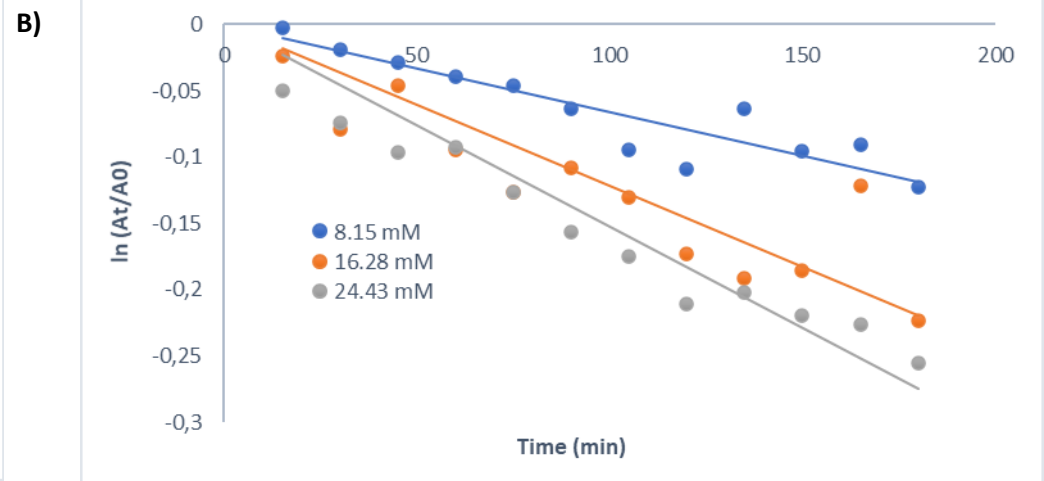
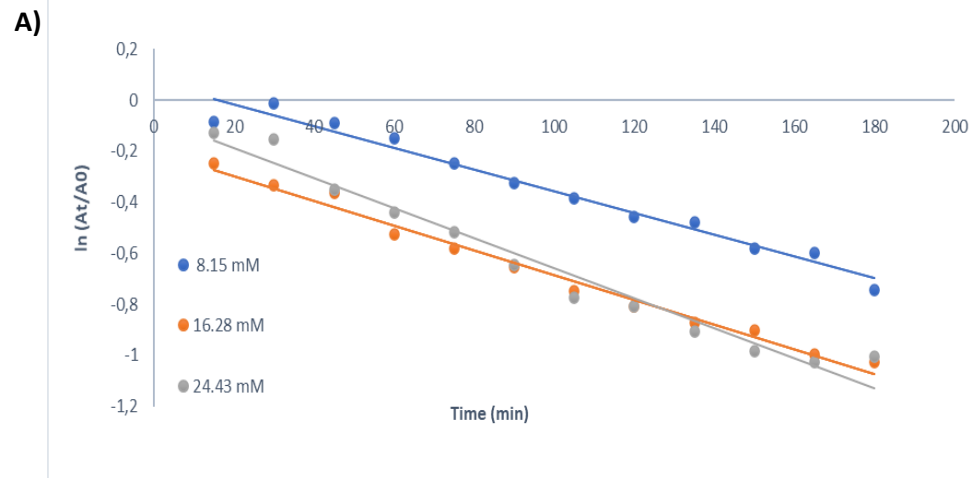
A: *P. grandiflorum* B: *P. × hortorum*, C: *P. zonale* hybrid, D: *S. aurea* × *dolomitica*, E: *S. dolomitica*, F: *P. zuluensis*, At: Absorbance of sample at 520 nm at t min, A0: Absorbance of sample at 520 nm at 0 min.

Table 5.4.3.2 Oxidative (H₂O₂) degradation kinetics parameters [degradation rate constant (k) and half-life (t_{1/2})] of pigmented flower extracts from some plant species of the Geraniaceae and Lamiaceae

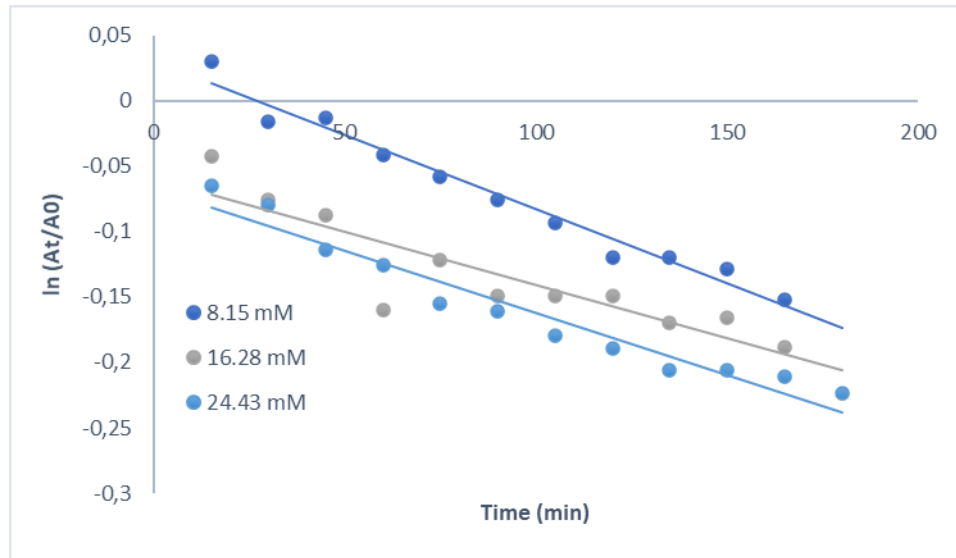
Sample	H ₂ O ₂ (mM)	-k x 10 ³ (min ⁻¹)	t _{1/2} (min)
Geraniaceae			
<i>Pelargonium</i> .	8.15	4.70 ^c ± 0.1	4.67 ^b ± 0.02
<i>grandiflorum</i>	16.28	4.80 ^d ± 0.00	4.65 ^a ± 0.00
(purple)	24.43	5.95 ^d ± 0.35	4.43 ^a ± 0.06
<i>Pelargonium</i> ×	8.15	0.65 ^a ± 0.05	6.58 ^d ± 0.14
<i>hortorum</i>	16.28	1.20 ^b ± 0.10	6.04 ^c ± 0.08
(scarlet)	24.43	1.50 ^b ± 0.20	5.82 ^c ± 0.13
<i>Pelargonium</i>	8.15	1.00 ^a ± 0.30	6.26 ^d ± 0.31
<i>zonale</i> hybrid	16.28	1.25 ^b ± 0.05	5.99 ^c ± 0.04
(pink)	24.43	1.15 ^b ± 0.25	6.10 ^c ± 0.22
		Mean = 2.47	Mean = 5.62
Lamiaceae			
<i>Salvia aurea</i> ×	8.15	1.10 ^a ± 1.00	6.24 ^d ± 0.49
<i>dolomitica</i> (dark	16.28	1.40 ^b ± 0.00	5.88 ^c ± 0.00
purple)	24.43	1.25 ^b ± 0.25	6.01 ^c ± 0.20
<i>Salvia</i>	8.15	0.75 ^a ± ± 0.05	6.50 ^d ± 0.07
<i>dolomitica</i> (light	16.28	0.75 ^b ± 0.15	6.52 ^c ± 0.02
purple)	24.43	0.95 ^b ± 0.05	6.27 ^c ± 0.05
<i>Plectranthus</i>	8.15	0.40 ^a ± 0.00	7.13 ^d ± 0.00
<i>zuluensis</i>	16.28	1.90 ^b ± 0.10	5.57 ^c ± 0.05
(purple)	24.43	1.25 ^b ± 0.25	6.01 ^c ± 0.20
		Mean = 1.08	Mean = 6.24

Values as mean ± SD

Different letters within the same column are significantly different (p ≤ 0.05)



E)



F)

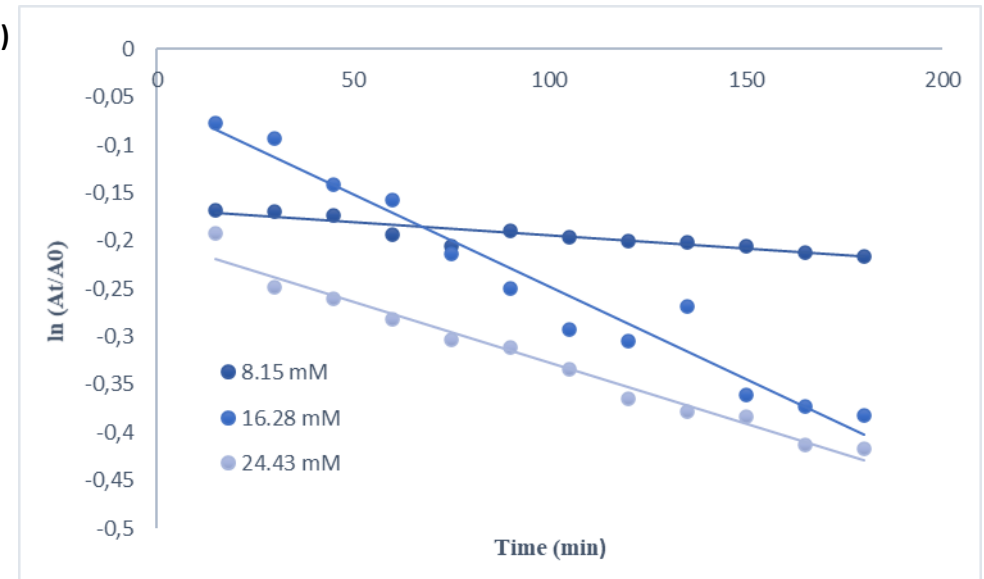


Figure 5.4.3.2 Effect of hydrogen peroxide concentration on anthocyanin degradation of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

A: *P. grandiflorum* B: *P. × hortorum*, C: *P. zonale* hybrid, D: *S. aurea* × *dolomitica*, E: *S. dolomitica*, F: *P. zuluensis*, At: Absorbance of sample at 520 nm at t min, A0: Absorbance of sample at 520 nm at 0 min.

5.5 Conclusion

Pigmented extracts from flowers of the Geraniaceae and Lamiaceae in this study contain mainly acetyl acylated and aromatic-malonyl acylated anthocyanins respectively. The pigmented extracts from the Lamiaceae contain high levels of rosmarinic acid and its derivatives, sagerinic acid and salvianolic acid which are absent in extracts from the Geraniaceae. The pigmented extracts from the Lamiaceae have higher thermal and oxidative stability as shown by lower degradation constants (k) and higher half-lives ($t_{1/2}$) compared to pigments from the Geraniaceae. The observed stability of the pigmented extracts from the Geraniaceae and Lamiaceae at a wide range of temperatures and oxidative environments suggests that they have potential as natural food colourants.

Chapter 6: Health-promoting properties of pigmented flower extracts of species from the Geraniaceae and Lamiaceae plant families

Abstract

This study investigated the potential health-promoting properties of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families found in South Africa. Pigmented flower extracts from the Geraniaceae (*Pelargonium grandiflorum* Willd. and *Pelargonium × hortorum* L.H. Bailey, *Pelargonium zonale* hybrid) had higher total phenolic content (TPC) and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity than extracts from the Lamiaceae (*Salvia aurea* × *dolomitica*, *Salvia dolomitica* Codd and *Plectranthus zuluensis* T. Cooke). Generally, all pigmented flower extracts, with the exception of *P. zonale* hybrid (Geraniaceae) showed strong protective effects in human colon adenocarcinoma (Caco-2) cells against radical damage. The same trend was observed with nitric oxide (NO) production in murine macrophages (RAW 264.7) cells. An increase in pigmented extract concentration resulted in increased NO production which could have application in anti-hypertension studies. Generally, all pigmented flower extracts showed the ability to reduce lipid accumulation in differentiated murine adipocytes (3T3-L1) cells with reduction in cell density after 14 days. Overall, although the *P. zonale* hybrid pigmented extracts showed low radical scavenging ability in Caco-2 and RAW 264.7 cells, these extracts were able to induce lipid reduction in murine adipocyte cells (which demonstrates their potential anti-obesity effects) while retaining their original cell density. Pigmented flower extracts showed anti-glycation activity comparable to that of aminoguanidine, especially extracts of *P. grandiflorum* and *P. × hortorum*, which suggests that these particular extracts could replace aminoguanidine as anti-diabetic medication. The pigmented flower extracts show a wide range of health-promoting properties which represents an additional benefit to their potential use as natural food colourants.

6.1 Introduction

Several research studies have shown possible negative health effects associated with the use of synthetic azo-based colourants in food such as hyperactivity in children and cancer (McCann *et al.* 2007, McCann *et al.* 1975). Such studies have driven consumer demand for more natural food ingredients and have prompted researchers to investigate potential sources of natural colourants. South Africa has a wide variety of plant species from the Geraniaceae (geranium) and Lamiaceae (mint) families. The plants from these families produce a wide range of brightly coloured flowers (due to the presence of anthocyanins), and therefore could be potential sources of natural colourants for food applications. Anthocyanins themselves are bioactive phenolic compounds and this presents the possibility of having anthocyanin-rich pigments from these plants applied as natural food colourants with added health-promoting properties.

Some studies have been conducted on various bioactive properties of different plants in the Geraniaceae and Lamiaceae families. Lis Balchin *et al* (1997) studied medicinal, anti-bacterial and anti-fungal properties of essential oils from Geranium plant species. The pharmacological properties of these plants were mainly attributed to tannins and the chemical components of the essential oils due to its use in traditional medicine (Scott *et al.*, 2004). Flavonoids from *Pelargonium* spp. are proposed to be responsible for several associated health benefits such as anti-carcinogenicity, hepatoprotection and modulation of immune and inflammatory function (Middleton, 1993). Several research efforts have aimed at evaluating the antioxidant and cancer cell cytotoxicity of *Pelargonium graveolens* (Geraniaceae) (Lalli, Van Zyl, Van Vuuren & Viljoen, 2008) as well as *Thymus algeriensis*, *Ocimum basilicum* and *Salvia* spp. (*Salvia stenophylla*, *Salvia repens* and *Salvia runcinata*) (Lamiaceae) (Rezzoug, Bakchiche, Gherib, Roberta, FlaminiGuido, Kilinçarslan, Mammadov and Bardaweel, 2019) essential oil and aqueous leaf extracts (Cavar and Maksimovic, 2012, Paltiyani, 2016). However, most of the South African species of the Geraniaceae and Lamiaceae plant families remain largely unexplored. The limited research on South African plant species have only focused on the medicinal properties such as antioxidant, anti-inflammatory activity of the essential oil extracts of the leaves (Elisha, Dzoyem, McGaw, Botha & Eloff, 2016; Ondua, Njoya, Abdalla & McGaw, 2019; Adebayo, Ondua, Shai & Lebelo, 2019).

Thus, the objective of this study was to evaluate the potential health-promoting properties of pigment rich extracts from flower species of Geraniaceae and Lamiaceae plant families in terms of their *in vitro* cellular antioxidant, anti-inflammatory anti-adipogenic and non-cellular anti-

diabetic activities with the aim of expanding the knowledge of the health-benefits of pigmented flower species for use as sources of natural food colourants.

6.2 Materials and methods

6.2.1 Samples

Flower samples of plants from the Geraniaceae family: *Pelargonium grandiflorum* Willd. (large-flowered pelargonium) and *P. × hortorum* L.H. Bailey were procured from Plant Land, Centurion, Gauteng, South Africa. *Pelargonium zonale* (L.) L'Hér. hybrids were procured in Lynwood Ridge, Pretoria, South Africa.

Flower samples of plants from the Lamiaceae family: *Salvia aurea* × *dolomitica* Bae's Blue and *Salvia dolomitica* Codd were procured from the botanical gardens at the Future Africa campus of the University of Pretoria, South Africa. *Plectranthus zuluensis* T. Cooke was procured from the Hatfield Campus, University of Pretoria, South Africa.\

Flower samples were air-dried at 27°C in an air convection oven for 24-48 hours. After drying, the dried flower petals were ground into a fine powder using a Waring hand blender. The flower powder was stored in airtight resealable bags (Ziplock) at 4°C.

6.2.2 Chemicals

Insulin, 3-isobutyl-1-methylxanthine (IBMX), rosiglitazone and dexamethasone, differentiation medium, fetal bovine serum (FBS) and 2, 2 Diphenyl-1-picrylhydrazyl (DPPH) were all purchased from Sigma Aldrich. Formic acid and Folin-Ciocalteu's phenol reagent were purchased from Merck.

6.2.3 Preparation of pigmented extracts

A weight of 500 mg of flower petal powder was added to 10 ml of 1% formic acid: water solution and extracted via magnetic stirring for 24 h. After extraction, the supernatants were filtered through a Buchner funnel under vacuum using Munktell filter paper (8-12 µm) and washed with 10 ml solvent. The extracts were stored in plastic tubes wrapped with aluminum foil at -20°C until use.

6.2.4 Determination of total phenolic content (TPC) using the Folin-Ciocalteu assay

The TPC was determined using the Folin-Ciocalteu assay modified for the use of a 96-well microplate as described by Ainsworth and Gillespie (2007). One hundred microliters of pigmented flower extracts and catechin standard solutions of different concentrations (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35 mg/ml) were prepared in distilled water and placed in Eppendorf tubes and 200 µl 10% v/v Folin-Ciocalteu reagent was added to each tube. A volume of 800 µl 250 mM Na₂CO₃ was added to each tube within 8 min after the addition of the Folin-Ciocalteu reagent. The solutions were vortexed and incubated for 2 h at room temperature. After incubation, 200 µl of the reaction mixture was placed into each well of a 96 well microplate and the absorbance read at 750 nm using a microplate reader. The total phenolic content was expressed as milligram catechin equivalents (CE) per g sample extract.

6.2.5 Determination of anti-glycation activity using the Advanced Glycation End Products (AGE) assay

Anti-glycation activity was done according to Siddiqui, Rasheed, Saquib, Al-Khedhairi, Al-Said, Musarrat and Choudhary (2016) with modification. A 100 µl aliquot of sample extract was added to 100 µl 40 mg/ml Bovine serum albumin (BSA), 100 µl methylglyoxal (MGO) and 100 µl phosphate buffer saline (PBS) (pH 7.4). A mixture of 100 µl pigmented flower extract, 200 µl PBS and 100 µl BSA was used as a sample control while 100 µl BSA with 100 µl MGO added to 200 µl PBS was used as a positive control. The negative control contained BSA and PBS. All mixtures were incubated at 37°C for 1 week. After incubation, samples were plated in fluorescence plates and read at 330 nm (excitation) and 420 nm (emission) fluorescence wavelength. Aminoguanidine bicarbonate was used as an AGE inhibitor standard at concentrations of 0.2-1.0 mM. The results were expressed as % AGE's formed.

6.2.6 Determination of cytotoxicity using the Crystal violet assay

Cells (Caco 2 and 3T3-L1) were detached from T75 storage flasks by addition of 2 ml Tryple express, and further incubation for 3-5 min. The enzyme was inactivated with 10 ml of growth media. The RAW 264.7 cells were detached from the T75 storage flask with a gentle scraping action and resuspended in 10 ml growth media. All cells were centrifuged at $3734 \times g$ for 1 minute to form a pellet of cells in a 15 ml sterile centrifuge tube. The supernatant was discarded and the pellet was redissolved in 5 ml of growth media for sub-culturing. For cell counting, 10 μ l of the cells were added to 90 μ l trypan blue, the staining dye (0.1% in 0.1 M PBS). Of this mixture, 10 μ l was placed on a haemocytometer and cell counting was done under a light microscope. For this study, 96-well micro plates were used and each well was filled with 90 μ l of cell suspended in growth media at a cell density of 10000 cells per 90 μ l. The cells were allowed to attach overnight prior to treatment with the phenolic extracts.

In each of two 96-well microplates, a volume of 90 μ l of Caco 2 and 3T3-L1 cell suspensions (cell density of 10×10^4 cells) and RAW 264.7 cell suspension (cell density of 10×10^6 cells) were treated with 10 μ l of flower extracts and incubated for 24, 48 and 72 hours respectively at 37°C. After incubation, the three types of cells were treated with 11 μ l of a 20% formaldehyde solution for fixation (final concentration 2%) and incubated for 30 min at 37°C. After incubation, the solutions were removed, dried and stained with 100 μ l of crystal violet (CV) staining solution and incubated at room temperature for 30 min. After incubation, the crystal violet solution was removed from the 96 well microplate by rinsing with water and 50 μ l of acetic acid was added and the microplate was read at 630 nm using a microplate reader. Cell viability was determined against a control (untreated samples) using the equation below:

$$\% \text{ Cell viability} = \frac{\text{Absorbance at 630 nm of cells exposed to sample}}{\text{Absorbance at 630 nm of control cells}} \times 100$$

6.2.7 Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity assay was done as described by Cheng, Moore & Yu, (2006) with some modifications for use of a 96-microplate. A 0.609 mM DPPH radical stock solution was prepared in distilled water. From the stock solution a 0.102 mM working solution was prepared. A volume of 10 μ l of pigmented flower extract and Trolox standards of different concentrations (0, 50, 100, 200, 300, 400 and 500 μ M) was reacted with 190 μ l of DPPH working solution in the 96-well microplate. The mixture was incubated in the dark at room temperature for 30 min and thereafter the absorbance was read at 570 nm using a (Omega, FLUOstar, BMG). microplate reader. The results was expressed as mmol Trolox Equivalents/g sample dry weight basis.

6.2.8 Determination of nitric oxide (NO) scavenging activity using the sodium nitroprusside assay

A 50 μ l volume of 5 mM sodium nitroprusside (in distilled water) was added to 50 μ l of pigmented flower extract and incubated for 1 h at room temperature, in the dark. The amount of NO was measured by adding 100 μ l Griess reagent and incubating for 10 min at room temperature followed by determination of absorbance at 570 nm. Results were expressed as NO_2^- equivalents. NaNO_2 (0 – 0.1 mM) was used as a standard.

6.2.9 Determination of cellular antioxidant activity in Caco-2 cells using the Dichlorofluorescein diacetate (DCFH-DA) assay

Caco-2 cells were plated in a 96-well plate at a cellular concentration of 10×10^4 cells after which they were incubated for 24 h at 37°C to allow cells to attach to the surface of the plate. Following incubation, 50 μ l 75 μ M DCFH-DA were added to each well, yielding a final concentration of 25 μ M. These were left to incubate for 45 min at 37°C. Following incubation, the DCFH-DA solution was discarded from the microplate and cells washed once with PBS. Fifty microlitres of pigmented flower extracts were added in triplicate to the wells. This was followed by addition of 50 μ l 8 mM AAPH. A positive control (cells exposed to DCFH-DA and AAPH only), and a negative control (cells exposed to DCFH-DA and PBS only) were included. The change in fluorescence was measured every 2 min over a period of 60 min at an excitation wavelength of 485 nm and emission wavelength of 530 nm. The gradient of the

change in fluorescence was calculated and the data obtained expressed as % oxidative damage relative to the 100% oxidative damage registered for the positive control (cells exposed to AAPH alone).

6.2.10 Lipopolysaccharide (LPS)-induced nitric oxide scavenging activity using RAW 264.7 cells

The method was conducted as done by Kim, Murakamib, Nakamura & Ohigashia (1998) with modifications. RAW 264.7 cells were made up to a concentration of $1.25 \times 10^6/\text{ml}$ in differentiation medium [Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (DMEM/FBS)]. A volume of 10 μl pigmented flower extract + 10 μl lipopolysaccharide (LPS) + 80 μl RAW 264.7 cells were all mixed in each well of a 96-well plate. This resulted in final concentrations of $1.0 \times 10^6/\text{ml}$ of cells and 0.1 $\mu\text{g}/\text{ml}$ LPS in each well. This mixture was then incubated at 37°C at 5% CO_2 for 24 h. After incubation, 50 μl of the liquid portion was assayed using 50 μl Griess reagent [mixture of 1% sulphanilamide and 0.1% N-(1-naphthyl-ethylenediamine)]. NaNO_2 (0 – 0.1 mM) was used as a standard. The mixture was read at 570 nm. Results were expressed as % NO produced relative to the positive control (the treatment consisting of RAW 264.7 cells with LPS only) which was indicated as having 100% NO produced.

6.2.11 Cellular anti-adipogenic activity using 3T3-L1 cells

Differentiation of 3T3-L1 cells – reduction of formed lipid droplets

Pre-adipocyte cells were seeded at a concentration of 0.1×10^4 per 100 μL in a 96-well plate and grown to 100% confluence for three days. On day 4, cells were induced to differentiate using 100 μl differentiation medium 1 (DMEM/FBS supplemented with final concentrations of 10 $\mu\text{g}/\text{ml}$ insulin, 0.25 mM IBMX, 1 μM Dexamethasone and 2 μM Rosiglitazone). Differentiation medium 1 was replenished every 3 days for 6 days. On day 10, differentiation medium 1 was replaced with differentiation medium 2 (DMEM with only 10 $\mu\text{g}/\text{ml}$ insulin) for 3 days. On day 14, differentiation medium 2 was replaced with DMEM (90 μL) only. Cells were exposed to 10 μl pigmented flower extracts and further incubated for 48 h at 37°C, 5% CO_2 . After incubation, cells were assessed for lipid droplet reduction using Oil Red O staining.

Differentiation of 3T3-L1 cells – prevention of lipid droplet accumulation

Differentiation of 3T3-L1 cells was undertaken as above. To assess prevention of lipid formation, with each addition of differentiation medium (90 µl DM1 or 90 µl DM2), 10 µl of pigmented flower extract was added for the duration of the differentiation process. Once the differentiation period was concluded, cells were assessed for lipid droplet prevention using Oil Red O staining.

Oil red O staining

The procedure was carried out according to the method by Theerakittayakorn and Bunprasert (2011) with modifications. Exposed cells were fixed with 2% (v/v) formaldehyde for 30 min at 37°C, 5% CO₂. After fixing, differentiation medium and formaldehyde were discarded and the plate was dried. Once dry, the cells were stained with freshly diluted Oil-Red O solution (three parts of 0.5% Oil-Red O (w/v) in 60% isopropyl alcohol and two parts water) for 30 min. After staining, the Oil- Red O staining solution was removed, and cells rinsed with water and dried. Images of the stained lipid droplets were collected using an Olympus microscope (Olympus, Tokyo, Japan).

Nile Red staining

The procedure was carried out according to the method by Theerakittayakorn and Bunprasert (2011) with modifications. Exposed cells were fixed with 2% (v/v) formaldehyde for 30 min at 37°C, 5% CO₂. After fixing, differentiation medium and formaldehyde were discarded, the plate was dried and cells washed with 300 µl PBS. After washing, the PBS was removed and 100 µl of PBS was added and a background reading done at 485 nm excitation and 590 nm emission wavelengths (fluorescence). A volume of 1 µl 100 µM Nile Red staining solution was added (final concentration of 1 µM) and incubated in the dark for 3 h. After incubation, the sample plates were washed once with 300 µl PBS. A volume of 100 µl PBS was added to the plates and read at 485 nm excitation and 590 nm emission wavelengths. Data was expressed as % lipid droplets formed.

6.3 Statistical analysis

Experiments were conducted triplicates. One-way analysis of variance (ANOVA) was conducted as required using SPSS software. Significant differences between means were determined at $p \leq 0.05$. Significant means were separated using the least significant difference

(LSD), Tukey and Scheffe tests at $p \leq 0.05$. Dependent variables were the measured values of total phenolic content, DPPH radical scavenging antioxidant activity, ORAC values, chemical nitric oxide scavenging activity and cellular % NO produced, % lipid droplet accumulation and % Advanced Glycation End products (AGE) formation. Independent variables were the concentrations of sample extracts used in all bioassays.

6.4 Results and discussion

6.4.1 Total phenolic content (TPC) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging antioxidant activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

The total phenolic content (TPC) and DPPH radical scavenging activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families are shown in Table 6.7.1. The pigmented flower extracts from the Geraniaceae plant family generally had a significantly ($p \leq 0.05$) higher total phenolic content and DPPH radical scavenging activity than the extracts of flower species from the Lamiaceae family. This is also reflected in the total anthocyanin content and flavonoid content where the Geraniaceae flower extracts had higher total anthocyanin and flavonoid content than pigmented flower extracts of the Lamiaceae family.

The high and positive correlation between the TPC and DPPH radical scavenging activity for the pigmented flower extracts from both plant families ($r = 0.94$ for the Geraniaceae and $r = 0.99$ for the Lamiaceae) indicates that the anthocyanins and other phenolic compounds were majorly responsible for their antioxidant activity.

Table 6.7.1 Total phenolic content (TPC) and DPPH radical scavenging antioxidant activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

Sample	Total phenolic content (TPC) mg CE/g extract	DPPH radical scavenging antioxidant activity ($\mu\text{mol TE/g}$)
Geraniaceae		
<i>Pelargonium grandiflorum</i>	$560^f \pm 3.25$	$2435^f \pm 1.25$
<i>Pelargonium \times hortorum</i>	$338^{ce} \pm 0.38$	$1746^e \pm 3.57$
<i>Pelargonium zonale hybrid</i>	$311^d \pm 1.46$	$1208^d \pm 5.95$
Lamiaceae		
<i>Salvia aurea \times dolomitica</i>	$309^c \pm 0.30$	$855^c \pm 0.09$
<i>Salvia dolomitica</i>	$222^b \pm 3.60$	$622^b \pm 0.89$
<i>Plectranthus zuluensis</i>	$108^a \pm 0.16$	$369^a \pm 0.14$

TE: Trolox equivalence; Values are mean $\pm$ SD; CE: catechin equivalence

Mean values with different superscript letters within columns differ significantly ($p \leq 0.05$)

6.7.2 Cytotoxicity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

6.7.2.1 Caco-2 cells

The cytotoxicity of pigmented flower extracts in Caco-2 cells is shown in Figure 6.7.1. No Caco-2 cell cytotoxicity was observed at the concentration of 125 $\mu\text{g/ml}$ for all flower extracts after 24- and 48-hour incubation. The Caco-2 cells showed a significant ($p \leq 0.05$) increase in viability (compared to the cell control) after treatment with the flower extracts at a concentration of 125 $\mu\text{g/ml}$. No significant ($p \leq 0.05$) difference in cell viability was observed for the 24- and 48-hour treatments.

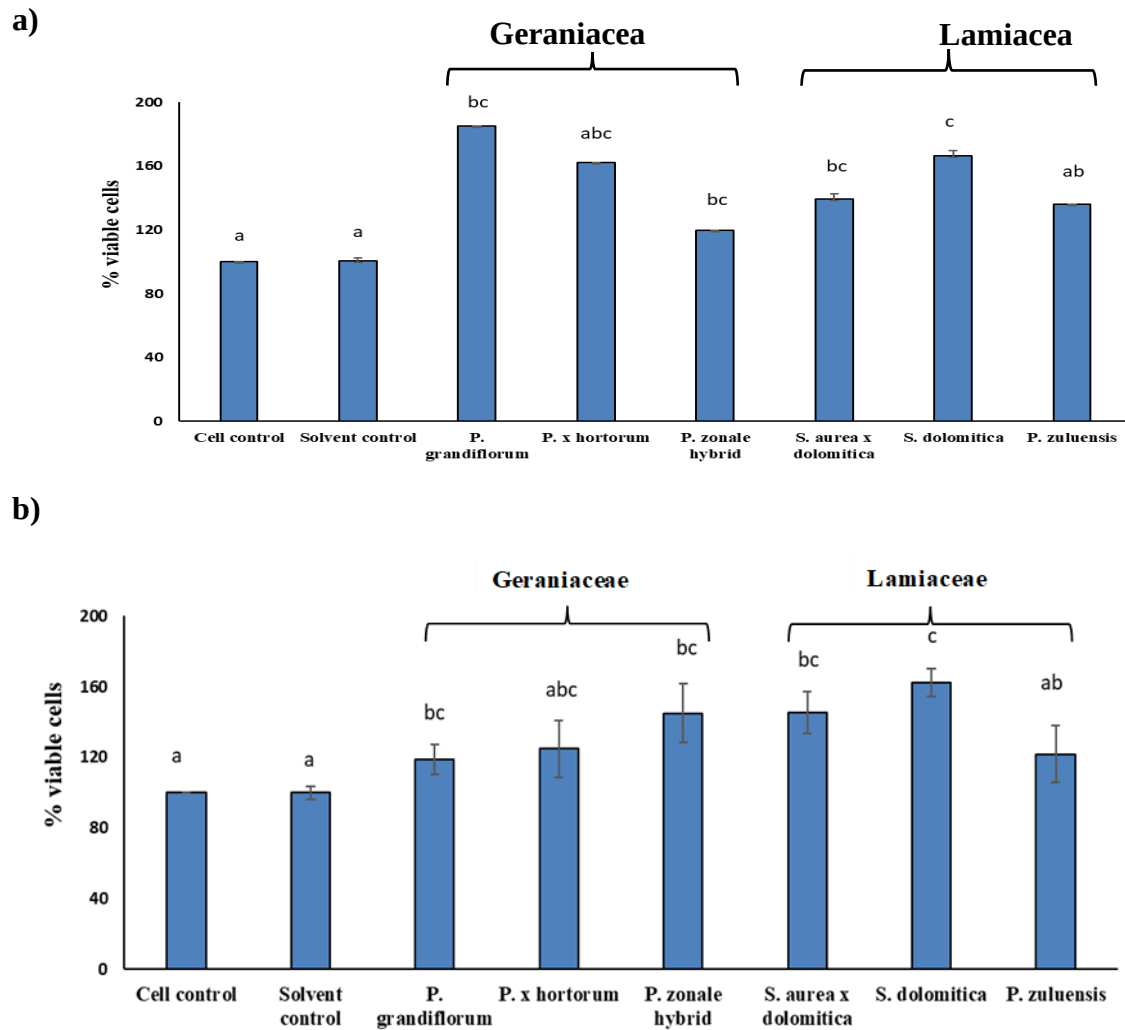


Figure 6.7.1 Cytotoxicity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families in Caco-2 cells after a) 24 hours and b) 48 hours at a concentration of 125 µg/ml

Bars (mean values) with different letters differ significantly ($p \leq 0.05$)

6.7.2.2 RAW 264.7 macrophage cells

The cytotoxicity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families at two different concentrations in RAW 264.7 cells is shown in Figure 6.7.2. No cytotoxicity of the RAW 267.4 cells was observed when the extracts were applied at a concentration of 250 µg/ml and also at the lower concentration of 125 µg/ml.

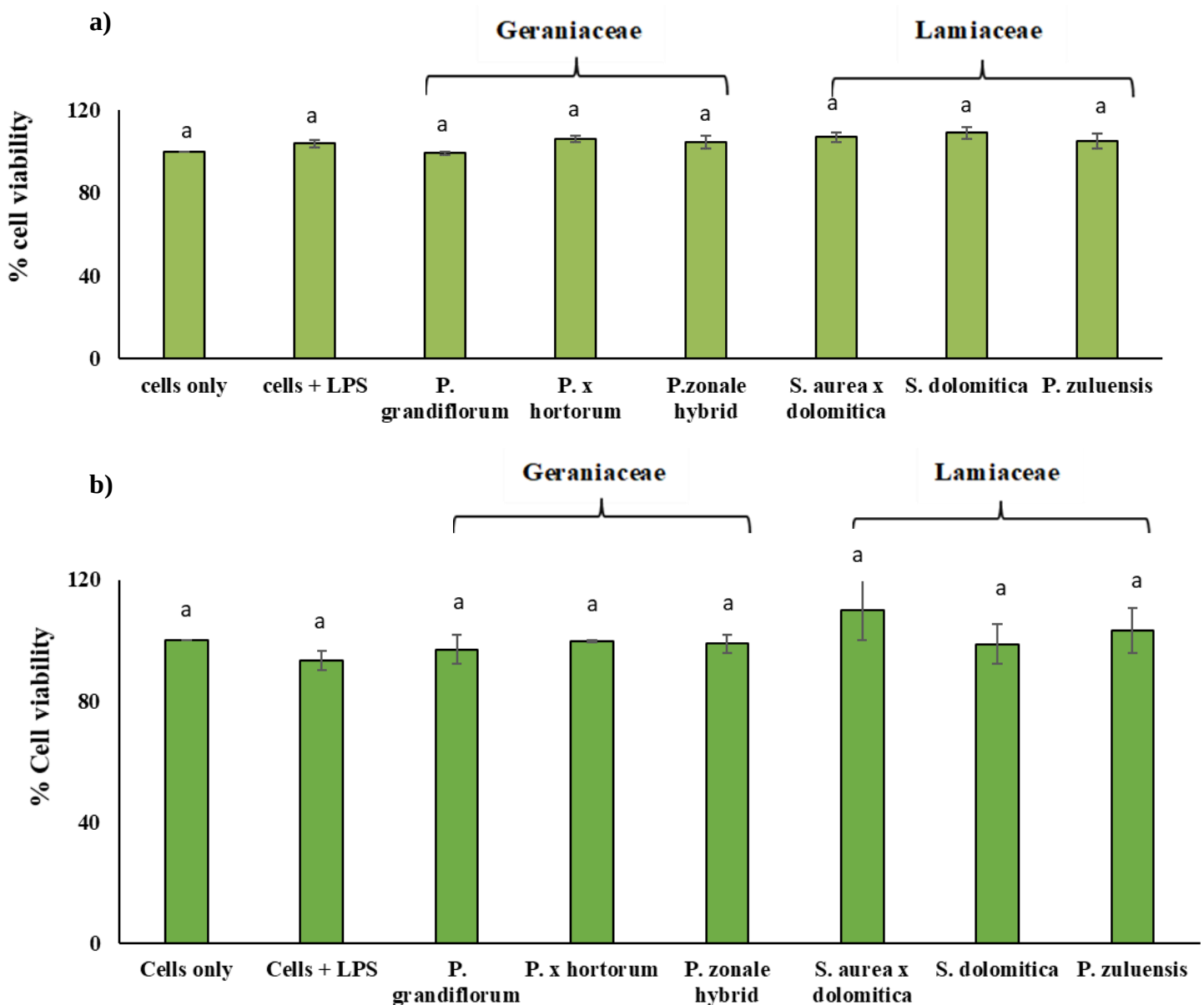


Figure 6.7.2 Cytotoxicity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families in RAW 264.7 macrophage cells after incubation for 24 hours at concentration of a) 250 µg/ml and b) 125 µg/ml

6.7.2.3 3T3-L1 adipocyte cells

The cytotoxicity of pigmented flower extracts in 3T3-L1 adipocyte cells at a concentration of 250 µg/ml over 72 hours is shown in Figure 6.7.3. The results show no significant ($p \leq 0.05$) reduction in cell viability for all extracts except for the *P. grandiflorum* (Geraniaceae) pigmented flower extract. The *P. grandiflorum* (Geraniaceae) pigmented flower extracts reduced cell viability by about 40 % after 72 hours.

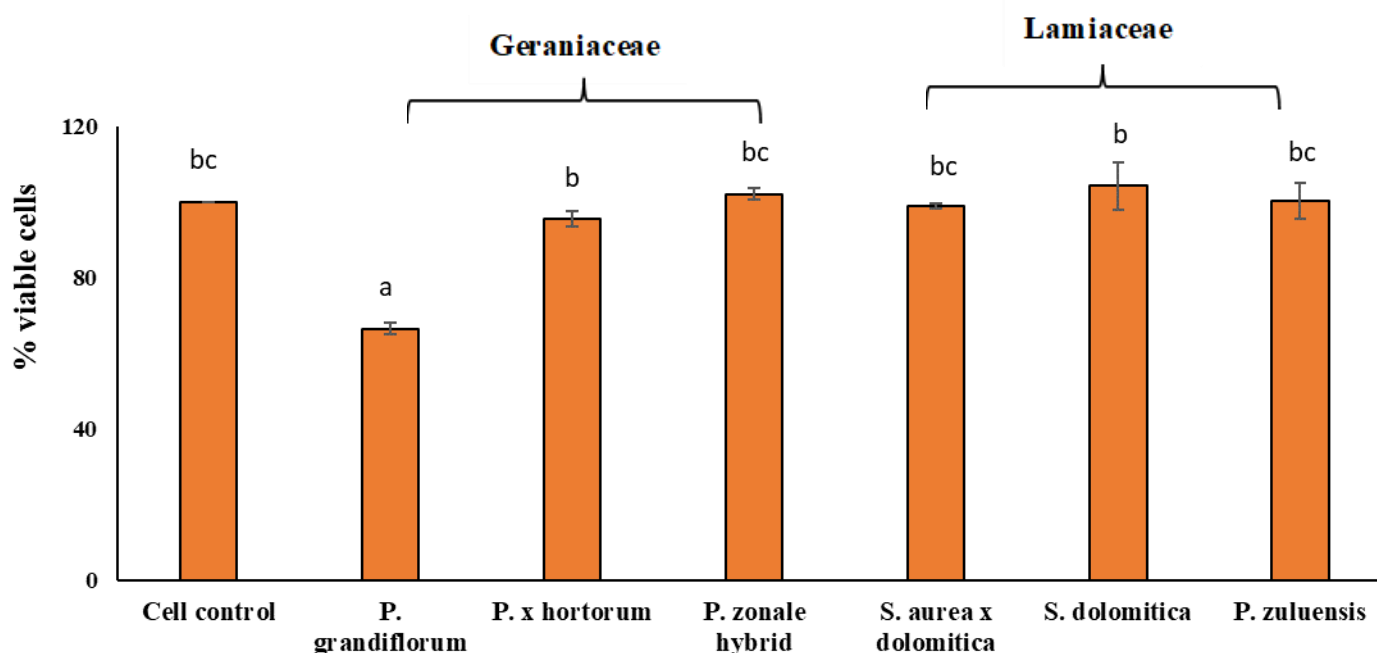


Figure 6.7.3 Cytotoxicity of 3T3-L1 cells treated with pigmented flower extracts from the Geraniaceae and Lamiaceae plant families following incubation for 72 hours. Bars (mean values) with different letters differ significantly ($p \leq 0.05$)

Based on the above results from the cytotoxicity studies, a non-toxic concentration of 125 µg/ml of the pigmented flower extracts was used in the Caco-2 antioxidant radical scavenging activity assays. For NO radical scavenging assays using RAW 264.7 cells and anti-adipogenic assays involving 3T3-L1 adipocytes, a higher non-toxic concentration of 250 µg/ml was used.

6.7.3 Cellular antioxidant activity and oxygen radical absorption capacity (ORAC) of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

The Caco-2 cellular antioxidant activity and oxygen radical absorption capacity (ORAC) of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families are shown in Figure 6.7.4. There was no significant difference ($p \leq 0.05$) in ORAC values of all the extracts.

With the exception of *P. zonale* hybrid (Geraniaceae), the pigmented flower extracts from all other plant families showed Caco-2 cellular antioxidant activity of at least 60%. For the pigments from plant species of the Geraniaceae family, that from *P. zonale* hybrid had relatively high levels of pelargonidin derivatives. The very low Caco-2 cellular antioxidant activity of the *P. zonale* hybrid pigment compared to pigments from *P. grandiflorum* and *P. × hortorum* among the Geraniaceae could be due to the lower bioactivity or stability of the pelargonidin derivatives in the Caco-2 cells resulting in their degradation (Yi, Akoh, Fischer and Krewer, 2006, Kay, Kroon and Cassidy, 2009). As the other non-anthocyanin phenolic compounds of *P. zonale* hybrid were very similar to that of the other species from the Geraniaceae family studied (*P. grandiflorum* and *P. × hortorum*), the anthocyanins in this specie could explain the reason for the low Caco-2 cellular antioxidant activity.

The high Caco-2 cellular antioxidant activity shown by the pigmented flower extracts from the Lamiaceae could be due to their enhanced stability and antioxidant activity as a result of possible intramolecular interactions of their aromatic acylated anthocyanins and intramolecular interactions between these aromatic acylated anthocyanins and phenolic acids (rosmarinic acid and its derivatives such as sagerinic acid and salvianolic acid). These interactions may confer stability to the anthocyanins from the Lamiaceae pigments and maintain their antioxidant activity in the Caco-2 cells physiological environment.

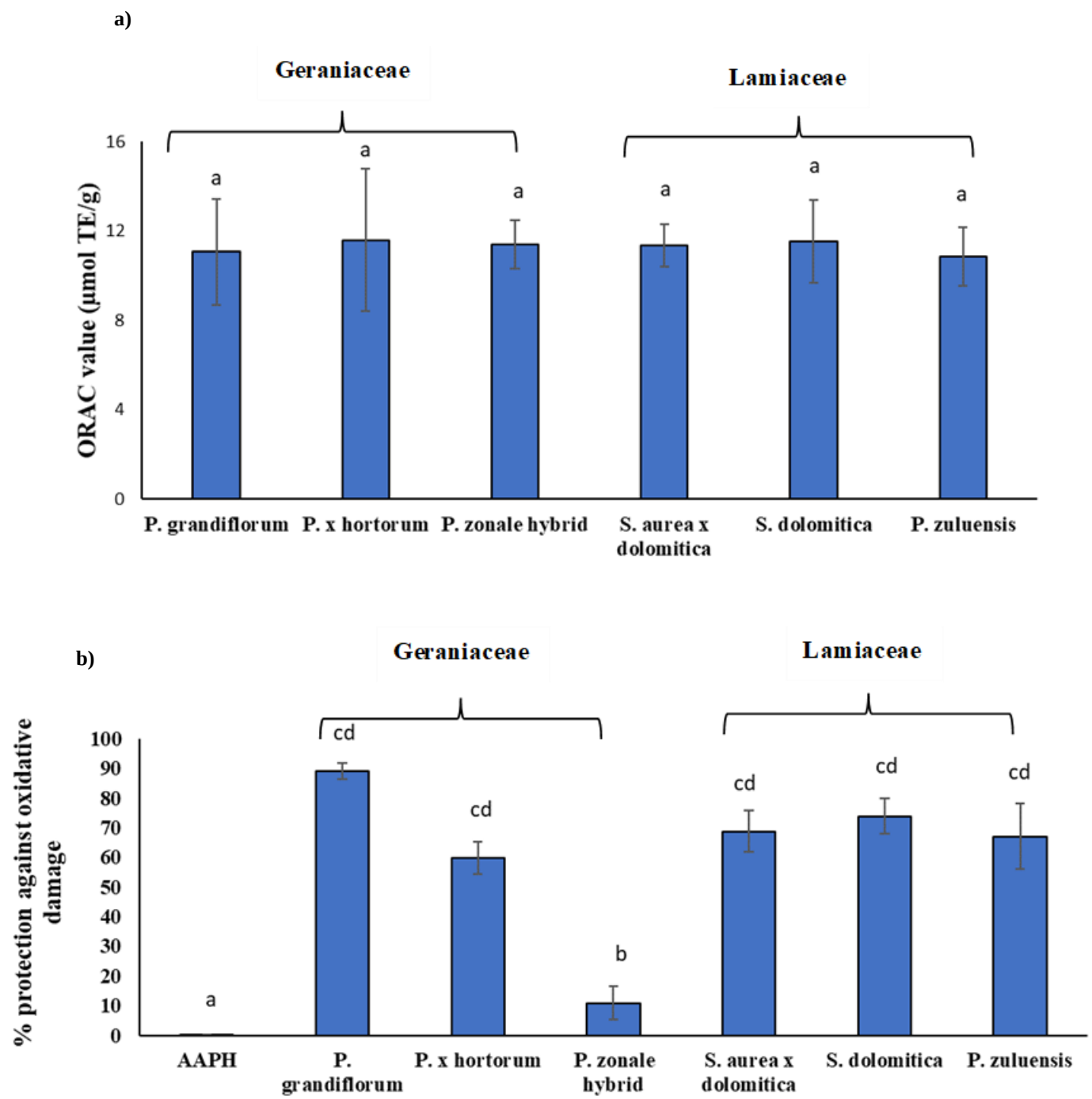


Figure 6.7.4 ORAC values (a and b) Caco-2 cellular antioxidant activity b) of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

TE: Trolox equivalents, AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride

Bars (mean values) with different letters differ significantly ($p \leq 0.05$)

6.7.4 Nitric oxide (NO) scavenging activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

The NO scavenging activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families is shown in Figure 6.7.5. Pigmented flower extracts of both families significantly reduced NO production as compared with the sodium nitroprusside control. There was a strong positive correlation ($r = 0.90$ for the Geraniaceae and $r = 0.99$ for the Lamiaceae) between TPC and NO radical scavenging activity, indicating that the anthocyanins and phenolic compounds in the pigmented extracts were major contributors to the observed NO radical scavenging activity.

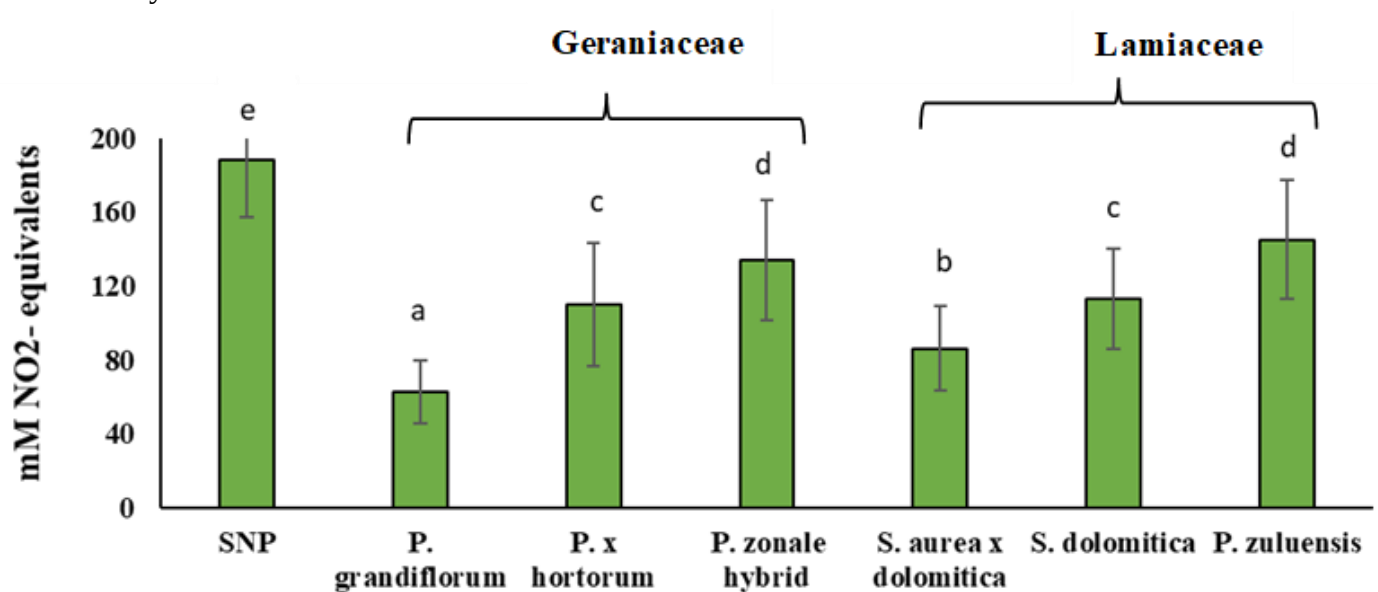


Figure 6.7.5 Nitric oxide (NO) scavenging activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant family

Bars (mean values) with different letters differ significantly ($p \leq 0.05$)

SNP: Sodium Nitroprusside

6.7.5 NO radical scavenging activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families in RAW 264.7 cells (Anti-inflammatory activity)

The NO radical scavenging activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families in RAW 264.7 cells (an indicator of anti-inflammatory activity) is shown in Figure 6.7.6a. All pigmented flower extracts of both families (with the exception of the *P zonale* hybrid pigment) significantly ($p \leq 0.05$) reduced the NO produced in RAW 264.7 cells as compared to the LPS + cell control at a concentration of 125 µg/ml. This is a similar trend to the observations on Caco-2 cellular antioxidant activity where the *P. zonale* hybrid pigment exhibited lowest Caco-2 cellular antioxidant activity. As explained earlier, the lower NO scavenging ability of *P. zonale* hybrid in the RAW 264.7 macrophage cells could be due to the lower bioactivity or stability of the pelargonidin derivatives identified as the main anthocyanin type in this specie.

When the pigmented extracts were applied at the higher concentration of 250 µg/ml, they exhibited no NO radical scavenging activity in the RAW 264.7 macrophage cells (Figure 6.7.6 a) which indicates that they showed no anti-inflammatory effects. In fact, all pigmented extracts exhibited prooxidant effects which resulted in slightly higher production of NO in the RAW 264.7 macrophage cells (Figure 6.7.6b). These results show that the concentration at which the extract is applied in these antioxidant assays is important in showing trends in the results and effects of different treatments. The increase in NO production induced by the pigmented flower extracts could find application in treatment of hypertension by modulation of the nuclear translocation and phosphorylation of NF-κβ (Song, Lee, Kim, Kim, Kim, Yoo and Park, 2008).

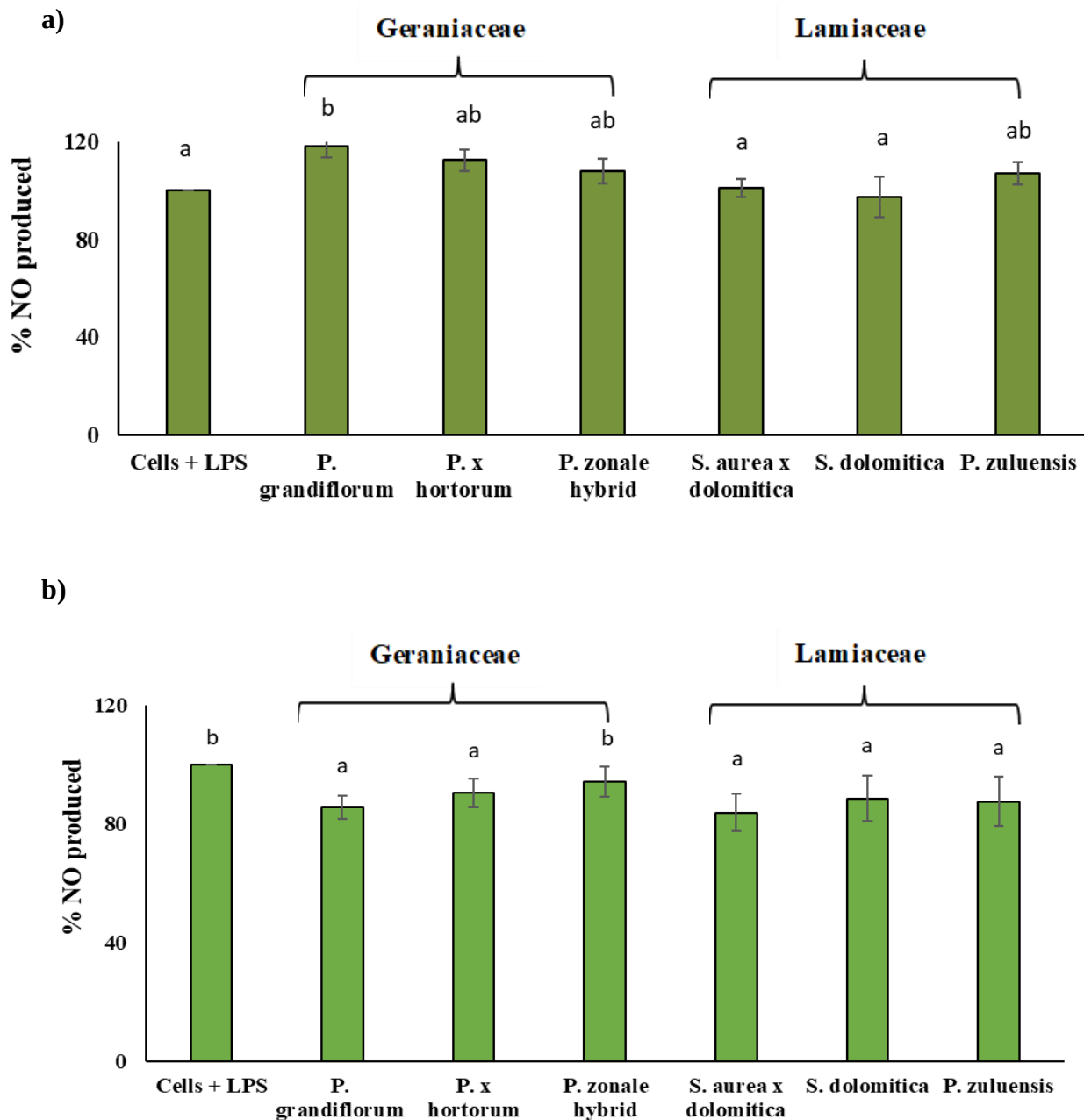


Figure 6.7.6 Nitric oxide (NO) radical scavenging activity of flower extracts from the Geraniaceae and Lamiaceae plant families at a concentration of a) 250 µg/ml and b) 125 µg/ml in RAW 264.7 macrophage cells
Bars (mean values) with different letters differ significantly ($p \leq 0.05$)

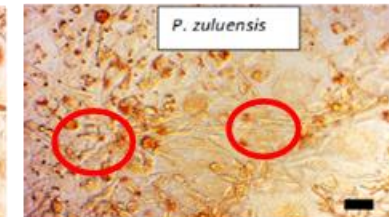
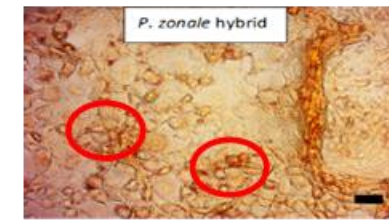
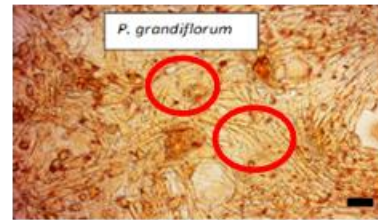
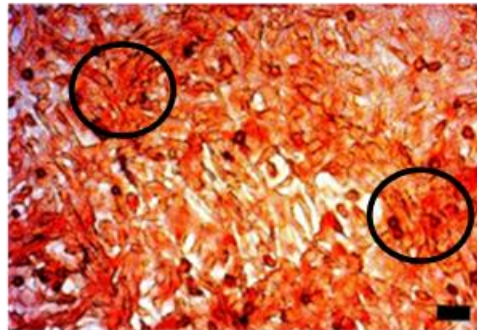
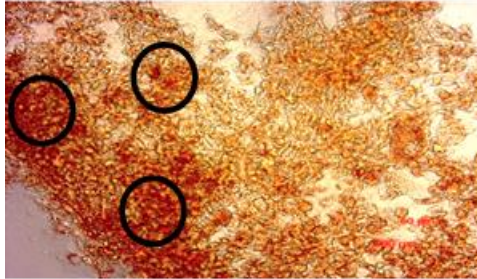
6.7.7 Anti-adipogenic activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families in 3T3-L1 adipocyte cells

Figure 6.7.7 shows microscopic images of 3T3 L-1 cells before and after treatment with pigmented flower extracts from the Geraniaceae and Lamiaceae plant families for 24 hours and 14 days under Oil Red O staining of lipids. The microscope images showed regions of reduction in concentration of lipid droplet accumulation (red circles) for cells treated with flower extracts for 24 hours and 14 days as compared to the differentiated control cells with areas of high lipid accumulation (black circles). However, cells treated with pigmented flower extracts showed decreased cell density after 14 days. The decreased cell density is caused by the chronic exposure of the cells with flower extracts over a period of 14 days thereby causing some cellular death to occur as seen by the changes in morphology. In this regard, the *P. zonale* hybrid (Geraniaceae) flower extract was an exception. Although 3T3 L-1 cells treated with *P. zonale* hybrid flower extract exhibited a reduction in concentration of lipid droplet accumulation, the cells themselves showed high fibroblast density (Figure 6.7.7). Overall, the *P. zonale* hybrid consistently showed low cellular radical scavenging activity but showed the ability to target lipid droplet reduction in adipocyte cells while still retaining the cell morphology with no cell death.

The potential of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families to reduce or prevent lipid droplet formation in 3T3-L1 cells (an indication of anti-adipogenic activity) at the non-toxic concentration of 250 µg/ml is shown in Figure 6.7.8. In general, pigmented flower extracts of both families caused a significant ($p \leq 0.05$) reduction in lipid accumulation after treatment for 14 days with the exception of the extract from *S. aurea* × *dolomitica* as compared to the differentiated control. There was no significant difference in reduction in lipid droplet accumulation in the cells after treatment for 24 hours with pigmented extracts from the Geraniaceae and Lamiaceae families. However, after 14 days treatment, the pigmented flower extracts from the Geraniaceae showed greater ability to reduce lipid droplet formation in the 3T3 L-1 cells. The superior ability of the pigmented extracts from the Geraniaceae family to reduce lipid accumulation after chronic exposure of 14 days compared to the Lamiaceae could be due to higher levels of anthocyanins and other flavonoids, as well as higher total phenolic content (TPC) and radical scavenging activity (Table 6.6.1) of the pigmented extracts from the Geraniaceae family. The high levels of anthocyanins and other

flavonoids in the pigmented extracts from the Geraniaceae family could reduce lipid accumulation over time in the cells potentially through prooxidant effects.

Differentiated



14 days

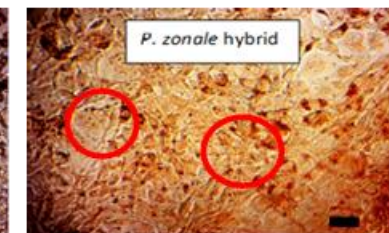
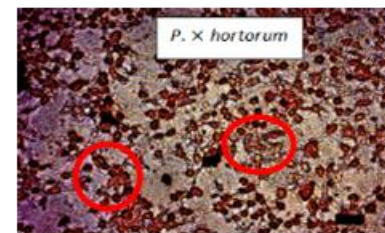


Figure 6.7.7 Light microscope images showing Oil Red O-stained lipid droplet reduction in 3T3-L1 adipocytes treated with extracts of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families for 24 hours and 14 days. Red arrows show regions of reduced lipid accumulation. Black Scale bar = 50 µm. Red circles show regions of reduced lipid accumulation. Black circles show regions of high lipid accumulation

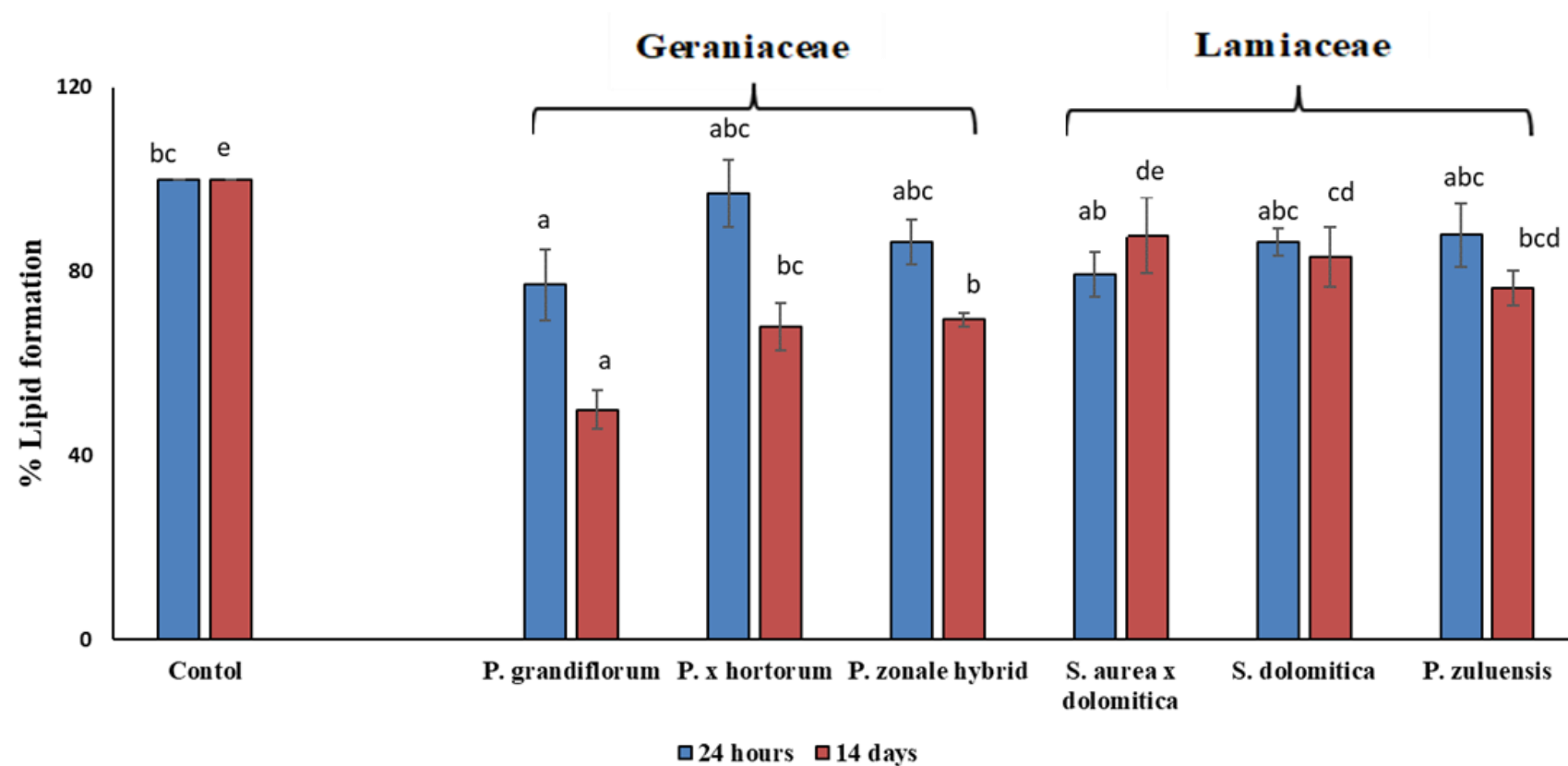


Figure 6.7.8 Effect of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families on reduction of lipid droplet formation (after 24 hours treatment and 14 days treatment) in 3T3-L1 cells using Nile Red fluorescent staining. For each treatment (24 hours and 14 days), bars (mean values) with different letters differ significantly ($p \leq 0.05$)

6.7.8 Anti-glycation activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

The anti-glycation activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families at two different concentrations are shown in Figure 6.7.9a and b. Generally, only *P. grandiflorum* and *P. × hortorum* (Geraniaceae) pigmented flower extracts, at a concentration of 125 µg/ml, showed a significant ($p \leq 0.05$) reduction in advanced glycation end-products (AGE's) formation as compared to the methylglyoxal/bovine serum albumin (MGO/BSA) mixture. With increase in concentration of flower extracts to 312.5 µg/ml, there were no observable difference in reduction in AGE formation and only *P. grandiflorum* (Geraniaceae) showed significant reduction in AGE formation. The potential of flower extracts of the Geraniaceae and Lamiaceae plant families to reduce the formation of AGE's were compared to a well known synthetic AGE inhibitor aminoguanidine at different concentrations (0.2-1.0 mM). Aminoguanidine reduced the formation of AGE's in a dose-dependent manner. The ability of *P. grandiflorum* (Geraniaceae) and *P. × hortorum* (Geraniaceae) at a concentration of 125 µg/ml to reduce AGE formation was comparable to the two highest aminoguanidine concentrations (0.8 and 1.0 mM) whereas the other flower extracts from both plant families were comparable to aminoguanidine concentrations of 0.2-0.8 mM.

A high and negative Pearson correlation (r) was obtained between total phenolic content (TPC) and AGEs produced for both the Geraniaceae (-0.98) and Lamiaceae (-0.95) plant families. This correlation suggest that the anthocyanins and other phenolic compounds present in the pigmented extracts could be responsible for the observed decreases of AGE formation. Due to the fact that pigments from the Lamiaceae family had lower levels of total anthocyanins and other phenolic compounds compared to pigments from the Geraniaceae, it would have been expected that the Lamiaceae pigmented extracts would exhibit a lower ability to reduce AGE formation. However, the AGE reduction ability of pigments from the Lamiaceae family was actually similar to that of the Geraniaceae pigments and the aminoguanidine standard. This could be attributed to higher stability of the Lamiaceae pigments due to the self-association via intramolecular interactions within aromatic acylated anthocyanins in combination with intermolecular interactions with phenolic acids identified in the Lamiaceae pigmented extracts such as rosmarinic acid and its derivatives (sagerinic and salvianolic acids).

The similarity in inhibition of AGE's formation by pigments of flower species of the Geraniaceae and Lamiaceae plant families as compared to aminoguanidine might suggest a mechanism wherein the anthocyanins and other phenolics in the pigments react with reactive carbonyls to form adducts thereby "trapping" the reactive carbonyls (Brownlee, Vlassara, Kooney, Ulrich and Cerami, 1986). Reactive carbonyls are free radical aldehyde or ketone species from sugars that are formed in the initial stages of glycation during the oxidation of Schiff bases which are generated by the reaction of sugars with proteins or amino acids (Wu *et al.*, 2011) These reactive carbonyls are important in the formation of AGE's. The pigmented flower extracts, especially those of the Lamiaceae, could therefore potentially replace aminoguanidine as natural AGE inhibitors.

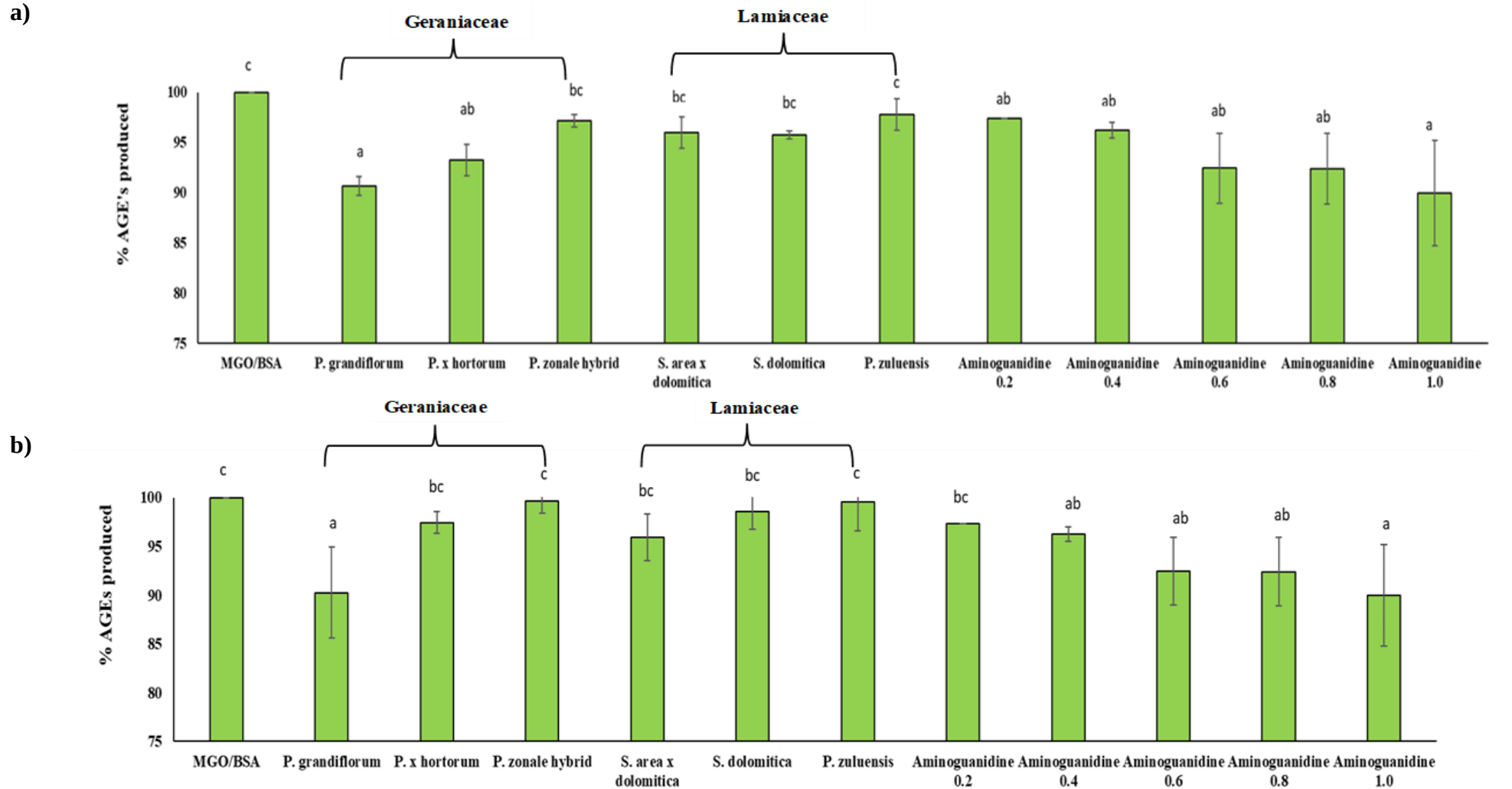


Figure 6.7.9 Anti-glycation activity of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families at a concentration of a) 125 µg/ml and b) 312.5 µg/ml AG: Aminoguanidine at concentrations of 0.2 – 1.0 mM, MGO/BSA: methylglyoxal/bovine serum albumin, Bars (mean values) with different letters differ significantly ($p \leq 0.05$)

6.8 Conclusion

The pigmented flower extracts from the Geraniaceae and Lamiaceae plant families showed strong DPPH radical scavenging activity and protective activity against radical damage in Caco-2 cells. The flower extracts of species from both plant families show good chemical and cellular NO radical scavenging activity in RAW 264.7 macrophage cells. Generally, all pigmented flower extracts showed significant reduction in lipid accumulation after 14 days treatment with some cytotoxic affects. *P. zonale* hybrid pigmented extract did not cause cytotoxicity after 14 days with targeted lipid droplet reduction and could be further investigated in anti-obesity studies. The pigmented flower extracts also show anti-glycation activity in its ability to reduce the formation of anti-glycation end-products (AGES) with comparable activity a commercially used anti-diabetic medication, aminoguanidine. The flower extracts of both families show a wide range of chemical and cellular antioxidant activity with variable activities and thus show promise as a food colourant with enhanced health-promoting properties.

Chapter 7. Colour stability of anthocyanin rich extracts from Geraniaceae and Lamiaceae plant families in different food systems

Abstract

This study set out to investigate the feasibility of applying pigments from flowers of plant species from the Geraniaceae and Lamiaceae families in different food systems as natural colourants. Pigmented flower petal powder from both families incorporated into plain yogurt showed browning after storage at 4°C for 7 days possibly due to enzymatic browning reactions. Pigmented flower extracts from both families showed superior storage stability as compared to E163 colourant in yogurt, fondant and gelatine candies and produced a variety of colours in these products. The yogurt, gelatine and fondant served as good vehicles for the extracts and were able to maintain the original colour of the extracts. Although the yogurt diluted the aqueous pigmented extracts and produced a lighter colour, there was clear evidence that the pigmented extracts did impart their colour. More concentrated versions of the pigmented extracts can be successfully applied in the yogurts with enhanced coloration. The pigmented flower extracts from the Geraniaceae and Lamiaceae plant families show promise as novel natural colourants for application in food systems.

7.1.1 Introduction

There is accumulating scientific evidence in the literature about potential negative health effects associated with the use of synthetic colourants in foods (McCann et al, 1975; Tsuda, Murakami, Matsusaka, Kano, Taniguchi & Sasaki, 2001; Chequer et al, 2012; Elbanna, Sarhan, Khider, Elmogy, Abulreesh, Shaaban, 2017). As a result, the last 20 years have seen a rise in consumer demand for healthier food ingredients in general such as colourants. In an attempt to satisfy this demand, food industries around the world are gradually moving away from the use of synthetic dyes and exploring the use of various natural colourants extracted from sources such as fruit and vegetables which contain various pigments including carotenoids, betalains, anthocyanins, bixin and chlorophyll (Downham and Collins, 2000).

As would be expected, the demand for natural colourants has driven research efforts on the feasibility of applying pigments from natural sources as colourants in foods. For example, Rodriguez-Saona, Giusti and Wrolstad (1999) researched the incorporation of pigments from red fleshed potato (*Solanum tuberosum*) and red radish (*Raphanus sativus*) into beverages and concluded that these pigments could be used as alternative colourants to FD and C Red #40 synthetic colourants.

Pigments from flowers of plants such as *Hibiscus sabdariffa* (Selim et al., 2008), blue butterfly pea (*Clitoria ternatea*) (Ab Rashid et al., 2021), *Thymus moroderi* (Díaz-García et al., 2014), rose (*Rosa*) and blue cornflower (*Centaurea cyanus* L.) (Pires et al., 2018) have been incorporated as colourants into various foods such as jams, hard candy, baked food products and yogurt. The chemistry of the pigments in these natural colourants plays an important role in contributing to their stability and feasibility of their use in food systems. For example, flowers of the blue butterfly pea contain aromatic acylated anthocyanins called ternatins (Chayaratanasin *et al*, 2019). The aromatic side chains could assist in the protection of anthocyanin nuclei against nucleophilic attack by water through intermolecular co-pigmentation with other phenolic compounds and intramolecular association (Francis, 1989, Dangles, Saito and Brouillard, 1993). This promotes and enhances stability of the colourant.

Plant species from the Geraniaceae and Lamiaceae families have a wide variety of uses including flavourants and medicinal uses (Watt & Breyer-Brandwijk, 1962; Raja, 2012). The plants from these families produce flowers that have a wide variety of bright colours due to their content of predominantly anthocyanin pigments. This presents an opportunity to study the potential application of pigmented extracts from the flowers of plant species from the

Geraniaceae and Lamiaceae as natural colourants in food systems. Thus, the objective of this research was to investigate the potential application of pigments from flowers of plant species of the Geraniaceae and Lamiaceae families as natural colourants in different food systems namely yogurt, high sugar food products such as fondant and a high protein food system such as gelatine.

7.2 Materials and methods

7.2.1 Samples

The flower samples from plant species of the Geraniaceae and Lamiaceae families that were used, as well as how they were dried and processed into powder form are as described in Section 5.2.1.

7.2.2 Chemicals

Formic acid was purchased from Merck. Commercial anthocyanin E163 colourant was kindly donated by ROHA Dyechem Limited, Centurion, South Africa.x`

7.2.3 Preparation of pigmented flower extracts

The aqueous flower extracts were prepared using a method by Díaz-García et al., (2014) with modifications. One gram of pigmented flower petal powder was extracted in 100 ml 1% formic acid: distilled water for 24 hours. After extraction, the extracts were filtered through a Buchner funnel under vacuum, decanted in plastic tubs and freeze-dried for 24-48 hours until dry. The solids yield of each extract (weight of freeze-dried solids residue expressed as a percentage of the weight of flower powder extracted) was then determined. Depending on the yield, 0.2 to 0.5 g of the freeze-dried pigmented extract powders were re-dissolved in the required amount of acidified water (1% formic acid in distilled water) to prepare aqueous extracts with similar concentrations. These aqueous extracts were used for the yogurt, fondant and gelatine applications.

7.2.3 Incorporation of pigmented flower petal powder and extracts in yogurt

Pigments from the flowers of the plant species of the Geraniaceae and Lamiaceae families were applied to yogurt either in the form of flower petal powder or freeze-dried pigmented aqueous extracts from the flowers. The incorporation of the extracts in yogurt was done according to

the method described by Díaz-García et al. (2014) and Pires et al. (2018) with modifications. Plain white low-fat yogurt was purchased from the local supermarket. The commercial anthocyanin colourant E163 was used as an external reference. The commercial powder colourant (E163) was applied in the yogurt at a concentration of 0.65 g/kg. The air-dried petal powder of flowers was added to 30 g of yogurt at a concentration of 1.54 g/kg yogurt for the Geraniaceae family and at 2.19 g/kg yogurt for Lamiaceae. The reconstituted freeze-dried pigmented flower extracts were added dropwise (approximately 5 drops) until a desired colour was obtained. In all cases, samples were taken out every day and colour parameters and pH analysed for 7 days. For each analysis, a small portion of yogurt was taken and put on a white paper on a glass slide. Three consecutive shots were taken with the colourimeter instrument. All assays were performed in triplicate for each sample.

7.2.4 Incorporation of pigmented flower extracts in fondant

The aqueous commercial colourant (E163) and the freeze-dried pigmented extracts were added as an aqueous extract to white fondant. Multiple drops as required of liquid extract were added to a small piece of fondant for all extracts and kneaded into the fondant until a colour change occurs with the addition of small amounts of powdered sugar to keep the fondant dry. The samples were maintained at room temperature. In all cases, samples were taken out after a week (7 days) and colour parameters analysed. All assays were performed in triplicate for each sample.

7.2.5 Incorporation of pigmented flower extracts in gelatine candies

The application of flower extracts in gelatine jelly was done according to Bloom (2004) with modification. Gelatine jelly was prepared by the addition of 5 envelopes (32 g) gelatine in 59.15 ml cold water. Sugar (64 g) and 85 g of corn syrup was dissolved in a saucepan over medium heat. The dissolved gelatine was added to the mixture and stirred for 5 min. The mixture was then removed from the heat and immediately poured into a mould. The gelatine was allowed to cool after which 2-4 drops of the flower extracts and commercial E163 colourant was added to the pre-set gelatine until the desired colour was obtained. The gelatine was left to set for 2-3 hours at room temperature. The colour parameters of the treated fondant were analysed. The fondant samples were stored at room temperature in the dark and analysed again after 15 days.

7.2.6 Colour parameter analysis of yogurt, gelatine candies and fondant

The colour parameters were calculated according to Díaz-García et al. (2014) with modifications. The CIELAB chroma system was used and parameters were measured with a Minolta colorimeter (CM-508i; Minolta, Osaka, Japan). Colour difference values were calculated to compare the samples treated with flower extracts or flower petal powder with samples treated with commercial food colourant E163. Each measurement was done directly on the sample surface and shot three times for each sample. ΔL^* (metric lightness difference), ΔC^* (metric chroma difference), C^* (metric chroma), ΔE^* (total colour difference) and h (hue angle) were defined as:

$$\Delta L^* = L_i^* - L_0^*$$

$$\Delta a^* = a_i^* - a_0^*$$

$$C^* = (a^{*2} + b^{*2})^{0.5}$$

$$\Delta C^* = C_i^* - C_0^*$$

$$\Delta E^* = [(L_i^* - L_0^*)^2 + (a_i^* - a_0^*)^2 + (b_i^* - b_0^*)^2]^{0.5}$$

$$h = \tan^{-1} (b^*/a^*)$$

$$h = 180 + \tan^{-1} (b^*/a^*)$$

$$h = 360 + \tan^{-1} (b^*/a^*)$$

where L_0^* , a_0^* , and b_0^* are the values of the samples at zero time and L_i^* , a_i^* and b_i^* are the measured values of each sample with time.

7.3 Statistical analysis

Experiments were conducted in triplicates. One-way analysis of variance (ANOVA) and pairwise comparison was conducted as required using SPSS software. Significant differences between means were determined using least significant difference (LSD), Tukey and Scheffe tests at $p \leq 0.05$. Independent variables were storage time (0-7 days) and flower types from Geraniaceae (*P. grandiflorum*, *P. × hortorum*, *P. zonale* hybrid) and Lamiaceae (*S. aurea* × *dolomitica*, *S. dolomitica* and *P. zuluensis*) families. Dependent variables were the measured values for L^* , a^* , b^* and C^* and calculated values for ΔL^* , ΔC^* , ΔE^* , h .

7.3 Results and discussion

7.3.1 Colour of yogurts with dried flower petal powder and extracts from the Geraniaceae and Lamiaceae plant families and E163 applied as colourants

The flower petal powders were applied as colourants by direct incorporation into yogurt to study the potential feasibility of their application in the simplest form possible i.e., as a dry powder. The colour parameters (L^* , a^* , b^* , C^* , h), chromaticity diagrams and images of yogurt incorporated with commercial E163 colourant and flower powder from the Geraniaceae and Lamiaceae families are shown in Table 7.1 and Figures 7.1, 7.2 and 7.3. Storage for 7 days at 4°C had a significant ($p \leq 0.05$) effect on the colour parameters of yogurts with pigmented flower powders and E163 incorporated as colourants.

Generally, the lightness (ΔL^*) of yogurts with flower powders from the Geraniaceae increased during storage to a greater extent than for the E163 colourant. In contrast, for the Lamiaceae, apart from yogurt with *S. aurea x dolomitica*, yogurts with flower powders of the other species recorded very small increases in lightness during storage. As indicated by the Δa^* , the redness of yogurts with E163 colourant increased slightly during storage. However, the trends in redness of yogurts with flower powders were mixed and showed either slight increases or decreases during storage. The observed slight increase in redness of the yogurt samples could suggest anthocyanin colour retention (Vieira, Marinho, Rocha, Barros and Stringheta, 2018; Quan, He, Lu, Yuan, Zeng, Gao, Qin, Chen and He, 2019) while small decreases could suggest oxidative degradation of anthocyanins (Patras *et al.*, 2010).

There were greater increases in yellowness (Δb^*) of yogurts with flower powders during storage compared to yogurts with E163. The increase in yellowness is also illustrated by the observed increase in chroma (C^*) of yogurts with flower powder during storage. This could be due to enzymatic browning and might suggest that there was residual activity of browning enzymes in the flower petal powders during storage. Some anthocyanins such as acylated anthocyanins and other polyphenols such as quercetin and kaempferol derivatives identified in these pigmented extracts may be used as substrates in the enzymatic browning reactions and could mask the effect of the small anthocyanin degradation. Molitor, Mauracher, Pargan, Mayer, Halbwirth and Rompel (2015) reported the action of polyphenol oxidase and aurone synthase in flower petals of the Asteraceae family which led to formation of aurone from chalcones. The total colour change (ΔE^*) generally shows that the browning of the flower petal

powder could mask the colour of the anthocyanins during storage and thus affect the overall colour.

Table 7.1 Effect of storage for 7 days at 4°C on colour parameters of yogurts with commercial E163 colourant and flower powder of plant species from the Geraniaceae and Lamiaceae families applied as colourants

		Geraniaceae						Lamiaceae						
Colourant E163		<i>P. grandiflorum</i>		<i>P. × hortorum</i>		<i>P. zonale</i> hybrid		<i>S. aurea</i> × <i>dolomitica</i>		<i>S. dolomitica</i>		<i>P. zuluensis</i>		
Days	0	7	0	7	0	7	0	7	0	7	0	7	0	7
<i>L</i> [*]	62.89 ^{ABa}	74.77 ^{lb}	52.91 ^{ABa}	81.00 ^{Db}	55.47 ^{ABa}	80.48 ^{Eb}	42.68 ^{Ba}	85.08 ^{Cb} ±	70.63 ^{ABa}	79.26 ^{Fa} ±	76.07 ^{Aa}	78.92 ^{Ga}	74.03 ^{ABa}	77.01 ^{Ha}
	± 0.90	± 0.26	± 6.36	± 0.73	± 1.67	± 0.36	± 14.37	2.41	± 11.89	1.78	± 6.97	± 2.17	± 0.44	± 1.78
<i>a</i> [*]	10.67 ^{Ea} ±	12.35 ^{Eb}	6.58 ^{CDa} ±	6.78 ^{CDa}	6.71 ^{Ca} ±	8.36 ^{Cb} ±	4.97 ^{ABa}	3.96 ^{ABa} ±	6.12 ^{BCa} ±	5.20 ^{BCa} ±	3.77 ^{Aa} ±	4.33 ^{Aa} ±	7.45 ^{CDb} ±	5.58 ^{CDa}
	0.12	± 0.07	1.17	± 0.25	0.53	0.31	± 1.59	0.35	0.55	0.29	0.37	0.09	0.65	± 0.12
<i>b</i> [*]	-0.37 ^{Aa} ±	1.40 ^{Cb} ±	-0.80 ^{Aa} ±	3.04 ^{Eb} ±	1.60 ^{CDa} ±	7.72 ^{GHb}	2.09 ^{Da} ±	7.97 ^{Hb} ±	1.06 ^{Ba} ±	12.29 ^{Kb} ±	5.71 ^{Fa} ±	10.76 ^{Jb} ±	2.01 ^{Ba} ±	8.51 ^{IKb} ±
	0.15	0.04	0.56	0.21	0.21	± 0.30	0.58	1.01	1.53	0.45	1.10	0.41	0.09	0.23
<i>C</i> [*]	10.67 ^{Ja} ±	12.43 ^{Mb}	6.64 ^{Ca} ±	7.32 ^{Fa} ±	6.90 ^{Ea} ±	11.38 ^{Kb}	5.39 ^{ACJa}	8.90 ^{FHMb}	6.39 ^{BEa} ±	13.35 ^{KNb}	6.85 ^{DEa}	11.60 ^{IKb}	7.72 ^{EGa} ±	10.18 ^{IKb}
	0.12	± 0.07	1.21	0.31	0.50	± 0.42	± 1.68	± 1.06	0.61	± 0.53	± 1.12	± 0.41	0.61	± 0.26
<i>h</i>	357.99 ^{Nb}	6.48 ^{Aa} ±	353.67 ^{Mb}	24.14 ^{Fa}	13.54 ^{Ba} ±	42.72 ^{Gb}	23.21 ^{Ea}	63.45 ^{Jb} ±	18.77 ^{DEa}	67.07 ^{KJb}	56.13 ^{Ha}	68.09 ^{Lb}	15.28 ^{Ca} ±	56.72 ^{lb} ±
	± 0.79	0.21	± 4.55	± 0.72	2.17	± 0.44	± 2.66	0.93	± 1.54	± 0.41	± 2.80	± 0.44	1.97	0.18
Δ <i>L</i> [*]	11.89		28.09		25.01		42.40		67.36		2.85		2.98	
Δ <i>a</i> [*]	1.64		0.20		1.63		-1.01		-0.92		0.56		-0.87	
Δ <i>b</i> [*]	1.78		3.84		6.12		5.88		11.23		5.05		6.49	
Δ <i>C</i> [*]	1.75		0.78		4.48		3.50		6.95		4.75		2.45	
Δ <i>E</i> [*]	12.12		28.35		25.80		42.82		68.30		5.83		7.38	

L^{*} (metric lightness), *a*^{*} (+ red; - green), *b*^{*} (+ yellow; - blue) *C*^{*} (metric chroma), *h* (hue angle), ΔL^* (metric lightness difference), ΔC^* (metric chroma difference), ΔE^* (total colour difference), A,B,C - Mean values in a row with different uppercase superscript letters differ significantly ($p \leq 0.05$); a,b: For each colour parameter, mean values with different lowercase superscript letters differ significantly ($p \leq 0.05$) by pairwise comparison between day 0 and day 7 of the storage period

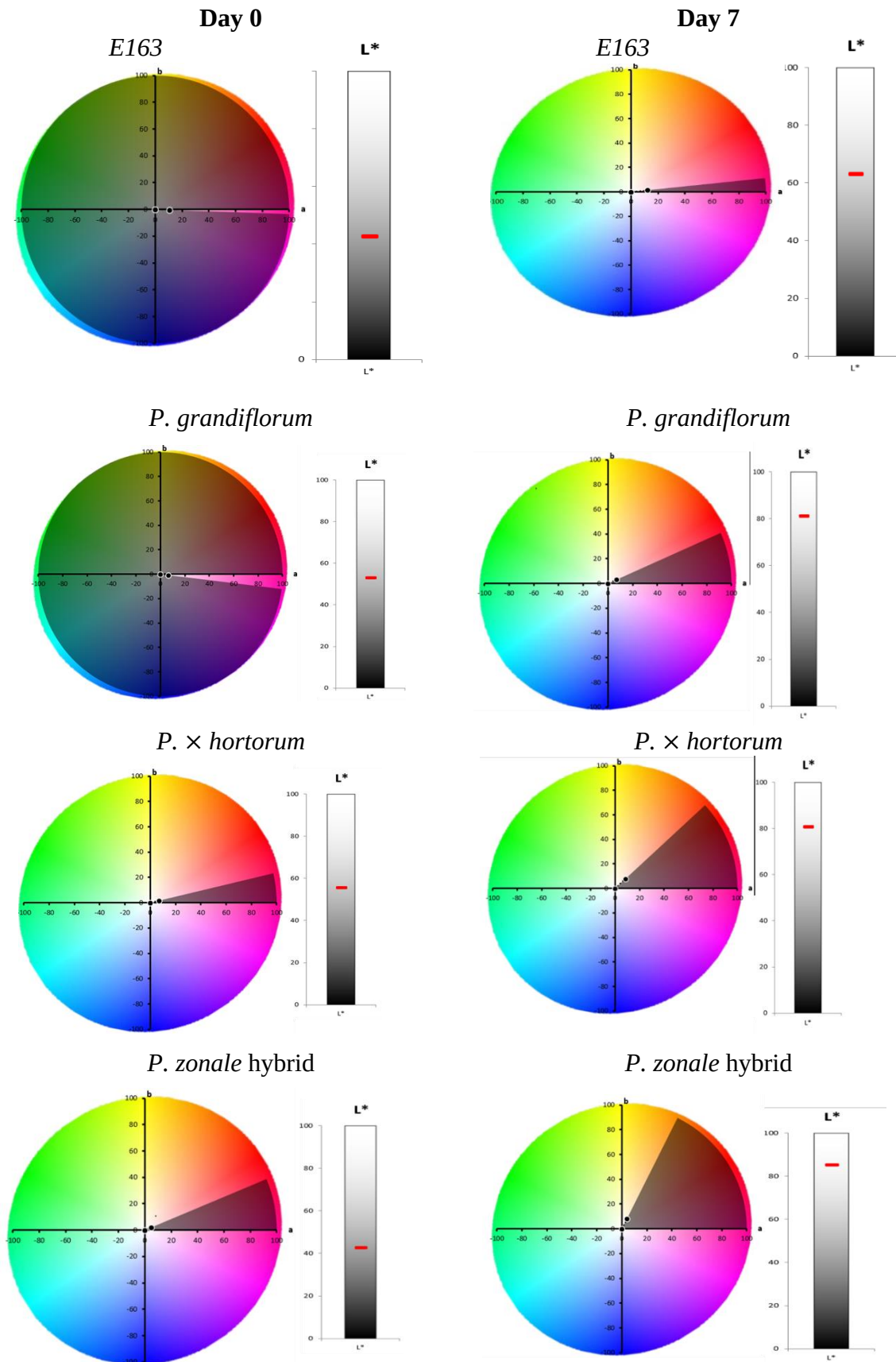
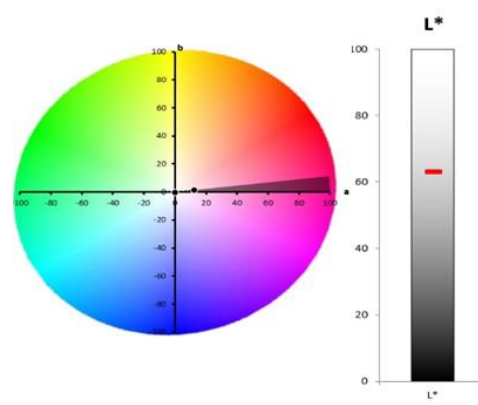
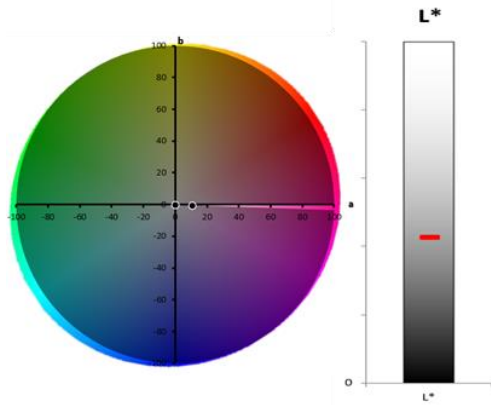
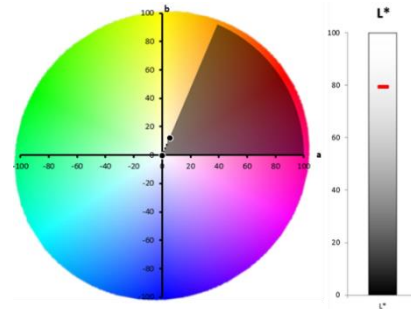
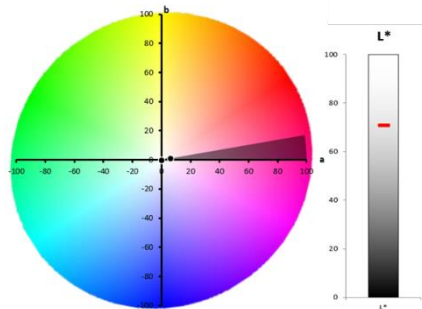


Figure 7.1 Chromaticity diagrams of yogurts incorporated with flower powder from the Geraniaceae plant family at days 0 and 7 at 4°C



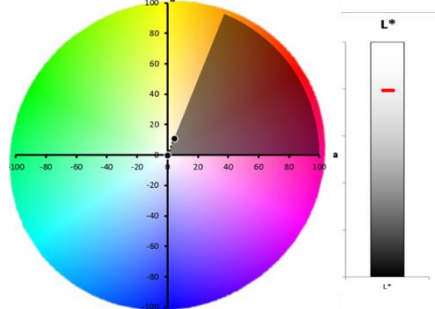
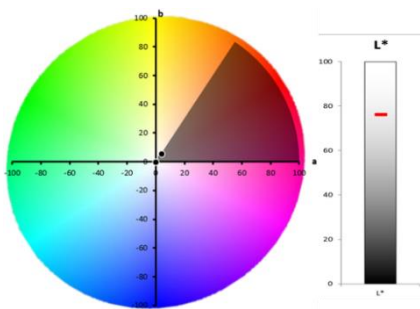
S. aurea × *dolomitica*

S. aurea × *dolomitica*



S. dolomitica

S. dolomitica



P. zuluensis

P. zuluensis

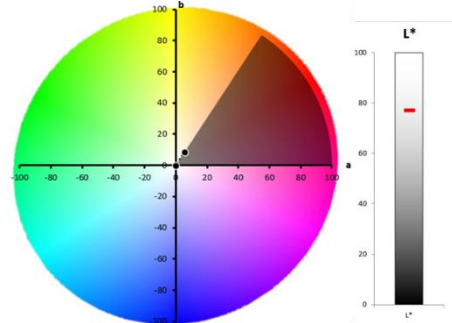
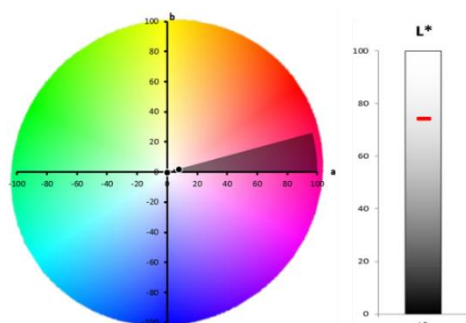


Figure 7.2 Chromaticity diagrams of yogurts incorporated with flower powder from the Lamiaceae plant family at days 0 and 7 at 4°C

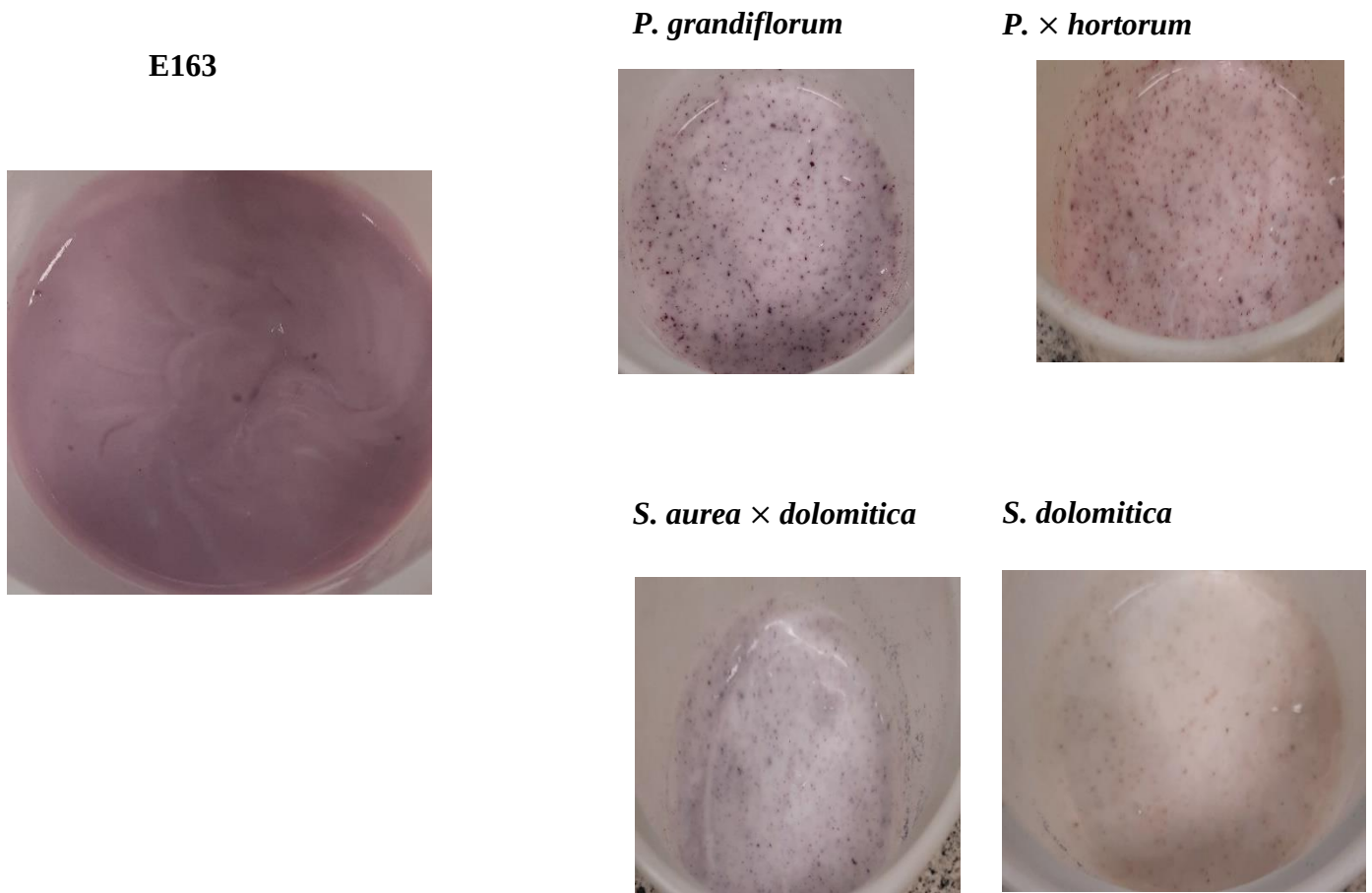


Figure 7.3 Images of yogurts incorporated with a) E163 colourant, b) *P. grandiflorum* powder, c) *P. × hortorum* powder, d) *P. zonale* hybrid powder, e) *S. aurea × dolomitica* powder, f) *S. dolomitica* powder, g) *P. zuluensis* powder at day 0

The colour parameters (L^* , a^* , b^* , C^* , h) and chromaticity diagrams of yogurts incorporated with commercial E163 colourant and pigmented flower extracts from the Geraniaceae and Lamiaceae are shown in Table 7.2 and Figures 7.4, 7.5 and 7.6. All the colour parameters changed significantly ($p \leq 0.05$) over the storage period of 7 days at 4°C.

Generally, the lightness (ΔL^*) of yogurts with E163 increased to a greater extent during storage than yogurts incorporated with pigmented flower extracts. The yogurt with *P. grandiflorum* from the Geraniaceae which produced a greater increase in lightness than yogurt with E163 was an exception in this regard. There was a decrease in redness (Δa^*) of yogurts incorporated with pigmented flower extracts during storage while yogurts with E163 colourant showed a small increase in redness during storage. The decrease in the redness of yogurts with pigmented flower extracts was accompanied by an increase in yellowness (Δb^*) which could be attributed to anthocyanin degradation (Quan et al., 2019). Despite this potential anthocyanin degradation, the yogurts incorporated with flower extracts generally showed lower change in total colour (ΔE^*) compared to yogurts with E163 commercial colourant (again, yogurt with *P. grandiflorum* from the Geraniaceae being an exception) possibly due to the smaller changes in lightness (ΔL^*). Overall, the yogurts incorporated with pigmented flower extracts from the three species of the Lamiaceae showed lower total decrease in redness (−6.56) as compared to the yogurts incorporated with pigmented flower extracts from the three species of the Geraniaceae (−9.94). This could be attributed to higher anthocyanin stability of the Lamiaceae pigmented extracts. As explained earlier based on results from the thermal and oxidative degradation kinetics studies, the superior stability of pigmented extracts from the Lamiaceae could be due to hydrophobic intramolecular self-stacking of the coumaroyl residues on the aromatic acylated anthocyanins as well as the proposed intermolecular interactions between aromatic acylated anthocyanins and rosmarinic acid and its derivatives sagerinic acid and salvianolic acid which were identified only in pigmented flower extracts of the Lamiaceae.

Overall, the pigmented flower extracts and petal powders of both families imparted a variety of colours to the yogurt ranging from blue berry purple, red and orange depending on the colour of the flowers. The flower extracts seem to perform better than the flower powder as colourants in yogurts in terms of the imparted colour and its stability. The performance of the flower powders as colourants in yogurt seems to be compromised by the potential enzymatic browning. On the other hand, even though enzymatic browning is not an issue when the pigmented extracts are used, the imparted colour is weak due to the fact that these are aqueous

pigmented extracts. The preparation of aqueous pigmented extracts of higher concentration for use as colourants could be a potential solution.

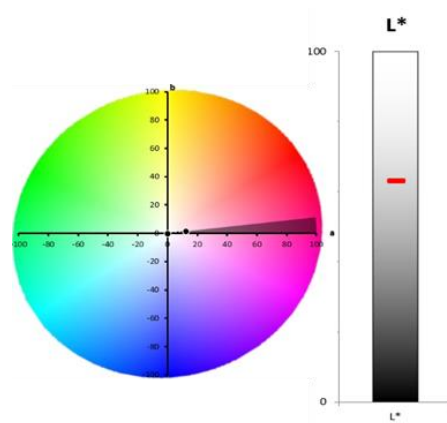
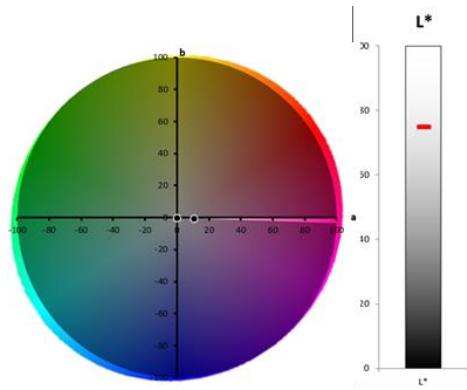
Other workers have also studied incorporation of pigmented flower extracts into yogurt as colourants. Pires, Dias, Barros, Barreira, Santos-Buelga and Ferreira (2018) incorporated different flower extracts (Rose, corn flower and *Dahlia*) into yogurt and compared the colour parameters to the commercial E163 colourant. They found that the rose extract showed comparable parameters in terms of colour and nutritional composition to the E163 colourant. Amr and Al-Tamimi (2007) used flower extracts of Persian buttercup (*Ranunculus asiaticus*) for application in yogurt. They reported that the flower extracts gave the yogurt a blue berry coloured appearance which were attributed to delphinidin.

Díaz-García, Castellar, Obón, Obón, Alcaraz and Rivera (2015) used flower extracts of *Thymus moroderi* (Lamiaceae) for application in yogurt and compared it to two commercial E163 colourants from grape skin and red carrot. The researchers found that yogurts incorporated with the flower extracts showed high colour strength and had pink tonalities. However, the grape skin and red commercial E163 colourants showed superior stability than the *T. moroderi* flower extract incorporated yogurts after 1 month of storage at 4°C.

Table 7.2 Effect of storage for 7 days at 4°C on colour parameters of yogurts with commercial E163 colourant and pigmented flower extracts from plant species of the Geraniaceae and Lamiaceae families applied as colourants

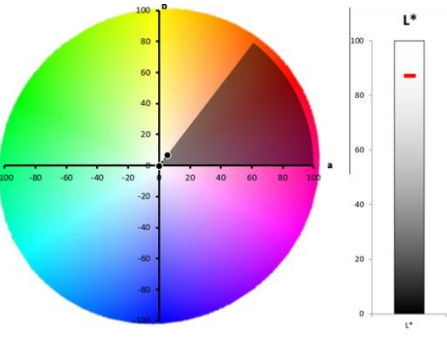
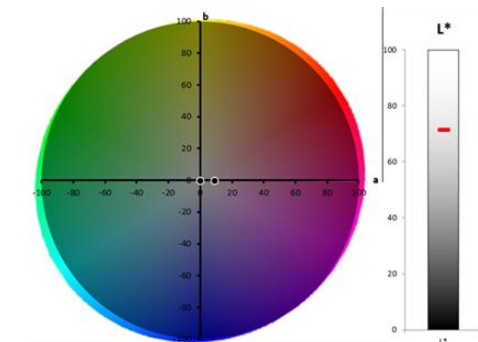
Geraniaceae										Lamiaceae					
Colourant E163			<i>P. grandiflorum</i>		<i>P. × hortorum</i>		<i>P. zonale</i> hybrid		<i>S. aurea</i>	×	<i>S. dolomitica</i>		<i>P. zuluensis</i>		
<i>dolomitica</i>															
Days	0	7	0	7	0	7	0	7	0	7	0	7	0	7	
<i>L</i> [*]	62.89 ^{Aa}	74.77 ^{Bb}	71.43 ^{Ca}	87.00 ^{Db}	86.12 ^{Ea} ±	86.80 ^{Fa} ±	83.22 ^{Ea}	88.63 ^{Fb}	82.79 ^{Ea}	87.27 ^{Fa}	80.62 ^{Ea}	89.22 ^{Fb} ±	81.65 ^{CEa}	85.53 ^{DFa}	
	± 0.90	± 0.26	± 5.97	± 1.53	0.92	0.37	± 3.08	± 0.54	± 1.76	± 0.97	± 5.63	0.96	± 0.92	± 0.73	
<i>a</i> [*]	10.67 ^{Fa} ±	12.35 ^{Ea}	9.06 ^{Db} ±	5.23 ^{Ca} ±	9.49 ^{Db} ±	5.87 ^{Ca} ±	7.82 ^{Db} ±	5.32 ^{Ca} ±	8.04 ^{Db} ±	4.93 ^{Ca} ±	3.38 ^{Ba} ±	3.27 ^{Fa} ±	8.78 ^{Db} ±	5.42 ^{Ca} ±	
	0.12	± 0.07	1.75	0.37	2.33	0.27	0.83	0.02	0.74	0.26	0.74	0.05	0.49	0.12	
<i>b</i> [*]	-0.37 ^{Ba} ±	1.40 ^{Ab} ±	-0.08 ^{Aa} ±	6.79 ^{Bb} ±	6.44 ^{Ea} ±	8.71 ^{Fb} ±	5.91 ^{Ea} ±	8.11 ^{Fb} ±	1.67 ^{Aa} ±	5.44 ^{Bb} ±	5.62 ^{Ea} ±	10.08 ^{Fb} ±	2.49 ^{Ca} ±	7.89 ^{Db} ±	
	0.15	0.04	1.43	0.68	0.17	0.47	0.80	0.05	0.62	0.45	0.43	0.52	0.70	0.35	
<i>C</i> [*]	10.67 ^{Da}	12.43 ^{Db}	9.16 ^{ABa}	8.57 ^{ABa}	11.55 ^{CDa}	10.50 ^{CDa}	9.87 ^{CDa}	9.70 ^{CDa}	8.24 ^{Aa} ±	7.35 ^{Aa} ±	6.56 ^{ABa}	10.60 ^{ABb}	9.15 ^{ABCa}	9.58 ^{ABCa}	
	± 0.12	± 0.07	± 1.77	± 0.77	± 1.90	± 0.54	± 0.28	± 0.04	0.66	0.51	± 0.50	± 0.51	± 0.41	± 0.35	
<i>h</i>	357.99 ^{Fb}	6.48 ^{Aa} ±	350.46 ^{Ib}	52.34 ^{Ea} ±	35.20 ^{Ca} ±	56.04 ^{Fb} ±	37.24 ^{Ca}	56.73 ^{Fb}	12.01 ^{Ba}	47.75 ^{Db}	58.94 ^{Ga}	71.97 ^{Hb}	15.88 ^{Ba} ±	55.49 ^{Db} ±	
	± 0.79	0.21	± 0.00	0.84	6.51	0.48	± 6.46	± 0.23	± 4.90	± 0.90	± 0.50	± 0.73	4.66	0.71	
Δ <i>L</i> [*]	11.89		15.58		0.68		5.41		4.48		8.61		3.88		
Δ <i>a</i> [*]	1.68		-3.83		-3.62		-2.49		-3.10		-0.11		-3.35		
Δ <i>b</i> [*]	1.78		6.87		2.28		2.20		3.77		4.46		5.41		
Δ <i>C</i> [*]	1.75		-0.59		-1.04		-0.16		-0.89		4.04		0.42		
Δ <i>E</i> [*]	12.14		17.45		4.33		6.35		6.63		9.70		7.45		

L^{*} (metric lightness difference), *a*^{*} (+ red; - green), *b*^{*} (+ yellow; - blue) *C*^{*} (metric chroma difference), *h* (hue angle), ΔL^* (metric lightness difference), ΔC^* (metric chroma difference), ΔE^* (total colour difference). A,B,C - Mean values in a row with different uppercase superscript letters differ significantly ($p \leq 0.05$); a,b: For each colour parameter, mean values with different lowercase superscript letters differ significantly ($p \leq 0.05$) by pairwise comparison between day 0 and day 7 of the storage period



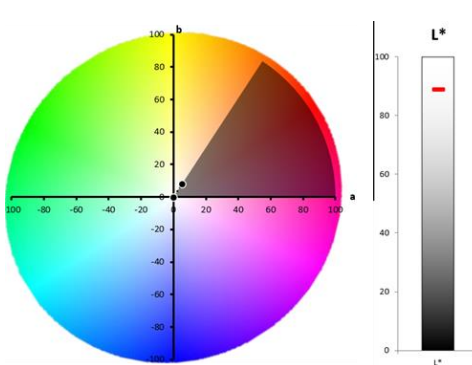
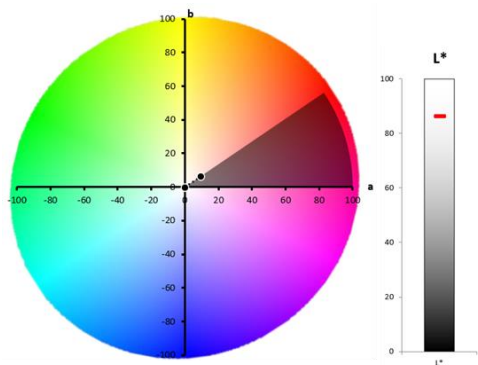
P. grandiflorum

P. grandiflorum



P. × hortorum

P. × hortorum



P. zonale hybrid

P. zonale hybrid

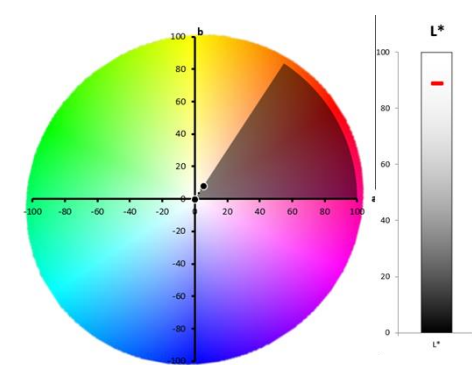
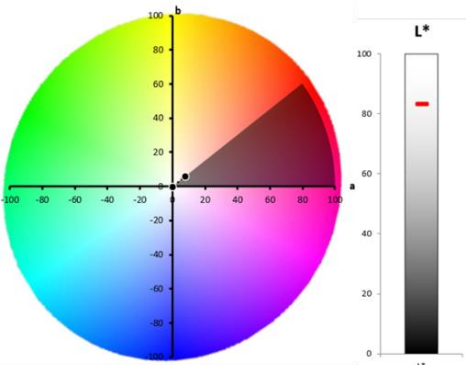
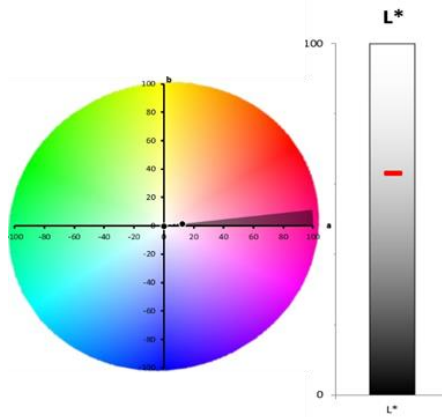
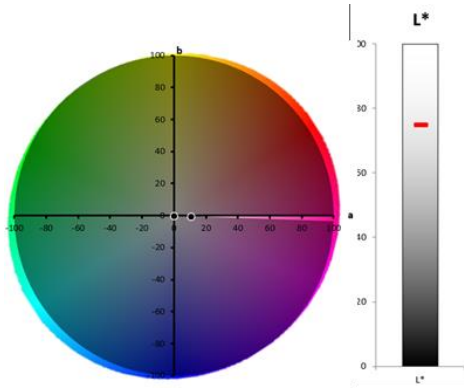
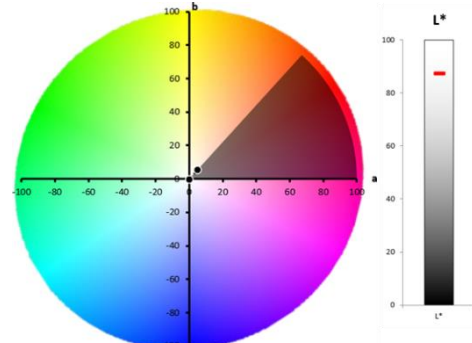
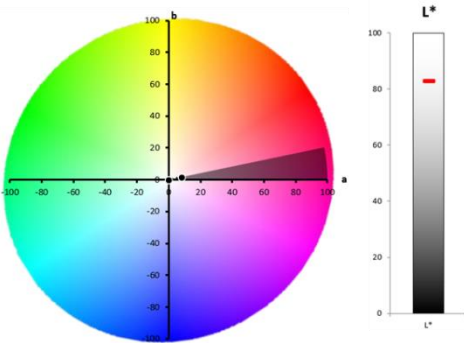


Figure 7. 4 Chromaticity diagrams of yogurts incorporated with flower extracts from the Geraniaceae plant family at days 0 and 7 at 4°C



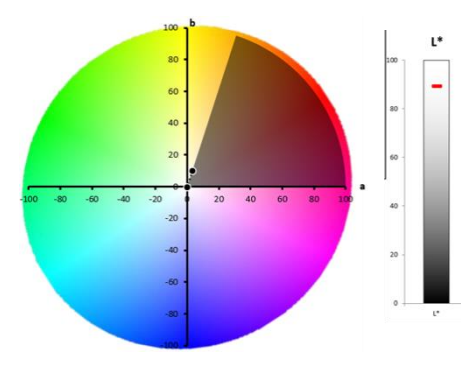
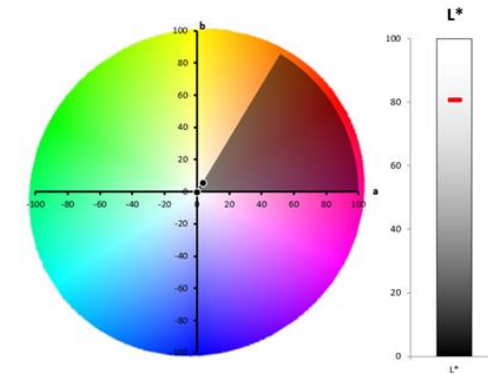
S. aurea × *dolomitica*

S. aurea × *dolomitica*



S. dolomitica

S. dolomitica



P. zuluensis

P. zuluensis

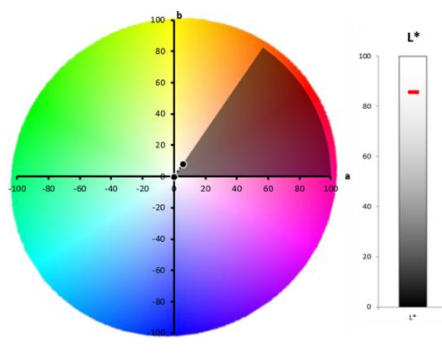
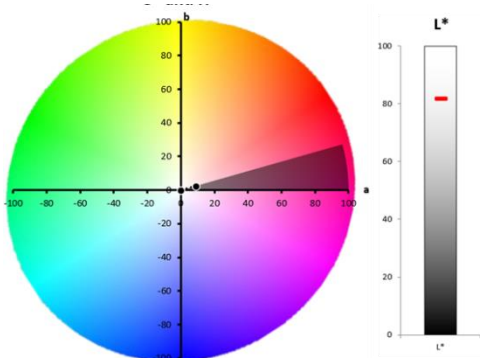


Figure 7.5 Chromaticity diagrams of yogurts incorporated with flower extracts from the Lamiaceae plant family at days 0 and 7 at 4°

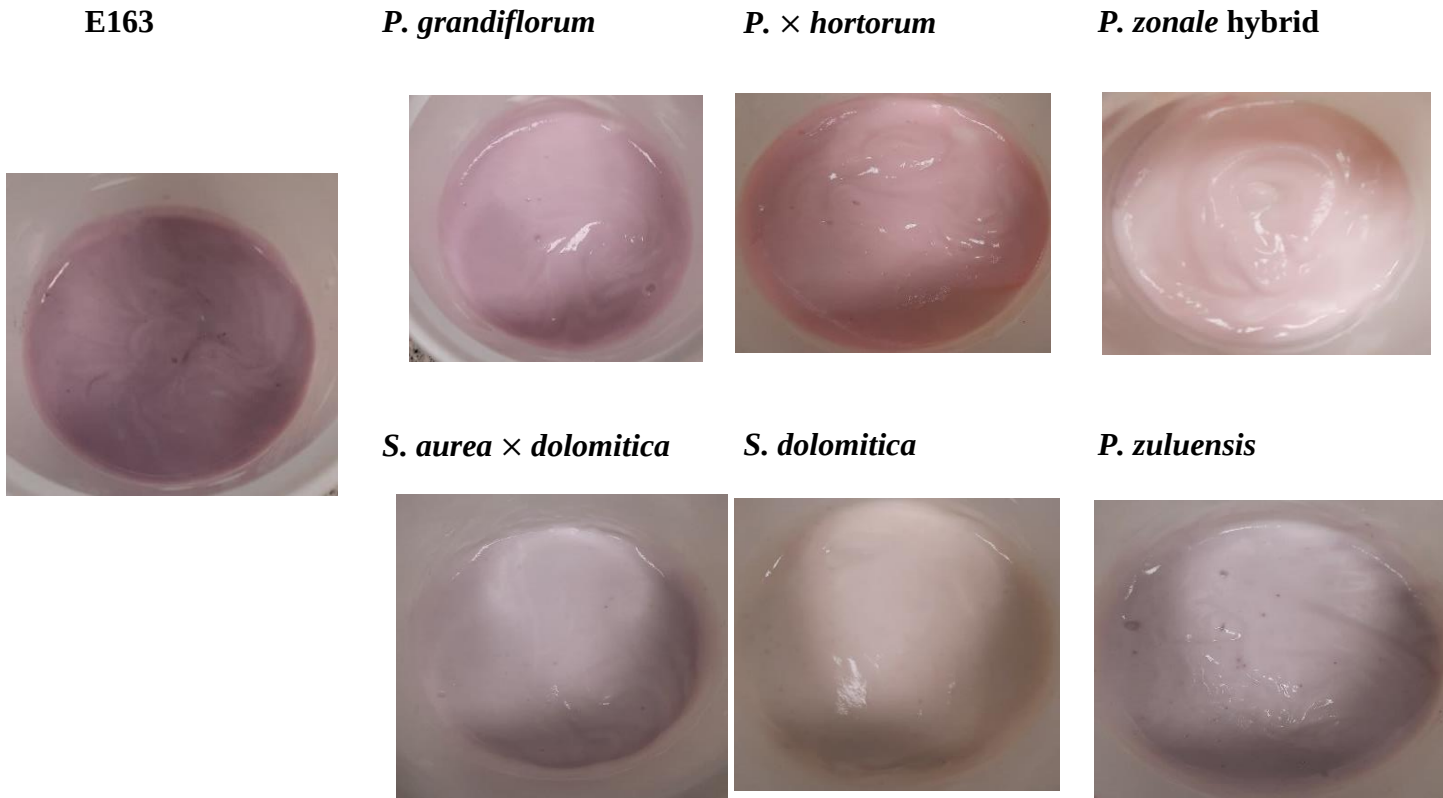


Figure 7.6 Images of yogurts incorporated with a) E163 colourant, b) *P. grandiflorum* powder, c) *P. × hortorum* extract, d) *P. zonale* hybrid extract, e) *S. aurea* × *dolomitica* extract, f) *S. dolomitica* extract, g) *P. zuluensis* extract at day 0

7.3.2 Colour of rolled fondant with pigmented extracts from the Geraniaceae and Lamiaceae plant families and E163 applied as colourants

The application of flower extracts in fondant was done to investigate their functionality in colouring of sugar-based food systems. The colour parameters (L^* , a^* , b^* , C^* , h), chromaticity diagrams and images of rolled fondant incorporated with pigmented flower extracts from the Geraniaceae and Lamiaceae plant families and commercial anthocyanin E163 colourant are shown in Table 7.3, Figures 7., 7.8 and 7.9, respectively.

All pigmented flower extract-incorporated fondant and E163-incorporated fondant generally showed significant ($p \leq 0.05$) changes in the colour parameters during storage for 15 days at room temperature except for lightness (L^*). Generally, over the 15-day storage period, fondant incorporated with pigmented flower extracts showed lower increases in lightness (L^*) and lower decreases in redness (Δa^*) compared to the fondant with E163 colourant. It must be noted that fondant with extracts from *P. zonale hybrid* and *S. dolomitica* showed rather small decreases in lightness although their actual L^* values were much higher than those of the fondant with E163.

More substantively, the observed lower increases in lightness over the storage period is an indication that the fondant with pigmented flower extracts exhibited lower loss of colour over the 15-day storage period, possibly due to lower anthocyanin degradation compared to fondant with E163. This is also reflected in the observation that fondant with the pigmented flower extracts showed a lower change in total colour (ΔE^*) as compared to the E163-coloured rolled fondant. Nonetheless, the fondant samples generally became less colourful {decreased chroma (ΔC^*)} over the 15-day storage period, had overall decreased redness and increase in yellowness (Δb^*).

These results indicate that the sugar-based system of the fondant is a good vehicle for the application of the pigmented extracts as natural colourants and may suggest a potential role played by the sugar in contributing to stability of the pigments. Wrolstad, Skrede, Lea and Enersen (1990) reported that sucrose could aid in anthocyanin stability by inhibition of condensation reactions involving anthocyanins. The pigmented flower extracts which were acidic in nature showed superior stability compared to the E163 colourant in the sugar system of the fondant and provides a variety of colours as seen in Figure 7.8. The anthocyanin composition of the flower extracts is what ultimately determines the overall colour at low pH. The flower extracts provide colours ranging from blue berry purple, red, pink to orange. The

P. zonale hybrid had an orange colour at acidic pH and might therefore substitute as a water-soluble orange commercial colourant.

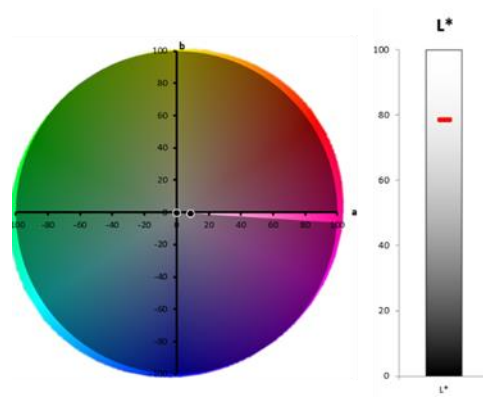
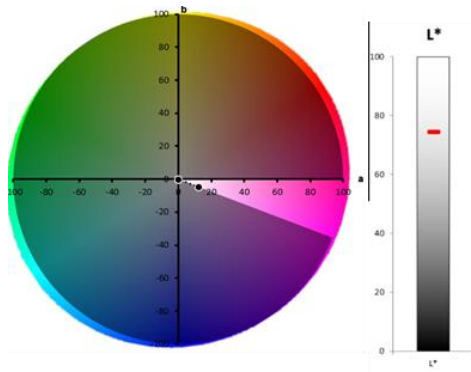
Table 7.3 Effect of storage for 15 days at room temperature on rolled fondant with commercial E163 colourant and pigmented flower extracts of plant species from the Geraniaceae and Lamiaceae families applied as colourants

	Geraniaceae								Lamiaceae					
	Commerical colourant E163		<i>P. grandiflorum</i>		<i>P. × hortorum</i>		<i>P. zonale hybrid</i>		<i>S. aurea × dolomitica</i>		<i>S. dolomitica</i>		<i>P. zuluensis</i>	
Days	0	15	0	15	0	15	0	15	0	15	0	15	0	15
<i>L</i> [*]	74.45 ^{Aa} ± 0.10	78.50 ^{Bb} ± 0.19	86.56 ^{Ba} ± 0.12	86.77 ^{Ca} ± 0.36	85.87 ^{Ba} ± 0.04	86.64 ^{Ca} ± 0.20	90.35 ^{Ia} ± 0.03	89.62 ^{Ha} ± 0.58	88.60 ^{Da} ± 0.05	88.83 ^{Ea} ± 0.60	91.94 ^{Fb} ± 0.31	90.29 ^{Ga} ± 0.86	89.39 ^{Ia} ± 0.47	90.21 ^{Hb} ± 0.06
<i>a</i> [*]	12.32 ^{Lb} ± 0.03	8.30 ^{Ha} ± 0.12	8.80 ^{Jb} ± 0.04	6.63 ^{Fa} ± 0.15	13.20 ^{Mb} ± 0.05	10.85 ^{Ka} ± 0.26	9.05 ^{Jb} ± 0.07	6.59 ^{Fa} ± 0.21	8.80 ^{Ib} ± 0.07	5.68 ^{Da} ± 0.25	5.19 ^{Bb} ± 0.11	4.88 ^{Aa} ± 0.07	7.48 ^{Gb} ± 0.17	5.60 ^{Ca} ± 0.03
<i>b</i> [*]	-4.69 ^{Aa} ± 0.23	-0.53 ^{Bb} ± 0.11	1.06 ^{Ca} ± 0.06	2.78 ^{Db} ± 0.48	3.85 ^{Ea} ± 0.03	4.71 ^{Fb} ± 0.19	6.14 ^{Ia} ± 0.03	7.13 ^{Jb} ± 0.37	2.92 ^{Ea} ± 0.02	4.68 ^{Fb} ± 0.48	7.29 ^{Ia} ± 0.25	7.46 ^{Ja} ± 0.04	4.98 ^{Ga} ± 0.05	7.47 ^{Hb} ± 0.04
<i>C</i> [*]	13.19 ^{Kb} ± 0.05	8.32 ^{Ca} ± 0.11	8.87 ^{Db} ± 0.03	7.20 ^{Aa} ± 0.06	13.75 ^{Lb} ± 0.04	11.83 ^{Ja} ± 0.16	10.94 ^{Ib} ± 0.07	9.71 ^{Ha} ± 0.13	9.27 ^{Gb} ± 0.06	7.38 ^{Ba} ± 0.12	8.95 ^{Ea} ± 0.25	8.91 ^{Fa} ± 0.07	8.98 ^{Ea} ± 0.13	9.34 ^{Fb} ± 0.01
<i>h</i>	339.18 ^{Ka} ± 0.99	356.36 ^{Lb} ± 0.82	6.86 ^{Aa} ± 0.41	22.70 ^{Db} ± 4.01	16.26 ^{Ca} ± 0.17	23.49 ^{Eb} ± 1.35	34.16 ^{Fa} ± 0.10	47.25 ^{Hb} ± 2.35	18.38 ^{Ba} ± 0.26	39.42 ^{Gb} ± 4.10	54.52 ^{Ia} ± 0.81	56.80 ^{Ja} ± 0.25	33.66 ^{Fa} ± 0.73	53.12 ^{Hb} ± 0.26
ΔL^*	4.05		0.21		0.76		-0.73		0.23		-1.65		0.83	
Δa^*	-4.02		-2.18		-2.35		-2.47		-3.12		-0.31		-1.87	
Δb^*	4.16		1.72		0.86		1.69		1.76		0.17		2.49	
ΔC^*	-4.87		-1.66		-1.92		-1.23		-1.90		-0.04		0.36	
ΔE^*	7.06		2.78		2.61		3.08		3.59		1.68		3.23	

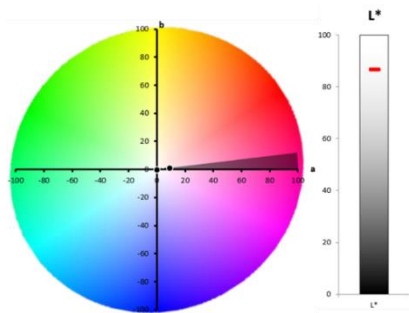
L^{*} (metric lightness), *C*^{*} (metric chroma), *h* (hue angle), ΔL^* (metric lightness difference), ΔC^* (metric chroma difference), ΔE^* (total colour difference), A,B,C - Mean values in a row with different uppercase superscript letters differ significantly ($p \leq 0.05$); a,b: For each colour parameter, mean values with different lowercase superscript letters differ significantly ($p \leq 0.05$) by pairwise comparison between day 0 and day 7 of the storage period.

Day 0
E163

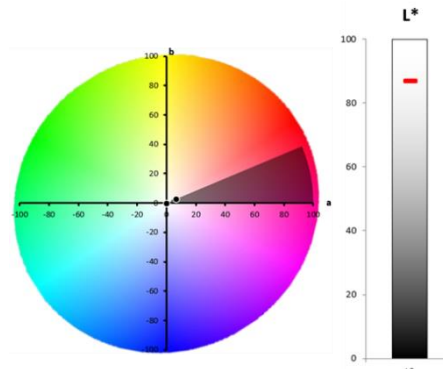
Day 15
E163



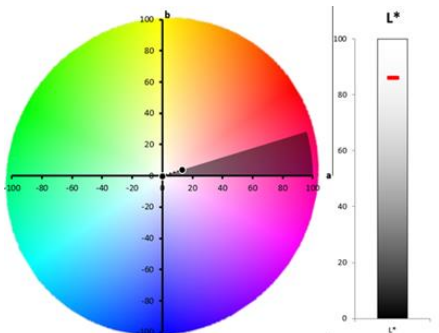
P. grandiflorum



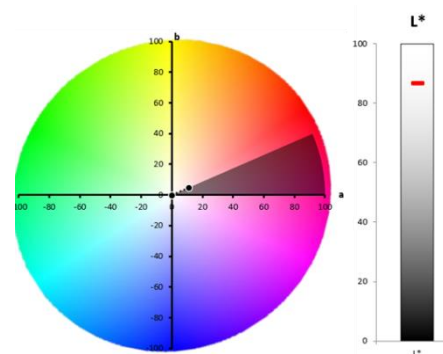
P. grandiflorum



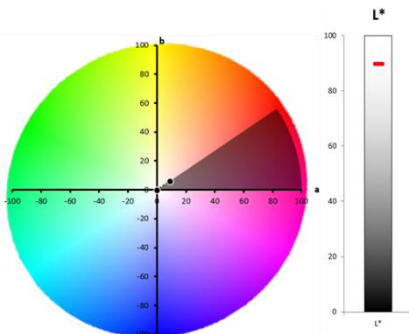
P. × hortorum



P. × hortorum



P. zonale hybrid



P. zonale hybrid

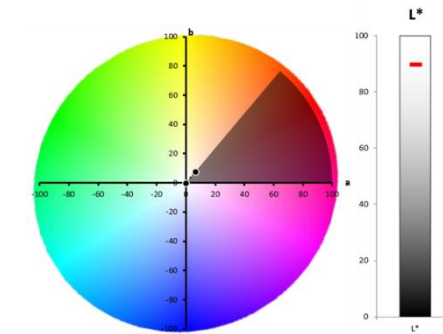
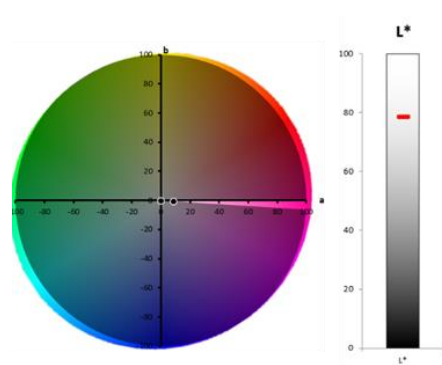
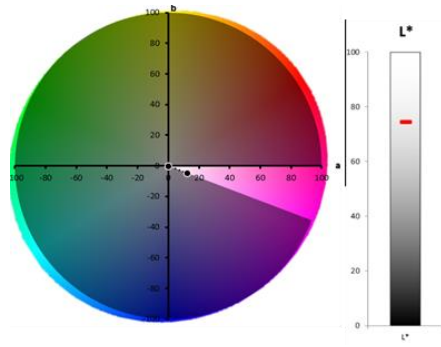


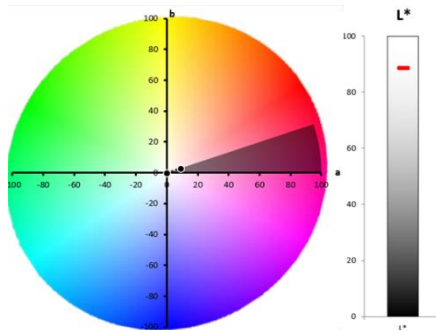
Figure 7.7 Chromaticity diagrams of rolled fondant incorporated with pigmented flower extracts from the Geraniaceae plant family at day 0 and 15 at room temperature

Day 0
E163

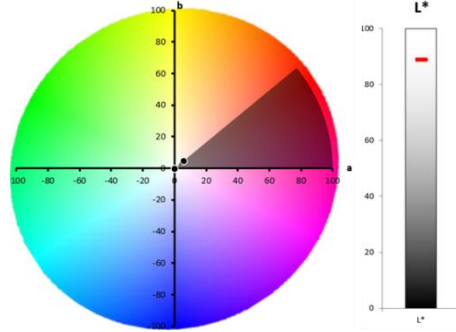
Day 15
E163



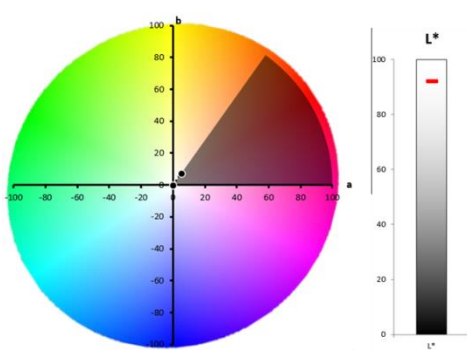
S. aurea × *dolomitica*



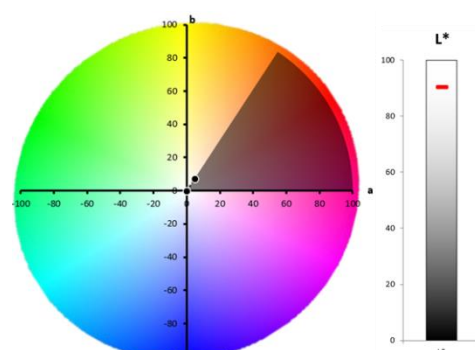
S. aurea × *dolomitica*



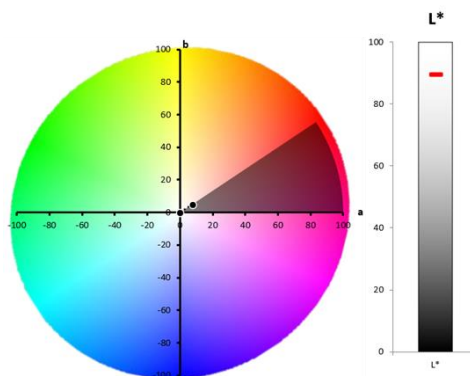
S. dolomitica



S. dolomitica



P. zuluensis



P. zuluensis

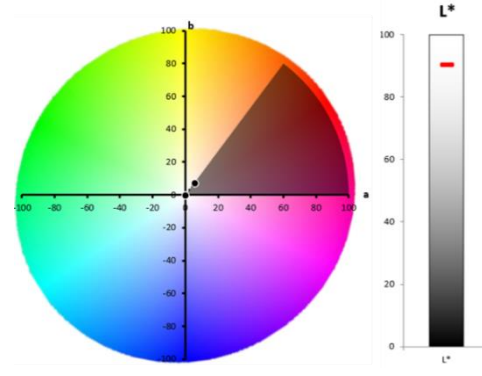


Figure 7.8 Chromaticity diagrams of rolled fondant incorporated with pigmented flower extracts from the Lamiaceae plant family at day 0 and 15 at room temperature

Geraniaceae

E163

P. grandiflorum

P. × hortorum

P. zonale hybrid



Lamiaceae

S. aurea × *dolomitica*

S. dolomitica

P. zuluensis



Figure 7.9 Rolled fondant treated with commercial E163 colourant and pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

7.3.3 Colour of gelatine candies with pigmented extracts from the Geraniaceae and Lamiaceae plant families and E163 applied as colourants

The flower extracts were incorporated into a gelatine candy to assess its success when applied as colourants in a protein-based system. The colour parameters (L^* , a^* , b^* , C^* , h), chromaticity diagrams and images of gelatine candies with pigmented flower extracts from the Geraniaceae and Lamiaceae plant families and commercial colourant E163 during storage for 7 days at room temperature are shown in Table 7.4 and Figures 7.10, 7.11 and 7.12 respectively.

All colour parameters for gelatine incorporated with pigmented flower extracts and E163 generally showed significant ($p \leq 0.05$) changes during storage for 7 days at room temperature. The gelatine incorporated with E163 generally showed higher change in lightness (ΔL^*) compared to the gelatine incorporated with pigmented flower extracts. The gelatine incorporated with *P. × hortorum* (Geraniaceae), *S. dolomitica* and *P. zuluensis* (Lamiaceae) pigmented flower extracts showed smaller decreases in redness (Δa^*) compared to the E163 colourant while gelatine incorporated with *P. grandiflorum*, *P. zonale* hybrid (Geraniaceae) and *S. aurea × dolomitica* (Lamiaceae) pigmented flower extracts showed relatively larger decreases in redness. While gelatine incorporated with *P. × hortorum*, *P. zonale* hybrid (Geraniaceae) and *S. dolomitica* (Lamiaceae) showed decreases in yellowness (Δb^*) during storage, gelatine incorporated with E163 and the other pigmented flower extracts increased in yellowness over the storage period.

The flower extracts showed a wide range of colours when incorporated into the gelatine food system whereas the E163 commercial colourant only provided a deep purple colour as seen in Figure 7.11. The colour variety ranged from deep purple (*P. grandiflorum*), dark red (*P. × hortorum*), dark orange (*P. zonale* hybrid) to light purple (*S. aurea × dolomitica*, *S. dolomitica* and *P. zuluensis*). These results show that in addition to the fondant, the gelatine also served as a good vehicle for the colourants. As observed for the fondant, the orange colour exhibited by the *P. zonale* hybrid could possibly be a water-soluble orange colourant for the food industry.

Table 7.4 Effect of storage for 7 days at room temperature on gelatine candies with commercial E163 colourant and pigmented flower extracts of plant species from the Geraniaceae and Lamiaceae families applied as colourants

	Geraniaceae								Lamiaceae							
	Commerical colourant E163		<i>P. grandiflorum</i>		<i>P. × hortorum</i>		<i>P. zonale hybrid</i>		<i>S. aurea</i>	×	<i>S. dolomitica</i>	<i>P. zuluensis</i>		<i>S. dolomitica</i>		
Days	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7
<i>L*</i>	28.50 ^{Aa} ± 2.19	35.99 ^{Ab} ± 6.92	34.51 ^{Aa} ± 1.69	32.56 ^{Aa} ± 1.67	36.65 ^{Aa} ± 2.50	36.09 ^{Aa} ± 2.47	39.86 ^{Ba} ± 1.54	45.73 ^{Bb} ± 2.41	46.30 ^{Ba} ± 0.23	56.14 ^{Bb} ± 0.77	63.41 ^{Cb} ± 2.10	53.58 ^{Ca} ± 0.08	50.30 ^{Ba} ± 2.89	53.08 ^{Ba} ± 0.84		
<i>a*</i>	7.13 ^{Bb} ± 2.02	2.37 ^{Aa} ± 0.79	18.82 ^{Db} ± 3.16	11.25 ^{Ca} ± 3.22	23.49 ^{Fa} ± 0.93	21.94 ^{Ea} ± 1.86	24.17 ^{Fb} ± 2.04	17.82 ^{Ea} ± 0.12	21.65 ^{Db} ± 0.57	4.66 ^{Ca} ± 0.39	4.09 ^{Ba} ± 0.51	2.45 ^{Aa} ± 0.07	6.34 ^{Ba} ± 0.07	4.58 ^{Aa} ± 0.17		
<i>b*</i>	-4.28 ^{Aa} ± 0.41	-2.17 ^{Ba} ± 1.02	-2.49 ^{Cb} ± 0.57	-2.35 ^{Da} ± 0.13	8.47 ^{Hb} ± 1.50	5.26 ^{Ga} ± 1.07	13.07 ^{Jb} ± 2.27	8.55 ^{Ia} ± 0.40	-2.46 ^{ACa} ± 0.37	-0.88 ^{BbDa} ± 0.27	8.93 ^{Ha} ± 0.77	8.02 ^{Ga} ± 0.06	1.64 ^{Ea} ± 0.38	4.84 ^{Fb} ± 0.36		
<i>C*</i>	8.35 ^{Bb} ± 1.91	3.22 ^{Aa} ± 1.26	19.00 ^{Fb} ± 3.12	11.52 ^{Ea} ± 3.13	24.99 ^{Ha} ± 1.38	22.57 ^{Ga} ± 2.04	27.50 ^{Hb} ± 2.85	19.77 ^{Ga} ± 0.28	21.79 ^{Fb} ± 0.54	4.75 ^{Ea} ± 0.42	9.83 ^{Da} ± 0.91	8.39 ^{Ca} ± 0.08	6.56 ^{Ba} ± 0.15	6.67 ^{Aa} ± 0.36		
<i>h</i>	327.65 ^{Jb} ± 6.02	320.05 ^{Ia} ± 6.74	7.80 ^{Ga} ± 2.31	347.11 ^{Hb} ± 4.11	19.70 ^{Ba} ± 2.56	13.35 ^{Aa} ± 1.73	28.17 ^{Da} ± 2.30	25.62 ^{Ca} ± 0.93	353.44 ^{La} ± 1.06	349.45 ^{Ka} ± 2.45	65.47 ^{Ea} ± 0.82	73.02 ^{Fb} ± 0.37	14.46 ^{Ca} ± 3.09	46.50 ^{Db} ± 1.36		
ΔL^*	7.48		-1.95		-0.56		5.87		9.83		-9.83		2.78			
Δa^*	-4.71		-7.57		-1.55		-6.35		-16.99		-1.64		-1.76			
Δb^*	2.11		-4.84		-3.21		-4.52		1.60		-0.91		3.20			
ΔC^*	-5.12		-7.48		-2.42		-7.73		-17.04		-1.44		0.10			
ΔE^*	9.12		9.19		3.61		9.76		19.69		10.01		4.59			

*L** (metric lightness), *C** (metric chroma difference), *E** (total colour difference) and *h* (hue angle), ΔL^* (metric lightness difference), ΔC^* (metric chroma difference), ΔE^* (total colour difference), A,B,C - Mean values in a row with different uppercase superscript letters differ significantly ($p \leq 0.05$); a,b: For each colour parameter, mean values with different lowercase superscript letters differ significantly ($p \leq 0.05$) by pairwise comparison between day 0 and day 7 of the storage period

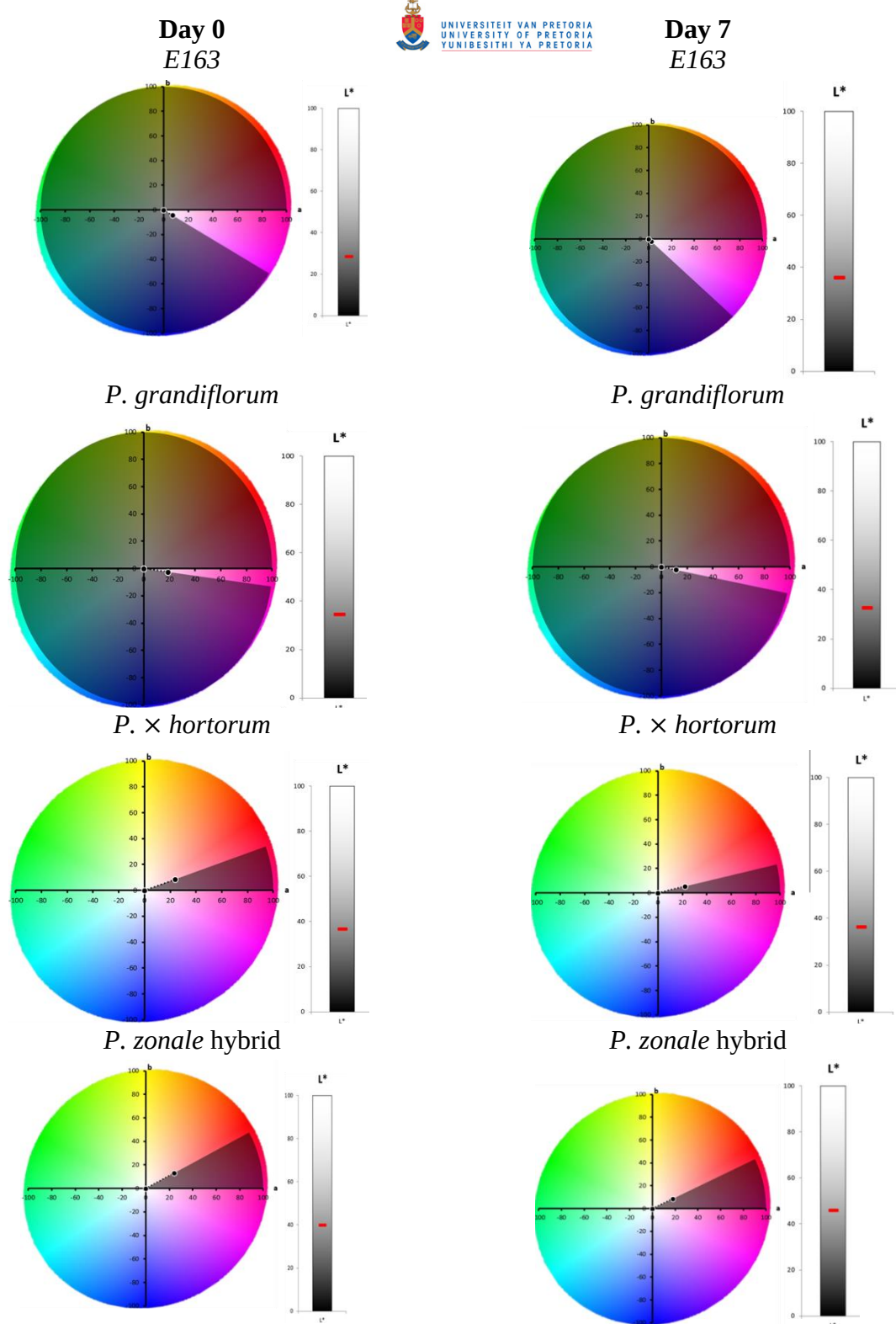


Figure 7.10 Chromaticity diagrams of gelatine candies incorporated with pigmented flower extracts from the Geraniaceae plant family at day 0 and 7 at room temperature

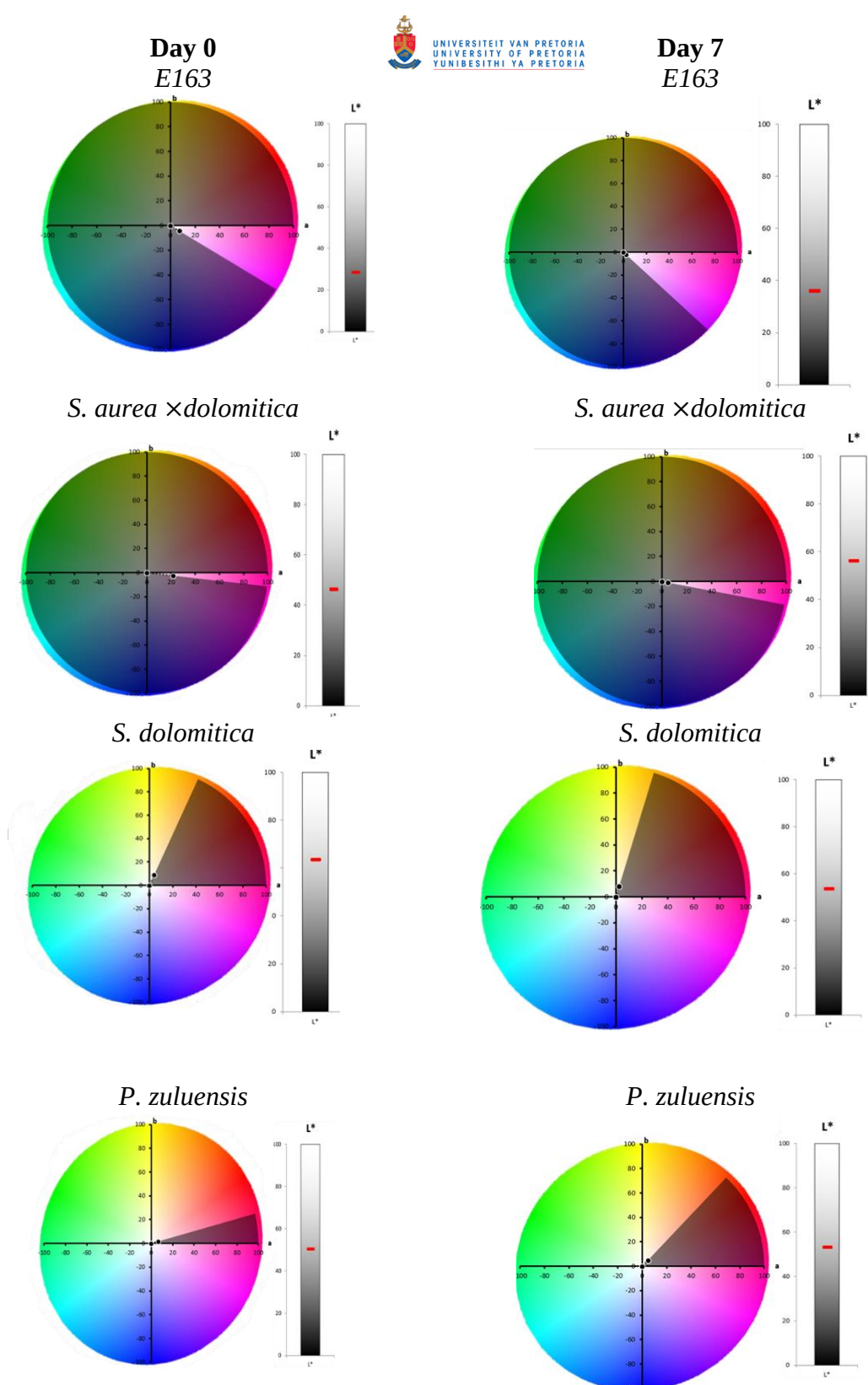


Figure 7.11 Chromaticity diagrams of gelatine candies incorporated with pigmented flower extracts from the Lamiaceae plant family at day 0 and 7 at room temperature

Geraniaceae

P. grandiflorum

P. × hortorum

P. zonale hybrid

E163



Lamiaceae

S. aurea × *dolomitica*

S. dolomitica

P. zuluensis

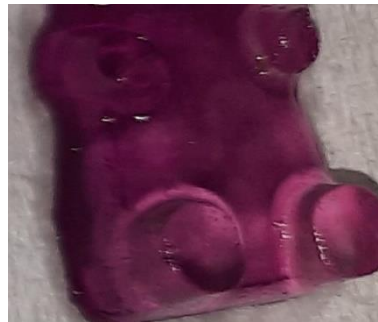


Figure 7.12 Gelatine incorporated with E163 commercial colourant and pigmented flower extracts from the Geraniaceae and Lamiaceae plant families

7.4 Conclusion

Pigmented petal powder and pigmented extracts of flower species from the Geraniaceae and Lamiaceae plant family show a variety of colours when incorporated in yogurt, fondant and gelatine. The flower powder and extracts incorporated in yogurt show colours ranging from pink, blue berry purple and orange compared with a dark purple colour from the E163 colourant. Although the colour of yogurts incorporated with flower extracts is lighter, it tends to be more stable during storage. The application of more concentrated pigmented flower extracts in the yogurt could enhance their colour. The use of flower powder as a colourant in the yogurt produces brown colour formation during the storage period possibly due to residual enzymatic browning. Application of *P. zonale* hybrid (Geraneaceae) pigmented extract in the yogurt, fondant and gelatine imparts an orange-colour to these products which can find use as a water-soluble pigment for the food industry. Overall, the yogurt, gelatine and fondant are good vehicles for application of the pigmented extracts as colourants. The flower extracts of plant species from both families show comparable stability compared to the commercial E163 colourant and therefore show promise as novel natural food colourants.

Chapter 8: General discussion

8.1 Discussion of research methodologies used

In this study, flowers were collected, petals separated and dried for preservation purposes for the duration of the study. The method for drying was chosen based on preliminary experiments to as best as possible retain the colour of the flowers and as a result, the anthocyanin content. Freeze drying was tested for preservation of colour based on previous research on drying of flowers. Chen, Gast and Smithey (2000) showed that freeze-drying preserved the colour of rose and coronation flower petals close to that of fresh flowers. Freeze-drying in the preliminary experiments in this study caused browning of flower petals possibly due to enzymatic activity or change in pH caused by rapid moisture loss during freeze-drying. Oven drying at 30°C overnight proved to be best for preservation of colour in flower petals. Oven drying allows for slow moisture loss thus resulting in better preservation of colour.

For extraction of anthocyanins from dried pigmented flower petal powder, acidified water (1% formic acid) was chosen based on preliminary experiments. Although acidified methanol is usually preferred for extraction of anthocyanins and has been shown to be 73% more effective in extraction of anthocyanins, water is non-toxic and proved to be very useful as a solvent choice during cell culture studies in this research. In other studies, acetone has been shown to be most efficient in extraction of anthocyanins in frozen strawberry fruit (Garcia-Viguera et al., 1998) but it was anticipated may become problematic during analysis in this research by for example, causing damage to the plastic material of the microplates used. An acidified medium was chosen for extraction as this increases the anthocyanin solubility and stability due to the fact that the flavylium cation form of anthocyanins is favoured in acidic conditions (Khoo, Azlan, Tang and Lim, 2017). Formic acid was chosen due to it being a weak acid and would minimize/prevent hydrolysis of acylated or glycosylated anthocyanins or degradation of anthocyanins and phenolic compounds (Dai and Mumper, 2010).

The total phenolic content (TPC) of pigmented flower extracts was determined using the Folin-Ciocalteu (FC) method. This method was adapted for wine analysis and is now widely used in the determination of total phenolic content due to its convenience and simplicity (Singleton, Orthofer and Lamuela-Raventós, 1999). It is based on the reduction of phosphomolybdate (Mo VI to Mo V) tungstate complexes in alkaline conditions to form a blue chromophore (Folin and

Ciocalteu, 1927). The assay is based on a reduction-oxidation reaction and therefore it is not specific for phenolic compounds. The Folin-Ciocalteu reagent can also react with any non-phenolic reducing substances such as ascorbic acid, sugars and proteins (Waterhouse, 2002) which present a source of interference.

The Folin-Ciocalteu method is unable to identify and quantify individual phenolic compounds, so it was necessary to use a chromatographic method for the characterisation and quantification of individual anthocyanins and phenolic compounds in extracts of pigmented flower species from the Geraniaceae and Lamiaceae plant families. Phenolic compounds were identified and quantified with an LC-MS system consisting of an ultra-performance liquid chromatograph (UPLC) coupled with a Quadrupole Time of Flight (QToF) mass spectrometer with electrospray ionisation in negative mode. Anthocyanins were identified and quantified using an LC-MS system consisting of an ultra-performance liquid chromatography (UPLC) coupled with a Quadrupole Time of Flight (QToF) electrospray ionisation mass spectrometer in positive mode. Quantification of anthocyanins was done by comparing their peak areas with that of external standards which was used to set up a calibration curve.

Pigmented flower extracts for the degradation kinetics studies were prepared to be as concentrated as possible in order to minimize the amount needed due to limited availability of flower samples. The extraction time was extended to 24 hours to allow for maximum diffusion of anthocyanin pigments from the flowers into the extraction solvent. After extraction, the aqueous extract was frozen and freeze-dried. A small defined amount of freeze-dried extract was resuspended in a defined volume of distilled water to give a new concentrated extract.

To assess the stability of pigmented flower extracts at different pH levels, flower extracts were added to solutions of different pH and incubated for 5 minutes and 72 hours respectively. After each incubation time, the ability of the anthocyanins in the pigmented flower extracts to resist pH change were determined spectrophotometrically at 520 nm which is the maximum absorption wavelength for anthocyanins. The method essentially tests the change in anthocyanin structure at changes of pH as the anthocyanins change from the more stable flavylium cation at pH of 1 to the quinoidal base at neutral pH and the anionic quinoidal base at alkaline pH (Brouillard and Dubois, 1977; Brouillard, Delaporte and Dubois, 1978, Brouillard, Iacobucci and Sweeny, 1982). The change in structure of anthocyanins in the flower extract with pH was confirmed with spectral wavelength scans.

The pH (pH = 1) which gave the most stable and most prominent colour of flower extracts was chosen for the degradation kinetics (temperature and oxidative stability) at a maximum absorption wavelength of ~520 nm (Figure 8.1). The final concentrations of extracts were so chosen to factor in sample availability. A wide range of temperatures and hydrogen peroxide concentrations were selected. The temperature range selected simulates different processing temperatures while the different hydrogen peroxide concentrations were selected to simulate different levels of nucleophilic hydroxyl and peroxy radicals generated through H₂O₂ degradation (De, Chaudhuri and Bhattacharjee, 1999).

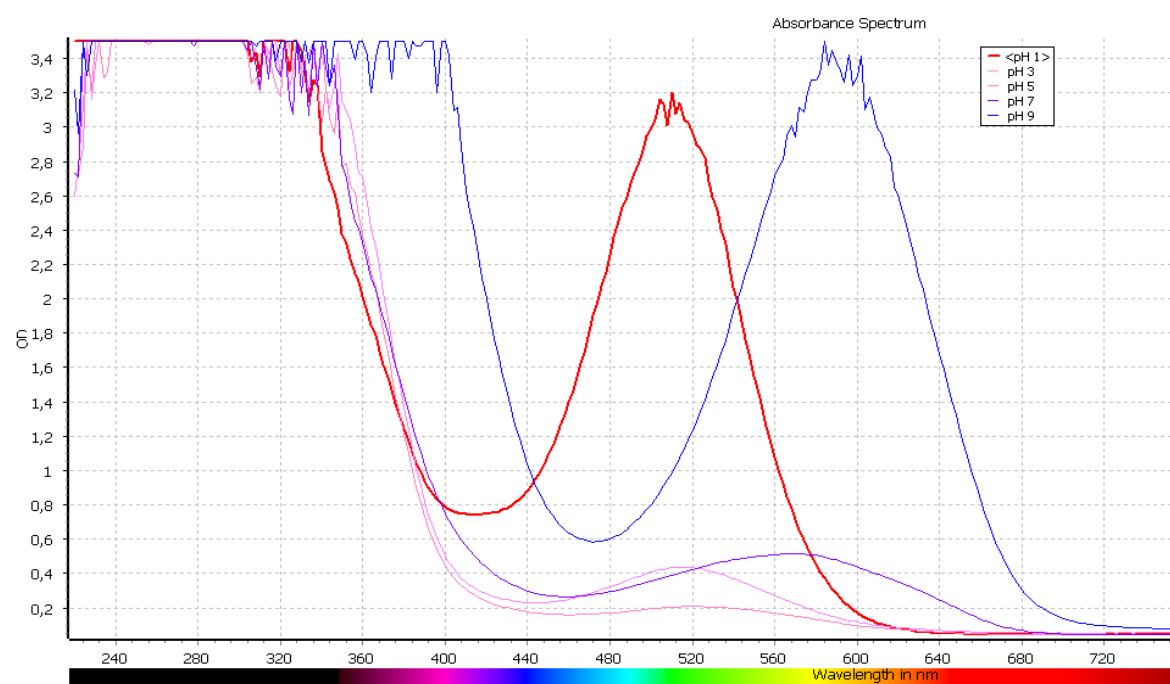


Figure 8.1 Wavelength scan of *P. grandiflorum* (as example) showing pH-dependent absorption maxima at different wavelengths

The DPPH radical scavenging assay was done to assess the radical scavenging ability of pigmented flower extracts in this study. This chemical assay was chosen due to its simplicity, repeatability, high sensitivity and reliability (Shahidi and Zhong, 2015, Romulo, 2020, Moon and Shibamoto, 2009). DPPH is a synthetic radical and thus does not necessarily give a representation of the extracts' radical scavenging ability in biological systems (Nwachukwu, Sarteshnizi, Udenigwe and Aluko, 2021).

Before assessing cellular antioxidant activity, cell cytotoxicity of the pigmented flower extracts was determined to pinpoint an appropriate non-toxic concentration to use for all cellular-based assays. Cellular toxicity was measured using the Crystal Violet (CV) method in 96-well

microplates. CV is a positively charged molecule that binds to negatively charged proteins and DNA of living cells. Dead cells detach from the microplate surface and are lost in the washing step thereby decreasing the amount of stained live cells, giving a quick and reliable method for cell toxicity (Feoktistova, Geserick and Leverkus, 2016). The advantage of the CV method is that large number of cells can be grown in microplates, fixed washed and dried and can be stored and processed at a later stage in comparison with other methods such as the tetrazolium method which requires immediate processing and measurement (Mosmann, 1983).

The Oxygen Radical Absorption Capacity (ORAC) assay was another chemical radical scavenging assay conducted in addition to DPPH radical scavenging. ORAC measures the radical scavenging ability of extracts by assessing the decay of fluorescent intensity of fluorescein, a non-protein fluorescent probe though its reaction with free radicals (Huang, Ou and Prior, 2005). The chemical radical scavenging assays do not always give correlated results due to differences in mechanisms of action and interactions between antioxidants and radicals between assays. It is therefore desirable that different types of radical scavenging assays with different mechanisms of action are conducted (Huang et al., 2005).

The chemical nitric oxide radical scavenging activity was determined using the Griess reagent method. The assay involves the use of sodium nitroprusside which releases NO under physiological conditions. The NO reacts with oxygen to produce nitrite ions which can react with the Griess reagent. Antioxidant compounds that can compete with oxygen for nitric oxide will reduce its production and subsequently less nitrite will cause a lighter pink colour after reaction with the Griess reagent (Marcocci, Maguire, Droy-Lefaix and Packer, 1994). The Griess reagent is nitrite specific and is simplistic, rapid and inexpensive (Tsiklauri, An, Ruszaj, Alaniya, Kemertelidze and Morris, 2011).

For a more biologically relevant assessment of the radical scavenging ability of the pigmented flower extracts, two cellular assays were done namely, cellular antioxidant activity using the Dichlorofluorescein-diacetate (DCFH-DA) assay in Caco-2 cells and nitric oxide radical scavenging activity in RAW 264.7 macrophage cells. In the DCFH-DA assay, non-fluorescent DCFH is formed in cells by the deacetylation of DCFH-DA. Fluorescent DCFH is then initiated through reaction with 2,2-azobis (2-methylpropionamidine) (AAPH) radicals. The assay relates the amount of fluorescence to antioxidant activity through an inverse relationship as radicals cause oxidation of DCFH resulting in fluorescence (Wolfe and Liu, 2007). The nitric oxide radical scavenging activity in RAW 264.7 macrophage cells assesses radical scavenging

and anti-inflammatory activity. Lipopolysaccharide (LPS) is used to induce the production of nitric oxide (NO) in the RAW 264.7 macrophage cells (McAleer and Vella, 2008). The NO radical scavenging activity is then measured using the Griess reagent (Kim, Murakami, Nakamura and Ohigashi, 1998).

Anti-obesity effect was determined by the ability of the pigmented flower extracts to prevent or reduce lipid accumulation in 3T3-cells using the Oil Red O (ORO) staining method. 3T3-L-1 adipocytes were grown and seeded in 96 well microplates. The cells were allowed to differentiate using a cocktail of differentiation media consisting of Insulin, Dexamethasone, IBMX and Rosiglitazone (DM 1) and insulin alone (DM 2). After the assay, the amount of lipid accumulation was determined using ORO staining, which can differentiate between adipocytes and other types of cells. The ORO-stained cells were then fixed and quantified by dissolving the dye in a 60% isopropyl alcohol solution and read at 405 nm in a spectrophotometer. The quantitative results obtained were accompanied by qualitative image analysis which provides a visual representation of the results. Quantification with ORO staining did not produce consistent results which could be due to the poor solubility of the ORO stain. The visual representation however proved to be useful because the spectrophotometric results obtained were sometimes inconsistent with what was observed in the images. This could also be due to the washing step after staining which in some cases visually removed cells. The removal of cells could result in significant changes in absorbance readings (Kraus, Ehebauer, Zapp, Rudolphi, Kraus and Kraus, 2016). However, this may not necessarily be the case always as Klaus et al. (2016) also showed that varying amounts of cells per well still yielded relatively similar absorbance or cell density.

The visual results from the ORO staining were supplemented with Nile Red staining which enables quantification of intracellular oil droplets. Nile Red fluorometrically detects and quantifies lipids and is highly sensitive towards changes in polarity of the environment (Fowler and Greenspan, 1985, Ghoneim, 2000). The Nile Red staining and fluorescence measurements seemed to work better than ORO staining and were able to give more accurate measurements of intracellular oil droplets.

To evaluate the potential of the pigmented extracts of flower species as natural food colourants, three different food systems were used, dairy, sugar and protein. Extractions were done by dissolving a defined amount of flower powder in 1% formic acid: distilled water and extracting for 24 hours. Extracts were then frozen and freeze-dried as previously described to determine

the yield for each flower specie. The yield per weight of each flower specie was used to prepare acidified extracts of similar concentration. Simple recipes were used to make the gelatine candies and fondant. The amount of each pigmented extract applied varied and were applied per drop until a satisfied visual colour change occurred.

8.2 Discussion of key research findings

The anthocyanin compounds identified in the pigmented flower extracts from plants in the two families were largely similar to anthocyanins reported in other plant species of the same families. There were a few exceptions in terms of the mode of acylation of some of the anthocyanins. In this study, *Pelargonium* species (Geraniaceae) were found to contain mainly 3,5-diglucosides of delphinidin, cyanidin, petunidin, peonidin and pelargonidin and acetyl acylated anthocyanins of delphinidin, malvidin, petunidin and peonidin. Similar anthocyanins have been reported in these plant species by Asen (1983) and Mitchell, Markham and Boase (1998). The purple *Salvia* species (Lamiaceae) studied were found to contain mainly rutinoides of pelargonidin, glucosides of petunidin, pelargonidin and p-coumaric acid- and malonyl-acylated delphinidin and malvidin. The dark purple *Plectranthus zuluensis* (Lamiaceae) used in this study contained p-coumaroylglucoside- and malonylglucoside-acylated malvidin derivatives. In contrast, Jordheim, Calcott, Gould, Davies, Schwinn and Andersen (2016) reported malonyl-, caffeic and p-coumaric acid-acylated peonidin derivatives in purple leaves of *P. ciliates* (Lamiaceae). Rutinoides derivatives of pelargonidin, cyanidin and delphinidin have also been reported in 11 different *Salvia* species by Haque (1981) and in 49 species of Lamiaceae flowers by Saito and Harborne (1992).

This is probably the first reported research describing the degradation kinetics of pigmented flower extracts from the Lamiaceae and Geraniaceae plant families in terms of their thermal and oxidative stability. Importantly, this study has highlighted the importance of phenolic acids for stability of anthocyanins in flower extracts.

In this research it has been shown that the degradation of pigments at different temperature and oxidative conditions follow first order kinetics. Solving first order degradation kinetics equations yields the equations in Figure 8.2 (Patras et al., 2010). The graphs show that on average for all the temperature (70, 80 and 90°C) and oxidative (hydrogen peroxide concentrations) (8.15, 16.28 and 24.43 mM) parameters, the decay curves for the Lamiaceae extracts had a less steep slope (indicative of low degradation rate constant k) than those of the

Geraniaceae. This translates to a higher half-life and thus higher thermal and oxidative stability of the Lamiaceae extracts compared to the Geraniaceae.

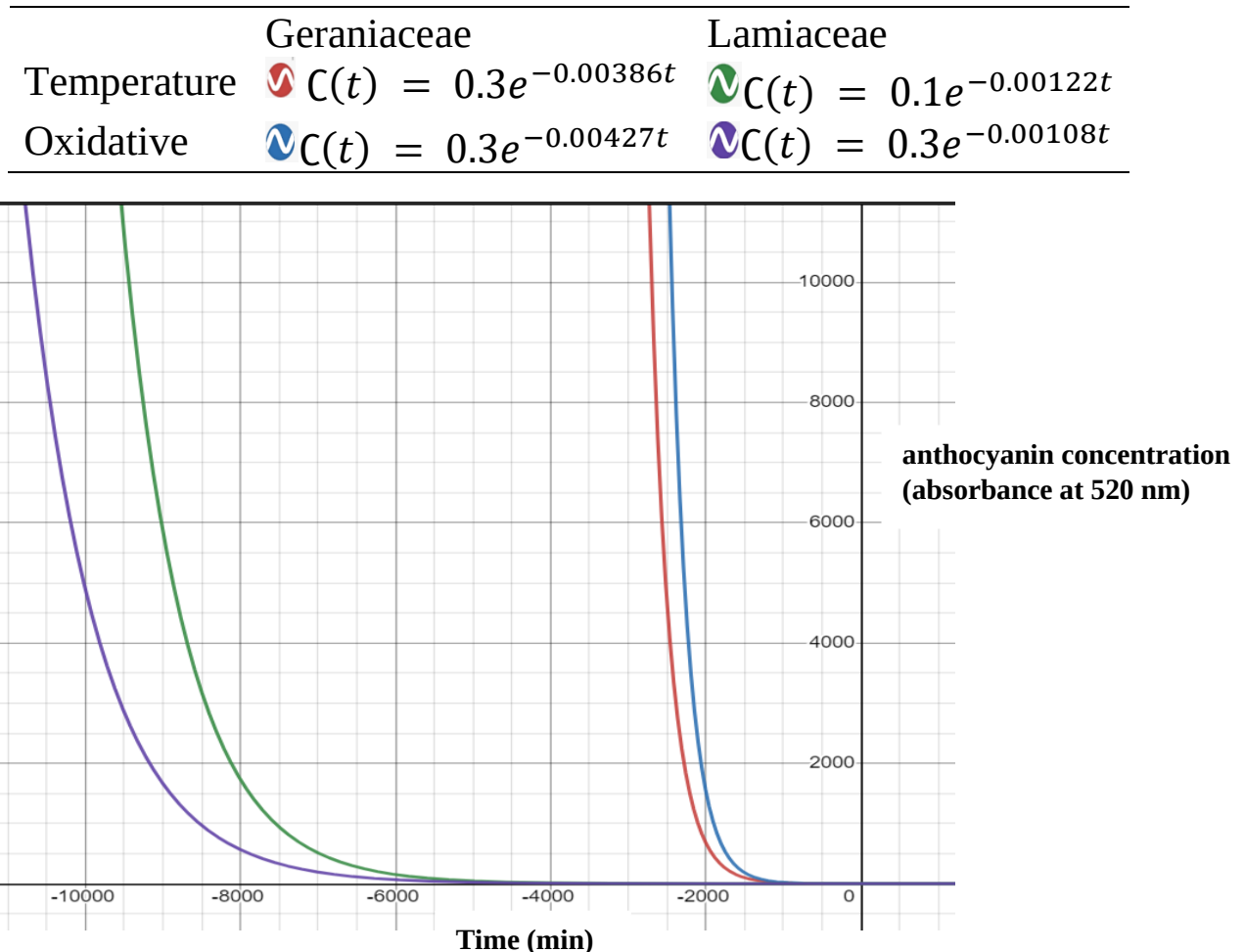


Figure 8.2 Degradation kinetics measured against time at different temperature and oxidative parameters of pigmented flower extracts of the Geraniaceae and Lamiaceae plant families. Graphs created with Desmos graph

While the pigmented flower extracts from the Lamiaceae contained high levels of phenolic acids specifically caffeic acid, rosmarinic acid and its derivatives sagerinic and salvianolic acids (Table 5.4.5), no such phenolic acids could be identified in the Geraniaceae pigments. It can be postulated that these phenolic acids could be involved in the stabilisation of anthocyanins in the Lamiaceae pigments. This proposed stabilisation of anthocyanins could then contribute to the observed higher thermal and oxidative stability of the Lamiaceae extracts compared to the Geraniaceae.

Figure 8.3 shows some proposed mechanisms of interaction between phenolic acids and anthocyanins which contribute to stabilisation of anthocyanins in the Lamiaceae pigments. The anthocyanin nucleus could interact via a sandwich stacking self-association (blue arrow) reaction via hydrophobic interactions (red lines) with its coumaroyl aromatic residues (Dangles et al., 1993, Dangles and Fenger, 2018) or intermolecular hydrogen bonding or hydrophobic interactions of the catechol groups on rosmarinic acid and its derivatives (blue zigzags) with the aromatic acylated anthocyanins or directly with the anthocyanin nucleus. Other researchers have also proposed that rosmarinic acid and aromatic residues could be involved in the stabilisation of acylated anthocyanins (Eiro and Meinonen, 2002; Hoshino, Matsumoto & Goto, 1980; Kein and Reilly, 2011).

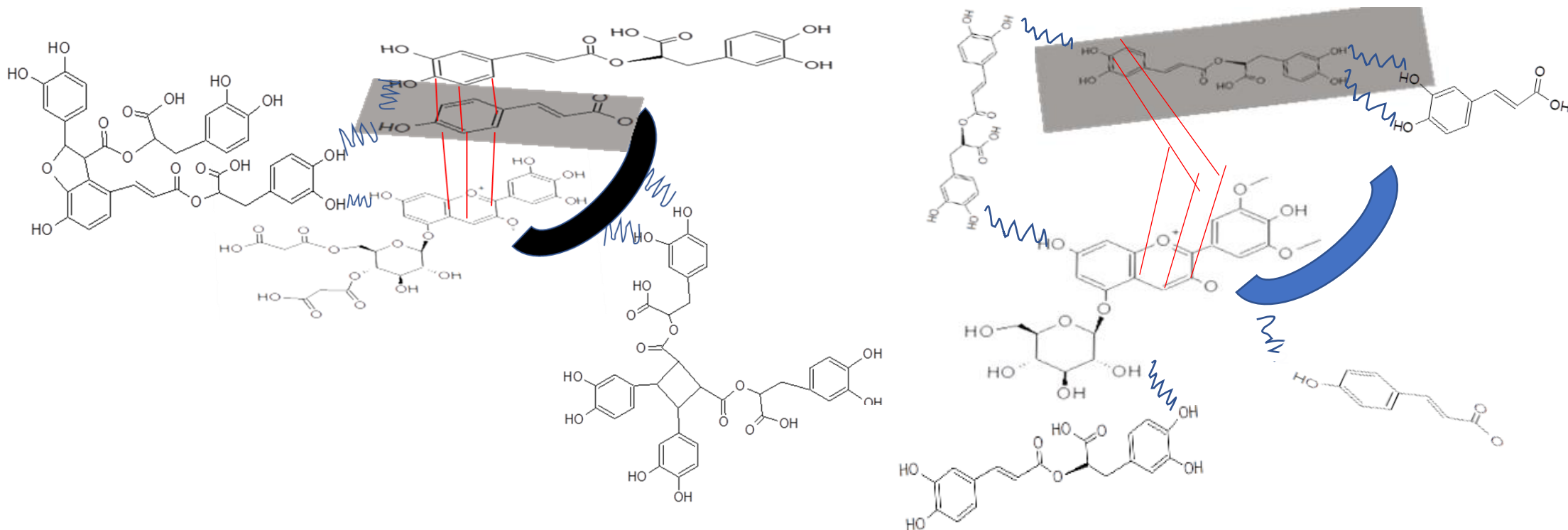


Figure 8.3 Proposed structure configuration of interactions of anthocyanins and phenolic acids (rosmarinic acid, sagerinic and salvianolic acid B) in *Salvia* species (left) and phenolic acids (rosmarinic acid and caffeic acid) in *P. zuluensis* (right) pigmented flower extracts of the Lamiaceae plant family. Light blue zigzag represents hydrogen bonding interactions. Red lines show regions of hydrophobic interactions of rosmarinic acid (top) with p-coumaric acid acylated group on anthocyanins and sandwich stacking self-association interactions with anthocyanin nucleus. Black and blue curves represent the sugar residue

The results from this research show that the pigmented flower extracts from the Geraniaceae had higher TPC and DPPH radical scavenging activity values than those of the Lamiaceae plant family. To an extent, this could be expected given that the Geraniaceae pigments had higher total anthocyanin and total flavonoid content than the Lamiaceae pigments. However, in contrast, generally, the pigmented flower extracts from the Lamiaceae plant family had comparable and, in some cases, higher nitric oxide radical scavenging activities and ORAC values than those of the Geraniaceae plant family. This latter trend of comparable or higher antioxidant properties of the Lamiaceae pigments compared to the Geraniaceae pigments could also be observed in the cellular assays (such as cellular antioxidant activity in Caco-2 cells and inhibition of NO production in LPS-stimulated RAW 267.4 macrophages).

The pigmented flower extracts from the Lamiaceae had lower total anthocyanin and total flavonoid contents and therefore their high ORAC and NO radical scavenging activities is noteworthy. This may be related to the fact that the Lamiaceae extracts contained phenolic acids while none were detected in the Geraniaceae extracts. Rosmarinic acid and its derivatives made up most of the phenolic acid content of the pigmented flower extracts of the Lamiaceae plant family especially the *Salvia* species. As postulated earlier, these phenolic acids could be involved in stabilising interactions with the aromatic acylated anthocyanins identified in the Lamiaceae species and this may contribute to their strong antioxidant properties. Similar strong radical scavenging activity of extracts from the aerial parts of *Salvia verticillate* (from the Lamiaceae family) which were also rich in rosmarinic acid and its derivatives have been reported by Katanić Stanković, Srećković, Mišić, Gašić, Imbimbo, Monti and Mihailović (2020).

The proposed mechanism by which anthocyanins and other phenolic compounds in the pigmented extracts could exert antioxidant effects in Caco-2 cells is shown in Figure 8.4. Anthocyanins in themselves can undergo spontaneous degradation at physiological conditions prevailing in Caco-2 cells (Kay et al., 2009). This brings into focus and highlights the proposed potential role of non-anthocyanin phenolic compounds such as phenolic acids in stabilising anthocyanins (particularly in the Lamiaceae pigments) which then contributes to the better-than-expected antioxidant properties of the Lamiaceae pigments.

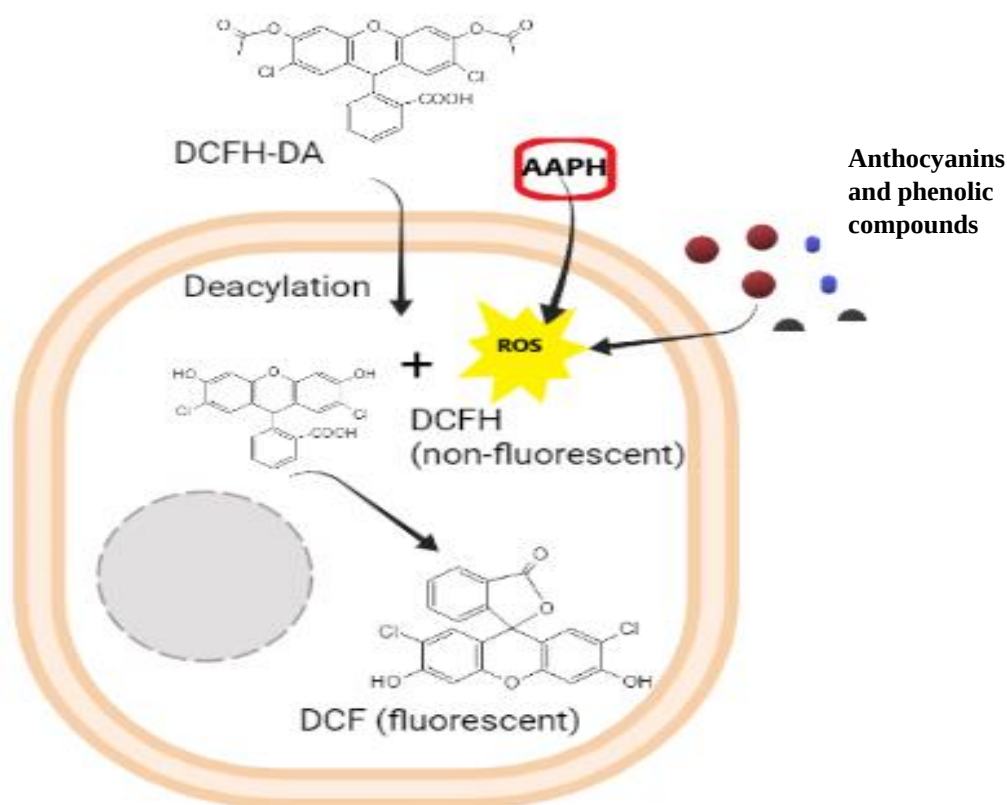


Figure 8.4 Proposed mechanism of radical scavenging activity in DCFH-DA assay in Caco-2 cells by pigmented flower extracts of the Geraniaceae and Lamiaceae plant families. DCFH-DA: dichlorofluorescein-diacetate, DCFH: dichlorofluorescein (non-fluorescent), DCF: dichlorofluorescein (fluorescent) Adapted from Nwachukwu, Sarteshnizi, Udenigwe & Aluko (2021)

The proposed mechanism of inhibition of NO production in LPS-stimulated RAW 267.4 macrophages is shown in Figure 8.5. The production of nitric oxide starts with the binding of LPS to the toll-like receptor 4 (TLR4). This allows for the activation of NADPH-oxidase which produces reactive oxygen species (ROS) (Morgan and Liu, 2011). The ROS in turn stimulate the nuclear translocation and phosphorylation of NF- κ B which further lead to the production of inducible nitric oxide synthase (iNOS) (Song et al., 2008).

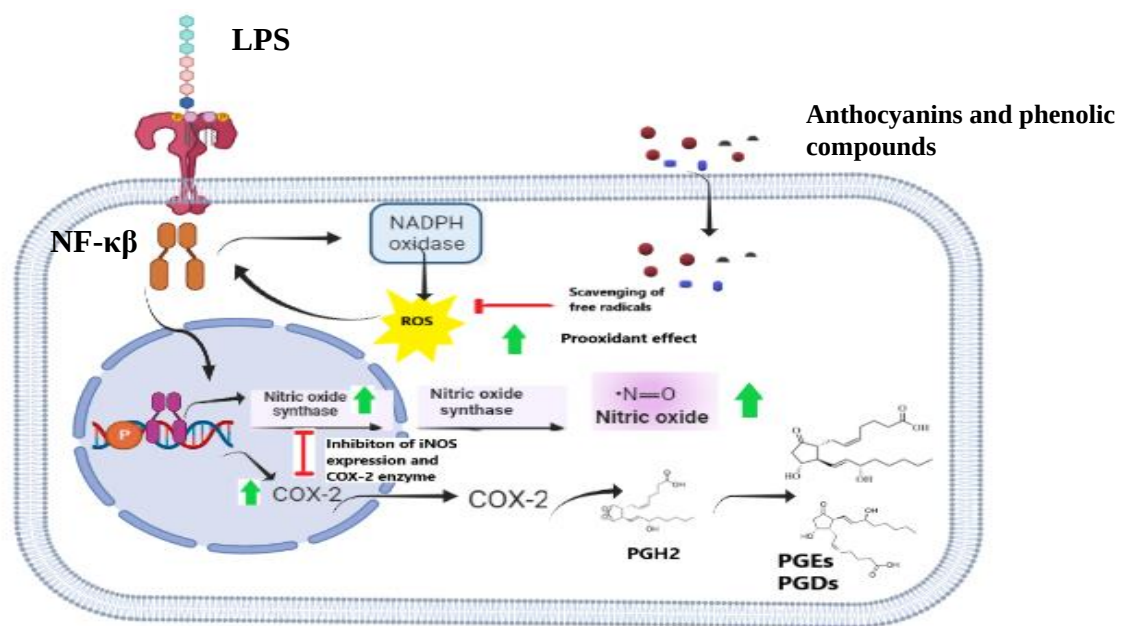


Figure 8.5 Proposed mechanisms of inhibition and induction of nitric oxide production in RAW 264.7 cells. Green arrows represent areas of induction or increased expression due to prooxidant effects. Red bars indicate areas of inhibition of NO production, COX-2: cyclooxygenase-2 enzyme, iNOS: inducible nitric oxide synthase, PGH₂: prostaglandin H₂, PGEs: prostaglandin E, PGD's: prostaglandin D Adapted from Nair et al. (2015)

There are several ways that antioxidants can prevent nitric oxide (NO•) radicals and the peroxynitrite (ONOO•), which arise from NO reacting with superoxide (O₂⁻), from reacting. The anthocyanins can neutralize the free radicals through single electron transfer (SET) and/or hydrogen atom transfer (HAT) or by sequential proton loss electron transfer (SPLET) (Lu, Qiang, Li, Zhang and Zhang, 2014). Another method described by Tsuda, Kato and Osawa (2000), involves the reaction of anthocyanins with peroxynitrite (ONOO⁻) to form nitrated degradation products of 4-hydroxy-3-nitrobenzoic acid and *p*-hydroxybenzoic acid.

NO production in RAW 267.4 macrophage cells could also be reduced by suppression of transcription factors such as nuclear factor (NF-κβ) translocation via ROS-mediated NADPH oxidase (NOX-1) activity and cytokines such as tumor necrosis factor (TNF-α) and interleukine (IL1-β) and inhibition of pro-inflammatory enzymes such as cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) activity and/or suppression of its production (Figure 8.5 red bars).

The anthocyanins and phenolic compounds of the pigmented flower extracts could neutralize the ROS production thereby limiting the translocation of NF-κβ resulting in a reduced iNOS and eventual NO production. Another mechanism could be that the anthocyanins and phenolic compounds inhibit other signalling factors associated with iNOS protein expression (Nair et al., 2015) or the inhibition of the COX-2 enzyme resulting in the reduction in prostaglandin production (Figure 8.5). In summary, the pigmented flower extracts from the Lamiaceae plant family might have multiple targets of inhibition which could explain their comparable activity with those of the Geraniaceae plant family.

Increasing the concentration of pigmented flower extracts to 250 µg/ml led to small observed increases in NO produced by the macrophages (Figure 8.5 green arrows) indicating potential prooxidant effects at the higher concentration. Due to the fact that increased NO production could lead to anti-hypertensive effects, the apparent prooxidant effect of the higher concentration of the extracts (especially for the *P. grandiflorum* pigmented extract) could be leveraged to produce anti-hypertensive effects in foods. The prooxidant effect could result in NF-κβ translocation and phosphorylation (Song et al., 2008) resulting in increased NO production thereby lowering hypertension. This study highlights the importance of extract concentration in producing specific effects in cells.

A proposed mechanism by which anthocyanins and other phenolic compounds in the pigmented extracts could reduce lipid droplet formation in 3T3-L1 adipocytes is shown in Figure 8.6. The differentiated cells, after treatment with the adipogenic mixture (insulin, dexamethasone, rosiglitazone and IBMX), increase the expression of transcription factors C/EBP β and C/EBP σ which in turn activates the hormone receptor peroxisome proliferator-activated receptor (PPAR γ) and C/EBP α (Rosen, 2005). As suggested by Aranaz et al. (2019), the reduction or prevention of lipid droplet formation could be due to suppression of the activation of the PPAR γ and C/EBP α transcription factors by phenolic compounds such as anthocyanins and other phenolic compounds. These compounds could also suppress genes for enzymes involved in adipogenesis such as fatty acid synthase (Fasn) and Stearoyl Coenzyme A desaturase (Scd1) in the early stages of adipocyte differentiation (Figure 8.6). Phenolic compounds have been shown to have the ability to bind to a ligand binding region of the PPAR γ receptor (Aranaz et al., 2019).

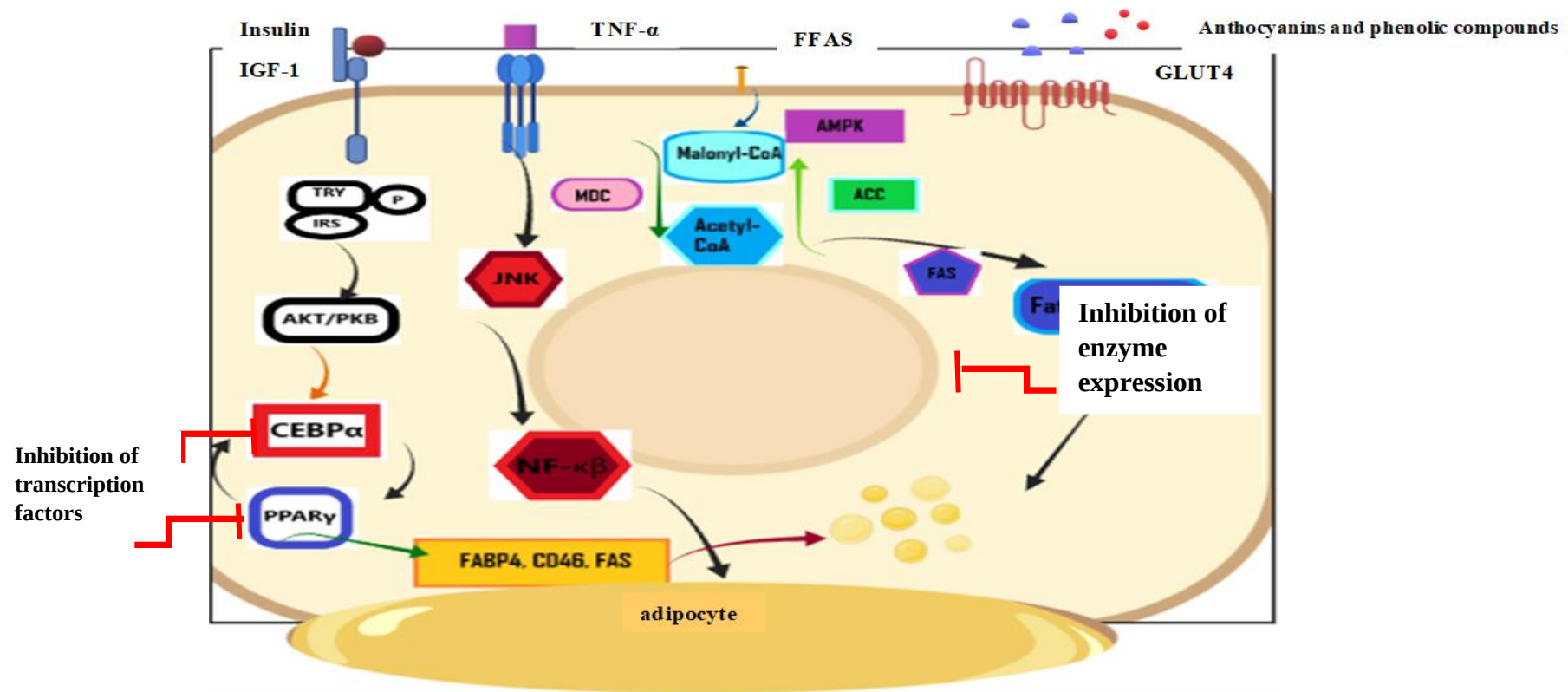


Figure 8.6 Proposed mechanisms of action of anthocyanins and other phenolic compounds of pigmented flower extracts from the Geraniaceae and Lamiaceae plant families for the reduction in lipid droplet formation in 3T3-L1 adipocytes. Created with BioRender.com. FFAS: free fatty acids, MDC: malonyl-CoA decarboxylase, ACC: acetyl-CoA carboxylase, FAS: fatty acid synthase, AMPK: AMP-activated protein kinase, JNK: c-Jun-N terminal Kinase, NF-κβ: nuclear factor-kappa beta, TRY: Tryptychon transcription factor, IRS: insulin receptor substrate, AKT/PKB: protein kinase B, CEBPα: CCAAT/enhancer-binding protein alpha, PPARγ: peroxisome proliferator-activated receptor gamma, FABP4: fatty acid binding protein 4, CD46: compliment regulatory protein, red spheres: anthocyanins, blue half-spheres: phenolic compounds Adapted from Chayaratanasin et al., 2019; Guru, Issac, Velayutham, Saraswathi, Arshad, Arockiaraj., 2020

The pigmented flower extracts demonstrated ability to prevent formation of advanced glycation end-products (AGE) which is an indication of anti-diabetic properties. Formation of AGEs begins with the reaction of reducing sugars with proteins which lead to formation of Amadori compounds (Helou, Marier, Jacolot, Abdennebi-Najar, Niquet-Léridon, Tessier and Gadonna-Widehem, 2014; Monnier, Nagaraj, Portero-Otin, Glomb, Elgawish, Sell and Friedlander, 1996). The Amadori compounds can undergo degradation to form reactive carbonyls such as methylglyoxal (Bierhaus, Hofmann, Ziegler and Nawroth, 1998), or they could also undergo oxidation to yield stable AGEs (Vasan, Foiles and Founds, 2003). The formation of AGEs could also be prevented through the ability of anthocyanins and phenolic compounds to scavenge free radicals produced during glycation as suggested by Peng, Ma, Chen and Wang (2011).

Reactive carbonyls could be scavenged or trapped by anthocyanins and phenolic compounds to prevent formation of AGEs by forming covalent adducts (Wang, Yagiz, Buran, Nunes and Gu, 2011; Lo, Li, Tan, Pan, Sang and Ho, 2006). Xiu-Yu, Huang, Lucy Sun, Chi-Tang, Shiming and Chih-Yu (2014) reported the trapping of reactive carbonyls such as methylglyoxal (MGO) with anthocyanins from Blackcurrent (*Ribes nigrum*) extracts and showed that the two main anthocyanins of blackcurrent extracts, delphinidin-3-O-rutinoside and cyanidin-3-O-rutinoside, caused a 36.75% decrease in MGO in an incubated mixture. The reaction products were analysed and found to contain delphinidin and cyanidin rutinoside-MGO adducts with preferred reactivity at C-6 and 8 on the A ring (Figure 8.7) (Li, Zheng, Sang and Lv, 2014). The anthocyanins “trap” methylglyoxal through hemiacetal reactions forming the products as illustrated in Figure 8.7.

It is proposed that the pigmented flower extracts from the Lamiaceae plant family could be more stable in the chemical environment due to the previously mentioned intermolecular and intramolecular complex formation of anthocyanins with other phenolic acids such as rosmarinic acid and its derivatives (Figure 8.7). The complex could prevent or decrease degradation rate thereby allowing the anthocyanins and phenolics to react with the BSA or methylglyoxal and prevent AGE formation. Overall, the observed ability of particularly the pigmented flower extracts from *P. grandiflorum* and *P. × hortorum* (Geraniaceae) to prevent AGEs formation in a comparable manner to the anti-diabetic drug aminoguanidine suggests the pigmented extracts from these two particular species could be considered as possible replacements for aminoguanidine in the treatment of diabetes.

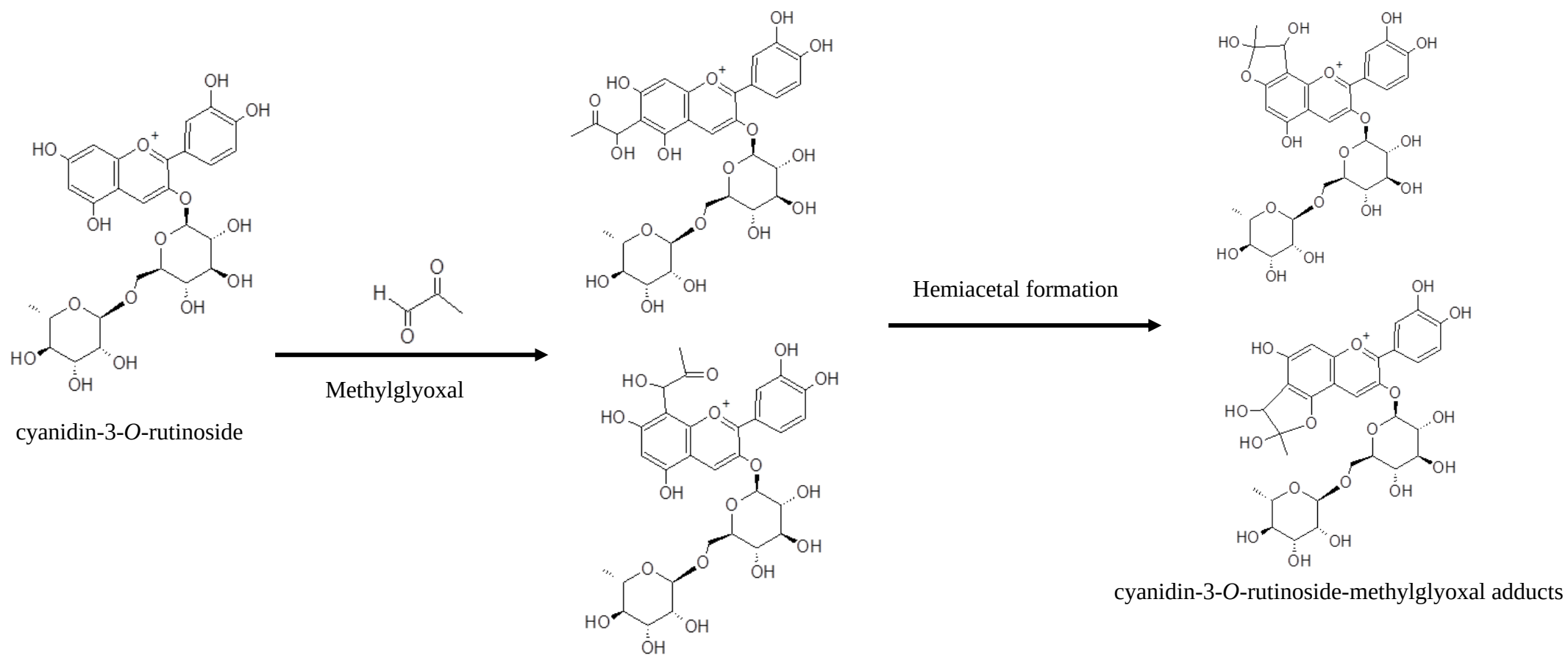


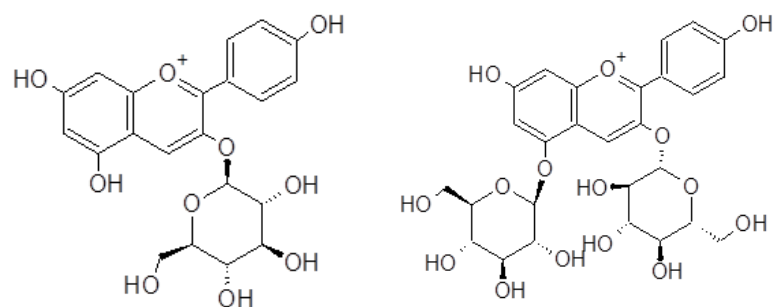
Figure 8.7 Proposed mechanism of the reaction of cyanidin-3-O-rutinoside (as example) with methylglyoxal to form cyanidin-3-O-rutinoside-methylglyoxal adducts Adapted from Li et al. (2014)

This research also investigated the possibility of applying the natural colourants directly to the foods in the form of dried flower powder instead of in extract form. As mentioned in chapter 6, after storage for 7 days at refrigeration (4°C) temperature, the yogurt incorporated with pigmented flower petal powder developed an unpleasant brown appearance as can be seen with yogurt incorporated with *P. zuluensis* pigmented flower powder as an example (Figure 8.8) (black circles). It is hypothesized that residual enzymatic activity in the petals could lead to formation of brown oxidation end-products. It may therefore not be advisable to use flower petal powder directly as a method of colour addition to food products but rather use concentrated aqueous extracts for this purpose.



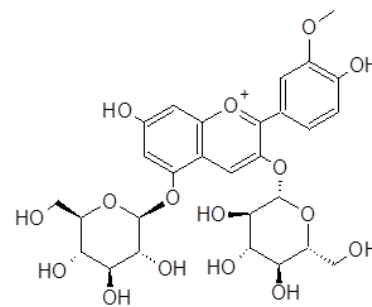
Figure 8.8 Images of yogurt incorporated with pigmented flower petals of *P. zuluensis* as example (left) and after 7 days storage at refrigeration temperature (right) showing browning spots (black circles) after storage

The production of an orange colour by the *P. zonale* hybrid pigment was an interesting observation and this could probably be the first report on the identification and application of an orange anthocyanin-based pigment extract in food systems. The *P. zonale* hybrid (Geraniaceae) extract contains 76% pelargonidin derivatives with 24% peonidin derivatives which could account for the orange colour (Figure 8.9). This could potentially be a water-soluble alternative to the natural orange lipid-soluble β -carotene used in the industry although pH will play an important role to ensure this colour.



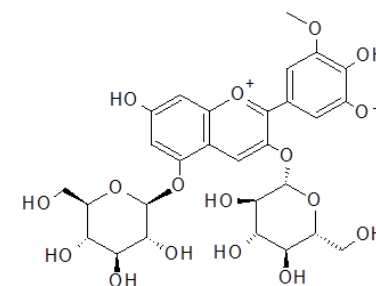
Pelargonidin derivatives

76%



Peonidin derivatives

24%



Malvidin derivative

4%



Figure 8. 9 Images of gelatine incorporated with *P. zonale* hybrid extract showing an orange colour that is mostly pelargonidin-glucoside based

The results of this study may suggest a double benefit effect of pigmented flower extracts from these two plant families as natural colourants in terms of product quality and health benefits. The stability of these extracts at a range of temperatures and oxidative conditions shows their possible applicability in baked goods while their storage stability shown in the food applications chapter points to good product quality. The radical scavenging, anti-inflammatory, anti-obesity and anti-diabetic effects demonstrates their potential in health-promotion for consumers.

Chapter 9: Conclusions and recommendations

This study has shown that pigmented flower extracts from the Geraniaceae and Lamiaceae plant families studied in this research differ in their anthocyanin profiles. Flower extracts from the Geraniaceae plant family mainly contain acetyl acylated anthocyanins whereas pigmented flower extracts from the Lamiaceae plant family contain aromatic malonyl acylated anthocyanins. The pigmented flower extracts from the Geraniaceae family contain significantly more total anthocyanins than the Lamiaceae family flower extracts. However, the Lamiaceae pigmented flower extracts contain high levels of rosmarinic acid and its derivatives, sagerinic acid and salvianolic acid which are not detected in the pigmented flower extracts of the Geraniaceae plant family.

The pigmented extracts from the Lamiaceae plant family studied in this research are more thermally and oxidatively stable as compared with the pigmented flower extracts from the Geraniaceae family. This is shown by the Lamiaceae pigments having lower temperature and oxidative degradation rate constants (k) and longer half-lives ($t_{1/2}$) at different temperatures and oxidizing agent (H_2O_2) concentrations. The greater stability of the Lamiaceae pigments is hypothesized to be due to the protective hydrophobic self-association interactions of the aromatic residue on the anthocyanins thereby preventing nucleophilic attack. It is also proposed that the greater stability is also due to intermolecular interactions between the aromatic acylated anthocyanins and the rosmarinic acid and its derivatives, sagerinic acid and salvianolic acid.

The pigmented flower extracts from the Geraniaceae and Lamiaceae plant family exhibit a range of health-promoting properties. All pigmented flower extracts from both families show powerful DPPH radical scavenging activity and AAPH radical scavenging activity in Caco-2 cells, an indication of ability to protect against oxidative stress. The pigmented flower extracts from both families also exhibit powerful chemical nitric oxide (NO) radical scavenging activity and are able to significantly reduce NO production by RAW 267.4 macrophage cells, which is an indication of potential anti-inflammatory effects. All extracts from both the Geraniaceae and Lamiaceae plant families show ability to significantly reduce lipid droplet accumulation in 3T3-L1 adipocyte cells after 14 days treatment, which indicates anti-obesity effects. Concerning anti-diabetic effects measured by ability to reduce formation of advanced glycation end-products (AGEs), the *P. grandiflorum* and *P. zonale* hybrid (Geraniaceae) pigmented flower extracts show similar ability to reduce the formation of AGEs compared to the

commercial anti-diabetic drug aminoguanidine at the two highest concentrations (0.8 and 1.0 mM). This suggests that these two extracts could potentially be a replacement for aminoguanidine as anti-diabetic medication.

This study shows the applicability of the pigmented flower extracts in three types of food systems: yogurt, fondant and gelatine as natural food colourants. The pigmented flower extracts from the Geraniaceae and Lamiaceae generally show higher storage stability compared to the commercial E163 colourant and have the added advantage of providing a wide range of colours such as red, purple and orange.

Overall, this study has demonstrated that pigmented flower extracts from the Geraniaceae and Lamiaceae plant families show promise as natural food colourants that could potentially be applied in the food industry as they provide a wide range of colours not shown by the commercial E163 colourant and have the added benefit of offering enhanced health-promoting properties. The observed thermal and oxidative stability of the pigmented extracts shows that they can be stable in a wide range of temperature and oxidative conditions and can be applied in food systems such as yogurts, sugar-based systems and protein-based food systems such as gelatine.

It is recommended that future research utilizes computational modelling to investigate the mechanism behind the thermal and oxidative stability of pigmented flower extracts from the Lamiaceae plant family and the role of rosmarinic acid and its derivatives in its stability as proposed in this study. The temperature and oxidative stability of the pigmented flower extracts could also be assessed at different pH values for a more accurate determination of its stability in food systems.

In terms of antioxidant activity, future research could use *in vitro* simulated gastrointestinal digestion of the pigmented extracts from the Geraniaceae and Lamiaceae plant families to better understand the bioaccessibility of their anthocyanins and possibly also find a link with their stability in terms of degradation kinetics. The bioaccessibility tests could be carried out with Caco-2 cells as a cellular model. The mechanism behind the ability of pigmented flower extracts to reduce lipid accumulation in 3T3-L-1 cells and reduction in nitric oxide production in RAW 264.7 cells could be confirmed through Western blotting analysis of transcription factor gene expression.

It is recommended that the anti-diabetic properties of flower pigments from both families, particularly the *P. grandiflorum* and *P. × hortorum* (Geraniaceae) pigmented flower extracts could be studied further. The pigmented extracts could be applied in *in vitro* cellular models to investigate their action in cells as well as the potential for them to be used as replacements for anti-diabetic medication such as aminoguanidine or others.

Future research could explore the application of the pigmented flower extracts in baked or cooked products to evaluate their stability during cooking or baking at high temperatures and oxidation conditions compared to the commercial E163 colourant or other synthetic colourants. Further research could also investigate the interaction of anthocyanins from these pigmented extracts with other food components such as starch and protein and how it affects the functional properties in these baked products. The encapsulation of the pigmented extracts to increase their stability and potential encapsulation materials could be studied. The encapsulated extracts could be exposed to different thermal and oxidative conditions to assess their stability.

In terms of production of the pigmented extracts future research could also investigate the optimization of the extraction of the pigments by investigating the type of solvent and extraction time. Ultimately, if pigmented extracts from these plants are to gain a status of significant commercial application as natural colourants, the production of these plants and their flowers will be of importance. Future research could also investigate optimization of the growth of the flowers with use of an aeroponics system as used in this study thereby upscaling the production of the plants and producing more concentrated extracts to match that of the commercial E163 anthocyanin extract.

Chapter 10 References

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Appendix A

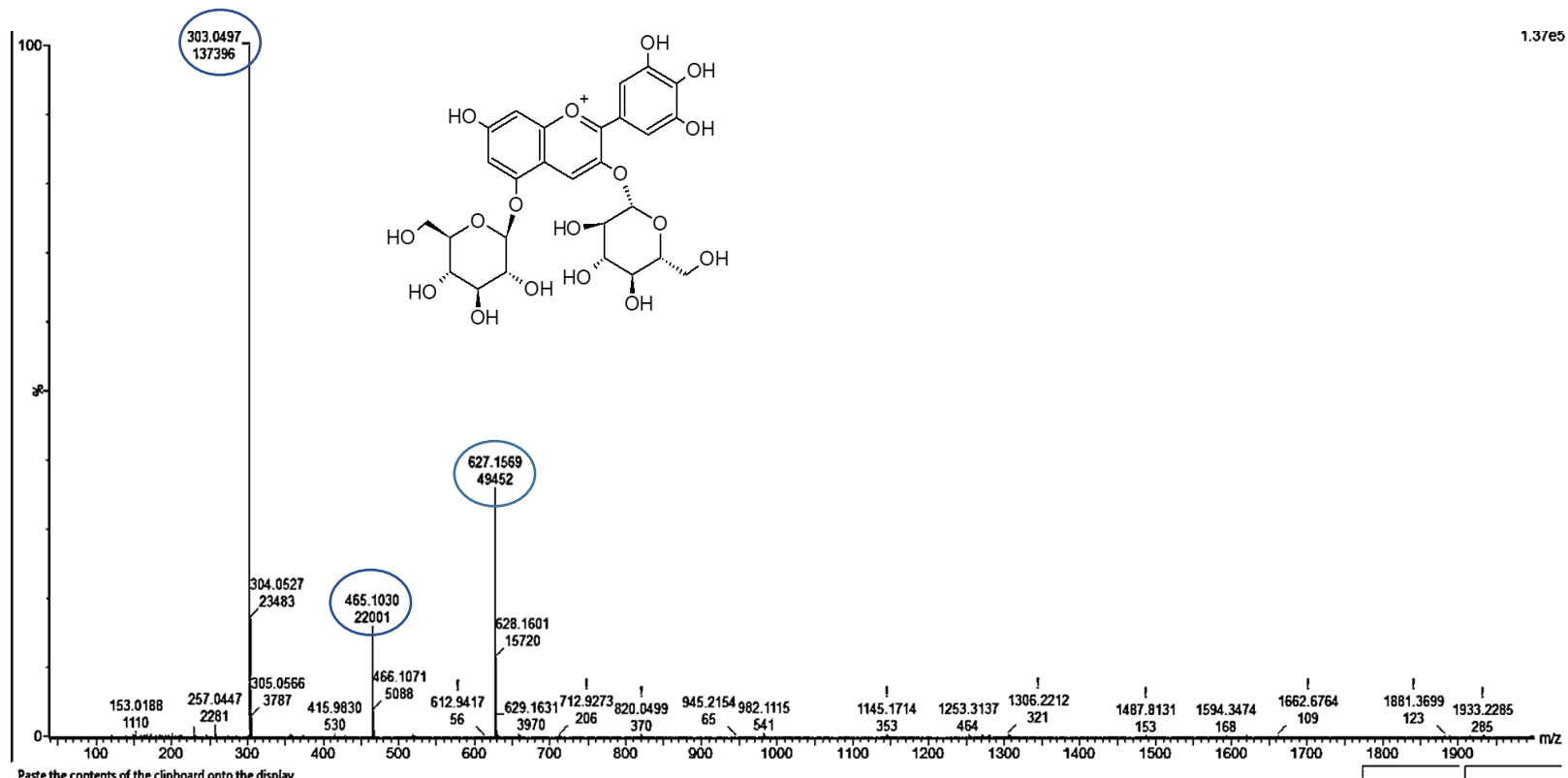


Figure A 1 Mass spectrum of delphinidin-3,5-diglucoside

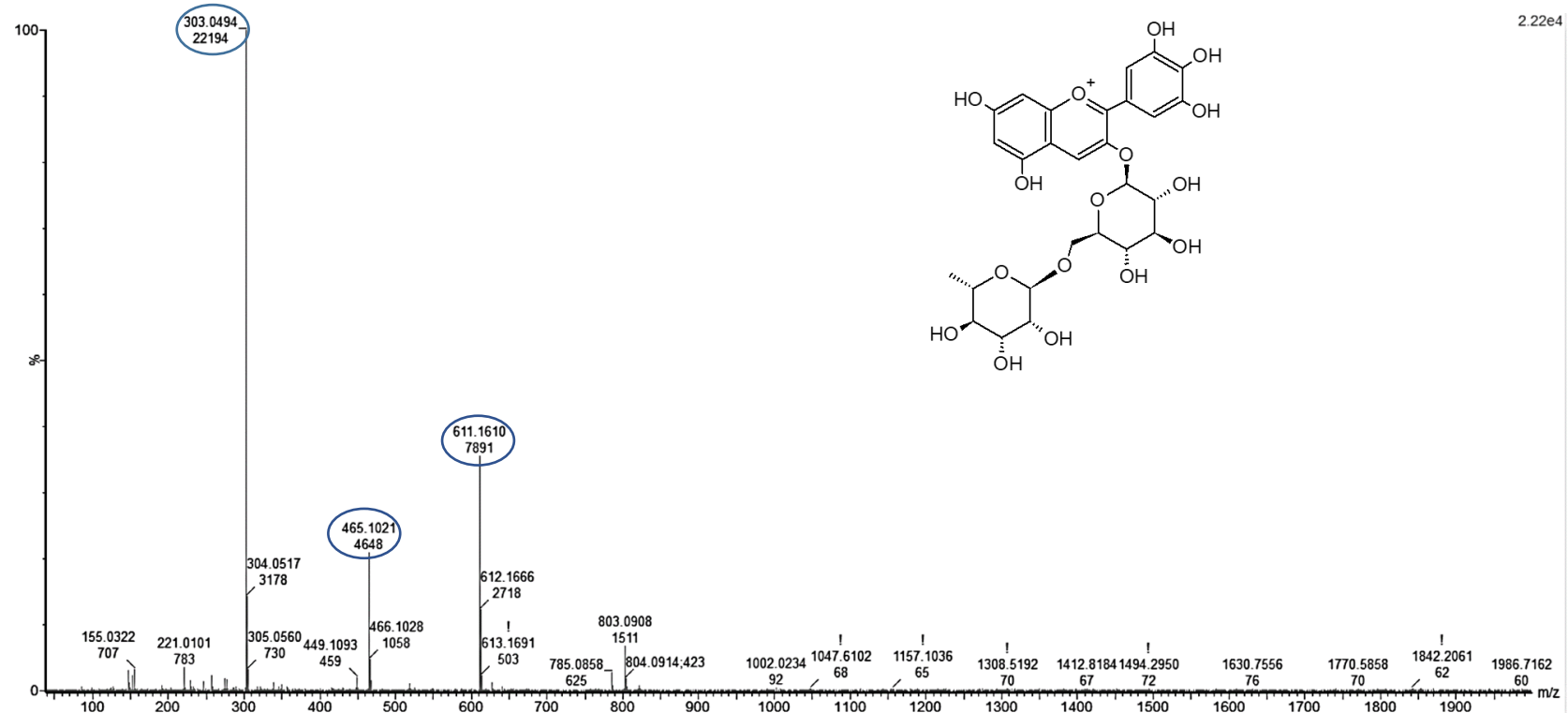


Figure A 2 Mass spectrum of delphinidin-3-O-rutinoside

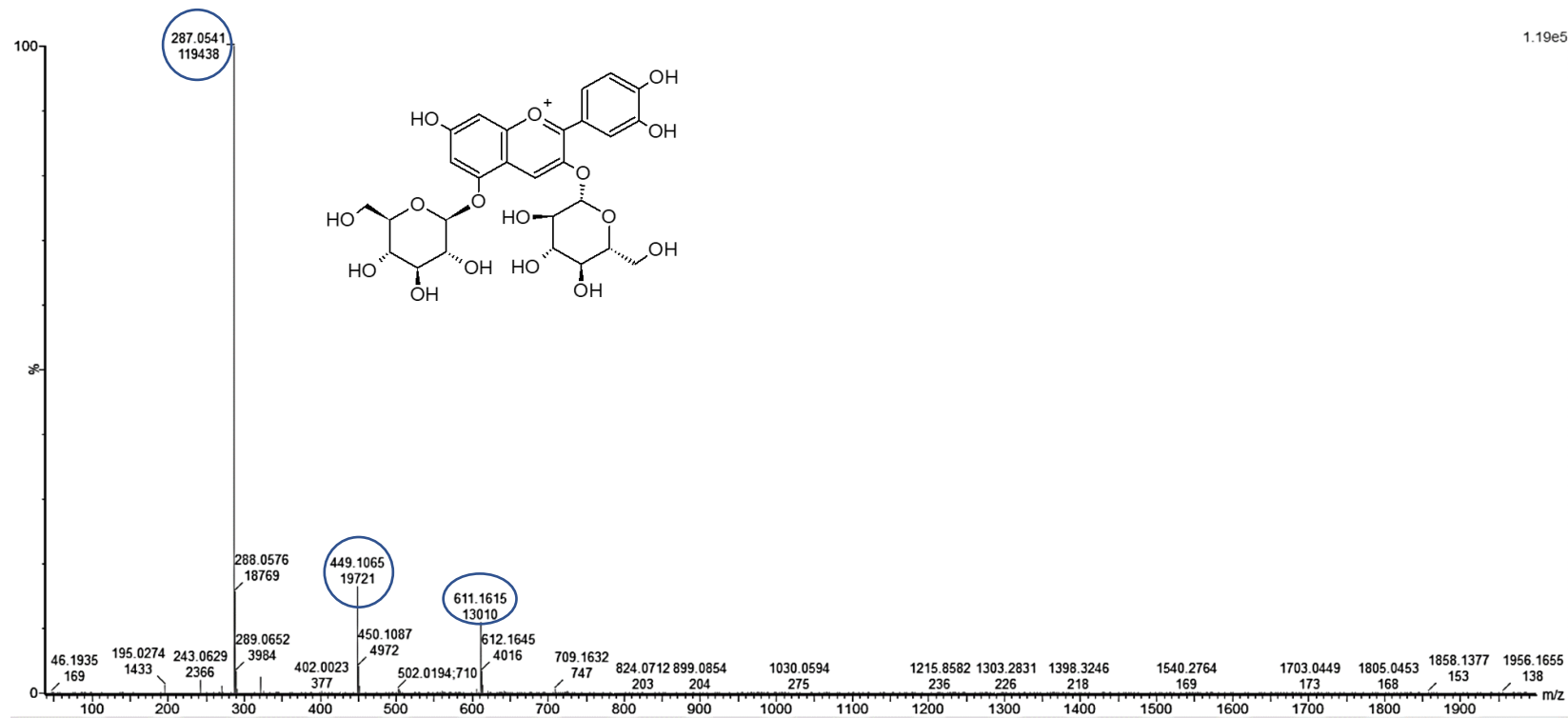


Figure A 3 Mass spectrum of cyanidin-3,5-diglucoside

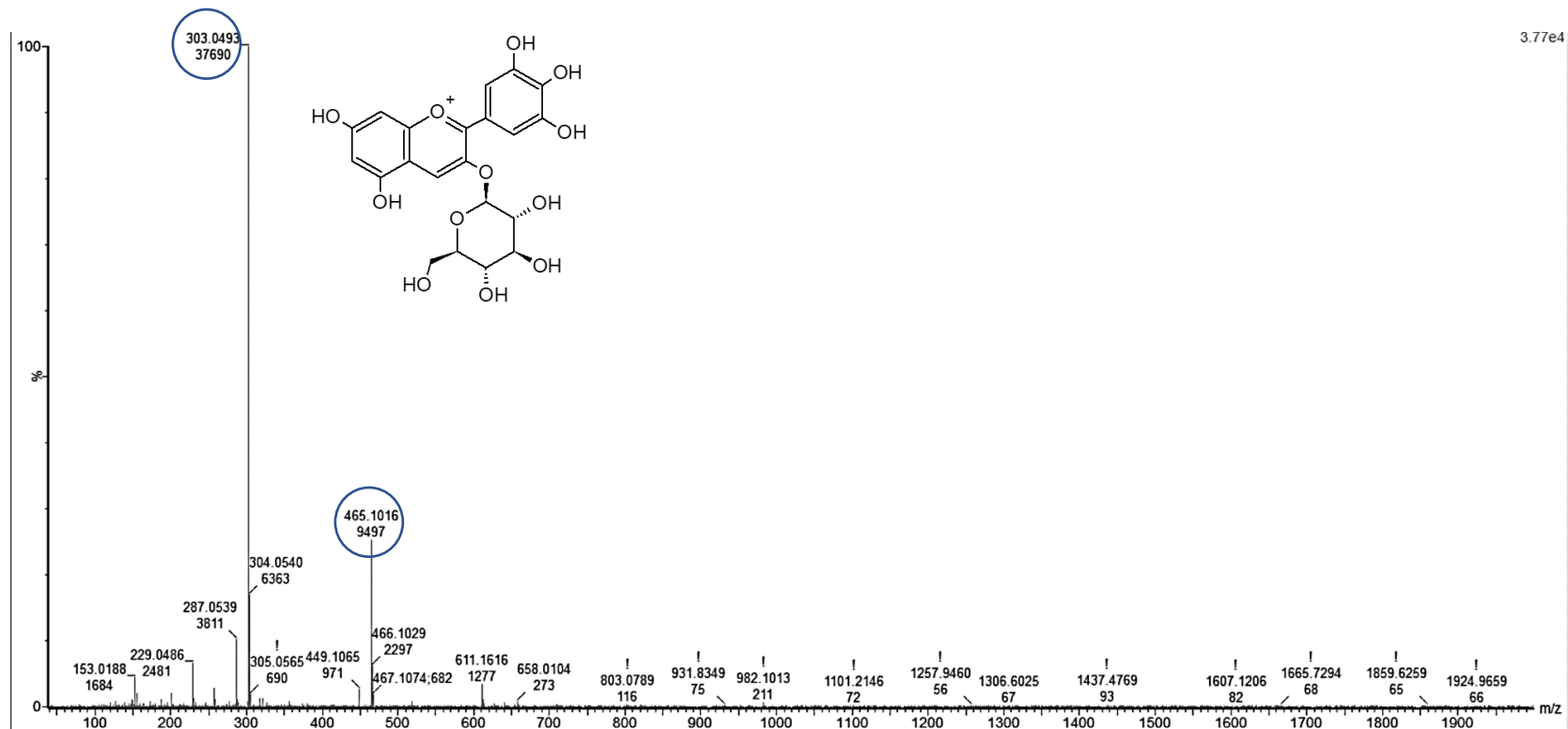


Figure A 4 Mass spectrum of delphinidin-3-O-glucoside

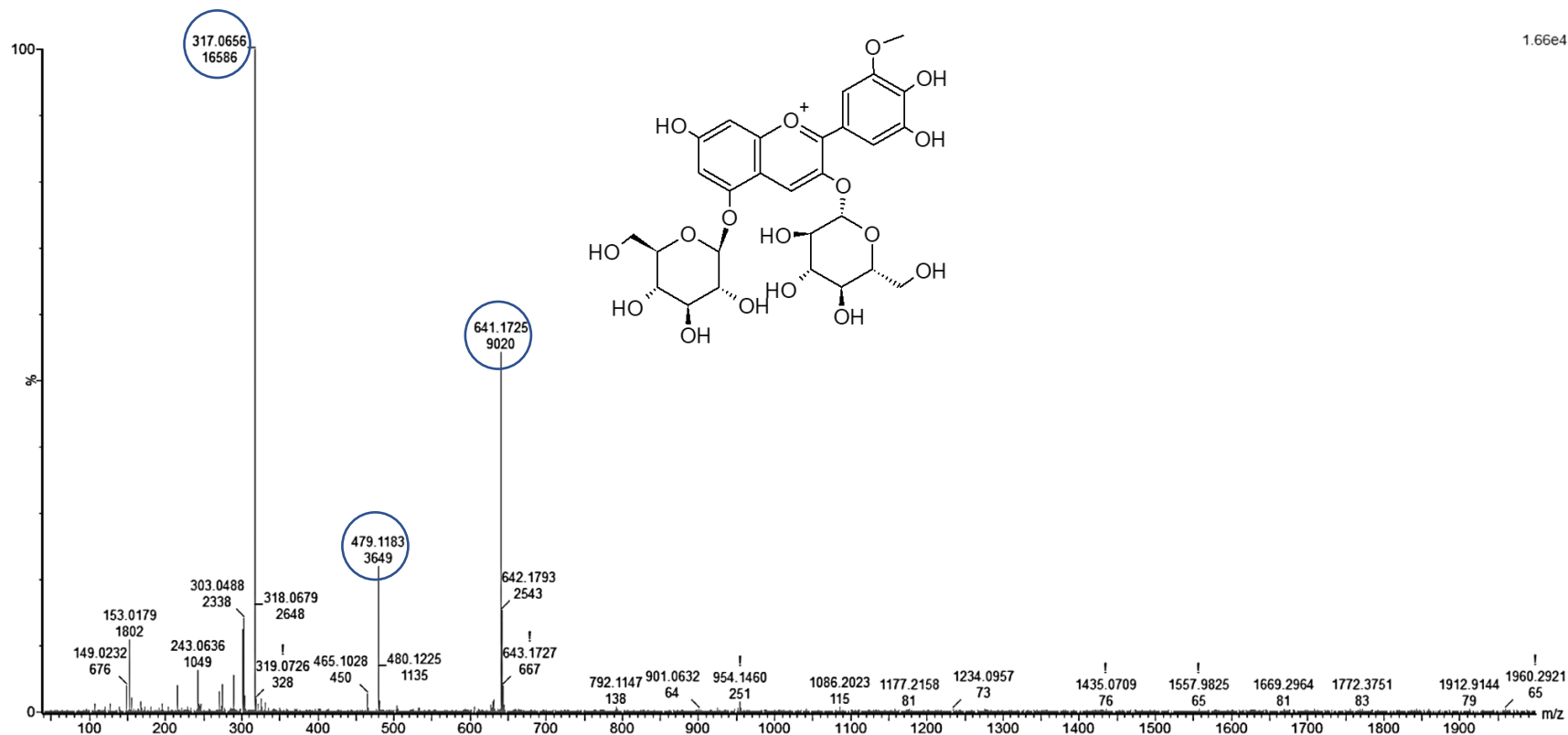


Figure A 5 Mass spectrum of petunidin-3,5-diglucoside

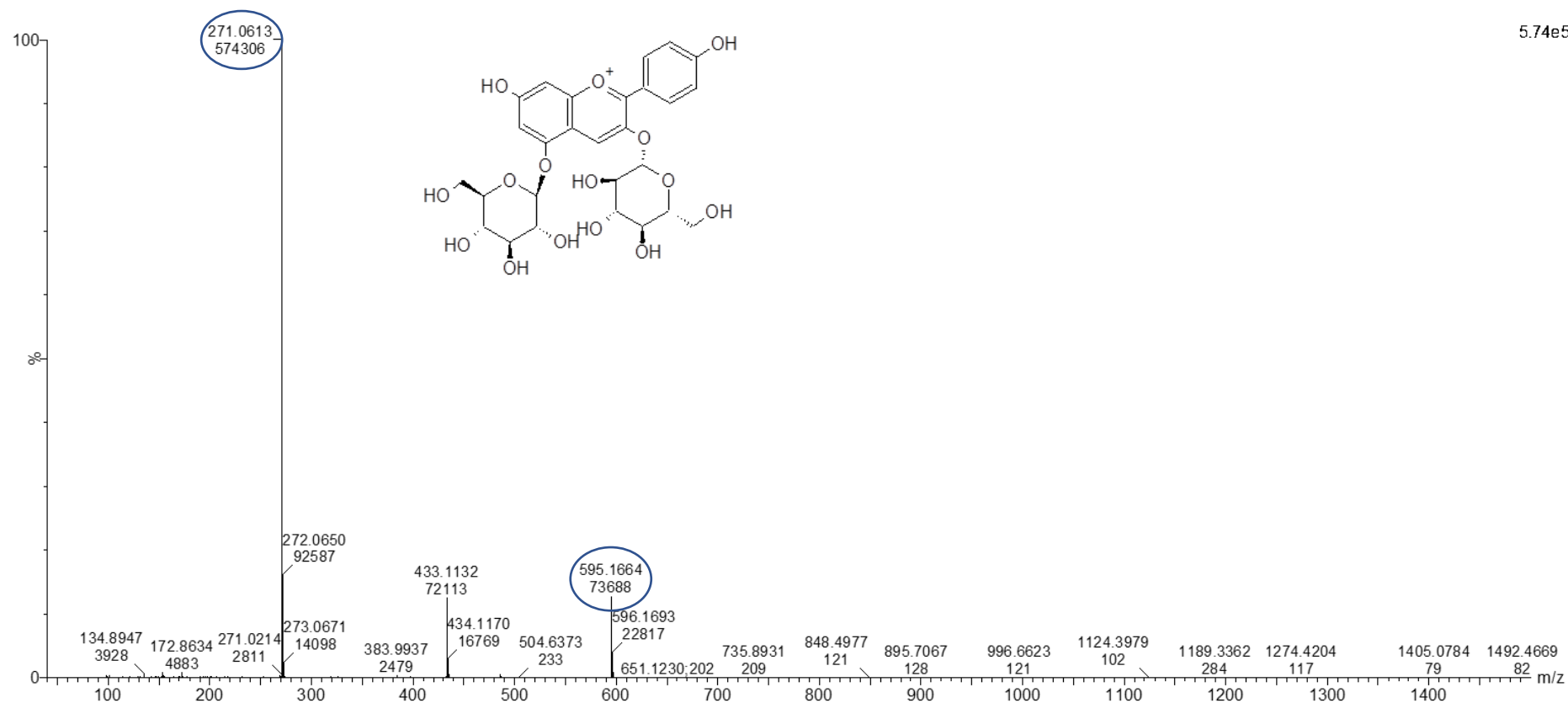


Figure A 6 Mass spectrum of Pelargonidin-3,5-diglucoside

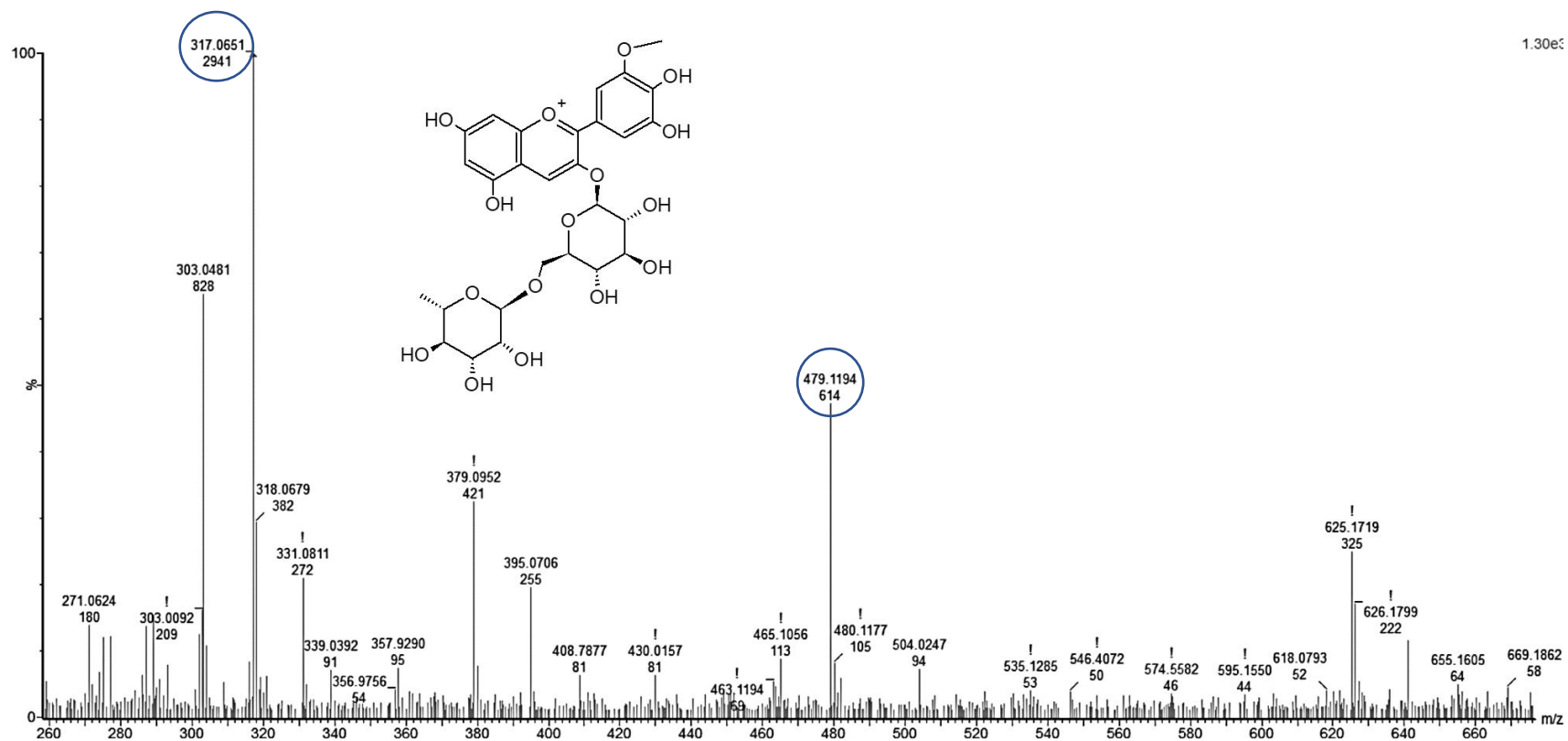


Figure A 7 Mass spectrum of *petunidin-3-O-rutinoside*

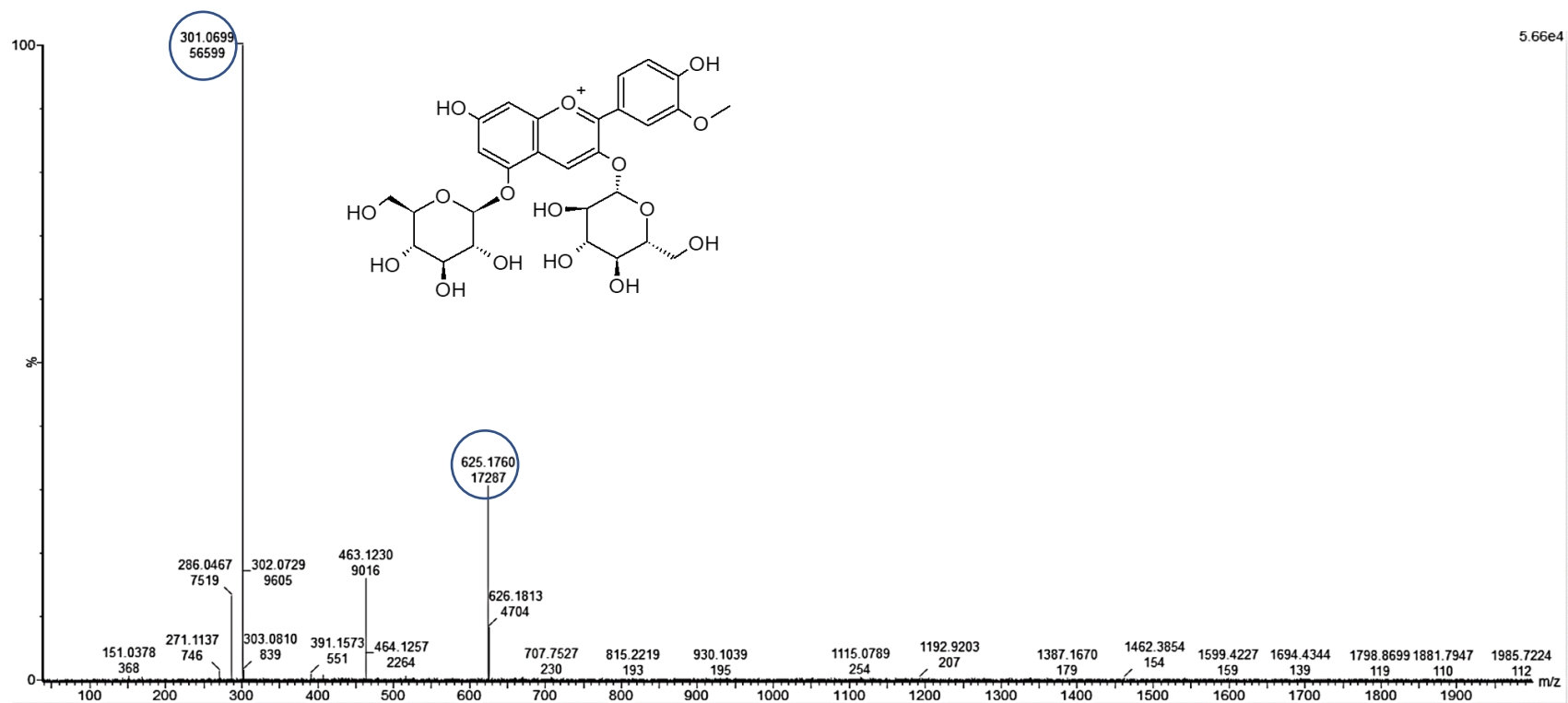


Figure A 8 Mass spectrum of peonidin-3,5-diglucoside

AV_UP_201102_7 1650 (12.840)

2: TOF MS ES+
4.44e3

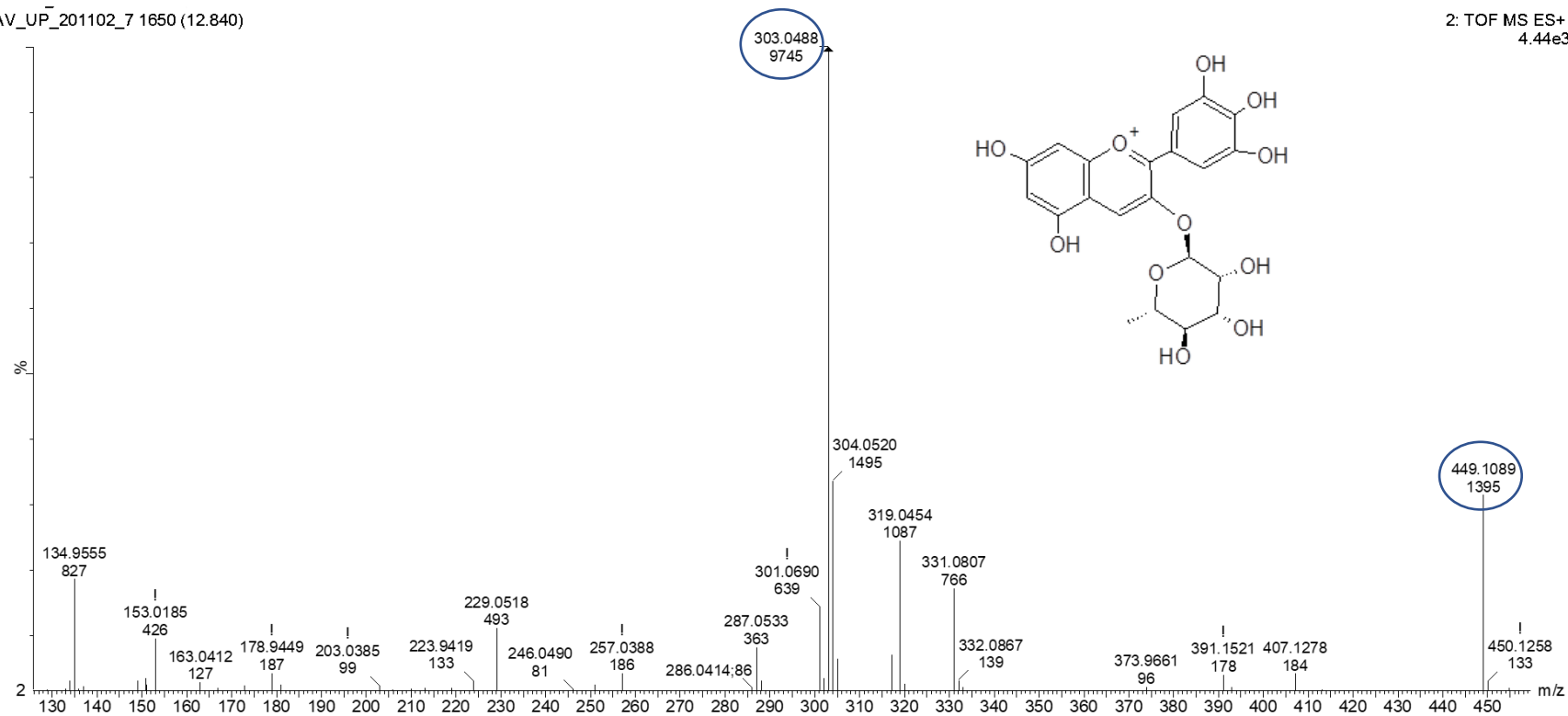


Figure A 9 Mass spectrum of delphinidin-3-O-rhamnoside

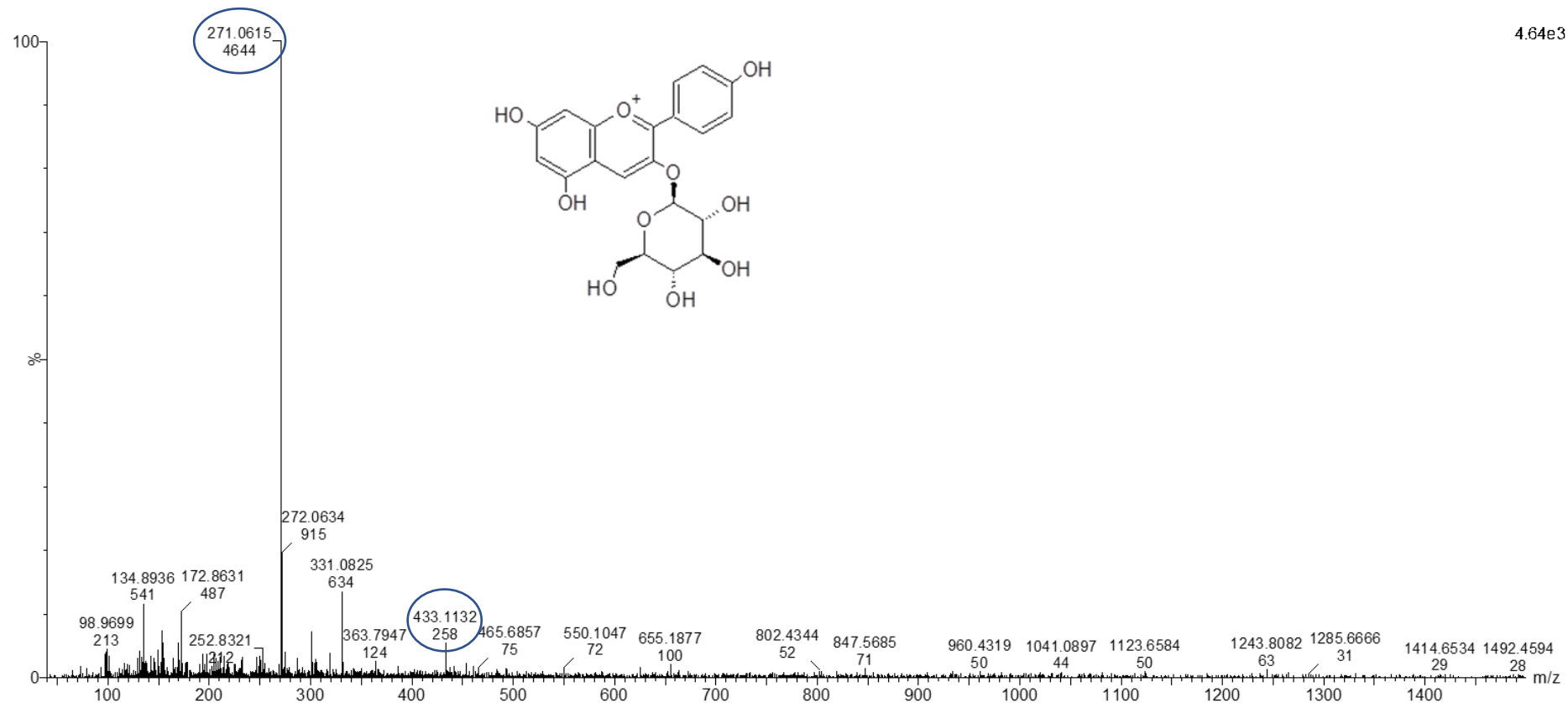


Figure A 10 Mass spectrum of *Pelargonidin-3-O-glucoside*

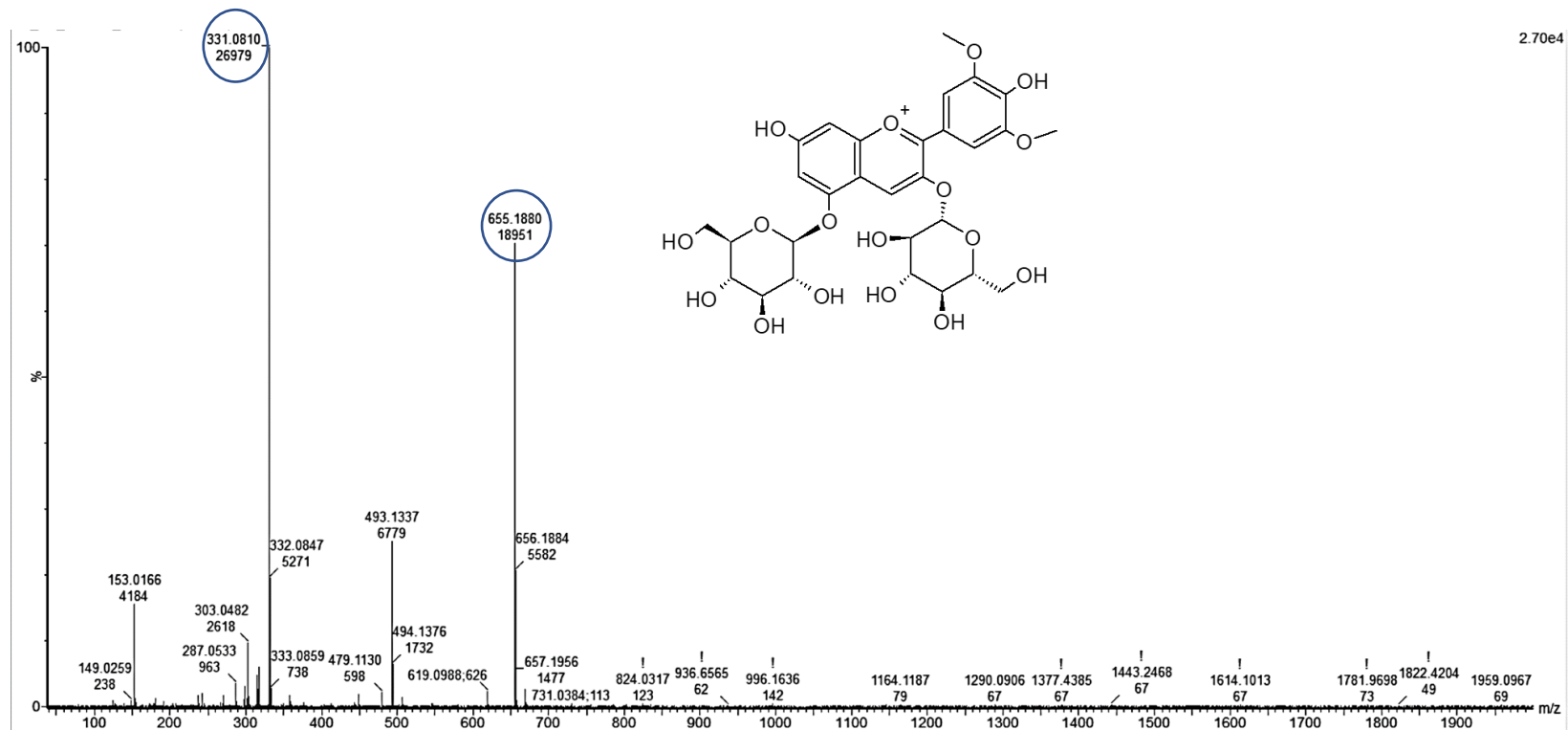


Figure A 11 Mass spectrum of malvidin-3,5-diglucoside

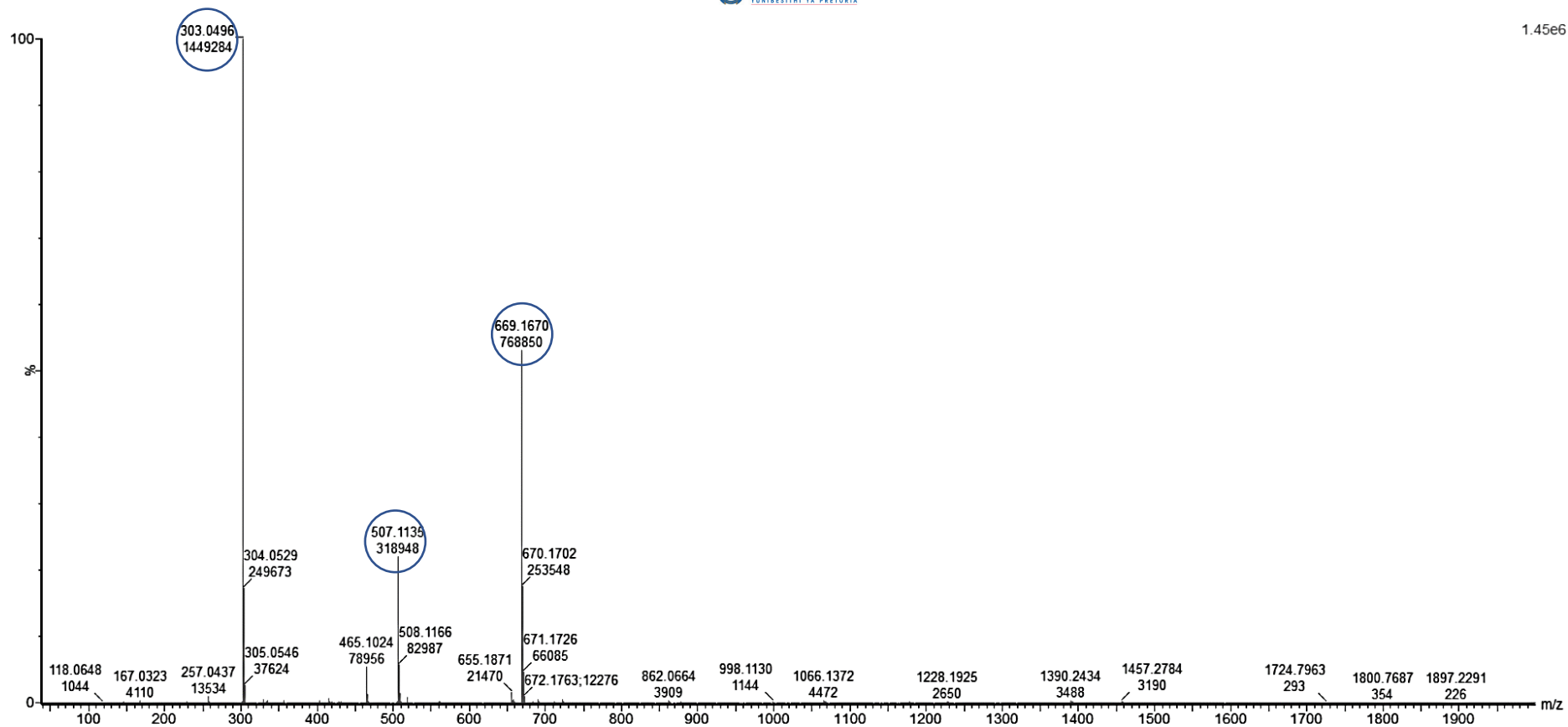


Figure A 12 Mass spectrum of delphinidin-3-O-(6-acetyl)-5-O-diglucoside)

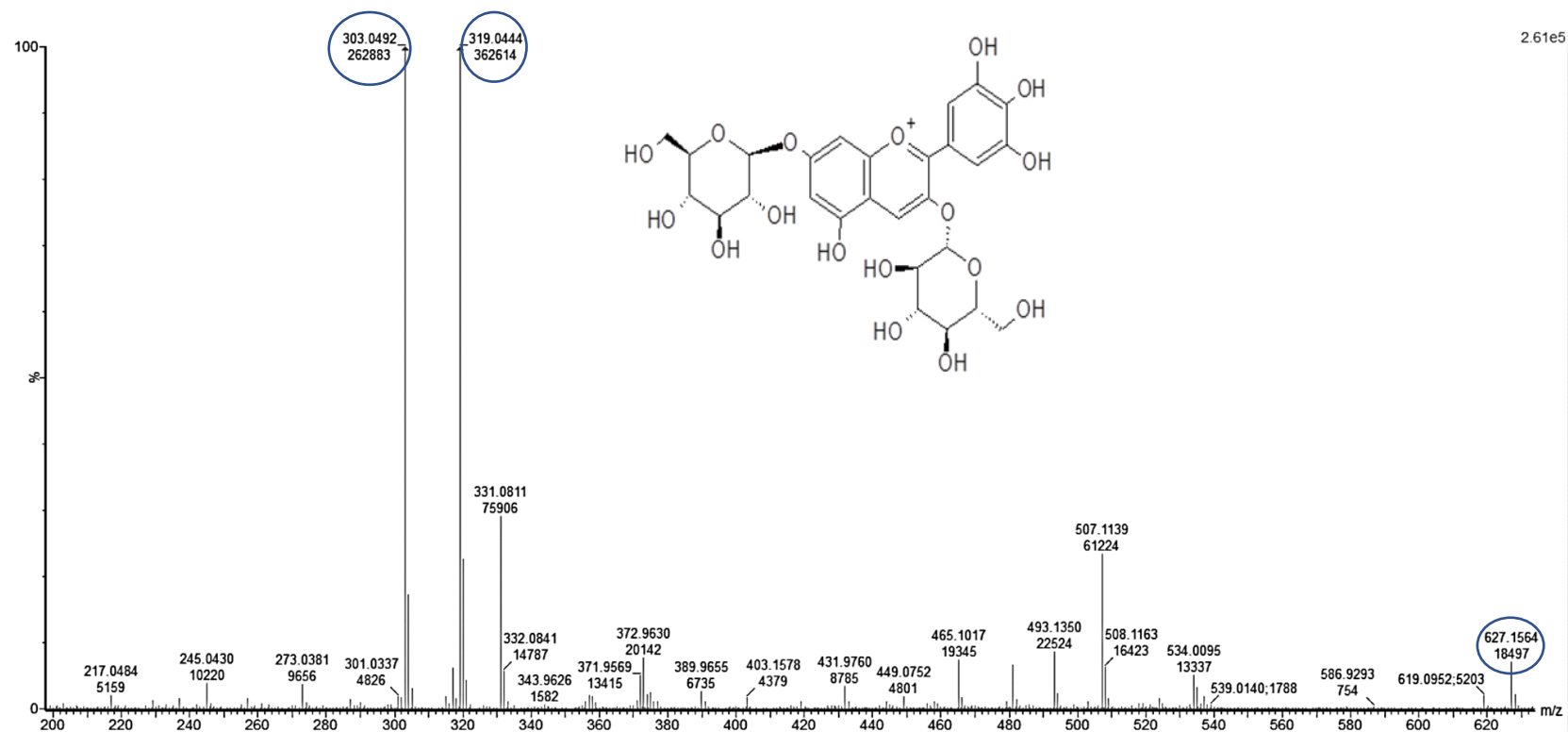


Figure A 13 Mass spectrum of delphinidin-3,7-diglucoside

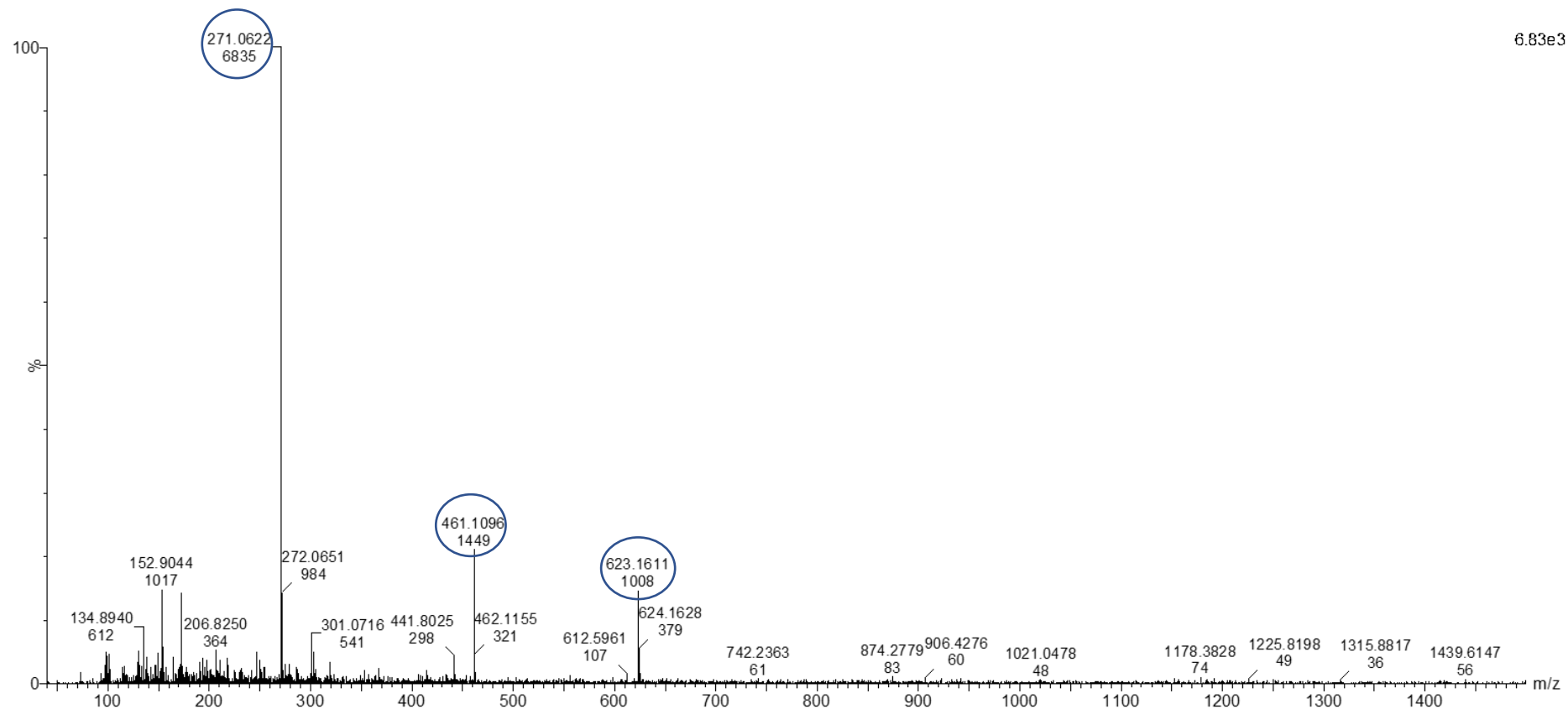


Figure A 14 Mass spectrum of pelargonidin glucoside derivative

8 - P. grandiflorum 2

AV_UP_201102_10 1945 (15.143) Cm (1926:1950)

2: TOF MS ES+
2.60e4

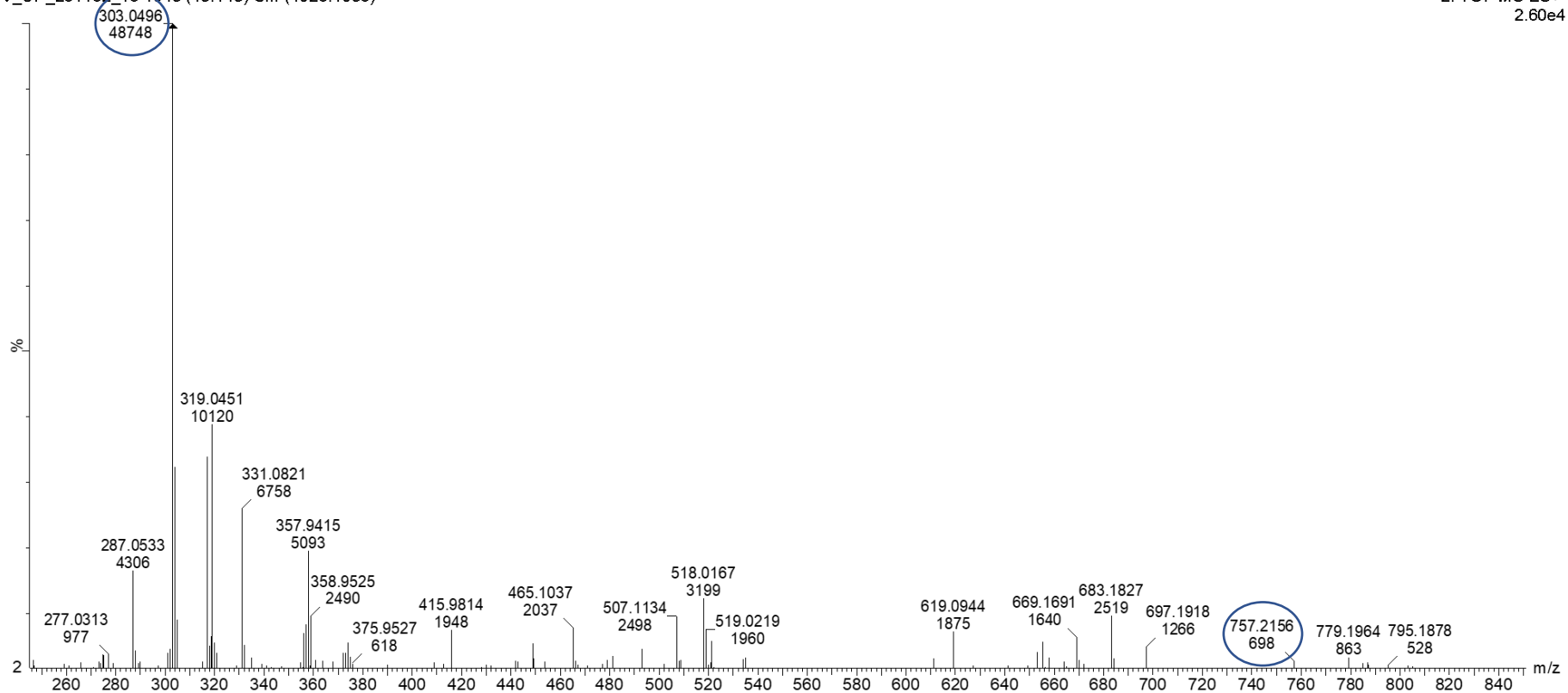


Figure A 15 Mass spectrum of Vincanin A (delphinidin-3-robinobiosido-5-rhamnoside)

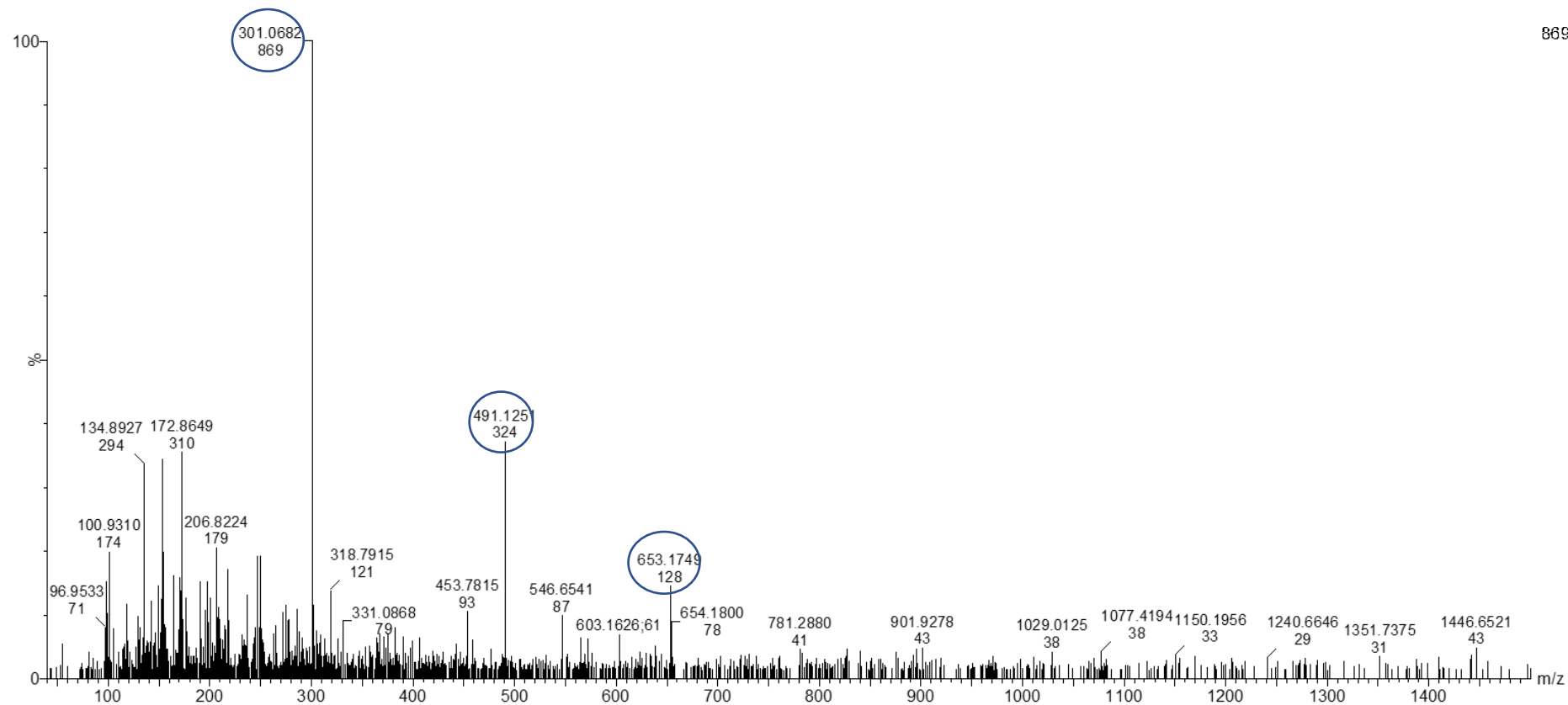


Figure A 16 Mass spectrum of peonidin glucoside derivative

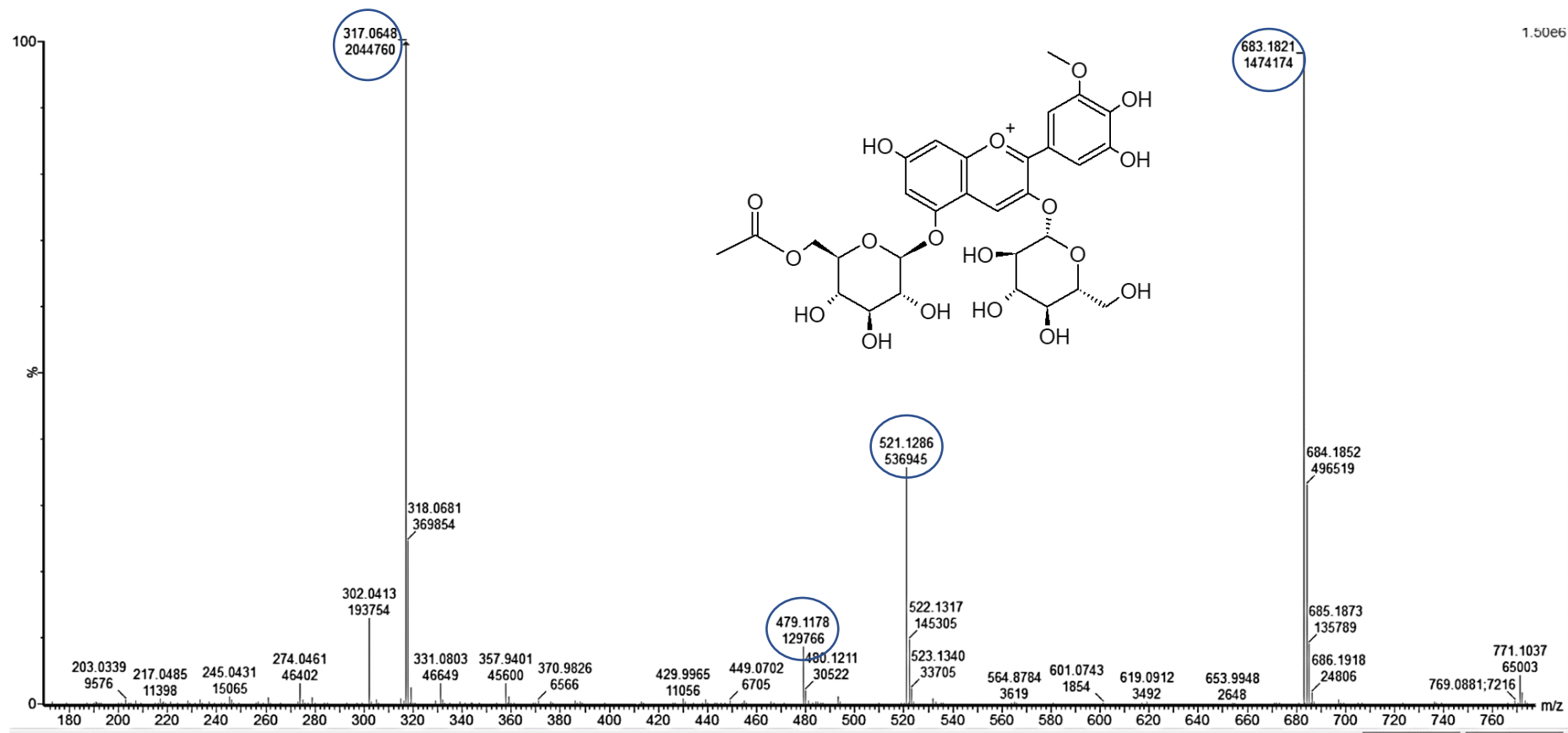


Figure A 17 Mass spectrum of petunidin-3-O-6-acetyl-5-O-diglucoside

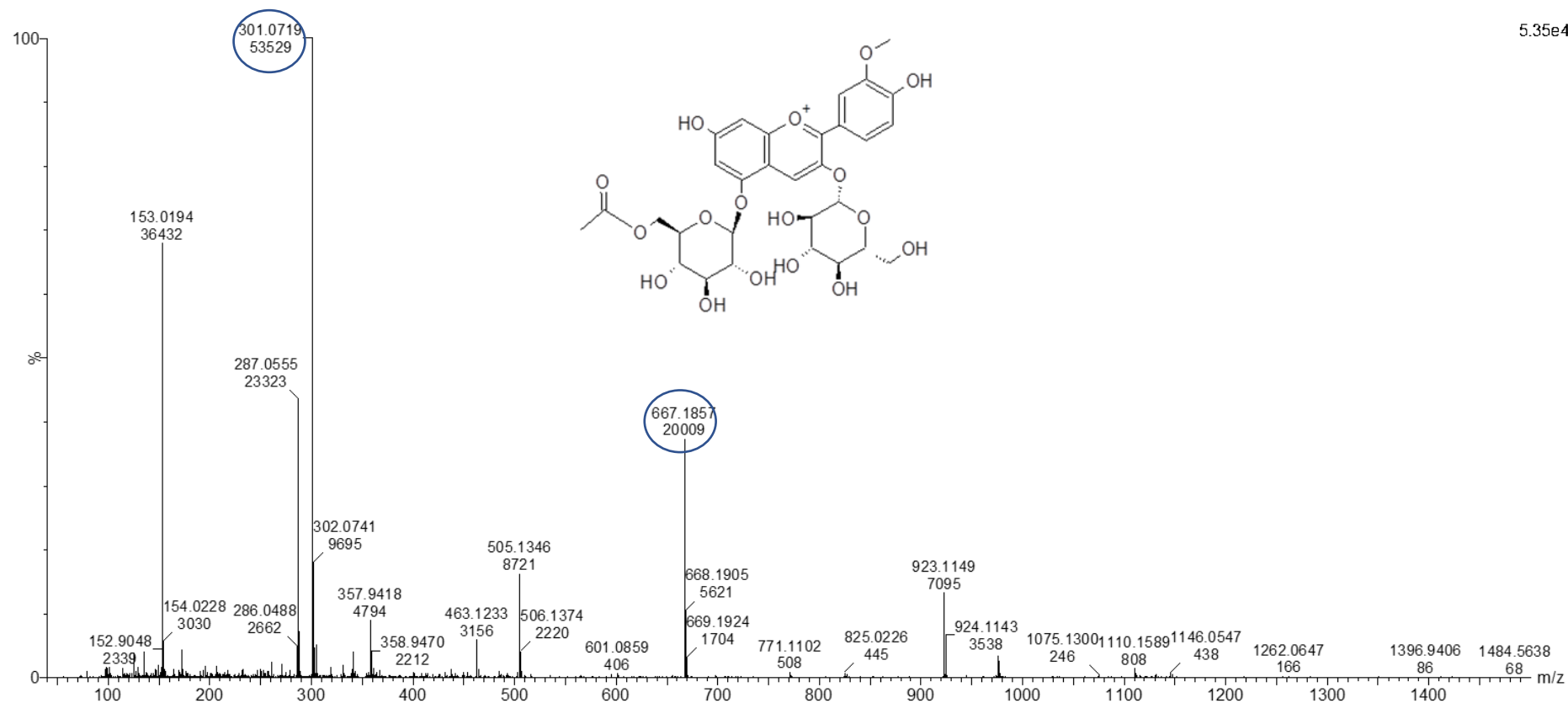


Figure A 18 Mass spectrum of peonidin-3-O-6-acetyl-5-O-diglucoside

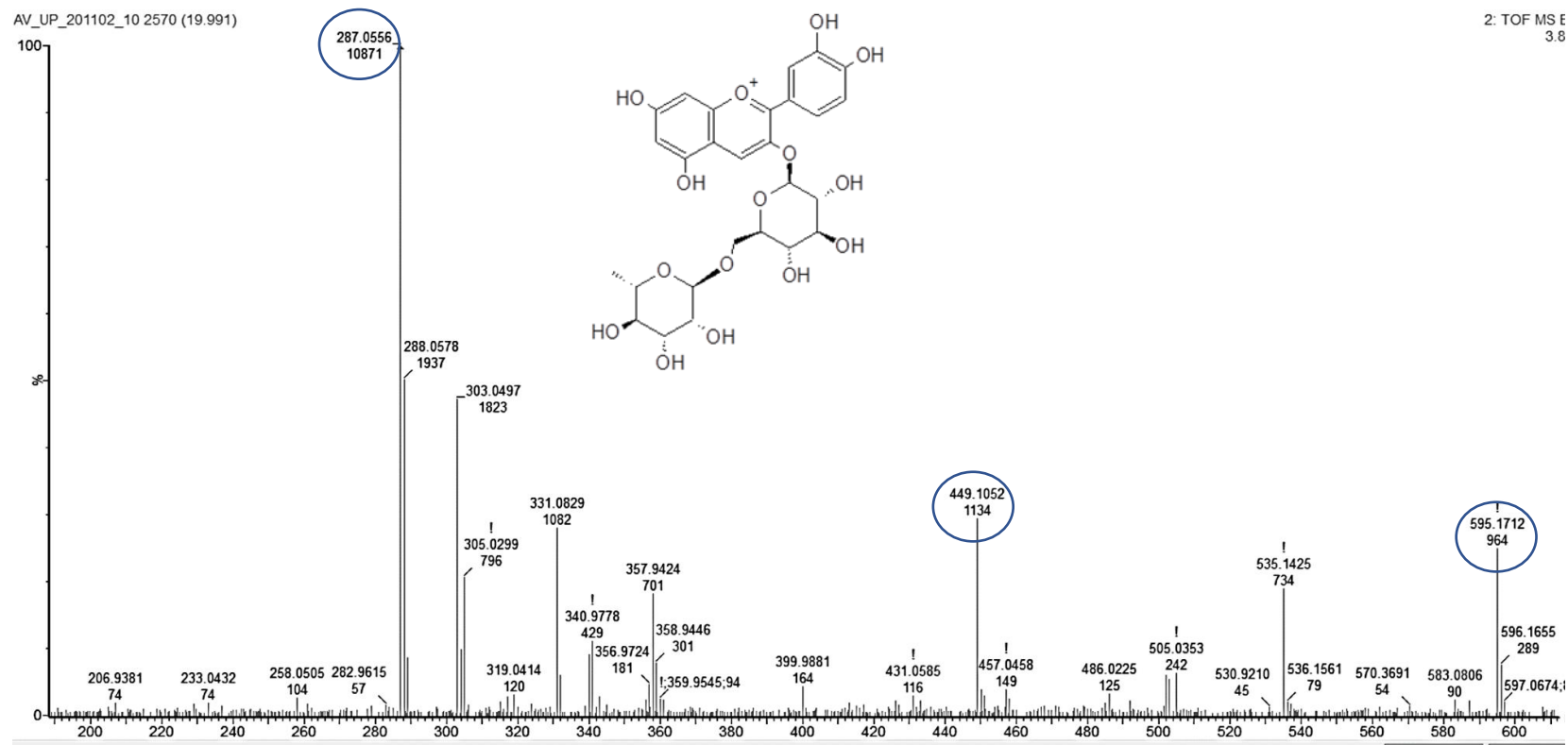


Figure A 19 Mass spectrum of cyanidin-3-O-rutinoside

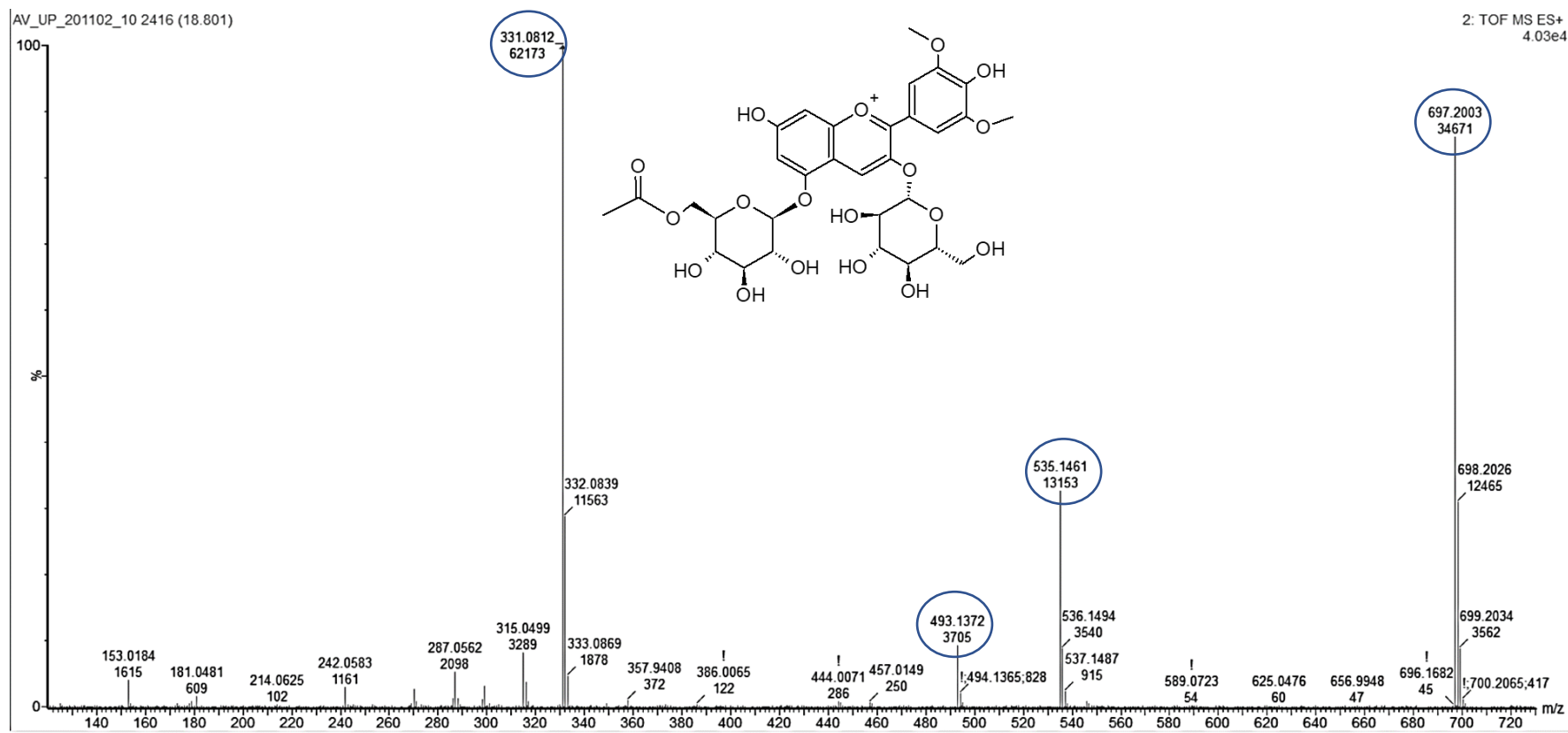


Figure A 20 Mass spectrum of malvidin-3-O-(6-O-acetyl)-5-O-diglucoside

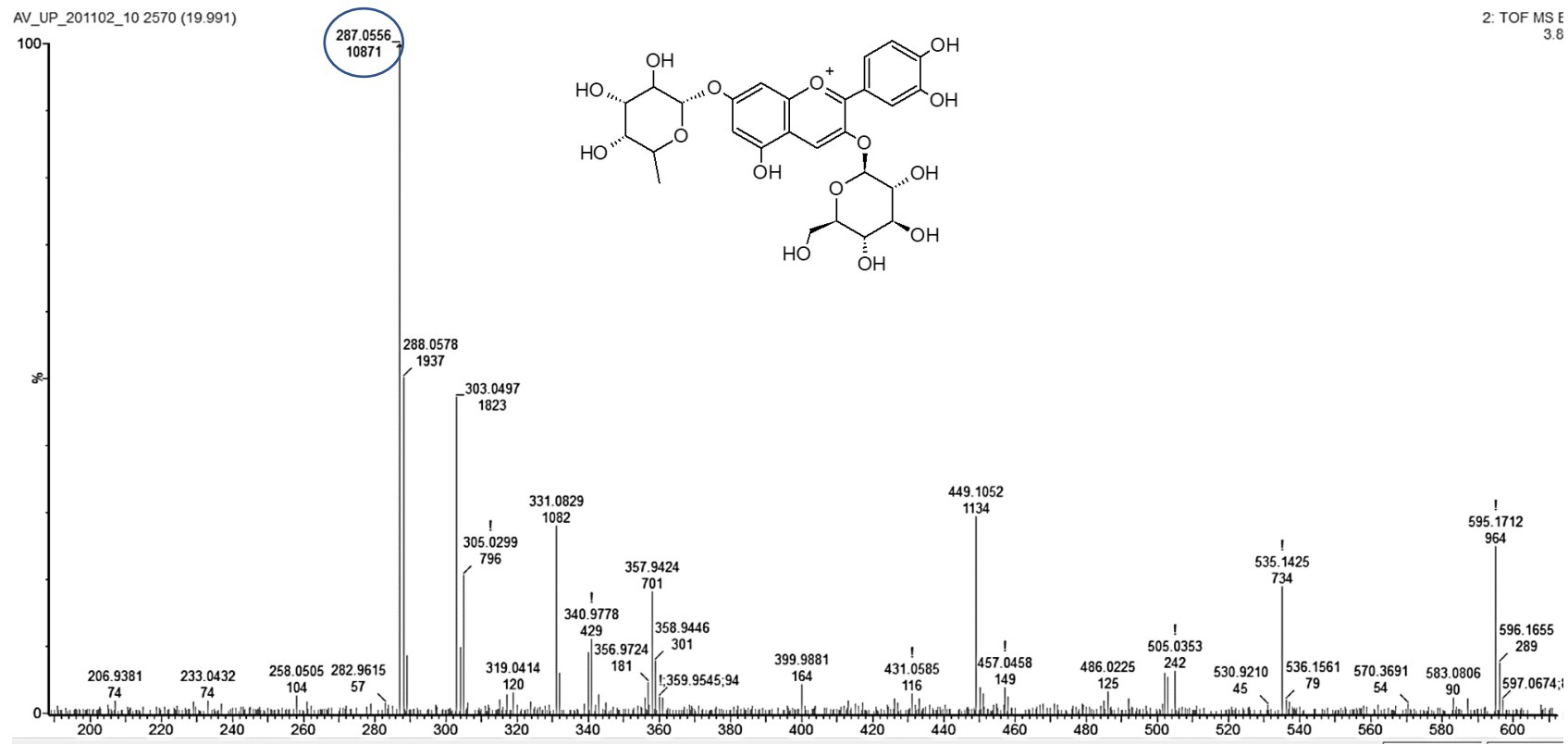


Figure A 21 Mass spectrum of cyanidin-3-(glucosyl)-7-rhamnoside

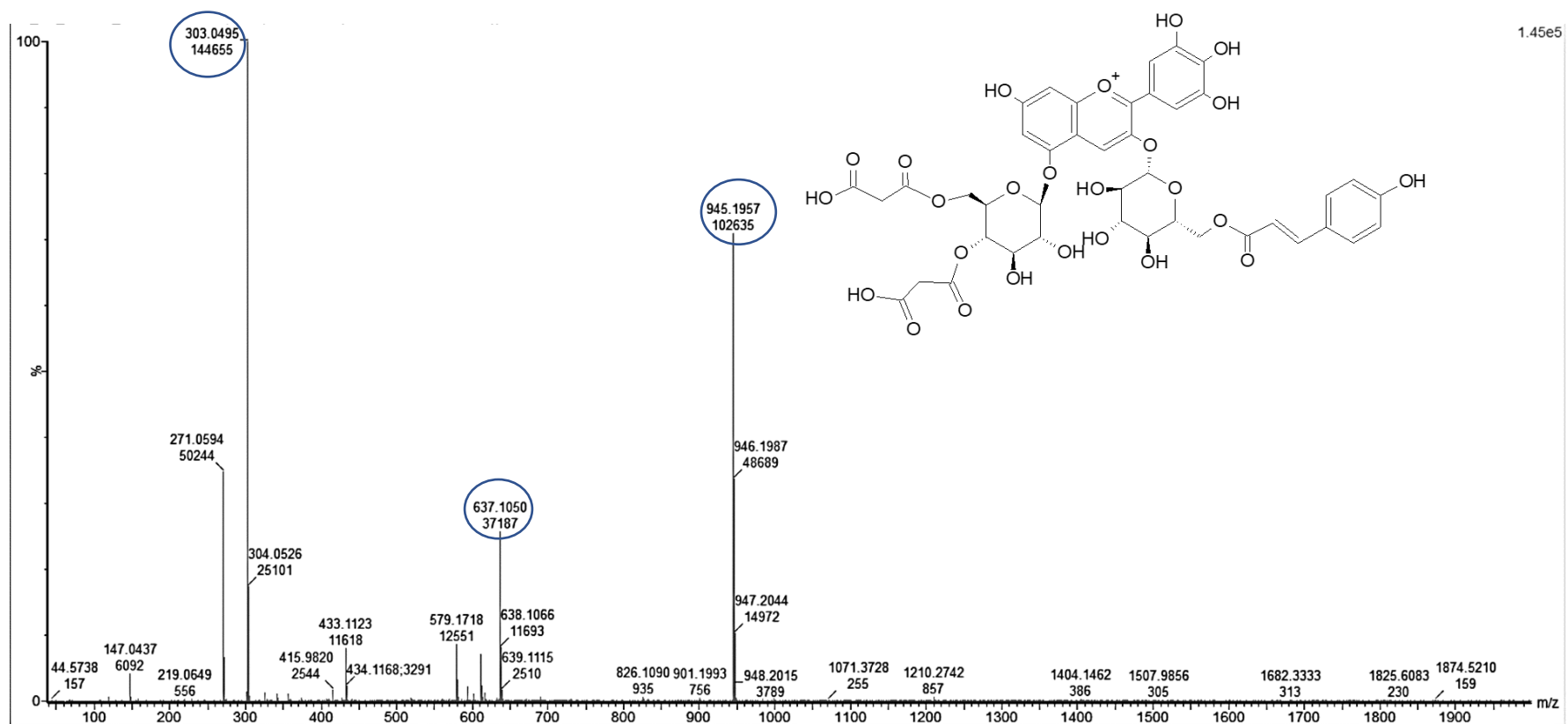


Figure A 22 Mass spectrum of Dimalonylawobanin (delphinidin-3-(6-p-coumaroylglucoside)-5-(4,6-dimalonylglucoside)

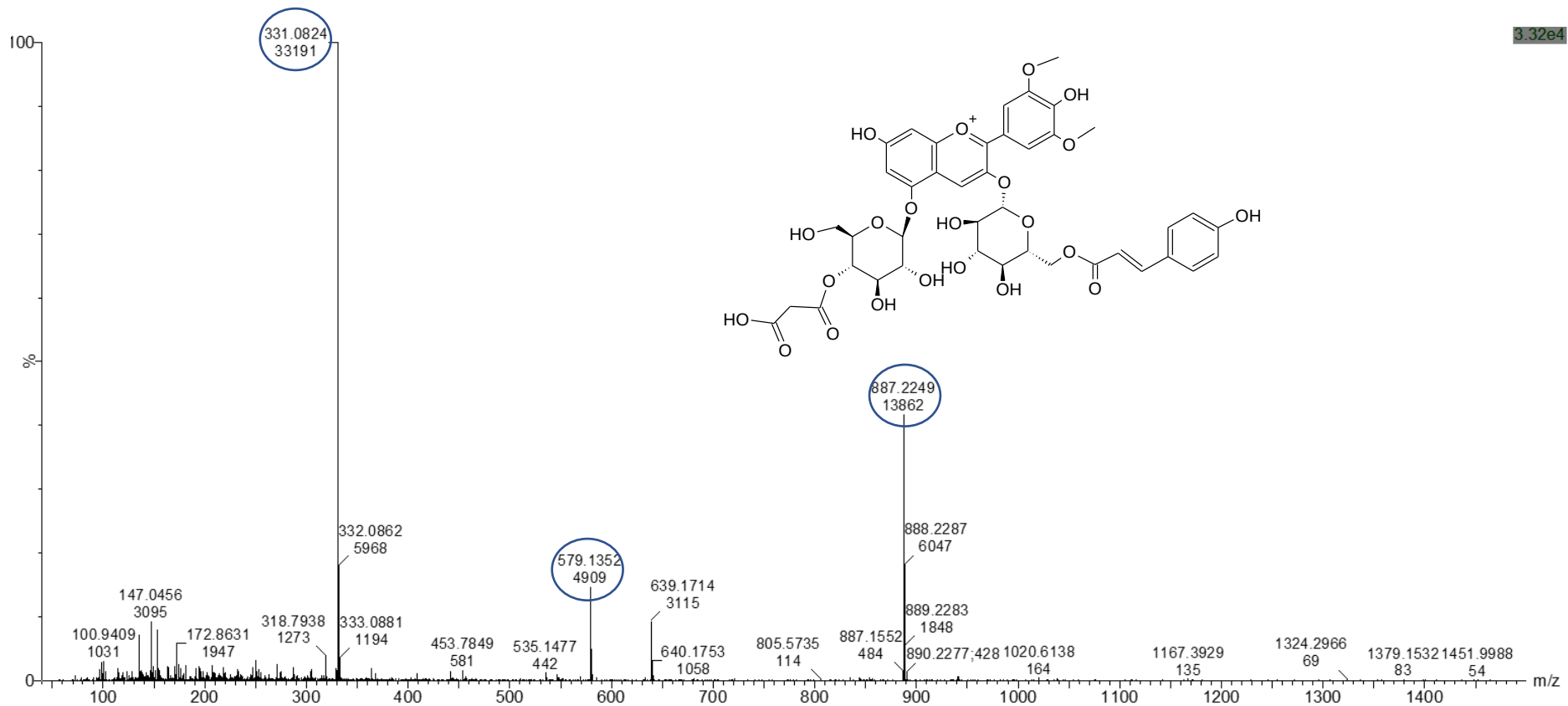


Figure A 23 Mass spectrum of malvidin-3-(6-p-coumaroylglucoside)-5-(4-malonylglucoside)

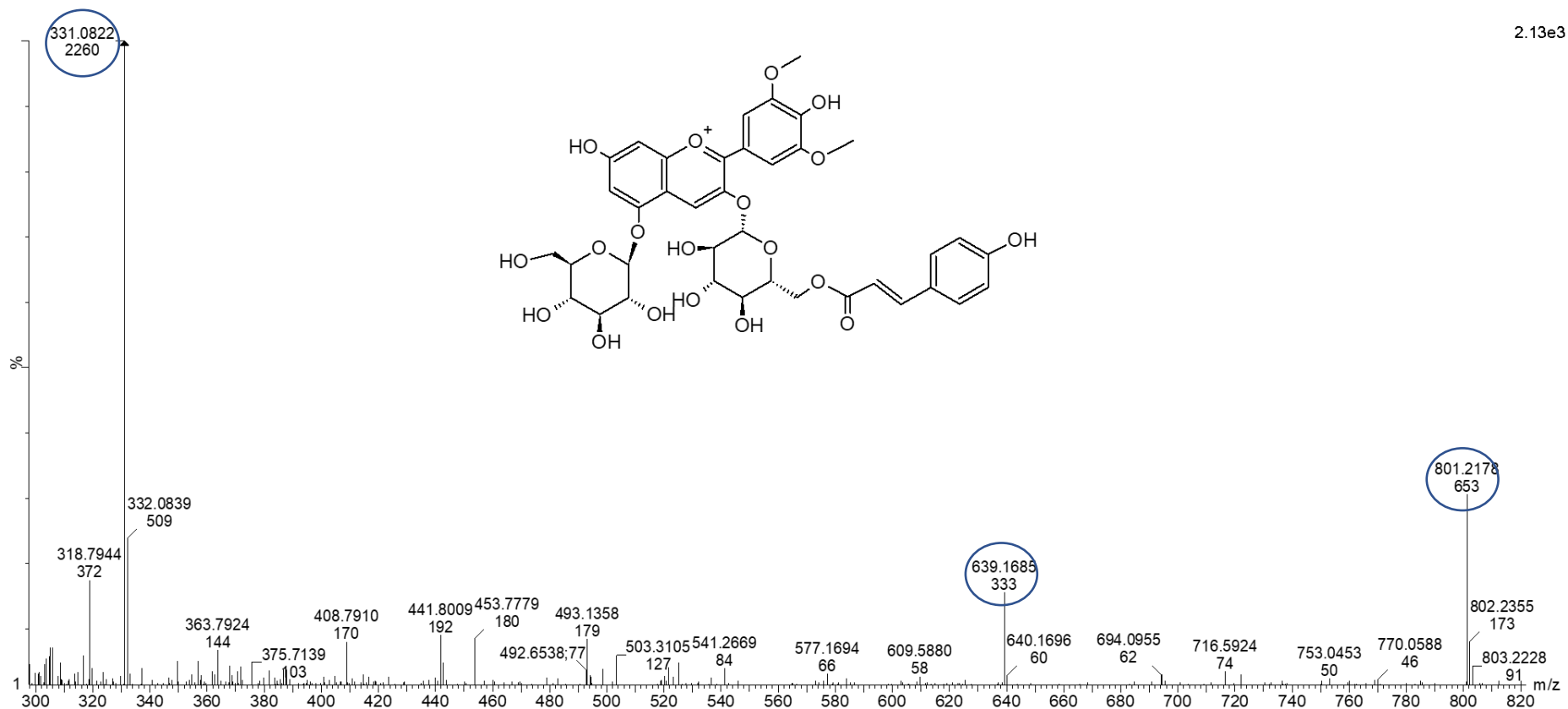


Figure A 24 Mass spectrum of Malvidin-3-(6-p-coumaroyl-glucoside)-5-glucoside

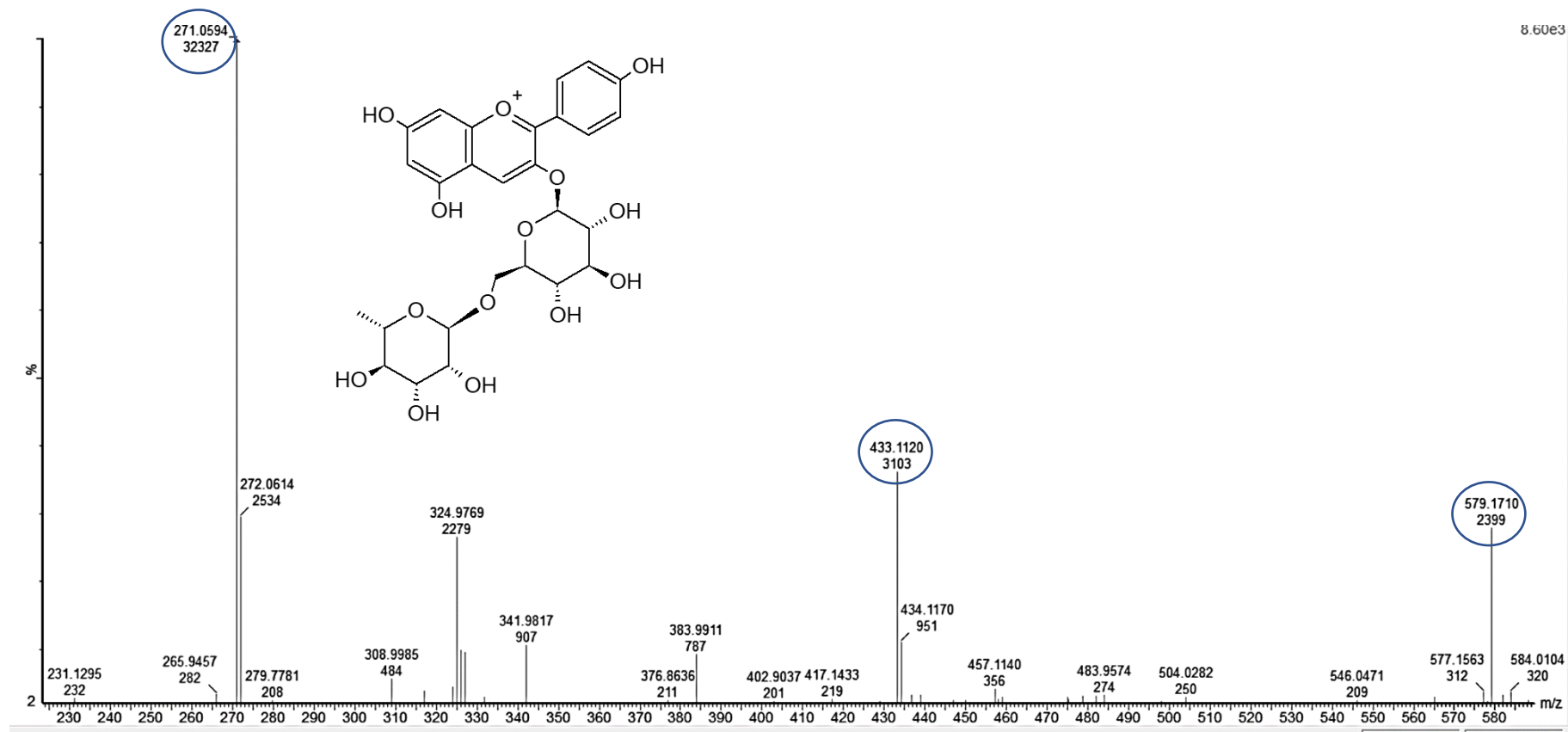


Figure A 25 Mass spectrum of Pelargonidin-3-O-rutinoside

Appendix B

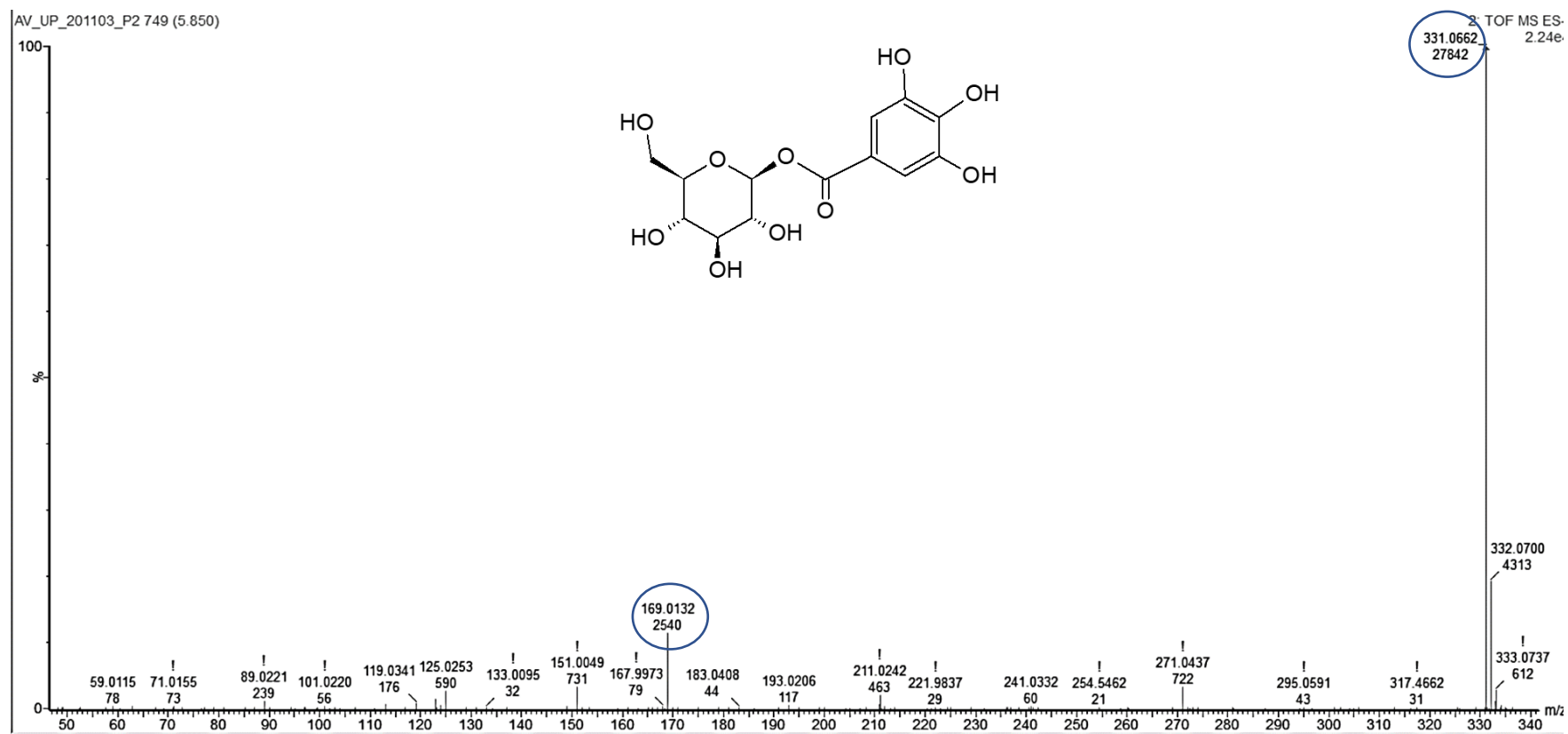


Figure B 1 Mass spectrum of β -glucogallin (galloyl- β -D-glucoside)

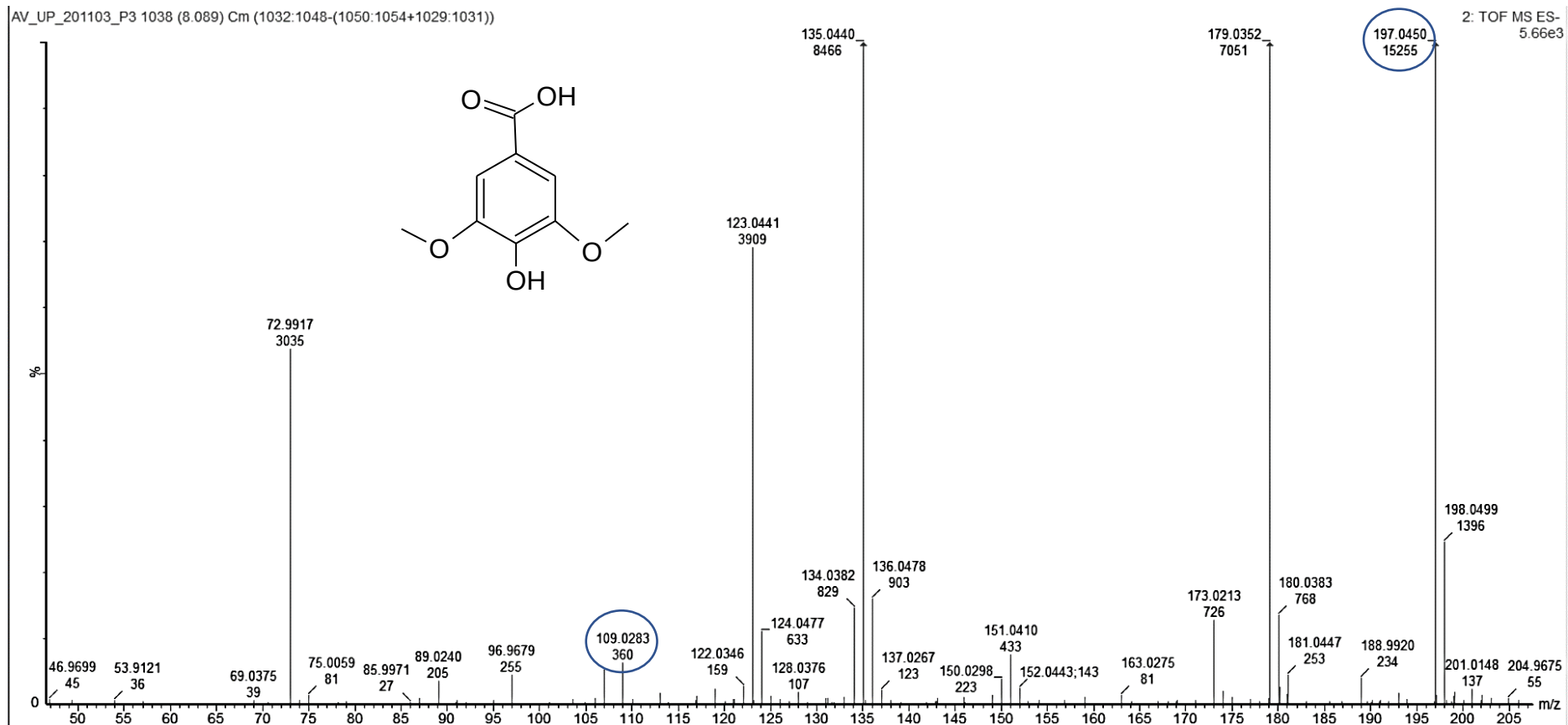


Figure B 2 Mass spectrum of Syringic acid

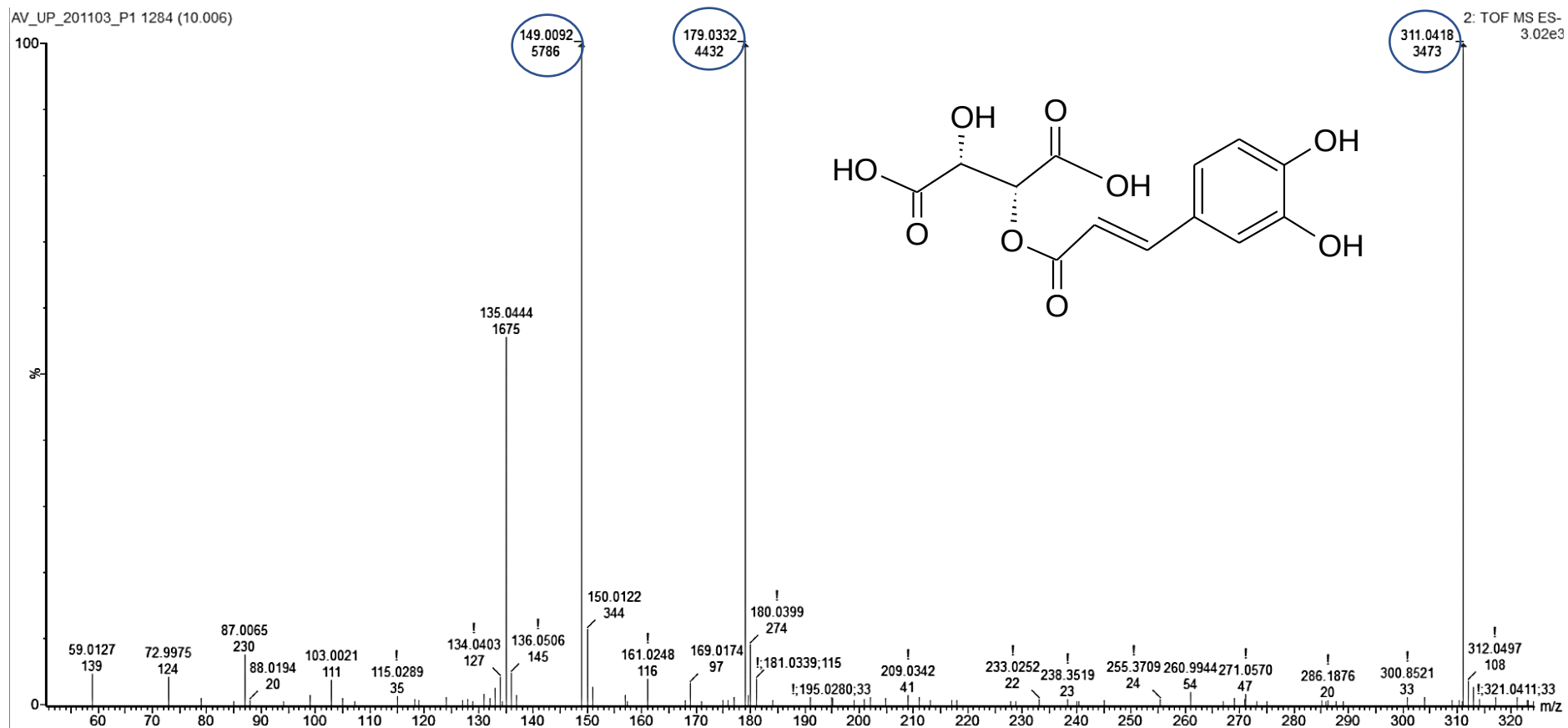


Figure B 3 Mass spectrum of Caffeoyltartaric acid

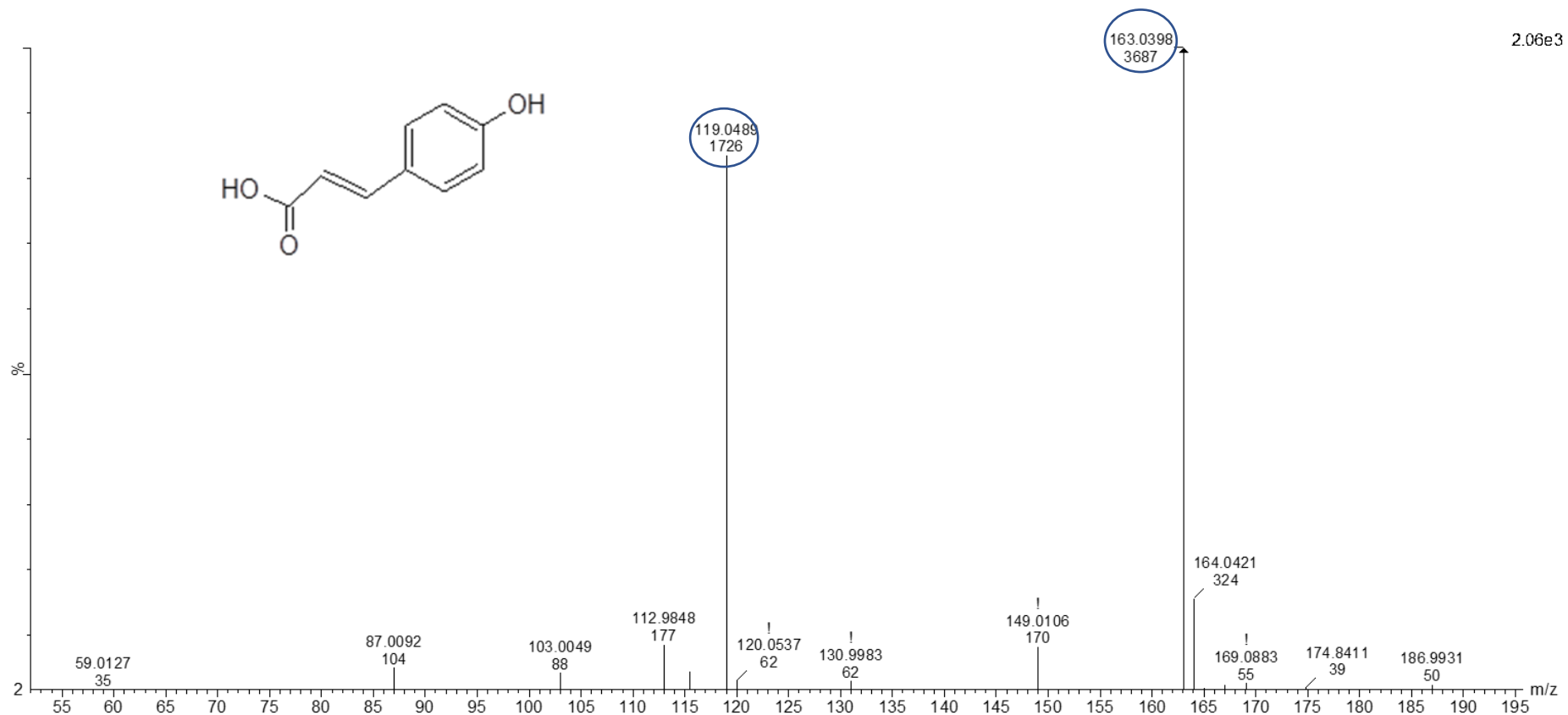


Figure B 4 Mass spectrum of p-coumaric acid

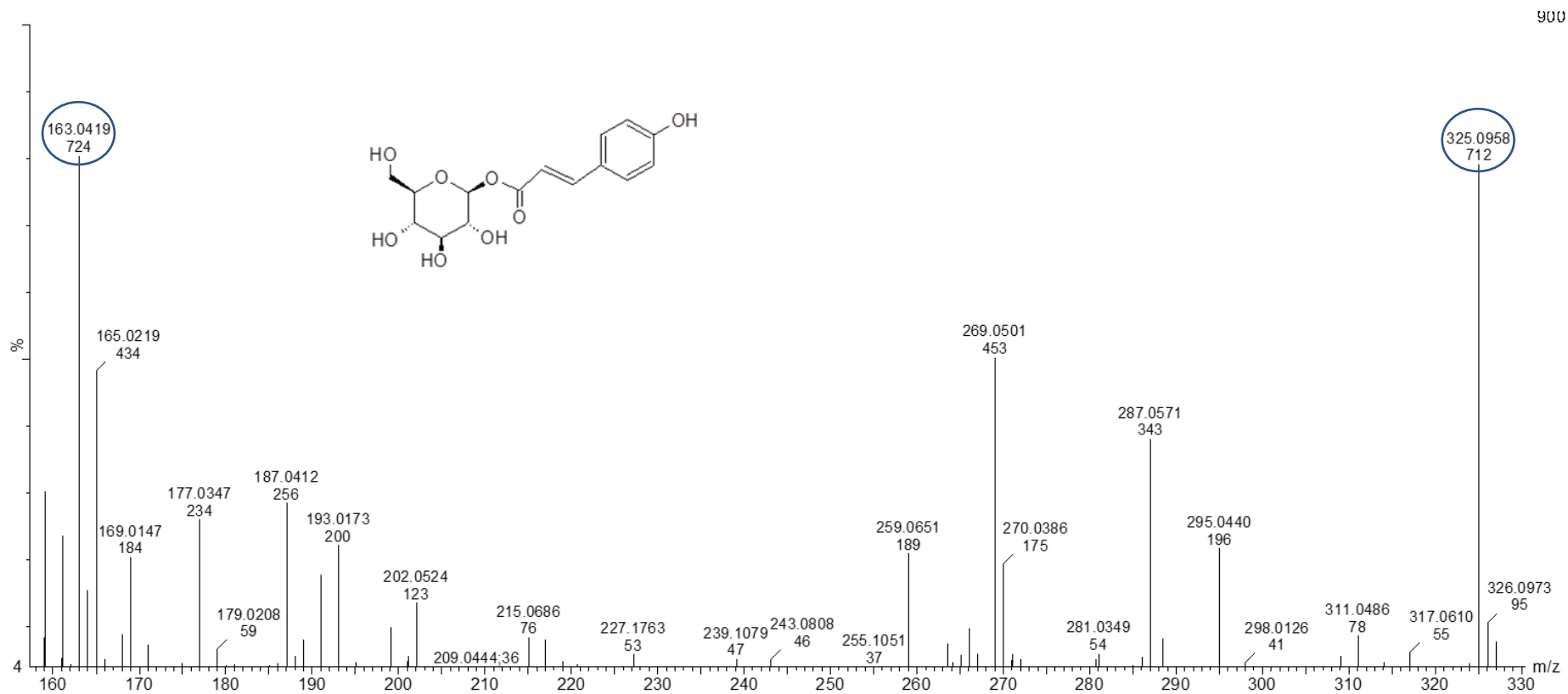


Figure B 5 Mass spectrum of *p*-coumaroylglucose

AV_UP_201103_P5 1609 (12.533) Cm (1601:1610)

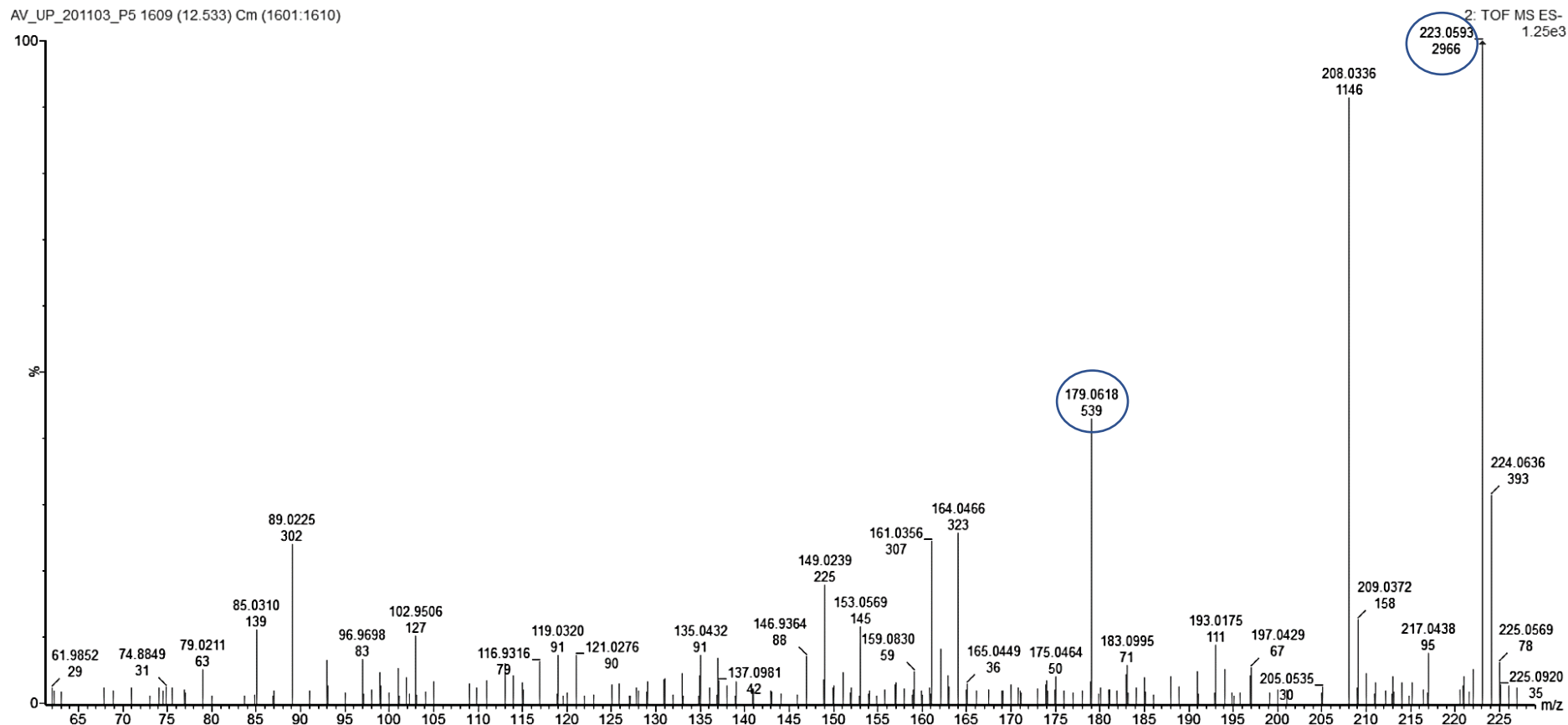


Figure B 6 Mass spectrum of Sinapic acid isomer

AV_UP_201103_P3 1650 (12.840) Cm (1645:1657)

2: TOF MS ES-
3.82e3

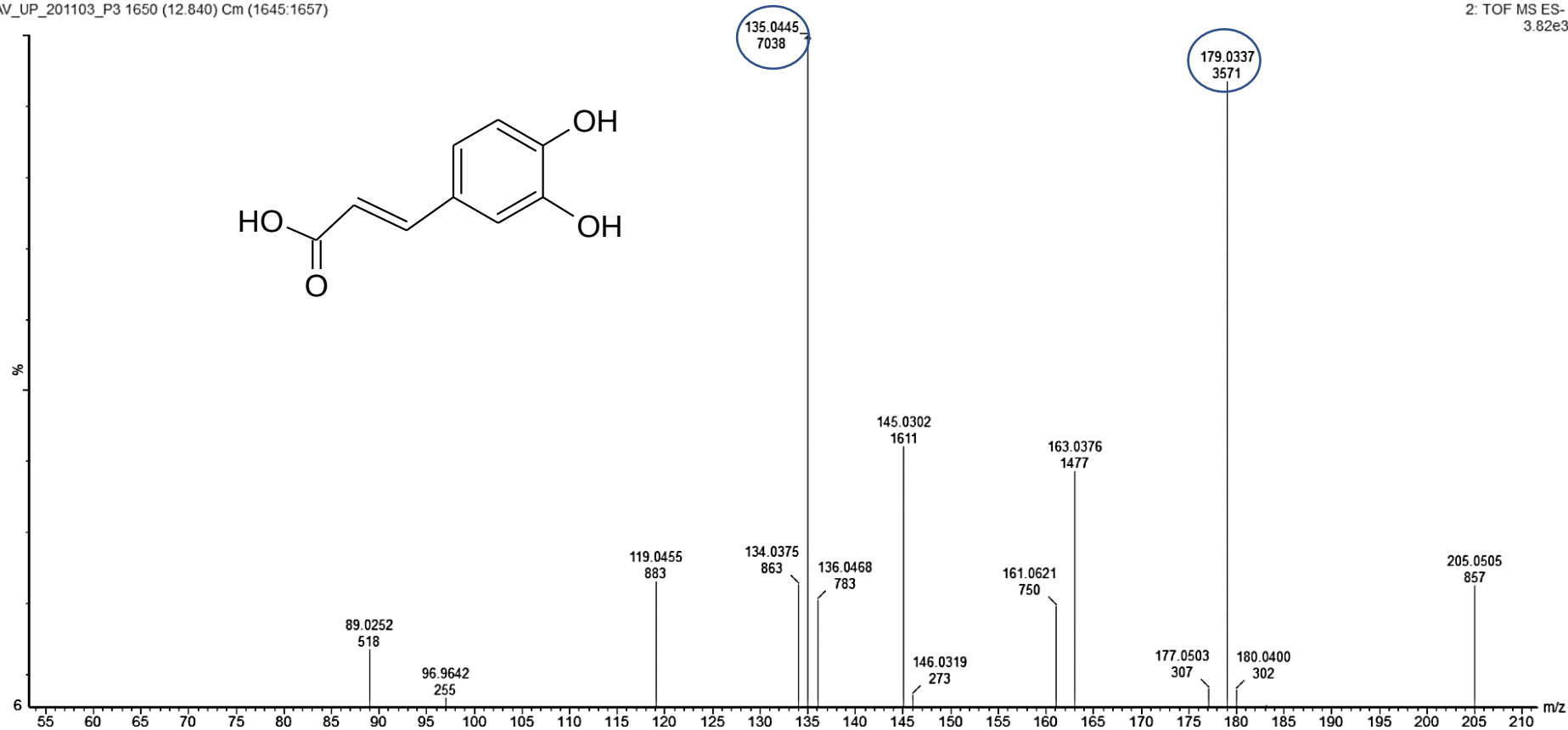


Figure B 7 Mass spectrum of Caffeic acid

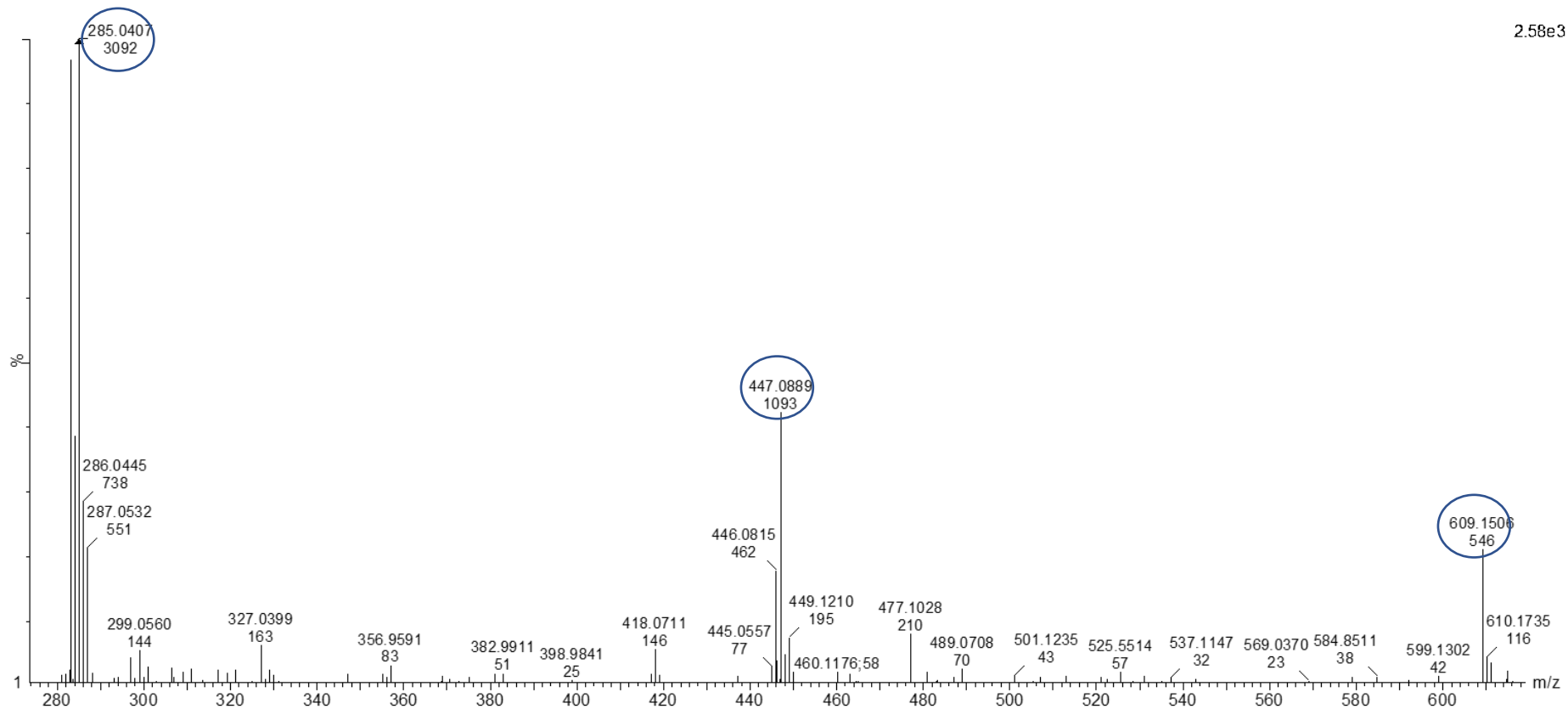


Figure B 8 Mass spectrum of kaempferol-3-O-glucosylgalactoside

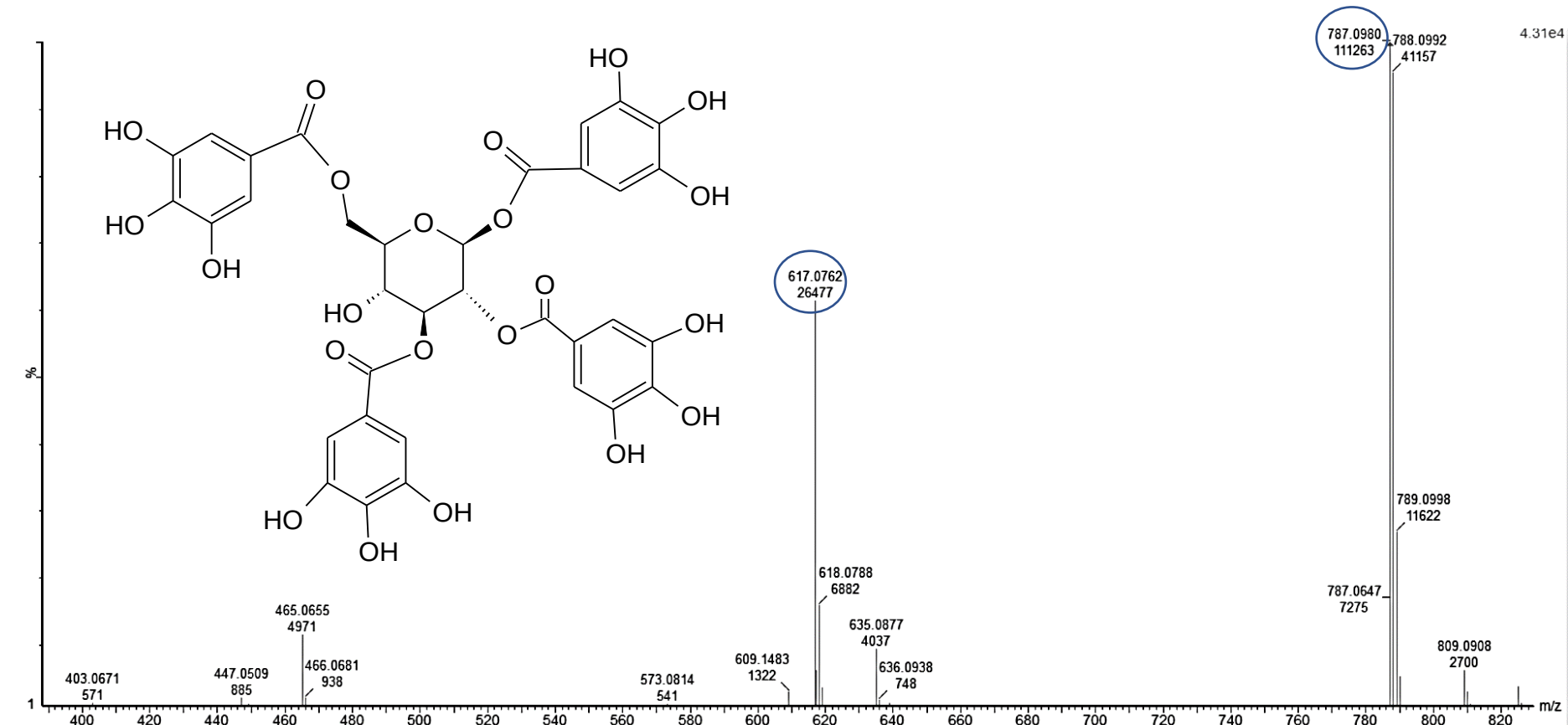


Figure B 9 Mass spectrum of tetra-O-galloyl-glucose

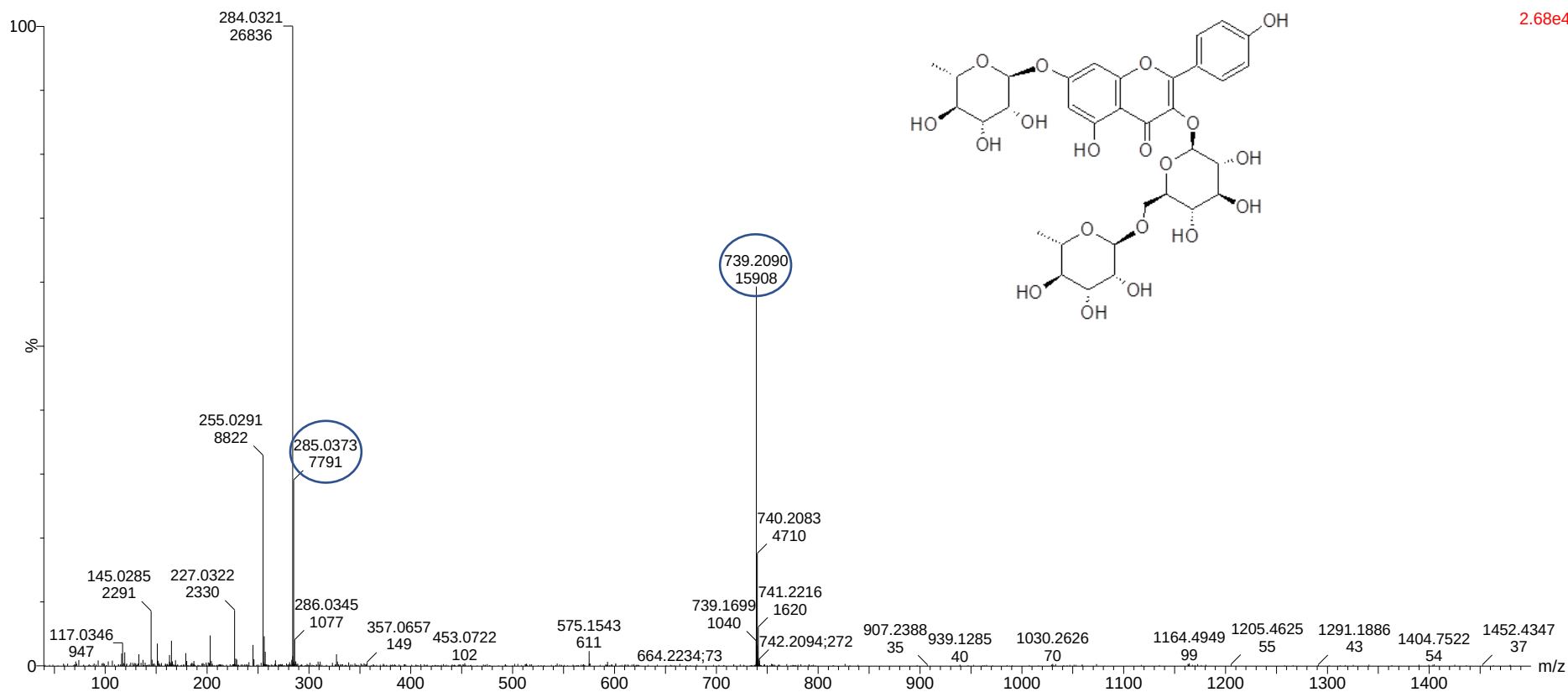


Figure B 10 Mass spectrum of Robinin (kaempferol-3-O-robinoside-7-O-rhamnoside)

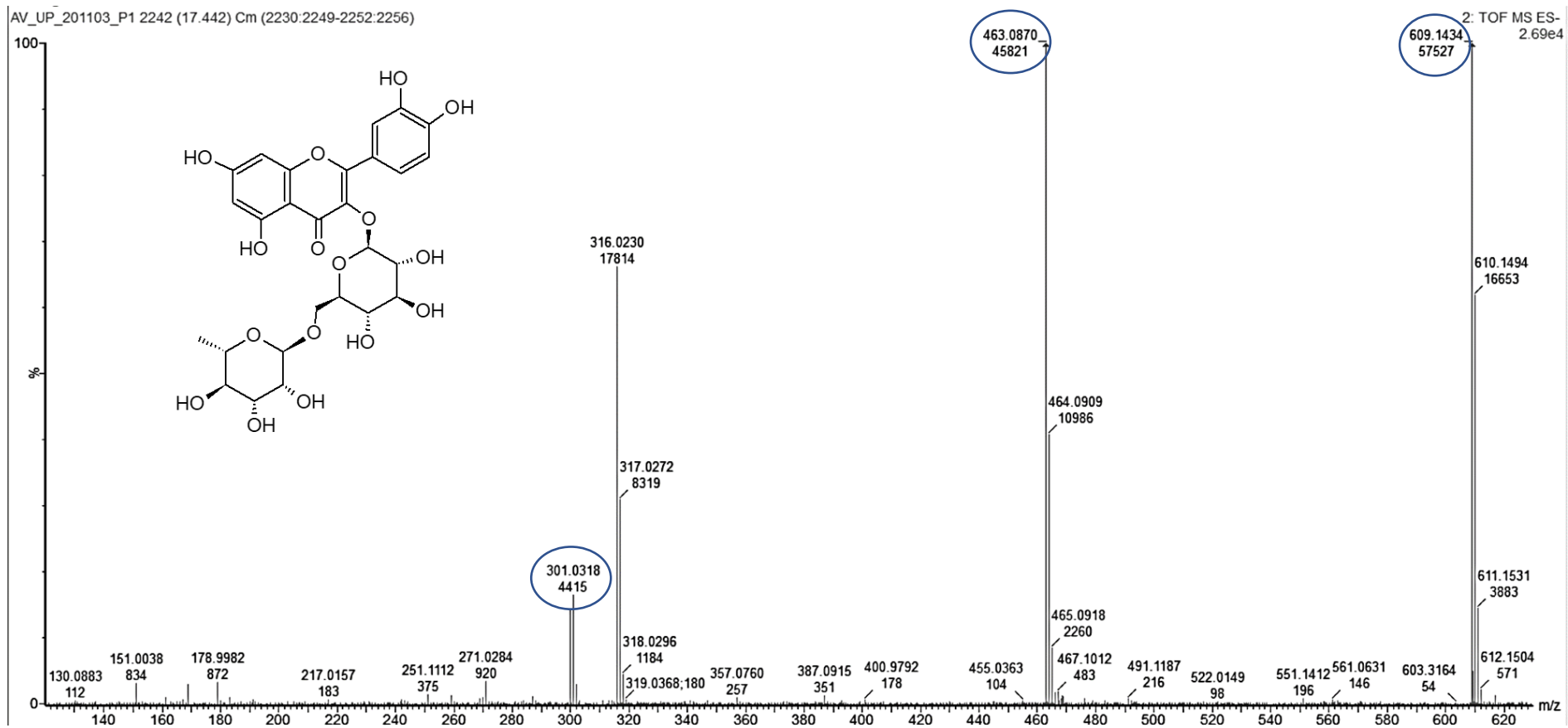


Figure B 11 Mass spectrum of Rutin (Quercetin-3-O-rutinoside)

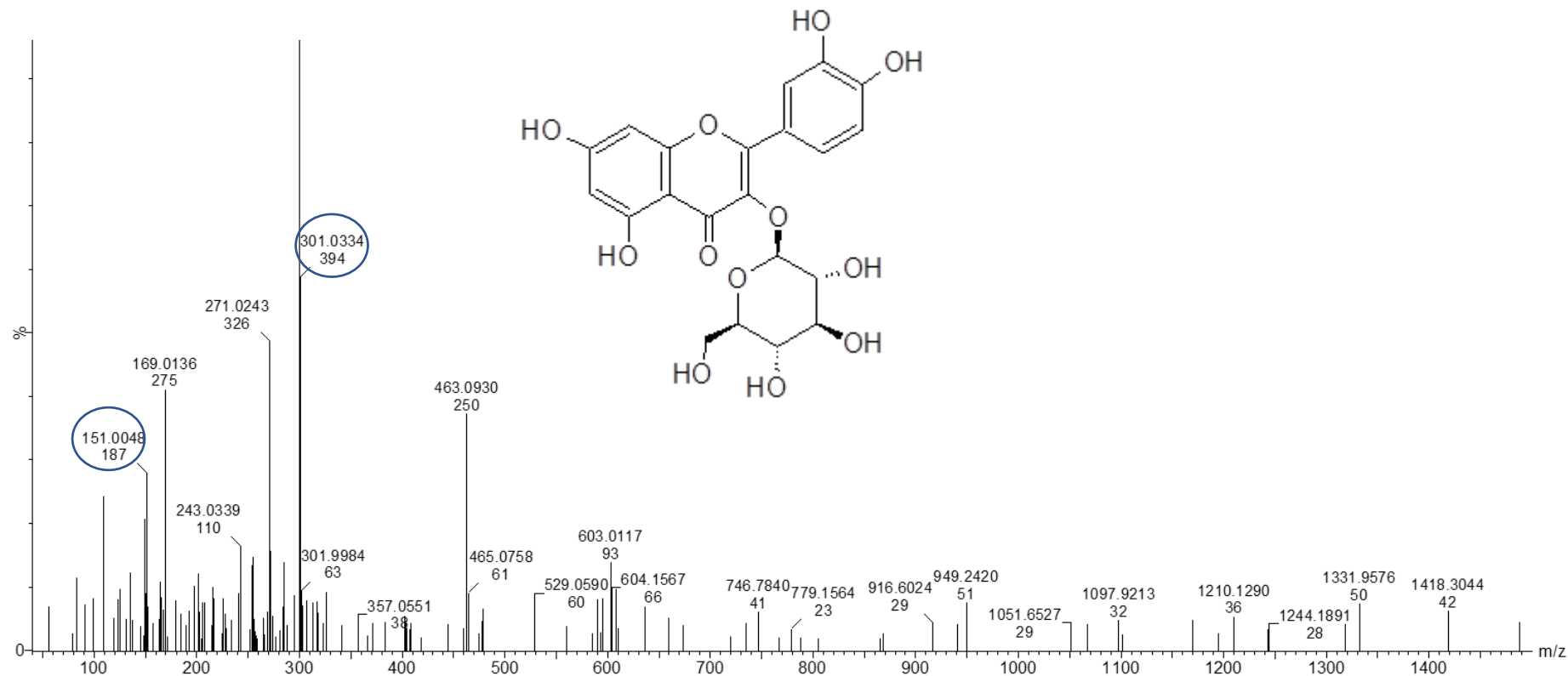


Figure B 12 Mass spectrum of Quercetin-3-O-glucoside (Isoquercetin)

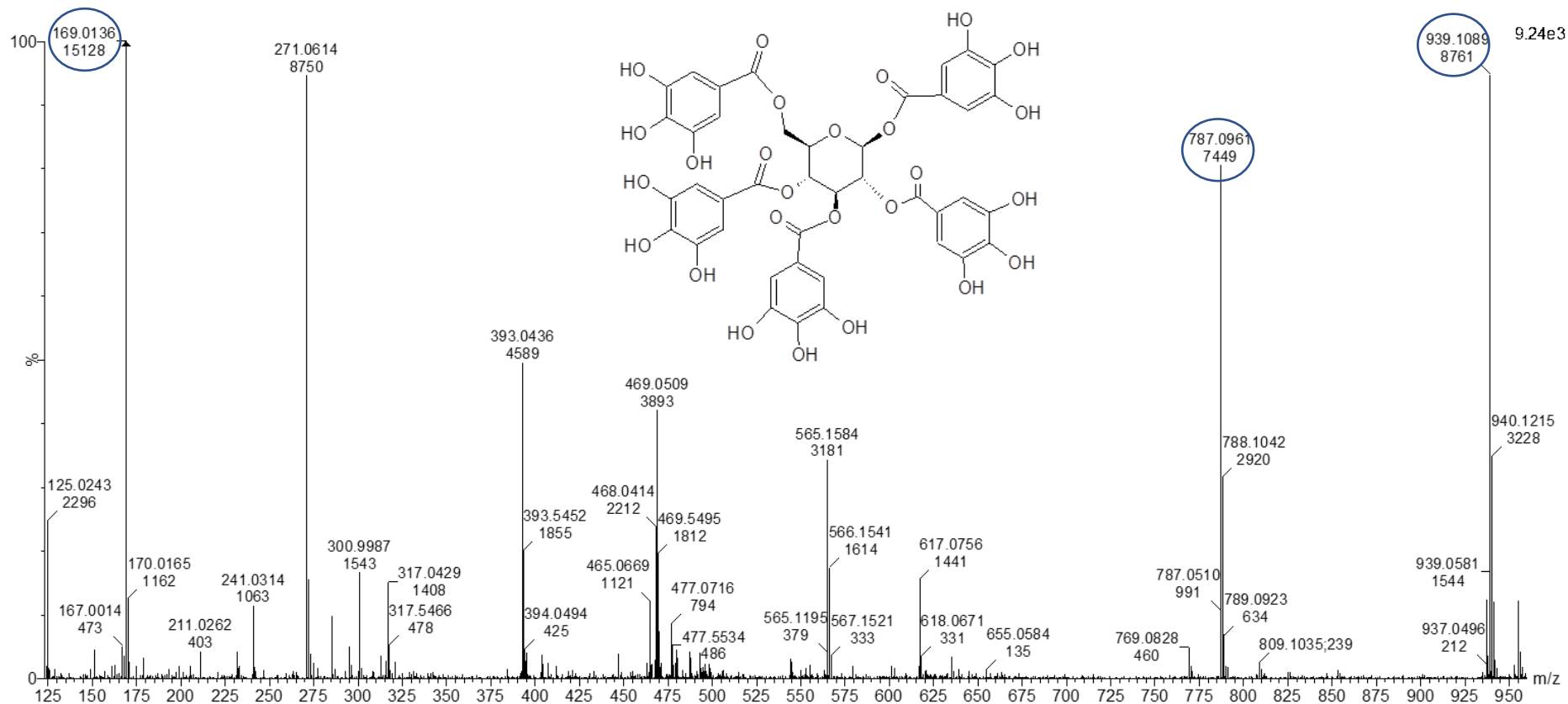


Figure B 13 Mass spectrum of *Penta-O-galloyl-glucose*

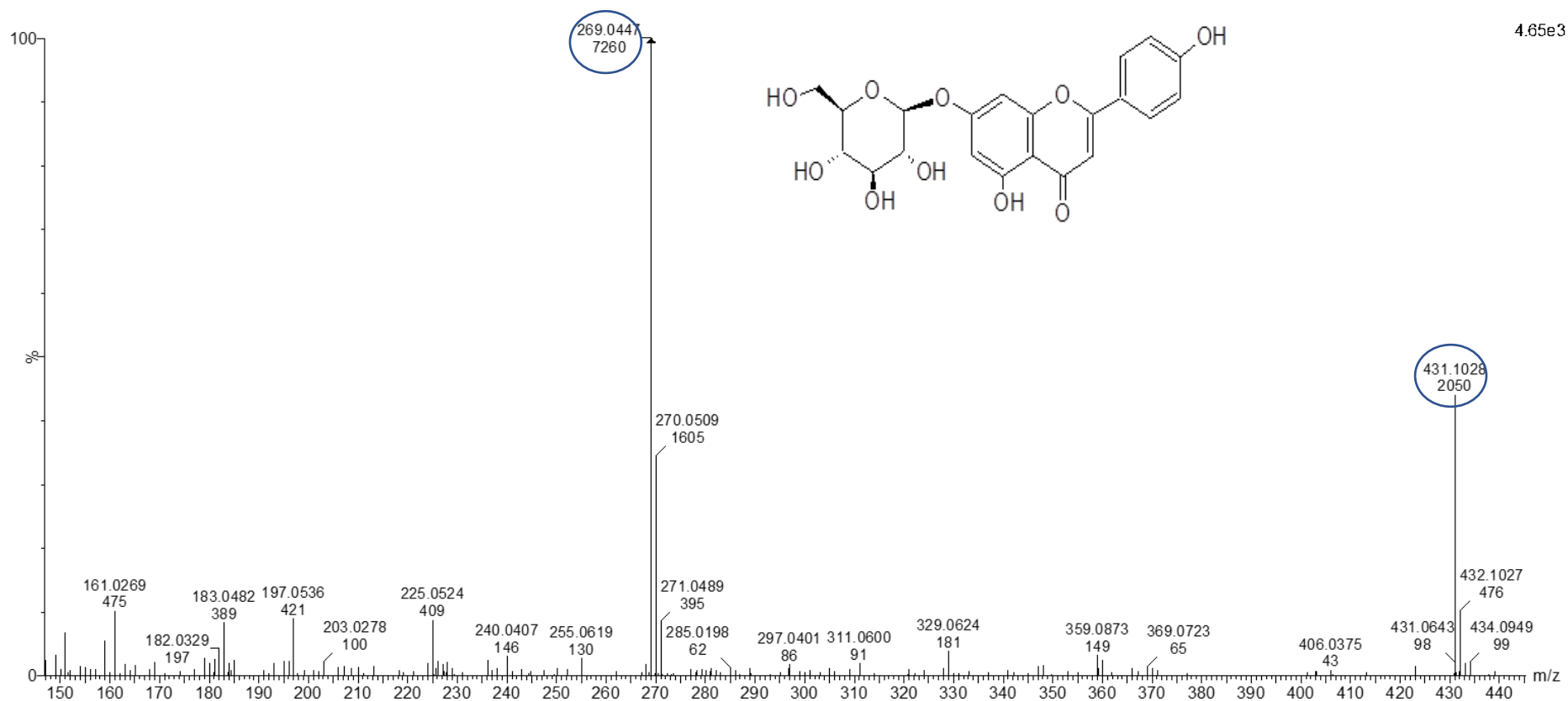


Figure B 14 Mass spectrum of Apigenin-7-O-glucoside

AV_UP_201103_P1 2444 (19.030) Cm (2443:2453)

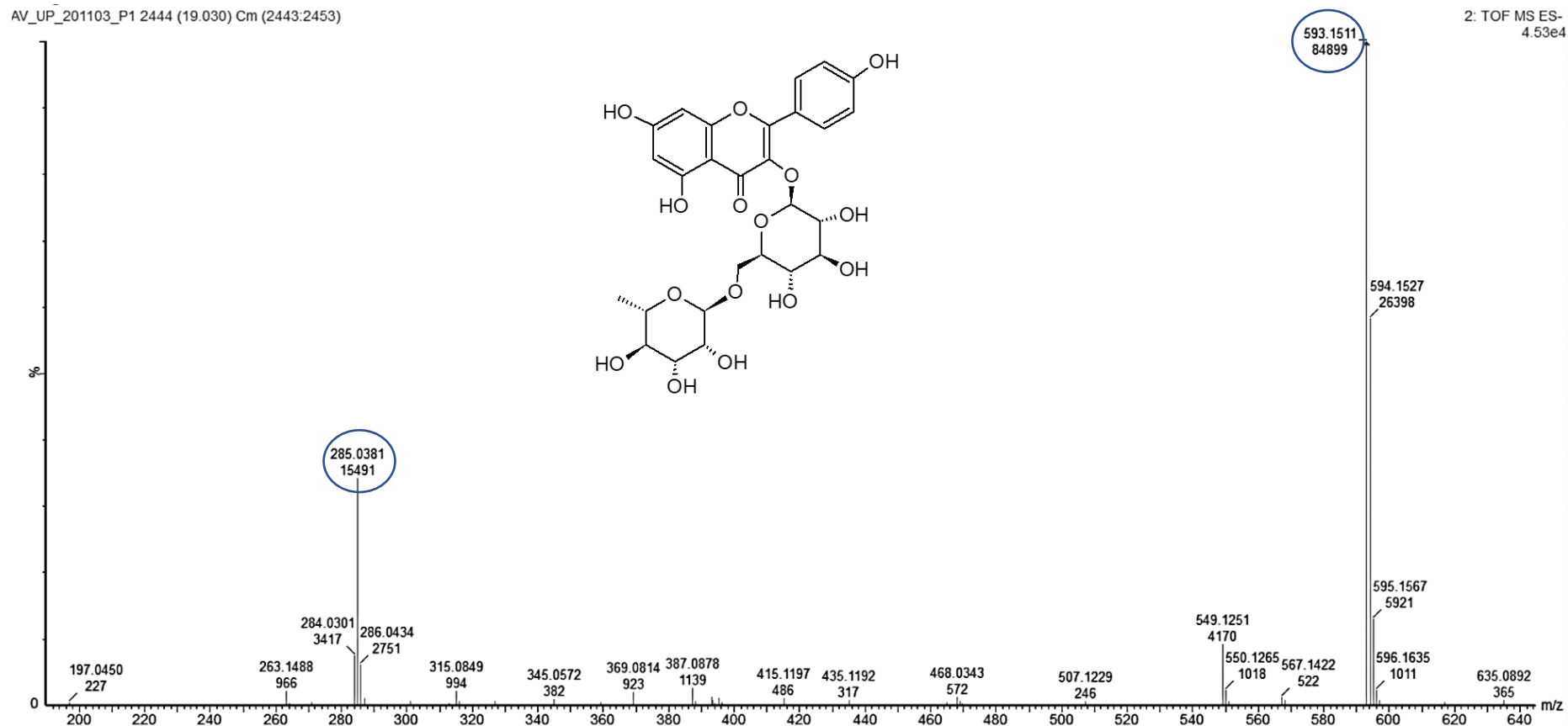


Figure B 15 Mass spectrum of Kaempferol-3-O-rutinoside

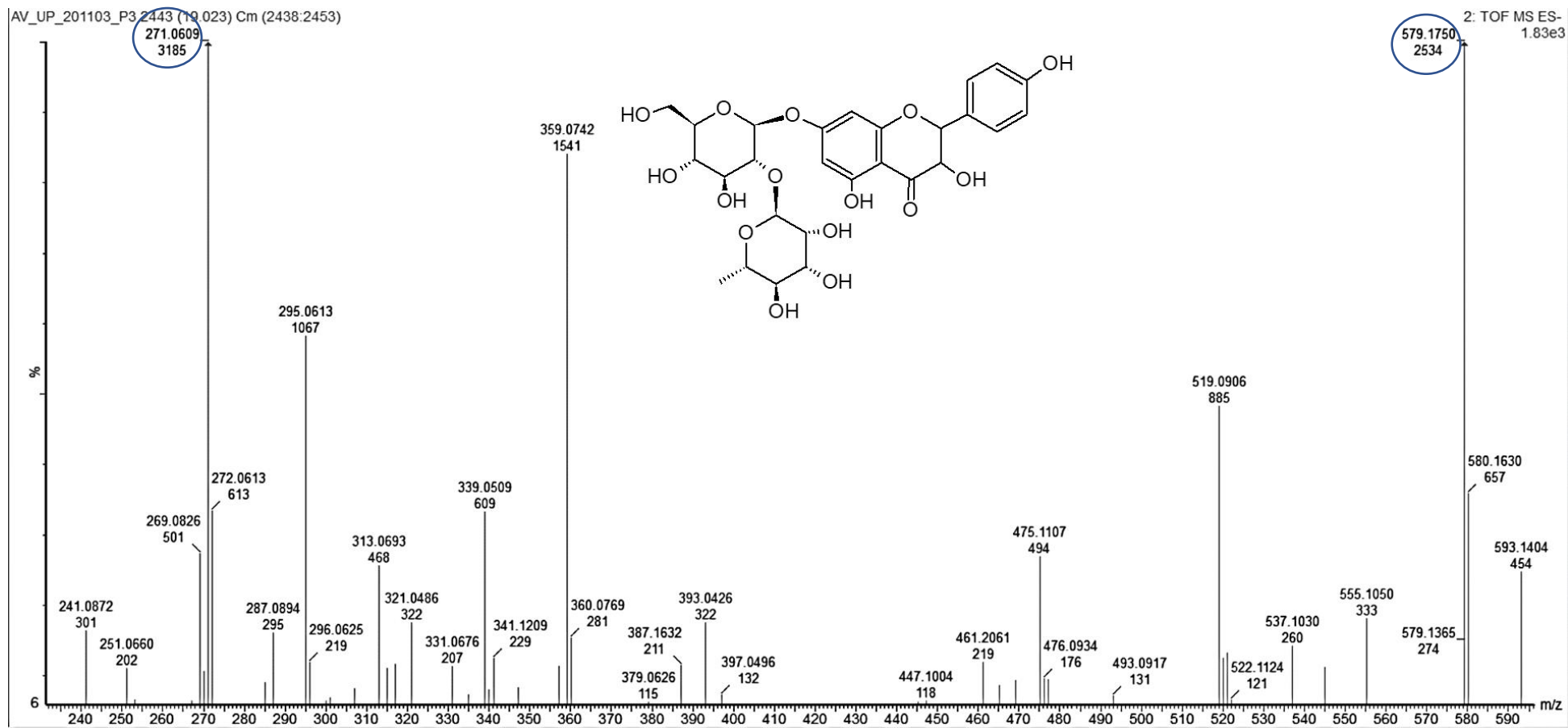


Figure B 16 Mass spectrum of Naringin

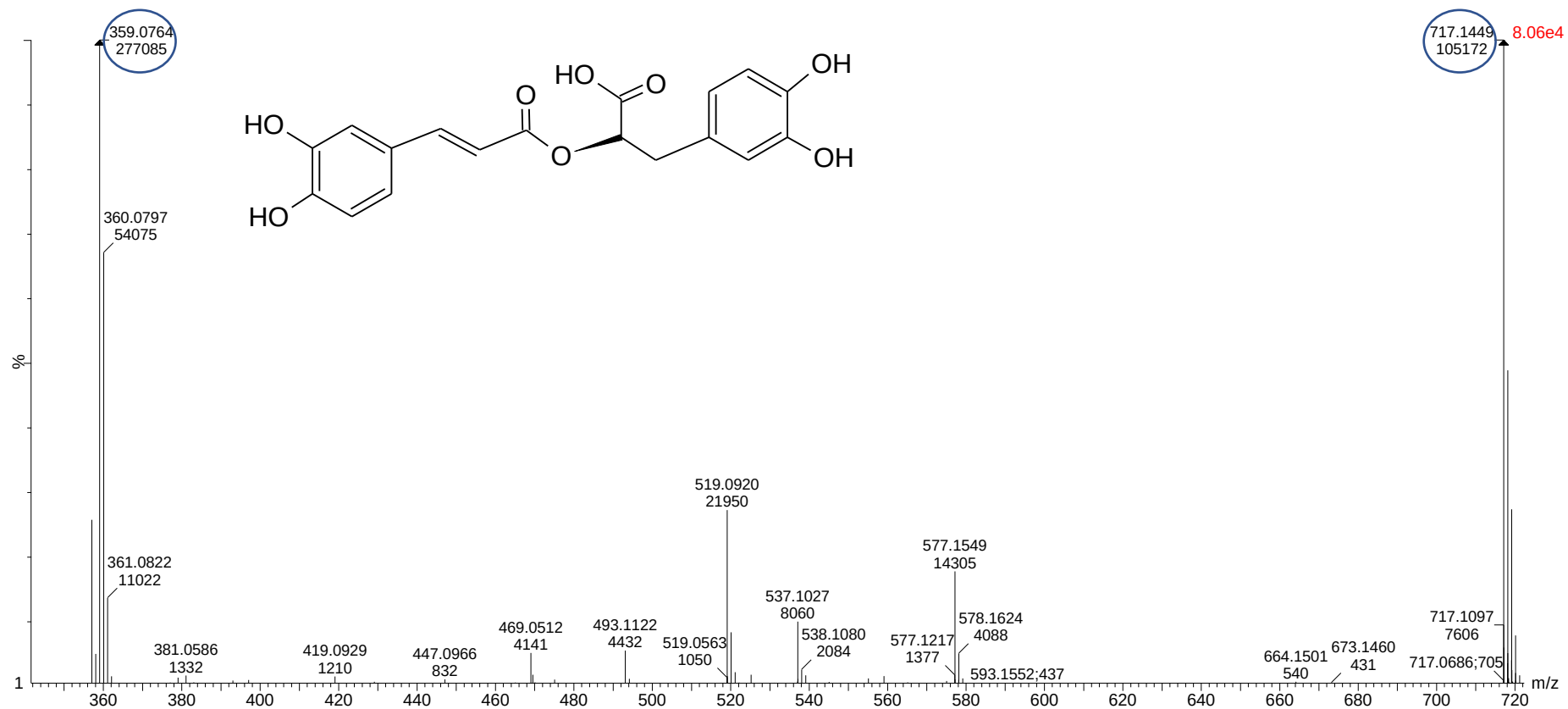


Figure B 17 Mass spectrum of Rosmarinic acid derivative

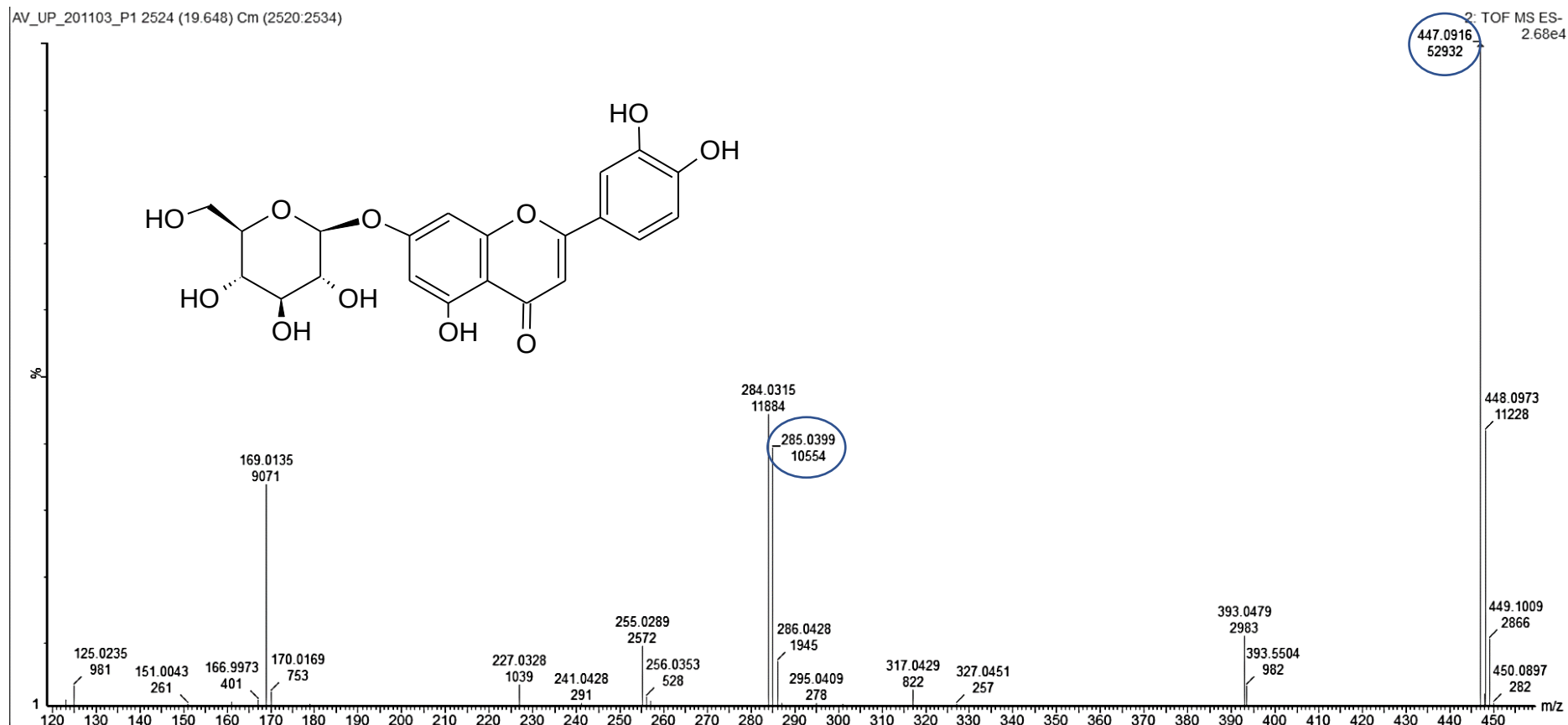


Figure B 18 Mass spectrum of Luteolin-7-O-glucoside

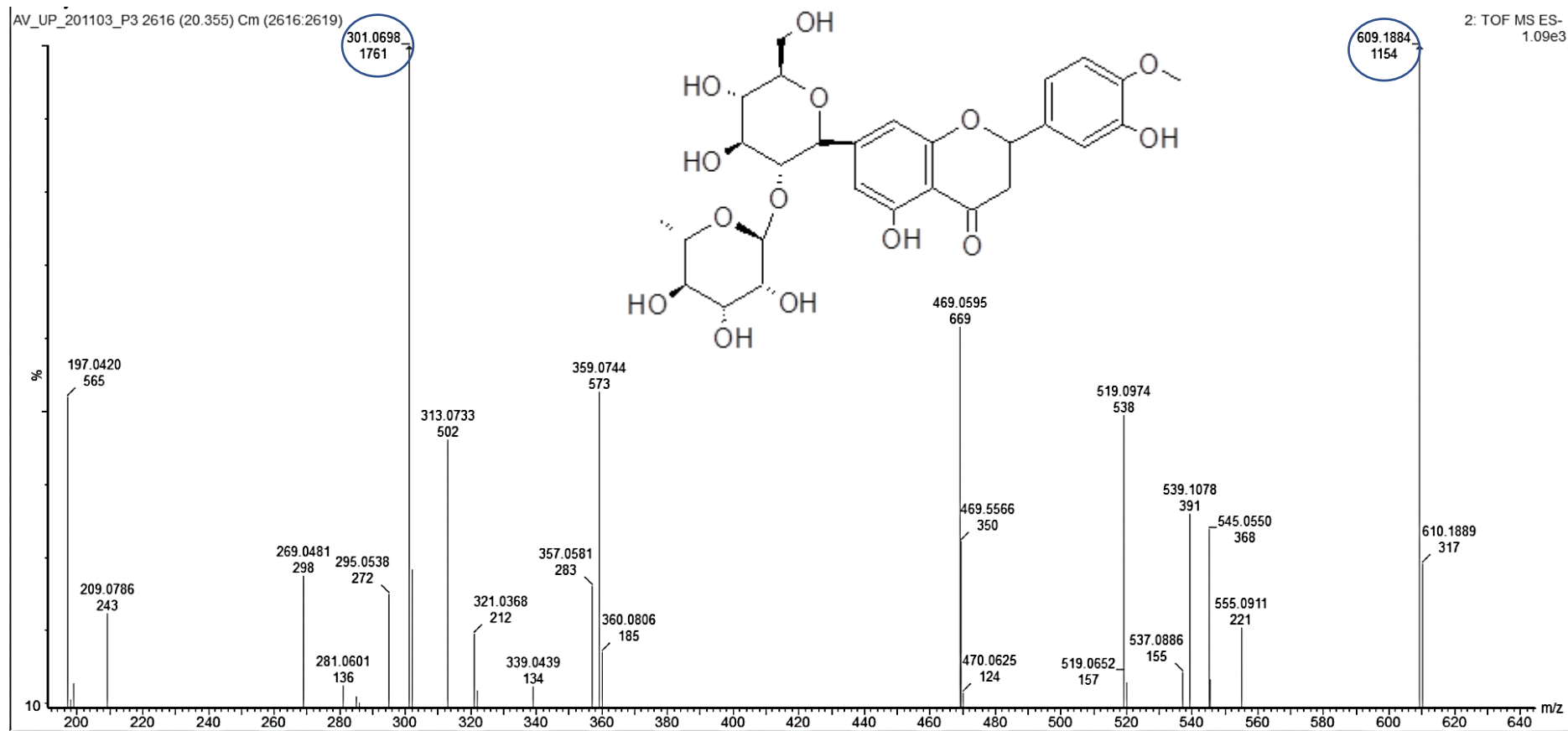


Figure B 19 Mass spectrum of Neohesperidin

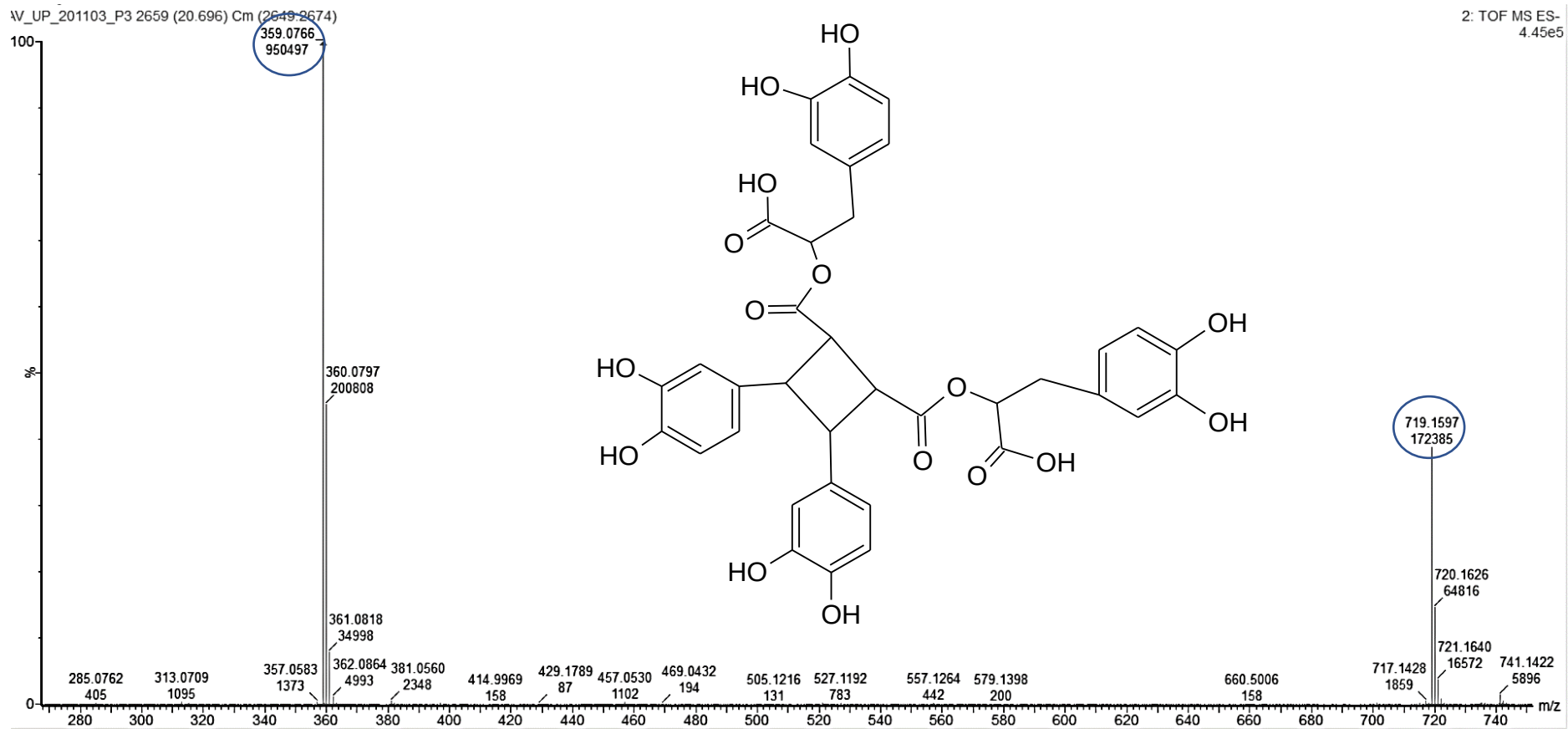


Figure B 20 Mass spectrum of Sagerinic acid

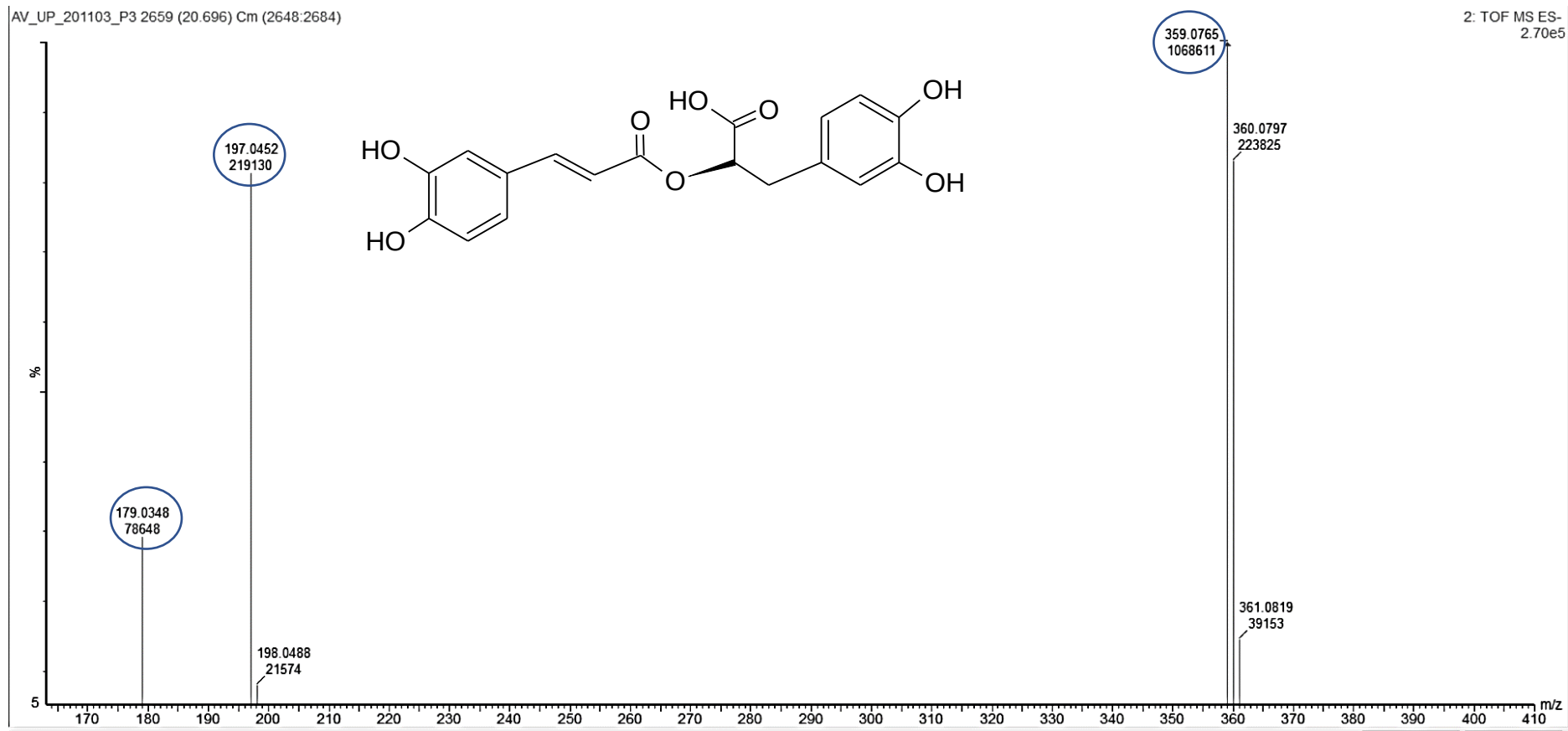
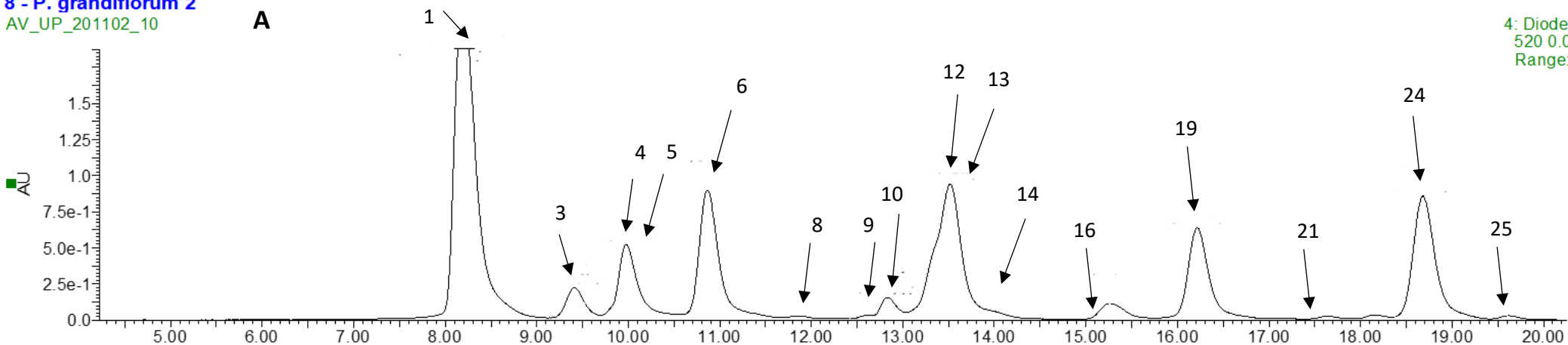


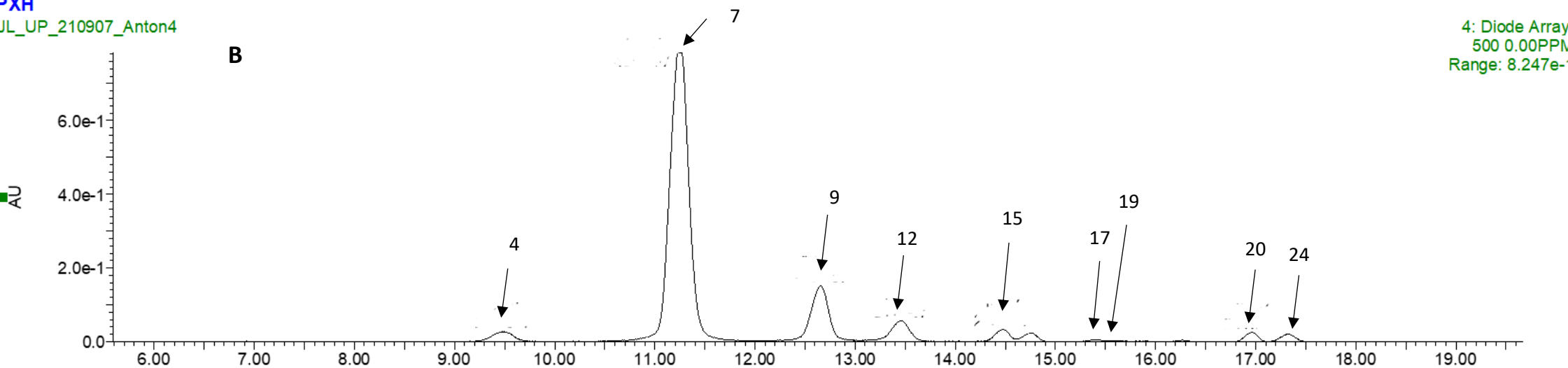
Figure B 21 Mass spectrum of Rosmarinic acid



8 - *P. grandiflorum* 2
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9XH
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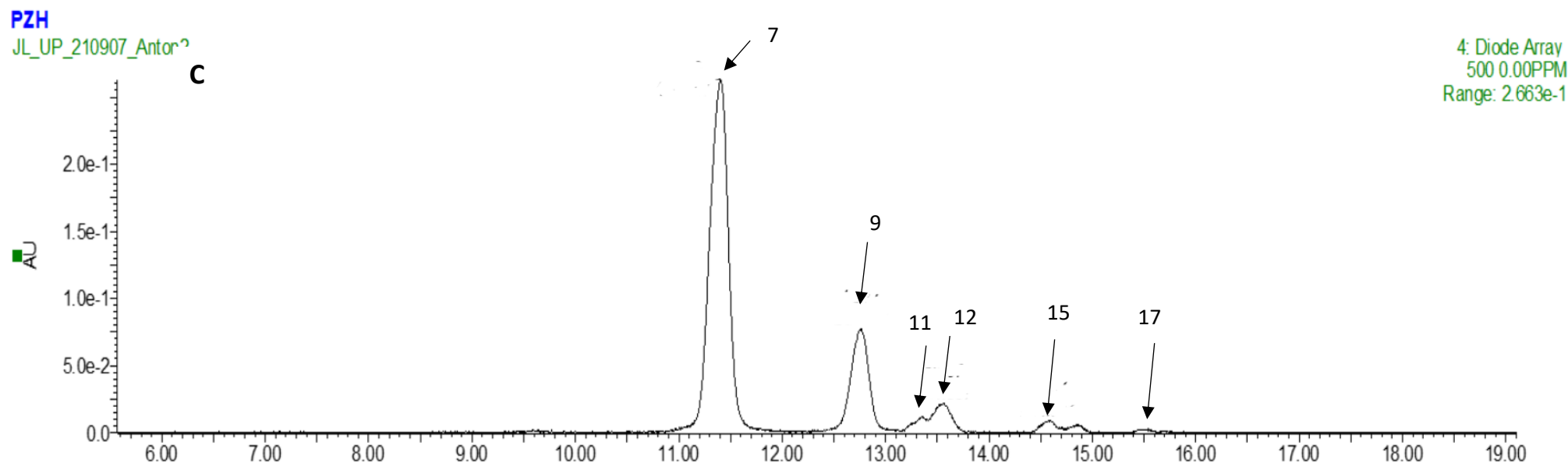
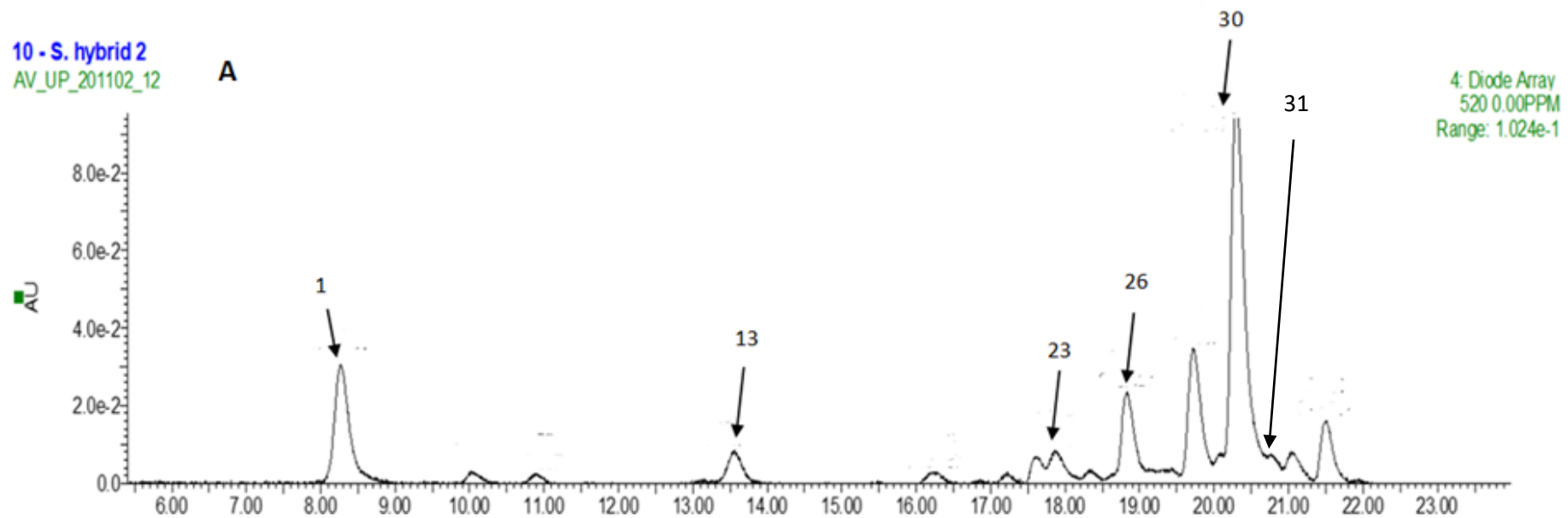


Figure C 1 Chromatograms of flower extracts of the Geraniaceae plant family at 520 nm. A: *P. grandiflorum*, B: *P. X hortorum*, C: *P. zonale* hybrid. 1: delphinidin-3,5-diglucoside, 3: delphinidin-3-O-rutinoside, 4: cyanidin-3,5-diglucoside, 5: delphinidin-3-O-glucoside, 6: petunidin-3,5-O-diglucoside, 7: pelargonidin-3,5-O-diglucoside, 8: petunidin-3-O-rutinoside, 9: peonidin-3,5-O-diglucoside, 10: delphinidin-3-O-rhamnoside, 11: pelargonidin-3-O-glucoside, 12: malvidin-3,5-O-diglucoside, 13: delphinidin-3-O-(6-acetyl)-5-O-diglucoside), 14: Delphinidin-3,7-O-diglucoside, 15: pelargonidin glucoside derivative, 16: Vincanin A (delphinidin-3-robinobiosido-5-rhamnoside), 17: peonidin glucoside derivative, 19: petunidin-3-O-(6-acetyl)-5-O-diglucoside), 20: peonidin-3-O-glucoside-5-(6-acetylglucoside), 21: delphinidin-3-O-rutinoside isomer, 24: malvidin-3-O-(6-O-acetyl)-5-O-diglucoside, 25: cyanidin-3-(glycosyl) rhamnoside

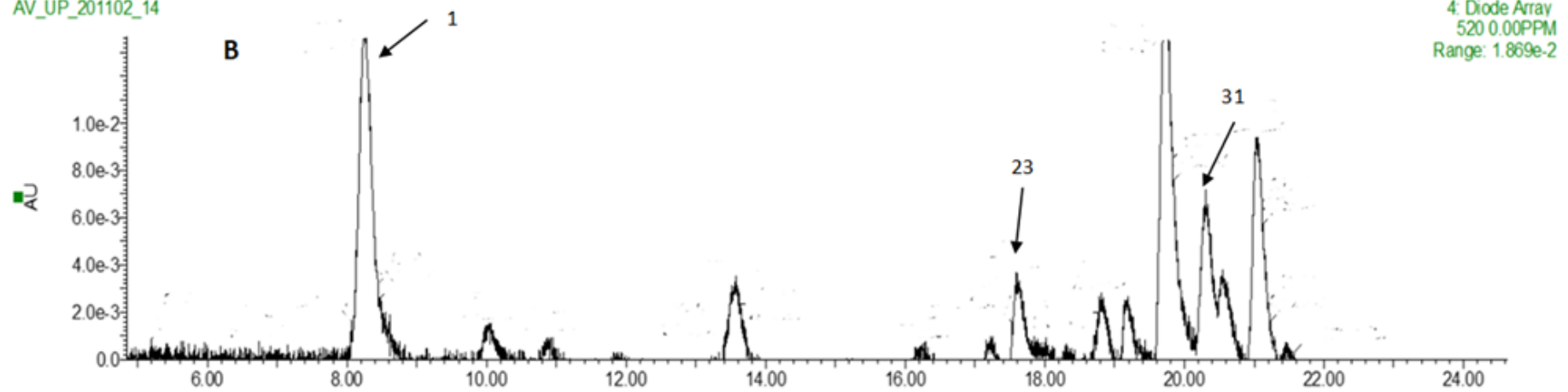
10 - S. hybrid 2
AV_UP_201102_12

A



12 - S. dolomitica 2
AV_UP_201102_14

B



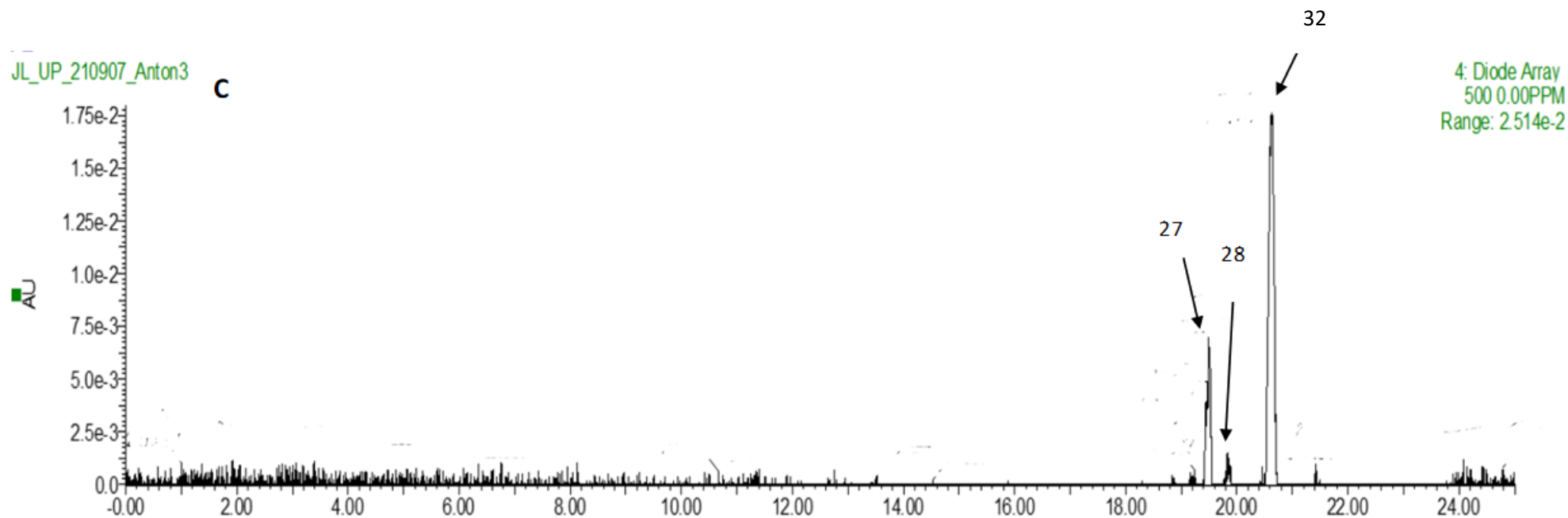
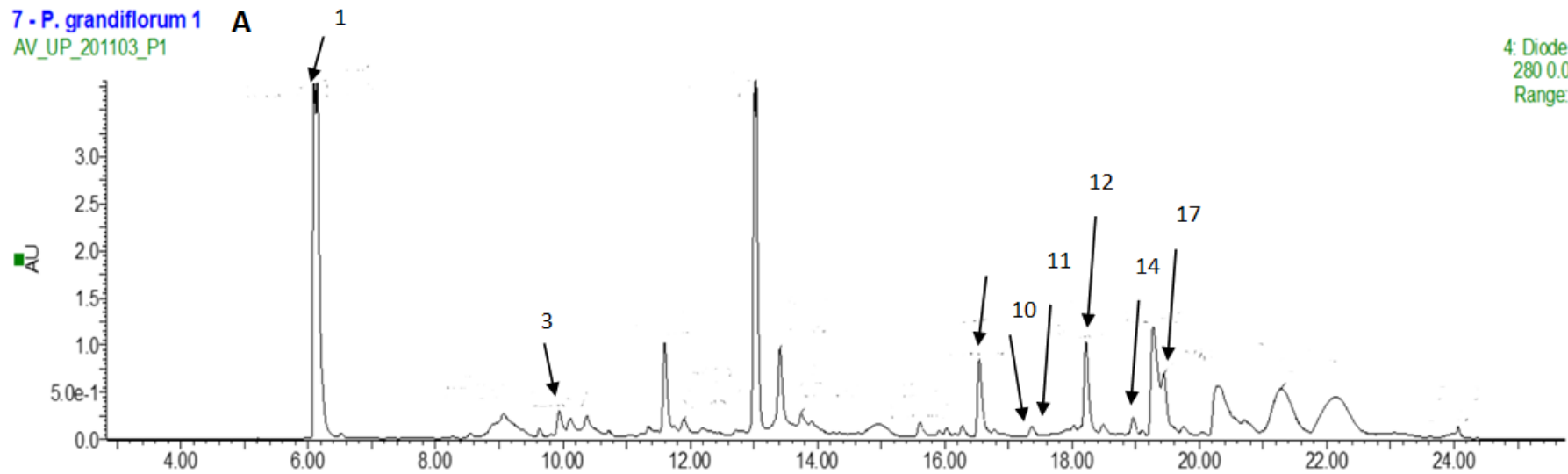
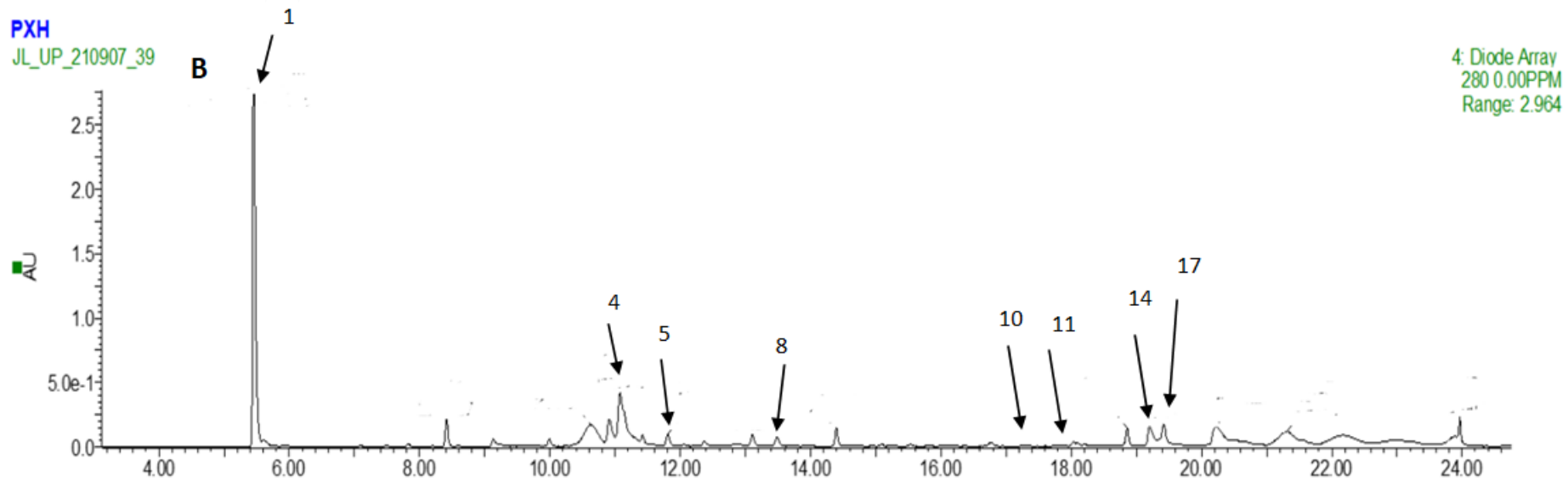


Figure C 2 Chromatograms of flower extracts of the Lamiaceae plant family at 500 nm. A: *S. aurea* × *dolomitica*, B: *S. dolomitica*, C: *P. zuluensis*. 1: delphinidin-3,5-O-diglucoside, 13: delphinidin-3-O-(6-acetyl)-5-O-diglucoside, 23: cyanidin-3-O-rutinoside, 26: Dimalonylawobanin (delphinidin-3-(6-p-coumaroylglucoside)-5-(4,6-dimalonylglucoside) isomer, 27: malvidin-3-(6-p-coumaroylglucoside)-5-(4-malonylglucoside) isomer, 28: malvidin 3-(6-coumaroylglucoside) 5-glucoside, 30: Dimalonylawobanin (delphinidin-3-(6-p-coumaroylglucoside)-5-(4,6-dimalonylglucoside), 31: pelargonidin-3-O-rutinoside, 32: malvidin-3-(6-p-coumaroylglucoside)-5-(4-malonylglucoside)

7 - *P. grandiflorum* 1
AV_UP_201103_P1



PXH
JL_UP_210907_39



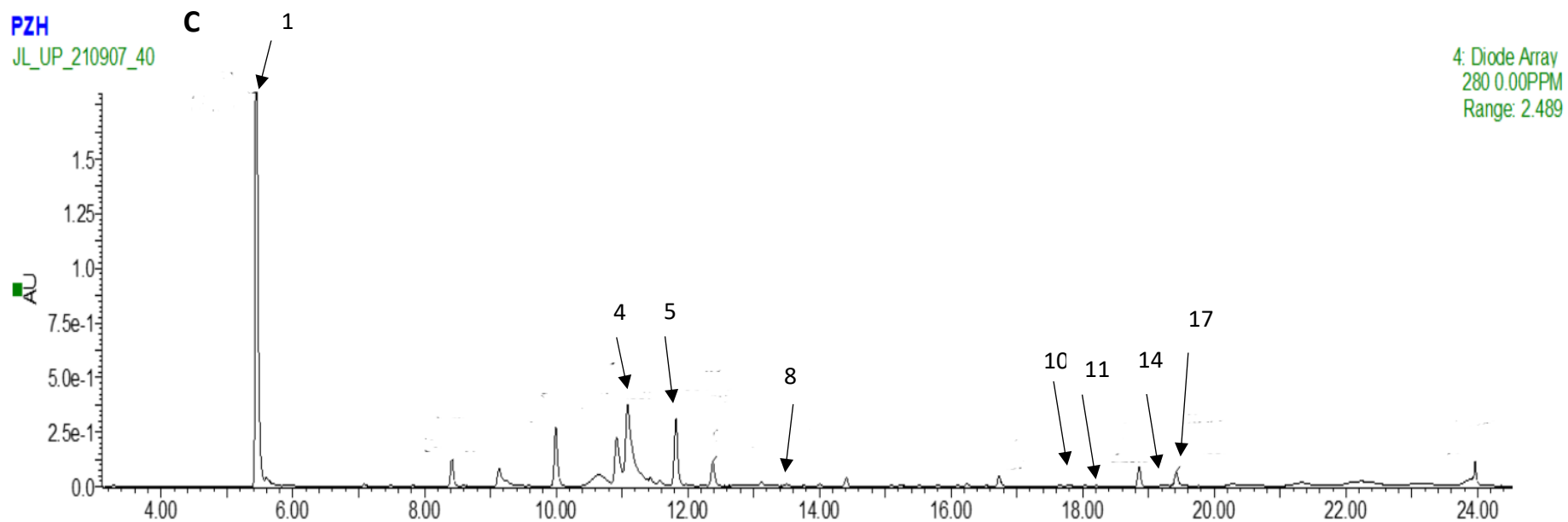
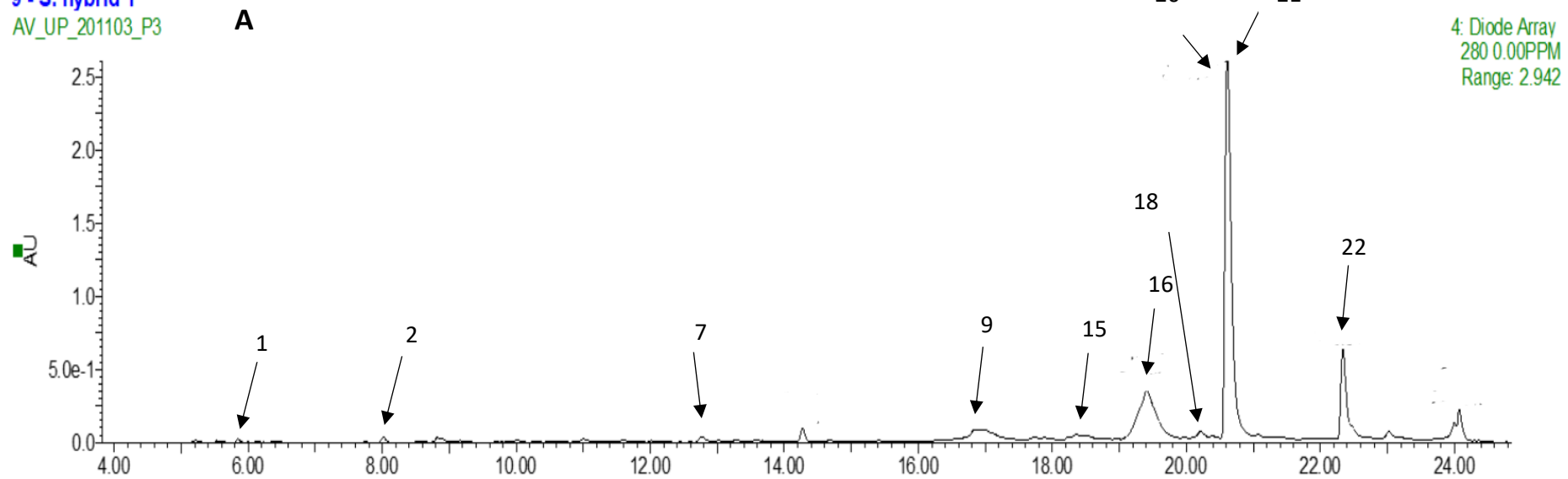
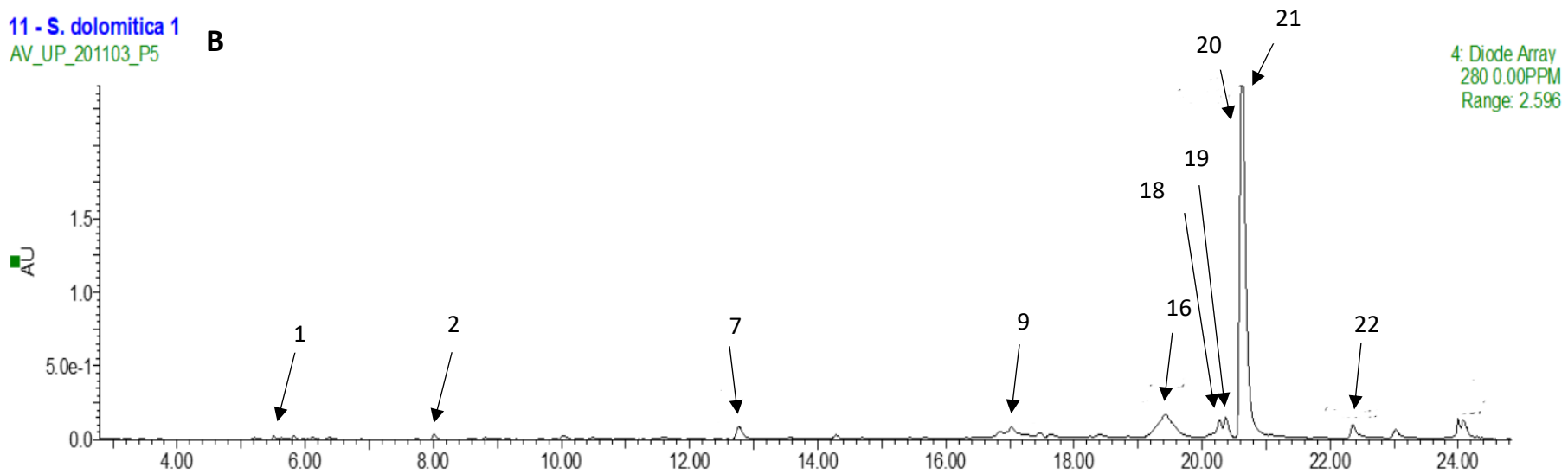


Figure D 1 Chromatograms of flower extracts of the Geraniaceae plant family at 280 nm. A: *P. grandiflorum*, B: *P. × hortorum*, C: *P. zonale hybrid*. 1: β -glucogallin (Galloyl- β -D-glucoside), 3: caffeoyltartaric acid, 4: p-coumaric acid, 5: p-coumaroylglucose, 8: Kaempferol-3-O-glucosylgalactoside, 9: Tetra-O-galloyl-glucose, 10: Robinin (kaempferol 3-O-robinoside-7-O-rhamnoside), 11: quercetin-3-O-rutinoside (Rutin), 12: quercetin-3-O-glucoside (Isoquercetin), 13: penta-O-galloyl-glucose, 15: kaempferol-3-O-rutinoside, 17: luteolin-7-O-glucoside

9 - S. hybrid 1
AV_UP_201103_P3



11 - S. dolomitica 1
AV_UP_201103_P5



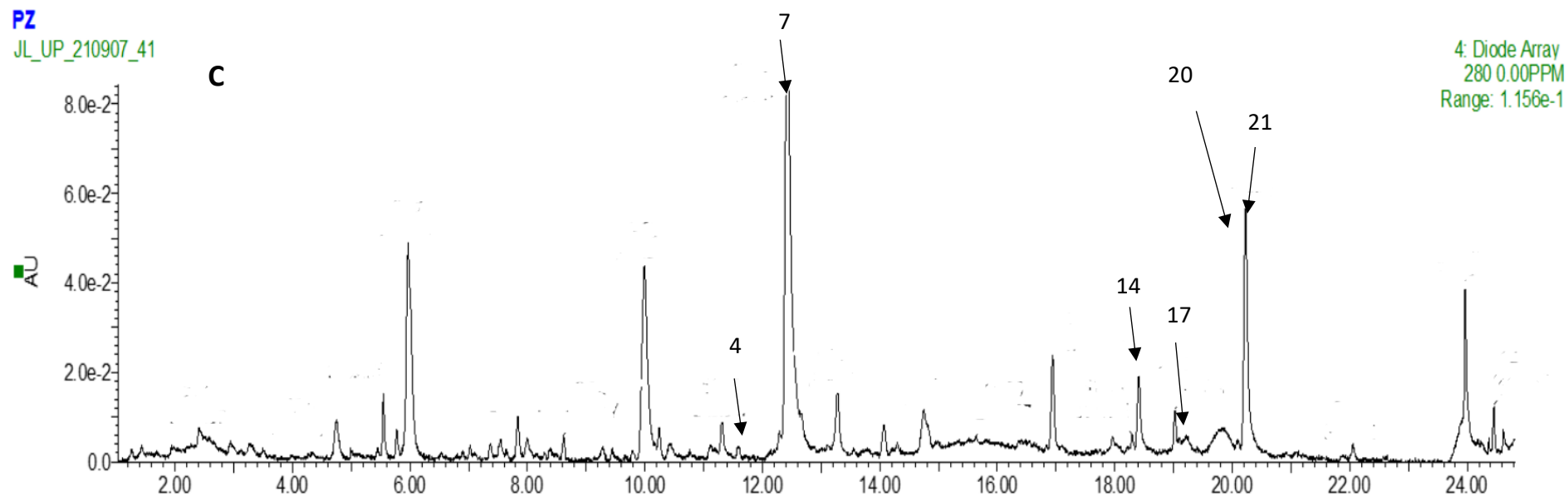


Figure D 2 Chromatograms of flower extracts of the Lamiaceae plant family at 280 nm. A: *S. aurea* × *dolomitica*, B: *S. dolomitica*, C: *P. zuluensis*. 1: β -Glucogallin (Galloyl- β -D-glucoside), 2: Syringic acid, 4: *p*-Coumaric acid, 6: sinapic acid isomer, 7: Caffeic acid, 9: Tetra-O-galloyl-glucose, 14: Apigenin-7-O-glucoside, 16: Naringin, 17: Luteolin-7-O-glucoside, 18: Rosmarinic acid derivative, 19: Neohesperidin, 20: Sagerinic acid, 21: Rosmarinic acid, 22: Salvianolic acid