ALTERNATIVE PATHWAY IMPLICATED AS AN INFLUENCING FACTOR IN THE SYNTHESIS OF THEAFLAVIN

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Abstract

The principal pigments present in black tea, theaflavins, have been indicated to be of potential clinical significance in various fields of research which has been hampered by the very low levels of theaflavins from black tea extractions, being the original method employed to acquire theaflavins. Forelle pear (44 µM Theaflavin/g dry weight/hr) and Yacon leaf (65 µM Theaflavin/g dry weight/hr) homogenates were tested for their theaflavin synthesis capacity and found to have a larger theaflavin synthesis capacity than a green tea leaf homogenate (26 µM Theaflavin/g dry weight/hr) based upon the flavognost method. In an incubation system of green tea leaf extract utilizing endogenous enzymes present in Forelle pear and Yacon homogenates to synthesise theaflavin, the formation of an unknown peak (*m/z* 563.1349; $(23.95)^5$; $C_{26}H_{28}O_{14}$) was detected by mass spectrometry with a molecular mass similar to theaflavin. This is in contrast to theaflavin being solely synthesised in an *in vitro* model incubation system using isolated catechins and purified Forelle pear polyphenol oxidase. The preferential formation of the unknown compound could explain the low levels of theaflavins in black tea.

Keywords Bioconversion · Polyphenol oxidase (PPO) · Theaflavins · Forelle pear · Yacon

1. Introduction

Tea is one of the most widely consumed beverages in the world. Tea can be grouped into three major categories: the nonfermented green teas, the partially fermented oolong and paochong teas, and the fully fermented black and pu-erh (red) based on the extent of fermentation [21]. Catechins; (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate are the major components present in green tea leaves, and can constitute up to 30% of the dry weight (DW) of young tea leaves [6]. In a review article by Ananingsih et al [2] the chemical changes which catechins may experience such as degradation, epimerization and polymerization as well as oxidation are described. Factors such as temperature, pH, metal ions, ingredients added as well as oxygen availability causing these changes in the catechins in green tea are described. During the fermentation stage in the manufacturing of black tea, monomeric flavan-3-ols (catechins) undergo polyphenol oxidase (PPO)-dependent oxidative polymerization producing various quinones that go through condensation reactions, resulting in dimeric compounds known as theaflavins (TF). Peroxidase utilizes hydrogen peroxide generated by PPO during the oxidation of catechins, and catalyses the oxidation of the products of the PPO catalysed reactions, amounting to the conversion of formed theaflavins into thearubigins [33].

The principal pigments present in black tea, theaflavins (theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate) possess a benzotropolone ring with di- or trihydroxy substitutions. Interest in theaflavins mainly arises from their potential benefit as natural dietary antioxidants. Furthermore, theaflavins have been implicated to have antiviral- [7], anticancer- [20], antimutagenic-, anticlastogenic- [13], antibacterial activity [25], antiobesity and lipid lowering effects [17] and even inhibit HIV-1 infection [42]. Despite their potential clinical significance in various fields, research on theaflavins has been hampered due to their unavailability, to such an extent that most previous research was focused on the use of theaflavins mixtures [29].

The original method employed to acquire theaflavins was the extraction of theaflavins from black tea, but the high cost associated with the low content and difficulty of recovering theaflavins from black tea, resulted in an industrially unfeasible approach [40]. From the 15-30% dry weight of catechins present in green tea shoots, only 0.4-1.9% dry weight of theaflavins are formed during black tea manufacture [29, 31]. For this reason, various approaches have been employed for the synthesis of theaflavins from substrate materials, including fresh tea leaves [34], tea leaf juice, processed green tea leaves, green tea slurry [11], liquid green tea extract [16] and *in vitro* modelling of fermentation with purified catechins [27]. These approaches included the utilization of potassium ferricyanide as a catalyst in chemical oxidation along with enzymatic oxidation [33, 39]. Enzymatic

oxidation approaches entail the exploitation of endogenous enzymes in liquid plant extracts or crude tea leaves containing PPO [27, 33], peroxidase-containing cultured plant cells such as cultured tea cells [34] and homogenates of various fruits [36]. Many fruit homogenates are capable of synthesising theaflavins, and 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. This is based on strong enzymatic activity with practically no oxidation products of their own detected during HPLC analysis [37, 38]. Japanese pear and loquat homogenates were reported to have a much larger theaflavin synthesis capacity in comparison to fresh tea leaves and were also the highest among 62 plants belonging to 49 families [38].

Investigations into pear PPO were mainly associated with the browning of puree, fresh-cut and juice of pear fruits [1, 12, 15]. PPO has been partially purified from Blanquilla pear [9] and purified from the pear varieties Ankara Armutu pear [44], d'Anjou pear [41], La France pear [3], Bartlett pear [8] and Williams pear [26]. The aim of the current study was to investigate the use of endogenous enzymes present in the homogenates of various pear varieties as well as endogenous enzymes from Yacon leaves to increase the synthesis of theaflavin starting from green tea. The study further aimed to purify the PPO from the identified source, and employing it in the increased synthesis of theaflavins in an *in vitro* model incubation system. To the best knowledge of the authors, no other research has been done on Yacon leaves as a source of PPO for conversion of catechins to theaflavins.

2. Materials and methods

2.1 Materials

Commercially available Lipton® Green tea and Lipton® Yellow label black tea were purchased from a local supermarket and ground to a fine powder. Tea (*Camellia sinensis*, leaves) and Yacon leaves (*Smallanthus sonchifolius*) were obtained from the experimental farm of the University of Pretoria, South Africa and stored at 4ºC until use. Fresh pear varieties, Forelle (class 1), Packman, Abet Beteil and Beurré Bosc, were obtained from a local fruit store and stored at 4ºC until use. Albumin Fraktion V (≥ 98% pulv. bovine) was purchased from Roth, Karlsruhe, Germany and the protein assay dye reagent concentrate was purchased from Biorad, California, United States. 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 a generous gift from Prof. Y. Hara from Tea Solutions, Hara Office Inc. Tokyo, Japan. The standards were also purchased from Sigma Aldrich (Pty) Ltd. Leucine enkephalin used in the UPLC-MS analysis was of pure LC-MS grade and purchased from ERA (Waters), Manshester, UK. The pure solvents; formic acid and acetonitrile were purchased from Honeywell, Burdick & Jackson, Muskegon, United States. Pure water was generated from a Millipore Elix 5 RO system and Millipore Advantage A10 Milli-Q system (Millipore SAS, Molsheim, France) as needed. 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. All other research chemicals were purchased from Sigma Aldrich (Pty) Ltd., St. Louis, United States unless otherwise stated.

2.2 Methods

2.2.1 Theaflavin synthesis capacity of different pear homogenates

One fruit of the different pear (*Pyrus communis*) varieties (Forelle 108.2 g ± 1.148 SD; Packman 146.5 g ± 2.209 SD; Abet Beteil 154.8 g ± 6.296 SD and Beurré Bosc 229.1 g ± 6.897 SD) were homogenized separately in 100 ml 0.1 M phosphate buffer (pH 5.9) in a household blender. Samples of the different varieties were added separately to ground Lipton® Green tea (1:1 *v/v* of 2% *w/v* stock solution in buffer) and stirred for an hour at room temperature using a magnetic stirrer. The samples were then placed in a water bath at 90 ºC for 10 minutes and subsequently centrifuged at 3,000 x g for 10 minutes. The theaflavin content of the supernatants was determined using the flavognost method [14].

2.2.2 Flavognost method

The organic solvent isobutyl methyl ketone (IBMK) was added to the supernatants produced in section 2.2.1 (1:1 *v/v*) and the theaflavins were extracted into the organic layer by vortexing four times 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* of 0.04 M diphenyl boric acid-2-amino ethyl ester made up in 96% ethanol) was added and vortexed. The complete development of the green theaflavin – flavognost reagent complex was achieved through a 12 ±1 minute incubation at 37 ±2 ºC. The green complex was spectrophotometrically measured at 625 nm against an IBMK/EtOH (1:2 *v/v*) blank. The theaflavin content was calculated on a dry weight basis with the formula:

$$\text{Theaflavin } (\mu M/g) = E_{625} \times \frac{47.9}{(DW/100)} \qquad [28]$$

where E_{625} is the absorbance at 625 nm, 47.9 is the micromolar absorbance coefficient of the theaflavin-flavognost reagent complex at 625 nm and DW is the fresh weight of the sample.

2.2.3 Bioconversion of catechins by Forelle pear and Yacon homogenates

The substrates, ground Lipton® Green tea (2 g) and Tea leaves (4 g) were separately homogenized in a blender in 100 ml potassium phosphate buffer (0.1 M, pH 5.9) for 2 minutes, with Yacon (*Smallanthus sonchifolius*) leaves and Forelle pear (cored) 2 g of each respectively, as enzyme sources. The samples were stirred for an hour at room temperature using magnetic stirrers. Samples were then filtered through muslin, placed in a water bath at 90 °C for 15 minutes and subsequently centrifuged at 3,000 x g for 10 minutes. Control samples of Tea, Yacon and Forelle pear (2 g) were prepared in the same manner but were placed in a water bath at 90 °C for 15 minutes directly after homogenation. The theaflavin content of the supernatants was determined using the flavognost method and UPLC-MS analysis.

2.2.4 UPLC-MS

Chromatographic separations of samples were done utilizing a Waters HSS T3 column (150 x 2.1 mm, 1.8 µm) and the temperature controlled at 65 ºC. 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, 0% eluent B at a 0.6 ml/min flow rate with a linear gradient to 13% eluent B at 31 minutes was employed. The conditions were changed to 21% eluent B at 41 minutes. From 43 to 45 minutes, the column was flushed with 100% eluent B and subsequently changed to initial conditions. The total run time was 50 minutes and depending on the concentration of the compounds of interest, the injection volume ranged between 1 to 5 µl. The photodiode array (PDA) detector 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. The reference calibrant, leucine enkephalin (50 ng/L) was used to obtain typical mass accuracies between 1 and 3 mDalton. 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 Dalton mass range. The source temperature was at 120 ºC, and the desolvation temperature was set at 450 ºC. Nitrogen gas was used as the nebulization at a flow rate of 800 l/hour. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.1 (SCN 704).

2.2.5 Extraction of crude enzyme from Forelle pear

A crude extract was prepared from frozen pear tissue (10 g) by homogenization in a household blender for 2 minutes 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.

2.2.6 Purification of PPO by affinity chromatography

A Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column was prepared as described by Arslan [4]. This entailed the initial activation of hydroxyl groups of Sepharose-4B gel by addition of cyanogen bromide. The gel was then washed with 0.1M sodium bicarbonate buffer pH 10. This was followed by coupling of the L-tyrosine to the cyanogen bromide activated gel over 16 hours at 4°C. Cold sodium nitrite (0.07M) was then added drop-wise to *p*-aminobenzoic acid (0.02M made up in HCl 0.1M) and the reaction was completed by stirring for 10 minutes. This solution was then added to 40ml of the Sepharose-4B-L-tyrosine suspension at pH 9.5 and stirred for 3 hours at room temperature. The gel was washed with water and then with disodium hydrogen orthophosphate pH 6 and packed into a column. 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. PPO was eluted with sodium phosphate buffer (5 mM; pH 8.5) containing 1 M NaCl. The PPO enzyme activity and protein concentration of the different fractions were determined.

2.2.7 PPO enzyme activity assay and protein concentration determination

PPO 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 reaction. One enzyme unit was defined as the amount of enzyme resulting in a 0.001 increase per minute in absorbance at 410 nm at 25 ºC. The protein concentration was determined with the Bradford assay with bovine serum albumin as standard [5].

2.2.8 Silver stained SDS-PAGE and native gel electrophoresis

A 12% SDS-PAGE gel was prepared as described by Laemmli [19] and an unstained protein molecular weight marker from Fermentas, Burlington, Canada was used. The silver stain kit, ProteoSilver^{TM} was used for staining of the SDS-PAGE gel. A 7% PAGE gel was prepared as described by Laemmli [19] and native gel electrophoresis was performed as described by Wissemann and Montgomery [41]. 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 slab gels were rinsed for 5 minutes with 1 mM ascorbic acid and soaked in water overnight.

2.2.9 Effect of pH on the activity and stability of PPO

The pH optimum of PPO 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 PPO was determined as adapted from Zhou *et al*. [43]. Enzyme reaction solutions of 45 µl enzyme in 810 µl of the various buffer solutions, ranging from pH 3 to pH 10 were incubated for 30 minutes at 30 ºC. Following the incubation period, the solutions were placed on ice and the residual enzyme activity was determined as described above.

2.2.10 Effect of temperature on the activity and stability of PPO

The temperature optimum of PPO was determined over the temperature range 25 ºC to 70 ºC 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 PPO activity was determined as described above. The effect of temperature on the stability of PPO was determined as adapted from Zhou *et al*. [43]. Enzyme reaction solutions of 45 µl enzyme in 810 µl citrate buffer (0.5 M, pH 4) were incubated for 30 minutes at the various temperatures. Following the incubation period, the solutions were placed on ice and the residual enzyme activity was determined as described above.

2.2.11 Bioconversion of catechins by Forelle pear PPO

The control mushroom tyrosinase (40 µl; 30 units/ml) and Forelle pear PPO (40 µl; 978 units/ml) were respectively added to a catechin mixture (2960 µl). The catechin mixture comprising of epicatechin (3 mg/ml) and epigallocatechin (3 mg/ml) was made in a phosphate buffer (0.2 M, pH 6.8). Samples were stirred for an hour using a magnetic stirrer. The reactions were stopped by incubating them at 90 ºC for 10 minutes followed by filtering (0.2 micron) and UPLC-MS sample analysis.

3. Results and discussion

The homogenates of different pear varieties available in South Africa were assessed for their theaflavin synthesis capacity. Forelle pear (0.147 (± 0.016) µM theaflavin/g FW pear/hour) was identified among the other

pear varieties tested, Packman (0.0086 (±0.00 μM theaflavin/g FW pear/hour 6)), Abet Beteil (0.094 (±0.005) μM theaflavin/g FW pear/hour) and Beurré Bosc (0.072 (±0.012) μM theaflavin/g FW pear/hour), as the pear variety with the highest theaflavin synthesis capacity based on the flavognost method. In addition to Forelle pear, a preliminary exploratory investigation into the theaflavin synthesis capacity of a homogenate of Yacon (*Smallanthus sonchifolius*) leaves was also performed.

The diphenolase activity of PPO present in the homogenates were determined in decreasing order of Yacon leaves (0.169 ± 0.007 gDW/gFW) (18.935 units/gDW), Tea leaves (0.498 ± 0.019 gDW/gFW) (13.521 units/gDW), and Forelle pear (0.208 ± 0.003 gDW/gFW) (9.936 units/gDW), where DW is dry weight and FW is fresh weight). The dry weight was determined by freezing samples at -80°C and subsequently freeze-drying the samples until dry. This was indicative of the homogenates' ability to synthesise theaflavin by the PPO-dependent oxidative polymerization during incubation in the manufacturing of black tea.

In an incubation system utilizing Lipton® Green tea extract as starting material, the theaflavin (TF) synthesis capacity of the homogenates were in decreasing order of; Yacon leaves (65 μM TF/gDW/hr), Forelle pear (44 μM TF/gDW/hr), and Tea leaves (26 μM TF/gDW/hr) (Figure 1). The high theaflavin content in the fresh green tea leaf sample (49 μM TF/gDW/hr) in comparison to the Tea leaf homogenate from Lipton® Green tea extract, could be as a result of the fast generation of theaflavins during the homogenation step (2 minutes) of the fresh green tea leaves, and the slower turnover of formed theaflavins into thearubigins during the bioconversion. Both Forelle pear and Yacon leaves were considered as excellent enzyme sources, based on the absence of an inherent theaflavin content (Figure 1) and practically no oxidation products of their own (as seen from the theaflavin results of the controls in figure 1). Forelle pear and Yacon leaf homogenates in comparison to the control Lipton® Yellow label black tea (48 μM TF/gDW/hr), utilizing Lipton® Green tea as starting material, respectively resulted in a 9% decreased and a 36% increased theaflavin content (Figure 1).

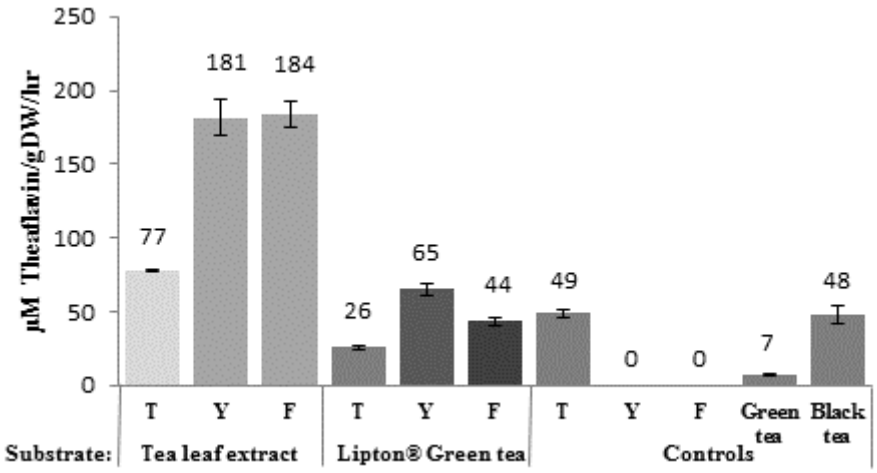

Figure 1. Bioconversion of respectively Tea leaf and Lipton® Green tea catechins into theaflavins following an hour incubation by the homogenates of Yacon, Forelle pear, and Tea leaf determined by the flavognost method. The controls for Tea, Yacon and Forelle as well as green tea and black tea values indicate the theaflavin values present in the samples before incubation with enzyme. Error bars indicate the standard error of the mean of the theaflavin synthesised per gram DW per hour of three independent repetitions performed in three biologically independent experiments. Abbreviations: T, Tea leaf, Y, Yacon and F, Forelle pear.

The substrate profile of the Lipton® Green tea may differ from that of fresh Tea leaf extract, based on the effect of processing and storage on the stability of the catechins. Although both extracts are rich sources of catechins, they can constitute up to 30% of the dry tea leaf weight [29, 31]. In an incubation system utilizing Tea leaf extract as starting material, the theaflavin synthesis capacity of the homogenates were in decreasing order ; Forelle pear (184 µM TF/gDW/hr), Yacon leaves (181 µM TF/gDW/hr), and Tea leaves (77 µM TF/gDW/hr) (Figure 1). The results could be influenced by the contributing effect of endogenous enzymes present in the extract, The UPLC-MS analysis of the catechin bioconversion efficiency was assessed based on the selective mass extracted data for theaflavins at *m/z* 563.14 (*m/z* = mass to charge ratio in Daltons). Prominent peaks were respectively identified for the control mushroom tyrosinase *m/z* 563.1400 (Rt 27.06 minutes), Forelle pear- *m/z* 563.1396 (Rt 26.95 min) and Yacon leaf homogenate *m/z* 563.1415 (Rt 26.95 min) (data not shown) where Rt refers to the retention time. The formation of theaflavin and other theaflavin-like analogues obtained for the incubation of Yacon with green tea using different analytical detection techniques is given in figure 2. Incubation of fresh green tea leaves and Lipton® Green tea extract using the other sources of polyphenol oxidase enzymes investigated in this study, gave similar results.

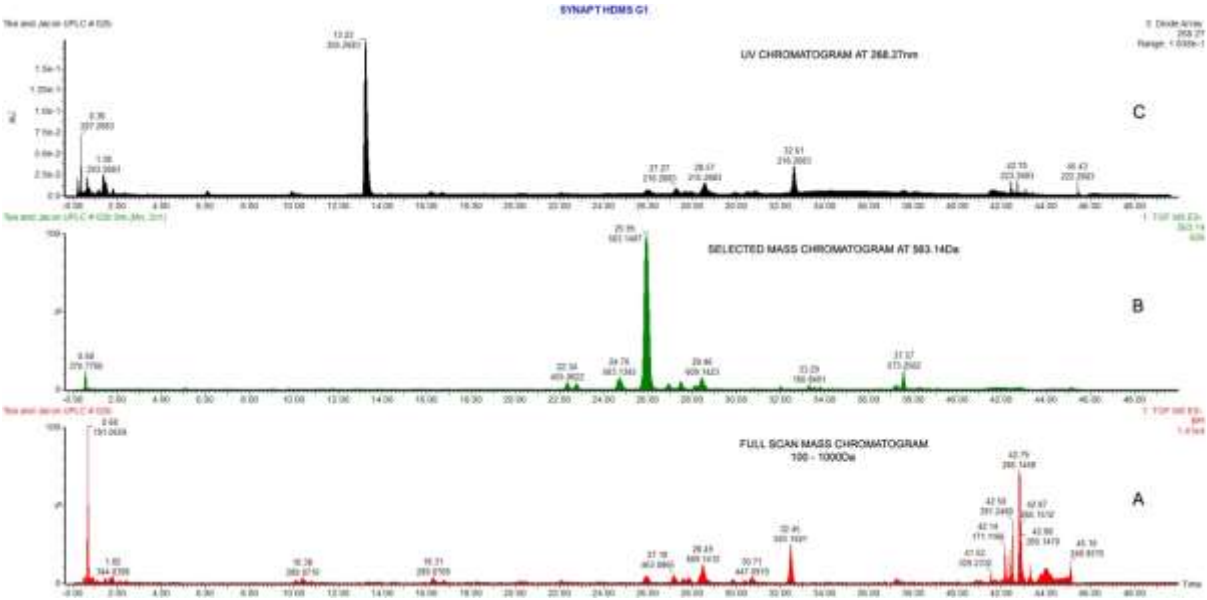

Figure 2. Analytical detection methods for theaflavin and theaflavin analogues using: (A) full scan mass spectral data (100 – 1000Da), (B) selected mass chromatogram at 563.14 Da and (C) selected UV chromatogram at 268.27nm. The results depicted were for green tea with Yacon but similar results were obtained with the other sources of the polyphenol oxidase enzyme

The biotransformation of tea leaf catechins by Yacon leaf homogenate produced a compound with a deprotonated pseudomolecular ion $[M-H]^-$ *m/z* 563.1415 (Rt 26.95 min) with a calculated elemental composition of $C_{26}H_{28}O_{14}$ (Figure 3e). The peaks at *m/z* 563.1396 (Rt 27.06 min) and *m/z* 563.1400 (Rt 26.95 min) respectively for the Forelle pear homogenate and the control mushroom tyrosinase samples, had corresponding mass fragmentation data, with base ion peaks, $[M-H]^-$, *m/z* 563.1396 and *m/z* 563.1400 respectively and an adduct ion at *m/z* 641.1364 and *m/z* 641.1356 respectively $[M-H+78]^-$ (Figures 3c and d). The peak with *m/z* 563.1415 (Rt 26.95 min) in the Yacon sample only had a corresponding base ion peak, $[M-H]^-$, *m/z* 563.1415 (Figure 3e). The tea leaf catechin biotransformation by Forelle pear homogenate produced a peak with *m/z* 563.1396 (Rt 26.03 min), an adduct ion *m/z* 641.1364 $[M-H+78]^-$ and a calculated elemental composition of $C_{26}H_{28}O_{14}$ and $C_{27}H_{30}O_{18}$ respectively (Figure 3d). The adduct ion, *m/z* 641.1364 $[M-H+78]^-$ corresponded to an increase of CH_2O_4. The tea leaf catechin biotransformation by control mushroom tyrosinase indicated the same results as that determined for the homogenate of Forelle pear (Figures 3c and 3d).

The mass fragmentation of peak *m/z* 563.1400 (Rt 26.03 min), corresponded to a peak determined in Lipton® Yellow label black tea with *m/z* 563.1349 (Rt 23.95 min), but in which the fragment ion of *m/z* 613.1141 $[M-H+50]^-$ (Rt 23.95 min) was not observed. The calculated elemental composition of the peak *m/z* 563.1349 (Rt 23.95 min) was determined as $C_{26}H_{28}O_{14}$, with the adduct ion *m/z* 613.1141 $[M-H+50]^-$ (Rt 23.95 min) as $C_{29}H_{26}O_{15}$ (Figure 3b). The peak with *m/z* 563.1349 (Rt 23.95 min) corresponding to $C_{26}H_{28}O_{14}$, might be one of

the sugar derivatives of apigenin as reported in literature. A very preliminary study on the unknown compound using extended run times on UPLC has indicated that there are at least 8 peaks with the same molecular mass (m/z 563) and which is not theaflavin. A paper by Lin et al (2008) [21] identified 3 peaks in a fermented tea sample which they identified as (a) apigenin 6-C glucosyl-8-C-arabinoside, (b) apigenin 6-C-arabinosyl-8-C-glucoside and (c) apigenin 6-C-pentosyl-8-C-hexoside. In another paper by Scoparo et al (2012) [30] a compound with m/z 563 in black tea was identified as apigenin 6-C-arabinosyl-8-C-glucose. Ku et al (2010) [18] also tentatively identified a peak with m/z 563 as apigenin glucosyl arabinoside. The identification of at least the major peaks of these unknown compounds with the molecular mass m/z 563 which is not theaflavin will be further investigated. The elemental composition of $C_{29}H_{26}O_{15}$ determined for the fragment ion *m/z* 613.1141 $[M-H+50]^-$ (Rt 23.95 min) has not been reported in literature. In Lipton® Yellow label black tea the expected presence of theaflavin was confirmed based on a peak observed with *m/z* 563.1415 (Rt 35.53 min) and a calculated elemental composition of $C_{29}H_{24}O_{12}$ (0.0 i-Fit value) (Figure 3a). The i-Fit score is a comparative measure of how well a cluster of peaks matches the pattern of each set of predicted isotope peaks. The lower the i-FIT value is, the better the fit. The identity of the abovementioned compound was confirmed with a theaflavins reference standard.

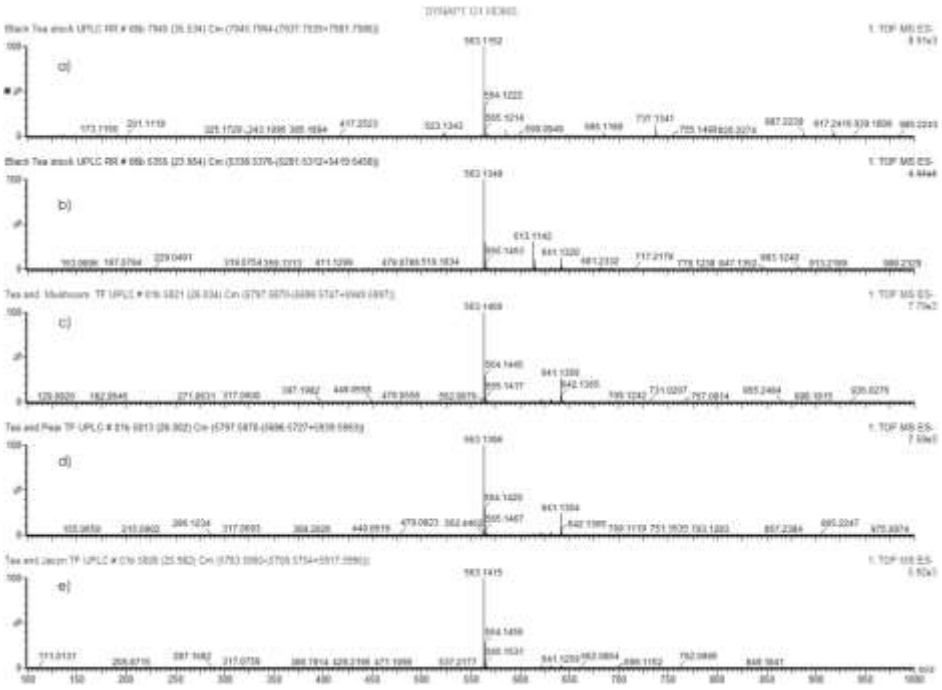

Fig 3 a – e. MS spectra of compound 4, [M-H]-, at *m/z* 563.1152 (Rt 35.53 min), $C_{29}H_{24}O_{12}$. b. MS spectra of compound 5, [M-H]-, at *m/z* 563.1349 (Rt 23.95 min), $C_{26}H_{28}O_{14}$. c. MS spectra of compound 1, [M-H]-, at *m/z* 563.1400 (Rt 26.03 min), $C_{26}H_{28}O_{14}$. d. MS spectra of compound 2, [M-H]-, at *m/z* 563.1396 (Rt 26.00 min), $C_{26}H_{28}O_{14}$. e. MS spectra of compound 3, [M-H]-, at *m/z* 563.1415 (Rt 25.98 min), $C_{26}H_{28}O_{14}$. All the elemental composition predictions had a 0.0 i-Fit value.

Further investigation into the oxidation of catechins during tea fermentation was done by an *in vitro* model incubation system based on the complexity associated with the use of fresh tea leaves as starting material and sample homogenates for biotransformation. Following a single affinity chromatography step using a Sepharose 4B-L-tyrosine-*p*-amino benzoic acid gel, a 3-fold purification of PPO from Forelle pear was achieved.

Silver stained SDS-PAGE of purified Forelle pear polyphenol established the presence of five prominent bands (56-, 27-, 25-, 18- and 14 kDa) associated with the purity of the enzyme and the presence of isoenzymes (Figure 4A). This was confirmed by a zymogram of the enzyme in which six active PPOs were detected in the crude sample (Figure 4B). The specific activity of the crude PPO was 14613 units/mg while the specific activity of the pooled fractions following affinity chromatography was 44059 units/mg and therefore resulting in a 3 fold purification of the PPO from Forelle pear. This was consistent with the identification of the presence of isoenzymes in PPO purified from d'Anjou pear and Williams pear in which three isoenzymes were detected [10, 41]. From the pH activity profile of Forelle pear PPO, 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 small shoulder was present at pH 6.5 (Figure 5). This was consistent with the pH profile of PPO 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 has been reported [43].

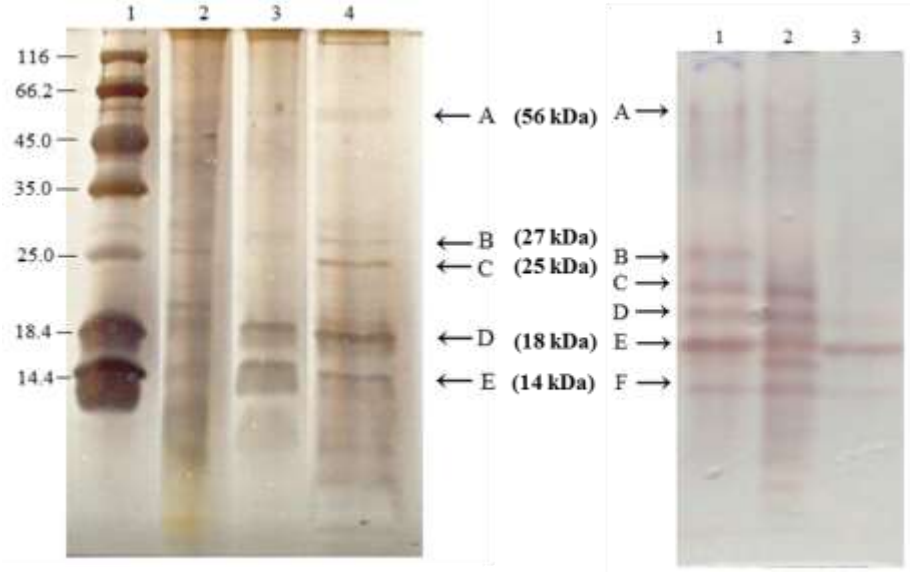

Figure 4. (A) Silver stained 12% SDS-PAGE gel of purified polyphenol oxidase from Forelle pears (*Pyrus communis* L.). Lane 1 protein marker, lane 2 crude (50.7 µg/µl), lane 3 unbound crude (42 µg/µl) and lane 4 bound fractions of purified polyphenol oxidase (3.3 µg/µl). (B) Zymogram of a 7% Native page stained for polyphenol oxidase activity by development in catechol. Lane 1 crude (50.7 µg/µl), lane 2 unbound crude (42 µg/µl) and lane 3 bound fractions of purified polyphenol oxidase (3.3 µg/µl).

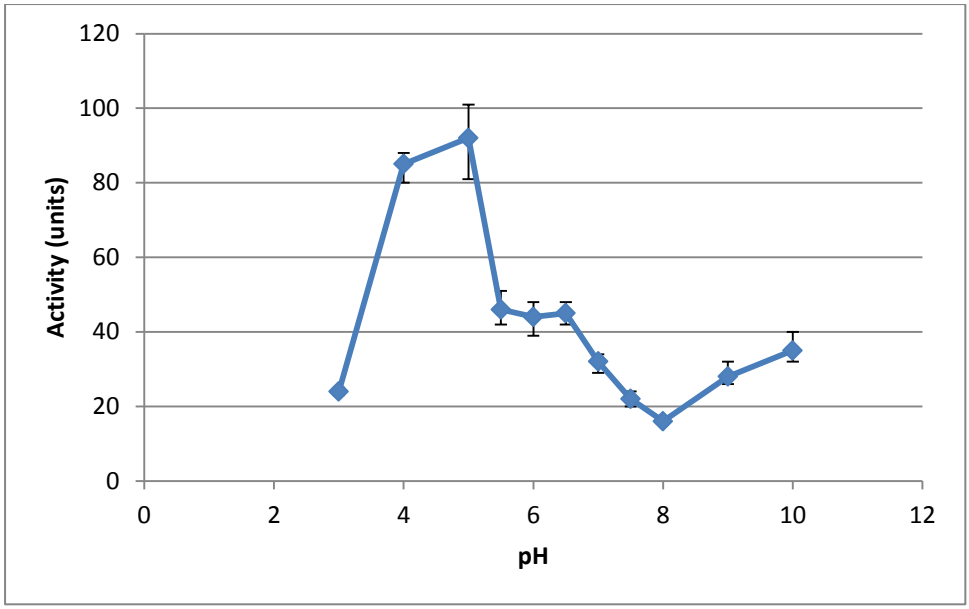

Figure 5. pH optimum determination of PPO with 4-methylcatechol in a citrate buffer pH 3 to 5.5, potassium phosphate buffer pH 6 to 7.5 and tris-HCl buffer pH 8 to 10. Error bars indicate the standard deviation of the activity (units) of three independent repetitions performed in three biologically independent experiments.

The pH stability curve of PPO activity indicated that the enzyme was most stable at pH 5, with a sharp decrease in activity from pH 5 to 5.5 followed by a small shoulder at pH 6.5 (data not included). This is in comparison to the pH optimum determination. The pH stability of PPO revealed that the pre-incubation of the enzyme had no apparent effect on the enzyme activity of Forelle pear PPO, which was also reported for PPO from sweet potato root [23].

PPO had an optimum temperature range between 25 ºC to 40°C. There was an approximate 91% retention of its maximum activity at 65ºC, which was higher than the reported 60% for both d'Anjou and Barlett pear PPO [32]. Forelle pear PPO, therefore, appeared more active over the determined temperature range than that reported for d'Anjou and Barlett pear PPO. The temperature stability of Forelle pear PPO was similar to that of Monroe apple PPO [41] (Figure 6). The amount of activity that was retained for an increase in temperature from 20 ºC to 40 ºC was 96% and decreased to 6% activity retention when the temperature was increased to 70 ºC. Despite the similarity of the PPO temperature stability data of Monroe apple and Forelle pear, the temperature optimum determination for Monroe apple PPO indicated a much larger effect of temperature on enzyme activity as only 35% of the maximum enzyme activity was retained at 70 ºC compared to 94% for Forelle pear PPO [43].

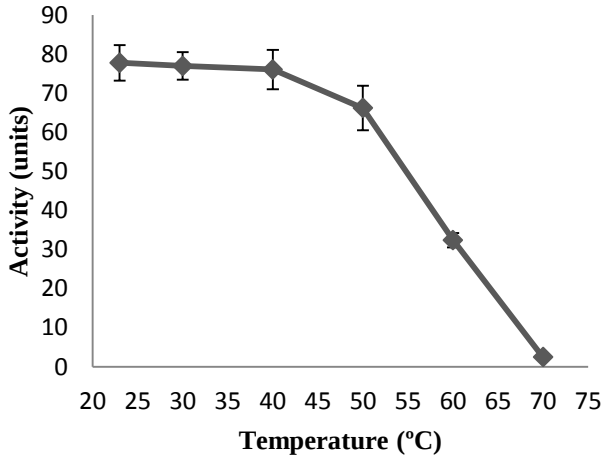

Figure. 6 The apparent stability of PPO after a 30-minute incubation period over a temperature range of 23ºC to 70ºC with 4-methylcatechol. Error bars indicate the standard deviation of the activity (units) of four independent repetitions performed in three biologically independent experiments.

In an *in vitro* model incubation system (catechin mixture of epicatechin and epigallocatechin), the bioconversion of catechins with Forelle pear PPO indicated the formation of two prominent peaks with *m/z* 563.1190 (Rt 35.54 min) and *m/z* 563.1199 (Rt 38.20 min) respectively (Figure 7a). Correspondingly the peaks with *m/z* 563.1196 (Rt 35.58 min) and *m/z* 563.1180 (Rt 38.21) were produced in the bioconversion with the control mushroom tyrosinase (Figure 7b). Based on the data obtained with the theaflavin standard ($C_{29}H_{23}O_{12}$: *m/z* 563.1190 (Rt 35.75 min)), the peaks *m/z* 563.1190 (Rt 35.54 min) and *m/z* 563.1196 (Rt 35.58 min) with corresponding elemental compositions ($C_{29}H_{23}O_{12,}$ 0.0 i-Fit values) were identified as theaflavin (Figures 8 and 9). In comparison to the control sample an increased number of peaks in the bioconversion with Forelle pear PPO were associated with the degree of purification.

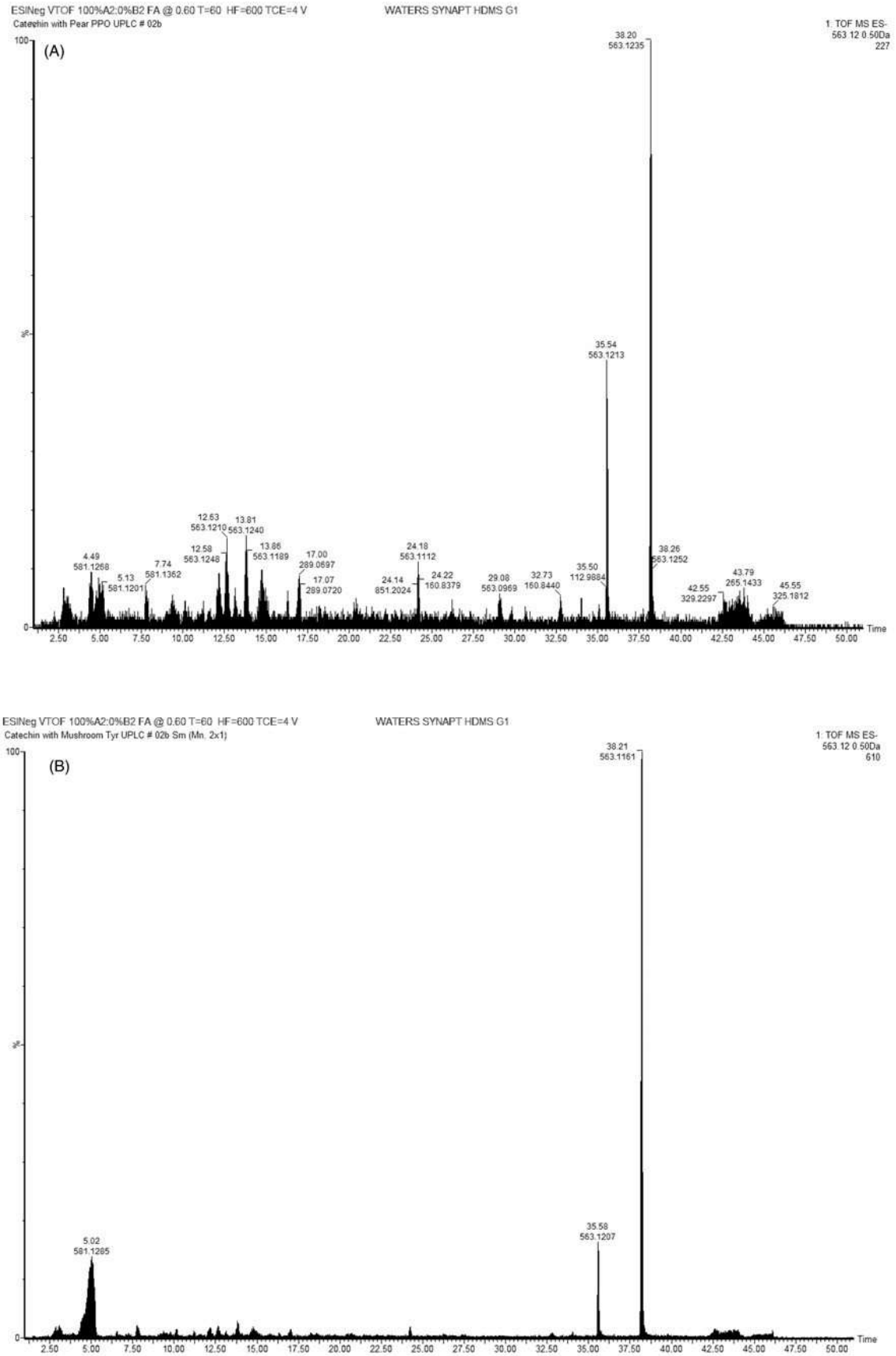

Figure 7. Theaflavin extracted mass (m/z 563.12) UPLC chromatograms of the bioconversion of a catechin mixture with (a) pear PPO (b) mushroom tyrosinase.

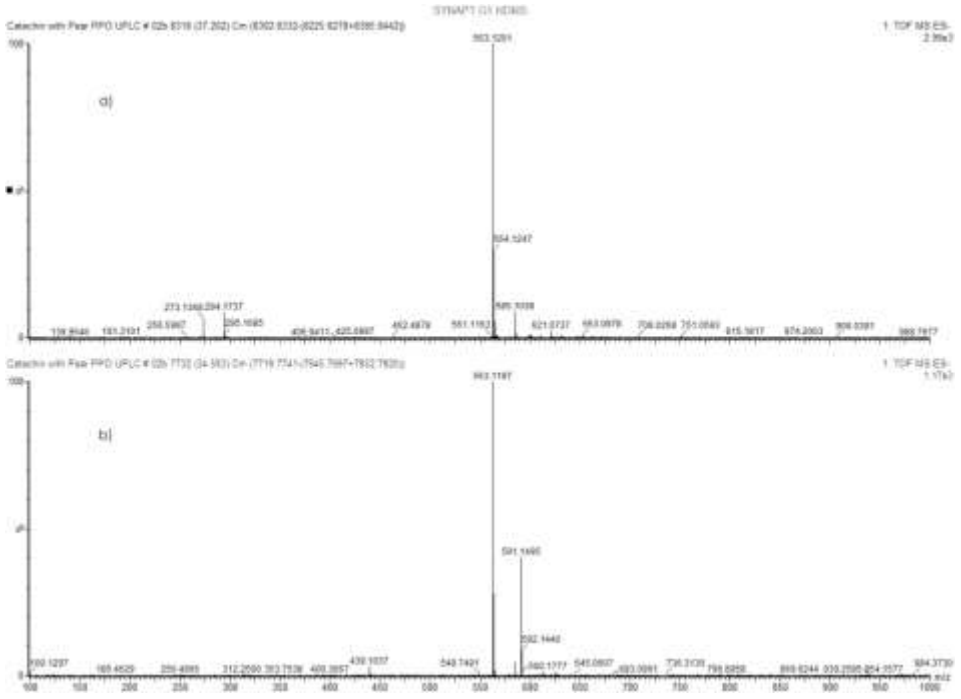

Figure 8 a. MS spectra of compound 6, [M-H]-, at *m/z* 563.1201 (Rt 37.20 min), $C_{29}H_{24}O_{12}$ (0.0 i-Fit value). **b**. MS spectra of compound 7, [M-H]-, at *m/z* 563.1187 (Rt 34.58 min), $C_{29}H_{24}O_{12}$ (0.1 i-Fit value)

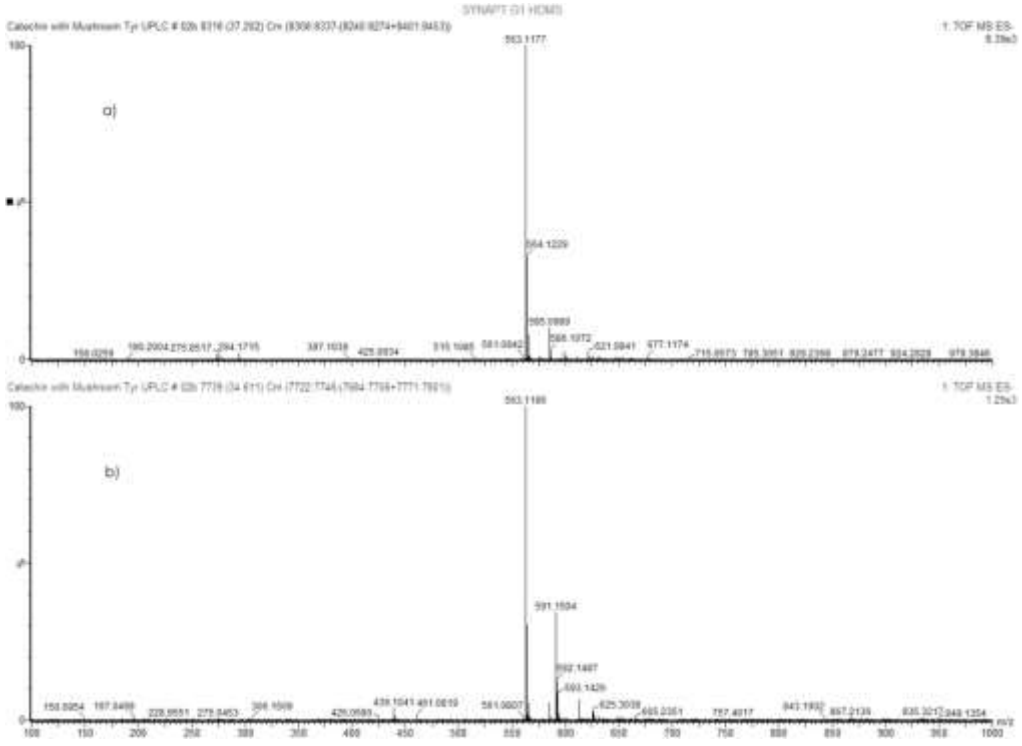

Figure 9 a. MS spectra of compound 8, [M-H]-, at *m/z* 563.1180 (Rt 37.20 min), $C_{29}H_{24}O_{12}$ (0.0 i-Fit value). **b**. MS spectra of compound 9, [M-H]-, at *m/z* 563.1196 (Rt 34.58 min), $C_{29}H_{24}O_{12}$, (0.0 i-Fit value)

In conclusion, based on the incubation studies of the bioconversion of Tea leaf extract in comparison to the *in vitro* model incubation results using extracted catechins, it can be deduced that the peaks *m/z* 563.1400 (Rt 26.95-27.06 min) ($C_{26}H_{28}O_{14}$) were preferentially synthesised to that of the expected theaflavin (eluting between 35.5 and 38.2 minutes). Forelle pear PPO and mushroom tyrosinase therefore displayed a lower affinity towards the catechins compared to the competing substrates present in fresh tea leaves. Furthermore, based on the presence of the peak with *m/z* 563.1349 (Rt 23.95 min) ($C_{26}H_{28}O_{14}$) in Lipton® Yellow label black tea, it can be deduced that the preferential formation could be an implicating factor in the low levels of theaflavins in black tea. Greater understanding of the conditions that modulate the formation of these unidentified compounds may increase the yield of theaflavins and/or the unknown compounds which may improve the health properties and the quality of black tea.

Acknowledgements UPLC-MS equipment used was funded by the National Research Foundation (Research Infrastructure Support Programmes National Equipment Programme) program as a joint venture between the CSIR Biosciences and the University of Johannesburg, Biochemistry department. CSIR Biosciences for funding the project. One author, M. vd Westhuizen is thankful to the NRF for funding to complete her MSc degree on the project.

Ethical Standards; The experiments comply with the current laws of South Africa in which the research was performed.

Conflict of interest. The authors declare that they have no conflict of interest

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