

**COMPARISON OF FREE AND IMMOBILIZED POLYPHENOL
OXIDASE ENZYMES FOR THE BIOCATALYTIC
CONVERSION OF CATECHINS INTO THEAFLAVINS**

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**COMPARISON OF FREE AND IMMOBILIZED POLYPHENOL
OXIDASE ENZYMES FOR THE BIOCATALYTIC
CONVERSION OF CATECHINS INTO THEAFLAVINS**

by
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SUBMISSION DECLARATION

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SUMMARY

The reusability of active enzyme is important in industrial bio-catalysis applications. This is not readily accomplished with free enzymes. The project investigated a new enzyme immobilization matrix, ReSyn™, and compared it to three widely used matrixes, Eugergit C, Eupergit C 250 L and DEAE Sephadex A-25. Initial comparative immobilization performed with BSA, determined ReSyn™ (covalent immobilization) to have an approximate 8 fold higher protein loading efficiency than the other support. The enzymes selected for immobilization were polyphenol oxidase (PPO) (first reported purification of PPO from Forelle pear) and laccase (Novozyme (Pty) Ltd) based upon their application potential. From the comparative assessment of immobilization based on BSA and the activity (units/mg support) attained by ReSyn™ immobilized laccase, it was deduced that ReSyn™ demonstrated performance superiority over the other supports.

The project aim was the application of immobilized PPO in the ice tea industry to increase the value of black iced tea based upon an increased theaflavin content. The unusually rich sub-group of polyphenols known as flavanols or catechins, may constitute up to 30% of the dry tea leaf weight. The oxidation of green leaf polyphenols is precluded in the preparation of green tea in contrast to the promotion of oxidation in the production of black tea. PPO catalyzes the controlled aerobic oxidation of catechins producing various quinones which undergo condensation reactions resulting in dimeric compounds known as theaflavins, whereas a peroxidase (PO) enzyme produces polymeric substances, known as thearubigins, during the fermentation stage of black tea production (Graham 1992). The production of theaflavins from flavan-3-ols (catechins), is catalysed by PPO (Bonnely *et al.* 2003). The black tea theaflavins are a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate.

The project focussed on the bioconversion of green tea leaf catechins into theaflavins, as the original method employed to acquire theaflavins, the extraction of theaflavins from black tea (only 0.4-1.85% of the dry weight of black tea), doesn't provide a cost-effective manner of recovering theaflavins, and amounts to an industrially unfeasible approach (Sharma *et al.* 2009, Wang & Li 2006). Preliminary exploratory investigation into the theaflavin synthesis capacity of Forelle pear and Yacon (*Smallanthus sonchifolius*) leaf homogenates, respectively determined a 136% and 98% theaflavin content to that present in Lipton® Yellow label black tea, after an hour of fermentation utilizing Lipton® Green tea as starting material. The homogenates of Forelle

pear, Yacon leaves and the control mushroom tyrosinase enzyme produced theaflavin in an *in vitro* model fermentation system, whereas the peak, m/z 563 (26.95-27.06)¹⁴⁶⁻⁸, C₂₆H₂₈O₁₄ was produced in a fermentation system on the basis of UPLC-MS analysis. Therefore it was concluded that the peak, m/z 563 (26.95-27.06)¹⁴⁶⁻⁸, C₂₆H₂₈O₁₄ was preferentially formed to theaflavin in the fermentation system. The presence of the fermentation oxidation product was confirmed in Lipton® Yellow label black tea, from which it was deduced that it could be an implicating factor in the synthesis of theaflavins.

The project succeeded in producing 0.010% and 0.006% yield of synthesized theaflavins, in an *in vitro* model fermentation system, by respectively employing free- and ReSyn™ immobilized laccase, in comparison to the reported 15-30% dry weight of catechins present in green tea shoots from which 0.4-1.85% dry weight of theaflavins are formed during black tea manufacturing corresponding to a yield between 2.7% and 6% of theaflavins (Sharma *et al.* 2009, Graham 1992). To the author's knowledge this is only the second reported incidence of laccase catalyzing the synthesis of theaflavins; theaflavin, theaflavin-3-*O*-gallate, theaflavin-3'-*O*-gallate and theaflavin-3,3-digallate.

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ABBREVIATIONS

ΔAbs_{410}	Change in absorbance measured at 410 nm
μm	Micrometer
$^{\circ}\text{C}$	Degrees celsius
$[\text{S}]_0$	Initial substrate concentration
$\text{\AA}$	Angstrom
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AR	Analytical Reagent
BSA	Bovine serum albumin
CMC	Carboxymethylcellulose
Corp	Corporation
Cr	Aminochrome
CTAB	Cetyltrimethylammonium bromide
C-terminal	Carboxyl-terminal
Cu	Copper
D	<i>o</i> -Diphenol
DEAE	Diethylaminoethyl
DOBA	3,4-Dihydroxybenzaldehyde
DOMA	Dihydroxymandelic acid
DOPA	Dihydroxyphenylalanine
(-)-EC	(-)-epicatechin
EC	Enzyme Commission
ECG	(-)-epicatechin gallate
E_d	Deoxy-tyrosinase
EDTA	Ethylenediaminetetraacetic acid
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin gallate (EGCG)
E_m	Met-tyrosinase
E_{ox}	Oxy-tyrosinase
ESI	Electrospray ionization
<i>g/g</i>	Gram per gram
H_2O_2	Hydrogen peroxide
His	Histidine
HLB	Hydrophile-lipophile balance
HPLC	High-performance liquid chromatography
HR	High Resolution
HTCCA	($\pm$)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acids

IBMK	Isobutylmethylketone
IC ₅₀	Half maximal inhibitory concentration
IF	Instability factor
Inc	Incorporated
kDa	kilo Dalton
kJ.mol ⁻¹	Kilojoule per mole
K _m ^D	Michaelis constant towards o-diphenol
K _m ^{O2}	Michaelis constant of tyrosinase towards oxygen
L	Leucoaminochrome
LDL	Low-density lipoprotein
Ltd	Limited
M	Methionine
MAC	Mesoporous activated carbons
MBTH	2-Hydrazono-3-methylbenzothiazolin
Min	Minute
MM	Methyl monomer
<i>m/m</i>	Mass per mass
MS/MS	Tandem mass spectrometry
<i>m/v</i>	Mass per volume
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NF-κB	Nuclear factor kappa B
N-terminal	Amino-terminal
(Pty) Ltd	Propriety Limited
PAA	Polyallylamine
PAGE	Polyacrylamide gel electrophoresis
PEDT	Poly 3,4-ethylenedioxythiophene
PEG	Polyethylene glycol
PEI	Polyethylenimine
pH	- log of H ⁺ concentration
PPO	Polyphenol oxidase
PQQ	Pyrroloquinoline quinone
PVP	Polyvinylpoly-pyrrolidone
Pulv	Pulverised
Q	Quinone
QH	Protonated o-quinone
Q-TOF	Quadrupole Time-of-Flight
R	Retention
Rpm	Revolutions per minute
RSA	Republic of South Africa
S	Serine

SARS-CoV	Severe acute respiratory syndrome coronavirus
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Std	Standard
SEM	Scanning electron microscopy
t_f	Final time
t_i	Initial time
TM	Trademark
TBARS	Thiobarbituric acid-reactive substrates
TBC	4-tert-butylcatechol
TEMED	N, N, N', N'-tetramethylenediamine
TF	Theaflavins
TF ₁	Theaflavin
TF ₂ A	Theaflavin-3-gallate
TF ₂ B	Theaflavin-3'-gallate
TF ₃	Theaflavin-3,3'-digallate
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris	Tris(hydroxymethyl)aminomethane
TRP	Tyrosinase related protein
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
V	Valine
v/v	Volume per volume
V _{max}	Maximum steady-state rate
V _O	Void volume
V _T	Total column volume
W	Concentration-independent term
w/v	Weight per volume
xg	Times gravity

CHAPTER 1 : LITERATURE REVIEW

1.1. BIOCATALYSIS

Biocatalysis can be utilized as a tool to improve a wide range of production processes by reducing their energy, raw material consumption and toxic side-product and waste generation (Alcalde *et al.* 2006). It takes advantage of the versatility of enzymes to catalyse processes for the production of natural and novel compounds, including both building blocks for chemical synthesis and consumer goods (Betancor & Luckarift 2008, Fernandes 2010). Biocatalysis exploits nature's highly efficient and selective means of transformation (Li & Trost 2008). In some cases, biocatalysis can allow reactions not easily conducted by classical organic chemistry or allow reactions that can replace several chemical steps (Petersen & Kiener 1999). Extremely high reaction rates and selectivity's that are beyond the reach of chemical catalysis are frequently achieved with biocatalysis (Li & Trost 2008). Enzymes also have the added advantage of functioning in mild operation conditions (Salis *et al.* 2005).

1.2. POLYPHENOL OXIDASE

Polyphenol oxidase (EC 1.14.18.1) is also commonly known as tyrosinase, catecholase, phenol oxidase and polyphenolase and these names are used interchangeably in this thesis (Burton 1994, Arnon 1949). Phenol oxidases are often used to refer to tyrosinases, catechol oxidases and laccases (Claus & Decker 2006).

1.2.1. Oxidizing enzymes

Oxidases and peroxidases generally produce reactive oxygen intermediates by reacting with molecular oxygen or peroxide, respectively. The reactive oxygen intermediates react further with reducing substrates; therefore, the reaction can produce several products or can be non-specific. Higher regiospecificity can be achieved with oxygenases, which introduce one or more oxygen atoms into their substrates (Burton 1994). Oxygenases are important for the recycling or catabolism of organic matter in the environment. This broad class of enzymes include both monooxygenases and dioxygenases (Figure 1.1) (Wackett 2002). Dioxygenases include heme-dependent iron-sulfur plant dioxygenases and Rieske iron-sulfur non-heme dioxygenases that incorporate dioxygen into substrates (Burton 1994, Wackett 2002).

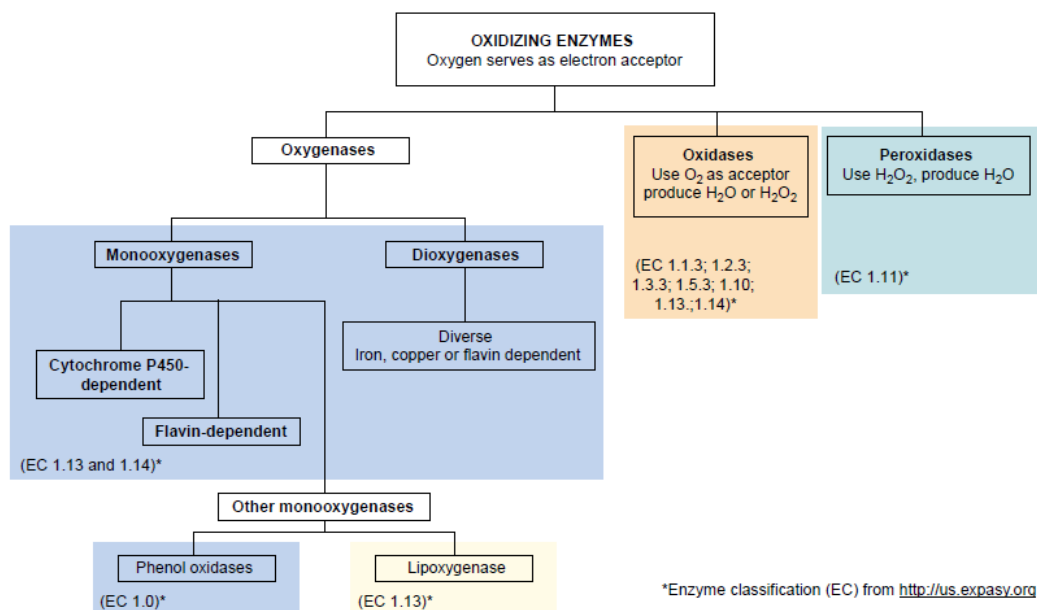


Figure 1.1: The classification of oxidizing enzymes (Burton 1994).

1.2.1.1. Monooxygenases

Monooxygenases catalyses the introduction of an oxygen atom into a substrate molecule with the reducing potential generally provided by NADH or NADPH for the supply of electrons to the substrate:



Monooxygenase can be flavin- (Langel & Ober 2011), heme- (Meyer *et al.* 1996) or metal-dependent enzymes (Duboc-Toia *et al.* 1999).

1.2.2. Metalloenzymes

Vanadium, manganese, iron, copper, nickel, molybdenum, tungsten and cobalt are all biologically essential transition metals, which bind to proteins in numerous ways. These metals occur in mononuclear, binuclear and multiple nuclear centres with the centres being homonuclear or heteronuclear. The transition metals are utilized for their capacity to bind and exchange ligands, redox activity and high charge density allowing the polarization of substrates, and the stabilization of transition-state intermediates (Ermler *et al.* 1998).

1.2.2.1. Copper proteins

Copper proteins are involved in reversible dioxygen binding (hemocyanin) (Solomon *et al.* 1994), proton pumping (cytochrome c oxidase) (Michel 1998), four-electron reduction to water coupled with substrate oxidation (Fet3p, laccase, ceruloplasmin and ascorbate oxidase), in two-electron reduction to peroxide coupled to the oxidation of substrates (amine-, galactose-, and catechol oxidases) (Klinman 1996) and in the activation for hydroxylation (dopamine β -hydroxylase, α -hydroxylating monooxygenase, particulate-methane monooxygenase and tyrosinase) (Klinman 1996, Klinman *et al.* 1984).

Three types of copper proteins are distinguished based upon the spectroscopic characteristics of their active sites (Malmstrom 1982). Type-1 copper centres are found in the electron-transfer proteins such as plastocyanin and azurin. These proteins are the so-called “blue copper centres” based upon the deep blue color resulting from an intense Cys S-Cu (II) charge-transfer transition. There is a “transition state” between the favoured coordination geometries of Cu (II) (square-planar) and Cu (I) (tetrahedral) (Shepard *et al.* 1990).

Type-2 copper proteins include oxygenases (dopamine- β -monooxygenase) and oxidases (galactose oxidases). These “non-blue copper centres” are characterized by a square-planar coordination sphere. Type-1 and -2 copper proteins have a mononuclear copper site whereas type-3 copper proteins have a binuclear copper site. Type-3 copper proteins are hemocyanin, tyrosinase and catechol oxidase (Gerdemann *et al.* 2002). Antiferromagnetically coupled copper ions (CuA and CuB) coordinated by six histidine residues characterize their active sites (Claus & Decker 2006).

Hemocyanin, tyrosinase and catechol oxidase differs in function, which seems to be largely depend upon the accessibility of the dioxygen binding site to potential substrates and selectivity effects that discriminate the type of substrates (Granata *et al.* 2006). Hemocyanin is an oxygen carrier protein and comprises of two classes namely Arthropoda and the Molluscan. Catechol oxidase is an *o*-diphenol oxidase and catalysis exclusively the oxidation of catechols to the corresponding *o*-quinones (catecholase activity). Tyrosinase also has a catecholase activity in addition to a cresolase activity enabling it to accept monophenols (like tyrosine or cresol) as substrates. The International Union of Biochemistry and Molecular Biology Nomenclature Committee distinguish between cresolase activity (E.C. 1.14.18.1) and catecholase activity (E.C. 1.10.3.1) and not between catechol oxidase and tyrosinase (Mayer & Harel 1979).

1.2.3. Structure of polyphenol oxidase

Tyrosinase has a helical structure with few β -structures as only the N- and C-terminal β -strands form a sheet structure (Figure 1.2). The core of tyrosinase is formed by a four-helix bundle with the catalytic binuclear copper center lodged in it (Matoba *et al.* 2006).

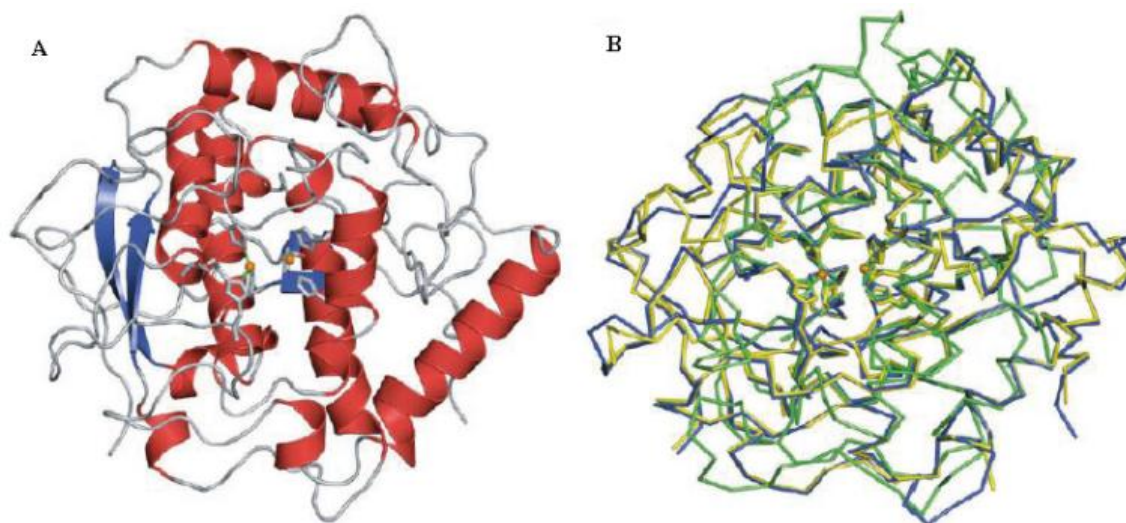


Figure 1.2: The X-ray structure of polyphenol oxidase from *Vitis vinifera*. (A) The overall ellipsoidal shape, dicopper center within a four-helix bundle and two β -sheets indicated by a ribbon model. (B) Ca representation of an overlap of the polyphenol oxidase from *V. vinifera* (blue), sweet potato (yellow) (Klabunde *et al.* 1998) and *Neurospora crassa* (green) (PDB code 1wx2). The superimposition of polyphenol oxidase from *V. vinifera* and sweet potato had a 0.76 Å root-mean-square deviation for 325 Ca pairs. Between polyphenol oxidase of *V. vinifera* and *N. crassa* a 1.11 Å deviation for 170 Ca pairs (Virador *et al.* 2009).

The overlapping of polyphenol oxidases from *V. vinifera*, sweet potato and *N. crassa* show few areas of slight difference in folding. These differences occur mostly on the surface and don't involve changes in the α -helices or β -strands (Virador *et al.* 2009). The overall structure of tyrosinase (*Streptomyces castaneoglobisporus*) is quite similar to that of hemocyanin (*Octopus dofleini*) and catechol oxidase (*Ipomoea batatas*). This is based upon the high degree of conservation present in the core domain composed of the α -bundle (Matoba *et al.* 2006).

1.2.3.1. Active site of tyrosinase

The copper-binding histidines are located in a helix bundle comprising of four densely packed helices (Figure 1.3). The CuA and CuB centres are connected by a loop containing residues M374, S375 and V377. The hydrogen acceptors for the NH-group of the imidazole rings of the copper-binding His367 and His180 are the peptide oxygen's of M374 and V377. This loop is essential for the active site architecture and stability of the enzyme (Schweikardt *et al.* 2007).

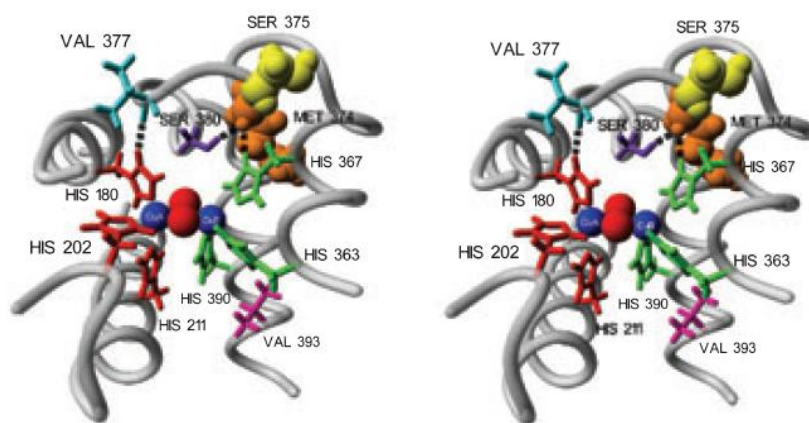


Figure 1.3: The stabilizing effect of S380 (purple) in the active site of mouse tyrosinase. The residues' CuA (red), CuB (green), coppers (blue), V377 (cyan), S375 (yellow), M374 (orange) and hydrogen bonds (black lines) are indicated (Schweikardt *et al.* 2007).

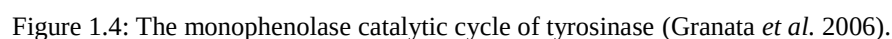
The dicopper center is located at the bottom of a large cavity formed by the hydrophobic residues as a putative substrate binding pocket (Matoba *et al.* 2006).

1.2.4. Monophenolase- and diphenolase activity

Tyrosinase is a mixed function oxidase having both a monophenolase- or cresolase-activity (*ortho*-hydroxylation of monophenols) and a diphenolase- or catecholase activity (oxidation of *o*-diphenols to *o*-diquinones). The binding of one molecule of atmospheric oxygen by the copper pair of tyrosinase enables this catalysis of two different enzymatic reactions. Catechol oxidases (EC 1.10.3.1) exert only the diphenolase activity and laccases (EC 1.10.3.2) are able to oxidize mono- and diphenols by a radical mechanism (Claus & Decker 2006). The monophenolase catalytic cycle is simpler than that of the diphenolase cycle. The two catalytic cycles can be shown as intermingled based upon the addition of limited amounts of diphenol removing the lag phase typically occurring in the monophenolase reaction. The diphenol reduces met-tyrosinase (E_m) and thereby gives rapid access to the oxy form (E_{ox}) (Land *et al.* 2003). Tyrosinase occurs in the oxy ($Cu^{II}_2O_2^{2-}$), met (Cu^{II}_2) and deoxy (Cu^I_2) forms (Jackman *et al.* 1992).

1.2.4.1. Monophenolase activity of tyrosinase

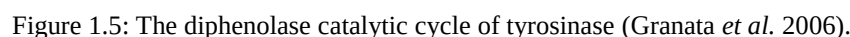
One atom of oxygen is incorporated into the aromatic ring (aromatic C-H bond) of the monophenol during the hydroxylation reaction (Figure 1.4). The phenol hydroxylation is carried out by the oxy form of tyrosinase and the oxidation of catechols by both the met – and oxy forms (Granata *et al.* 2006).



The rate limiting step has been reported to be associated with the cleavage of the peroxide O-O bond (60.2 kJ.mol⁻¹), although the rate-limiting step has also been reported to be connected with phenol deprotonation and binding to the copper-peroxo intermediate (Espín & Wichers 2001).

1.2.4.2. Diphenolase activity of tyrosinase

The diphenolase- or catecholase activity of tyrosinase oxidizes *o*-diphenols to *o*-diquinones (Figure 1.5) (Pierpoint 1969).



The highly reactive produced quinones can react with amino acids and peptides (Pierpoint 1969) and also polymerize to form red, brown or black pigments (Martinez & Whitaker 1995).

1.2.5. Lag phase of tyrosinase phenolase activity

The oxidation of monohydric phenol substrates by tyrosinase exhibits usual kinetic properties of a lag period which increases with increasing substrate concentrations. The lag phase during the oxidation of monophenols can be interpreted as arising in the decay of E_{ox} to E_m . The dissociation of the enzyme-diphenol complex also generates the E_m which is capable of reacting with the monophenol substrates. Therefore, at the beginning of the cresolase reaction only a fraction of the total possible activity is observed. The lag phase can be abolished by the addition of diphenols or H_2O_2 (Wilcox *et al.* 1985).

1.2.6. Substrates for polyphenol oxidase

The range of substrates which can be transformed by tyrosinase is potentially very broad in particularly that of tyrosinase from plant sources (Burton 1994). Due to the wide specificity of polyphenol oxidase, several types of substrates based upon the stability of the corresponding quinone have been suggested (Garcia-Molina *et al.* 2007). The first type is substrates yielding stable *o*-quinones for example, 4-*tert*-butylcatechol (TBC) yielding a very stable *o*-quinone (*o*-TBQ) (Figure 1.6) (Munoz *et al.* 2006, Peñalver *et al.* 2002).

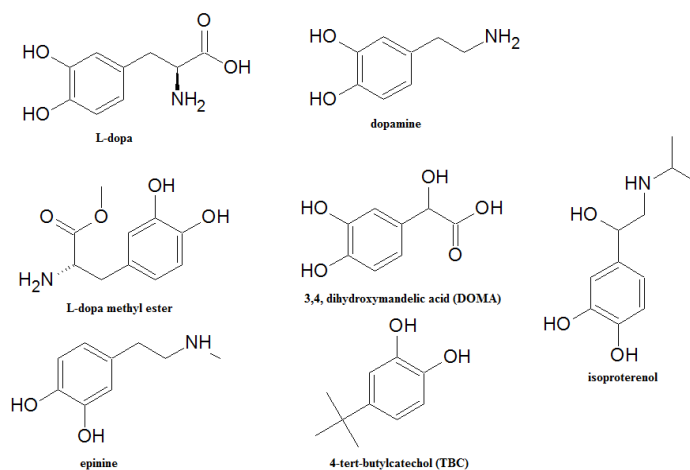


Figure 1.6: The structures of the substrates L-dopa, dopamine, L-dopa methyl ester, 3,4 dihydroxymandelic acid (DOMA), epinine, 4-*tert*-butylcatechol (TBC) and isoproterenol.

The second type is substrates yielding a stable coupling product. These types of substrates produce a very unstable *o*-quinone that through a first-order reaction evolves to a stable product. An example is 3,4-dihydroxymandelic acid (DOMA) with its *o*-quinone evolving to 3,4-dihydroxybenzaldehyde (DOBA) (Sugumaran *et al.* 1992). Other examples are substrates

whose *o*-quinone evolves to aminochrome like isoproterenol, L-dopa methyl ester, L-dopa (Espín *et al.* 1998), epinine (Peñalver *et al.* 2002) and dopamine (Montgomery & Sgarbieri 1975).

The third type of substrate of polyphenol oxidase is those substrates yielding a stable nucleophile adduct (Figure 1.7). The oxidation of the substrate produces a chromophoric adduct with a clear stoichiometry by giving rise to an unstable *o*-quinone that is attacked by a potent nucleophilic reagent (MBTH) (Espín *et al.* 1997).

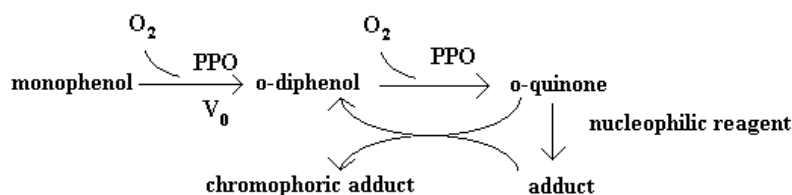


Figure 1.7: *o*-Quinone evolves through a nucleophilic attack yielding a stable nucleophile adduct (Garcia-Molina *et al.* 2007).

Substrates measured utilizing ascorbic acid is the fourth type of tyrosinase substrates (Figure 1.8). A very unstable *o*-quinone that evolves with no clear stoichiometry even through MBTH attack is produced. The product can only be characterized through reduction by ascorbic acid (El-Bayoumi & Frieden 1957).

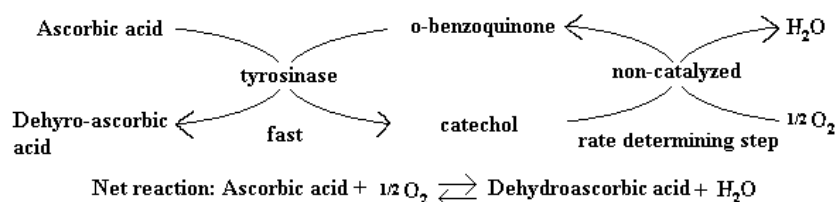


Figure 1.8: Ascorbic acid reduction of *o*-benzoquinone formed from catechol by tyrosinase (El-Bayoumi & Frieden 1957).

1.2.7. Inhibitors of polyphenol oxidase

There are three main groups of polyphenol oxidase inhibitors: (i) ions or small molecules that bind to the copper centre in the active site, (ii) aromatic inhibitors competing with phenolic substrates for binding to the active site and (iii) compounds oxidizing or reducing the copper ions. Aromatic carboxylic acids such as benzoic acids (benzoic, *p*-hydroxybenzoic, vanillic and syringic), cinnamic acids (cinnamic, *p*-coumaric, ferulic and sinapic), phenylacetic acid, phenylpropionic acid (*p*-hydroxyphenylpropionic) and sorbic acid are all competitive inhibitors of polyphenol oxidase (Janovitz-Klapp *et al.* 1990).

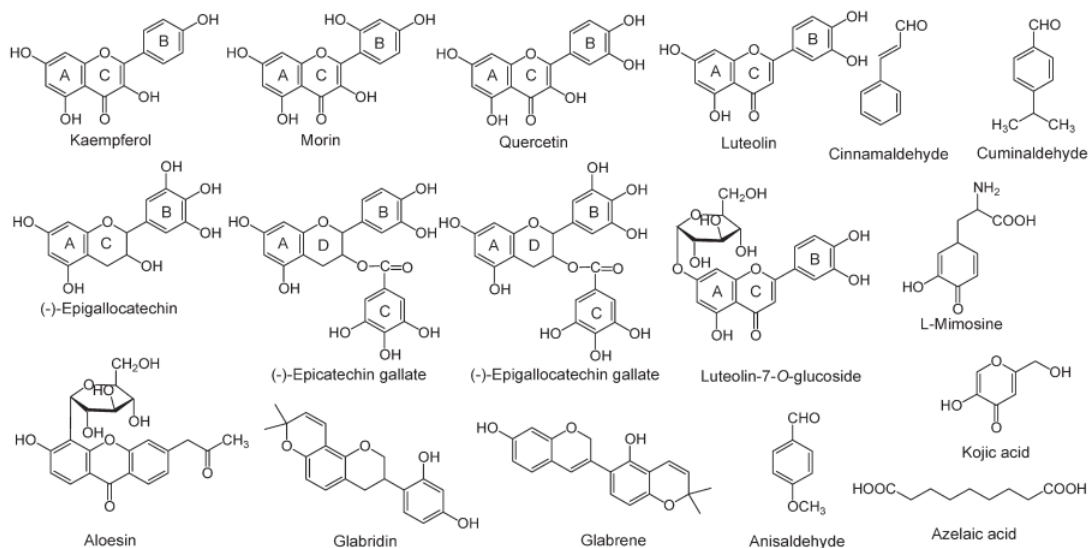


Figure 1.9: Structures of tyrosinase inhibitors from natural sources (Kim & Uyama 2005).

The flavanols (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) are all competitive inhibitors of the monophenolase activity of tyrosinase (No *et al.* 1999). The flavonols quercetin, kaempferol and morin are all competitive inhibitors of the diphenolase activity of tyrosinase. The flavones luteolin and luteolin 7-*O*-glucoside are noncompetitive inhibitors of diphenolase activity of tyrosinase (Kubo *et al.* 2000). The isoflavans glabrene and isoliquiritigenin are mixed-type inhibitors of the diphenolase activity of tyrosinase, and glabridin is a noncompetitive inhibitor thereof (Nerya *et al.* 2003). Other inhibitors of the diphenolase activity of tyrosinase are anisaldehyde, cuminaldehyde (Kubo & Kinst-Hori 1998) and cinnamaldehyde (Kubo & Kinst-Hori 1999) are noncompetitive inhibitors, and kojic acid is a mixed type inhibitor (Figures 1.9 and 1.10) (Chen *et al.* 1991).

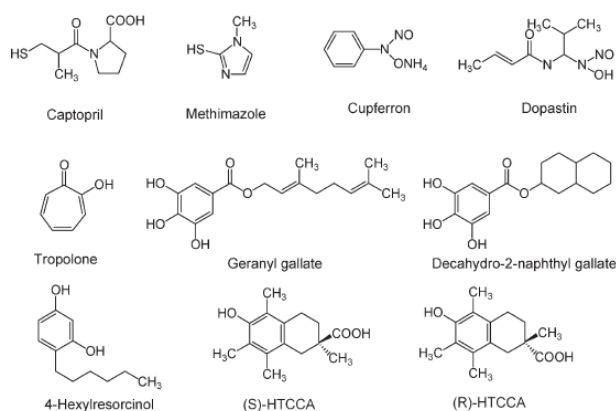
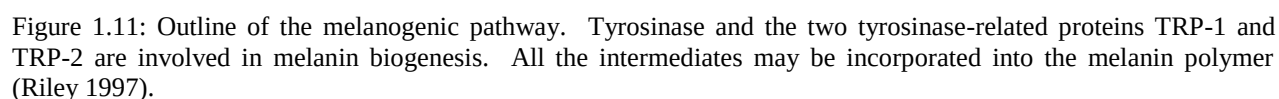
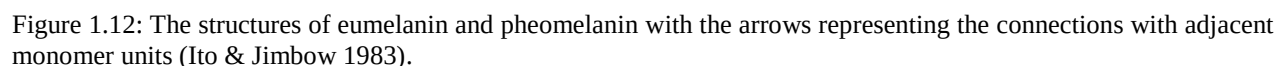


Figure 1.10: Tyrosinase inhibitors from synthetic sources and derivatives of natural compounds include captopril (Espín & Wichers 2001), methimazole (Andrawis & Kahn 1986), cupferron (Xie *et al.* 2003), dopastin (Shiino *et al.* 2001), tropolone (Espín & Wichers 1999), geranyl gallate, decahydro-2-naphthyl gallate (Kubo *et al.* 2003), 4-hexylresorcinol (Jiménez & García-Carmona 1997), (S)-HTCCA and (R)-HTCCA ((±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acids) (Yu 2003).

Melanin is an irregular light-absorbing polymer that is widely dispersed in the plant and animal kingdoms. Tyrosinase oxidation of tyrosine is the critical step in the melanin biogenesis as melanin is derived from indoles and other intermediate products of the oxidation (Riley 1997) (Figure 1.11). The substrates tyrosine, DOPA and DHI can be catalyzed by tyrosinase in three different reactions (Hearing & Tsukamoto 1991). In vertebrates, melanin is the major pigment present in their surface structures with the enzyme tyrosinase only being active in specialized organelles in retinal pigment epithelium and melanocytes. In mammals, melanin forms the predominant pigment of hair and epidermis. The melanin is formed in intracellular granules that are transferred from melanocytes to the epithelial cells (Riley 1997).



The two chemically distinct types of melanins produced in melanocytes are pheomelanins, which are red or yellow and eumelanins, which are brown or black (Figure 1.12). Mixtures of pheomelanins and eumelanins are typically found in mammals (Hearing & Tsukamoto 1991).



Melanin can act as a free-radical sink and functions as a strengthening element of plant cell walls and insect cuticles. The light absorption ability of melanin is employed in thermoregulation, photo protection, photoreceptor shielding, camouflage and display (Riley 1997).

1.2.9. The various sources of polyphenol oxidase

The distribution of tyrosinase in all domains of life is nearly ubiquitous (Claus & Decker 2006) (Figure 1.13). Polyphenol oxidase is found in groups as diverse as plants (most fruits and vegetables) (Van Gelder *et al.* 1997, Flurkey 1986), fungi (Van Gelder *et al.* 1997, Halaouli *et al.* 2006), amphibia (Miranda *et al.* 1985, Chen & Chavin 1968), arthropods (Martinez & Whitaker 1995) and mammals (Miranda *et al.* 1985). The genes coding for the enzyme are tissue specifically expressed and also developmentally controlled (Mayer 2006). Polyphenol oxidase is encoded from a divergent multigene family, and those in plants and fungi have multiple forms (Mayer 2006, Boss *et al.* 1995, Thipyapong & Steffens 1997). The expression of polyphenol oxidase activity in bacterial organisms (*Marinomonas mediterranea*) is growth-phase regulated with a maximum in the stationary phase of bacterial growth (Fernandez *et al.* 1999). In plants transcriptional control of polyphenol oxidase expression after wounding or the manifestation of a physiological disorder was shown by the induction of polyphenol oxidase mRNA (Boss *et al.* 1995).

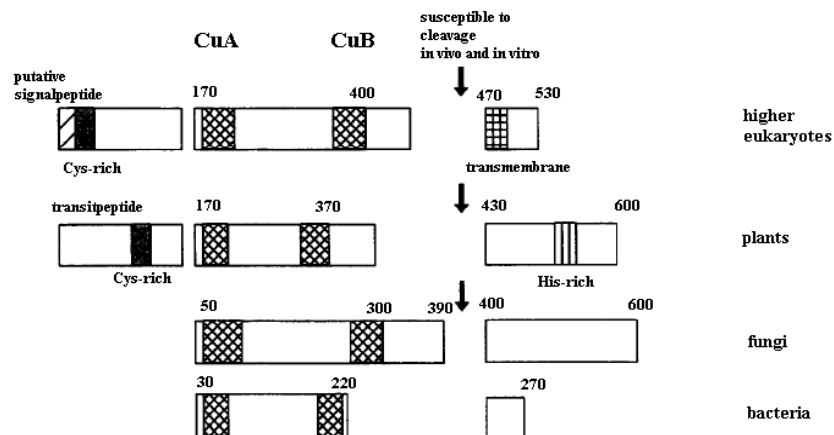


Figure 1.13: The domain structure of tyrosinases from higher eukaryotes, plants, fungi and bacteria. Conserved domains, including the CuA and CuB are indicated as well as the predicted cleavage site for the generation of the protease-resistant fragment from that of the mature tyrosinase (Van Gelder *et al.* 1997).

Plant and animal tyrosinase has a conserved cysteine-rich region in the N-terminal region (Van Gelder *et al.* 1997). A third histidine-rich region postulated to be a putative Cu binding domain in plant tyrosinase has been identified (Joy IV *et al.* 1995).

1.2.10. The location of polyphenol oxidase

Mammalian tyrosinase is found specifically in the melanocytes which produce and secrete pigment granules. Melanocytes primarily occur in the skin, hair bulbs and eyes. The tyrosinase is synthesized on the ribosomes and transported to the Golgi apparatus through the smooth endoplasmic reticulum. The enzyme is subsequently packaged into vesicles and delivered to the melanosomes. In the melanosomes, tyrosinase becomes membrane bound (Hearing & Jiménez 1987). In plant leaves polyphenol oxidase is mainly located in the lumen of the chloroplasts (Tolbert 1973, Trebst & Depka 1995). After translation precursor polyphenol oxidase is routed to the thylakoid lumen in two steps. This two-step targeting is observed in a variety of plants, including maize (*Zea mays* L.), pea (*Pisum sativum* L.) and tomato. Firstly, in an ATP-dependent step the precursor (67 kDa) is imported into the stroma. It is processed to an intermediate (62 kDa) by a stromal peptidase. Translocation into the lumen was found to be light dependent and involved the intermediate polyphenol oxidase being processed into a mature form (59 kDa). In the lumen mature polyphenol oxidase was soluble and not bound to the thylakoids (Sommer *et al.* 1994). Polyphenol oxidase in plant leaves has been reported to be tightly bound to the chloroplast lamellar structure (Tolbert 1973). Apple polyphenol oxidase is uniformly distributed in both mature and immature apples (Murata *et al.* 1995). During ripening and storage, some apple (*Malus pumila*) polyphenol oxidase was found to be solubilized and proteolyzed (Murata *et al.* 1997).

Sub cellular redistribution of potato (*Solanum tuberosum* L.) tuber polyphenol oxidase takes place after impact injury, and this coincides with the loss of membrane integrity (Partington *et al.* 1999). It was also reported that polyphenol oxidase activity increases dramatically in leaves of wounded plants, although the increased level of polyphenol oxidase was attributed to the expression of polyphenol oxidase being induced (Boss *et al.* 1995, Thipyapong & Steffens 1997, Constabel *et al.* 2000). Bacterial (*M. mediterranea*) polyphenol oxidase is multipotent with the enzyme mostly being membrane-bound but also being present in the soluble fraction (Fernandez *et al.* 1999).

1.2.11. Activation of polyphenol oxidase

The activation of polyphenol oxidase has been suggested to take place through either the release of free fatty acids or acidification (Hutcheson *et al.* 1980). In amphibian skin, tyrosinase is activated by a proteolytic activation system that doesn't seem to function in the activation of

mammalian tyrosinase or that of fungal tyrosinase (*Agaricus bisporus*) (Hearing 1973, McGuire 1970, Soler-Rivas *et al.* 1997). Active apple polyphenol oxidase is mainly localized around the core of the mature apple (Murata *et al.* 1995). Polyphenol oxidase in leaves has been reported to have immense latent activity (Tolbert 1973). C₁₈ fatty acids (linolenic acid) are potent activators of the enzyme activity, although the isolated enzyme slowly activates spontaneously over time (Golbeck & Cammarata 1981).

1.2.12. Biological function and uses of tyrosinase

1.2.12.1. Role of tyrosinase in melanogenesis

Throughout the phylogenetic spectrum, tyrosinase is responsible for the production of the pigment melanin. Tyrosinase is the critical factor that regulates melanogenesis (Hearing & Jiménez 1987). The melanogenic pathway was previously described in section 1.2.8. Tyrosinase inhibitors are utilized as skin-whitening agents (Baurin *et al.* 2002). A composition comprising of dye precursor and tyrosinase has been employed to tan the skin by the formation of melanin-like dyes on the skin (Herlihy 1985).

The autoimmune disorder vitiligo is due to the generation and presence of auto-antibodies directed against melanocyte antigens in patient's sera. It was found that tyrosinase is an autoantigen in vitiligo, which suggested that anti-tyrosinase titres may serve as a marker for disease activity (Baharav *et al.* 1996). In Allelism, genes linked with skin pigment synthesis have been demonstrated to be associated with the risk of prostate cancer. This is possibly based upon it mediating the protective effects of UV (Luscombe *et al.* 2001). The fungal pigment melanin promotes the infectivity of zoo pathogenic fungi (Jacobson 2000). The contribution of melanin to virulence of melanized microbes appears to be due to a reduction of susceptibility to the host defense mechanisms (Nosanchuk & Casadevall 2003).

1.2.12.2. Polyphenol oxidase and defence

During the attack of fungi on other organisms, polyphenol oxidase may be a defence factor and may be involved in the defence against pathogens (Mayer 2006). Polyphenol oxidase mediated phenolic oxidation is important in restricting plant disease development (Li & Steffens 2002) and the enzyme plays a role in plant's defence against insects (Haruta *et al.* 2001). A positive

correlation between the levels of polyphenol oxidase in plants and their resistance to pathogens and herbivores is observed (Figure 1.14) (Mayer 2006).

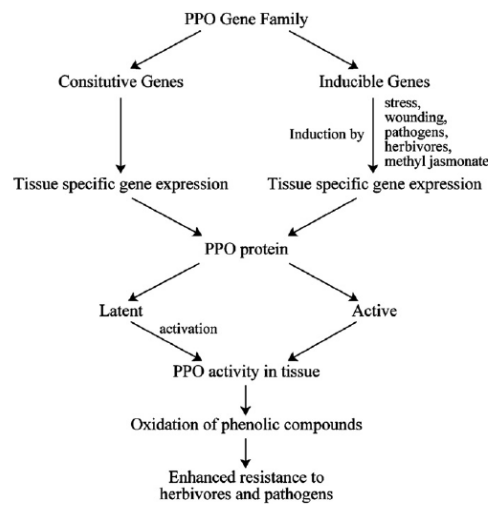


Figure 1.14: The correlation between the functions of polyphenol oxidase and the levels of the enzyme activity (Mayer 2006).

1.2.12.3. Enzymatic browning

In the food industry, the problem of spoilage caused by enzymatic browning is a problem of particular importance to food growers, processors and retailers (Bachem *et al.* 1994). Discoloration caused by enzymatic browning is a result of the action of endogenous polyphenol oxidase and subsequent polymerization of quinonoid compounds with other components present in food (Figure 1.15). In harvested litchi peel as an example, browning is largely attributed to the rapid degradation of anthocyanin pigments causing the subsequent loss of the bright-red skin colour (Jiang *et al.* 2004).

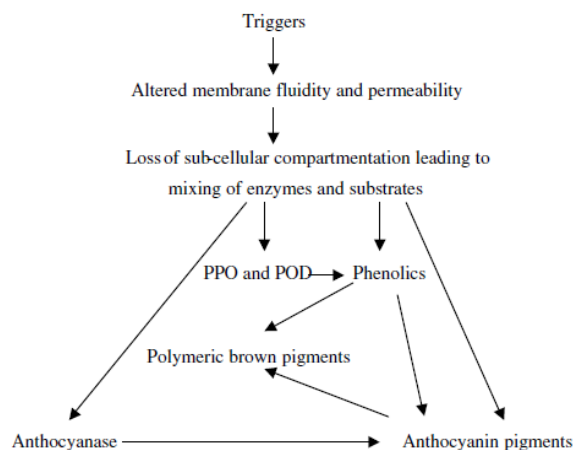


Figure 1.15: The proposed mechanism of enzymatic browning of the pericarp of harvested litchi fruit (Jiang *et al.* 2004).

Anti-browning agents primarily target either the polyphenol oxidase activity or the intermediates for pigment formation (McEvily *et al.* 1992). Tyrosinase inhibitors are therefore, examples of anti-browning agents utilized in food systems (Baurin *et al.* 2002, Zheng *et al.* 2008). Constraints on anti-browning agents include toxicity, colour, texture, cost, and effects on taste (McEvily *et al.* 1992).

1.2.12.4. Other applications of polyphenol oxidase

Immobilized tyrosinase has been utilized for the determination of phenolics in red wines (Yildiz *et al.* 2005, Kıralp *et al.* 2003) and cyanide in samples (Shan *et al.* 2004) as well as for the detection of polyphenols in tea (Abhijith *et al.* 2007) and pollutants in water (Russell & Burton 1999) (Edwards *et al.* 1999). Polyphenol oxidase is also employed for the removal of phenols from industrial effluents (Edwards *et al.* 1999). The biotransformation of tea catechins into theaflavins with immobilized polyphenol oxidase has also been explored (Sharma *et al.* 2009).

1.2.13. Polyphenol oxidase and tea

1.2.13.1. Polyphenol oxidase during tea manufacturing

Tea can be grouped into three types namely black tea (fermented tea), oolong tea (partially fermented tea) and green tea (non-fermented tea) (Figure 1.16) (Leung *et al.* 2001).

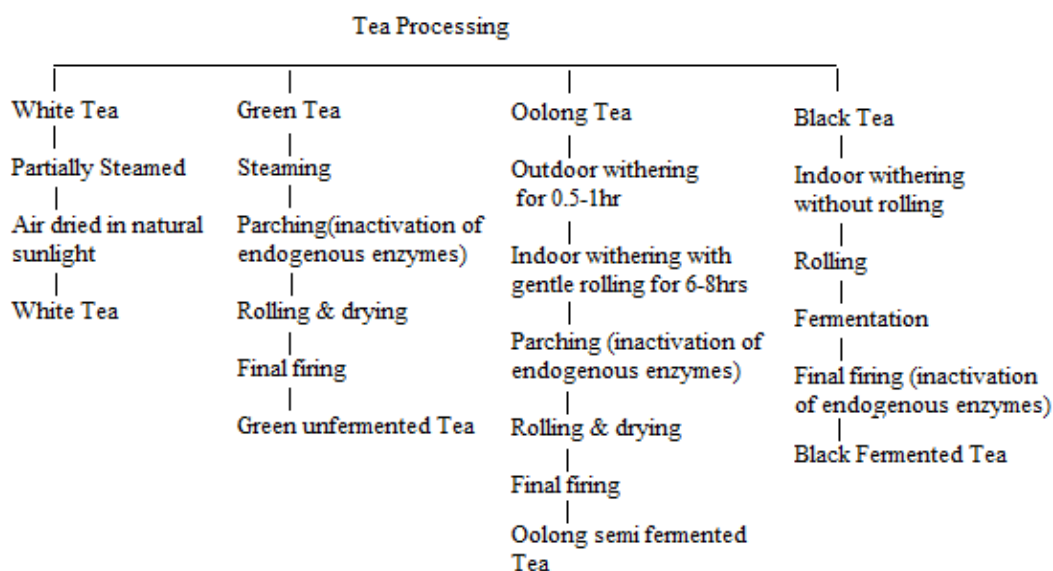


Figure 1.16: The conventional manufacturing processes of white, green, oolong and black tea (Karori *et al.* 2010).

Catechins constitute the major components of green tea leaves (Leung *et al.* 2001). The total content of catechins [(-)-epicatechin (EC), EGC and EGCG], gallic acid and caffeine is related to the degree of fermentation during tea manufacturing and the quality of the tea leaves. These contents function as sufficient descriptors to distinguish between green-, red-, black- and oolong teas despite large variability observed between different teas as well as within the same type of tea sample (Cabrera *et al.* 2003).

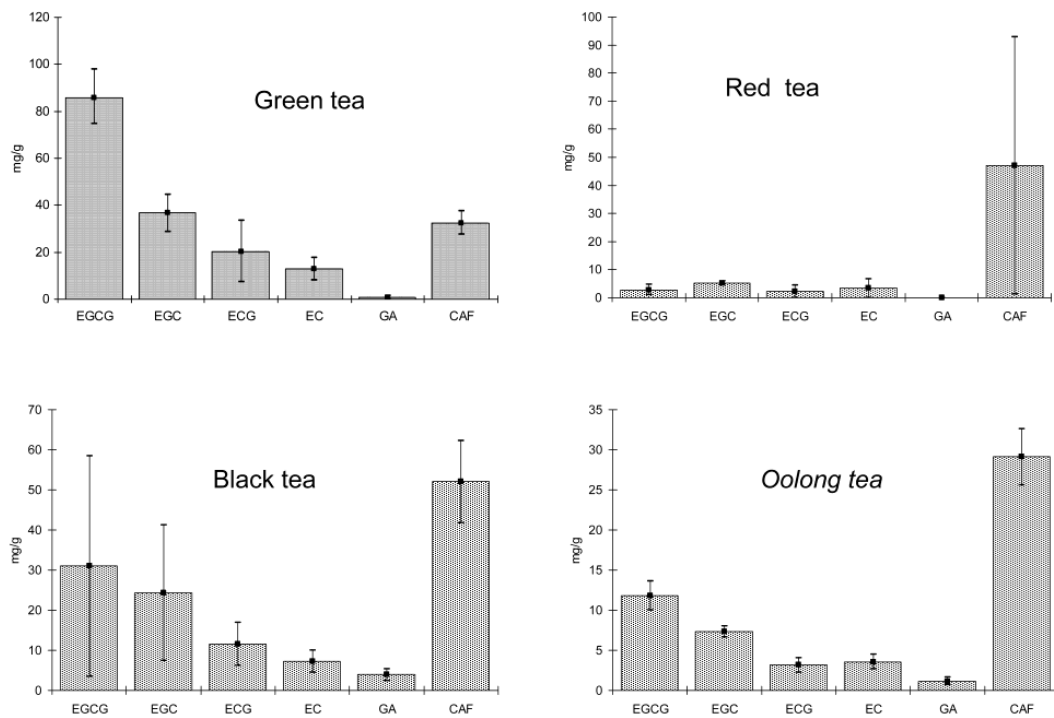


Figure 1.17: The levels of catechins [(-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin ((-)-EC)] in green-, red-, black and oolong tea respectively. The error bars represent the standard deviation of three replicates ($n = 3$) (Cabrera *et al.* 2003).

A higher content of catechins is present in green tea in comparison to that of both oolong and fermented teas (Figure 1.17). This is due to the fermentation process in black and red teas resulting in a significant reduction in the levels of catechins. The fermentation process in black teas is also responsible for a remarkably elevated level of gallic acid. In fermented teas, caffeine is present in higher amounts in comparison to the moderate amount in green and oolong teas (Figure 1.17) (Cabrera *et al.* 2003).

During the manufacturing of black tea and oolong tea, the green tea catechins (EC, ECG, EGC and EGCG) are oxidized and dimerized to form theaflavins [theaflavin (TF₁), theaflavin-3-gallate (TF_{2A}), theaflavin-3'-gallate (TF_{2B}) and theaflavin-3,3'-digallate (TF₃)] (Figure 1.18) (Leung *et al.* 2001).

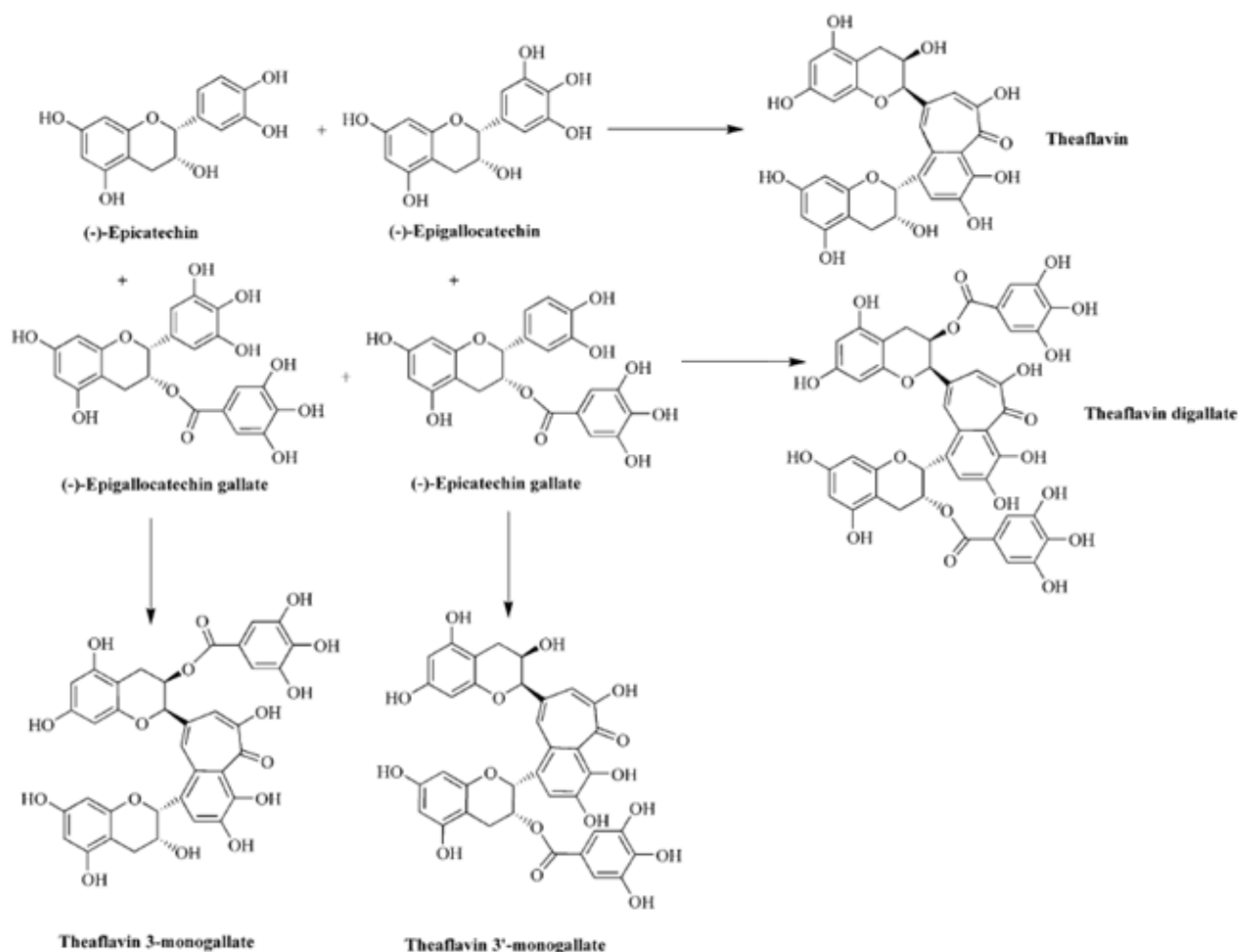


Figure 1.18: Reactions involved in theaflavin production (Bonnely *et al.* 2003).

Green tea catechins are also polymerized into thearubigins during the fermentation process (Leung *et al.* 2001). EGCG, theaflavin and thearubigin are all significant flavan-3-ols in tea (Peterson *et al.* 2005).

1.2.13.2. Antioxidant activity of tea constituents

The antioxidant activity of the catechins and theaflavins is based upon an analysis of the conjugated dienes and thiobarbituric acid-reactive substrates (TBARS) produced during low-density lipoprotein (LDL) oxidation revealing the order: $TF_3 > ECG > EGCG \geq TF_2B \geq TF_2A > TF_1 \geq (-)EC > EGC$. At concentrations of 5-40 $\mu M/L$ of the theaflavin derivatives indicated a dose-dependent antioxidant activity in Cu^{+2} -mediated LDL oxidation (Leung *et al.* 2001). Based upon a modified Oxygen-Radical Absorbance Capacity method, the radical scavenging activity in the order of: $TF_3 \approx TF_2A \approx TF_2B \approx TF_1 > epitheafavic\ acid > EGCG > purpurogallin$ was found (Jhoo *et al.* 2004).

1.2.14. Theaflavin synthesis capacity of plant polyphenol oxidase

Plant polyphenol oxidase has been purified from various sources, including that from pineapple (Das *et al.* 1997), Loquat (Sellés-Marchart *et al.* 2006), butter lettuce (Gawlik-Dziki *et al.* 2008), green bean (Guo *et al.* 2009), Barbados cherry (Kumar *et al.* 2008), spinach (Sánchez-Ferrer *et al.* 1989), *Ferula* sp. (Erat *et al.* 2006), tobacco (Shi *et al.* 2002), Red Swiss chard (Gao *et al.* 2009), Mamey (Palma-Orozco *et al.* 2011) and litchi (Yue-Ming *et al.* 1997). In particular, polyphenol oxidase from pear has been purified from d'Anjou pears (Smith & Montgomery 1985, Wissemann & Montgomery 1985), Barlett pears (de Jesus Rivas & Whitaker 1973) and Williams pears (Richard-Forget & Gaillard 1997). Japanese pear and loquat homogenates have a much greater theaflavin synthesis capacity than that of fresh tea leaves and were also the highest among 62 plants belonging to 49 families. The addition of Japanese pear- and loquat homogenates into green tea infusions produced the black tea pigments theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate (Tanaka *et al.* 2002). The biotransformation of tea catechins into theaflavins with immobilized polyphenol oxidase has been explored (Sharma *et al.* 2009).

1.3. IMMOBILIZATION

At an industrial scale, the desirability of many cycles of high-yield processes usually requires enzyme characteristics to be improved despite of their excellent catalytic properties (Mateo *et al.* 2007). The improvement of activity, stability, selectivity towards non-natural substrates, the inhibition by reaction products and prohibitive enzyme cost, may be required, offering in general drawbacks to the use of biocatalysts to that of classical chemical catalysts (Mateo *et al.* 2007, Villeneuve *et al.* 2000). Enzyme immobilization offers a means of enzyme engineering that is compatible with other biological or chemical approaches to improve enzyme functions (Mateo *et al.* 2007).

Immobilization is the process of attaching an enzyme onto an insoluble solid support (Villeneuve *et al.* 2000). Enzyme immobilization enhances the applicability of biomolecules as robust and reusable biocatalysts due to the increase in stability it confers to enzymes (Betancor & Luckarift 2008). The enzyme structure is more rigid after immobilization, which increases the enzyme's thermal stability (Villeneuve *et al.* 2000). Further advantages include enhanced enzyme activity, modification of substrate selectivity and enantioselectivity, multi-enzyme reactions, the possibility to modulate enzyme catalytic properties, easy separation from the reaction mixture, easier prevention of microbial contaminations, prevention of protein contamination of the product

and continuous or repeated use (Petersen & Kiener 1999, Brady & Jordaan 2009). Therefore, immobilization overcomes the lack of long-term stability under process conditions and difficulties in recycling and recovering of the enzyme which hampers the application of enzymes for a given reaction (Petersen & Kiener 1999).

1.3.1. ReSynTM

ReSynTM is a novel protein immobilization matrix functioning as a carrier material during immobilization. This polyethylene imine (PEI) based resin is found on a newly developed method in which PEI dissolves in the aqueous phase of a water-in-oil emulsion. Spherical particles of cross-linked PEI are formed by the addition of a limited quantity of water-soluble cross-linker (for example, glutaraldehyde) and the particles can subsequently be isolated from the oil phase (Figure 1.19) (Jordaan *et al.* 2009).

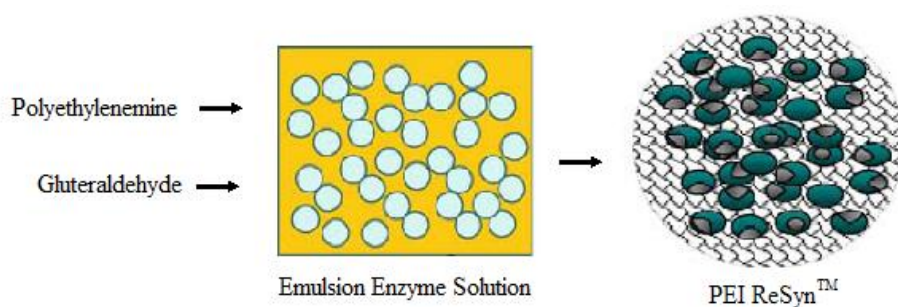


Figure 1.19: Synthesis of the PEI based ReSynTM (Brady & Jordaan 2009).

This results in emulsion-derived particle comprising of a lattice of cross-linked polymeric strands, interstitial openings around and adjacent to the strands and functional groups on the lattice by means of which immobilization is achieved (Jordaan *et al.* 2009). Anionic binding of proteins can be achieved through the very high density of amine groups, although these can also be utilized as a basis for the dialdehyde covalent cross-linking of the enzymes. Immobilization through hydrophobic and affinity bonding can also be achieved through the modification of lattice for this functionality (Jordaan *et al.* 2009). Extremely high enzyme loads of more than 30% *g/g* can be attained by ReSynTM as a result of the open fibre network and high amine density (Brady & Jordaan 2009). Additional stability of enzymes in extremes of temperature or pH can also be realized by the immobilization support (Jordaan *et al.* 2009).

1.4. RESEARCH HYPOTHESES AND AIMS

1.4.1. Hypotheses

H₁₀: There will be no statistical significant difference, in the enzyme activity binding capacity (enzyme units/mg support), between the supports; ReSynTM, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25, at the 95% level of confidence.

H₁: There will be a statistical significant difference, in the enzyme activity binding capacity (enzyme units/mg support), between the supports; ReSynTM, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25, at the 95% level of confidence.

These hypotheses were set up between the various supports at four different reaction times and two different reaction temperatures.

H₂₀: There will be no statistical significant difference in the yield of theaflavins/mg enzyme between the free- and ReSynTM immobilized polyphenol oxidase at the 95% level of confidence.

H₂: There will be a statistical significant difference in the yield of theaflavins/mg enzyme between the free- and ReSynTM immobilized polyphenol oxidase at the 95% level of confidence.

1.4.2. Aims

1.4.2.1. Purification of pear polyphenol oxidase

1.4.2.1.1. Identification of the pear variety with the highest theaflavin synthesis capacity in green tea based upon the flavognost method.

1.4.2.1.2. Preliminary investigation of the theaflavin synthesis capacity of the identified pear variety homogenate based upon:

1.4.2.1.2.1. Diphenolase activity of polyphenol oxidase in sample extracts.

1.4.2.1.2.2. Theaflavin synthesis capacity in a fermentation system based upon the flavognost method.

1.4.2.1.2.3. TLC detection of inherent catechins and theaflavins present in sample extracts.

1.4.2.1.3. Purification of polyphenol oxidase with concurrent analysis of:

1.4.2.1.3.1. Enzyme activity (units) and protein concentration (mg/ml).

1.4.2.1.3.2. Molecular weight determination based upon silver stained SDS-PAGE.

1.4.2.1.3.3. Temperature and pH optimum determination of purified polyphenol oxidase.

1.4.2.2. Comparative assessment of immobilization of polyphenol oxidase

1.4.2.2.1. Immobilization on the supports Eupergit C, Eupergit C250L, DEAE Sephadex A-25 and ReSynTM:

1.4.2.2.1.1. ReSynTM synthesis and determination of the particle size and dry weight.

1.4.2.2.1.2. Preliminary assessment of immobilization with bovine serum albumin.

1.4.2.2.1.3. Analysis of the loading capacity, binding efficiency and nature of binding.

1.4.2.3. Biotransformation of tea catechins into theaflavins

1.4.2.3.1. Theaflavin synthesis with both free- and ReSynTM immobilized polyphenol oxidase:

1.4.2.3.1.1. UPLC-MS analysis of the biotransformation of catechins into theaflavins.

CHAPTER 2 : CHARACTERISATION OF POLYPHENOL OXIDASE

2.1. PURIFICATION OF POLYPHENOL OXIDASE

The first tyrosinase was discovered 1895 in mushroom *Russula nigricans* with the observance of a black pigment (Bourquelot & Bertrand 1895). The name tyrosinase was derived from that of the substrate tyrosine (Bertrand 1896). The distribution of tyrosinase in all domains of life is nearly ubiquitous (Claus & Decker 2006). Polyphenol oxidase is found in groups as diverse as plants (most fruits and vegetables) (Van Gelder *et al.* 1997, Flurkey 1986), fungi (Van Gelder *et al.* 1997, Halaouli *et al.* 2006), amphibia (Miranda *et al.* 1985, Chen & Chavin 1968), arthropods (Martinez & Whitaker 1995) and mammals (Miranda *et al.* 1985).

2.1.1. Reported purification of polyphenol oxidase

The spoilage of food caused by enzymatic browning is a problem of particular importance to food growers, processors and retailers (Bachem *et al.* 1994). In the food industry, the browning of raw fruits and vegetables during storage and processing is believed to be the main cause of quality loss during post harvest handling (Coetzer *et al.* 2001). Discoloration caused by enzymatic browning is a result of the action of endogenous polyphenol oxidase, and therefore, many investigations have been conducted to characterize polyphenol oxidase from many fruits and vegetables (Jiang *et al.* 2004).

Polyphenol oxidase has been purified from various fruit sources including coffee (leaf and fruit endosperm) (Mazzafera & Robinson 2000), medlar fruits (Dincer *et al.* 2002), blueberry (Kader *et al.* 1998), blackberry (Gonzalez *et al.* 2000), raspberry (González *et al.* 1999), mulberry (Arslan *et al.* 2004), marula (Mdluli 2005), grapes (Dry & Robinson 1994), apricot (Chevalier *et al.* 1999, Arslan *et al.* 1998), banana (peel and pulp) (Yang *et al.* 2001a, Yang *et al.* 2000), litchi (pericarp) (Yue-Ming *et al.* 1997), persimmon fruit (Núñez-Delicado *et al.* 2003), dates (Hasegawa & Maier 1980), strawberry (Serradell *et al.* 2000), apple (peel and leaf) (Zhou *et al.* 1993, Ridgway & Tucker 1999), quince (Hülya & Ayten 2002), longan fruit (Jiang 1999), pineapple (Das *et al.* 1997) and pear (de Jesus Rivas & Whitaker 1973). The purification of polyphenol oxidase from vegetables has been reported to include mushrooms (Keilin & Mann 1938), broad bean (leaf) (Robinson & Dry 1992), artichoke (Dogan *et al.* 2005b), beet (root and leaf) (Escribano *et al.* 1997, Gandía-Herrero *et al.* 2004), lettuce

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(Chazarra *et al.* 2001), field bean (Kanade *et al.* 2006), sweet potato (root) (Fatibello-Filho & da Cruz Vieira 1997), potato (Sanchez-Ferrer *et al.* 1993b) avocado (Espín *et al.* 1997), cabbage (Nagai & Suzuki 2001, Fujita *et al.* 1995) and desert truffle (Pérez-Gilabert *et al.* 2001).

Other plant sources from which polyphenol oxidase has been purified were orchid (Ho 1999), thyme (*Thymus longicaulis* subsp. *Chaubardii* var. *chaubardii*) (Dogan & Dogan 2004), sugarcane (Bucheli *et al.* 1996), creosote bush (Cho *et al.* 2003), peppermint (Kavrayan & Aydemir 2001), basil (Dogan *et al.* 2005a), *Allium* sp. (Arslan *et al.* 1997), *Hevea brasiliensis* (Wititsuwannakul *et al.* 2002), wild rice (Owusu-Ansah 1989), chestnuts (Xu *et al.* 2004), *Ferula* sp. (Erat *et al.* 2006), tobacco (Shi *et al.* 2002) and tea (leaf and shoots) (*Camellia sinensis*) (Halder *et al.* 1998, Gregory & Bendall 1966). The purification of polyphenol oxidase has also been reported from hamster melanoma (Pomerantz & Li 1973), the ink of squid (Naraoka *et al.* 2003), shrimp (Chen *et al.* 1997), *Neurospora crassa* (Horowitz & Fling 1953) and various species of *Streptomyces* (Suzuki *et al.* 2003, Kohashi *et al.* 2004, Lerch & Ettlinger 1972).

2.1.2. Purification of polyphenol oxidase from pears

2.1.2.1. Classification of pears

Pears are classified within the family Rosaceae and form part of the apple subfamily Maloideae. The subfamily Maloideae comprises of approximately a 1000 species in 30 genera. This subfamily is characterized by the pome, a distinctive fruit and a base chromosome number (x) of 17. The other subfamilies Rosoideae (plants such as raspberries, strawberries and roses), Amygdaloideae (including apricots, peaches, plums and cherries) and Spiraeoideae or the bridlewreath subfamily have $x = 7, 8$ or 9 respectively with few genera having $x = 15$ or 17 (Goldblatt 1976). Pears are further classified in the super tribe Pyrodae, tribe Pyreae, subtribe Pyrinae and genus *Pyrus communis* L. (Potter *et al.* 2007).

2.1.2.2. Pear polyphenol oxidase

Interest in pear polyphenol oxidase is attributed to the propensity of fresh-cut pears to enzymatic browning and softening during storage (Dong *et al.* 2000). Polyphenol oxidase is responsible for the browning of fresh horticultural products after cutting, bruising or other cellular damage (Martinez & Whitaker 1995). Various efforts have been made to investigate the browning of

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puree, fresh-cut and the juice of pear fruits (Ibarz *et al.* 1999, Gorny *et al.* 2002, Amiot *et al.* 1995). In this project, the interest in pear polyphenol oxidase pertains to its high theaflavin synthesis capacity. It was found that Japanese pear and loquat homogenates have a much greater theaflavin synthesis capacity than that of fresh tea leaves and was also the highest among 62 plants belonging to 49 families. The addition of Japanese pear- and loquat homogenates into green tea infusions produced the black tea pigments theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate (Tanaka *et al.* 2002).

2.1.2.3. Reported purified pear polyphenol oxidase

Pear polyphenol oxidase has been extracted from the cultivars Durondeau (Weemaes *et al.* 1998), Blanquilla (Espín *et al.* 1997), Williams (Carbonaro & Mattera 2001, Gauillard & Richard-Forget 1997), d'Anjou and Bartlett pears (Siddiq & Cash 2000). Polyphenol oxidase has also been reported to have been partially purified from the cultivars Blanquilla (Espín *et al.* 1997, Espin *et al.* 1996). Polyphenol oxidase has been purified from the pear varieties Ankara Armutu (Ziyan & Pekyardimci 2004), d'Anjou (Wisseman & Montgomery 1985), La France (Asaka *et al.* 1994), Bartlett (de Jesus Rivas & Whitaker 1973) and Williams pears (Richard-Forget & Gauillard 1997).

2.1.2.4. The extraction medium

Distilled water has been used as the extraction medium for polyphenol oxidase from the Durondeau pear cultivar (Weemaes *et al.* 1998). Changes in absorbance, browning and loss of enzyme activity over time in a water extract of polyphenol oxidase from d'Anjou pear is attributed to the interactions between the enzyme and endogenous pear phenolic compounds (Smith & Montgomery 1985). Buffers utilized for the extraction of pear polyphenol oxidase include tris hydroxymethyl aminomethane (Siddiq & Cash 2000), sodium phosphate EDTA (Espin *et al.* 1996), phosphate (Ziyan & Pekyardimci 2004, Asaka *et al.* 1994), citrate sodium phosphate (Wisseman & Montgomery 1985) and sodium phosphate buffer (de Jesus Rivas & Whitaker 1973). The extraction media may contain phenol-absorbing agents and polyphenol oxidase activators such as detergents and proteases (Tolbert 1973, Sánchez-Ferrer *et al.* 1989, Smith & Montgomery 1985, Robinson & Dry 1992).

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2.1.2.4.1. Phenol-absorbing agents

Phenol-absorbing agents (polyvinylpyrrolidone, AG 2-X8, anion exchange resins, polycaprolactam powder, Amberlite XAD resins and polyethylene glycol) are used to prevent phenol-protein interactions based upon the phenolics binding ability, which inactivate or modify the enzyme (Figure 2.1) (Wissemann & Montgomery 1985, de Jesus Rivas & Whitaker 1973, Ziyen & Pekyardimci 2004, Wuyts *et al.* 2006).

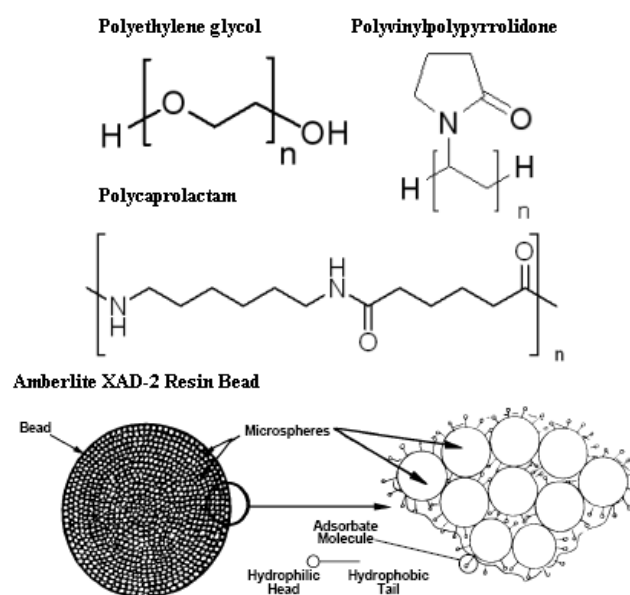


Figure 2.1: The structure of polyethylene glycol, polyvinylpyrrolidone, polycaprolactam and the structure of a hydrophobic Amberlite XAD-2 resin bead.

The polymer complexes with endogenous polyphenols and prevents or slows down the crude extracts from browning (de Jesus Rivas & Whitaker 1973, Paul & Gowda 2000). The best combination of phenol-absorbing agents is dependent upon the types and quantities of phenols, therefore, differing for each plant (Sánchez-Ferrer *et al.* 1989). Additional isoforms of the enzyme can be produced by the interaction between phenolic substrates and polyphenol oxidase if the endogenous phenolic substrates are not removed during the extraction process (Smith & Montgomery 1985).

2.1.2.4.2. Detergents

Latent polyphenol oxidase present in the chloroplast of plants can be solubilized and activated by detergents (Figure 2.2) (Mayer & Harel 1979, Kenten 1958, Kenten 1957). The most efficient detergent was determined to be non-ionic with a hydrophile-lipophile balance (HLB) between 12.4 and 13.5 for the extraction of polyphenol oxidase from grape berries. The presence of cationic [cetyltrimethylammonium bromide (CTAB)] and anionic (SDS) detergents extracts

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polyphenol oxidase with the same low efficiency as that of the buffer. Sodium deoxycholate is the exception which is possibly explained by its low aggregation number (Sánchez-Ferrer *et al.* 1989).

Ionic detergents present in the extraction buffer are good activators whereas the non-ionic detergent Triton X-114 was found to be an ineffective activator of polyphenol oxidase from several wheat cultivars (Okot-Kotber *et al.* 2002). The non-ionic detergent Triton X-100 results in a 30% increase in the activity of polyphenol oxidase from D’Anjou pears (Wissemann & Montgomery 1985). Triton X-114 and Triton X-100 solubilise proteins and chlorophyll with the same efficiency (Sánchez-Ferrer *et al.* 1989).

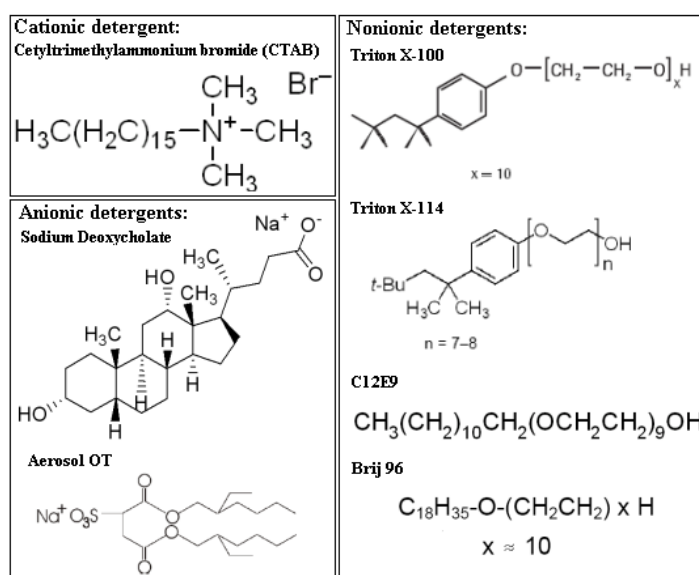


Figure 2.2: The structures of cationic, anionic and nonionic detergents (Sánchez-Ferrer *et al.* 1989).

The Triton-114 method utilizes the ineffective activator of polyphenol oxidase, Triton-114, for the extraction of proteins and subsequently temperature-induced phase partitioning is performed. Triton-114 offers the advantage of failing to maintain all the proteins and chlorophyll in solution, forming large aggregated mixed micelles of Triton-114. These contain membrane proteins, phospholipids, phenols and chlorophyll. The Triton-114 method is able to remove the chlorophyll and phenolic compounds present in the extracts, thereby removing chlorophyll and phenols from green plant extracts without the use of drastic extraction methods to clarify the extracts such as acetone powders or other organic solvents. Polyphenol oxidase is twice as readily activated by Triton-114 purified in Tris-HCl than in sodium phosphate buffer (Sánchez-Ferrer *et al.* 1989).

2.1.2.5. Proteases

A very active polyphenol oxidase is generally present in a latent form in the chloroplast of all plants. The treatment with peptides from autolyzed trypsin or aging releases the latent polyphenol oxidase in the chloroplast of some plants (Tolbert 1973). Polyphenol oxidase is synthesized in 60 kDa form which is sensitive to proteolytic cleavage at the C-terminal end resulting in an active 45 kDa form (Robinson & Dry 1992). Trypsin itself is able to extract little enzyme, but in the presence of detergents that digest the membrane, its action is enhanced due to the synergic effect. The activity of polyphenol oxidase is greater when the enzyme is first extracted with Triton-100 and subsequently activated by trypsin. In the Triton-114 method the activation of the enzyme by trypsin increased during purification as opposed to the decrease during the ammonium sulphate method. The same increase in enzyme activity with trypsin is also observed with the supernatant of Triton-100 (Sánchez-Ferrer *et al.* 1989).

2.1.2.6. Methods for purification of plant polyphenol oxidase

The multi sub-unit nature and the presence of several polyphenol oxidase isoenzymes hamper the complete purification of polyphenol oxidase, although affinity chromatography methods, particularly using the affinity ligand 4-amino-benzoate, have proven successful (Arslan *et al.* 2004, Wong *et al.* 1971). Other methods reported for the preparation and purification of plant polyphenol oxidase include hydroxylapatite – (Wissemann & Montgomery 1985, Yang *et al.* 2000, Gregory & Bendall 1966), hydrophobic – (Das *et al.* 1997, Zhou *et al.* 1993, Lopez-Serrano & Barceló 2002) and Concanavalin-A-Sepharose chromatography (Kwon & Kim 1996) as well as detergent-mediated two-phase partitioning (Sanchez-Ferrer *et al.* 1993a, Vaidya *et al.* 2006) and gel filtration with ion exchange (Sakiroglu *et al.* 1996).

2.1.2.6.1. Affinity chromatography

Enzyme purification through affinity chromatography is achieved by means of a cross-linked polymer or gel to which a specific competitive inhibitor of the enzyme of interest is covalently attached. Proteins with affinity towards the bound inhibitor are retarded in proportion to their affinity constant and eluted with the change of parameters (pH or salt concentration) or the presence of a competitive inhibitor. The success of affinity chromatography requires that the unsubstituted matrix or gel has minimal interactions with proteins in general, and the adsorbent must permit easy entry and exit of macromolecules and retain favourable flow properties.

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Convenient and extensive attachment of the specific ligand through chemical bonds stable under the conditions of adsorption and elution must be permitted by the supporting material. Lastly successful affinity chromatography requires a sufficient distance between the inhibitor groups and the solid matrix to minimize steric interference during the binding processes (Cuatrecasas *et al.* 1968, Cuatrecasas 1970).

Polyphenol oxidase purification by affinity chromatography from mulberry (*Morus alba* L.) fruit using a Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column has been reported (Figure 2.3). Mulberry polyphenol oxidase was extracted in phosphate buffer containing polyethylene glycol (PEG) and ascorbic acid. The enzyme solution was filtered, fractionated with solid ammonium sulphate, dialyzed and applied to the affinity column (Arslan *et al.* 2004).

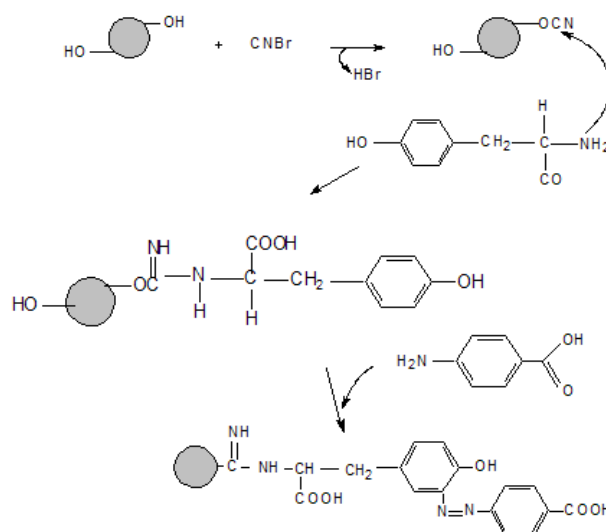


Figure 2.3: Preparation of a Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column (Arslan & Erzengin 2002).

Utilizing the affinity column Sepharose 4B-L-tyrosine-*p*-amino benzoic acid a 74 fold purification of mulberry polyphenol oxidase was achieved. The fold purification is higher than 9.3-, 8.7- and 10 fold obtained for pear (Espin *et al.* 1996, Ziyan & Pekyardimci 2004, Asaka *et al.* 1994) and 58-, 38.1- and 21 fold respectively obtained for marula, apricot and litchi (Yue-Ming *et al.* 1997, Mdluli 2005, Chevalier *et al.* 1999).

2.2. KINETIC ANALYSIS OF POLYPHENOL OXIDASE

The type-3 copper enzyme polyphenol oxidase (EC 1.14.18.1) has a binuclear copper centre (Gerdemann *et al.* 2002, Matoba *et al.* 2006). Polyphenol oxidase has both a monophenolase- or cresolase-activity (*ortho*-hydroxylation of monophenols) and a diphenolase- or catecholase

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activity (oxidation of *o*-diphenols to *o*-diquinones). The binding of one molecule of atmospheric oxygen by the copper pair of tyrosinase enables the catalysis of two different enzymatic reactions (Land *et al.* 2003). During the monophenolase activity of polyphenol oxidase, one atom of oxygen is incorporated into the aromatic ring (aromatic C-H bond) of the monophenol during the hydroxylation reaction. The phenol hydroxylation is carried out by E_{ox} and the oxidation of catechols by both E_m and E_{ox} (Granata *et al.* 2006). The diphenolase- or catecholase activity of tyrosinase oxidizes *o*-diphenols to *o*-diquinones. The highly reactive produced quinones can react with amino acids and peptides and also polymerize to form brown or black pigments (Pierpoint 1969, Martinez & Whitaker 1995).

2.2.1. Reaction mechanism of polyphenol oxidase

In the native form of tyrosinase, it can act on monophenols because of the mixture of E_m and E_{ox} , these forms respectively being not active and active on monophenols (Figure 2.4) (Fenoll 2000). The turnover is started by E_{ox} acting on the monophenol (M) hydroxylating it to E_mD at which point the enzyme may oxidise *o*-diphenol (D) to quinone (Q) generating deoxy-tyrosinase (E_d). Alternatively, the *o*-diphenol is released producing E_m which would bind to monophenol producing the inactive form E_mM . The *o*-diphenol is recycled to the reaction medium in cases where the quinone is very unstable. This is achieved through intramolecular cyclization and further redox steps involving the transformation of E_m which is inactive towards monophenols to E_{ox} . This transformation gives rise to the characteristic lag period of the activity of tyrosinase. The steady state would be reached in such cases (Cabanès *et al.* 1987).

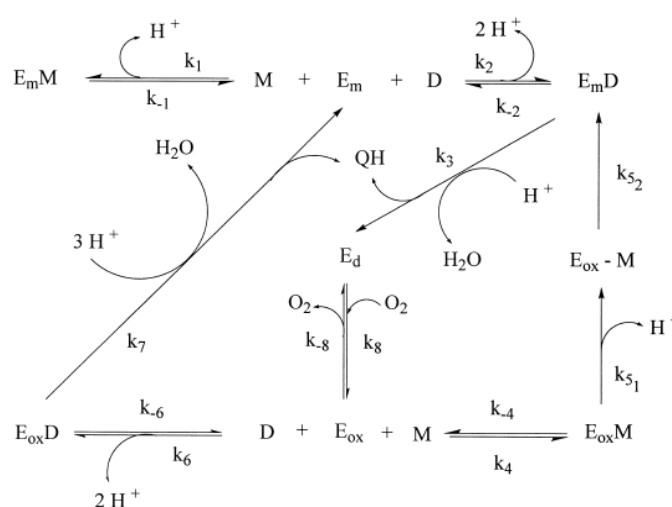


Figure 2.4: The action mechanism of tyrosinase on monophenol (M) and the release of *o*-diphenol (D) considering an unstable *o*-quinone (Q). Abbreviations: E_m , Met-tyrosinase (with Cu^{2+} - Cu^{2+} in the active site); E_{ox} , Oxy-tyrosinase (with Cu^{2+} -O22- Cu^{2+} in the active site); E_d , Deoxy-tyrosinase (with Cu^{1+} - Cu^{1+} in the active site); and QH, *o*-quinone protonated (Cabanès *et al.* 1987).

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Due to the instability of the protonated *o*-quinone (QH) it evolves to aminochrome (Cr) by the deprotonation of the amino group of QH to carry out a nucleophilic attack giving rise to a leucoaminochrome (L) (Figure 2.5). This in turn is oxidized by another molecule of the protonated *o*-quinone forming aminochrome and regenerating *o*-diphenol (Cabanès *et al.* 1987).

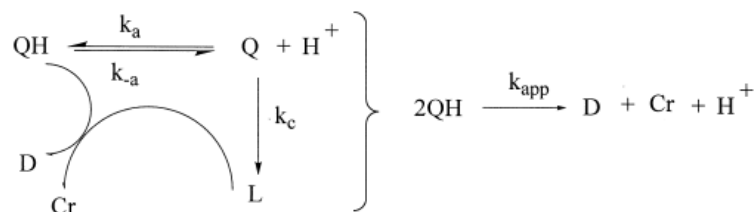


Figure 2.5: The evolution of *o*-quinones (Q) through deprotonation, cyclization and subsequent oxidation-reduction. Abbreviations: QH, *o*-quinone protonated; D, *o*-diphenol; Cr, aminochrome and L, leucoaminochrome (Cabanès *et al.* 1987).

The kinetics is complicated by monophenols being able to bind to both enzyme forms. This is further complicated by the *o*-quinone products being unstable and continual evolving to generate *o*-diphenols (Fenoll *et al.* 2000).

2.3. MATERIALS AND METHODS

2.3.1. Materials

Hydrochloric acid (AR, 32%) and Methanol (AR, 99.5%) were purchased from Radchem Products Inc, Orland Park, United States. The buffers used were prepared from; tris(hydroxymethyl)aminomethane (Trizma® base, minimum 99.9% titration), sodium phosphate dibasic and sodium phosphate monobasic purchased from Sigma Aldrich (Pty) Ltd, St. Louis, United States. Furthermore, dipotassium phosphate and potassium orthophosphate were respectively purchased from Saarchem (Pty) Ltd, Gauteng, South Africa and Labchem (Pty) Ltd, Gauteng, South Africa. Commercially available Lipton® Green Tea and Lipton® Yellow label black tea were purchased from a local supermarket, ground to a fine powder with a high speed blender and sifted through a 100 micron sieve. Tea (*Camellia sinensis*, frozen fresh leaves) and Yacon (*Smallanthus sonchifolius*, leaves) were obtained from the experimental farm of the University of Pretoria and stored at 4°C until use. Fresh pear varieties Forelle (class 1), Packman, Abet Beteil and Beurré Bosc were obtained from a local fruit store and stored at 4°C until use within five days. Polyvinylpyrrolidone (PVP) and ammonium sulphate were purchased from Sigma Aldrich (Pty) Ltd, St. Louis, United States. The chromatography columns HiPrep 26/60 Sephacryl S-200 HR column (6 x 2.6 cm) and Superdex 200 10/300 GL column (19 x 2.7 cm) were purchased from GE Healthcare Ltd, Gloucestershire, United Kingdom and the SnakeSkin pleated dialysis tubing (molecular weight cut off 10,000) from Thermo Fisher Scientific Corp, Waltham, United States. Albumin Fraktion V ($\geq 98\%$ pulv. bovine) was purchased from Roth, Karlsruhe, Germany and the protein assay dye reagent concentrate was purchased from Biorad, California, United States. The substrate 4-methylcatechol was purchased from Sigma Aldrich (Pty) Ltd, St. Louis, United States. For the TLC separation of catechins and theaflavins the compounds; iron(III)chloride from Sigma Aldrich (Pty) Ltd, St. Louis, United States and chloroform from E-LAB Direct Ltd, Huddersfield, United Kingdom were used. Furthermore, also ethyl acetate (HPLC-grade) was purchased from Analytical Sciences, Cambridge, England and anhydrous sodium sulphate was purchased from Merck, Damstadt, Germany. Nylon plates pre-coated with polyamide were purchased from MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany. The catechin standards [catechin (C), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG)] and theaflavin standards [theaflavin (TF), theaflavin-3-gallate (T3G), theaflavin-3'-gallate (T3'G) and theaflavin-3,3'-digallate(TdG)] were generous gifts from Prof. Y. Hara from Tea Solutions, Hara

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Office Inc Tokyo, Japan. Silver stained SDS-PAGE and zymogram was performed using sodium dodecyl sulphate (SDS), acrylamide / bis-acrylamide (30% solution electrophoresis reagent), N, N, N', N'-tetramethylethylenediamine (TEMED, Stratagene electrophoresis purity reagent), N', N-methylene bisacrylamide, ammonium persulphate ($\geq 98\%$), mercaptoethanol and glycerol purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. Furthermore, bromophenol blue, acetic acid and glycine were purchased from Merck, Darmstadt, Germany and the unstained protein molecular weight marker was purchased from Fermentas, Burlington, Canada. The ProteoSilverTM Silver Stain kit (silver solution, sensitizer, developer 1 & 2 and stop solution) and *p*-phenylenediamine were purchased from Sigma Aldrich (Pty) Ltd, St. Louis, United States. Acetic acid and ascorbic acid were respectively purchased from Merck, Darmstadt, Germany and Saarchem (Pty) Ltd, Gauteng, South Africa. For the Flavonoid determination of theaflavins; diphenylboric acid-2-aminoethyl ester and isobutylmethylketone were purchased from Sigma Aldrich (Pty) Ltd, St. Louis, United States. Ethanol (96% rectified) was purchased from MINEMA Chemicals (Pty) Ltd, Gauteng, South Africa.

2.3.2. List of equipment

The equipment used was a Waring commercial blender, Titramax 101 vibrating platform shaker (Heidolph Instruments, Schwabach, Germany), Sunflower mini-shaker 3D (BOECO, Hamburg, Germany), AccuBlockTM digital dry baths (Labnet International Inc, New Jersey, USA), SevenEasyTM bench top pH meter (Mettler Toledo Ltd, Leicester, United Kingdom), water bath, thin layer chromatography development chamber and magnetic stirrers. The centrifuges used were an AllegraTM X-22R centrifuge with a SX4250 rotor and an AvantiTM J-25I centrifuge with a JA-14 rotor (Beckman Coulter Inc, California, USA) and a Heraeus Pico 17 centrifuge (Thermo Fisher Scientific Corp, Waltham, United States). The ultrafiltration system used comprised of a Masterflex console drive (Cole-Parmer Instrument Company, Illinois, USA) with a MinimateTM TFF capsule and an OmegaTM 10k membrane (Pall Corporation, Port Washington, New York). An AS 220/X analytical balance and a WPS 2100/C2 balance (RADWAG®, Random, Poland) were used. The gel electrophoresis system used was a Power StationTM 300 (Labnet International Inc) with the gel electrophoresis tank equipment (Bio-Rad, California, United States). The vacuum pump system that was used comprised of a vacuum pump (Alcatel) with glassware and 0.45 μ m filters (Millipore, Massachusetts, United States). A rotary evaporator (Rotavapor RII) (BUCHI, Flawil, Switzerland), PowerWave HT spectrophotometer (Biotek Instruments Inc, Winooski, United States), AKTA prime plus chromatographic system

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(GE Healthcare Ltd, Gloucestershire, UK) and a Virtis Genesis 25 freeze drying system (SP Scientific Inc, New York, USA) were used.

2.3.3. Methods

2.3.3.1. Measurement of the theaflavins produced by extracts of different pear varieties

Ground Lipton® Green tea (2% w/v) was made up in 0.1 M phosphate buffer (pH 5.9). One fruit of the different pear (*Pyrus communis*) varieties (Forelle 108.2 g $\pm$ 1.148 SD, Packman 146.5 g $\pm$ 2.209 SD, Abet Beteil 154.8 g $\pm$ 6.296 SD and Beurré Bosc 229.1 g $\pm$ 6.897 SD) were homogenized in 100 ml 0.1 M phosphate buffer (pH 5.9) separately in a household fruit blender. The different variety samples were respectively added to tea samples (1:1 v/v) and stirred using a magnetic stirrer for an hour. The samples were then placed in a water bath at 90°C for 10 min and subsequently centrifuged at 3000xg for 10 min. The theaflavin content of the supernatants was determined using the flavognost method (Hilton 1973) which is a rapid procedure producing good reproducible results (Steinhaus & Engelhardt 1989).

2.3.3.1.1. Flavognost method

Theaflavins in the samples were extracted in the organic solvent isobutyl methyl ketone (IBMK) by the addition of the sample to IBMK (1:1 v/v) and vortexing four times respectively for 30 seconds. The layers were allowed to separate, and the top (IBMK) layer was transferred to a new Eppendorf tube in which the flavognost reagent (1:2 v/v) was added and vortexed. The flavognost reagent consists of diphenyl boric acid-2-amino ethyl ester (0.04 M made up in 96% ethanol) that complexes with the theaflavins forming a green complex. The reaction forming the green theaflavin – flavognost reagent complex was allowed to develop for 12 $\pm$ 1 min at 37 $\pm$ 2°C after which the green complex was spectrophotometrically measured at 625 nm with an IBMK/EtOH (1:2 v/v) blank. The theaflavin content was calculated on a dry weight (DW) basis with the formula:

———— (Robertson & Hall 1989)

Where E_{625} is the absorbance at 625 nm, 47.9 is the micro molar absorbance coefficient of the theaflavin-flavognost reagent complex at 625 nm and DW the dry weight of the sample.

2.3.3.2. Dry weight determination of Forelle pear, Tea and Yacon

The dry weight of fresh Forelle pear (cored), Tea (*Camellia sinensis*, frozen fresh leaves) and Yacon (*Smallanthus sonchifolius*, leaves) were determined by freezing samples at -80 °C and subsequently freeze drying the samples until dry.

2.3.3.3. Polyphenol oxidase activity determination of Forelle pear, Tea and Yacon

2.3.3.3.1. Sample preparation for polyphenol oxidase activity assay

The extraction of polyphenol oxidase from the samples [Forelle pear (cored), Tea (frozen fresh leaves) and Yacon (frozen fresh leaves)] were performed as described by (Wesche-Ebeling & Montgomery 1990). The crude extract was prepared by 10 g of sample homogenized in a blender for 2 min using 100 ml of potassium phosphate buffer (0.1 M, pH 6.5) containing 5% polyethylene glycol (PEG) and ascorbic acid (10 mM). Polyethylene glycol a phenol-absorbing agent was used to prevent phenol-protein interactions, which inactivate or modify the enzyme based upon their phenolics binding ability (Wisseemann & Montgomery 1985, de Jesus Rivas & Whitaker 1973, Ziyan & Pekyardimci 2004). The homogenates were filtrated through muslin and subsequently assayed for polyphenol oxidase activity.

2.3.3.3.2. Polyphenol oxidase activity assay

The determination of enzyme activity was performed as described by Mydlarz & Palmer (2011). The activity assay was slightly modified to obtain a decrease in the absorbance values. Polyphenol oxidase activity was measured from 10 µl sample with the addition of 80 µl potassium phosphate buffer (50 mM, pH 7.5) and 40 µl trypsin (0.1 mg/ml) that were allowed to incubate for 10 min. The reaction was started with the addition of 70 µl 4-methylcatechol (100 mM stock, 35 mM final). The development of the coloured oxidative product was followed at 410 nm for 15 min during which time all the reactions were linear. The data is presented as the change in absorbance (final – initial) per min (ΔAbs_{410}). One unit of enzyme activity corresponds to the amount of enzyme which increases the absorbance measured at 410 nm by 0.001 per min at pH 7.5 and 25°C with 4-methylcatechol substrate in a 200 µl reaction mixture.

2.3.3.4. Measurement of theaflavins produced by extracts of Forelle pear, Tea and Yacon

Tea (frozen fresh leaves), Yacon (frozen fresh leaves) and Forelle pear (kept at 4°C and cored) 2 g of each was respectively homogenized in a blender in 100 ml potassium phosphate buffer (0.1 M,

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pH 5.9) for 2 min with ground Lipton® green tea (2 g). The samples in 100 ml potassium phosphate buffer (0.1 M, pH 5.9) were also respectively homogenized for 2 min with fresh Tea (4 g frozen fresh leaves). The samples were stirred for an hour at room temperature using a magnetic stirrer. Samples were then filtered through muslin, placed at 90 °C for 15 min and subsequently centrifuged at 3,000xg for 10 min. Control samples of Tea, Yacon and Forelle pear (2 g) were prepared in the same manner but were placed at 90 °C for 15 min directly after being homogenized. The theaflavin content of the supernatants was determined using the flavognost method (as described in 2.3.3.1.1).

2.3.3.5. Thin layer chromatography separation of catechins and theaflavins

The separation of catechins and theaflavins from Tea (frozen fresh leaves) Yacon (frozen fresh leaves) and Forelle pear (kept at 4°C and cored) samples were performed as described by Wang *et al.* (2009).

2.3.3.5.1. Plant extracts preparation

Tea (frozen fresh leaves), Yacon and Forelle pear extracts were prepared by extracting 10 g of plant material with 150 ml boiling water. The samples were filtered and 100 ml of the water extract was further extracted with ethyl acetate (2 x 100 ml) in a separating funnel. After collection of the ethyl acetate phase, it was filtered through anhydrous sodium sulphate and evaporated to dryness.

2.3.3.5.2. Thin layer chromatography

Thin layer chromatography was performed on Nylon plates pre-coated with polyamide (initially 10 cm x 5 cm and finally 20 cm x 5 cm). Solution of standards and samples were applied initially as spots and finally as 8 mm bands, 10 mm from the bottom of the plates and initially 13 mm from the sides and 12 mm in between and finally as 9 mm from the sides.

Standard solutions of CM (catechin mixture) containing all five catechins [catechin (C), epicatechin ((-)EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG)] each 0.2 mg/ml in methanol, TM (theaflavin mixture) containing all four theaflavins [theaflavin (TF), theaflavin-3-gallate (T3G), theaflavin-3'-gallate (T3'G) and theaflavin-3,3'-digallate(TdG)] each 0.25 mg/ml in methanol and M (mixture) containing all of the five catechin standards and four theaflavin standards, 0.1 mg/ml of each in methanol, were prepared. The samples; Tea (frozen fresh leaves) (25 mg/ml FW in methanol), Yacon (24 mg/ml

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FW in methanol) and Forelle pear (2.5 mg/ml FW in methanol) were prepared and 5 µl of each was applied.

The mobile phase contained chloroform-methanol 2:3 (v/v) in a developing chamber saturated by preconditioning for 15 min. The plates were initially developed over a distance of 8 cm and finally 18 cm. The developed plates were dried under a stream of warm air for 2 min and then evenly sprayed (without soaking the plate) with an iron (III) chloride solution, 5% (m/v) in ethanol. The drying of the plates in a stream of warm air for 2 min furnished coloured bands for the separated compounds.

2.3.3.6. Protein concentration determination

The protein concentrations were determined according to the dye binding method (Bradford 1976) with bovine serum albumin (Fraktion V) as standard. A bovine serum albumin standard curve was constructed by diluting one part protein assay dye reagent concentrate (solution containing dye, phosphoric acid and methanol) with four parts double distilled water. The prepared dye reagent was filtered through a Whatman #1 filter. Bovine serum albumin dilution within the linear range of 0.05 mg/ml to approximately 0.5 mg/ml was prepared. The protein dilution (10 µl) and the diluted dye reagent (200 µl) were aliquoted into the wells of a 96 well microtiter plate, mixed thoroughly and incubated at room temperature for at least 5 min and no more than one hour. The absorbance was measured at 595 nm and a bovine serum albumin standard curve constructed.

2.3.3.7. Purification of polyphenol oxidase with gel filtration

2.3.3.7.1. Enzyme extraction from Forelle pears

Polyphenol oxidase was extracted and purified according to a modified method of Ziyan & Pekyardimci (2004). Partially purified enzyme was prepared from 315g fresh pear tissue obtained from 6 pears being halved, cored and cut into slices. The pear tissue was homogenized for 3 min with 300 ml potassium phosphate buffer (0.2 M, pH 6.8) containing 0.05 M ascorbic acid and 0.5% polyvinyl poly-pyrrolidone (PVP). The homogenate was filtered through a cheese cloth and subsequently centrifuged at 30 000xg for 30 min. The enzyme solution was fractionated with solid ammonium sulphate at 30-80% saturation, and the precipitate collected through centrifugation at 15 000xg for 20 min. The pellet was re-dissolved in and dialysed against

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phosphate buffer (0.2 M, pH 6.8) at 4°C in a SnakeSkin pleated dialysis tubing (10,000 molecular weight cut off). The dialysed solution was ultra filtrated.

2.3.3.7.2. Gel filtration

A sample was initially loaded onto a Superdex 200 10/300 GL column (1 x 30 cm, 8 ml V_O and 24 ml V_T) from GE Healthcare Ltd, Gloucestershire, UK which is prescribed for the high-resolution separations of proteins, peptides and other biomolecules according to size. The flow rate was adjusted to 2 ml/min and 10 ml fractions were collected. The fractions with enzyme activity were pooled. The elution process continued until no absorbance was observed at 280 nm. Both the enzyme activity and protein concentration of the fraction were determined. The rest of the sample (0.5 ml) was subsequently loaded onto a HiPrep 26/60 Sephacryl S-200 HR column (2.6 x 60 cm, 100 ml V_O and 320 ml V_T) from GE Healthcare Ltd, Gloucestershire, UK which is prescribed for the applications of purifying antibodies, serum proteins and mid-sized proteins. The column was equilibrated with phosphate buffer (0.1 M, pH 6.8) containing 10% glycerin. The column flow rate was 0.4 ml/min and 1 ml fractions were collected.

2.3.3.7.3. Detection of polyphenol oxidase isozymes

The procedure followed for the detection of polyphenol oxidase in electrophoresis gel slabs was that of Wissemann & Montgomery (1985). Native PAGE was performed using a stacking gel (Tris-HCl 0.5 M, pH 6.8) and a 7% separating gel (Tris-HCl 1.5 M, pH 8.8) which were prepared from an acrylamide stock (30% acrylamide, 0.8% N', N'-methylene bisacrylamide) and were polymerized with the addition of 20% ammonium persulphate (respectively 25 μ l and 75 μ l) and tetramethylethylenediamine (TEMED) (respectively 5 μ l and 7.5 μ l). The samples were prepared in the sample buffer [Tris-HCl (0.125 M, pH 6.8), glycerol (20% v/v), bromophenol blue (0.2% w/v) and mercaptoethanol (25 mM)] in a 1:1 ratio. Electrophoresis was carried out in an electrophoresis system with running buffer [Tris (0.25 M) and glycine (2 M)] at 100 V. The native PAGE gel was developed in 15 mM catechol in 0.1 M citrate-0.2 M sodium phosphate buffer (pH 5.0) containing 0.05% *p*-phenylenediamine for an hour. The slabs were rinsed for 5 min with 1 mM ascorbic acid, soaked in water overnight.

2.3.3.8. Purification of polyphenol oxidase with affinity chromatography

2.3.3.8.1. Affinity column preparation

2.3.3.8.1.1. Activation of hydroxyl groups of Sepharose-4B by cyanogen bromide and coupling of L-tyrosine

The Sepharose-4B gel (10 ml) was washed with distilled water and filtered on a Buchner funnel. Distilled water (10 ml) was added to the gel and subsequently cyanogen bromide (4 g) was added to the mixture with the pH being kept at 11 with sodium hydroxide. The mixture was washed with sodium bicarbonate buffer (250 ml, 0.1 M and pH 10.0). L-tyrosine (20 ml, 4 mM made up in the sodium bicarbonate buffer) was added to the cyanogen bromide activated gel and stirred for 90 min and left for 16 h at 4°C. Excess L-tyrosine was removed from the Sepharose-4B-L-tyrosine gel by washing with distilled water (until no absorbance was present at 280 nm). The washing was completed with sodium bicarbonate buffer (100 ml, 0.2 M and pH 8.80) and the gel was made up to 40 ml in the buffer (Arslan *et al.* 2004).

2.3.3.8.1.2. Diazotization *p*-amino benzoic acid and coupling of the compound to the Sepharose-4B-L-tyrosine

Cold sodium nitrite (0.07 M) was added drop-wise to *p*-aminobenzoic acid (0.02 M made up in hydrogen chloride 0.1 M) and the reaction was completed by stirring for 10 min. This solution was added to the 40 ml Sepharose-4B L-tyrosine suspension at pH 9.5 and stirred for 3 h at room temperature. The gel was washed with distilled water (1 litre) and subsequently with disodium hydrogen orthophosphate buffer (200 ml and pH 6.0) in which it was kept. The gel was packaged into a column (1 x 10 cm) and pre-equilibrated with phosphate buffer (5 mM and pH 5.0) (Arslan 2004).

2.3.3.8.2. Extraction and purification of polyphenol oxidase

The extraction and purification of polyphenol oxidase from Forelle pear was performed as described by Yerlitürk *et al.* (2008). A crude extract was prepared from frozen pear tissue (10 g) by homogenization in a household blender for 2 min with 100 ml sodium phosphate buffer (0.1 M, pH 6.5) containing 5% poly(ethylene glycol). The homogenate was filtered (45 micron) and purified with affinity chromatography. The Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column was synthesized as described above. The affinity column (1.5 x 12 cm) was equilibrated with sodium phosphate buffer (5 mM, pH 5) and the enzyme solution applied. The affinity column was washed with the same buffer until the absorbance at 280 nm was constant. Polyphenol oxidase was eluted with sodium phosphate buffer (5 mM, pH 8.5) containing 1 M

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NaCl and 1 ml fractions were collected. The polyphenol oxidase enzyme activity (2.3.3.3.2) and protein concentration (2.3.3.3.6) of the different fractions were determined.

2.3.3.8.3. Silver stained SDS-PAGE and detection of isozymes

The detection of polyphenol oxidase isozymes were performed as described above in 2.3.3.7.3. SDS-PAGE was performed using a stacking gel [Tris-HCl (0.5 M, pH 6.8) and SDS (20% w/v)] and a 12% separating gel [Tris-HCl (1.5 M, pH 8.8) and SDS (20% w/v)] which were prepared from an acrylamide stock (30% acrylamide, 0.8% N', N'-methylene bisacrylamide) and were polymerized with the addition of 20% ammonium persulphate (respectively 25 µl and 75 µl) and tetramethylethylenediamine (TEMED) (respectively 5 µl and 7.5 µl). The samples were prepared in reducing sample buffer [Tris-HCl (0.125 M, pH 6.8), glycerol (20% v/v), SDS (4% w/v), bromophenol blue (0.2% w/v) and mercaptoethanol (25 mM)] in a 1:1 ratio and boiled at 95°C for 5 min. An unstained protein molecular weight marker from Fermentas, Burlington, Canada was used. Electrophoresis was carried out in an electrophoresis system with running buffer [Tris (0.25 M), SDS (1% w/v) and glycine (2 M)] at 100 V. Following electrophoresis the gel was silver stained using the ProteoSilverTM Silver stain kit that provides for exceptionally sensitive protein detection from Sigma Aldrich (Pty) Ltd, St. Louis, USA. Direct silver staining was performed at room temperature on an orbital shaker at 60 rpm. After electrophoresis, the gel was stained in fixing solution [ethanol (50% v/v) and acetic acid (10% v/v)] for 20 min and respectively washed in ethanol (30% v/v) and water for 10 min. The gel was incubation for 10 min in sensitizer solution (constituents not disclosed), washed twice in water for 10 min and equilibrated for 10 min in silver solution (constituents not disclosed). Washing in water was performed for 1 min not to decrease the sensitivity, followed by gel development in developer solution (constituents not disclosed) and when the desired staining intensity was reached (5 to 7 min) the developing reaction was stopped by 5 min incubation in stop solution (constituents not disclosed). In principle, the kit utilizes silver nitrate to bind to selective amino acids on the protein under weakly acidic or neutral pH conditions. Formaldehyde was used to reduce the protein bound silver ions at alkaline pH to form metallic silver in the gel.

2.3.3.9. Effect of pH on the activity of polyphenol oxidase

The pH optimum of polyphenol oxidase was determined over the pH range of 3 to 10 utilizing three different buffer systems as follows; citrate buffer (0.5 M, pH 3, 4, 5 and 5.5), potassium phosphate buffer (0.5 M, pH 6, 6.5, 7 and 7.5) and Tris-HCl buffer (0.5 M, pH 8, 9 and 10). The activity was determined over the pH range as described above.

The effect of pH on the stability of polyphenol oxidase was determined as adapted from Zhou *et al.* (1993). Enzyme reaction solutions of 45 µl enzyme in 810 µl of the various buffer solutions ranging from pH 3-10 was incubated for 30 min at 30°C. After the lapse of 30 min, the solutions were placed on ice, and the residual enzyme activity was determined as described above.

2.3.3.10. Auto-oxidation of 4-methylcatechol

2.3.3.10.1. Effect of pH on 4-methylcatechol

The change in absorbance of 4-methylcatechol at 410 nm was monitored in a pH 4 citrate buffer (0.5 M), pH 6.5 potassium phosphate buffer (0.5 M) and pH 9 Tris-HCl buffer (0.5 M). The reaction comprised of 10 µl 4-methylcatechol (0.07 M, made up in ddH₂O) and started with the addition of 190 µl of the respective buffers.

2.3.3.10.2. Absorption spectrum of 4-methylcatechol

The absorption spectrum of 4-methylcatechol (0.07 M) made up in a citrate buffer (0.5 M, pH 4), potassium phosphate buffer (0.5 M, pH 6.5) and Tris-HCl buffer (0.5 M, pH 9) was determined over the range of 200 nm to 400 nm.

2.3.3.11. Effect of temperature on the activity of polyphenol oxidase

The temperature optimum of polyphenol oxidase was determined over the temperature range of 25°C to 70°C in a citrate buffer (0.5 M, pH 4). The buffer solution was incubated at the various temperatures and allowed to equilibrate after which the polyphenol oxidase activity was determined as described above (2.3.3.3.2).

The effect of temperature on the stability of polyphenol oxidase was determined as adapted from Zhou *et al.* (1993). Enzyme reaction solutions of 45 µl enzyme in 810 µl citrate buffer (0.5 M, pH 4) was incubated for 30 min at the various temperatures. After the lapse of 30 min, the solutions were placed on ice, and the residual enzyme activity was determined as described above (2.3.3.3.2).

2.3.3.12. Determination of K_M and V_{max} of polyphenol oxidase

The kinetic parameters K_M (Michaelis Menten constant) and V_{max} (maximum reaction velocity) of pear polyphenol oxidase were determined using the substrate 4-methylcatechol at five substrate concentrations (5 mM, 2.5 mM, 1.7 mM, 1.25 mM and 1 mM), determined at the optimum pH value of 5 and temperature of 30°C. Polyphenol oxidase activity assay was performed as described in 2.3.3.3.2. Data was plotted at $1/V$ and $1/[S]$ concentration as according to the method of Lineweaver and Burk (1934) and plotted at $[S]$ concentration/ V and $[S]$ concentration in a Hanes-Woolf plot (Wilkinson 1961).

2.4. RESULTS

2.4.1. Measurement of the theaflavins produced by extracts of different pear varieties

The theaflavin synthesis capabilities of the different pear varieties Forelle, Packman, Abet Beteil and Beurré Bosc were assessed based upon the flavognost method (Table 2.1).

Table 2.1: Determination of the theaflavin synthesis capabilities of different pear varieties from the catechin content of Lipton® Green tea based upon the Flavognost method after a 1 h incubation period.

Pear Variety	Enzyme activity per gram FW pear (μ mole TF/ h)	
Forelle	Mean (n = 3)	0.147
	SD	0.028
	CV	5.284
Packham	Mean (n = 3)	0.085
	SD	0.010
	CV	8.116
Abet Beteil	Mean (n = 3)	0.094
	SD	0.009
	CV	10.320
Beurré Bosc	Mean (n = 3)	0.072
	SD	0.021
	CV	3.417
μ mole TF/ h = micromole theaflavin produced per hour n = number of biological independent experiments		

The enzyme activity of the Forelle pear variety had the highest per gram fresh weight synthesis capability and produced 0.147 μ mole theaflavin per hour. Forelle pears (*Pyrus communis* L.) were therefore further investigated for the theaflavin synthesis enzyme activity.

2.4.2. Dry weight determination of Forelle pear, Tea and Yacon

The dry weight of fresh Forelle pear (kept at 4°C and cored), Tea (frozen fresh leaves) and Yacon (frozen fresh leaves) were determined (Table 2.2). The ratio of gram dry weight per gram fresh weight for Tea (frozen fresh leaves), Forelle pear and Yacon was determined as 0.498, 0.169 and 0.208 respectively.

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Table 2.2: Comparison of the fresh weight and dry weight respectively of Tea (frozen fresh leaves), Forelle pear and Yacon.

	Tea (frozen fresh leaves)			Yacon			Forelle pear		
	FW	DW	g DW/g FW	FW	DW	g DW/g FW	FW	DW	gDW/gFW
Bio 1	8.657	4.500	0.520	10.887	1.923	0.177	11.699	2.442	0.209
Bio 2	8.274	4.020	0.486	10.080	1.648	0.164	10.938	2.308	0.211
Bio 3	8.239	4.025	0.489	10.030	1.669	0.166	11.795	2.414	0.205
Mean (n = 3)			0.498			0.169			0.208
SD			0.019			0.007			0.003
CV			3.788			4.073			1.541
FW = gram fresh weight				Mean = change in absorbance per 15 min					
DW = gram dry weight				n = number of biological independent experiments					
gDW/gFW = gram dry weight per gram fresh weight				SD = Standard deviation					
Bio = Biological independent experiment				CV = Coefficient of variation					

2.4.3. Polyphenol oxidase activity determination of Forelle pear, Tea and Yacon

The polyphenol oxidase activity was determined using 4-methylcatechol as a substrate by measuring the change in absorbance over the linear region of the rate of the reaction. Under the experimental conditions, the linear region was determined as the initial 15 min of the reaction ($R^2 = 0.994$) (Figure 2.6). The polyphenol oxidase enzyme activity was subsequently determined over the initial 15 min.

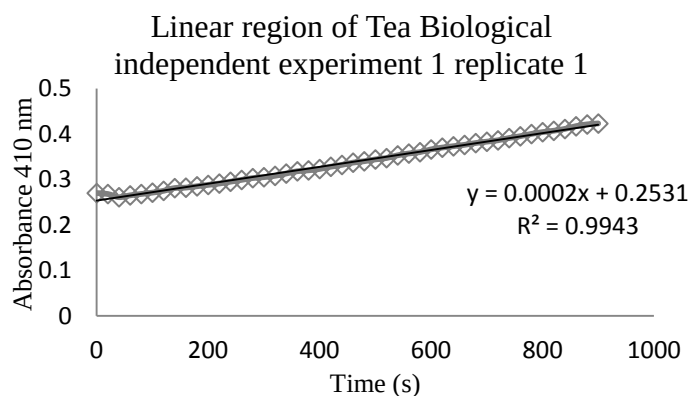


Figure 2.6: Initial 15 min linear region indicated for Tea (frozen fresh leaves) biological independent experiment 1 replicate 1.

The polyphenol oxidase activity of Tea (frozen fresh leaves), Forelle pear (kept at 4°C and cored), and Yacon (frozen fresh leaves) was assessed by the extraction of polyphenol oxidase from the samples by homogenization in potassium phosphate buffer containing polyethylene glycol (PEG) and ascorbic acid.

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Table 2.3: Polyphenol oxidase activity of Tea (frozen fresh leaves), Forelle pear and Yacon.

		Bio 1	Bio 2	Bio 3	Overall	$\Delta \text{Abs}_{410}/\text{gFW}/\text{min}$	$\Delta \text{Abs}_{410}/\text{gDW}/\text{min}$
Tea (frozen fresh leaves)	Mean (n = 3)	0.193	0.196	0.218	0.202	0.007	0.014
	SD	0.016	0.016	0.016	0.014		
	CV	8.375	8.263	7.209	6.746		
Yacon	Mean (n = 3)	0.096	0.088	0.104	0.096	0.003	0.019
	SD	0.008	0.010	0.007	0.008		
	CV	8.640	11.78	6.400	8.333		
Forelle pear	Mean (n = 3)	0.058	0.061	0.067	0.062	0.002	0.010
	SD	0.003	0.002	0.003	0.005		
	CV	5.270	2.490	4.470	7.391		
Bio = Biological independent experiment					$\Delta \text{Abs}_{410}/\text{gFW}/\text{min}$ = change in absorbance per g fresh weight sample per min		
Mean = change in absorbance per 15 min							
SD = Standard deviation					$\Delta \text{Abs}_{410}/\text{gDW}/\text{min}$ = change in absorbance per g dry weight sample per min		
CV = Coefficient of variation							

From the comparative assessment of the polyphenol oxidase activity of the different samples (Table 2.3) Tea (frozen fresh leaves) (0.007) has the highest polyphenol oxidase activity per gram fresh weight sample per min, secondly Yacon (0.003) and lastly Forelle pears (0.002). However, based upon the change in absorbance per gram dry weight sample per min Yacon (0.019) has the highest polyphenol oxidase activity, secondly Tea (frozen fresh leaves) (0.014) and lastly Forelle pears (0.010).

2.4.4. Measurement of theaflavins produced by extracts of Forelle pear, Tea and Yacon

The theaflavin synthesis capabilities of Tea (frozen fresh leaves), Yacon and Forelle pear were assessed with both Lipton® Green tea and Tea (frozen fresh leaves) over an hour of fermentation based upon the flavognost method (Table 2.4). Based upon the determined ratio of 0.498 (± 0.019) gDW/gFW of Tea (frozen fresh leaves) (Table 2.2), 1% w/v Lipton® Green tea and 2% w/v Tea (frozen fresh leaves) was used to enable more accurate between sample comparisons.

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Table 2.4: Theaflavin content of Tea (frozen fresh leaves), Yacon and Forelle pear after an hour fermentation with Lipton® Green tea and Tea (frozen fresh leaves) respectively.

Controls:													
	Lipton® Green tea (1% w/v):				Tea (frozen fresh leaves, 2% w/v)				Yacon (2% w/v)				
	Bio 1	Bio 2	Bio 3	Overall	Bio 1	Bio 2	Bio 3	Overall	Bio 1	Bio 2	Bio 3	Overall	
	Mean	0.006	0.007	0.008	0.007	0.046	0.056	0.051	0.051	0	0	0	0
	SD	0.002	0.002	0.003	0.001	0.001	0.003	0.002	0.005	0	0	0	0
	CV	30.29	34.99	40.66	14.29	2.721	5.976	3.365	9.804	-	-	-	-
	µmole TF/g FW	Na.				24.43				0			
	µmole TF/g DW	6.706				49.05				0			
	Forelle pear (2% w/v)				Lipton® Yellow label black tea (1% w/v):								
	Bio 1	Bio 2	Bio 3	Overall	Bio 1	Bio 2	Bio 3	Overall					
	Mean	0	0	0	0	0.040	0.047	0.062	0.050				
	SD	0	0	0	0	0.001	0.003	0.001	0.011				
	CV	-	-	-	-	5.292	3.797	1.020	22.75				
	µmole TF/gFW/h	0				Na.							
	µmole TF/gDW/h	0				47.90							
Substrate: Lipton® Green tea (1% w/v):													
	Enzyme source: Tea (frozen fresh leaves, 2% w/v)				Enzyme source: Yacon (2% w/v)				Enzyme source: Forelle pear (2% w/v)				
	Bio 1	Bio 2	Bio 3	Overall	Bio 1	Bio 2	Bio 3	Overall	Bio 1	Bio 2	Bio 3	Overall	
	Mean	0.024	0.029	0.029	0.027	0.021	0.026	0.022	0.023	0.017	0.018	0.021	0.019
	SD	0.002	0.003	0.001	0.003	0.002	0.001	0.001	0.003	0.001	0.01	0.001	0.002
	CV	7.37	10.129	2.026	10.561	7.37	5.063	6.428	11.503	8.449	5.394	5.921	11.152
	µmole TF/gFW/h	12.93				11.02				9.101			
	µmole TF/gDW/h	25.97				65.19				43.75			
Substrate: Tea (frozen fresh leaves, 2% w/v)													
	Enzyme source: Tea (frozen fresh leaves, 2% w/v)				Enzyme source: Yacon (2% w/v)				Enzyme source: Forelle pear (2% w/v)				
	Bio 1	Bio 2	Bio 3	Overall	Bio 1	Bio 2	Bio 3	Overall	Bio 1	Bio 2	Bio 3	Overall	
	Mean	0.08	0.081	0.082	0.081	0.061	0.072	0.058	0.064	0.082	0.086	0.073	0.080
	SD	0.003	0.005	0.002	0.001	0.001	0.003	0.001	0.007	0.002	0.001	0.002	0.007
	CV	4.268	6.014	2.696	1.235	0.823	3.52	1.658	11.578	2.634	1.117	3.283	8.288
	µmole TF/gFW/h	38.80				30.66				38.32			
	µmole TF/gDW/h	77.91				181.4				184.2			
Bio = Biological independent experiment				µmole TF/g FW = µmole theaflavin produced per gram fresh weight at time point zero									
Mean = change in absorbance per 15 min				µmole TF/g DW = µmole theaflavin produced per gram dry weight at time point zero									
SD = Standard deviation				µmole TF/gFW/h = µmole theaflavin produced per gram fresh weight per hour									
CV = Coefficient of variation				µmole TF/gDW/h = µmole theaflavin produced per gram dry weight per hour									

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The micromole theaflavin per gram dry weight content of Lipton® Green tea (6.706 $\mu\text{mole TF/g DW}$) (Table 2.4) was low and that of Lipton® Yellow label black tea (black tea) (47.90 $\mu\text{mole TF/g DW}$) high respectively corresponding to the absence and presence of fermentation during the manufacturing process. The Tea (frozen fresh leaves) micromole theaflavin per gram fresh weight content was unexpectedly high (49.05 $\mu\text{mole TF/g DW}$) based upon the absence of fermentation by directly after homogenization performing a heat inactivation step. No theaflavin content was observed in both the Yacon and Forelle pear control samples. Utilizing Lipton® Green tea as substrate, Tea (frozen fresh leaves) (12.93 $\mu\text{mole TF/gFW/h}$) produced the highest micromole theaflavin content after an hour fermentation per gram fresh weight sample, secondly Yacon (11.02 $\mu\text{mole TF/gFW/h}$) and lastly Forelle pear (9.101 $\mu\text{mole TF/gFW/h}$). Yacon (65.19 $\mu\text{mole TF/gDW/h}$) produced the highest micromole theaflavin per gram dry weight per hour although Forelle pear (43.75 $\mu\text{mole TF/gDW/h}$) produced the second highest and lastly Tea (frozen fresh leaves) (25.97 $\mu\text{mole TF/gDW/h}$). The micromole theaflavin per gram dry weight per hour produced by Yacon (65.19 $\mu\text{mole TF/gDW/h}$) from Lipton® Green tea was higher than that determined in Lipton® Yellow label black tea (black tea) (47.90 $\mu\text{mole TF/g DW}$). Utilizing Tea (frozen fresh leaves) as the substrate in contrast to using Lipton® Green tea as substrate, Tea (frozen fresh leaves) (38.80 $\mu\text{mole TF/gFW/h}$) produced the highest micromolar theaflavin per gram fresh weight. The second highest amount of theaflavin per gram fresh weight was Forelle pear (38.32 $\mu\text{mole TF/gFW/h}$) and lastly Yacon (30.66 $\mu\text{mole TF/gFW/h}$). In terms of micromole theaflavin produced per gram dry weight sample per hour Forelle pear (184.2 $\mu\text{mole TF/gDW/h}$) and Yacon (181.4 $\mu\text{mole TF/gDW/h}$) produced similar amounts and Tea (frozen fresh leaves) (77.91 $\mu\text{mole TF/gDW/h}$) the lowest amount.

With reference to the control samples of Lipton® Green tea (6.706 $\mu\text{mole TF/gDW/h}$) and Tea (frozen fresh leaves) (49.05 $\mu\text{mole TF/gDW/h}$) the Lipton® Green tea as the substrate with the Tea (frozen fresh leaves) sample (25.97 $\mu\text{mole TF/gDW/h}$) produced unexpectedly low amounts of theaflavins [$\geq 56 \mu\text{mole TF/gDW/h}$ (6.706 + 49.05)]. Similarly low amounts of theaflavins were present in the Tea (frozen fresh leaves) substrate with the Tea (frozen fresh leaves) sample [77.91 $\mu\text{mole TF/gDW/h}$ in comparison to the expected $\geq 98 \mu\text{mole TF/gDW/h}$ (49.05 + 49.05)]

2.4.5. Thin layer chromatography separation of catechins and theaflavins

Simultaneous analysis of the catechins and theaflavins in Tea (frozen fresh leaves), Yacon and Forelle pear were performed. From the flavognost (Table 2.4) of the theaflavin content in the control samples, it is expected that theaflavins will only be observed in Tea (frozen fresh leaves).

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The catechin standards (Figure 2.7) were clearly separated on the polyamide TLC plates (10 cm x 5 cm) with chloroform-methanol 2:3 (v/v) and the R_f values determined (Table 2.5). The migrations of the theaflavin standards (Figure 2.7) on polyamide TLC plates (10 cm x 5 cm) were very slow and unclear. Subsequently, separations on larger polyamide TLC plates (20 cm x 5 cm) were performed.

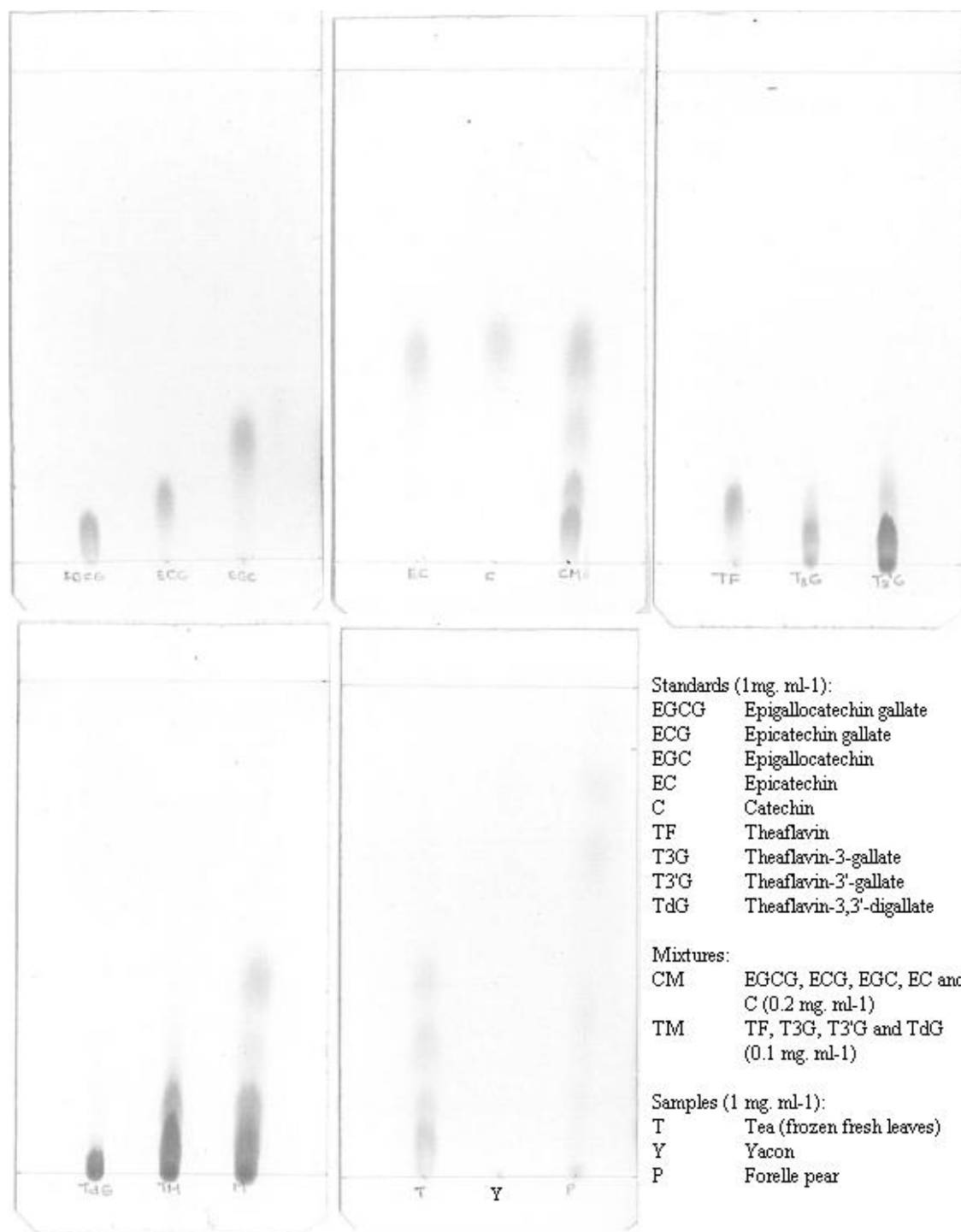


Figure 2.7: (A) Separation of standards, mixtures and samples on polyamide TLC plates (10 cm x 5 cm) with chloroform-methanol 2:3 (v/v) in a developing chamber.

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From the samples Tea (frozen fresh leaves), Yacon and Forelle pear (1 mg/ml) separation on polyamide TLC plates (10 cm x 5 cm) only light spots were observed (Figure 2.7). From the subsequent separation of Tea (frozen fresh leaves), Yacon and Forelle pear (25-, 24- and 2.5 mg/ml FW respectively) on polyamide TLC plates (20 cm x 5 cm), resulted in darker bands (Figure 2.8) and R_f values were determined (Table 2.5).

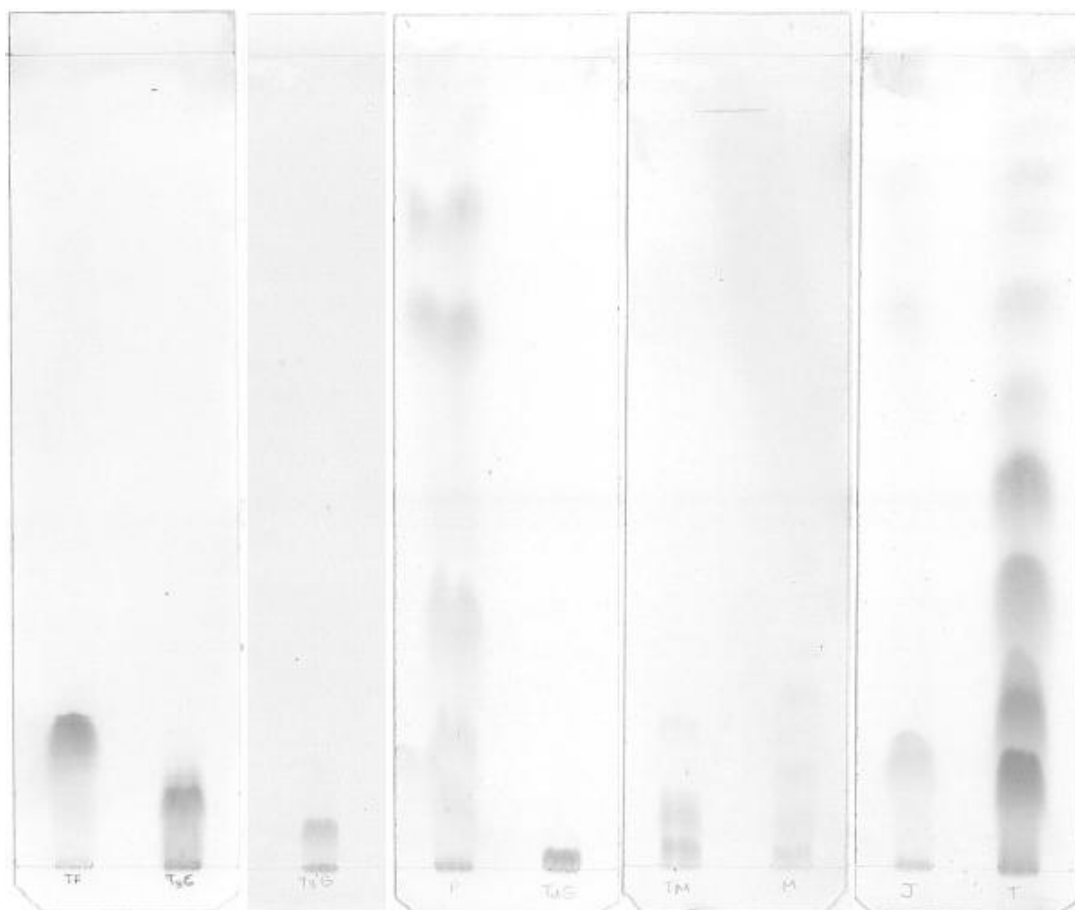


Figure 2.8: (B) Separation of theaflavin standards (1 mg/ml), T (Tea frozen fresh leaves), Y (Yacon) and P (Forelle pear) stock solution of respectively 25 mg/ml FW, 24 mg/ml FW and 2.5 mg/ml FW respectively on polyamide TLC plates (20 cm x 5 cm) with chloroform-methanol 2:3 (v/v) in a vertical developing chamber.

Based upon the R_f values (Table 2.5) the possible presence of all the catechin standards (epigallocatechin gallate, epicatechin gallate, epigallocatechin, epicatechin and catechin) and theaflavin can be identified in the Tea (frozen fresh leaves) (1 mg/ml) on polyamide TLC plates (10 cm x 5 cm). This was also observed in the separation of Tea (frozen fresh leaves) (25 mg/ml) on a polyamide TLC plate (20 cm x 5 cm), except for the possible presence of epigallocatechin gallate and epicatechin gallate. Six spots with unknown R_f values were observed in the Tea (frozen fresh leaves) (25 mg/ml FW). In the Yacon (24 mg/ml FW) and Pear (2.5 mg/ml FW) samples separated on a polyamide TLC plate (20 cm x 5 cm) the possible presence of epicatechin gallate was identified. In both samples, four spots with unknown R_f values were observed. In the

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Pear (1 mg/ml) separated on a polyamide TLC plate (10 cm x 5 cm) the only the possible presence of epicatechin gallate and three unknown spot were observed, which can be explained by the lower sample concentration. The absence of theaflavins in the Yacon and Forelle pear samples were expected from the theaflavin content determined (Table 2.4) based upon the flavonost method. The presence or absence of the catechin and theaflavin standards will be confirmed based upon UPLC-MS data.

Table 2.5: R_f values of the catechin standards, theaflavin standards, Tea (frozen fresh leaves), Yacon and Forelle pear samples.

Polyamide TLC plates (10 cm x 5 cm)						Polyamide TLC plates (20 cm x 5 cm)			
Standards	CM	TM	M	T	P	Standards	T	Y	P
EGCG 0.075	0.088		0.100	0.088					
ECG 0.138	0.163		0.138	0.150	0.175			0.145	0.162
EGC 0.25	0.288			0.275			0.266		
(-)EC 0.413	0.419		0.388	0.400			0.595		
C 0.438				0.400			0.595		
TF 0.125		0.088	0.100	0.088		TF 0.178	0.127		
T3G 0.063		0.163	0.063			T3G 0.061			
T3'G 0.063		0.288	0.063			T3'G 0.051			
TdG 0.025		0.419	0.038			TdG 0.047			
Unknown						Unknown			
P 0.313, 0.676, 0.676, 0.815						T 0.208, 0.364, 0.486, 0.705, 0.798, 0.861			
CM = Catechin mixture						Y 0.688, 0.688, 0.786, 0.850			
TM = Theaflavin mixture						P 0.329, 0.676, 0.676, 0.815			
M = Mixture of all the catechin and theaflavin standards									

2.4.6. Purification of polyphenol oxidase with gel filtration

Polyphenol oxidase crude extract was prepared and subsequently fractionated with solid ammonium sulphate. Ultra filtration was performed to reduce the sample volume before gel filtration. A small sample was initially separated on Superdex 200 10/300 GL column (1 x 30 cm, 8 ml V_O and 24 ml V_T) from which only a single peak (fraction 9-20) in protein concentration was determined in the elution profile (Figure 2.9).

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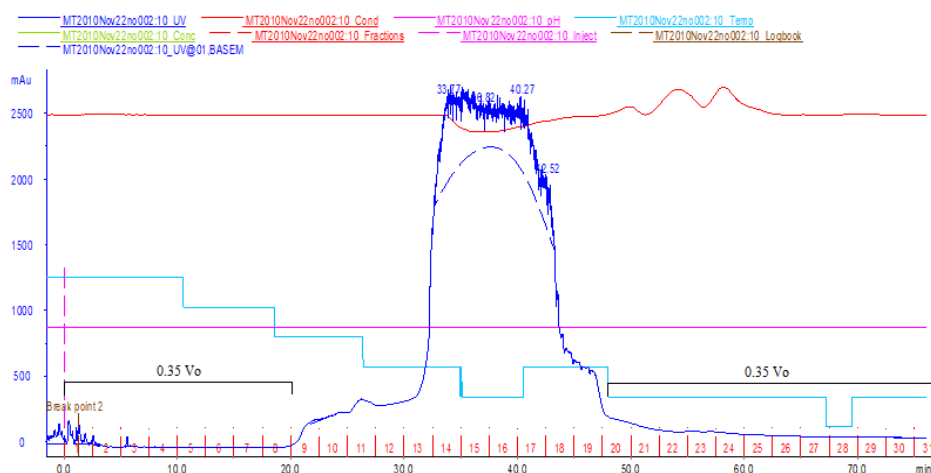


Figure 2.9: Superdex 200 10/300 GL chromatogram (1 x 30 cm, 8 ml V_O and 24 ml V_T) chromatogram at a 0.4 ml/min flow rate with 1 ml fractions taken. A single large peak (dark blue) of protein was determined by UV absorption at 280 nm. These fractions were pooled and were the only fractions with polyphenol oxidase activity. In the curve plane display the conductivity (red), UV absorption (dark blue), pH (pink) and temperature (light blue) was indicated.

Gel filtration was subsequently performed on a HiPrep Sephacryl S-200 HR column (2.6 x 60 cm, 100 ml V_O and 320 ml V_T) in which similarly, a single peak was observed (Figure 2.10).

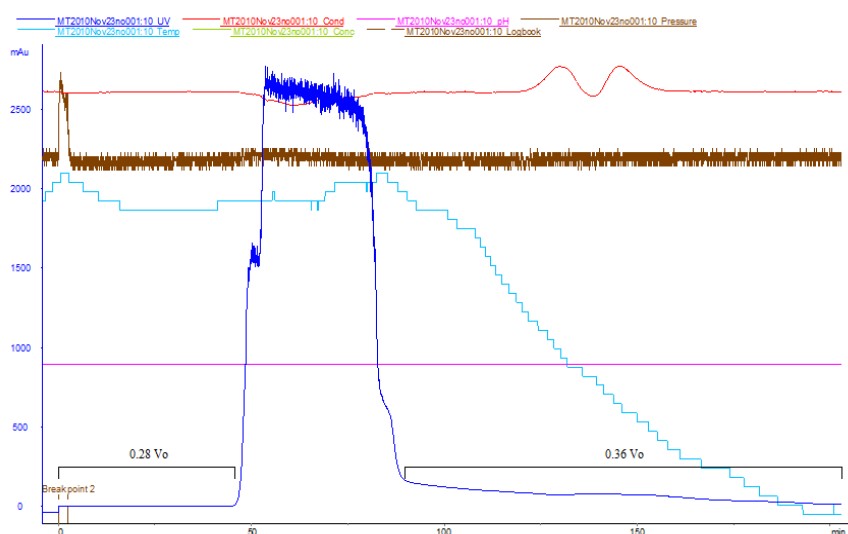


Figure 2.10: Sephacryl S-200 HR (2.6 x 60 cm, 100 ml V_O and 320 ml V_T) chromatogram at a 2 ml/min flow rate with 10 ml fractions taken. A single large peak (dark blue) of protein was determined by UV absorption at 280 nm. These fractions were pooled and were the only fractions with polyphenol oxidase activity. In the curve plane display the conductivity (red), pressure (brown), UV absorption (dark blue), pH (pink) and temperature (light blue) was indicated.

From the purification table, it can be seen that most of the enzyme specific activity (units/mg) was lost during the ammonium sulphate precipitation step and an increase in specific activity was subsequently observed during the gel filtration step (Table 2.6). The total amount of protein (mg) decreased throughout the purification steps as expected. A 0.2 fold purification of polyphenol oxidase was achieved.

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Table 2.6: Purification of polyphenol oxidase.

Purification steps	Volume (ml)	Conc. (mg/ml)	Total amount (mg)	Conc. (units/ml)	Total amount (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	745	0.512	381.4	3061	2.281E +6	5981	100	1
30-80% ammonium sulphate	27	6.369	172.0	4733	1.278E +5	743.2	560.3	0.124
Ultra filtration	7	25.54	178.8	15033	1.052E+5	588.7	461.2	0.098
Gel filtration Superdex 200 10/300GL	107	0.684	73.21	977.8	1.332E+5	1430	584.0	0.239

2.4.6.1. Detection of polyphenol oxidase isozymes

From the native PAGE stained for polyphenol oxidase activity it was observed that activity was retained throughout the extraction and purification. Four isozymes can be identified from the partially purified polyphenol oxidase from Forelle pears (*Pyrus communis* L.) (Figure 2.11)



Figure 2.11: Native PAGE stained for PPO activity of partially purified PPO from Forelle pears (*Pyrus communis* L.): crude extract (lane 1), 30-80% ammonium sulphate fraction (lane 2), Superdex 200 10/300 GL fraction 38-44 (lane 3) and Superdex 200 10/300 GL fraction 77-87 (lane 4).

2.4.7. Purification of polyphenol oxidase with affinity chromatography

A Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid affinity column was prepared as described by Arslan *et al.* (2004). A single affinity chromatography purification step of Forelle pear polyphenol oxidase was performed. The polyphenol oxidase enzyme activity and protein concentration of the different fractions were determined.

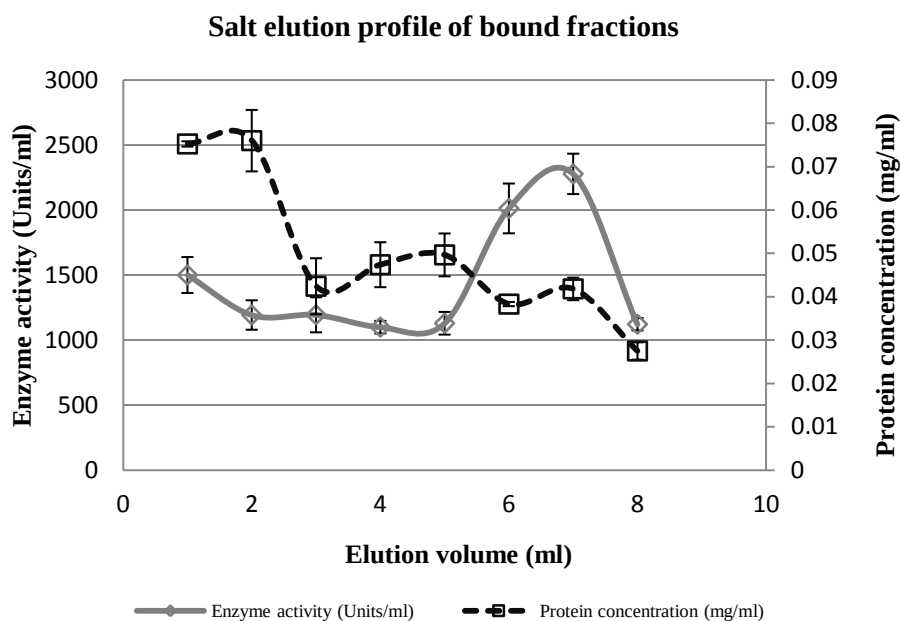


Figure 2.12: Salt elution profile of the bound fractions from the Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid affinity column. Error bars respectively indicate the standard deviation of the enzyme activity (units/ml) and protein concentration (mg/ml) of three biological independent experiments (4 replicates each).

An expected decrease in protein concentration was observed over all the fractions (Figure 2.12). A high enzyme activity was observed at fraction 1 and a significant peak between fraction 5 and 8. These two peak areas of enzyme activity had corresponding peaks in protein concentration. A peak in protein concentration between fraction 3 and 5 was not associated with a corresponding peak in polyphenol oxidase enzyme activity.

Table 2.7: The protein concentration mass balance during polyphenol oxidase purification.

	Crude	Unbound	Wash		Fraction							
			1	2	1	2	3	4	5	6	7	8
Protein conc. (mg/ml) (SD)	0.557 (0.015)	0.248 (0.008)	0.070 (0.008)	0.027 (0.001)	0.075 (0.0006)	0.076 (0.007)	0.042 (0.006)	0.047 (0.005)	0.050 (0.005)	0.038 (0.0005)	0.042 (0.003)	0.027 (0.002)
Volume	7	7	7	7	1	1	1	1	1	1	1	1
Protein (mg)	3.900	1.738	0.493	0.191	0.075	0.076	0.042	0.047	0.050	0.038	0.042	0.027
Subtotal	3.900 (100%)	1.738 (45%)	0.684 (18%)		0.397 (10%)							
Total	3.900 (100%)	2.820 (72%)										

From the protein concentration mass balance during the purification of polyphenol oxidase a 72% protein recovery was determined based upon the initial protein concentration of the crude (Table 2.7).

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Table 2.8: The enzyme activity recovery during polyphenol oxidase purification.

	Crude	Unbound	Wash		Fraction							
			1	2	1	2	3	4	5	6	7	8
Activity units/ml (SD)	8142 (111.8)	2977 (74.45)	914.0 (88.13)	457.9 (75.90)	1598 (138.1)	1193 (113.2)	1194 (134.6)	1099 (48.16)	1129 (87.18)	2012 (191.4)	2278 (115.4)	1122 (47.83)
Volume	7	7	7	7	1	1	1	1	1	1	1	1
Activity (units)	56995	20838	6398	3205	1598	1193	1194	1099	1129	2012	2278	1122
Subtotal	56995 (100%)	20838 (37%)	9603 (17%)		11625 (20%)							
Total	56995 (100%)	42066 (74%)										

A 74% recovery of polyphenol oxidase activity was determined from the enzyme activity mass balance during the purification of polyphenol oxidase (Table 2.8). From the assessment of the specific activity (units/mg) of polyphenol oxidase during purification determined the highest fold purification (3.62) in fraction 7 (Table 2.9).

Table 2.9: The specific activity of polyphenol oxidase during purification.

	Crude	Unbound	Wash		Fraction							
			1	2	1	2	3	4	5	6	7	8
Specific activity (units/mg)	14614	11990	12978	16780	21307	15697	28429	23383	22580	52947	54238	41556
Purification n fold	1.00	0.82	0.89	1.10	1.46	1.07	1.95	1.60	1.55	3.62	3.71	2.84
Average purification n fold	1.00	0.82	1.00		2.23							

A 3 fold purification of polyphenol oxidase from Forelle pear (pooled fractions 1 to 7) was achieved in the single affinity chromatography purification step (Table 2.10).

Table 2.10: The purification table of polyphenol oxidase from Forelle pear.

Purification step	Volume (ml)	Activity (units/ml)	Total activity	Protein conc. (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification n fold
Crude	7	8142	56995	0.557	3.900	14613	100	1
Affinity chromatography	7	2334	16336	0.053	0.371	44059	28.66	3.015

2.4.7.1. Silver stained SDS-PAGE and detection of isozymes

Silver stained SDS-PAGE of the purified Forelle pear polyphenol oxidase showed the presence of several bands with five prominent bands at ~56 kDa, ~27 kDa, ~25 kDa, ~18 kDa and ~14 kDa (Figure 2.13 A). The zymogram of purified pear polyphenol oxidase confirmed the presence of isoenzymes. Six active polyphenol oxidase isoenzymes were detected with 4-methylcatechol in the crude sample (Figure 2.13 B).

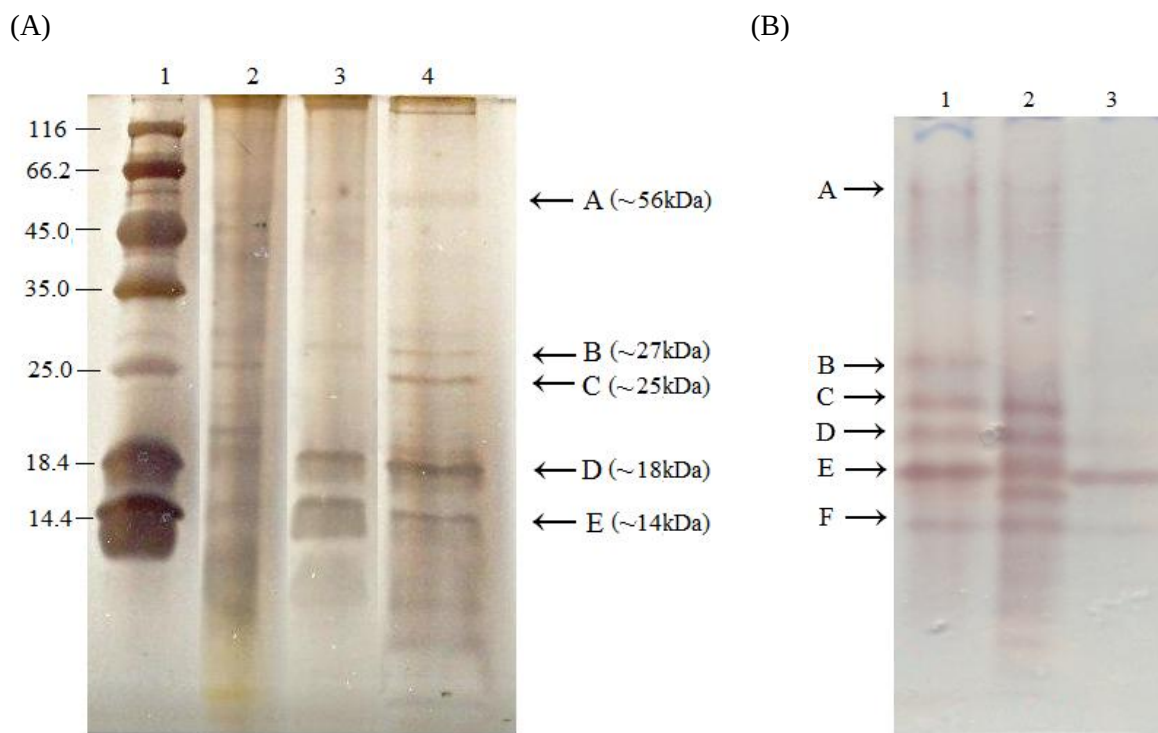


Figure 2.13: (A) Silver stained 12% SDS-PAGE gel of purified polyphenol oxidase from Forelle pears (*Pyrus communis* L.). Lane 1 protein marker, lane 2 crude (9.13 µg), lane 3 unbound crude (3.78 µg) and lane 4 bound fractions of purified polyphenol oxidase (0.85 µg). (B) Zymogram of a 7% Native page stained for polyphenol oxidase activity by development with catechol as substrate. Lane 1 crude (9.13 µg), lane 2 unbound crude (3.78 µg) and lane 3 bound fractions of purified polyphenol oxidase (0.85 µg).

2.4.8. Effect of pH on the activity of polyphenol oxidase

The pH-activity profile of Forelle pear polyphenol oxidase based upon the oxidation of 4-methylcatechol in a citrate buffer pH 3 to 5.5, potassium phosphate buffer pH 6 to 7.5 and Tris-HCl buffer pH 8 to 10 is shown in Figure 2.14. Polyphenol oxidase had an observed optimum pH at 5, followed by a sharp decrease in activity (units) between pH 5 and 5.5, and a subsequent small shoulder was present at pH 6.5.

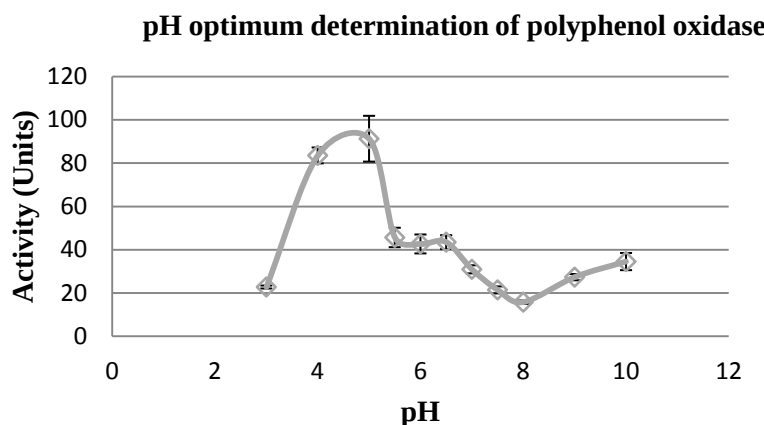


Figure 2.14: pH optimum determination of polyphenol oxidase with 4-methylcatechol in a citrate buffer pH 3 to 5.5, potassium phosphate buffer pH 6 to 7.5 and Tris-HCl buffer pH 8 to 10. Error bars indicate the standard deviation of the activity (units) of three biological independent experiments (3 replicates each).

The pH stability curve of polyphenol oxidase activity indicated that the enzyme was most stable at pH 5, with a sharp decrease in activity (units) from pH 5 to 5.5 followed by a small shoulder at pH 6.5 (Figure 2.15).

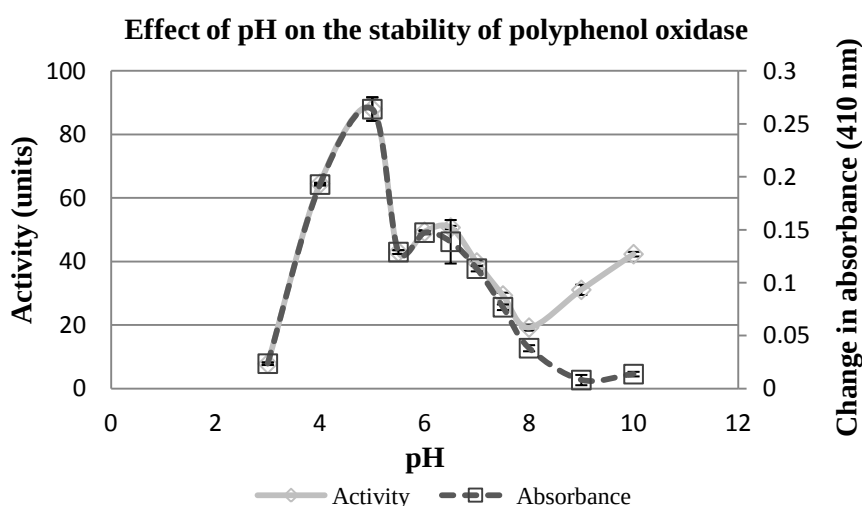


Figure 2.15: The apparent stability of polyphenol oxidase over a pH range of 3-10 after incubation for 30 min at 30°C with 4-methylcatechol (solid line). The absorbance (410 nm) of the apparent stability of polyphenol oxidase excluding the contributing effect of the non-enzymatic conversion of 4-methylcatechol over the pH range of 3 to 10 (dashed line). Error bars indicate the standard deviation of the activity (units) of three biological independent experiments (3 replicates each).

2.4.9. Auto-oxidation of 4-methylcatechol

The effect of the autoxidation rate of the non-enzymatic conversion of 4-methylcatechol to 2-hydroxy-5-methyl-1,4-benzoquinone was investigated as a function of pH. From the absorption spectrum, the presence of the autoxidation product was absent at pH 4 and was clearly visible at pH 9 with a peak at 487 nm (Figure 2.16). The contributing effect of the autoxidation of

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4-methylcatechol over the pH range of 6 to 10 was excluded from the change in absorbance at 410 nm of the pH stability analysis of polyphenol oxidase (Figure 2.15).

Absorption spectrum of 4-methylcatechol at different pH values

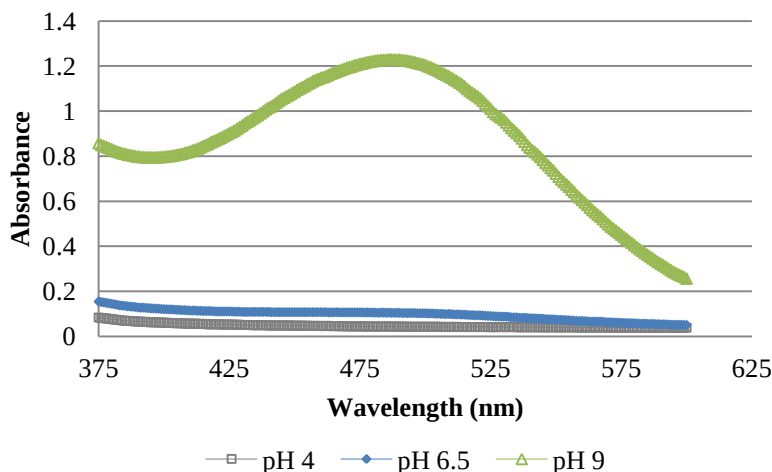


Figure 2.16: Absorption spectrum of 4-methylcatechol made up in a citrate buffer pH 4, potassium phosphate buffer pH 6.5 and Tris-HCl buffer pH 9.

2.4.10. Effect of temperature on the activity of polyphenol oxidase

Pear polyphenol oxidase activity was stable over the temperature range of 25°C to 70°C with slight variations in enzyme activity. This was based upon the results obtained for the temperature optimum determination at both pH 4 (citrate buffer) and pH 6.5 (potassium phosphate buffer) (Figure 2.17). The optimum temperature for pear polyphenol oxidase was 25°C (determined at pH 6.5) and 30°C (determined at pH 4) (Figure 2.17).

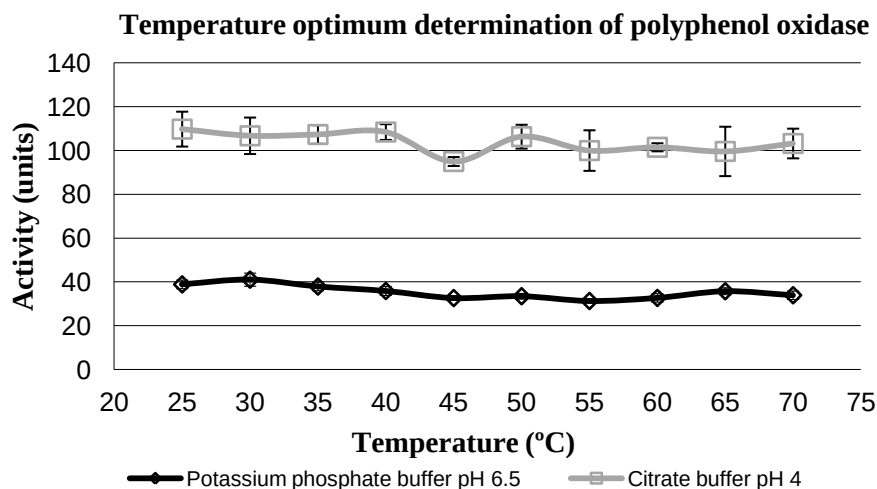


Figure 2.17: Temperature optimum determination of polyphenol oxidase with 4-methylcatechol respectively in a potassium phosphate buffer pH 6.5 and citrate buffer pH 4. Error bars indicate the standard deviation of the activity (units) of three biological independent experiments (3 replicates each).

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The temperature stability of pear polyphenol was higher at lower temperatures (Figure 2.18). This was based upon 98% of the activity being retained with a temperature increase from 23°C to 40°C, compared to only 3% of the activity that was retained at 70°C.

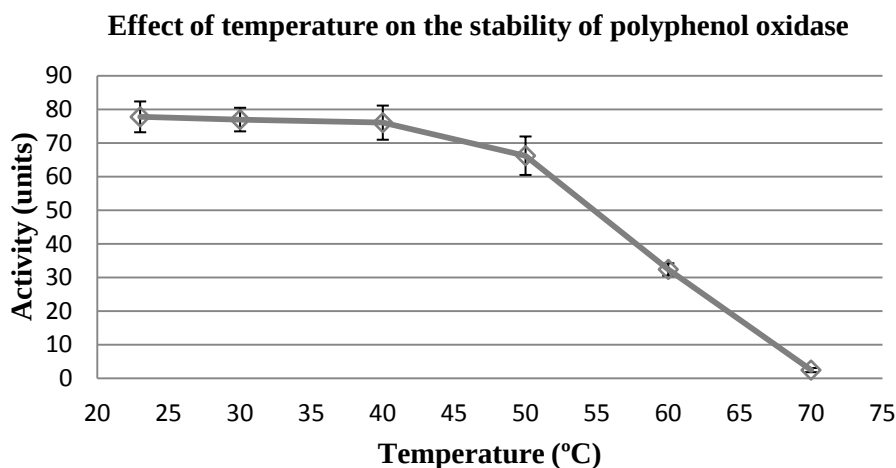


Figure 2.18: The apparent stability of polyphenol oxidase after a 30 min incubation period over a temperature range of 23°C to 70°C with 4-methylcatechol. Error bars indicate the standard deviation of the activity (units) of three biological independent experiments (4 replicates each).

2.4.11. Determination of K_M and V_{max} of polyphenol oxidase

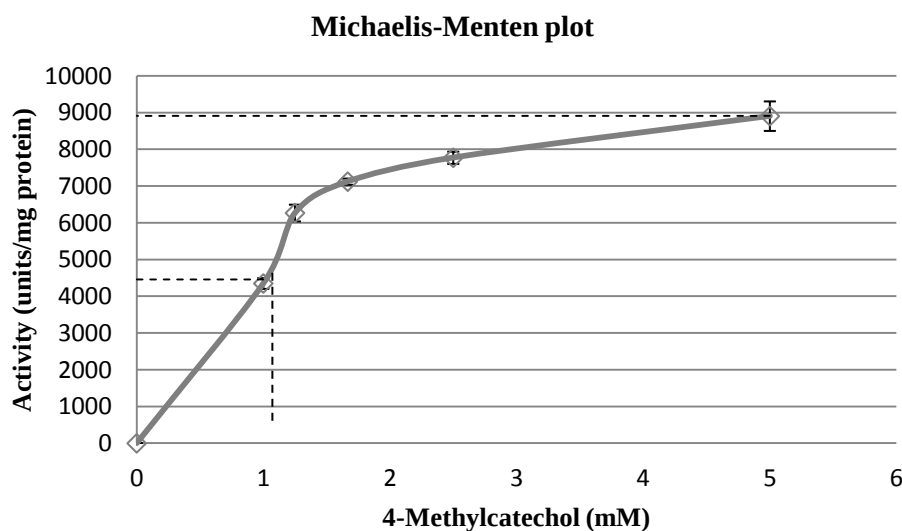


Figure 2.19: Michaelis-Menten plot of Forelle pear polyphenol oxidase determined using 4-methylcatechol as substrate in five different concentrations under optimum conditions (pH 5 and 30°C). Error bars indicate the standard deviation of the activity (units/mg protein) of three biological independent experiments (4 replicates each).

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From the double-reciprocal Lineweaver-Burk plot ($-1/K_m$ x-axis intercept, and $1/V_{max}$ y-axis intercept) the kinetic parameters of K_M and V_{max} was determined as 14 mM and 1.4×10^5 units/mg protein respectively (Figure 2.20).

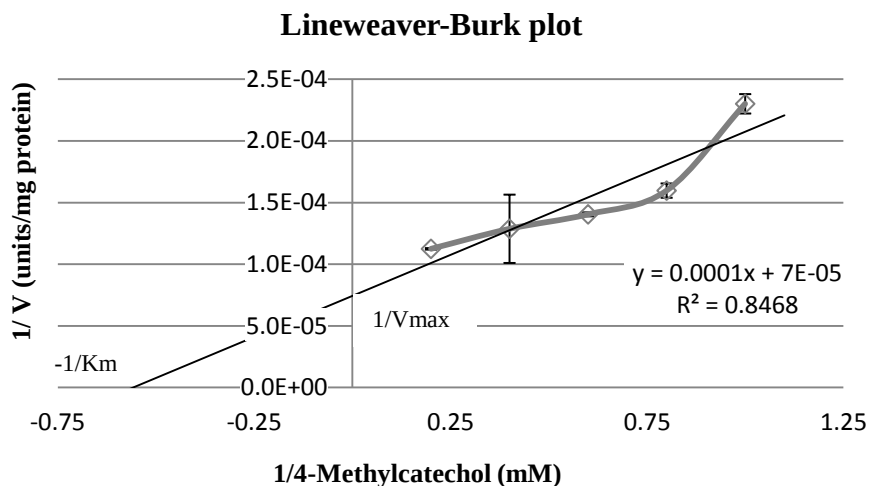


Figure 2.20: Lineweaver-Burk plot determined from the Michaelis-Menten plot of Forelle pear polyphenol oxidase. Error bars indicate the standard deviation of the velocity (units/mg protein) of three biological independent experiments (4 replicates each).

From the alternative linear form of the Hanes-Woolf plot ($-K_M$ x-axis intercept, and K_M/V_{max} y-axis intercept) the kinetic parameters of K_M and V_{max} was determined as 0.9 mM and 1.0×10^4 units/mg protein respectively (Figure 2.21).

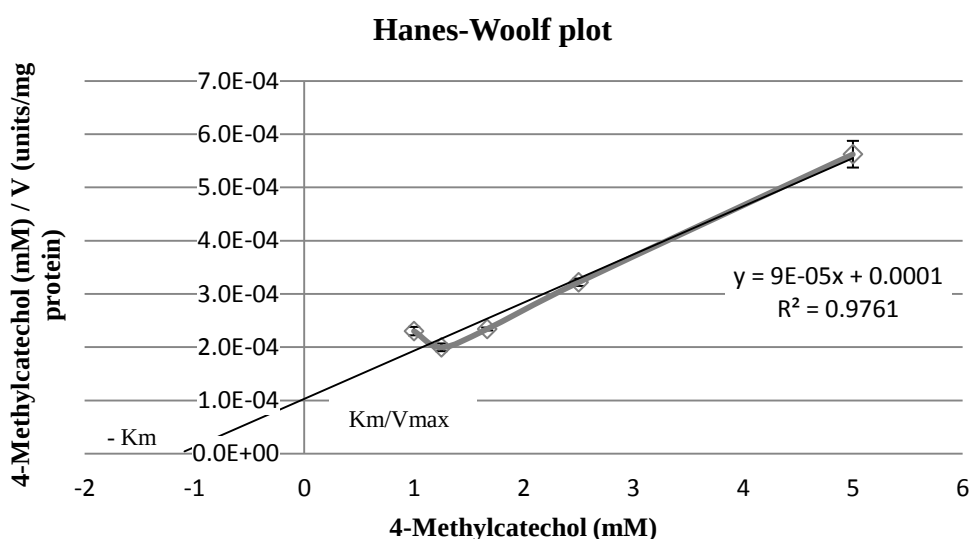


Figure 2.21: Hanes-Woolf plot determined from the Michaelis-Menten plot of Forelle pear polyphenol oxidase determined using 4-methylcatechol as substrate in five different concentrations under optimum conditions (pH 5 and 30°C). Error bars indicate the standard deviation of the velocity (units/mg protein) of three biological independent experiments (4 replicates each).

2.5. DISCUSSION

The pear varieties were assessed for their theaflavin synthesis capacity based upon literature indicating that Japanese pear homogenates had a much greater theaflavin synthesis capacity than that of fresh tea leaves. Loquat and Japanese pear homogenates were also the highest among 62 plants belonging to 49 families (Tanaka *et al.* 2002). Lipton® Green tea (non-fermented) was used as a substrate with the addition of the respective pear homogenates that was stirred for an hour at room temperature to simulate a fermentation system. During this reaction, biochemical oxidations mediated by polyphenol oxidase and or peroxidase enzymes present in the pear varieties along with the direct chemical combinations of reactive species took place leading to the formation of theaflavins and thearubigins (Hodges 2008). Forelle pear was identified among the pear varieties Forelle, Packman, Abet Beteil and Beurré Bosc as the pear variety with the highest theaflavin synthesis capacity (0.147 μ mole theaflavin/hour/gram fresh weight pear) (Table 2.1) based upon the flavognost method. The level of theaflavin detected by the flavognost method represent complexation between the boric acid (flavognost reagent) and cis-1,2-dihydroxy groups attached to the benzene ring of the theaflavins. In an article by Spiro and Price 1986, it was hypothesised that the 3,4,5-trihydroxybenzoyl side groups of theaflavin gallates could themselves complex with the flavognost reagent, but according to the experimental results, it was concluded that the flavognost can only complex with the cis 1,2-dihydroxy groups of the theaflavins and not with the gallate side groups. Preliminary exploratory investigation into the theaflavin synthesis capacity of Yacon (*Smallanthus sonchifolius*) leaves was also performed. The theaflavin synthesis capacities of both Forelle pear and Yacon have not been reported in literature.

Comparative assessment of the diphenolase activity ($\Delta\text{Abs}_{410}/\text{g FW/min}$) of polyphenol oxidase of the sample extracts was determined with 4-methylcatechol as substrate, by measuring the change in absorbance over the linear region of the rate reaction (Figure 2.6). The phenol-absorbing agent polyethylene glycol (PEG) was used in the extraction buffer to prevent phenol-protein interactions, which inactivate or modify enzymes (Loomis 1974). The diphenolase activity ($\Delta\text{Abs}_{410}/\text{g FW/min}$) of polyphenol oxidase was in the decreasing order of Tea (frozen fresh leaves) (0.007 $\Delta\text{Abs}_{410}/\text{g FW/min}$), Yacon (0.003 $\Delta\text{Abs}_{410}/\text{g FW/min}$) and lastly, Forelle pear (0.002 $\Delta\text{Abs}_{410}/\text{g FW/min}$) (Table 2.3). The ratio of gram dry weight per gram fresh weight for Tea (frozen fresh leaves), Forelle pear and Yacon was determined as 0.498, 0.169 and 0.208 respectively (Table 2.2). Based upon the difference between samples the ratio of gram dry weight per gram fresh weight enabled a more accurate result between sample comparisons. In terms of

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dry weight, the activity ($\Delta\text{Abs}_{410}/\text{g DW}/\text{min}$) was the highest for Yacon ($0.019 \Delta\text{Abs}_{410}/\text{g DW}/\text{min}$), secondly Tea (frozen fresh leaves) ($0.014 \Delta\text{Abs}_{410}/\text{g DW}/\text{min}$) and lastly, Forelle pears ($0.010 \Delta\text{Abs}_{410}/\text{g DW}/\text{min}$) (Table 2.3). The diphenolase enzyme activity is indicative of the different sample homogenates' ability to synthesize theaflavin, as polyphenol oxidase is the enzyme responsible for the oxidative polymerization of monomeric flavan-3-ols (catechins) during fermentation leading to the formation of bisflavanols (theaflavins, thearubigins and other oligomers) (Pan *et al.* 2000).

The theaflavin synthesis capabilities of Tea- (frozen fresh leaves), Yacon- and Forelle pear extracts were assessed with both Lipton® Green tea and Tea (frozen fresh leaves) over an hour of fermentation based upon the flavognost method (Table 2.4). In the Tea (frozen fresh leaves), the micromole theaflavin per gram dry weight content was unexpectedly high ($49.05 \mu\text{mole TF}/\text{g DW}$) based upon the absence of fermentation by performing a heat inactivation step directly after homogenization. Factors that could have contributed to the unexpected high level of theaflavin include the saturation of the polyphenol oxidase with oxygen during homogenization increasing the rate of theaflavin synthesis, the pH stabilizing effect of the buffer in an environment near the optimum pH of polyphenol oxidase, the decreased significance of the conversion of theaflavins to thearubigins at high temperatures due to low level of Tea (frozen fresh leaves) 2% w/v and associated low level of exothermic heat that was generated and also the increased rate of oxygen transfer in Tea (frozen fresh leaves) based upon a 70% moisture content. Two different substrate extracts, ground Lipton® Green tea and Tea (frozen fresh leaves) were used as starting materials to assess the theaflavin synthesis capacity of Tea (frozen fresh leaves), Yacon and Forelle pear homogenates in a fermentation system based upon the flavognost method. Both extracts are rich sources of catechins as they can constitute up to 30% of the dry tea leaf weight, but the substrate profiles may differ based upon the effect of processing and storage on the stability of the catechins (Sharma *et al.* 2009, Sang *et al.* 2004a). The Lipton® Yellow label black tea control had a $47.90 \mu\text{mole theaflavin per gram dry weight}$ content, which was calculated as 2.7% theaflavins based upon the molecular weight of theaflavin, correlating with the report the 0.4 - 1.85% theaflavins formed during the manufacturing of black tea from the 15 - 30% of catechins present in green tea shoots (Sharma *et al.* 2009, Sang *et al.* 2004a).

Utilizing the ground, Lipton® Green tea extracts as substrate material, Yacon ($65.19 \mu\text{mole TF}/\text{g DW}/\text{h}$) has the highest theaflavin synthesis capacity, secondly Forelle pear ($43.75 \mu\text{mole TF}/\text{g DW}/\text{h}$) and lastly, Tea (frozen fresh leaves) ($25.97 \mu\text{mole TF}/\text{g DW}/\text{h}$) (Table 2.4). From this Yacon and Forelle pear respectively had a 151% and 68% greater

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theaflavin synthesis capacity than that of Tea (frozen fresh leaves). This theaflavin synthesis capacity of Yacon and Forelle pear cannot be attributed to an inherent theaflavin content based upon the absence of theaflavin detected in the respective control samples (Table 2.4). This was confirmed by thin layer chromatography in which no theaflavins were detected in both Yacon and Forelle pear whereas the possible presence of theaflavin and theaflavin-3'-gallate was indicated in Tea (frozen fresh leaves) (Table 2.5). The decreased amount of detected theaflavin present in the Tea (frozen fresh leaves) sample with the Lipton® Green tea extract in comparison to that of the control samples, can be attributed to an increased amount of catechins initiating the destruction of theaflavins by their conversion into thearubigins, which also takes place through the catechin quinones utilized in the dimerization of catechins into theaflavins (Hodges 2008). The generation of theaflavins was reported to be a fast process while the turnover thereof takes place at a slower rate with a progressive decrease in the content of theaflavins (Bonnely *et al.* 2003). Therefore, during the two-minute homogenization of the Tea (frozen fresh leaves) control sample the fast synthesis of theaflavins (49.05 $\mu\text{mole TF/g DW}$) was detected whereas during the hour fermentation of the Tea (frozen fresh leaves) utilizing the Lipton® Green tea extract as substrate the slower rate turnover of theaflavins was possibly observed. This theaflavin turnover rate was increased due to the inherent catechin content of the Tea (frozen fresh leaves) sample initiating the destruction. The turnover of theaflavins was expectedly lower in that of both the Yacon and Forelle samples based upon the decreased or absence of an inherent catechin content in the respective sample homogenates.

This was confirmed by thin layer chromatography in which the presence of the inherent substrates in Forelle pear, Yacon and Tea (fresh frozen leaves) was determined as an indication of their influence on the different samples' theaflavin synthesis capacity. Based upon R_f value the simultaneous analysis of the catechins and theaflavins by thin layer chromatography indicated the possible presence of epigallocatechin gallate, epicatechin gallate, epigallocatechin, epicatechin and catechin in Tea (frozen fresh leaves). This is expected based upon the reported catechin content that can constitute up to 30% of the dry tea leaf weight. In Yacon and Forelle pear only epicatechin gallate's possible presence was indicated (Table 2.5).

In comparison to the Lipton® Yellow label black tea control (47.90 $\mu\text{mole TF/g DW}$) (Table 2.4), Yacon and Forelle pear utilizing Lipton® Green tea extract as substrate material, after an hour of fermentation respectively had a 8.664% decreased and a 36.10% increased theaflavin content ($\mu\text{mole TF/g DW}$). Utilizing the Tea (frozen fresh leaves) extract as substrate material the theaflavin synthesis capacity was in decreasing order of Forelle pear (184.2 $\mu\text{mole TF/g DW/h}$),

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Yacon (181.4 $\mu\text{mole TF/g DW/h}$) and lastly, Tea (frozen fresh leaves) (77.91 $\mu\text{mole TF/g FW/h}$) (Table 2.4). The overall increase in theaflavin synthesis capacity over the three samples when Tea (frozen fresh leaves) extract is used as starting material in comparison to the utilization of Lipton® Green tea extract, can be attributed to the inherent Tea leaf polyphenol oxidase activity that was absent in the Lipton® Green tea extract based upon the enzyme inactivation step by steaming during the processing of green tea. Furthermore, this can be attributed to the difference in substrate profiles based upon the effect of processing and storage on the stability of the catechins (Sharma *et al.* 2009, Sang *et al.* 2004a). In sample comparison, when Tea (frozen fresh leaves) extract is utilized as starting material, Yacon and Forelle pear respectively had a 132% and 136% greater theaflavin synthesis capacity than that of Tea (frozen fresh leaves). The theaflavin synthesis capacity of Forelle pear in comparison to that of Tea (frozen fresh leaves) doubled when Tea (frozen fresh leaves) extract was utilized as starting material instead of Lipton® Green tea extract. This can be attributed to a more favourable substrate- and enzymatic profile for the synthesis of theaflavin when Tea (frozen fresh leaves) extract and Forelle pear extract are combined, which is not observed to such an extent with the Yacon extract. In comparison to the Lipton® Yellow label black tea control (47.90 $\mu\text{mole TF/g DW}$) (Table 2.4), Yacon and Forelle pear utilizing Lipton® Green tea (no enzymatic activity) as substrate material, respectively had 98% and 136% theaflavin content ($\mu\text{mole TF/g DW}$) after an hour of fermentation.

Preliminary exploratory investigation into the theaflavin synthesis capacity of Forelle pear and Yacon (*Smallanthus sonchifolius*) leaves were compared to Tea (frozen fresh leaves). The study was based upon (1) assessing the diphenolase activity of polyphenol oxidase in the sample extracts, (2) theaflavin synthesis capacity of the extracts in a fermentation system respectively utilizing Lipton® Green tea and Tea (frozen fresh leaves) as starting material and (3) thin layer chromatography detection of the inherent presence of catechins and theaflavins in the sample extracts. Following the results, it was clear that both Forelle pear and Yacon would be ideal candidates for further investigation into their theaflavin synthesis capacity. Further investigation was, however, only carried out on Forelle pear polyphenol oxidase.

Forelle pear polyphenol oxidase purification procedure included the steps of fractionation with solid ammonium sulphate at 30-80% saturation, dialyses, gel filtration and ultra-filtration. Based upon the inclusion of the inhibitor ascorbic acid in the extraction buffer dialysis was performed but only after the ammonium sulphate fractionation step. Following the gel filtration step and removal of the inhibitor through dialysis, a subsequent increase in specific activity was observed. A similar inhibitory effect on the crude extract would, however, then be expected. Therefore, the

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enzyme could possibly have been activated during the gel filtration step. A 0.2 fold partial purification of polyphenol oxidase was achieved (Table 2.6). The native PAGE stained for polyphenol oxidase activity of the partially purified polyphenol oxidase indicated the retention of enzyme activity throughout extraction and purification as well as the presence of four isozymes (Figure 2.11). The presence of three or at least two polyphenol oxidase isoenzymes was respectively confirmed by literature in d'Anjou pear (Wisseman & Montgomery 1985) and Williams pear (Gauillard & Richard-Forget 1997).

Prior gel filtration was performed on a Superdex 200 10/300 GL column (1 x 30 cm, 8 ml V_O and 24 ml V_T) which is prescribed for the high-resolution separation of proteins, peptides and other biomolecules according to size. Only a single peak of high intensity was observed indicating the overloading of the column (Figure 2.9). As described, gel filtration was subsequently performed on a larger Sephacryl S-200 HR column (2.6 x 60 cm, 100 ml V_O and 320 ml V_T) and 3.18 mg protein was loaded onto the column. As can be seen from the single peak of high intensity, the column was also overloaded (Figure 2.10). Based upon the high binding capacity of ReSynTM that can attain extremely high enzyme loads of more than 30% *g/g*, that is to be utilized for the immobilization of the purified polyphenol oxidase, a larger amount of enzyme would be required. Therefore, based upon the small amount of protein that can be loaded onto the gel filtration column and the multiple step purification procedure, Forelle pear polyphenol oxidase purification was subsequently performed and compared to a single step affinity chromatography purification step as described by Arslan *et al.* (2004).

Sepharose 4B-L-tyrosine -*p*-amino benzoic acid affinity column was subsequently employed for the purification of polyphenol oxidase and also offers greater specificity in comparison to that of gel filtration for the purification of polyphenol oxidase. Polyphenol oxidase in the crude was retarded on the column by an interaction with the bound L-tyrosine and eluted with a simultaneous change in both pH and salt concentration. From the salt elution profile of the bound fractions, high enzyme activity was observed at fraction 1 and a significant peak between fraction 5 and 8, which had corresponding peaks in protein concentration (Figure 2.12). A peak in protein concentration between fraction 3 and 5 without a corresponding peak in enzyme activity is indicative of undesired enzyme interaction with the bound L-tyrosine. Although this undesired enzyme interacted with L-tyrosine, it lacked specificity for 4-methyl catechol with which the polyphenol oxidase enzyme activity was determined. Both protein and enzyme activity mass balances were determined during the purification which respectively corresponded to a 72% protein recovery and 74% recovery of polyphenol oxidase activity (Table 2.7 and Table 2.8). The

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unrecovered polyphenol oxidase activity (26%) and protein (28%) was bound irreversibly to the column. This included both enzymes that interacted with the bound L-tyrosine and possibly remaining cyanogen bromide groups or other non-specific interactions. The loss of polyphenol oxidase activity during purification can be attributed to enzyme still bound to the column after eight salt washes and the denaturation of the enzyme (Table 2.8).

The highest specific activity (54238 units/mg) was determined for fraction 7 during the purification of polyphenol oxidase (Table 2.9). Salt washes 1 to 7 were pooled and a 3 fold purification of polyphenol oxidase from Forelle pear was achieved in the single affinity chromatography purification step (Table 2.10). The method followed was as described by Yerlitürk *et al.* (2008) in which a 31.50 fold purification of polyphenol oxidase from wild pear (*Pyrus Elaeagnifolia*) was obtained. In comparing the yield calculated from the total activity reported by Yerlitürk *et al.* (2008) and the present study the yield is 127% and 29% respectively. The more than 100% yield can be contributed to either an activation of polyphenol oxidase during purification or the inhibition of polyphenol oxidase in the crude. The inhibition of polyphenol oxidase in crude can be attributed to the presence of ascorbic acid in the extraction buffer. Ascorbic acid falls within the group of polyphenol oxidase inhibitors that reduces the Cu^{2+} of polyphenol oxidase to Cu^+ (Hsu *et al.* 1988) and therefore, excluded from the extraction buffer in the present study. The total activity of the crude reported by Yerlitürk *et al.* (2008) is likely to be underestimated based upon the inhibitory effect of ascorbic acid on polyphenol oxidase in the crude. The purified polyphenol oxidase was not, or to a significantly lesser extent inhibited by ascorbic acid based upon the washing of the affinity column for the removal of unbound protein. The percentage of the total amount of protein (mg) eluted from the affinity column for the results reported in Yerlitürk *et al.* (2008), and the present study was respectively 4.2% and 9.5%. Based upon the similarity of the source material between the studies, the larger amount of protein retained on the affinity column in the present study could be as a result of insufficient removal of unbound protein from the column during washing or surface variability between the batches of synthesized Sepharose 4B-L-tyrosine –*p*-amino benzoic acid affinity columns.

Polyphenol oxidase has also been reported to have been purified according to a purification procedure that comprised of the Sepharose 4B-L-tyrosine –*p*-amino benzoic acid affinity chromatography step with a prior ammonium sulphate precipitation step. Here a 7 fold purification was reported for polyphenol oxidase from *Lactaris salmonicolor* (Dedeoglu & Guler 2009) and a 74 fold purification of polyphenol oxidase from mulberry (Arslan *et al.* 2004),

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compared to the 3 fold purification of Forelle pear polyphenol oxidase, in the present study without the prior precipitation step.

Silver stained SDS-PAGE of the purified Forelle pear polyphenol oxidase showed the presence of several bands with five prominent bands at ~56 kDa, ~27 kDa, ~25 kDa, ~18 kDa and ~14 kDa (Figure 2.13 A). SDS-PAGE of polyphenol oxidase purified by Sepharose 4B-L-tyrosine -*p*-amino benzoic acid affinity chromatography from *Lactaris salmonicolor* (Dedeoglu & Guler 2009) and mulberry (Arslan *et al.* 2004) indicated the presence of a single band at respectively 35 kDa and 65 kDa. In both instances, SDS-PAGE was stained with coomassie blue. The increased amount of bands can be explained by the increased sensitivity of silver staining and the presence of isoenzymes. The increased sensitivity of silver staining indicated the presence of undesired proteins corresponding to the peak area of protein concentration that doesn't correspond with polyphenol oxidase activity for the substrate 4-methyl catechol (Figure 2.13) and the increased amount of retained protein during purification (9.5% vs. 4.2% as discussed above). The zymogram of purified pear polyphenol oxidase confirmed the presence of isoenzymes. Six active polyphenol oxidase isoenzymes were detected with 4-methylcatechol in the crude sample (Figure 2.13 B). A loss of active enzyme is evident in the unbound fraction corresponding to the amount of activity of the unbound fraction (Table 2.8). This loss of activity can be attributed to the overloading of the affinity column. A prominent enzyme activity band (E) with two light bands (D and F) is present in the purified polyphenol oxidase sample. The presence of three and at least two polyphenol oxidase isoenzymes was respectively confirmed by literature in d'Anjou pear (Wisseemann & Montgomery 1985) and Williams pear (Gauillard & Richard-Forget 1997).

From the pH activity profile of Forelle pear polyphenol oxidase, the enzyme had an observed optimum pH at 5, followed by a sharp decrease in activity (units) between pH 5 and 5.5, and a subsequent small shoulder was present at pH 6.5 (Figure 2.14). This was consistent with the pH profile of polyphenol oxidase purified from Monroe apple peel. A bell-shaped curve between pH 3.5 and 5.5 with an optimum at pH 4.6, a rapid decrease to pH 5.5, followed by a small shoulder at pH 6.5 was reported (Zhou *et al.* 1993).

The pH stability curve of polyphenol oxidase activity indicated that the enzyme was most stable at pH 5, with a sharp decrease in activity (units) from pH 5 to 5.5 followed by a small shoulder at pH 6.5 (Figure 2.15). In comparison to the pH optimum determination (Figure 2.14) the pH stability of polyphenol oxidase (Figure 2.15) revealed that the preincubation of the enzyme had no

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apparent effect on the enzyme activity of pear polyphenol oxidase. This is confirmed by literature in the determination of the pH stability of polyphenol oxidase from a sweet potato root in which no apparent effect on enzyme activity was also determined in comparison the to the pH optimum determination with chlorogenic acid and catechol as substrates (Lourenco *et al.* 1992).

The effect of the autoxidation rate of the non-enzymatic conversion of 4-methylcatechol to 2-hydroxy-5-methyl-1,4-benzoquinone investigated as a function of pH, indicated a significantly increased effect at a high pH value (Figure 2.16). The substrate 4-methylcatechol (0.07 M) had a clear colour at pH 4, a light pink colour at pH 6.5 and a characteristic dark pink colour at pH 9. From the absorption spectrum, the presence of the autoxidation product was absent at pH 4 and was clearly visible at pH 9 with a peak at 487 nm (Figure 2.16). The absorption spectrum at pH 9 indicated the influence of the autoxidation product of 4-methylcatechol at 410 nm, at which the polyphenol oxidase activity assay was performed. This was consistent with literature indicating an increased autoxidation rate of 4-methylcatechol at higher pH values (Rinaldi *et al.* 1995). The interference of the non-enzymatic autoxidation product at high pH values was evident from the pH optimum determination (Figure 2.14) and pH stability (Figure 2.15) of polyphenol oxidase. Therefore, the apparent increase in polyphenol oxidase activity from pH 8 to 10 (Figures 2.14 and 2.15) was not as a result of the effect of pH on the activity of polyphenol oxidase, but the effect of pH on the substrate. The contributing effect of the autoxidation 4-methylcatechol over the pH range of 6 to 10 was subsequently exclude from the change in absorbance at 410 nm of the pH stability analysis of polyphenol oxidase (Figure 2.15).

The autoxidation of 4-methylcatechol may also have a dramatic effect on the apparent polyphenol oxidase activity around neutral pH. This is based upon literature indicating that the presence of Cu^{2+} has an accelerating effect on the autoxidation rate of 4-methylcatechol, with a dramatic effect around neutral pH (Rinaldi *et al.* 1995). Polyphenol oxidase has a dicopper centre connected by a loop essential for the active site architecture and stability of the enzyme (Schweikardt *et al.* 2007). This dicopper centre is located at the bottom of a large cavity formed by the hydrophobic residues as a putative substrate binding pocket (Matoba *et al.* 2006).

Pear polyphenol oxidase activity was stable over the temperature range of 25°C to 70°C with slight variations in enzyme activity. This was based upon the results obtained for the temperature optimum determination at both pH 4 (citrate buffer) and pH 6.5 (potassium phosphate buffer) (Figure 2.17). The optimum temperature for pear polyphenol oxidase was 25°C (determined at pH 6.5) and 30°C (determined at pH 4) (Figure 2.17). Forelle pear polyphenol oxidase exhibited

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about 91% of its maximum activity at 65°C, which was higher than the reported 60% activity retention for both d'Anjou and Barlett pear polyphenol oxidase (Siddiq & Cash 2000). Forelle pear polyphenol oxidase, therefore, appeared more active over the determined temperature range than that reported for d'Anjou and Barlett pear polyphenol oxidase.

The temperature stability of pear polyphenol was higher at lower temperatures (Figure 2.18). This was based upon 98% of the activity being retained with a temperature increase from 23°C to 40°C, compared to only 3% of the activity that was retained at 70°C. The temperature stability of Forelle pear polyphenol oxidase was similar to that of Monroe apple polyphenol oxidase. The amount of activity that was retained for an increase in temperature from 20°C to 40°C was 96% and decreased to 6% activity retention when the temperature was increased to 70°C. Despite the similarity of the polyphenol oxidase temperature stability data of Monroe apple and Forelle pear, the temperature optimum determination of Monroe apple polyphenol oxidase indicated a much larger effect of temperature on enzyme activity. As only 35% of the maximum enzyme activity was retained at 70°C compared to the 94% activity retention determined for Forelle pear polyphenol oxidase (Zhou *et al.* 1993).

The kinetic parameters were determined from the initial velocity data of the Michaelis-Menten plot (Figure 2.19) by the linear transformation of the data in Lineweaver-Burk and Hanes-Woolf methods to reduce bias introduced by non-linearity. From the double-reciprocal Lineweaver-Burk plot ($-1/K_M$ x-axis intercept and $1/V_{max}$ y-axis intercept) the kinetic parameters of K_M and V_{max} was determined as 14 mM and 1.4×10^5 units/mg protein respectively (Figure 2.20). The determined K_M corresponded to the 10 mM reported for litchi fruit peel polyphenol oxidase, although a increased V_{max} value of 1×10^4 units/per mg was indicated, also utilizing 4-methylcatechol as substrate (Yue-Ming *et al.* 1997). It was suggested that based upon statistical considerations that compared to the double reciprocal form (Lineweaver-Burk plot) more accurate subjective estimates could be obtained by fitting the alternative linear form in S/V and S (Wilkinson 1961). From the alternative linear form of the Hanes-Woolf plot ($-K_M$ x-axis intercept, and K_M/V_{max} y-axis intercept) the kinetic parameters of K_M and V_{max} was determined as 0.9 mM and 1.0×10^4 units/mg protein respectively (Figure 2.21).

2.6. CONCLUSION

Forelle pear was identified among the pear varieties Forelle, Packman, Abet Beteil and Beurré Bosc as the pear variety with the highest theaflavin synthesis capacity (0.147 $\mu\text{mole theaflavin/hour/gram fresh weight pear}$) in a fermentation system utilizing Lipton® Green tea as starting material and assessment based upon the flavognost method. Preliminary exploratory investigation into the theaflavin synthesis capacity of Forelle pear and Yacon (*Smallanthus sonchifolius*) leaves was based upon the comparative assessment with Tea (frozen fresh leaves). The synthesis capacity was assessed in terms of the diphenolase activity of polyphenol oxidase, which is indicative of the sample homogenates ability to synthesize theaflavin and was determined in the decreasing order of Yacon (0.019 $\Delta\text{Abs}_{410}/\text{g DW/min}$), Tea (frozen fresh leaves) (0.014 $\Delta\text{Abs}_{410}/\text{g DW/min}$) Forelle pears (0.010 $\Delta\text{Abs}_{410}/\text{g DW/min}$). The theaflavin synthesis capabilities in a fermentation system based upon the flavognost method utilizing Lipton® Green tea as starting material was subsequently determined in the decreasing order of Yacon (65.19 $\mu\text{mole TF/g DW/h}$), Forelle pear (43.75 $\mu\text{mole TF/g DW/h}$), Tea (frozen fresh leaves) (25.97 $\mu\text{mole TF/g DW/h}$). When Tea (frozen fresh leaves) was used as starting material, the theaflavin synthesis capacity was in the order of Forelle pear (184.2 $\mu\text{mole TF/g DW/h}$), Yacon (181.4 $\mu\text{mole TF/g DW/h}$) and lastly, Tea (frozen fresh leaves) (77.91 $\mu\text{mole TF/g FW/h}$). This theaflavin synthesis capacity of Yacon and Forelle pear couldn't be attributed to an inherent theaflavin content based upon the control samples and which was confirmed by thin layer chromatography. A lower than expected theaflavin synthesis capacity of Tea (frozen fresh leaves) with reference to the control sample was due to an increased amount of catechins initiating the destruction of theaflavin. In comparison to the Lipton® Yellow label black tea control (47.90 $\mu\text{mole TF/g DW}$), Yacon and Forelle pear utilizing Tea (frozen fresh leaves) extract as substrate material after an hour of fermentation respectively had a 178.7% and 184.6% increased theaflavin content ($\mu\text{mole TF/g DW}$). Both Forelle pear and Yacon (*Smallanthus sonchifolius*) leaves were identified as ideal candidates for further investigation into their theaflavin synthesis capacity, but further investigation was only carried out on Forelle pear polyphenol oxidase.

A 0.2 fold purification of polyphenol oxidase from Forelle pear was achieved by gel filtration with the identification of four isoenzymes on native PAGE stained for polyphenol oxidase activity. Based upon the quantity of purified polyphenol oxidase required for subsequent

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immobilization and higher specificity, polyphenol oxidase was subsequently purified with a Sepharose 4B-L-tyrosine –*p*-amino benzoic acid affinity column. A 3 fold purification of polyphenol oxidase from Forelle pear was achieved in a single affinity chromatography step. The presence of five prominent bands at ~56 kDa, ~27 kDa, ~25 kDa, ~18 kDa and ~14 kDa were detected with silver stained SDS-PAGE, and the presence of three isoenzymes detected on native PAGE. The polyphenol oxidase has an optimum and was most stable at pH at 5. Analysis of the effect of the autoxidation rate at 410 nm of the non-enzymatic conversion of 4-methylcatechol as a function of pH, indicated an increase in pH over the pH range of 6 to 10 with a peak at 487 nm. Polyphenol oxidase was stable over the temperature range of 25°C to 70°C with an optimum temperature at 25°C (determined at pH 6.5) and 30°C (determined at pH 4). The enzyme was more stable at lower temperatures with 98% activity retention with a temperature increase from 23°C to 40°C, compared to only 3% of the activity that was retained at 70°C. The kinetic parameters K_M and V_{max} were determined as 14 mM and 1.4×10^5 units/mg protein respectively with Lineweaver Burk and as 0.9 mM and 1.0×10^4 units/mg protein respectively with the Hanes-Woolf plot.

CHAPTER 3

IMMOBILIZATION OF POLYPHENOL OXIDASE AND LACCASE

3.1. PROCESS OF IMMOBILIZATION

Immobilization is the process of attaching an enzyme onto an insoluble solid support (Villeneuve *et al.* 2000). Enzyme immobilization enhances the applicability of biomolecules as robust and reusable biocatalysts due to the increase in stability it confers to enzymes (Betancor & Luckarift 2008). The enzyme structure is more rigid after immobilization, which decreases the enzyme's sensitivity to thermal deactivation resulting in the increased stability (Villeneuve *et al.* 2000). Further advantages include enhanced enzyme activity, modification of substrate selectivity and enantioselectivity, multi-enzyme reactions, the possibility to modulate enzyme catalytic properties, easy separation from the reaction mixture, easier prevention of microbial contaminations, prevention of protein contamination of the product and continuous or repeated use (Petersen & Kiener 1999, Brady & Jordaan 2009). Therefore, immobilization overcomes the lack of long-term stability under process conditions and difficulties in recycling and recovering of the enzyme which hampers the application of enzymes for a given reaction (Petersen & Kiener 1999). The most frequently used immobilization techniques can be divided into noncovalent adsorption or deposition, covalent attachment, cross-linking of an enzyme and entrapment in a polymeric gel, membrane or capsule (Bornscheuer 2003).

3.1.1. Noncovalent adsorption or deposition

3.1.1.1. Adsorption

Physical adsorption on a carrier material is one of the most widely used methods of enzyme immobilization due to the simplicity of the procedures involved (Villeneuve *et al.* 2000). Enzymes immobilized by adsorption used in aqueous media are significantly disadvantaged by the tendency of the enzyme to readily leach off the carrier, although in organic solvents this is not the case due to the intrinsic insolubility of the enzyme. Therefore, to avoid leaching enzyme immobilization through adsorption should be applied in organic solvents or in pure hydrophobic reactants (Hanefeld *et al.* 2009).

Several parameters determine the success and efficiency of physical adsorption of the enzyme on a solid support. These parameters include the specific area and size of the protein to be absorbed

Chapter 3: Immobilization of Polyphenol Oxidase and Laccase - Introduction

and nature of the surface of the carrier based upon porosity and pore size (Villeneuve *et al.* 2000). The pore size should enable immobilization on the outer pore surface area as well as inside the pores as was demonstrated with polyphenol oxidase adsorbed on mesoporous activated carbon matrices (MAC) (Figure 3.1) (John Kennedy *et al.* 2007).

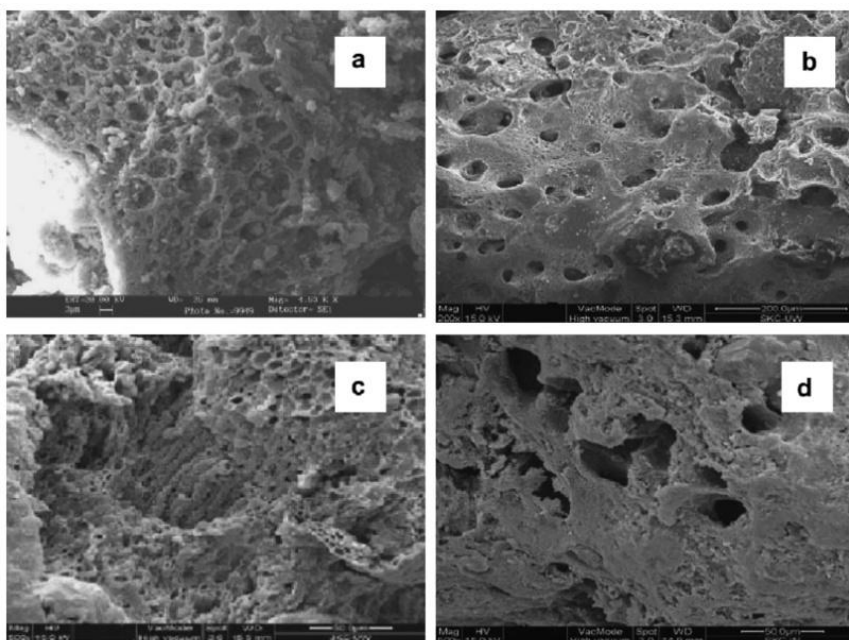


Figure 3.1: Scanning electron microscopy (SEM) micrograph showing the surface morphology (a) MAC400 (b) MAC200 (a decreased average pore size of 21.4 Å compared to the 35.3 Å of MAC 400) as well as that of polyphenol oxidase immobilized on (c) MAC400 resulting in a fully modified pore wall surface and on (d) MAC200 from which the same extent of polyphenol oxidase pore penetration was not observed based upon some completely vacant pores (John Kennedy *et al.* 2007).

During absorption, the enzyme concentration is important in terms of saturating the carrier as was noted in the absorption of laccase from *Trametes versicolor* on spectrographic graphite electrodes (Haghighi *et al.* 2003). Adsorption was reported to be pH dependent in the case of potato polyphenol oxidase on Celite 545, as the immobilization was significantly affected by altering the pH of the buffer. The enzyme was maximally adsorbed at pH 7.0 with decreased binding above and below this pH (Khan *et al.* 2006).

The hydrophobic or hydrophilic nature of the support plays a role as hydrophilic supports may compete with the enzyme for available water in the reaction (Villeneuve *et al.* 2000) furthermore, influence the reaction product mixture. As illustrated by the adsorption of polyphenol oxidase with glutaraldehyde cross-linking onto a hydrophilic nylon flat-sheet membrane and onto a hydrophobic polyethersulphone capillary membrane as in the prior, the intermediate reaction product was detected and in the latter only the final product (Boshoff *et al.* 1998).

3.1.1.2. Deposition

In enzyme immobilization by deposition, the support is added to the aqueous solution of enzyme, and the enzyme is deposited on the solid support. No hydrophobic or entropic driving factor is involved in the enzyme immobilization by deposition. The biocatalyst is recovered by the evaporation of the aqueous phase or by precipitation (Hanefeld *et al.* 2009). Immobilization onto a solid support is achieved by low energy binding forces, which include Van der Waals interactions, hydrogen bonds, ionic bonds and hydrophobic interactions (Villeneuve *et al.* 2000).

3.1.1.2.1. Van der Waals interactions / entropy changes

Both the enzyme and the carrier have to have large lipophilic surface areas for efficient enzyme immobilization via van der Waals interactions or entropy changes. When an enzyme is immobilized on a hydrophobic carrier, the only forces between the enzyme and the carrier are van der Waals forces. The true driving force involved in this immobilization is entropy due to the weak nature of van der Waals forces. A large number of water molecules are displaced by an enzyme from its own as well as the surface of the carrier. The gain in entropy is due to the interaction between the two molecules and is also known as hydrophobic interaction (Hanefeld *et al.* 2009).

3.1.1.2.2. Hydrogen bonds

Most enzymes have hydrophilic amino acid residues on the surface of the enzyme, and some enzymes may be glycosylated, which further increases the enzymes hydrophilicity. Due to this hydrophilicity they can be immobilized on hydrophilic carriers by easily forming hydrogen bonds. Often polar compounds such as sugars, albumin or PEG are included to stabilize the enzyme. It is thought that the stabilization is achieved through the favourable influence of the polar compounds on the distribution of water in the enzyme's microenvironment, and that they protect enzymes against the negative influences of organic solvents (Hanefeld *et al.* 2009).

3.1.1.2.3. Ionic interactions

Absorption on ion exchangers is based upon electrostatic interactions, and in essence, any ion exchanger can act as a carrier (Hanefeld *et al.* 2009, Tyagi & Gupta 1994). To facilitate adequate binding of the enzyme to the ion exchanger, the enzyme can be chemically modified to increase its surface charge. For example, enzymes can be chemically modified by acid anhydrides to enhance its negative surface charge by acylation and subsequently result in stronger binding to an

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anion exchanger such as DEAE-cellulose (Tyagi & Gupta 1994). The application and immobilization by means of ionic interactions are strongly dependent on the pH value and salt concentration. High salt concentrations in ionic immobilization can lead to ion exchange and washing out of the immobilized enzyme (Hanefeld *et al.* 2009).

Immobilization of polyphenol oxidase by ionic interactions to produce a novel bioelectrode was achieved by self-assembled multi-layers of the enzyme intercalated with cationic polyallylamine (PAA) built up on a thiol-modified gold surface (Forzani *et al.* 2000).

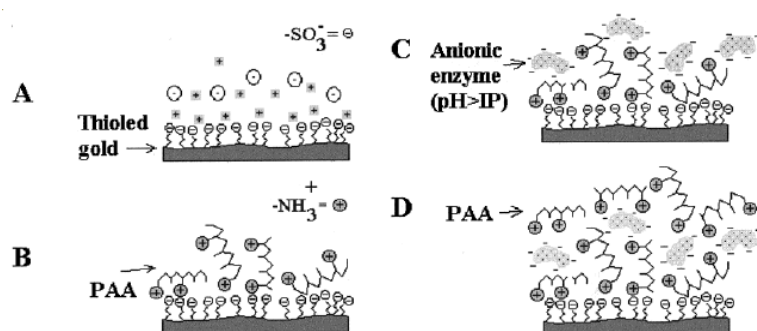


Figure 3.2: Schematic representation of the self-assembled PPO-PAA electrode (Forzani *et al.* 2000).

In the preparation of PPO-PAA assemblies, the first step entails the derivatization of the gold surface with a thiol solution. The exposed sulfonate groups bring about a negative charged surface (Figure 3.2 A). Immersion in a cationic polyelectrolyte solution generates a poly cation of the PPA protonated by water, which is readily absorbed on the thiol layer resulting in a positive residual charge on the exposed surface (Figure 3.2 B). Subsequent immersion in an anionic enzyme solution resulted in the anionic PPO being adsorbed on the positively charged surface, and a decreased protonated amine fraction (Figure 3.2 C). The polyanion nature of PPO favoured the adsorption of a second PAA layer (Figure 3.2 D). The previous two steps were repeated depending on the desired number of bi-layers, which is proportionate to the amount of biocatalyst (Forzani *et al.* 2000).

3.1.2. Covalent attachment

Covalent immobilization leads to an enzyme that is tightly fixed to the solid support and subsequently minimizes leaching of the enzyme in an aqueous medium. Therefore, it is the preferred method when working in aqueous solution. This was evident from the covalent immobilization of laccase on magnetic mesoporous silica spheres in which almost no leaching after ten consecutive operations was observed (Zhu *et al.* 2007). Covalent immobilization is also

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preferred if denaturing factors exist as the conformational flexibility and thermal vibrations are reduced. The reduction is the result of multiple covalent bonds formed between the enzyme and the carrier preventing the unfolding and denaturation of the enzyme. Covalent immobilization also prevents protein contamination of the product. This type of immobilization is distinctly disadvantaged by the chemical modification of the enzyme resulting from the covalent binding (Hanefeld *et al.* 2009).

Both hydrophilic and hydrophobic carriers as in the case of non-covalent enzyme immobilization can be used. The carrier can be attached via a spacer arm to the support (Figure 3.3). Long spacers can be advantageous for the immobilization of enzymes, which undergo significant conformational changes during the interaction with the substrate. Longer spacers allow a wider conformational flexibility to the protein. Shorter spacers restrict enzyme mobility and prevent unfolding and thereby provide higher thermal stability (Hanefeld *et al.* 2009).

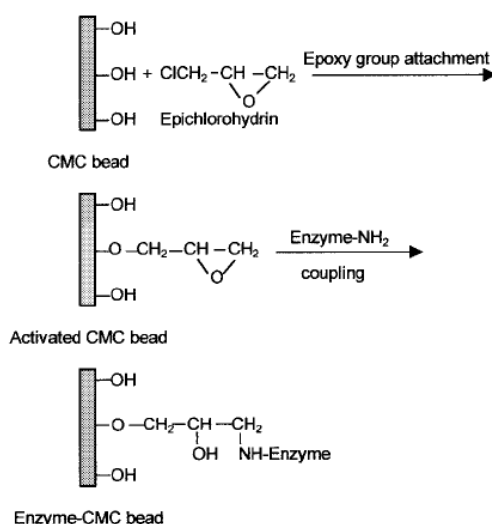


Figure 3.3: The attachment of epoxy groups on the CMC beads and subsequent covalent immobilization of polyphenol oxidase (Yakup Arica 2000).

The covalent immobilization of an enzyme could provide washing and reusing possibilities without the loss of activity. This was observed with the enzymes glucose oxidase, α -amylase and urease covalently immobilized on paramagnetic non-porous poly acrolein beads (Varlan *et al.* 1996).

3.1.3. Entrapment in a polymeric gel, membrane or capsule

Enzyme immobilization by entrapment is achieved by means of polymerization (Villeneuve *et al.* 2000). Entrapment-based enzyme immobilization can immobilize large amounts of enzyme without chemical alteration (Subramanian *et al.* 1999a). Encapsulation is the best means to avoid any negative influence on the structure of an enzyme (Hanefeld *et al.* 2009). The enzyme doesn't chemically interact with the polymer, which is advantageous as denaturation is usually avoided (Villeneuve *et al.* 2000).

A common approach is the entrapment of a hydrophilic catalyst in a polar interior of an amphiphilic macromolecular support. If immobilization by entrapment is employed the binding sites are less defined (Van de Coevering *et al.* 2005). Enzyme entrapment can be achieved with a gelatine layer where a thicker layer would entrap more enzyme, but this becomes impractical as the layer is quite fragile. Furthermore, the technique is hindered by great variation in the amount of immobilized enzyme and peel of the gelatine layer with extensive washes subsequently resulting in the loss of enzyme activity (Subramanian *et al.* 1999a).

Polyphenol oxidase was immobilized via entrapment in conducting copolymers of thiophene functionalized menthyl monomer (MM), during electrochemical polymerization of pyrrole (Figure 3.4).

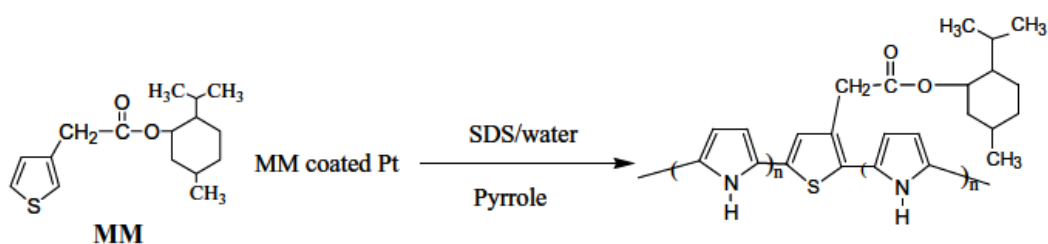


Figure 3.4: Synthesis of the copolymer of MM with pyrrole (Kıralp *et al.* 2003).

Polyphenol oxidase has also been immobilized by entrapment in an electrogenerated film of the conducting polymer poly 3,4-ethylenedioxythiophene (PEDT) (Védrine *et al.* 2003). The physical entrapment of tyrosinase in a membrane on the surface of solid graphite and carbon paste electrodes has also been demonstrated (Nistor *et al.* 1999).

Microencapsulation differs from entrapment as the enzyme and its whole environment is immobilized (Figure 3.5). Artificial cells are created in microencapsulation that are delimited by

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a membrane that large enzyme molecules can't cross but smaller substrate and product molecules are able to (Villeneuve *et al.* 2000). Microencapsulation is limited by the diffusion rate of substrate and product across the membrane.

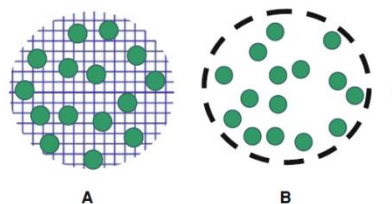


Figure 3.5: The enzyme immobilization strategies of entrapment (A) and encapsulation (B) (Bayramoglu *et al.* 2003).

3.1.4. Different immobilization supports

3.1.4.1. Eupergit C

The carrier Eupergit C consists of macro porous beads on which enzymes can be covalently immobilized. The carrier (100-250 μm) is synthesized through the copolymerization of N, N'-methylene-bis-methacrylamide, glycidyl methacrylate, allyl glycidyl ether and methacrylamide (Figure 3.6) (Katchalski-Katzir & Kraemer 2000).

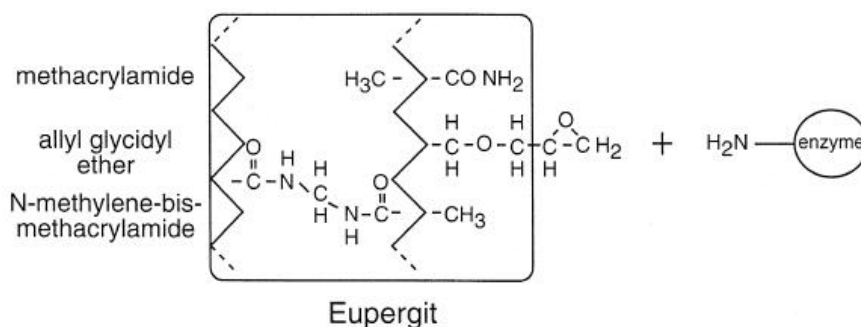


Figure 3.6: The structure of Eupergit C and the covalent immobilization of enzymes (Katchalski-Katzir & Kraemer 2000).

Proteins are bound to Eupergit C via epoxide groups, which react with the different nucleophiles on the protein (amino, sulfhydryl, hydroxyl, phenolic groups) as a function of pH. At neutral pH thiol groups, at pH > 8 the amine groups, at pH > 11 the phenolic groups and at slightly acidic pH the carboxyl groups are involved in the covalent bonding of the amino acid side chain groups (Boller *et al.* 2002). Eupergit C is mechanically and chemically stable in the pH range of 1 to 12 (Katchalski-Katzir & Kraemer 2000).

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Enzymes' amino, sulfhydryl, hydroxyl and phenolic groups could be essential for the catalytic activity of the enzyme. Immobilization through these groups could therefore lead to difficulty in achieving stable binding and high levels of activity. Based upon the substrate being blocked to access the active site, enzymes may denature or multiple point-binding may occur (Knezevic *et al.* 2006).

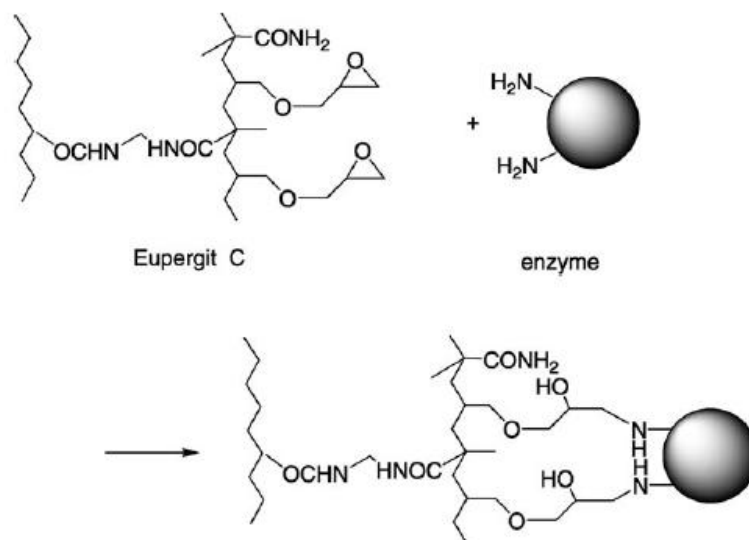


Figure 3.7: The immobilization of enzymes on Eupergit C (Sheldon 2007).

It is proposed that, firstly, the enzyme is physically absorbed on the carrier by hydrophobic interactions. This brings the amino and thiol groups on the surface of the enzyme and the oxirane groups of the carrier in close proximity. Secondly, these groups react with oxirane groups by nucleophilic attack, forming very stable C-N and C-S bonds (Mateo *et al.* 2000). Multipoint attachment is promoted by the high density of oxirane groups on the surface of the support. This is assumed to lead to an increase in conformational stability of the enzyme which improves long term operational stability (Figure 3.7) (Mateo *et al.* 2000).

3.1.4.2. Eupergit C 250L

Eupergit C and Eupergit C 250L are both microporous, epoxy-activated acrylic beads with the same composition, reactive groups and a diameter of 100-250 μm . Eupergit C and Eupergit C 250L differ in their porosity with an average pore size of $r = 10 \text{ nm}$ and $r = 100 \text{ nm}$ respectively. The content of oxirane also differs with an oxirane density of 600 and 300 $\mu\text{mole/g}$ dry beads for Eupergit C and Eupergit C 250L respectively (Boller *et al.* 2002). Eupergit C 250L has fewer epoxide groups, subsequently resulting in a larger quantity of polymer required to achieve the same extend of protein binding. The presence of more polymer matrix with a lower

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distribution of active groups ($> 250 \mu\text{mole/g}$ dry weight) of Eupergit C 250L is beneficial in terms of enzyme activity (Hernaiz & Crout 2000).

3.1.4.3. DEAE Sephadex A-25

Enzymes are immobilized on the anion exchanger DEAE-Sephadex support through adsorption by electrostatic interaction. DEAE Sephadex A-25 and A-50 are used in the molecular weight ranges of $\leq 30,000$ and $30,000\text{--}200,000$ respectively with the possibility of the inclusion of enzymes within these molecular weight ranges (Baron *et al.* 1997).

3.1.4.4. ReSynTM

ReSynTM are emulsion-derived particles comprising of a lattice of cross-linked polymeric strands, interstitial openings around and adjacent to the strands and functional groups on the lattice by means of which immobilization is achieved (Jordaan *et al.* 2009).

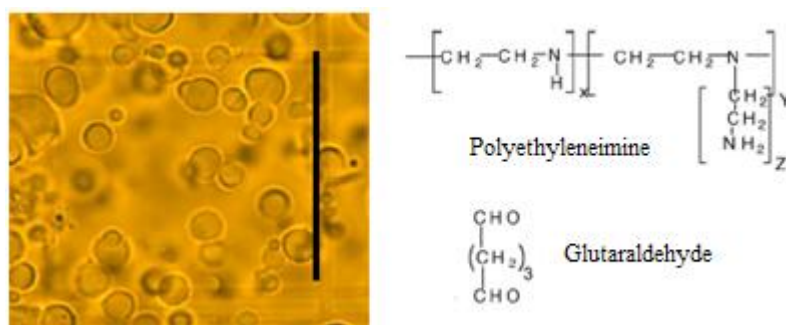


Figure 3.8: Support lattice of polymeric stands/fibres or network backbone and the structures of the polymer polyethylene imine (PEI) and cross-linking agent glutaraldehyde (Jordaan *et al.* 2009).

The polymer can be polyethylene imine (PEI) while the cross-linking agent can be glutaraldehyde (Figure 3.8). The cross-linker can influence the activity of the enzyme through the accessibility of the particles to substrates and products or the degree of enzyme denaturation. The protein reacts with the functional groups on the polymeric strands or fibres and/or that of the cross-linking agent. Immobilization by covalent bonding can be achieved by the interaction of epoxide or aldehyde with amine groups of the protein. Anionic binding of proteins can be achieved through the very high density of amine groups, although these can also be utilized as a basis for the dialdehyde covalent cross-linking of the enzymes. Immobilization through hydrophobic and affinity bonding can also be achieved through the modification of lattice for this functionality (Jordaan *et al.* 2009).

The particle size of the emulsion-derived particles, ReSynTM can be modified based upon the method of synthesis. Factors such as the oil phase (presumably influencing emulsion droplet size), surfactant and/or cross-linker used, single emulsion or dual emulsion synthesis of particles and various post treatment techniques (drying or sonication) influences the particle size of the synthesized ReSynTM particles (Jordaan *et al.* 2009).

A range of enzyme classes such as glucose oxidase, lipase and laccase has been immobilized on ReSynTM with a range between 30%- and 36% *m/m* protein loading. The extremely high enzyme loads of more than 30% *g/g* can be attained by ReSynTM as a result of the open fibre network and high amine density (Brady & Jordaan 2009). Additional stability of enzymes in extremes of temperature or pH can also be realized by the immobilization support (Jordaan *et al.* 2009).

3.2. IMMOBILIZATION AND CATALYTIC REACTIONS

The approaches to enzyme immobilization can all be considered as a compromise between the maintenance of high catalytic activity while achieving the advantages of immobilization (Bornscheuer 2003). The advantages of enzyme immobilization include easy separation from the reaction mixture, easier prevention of microbial contaminations, prevention of protein contamination of the product and continuous or repeated use (Petersen & Kiener 1999, Brady & Jordaan 2009). Enzyme immobilization can increase the enzyme stability (less sensitive to thermal deactivation), enhance the enzyme activity, enable the modification of substrate selectivity and enantioselectivity, multi-enzyme reactions and the possibility to modulate enzyme catalytic properties (Petersen & Kiener 1999, Villeneuve *et al.* 2000, Brady & Jordaan 2009).

3.2.1. Immobilized enzyme with increased stability

The stability of immobilized enzymes is dependent on factors such as the number of bonds and the nature of these bonds (i.e. non-covalent, covalent or different types of these bonds) between the carrier and the enzyme, the microenvironment of the enzyme and the carrier, the degree of enzyme molecule confinement in the carrier and the immobilization conditions (Cao 2005).

3.2.2. Immobilized enzyme with enhanced activity

Immobilization *Bacillus licheniformis* protease had activity retention of almost double with silica derivatives having twice the pore size, regardless of comparable enzyme loading. The specific

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activity on the parent carrier can also be enhanced by the use of different spacers or binding chemistry (Ferreira *et al.* 2003). Imprinting-immobilization utilizes substrates or inhibitor during enzyme immobilization. It has proved to be a powerful technique for preventing the loss of enzyme activity based upon the tightening of the enzyme active centre (Cao 2005, Ferreira *et al.* 2003, Fishman & Cogan 2003).

3.2.3. Immobilized enzyme with improved selectivity

In many occasions, enzymes present very low selectivity towards non-natural or artificial compounds. With immobilization techniques such as entrapment, covalent and simple adsorption, it is possible to strongly modulate enzyme selectivity. Different immobilization strategies may permit the immobilization of enzymes with different rigidity, orientations or generating different environments (Cao 2005, Palomo 2009). The enantioselectivity of lipase is strongly modified by immobilization performed by the promotion of different degrees of multipoint covalent immobilization, involving different areas of the enzyme surface or the creation of different environments surrounding different enzyme areas (Fernandez-Lorente *et al.* 2001).

3.3. MATERIALS AND METHODS

3.3.1. Materials

Sulphuric acid (98.8%) was purchased from Saarchem (Pty) Ltd, Gauteng, South Africa and hydrochloric acid (99%), citric acid (99%) and boric acid were purchased from and Sigma Aldrich (Pty) Ltd, St Louis, United States. Methanol (AR, 99.5%) was purchased from Radchem Products Inc, Orland Park, United States. The buffers used were prepared from; sodium phosphate dibasic, sodium phosphate monobasic, tris (hydroxymethyl) aminomethane (Trizma® base, minimum 99.9% titration) purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. Furthermore, succinic acid and sodium lactate (about 50% extra pure) were purchased from Merck, Damstadt, Germany and dipotassium phosphate and potassium orthophosphate were respectively purchased from Saarchem (Pty) Ltd, Gauteng, South Africa and Labchem (Pty) Ltd, Gauteng, South Africa. ReSyn™ particles were synthesized using; glutaraldehyde (grade II, 25%), nonoxyl-4, polyethylene imine (polyamine) (PEI) purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States and Enerpar oil was obtained from BP Midrand, South Africa. The immobilization supports Eupergit C and Eupergit C 250L were purchased from Evonik Röhm GmbH Pharma Polymers, Darmstadt, Germany and DEAE Sephadex A-25 was purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. Sodium chloride used in the salt washing steps after immobilization was purchased from Merck, Damstadt, Germany. Ethanolamine ($\geq 98\%$) used as a post-treatment after immobilization was purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. Laccase (Suberase) enzyme was a generous gift from Novozyme (Pty) Ltd, Gauteng, South Africa. Albumin Fraktion V ($\geq 98\%$ pulv. bovine) was purchased from Roth, Karlsruhe, Germany and the protein assay dye reagent concentrate was purchased from Biorad, California, United States. The substrate 4-methylcatechol was purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. Coomassie stained SDS-PAGE was performed using sodium dodecyl sulphate (SDS), acrylamide/bis-acrylamide (30% solution electrophoresis reagent), N, N, N', N'-tetramethylethylenediamine (TEMED, Stratagene electrophoresis purity reagent), N', N-methylene bisacrylamide, ammonium persulphate ($\geq 98\%$), mercaptoethanol and glycerol purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. Furthermore, bromophenol blue, acetic acid and glycine were purchased from Merck, Damstadt, Germany and the unstained protein molecular weight marker was purchased from Fermentas, Burlington, Canada.

3.3.2. List of equipment

The equipment used was a Titramax 101 vibrating platform shaker (Heidolph Instruments, Schwabach, Germany), Digital Dry Baths (Labnet International Inc), water bath, SevenEasyTM bench top pH meter (Mettler Toledo Ltd, Leicester, United Kingdom), PowerWave HT spectrophotometer (Biotek Instruments Inc, Winooski, United States), Virtis Genesis 25 freeze drying system (SP Scientific Inc, New York, United States), the centrifuges that were used was an AllegraTM X-22R centrifuge with a SX4250 rotor and an AvantiTM J-25I centrifuge with a JA-14 rotor (Beckman Coulter Inc, California, United States) and the balances that were used, an AS 220/X analytical balance and a WPS 2100/C2 balance (RADWAG®, Random, Poland). The ultrafiltration system used comprised of a Masterflex console Drive (Cole-Parmer Instrument Company, Illinois, United States) with a MinimateTM TFF Capsule and an OmegaTM 10 k molecular weight cut off membrane (Pall Corporation, Port Washington, New York). The gel electrophoresis system used was a Power StationTM 300 (Labnet International Inc) with the gel electrophoresis tank equipment (Bio-Rad, California, United States). The shakers used, were a Belly Dancer orbital shaker (Stovall Life Science Inc, North Carolina, United States) and a micro titre plate shaker (Heidolph Instruments, Schwabach, Germany). The vortexes used, was a Vortex-2 Genie (Scientific Industries Inc, New York, United States) and a MT 19 Auto Vortex mixer (Chiltern Scientific, London, United Kingdom). A Glass-Col Tube Rotor (New Brunswick Scientific Edison, New Jersey, United States) and a Bio RS-24 rotor (BOECO, Hamburg, Germany) were used. The automated pipetting system epMotion® 5075 (Eppendorf, New York, United States) was used when the protein concentration of large amounts of samples were determined. The statistical analysis; one-way ANOVA, Duncan's multiple range test and summary statistical analysis was performed using the statistical software JMP 10.0 (SAS Institute Inc, Cary, North Carolina, United States). Particle size of synthesized ReSynTM was determined with a DelsaTM Nano C (Beckman Coulter Inc, California, USA).

3.3.3. Methods

3.3.3.1. Synthesis of ResynTM particles

ReSynTM particles were synthesized according to the method of Jordaan *et al.* (2009) modified as follows:

Emulsion A composition:

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20 ml Enpar Oil (oil phase)

0.2 ml nonoxyl-4 (surfactant)

4 ml Polyethyleneimine (polyamine), (10% *m/v* aqueous solution) pH 9

Emulsion B composition:

20 ml Enpar Oil (oil phase)

0.2 ml nonoxyl-4 (surfactant)

4 ml glutaraldehyde, (25% *m/v*, grade II)

Emulsions A and B were briefly vortexed (30 seconds) after the addition of nonoxynol-4 to the Enpar oil and again after the addition of polyethylene imine and glutaraldehyde respectively. Instead of end-over-end mixing Emulsion A was added to Emulsion B while stirring using a magnetic stirrer bar at approximately 700 rpm for one hour. To recover the particles the emulsion was centrifuged at 3000 rpm for 20 min (in a Beckman-Coulter AllegraTM X-22R centrifuge fitted with a SX4250 rotor). The pellet was resuspended in deionised water and diluted to 40 to 50 ml, and centrifuged again. The washing process was repeated twice more with the final supernatant being clear. The final pellet was resuspended in of potassium phosphate buffer (0.05 M, pH 7.0). The ReSynTM was synthesized in duplicate and combined. The dry weight of the synthesized ReSynTM was determined by lyophilisation (of 1 ml samples).

3.3.3.1.1.1. Protein concentration determination

The protein concentration was determined according to the dye binding method (Bradford 1976) with bovine serum albumin (Fraction V) as standard. A bovine serum albumin standard curve was constructed by diluting one part dye reagent concentrate with four parts double distilled water. The prepared dye reagent was filtered through a Whatman #1 filter. Bovine serum albumin dilution within the linear range of 0.05 mg/ml to approximately 0.5 mg/ml was prepared. The protein dilution (10 μ l) and the diluted dye reagent (200 μ l) were plated in a 96 well Elisa plate, mixed thoroughly and incubated at room temperature for at least 5 min and no more than one hour. The absorbance was measured at 595 nm and a bovine serum albumin standard curve constructed.

3.3.3.2. BSA immobilization on ReSynTM, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25

Immobilization was carried out as modified from Baron *et al.* (2007) at 25°C and 4°C respectively. BSA (20 mg/ml) dissolved in a potassium phosphate buffer (0.05 M, pH 7.0) was

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added (1 ml) to 100 mg of the supports Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 dissolved in 1 ml potassium phosphate buffer (0.05 M, pH 7.0) as well as to 1 ml (65.67 mg/ml) of the synthesized ReSynTM. The suspensions were gently shaken by horizontal rotation and immobilization was stopped by centrifugation at 3000xg for 5 min at the time points 4-, 8-, 24- and 48 h. After immobilization, the supports were washed six consecutive times, each time with 2 ml deionised water. The protein concentration present in the supernatant was determined by Bradford's assay. The concentration bound BSA was determined by subtracting the amount of BSA in the supernatant from the initial 20 mg/ml BSA sample.

3.3.3.3. Salt washing

Salt washing was performed on the 4°C samples taken at time points 4 and 48 h of the various supports. This was achieved by the addition of 1 ml of NaCl (2 M) and brief (30 seconds) vortexing and subsequent horizontal rotation for one hour at 4°C. The salt washing step was repeated twice. The protein concentration present in the supernatants was determined by Bradford's assay, which represent the non-covalently bound BSA. The covalently bound BSA was calculated as the non-covalently bound BSA subtracted from the bound BSA.

3.3.3.4. PPO immobilization on ReSynTM

Polyphenol oxidase purified from Forelle pear was immobilized on ReSynTM as modified from Baron *et al.* (2007) at 4°C. ReSynTM particles were manufactured as described above and 500 µl used for immobilization. The particles were equilibrated and resuspended in 500 µl potassium phosphate buffer (0.1 M, pH 5). Polyphenol oxidase (0.26 mg, 0.052 mg/ml) was immobilized for 4 h by horizontal rotation and stopped by centrifugation. The particles were washed six times consecutively with deionised water (10 ml). The protein concentration respectively present in the pooled supernatants was determined by the Bradford assay. The concentration of bound polyphenol oxidase was determined as the difference in the amount of polyphenol oxidase in the supernatant from the initial polyphenol oxidase (0.052 mg/ml) sample. The enzyme activity of the ReSynTM immobilized polyphenol oxidase was analysed as described below (3.3.3.4.1). The polyphenol oxidase immobilized on ReSynTM was post-treated with potassium phosphate buffer (0.1 M, pH 5.9) containing 1 mM ethanolamine for 30 min with end over end rotation at 4°C. The immobilized system was washed 6 times with water to remove unreacted ethanolamine (Jordaan *et al.* 2009).

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3.3.3.4.1. Polyphenol oxidase activity assay

Immobilized polyphenol oxidase activity was measured for 10 µl sample in 120 µl potassium phosphate buffer (0.5 M, pH 6.5) and the reaction was started by the addition of 70 µl 4-methylcatechol (0.01 M stock, 3.5 mM final concentration). The development of the coloured oxidative product was followed at 410 nm in the linear region of the rate reaction. One unit of enzyme activity corresponds to the amount of enzyme which increases the absorbance measured at 410 nm by 0.001 per min at pH 6.5 and 25°C with 4-methylcatechol substrate in a 200 µl reaction mixture.

3.3.3.5. Laccase sample preparation

Laccase (Novozymes® Suberase), a fungal laccase from *Myceliophthora thermophila* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* strain, was concentrated to 10 mg/ml and a buffer exchange to succinate-lactate buffer (0.1 M, pH 4.5) was performed by ultrafiltration (Cole-Parmer Instrument Company apparatus, OmegaTM 10 k molecular weight cut off membrane). The purity of concentrated enzyme was verified by SDS-PAGE.

3.3.3.5.1. Coomassie stained SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a stacking gel [Tris-HCl (0.5 M, pH 6.8) and SDS (20% w/v)] and a 12% separating gel [Tris-HCl (1.5 M, pH 8.8) and SDS (20% w/v)] which were prepared from an acrylamide stock (30% acrylamide, 0.8% N', N'-methylene bisacrylamide) and were polymerized with the addition of 20% ammonium persulphate (respectively 25 µl and 75 µl) and tetramethylethylenediamine (TEMED) (respectively 5 µl and 7.5 µl). The samples were prepared in reducing sample buffer [Tris-HCl (0.125 M, pH 6.8), glycerol (20% v/v), SDS (4% w/v), bromophenol blue (0.2% w/v) and mercaptoethanol (25 mM)] in a 1:1 ratio and boiled at 95°C for 5 min. An unstained protein molecular weight marker from Fermentas, Burlington, Canada was used to determine the molecular weight of the proteins. Electrophoresis was carried out in an electrophoresis system with running buffer [Tris (0.25 M), SDS (1% w/v) and glycine (2 M)] at 100 V. SDS-PAGE gel was stained with Coomassie staining solution I [10% (v/v) acetic acid, 0.125% (w/v) Coomassie Brilliant Blue G and 25% (v/v) isopropanol] for 30 min. The staining solution was decanted, and the gel placed in Coomassie staining solution II [10% (v/v) acetic acid, 0.003% (w/v) Coomassie Brilliant Blue G and 10% (v/v) isopropanol] for 60 min. The staining solution was decanted, and the gel was subsequently destained overnight in Coomassie destaining solution [10% (v/v) acetic acid and 5% isopropanol]. Staining and destaining was performed on an orbital shaker at 60 rpm.

3.3.3.6. Laccase immobilization on ReSynTM, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25

ReSynTM particles were manufactured as described above and 500 µl used for immobilization. The supports, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 were weighed out (14 mg) and equilibrated in a succinate-lactate buffer (0.1 M, pH 4.5). All the supports were centrifuged for 10 min using an Allegra X22R centrifuge (2000xg) and resuspended in 500 µl succinate-lactate buffer (0.1 M, pH 4.5). Laccase (5 mg, 10 mg/ml) was immobilized on the different supports by horizontal rotation and stopped at the time points 1-, 4-, 7- and 24 h by centrifugation. The particles were washed six times consecutively with deionised water (2 ml). The protein concentration respectively present in the pooled supernatants was determined by the Bradford assay. The concentration of bound laccase was determined as the difference in the amount of laccase in the supernatant from the initial laccase (10 mg/ml) sample. The enzyme activities of the immobilized laccase on the different particle were analysed as described below.

3.3.3.6.1. Laccase activity determination

Laccase activity was determined with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mM) as the substrate in succinate-lactate buffer (0.1 M, pH 4.5). The reaction was started with the addition of 180 µl ABTS to 20 µl sample in a microtiter plate, and the product formation was followed spectrophotometrically at 420 nm with a PowerWave HT (Biotek Instrument) (Jordaan 2009a). One unit of enzyme activity corresponds to the amount of enzyme which increases the absorbance measured at 420 nm by 0.001 per min at pH 4.5 and 25°C with ABTS substrate in a 200 µl reaction mixture.

3.3.3.6.2. Statistical analysis

ANOVA (analysis of variance) enables the comparison of the means of two or more groups, under the assumption that the sampled population is normally distributed, by using *F*-statistics (Snedecor & Cochran 1989). One-way ANOVA was performed using the statistical software JMP 10.0 (SAS Institute Inc, Cary, North Carolina, United States). One-way ANOVA was used to determine whether an independent variable has a significant effect in the mean values of any groups in the data. The following null hypothesis that there is no difference in the enzyme activity binding capacity between matrix for immobilization performed at specific temperature and time points with respect to the mean units activity per mg support was tested by one-way ANOVA. The null hypotheses can be indicated as

$$H_{0(1 \text{ to } 8)}: \mu_1 = \mu_2 = \mu_3 = \mu_4$$

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where μ_1 represent the mean units activity per mg support shown by the ReSynTM group, μ_2 the mean units activity per mg support shown by the Eupergit C 250L group, μ_3 the mean units activity per mg support shown by the DEAE Sephadex A-25 group and μ_4 represent the mean units activity per mg support shown by the Eupergit C group. The null hypotheses (1) to (4) respectively relate to immobilization performed over the time points; 1-, 4-, 7- and 24 h for immobilization at 4°C. Correspondingly the null hypothesis (5) to (8) respectively relate to immobilization performed over the time points; 1-, 4-, 7- and 24 h for immobilization at 25°C. The null hypothesis were tested by the F statistic, if the F statistic is sufficiently large (and the associated p value is sufficiently small) the null hypothesis can be rejected. For the first null hypothesis, this would conclude that in the mean units activity per mg support, that immobilization on at least one of the matrixes differs from one of the other matrixes for immobilization performed for 1 h at 4°C. Subsequently, the alternative hypothesis ($H_{1(1 \text{ to } 8)}$) is that there is a difference in enzyme activity binding capacity between at least two immobilization matrixes under the specific immobilization conditions. One-way ANOVA requires a post-hoc test to be performed in order to determine which group(s) was different from the rest.

Duncan's multiple range test (DMRT) using the statistical software JMP 10.0 (SAS Institute Inc, Cary, North Carolina, United States) was employed as a multiple comparison method in order to assess which of the immobilization matrixes were significantly different from one another for immobilization performed at the different time points and respectively performed at 4°C and 25°C. The DMRT was therefore used to test for variation in the mean units activity per mg support between immobilization performed on the different matrixes. In the DMRT, the means were separated at a significance level of $p = 0.05$ when there were significant differences between the mean units activity per mg support on the different supports under specific immobilization conditions.

Summary statistical analysis of the mean units activity per mg support of laccase respectively immobilized on ReSynTM, Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C was also performed on the basis of standard deviation, standard error, lower and upper 95% confidence limit and coefficient of variation using the statistical software JMP 10.0 (SAS Institute Inc, Cary, North Carolina, United States).

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3.3.3.6.3. Optimum pH and temperature determination for free- and ReSynTM immobilized laccase

The pH optimum of free- and ReSynTM immobilized laccase was determined over the pH range of 1.5 to 7 with 0.5 pH intervals in a succinate-lactate buffer (0.1 M). The temperature optimum of free- and ReSynTM immobilized laccase was determined over the temperature range of 25°C to 70°C with 5°C intervals in universal buffer (pH 2). The universal buffer stock (10 X) comprised of Tris (0.5 M), Boric acid (0.5 M), Citric acid (0.33 M) and disodium phosphate (0.5 M) (Perrin & Dempsey 1974). The free- and ReSynTM immobilized laccase activity for both pH and temperature optimum determination was analysed as described above (3.3.3.6.1).

3.4. RESULTS

3.4.1. Synthesis of ReSyn™ particles

ReSyn™ particles were synthesized as modified from Jordaan *et al.* (2009) as described in 3.3.3.1 and resuspended in a potassium phosphate buffer (0.05 M, pH 7.0). The homogeneously resuspended samples of the emulsion derived particles were lyophilized in order to determine the dry weight of the particles.

Table 3.1: The determination of the dry weight of 1 ml synthesized ReSyn™ by lyophilisation.

Weight (mg)	70.30
	62.30
	64.40
Mean (n = 3)	65.67
SD	4.148
CV	6.316

The dry weight of the synthesized ReSyn™ was determined by lyophilisation as 65.67 mg/ml. The other supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 were made up to 100 mg/ml.

The particle size of a batch of ReSyn™ particles synthesized as described in the method section was determined to have a diameter in the range of 1.5 to 4.0 µm (Appendix B, Table 6.4). Delsa™ Nano C from Beckman Coulter Inc, California, USA which employed photon correlated spectroscopy, was used to determine the particle size based upon the measurement of the rate of fluctuations in laser light intensity scattered by the particles diffusing through water.

3.4.2. Immobilization

BSA was immobilized on the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ at 4° C and 25° C over the time points 1-, 4-, 8-, 24- and 48 h. In Appendix A two examples of BSA standard curves used for the determination of protein concentration are included. The bound BSA (mg/ml) was calculated as the difference between the BSA control and the unbound BSA.

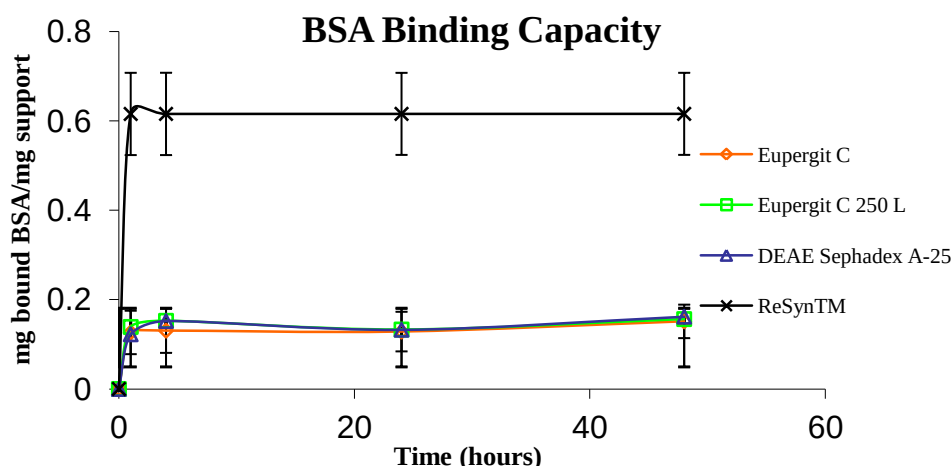


Figure 3.9: Comparative assessment of the BSA (bovine serum albumin) binding capacity of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM. The binding capacity of the different supports represents both non-covalent and covalent bound BSA. Immobilization was performed at 4°C in a potassium phosphate buffer (50 mM, pH 7.0) with horizontal rotation and stopped respectively by centrifugation at the time points 0-, 1-, 4-, 24- and 48 h. Error bars represent standard deviation (SD) of the mg bound BSA per mg support of three biological independent experiments (six replicates each).

ReSynTM enabled the rapid immobilization of BSA within the first hour. The supports Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 resulted in most of the BSA being bound within the first hour, although an increase was observed over the 48 h immobilization time period. After 48 h of immobilization 0.151-, 0.156-, 0.162- and 0.616 mg BSA per mg of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM was immobilized respectively at 4°C (Figure 3.9).

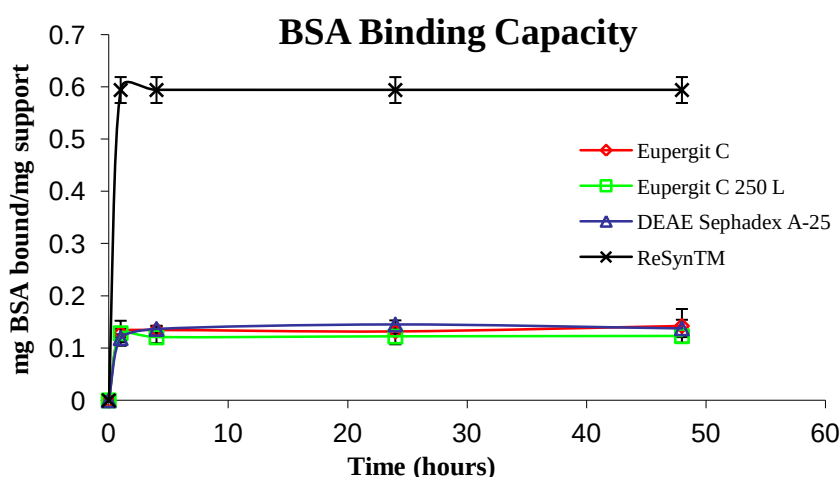


Figure 3.10: Comparative assessment of the BSA (bovine serum albumin) binding capacity of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM. The binding capacity of the different supports represents both non-covalent and covalent bound BSA. Immobilization was performed at 25°C in a potassium phosphate buffer (50mM, pH 7.0) with horizontal rotation and stopped respectively by centrifugation at the time points 0-, 1-, 4-, 24- and 48 h. Error bars represent standard deviation (SD) of the mg bound BSA per mg support of three biological independent experiments (n = 6).

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Under the same conditions, immobilization at 25°C resulted in 0.142-, 0.123-, 0.138- and 0.594 mg BSA respectively being immobilized per mg of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ (Figure 3.10). In comparison immobilization at 4°C resulted in a slight increase in the amount of BSA being bound per mg support. From these results at both temperatures, it can be seen that ReSyn™ binds approximately 4 fold more BSA per mg support than Eupergit C, Eupergit C 250L and DEAE Sephadex A-25.

3.4.3. Salt washing

The immobilized BSA on the various supports was washed twice with salt (2 M NaCl) to remove any non-covalently bound BSA.

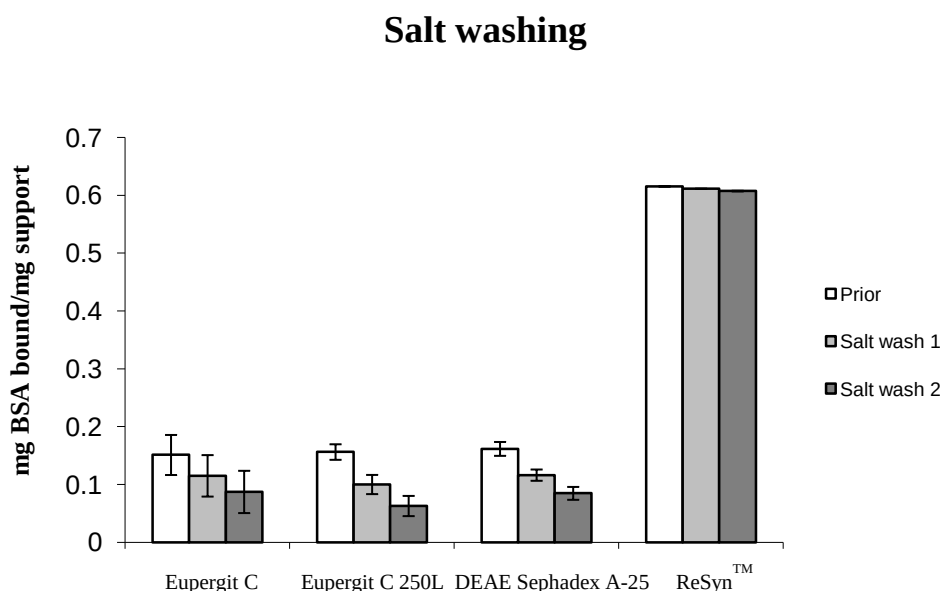


Figure 3.11: The amount of BSA bound after subsequent salt washing steps of 2M NaCl of the different supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™. BSA immobilization was performed for 48 h at 4°C. Error bars represent standard deviation (SD) of the mg bound BSA per mg support after salt washing of three biological independent experiments (n = 6).

The amount of BSA bound per mg of ReSyn™ after 48 h of immobilization at 4°C was not significantly affected (1.5% decrease), whereas a significant decrease of 42-, 60- and 48% respectively in the amount of BSA bound per mg of the supports Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 (Figure 3.11) was observed. Similar results were obtained after two salt washes of BSA immobilized for 4 h at 4°C (Figure 3.12).

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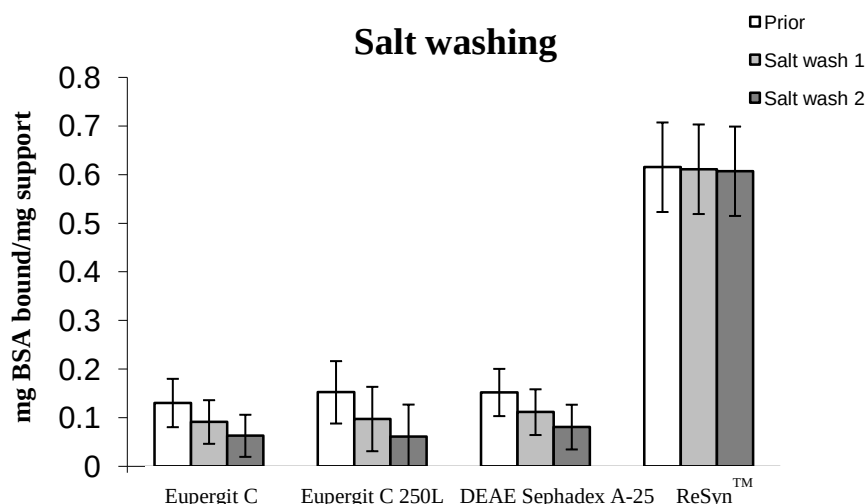


Figure 3.12: The amount of BSA bound after subsequent salt washing steps of 2 M NaCl of the different supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™. BSA immobilization was performed for 4 h at 4°C. Error bars represent standard deviation (SD) of the mg bound BSA per mg support after salt washing of three biological independent experiments (n = 6).

The BSA loading efficiency (mg BSA covalently bound/mg dry weight of support x 100) of Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ respectively was 8.7, 6.3, 8.5 and 60.7 (Figure 3.13). Therefore, ReSyn™ has approximately an 8 fold higher BSA loading efficiency than that of Eupergit C, Eupergit C 250L and DEAE Sephadex A-25.

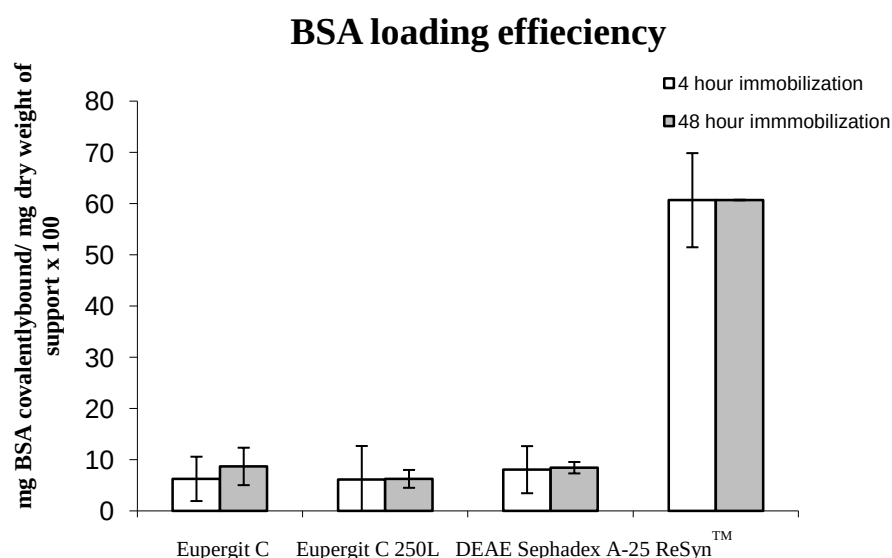


Figure 3.13: The BSA loading efficiency of the different supports determined for 4 and 48 h immobilization at 4°C. The BSA loading efficiency was calculated as the mg BSA covalently bound after two salt wash steps per mg dry weight of the support multiplied by one hundred. Error bars represent standard deviation (SD) of the mg BSA covalently bound after two salt wash steps per mg dry weight of the support multiplied by one hundred of three biological independent experiments (n = 6).

3.4.4. PPO immobilization on ReSyn™

Polyphenol oxidase purification utilizing a Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column was repeated and pooled to obtain a stock polyphenol oxidase sample (38 ml, 0.052 mg/ml) (Table 3.2).

Table 3.2: Summary of polyphenol oxidase immobilization on ReSyn™.

Average activity of pooled fractions (units)	Stock PPO con. (mg/ml)	PPO (mg) bound on ReSyn™ (mg)	Immobilized PPO activity (units/mg ReSyn™)
1453	0.052 (± 0.002)	0.0015 (± 0.0002)	0.074 (± 0.074)
	0.051 (± 0.003)	0.0014 (± 0.0002)	0.066 (± 0.009)
	0.053 (± 0.003)	0.0014 (± 0.0007)	0.067 (± 0.022)
Mean (n = 3)	0.052	0.0014	0.069
SD.	0.001	0.00005	0.005
CV	2.552	3.613	6.744

As a result of the limited quantity of purified enzyme, polyphenol oxidase was only immobilized on ReSyn™, in terms of which 0.0014 mg polyphenol oxidase bound per mg ReSyn™ (Table 3.2). The polyphenol oxidase immobilized on ReSyn™ system was post-treated with ethanolamine. Immobilized polyphenol oxidase activity was determined as 0.069 units enzyme activity per mg ReSyn™ with 4-methylcatechol as the substrate (Table 3.2).

3.4.5. Laccase sample preparation

Laccase (Novozymes® Suberase) was concentrated to 11.74 mg/ml stock solution and a buffer exchange to succinate-lactate (0.1 M, pH 4.5) was performed by ultrafiltration prior to immobilization (Table 3.3)

Table 3.3: The protein concentration determination of concentrated laccase stock.

Concentration (mg/ml)	12.06
	11.93
	12.94
	9.797
	10.80
	11.74
Mean (n = 6)	11.55
SD	1.095
CV	9.487

Assessment of the purity of the laccase stock solution by Coomassie stained SDS-PAGE identified the presence of two bands for which the molecular weight was respectively calculated as 81 kDa and 72 kDa based upon the linear relationship between the log of the molecular weight of marker bands (logkDa) and distance migrated (mm) in terms of the 100 kDa band of the marker (Figure 3.14 A and B).

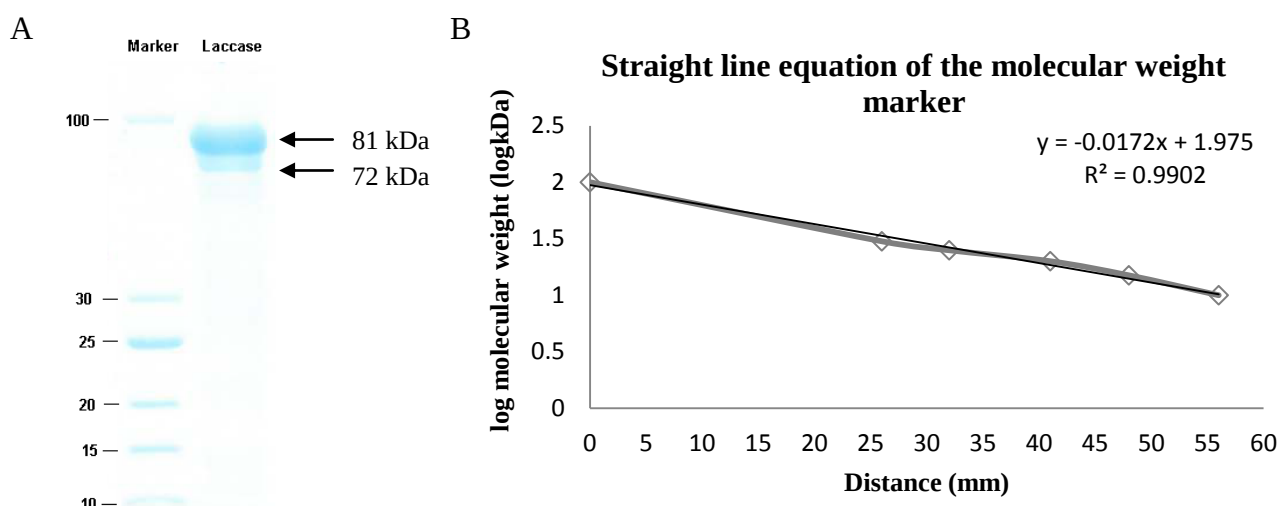


Figure 3.14: A. Coomassie stained 12% SDS-PAGE gel of ultrafiltrated laccase. Lane 1 PageRuler™ Unstained Low Range Protein Ladder marker and lane 2 laccase (58 µg) B. Straight line equation of the molecular weight marker plotted as the log of the molecular weight (kDa) against the band migration distance (mm) in terms of the 100 kDa band.

3.4.6. Laccase immobilization on ReSyn™, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25

Laccase was immobilized on the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ at 4° C and 25° C over the time points 1-, 4-, 7- and 24 h. The bound laccase (mg) was calculated based upon the difference between the laccase stock and the unbound laccase.

The various supports resulted in most of the laccase being bound within the first hour as observed in the BSA immobilization studies (Figures 3.9 and 3.15). The amount of laccase bound per mg of the supports; Eupergit C, Eupergit C 250L and ReSyn™ was constant between 1- and 24 h of immobilization at 4°C, whereas a 23% increase in the amount of laccase bound per mg DEAE Sephadex A-25 was observed.

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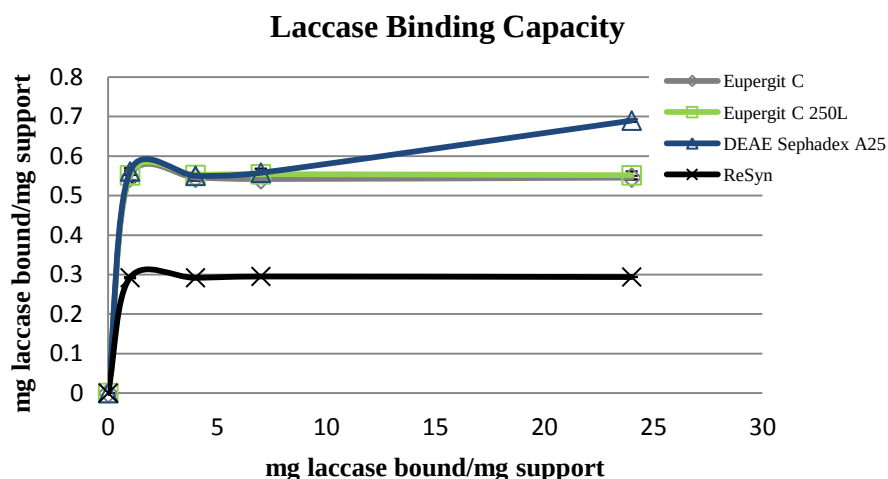


Figure 3.15: Comparative assessment of the laccase binding capacity of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM. The binding capacity of the different supports represents both non-covalent and covalent bound laccase. Immobilization was performed at 4°C in a succinate-lactate buffer (0.1 M, pH 4.5) with horizontal rotation and stopped respectively by centrifugation at the time points 0, 1, 4, 7 and 24 h. Error bars represent standard deviation (SD) of the mg bound laccase per mg support of three biological independent experiments (n = 3).

In contrast to immobilization performed at 4°C, most laccase was only bound after 4 h of immobilization when immobilization was performed at 25°C (Figures 3.15 and 3.16). The amount of laccase bound per mg of the supports; Eupergit C, Eupergit C 250L and ReSynTM was relatively constant between 4- and 24 h of immobilization performed at 25°C, similarly the amount of laccase bound per mg DEAE Sephadex A-25 remained constant in contrast to the 23% increase observed during immobilization at 4°C (Figures 3.15 and 3.16).

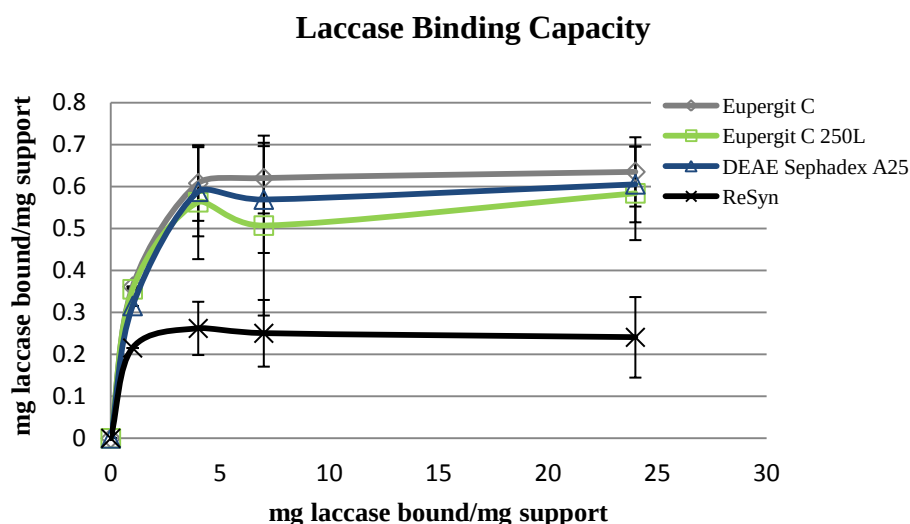


Figure 3.16: Comparative assessment of the laccase binding capacity of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM. The binding capacity of the different supports represents both non-covalent and covalent bound laccase. Immobilization was performed at 25°C in a succinate-lactate buffer (0.1 M, pH 4.5) with horizontal rotation and stopped respectively by centrifugation at the time points 0, 1, 4, 7 and 24 h. Error bars represent standard deviation (SD) of the mg bound laccase per mg support of three biological independent experiments (n = 3).

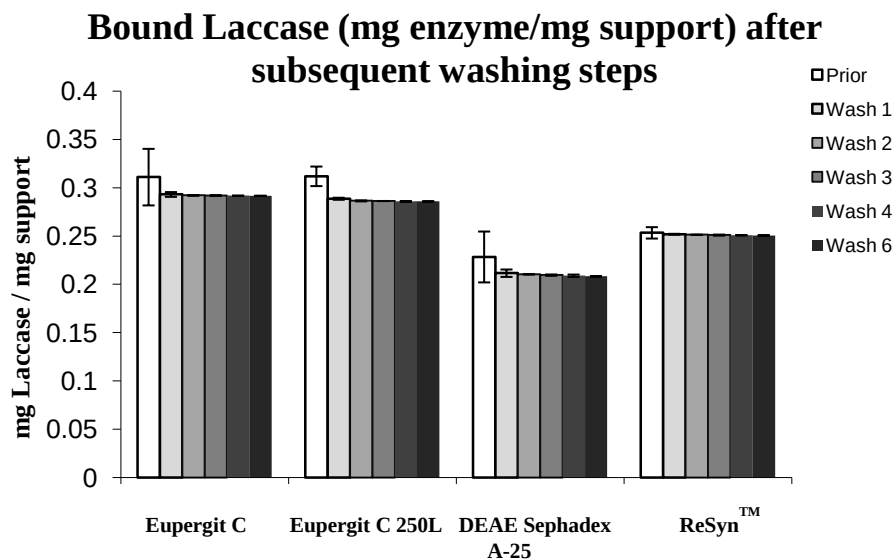


Figure 3.17: The amount of bound laccase (mg laccase/mg support) after subsequent water washing steps of the different supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™. Laccase immobilization was performed for 1 h at 25°C. Error bars represent standard deviation (SD) of the mg bound laccase per mg support after water washing of three biological independent experiments (n = 3).

Laccase immobilized on the various supports were washed six times consecutively with deionised water (2 ml). Results were determined for laccase immobilized for an hour at 25°C. After six consecutive washes, the amount of laccase per mg support decreased by 6.22%, 8.36%, 8.83% and 0.99% respectively for the supports; Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ (Figure 3.17).

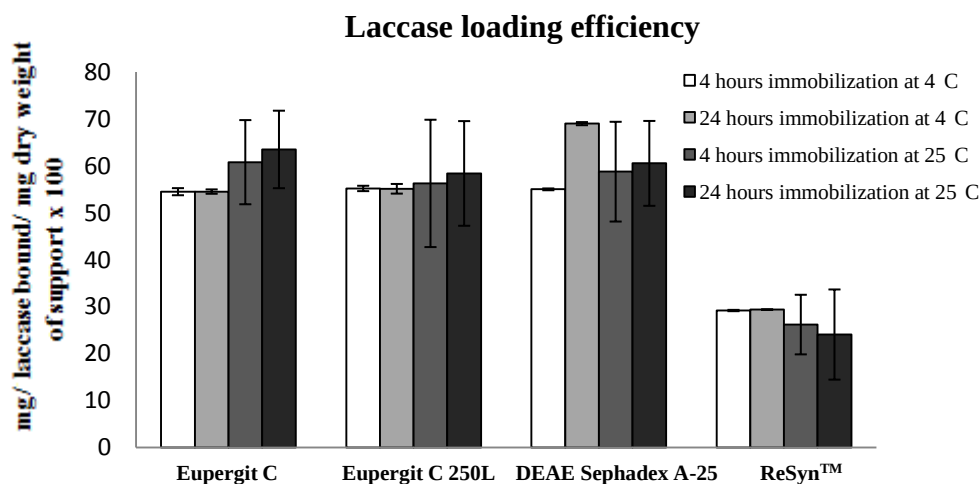


Figure 3.18: The laccase loading efficiency of the different supports determined for 4 and 48 h immobilization at 4°C and 25°C. The laccase loading efficiency was calculated as the mg/ml laccase bound after six consecutive deionised water washing steps per mg dry weight of the support multiplied by one hundred. Error bars represent standard deviation (SD) of the mg/ml laccase bound after six consecutive deionised water washing steps per mg dry weight of the support multiplied by one hundred of three biological independent experiments (n = 6).

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The laccase loading efficiency (mg laccase bound/mg dry weight of support x 100) of Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM after 4 h of immobilization at 4°C respectively was 54.50, 55.19, 55.00 and 29.19 (Figure 3.18). The laccase loading efficiencies of the various supports remained similar for immobilization performed for 24 h at 4°C, except for a 25% increase in laccase loading efficiency of DEAE Sephadex A-25. An increase of respectively 4%, 4% and 3% in the laccase loading efficiency between 4- and 24 h of immobilization at 25°C was determined for the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and a decrease of 8% was determined for ReSynTM.

3.4.6.1. Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate significant differences in the mean units activity per mg support (enzyme activity binding capacity) for the different immobilization matrixes; Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM respectively at the different immobilization time points; 1-, 4-, 7- and 24 h of immobilization separately at 4°C and 25°C (Appendix B Table 6.3). The one-way ANOVA analysis revealed a significant effect for the type of immobilization matrix, $F(3, 86) = 131.05$, $p < 0.001$ for immobilization performed for 1 h at 4°C. Furthermore, a significant effect for the type of immobilization matrix, $F(3, 92) = 104.13$ ($p < 0.001$), $F(3, 80) = 386.95$ ($p < 0.001$) and $F(3, 92) = 213.40$ ($p < 0.001$) were respectively observed for immobilization performed for 4-, 7- and 24 h at 4°C. Similarly, a significant effect for the type of immobilization matrix, $F(3, 81) = 162.72$ ($p < 0.001$), $F(3, 92) = 197.44$ ($p < 0.001$), $F(3, 92) = 480$ ($p < 0.001$) and $F(3, 92) = 50.390$ ($p < 0.001$) were respectively observed for immobilization performed at the time points; 1, 4, 7 and 24 h of immobilization at 25°C.

Duncan's multiple range test (DMRT) was employed as a multiple comparison method in order to assess which of the immobilization matrixes were significantly different from one another for immobilization performed at the different time points and respectively performed at 4°C and 25°C. Utilizing the Duncan's multiple range test the mean enzyme activity binding capacity (units enzyme activity/mg support) were separated at a significance level of $p = 0.05$ when there were significant differences between the mean activity on the different supports under specific immobilization conditions. The mean grouping from a to d was in decreasing order of the enzyme activity binding capacity (units enzyme activity/mg support). It can be concluded that the statistical significant difference in all the mean activities of laccase immobilized on ReSynTM (a),

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Eupergit C 250L (b), Eupergit C (c) and DEAE Sephadex A-25 (d) over the time points; 1-, 4-, 7- and 24 h was respectively determined at a 95% level of confidence for immobilization performed at 4°C (Figure 3.19). Similarly, the mean activities of laccase immobilized on ReSyn™ (a), Eupergit C 250L (b), Eupergit C (c) and DEAE Sephadex A-25 (d) respectively over the time points; 7- and 24 h at 25°C were statistically different at a 95% level of confidence. No statistical significant difference at a 95% level of confidence was observed in the mean activity of laccase immobilized on Eupergit C 250L (b) and DEAE Sephadex (b) for immobilization respectively performed for 1- and 4 h at 25°C. Only for immobilization performed for 7 h at 4°C was the mean enzyme activity binding capacity of laccase immobilized on Eupergit C higher than that on DEAE Sephadex A-25.

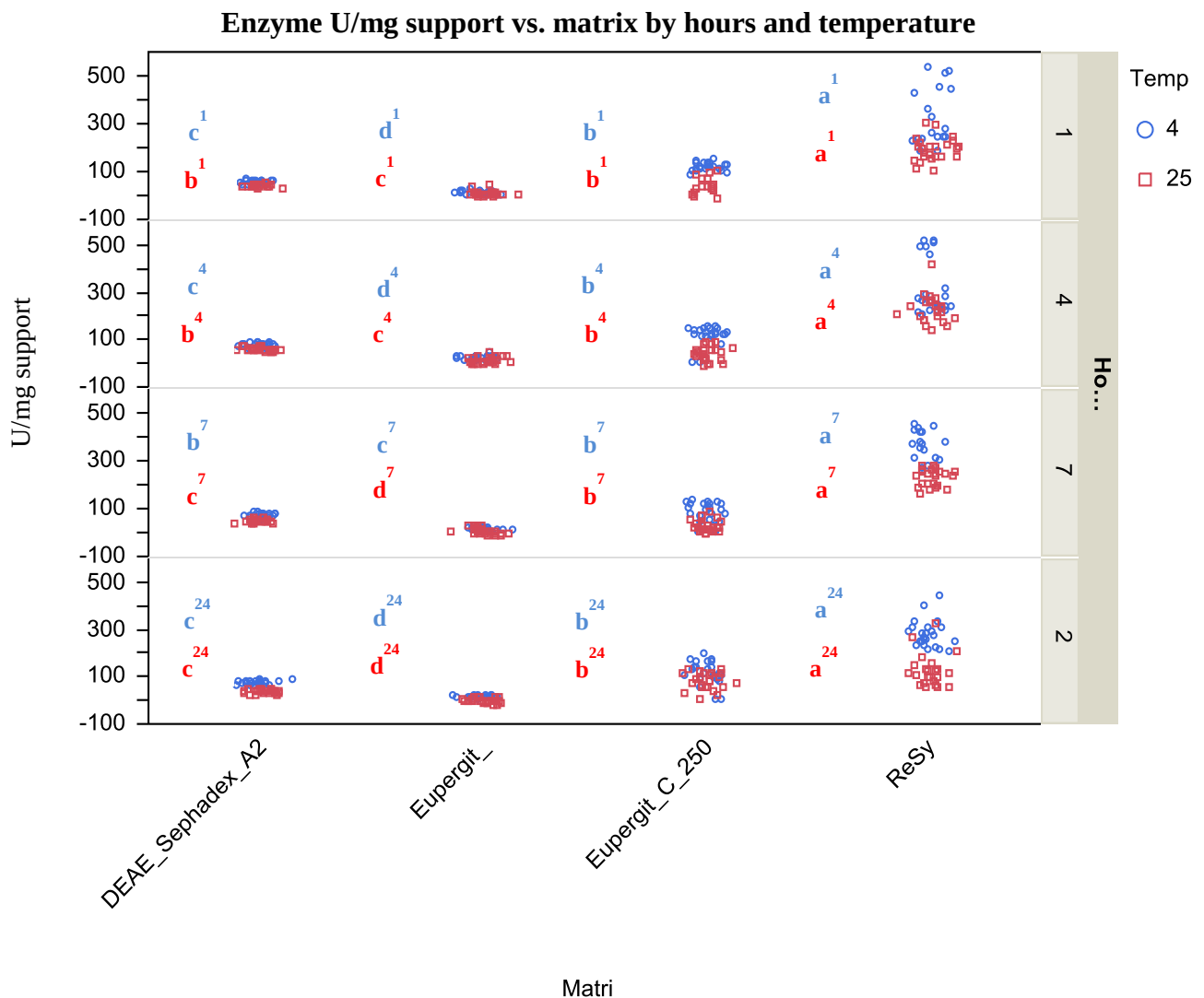


Figure 3.19: The enzyme activity binding capacity (units enzyme activity/mg support) distribution of immobilized laccase on the various supports; DEAE Sephadex A-25, Eupergit C, Eupergit C 250L and ReSyn™. Blue and red respectively represent immobilization performed at 4°C and 25°C. The time points; 1-, 4-, 7- and 24 h are indicated as superscripted values. The group activity distributions under specific immobilization conditions followed by a common letter (a-d) with a common superscript immobilization time point are not significantly different ($P > 0.05$) according to the Duncan's multiple range test (DMRT), performed utilizing the statistical software JMP 10.0 (SAS Institute Inc, Cary, North Carolina, United States).

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Summary statistical analysis of the mean units activity per mg support of laccase respectively immobilized on ReSynTM, Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C was performed on the basis of standard deviation, lower and upper 95% confidence limit and coefficient of variation (Appendix B Figure 6.3). On average the coefficient of variation for immobilization respectively over the time points at both 4°C and 25°C, was in decreasing order of 117 ($\pm$ 121), 51 ($\pm$ 23), 28 ($\pm$ 12) and 13 ($\pm$ 12) respectively for the supports; Eupergit C, Eupergit C 250L, ReSynTM and DEAE Sephadex A-25.

The comparative assessment of laccase activity on the different supports was based upon the comparison of the lower 95% confidence level of the mean (units enzyme activity per mg immobilization support) determined for ReSynTM with the upper 95% confidence level the mean of the other supports under corresponding conditions of immobilization. The lower 95% confidence level of the mean for ReSynTM was determined as 266, 262, 339 and 252 respectively, corresponding to the time points; 1-, 4-, 7- and 24 h of immobilization at 4°C (Appendix B, Figure 6.3). Under the same immobilization conditions, the upper 95% confidence level of the mean for the supports; Eupergit C 250L respectively corresponded to 124, 133, 100 and 135, DEAE Sephadex A-25 was respectively determined as 54, 79, 73 and 74 and for Eupergit C it respectively was 10, 25, 15 and 14. Under immobilization conditions of 25°C over the time points; 1-, 4-, 7- and 24 h the lower 95% confidence level of the mean for ReSynTM was respectively determined as 175, 207, 219 and 102. Correspondingly, under the same immobilization conditions the upper 95% confidence level of the mean for the supports; Eupergit C 250L respectively was 65, 56, 43 and 99, for DEAE Sephadex A-25 this was respectively determined as 47, 66, 55 and 44 and for Eupergit C it respectively corresponded to 16, 23, 15 and 7.

3.4.6.2. Optimum pH and temperature determination for free- and ReSynTM immobilized laccase

The pH-activity profile of free- and ReSynTM immobilized laccase was determined over the pH range of 1.5 to 7 with 0.5 pH intervals in succinate-lactate buffer (0.1 M) and is shown in Figure 3.20. Free laccase has an observed optimum pH at 2, followed by a sharp decrease in activity (units) between pH 2 to 7. ReSynTM immobilized laccase had a corresponding observed optimum pH at 2, with additional peaks between pH 4 to 5.5 (at pH 4.5) and between pH 5.5 to 6.5 (at pH 6).

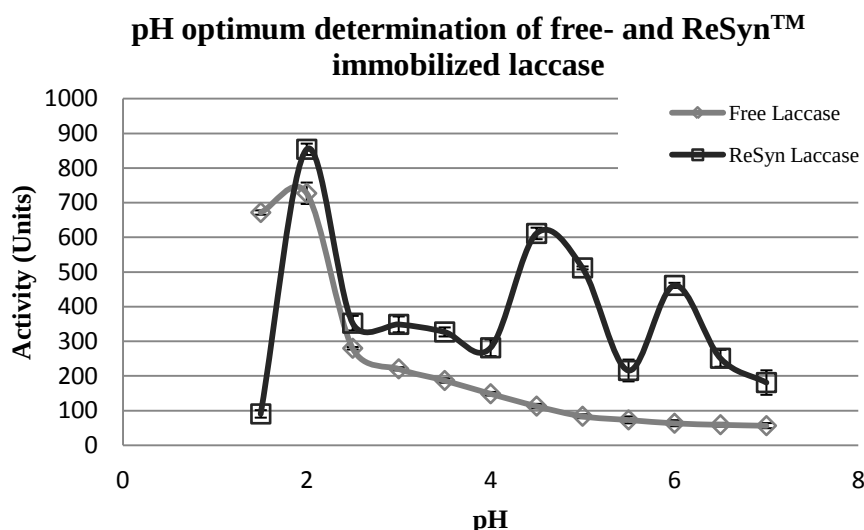


Figure 3.20: The pH optimum determination of ReSyn™ immobilized laccase and free laccase over a pH range of 1.5 to 7 with 0.5 pH intervals, with ABTS as the substrate in succinate-lactate buffer (0.1 M). Error bars indicate the standard deviation of the activity (units) of three biological independent experiments (3 replicates each).

The temperature optimum of free- and ReSyn™ immobilized laccase was determined over the temperature range of 25°C to 70°C with 5°C intervals in universal buffer (pH 2) as indicated in Figure 3.21. Free laccase indicated a broad peak in activity between 35°C and 70°C which was associated with slight variations in enzyme activity. ReSyn™ immobilized laccase had an optimum temperature at 50°C, with a small shoulder at 35°C.

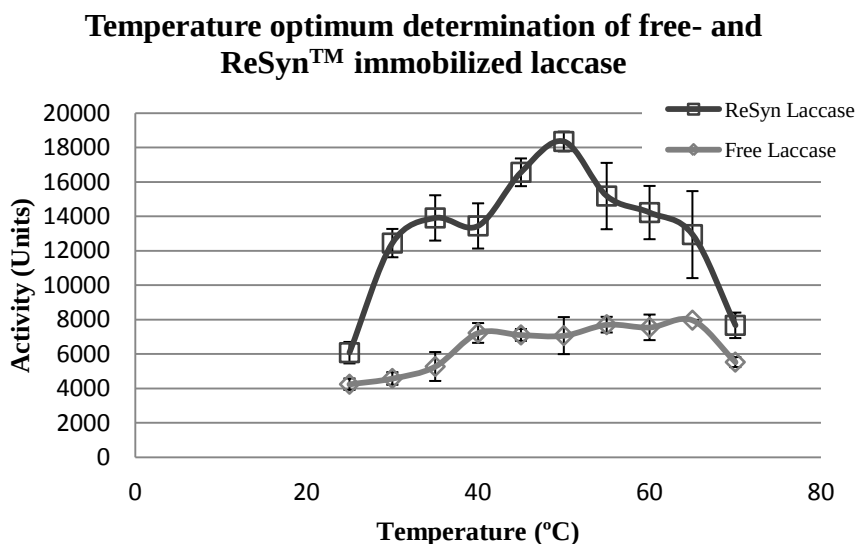


Figure 3.21: Temperature optimum determination of ReSyn™ immobilized laccase and free laccase was determined over a temperature range of 25°C to 70°C with 5°C intervals, with ABTS as substrate in universal buffer (0.1 M, pH 2). Error bars indicate the standard deviation of the activity (units) of three biological independent experiments (3 replicates each).

3.5. DISCUSSION

Initial comparative assessment of immobilization on the different supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM was performed with BSA. ReSynTM particles were synthesized using the method of Jordaan *et al.* (2009) but modified as described in the text, by employing a stirring method as opposed to vortexing when the two emulsions are added together, as this would be a more applicable method for up scaled particle synthesis. ReSynTM particles are disadvantaged by their inability to be dried as the integrity of the emulsion derived lattice with interstitial openings around and adjacent to the stands would be lost during drying. Therefore, the dry weights of homogenously resuspended samples of emulsion derived particles were determined to enable accurate between support comparisons. The dry weight of the synthesized ReSynTM particles was determined by lyophilisation as 65.67 mg/ml.

Immobilization was performed at 4°C and 25°C over the time points 1-, 4-, 8-, 24- and 48 h. Comparative assessment of the binding capacity of the different supports indicated that ReSynTM enabled the rapid immobilization of BSA within the first hour and was stable between 4- and 48 h of immobilization. The other supports, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25, also resulted in most of the BSA being bound within the first hour, although an increase was observed over the 48 h immobilization time period. After 48 h of immobilization respectively 0.151-, 0.156-, 0.162- and 0.616 mg BSA per mg of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM was immobilized at 4°C (Figure 3.9). On average the amount of BSA bound by ReSynTM was 3.9 fold higher per mg support than that of the other supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25. This extremely high load of BSA attained by ReSynTM was as a result of the open fibre network and high amine density (Brady & Jordaan 2009). BSA immobilization at 25°C resulted in 0.142-, 0.123-, 0.138- and 0.594 mg BSA respectively being immobilized per mg of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM (Figure 3.10). This corresponds to 94-, 78-, 85-, and 96% of the amount of BSA that was bound at 4 °C respectively for Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM. Eupergit C 250L bound less BSA per mg support than Eupergit C, this was due to Eupergit C 250L having fewer epoxide groups subsequently resulting in a larger quantity of polymer required to achieve the same extent of protein binding. The presence of a more polymer matrix with a lower distribution of active groups (> 250 µmole/g dry weight) of Eupergit C 250L is beneficial in terms of enzyme activity (Hernaiz & Crout 2000).

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BSA immobilized for 48 h at 4°C on the various supports was washed twice with salt (2 M NaCl) in order to assess the strength of the bonds formed between the BSA and the various supports. The amount of bound BSA decreased after each wash resulting in a significant decrease of 42-, 60- and 48% respectively in the amount of BSA bound per mg of the supports Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 after two salt washes (Figure 3.11). These results indicate that the nature of the binding between the BSA, and these supports are non-covalent. Covalent bonds are much stronger than non-covalent bonds (ionic interactions, hydrogen bonds and van der Waals interactions) and subsequently, the presence of salt would not be able to out-compete the BSA bound to the supports. The 48% decrease in the amount of BSA bound per mg DEAE Sephadex A-25 corresponds to literature indicating that electrostatic interactions are formed between enzymes and the anion exchanger DEAE-Sephadex support (adsorption) (Baron *et al.* 1997). This demonstrates the disadvantage of protein immobilized by adsorption where high salt concentrations can lead to ion exchange and washing out of the immobilized protein. Immobilization was performed at the optimum pH of polyphenol oxidase (pH 7) for subsequent immobilization analysis (Kazandjian & Klibanov 1985). The overall surface charge of BSA would be negative at pH 7, as the pH value is larger than the protein's pI value of 4.7, subsequently amounting to favourable conditions for the formation of electrostatic interactions between BSA and the anion exchanger.

The data for the Eupergit resins does not correspond to literature indicating that enzymes can be covalently immobilized on Eupergit C and Eupergit C 250L based upon the indicated contribution of non-covalent bonding (Katchalski-Katzir & Kraemer 2000). BSA was immobilized at pH 7 and therefore, should be bound to Eupergit C and Eupergit C 250L via epoxide groups that react with thiol groups on the protein. The immobilization on these supports was a function of pH, as the epoxide groups would react with amine groups at pH 8, phenolic groups at pH 11 and with carboxyl groups at slightly acidic pH (Boller *et al.* 2002). The loss in bound BSA corresponds to non-covalently bound BSA, although covalent interactions as described above between the Eupergit C and Eupergit C 250L supports may still be present. The influence of initial BSA not bound to the supports but merely still in solution between the particles is very minimal due to the six consecutive washes with deionised water of 2 ml each and is evident from the results obtained for ReSynTM. After two salt washes, only a 1.5% decrease in the amount of BSA bound per mg ReSynTM was determined, indicating that the BSA is covalently bound to the support. Covalent bonds are formed by the interaction of epoxide or aldehyde groups of ReSynTM with amine groups of the protein. Based upon the ratio of the polymer (polyethylene imine) and the crosslinker (glutaraldehyde) the synthesized ReSynTM particles would have aldehyde functionality, as the

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particles were synthesized under limiting conditions of the crosslinker. The BSA is tightly fixed onto ReSynTM through the covalent immobilization and subsequently minimizes leaching of the enzyme in an aqueous medium. Although ReSynTM, Eupergit C and Eupergit C 250L are reported to achieve protein immobilization via covalent bond formation, these results indicate that covalent bonds are formed to a much larger extent between ReSynTM and BSA. These results were confirmed by two consecutive salt (2 M NaCl) washes of BSA immobilized for 4 h at 4°C on the various supports (Figure 3.12).

The BSA loading efficiency (mg BSA covalently bound/mg dry weight of support x 100) of Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM respectively was 8.7, 6.3, 8.5 and 60.7 (Figure 3.13). Therefore, ReSynTM has approximately an 8 fold higher BSA loading efficiency than that of Eupergit C, Eupergit C 250L and DEAE Sephadex A-25. Based upon DEAE Sephadex A-25 achieving immobilization by means of adsorption by electrostatic interaction, a low BSA loading efficiency (mg BSA covalently bound/mg dry weight of support x 100) was expected. This was, however, not expected for Eupergit C and Eupergit C 250L based upon the literature reported covalent nature of immobilization using these supports (Boller *et al.* 2002). The extremely high BSA load (mg BSA covalently bound/mg dry weight of support x 100) attained by ReSynTM is as a result of the open fibre network and high amine density (Brady & Jordaan 2009).

Immobilization of purified polyphenol oxidase resulted in the binding of a low amount of enzyme onto the support, which was associated with the purity of the enzyme and subsequent undesirable binding onto the support. In order to prevent further undesirable binding onto the support the ReSynTM the immobilized system was post-treated with ethanolamine. The post-treatment step enabled unreacted epoxide and aldehyde groups present on ReSynTM to be removed by covalent bond formation with ethanolamine, preventing subsequent immobilization reactions onto the support. Based upon the small molecular nature of ethanolamine (C₂H₇NO) steric influences with the immobilized polyphenol oxidase should be minimal. Immobilized polyphenol oxidase activity was determined as 0.069 units enzyme activity per mg ReSynTM with 4-methylcatechol as the substrate (Table 3.2). The ethanolamine post-treatment has been reported to amount to a reduction in immobilized enzymatic activity, but also to offer the advantages of improved stability towards oxidative denaturation by mediators and enhanced temperature and acidic pH stability (Jordaan *et al.* 2009).

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The subsequent immobilization studies were performed by employing laccase, as it is also capable of converting catechins into theaflavins (Itoh *et al.* 2007). Suberase® a fungal laccase from *Myceliophthora thermophila* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* strain gifted from Novozyme (Pty) Ltd, Gauteng, South Africa was used to comparatively assess immobilization on the different supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™. The laccase was concentrated to an 11.74 mg/ml stock solution and a buffer exchange to succinate-lactate (0.1 M, pH 4.5) was performed by ultrafiltration (Table 3.3). Coomassie stained SDS-PAGE assessment of the purity of the laccase stock solution determined two bands respectively calculated as 81 kDa and 72 kDa, based upon the straight line equation determined from the molecular weight marker, were consistent with the reported molecular mass of 75 to 95 kDa (Berka *et al.* 1997).

Laccase was immobilized on the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ over the time points 1-, 4-, 7- and 24 h respectively at 4°C and 25°C. Immobilization performed at 4°C, resulted in most of the laccase being bound within the first hour, as was observed in the BSA immobilization studies (Figures 3.9 and 3.15). The amount of laccase bound per mg support; Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ corresponded to 0.546, 0.551, 0.561 and 0.292 after one hour of immobilization at 4°C. The amount of laccase bound per mg of the supports; Eupergit C, Eupergit C 250L and ReSyn™ was constant between 1- and 24 h of immobilization at 4°C, which respectively corresponded to 0.545, 0.551 and 0.294 mg laccase bound per mg support. In contrast, a significant increase of 23% in the amount of laccase bound per mg DEAE Sephadex A-25 was observed between 1- and 24 h under the same immobilization conditions. Immobilization was performed at the optimum pH of laccase of pH 4.5 (Jordaan *et al.* 2004). Laccase from *Myceliophthora thermophila* produced by *Aspergillus oryzae* was reported to have a pI value of 4.2, resulting in a slight overall negative protein surface charge at a pH of 4.5 (Berka *et al.* 1997). As explained above, this will amount to favourable conditions for the formation of electrostatic interactions between laccase and the anion exchanger, DEAE Sephadex A-25. The conditions for electrostatic bond formation would be more favourable at an increased pH value. Immobilization of laccase on Eupergit C and Eupergit C 250L is as a function of pH, which at a pH of 4.5 was based upon the formation of covalent bonds between the epoxide groups on the support and carboxylic acid groups (Boller *et al.* 2002). In comparison to the other supports, immobilization on ReSyn™ would be to a lesser extent be affected by pH, as immobilization is based upon the formation on covalent bonds between aldehyde groups of ReSyn™ with amine groups of the protein.

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Immobilization performed at 25°C, resulted in most of the laccase only being bound after 4 h of immobilization, in contrast to the rapid immobilization after an hour at 4°C (Figures 3.16 and 3.15). The decreased temperature favoring an increased immobilization rate was in contrast to literature in which case it was indicated that rate of immobilization by adsorption increased with temperature (Lei *et al.* 2004) and that temperature has no effect on the amount of covalently bound protein (for long enough immobilization periods such as 120 min) (Bahar & Celebi 1999). The difference in the rate of immobilization between 4°C and 25°C can't be attributed to a difference in the exposure of the supports to the protein solution as immobilization was performed under the same conditions. The amount of laccase bound per mg support; Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM corresponded respectively to 0.361, 0.355, 0.315 and 0.215 after an hour and 0.608, 0.563, 0.588 and 0.262 after 4 h of immobilization at 25°C. The amount of laccase bound per mg of the supports; Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM was relatively constant between 4- and 24 h of immobilization at 25°C, which respectively corresponded to 0.635, 0.584, 0.605 and 0.241 mg laccase bound per mg support. This was in contrast to the 23% increase in the amount of laccase bound per mg DEAE Sephadex A-25 at 4°C. The absence of the observed increase at 4°C of the amount of laccase bound per mg DEAE Sephadex A-25 can be attributed to the decreasing stability of the enzyme at room temperature for a prolonged period of time and the associated much weaker nature of non-covalent bonds (ionic interactions) between laccase and the support. This was consistent with literature indicating that the stability of immobilized enzymes was dependent on the nature of the bonds between the carrier and the enzyme (Cao 2005).

A significantly decreased amount of laccase was immobilized on ReSynTM in comparison to the determined BSA loading capacity of ReSynTM (for example 0.29 mg laccase/mg ReSynTM as opposed to 0.62 mg BSA/mg ReSynTM for immobilization performed for 1 h at 4°C) (Figure 3.9 and 3.15). The decreased amount of bound laccase was attributed to the increased size of the protein, determined as 81 kDa and 72 kDa (Figure 3.14) as opposed to the reported 66 kDa of BSA, and the presence of a carbohydrate moiety on laccase, as all described laccase to date have been shown to be glycoproteins (Mayer & Staples 2002).

In order to ensure the removal of unbound laccase but not to adversely affect the immobilized laccase activity, the laccase immobilized on the various supports were washed six consecutive times with deionised water (instead of sodium chloride). Results were indicated for laccase immobilized for an hour at 25°C, but after six consecutive washes, the amount of laccase per mg support decreased by 6.22%, 8.36%, 8.83% and 0.99% respectively for the supports; Eupergit C,

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Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ (Figure 3.17). From the small decrease in the percentage bound laccase per mg support; Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ respectively correspond to 0.03%, 0.01%, 0.36% and 0.10% between the fifth and sixth wash, it can be concluded that almost all the unbound laccase has been removed.

The laccase loading efficiency (mg laccase bound/mg dry weight of support x 100) of Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ after 4 h of immobilization at 4°C respectively was 54.50, 55.19, 55.00 and 29.19 (Figure 3.18) as opposed to the BSA loading efficiency (mg BSA covalently bound/mg dry weight of support x 100) which was respectively determined as 8.7, 6.3, 8.5 and 60.7 (Figure 3.13). The ReSyn™ laccase loading efficiency was consistent with the between 30%- and 36% *m/m* protein loading reported for a range of enzyme classes (Jordaan *et al.* 2009). The difference in the laccase and BSA loading efficiency of the various supports was dependent on the favourable or non-favourable combination between the proteins and supports as well as the immobilization conditions, where factors such as pH influenced the efficiency of immobilization. The laccase loading efficiencies of the various supports remained similar for immobilization performed for 24 h at 4°C, except a 25% increase in laccase loading efficiency of DEAE Sephadex A-25. The increase in the amount of bound laccase per mg DEAE Sephadex A-25 and the corresponding increase in laccase loading efficiency can only be contributed to the combination of the variables of time (24 h) and temperature (4°C) which amounted to more favourable conditions for laccase immobilization (by electrostatic interactions) on the anion exchanger. An slight increase of respectively 4%, 4% and 3% in the laccase loading efficiency between 4- and 24 h of immobilization at 25°C was determined for the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and a decrease of 8% of the laccase loading efficiency was determined for ReSyn™.

One-way analysis of variance (ANOVA) was used to evaluate significant differences in the mean units activity per mg support for the different immobilization matrixes; Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSyn™ respectively at the different immobilization time points; 1-, 4-, 7- and 24 h of immobilization separately performed at 4°C and 25°C (Appendix B Table 6.3). From the one-way ANOVA analysis, the model is the predictor variable and its associated degrees of freedom (DF) are equal to $k-1$, where k is the number of experimental groups. Comparison of the sum of squares (SS) of the predictor with the total sum of squares gives an indication of the variation in the enzyme activity binding capacity data that is accounted for by the predictor variable.

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R^2 is the ratio of the predictor sum of squares to the corrected sum of squares. The R^2 statistic represents the percentage of variance in the response that corresponds to or is explained by the variability in the predictor variable. It is a measure of the strength of the relationship between the predictor and the response, with values closer to 0 indicating a weaker relationship and values closer to 1 a stronger relationship. If the independent variable (immobilization matrix) has a very weak effect on the activity of the immobilized enzyme, R^2 will be small and vice versa. Relatively large R^2 values for the various one-way ANOVA analyses were determined as 0.82, 0.77, 0.94 and 0.87 for immobilization performed at 4°C and 0.86, 0.87, 0.94 and 0.62 for immobilization performed at 25°C respectively over the time points; 1-, 4-, 7- and 24 h of immobilization, indicating strong relationships between the predictor and the response. The R^2 value of 0.82 meant that 82% of the total variance of the activity units activity per mg support is accounted for by the between-groups variation of the immobilization matrix variable, for immobilization performed for an hour at 4°C. Based upon the determined strength of the relationships between the predictor variable and the response, indicated by the R^2 values, it can be deduced that the results are truly meaningful. The mean square of the model (mean square between groups) was equal to the sum of squares divided by its associated degrees of freedom.

In order to test the null hypothesis the F statistic (F ratio) was calculated as the ratio of the model mean square to the error mean square. The one-way ANOVA analyses revealed a significant effect for the type of immobilization matrix, $F(3, 86) = 131.05$ ($p < 0.001$) for immobilization performed for 1 h at 4°C. Furthermore, significant effects for the type of immobilization matrix, $F(3, 92) = 104.13$ ($p < 0.001$), $F(3, 80) = 386.95$ ($p < 0.001$) and $F(3, 92) = 213.40$ ($p < 0.001$) were respectively observed for immobilization performed for 4-, 7- and 24 h at 4°C. Similarly, a significant effect for the type of immobilization matrix, $F(3, 81) = 162.72$ ($p < 0.001$), $F(3, 92) = 197.44$ ($p < 0.001$), $F(3, 92) = 480$ ($p < 0.001$) and $F(3, 92) = 50.390$ ($p < 0.001$) was respectively observed for immobilization performed at the time points; -1, -4, -7 and 24 h of immobilization at 25°C. The probability ($Pr > F$) associated with the F ratio, is the probability of obtaining (by chance alone) an F statistic in the analysis that is larger or as large if the null hypothesis were true. The probability associated to all the F ratios was determined as $p < 0.001$. The significant F statistics (with the sufficiently small associated p values) amounted to the rejection of the null hypotheses, that there is no difference in the enzyme activity binding capacity between matrix for immobilization performed at specific temperature and time points with respect to the mean units activity per mg support. Therefore, the rejection of null hypotheses (1) to (4) respectively relates to immobilization performed over the time points; 1-, 4-, 7- and 24 h immobilization at 4°C. Correspondingly the null hypothesis (5) to (8) that respectively relate to

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immobilization performed over the time points; 1-, 4-, 7- and 24 h for immobilization at 25°C were also rejected. For the first null hypothesis, this would conclude that in the mean units activity per mg support, immobilization on at least one of the matrixes differs from one of the other matrixes for immobilization performed for 1 h at 4°C. Subsequently, the alternative hypothesis ($H_{1(1 \text{ to } 8)}$) that there is a difference in enzyme activity binding capacity between at least two immobilization matrixes under the specific immobilization conditions can be accepted.

A post-hoc test was performed as one-way ANOVA doesn't enable the determination of which group(s) was different from the rest. Duncan's multiple range test (DMRT) was employed as a multiple comparison method in order to assess which of the immobilization matrixes were significantly different from one another for immobilization performed at the different time points and respectively performed at 4°C and 25°C. The DMRT was therefore used to test for variation in the mean units activity per mg protein between immobilization performed on the different matrixes. Utilizing the Duncan's multiple range test the mean activity (units enzyme activity per mg support) were separated at a significance level of $p = 0.05$ when there were significant differences between the mean activity on the different supports under specific immobilization conditions. The mean grouping from a to d was in decreasing order of the activity (units enzyme activity per mg support). It can be concluded that the statistical significant difference in all the mean specific activities of laccase immobilized on ReSynTM (a), Eupergit C 250L (b), Eupergit C (c) and DEAE Sephadex A-25 (d) over the time points; 1-, 4-, 7- and 24 h was respectively determined at a 95% level of confidence for immobilization performed at 4°C (Figure 3.19). Similarly, the mean activities of laccase immobilized on ReSynTM (a), Eupergit C 250L (b), Eupergit C (c) and DEAE Sephadex A-25 (d) respectively over the time points; 7- and 24 h at 25°C were statistically different at a 95% level of confidence. No statistical significant difference at a 95% level of confidence was observed in the mean activity of laccase immobilized on Eupergit C 250L (b) and DEAE Sephadex (b) for immobilization respectively performed for 1- and 4 h at 25°C. Only for immobilization performed for 7 h at 4°C was the mean activity of laccase immobilized on Eupergit C higher than that on DEAE Sephadex A-25.

Summary statistical analysis was performed to assess whether the activity distribution plot correctly reflected the apparent increased variation in the ReSynTM immobilized laccase activity data points in comparison to the other supports (Figure 3.19). Summary statistical analysis of the mean units activity per mg support of laccase respectively immobilized on ReSynTM, Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C was performed on the basis of standard deviation, standard error, lower and upper 95% confidence limits and coefficient of variation. On

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average the coefficient of variation for immobilization overall the time points at both 4°C and 25°C, was in the decreasing order of 117 ($\pm$ 121), 51 ($\pm$ 23), 28 ($\pm$ 12) and 13 ($\pm$ 12) respectively for the supports; Eupergit C, Eupergit C 250L, ReSynTM and DEAE Sephadex A-25 (Appendix B Figure 6.3). The overall high coefficient of variation values was attributed to an inherent difficulty associated with the accurate and reproducible removal of the same amount of particles with immobilized enzyme for activity determination, which is directly influenced by the distribution of the particles in solution. Determining factors influencing the distribution of the particles in solution are associated with the nature and size of the particles.

The particle size of the epoxy-activated acrylic beads; Eupergit C and Eupergit C 250L ranges between 100-250 μ m (Boller *et al.* 2002) and DEAE Sephadex A-25 between 40-120 μ m. The nature and larger particle size of the epoxy-activated acrylic beads amounted to the settling of the particles in solution and correspondingly a higher associated coefficient of variation in the activity determination of laccase immobilized on the supports. The low coefficient of variation of enzymatic activity determined on DEAE Sephadex A-25 particles comprising of cross-linked dextran, was attributed to the gel-like nature of the particles enabling a disperse colloidal suspension of particles. From the distribution results three peak intensities with diameters of 173.5 nm ($\pm$ 51.5), 2729.8 nm ($\pm$ 1239.8) and 49,803.8 nm ($\pm$ 24 072.0) was determined, which respectively corresponded to the fragmentation of cross-linked polymer, distribution of synthesized ReSynTM particles and aggregated particles. In comparison to the epoxy-activated acrylic beads, the lower coefficient of variation for activity determination of laccase on ReSynTM is attributed to the smaller particle size (3 μ m) that was associated with a decreased rate of settling out of solution and the colloidal nature of the particles in solution.

The comparative assessment of laccase activity on the different supports could not accurately be based upon the mean activity (units enzyme activity per mg immobilization support) that estimated the expected value of the underlying distribution, due to the large associated coefficient of variation values. Therefore, the comparative assessment was based upon the comparison of the lower 95% confidence level of the mean determined for ReSynTM with the upper 95% confidence level the mean of the other supports under corresponding conditions of immobilization. The lower 95% confidence level of the mean for ReSynTM was determined as 266, 262, 339 and 252 respectively corresponding to the time points; 1-, 4-, 7- and 24 h of immobilization at 4°C (Appendix B, Figure 6.3). Under the same immobilization conditions, the upper 95% confidence level of the mean for the supports; Eupergit C 250L respectively corresponded to 124, 133, 100 and 135, DEAE Sephadex A-25 was respectively determined as 54, 79, 73 and 74 and for

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Eupergit C it respectively was 10, 25, 15 and 14. It can be concluded that the lower 95% confidence level of the mean determined for ReSynTM was higher than the upper 95% confidence level of the mean of the supports; Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C respectively over the time points; 1-, 4-, 7- and 24 h of immobilization at 4°C. Therefore, based upon the expectation that the lower and upper confidence intervals contain the true population mean at a 95% level of confidence it can be deduced that an overlap between the lower confidence interval of ReSynTM and the upper confidence interval of Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C was not expected at a 95% level of confidence. These results were consistent under immobilization conditions of 25°C over the time points; 1-, 4-, 7- and 24 h were the lower 95% confidence level of the mean for ReSynTM was respectively determined as 175, 207, 219 and 102. Correspondingly under the same immobilization conditions the upper 95% confidence level of the mean for the supports; Eupergit C 250L respectively was 65, 56, 43 and 99, while for DEAE Sephadex A-25 this was respectively determined as 47, 66, 55 and 44 and for Eupergit C it respectively corresponded to 16, 23, 15 and 7. On an average over all the time points, the lower 95% confidence interval of the mean of ReSynTM was respectively 3-, 3-, and 11 fold higher than the upper 95% confidence interval of the mean of the supports; Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C at 25°C. The increased activity of laccase immobilized on ReSynTM can be attributed to a favourable microenvironment created in the open fibre network of the support. Similar amounts of laccase (mg enzyme per mg support) as indicated previously was achieved at 4°C and 25°C where as an overall decrease based upon the lower- and upper 95% confidence limit of the mean units enzyme activity per mg immobilization support is apparent at 25°C. The decreased enzymatic activity can be explained by the denaturation of the enzyme at room temperature and subsequent corresponding loss of activity. A corresponding increase of 2-, 4- and 17 fold was respectively observed at 4°C. It can be deduced that the apparent increased variation in the activity data points of laccase immobilized on ReSynTM in the activity distribution plot was attributed to the magnitude of the activity values and was not due to an increased variation in the data (Figure 3.19).

The lower- (LCL) and upper 95% confidence limit (UCL) of the mean of Eupergit C 250L was 93 (LCL) and 135 (UCL) and for Eupergit C was 11 (LCL) and 14 (UCL) for 24 h of immobilization at 4°C. The increased mean confidence limits of Eupergit C 250L is consistent with the presence of a more polymer matrix with a lower distribution of active groups (> 250 µmole/g dry weight), which is reported to be beneficial in terms of enzyme activity (Hernaiz & Crout 2000).

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The pH optimum of free- and ReSynTM immobilized laccase was determined over the pH range of 1.5 to 7 with ABTS as the substrate in succinate-lactate buffer (0.1 M) (Figure 3.20). Free laccase had an observed optimum pH at 2, followed by a sharp decrease in activity (units) between pH 2 to 7. The pH optimum of laccase was indicated to be dependent to a large extent on the substrate, ABTS as the substrate consistently reported rather acidic pH optimum values (pH 3 to 5) (Heinzkill *et al.* 1998). ReSynTM immobilized laccase had a corresponding observed optimum pH at 2, with additional peaks between pH 4 to 5.5 (at pH 4.5) and between pH 5.5 to 6.5 (at pH 6). Despite the uncharacteristic three peak pH activity profile, the additional optimum pH peaks correspond to reported optimum activity respectively around pH 4.5 and pH 6, for laccase from the same source which was also determined with ABTS as the substrate (Forde *et al.* 2010, Hollmann *et al.* 2008). The immobilization on ReSynTM, therefore, provided increased functionality to the laccase at pH 4.5 and 6, increasing the industrial applicability of the enzyme.

The temperature optimum of free- and ReSynTM immobilized laccase was determined over the temperature range of 25°C to 70°C in a universal buffer (pH 2) with ABTS as the substrate (Figure 3.21). Free laccase indicated a broad peak in activity between 35°C and 70°C which was associated with slight variations in enzyme activity. ReSynTM immobilized laccase had a corresponding broad peak in activity between 40°C and 70°C (optimum activity at 50°C) with an additional shoulder between 25°C and 40°C with a peak at 35°C. ReSynTM immobilized laccase indicated a much larger peak height in enzyme activity (bell-shaped profile) as a function of pH in comparison to the free enzyme. Consistent with the results obtained, an optimum temperature around 50°C was reported for laccase from the same source with ABTS as the substrate (Hollmann *et al.* 2008). Furthermore, the temperature optimum determination reported for free and covalently immobilized laccase from the same source with ABTS as the substrate had an optimum activity at 60°C with a bell-shaped profile (Kunamneni *et al.* 2008).

3.6. CONCLUSION

Initial comparative assessment of immobilization on different supports was performed with BSA. After 48 h of immobilization respectively 0.151-, 0.156-, 0.162- and 0.616 mg BSA per mg of the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM was determined at 4°C with corresponding slightly decreased values at 25°C, with the extremely high load of BSA attained by ReSynTM attributed to the open fibre network and high amine density. Assessment of the strength of BSA bonding onto the supports Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 indicated a non-covalent nature of immobilization in contrast to a covalent nature for ReSynTM. ReSynTM had an approximate 8 fold higher BSA loading efficiency than the other supports.

Immobilization of polyphenol oxidase purified from Forelle pear amounted to the binding of a very low amount of protein onto ReSynTM. Subsequent comparative assessment of immobilization was performed with laccase from *Myceliophthora thermophila*, the amount of laccase bound per mg of the supports; Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM corresponded to 0.546, 0.551, 0.561 and 0.292 after one hour of immobilization at 4°C, which remained constant to 24 h, except for an increase of 23% in the amount of laccase bound per mg DEAE Sephadex A-25. This observed increased amount of laccase bound per mg DEAE Sephadex A-25 was not determined for immobilization performed at 25°C, which was due to the decreasing stability of the enzyme at room temperature for a prolonged period of time and the associated much weaker nature of non-covalent bonds (ionic interactions). Immobilization performed at 25°C resulted in similar amounts of the laccase only being bound after 4 h of immobilization. The laccase loading efficiency of the other supports was approximately 2 fold higher than that of ReSynTM.

One-way analysis of variance (ANOVA) determined significant differences among the mean units activity per mg support for the different immobilization matrixes respectively over time points; 1-, 4-, 7- and 24 h of immobilization separately performed at 4°C and 25°C. The significant *F* statistics (with the sufficiently small associated *p* values) amounted to the rejection of the null hypotheses ($H_{0(1-8)}$). Duncan's multiple range test (DMRT) determined that the statistical significant difference (at $p = 0.05$) in all the mean enzyme activity binding capacity was between all the supports over all the time points at both 4°C and 25°C. Summary statistical analysis indicated on average that the coefficient of variation for immobilization was in the decreasing

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order of the supports; Eupergit C, Eupergit C 250L, ReSynTM and DEAE Sephadex A-25. The overall high determined coefficient of variation values was attributed to an inherent difficulty associated with the accurate and reproducible removal of the same amount of particles with immobilized enzyme for activity determination, which is influenced by the support's particle size. The comparative assessment of laccase activity revealed that the lower 95% confidence level of the mean determined for ReSynTM was (respectively 2-,4-, and 17 fold at 4°C) higher than the upper 95% confidence level of the mean of other supports; Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C.

The industrial applicability of laccase was increased by immobilization on ReSynTM based upon pH optimum determination of 2 for the free enzyme with additional peaks at 4.5 and 6 for the immobilized laccase. Furthermore, the temperature optimum determination indicated a significantly increased activity temperature profile for the immobilized laccase.

CHAPTER 4 : BIOTRANSFORMATION OF TEA CATECHINS INTO THEAFLAVINS

4.1. THEAFLAVINS

After water, tea is the most consumed beverage in the world. Tea can be grouped into three types namely black tea (fermented tea), oolong tea (partially fermented tea) and green tea (non-fermented tea). Catechins; (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate constitute the major components of green tea leaves with a higher content present in green tea in comparison to both that of black - and oolong tea (Leung *et al.* 2001, Cabrera *et al.* 2003). The monomeric flavan-3-ols (catechins) undergo polyphenol oxidase-dependent oxidative polymerization during fermentation in black - and oolong tea leading to the formation of bisflavanols (theaflavins, thearubigins and other oligomers) (Pan *et al.* 2000). Theaflavins possess a system of benzotropolone rings with di- or trihydroxy substitutions. In black tea, theaflavins (theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin 3,3'-digallate) are the principal pigments, important in the initiation of black tea creaming and accounts for approximately 1-2% of the total dry weight (Figure 4.1) (Pan *et al.* 2000, Collier *et al.* 1973, Jöbstl *et al.* 2005).

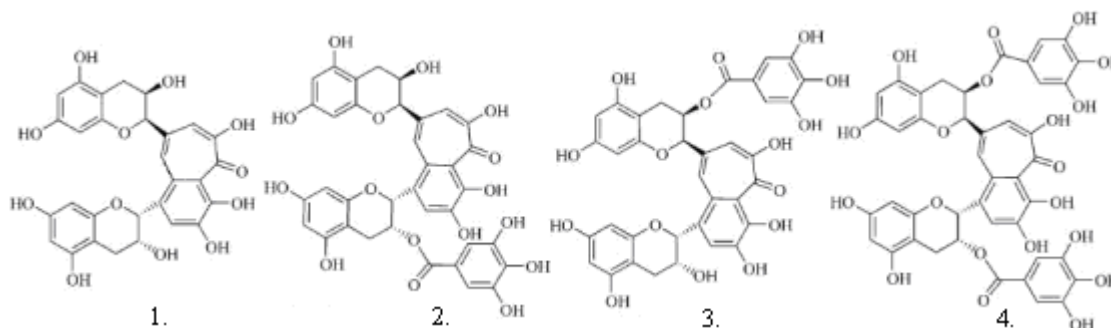


Figure 4.1: The structures of theaflavin (1), theaflavin-3'-gallate (2), theaflavin-3-gallate and (3) theaflavin-3,3'-digallate (4) (Bonnely *et al.* 2003).

4.1.1. Theaflavins as potential therapeutics

Interest in theaflavins arises from their potential benefits as a natural dietary antioxidant. Theaflavins act as *in vitro* antioxidants by chelating redox-active transition metal ions (Miller *et al.* 1996) and scavenging reactive oxygen species (Lin *et al.* 2000). Indirectly, theaflavins function as antioxidants firstly through the activation of phase II (detoxification-) and antioxidant enzymes. These enzymes include superoxide dismutase and glutathione S-transferases (Saha & Das 2003). Secondly, theaflavins function indirectly as antioxidants

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through the inhibition of nuclear factor- κ B (Lin *et al.* 1999) and activator protein-1 (12-O-tetradecanoylphorbol 13-acetate induced binding activity) (Chen *et al.* 1991). Thirdly, the inhibition of “pro-oxidant” enzymes such as lipoxygenase (Hong *et al.* 2001) and xanthine oxidase (Lin *et al.* 2000) enable theaflavins to function indirectly as antioxidants. The expression of the “pro-oxidant” enzymes cyclooxygenases and inducible nitric oxide synthase was respectively inhibited and reduced by theaflavin (Lin *et al.* 1999, Cai *et al.* 2006).

Theaflavins have been implicated to have antiviral activity by the identification of theaflavin-3'-gallate, theaflavin-3,3'-digallate and tannic acid, as effective inhibitors of the virally encoded 3C-like protease ($IC_{50} < 10 \mu M$), from the screening of 720 natural products and subsequent analysis of well-known components present in tea (Chen *et al.* 2005). The potential clinical significance of theaflavins has also been suggested based upon their reported anticancer- (Leone *et al.* 2003), antimutagenic-, anticlastogenic- (Halder *et al.* 2005) and antibacterial activity (Nashimoto & Tashiro 1999). Research on theaflavins has been hampered, to such an extent that most previous research was focused on the use of mixtures of theaflavins, due to their low abundance and challenging purification procedure (Sang *et al.* 2004a).

4.1.2. Production of theaflavins

The extraction of theaflavins from black tea was the original method employed to acquire theaflavins, this didn't provide a cost-effective manner of recovering theaflavins, as it was hampered by high costs. The high cost was associated with the low content and difficulty of recovering theaflavins from black tea, resulting in the extraction of theaflavins from black tea being an industrially unfeasible approach (Wang & Li 2006). From the 15-30% dry weight of catechins present in green tea shoots, only 0.4-1.85% dry weight of theaflavins are formed during black tea manufacturing (Sharma *et al.* 2009, Graham 1992). Subsequently, various approaches have been employed for the synthesis of theaflavins from substrate materials including raw tea leaves (not wilted) (Takemoto 2009a), tea leaf juice, processed green tea leaves, green tea slurry (Goodsall *et al.* 2001), liquid green tea extract (Itoh *et al.* 2007) and *in vitro* modelling of fermentation with purified catechins (Robertson & Bendall 1983). These approaches included the utilization of potassium ferricyanide as a catalyst in chemical oxidation along with enzymatic oxidation (Sharma *et al.* 2009, Wan *et al.* 1997). Enzymatic oxidation approaches entail the exploitation of endogenous enzymes in liquid plant extracts or crude tea leaves containing polyphenol oxidase (Robertson & Bendall 1983, Subramanian *et al.* 1999b), peroxidase-containing cultured plant cells such as cultured tea cells (Takemoto 2009b) and homogenates of

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various fruits (Tanaka *et al.* 2000). Many fruit homogenates are capable of synthesizing theaflavins, of which the mechanism was suggested to be similar to that present in tea fermentation. These plants include loquat, banana, apple and Japanese pear of which banana and Japanese pear were identified as excellent enzyme sources, based upon strong enzymatic activity with practically no oxidation products of their own detected on HPLC analysis (Tanaka *et al.* 2002, Tanaka & Kouno 2003). Furthermore, approaches entailing exogenously purified peroxidase-, polyphenol oxidase- and laccase enzymes have been employed.

The oxidation enzyme, peroxidase, catalyzes the oxidation of polyphenols in the presence of hydrogen peroxide. The generation of hydrogen peroxide during catechin oxidation has implicated peroxidase in tea fermentation, although its role was not clear (Subramanian *et al.* 1999b, Nakayama *et al.* 2002). Horseradish peroxidase has been shown to oxidize tea catechins leading to the formation of theaflavin-type compounds, but also that it oxidizes theaflavins into thearubigins (Subramanian *et al.* 1999b, Sang *et al.* 2004b). It has been suggested that catechins act as substrates for peroxidases at low concentrations, but possibly inhibits peroxidase at the high concentrations present in green tea leaf (Robertson & Bendall 1983). The presence of catalase in tea leaf, that acts rapidly to remove peroxides, possess a limit to the possible role of peroxidase. Peroxidase and polyphenol oxidase have been shown to have a coupled nature of action, with polyphenol oxidase indicated to be of prime importance in the oxidation of flavan-3-ols (Robertson & Bendall 1983, Finger 1994).

Polyphenol oxidase is the enzyme responsible for the oxidation of catechins, utilizing flavanol polyphenols such as (-)-epigallocatechin gallate, (-)-epicatechin, (-)-epicatechin gallate and (-)-epigallocatechin as substrates. Highly electrophilic *o*-quinones are formed by the oxidation of the catechol moiety on the B-ring. These *o*-quinones subsequently react with nucleophilic flavanols forming a C-C bond, that undergoes a cascade of reactions yielding dimeric species; theaflavins, theanaphthoquinones, theacitrins and theasinensins (Gregory & Bendall 1966, Drynan *et al.* 2010). Hydrogen peroxide generated by polyphenol oxidase during the oxidation of catechins, was available to peroxidase for subsequent oxidations of the products of polyphenol oxidase catalyzed reactions, amounting to the turnover of formed theaflavins into thearubigins (Subramanian *et al.* 1999b). Based upon tea-leaf polyphenol oxidases, reported inability to oxidize monohydric phenols by dehydrogenation or hydroxylation, distinguished the polyphenol oxidase [PPO, EC 1.14.18 monophenol mono-oxygenase (tyrosinase) or EC 1.10.3.2 *o*-diphenol: O₂ oxidoreductase] from tyrosinase according to Gregory and Bedall, 1966. Furthermore, tea-leaf polyphenol oxidase possessed low activities towards the substrates;

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p-phenylene-diamine and quinol, which is a classical distinctive attribute of laccases (EC 1.10.3.2) (Gregory & Bendall 1966).

The oxidative polymerization of phenols can also be catalyzed by laccase (benzenediol/oxygen oxidoreductase, EC 1.10.3.2) (Mita *et al.* 2003). Both laccase and polyphenol oxidase utilizes oxygen as an oxidizing agent, amounting to an environmentally benign process in air without the use of hydrogen peroxide as an oxidizing agent, as in the case of peroxidase (Kurisawa *et al.* 2003). Epitheaflagallin and epitheaflagallin 3-O-gallate have been reported to be preferentially synthesised by laccase from epigallocatechin and epigallocatechin gallate in the presence of gallic acid (Itoh *et al.* 2007).

4.1.3. Bioconversion by immobilized enzyme systems

Freshly extracted tea leaf polyphenol oxidase has been covalently immobilized on activated cellulose for the bioconversion of catechins into theaflavins in an *in vitro* fermentation system. Immobilization enabled the bioconversion of catechins into theaflavins over the first 14 turnovers of batches with the same conversion efficiency, after which a linear loss of efficiency occurred, with almost complete loss of activity at 22 batches. The bioconversion of catechins into theaflavins by the immobilized polyphenol oxidase was therefore stable after repeated use and over a long period of storage (Sharma *et al.* 2009). Furthermore, an immobilized enzyme system created from unprocessed tea leaves utilizing endogenous tea enzymes has been reported. This system was prepared by the removal of caffeine and phenolic compounds in such a manner that the activities of peroxidase and polyphenol oxidase remained unaffected (Bonnely *et al.* 2003). No reported instances of the application of immobilized laccase for the biotransformation of tea catechins could be found.

4.1.4. Factors affecting theaflavins syntheses

Complex compositional changes of polyphenols occur during black tea leaf fermentation, which are primarily affected by the raw material and process conditions (Lian *et al.* 2002). Utilization of raw material such as macerated tea shoot systems has amounted to difficulty in interpreting results, as these systems are associated with considerable variation between different clones in both enzyme concentration and polyphenol composition, as well as between the same clones under constantly changing growth conditions (Robertson & Bendall 1983). Furthermore, these systems are also physically heterogeneous, differing in oxygenation of the reacting cell contents

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and temperature (Forrest & Bendall 1969). Problems associated with raw material such as heterogeneous tea shoot macerates have been overcome by *in vitro* model fermentation systems (Robertson & Bendall 1983). Model fermentation provides a very useful method for the study of catechin oxidation during tea fermentation, but the presence of most of the oxidation products obtained by *in vitro* oxidation of catechins, has not been confirmed in commercial black tea (Tanaka & Kouno 2003).

A larger amount of theaflavins are formed at pH 4.5 in comparison to pH 5.5, which is the optimum pH of polyphenol oxidase activity. The decrease in synthesized theaflavins was attributed to an increased production of peroxide during oxidation of catechins by polyphenol oxidase at pH 5.5, and an associated increased turnover of theaflavins into thearubigins (Subramanian *et al.* 1999b). At pH 6.5 the product formation was reported to be largely affected by the auto-oxidation thereof (Sharma *et al.* 2009).

As theaflavins are not the end products of fermentation, their accumulation was a balance between synthesis and oxidation, therefore, based upon the net effect of both theaflavin formation (from oxidized catechin quinones) and further degradation (into thearubigins) (Hilton 1972). Theaflavins have been reported to be degraded into thearubigins by a coupled oxidation with (-)-epicatechin-quinone (Opie *et al.* 1993). Therefore, the complexity during polymerization was increased by the catechins initiating the destruction of theaflavins, which also takes place through the catechin quinones utilized in the dimerisation of catechins into theaflavins (Hodges 2008). The generation of theaflavins was a fast process while the turnover thereof takes place at a slower rate, with a progressive decrease in the content of theaflavins, possibly due to rearrangement into more complex molecules (Bonnely *et al.* 2003). At the maximum level of theaflavins in a batch fermentation system, it was reported to still contain active reagents that could be converted to theaflavins, subsequently amounting to the degradation of theaflavins during storage (Hodges 2008). The degradation of individual theaflavins varies, with the decline in their levels being influenced by both duration and temperature, coinciding with a decline in brightness and briskness (Obanda *et al.* 2001).

Temperature affects both formation and degradation reaction rates, amounting in a complex effect of temperature on the accumulation of theaflavins. In the early stage of fermentation, lower temperature results in a slower rate of theaflavin accumulation, which becomes less significant at a later stage of fermentation (Lian *et al.* 2002). An increased temperature speeds up the rate of the fermentation reaction with a faster depletion of all the catechins. As the catechin substrate was

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depleted, the degradation of theaflavin becomes dominant (Obanda *et al.* 2001). Furthermore, high catechin substrate concentrations amount to enzyme inhibition, therefore a specific weight ratio of catechins to theaflavins in the reaction mixture must be contracted (Sharma *et al.* 2009, Hodges 2008). Individual theaflavins decline to various degrees. The direction and change of theaflavin levels are both time- and temperature-dependent (Obanda *et al.* 2001).

4.2. MATERIALS AND METHODS

4.2.1. Materials

The hydrochloric acid (99%) was purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. The buffers used were prepared from; dipotassium phosphate and potassium orthophosphate which were respectively purchased from Saarchem (Pty) Ltd, Gauteng, South Africa and Labchem (Pty) Ltd, Gauteng, South Africa. Furthermore, succinic acid and sodium lactate (about 50% extra pure) were purchased from Merck, Damstadt, Germany. The catechin standards [catechin (C), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG)] and theaflavin standards [theaflavin (TF), theaflavin-3-gallate (T3G), theaflavin-3'-gallate (T3'G) and theaflavin-3,3'-digallate (TdG)] and Theaphenon E were generous gifts from Prof. Y. Hara from Tea Solutions, Hara Office Inc Tokyo, Japan. Commercially available Lipton® Green Tea and Lipton® Yellow label black tea were purchased from a local supermarket, ground to a fine powder with a high speed blender and sifted through a 100 micron sieve. Forelle pear (class 1) was purchased from a local fruit store and stored at 4°C until use within five days. Tea (*Camellia sinensis*, frozen fresh leaves) and Yacon (*Smallanthus sonchifolius*, leaves) were obtained from the experimental farm of the University of Pretoria and stored at 4°C until use. The enzymes used were polyphenol oxidase purified from Forelle pear, laccase (Suberase) enzyme a generous gift from Novozyme (Pty) Ltd, Gauteng, South Africa and mushroom tyrosinase purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. ReSynTM particles were synthesized using; glutaraldehyde (grade II, 25%), nonoxyl-4, polyethylene imine (polyamine) (PEI) purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States and Enerpar oil was obtained from BP Midrand, South Africa. Ascorbic acid and ethylenediaminetetraacetic acid (EDTA) were respectively purchased from Saarchem (Pty) Ltd, Gauteng, South Africa and Merck, Damstadt, Germany. Leucine enkephalin used in the UPLC-MS analysis was of ultra-pure LC-MS grade and purchased from Fluka, Steinheim, Germany. The ultra-pure solvents; formic acid and acetonitrile were purchased from Honeywell, Burdick & Jackson, Muskegon, United States. Furthermore, ultra-pure water and nitrogen gas was used in the UPLC-MS analysis. A Waters HSS T3 column (150 mm x 2.1 mm, 1.8 µm) from Waters, Milford, United States was used in the chromatographic separations.

4.2.2. List of equipment

The equipment used was; Digital Dry Baths (Labnet International Inc), water bath, SevenEasy™ bench top pH meter (Mettler Toledo Ltd, Leicester, United Kingdom) and an orbital shaker incubator. The centrifuges used were a Heraeus Pico 17 microcentrifuge with a 24 x 1.5/2.0 ml rotor (Thermo Fisher Scientific Corp, Waltham, United States) and an Allegra™ X-22R centrifuge with a SX4250 rotor (Beckman Coulter Inc, California, United States). The balances used were an AS 220/X analytical balance and a WPS 2100/C2 balance (RADWAG®, Random, Poland). Samples were filtered through Arodisc® PSF syringe filters (Pall Corporation, Port Washington, New York) prior to UPLC-MS analysis. Ultra-pure water was generated from a Millipore Elix 5 RO system and a Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France). The generation of accurate mass data was obtained by a Waters UPLC coupled in tandem to a Waters photo diode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer. The software used to control the hyphenated system and to do all data manipulation was MassLynx 4.1 (SCN 704). The UPLC-MS equipment was funded by the NRF (RISP NEP) program as a joint venture between the CSIR Biosciences and University of Johannesburg, Department of Biochemistry.

4.2.3. Methods

4.2.3.1. Catechin sample and Theaphenon E analysis by UPLC-MS

Sample analysis was performed with ultra performance liquid chromatography coupled to mass spectrometry. Ultra performance liquid chromatography (UPLC) retains the practicality and principles of HPLC, while through employing sub-2.0 µm particles and higher pressures up to an approximate 14,000 psi, it achieved improvements in analysis time (associated with reduced organic waste production), resolution (not compromised at high speed) and detection sensitivity (ensures an increased metabolite detection as well as that of more low concentration metabolites) (Mazzeo *et al.* 2005, Swartz & Murphy 2004, Nguyen *et al.* 2006). In traditional liquid chromatography the particle size packed into the analytical column was 5 or 3.5 µm. Based upon the van Deemter equation, according to which a reduction in particle size to less than 2.5 µm significantly increases efficiency, which doesn't diminish at increased flow rates or linear velocities. The combination of small particle size and high pressure enabled UPLC to attain a much greater efficiency. An analyte's diffusion path can effectively be shortened by the smaller

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particles, thereby significantly improving the efficiency of separation (Nassar 2010). Since UPLC relies on the same selectivity and retentivity as HPLC, efficiency was the primary separation parameter behind UPLC. In comparison to HPLC, a substantial improvement in performance for complex mixture analysis was provided by UPLC-MS. Tandem mass spectrometry in the form of tandem-in-space, quadrupole time of flight (Q-TOF) was employed in order to perform multiple mass (m/z) analyses (Ramanathan 2011). UPLC significantly enhances mass spectrometry detection (MS) by promoting increased source ionization efficiencies (reduced ion suppression) for improved sensitivity based upon increased peak concentrations with reduced chromatographic dispersion at lower flow rates (no flow splitting). Furthermore, an additional advantage is provided by the simpler non-buffered mobile phase in UPLC, in contrast to typical HPLC analysis requiring viscous, buffered mobile phases operated at high temperatures. Electrospray ionization (ESI) was employed to realize the transformation of solution molecules from liquid phase to gas phase in UPLC-MS. A mass spectrometer measures the mass-to-charge ratio (m/z) of gas-phase ions and provides a measure of the abundance of ionic species. The mass-to-charge (m/z) ratios of ions are measured by mass analyzers based upon the interaction of charged particles with electric or magnetic fields (Nassar 2010). The mass analyzer and detector must be calibrated with ions with known m/z and be maintained under high-vacuum conditions. UPLC was coupled with a time-of-flight (TOF) mass analyzer, capable of high-speed scanning in order to acquire the data at the rate of the chromatographic output. From the source region, ions are “gated” by an electrical field pulse and accelerated down the TOF flight tube. The detector is reached quicker by low m/z ions travelling at a higher velocity in contrast to slower high m/z ions. Mass analysis of unknown compounds can be achieved by the calibration of the accelerating field and resulting flight times (Ramanathan 2011).

A catechin mixture comprising of epicatechin (3 mg/ml) and epigallocatechin (3 mg/ml) was made up in a phosphate buffer (0.2 M, pH 6.8) and Theaphenon E (20 mg/ml) was made up in succinate lactate buffer (100 mM, pH 5.9) for subsequent UPLC-MS analysis. Chromatographic separations of samples were done utilizing a Waters HSS T3 column (150 mm x 2.1, 1.8 μ m) thermostated at 65°C. A binary solvent mixture consisting of water (Eluent A) containing 10 mM formic acid (pH 2.3) and acetonitrile (Eluent B) containing 10 mM formic acid was used. Initially, 100% eluent A at a 0.6 ml/min flow rate with a linear gradient to 87% eluent A at 31 min was employed. The conditions were changed to 69% eluent A at 41 min. From 43 to 45 min, the column was flushed with 100% eluent B and subsequently changed to initial conditions. The total run time was 50 min and depending on the concentration of the compounds of interest the

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injection volume ranged between 1 to 5 µl. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) and collected 20 spectra per second.

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode to detect the compounds of interest. The reference calibrant, leucine enkephalin (50 µg/ml) was used to obtain typical mass accuracies between 1 and 3 mDa. The mass spectrometer was operated in the negative and positive mode with a capillary voltage of 2.0 kV, the extraction cone at 5 V and the sampling cone at 30 V. The scan time was 0.1 seconds covering the 100 to 1000 Da mass range. The source temperature was at 120°C, and the desolvation temperature was set at 450°C. Nitrogen gas was used as the nebulisation at a flow rate of 800 l/h.

4.2.3.2. Theaflavins standards UPLC-MS analysis

The theaflavin standards; theaflavin, theaflavin-3-*O*-gallate, theaflavin-3'-*O*-gallate and theaflavin-3,3-di-gallate were purchased from Sigma Aldrich (Pty) Ltd, St Louis, United States. The theaflavins standards were respectively dissolved in methanol (1 mg/ml) and UPLC-MS analysis was performed in order to subsequently identify the presence of theaflavins in various samples.

4.2.3.3. Tea (*Camellia sinensis*, frozen fresh leaves), Lipton® Green tea and Lipton® Yellow label black tea content analysis

Tea (*Camellia sinensis*, frozen fresh leaves, 4 g), Lipton® Green tea (ground, 2 g) and Lipton® Yellow label black tea (ground, 2 g) were respectively homogenized in a blender with 100 ml potassium phosphate buffer (0.1 M, pH 5.9) for 2 min. The Tea (*Camellia sinensis*, frozen fresh leaves) fresh frozen leaves sample was subsequently placed at 90°C for 10 min to inactivate any enzymatic activity. Boiled Lipton® Green tea and Lipton® Yellow label black tea samples (ground, 2 g each) were also prepared in the same manner. Samples were filtered through a 0.2 µm filter and UPLC-MS sample analysis was performed as described above (4.2.3.1). UPLC chromatogram mass extraction was performed based upon correspondingly known compounds, catechin mass extraction (288-292+308-308+440-460+576-580+590-596 Da) was respectively based upon catechin (/epicatechin), gallic acid (/epigallocatechin) and catechin gallate (/epicatechin gallate) with the inclusion of procyanidin B and procyanidin. Theaflavin mass extraction (562-566+714-717+866-869 Da) was respectively based upon theaflavin, theaflavin-3-*O*-gallate or theaflavin-3'-*O*-gallate and theaflavin-3,3-di-*O*-gallate and

Chapter 4: Biotransformation of tea catechins into theaflavins – Materials and Methods

the theasinensin mass extraction (608-612+760-74+896-900+914-917 Da) was respectively based upon theasinensin C/E, theasinensin B, theasinensin F/G and theasinensin A/D.

The MassLynx 4.1 software was used for the accurate mass and composition calculations of some of the precursor ions and fragment ions. Both isotopic distribution and accurate masses are taken into account in the calculation of the i-Fit value (Ibáñez *et al.* 2009). The i-Fit value enabled the discrimination between the correct elemental composition and the second-ranked formula, with lower values indicating a more plausible elemental composition.

4.2.3.4. Bioconversions of Tea (frozen fresh leaves) catechins into theaflavins with pear, yacon and mushroom tyrosinase

Tea (frozen fresh leaves, 2 g), Yacon (frozen fresh leaves, 2 g) and Forelle pear (kept at 4°C and cored, 2g) were respectively homogenized in a blender with 100 ml potassium phosphate buffer (0.1 M, pH 5.9) for 2 min with fresh Tea (frozen fresh leaves, 4 g). Mushroom tyrosinase (40 µl, 30 units/ml) was added to homogenized fresh Tea (frozen fresh leaves, 0.1 g). The samples were stirred for an hour at room temperature using a magnetic stirrer. Samples were then filtered through muslin, placed at 90 °C for 10 min and subsequently centrifuged at 3,000xg for 10 min. Control samples of Tea, Yacon and Forelle pear (2 g of each) were prepared in the same manner but were placed at 90 °C for 15 min directly after being homogenized. Samples were filtered (0.2 micron), and subsequent sample analyses were performed by UPLC-MS as described above (4.2.3.1). UPLC chromatogram theaflavin mass extraction (562-566+714-717+866-869 Da) of the data was also performed.

4.2.3.5. Bioconversions of a catechin mixture into theaflavin with pear polyphenol oxidase and mushroom tyrosinase

Mushroom tyrosinase (40 µl, 30 units/ml) and Forelle pear polyphenol oxidase (40 µl, 978 units/ml) were respectively added to a catechin mixture (2960 µl). The catechin mixture comprised of epicatechin (3 mg/ml) and epigallocatechin (3 mg/ml) was made up in a phosphate buffer (0.2 M, pH 6.8). Samples were stirred using a magnetic stirrer for an hour. The reactions were stopped at 90°C for 10 min, filtered (0.2 micron) and UPLC-MS sample analysis was performed as described above (4.2.3.1). Theaflavin (563.1205 Da) mass extraction of the UPLC data was also performed.

4.2.3.6. Immobilized- and free laccase biotransformation of tea catechins into theaflavins utilizing a catechin mixture

The biotransformation was performed based upon the results obtained by Sharma *et al.* (2009). Laccase (Suberase from Novozyme (Pty) Ltd) from *Myceliophthora thermophila* immobilized on ReSynTM was used for the conversion of the catechin mixture (respectively 80-, 40- and 20 mg). The immobilized system was resuspended in a succinate lactate buffer (100 mM, pH 5.9) and comprised of 4.8 mg immobilized laccase enzyme in a total volume of 1 ml per reaction. Free laccase was made up to 4.8 mg/ml in a succinate lactate buffer (100 mM, pH 5.9) and volume of 1 ml per reaction was used. The biotransformation was performed at 37.5°C with 150 rpm rotation. The reactions were respectively stopped at the time intervals of 0-, 30-, 60-, 90-, 120- and 150 min as described by Bonnely *et al.* (2003). This was performed by centrifugation of the samples (to remove the immobilized enzyme system) and stabilization of 100 µl of the supernatant by the addition of 14 µl of a mixture of ascorbic acid (250 µg/ml) and EDTA (250 µg/ml). The samples were filtered (0.2 micron) and analyzed by UPLC-MS as described above (4.2.3.1). Theaflavin mass extraction (562-566+714-717+866-869 Da) and theasinensin mass extraction (608-612+760-74+896-900+914-917 Da) aided assessment of the UPLC chromatogram data. The MassLynx 4.1 software was used for the accurate mass and composition calculations of some of the precursor ions and fragment ions. Theaflavin standards curves; theaflavin (10-, 20-, 40 µg/ml), theaflavin-monogallate (100- to 700 ng/ml at 100 ng/ml intervals) and theaflavin-digallate (100- to 700 ng/ml at 100 ng/ml intervals and 1000 ng/ml) were constructed for the quantitative determination of peaks corresponding to synthesised theaflavins. Quantification of theaflavin in Lipton® Yellow label black tea was used as a control sample.

4.2.3.7. Immobilized polyphenol oxidase biotransformation of tea catechins into theaflavins utilizing a catechin mixture

The biotransformation as described above was followed. Polyphenol oxidase purified from Forelle pear immobilized on ReSynTM was resuspended in a potassium phosphate buffer (100 mM, pH 5.9). Biotransformation was performed with 0.019 mg of bound polyphenol oxidase per reaction, over the time points; 0-, 60-, 120-, 240-, 360-, 480- and 1440 min. Prior to UPLC-MS analyses of the samples as described (4.2.3.1), the samples were filtered (0.2 micron). Theaflavin mass extraction (562-566+714-717+866-869 Da) of the UPLC chromatogram data was also performed.

4.3. RESULTS

4.3.1. Catechin mixture and Theaphenon E analysis by UPLC-MS

Initial assessment of the substrates; epicatechin ((-)-EC) and epigallocatechin (EGC) stability in a potassium phosphate buffer (0.2 M, pH 6.8) as well as the substrate profile of a commercially available Theaphenon E (a reported almost 70% epigallocatechin and a total catechin content of > 90%) was determined with UPLC-MS analysis (Figures 4.2, 6.5, 6.6, 6.8 and 6.9 and Table 4.1). From the UPLC chromatograms, peaks were numerically labelled and MS data of peaks of interest were extracted. The MS data was reported in the ESI negative mode, based upon the catechin peak detection in the ESI negative mode as opposed to the ESI positive mode of catechins present in the catechin mixture [epicatechin ((-)-EC) and epigallocatechin (EGC)] (Figure 4.2 A and 6.4). The MS spectra and summary analysis (Table 6.5) of the MS data was included in Appendix C.

Table 4.1: UPLC chromatogram peak summary of the catechin mixture and Theaphenon E

Catechin mixture (epicatechin and epigallocatechin) (Figure 4.2 A)
m/z 265 (43.78) ¹ , m/z 289 (17.27) ^{2*} , m/z 289 (11.38) ^{3*} , m/z 305 (5.76) ^{4*} , m/z 609 (3.15) ^{5*} and m/z 176 (1.61) ⁶
Commercial catechin mix, Theaphenon E (Figure 4.2 B).
m/z 447 (34.01) ⁷ , m/z 441 (28.86) ⁸ , m/z 883 (26.84) ^{9*} , m/z 457 (21.70) ^{10*} , m/z 915 (18.50) ^{11*} , m/z 289 (18.25) ^{12*} , m/z 305 (12.05) ^{13*} , m/z 305 (6.14) ^{14*} , m/z 169 (3.12) ^{15*} and m/z 176 (1.57) ¹⁶
(retention time (min)) ^{compound number}
* = Mass spectrometry data extracted

4.3.2. Theaflavins standards UPLC-MS analysis

To enable the accurate determination of the presence of theaflavins in various samples, UPLC-MS analysis of the standards; theaflavin, theaflavin-3-O-gallate, theaflavin-3'-O-gallate and theaflavin-3,3-di-gallate was performed. UPLC and mass spectra chromatograms (Figures 6.10 and 6.11) and summary of the analysis (Table 6.5) were included in Appendix C.

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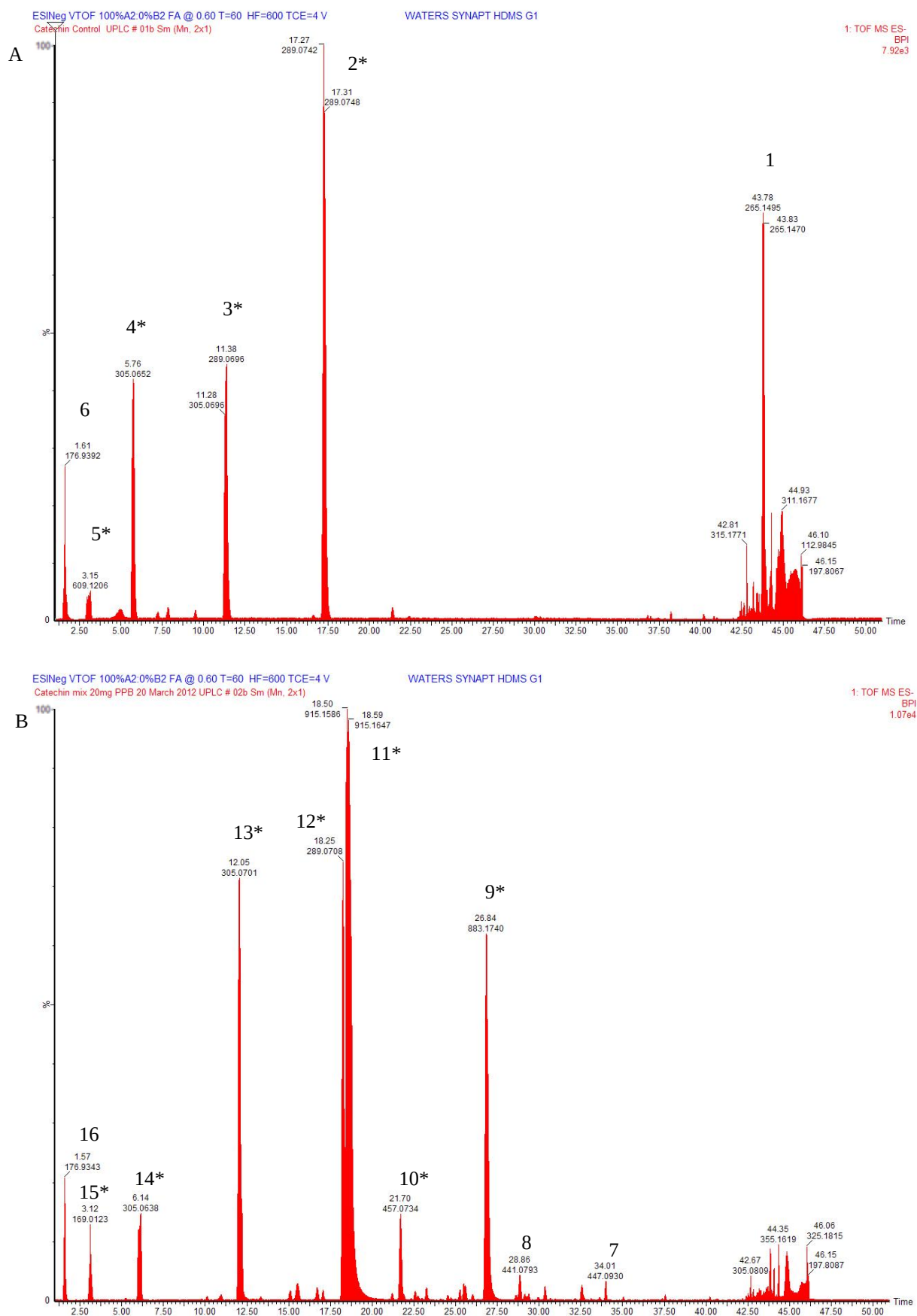


Figure 4.2: UPLC chromatogram of A. catechin mixture ((-)EC and EGC) and B. Theaphenon E as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of respectively 1-6 and 7-15 was indicated, and a peak summary included in Table 4.1. MS data were extracted for peaks*; 2 (epicatechin), 3 (catechin), 4 (epigallocatechin/ gallic acid) (Figure 6.5), 5 (theasinensin C and E) (Figure 6.6), 13 (epigallocatechin), 14 (gallic acid) and 15 (gallic acid) (Figure 6.9).

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4.3.3. Tea (*Camellia sinensis*, fresh frozen leaves), Lipton® Green tea and Lipton® Yellow label black tea content analysis

The catechin substrate availability as well as the presence of theaflavins and theasinensins in Tea (*Camellia sinensis*, fresh frozen leaves), Lipton® Green tea and Lipton® Yellow label black tea was determined with UPLC-MS and was respectively based upon the UPLC chromatogram catechin (288-292+308-308+440-460+576-580+590-596 Da), theaflavin (562-566+714-717+866-869 Da) and theasinensin (608-612+760-74+896-900+914-917 Da) mass extraction data. The effect of boiling Lipton® Green tea and Lipton® Yellow label black tea was also determined by UPLC-MS.

Tea (*Camellia sinensis*, frozen fresh leaves) analysis was based upon inherent catechin, theaflavin and theasinensin content indicated in Figure 4.3 and 4.4 with a UPLC chromatogram summary in Table 4.2.

Table 4.2: UPLC chromatogram peak summary of Tea (*Camellia sinensis*, frozen fresh leaves)

Tea (<i>Camellia sinensis</i> , frozen fresh leaves)
Total ion chromatogram (Figure 4.3 A)
m/z 255 (43.79) ¹⁷ , m/z 593 (33.43) ¹⁸ , m/z 447 (31.53) ¹⁹ , m/z 609 (29.45) ^{20*} , m/z 463 (28.11) ^{21*} , m/z 563 (26.85) ^{22*} , m/z 783 (20.99) ²³ , m/z 289 (17.21) ^{24*} , m/z 289 (11.33) ^{25*} , m/z 609 (9.51) ²⁶ , m/z 305 (5.77) ^{27*} , m/z 581 (4.44) ²⁸ , m/z 609 (3.06) ²⁹ and m/z 191 (1.68) ³⁰
Catechin mass extraction (Figure 4.3 B)
m/z 248 (46.10) ³¹ , m/z 305 (42.65) ³² , m/z 593 (33.43) ¹⁸ , m/z 447 (31.53) ¹⁹ , m/z 593 (28.56) ³³ , m/z 289 (17.21) ^{24*} , m/z 577 (14.76) ³⁴ , m/z 289 (11.33) ^{25*} , m/z 305 (5.77) ^{27*} and m/z 439 (1.71) ³⁴
Theaflavin mass extraction (Figure 4.4 A)
m/z 248 (46.10) ³¹ , m/z 297 (43.68) ³⁶ , m/z 715 (39.22) ³⁷ , m/z 563 (38.26) ³⁸ , m/z 563 (35.67) ³⁹ , m/z 489 (34.27) ⁴⁰ , m/z 609 (29.45) ^{20*} , m/z 563 (26.85) ^{22*} , m/z 441 (25.65) ⁴¹ , m/z 173 (17.82) ⁴² , m/z 581 (4.44) ²⁸ and m/z 370 (1.58) ⁴³
Theasinensin mass extraction (Figure 4.4 B)
m/z 609 (29.45) ^{20*} , m/z 463 (28.11) ^{21*} , m/z 289 (17.21) ^{24*} , m/z 609 (7.88) ^{44*} , m/z 607 (5.64) ⁴⁵ and m/z 609 (3.06) ²⁹
(retention time (min)) ^{compound number}
* = Mass spectrometry data extracted

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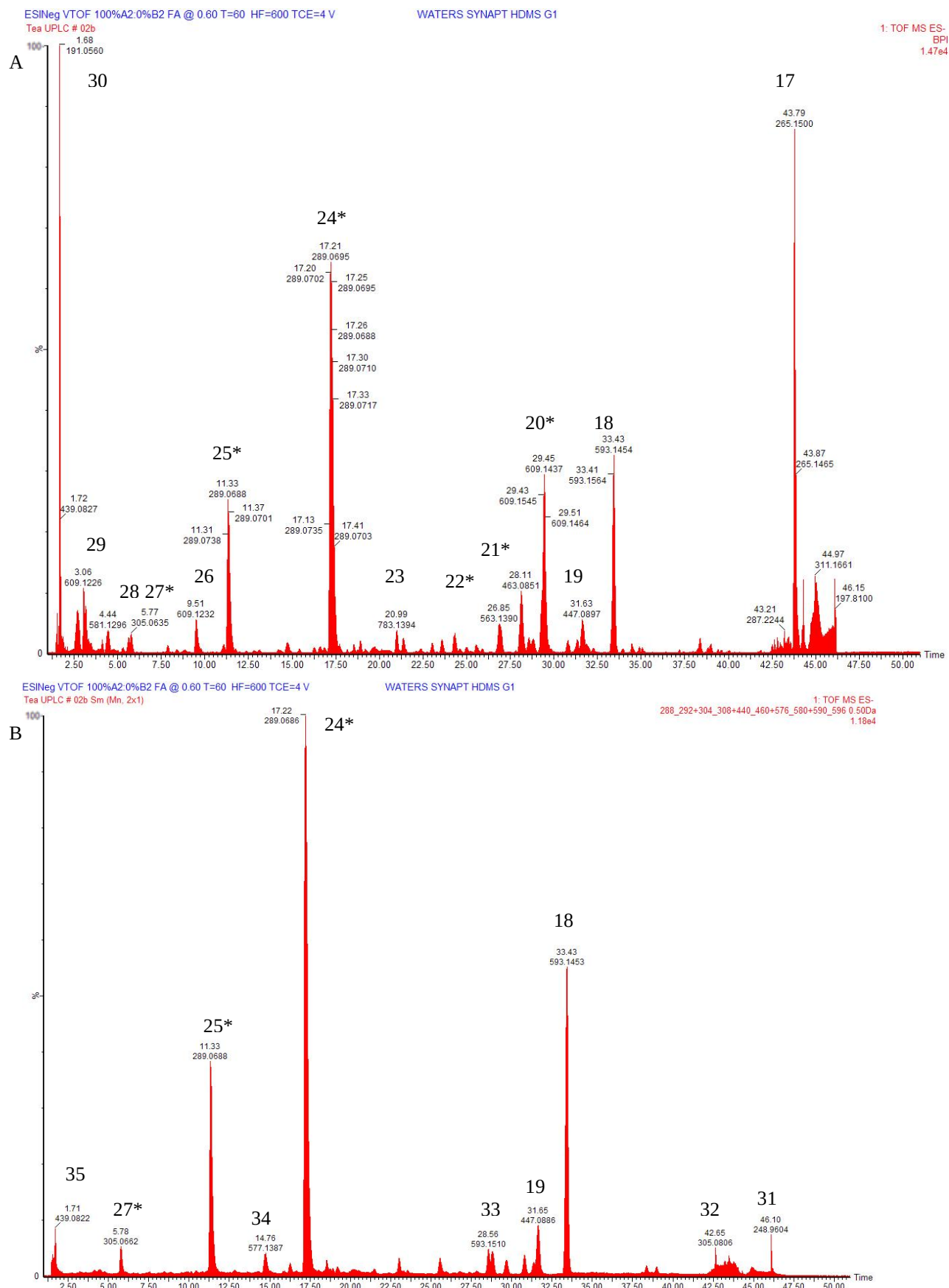


Figure 4.3: UPLC chromatogram of Tea (*Camellia sinensis*, frozen fresh leaves) A. total ion chromatogram and B. catechin mass extracted data. The numerical peak allocation of respectively 17-30 and 18, 19, 24, 25, 27, 31-35 was indicated and a peak summary included in Table 4.2. MS data were extracted for peaks*; 21 (unknown), 24 (epicatechin), 25 (catechin), 27 (gallocatechin) (Figure 6.12), 22 (unknown) (Figure 6.7) and 20 (unknown) (Figure 6.13).

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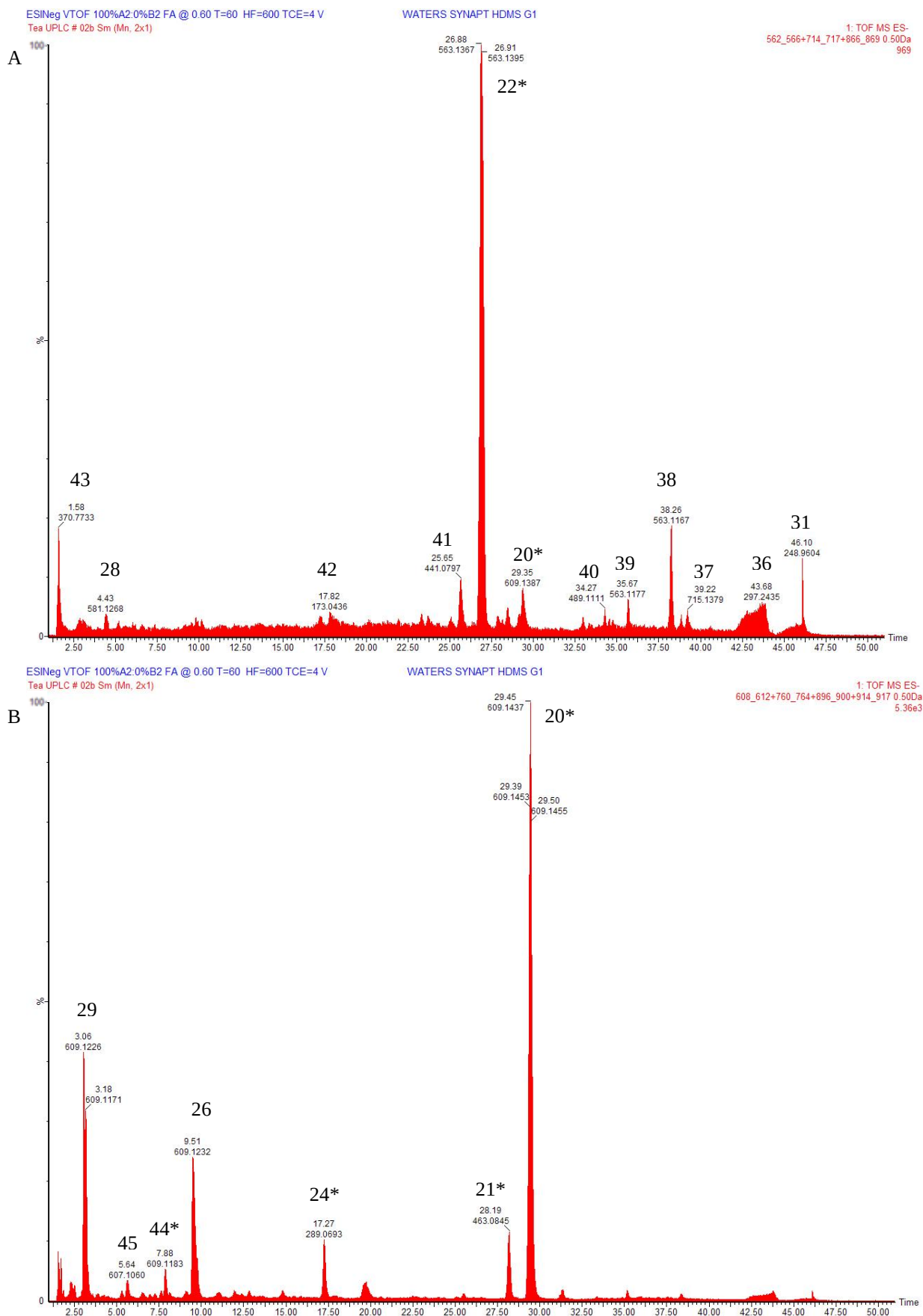


Figure 4.4: UPLC chromatogram of Tea (*Camellia sinensis*, frozen fresh leaves) A. theaflavin and B. theasinensin mass extracted data. The numerical peak allocation of respectively 20, 22, 28, 31 and 20, 21, 24, 26, 29, 44, 45 was indicated and a peak summary included in Table 4.2. MS data were extracted for peaks*; 21 (unknown) (Figure 6.12), 22 (unknown) (Figure 6.7), 20 (unknown) and 44 (unknown) (Figure 6.13).

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Lipton® Green tea analysis was based upon inherent catechin, theaflavin and theasinensin content as well as the effect of boiling was indicated in Figure 4.5, 4.6 and 4.7 with a UPLC chromatogram summary in Table 4.3.

Table 4.3: UPLC chromatogram peak summary of Lipton® Green tea

Lipton® Green tea Total ion chromatogram (Figure 4.5 A) m/z 309 (44.24)⁴⁶, m/z 265 (43.80)⁴⁷, m/z 447 (37.64)⁴⁸, m/z 755 (37.00)⁴⁹, m/z 901 (36.72)⁵⁰, m/z 431 (34.77)⁵¹, m/z 447 (31.92)⁵², m/z 447 (31.66)⁵³, m/z 593 (31.42)⁵⁴, m/z 533 (29.41)⁵⁵, m/z 463 (28.04)⁵⁶, m/z 609 (27.81)⁵⁷, m/z 883 (25.39)⁵⁸, m/z 563 (25.27)⁵⁹, m/z 479 (23.78)⁶⁰, m/z 457 (20.90)^{61*}, m/z 915 (18.42)^{62*}, m/z 289 (17.96)⁶³, m/z 577 (15.65)⁶⁴, m/z 353 (15.02)⁶⁵, m/z 305 (12.38)^{66*}, m/z 609 (8.86)⁶⁷, m/z 305 (6.97)⁶⁸, m/z 687 (4.92)⁶⁹ and m/z 191 (1.82)⁷⁰ Catechin mass extraction (Figure 4.5 B) m/z 447 (37.64)⁴⁸, m/z 901 (36.72)⁵⁰, m/z 447 (31.92)⁵², m/z 593 (31.42)⁵⁴, m/z 593 (26.86)⁷¹, m/z 883 (25.39)⁵⁸, m/z 441 (25.33)⁷², m/z 453 (22.49)⁷³, m/z 593 (22.01)⁷⁴, m/z 457 (20.90)^{61*}, m/z 915 (18.42)^{62*}, m/z 289 (17.96)⁶³, m/z 577 (15.65)⁶⁴, m/z 289 (12.72)⁷⁵, m/z 305 (12.38)^{66*}, m/z 593 (11.63)⁷⁶, m/z 593 (8.67)⁷⁷, m/z 305 (6.97)⁶⁸ and m/z 191 (1.82)⁷⁰ Theaflavin mass extraction (Figure 4.6 A) m/z 887 (36.79)⁷⁸, m/z 563 (27.33)⁷⁹, m/z 563 (25.27)⁵⁹, m/z 563 (24.24)⁸⁰, m/z 563 (22.40)⁸¹, m/z 865 (18.21)⁸² and m/z 165 (1.74)⁸³ Theasinensin mass extraction (Figure 4.6 B) m/z 609 (27.81)⁵⁷, m/z 609 (26.91)⁸⁴, m/z 609 (24.32)⁸⁵, m/z 915 (18.42)^{62*}, m/z 609 (17.61)⁸⁶, m/z 761 (13.47)^{87*}, m/z 305 (12.38)^{66*}, m/z 761 (10.70)^{88*}, m/z 609 (8.86)⁶⁷, m/z 305 (6.99)⁶⁸, m/z 609 (5.96)⁸⁹ and m/z 169 (3.68)⁹⁰ Boiled Lipton® Green tea (Figure 4.7) m/z 265 (43.80)⁴⁷, m/z 901 (36.72)⁵⁰, m/z 447 (31.66)⁵³, m/z 593 (31.42)⁵⁴, m/z 533 (29.41)⁵⁵, m/z 463 (28.04)⁵⁶, m/z 609 (27.81)⁵⁷, m/z 441 (25.36)⁷², m/z 883 (25.39)⁵⁸, m/z 479 (23.78)⁶⁰, m/z 593 (21.92)⁷⁴, m/z 915 (18.42)^{62*}, m/z 577 (15.65)⁶⁴, m/z 633 (15.23)⁹¹, m/z 353 (15.02)⁶⁵, m/z 289 (12.70)⁷⁵, m/z 305 (12.38)^{66*}, m/z 593 (11.66)⁷⁶, m/z 609 (8.86)⁶⁷, m/z 305 (6.97)⁶⁸, m/z 687 (4.92)⁶⁹ and m/z 191 (1.82)⁷⁰ (retention time (min))^{compound number} * = Mass spectrometry data extracted

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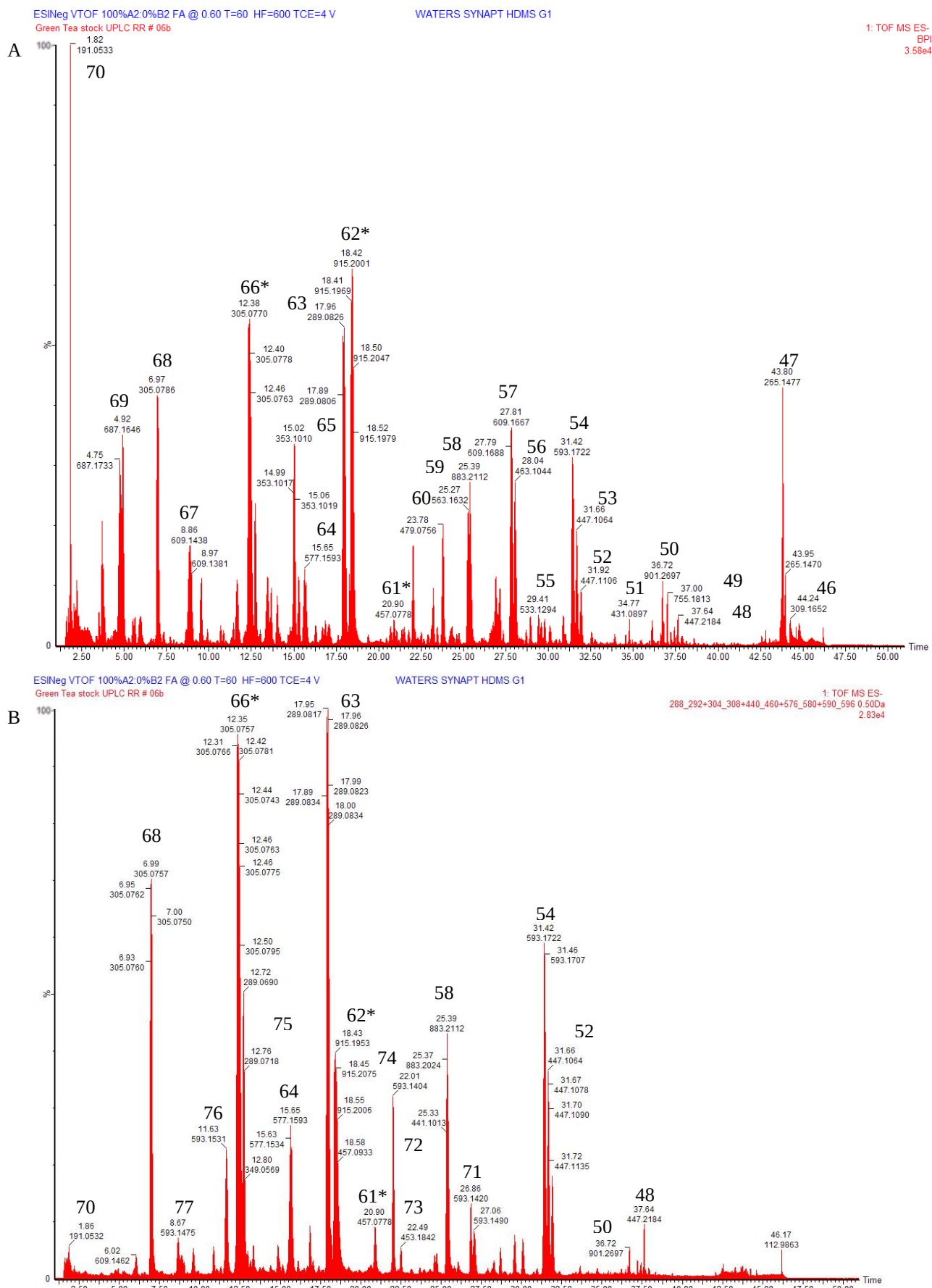


Figure 4.5: UPLC chromatogram of Lipton® Green tea A. total ion chromatogram and B. catechin mass extracted data. The numerical peak allocation of respectively 46-70 and 48, 50, 52, 54, 58, 61-64, 66, 68, 70-77 was indicated and a peak summary included in Table 4.3. MS data were extracted for peaks*; 61 (epigallocatechin gallate/gallocatechin gallate), 66 (epigallocatechin) (Figure 6.14) and 62 (assamicain A, B or C) (Figure 6.15).

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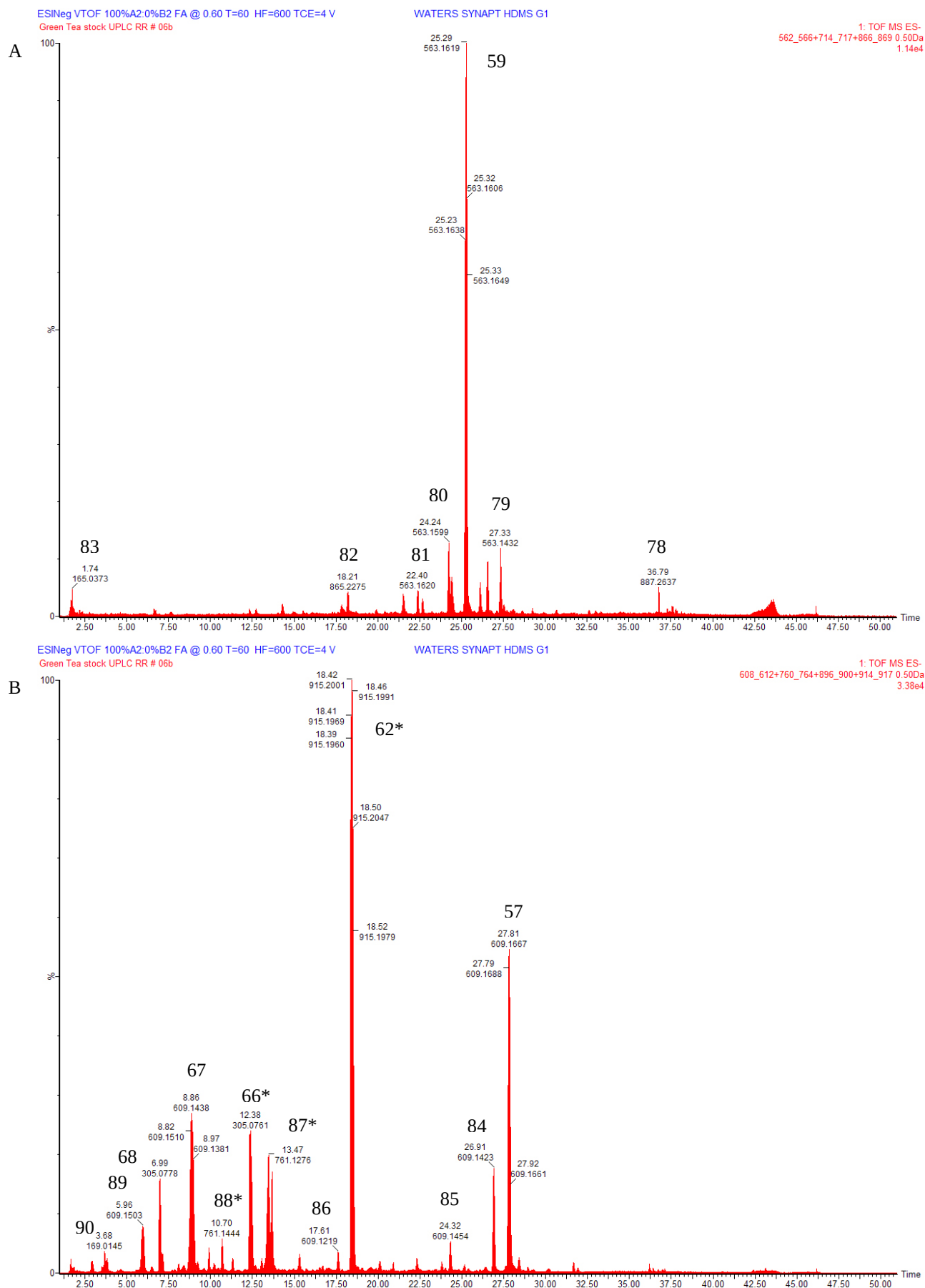


Figure 4.6: UPLC chromatogram of Lipton® Green tea A. theaflavin and B. theasinensin mass extracted data. The numerical peak allocation of respectively 59, 78-83 and 57, 62, 66-68, 84-90 was indicated and a peak summary included in Table 4.3. MS data were extracted for peaks*; 62 (assamian A, B or C), 87 (EGCG-ECG heterodimer) and 88 (EGCG-ECG heterodimer) (Figure 6.15).

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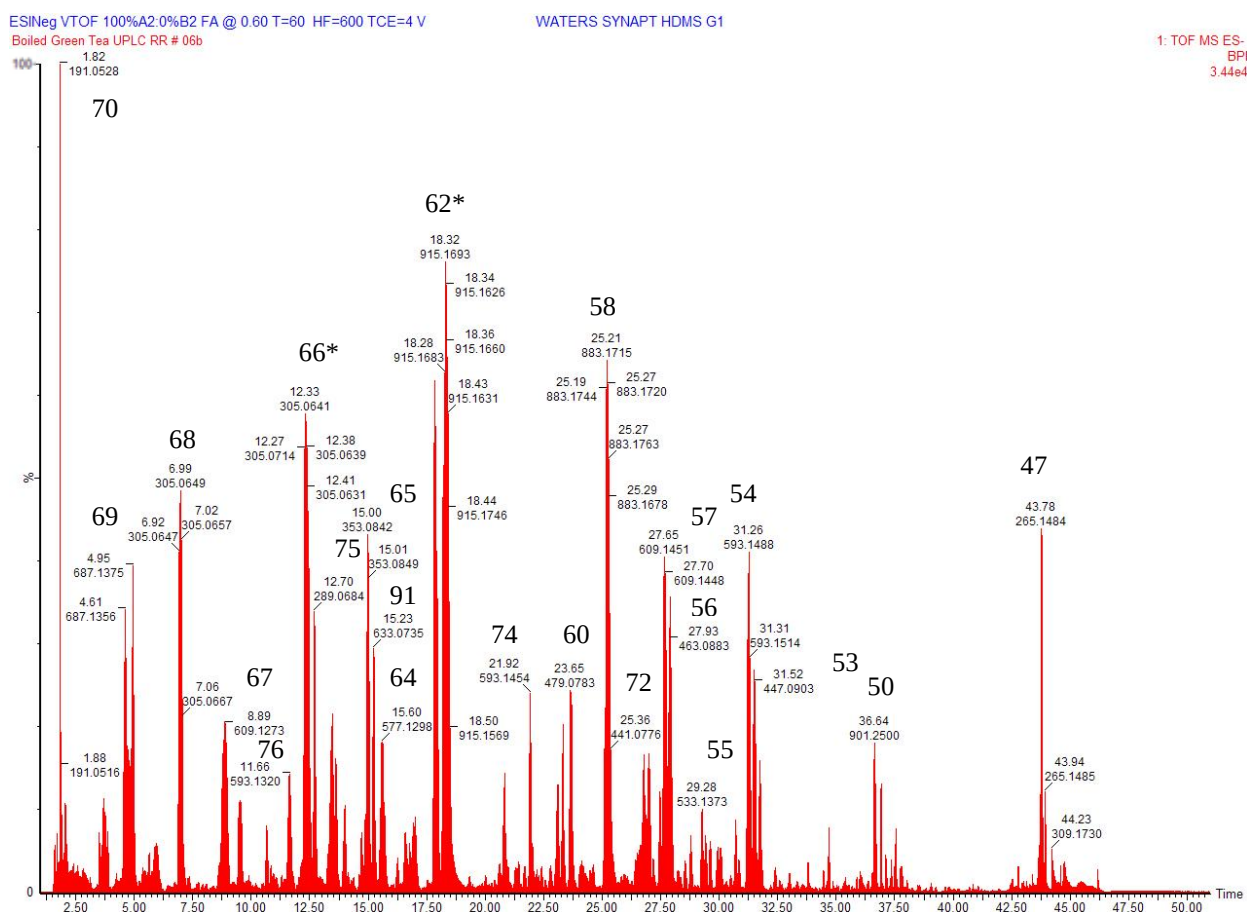


Figure 4.7: UPLC chromatogram of boiled Lipton® Green tea as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 47, 50, 53-58, 60, 62, 64-70, 72, 74-76 and 91 was indicated and a peak summary included in Table 4.3. MS data were extracted for peaks*; 62 (assamian A, B or C) (Figure 6.15) and 66 (epigallocatechin) (Figure 6.14).

Lipton® Yellow label black tea analysis was based upon inherent catechin, theaflavin and theasinensin content as well as the effect of boiling was indicated in Figure 4.8, 4.9 and 4.10 with a UPLC chromatogram summary in Table 4.4.

Table 4.4: UPLC chromatogram peak summary of Lipton® Yellow label black tea

Lipton® Yellow label black tea
Total ion chromatogram (Figure 4.8)
m/z 265 (43.77) ⁹² , m/z 901 (37.38) ⁹³ , m/z 901 (36.51) ⁹⁴ , m/z 489 (33.63) ⁹⁵ , m/z 447 (31.22) ⁹⁶ , m/z 593 (31.00) ⁹⁷ , m/z 739 (30.43) ^{98*} , m/z 609 (27.46) ⁹⁹ , m/z 463 (26.78) ¹⁰⁰ , m/z 609 (26.59) ¹⁰¹ , m/z 563 (24.95) ¹⁰² , m/z 191 (21.23) ¹⁰³ , m/z 595 (18.52) ¹⁰⁴ , m/z 337 (18.24) ¹⁰⁵ , m/z 289 (17.87) ¹⁰⁶ , m/z 173 (16.21) ¹⁰⁷ , m/z 353 (14.96) ¹⁰⁸ , m/z 289 (12.73) ¹⁰⁹ , m/z 337 (12.22) ¹¹⁰ , m/z 353 (9.54) ¹¹¹ , m/z 411 (5.64) ¹¹² , m/z 343 (4.95) ¹¹³ and m/z 191 (1.83) ¹¹⁴
Catechin mass extraction (Figure 4.9 A)
m/z 447 (31.22) ⁹⁶ , m/z 593 (31.00) ⁹⁷ , m/z 595 (18.52) ¹⁰⁴ , m/z 289 (12.73) ¹⁰⁹ , m/z 191 (1.83) ¹⁹¹ , m/z 112 (46.18) ¹¹⁵ , m/z 885 (36.99) ¹¹⁶ , m/z 447 (29.72) ¹¹⁷ , m/z 593 (29.20) ¹¹⁸ , m/z 593 (26.71) ¹¹⁹ , m/z 453 (22.31) ¹²⁰ , m/z 593 (21.80) ¹²¹ , m/z 289 (17.87) ¹²² and m/z 577 (15.57) ¹²³

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Table 4.4 continue: UPLC chromatogram peak summary of Lipton® Yellow label black tea

Lipton® Yellow label black tea
Theaflavin mass extraction (Figure 4.9 B)
m/z 265 (43.77) ⁹² , m/z 901 (37.38) ⁹³ , m/z 563 (24.95) ¹⁰² , m/z 112 (46.18) ¹¹⁵ , m/z 901 (37.08) ¹²⁴ , m/z 563 (36.57) ^{125*} , m/z 563 (34.23) ^{126*} , m/z 565 (28.17) ¹²⁷ , m/z 581 (27.05) ¹²⁸ , m/z 563 (23.94) ¹²⁹ , m/z 491 (14.56) ¹³⁰ , m/z 581 (8.39) ¹³¹ , m/z 581 (5.75) ¹³² , m/z 344 (3.45) ¹³³ and m/z 565 (1.75) ¹³⁴
Theasinensin mass extraction (Figure 4.10 A)
m/z 447 (31.22) ⁹⁶ , m/z 609 (27.46) ⁹⁹ , m/z 609 (26.59) ¹⁰¹ , m/z 755 (28.54) ¹³⁵ , m/z 611 (19.40) ¹³⁶ , m/z 759 (15.26) ¹³⁷ , m/z 759 (12.04) ¹³⁸ , m/z 761 (10.65) ¹³⁹ and m/z 609 (3.68) ¹⁴⁰
Boiled Lipton® Yellow label black tea (Figure 4.10 B)
m/z 265 (43.77) ⁹² , m/z 901 (37.38) ⁹³ , m/z 901 (36.51) ⁹⁴ , m/z 489 (33.63) ⁹⁵ , m/z 593 (31.00) ⁹⁷ , m/z 739 (30.43) ⁹⁸ , m/z 609 (27.46) ⁹⁹ , m/z 463 (26.78) ¹⁰⁰ , m/z 595 (18.52) ¹⁰⁴ , m/z 337 (18.24) ¹⁰⁵ , m/z 353 (14.96) ¹⁰⁸ , m/z 353 (9.54) ¹¹¹ , m/z 411 (5.64) ¹¹² , m/z 343 (4.95) ¹¹³ , m/z 191 (1.83) ¹¹⁴ , m/z 885 (37.86) ¹¹⁶ , m/z 593 (21.73) ¹²¹ , m/z 289 (17.86) ¹²² , m/z 761 (10.68) ¹³⁹ , m/z 885 (36.96) ¹⁴¹ , m/z 479 (23.48) ¹⁷ and m/z 337 (16.21) ¹⁴³
(retention time (min)) ^{compound number}
* = Mass spectrometry data extracted

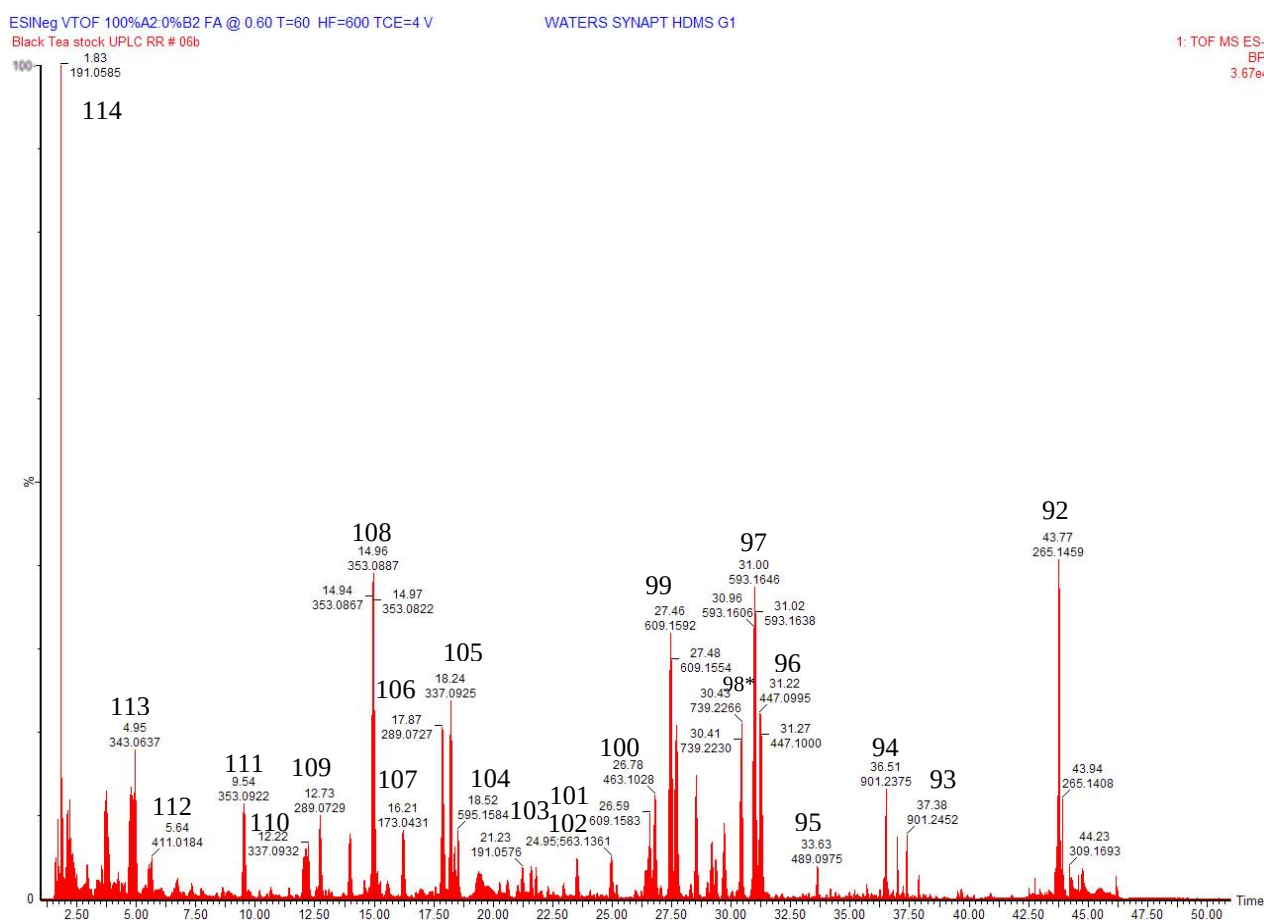


Figure 4.8: Total ion UPLC chromatogram of Lipton® Yellow label black tea as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 92-114 was indicated, and a peak summary included in Table 4.4. MS data were extracted for peak*, 98 (unknown) (Figure 6.16).

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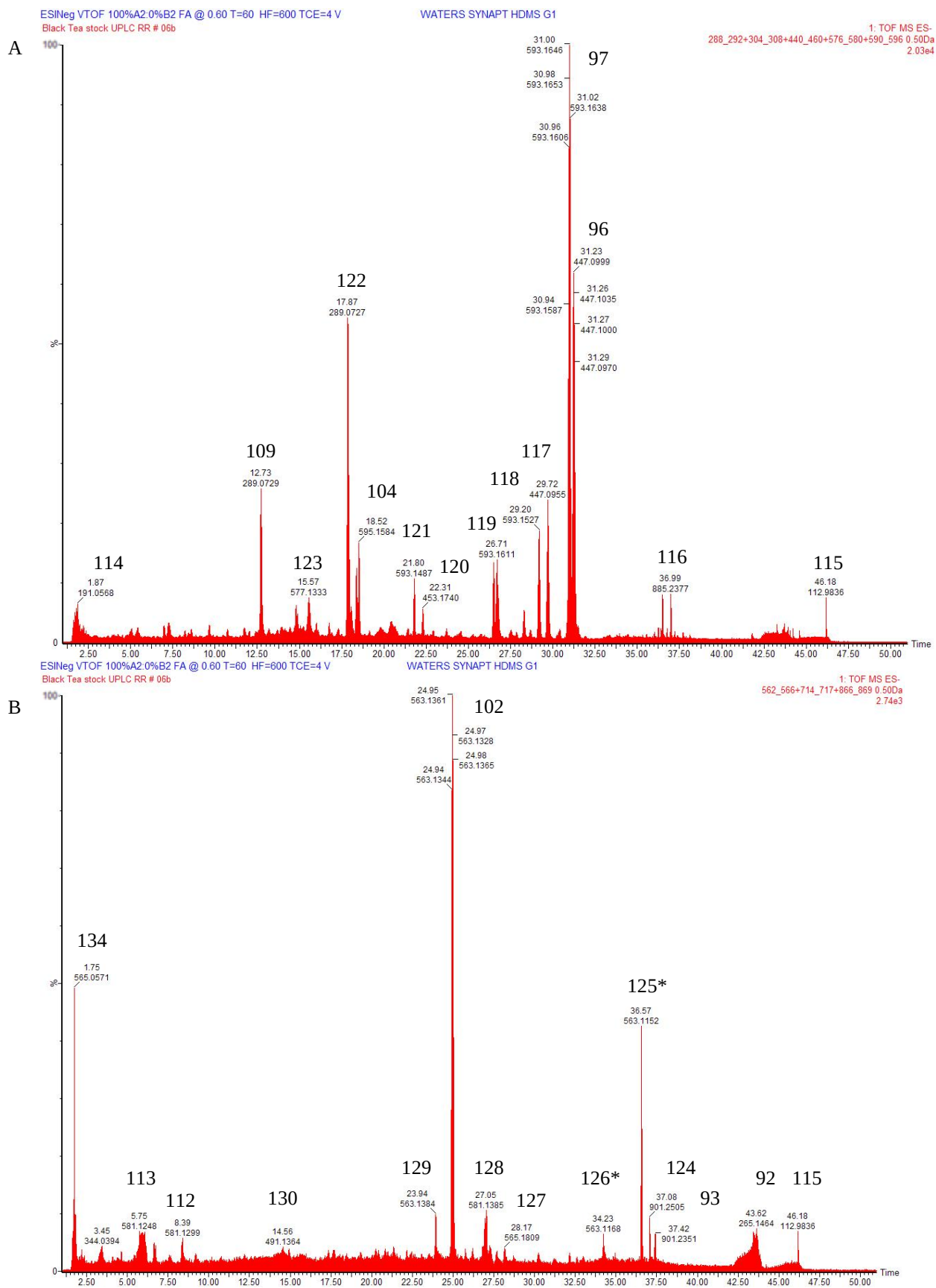


Figure 4.9: UPLC chromatogram of Lipton® Yellow label black tea A. catechin and B. theaflavin mass extracted data. The numerical peak allocation of respectively 96, 97, 104, 109, 114-123 and 92, 93, 102, 115, 124-134 was indicated and a peak summary included in Table 4.4. MS data were extracted for peaks*; 125 (unknown quercetin conjugate) and 126 (theaflavin) (Figure 6.17).

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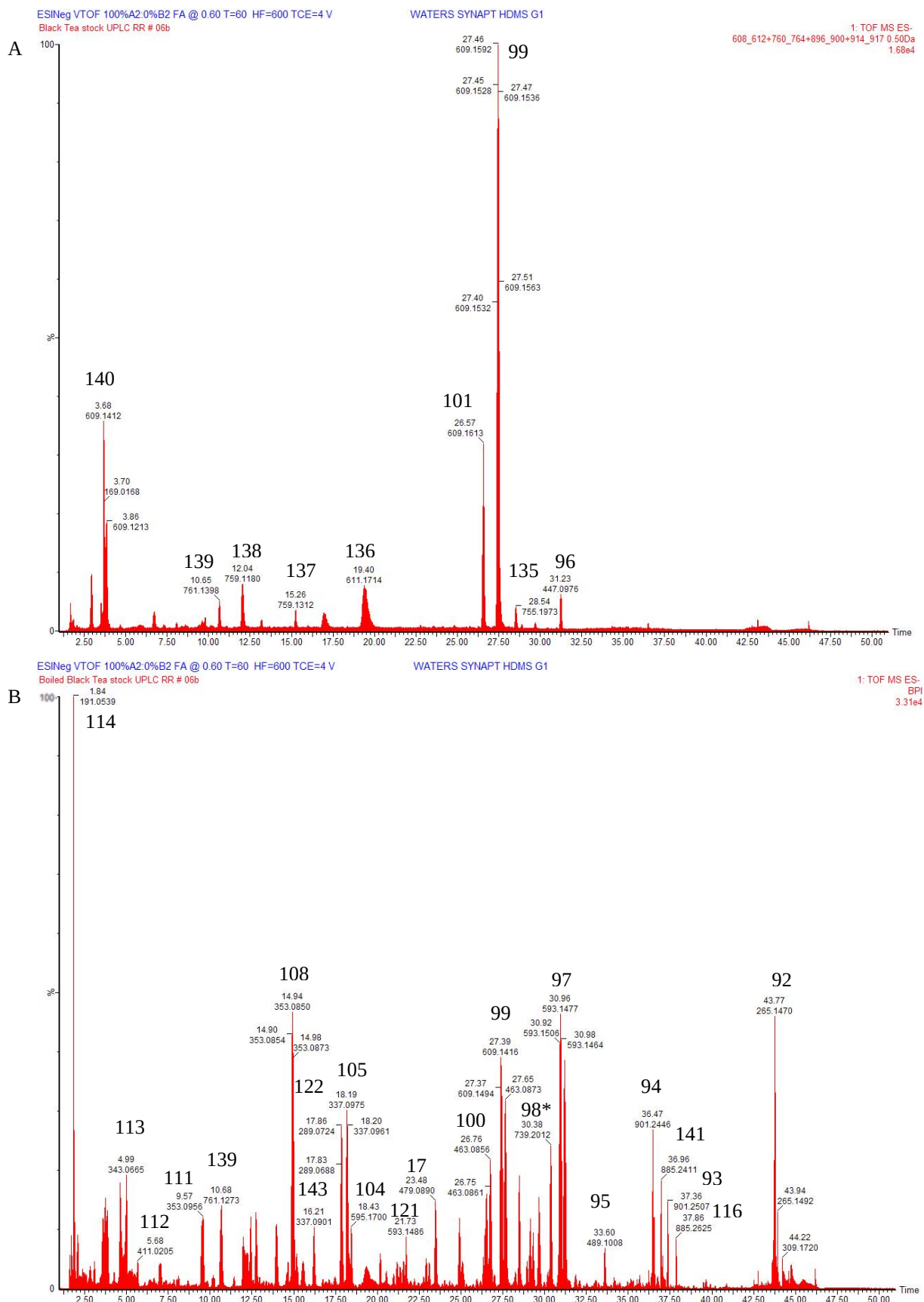


Figure 4.10: UPLC chromatogram of Lipton® Yellow label black tea A. thesinsensin mass extracted data, and B. boiled. The numerical peak allocation of respectively 96, 99, 101, 135-140 and 92-95, 97-100, 104, 105, 108, 111-114, 116, 121, 122, 141, 143 was indicated and a peak summary included in Table 4.4. MS data were extracted for peak*, 98 (unknown) (Figure 6.16).

4.3.4. Bioconversions of fresh Tea leaf catechins into theaflavins with pear, yacon and mushroom tyrosinase

Tea (*Camellia sinensis*, frozen fresh leaves) was utilized as the substrate for syntheses of theaflavins, respectively by the bioconversion with Forelle pear (*Pyrus communis*)-, Yacon (*Smallanthus sonchifolius*, leaves) homogenates and mushroom tyrosinase (Table 4.5).

Table 4.5: UPLC chromatogram peak summary of the bioconversion of Tea leaf catechins into theaflavins with pear, yacon and mushroom tyrosinase.

Pear (Figure 4.11 A)
m/z 265 (43.83) ¹⁷
Yacon (Figure 4.11 B)
m/z 265 (43.83) ¹⁷
Tea (<i>Camellia sinensis</i> , frozen fresh leaves) and Pear (Figure 4.12 A)
m/z 265 (43.84) ¹⁷ , m/z 287 (43.21) ¹⁴² , m/z 505 (34.50) ¹⁴³ , m/z 593 (33.48) ¹⁸ , m/z 609 (29.51) ²⁰ , m/z 289 (17.28) ²⁴ , m/z 289 (11.37) ²⁵ , m/z 169 (3.12) ¹⁴⁴ , m/z 169 (2.68) ¹⁴⁵ and m/z 191 (1.67) ³⁰
Tea (<i>Camellia sinensis</i> , frozen fresh leaves) and Mushroom tyrosinase (Figure 4.12 B)
m/z 265 (43.84) ¹⁷ , m/z 593 (33.48) ¹⁸ , m/z 447 (30.71) ¹⁹ , m/z 609 (29.51) ²⁰ , m/z 563 (28.29) ^{146*} , m/z 463 (27.18) ²¹ , m/z 289 (17.28) ²⁴ , m/z 289 (11.37) ²⁵ , m/z 169 (3.12) ¹⁴⁴ , m/z 169 (2.68) ¹⁴⁵ and m/z 191 (1.67) ³⁰
Tea (<i>Camellia sinensis</i> , frozen fresh leaves) and Yacon (Figure 4.13)
m/z 265 (43.84) ¹⁷ , m/z 593 (33.48) ¹⁸ , m/z 609 (29.51) ²⁰ , m/z 289 (17.28) ²⁴ , m/z 289 (11.37) ²⁵ , m/z 191 (1.67) ³⁰ , m/z 447 (30.71) ¹⁹ and m/z 463 (27.18) ²¹
Theaflavin mass extraction of Tea (<i>Camellia sinensis</i> , frozen fresh leaves) with respectively with Mushroom tyrosinase, Pear- and Yacon homogenate (Figure 4.14)
m/z 271 (43.57) ¹⁷ , m/z 563 (34.29) ¹⁸ , m/z 609 (29.46) ²⁰ , m/z 289 (17.28) ²⁴ , m/z 191 (1.65) ³⁰ and respectively m/z 563 (27.06) ^{146*} , m/z 563 (27.00) ^{147*} and m/z 563 (26.95) ^{148*}
(retention time (min)) ^{number}
* = Mass spectrometry data extracted

The efficiency of the bioconversion of Tea (*Camellia sinensis*, frozen fresh leaves) catechins respectively by mushroom tyrosinase, pear- and yacon homogenate, was assessed based upon the theaflavin mass extraction (562-566+714-717+866-869) of the UPLC-MS data (Figure 4.14). The mass spectra of the respectively identified peaks; m/z 563 (26.03)¹⁴⁶, m/z 563 (26.00)¹⁴⁷ and m/z 563 (25.98)¹⁴⁸ corresponding to the monoisotopic mass of theaflavin were extracted and comparatively evaluated based upon those correspondingly present in Lipton® Yellow label black tea [m/z 563 (35.53)¹⁴⁷ and m/z 563 (23.95)¹⁰²] (Figure 4.15).

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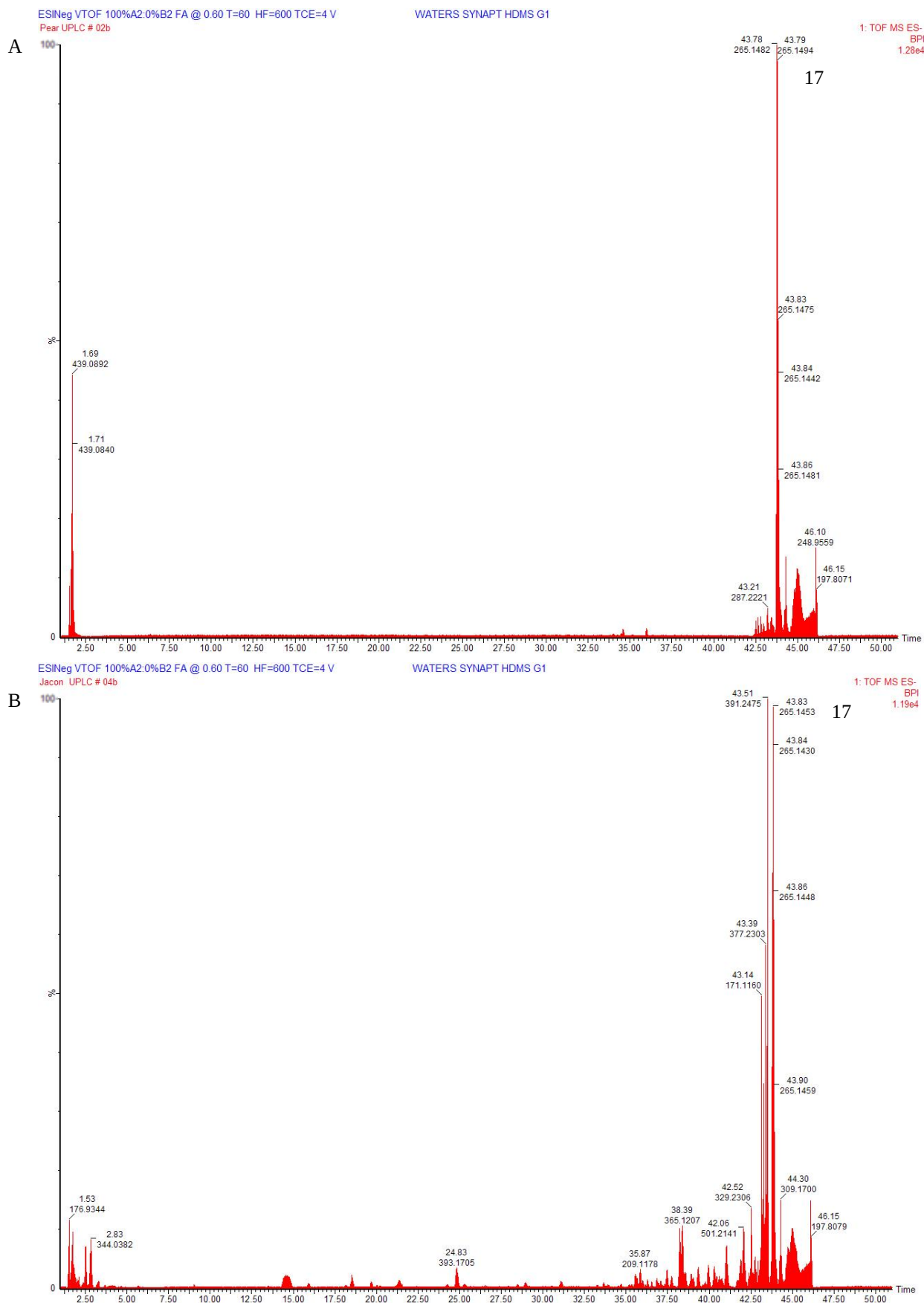


Figure 4.11: UPLC chromatogram of the control samples A. Pear- B. Yacon homogenate as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 17 was indicated, and a peak summary included in Table 4.5.

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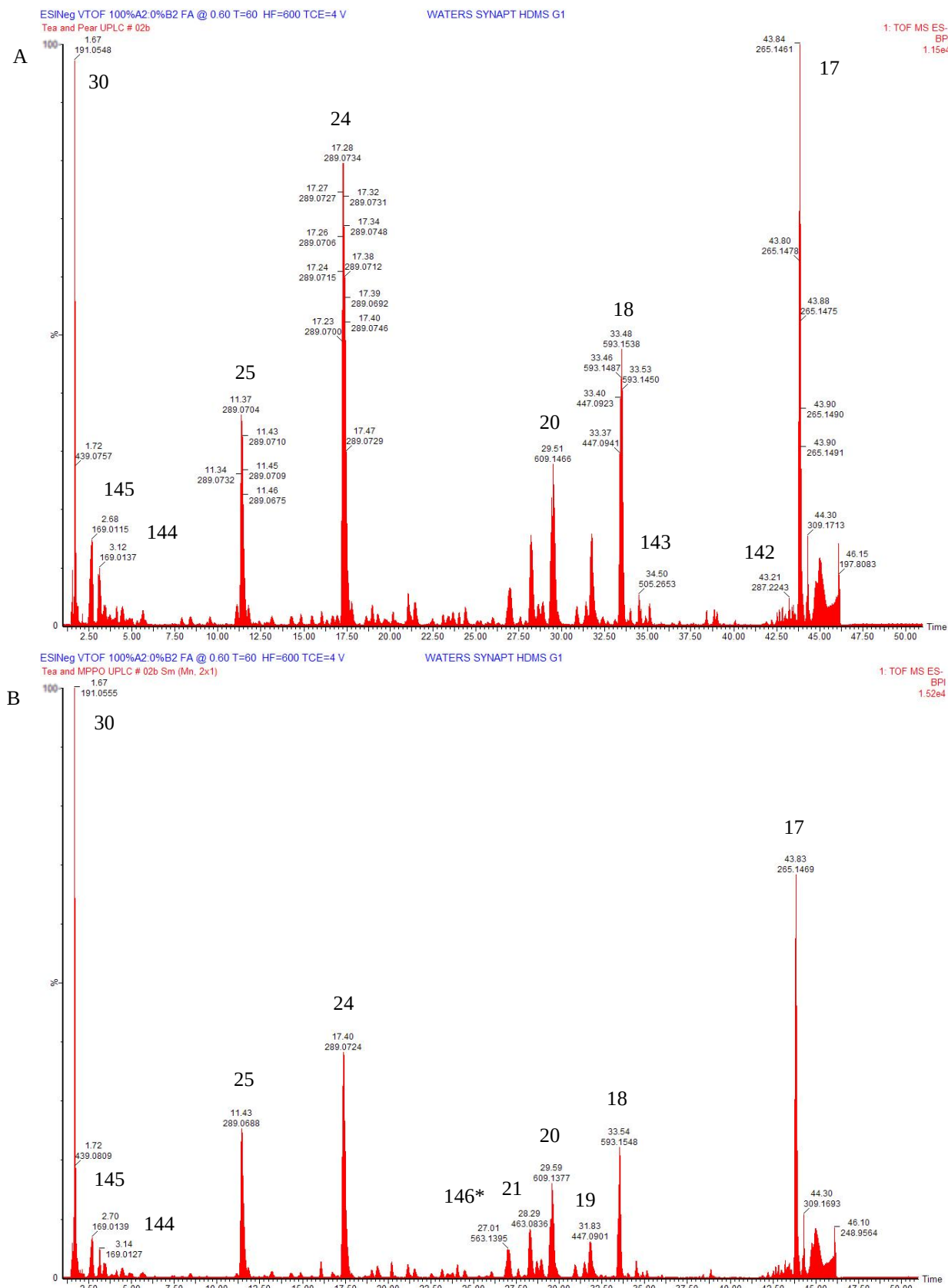


Figure 4.12: UPLC chromatogram of bioconversion of Tea (*Camellia sinensis*, frozen fresh leaves) with A. Pear homogenate B. mushroom tyrosinase as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of respectively 17, 18, 20, 24, 25, 30, 142-145 and 17-21, 24, 25, 30, 144-146 was indicated and a peak summary included in Table 4.5. MS data were extracted for peak*, 146 (*O*-pentosyl 8-*C*-hexosyl apigenin or apigenin-7-*O*- β -D-apiofuranosyl- β -D-glucopyranoside or isoschaftoside (apigenin 6-*C*- α -L-arabinopyranosyl-8-*C*- β -D-glucopyranoside)) (Figure 4.15).

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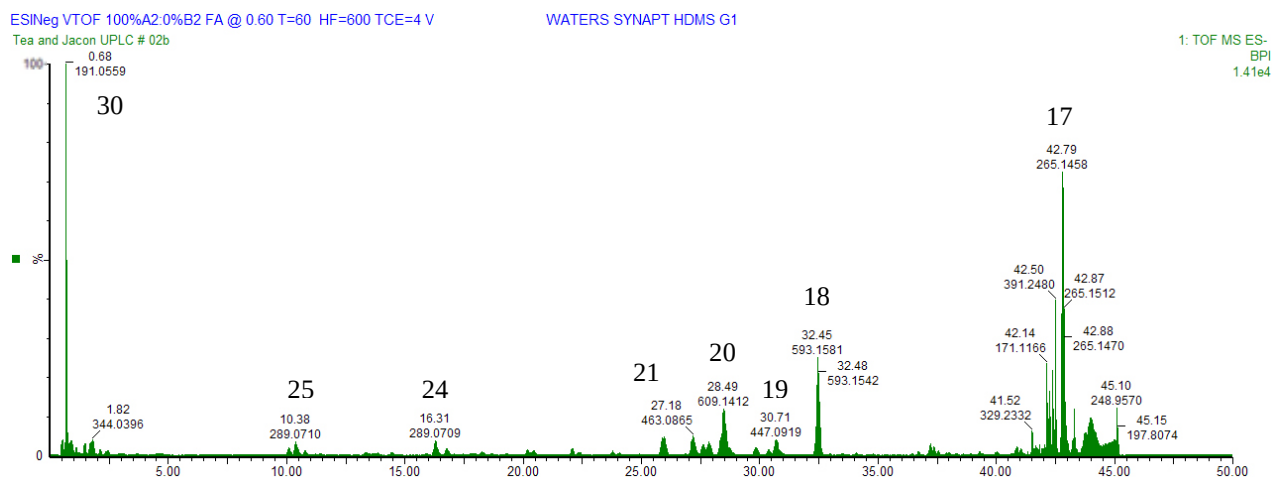


Figure 4.13: UPLC chromatogram of bioconversion of Tea (*Camellia sinensis*, frozen fresh leaves) with Yacon homogenate as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 17-21, 24, 25, 30 was indicated and a peak summary included in Table 4.5.

The elemental compositions of the peaks and fragment ions; m/z 563 (35.53)¹²⁶, m/z 563 (23.95)¹⁰², m/z 613 [M-H+50]⁻ (23.95)¹⁰², m/z 563 (26.03)¹⁴⁶, m/z 641 [M-H+78]⁻ (26.03)¹⁴⁶, m/z 563 (26.00)¹⁴⁷, m/z 641 [M-H+78]⁻ (26.00)¹⁴⁷ and m/z 563 (25.98)¹⁴⁸ were determined and based upon the i-Fit values the most confident assignments respectively C₂₉H₂₃O₁₂, C₂₆H₂₇O₁₄, C₂₉H₂₅O₁₅, C₂₆H₂₇O₁₄, C₂₇H₂₉O₁₈, C₂₆H₂₇O₁₄, C₂₇H₂₉O₁₈ and C₂₆H₂₇O₁₄ were included in Table 6.6, Appendix C (Figure 4.15).

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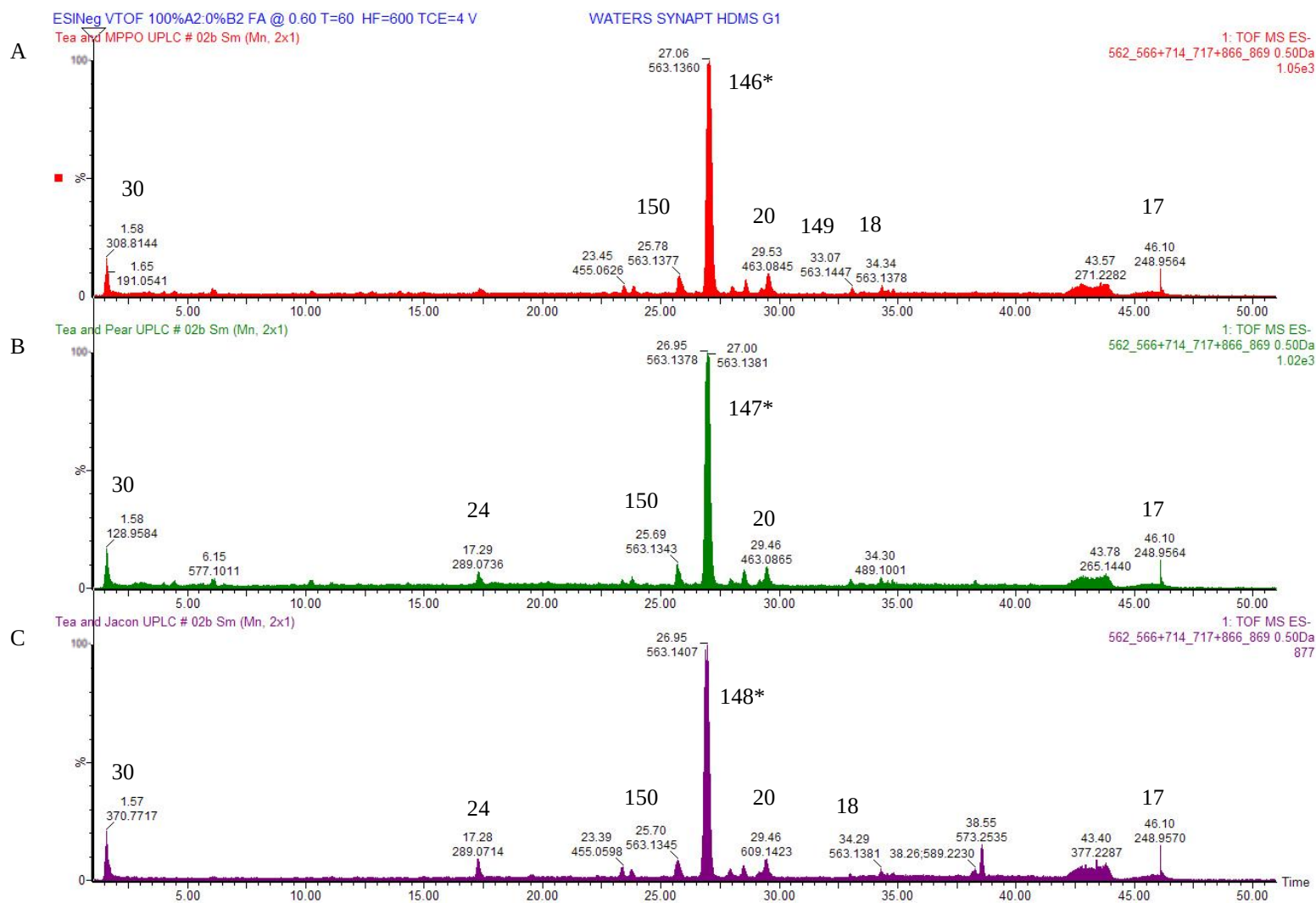


Figure 4.14: Theaflavin mass extracted UPLC chromatogram of bioconversion of Tea (*Camellia sinensis*, frozen fresh leaves) with A. mushroom tyrosinase B. Pear- C. Yacon homogenate. The numerical peak allocation of 17, 18, 20, 24, 30, 150 and respectively 146, 147 and 148 was indicated and a peak summary included in Table 4.6. MS data were extracted for peaks*, 146, 147 and 148 (O-pentosyl 8-C-hexosyl apigenin or apigenin-7-O-β-D-apiofuranosyl-β-D-glucopyranoside or isoschaftoside (apigenin 6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranoside)) (Figure 4.15).

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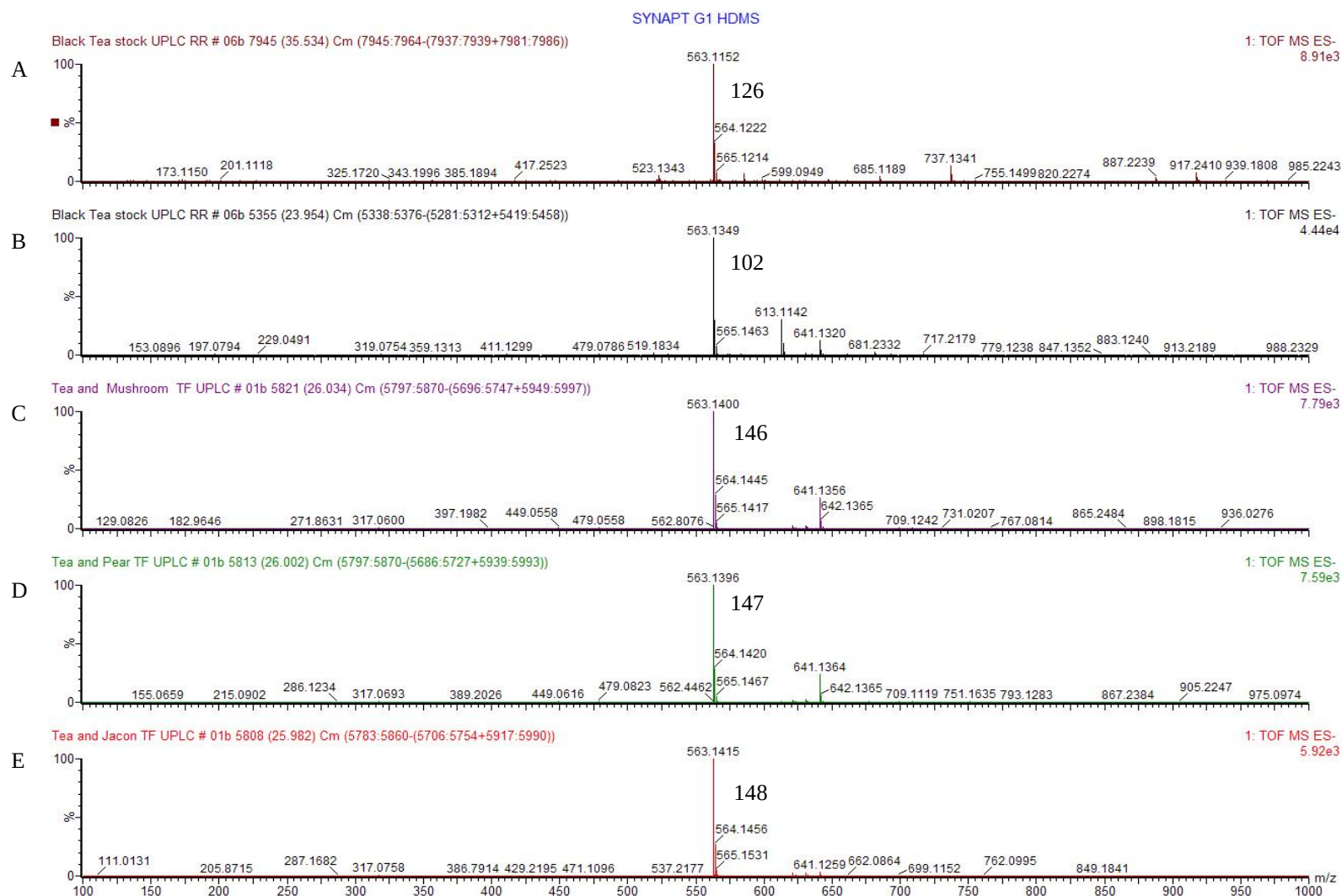


Figure 4.15: A. MS spectra of compound 126 (Theaflavin), $[M-H]^-$, at m/z 563 (35.53). B. MS spectra of compound 102, $[M-H]^-$, at m/z 563 (23.95). C. MS spectra of compound 146, $[M-H]^-$, at m/z 563 (26.03). D. MS spectra of compound 147, $[M-H]^-$, at m/z 563 (26.00). E. MS spectra of compound 148, $[M-H]^-$, at m/z 563 (25.98). Peaks 146-148 and 102 corresponded to *O*-pentosyl 8-*C*-hexosyl apigenin or apigenin-7-*O*- β -D-apiofuranosyl- β -D-glucopyranoside or isoschaftoside (apigenin 6-*C*- α -L-arabinopyranosyl-8-*C*- β -D-glucopyranoside).

4.3.5. Bioconversions of a catechin mixture into theaflavin with pear polyphenol oxidase and mushroom tyrosinase

In order to respectively assess the synthesis of the peaks; m/z 563 (26.00)¹⁴⁷ and m/z 563 (26.03)¹⁴⁶ a catechin mixture containing epicatechin ((-)EC) and epigallocatechin (EGC) was utilized as starting material for the synthesis of theaflavin by purified Forelle pear polyphenol oxidase and mushroom tyrosinase (Table 4.6).

Table 4.6: UPLC chromatogram peak summary of the bioconversion of a catechin mixture into theaflavin with pear polyphenol oxidase and mushroom tyrosinase.

Catechin mixture (epicatechin and epigallocatechin) (Figure 4.2 A) m/z 265 (43.78)¹, m/z 289 (17.27)^{2*}, m/z 289 (11.38)^{3*}, m/z 305 (5.76)^{4*}, m/z 609 (3.15)^{5*}, m/z 176 (1.61)⁶ Theaflavin mass extracted data: Catechin mixture with pear polyphenol oxidase (Figure 4.16 A) m/z 265 (43.79)¹, m/z 563 (38.20)^{149a*}, m/z 563 (35.54)^{150a*}, m/z 160 (32.73)¹⁵¹, m/z 563 (29.08)¹⁵², m/z 563 (24.18)¹⁵³, m/z 289 (17.00)², m/z 563 (13.81)¹⁵⁴, m/z 563 (12.63)¹⁵⁵, m/z 581 (7.74)¹⁵⁶ and m/z 581 (4.49)¹⁵⁷ Catechin mixture with Mushroom tyrosinase (Figure 4.16 B) m/z 563 (38.21)^{149b*} and m/z 563 (35.58)^{150b*} (retention time (min))^{number} * = Mass spectrometry data extracted
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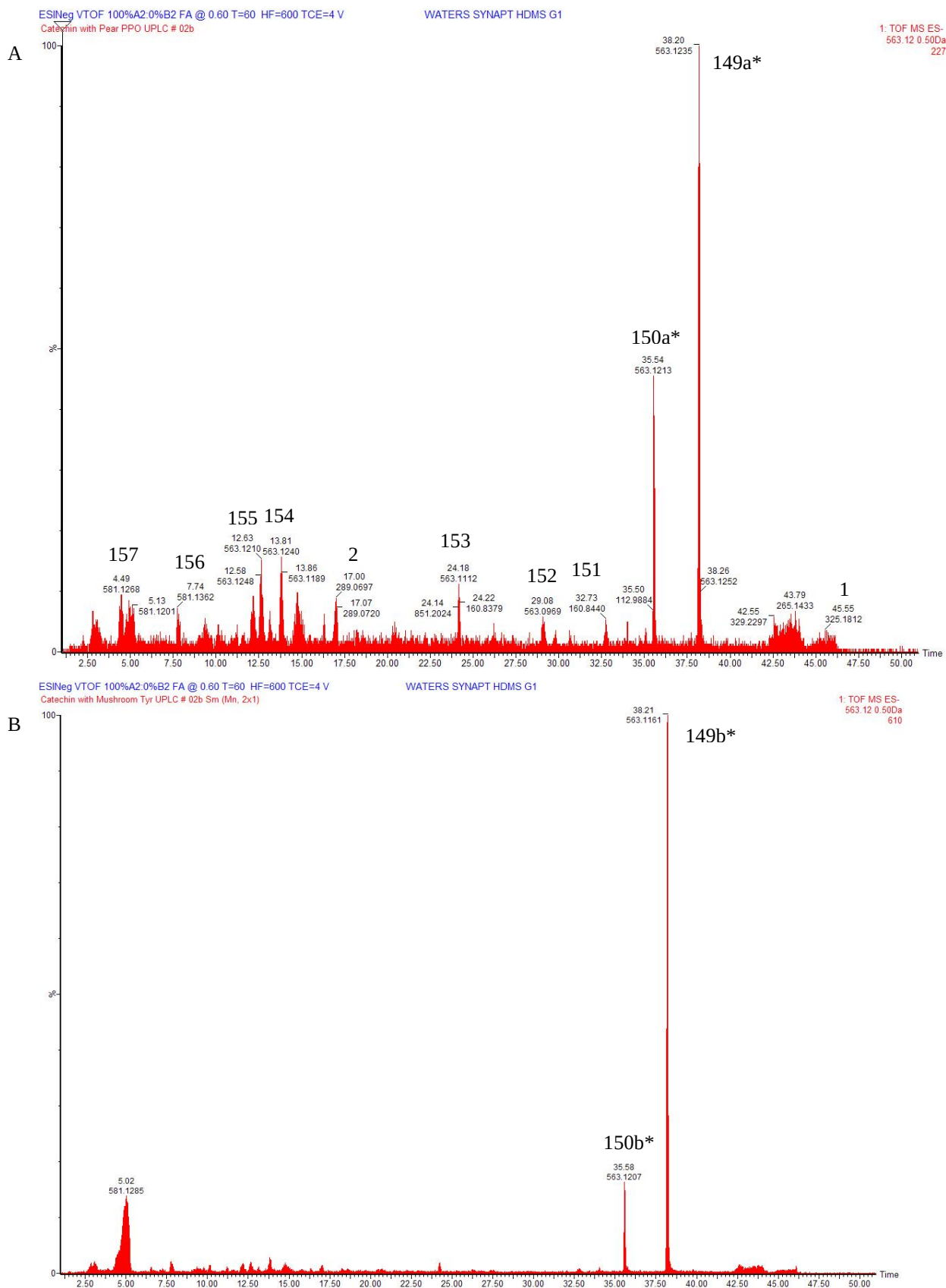


Figure 4.16: Theaflavin mass extracted (563) UPLC chromatogram of the bioconversion of a catechin mixture with A. pear polyphenol oxidase B. mushroom tyrosinase. The numerical peak allocation respectively of 1, 2, 149a, 150a, 151-157 and 149b, 150b was indicated and a peak summary included in Table 4.6. MS data were extracted for peaks*, 149a (unknown), 150a (theaflavin) (Figure 4.17), 149b (unknown) and 150b (theaflavin) (Figure 4.18).

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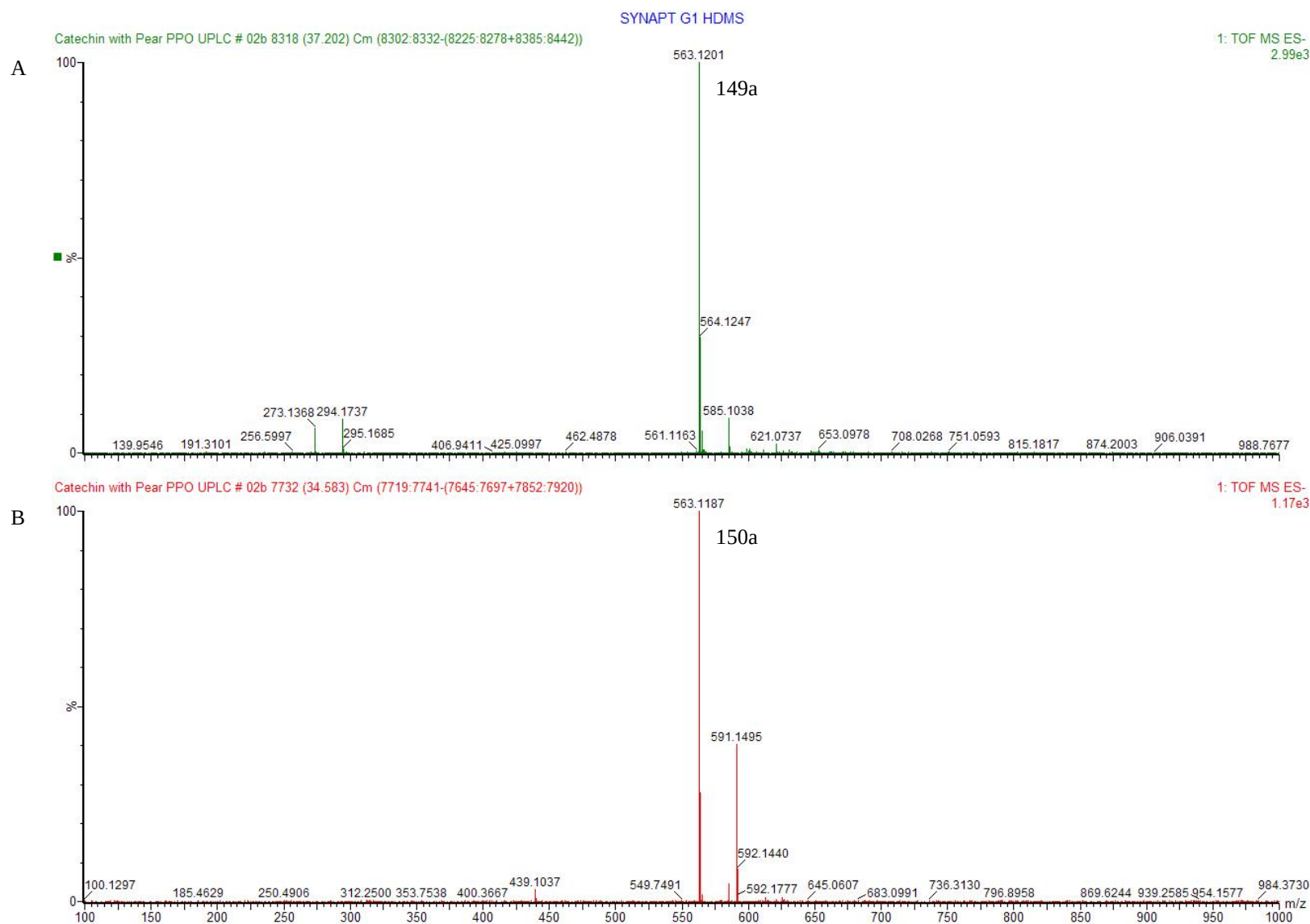
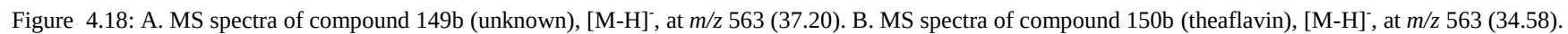


Figure 4.17: A. MS spectra of compound 149a (unknown), $[M-H]^-$, at m/z 563 (37.20). B. MS spectra of compound 150a (theaflavin), $[M-H]^-$, at m/z 563 (34.58).



4.3.6. Immobilized- and free laccase biotransformation of tea catechins into theaflavins utilizing a catechin mixture

Laccase (Suberase from Novozyme (Pty) Ltd) from *Myceliophthora thermophila* respectively as a free- and ReSyn™ immobilized enzyme was utilized for the synthesis of theaflavins performed at 37.5°C for 150 min utilizing the catechin mixture, Theaphenon E (respectively 80-, 40- and 20 mg) as the substrate (Figures 4.19 and 4.20). Subsequent biotransformation reactions were performed utilizing 20 mg of starting material. Theaflavins syntheses were also assessed as a function of time over the intervals of 0-, 30-, 60-, 90-, 120- and 150 min. In addition, ReSyn™ immobilized laccase catechin bioconversion was also performed over the time periods; 0-, 60-, 120-, 240-, 360-, 480- and 1440 min (Figure 4.27).

Table 4.7: UPLC chromatogram peak summary of the bioconversion of a catechin mixture, respectively 80-, 40- and 20 mg into theaflavin with free- and ReSyn™ immobilized laccase.

Commercial catechin mix, Theaphenon E (Figure 4.2 B).
m/z 447 (34.01) ⁷ , m/z 441 (28.86) ⁸ , m/z 883 (26.84) ^{9*} , m/z 457 (21.70) ^{10*} , m/z 915 (18.50) ^{11*} , m/z 289 (18.25) ^{12*} , m/z 305 (12.05) ^{13*} , m/z 305 (6.14) ^{14*} , m/z 169 (3.12) ^{15*} , m/z 176 (1.57) ¹⁶
Free laccase:
Catechin mixture with free laccase (80 mg) (Figure 4.19 A)
m/z 265 (44.63) ¹ , m/z 551 (36.84) ¹⁵⁸ , m/z 911 (33.01) ¹⁵⁹ , m/z 913 (29.20) ^{160*} , m/z 883 (25.79) ^{9*} , m/z 635 (24.65) ¹⁶¹ , m/z 457 (20.77) ¹⁰ , m/z 915 (17.76) ¹¹ , m/z 913 (15.25) ^{162*} , m/z 305 (11.44) ¹³ , m/z 761 (9.07) ¹⁶³ , m/z 305 (5.76) ⁴ , m/z 169 (2.89) ¹⁶⁴ and m/z 181 (1.61) ¹⁶⁵
Catechin mixture with free laccase (40 mg) (Figure 4.19 B)
m/z 883 (25.79) ^{9*} , m/z 915 (17.76) ¹¹ , m/z 913 (15.25) ^{162*} , m/z 305 (11.44) ¹³ , m/z 761 (9.07) ¹⁶³ , m/z 305 (5.76) ⁴ and m/z 181 (1.61) ¹⁶⁵
Catechin mixture with free laccase (20 mg) (Figure 4.19 C)
m/z 883 (25.79) ^{9*} , m/z 915 (17.76) ¹¹ , m/z 913 (15.25) ^{162*} , m/z 761 (9.07) ¹⁶³ and m/z 181 (1.61) ¹⁶⁵
ReSyn™ laccase
Catechin mixture with ReSyn™ laccase (80 mg) (Figure 4.20 A)
m/z 265 (44.63) ¹ , m/z 551 (36.84) ¹⁵⁸ , m/z 913 (34.24) ¹⁶⁶ , m/z 913 (29.20) ^{160*} , m/z 883 (25.79) ^{9*} , m/z 913 (24.41) ¹⁶⁷ , m/z 457 (20.77) ¹⁰ , m/z 915 (17.76) ¹¹ , m/z 913 (15.25) ^{162*} , m/z 289 (11.54) ³ , m/z 305 (11.42) ¹³ , m/z 761 (9.15) ¹⁶³ , m/z 305 (5.79) ⁴ and m/z 169 (2.88) ¹⁵
Catechin mixture with ReSyn™ laccase (40 mg) (Figure 4.20 B)
m/z 883 (25.79) ^{9*} , m/z 913 (22.01) ¹⁶⁸ , m/z 457 (20.89) ¹⁰ , m/z 915 (17.87) ¹¹ , m/z 289 (17.60) ^{2*} , m/z 305 (11.50) ¹³ , m/z 761 (9.29) ¹⁶³ and m/z 305 (5.83) ^{4*}
Catechin mixture with ReSyn™ laccase (20 mg) (Figure 4.20 C)
m/z 883 (25.79) ^{9*} , m/z 913 (21.95) ^{169*} , m/z 289 (17.60) ^{2*} , m/z 289 (11.58) ¹⁷⁰ and m/z 761 (9.21) ¹⁶³
(retention time (min)) ^{number}
* = Mass spectrometry data extracted

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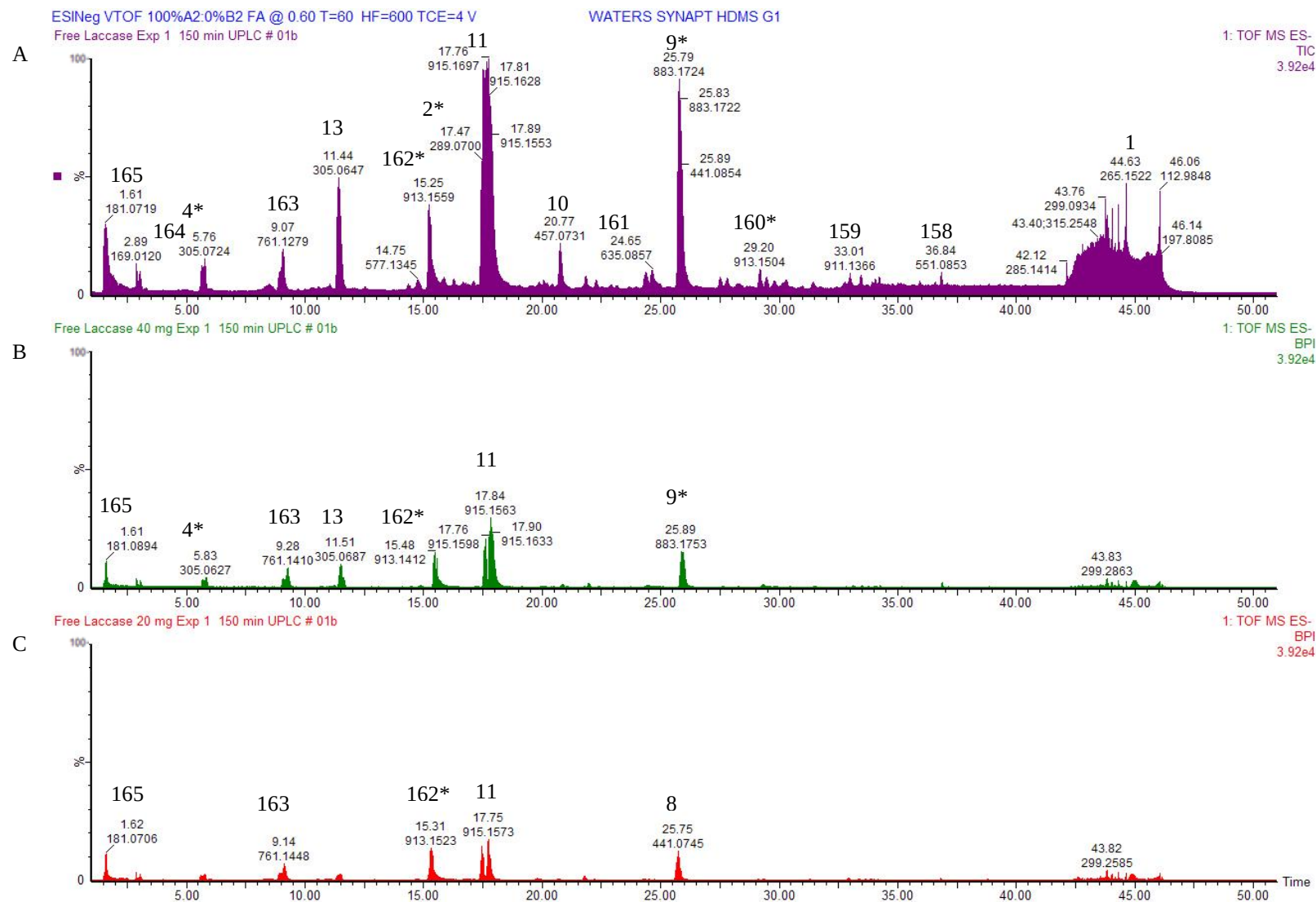


Figure 4.19: UPLC chromatogram of bioconversion of catechin mixture with free laccase from *Myceliophthora thermophila* A. 80 mg B. 40 mg and C. 20 mg as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 1, 4, 8-11, 13 and 158-165 was indicated and a peak summary included in Table 4.7. MS data were extracted for peaks*; 2 (epicatechin) (Figure 6.5 A), 4 (epigallocatechin/ gallocatechin) (Figure 6.5 C), 9 (epicatechin gallate/ catechin gallate) (Figure 6.8), 160 (theasinensin A or D) and 162 (theasinensin A) (Figure 6.18).

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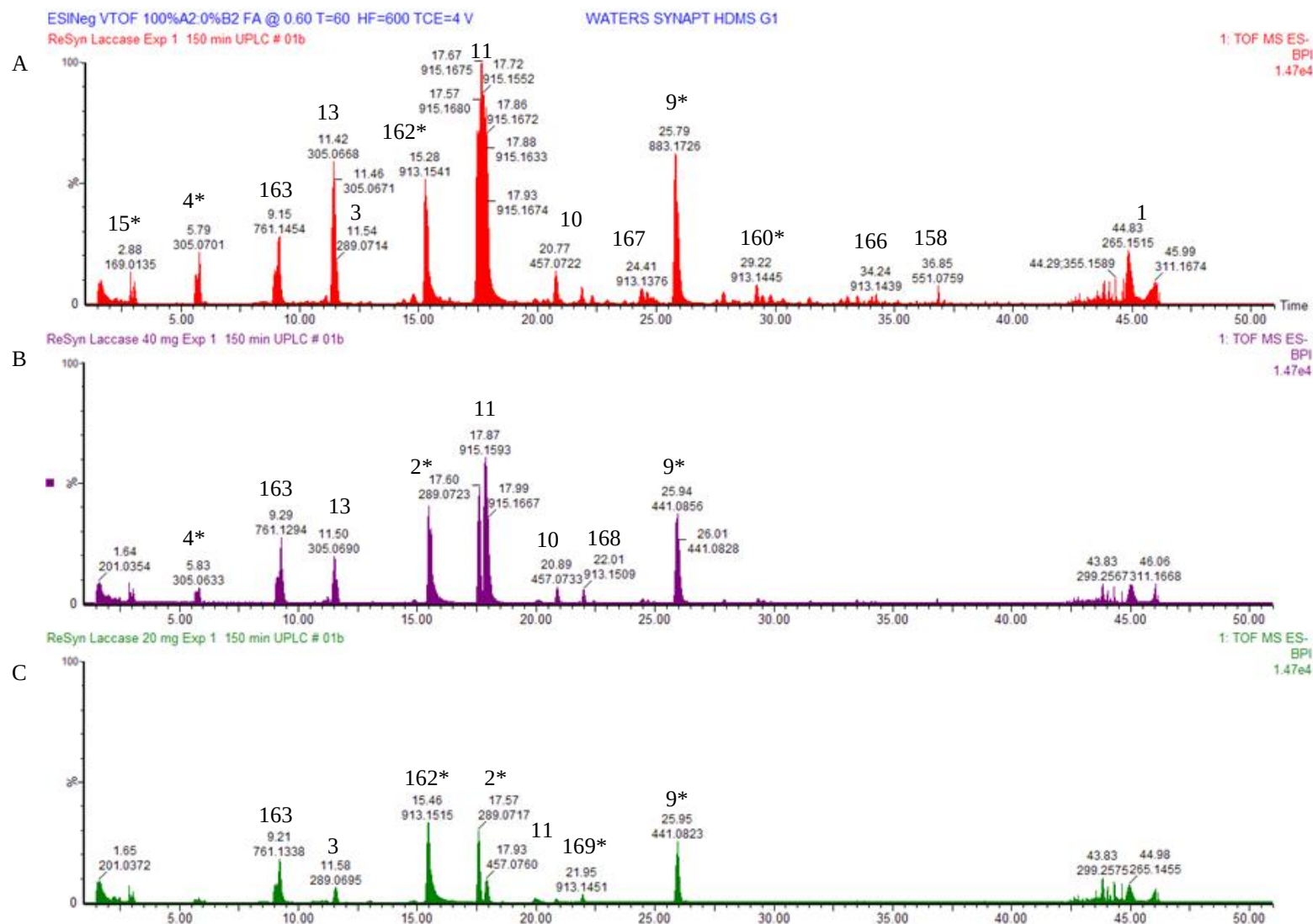


Figure 4.20: UPLC chromatogram of bioconversion of catechin mixture with ReSyn™ immobilized laccase from *Myceliophthora thermophila* A. 80 mg B. 40 mg and C. 20 mg as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 1, 3, 4, 9-11, 13, 15, 162, 163, 166, 167, 168 and 169 was indicated and a peak summary included in Table 4.7. MS data were extracted for peaks*; 2 (epicatechin) (Figure 6.5 A), 4 (epigallocatechin/ gallic acid) (Figure 6.5 C), 9 (epicatechin gallate/ catechin gallate) (Figure 6.8), 15 (gallic acid) (Figure 6.9), 162 (theasinensin A) (Figure 6.18) and 169 (theasinensin D) (Figure 6.16).

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Table 4.8: UPLC chromatogram peak summary of the bioconversion of a catechin mixture (20 mg) into theaflavin with free- and ReSyn™ immobilized laccase.

Commercial catechin mix, Theaphenon E (Figure 4.2 B).
m/z 447 (34.01) ⁷ , m/z 441 (28.86) ⁸ , m/z 883 (26.84) ^{9*} , m/z 457 (21.70) ^{10*} , m/z 915 (18.50) ^{11*} , m/z 289 (18.25) ^{12*} , m/z 305 (12.05) ^{13*} , m/z 305 (6.14) ^{14*} , m/z 169 (3.12) ^{15*} , m/z 176 (1.57) ¹⁶
Catechin mixture (20 mg) with free laccase
Total ion chromatogram after 150 min (Figure 4.21)
m/z 441 (25.75) ^{9*} , m/z 913 (21.77) ^{169*} , m/z 915 (17.76) ¹¹ , m/z 913 (15.25) ^{162*} , m/z 289 (11.48) ³ , m/z 761 (9.14) ¹⁶³ and m/z 169 (2.88) ^{15*}
Theaflavin mass extraction (Figure 4.23)
m/z 867 (39.29) ¹⁷⁰ , m/z 715 (38.80) ¹⁷¹ , m/z 563 (38.21) ¹⁷² , m/z 715 (26.55) ¹⁷³ and m/z 713 (24.88) ¹⁷⁴
Theasinensin mass extraction (Figure 4.25)
m/z 915 (17.75) ¹¹ , m/z 913 (15.31) ^{162*} and m/z 761 (9.14) ¹⁶³
Catechin mixture (20 mg) with ReSyn™ laccase
Total ion chromatogram after 150 min (Figure 4.22)
m/z 441 (25.75) ^{9*} , m/z 913 (21.77) ^{169*} , m/z 915 (17.76) ¹¹ , m/z 289 (17.57) ^{2*} , m/z 913 (15.25) ^{162*} , m/z 289 (11.48) ³ , m/z 761 (9.14) ¹⁶³ and m/z 305 (5.75) ^{4*}
Total ion chromatogram after 1140 min (Figure 4.27)
m/z 441 (27.07) ⁸ , m/z 289 (18.47) ^{2*} , m/z 913 (16.52) ^{162*} , m/z 289 (12.33) ³ , m/z 761 (10.01) ¹⁶³ and m/z 169 (3.00) ¹⁶⁴
Theaflavin mass extraction (Figure 4.24)
m/z 867 (39.29) ¹⁷⁰ , m/z 715 (38.80) ¹⁷¹ , m/z 563 (38.21) ¹⁷² and m/z 713 (24.88) ¹⁷⁴
Theasinensin mass extraction (Figure 4.26)
m/z 913 (21.96) ^{169*} , m/z 915 (17.75) ¹¹ , m/z 913 (15.31) ^{162*} , m/z 761 (9.14) ¹⁶³ and m/z 609 (2.93) ^{5*}
(retention time (min)) ^{number}
* = Mass spectrometry data extracted

The assessments of the efficiency of the biotransformations were aided by both theaflavin mass extraction (562-566+714-717+866-869 Da) and theasinensin mass extraction (608-612+760-74+896-900+914-917 Da) of the UPLC chromatogram data (Table 4.8). Peaks corresponding to theaflavin-3,3-di-gallate (m/z 867 (39.29)¹⁷⁰), theaflavin-3-*O*-gallate or theaflavin-3'-*O*-gallate (m/z 715 (38.80)¹⁷¹) and theaflavin (m/z 563 (38.21)¹⁷²), were quantified based upon the respective standard curves, as 637.0-, 998.4- and 282.8 ng/ml after 150 min when free laccase was employed (Figure 4.23). Similarly the peaks corresponding to theaflavin-3,3-di-gallate (m/z 867 (39.29)¹⁷⁰), theaflavin-3-*O*-gallate or theaflavin-3'-*O*-gallate (m/z 715 (38.80)¹⁷¹) and theaflavin (m/z 563 (38.21)¹⁷²), were quantified as 414.1-, 707.1- and 120.2 ng/ml after 150 min when ReSyn™ immobilized laccase was employed (Figures 4.24 and 6.19 and Table 6.7). The peak, m/z 563 (34.23)^{126*} corresponding to theaflavin, present in the control Lipton® Yellow label black tea was quantified as 20500 ng/ml (Figures 4.9 B and Table 6.7).

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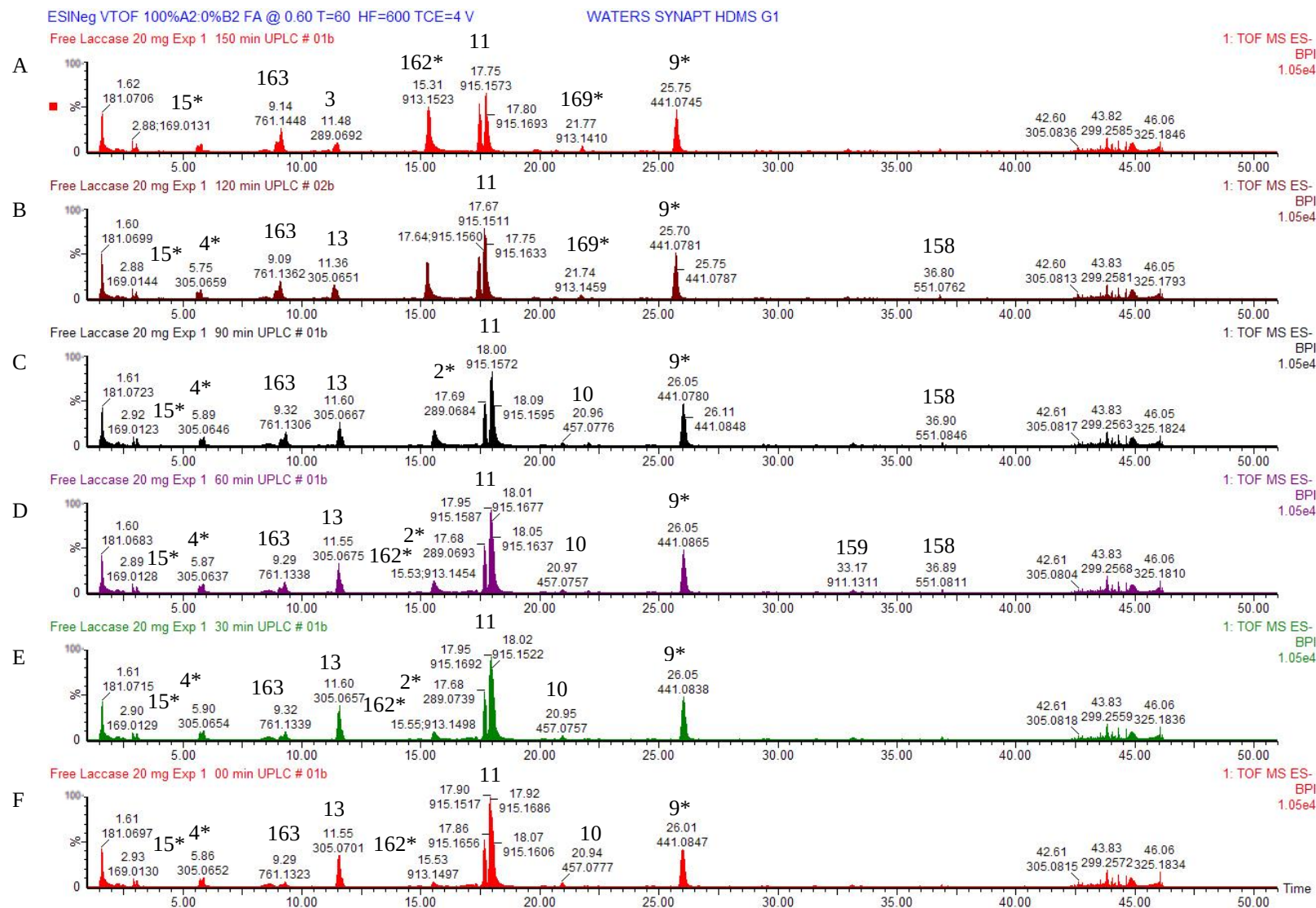


Figure 4.21: UPLC chromatogram of bioconversion of catechin mixture with free laccase from *Myceliophthora thermophila* over the time points; A. 150-, B. 120-, C. 90-, D. 60-, E. 30- and F. 0 min as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 3, 4, 9-11, 13, 15, 158, 159, 162 and 163 was indicated and a peak summary included in Table 4.8. MS data were extracted for peaks*; 2 (epicatechin) (Figure 6.5), 4 (epigallocatechin/ gallic acid) (Figure 6.5), 9 (epicatechin gallate/ catechin gallate) (Figure 6.8), 15 (gallic acid) (Figure 6.9) and 162 (theasinensin A) (Figure 6.18).

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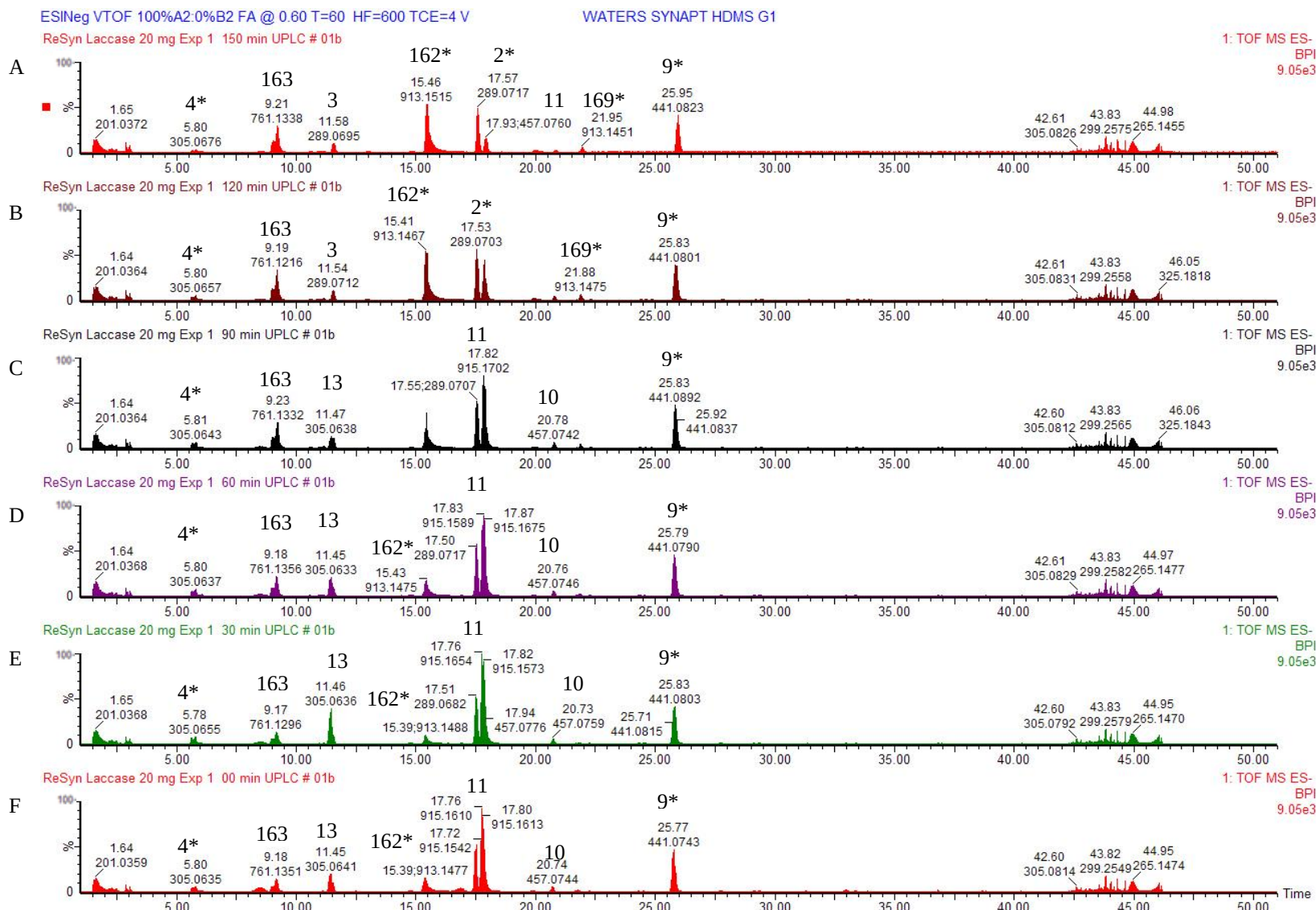
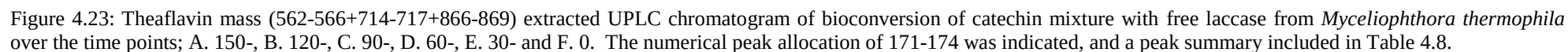


Figure 4.22: UPLC chromatogram of bioconversion of catechin mixture with ReSyn™ immobilized laccase over the time points; A. 150-, B. 120-, C. 90-, D. 60-, E. 30- and F. 0 min as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 2, 3, 4, 9-11, 13, 162 and 163 was indicated and a peak summary included in Table 4.8. MS data were extracted for peaks*; 2 (epicatechin) (Figure 6.5), 4 (epigallocatechin/ gallic catechin) (Figure 6.5), 9 (epicatechin gallate/ catechin gallate) (Figure 6.8) and 162 (theasinensin A) (Figure 6.18).



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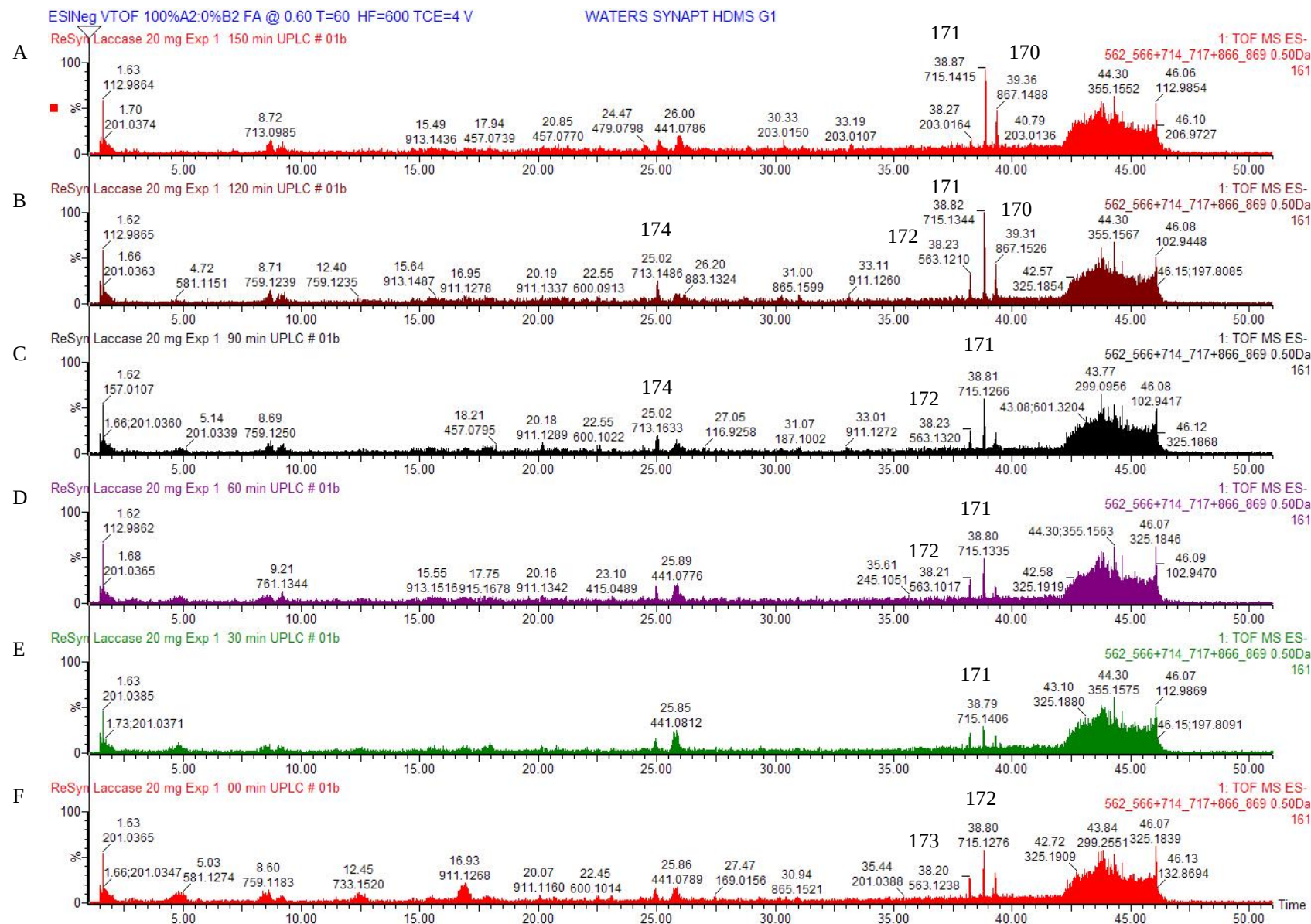


Figure 4.24: Theaflavin mass (562-566+714-717+866-869) extracted UPLC chromatogram of bioconversion of catechin mixture with ReSynTM immobilized laccase over the time points; A. 150-, B. 120-, C. 90-, D. 60-, E. 30- and F. 0 min. The numerical peak allocation of 170-173 and 174 was indicated, and a peak summary included in Table 4.8.

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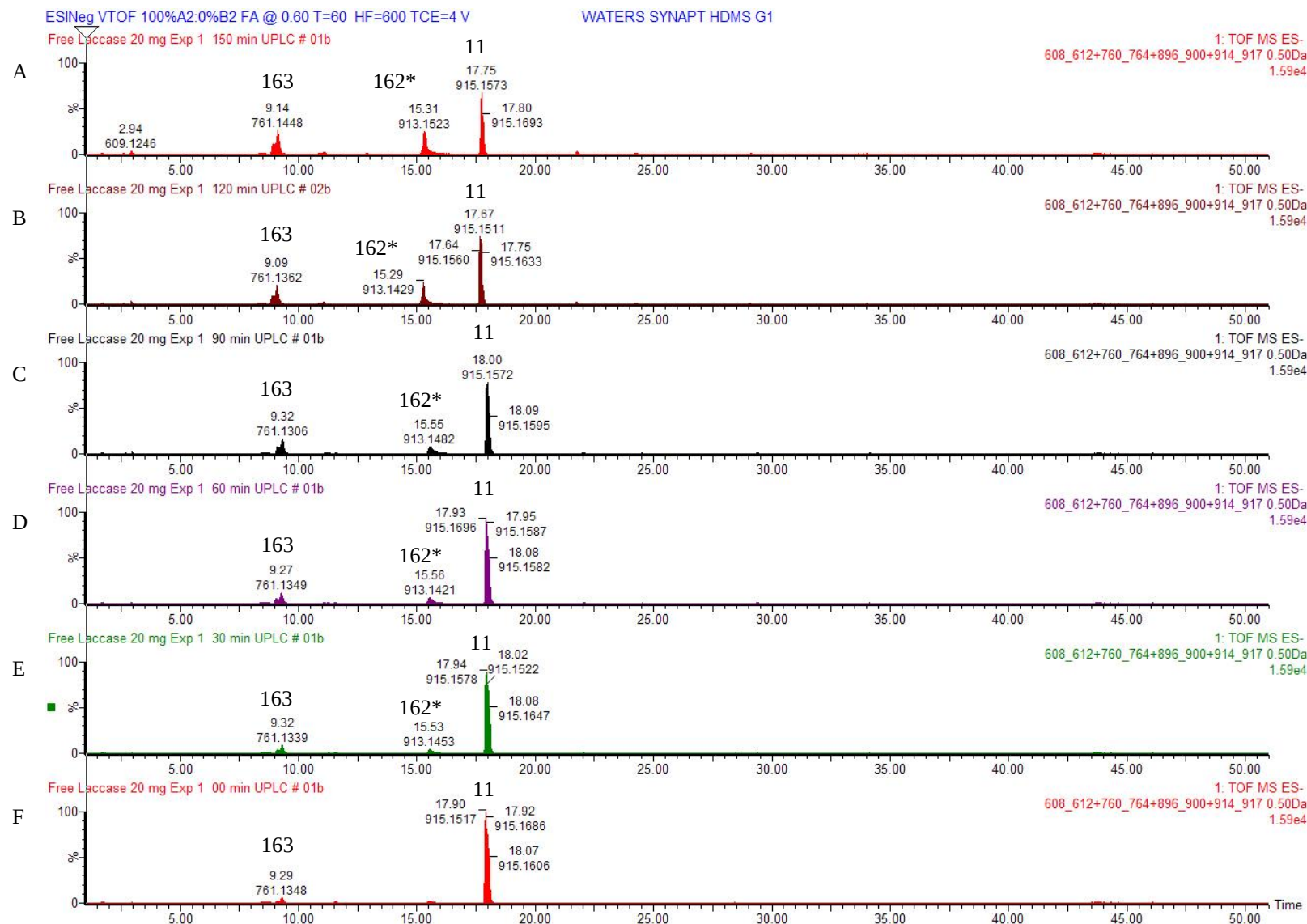


Figure 4.25: Theasinensin mass extraction (608-612+760-74+896-900+914-917) extracted UPLC chromatogram of bioconversion of catechin mixture with free laccase from *Myceliophthora thermophila* over the time points; A. 150-, B. 120-, C. 90-, D. 60-, E. 30- and F. 0 min. The numerical peak allocation of 11, 162 and 163 was indicated and a peak summary included in Table 4.8. MS data were extracted for peak* 162 (theasinensin A) (Figure 6.18).

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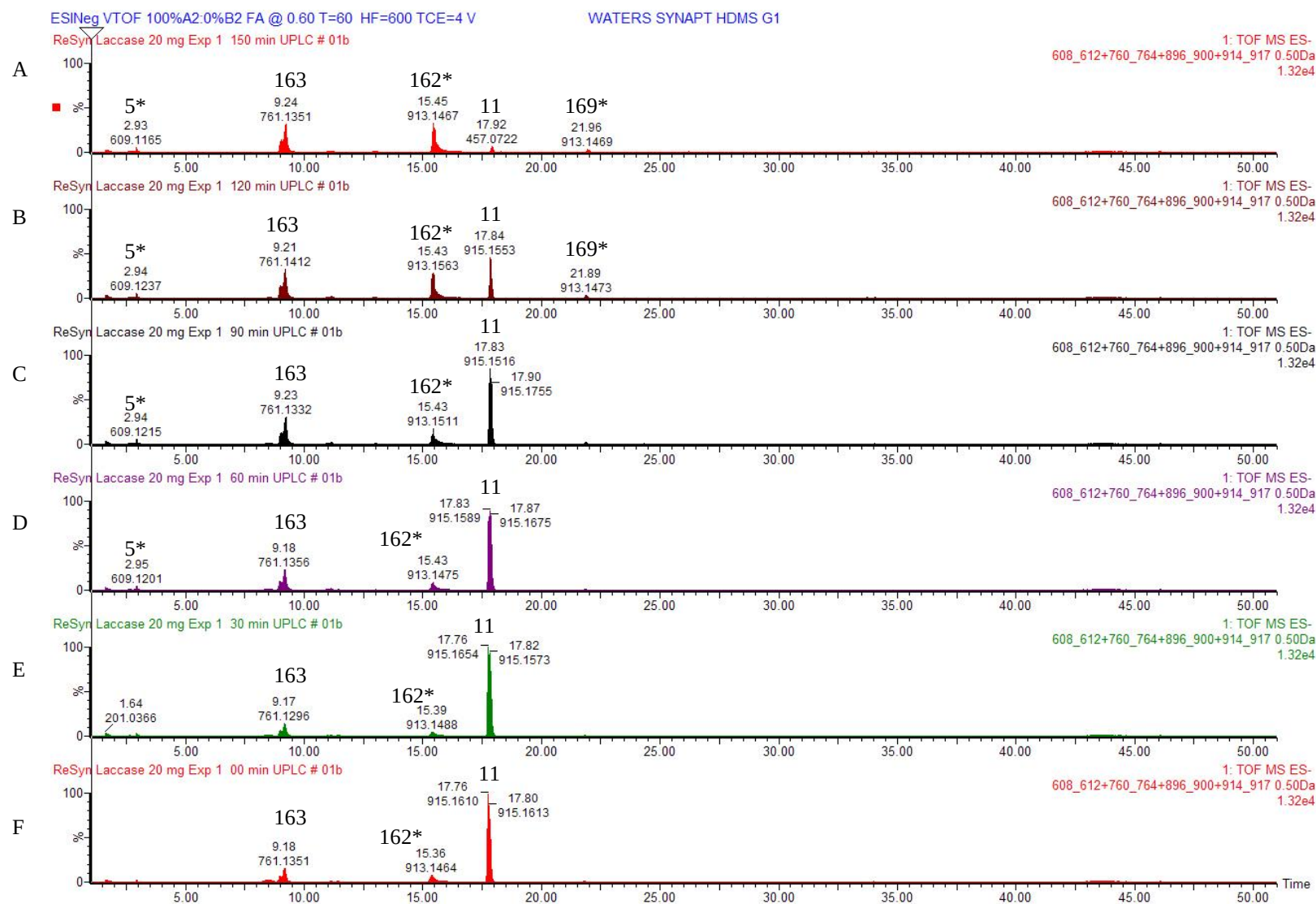


Figure 4.26: Theasinensin mass extraction (608-612+760-74+896-900+914-917) extracted UPLC chromatogram of bioconversion of catechin mixture with ReSyn™ immobilized laccase from *Myceliophthora thermophila* over the time points; A. 150-, B. 120-, C. 90-, D. 60-, E. 30- and D. 0 min. The numerical peak allocation of 5, 11, 162 and 169 was indicated and a peak summary included in Table 4.8. MS data were extracted for peak*; 5 (theasinensin C or E) (Figure 6.6), 162 (theasinensin A) (Figure 6.18) and 169 (theasinensin D) (Figure 6.16).

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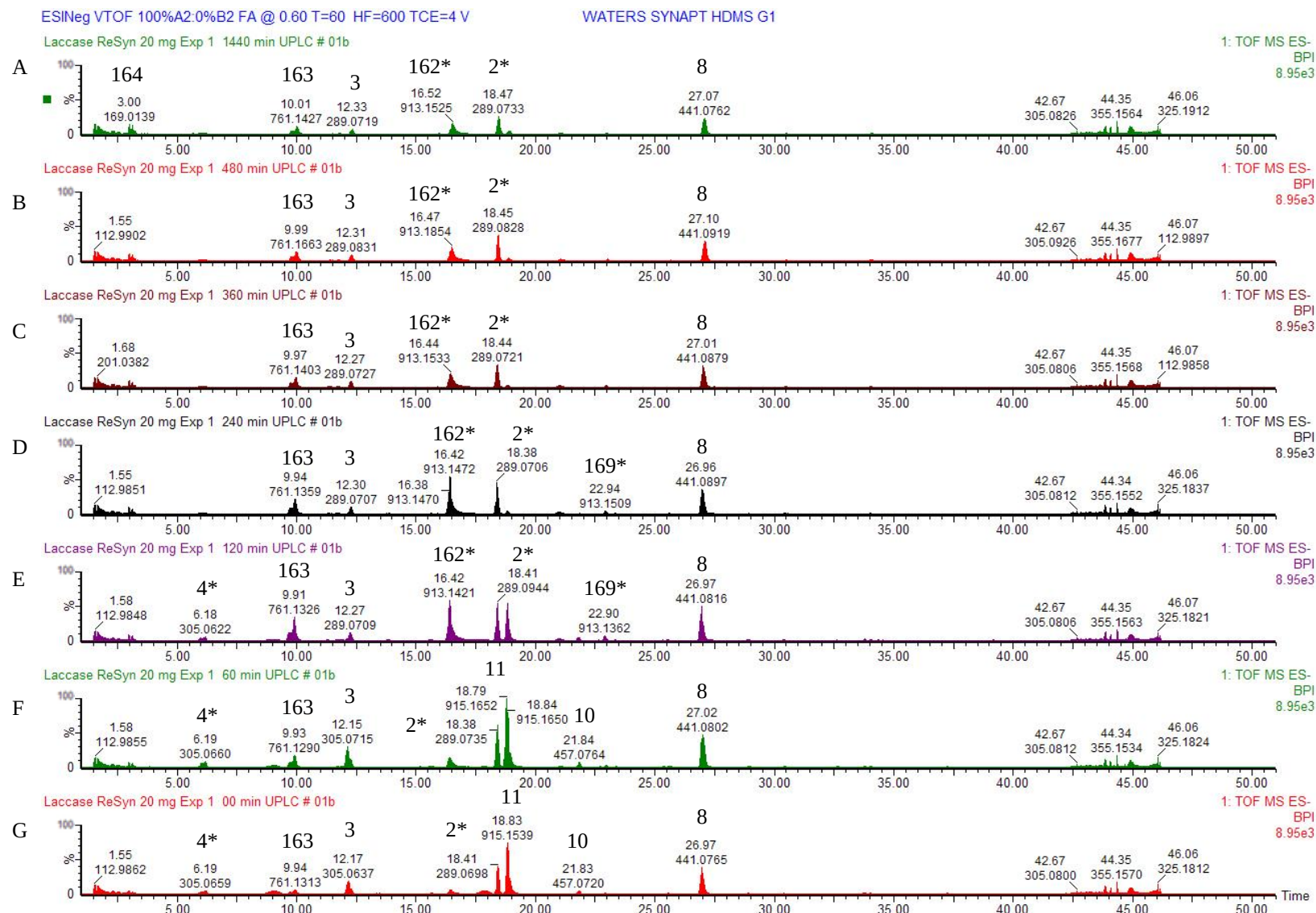


Figure 4.27: UPLC chromatogram of bioconversion of catechin mixture with ReSyn™ immobilized laccase from *Myceliophthora thermophila* over the time points; A. 1440-, B. 480-, C. 360-, D. 240-, E. 120-, F. 60- and G. 0 min as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 2-4, 8, 162 and 163 was indicated and a peak summary included in Table 4.8. MS data were extracted for peak*; 2 (epicatechin) (Figure 6.5), 4 (epigallocatechin/ gallocatechin) (Figure 6.5), 162 (theasinensin A) (Figure 6.18) and 169 (theasinensin D) (Figure 6.16).

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The elemental compositions of the peaks; m/z 915 (16.76)¹¹ and m/z 913 (14.25)^{162*} were determined and based upon the i-Fit values the most confident assignments respectively C₄₄H₃₅O₂₂ and C₄₄H₃₃O₂₂ were included in Table 6.6, Appendix C.

4.3.7. Immobilized polyphenol oxidase biotransformation of tea catechins into theaflavins utilizing a catechin mixture

ReSynTM immobilized polyphenol oxidase purified from Forelle pear was also employed in the synthesis of theaflavins, utilizing Theaphenon E as starting material. The bioconversion as a function of time was performed over the time points; 0-, 60-, 120-, 240-, 360-, 480- and 1440 min (Figure 4.28). Theaflavin mass extraction (562-566+714-717+866-869 Da) of the UPLC chromatogram data was performed to aid the assessment of the bioconversion efficiency (Figure 4.29 and Table 4.9).

Table 4.9: UPLC chromatogram peak summary of the bioconversion of a catechin mixture (20 mg) into theaflavin with ReSynTM immobilized polyphenol oxidase.

Commercial catechin mix, Theaphenon E (Figure 4.2 B).
m/z 447 (34.01) ⁷ , m/z 441 (28.86) ⁸ , m/z 883 (26.84) ^{9*} , m/z 457 (21.70) ^{10*} , m/z 915 (18.50) ^{11*} , m/z 289 (18.25) ^{12*} , m/z 305 (12.05) ^{13*} , m/z 305 (6.14) ^{14*} , m/z 169 (3.12) ^{15*} , m/z 176 (1.57) ¹⁶
Catechin mixture (20 mg) with ReSyn TM polyphenol oxidase
Total ion chromatogram after 1440 min (Figure 4.28)
m/z 441 (26.95) ⁸ , m/z 457 (21.74) ¹⁰ , m/z 915 (18.69) ¹¹ , m/z 289 (18.33) ^{2*} , m/z 305 (12.11) ¹³ and m/z 305 (6.15) ^{4*}
Theaflavin mass extraction (Figure 4.29)
m/z 715 (39.16) ¹⁷¹ , m/z 563 (38.55) ¹⁷² and m/z 713 (26.04) ¹⁷⁴
(retention time (min)) ^{number}
* = Mass spectrometry data extracted

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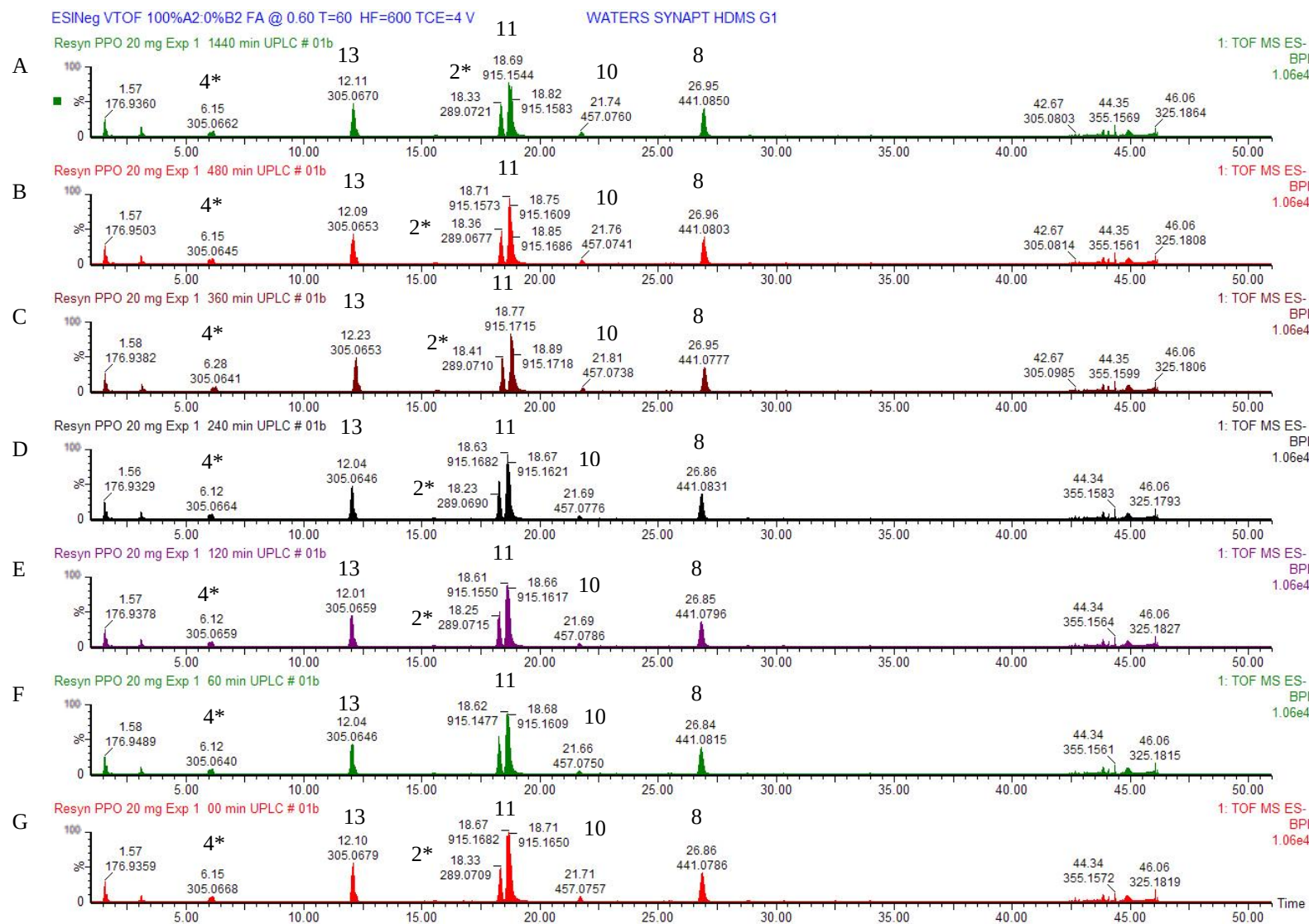


Figure 4.28: UPLC chromatogram of bioconversion of catechin mixture with ReSynTM immobilized polyphenol oxidase over the time points; A. 1440-, B. 480-, C. 360-, D. 240-, E. 120-, F. 60- and G. 0 min as visualized by the total ion current of the mass spectrometer in the negative ion mode. The numerical peak allocation of 2, 4, 8, 10 and 11 was indicated and a peak summary included in Table 4.9. MS data were extracted for peak^{*}; 2 (epicatechin) (Figure 6.5) and 4 (epigallocatechin/ galliccatechin) (Figure 6.5).

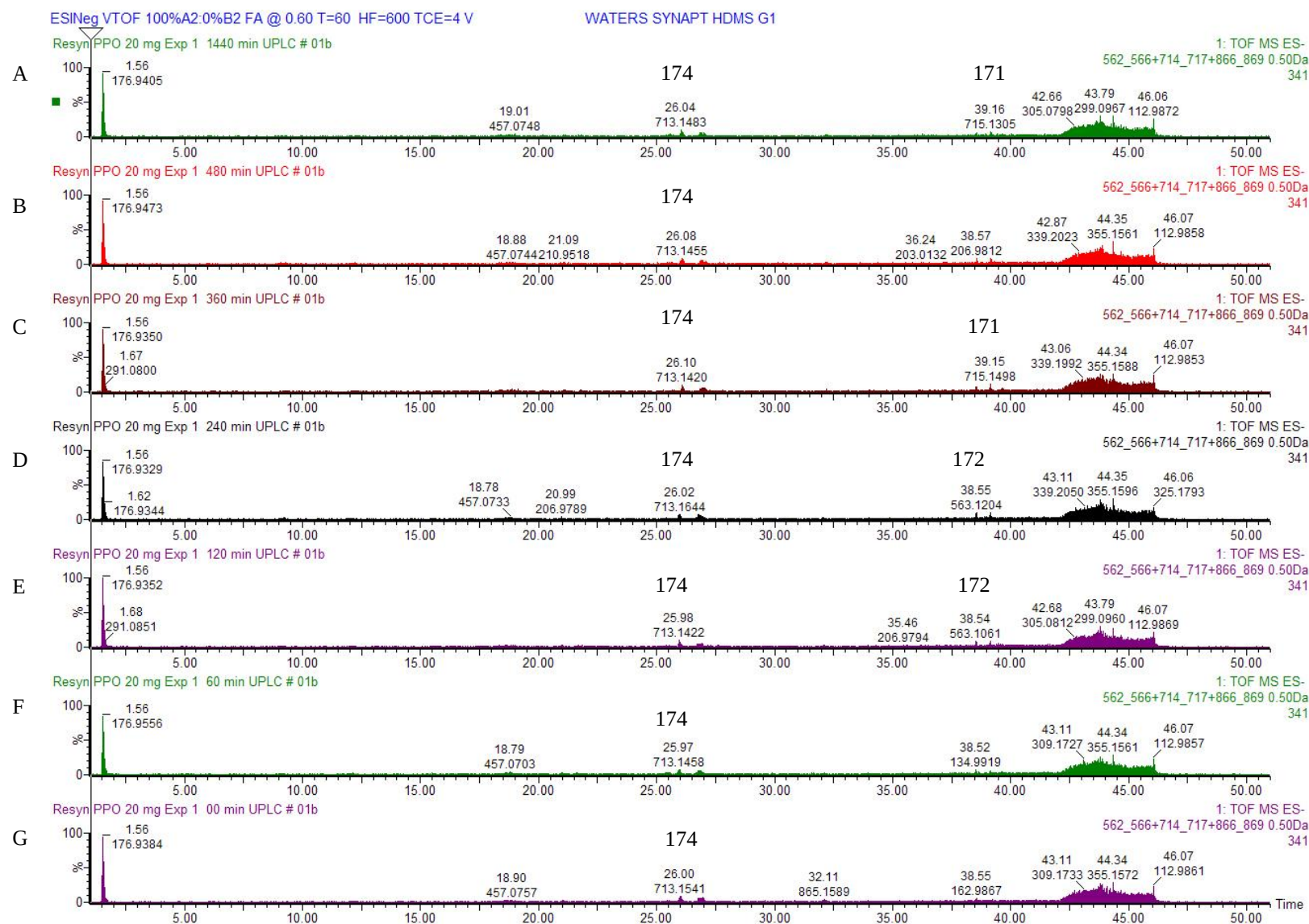


Figure 4.29: Theaflavin mass extracted UPLC chromatogram of bioconversion of catechin mixture with ReSyn™ immobilized polyphenol oxidase over the time points; A. 1440-, B. 480-, C. 360-, D. 240-, E. 120-, F. 60- and G. 0 min. The numerical peak allocation of 171, 172 and 174 was indicated and a peak summary included in Table 4.9.

4.4. DISCUSSION

Initial UPLC-MS assessment of the stability of the substrates; epicatechin ((-)EC) and epigallocatechin (EGC) in a potassium phosphate buffer (0.2 M, pH 6.8) at 25°C determined the presence of six peaks corresponding to m/z 265 (43.78)¹, m/z 289 (17.27)^{2*}, m/z 289 (11.38)^{3*}, m/z 305 (5.76)^{4*}, m/z 609 (3.15)^{5*} and m/z 176 (1.61)⁶ (Figure 4.2 A and Table 4.1). The ESI mass fragmentation of the peak, m/z 289 (17.27)^{2*} indicated the base peak ion, $[M-H]^-$, at m/z 289 and was identified as catechin or epicatechin. This was confirmed by the fragment ions at m/z 245 $[M-H-44]^-$, 205 $[M-H-84]^-$ and 179 $[M-H-110]^-$ that were present for peak 2 (Figure 6.5 A). The fragment ions respectively correspond to a loss of $C_2H_4O_1$, $C_4H_4O_2$ and $C_6H_6O_3$. The loss of $C_4H_4O_2$ was attributed to cleavage of the A ring of flavan-3-ol (Dou *et al.* 2007). Furthermore, the loss of $C_6H_6O_3$ was due to heterocyclic ring fission (Gu *et al.* 2003). The presence of a mass fragment ion at m/z 579 $[2M-H]^-$ was attributed to the formation of a gas phase dimer (Figure 6.5 A).

The peak, m/z 289 (11.38)^{3*} also indicated as a base peak at a m/z of 289, therefore, corresponding to catechin or epicatechin. Fragmentation ions at m/z of 245 $[M-H-44]^-$, 219 $[M-H-70]^-$ and 179 $[M-H-110]^-$ were present for peak 3 (Figure 6.5 B). The presence of fragments at 245 $[M-H-44]^-$ and 179 $[M-H-110]^-$ corresponds to a loss of $C_2H_4O_1$ and $C_6H_6O_3$ as explained for m/z 289 (17.27)^{2*} above. The loss of $C_4H_6O_2$ corresponding to the mass fragment at 219 $[M-H-70]^-$, which was attributed to cleavage of the A ring of flavan-3-ol (Gu *et al.* 2003). Based upon the difference in retention time it can be deduced that m/z 289 (17.27)^{2*} corresponds to epicatechin and m/z 289 (11.38)^{3*} to catechin.

Gallocatechin or epigallocatechin was identified as the peak, m/z 305 (5.76)^{4*} based upon the base peak, $[M-H]^-$, m/z 305 in the ESI analyses (Figure 6.5 C). The peak at m/z 305 (5.76)^{4*} had mass fragment ions at 261 $[M-H-44]^-$, 219 $[M-H-86]^-$ and 179 $[M-H-126]^-$, which respectively correspond to the loss of $C_2H_4O_1$, $C_4H_6O_2$ and $C_6H_6O_3$. The loss of $C_4H_6O_2$ was attributed to cleavage of the A ring of flavan-3-ol and the loss of $C_6H_6O_3$ was due to heterocyclic ring fission (HRF) (Gu *et al.* 2003). Furthermore, the loss of $C_7H_8O_3$ is attributed to retro-Diels-Alder (RDA) fusion (Dou *et al.* 2007). In addition, the mass fragment ion at m/z of 125 $[M-H-180]^-$ relates to trihydroxy benzene (Lee *et al.* 2010). Mass fragment at m/z 611 $[2M-H]^-$ was attributed to the formation of a gas phase dimer.

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The ESI analyses of peak, m/z 609 (3.15)^{5*} determined a deprotonated molecular ion at a m/z 609 and fragment ions at m/z 471 [M-H-138]⁻, 169 [M-H-440]⁻ and 125 [M-H-484]⁻ (Figure 6.6). The presence of the fragment m/z 471 corresponding to methylated EGCG (m/z 471), suggested that the peak was an EGC homo dimer and tentatively identified collectively as theasinensin C and E (Neilson *et al.* 2007). Based upon the absence of a fragment at a m/z 457, the formation of the peak can't be explained by the addition of a gallic acid to EGCG resulting in the formation of epigallocatechin-3,5-digallate m/z 609 (Wang *et al.* 2008). The fragments at 169 [M-H-440]⁻ and 125 [M-H-484]⁻ respectively corresponded to gallic acid and trihydroxy benzene (Lee *et al.* 2010).

The peaks; m/z 289 (17.27)^{2*}, m/z 289 (11.38)^{3*}, m/z 305 (5.76)^{4*} and m/z 609 (3.15)^{5*} respectively indicated the presence of epicatechin, catechin, epigallocatechin or gallocatechin, and theasinenesin C and E. From the results it can be deduced that epicatechin underwent epimerization leading towards the formation of catechin and that epigallocatechin dimerized forming the homodimers, theasinensin C and E. The catechin mixture was subjected to 10 min at 90°C to simulate a possible enzyme inactivation step in subsequent catechin biotransformation experiments. Catechins were reported to undergo epimerisation at the high temperature with the heating time being an influencing factor, and in alkaline solutions (catechins were stable in acidic solutions, pH < 4), where the associated complexity of the ionic environment also affects the stability of catechins (Wang & Helliwell 2000, Chen *et al.* 1998). The production of theasinensins (reduction product) was attributed to the degradation of the heat-susceptible intermediate, dehydrotheasinensin C. Dehydrotheasinensin C on hydrogenation was reported to be converted to theasinensin C and in a neutral phosphate buffer (such as under our reaction conditions) to be decomposed to theasinensin E (Matsuo *et al.* 2006). As dehydrotheasinensins are produced by the enzymatic oxidation of the corresponding catechin, based upon the absence of enzymatic activity, it was deduced that the dehydrotheasinensin C was present in one of the source samples. The reaction conditions could have amounted to the reduction of the unstable dehydrotheasinensins into the corresponding theasinensins, although based upon the instability thereof it could also have been present in the source samples (Tanaka *et al.* 2003).

The substrate profile of a commercially available mixture of catechins, Theaphenon E which was reported to consist of almost 70% epigallocatechin and a total catechin content of > 90% was assessed. Peaks corresponding to m/z 447 (34.01)⁷, m/z 441 (28.86)⁸, m/z 883 (26.84)^{9*}, m/z 457 (21.70)^{10*}, m/z 915 (18.50)^{11*}, m/z 289 (18.25)^{12*}, m/z 305 (12.05)^{13*}, m/z 305 (6.14)^{14*}, m/z 169 (3.12)^{15*} and m/z 176 (1.57)¹⁶ were determined by UPLC-MS (Figure 4.2 B). The peak at m/z 883 (26.84)^{9*} had a base peak at a m/z 441 and fragmentation ions at m/z 883 [M-H+442]⁻,

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881 [M-H+440]⁻, 289 [M-H-152]⁻, 169 [M-H-272]⁻ and 125 [M-H-316]⁻ (Figure 6.8 A). The base peak at m/z 441 indicated that the peak at m/z 883 corresponded to a dimer formed during analysis. The peak 9 with an apparent mass at m/z 883 but a base peak at a m/z 441 was identified as either epicatechin gallate or catechin gallate, the epimers couldn't be distinguished without a corresponding authentic standard. The fragments; 883 [M-H+442]⁻ and 881 [M-H+440]⁻ respectively correspond to the epigallocatechin gallate B-ring condensation-elimination homodimer P-2 and its quinone (Neilson *et al.* 2007). Furthermore, the fragments; 289 [M-H-152]⁻, 169 [M-H-272]⁻ and 125 [M-H-316]⁻ correspond to catechin or epicatechin, gallic acid and trihydroxy benzene (Lee *et al.* 2010).

A base peak, [M-H]⁻, at m/z 457 was determined for the peak m/z 457 (21.70)^{10*} from which the presence of galocatechin gallate or epigallocatechin gallate can be deduced. Fragment ions at m/z 331 [M-H-126]⁻, 305 [M-H-152]⁻, 169 [M-H-288]⁻, 125 [M-H-332]⁻ and 915 [2M-H]⁻ were present for peak 10 (Figure 6.8 B). The base peak ion at m/z 169 corresponds to the loss of a 288 Da fragment, associated with an epigallocatechin or galocatechin (m/z 306) moiety that has lost a H₂O group. The m/z 169 and m/z 125 fragments correspond to the identified gallic acid and the loss of a C₂H₄O₁ or the CO₂ group from the carboxylic acid moiety of gallic acid, trihydroxy benzene. Fragment m/z 331 was formed as a result of the neutral loss of trihydroxy benzene (m/z 125) from epigallocatechin gallate or galocatechin gallate (m/z 457). Fragment ion m/z 305, epigallocatechin or galocatechin is formed by retro-Diels-Alder (RDA) fission during which a neutral fragment of 152 Da, a galloyl group (C₈H₈O₃) is lost from epigallocatechin gallate or galocatechin gallate (m/z 457) (Lee *et al.* 2010, Sang *et al.* 2007). The fragment ion 915 [2M-H]⁻ was observed as a result of a gas phase dimer of epigallocatechin gallate or galocatechin gallate (m/z 457) formed during ESI analysis.

Based upon the deprotonated molecular ion, [M-H]⁻, at m/z of 915 in the negative ESI analysis, the peak, m/z 915 (18.50)^{11*}, was tentatively identified as a chalcane-flavan dimer, assamicain A, B or C (Figure 6.8 C) (Hashimoto *et al.* 1989). This was confirmed by the presence of mass fragments at m/z 457 [0.5M-H]⁻, 305 [M-H-610]⁻, 169 [M-H-746]⁻ and 125 [M-H-790]⁻, which as explained above for peak 10, galocatechin gallate or epigallocatechin gallate respectively correspond to epigallocatechin gallate, epigallocatechin, gallic acid and trihydroxy benzene. The mass fragments at m/z 913 [M-H-2]⁻ correspond to the quinone of assamicain A, B or C (Sang *et al.* 2007).

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The peak at m/z 289 (18.25)^{12*} had a deprotonated molecular ion at m/z 289 and fragmentation ions at 579 [M-H+290]⁻, 205 [M-H-84]⁻ and 179 [M-H-110]⁻. Based upon the deprotonated molecular ion and corresponding fragmentation pattern of peak m/z 289 (18.25)^{12*} as determined for peak m/z 289 (17.27)^{2*} the compound can be identified as catechin or epicatechin (Figure 6.8 D). The peak, m/z 305 (12.05)^{13*} had a corresponding deprotonated molecular ion, m/z 305 and fragmentation pattern; 289 [M-H-16]⁻, 219 [M-H-86]⁻, 179 [M-H-126]⁻ and 125 [M-H-180] as the peak m/z 305 (5.76)^{4*} and therefore was also identified as gallocatechin or epigallocatechin (Figures 6.5 C and 6.9 A). The fragment at 611 [M-H-306]⁻ indicated the formation of a gas phase dimer during ESI. Similarly based upon the deprotonated molecular ion and fragmentation pattern peak m/z 305 (6.14)^{14*} was identified as gallocatechin or epigallocatechin (Figure 6.9 B). According to the retention time peak m/z 305 (6.14)^{14*} and m/z 305 (12.05)^{13*} can tentatively be identified as gallocatechin and epigallocatechin respectively. Gallic acid was identified as corresponding to peak m/z 169 (3.12)^{15*} with a base peak at m/z 169 and a daughter ion at 125 [M-H-180]⁻ which as discussed above relates to a trihydroxy benzene (Figure 6.9 C) (Lee *et al.* 2010).

The substrate profile of Theaphenon E indicated the presence of epicatechin gallate or catechin gallate, gallocatechin gallate or epigallocatechin gallate, assamicain A, B or C, catechin or epicatechin, epigallocatechin, gallocatechin and gallic acid, respectively based upon the peaks; m/z 883 (26.84)^{9*}, m/z 457 (21.70)^{10*}, m/z 915 (18.50)^{11*}, m/z 289 (18.25)^{12*}, m/z 305 (12.05)^{13*}, m/z 305 (6.14)^{14*} and m/z 169 (3.12)^{15*} (Figure 4.2 B). Theaphenon E was reported to consist of almost 70% epigallocatechin and a total catechin content of > 90%, but as no quantitative analysis was performed this could not be assessed, although based upon intensity of assamicain A, B or C, m/z 915 (18.50)^{11*} was indicated as the most prominent peak (Figure 4.2 B). Assamicains A, B and C have been isolated from the fresh leaves of *Camellia sinensis* var. *assamica* (Camelliaceae) (Hashimoto *et al.* 1989).

In order to authoritatively determine the presence of the theaflavins in subsequent analyses, UPLC-MS analysis was performed on the standards; theaflavin, theaflavin-3-*O*-gallate, theaflavin-3'-*O*-gallate and theaflavin-3,3-di-gallate which respectively had a deprotonated molecular ion at a m/z 563 (36.75), m/z 715 (37.02), m/z 715 (37.59) and m/z 867 (37.36) (Figures 6.10 and 6.11).

The catechin substrate availability as well as the presence of theaflavins and theasinensins in Tea (*Camellia sinensis*, frozen fresh leaves) were determined based upon UPLC-MS analysis. Based

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upon the catechin mass extracted data peaks corresponding to a m/z 248 (46.10)³¹, m/z 305 (42.65)³², m/z 593 (33.43)¹⁸, m/z 447 (31.53)¹⁹, m/z 593 (28.56)³³, m/z 289 (17.21)^{24*}, m/z 577 (14.76)³⁴, m/z 289 (11.33)^{25*}, m/z 305 (5.77)^{27*} and m/z 439 (1.71)³⁴ were detected (Figure 4.3 B). The peaks m/z 289 (17.21)^{24*} and m/z 289 (11.33)^{25*} based upon the determined corresponding base peak at m/z 289 and fragmentation pattern; 579 [M-H+290]⁻, 205 [M-H-84]⁻ and 179 [M-H-110]⁻, as discussed for peaks m/z 289 (17.27)^{2*} and m/z 289 (11.38)^{3*} can be respectively identified as epicatechin and catechin (Figures 6.5 A, 6.5 B, 6.12 B and 6.12 C) (Dou *et al.* 2007, Gu *et al.* 2003). Similarly, the peak m/z 305 (5.77)^{27*} was identified as gallocatechin or epigallocatechin based upon peak m/z 305 (5.76)^{4*} as described above (Figures 6.5 C and 6.12 A). Tentatively based upon retention time, peak m/z 305 (5.77)^{27*} corresponds to gallocatechin as was indicated for peaks m/z 305 (6.14)^{14*} and m/z 305 (12.05)^{13*} (Figures 6.9 A, 6.9 B and 6.12 A).

The presence of the catechins; epicatechin, catechin and gallocatechin was identified in Tea (*Camellia sinensis*, frozen fresh leaves) based upon the peaks respectively corresponding to m/z 289 (17.21)^{24*}; m/z 289 (11.33)^{25*} and m/z 305 (5.77)²⁷. The tea catechins make up between 10-30% of the dry weight of tea leaves. The major catechins present in tea leaves are epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin, which account for 86% to 97% of the total catechin content. The absence of a peak corresponding to epigallocatechin and epigallocatechin gallate was attributed to the epimerization of epigallocatechin to gallocatechin and the efficiency of extraction. In order to inactivate any enzymatic activity the Tea (*Camellia sinensis*, frozen fresh leaves) were subjected to a heat inactivation step (90°C for 10 min), during which the high temperature resulted in the epimerization reaction (Wang & Helliwell 2000, Chen *et al.* 1998). The extraction efficiency can be influenced by factors such as tea and solvent type, time, pH, temperature and ratio of solvent to material (Perva-Uzunalić *et al.* 2006). The extractions were performed in aqueous solutions despite the reported increased catechins extraction efficiency in ethanol, based upon the detrimental effect the solvent would have on enzymatic activity in subsequent bioconversion reactions (Lin *et al.* 2003).

The inherent theaflavin content present in Tea (*Camellia sinensis*, frozen fresh leaves) was determined based upon the theaflavin mass extracted data, in terms of which the peaks; m/z 248 (46.10)³¹, m/z 297 (43.68)³⁶, m/z 715 (39.22)³⁷, m/z 563 (38.26)³⁸, m/z 563 (35.67)³⁹, m/z 489 (34.27)⁴⁰, m/z 609 (29.45)^{20*}, m/z 563 (26.85)^{22*}, m/z 441 (25.65)⁴¹, m/z 173 (17.82)⁴², m/z 581 (4.44)²⁸ and m/z 370 (1.58)⁴³ were detected (Figure 4.4 A). The peak at

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m/z 563 (26.85)^{22*} had a corresponding deprotonated molecular ion, $[M-H]^-$ at m/z 563 as was determined for the theaflavin standard, m/z 563 (36.75), although a significant reduction in retention time was determined (Figures 6.7 and 6.10). Therefore, the peak couldn't be tentatively identified as theaflavin. Furthermore, the inherent theasinensin content of Tea (*Camellia sinensis*, frozen fresh leaves) indicated peaks at m/z 609 (29.45)^{20*}, m/z 463 (28.11)^{21*}, m/z 289 (17.21)^{24*}, m/z 609 (7.88)^{44*}, m/z 607 (5.64)⁴⁵ and m/z 609 (3.06)²⁹ based upon the theasinensin mass extracted data (Figure 4.4 B). The peak at m/z 609 (29.45)^{20*} had a base peak at m/z 609 and fragment ions at m/z 913 $[M-H+304]^-$, 563 $[M-H-46]^-$, 441 $[M-H-168]^-$ and 303 $[M-H-306]^-$ and the peak at m/z 609 (7.88)^{44*} had corresponding base peak but fragment ions at m/z 563 $[M-H-46]^-$, 463 $[M-H-146]^-$ and 301 $[M-H-308]^-$ (Figures 6.13 A and B). The mass fragmentation of the peaks; m/z 609 (29.45)^{20*} and m/z 609 (7.88)^{44*} didn't correlate with the peak m/z 609 (3.15)^{5*} that was tentatively identified as theasinensin C and E (Figure 6.6). The absence of peaks corresponding to theaflavins and theasinensin was consistent with the heat inactivation of any enzymatic activity present in the Tea (*Camellia sinensis*, frozen fresh leaves) and associated absence of catechin oxidation products.

The catechin, theaflavin and theasinensin profile of Lipton® Green tea was also determined by UPLC-MS analysis. The inherent catechin content was determined based upon the catechin mass extracted data from which the peaks; m/z 447 (37.64)⁴⁸, m/z 901 (36.72)⁵⁰, m/z 447 (31.92)⁵², m/z 593 (31.42)⁵⁴, m/z 593 (26.86)⁷¹, m/z 883 (25.39)⁵⁸, m/z 441 (25.33)⁷², m/z 453 (22.49)⁷³, m/z 593 (22.01)⁷⁴, m/z 457 (20.90)^{61*}, m/z 915 (18.42)^{62*}, m/z 289 (17.96)⁶³, m/z 577 (15.65)⁶⁴, m/z 289 (12.72)⁷⁵, m/z 305 (12.38)^{66*}, m/z 593 (11.63)⁷⁶, m/z 593 (8.67)⁷⁷, m/z 305 (6.97)⁶⁸ and m/z 191 (1.82)⁷⁰ were indicated (Figure 4.5 B). The peak m/z 457 (20.90)^{61*} was identified as epigallocatechin gallate or gallic acid based upon a corresponding retention time, base peak (m/z 457) and fragmentation pattern (331 $[M-H-126]^-$, 305 $[M-H-152]^-$, 169 $[M-H-288]^-$ and 125 $[M-H-332]^-$) as for peak m/z 457 (20.70)^{10*}, discussed previously (Figures 6.8 B and 6.14 B). Similarly peak, m/z 305 (12.38)^{66*} was identified as epigallocatechin as determined for peak m/z 305 (11.05)^{13*} (Figures 6.9 A and 6.14 A). Therefore, the presence of the catechins; epigallocatechin gallate or gallic acid and epigallocatechin was identified in Lipton® Green tea. Furthermore, the peaks; m/z 441 (25.33)⁷², m/z 289 (17.96)⁶³, m/z 289 (12.72)⁷⁵, m/z 305 (6.97)⁶⁸ were respectively and tentatively identified as epicatechin gallate or catechin gallate, epicatechin, catechin and gallic acid based upon the previously identified peaks; m/z 441 (25.85)^{9*}, m/z 289 (17.27)^{2*}, m/z 289 (11.38)^{3*} and m/z 305 (5.76)^{4*} respectively (Figures 4.5 B, 6.5 A, 6.5 B, 6.5 C and 6.8 A). Despite the absence of quantitative analysis of the amount of catechins, based upon the increased incidence of identified catechins,

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the increased total catechin content of green tea in comparison to Tea (frozen fresh leaves) was consistent with literature. A catechin content of 12-14% (w/w) and 29-39% (w/w) was respectively reported for green tea leaves and green tea dry extracts (Wang *et al.* 2003). The increased level of catechin present in green tea was associated with an increased level of catechins released from tea leaves during the manufacturing of green tea (Lin *et al.* 2003).

Peaks corresponding to m/z 887 (36.79)⁷⁸, m/z 563 (27.33)⁷⁹, m/z 563 (25.27)⁵⁹, m/z 563 (24.24)⁸⁰, m/z 563 (22.40)⁸¹, m/z 865 (18.21)⁸² and m/z 165 (1.74)⁸³ were determined in the theaflavin mass extracted data of Lipton® Green tea (Figure 4.6 A). The peaks; m/z 563 (27.33)⁷⁹, m/z 563 (25.27)⁵⁹, m/z 563 (24.24)⁸⁰ and m/z 563 (22.40)⁸¹ in comparison to the theaflavin standard, m/z 563 (36.75) had a significantly decreased retention time and subsequently couldn't be identified as theaflavin (Figures 4.6 A and 6.10 A). Similarly, the peak, m/z 865 (18.21)⁸² could not be identified as theaflavin digallate based upon the standard, m/z 867 (37.36) (Figures 4.6 A and 6.11 C).

Based upon the theasinensin mass extracted data the peaks; m/z 609 (27.81)⁵⁷, m/z 609 (26.91)⁸⁴, m/z 609 (24.32)⁸⁵, m/z 915 (18.42)^{62*}, m/z 609 (17.61)⁸⁶, m/z 761 (13.47)^{87*}, m/z 305 (12.38)^{66*}, m/z 761 (10.70)^{88*}, m/z 609 (8.86)⁶⁷, m/z 305 (6.99)⁶⁸, m/z 609 (5.96)⁸⁹ and m/z 169 (3.68)⁹⁰ were indicated in Lipton® Green tea (Figure 4.6 B). The peak at m/z 915 (18.42)^{62*} can be identified as an assamicain A, B or C based upon a deprotonated molecular ion, $[M-H]^-$, m/z 915 and fragment ions at m/z 457 $[0.5M-H]^-$, 305 $[M-H-610]^-$, 169 $[M-H-746]^-$ and 125 $[M-H-790]^-$, respectively corresponding to epigallocatechin gallate, gallic acid and trihydroxybenzene (Figure 6.15 A) (Lee *et al.* 2010). Based upon the deprotonated molecular ion, $[M-H]^-$, m/z 761 both peaks m/z 761 (13.47)^{87*} and 761 (10.70)^{88*} were suggested to be EGCG-ECG heterodimers, analogous to theasinensins (Figures 6.15 C and D) (Neilson *et al.* 2007).

Comparative assessment of the total ion chromatogram peak intensities between the Lipton® Green tea and boiled sample thereof, revealed slight variations in peak intensities with the main difference attributed to the peak, m/z 883 (25.39)⁵⁸ (Figures 4.5 A and 4.7). Boiling of the Lipton® Green tea sample corresponded to a very large increase in the peak intensity of the peak, m/z 883 (25.39)⁵⁸, which can tentatively be identified as the epigallocatechin gallate B-ring condensation-elimination homodimer P-2, based upon the identified peak, m/z 883 (26.84)^{9*}. P-2 was produced from the further oxidation of theasinensin A quinone, proposed to be generated by the dimer formed between two epigallocatechin gallate quinones or alternatively by the

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autoxidation of theasinensin A (Neilson *et al.* 2007). The autoxidation of epigallocatechin gallate could have been promoted under the reaction conditions such as boiling, associated with the level of dissolved oxygen. Furthermore, epigallocatechin gallate and epigallocatechin homo- and heterodimers were reported to form homo- and heterodimers at pH close to neutral (Henning *et al.* 2008). Therefore, it can be deduced that the increased temperature could have been a determinate factor in the formation of P-2.

The catechin substrate availability as well as the presence of theaflavins and theasinensins in Lipton® Yellow label black tea was also determined based upon UPLC-MS analysis. Based upon the catechin mass extracted data peaks corresponding to a m/z 447 (31.22)⁹⁶, m/z 593 (31.00)⁹⁷, m/z 595 (18.52)¹⁰⁴, m/z 289 (12.73)¹⁰⁹, m/z 191 (1.83)¹⁹¹, m/z 112 (46.18)¹¹⁵, m/z 885 (36.99)¹¹⁶, m/z 447 (29.72)¹¹⁷, m/z 593 (29.20)¹¹⁸, m/z 593 (26.71)¹¹⁹, m/z 453 (22.31)¹²⁰, m/z 593 (21.80)¹²¹, m/z 289 (17.87)¹²² and m/z 577 (15.57)¹²³ were detected (Figure 4.9 A). The peaks; m/z 289 (12.73)¹⁰⁹ and m/z 289 (17.87)¹²² were tentatively identified as catechin and epicatechin based upon the previously identified peaks m/z 289 (11.38)^{3*} and m/z 289 (17.27)^{2*} respectively (Figures 4.9 A, 6.5 A and 6.5 B). The lower incidence of catechins in Lipton® Yellow label black tea (black, fermented tea) in comparison to Lipton® Green tea (non-fermented tea) corresponds to the level of fermentation, during which most of the catechins were altered to form bisflavanols, theaflavins, thearubigins and other oligomers. Only 3-10% (w/w) of the flavanols was reported to remain in black tea (Yang *et al.* 2001b).

The peaks corresponding to m/z 265 (43.77)⁹², m/z 901 (37.38)⁹³, m/z 563 (24.95)¹⁰², m/z 112 (46.18)¹¹⁵, m/z 901 (37.08)¹²⁴, m/z 563 (36.57)^{125*}, m/z 563 (34.23)^{126*}, m/z 565 (28.17)¹²⁷, m/z 581 (27.05)¹²⁸, m/z 563 (23.94)¹²⁹, m/z 491 (14.56)¹³⁰, m/z 581 (8.39)¹³¹, m/z 581 (5.75)¹³², m/z 344 (3.45)¹³³ and m/z 565 (1.75)¹³⁴ were determined by theaflavin mass data extraction (Figure 4.9 B). The peak, m/z 563 (36.57)^{125*} had a base peak at m/z 901 with fragment ions; m/z 755 [M-H-146]⁻, 609 [M-H-2(146)]⁻, 301 [M-H -2(146) - 308] which correspond to that reported for an unknown quercetin conjugate (Figure 6.17 B). The loss of 146, 308 and 301 respectively correspond to the cleavage of a rhamnosyl, rutinosyl and aglycone quercetin, tentatively indicating that peak 125 was quercetin (Del Rio *et al.* 2004). As indicated for Lipton® Green tea the peaks; m/z 563 (24.95)¹⁰², m/z 565 (28.17)¹²⁷ and m/z 563 (23.94)¹²⁹ in comparison to the theaflavin standard, m/z 563 (36.75) had a significantly decreased retention time and subsequently couldn't be identified as theaflavin (Figures 4.9 B and 6.10 A). With reference to the theaflavin standard, m/z 563 (36.75) the peak, m/z 563 (34.23)^{126*} was identified as theaflavin based upon the deprotonated molecular ion, [M-H]⁻, m/z 563 and similar retention

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times (Figures 6.10 A, 6.10 B and 6.17 A). Theaflavin was synthesised by oxidative polyphenol oxidase dependent polymerization from epicatechin and epigallocatechin during fermentation (Leung *et al.* 2001). The theaflavin peak, m/z 563 (34.23)^{126*} was quantified as 20500 ng/ml based upon the theaflavin standard curve, this was calculated as 0.1% (w/w) theaflavin present in Lipton® Yellow label black tea (Table 6.7). This was lower than the reported 0.4 - 1.85% theaflavins formed during the manufacturing of black tea, which was associated with the extraction efficiency (Sharma *et al.* 2009).

The theasinensin mass extracted data of Lipton® Yellow label black tea indicated peaks at m/z 447 (31.22)⁹⁶, m/z 609 (27.46)⁹⁹, m/z 609 (26.59)¹⁰¹, m/z 755 (28.54)¹³⁵, m/z 611 (19.40)¹³⁶, m/z 759 (15.26)¹³⁷, m/z 759 (12.04)¹³⁸, m/z 761 (10.65)¹³⁹ and m/z 609 (3.68)¹⁴⁰ (Figure 4.10 A). Peak m/z 609 (3.68)¹⁴⁰ was tentatively identified collectively as theasinensin C and E but based upon the increased retention time the peaks; m/z 609 (27.46)⁹⁹ and m/z 609 (26.59)¹⁰¹ could not correspondingly be identified. Theasinensins are secondary polyphenols, which were derived from flavan-3-ols by polyphenol oxidase during fermentation in the manufacturing of black tea (Nakai *et al.* 2005).

Variations in peak intensities between Lipton® Yellow label black tea and the boiled sample was observed, with the most significant difference attributed to peak, m/z 761 (10.68)¹³⁹ (Figures 4.8 and 4.10 B). Boiling of Lipton® Yellow label black tea amounted to a significant increase in the peak, m/z 761 (10.68)¹³⁹, which can tentatively be identified as an EGCG-ECG heterodimers, analogous to theasinensins (Neilson *et al.* 2007). This corresponded to the observed increase of the epigallocatechin gallate homodimer, P-2 in the boiled Lipton® Green tea sample as discussed.

Bioconversion of catechins into theaflavin was performed utilizing Tea (*Camellia sinensis*, frozen fresh leaves) as starting material. The endogenous enzymes present in the homogenates of Forelle pear, and Yacon (*Smallanthus sonchifolius*) leaves were exploited based upon the high theaflavin synthesis capacity determined with the flavognost reagent (2.4.4). Furthermore, the UPLC chromatograms of both Forelle pear- and Yacon homogenates indicated practically no oxidation products of their own (Figure 4.11). Therefore, both Forelle pear and Yacon can be considered as excellent enzyme sources. Mushroom tyrosinase (from Sigma Aldrich (Pty) Ltd) was also employed as a control for the bioconversion of tea leaf catechins. Fermentation was promoted for 1 h at room temperature after which any enzymatic activity was inactivated by a heat inactivation step. The catechin bioconversion efficiency was assessed based upon the theaflavin mass extracted data, in terms of which the prominent peaks; m/z 563 (27.06)^{146*}, m/z 563 (26.95)^{147*}

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and m/z 563 (26.95)^{148*} were respectively detected in the Tea (*Camellia sinensis*, frozen fresh leaves) with mushroom tyrosinase, Forelle pear homogenate and Yacon homogenate (Figure 4.14). The peaks; m/z 563 (27.06)^{146*} and m/z 563 (26.95)^{147*}, respectively for the mushroom tyrosinase and Forelle pear homogenate samples, had corresponding mass fragmentation data, with a base peak, $[M-H]^-$, m/z 563 and a fragmentation ion at m/z 641 $[M-H+78]^-$ (Figures 4.15 C and D). The peak, m/z 563 (26.95)^{148*} in the Yacon sample only had a corresponding base peak, $[M-H]^-$, m/z 563 (Figure 4.15 E). Two peaks; m/z 563 (35.53)^{126*} and m/z 563 (23.95)^{102*} with corresponding m/z were determined in Lipton® Yellow label black tea. Similarly the peaks had a base peak at m/z 563, but peak m/z 563 (35.53)^{126*} had a fragmentation ion at, m/z 737 $[M-H+174]^-$ and peak, m/z 563 (23.95)^{102*} had fragmentation ions at, m/z 641 $[M-H+78]^-$ and m/z 613 $[M-H+50]^-$ (Figures 4.15 A and B). Based upon similar retention times and mass spectrometry data peak, m/z 563 (35.53)^{126*} present in the Lipton® Yellow label black tea, was identified as theaflavin in terms of the theaflavin standard, m/z 563 (36.75). The deduction made was confirmed by the elemental composition of peak, m/z 563 (35.53)^{126*} determined as $C_{29}H_{24}O_{12}$ (0.0 i-Fit value) corresponding to that of theaflavin (Table 6.6). The elemental composition of the other corresponding peak, m/z 563 (23.95)^{102*} identified in Lipton® Yellow label black tea, was determined as $C_{26}H_{28}O_{14}$, furthermore the fragment peak, m/z 613 $[M-H+50]^-$ (23.95)¹⁰² was determined as $C_{29}H_{26}O_{15}$ (Figure 4.15 B and Table 6.6). The determined elemental composition, $C_{26}H_{28}O_{14}$, identified peak, m/z 563 (23.95)¹⁰², as *O*-pentosyl 8-C-hexosyl apigenin (m/z $[M-H]^-$ 563, $C_{26}H_{28}O_{14}$) (Lv *et al.* 2012) or apigenin-7-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (m/z $[M-H]^-$ 563, $C_{26}H_{28}O_{14}$) (Mencherini *et al.* 2007) or isoschaftoside (apigenin 6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranoside) (m/z $[M-H]^-$ 563, $C_{26}H_{28}O_{14}$) (Du *et al.* 2012, Krenna *et al.* 2003, Hussein *et al.* 2012). The fragment ion, m/z 613 $[M-H+50]^-$ (23.95)^{102*} indicated an increase of 3 carbon atoms and 1 oxygen with a loss of 2 hydrogen atoms. The elemental composition, $C_{29}H_{26}O_{15}$ determined for the fragment ion, m/z 613 $[M-H+50]^-$ (23.95)^{102*} has not been reported in literature.

The tea leaf catechin biotransformation by mushroom tyrosinase produced peak, m/z 563 (26.03)^{146*} with a fragment ion m/z 641 $[M-H+78]^-$ for which the elemental composition was respectively determined as $C_{26}H_{28}O_{14}$ and $C_{27}H_{30}O_{18}$ (Figure 4.15 C and Table 6.6). The mass fragmentation of peak m/z 563 (26.03)^{146*}, corresponded to the peak, m/z 563 (23.95)^{102*} in the Lipton® Yellow label black tea but the fragment ion, m/z 613 $[M-H+50]^-$ (23.95)^{102*} was not determined. The fragment ion, m/z 641 $[M-H+78]^-$ corresponded to an increase of 1 carbon atom, 2 hydrogen and 4 oxygen atoms. The tea leaf catechin biotransformation by Forelle pear

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indicated the same results as that determined for mushroom tyrosinase (Figures 4.15 C and 4.15 D and Table 6.6).

The biotransformation of tea leaf catechins by Yacon homogenate, indicated the peak, m/z 563 (26.95)^{148*} with a deprotonated peak, $[M-H]^-$, m/z 563 for which the elemental composition was determined as $C_{26}H_{28}O_{14}$ (Figure 4.15 E and Table 6.6). The base peak corresponded to both that determined during the catechin biotransformation by both mushroom tyrosinase and Forelle pear.

Due to the complexity associated with using fresh tea leaves as starting material, an *in vitro* model fermentation system (catechin mixture of epicatechin and epigallocatechin) was employed to further study the catechin oxidation during tea fermentation. In order to further decrease the complexity of reaction conditions, purified polyphenol oxidase from Forelle pear was employed. As previously mentioned, mushroom tyrosinase was used as a control in the study. Assessment of catechin bioconversion based upon theaflavin mass extracted data revealed the formation of two prominent peaks by respectively employing polyphenol oxidase and mushroom tyrosinase. The polyphenol oxidase formed the peaks; m/z 563 (38.20)^{149a*} and m/z 563 (35.54)^{150a*}, while correspondingly the peaks; m/z 563 (38.21)^{149b*} and m/z 563 (35.58)^{150b*} was produced by mushroom tyrosinase (Figure 4.16). The peaks; m/z 563 (35.54)^{150a*} and m/z 563 (35.58)^{150b*} were tentatively identified as theaflavin based upon the mass fragmentation data and retention times correlating to that of the theaflavin standard, m/z 563 (35.75) (Figures 4.17 B, 4.18 B, 6.10 A and 6.10 B). Based upon the similarity of the mass fragmentation data to that of theaflavin but associated increased retention time of the prominent peaks; m/z 563 (38.20)^{149a*} and m/z 563 (38.21)^{149b*}, the elemental composition of the peaks would have to be determined in order for peak identification (Figures 4.17 A and 4.18 A). The increased number of minor peaks detected in the Forelle pear polyphenol oxidase sample was associated with the degree of purification (Figure 4.16 A). From the *in vitro* model fermentation results, it can be deduced that peak, m/z 563 (26.95-27.06)^{146*, 147* and 148*} ($C_{26}H_{28}O_{14}$) was preferentially synthesised to that of theaflavin when tea fresh leaves was used as starting material. Therefore, it can be concluded that pear polyphenol oxidase and mushroom tyrosinase have a lower affinity towards catechins, to that of competing substrates present in fresh tea leaves. Based upon the identified presence of peak, m/z 563 (23.95)^{102*} ($C_{26}H_{28}O_{14}$) in Lipton® Yellow label black tea it can be deduced that the preferential formation, could be an implicating factor in the synthesis of theaflavins.

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Subsequent *in vitro* model fermentation studies were performed utilizing laccase (Suberase from Novozyme (Pty) Ltd), in both the free- and ReSynTM immobilized form, which is also capable of oxidatively polymerizing phenols. Initial fermentation studies were performed on both free- and ReSynTM immobilized laccase, utilizing various amounts (20-, 40- and 80 mg) of the substrate, Theaphenon E (Figures 4.19 and 4.20). As adequate peak intensities were still detectable with the lowest amount of substrate, subsequent catechin biotransformation studies were performed at this level of substrate, as high catechin substrate concentrations could amount to enzyme inhibition (Sharma *et al.* 2009). In order to assess the formation of *in vitro* fermentation products and to avoid assessment at a level of substrate depletion, at which the degradation of theaflavins become dominant, assessment was performed over time, at the time points; 0 to 150 min at 30 min intervals (Obanda *et al.* 2001). Initially the peaks corresponding to epicatechin gallate or catechin gallate (m/z 441 (25.01)^{9*}), epigallocatechin gallate or galocatechin gallate (m/z 457 (20.94)^{10*}), assamicain A, B or C (m/z 915 (17.90)^{11*}), theasinensin A (m/z 913 (15.53)^{162*}), epigallocatechin (m/z 305 (11.55)^{13*}), C₂'-C₂' heterodimers of epigallocatechin gallate and epigallocatechin (m/z 761 (9.29)¹⁶³), epigallocatechin or galocatechin (m/z 305 (5.86)^{4*}) and gallic acid (m/z 169 (293)^{15*}) were present in both the free- and ReSynTM immobilized laccase in the *in vitro* model fermentation systems. This correlated to the control Theaphenon E sample, but indicated that the peaks; m/z 913 (15.31)¹⁶² and m/z 761 (9.14)¹⁶³ were either absent or detected at a much lower intensity, similarly this was observed for the peaks; m/z 447 (34.01)⁷ (unidentified) and m/z 441 (28.86)⁸ (likely to correspond to epicatechin gallate based upon peak, m/z 441 (25.85)^{9*}). Discrepancies between the Theaphenon E and initial (0 min) samples were associated with the short lapse in time to inactivate the enzyme activity. Over the course of the *in vitro* fermentation a significant decrease in the intensity of the substrate; epigallocatechin (m/z 305 (11.55)^{13*}) was observed.

The efficiency of catechin biotransformation was determined based upon the theaflavin mass extracted data. Free laccase amounted to the formation of the peaks; m/z 867 (39.29)¹⁷⁰, m/z 715 (38.80)¹⁷¹ and m/z 563 (38.21)¹⁷², while correspondingly the peaks; m/z 867 (39.36)¹⁷⁰ and m/z 715 (38.87)¹⁷¹ were formed, when ReSynTM laccase was employed. These peaks were associated with a shift in retention time, but respectively corresponded to the theaflavin standards; theaflavin-3,3-di-gallate (m/z 867 (37.36)), theaflavin-3-O-gallate (m/z 715 (37.02)) or theaflavin-3'-O-gallate (m/z 715 (37.59) and theaflavin (m/z 563 (36.75) (Figures 6.10 and 6.11). The peaks identified in the free laccase samples, were respectively quantified as 637-, 998- and 283 ng/ml after 150 min, base upon the corresponding theaflavin standard curves (Figures 4.23 and 6.19 and Table 6.7). Similarly the peaks in the ReSynTM immobilized sample were

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respectively quantified as 414-, 707- and 120 ng/ml after 150 min (Figures 4.24 and 6.19 and Table 6.7). This corresponded to the formation of 0.064- and 0.007% (w/w) theaflavins from Theaphenon E, respectively by free- and ReSynTM immobilized laccase, which was lower than the report the 0.4 - 1.85% theaflavins formed during the manufacturing of black tea (Sharma *et al.* 2009).

The theasinensin mass extracted data indicated the formation of peaks; m/z 913 (15.31)^{162*} and m/z 761 (9.14)¹⁶³ by free laccase as well as a slight decrease in the intensity of peak, m/z 915 (17.75)^{11*} (Figure 4.25). Similarly by utilizing ReSynTM immobilized laccase the formation of the peaks; m/z 913 (15.31)^{162*} and m/z 761 (9.14)¹⁶³ were observed, but after 90 min the peak intensity of m/z 915 (17.84)^{11*} significantly decreased with an associated increase in the peaks; m/z 913 (15.31)^{162*} and m/z 913 (21.92)^{169*}. The difference between the free- and ReSynTM immobilized laccase was attributed to the effect that immobilization could have on an enzyme's affinity towards various substrates.

The peaks; m/z 913 (14.25)^{162*} and m/z 913 (21.92)^{169*} were identified as theasinensin A and D, EGCG C₂'-C₂' homodimers, based upon the deprotonated molecular ion, [M-H]⁻, m/z 913 and corresponding fragment ions; m/z 743 [M-H-170]⁻, m/z 591 [M-H-322]⁻ and m/z 573 [M-H-340]⁻ (Figures 6.16 and 6.18 B) (Neilson *et al.* 2007). This was confirmed in terms of the peak, m/z 913 (14.25)^{162*} based upon the determined elemental composition, C₄₄H₃₄O₂₂ corresponding to that of theasinensin A and D (Table 6.6). A further deduction can be made that peak, m/z 913 (14.25)^{162*} was likely to correspond to theasinensin A and peak, m/z 913 (21.92)^{169*} to that of theasinensin D based upon retention time (Neilson *et al.* 2007).

The peak, m/z 761 (9.14)¹⁶³ were suggested to correspond to C₂'-C₂' heterodimers of epigallocatechin gallate and epigallocatechin, that are structurally analogous to the theasinesins (Neilson *et al.* 2007). Furthermore the peak, m/z 915 (17.75)¹¹ was tentatively identified as assamicain A, B or C, which was confirmed by the determined elemental composition, C₄₄H₃₆O₂₂ (Table 6.6) (Lee *et al.* 2010).

Therefore, free laccase can be implicated in the synthesis of theasinensin A (homodimer of EGCG) as well as the EGCG-EGC heterodimer. Furthermore, overtime a slight decrease in the intensity of the assamicain A, B or C, m/z 915 (17.75)¹¹ was observed. The ReSynTM immobilized laccase similarly amounted to the formation of theasinensin A (homodimer of EGCG) and EGCG-EGC heterodimer. After 90 min the assamicain A, B or C degraded which could be associated

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with the formation of the EGCG heterodimers corresponding to theasinensin A and D, based upon the release of an epigallocatechin gallate moiety. Furthermore, a preference for the formation of theasinensin A was observed based upon the peak intensities. An *in vitro* assessment of ReSynTM immobilized laccase over an increased period of time, 1440 min, indicated an almost complete depletion of assamicain A, B or C (m/z 915 (17.75)¹¹) as was observed at 240 min, which was associated with the maximum intensity of theasinensin A (m/z 913 (14.25)¹⁶²). Between 240 and 360 min the peak intensity of theasinensin A (m/z 913 (14.25)¹⁶²) significantly decreased, after which it seemed to remain stable. Similar results were observed for the C₂'-C₂' heterodimers of epigallocatechin gallate and epigallocatechin, that are structurally analogous to the theasinesins (m/z 761 (9.14)¹⁶³) (Figure 4.27).

ReSynTM immobilized polyphenol oxidase purified from Forelle pear was also employed in the synthesis of theaflavins, utilizing Theaphenon E as starting material. Over time (1440 min) seemingly no significant change in peak intensity from that at the start of the *in vitro* fermentation was observed (Figure 4.28). Due to the difficulty associated with the purification of polyphenol oxidase and high cost of the Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column only 0.019 mg bound polyphenol oxidase was employed per *in vitro* model fermentation reaction. Due to the low amount of polyphenol oxidase, the immobilized polyphenol oxidase system was post treated with ethanolamine, to prevent subsequent immobilization reactions onto the support. The ethanolamine post-treatment has been reported to amount to a reduction in immobilized enzymatic activity (Jordaan *et al.* 2009). The absence of any observed changes in peak intensity was therefore, attributed to the low level of enzyme with possible enzyme inhibition and associated decreased activity due to the ethanolamine post-treatment.

4.5. CONCLUSION

Initial UPLC-MS assessment of the stability of the substrates; epicatechin and epigallocatechin in potassium phosphate buffer (0.2 M, pH 6.8) at 25°C, indicated that epicatechin underwent epimerization leading towards the formation of catechin, which was associated with the heat enzymatic activity inactivation step, to be employed in subsequent *in vitro* fermentation modelling. Furthermore, this was also implicated in the observed dimerization of epigallocatechin into theasinensin C and E, associated with the degradation of the heat-susceptible intermediate, dehydrotheasinensin C. The presence of the dehydrotheasinensins, responsible for the formation of theasinensin A and D, was implicated to be in the source sample of the catechin mixture. The determination of the substrate profile of a commercially available mixture of catechins, Theaphenon E, indicated the presence of epicatechin gallate or catechin gallate, gallocatechin gallate or epigallocatechin gallate, assamicain A, B or C, catechin or epicatechin, epigallocatechin, gallocatechin and gallic acid. Despite the absence of quantitative assessment, assamicain A, B or C was indicated as the most prominent peak based upon peak intensity, which was inconsistent with the reported almost 70% epigallocatechin and a total catechin content of > 90%.

The presence of the catechins; epicatechin, catechin and gallocatechin was identified in Tea (*Camellia sinensis*, frozen fresh leaves). The absence of peaks corresponding to the major catechins, epigallocatechin and epigallocatechin gallate was attributed to the epimerization of epigallocatechin to gallocatechin (associated with the heat enzyme activity inactivation step) and the efficiency of extraction. Peaks corresponding to the theaflavins and theasinensins were absent, consistent with the heat inactivation of any enzymatic activity present in the Tea (*Camellia sinensis*, frozen fresh leaves) and associated absence of catechin oxidation products. Assessment of Lipton® Green tea indicated the presence of the catechins; epigallocatechin gallate or gallocatechin gallate and epigallocatechin, furthermore tentatively that of epicatechin gallate or catechin gallate, epicatechin, catechin and gallocatechin were determined. The increased incidence of catechins in Lipton® Green tea corresponded to the reported 29-39% (w/w) in comparison to the 12-14% (w/w) in green tea leaves. The presence of EGCG-EGC heterodimers analogous to theasinensin and that of theasinensin A and D was indicated in Lipton® Green tea. Boiling of Lipton® Green tea was associated with a large increase in the peak intensity of the epigallocatechin gallate B-ring condensation-elimination homodimer, P-2. In Lipton® Yellow label black tea only the catechins; catechin and epicatechin were detected corresponding to the

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level of fermentation. The presence of the polyphenol oxidase dependent polymerization product, theaflavin, formed during fermentation, was detected in Lipton® Yellow label black tea, consistent with the 1-2% (w/w) reported content of theaflavins in black tea. Furthermore, the presence of the theasinensin C and E also formed during fermentation was detected. Boiling of Lipton® Yellow label black tea amounted to a significant increase in the peak intensity of EGCG-ECG heterodimer analogous to theasinensins.

Bioconversion of catechins present in Tea (*Camellia sinensis*, frozen fresh leaves) utilizing endogenous enzymes present in the homogenates of Forelle pear, and Yacon (*Smallanthus sonchifolius*) leaves was performed. Both Forelle pear and Yacon homogenates indicated practically no oxidation products of their own. The catechin bioconversion utilizing both enzyme sources as well as a mushroom tyrosinase (from Sigma Aldrich (Pty) Ltd) as a control, respectively indicated the formation of a prominent peak at m/z 563 (26.95-27.06)¹⁴⁶⁻⁸, C₂₆H₂₈O₁₄ possibly corresponding to *O*-pentosyl 8-C-hexosyl apigenin (Lv *et al.* 2012) or apigenin-7-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (Mencherini *et al.* 2007) or isoschaftoside (apigenin 6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranoside) (Du *et al.* 2012, Krenna *et al.* 2003, Hussein *et al.* 2012).

In vitro model fermentation was subsequently employed with purified Forelle pear polyphenol oxidase and the control mushroom tyrosinase, in order to decrease the complexity of the catechin bioconversion assessment. Theaflavin was synthesized respectively by both enzyme sources, leading to the conclusion that both Forelle pear polyphenol oxidase and mushroom tyrosinase, has a lower affinity towards catechins, to that of the competing substrates present in fresh tea leaves. Based upon the identification of a corresponding peak, m/z 563 (23.95)¹⁰² (C₂₆H₂₈O₁₄) in Lipton® Yellow label black tea, it can be deduced that the preferential formation, could be an implicating factor in the synthesis of theaflavins.

Subsequent *in vitro* model fermentation studies were performed utilizing laccase (Suberase from Novozyme (Pty) Ltd), in both the free- and ReSyn™ immobilized form. The catechin mixture, Theaphenon E was employed, and before the start of the biotransformation peaks corresponding to epicatechin gallate or catechin gallate, epigallocatechin gallate or galocatechin gallate, assamicain A, B or C, theasinensin A, epigallocatechin, C₂'-C₂' heterodimers of epigallocatechin gallate and epigallocatechin, epigallocatechin or galocatechin and gallic acid were determined. Over time the peak intensity of epigallocatechin significantly decreased. Both free- and ReSyn™ immobilized laccase catalysed the synthesis of the theaflavins; theaflavin, theaflavin-3-*O*-gallate,

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theaflavin-3'-O-gallate and theaflavin-3,3-di-gallate, which were respectively quantified as 637-, 998- and 283 ng/ml in the free laccase sample, and 414-, 707- and 120 ng/ml in the ReSynTM immobilized laccase sample, after 150 min. This corresponded to the formation of 0.064- and 0.007% (w/w) theaflavins from Theaphenon E, respectively by free- and ReSynTM immobilized laccase, which was lower than the report the 0.4 - 1.85% theaflavins formed during the manufacturing of black tea (Sharma *et al.* 2009).

In both the free- and ReSynTM immobilized laccase samples, the formation of theasinsin A and D as well as that of the C₂'-C₂' heterodimers of epigallocatechin gallate and epigallocatechin, that are structurally analogous to the theasinesins were determined. A decrease in the peak intensity of assamicain A, B or C was observed, which was to a large extent observed after 90 min *in vitro* fermentation with ReSynTM immobilized laccase. The degradation of assamicain A, B or C was implicated in the formation of theasinsin A and D, with preferential formation of theasinensin A. Over an increased period of time, assamicain A, B or C was almost completely depleted after 240 min, which was associated with the maximum intensity of theasinensin A. A significant decrease in peak intensity between 240 and 360 min was observed for theasinensin A, which seemingly remained stable over the remaining course of fermentation. Similar results were observed for the C₂'-C₂' heterodimers of epigallocatechin gallate and epigallocatechin.

In vitro bioconversion of catechins by ReSynTM immobilized polyphenol oxidase was hampered by the amount of immobilized enzyme, and subsequent ethanolamine post-treatment of the immobilized enzyme, respectively associated with enzyme inhibition and decreased enzymatic activity.

CHAPTER 5 : CONCLUDING DISCUSSION

Catechins (monomeric flavan-3-ols), an unusually rich sub-group of polyphenols, are the major components present in green tea leaves and can constitute up to 30% of the dry tea leaf weight (Sharma *et al.* 2009). In the manufacturing of black tea, the oxidation of green tea catechins; (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate, is promoted during fermentation (Leung *et al.* 2001). Polyphenol oxidase catalyzes the controlled aerobic oxidation of catechins producing various *o*-quinones which undergo condensation reactions, resulting in the formation of the dimeric compounds known as theaflavins; theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate (Leung *et al.* 2001). Hydrogen peroxide generated in the polyphenol oxidase dependent oxidative polymerization of catechins, is utilized by peroxidase for subsequent oxidation, amounting to the conversion of formed theaflavins into thearubigins (Pan *et al.* 2000, Subramanian *et al.* 1999b). In contrast the oxidation of green leaf polyphenols is precluded in the preparation of green tea (non-fermented tea) (Leung *et al.* 2001).

The project focussed on the bioconversion of green tea leaf catechins into theaflavins, as the original method employed to acquire theaflavins, the extraction of theaflavins from black tea (only 0.4-1.85% of the dry weight of black tea), doesn't provide a cost-effective manner of recovering theaflavins, and amounts to an industrially unfeasible approach (Sharma *et al.* 2009, Wang & Li 2006). Enzymatic oxidation was employed to exploit nature's highly efficient and selective means of transformation, which can allow reactions not easily conducted by classical organic chemistry (Li & Trost 2008, Petersen & Kiener 1999). The approach of biocatalysis was utilized as a tool for the production of theaflavins, by taking advantage of the versatility of enzymes, and their functioning under mild operation conditions (Betancor & Luckarift 2008, Fernandes 2010, Salis *et al.* 2005). Initial catechin bioconversion studies exploited the endogenous enzymes present in the homogenate of fruit, as the mechanism of theaflavin synthesis was suggested to be similar to that present in tea fermentation. Specifically pear homogenate, as Japanese pear homogenate was reported with much greater theaflavin synthesis capacity of than that of fresh tea leaves, which was also higher than 62 plants belonging to 49 families (Tanaka *et al.* 2002, Tanaka & Kouno 2003). Forelle pear was identified among the pear varieties Forelle, Packman, Abet Beteil and Beurré Bosc available in South Africa as the pear variety with the highest theaflavin synthesis capacity in a fermentation system utilizing Lipton® Green.

Preliminary exploratory investigation into the theaflavin synthesis capacity of Forelle pear and as well as Yacon (*Smallanthus sonchifolius*) leaf homogenates, determined diphenolase activities in the same order of magnitude as that of Tea (frozen fresh leaves homogenate), with the Yacon homogenate activity determined as the highest. Furthermore in a fermentation system the theaflavin synthesis capacity based upon the flavognost method was in the decreasing order of Forelle pear, Yacon and Tea (frozen fresh leaves) respectively utilizing Lipton® Green tea and Tea (frozen fresh leaves) as the source of catechins. Control samples confirmed that the theaflavin synthesis capacity was not attributed to an inherent theaflavin content. In comparison to Lipton® Yellow label black tea, Forelle pear and Yacon homogenates had a 136% and 98% theaflavin content after an hour of fermentation utilizing Lipton® Green tea (no enzymatic activity) as starting material. From the preliminary exploratory investigation, both Forelle pear and Yacon leaves were identified as ideal candidates for further investigation into their theaflavin synthesis capacity. Further investigation was only performed on Forelle pear polyphenol oxidase, but the indicated theaflavin synthesis capacity of Yacon leaves warrants further investigation. As the enzyme responsible for the bioconversion of catechin into theaflavins, polyphenol oxidase has not been purified from Yacon (*Smallanthus sonchifolius*), this would entail the purification and characterization of the enzyme in addition to the evaluation of the theaflavin synthesis ability of the enzyme in a fermentation system.

No instance of the purification of polyphenol oxidase from the Forelle pear cultivar has previously been reported. Furthermore, although polyphenol oxidase has been purified from various sources, no uniform purification approach in terms of extraction medium, phenol-absorbing agents, possible use of detergents and proteases as well as the method employed has been reported. Initial purification was performed based upon a gel filtration method, after which a single step affinity chromatography step was employed, based upon the greater specificity of the approach for the purification of polyphenol oxidase. A 3 fold purification of Forelle pear polyphenol oxidase was attained in a single Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column purification step. The complete purification of polyphenol oxidase is hampered by the multi sub-unit nature and presence of several polyphenol oxidase isoenzymes. The purification of polyphenol oxidase could be improved based upon optimization studies of the level of polyphenol oxidase in the source and that of the approach of affinity chromatography. Factors that affect the level of polyphenol oxidase in the Forelle pears are the stage of ripening or storage of the fruits which could be associated with solubilisation and proteolysis of the enzyme (Murata *et al.* 1997). Further factors include the redistribution or induction of the expression of the enzyme after wounding, coinciding with the loss of membrane integrity as well as the localization of active

polyphenol oxidase in mature fruit (Boss *et al.* 1995, Thipyapong & Steffens 1997, Murata *et al.* 1995, Partington *et al.* 1999, Constabel *et al.* 2000). The single affinity chromatography step could be supplemented by a prior ammonium sulphate precipitation step, reported to respectively amount to a 7- and 74 fold purification of polyphenol oxidase from *Lactaris salmonicolor* and mulberry (Arslan *et al.* 2004, Dedeoglu & Guler 2009). The Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column itself could be optimized based upon the observed bound protein without polyphenol oxidase activity, as the success of affinity chromatography requires that the unsubstituted matrix or gel has minimal interactions with proteins in general.

A polyphenol oxidase purification approach, from fruits in general and more specifically pear, is required to yield a large quantity of purified enzyme in an industrially feasible manner, because in general only mushroom tyrosinase is commercially available. This is required in order to aid investigations into the browning of fruits during storage and processing in the food industry, as this is believed to be the main cause of quality loss during post harvest handling (Coetzer *et al.* 2001). In general, interest in pear polyphenol oxidase is attributed to the propensity of fresh-cut pears to enzymatic browning and softening during storage (Dong *et al.* 2000). Furthermore polyphenol oxidase can be employed for the determination of phenolics in red wines (Yildiz *et al.* 2005, Kiralp *et al.* 2003) and cyanide in samples (Shan *et al.* 2004), detection of polyphenols in tea (Abhijith *et al.* 2007) and pollutants in water (Russell & G Burton 1999, Edwards *et al.* 1999) and for the removal of phenols from industrial effluents (Edwards *et al.* 1999).

Polyphenol oxidase characterisation studies determined the enzyme to have an optimum pH and to be most stable at pH 5, and an associated autoxidation rate of the substrate, 4-methylcatechol that becomes increasing significant at higher pH values. Polyphenol oxidase was stable over the temperature range of 25°C to 70°C with an optimum temperature at 25°C (determined at pH 6.5) and 30°C (determined at pH 4). Therefore, based upon pH and temperature studies, the enzyme was revealed to function under mild operation conditions, in contrast to what would be required if a chemical catalyst was to be employed for the production of theaflavins (Salis *et al.* 2005). The kinetic parameters K_M and V_{max} was determined as 14 mM and 1.4×10^5 units/mg protein respectively with Lineweaver Burk and as 0.9 mM and 1.0×10^4 units/mg protein respectively with Hanes-Woolf plot, utilizing 4-methylcatechol as substrate. The kinetic parameters of polyphenol oxidase should also be determined utilizing the various catechins as substrate, in order to assess the affinity of the enzyme for the substrates in the synthesis of theaflavins.

Despite excellent catalytic properties, at an industrial scale, the desirability of many cycles of high-yield processes usually requires enzyme characteristics to be improved (Mateo *et al.* 2007). The improvement of activity, stability, selectivity towards non-natural substrates, the inhibition by reaction products and prohibitive enzyme cost, may be required, offering in general drawbacks to the use of biocatalysts to that of classical chemical catalysts (Mateo *et al.* 2007, Villeneuve *et al.* 2000). Therefore, the approach of enzyme immobilization was employed to, enhances the applicability of polyphenol oxidase and laccase (Sigma Aldrich (Pty) Ltd) as robust and reusable biocatalysts due to the increase in stability it confers to enzymes (Betancor & Luckarift 2008).

The oxidative polymerization of phenols can also be catalyzed by laccase (benzenediol/oxygen oxidoreductase, EC 1.10.3.2) (Mita *et al.* 2003). Both laccase and polyphenol oxidase utilizes oxygen as an oxidizing agent, amounting to an environmentally benign process in air without the use of hydrogen peroxide as an oxidizing agent, as in the case of peroxidase (Kurisawa *et al.* 2003).

The project investigated a novel enzyme immobilization matrix, ReSynTM and compared it to three widely used matrixes, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 in terms of protein binding capacity and enzyme activity. Extremely high enzyme loads can be attained by ReSynTM, emulsion-derived particles, as a result of an open fibre network and high amine density, enabling the penetration of protein molecules throughout the volume of the microspheres in contrast to current solid or porous/cracked microspheres (Brady & Jordaan 2009). Initial comparative assessment of immobilization on the different supports, was performed with BSA, which determined an approximate 8 fold higher protein loading efficiency than the other supports. The strength of BSA bonding onto the supports Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 indicated a non-covalent nature of immobilization in contrast to a covalent nature for ReSynTM. Based upon the strength of bonding, covalent immobilization leads to an enzyme that is tightly fixed to the solid support and subsequently minimizes leaching of the enzyme in an aqueous medium, preventing protein contamination of the product (Zhu *et al.* 2007). Furthermore, covalent immobilization of an enzyme could provide washing and reusing possibilities without the loss of activity (Varlan *et al.* 1996). Immobilization of Forelle pear polyphenol oxidase on ReSynTM, amounted to the binding of a low amount of protein, which could have been associated with the purity of the enzyme. Furthermore, the functionality of the lattice can be modified in order to optimize binding of the polyphenol oxidase enzyme onto the support. Immobilization of laccase from *Myceliophthora thermophila* determined an approximate

2 fold higher protein loading efficiency for the other supports in comparison to the utilization of ReSynTM. Similarly the functionality of the lattice can be modified in order to optimize binding of the laccase enzyme onto the support. A significant difference among the mean units activity per mg support for the different immobilization matrixes respectively over the time points; 1-, 4-, 7- and 24 h of immobilization separately performed at 4°C and 25°C was determined by one-way analysis of variance (ANOVA). ANOVA enabled the comparison of the mean enzyme activity binding capacity by utilizing *F*-statistics, but in order to determine which group(s) was different from the rest, Duncan's multiple range test (DMRT) was performed as a post-hoc test. Based upon DMRT it was concluded that there was a statistical significant difference (at $p = 0.05$) in all the mean enzyme activity binding capacities between all the supports over all the time points at both 4°C and 25°C. Summary statistical analysis indicated an overall high coefficient of variation, which was in the decreasing order of the supports; Eupergit C, Eupergit C 250L, ReSynTM and DEAE Sephadex A-25. This was attributed to the inherent difficulty of determining immobilized enzyme activity in an accurate and reproducible manner, which is associated with the particle size of the immobilization matrixes. It was concluded that the lower 95% confidence level of the mean immobilized laccase activity binding capacity determined for ReSynTM was higher than the upper 95% confidence level of the mean of other supports; Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C, which was respectively 2-, 4- and 17 fold higher after 24 h of immobilization at 4°C. From the comparative assessment of immobilization it was deduced that ReSynTM demonstrated performance superiority over the other supports.

The industrial applicability of laccase was increased by immobilization on ReSynTM based upon pH optimum determination of 2 for the free enzyme with additional peaks at 4.5 and 6 for the immobilized laccase. Furthermore, the temperature optimum determination indicated a significantly increased activity temperature profile for the immobilized laccase. Based upon the considerable importance of the reusability of an enzyme for technical applications, an assessment of the retention of enzymatic activity after repeated catalytic cycles, and long-term storage, pH- and temperature stability of the immobilized enzyme should be performed. This would enable validating that immobilization enhances the applicability of laccase as a robust and reusable biocatalyst. Laccases are exceptionally versatile enzymes, which are increasingly investigated for a variety of practical applications, which are ascribed to its ability to produce a free radical form of a suitable substrate (Mayer & Staples 2002). Investigation into the application of ReSynTM immobilized laccase in the paper and pulp industry for enzymatic bleaching (Camarero *et al.* 2004, Bauer *et al.* 1999), in drug analysis as forming part of a morphine sensor (Bauer *et al.* 1999), removal of phenolic compounds (Tsioulpas *et al.* 2002) as well as the

application of laccase in bioremediation processes could be explored (Mayer & Staples 2002). Immobilization of polyphenol oxidase and laccase respectively could realize the application these enzymes have in the bioconversion of catechins into theaflavins.

In vitro model fermentation was employed as a very useful method for the study of catechin oxidation during tea fermentation (Tanaka & Kouno 2003). The catechin substrate profile of a commercially available catechin mixture, Theaphenon E was determined as well as that of Tea (*Camellia sinensis*, frozen fresh leaves) in order to assess the compatibility between the substrate profiles. The presence of epicatechin gallate or catechin gallate, gallocatechin gallate or epigallocatechin gallate, assamicain A, B or C, catechin or epicatechin, epigallocatechin, gallocatechin and gallic acid was determined in Theaphenon E. Therefore, the substrate profile of Theaphenon E indicated the presence of the major catechins present in tea leaves; epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin, which account for 86% to 97% of the total catechin content. Furthermore, only the presence of the catechins; epicatechin, catechin and gallocatechin was identified in Tea (*Camellia sinensis*, frozen fresh leaves), which was associated with the efficiency of extraction. No theaflavins were detected in the Tea (*Camellia sinensis*, frozen fresh leaves) consistent with the absence of catechin oxidation products, based upon the inactivation of enzymatic activity, as in the manufacturing of green tea (non-fermented). In Lipton® Green tea an increased incidence of catechins was detected, corresponding to the reported 29-39% (w/w) in comparison to the 12-14% (w/w) in green tea leaves. Boiling of Lipton® Green tea and Lipton® Yellow label black tea were respectively associated with a large increase in the peak intensity of the epigallocatechin gallate B-ring condensation-elimination homodimer, P-2 and an EGCG-ECG heterodimer analogous to theasinensins. In Lipton® Yellow label black tea (fermented) a decreased incidence of catechins, and the polymerization products; theaflavin and theasinensin C and E were detected, consistent with the extent of fermentation.

The bioconversion of Tea (*Camellia sinensis*, frozen fresh leaves) utilizing endogenous enzymes present in the homogenates of Forelle pear, Yacon (*Smallanthus sonchifolius*) leaves and control mushroom tyrosinase enzyme, respectively indicated the formation of a prominent peak corresponding to the m/z of theaflavin. These peaks, at m/z 563 (26.95-27.06)¹⁴⁶⁻⁸, C₂₆H₂₈O₁₄ possibly corresponded to *O*-pentosyl 8-C-hexosyl apigenin (Lv *et al.* 2012) or apigenin-7-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (Mencherini *et al.* 2007) or isoschaftoside (apigenin 6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranoside) (Du *et al.* 2012, Krenna *et al.* 2003, Hussein *et al.* 2012). The presence of a corresponding peak, m/z 563 (23.95)¹⁰² (C₂₆H₂₈O₁₄) was

detected in Lipton® Yellow label black tea. Further investigation was performed at a decreased level of complexity, by employing an *in vitro* model fermentation system with purified polyphenol oxidase from Forelle pear and the control mushroom tyrosinase enzyme. The *in vitro* model fermentation systems respectively lead to the synthesis of theaflavin. Based upon a comparison of the oxidation products obtained from the fermentation and *in vitro* model fermentation studies, it was concluded that the peak, m/z 563 (26.95-27.06)¹⁴⁶⁻⁸, C₂₆H₂₈O₁₄, was preferentially formed to theaflavin, from which it was deduced that it could be an implicating factor in the synthesis of theaflavins.

An anomaly was apparent between the high theaflavin synthesis capacity determined based upon flavognost method and the UPLC-MS analysis in terms of which no apparent theaflavins were detected, in fermentation systems utilizing endogenous enzymes present in the homogenates Forelle pear and Yacon in the bioconversion of Tea (*Camellia sinensis*, frozen fresh leaves) catechins. The flavognost method determined the theaflavin synthesis capacity in the decreasing order of Forelle pear (184.2 µmole TF/g DW/h), Yacon (181.4 µmole TF/g DW/h) and Tea (frozen fresh leaves) (77.91 µmole TF/g FW/h). This is in contrast to the UPLC-MS analysis which indicated the formation of a prominent peak, possibly corresponding to *O*-pentosyl 8-C-hexosyl apigenin (Lv *et al.* 2012) or apigenin-7-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (Mencherini *et al.* 2007) or isoschaftoside (apigenin 6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranoside) (Du *et al.* 2012, Krenna *et al.* 2003, Hussein *et al.* 2012). Further investigation needs to be conducted in order to explain the apparent anomaly.

In vitro model fermentation respectively with free- and ReSynTM immobilized laccase, catalyzed the synthesis of the theaflavins; theaflavin, theaflavin-3-*O*-gallate, theaflavin-3'-*O*-gallate and theaflavin-3,3-di-gallate, which was quantified as 637-, 998- and 283 ng/ml in the free laccase sample and 414-, 707- and 120 ng/ml in the ReSynTM immobilized sample, after 150 min. The total amount of theaflavins (1.241 µg/ml) synthesized from the catechin mixture, Theaphenon E (20 mg/ml) by free- and ReSynTM immobilized respectively corresponds to a 0.010% and 0.006% yield of synthesized theaflavins, in comparison to the reported 15-30% dry weight of catechins present in green tea shoots from which 0.4-1.85% dry weight of theaflavins are formed during black tea manufacturing corresponding to a yield between 2.7% and 6% of theaflavins (Sharma *et al.* 2009, Graham 1992). In both instances fermentation was associated with the formation of theasinsin A and D as well as that of the C₂'-C₂' heterodimers of epigallocatechin gallate and epigallocatechin, that are structurally analogous to the theasinesins. The degradation

of assamicain, associated with the release of an epigallocatechin gallate moiety, was implicated in the formation of theasinensin A and D, with preferential formation of theasinesin A.

Optimization studies of the bioconversion of catechin into theaflavins must be performed based upon reaction conditions such as the optimum pH with consideration of the associated production of peroxide during the oxidation of catechins, which is linked to the turnover of theaflavins into thearubigins by peroxidase (Subramanian *et al.* 1999b). Therefore, determining reaction conditions at which a balance between the synthesis and oxidation of theaflavins are achieved. Furthermore, a specific weight ratio of catechins to theaflavins must be determined, based upon high catechin substrate concentration amounting to enzyme inhibition, and the implication of catechins in initiating the destruction of theaflavins (Sharma *et al.* 2009, Hodges 2008). Temperature affects both formation and degradation reaction rates, therefore assessment of the complex effect of temperature on the accumulation of theaflavins must be performed with a determined optimum temperature for the synthesis of theaflavins (Lian *et al.* 2002, Obanda *et al.* 2001).

To the author's knowledge this is the second reported incidence of laccase catalyzing the synthesis of theaflavins; theaflavin, theaflavin-3-*O*-gallate, theaflavin-3'-*O*-gallate and theaflavin-3,3-di-gallate. The other reported incident also determined the synthesis of theaflavins from tea polyphenol by using laccase enzyme-catalysing oxidation (Kun 2008). Previous research has implicated laccase in the synthesis of two new catechin-hydroquinone adducts (Hosny & Rosazza 2002), conversion of procyanidin B-2 (epicatechin dimer) to the procyanidin A-2 dimer (Osman & Wong 2007). The laccase-dependent preferential oxidation of gallic acid and the pyrogallol group in epigallocatechin and epigallocatechin gallate in crude tea catechins into epitheafalgallin and epitheafalgallin 3-*O*-gallate, which was accelerated with the addition of gallic acid was also noted (Itoh *et al.* 2007). An investigation into the oxidation of (+)-catechin catalyzed by laccase determined the formation of dimers of the type dehydrocatechin B (hydrophilic) and hydrophobic dimers of type-A as well as a trimer, tetramer and their oxidized forms were reported (Ma *et al.* 2009, Osman *et al.* 2007). Laccase-catalysed oxidation and autoxidation of (±)-catechin in the presence and absence of sodium benzenesulphinate was investigated (Brown & Whiteoak 1964). Laccase has been employed to catalyze the oxidative polymerization of catechin in aqueous organic solvents, although the identities of the formed poly(catechin)s were not determined (Kurisawa *et al.* 2003).

Interest in theaflavins arises from their potential benefits as a natural dietary antioxidant (Miller *et al.* 1996, Saha & Das 2003, Lin *et al.* 1999, Cai *et al.* 2006). Theaflavins have been implicated to have antiviral activity by the identification of theaflavin-3'-gallate, theaflavin-3,3'-digallate and tannic acid, as effective inhibitors of the virally encoded 3C-like protease ($IC_{50} < 10 \mu M$), from the screening of 720 natural products and subsequent analysis of well-known components present in tea (Chen *et al.* 2005). The potential clinical significance of theaflavins has also been suggested based upon their reported anticancer- (Leone *et al.* 2003), antimutagenic-, anticlastogenic- (Halder *et al.* 2005) and antibacterial activity (Nashimoto & Tashiro 1999). Despite the implication of theaflavins as potential therapeutics in various fields of research, research on theaflavins has been hampered, to such an extent that most previous research was focused on the use of mixtures of theaflavins. It is of fundamental importance that an industrially feasible approach should be developed for the synthesis of theaflavins, to overcome their low abundance and challenging purification procedure. Furtherance of this project could amount to the development of a ReSynTM immobilized laccase or polyphenol oxidase systems for the bioconversion of tea catechins into theaflavins in an industrially feasible manner. The immobilized enzyme system could be applied in the ice tea industry to yield a product with increased theaflavin content, and associated increased antioxidant activity. This would enable the exploitation of the lucrative tea industry, with tea being the most consumed beverage in the world, second only to water.

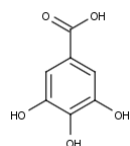
In conclusion, in the comparison of free and immobilized polyphenol oxidase enzymes for the biocatalytic conversion of catechins into theaflavins, the various aims were achieved. The project succeeded in the purification of Forelle pear polyphenol oxidase based upon (1) the identification of the pear variety with the highest theaflavin synthesis capacity, (2) preliminary investigation of the theaflavin synthesis capacity of the identified pear variety in terms of the diphenolase activity, theaflavin synthesis capacity in a fermentation system, and an inherent catechin and theaflavin content and (3) the purification of pear polyphenol oxidase. Comparative assessment of immobilization was performed with laccase, based upon an insufficient amount of purified Forelle pear polyphenol oxidase. Immobilization on the supports Eupergit C, Eupergit C 250L, DEAE Sephadex A-25 and ReSynTM was comparatively assessed based upon a preliminary assessment with bovine serum albumin in terms of loading capacity, binding efficiency and the nature of binding onto the support. Comparative assessment of laccase immobilized on the various supports amounted to the rejection of the null hypothesis (H_{10}), that there will be no statistical significant difference, in the enzyme activity binding capacity (enzyme units/mg support), between the supports; ReSynTM, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25, at the

95% level of confidence. Subsequently the alternative hypothesis (H1) was accepted, that there will be a statistical significant difference, in the enzyme activity binding capacity (enzyme units/mg support), between the supports; ReSynTM, Eupergit C, Eupergit C 250L and DEAE Sephadex A-25, at the 95% level of confidence, for all the hypotheses that were set up between the various supports at four different reaction times and two different reaction temperatures. The aim to biotransform tea catechins into theaflavins was successfully achieved with both free laccase and ReSynTM immobilized laccase in an *in vitro* model fermentation system. We were unable to accept or reject the null hypothesis (H2₀), that there will be no statistical significant difference in the yield of theaflavins/mg enzyme between the free- and ReSynTM immobilized laccase at the 95% level of confidence. Due to time constraints the quantification of theaflavin peak height intensities was only performed on a single replicate, which provided insufficient data for statistical analysis of the yield of theaflavins/mg enzyme between the free- and ReSynTM immobilized laccase at the 95% level of confidence.

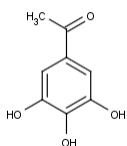
CHAPTER 6 : APPENDIXES

6.1. APPENDIX A

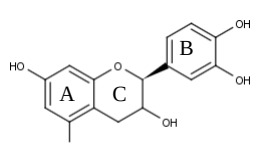
Structures:



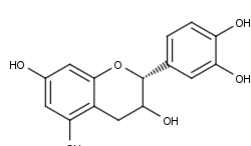
Gallic acid



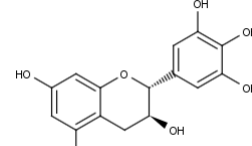
Galloyl



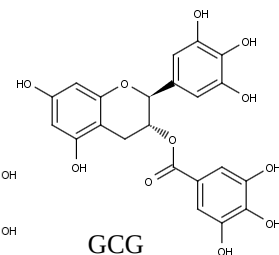
Catechin



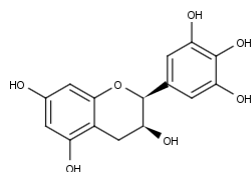
Epicatechin



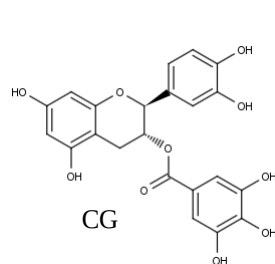
GC



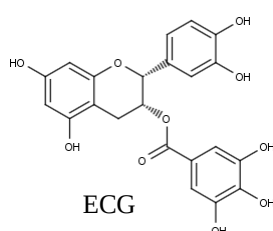
GCG



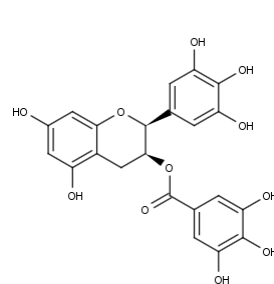
EGC



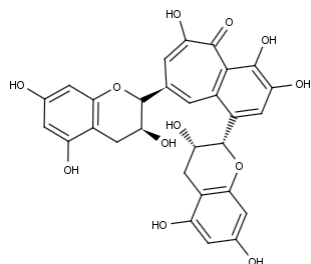
CG



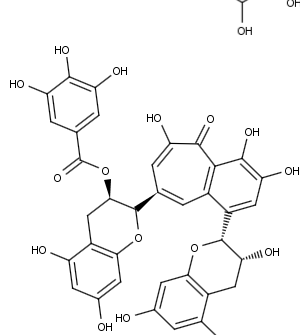
ECG



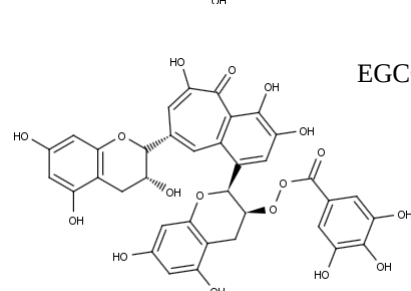
EGCG



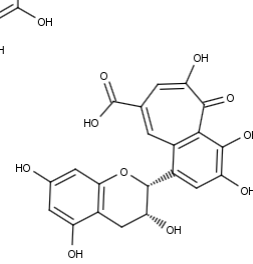
Theaflavin



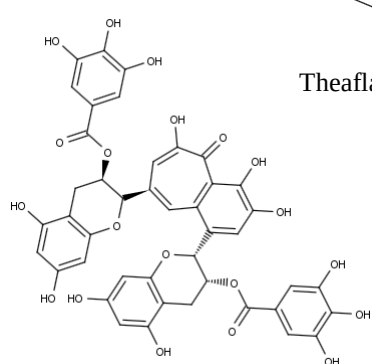
Theaflavin-3-O-gallate



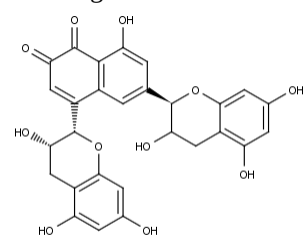
Theaflavin-3'-O-gallate



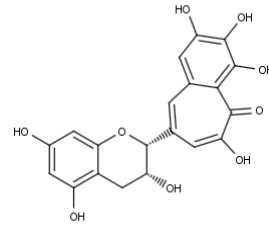
Epitheaflavonic acid



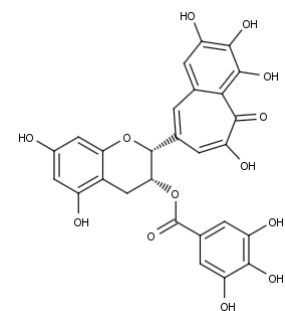
Theaflavin-3,3'-digallate



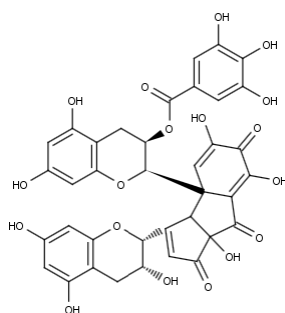
Theanaphthoquinone



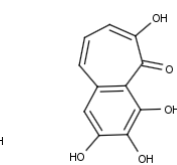
Epitheaflagallin



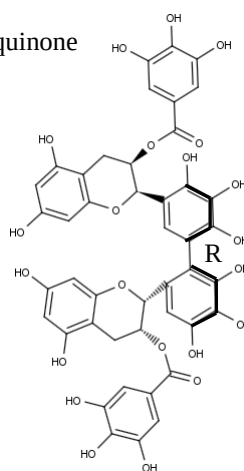
Epitheaflagallin 3-O-gallate



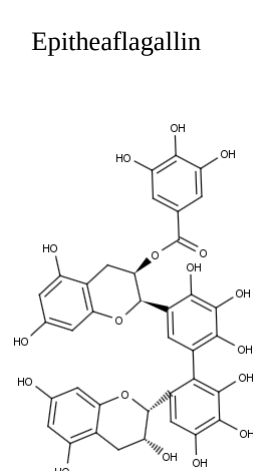
Theacitrin A



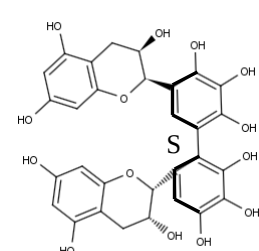
Purpurogallin



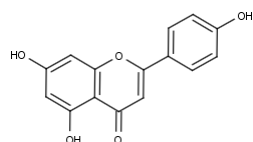
Theasinensin A (R) or
Theasinensin D (S)



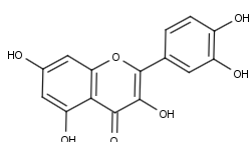
Theasinensin B



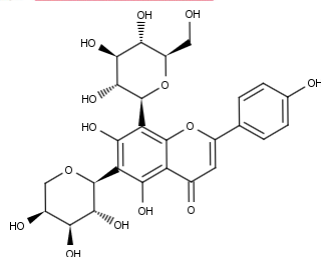
Theasinensin C (R) or
Theasinensin E (S)



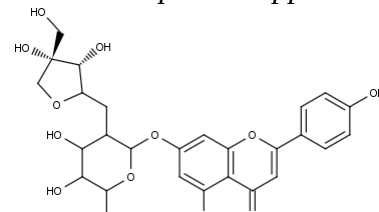
Apigenin



Quercetin



Isoschaftoside (6-C- α -L-arabinopyranosyl-8-C- β -D-glucopyranoside)



Apiin (apigenin-7-O- β -D-apiofuranosyl- β -D-glucopyranoside)

Protein concentration determination:

The first example of a BSA standard curve constructed for protein concentration determination (Table 6.1 and Figure 6.1).

Table 6.1: BSA standard curve data utilized for the determination of the protein concentration during polyphenol oxidase extraction and purification as well as in the immobilization of BSA.

Conc. (μ M)	Blank	0.000	0.050	0.100	0.149	0.200	0.400	0.435	0.500
	0.292	0.291	0.350	0.410	0.482	0.538	0.740	0.774	0.849
	0.282	0.297	0.362	0.420	0.484	0.562	0.802	0.836	0.927
	0.288	0.287	0.350	0.420	0.466	0.512	0.785	0.835	0.927
	0.289	0.283	0.349	0.405	0.474	0.524	0.763	0.833	0.920
	0.304	0.306	0.374	0.447	0.522	0.570	0.782	0.763	0.850
	0.305	0.309	0.370	0.416	0.479	0.534	0.762	0.821	0.853
	0.297	0.290	0.368	0.412	0.467	0.525	0.716	0.754	0.854
	0.287	0.290	0.374	0.411	0.472	0.530	0.777	0.832	0.933
Mean (n = 8)	0.293	0.294	0.362	0.418	0.481	0.537	0.766	0.806	0.889
M 595 Corr.	0.000	0.001	0.069	0.125	0.188	0.244	0.473	0.513	0.596
SD	0.008	0.009	0.011	0.013	0.018	0.020	0.027	0.036	0.040
CV	Na.	8.153	0.159	0.104	0.095	0.081	0.058	0.070	0.068
Conc. = Concentration M 595 Corr. = Corrected absorbance value after the absorbance of the blank at 595nm is subtracted SD = Standard deviation CV = Coefficient of variation Na. = Not applicable									

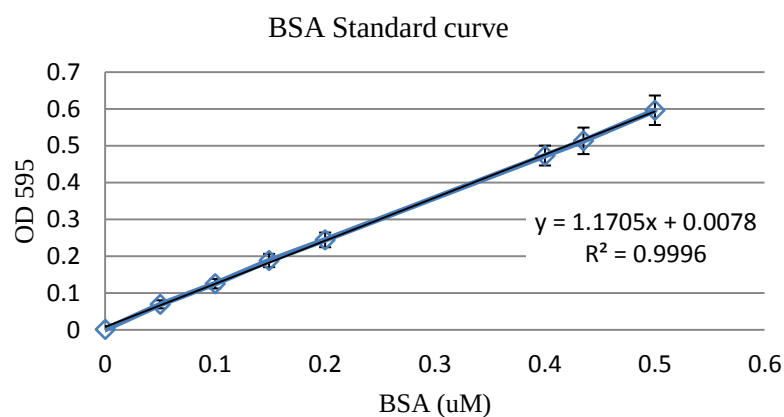


Figure 6.1: Bovine serum albumin standard curve. Error bars represent standard deviation (SD) of the absorbance at 595 nm calculated from eight replicates.

A second example of a BSA standard curve constructed for protein concentration determination (Table 6.2 and Figure 6.2).

Table 6.2: BSA standard curve data utilized for the determination of the protein concentration during the salt washing steps.

Conc. (μM)	Blank	0.000	0.050	0.149	0.250	0.294	0.345
	0.374	0.374	0.489	0.631	0.804	0.834	0.894
	0.376	0.376	0.446	0.633	0.808	0.804	0.887
	0.372	0.372	0.497	0.617	0.746	0.838	0.869
	0.374	0.374	0.475	0.652	0.802	0.807	0.890
	0.368	0.368	0.495	0.657	0.784	0.886	0.885
	0.368	0.368	0.49	0.632	0.795	0.883	0.885
	0.418	0.418	0.489	0.627	0.739	0.795	0.919
	0.368	0.368	0.475	0.614	0.767	0.842	0.984
Mean (n = 8)	0.361	0.361	0.461	0.622	0.757	0.799	0.869
M 595 Corr.	0.000	0.000	0.100	0.261	0.396	0.438	0.502
SD	0.017	0.017	0.017	0.015	0.027	0.034	0.036
CV	Na.	Na.	16.69	5.783	6.808	7.861	7.102
Conc. = Concentration M 595 Corr. = Corrected absorbance value after the absorbance of the blank at 595nm is subtracted SD = Standard deviation CV = Coefficient of variation Na. = Not applicable							

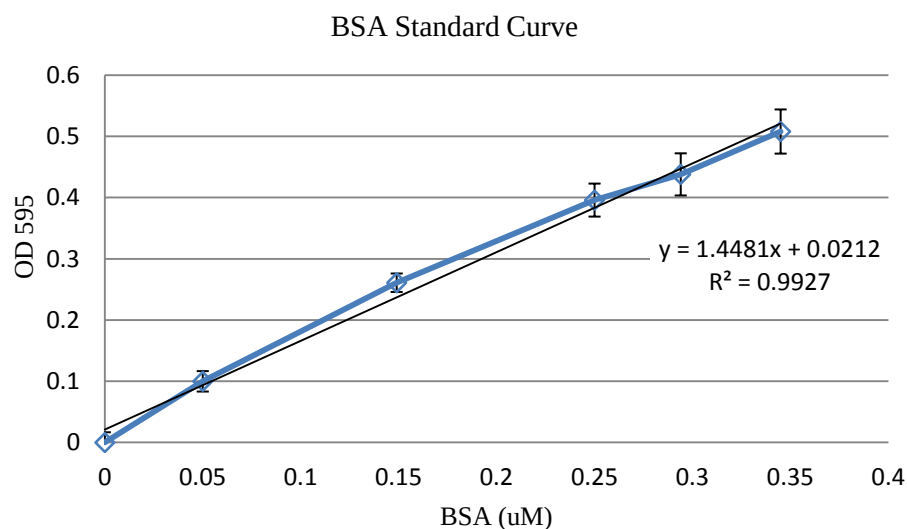


Figure 6.2: Bovine serum albumin standard curve. Error bars represent standard deviation (SD) of the absorbance at 595 nm calculated from eight replicates.

6.2. APPENDIX B

Table 6.3: One-way ANOVA analysis of the activity of laccase respectively immobilized on ReSynTM, Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C.

4°C						25°C					
Source	DF	Sum of Square (SS)	Mean Square (MS)	F Value	R ²	Source	DF	Sum of Square (SS)	Mean Square (MS)	F Value	R ²
1 h						1 h					
Model	3	1178586.6	392862	131.05	0.82	Model	3	485238.56	161746	162.72	0.86
Error	86	257804.68	2997.73			Error	81	80515.041	994.013		
Corrected Total	89	1436391.3				Corrected Total	84	565753.60			
4 h						4 h					
Model	3	1127607.5	375869	104.13	0.77	Model	3	684481.72	228161	197.44	0.87
Error	92	332095.62	3609.74			Error	92	106313.02	1155.58		
Corrected Total	95	1459703.1				Corrected Total	95	790794.74			
7 h						7 h					
Model	3	1477801.4	492600	386.95	0.94	Model	3	758312.32	252771	480.01	0.94
Error	80	101841.58	1273.02			Error	92	48446.284	526.590		
Corrected Total	83	1579643.0				Corrected Total	95	806758.60			
24 h						24 h					
Model	3	923175.92	307725	213.40	0.87	Model	3	217050.18	72350.06	50.390	0.62
Error	92	132664.79	1442.01			Error	92	132091.82	1435.781		
Corrected Total	95	1055840.7	=			Corrected Total	95	349141.99			
Model = predictor variable p < 0.001						N = between 16 to 24					

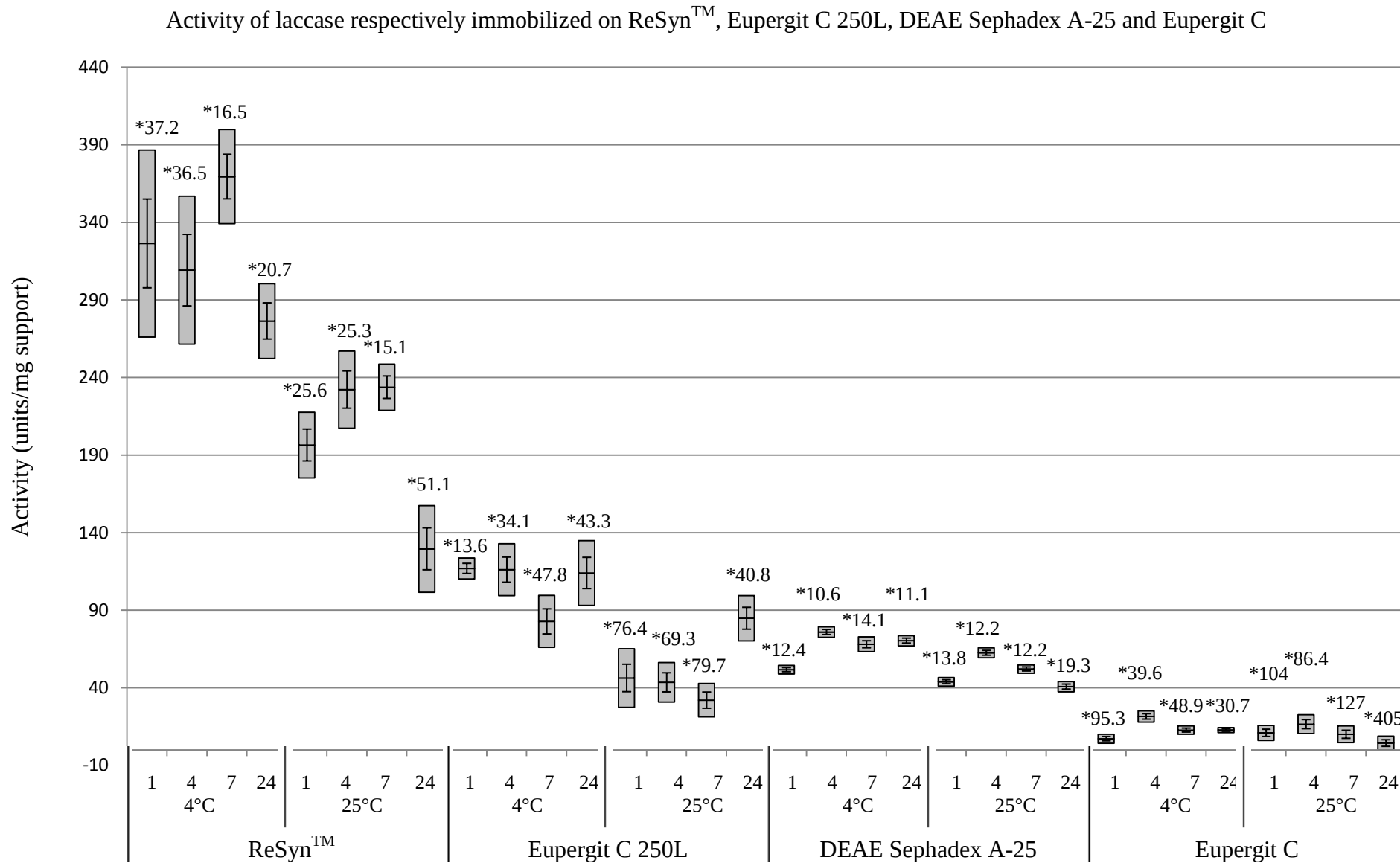


Figure 6.3: Box plot of the activity (units/mg support) representing the upper 95% CL, mean and lower 95% CL of laccase respectively immobilized on ReSyn™, Eupergit C 250L, DEAE Sephadex A-25 and Eupergit C respectively over the time points; 1, 4, 7 and 24 hours at both 4°C and 25°C. The error bars represent the standard error of the activity (units/mg support) and the coefficient of variation of the activity for the respective laccase immobilizations was represented by the asterisk (*).

Table 6.4: Synthesised ReSyn™ particle size determination on a Delsa™ Nano C.

Distribution results:	
Peak	Diameter (nm)
1	173.5 ($\pm$ 51.5)
2	2,729.8 ($\pm$ 1,239.8)
3	49,803.8 ($\pm$ 17,049.4)
Average	18,197.8 ($\pm$ 24, 072.0)
Residual	1.100e-002
Cumulants results:	
Diameter (d)	2943.1 nm
Polydispersity Index (P.I.)	0.908
Diffusion Const. (D)	1.671e-009 cm ² /sec
Measurement condition:	
Temperature	25.0°C
Diluent	Water
Refractive index	1.3328
Viscosity	0.8878 cP
Scattering intensity	12157 cps
nm = nanometer $\pm$ = standard deviation cm ² /sec = square position in centimeters per second	
cP = centipoise cps = counts per second	

6.3. APPENDIX C

Table 6.5: Summary of ESI mass fragmentation data.

C	RT	[M-H] ⁻	Fragmentation	Identified	Ref.
Std	36.75	563	-	Theaflavin	-
Std	37.02	715	-	Theaflavin-3-O-gallate	-
Std	37.59	715	-	Theaflavin-3'-O-gallate	-
Std	37.36	867	-	Theaflavin-3,3-di-gallate	-
2	17.27	289	579 [M-H] ⁻ , 245 [M-H-44] ⁻ , 205 [M-H-84] ⁻ and 179 [M-H-110] ⁻	Epicatechin	(Dou <i>et al.</i> 2007)
3	11.38	289	245 [M-H-44] ⁻ , 219 [M-H-70] ⁻ and 179 [M-H-110] ⁻	Catechin	(Gu <i>et al.</i> 2003)
4	5.76	305	261 [M-H-44] ⁻ , 219 [M-H-86] ⁻ , 179 [M-H-126] ⁻ and 125 [M-H-180] ⁻	Epigallocatechin/gallocatechin	(Dou <i>et al.</i> 2007)
5	2.11	609	471 [M-H-138] ⁻ and 125 [M-H-484] ⁻	Theasinensin C and E	(Neilson <i>et al.</i> 2007)
9	25.85	441	883 [M-H+442] ⁻ , 881 [M-H+440] ⁻ , 289 [M-H-152] ⁻ , 169 [M-H-272] ⁻ and 125 [M-H-316] ⁻	Epicatechin gallate/catechin gallate	(Neilson <i>et al.</i> 2007)
10	20.70	457	331 [M-H-126] ⁻ , 305 [M-H-152] ⁻ , 169 [M-H-288] ⁻ , 125 [M-H-332] ⁻ and 915 [2M-H] ⁻	Epigallocatechin gallate/gallocatechin gallate	(Lee <i>et al.</i> 2010, Sang <i>et al.</i> 2007)
11	17.59	915	913 [M-H-2] ⁻ , 457 [M-H-458] ⁻ , 305 [M-H-610] ⁻ , 169 [M-H-746] ⁻ and 125 [M-H-790] ⁻	Assamicain A, B or C	(Hashimoto <i>et al.</i> 1989)
12	17.25	289	579 [M-H+290] ⁻ , 205 [M-H-84] ⁻ and 179 [M-H-110] ⁻	Epicatechin	(Dou <i>et al.</i> 2007)
13	11.05	305	611 [M-H-306] ⁻ , 289 [M-H-16] ⁻ , 219 [M-H-86] ⁻ , 179 [M-H-126] ⁻ and 125 [M-H-180] ⁻	Epigallocatechin	(Dou <i>et al.</i> 2007)
14	5.12	305	611 [M-H-306] ⁻ , 219 [M-H-86] ⁻ , 179 [M-H-126] ⁻ and 125 [M-H-180] ⁻	Gallocatechin	(Dou <i>et al.</i> 2007)
15	2.17	169	125 [M-H-180] ⁻	Gallic acid	(Lee <i>et al.</i> 2010)
20	7.88	609	913 [M-H+304] ⁻ , 563 [M-H-46] ⁻ , 441 [M-H-168] ⁻ and 303 [M-H-306] ⁻	Unknown	-
21	25.76	463	927 [M-H+464] ⁻ , 431 [M-H-32] ⁻ , 401 [M-H-62] ⁻ and 291 [M-H-172] ⁻	Unknown	-
22	26.85	563	-	Unknown	-
24	16.21	289	611 [M-H+233] ⁻ , 579 [M-H+290] ⁻ , 205 [M-H-84] ⁻ and 179 [M-H-110] ⁻	Epicatechin	(Dou <i>et al.</i> 2007)
25	10.33	289	579 [M-H+290] ⁻ , 205 [M-H-84] ⁻ and 179 [M-H-110] ⁻	Catechin	(Gu <i>et al.</i> 2003)
27	4.78	305	179 [M-H-126] ⁻	Gallocatechin	(Dou <i>et al.</i> 2007)
44	28.45	609	563 [M-H-46] ⁻ , 463 [M-H-146] ⁻ and 301 [M-H-308] ⁻	Unknown	-
61	19.76	457	479 [M-H+2] ⁻ , 331 [M-H-126] ⁻ , 305 [M-H-152] ⁻ , 169 [M-H-288] ⁻ and 125 [M-H-332] ⁻	Epigallocatechin gallate/gallocatechin gallate	(Lee <i>et al.</i> 2010, Sang <i>et al.</i> 2007)
62	17.40	915	457 [0.5M-H] ⁻ , 305 [M-H-610] ⁻ , 169 [M-H-746] ⁻ and 125 [M-H-790] ⁻	Assamicain A, B or C	(Hashimoto <i>et al.</i> 1989)(Lee <i>et al.</i> 2010)

66	11.31	305	611 [M-H+306] ⁻ , 219 [M-H-86] ⁻ , 179 [M-H-126] ⁻ and 125 [M-H-180] ⁻	Epigallocatechin	(Dou <i>et al.</i> 2007)
87	12.47	761	-	EGCG-ECG heterodimers	(Neilson <i>et al.</i> 2007)
88	8.07	761	-	EGCG-ECG heterodimers	(Neilson <i>et al.</i> 2007)
98	29.41	739	-	Unknown	-
125	36.57	563	755 [M-H-146] ⁻ , 609 [M-H-2(146)] ⁻ , 301 [M-H-2(146)-308]	Unknown Quercetin conjugate	(Del Rio <i>et al.</i> 2004)
126	34.23	563	-	Theaflavin	Standard
160	28.20	913	-	Theasinensin A or D	(Neilson <i>et al.</i> 2007)
162	14.25	913	<i>m/z</i> 743 [M-H-170] ⁻ , <i>m/z</i> 591 [M-H-322] ⁻ and <i>m/z</i> 573 [M-H-340] ⁻	Theasinensin A	(Neilson <i>et al.</i> 2007)
169	21.92	913	<i>m/z</i> 743 [M-H-170] ⁻ , <i>m/z</i> 591 [M-H-322] ⁻ and <i>m/z</i> 573 [M-H-340] ⁻	Theasinensin D	(Neilson <i>et al.</i> 2007)

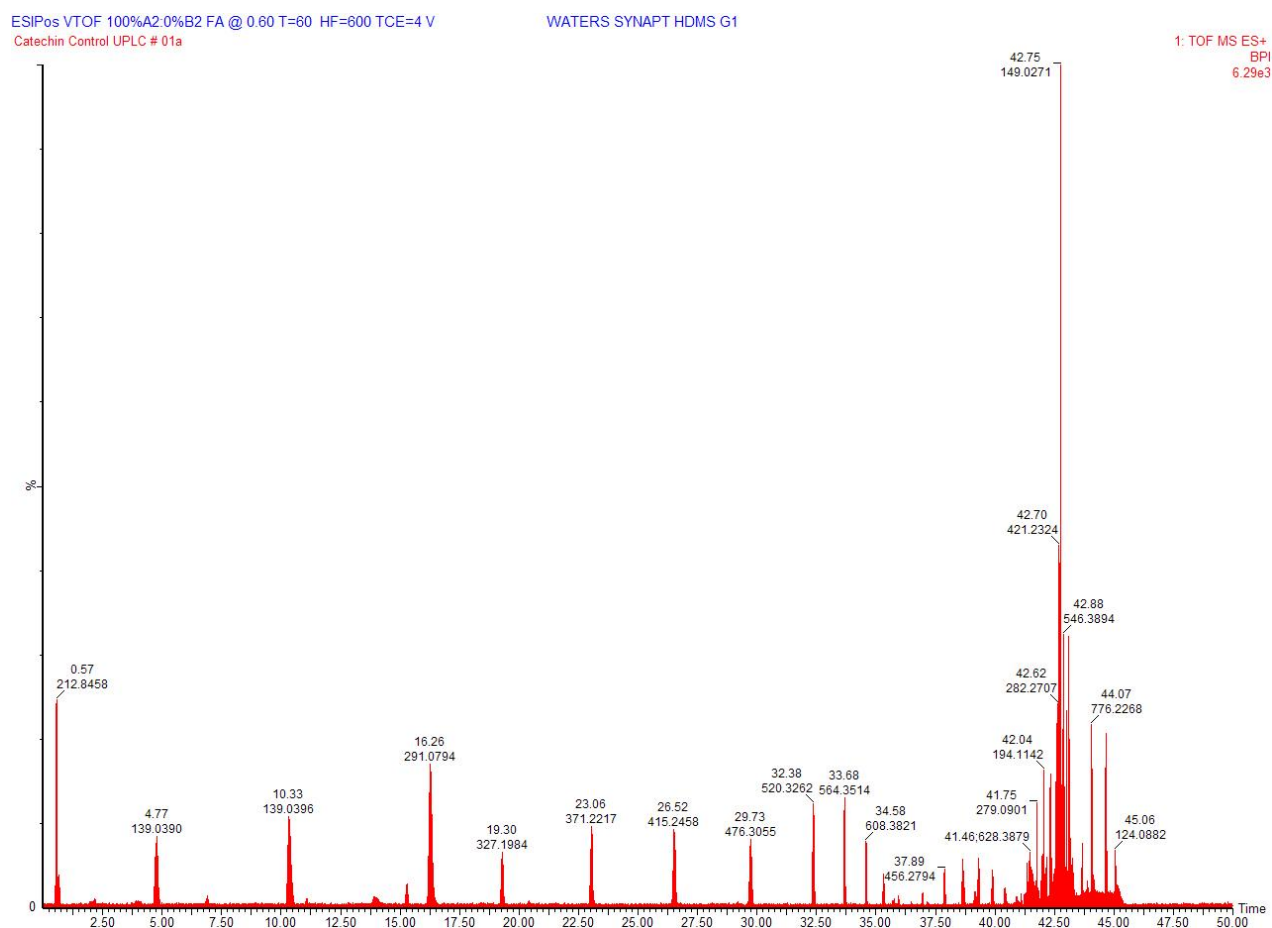


Figure 6.4: Total ion current UPLC chromatogram of the catechin mixture ((-)-EC and EGC) as visualized by the total ion current of the mass spectrometer in the positive ion mode.

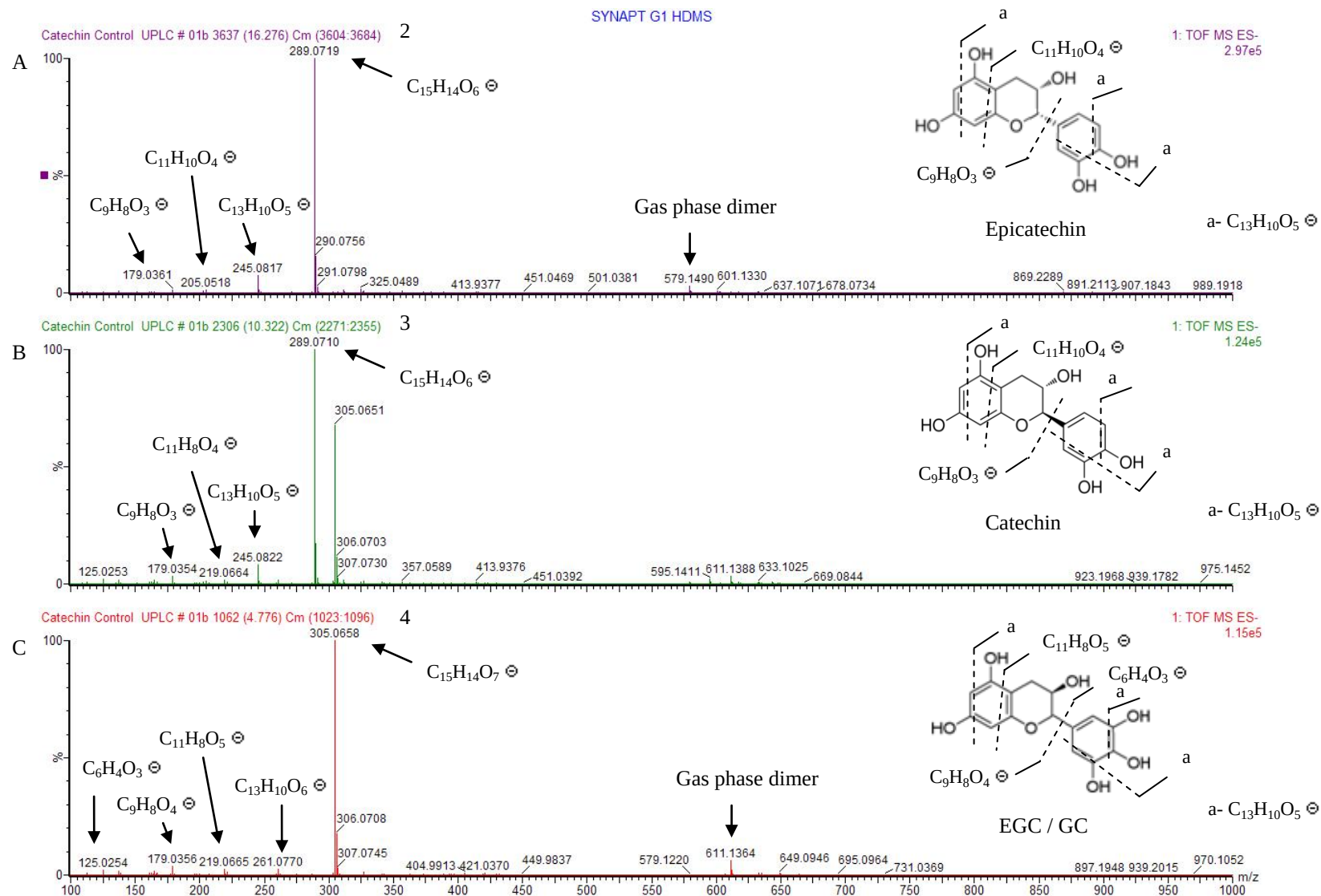


Figure 6.5: ESI mass fragmentation of the ions present in the catechin control sample. A. MS spectra of compound 2, $[M-H]^-$, at m/z 289 (17.27). B. MS spectra of compound 3, $[M-H]^-$, at m/z 289 (11.38). C. MS spectra of compound 4, $[M-H]^-$, at m/z 305 (5.76).

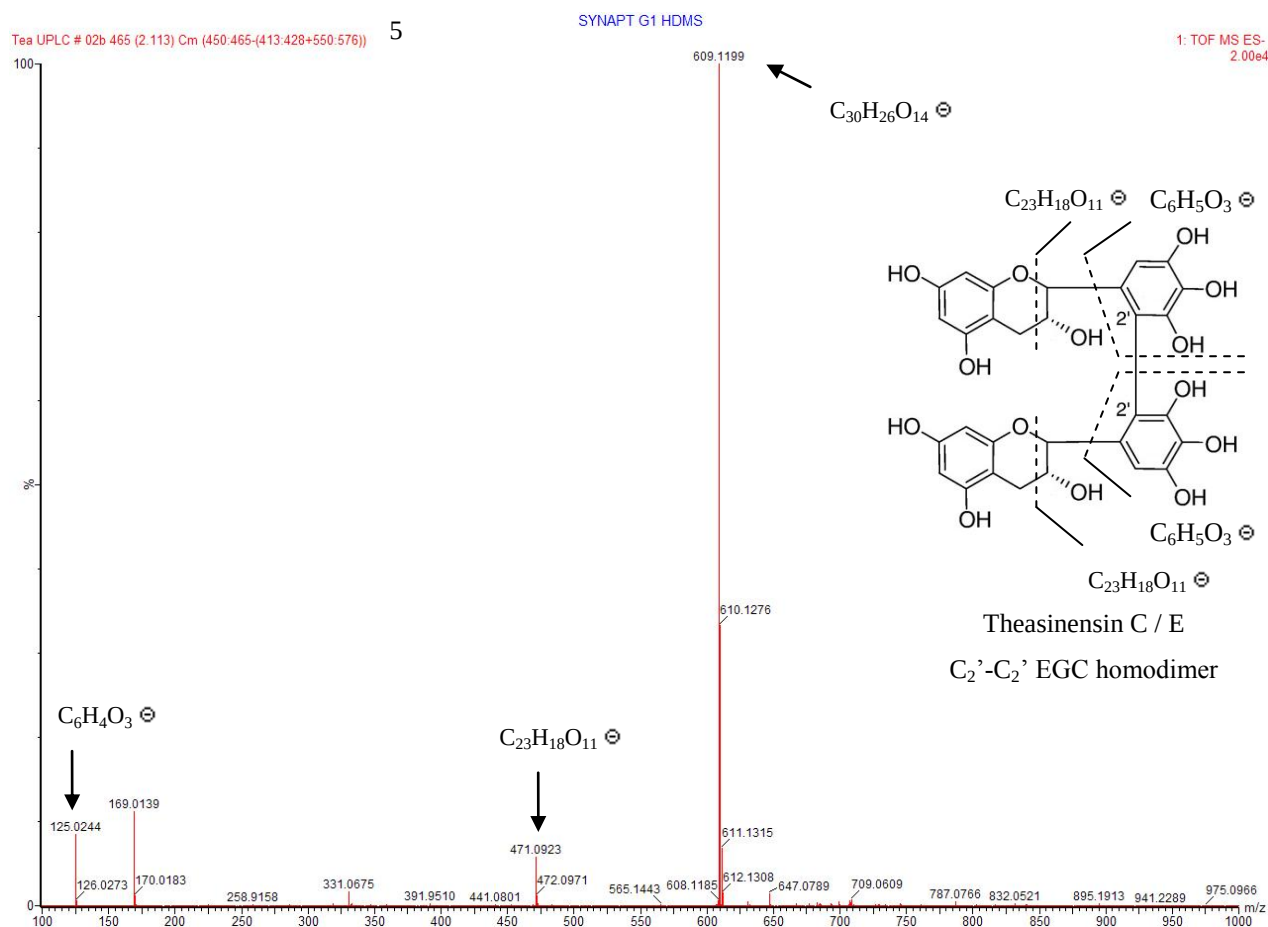


Figure 6.6: ESI mass fragmentation of the ions present in the catechin control sample. MS spectra of compound 5, $[M-H]^-$, at m/z 609 (2.11).

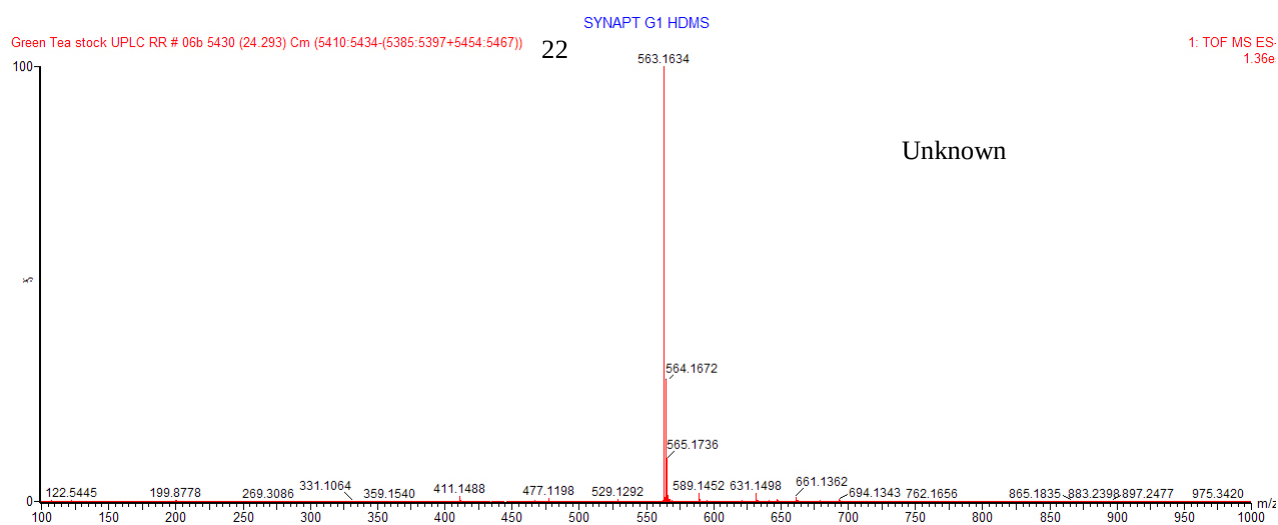


Figure 6.7: A. MS spectra of compound 22, $[M-H]^-$, at m/z 563 (26.85)

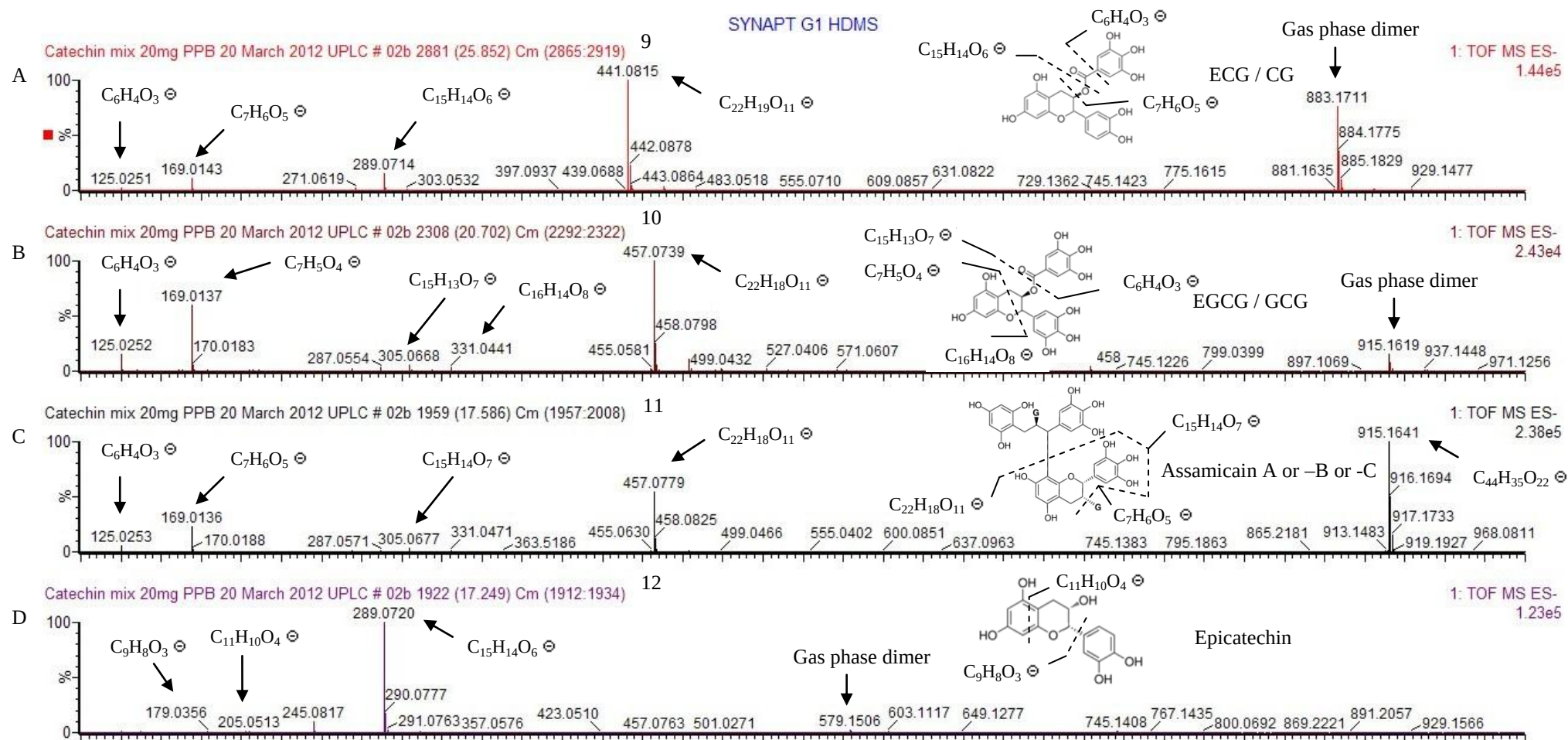


Figure 6.8: ESI mass fragmentation of the ions present in the catechin mixture sample. A. MS spectra of compound 9, $[M-H]^-$, at m/z 883 (25.85). B. MS spectra of compound 10, $[M-H]^-$, at m/z 457 (20.70). C. MS spectra of compound 11, $[M-H]^-$, at m/z 915 (17.59). D. MS spectra of compound 12, $[M-H]^-$, at m/z 289 (17.25).

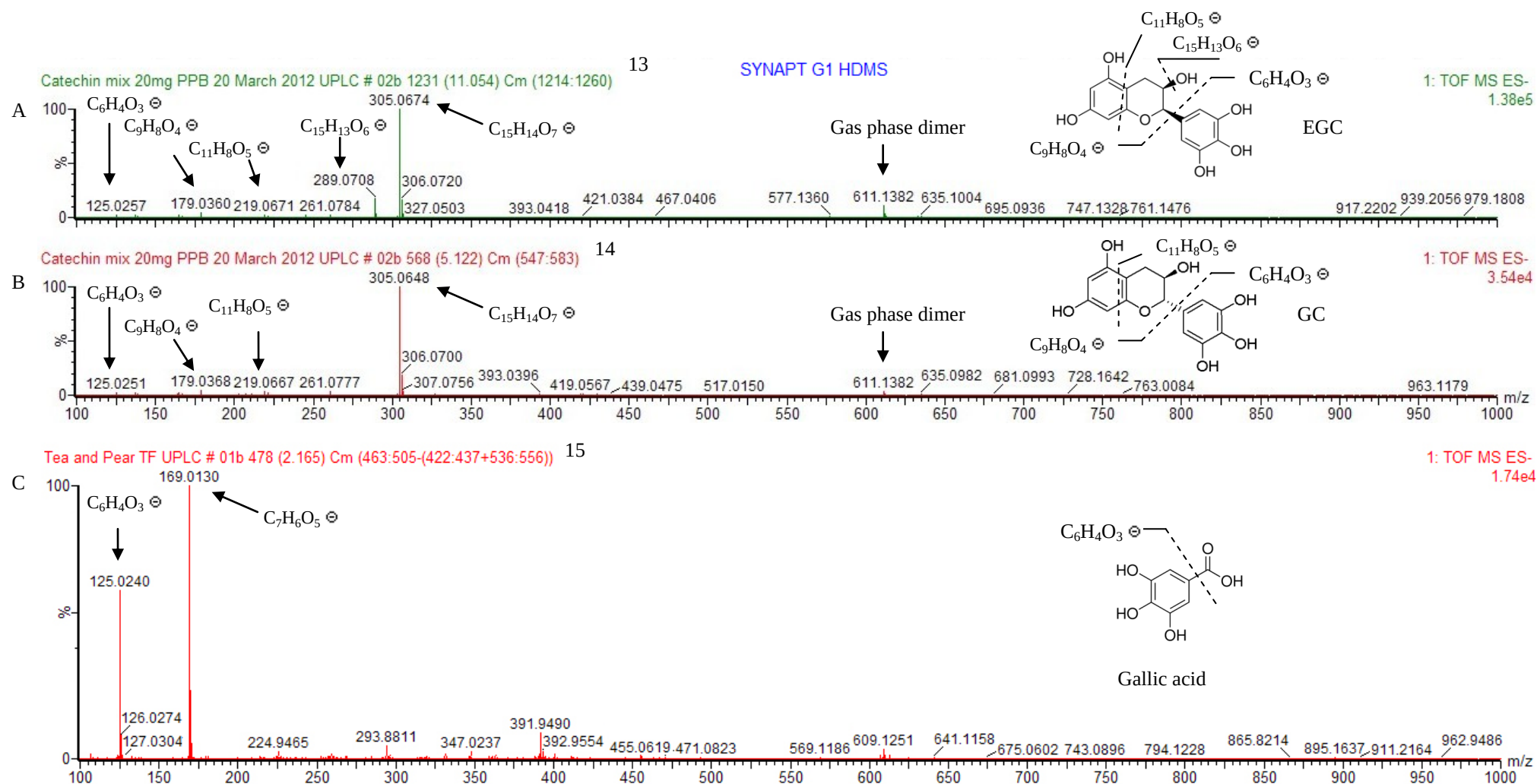


Figure 6.9: A. MS spectra of compound 13, $[M-H]^-$, at m/z 305 (11.05). B. MS spectra of compound 14, $[M-H]^-$, at m/z 305 (5.12). C. MS spectra of compound 15, $[M-H]^-$, at m/z 169 (2.17).

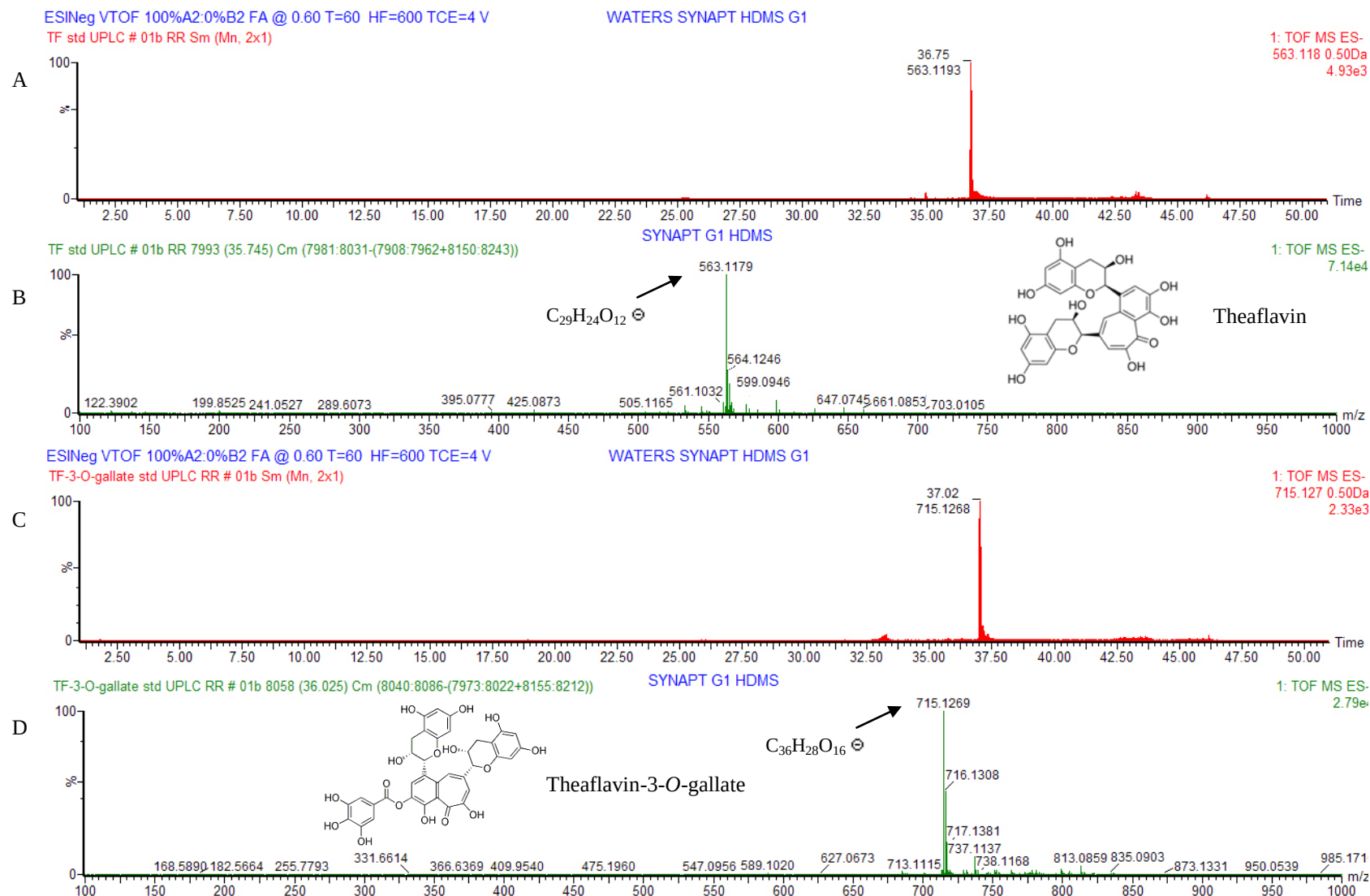


Figure 6.10: UPLC chromatogram and ESI mass fragmentation of the theaflavin and theaflavin-3-O-gallate standards were determined. A. UPLC peak of theaflavin at 563 (36.75). B. MS spectra of theaflavin-3-O-gallate at m/z 563 (35.75). C. UPLC peak of theaflavin at 715 (37.02). D. MS spectra of theaflavin-3-O-gallate at m/z 715 (36.03).

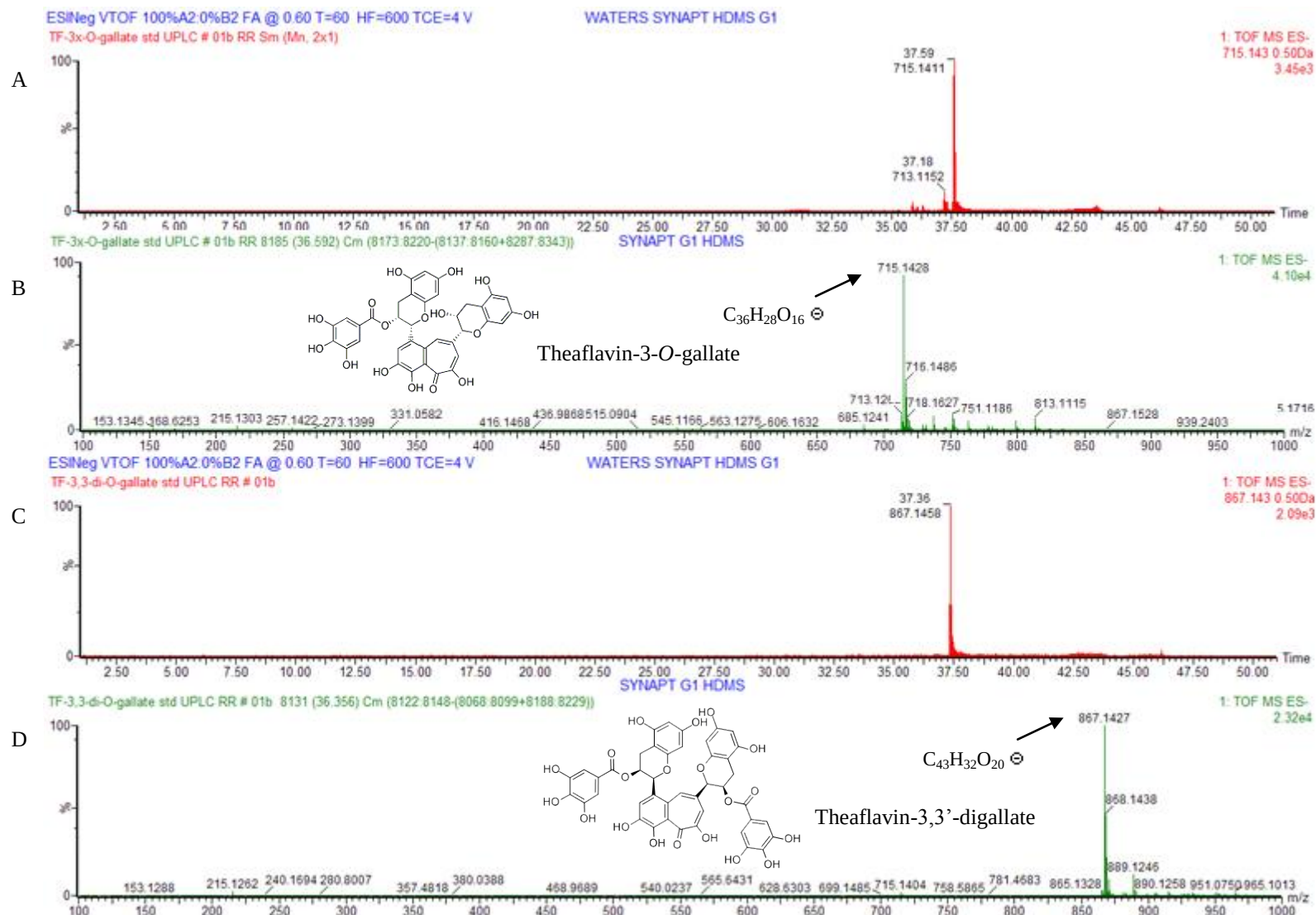


Figure 6.11: UPLC chromatogram and ESI mass fragmentation of the theaflavin-3'-O-gallate and theaflavin-3,3-di-gallate standards were determined. A. UPLC peak of theaflavin-3'-O-gallate at 715 (37.59). B. MS spectra of theaflavin-3'-O-gallate at m/z 715 (36.59). C. UPLC peak of theaflavin-3,3-di-gallate at 867 (37.36). D. MS spectra of theaflavin-3,3-di-gallate at m/z 867 (36.36).

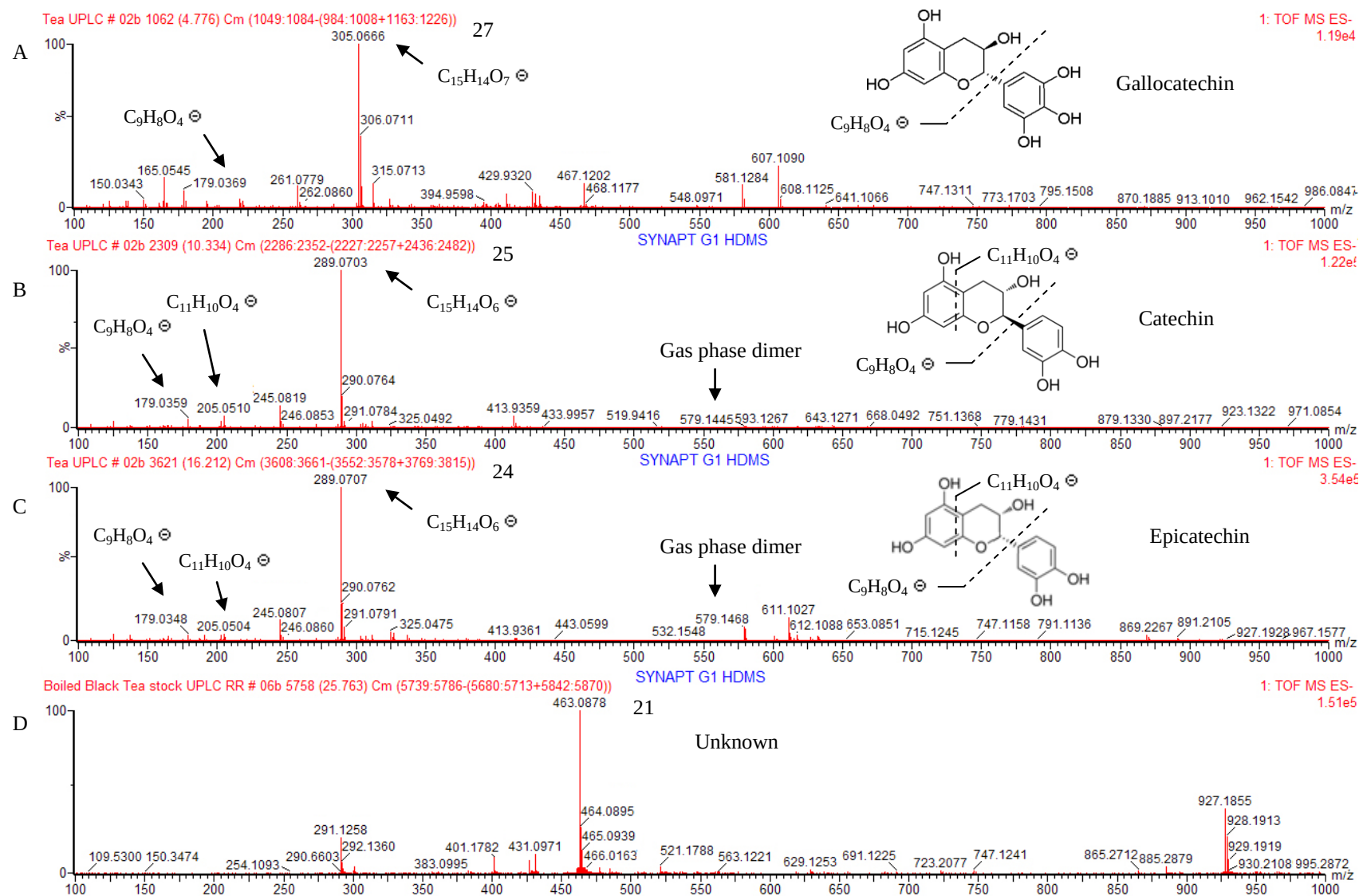


Figure 6.12: A. MS spectra of compound 27, $[M-H]^-$, at m/z 305 (4.78). B. MS spectra of compound 25, $[M-H]^-$, at m/z 289 (10.33). C. MS spectra of compound 24, $[M-H]^-$, at m/z 289 (16.21). D. MS spectra of compound 21, $[M-H]^-$, at m/z 463 (25.76).

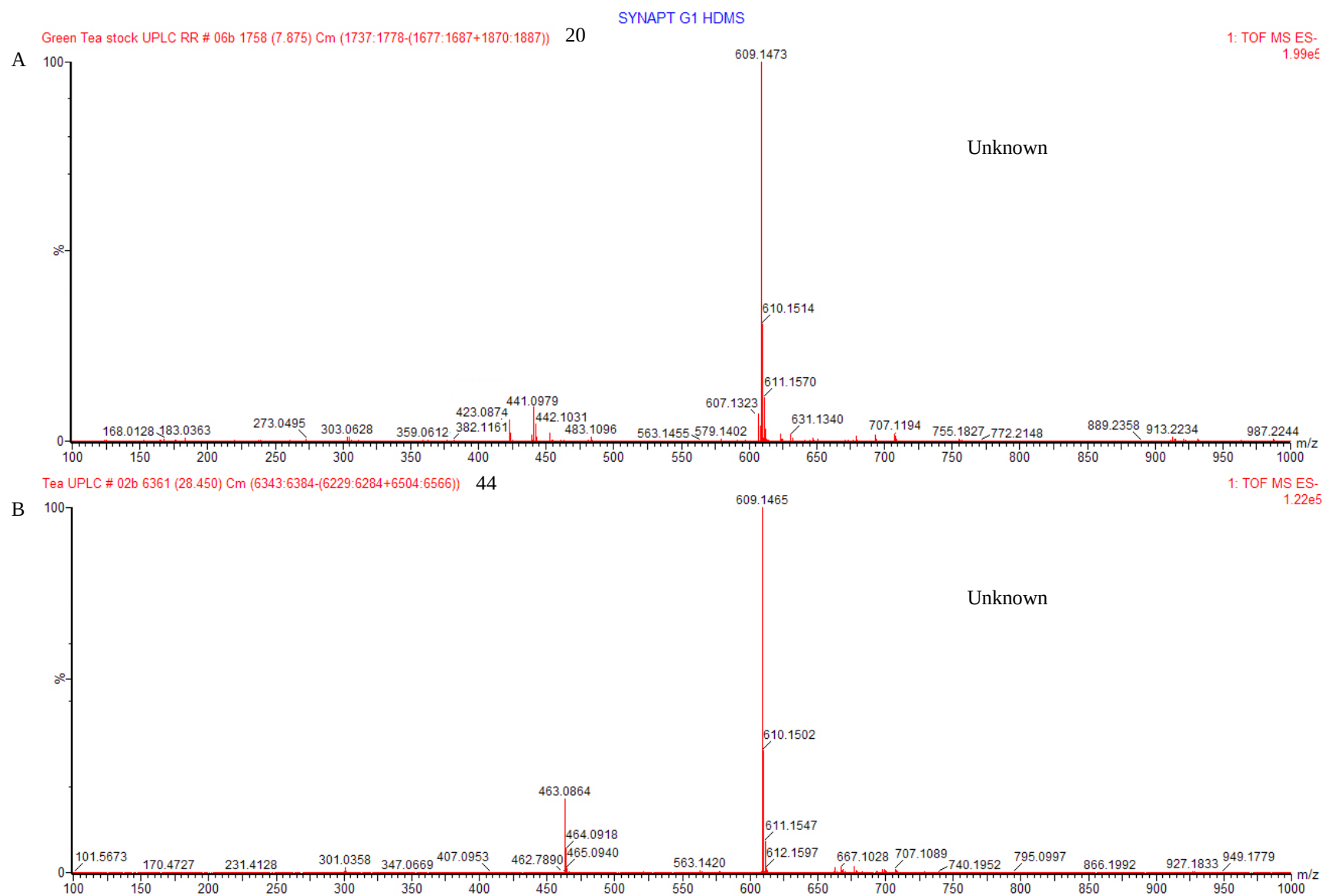


Figure 6.13: A. MS spectra of compound 20, $[M-H]^-$, at m/z 609 (7.88). B. MS spectra of compound 44, $[M-H]^-$, at m/z 609 (28.45).

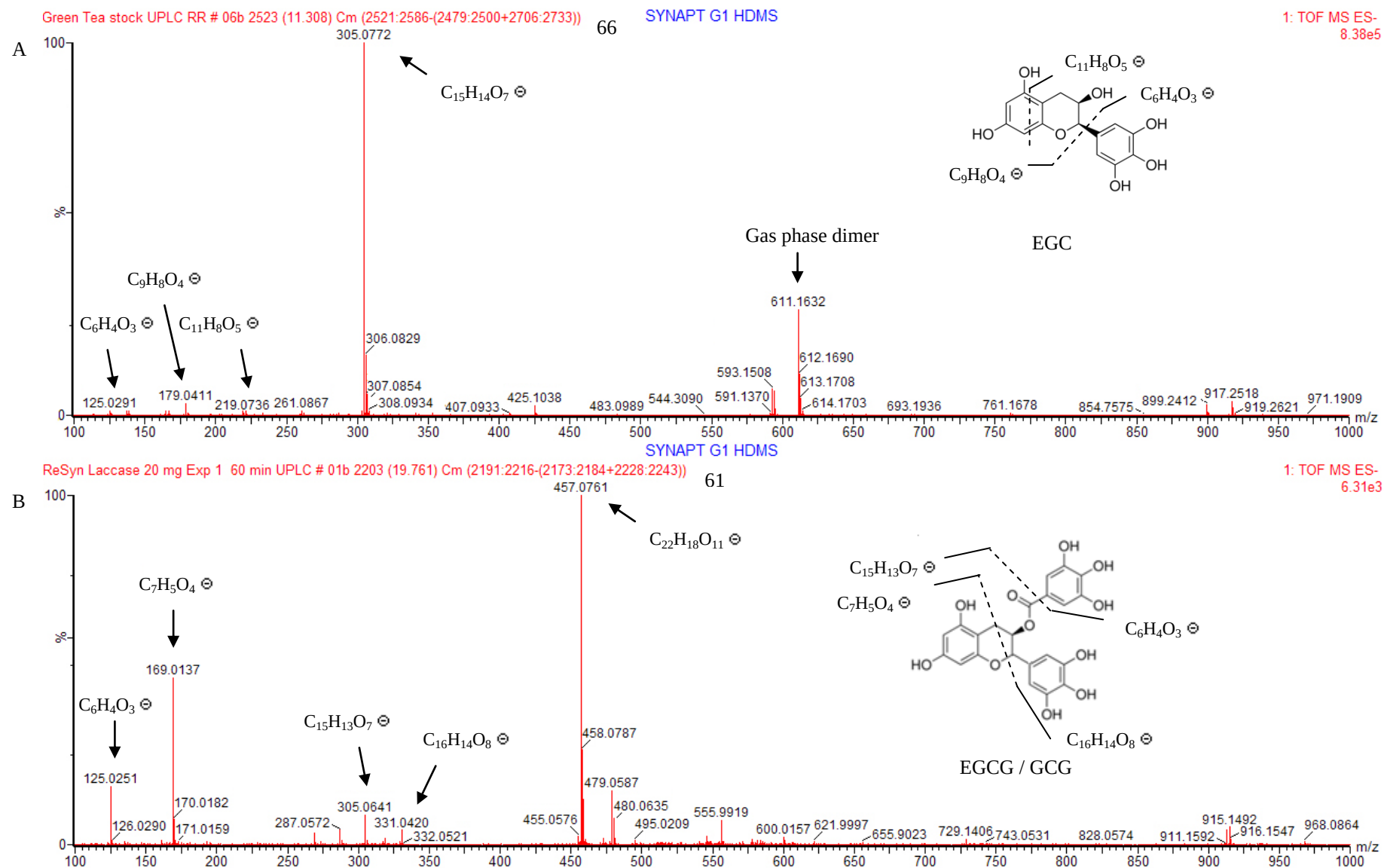


Figure 6.14: A. MS spectra of compound 66, $[M-H]^-$, at m/z 305 (11.31). B. MS spectra of compound 61, $[M-H]^-$, at m/z 457 (19.76).

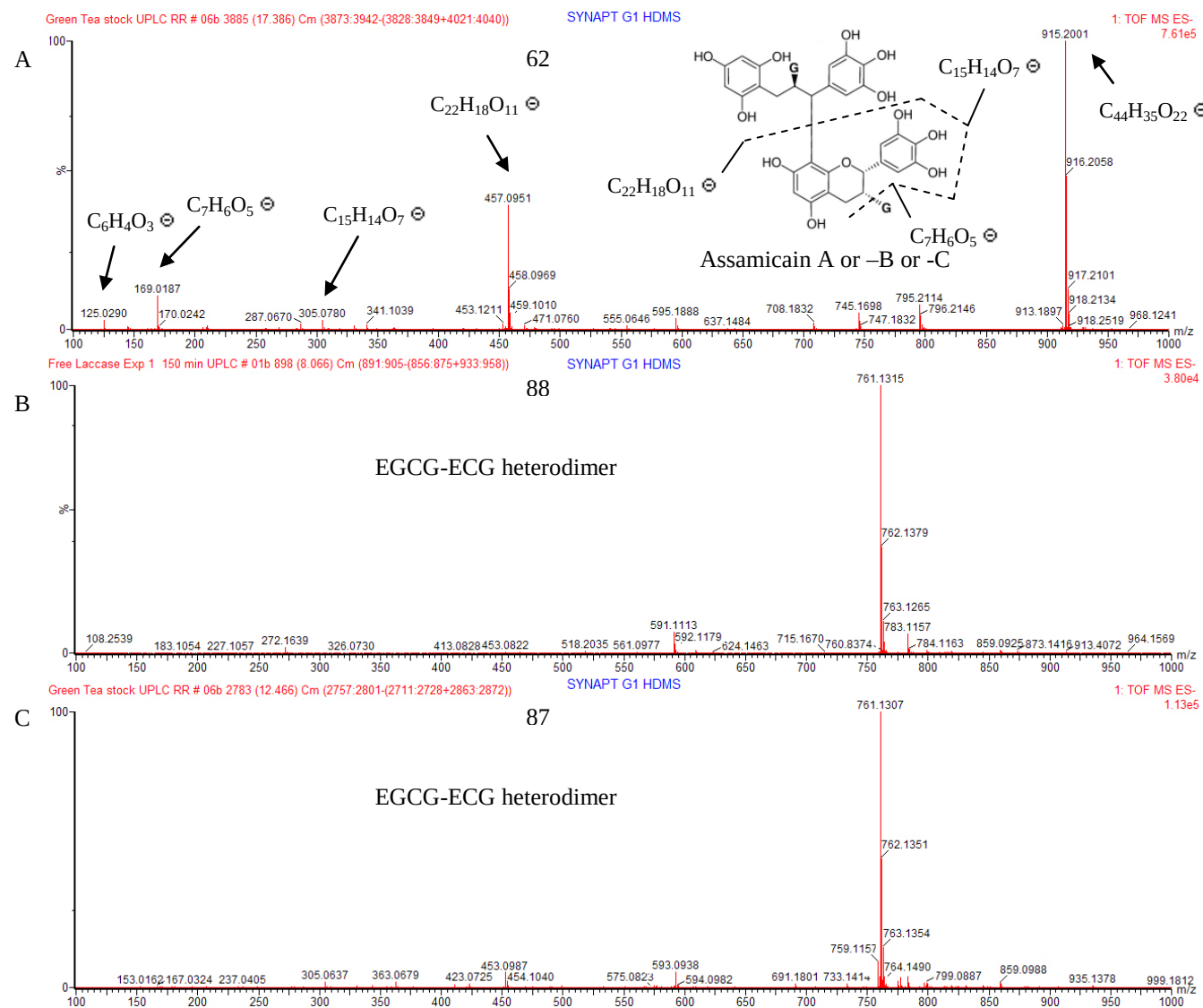


Figure 6.15: A. MS spectra of compound 62, $[M-H]^-$, at m/z 915 (17.40). B. MS spectra of compound 88, $[M-H]^-$, at m/z 761 (8.07). C. MS spectra of compound 87, $[M-H]^-$, at m/z 761 (12.47).

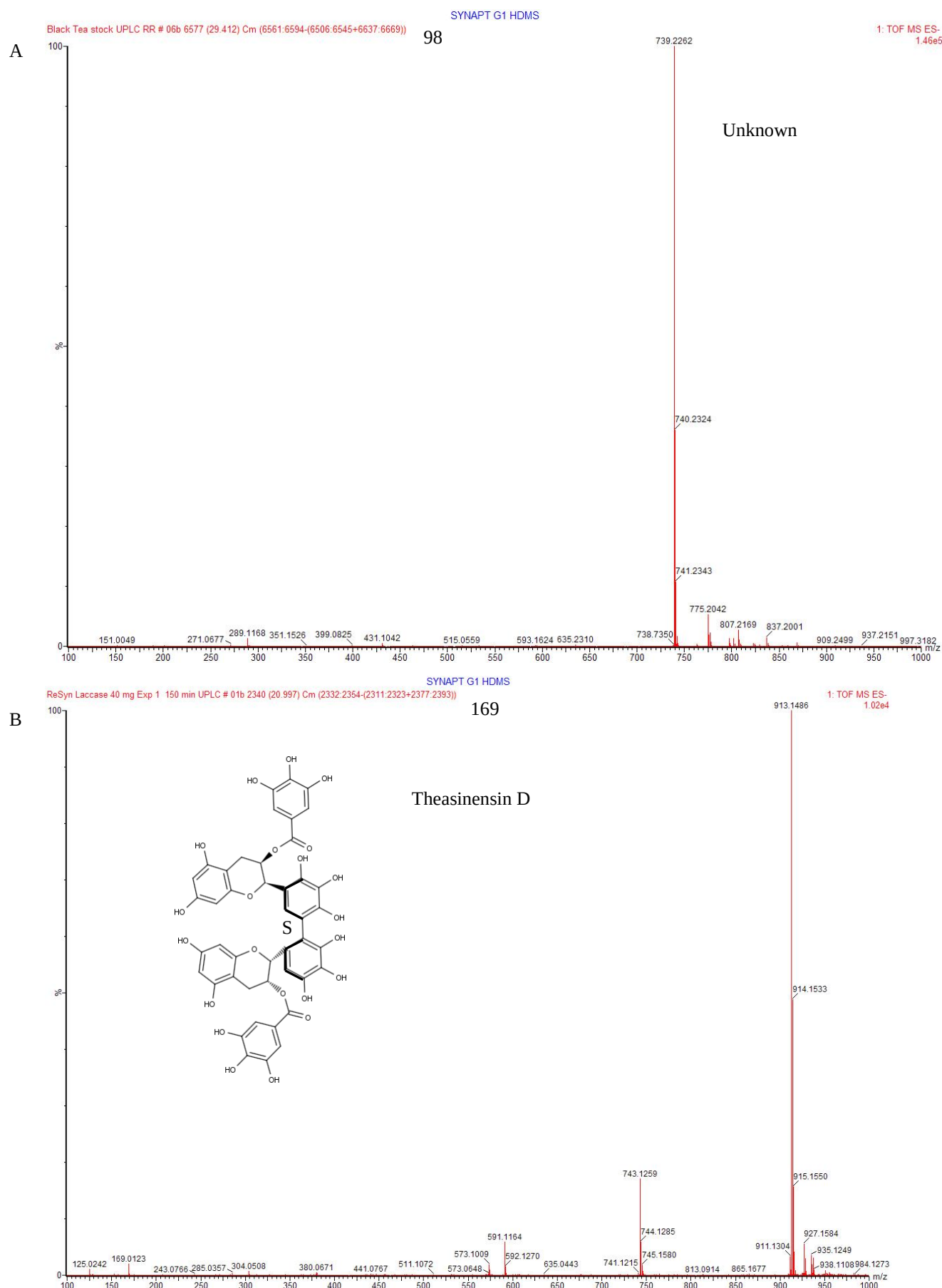


Figure 6.16: A. MS spectra of compound 98, [M-H]⁻, at m/z 739 (30.43). B. MS spectra of compound 169, [M-H]⁻, at m/z 913 (21.00).

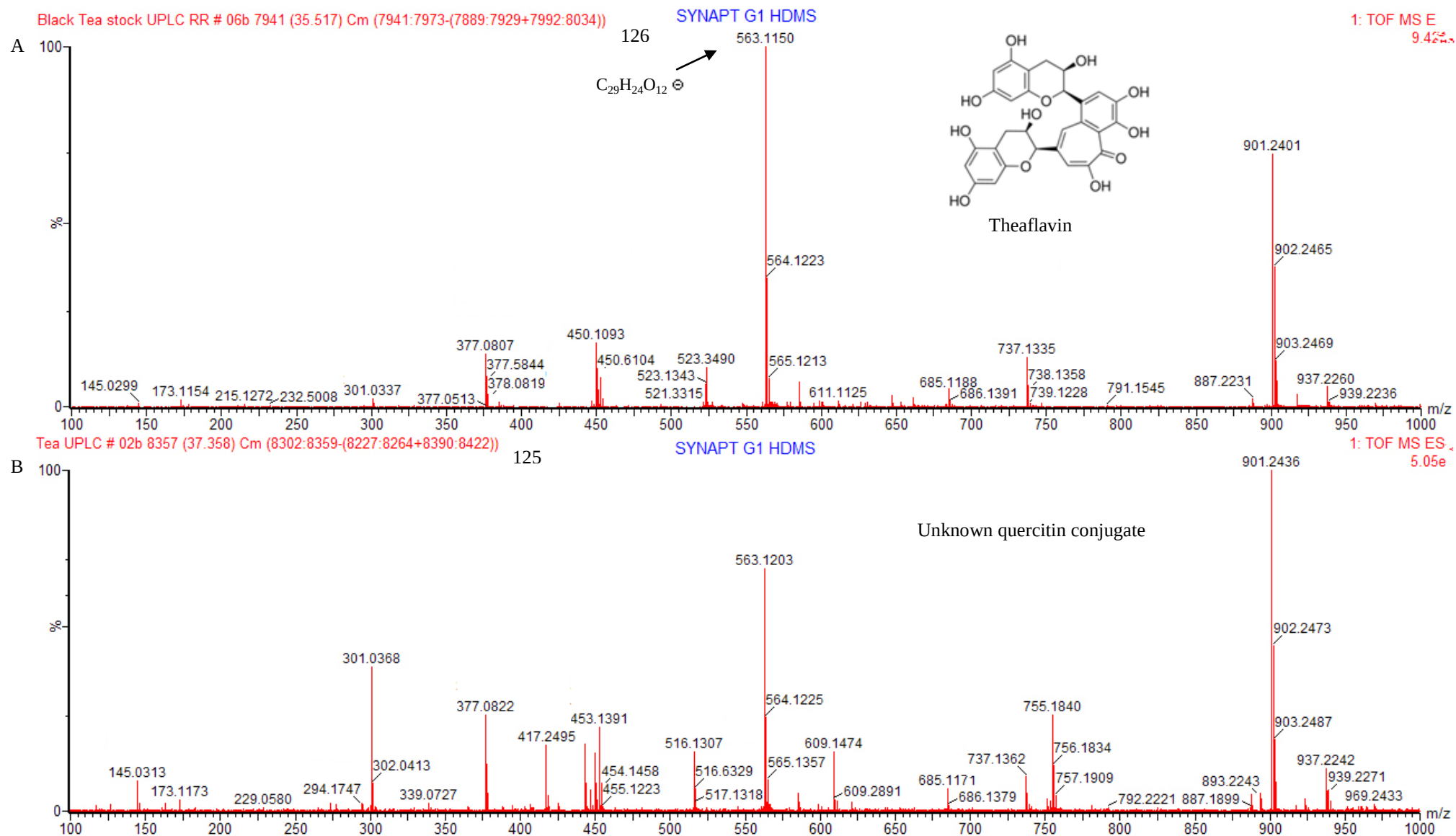


Figure 6.17: A. MS spectra of compound 126, [M-H]⁻, at m/z 563 (35.52). B. MS spectra of compound 125, [M-H]⁻, at m/z 563 (37.36).

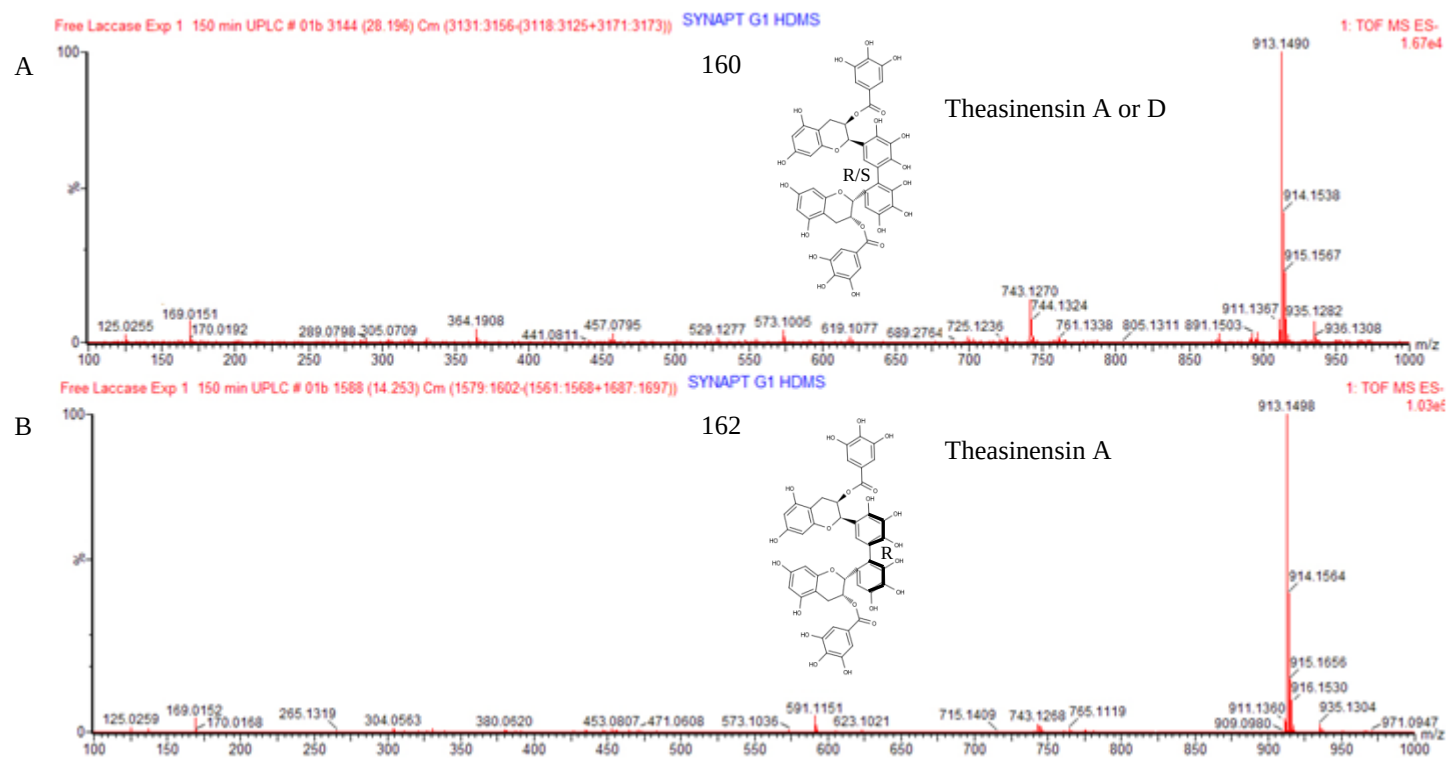


Figure 6.18: A. MS spectra of compound 160, $[M-H]^-$, at m/z 913 (28.20) B. MS spectra of compound 162, $[M-H]^-$, at m/z 913 (14.25).

Table 6.6: Elemental composition report of peaks and peak fragments; 126, 102, 102 m/z 563 [M-H]⁻, 146, 146 m/z 641 [M-H+78]⁻, 147, 147 m/z 641 [M-H+78]⁻, 148, 11 and 162.

Peak	RT	Mass	Calc. Mass	mDa	PPM	DBE	i-Fit (Norm)	Formula	Figure
126, m/z 563 [M-H] ⁻	35.53	563.1152	563.1190	-3.8	-6.7	18.5	0.0	C ₂₉ H ₂₃ O ₁₂	4.15 A
102, m/z 563 [M-H] ⁻	23.95	563.1352	563.1401	-4.9	-8.7	13.5	0.0	C ₂₆ H ₂₇ O ₁₄	4.15 B
102, m/z 613[M-H+50] ⁻	23.95	613.1138	613.1193	-5.5	-9.0	17.5	0.0	C ₂₉ H ₂₅ O ₁₅	4.15 B
146, m/z 563 [M-H] ⁻	26.03	563.1398	563.1401	-0.3	-0.5	13.5	0.0	C ₂₆ H ₂₇ O ₁₄	4.15 C
146, m/z 641 [M-H+78] ⁻	26.03	641.1351	641.1354	-0.3	-0.5	13.5	0.0	C ₂₇ H ₂₉ O ₁₈	4.15 C
147, m/z 563 [M-H] ⁻	26.00	563.1396	563.1401	-0.5	-0.9	13.5	0.0	C ₂₆ H ₂₇ O ₁₄	4.15 D
147, m/z 641 [M-H+78] ⁻	26.00	641.1356	641.1354	0.2	0.3	13.5	0.0	C ₂₇ H ₂₉ O ₁₈	4.15 D
148, m/z 563 [M-H] ⁻	25.98	563.1416	563.1401	1.5	2.7	13.5	0.0	C ₂₆ H ₂₇ O ₁₄	4.15 E
11, m/z 915 [M-H] ⁻	16.76	915.1636	915.1620	1.6	1.7	27.5	0.1	C ₄₄ H ₃₅ O ₂₂	4.21
162, m/z 913 [M-H] ⁻	14.25	913.1500	913.1463	3.7	4.1	28.5	0.0	C ₄₄ H ₃₃ O ₂₂	6.18 B
Elemental composition report; single mass analysis; tolerance = 5.0 mDa/DBE: min = 0.0, max = 50.0; monoisotopic mass, even electron ions; respectively 55-, 55-, 129-, 114-, 132-, 114-, 132, 114-, 312 and 323 formula(e) evaluated with respectively 1-, 1-, 5-, 2-, 4-, 2-, 2-, 3- and 6 results within limits (respectively up to 10 closest results and up to 10 best isotopic matches for each mass). RT = Retention time (min) Calc. = calculated mDa = milliDalton PPM = parts-per-million DBE = double bond equivalents i-Fit = isotope-ratio fit									

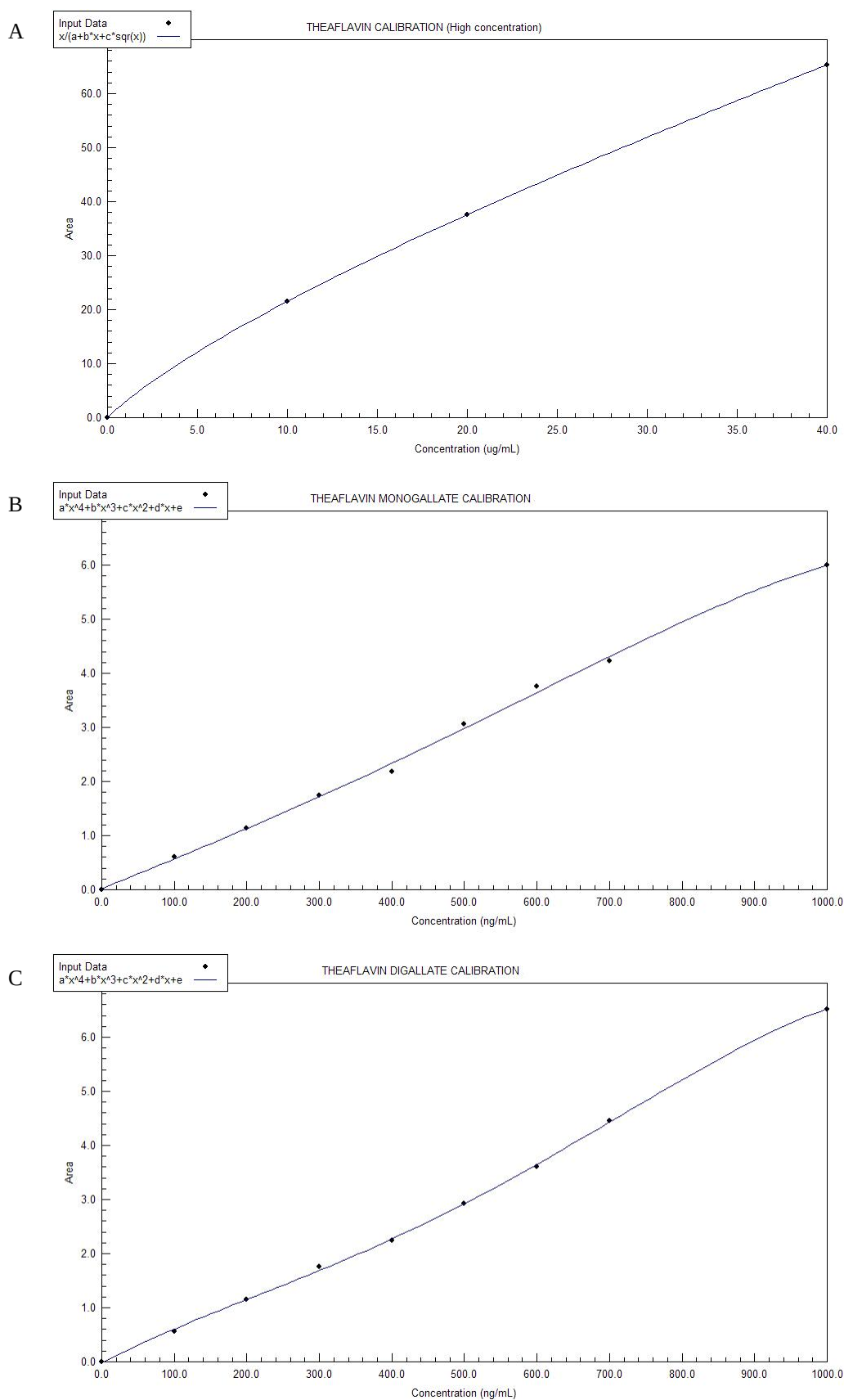


Figure 6.19: Peak quantification standard curves ($R^2 \geq 0.999$) of the standards; A. Theaflavin, B. Theaflavin-monogallate and C. theaflavin-digallate.

Table 6.7: *In vitro* modelling of fermentation with free- and ReSyn™ immobilized laccase.

ReSyn™ immobilized laccase

	Theaflavin <i>m/z</i> 563 (38.21) ¹⁷²	Theaflavin-monogallate <i>m/z</i> 715 (38.80) ¹⁷¹	Theaflavin-digallate <i>m/z</i> 867 (39.29) ¹⁷⁰
Time	(ng/ml)	(ng/ml)	(ng/ml)
0	212.1	525.2	252.5
30	141.4	242.4	101.5
60	127.2	404.0	111.1
90	162.6	515.1	181.8
120	190.9	747.4	353.4
150	120.2	707.1	414.1

Free laccase

	Theaflavin <i>m/z</i> 563 (38.21) ¹⁷²	Theaflavin-monogallate <i>m/z</i> 715 (38.80) ¹⁷¹	Theaflavin-digallate <i>m/z</i> 867 (39.29) ¹⁷⁰
Time	(ng/ml)	(ng/ml)	(ng/ml)
0	155.5	222.2	160.6
30	198.0	401.0	198.6
60	183.8	484.2	293.0
90	254.5	626.3	329.2
120	226.2	787.8	495.0
150	282.8	998.4	637.0

Lipton® Yellow label black tea
 Theaflavin, *m/z* 563 (34.23)^{126*} 20500 ng/ml
 ng/ml = nanogram per milliliter

CHAPTER 7 : REFERENCES

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