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A model oxidation system to study oxidised phenolic compounds present in black tea

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Abstract

A model oxidation system was developed and optimised in order to provide a relevant tool to study oxidation products generated during the manufacture of black tea. The system utilised endogenous tea enzymes prepared by removing the phenolic compounds and caffeine from unprocessed tea leaves in such a way that the polyphenoloxidase (PPO) and peroxidase (POD) activities remained unaffected. This immobilised enzyme system was then used to oxidise the flavan-3-ols obtained from an ethyl acetate extract of green tea. The disappearance of the flavan-3-ols and the appearance of the different theaflavins were monitored by HPLC-UV. The theaflavin species detected were shown to be similar to those already described in black tea. The ^{1}H NMR spectra of more oxidised compounds produced by the model system were compared with those observed in black tea. The ^{1}H NMR spectra of the products generated by the model system and of the black tea extract were similar, suggesting that the washed leaf, heterogeneous catalysis system can successfully simulate the molecular changes that occur during tea production.

Keywords: Black Tea; Model oxidation system; Flavan-3-ols; Thearubigins; Theaflavins; Polyphenoloxidase; Peroxidase

1. Introduction

Theaflavins and thearubigins are oxidation products of tea polyphenols, which are formed when black tea is manufactured from freshly plucked green leaves. These are key compounds which contribute significantly to the colour and organoleptic characteristics of black tea infusions (Bailey, Nursten, & McDowell, 1994a, 1994b, 1994c; Charlton et al., 2000; Hilton, 1972; Roberts & Smith, 1963). The synthesis and the molecular structure of the theaflavins are now well described (Collier et al., 1973; Davis, Cai, & Davies, 1995). However, the thearubigins, which constitute 10–15% of the black tea dry weight, still need further study as their chemical structure and mechanism of formation remain unclear.

Several strategies have been used to investigate the thearubigins. Many authors have attempted to characterise thearubigins extracted from black tea (Bailey et al., 1994c; Cattell & Nursten, 1977; Collier et al., 1973; Davis et al., 1995; Degenhardt, Engelhardt, Wendt, & Winterhalter, 2000; Zeeb, Nelson, Albert, & Dalluge, 2000; Ozawa, Kataoka, Morikawa, & Negishi, 1996). In general, this approach, which requires a series of purification steps, has produced low yields of a heterogeneous family of compounds rather than a single thearubigin-like molecule which might be relatively easy to characterise. An alternative approach, using a model oxidation system, has thus been developed, in order to generate a better yield of oxidation products that are easier to separate. For example, oxidation in model systems has been carried out chemically by using ferrous sulphate (Oszmianski, Cheynier, & Moutounet, 1996) or hydrogen peroxide (Zhu, Huang, Yu, LaVoie, Yang, & Ho, 2000) or enzymatically with, for example, grape polyphenoloxidase (PPO) (Cheynier & Dasilva, 1991; Cheynier, Osse, & Rigaud, 1988; Guyot, Vercauteren, & Cheynier, 1996). PPO and peroxidase (POD) are key enzymes for pigment generation during the black tea process (Dix, Fairley, Millin, & Swaine, 1981). The in vitro study of the tea PPO-catalysed formation of black tea oxidation products has been carried out by a number of researchers (Opie, Clifford, & Robertson, 1993, 1995; Opie Robertson, & Clifford, 1990; Robertson, 1983a, 1983b; Robertson & Bendall, 1983). Model oxi-

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dation systems have also been used to compare the oxidation products obtained with tea PPO and with horseradish POD (Finger, 1994).

Whilst the enzyme model systems so far developed have been informative, they do present some disadvantages. First, in some cases, the relevance of the model oxidation system to the real oxidation process occurring in tea leaves was not established. Second, the use of buffer in the oxidation system may interfere with some analyses (e.g. ^{1}H NMR spectroscopy). Recently, Tanaka, Mine, Inoue, Matsuda, & Kouno (2002) used plant homogenates (including tea leaves) to generate theaflavins from epicatechin (EC) and epigallocatechin (EGC) and demonstrated that the production of theaflavins without buffer was possible. However, the authors recommend use of banana rather than tea homogenates to avoid the appearance of many minor and complex products from the endogenous flavonoids in the tea leaves (Tanaka et al., 2002).

In this work, the optimisation of the model oxidation system, using washed tea leaves as an immobilised enzyme source, is described. The chemical characterisation of the oxidation products and the relevance of the model oxidation system to the chemical changes that occur during black tea production are also discussed.

2. Materials and methods

2.1. Materials

Tea leaves were plucked after 15 days growth and withered in Kenya, then stored at −80 °C. All chemical compounds were obtained from Sigma, Dorset, UK. The ethyl acetate fraction of green tea was been obtained as described by Davis et al. (1995).

2.2. Methods

2.2.1. Preparation of enzyme extract

Tea leaves (1.0 g), which had been ground to a fine powder in liquid nitrogen, were homogenised with polyvinylpolypyrolidone (PVPP) (0.8 g) on ice. Buffer solution (30 ml, 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), 0.2 M Na_2SO_4, 1 mM benzamidine, 0.3% Triton X-100, pH 5.5) was added and the grinding continued for a further 1 min. The mixture was stirred on ice for 30 min and centrifuged at 4 °C at 10,000 *g* for 20 min in a Beckman Avanti J-25 centrifuge. The supernatant corresponded to the crude enzyme extract.

2.2.2. Determination of enzyme activity

The enzyme activity was determined by monitoring the formation of quinones spectrophotometrically (Perkin-Elmer Lambda 40 at 400 nm) with time and at constant temperature. The cuvette inside the spectrophotometer was thermostatted using a Haake F3 water bath.

2.2.2.1. Determination of the PPO activity. Crude extract (100 µl) was mixed with the reaction buffer (2.75 ml, 100 mM sodium phosphate–citrate buffer, pH 5.5) and with (+)-catechin (150 µl, 60 mM). The mixture was then vortexed and the absorbance at 400 nm was read against water at 40 °C.

For the enzyme blank, the enzyme activity was inhibited by adding a solution (200 µl of acetonitrile/acetic acid/water (60/10/30, v/v/v) before the incubation at 40 °C. Blank values were subtracted from the assay values.

2.2.2.2. Determination of the POD activity. The PPO procedure was repeated (including the enzyme blank), but the incubation occurred at 30 °C and in presence of hydrogen peroxide (50 µl, 1.5% v/v solution).

The dry matter of the enzyme extract was determined after 12 h of dialysis against water in dialysis tubing (Sigma, Dorset, UK) with a molecular weight cut-off of 12,000 Da. The rate of appearance of the quinones was defined by the slope of the linear part of the curve and expressed in absorbance units per minute (uA min^{-1}). The enzyme activity was expressed as the rate per dry matter over 12,000 Da (uA min^{-1} g^{-1}) (Fig. 1).

2.2.3. The washing protocol of the leaves

Tea leaves (25 g) were homogenised in a domestic blender (3×10 s) with water (250 ml). The mixture was transferred to a 10 l container and water (4.8 l) was added. The mixture was stirred (using a Heidolph ST1 stirrer, speed 3) and continuously sparged with helium for 2 h. The mixture was filtered and the leaf residue washed a further three times.

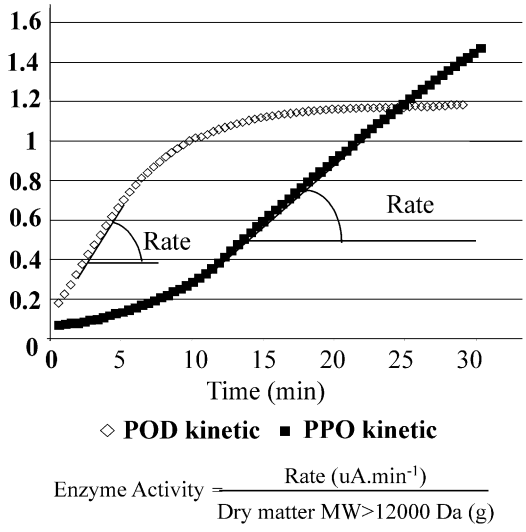

Fig. 1. Determination of the polyphenoloxidase (PPO) and peroxidase (POD) activity.

2.2.4. Oxidation procedure

Washed leaves (25 g) were added to an ethyl acetate fraction of green tea (400 mg dissolved in 400 ml of water) and the mixture stirred (using a Heidolph ST1 stirrer, speed 2). PPO-catalysed oxidation was completed by sparging the mixture with air at a flow rate of 1.5 l min^{-1}. POD-catalysed oxidation was carried out under helium (to prevent PPO oxidation) and hydrogen peroxide (6.5 ml, 1.5% v/v) was added to the medium. Samples (5 ml) were taken during the oxidation process and stabilised by adding 0.7 ml of a mixture of ascorbic acid (250 μg ml^{-1}) and of EDTA (250 μg ml^{-1}) before HPLC analysis. After 24 h of oxidation, the mixture was filtered and freeze-dried for NMR analysis.

2.2.5. Methanol extraction of phenolic compounds

Samples of ground washed leaves or commercial black tea (200 mg) were extracted with methanol/water (5 ml, 70/30, v/v) at 70 °C for 10 min. Samples were vortexed every 5 min and centrifuged at 2500 rpm for 10 min, with a Sanyo Mistral 3000i centrifuge. The extraction was repeated twice and the combined supernatants were collected, filtered through an Acrodisc 0.45-μm filter (Pall, Gelman) and stabilised by adding 0.7 ml of a mixture of ascorbic acid (250 μg m^{-1}) and of EDTA (250 μg ml^{-1}).

2.2.6. HPLC analysis of flavan-3-ols

The HPLC system was a Dionex, including GP40 gradient pump with a Gina 50 autosampler, coupled with a PDA100 photodiode array detector (HPLC-DAD). The separation was performed on a Luna (Phenomenex) phenyl hexyl column (250 mm×4.6 mm i.d.; particle size, 5 μm) at 30 °C. The flow rate was 1 ml min^{-1}. The gradient elution was as follows: solvent A, 2% acetic acid in acetonitrile; solvent B, 2% acetic acid in water; from 0 to 10 min, isocratic 95% B; from 10 to 40 min 95 to 82% B; from 40 to 50 min, isocratic 82% B.

2.2.7. HPLC analysis of theaflavins

The separation was performed on a Hypersil (Phenomenex) C_{18} ODS column (100 mm×4.6 mm i.d.; particle size, 3 μm) at 30 °C. The flow rate was 1.8 ml min^{-1}. The elution was performed isocratically using 80% solvent B.

2.2.8. Nuclear magnetic resonance spectroscopy

NMR spectra were measured on a Brüker AMX400 spectrometer operating at a probe temperature of 300 K, using either a dual $^{1}H/^{13}C$ 5 mm probe or a multinuclear 5 mm inverse probe, as appropriate. The solvent was methanol-d_4 and spectra were referenced relative to internal trimethylsilane (TMS). Sample concentrations were typically ca. 8 mg ml^{-1}.

3. Results and discussion

3.1. Optimisation of the model oxidation system

The first aim of this work was to prepare an immobilised enzyme system by removing the endogenous flavonoids from tea leaves without affecting the PPO or the POD activities. Different procedures were tested to determine which efficiently removed most of the epigallocatechin gallate (EGCG) and the epicatechin gallate (ECG) (Fig. 2). Thus, 97% of the endogenous flavan-3-ols are removed by washing for 15 min with 400 ml of water, 12 times. However, even so, the small residual quantities of flavan-3-ols can interfere with the oxidation reactions that we wish to characterise. Increasing the temperature of the washing to 30 °C reduces the residual amounts 10-fold, only 0.2% of the flavan-3-ols remaining in the leaves. However, washing the leaves at ambient temperature is preferable to be sure not to affect the enzyme activity. Extracting the leaves 15 times at ambient temperature was shown to remove more flavan-3-ols than 12 extractions at 30 °C. The most efficient extraction was found to occur with three 5 l, 2 h extractions. This removed all the caffeine and produced leaves containing less than 0.2% of flavan-3-ols. This last protocol was therefore adopted as the preparation method of the immobilised enzyme.

The enzyme activities before and after the washing, were checked to determine whether any enzyme was extracted during the washing of the leaves or whether their oxidative efficiencies were affected. The crude enzyme extract was prepared in order to obtain the optimum activity of PPO and POD. This required the use of PVPP to remove polyphenolic compounds able to interfere with the activity measurement. Benzamidine was also added to the extraction buffer to prevent the action of protease able to degrade PPO and POD. Fig. 3 shows a comparison of the POD and the PPO activities before and after washing. For both enzymes, no loss of activity was observed. This is not surprising, as PPO is localised in the chloroplast and, to extract it, the use of a detergent is necessary (Halder, Tamuli, & Bhaduri 1998). As PPO and POD are known to be strongly associated (Coggon, Moss, & Sanderson, 1973), it would be expected that POD activity would also be preserved. In conclusion, stirring ground leaves in water does not affect the enzyme activity, but is efficient enough to remove most of the endogenous flavonoids.

3.2. Validation of the model oxidation system

The immobilised oxidative enzyme system, prepared as above, was used to simulate the fermentation occurring during the black tea process. A green tea ethyl acetate fraction, obtained as described by Davis et al. (1995), contains all the flavan-3-ols present in the tea

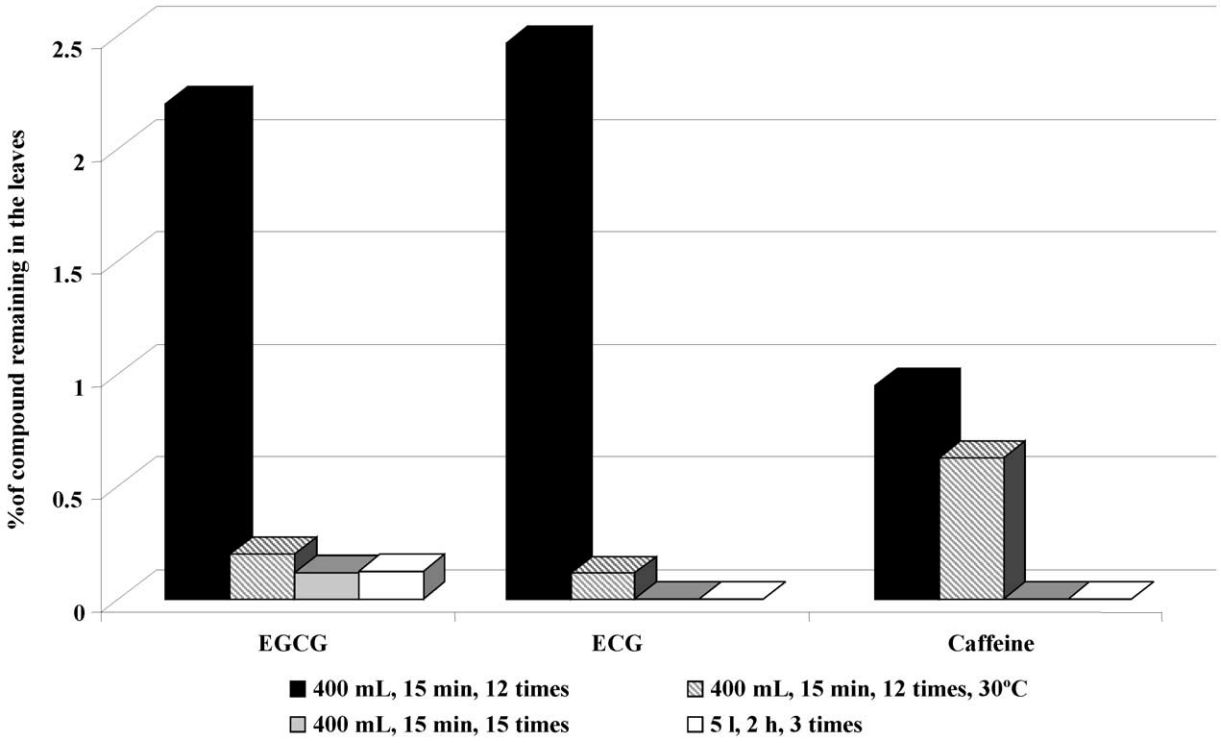

Fig. 2. Epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and caffeine contents remaining in tea leaves after different washing protocols.

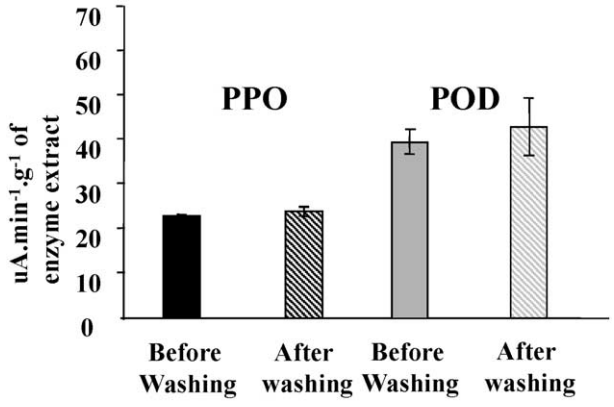

Fig. 3. Comparison of the polyphenoloxidase (PPO) and peroxidase (POD) activities before and after the washing of the tea leaves.

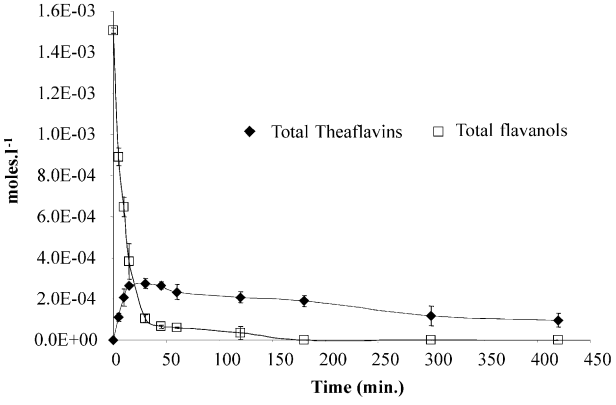

Fig. 4. Total flavan-3-ol and total theaflavin contents against time during PPO oxidation.

leaves in the same amounts as occur in planta. The main flavan-3-ols present are the gallated catechins, EGCG and ECG representing respectively, 57 and 24% of the extract. Epicatechin (EC) and catechin (C) are present at lower levels, i.e. 13.5 and 5%, respectively.

This ethyl acetate extract was used as a substrate for the PPO present in the washed tea leaves. Fig. 4 shows the disappearance of the flavan-3-ols and the appearance of the theaflavins in this model system. It is apparent that the flavan-3-ols present in the mixture are not necessarily transformed into theaflavins. The maximum amount of theaflavins obtained represents only 20% of the theoretical amount, based upon the initial flavan-3-ols content. This observation has already been reported an for in vitro system (Robertson, 1983b). PPO oxidations of flavan-3-ols lead to the formation of highly reactive quinones, which are able to oxidise other flavan-3-ols, including the theaflavins. However, the yield of this reaction is difficult to determine as some theaflavins are being generated at the same time as others are being transformed into other species.

The disappearance of the flavan-3-ols occurs quickly: after 30 min of oxidation more than 90% of the starting materials have reacted. Theaflavins appear during the first 5 min of the oxidation and reach a maximum yield after 15 min. When the oxidation is continued, the amount of theaflavins progressively decreases. Thus, after 3 h, about 70% of the theaflavins remain in the

medium and, even after 7 h of oxidation, their disappearance is not complete, as 30% of the total theaflavins are still in the medium. Theaflavins generation is a fast process, while their turnover is a slower phenomenon. It has been already reported that theaflavins are not a good substrate for PPO (Opie et al., 1993; Subramanian, Venkatesh, Ganguli, & Sinkar, 1999). However, their rearrangement into more complex molecules is possible and could explain the slow decrease of the theaflavin content of the medium.

Fig. 5a and b show the consumption of each flavan-3-ol and the generation of each theaflavin, respectively. The disappearance of the gallated flavan-3-ols occurs more rapidly than the disappearances of the non-gallated species. At the same time the gallated theaflavins are generated faster than the non-gallated ones. The oxidation of catechin occurs relatively slowly and 80% of the initial amount remains in the medium after 10 min, whilst only 35% of EGCG and of ECG remain. Catechin totally disappears after 5 h of oxidation, whilst the other flavan-3-ols are fully oxidised after only 1 h. Catechin is the only flavan-3-ol present in tea not involved in the formation of theaflavins and this could explain its slow oxidation. However, its observed disappearance confirms that several oxidation pathways are possible for flavan-3-ols. Coggon et al. (1973) reported that the tea PPO oxidises the non-gallated species more efficiently, which suggests that, in our system, both chemical and enzymatic oxidation reactions occur.

Fig. 6 illustrates the reactions that lead to the formation of different theaflavins. The high levels of EGCG and ECG would lead us to expect that theaflavin digallate would be the main theaflavin generated. The generation of theaflavin-3-monogallate would also be expected. More surprisingly, theaflavin-3′-monogallate was detected. This was not expected as no EGC was found in the ethyl acetate extract of green tea (Fig. 5a). The monitoring of the gallic acid content against time shows the generation of this phenolic compound in the medium during the oxidation of the flavan-3-ols (Fig. 5c). This observation is consistent with previous observations (Coggon et al., 1973). Two hypotheses are possible. First, leaf tea tannase activity could have partially de-gallated the EGCG to produce EGC and gallic acid. The EGC thus formed could have reacted further to form the theaflavin-3′-monogallate. However, the absence in the reaction mixture of any detectable EGC or one of its reaction products, theaflavin (Fig. 6), effectively rules out this hypothesis. A second more likely explanation for the formation of theaflavin-3′-monogallate is via a chemical rearrangement during the oxidation of EGCG. Loss of a gallated residue during

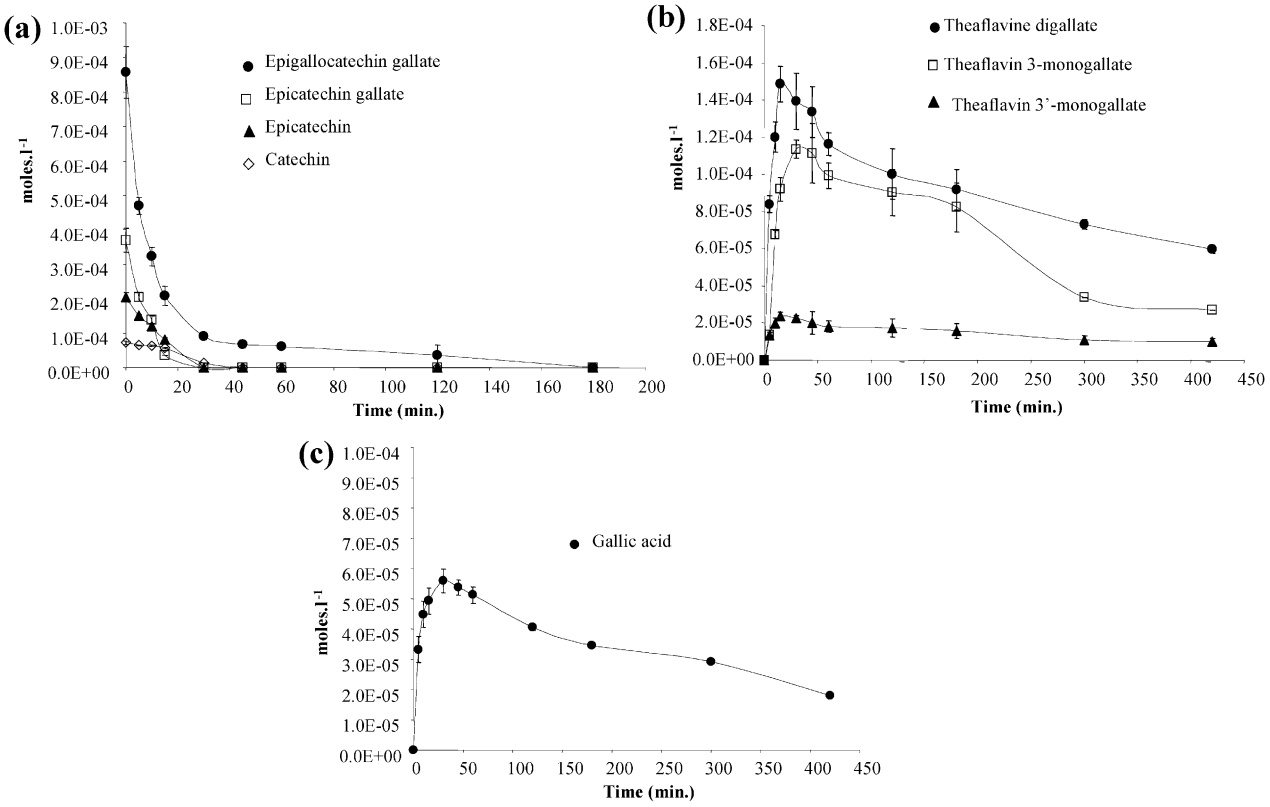

Fig. 5. (a) Epigallocatechin gallate, epicatechin gallate, epicatechin, catechin contents, (b) theaflavin digallate, theaflavin 3-monogallate, theaflavin 3′-monogallate contents and (c) gallic acid contents against time during a PPO oxidation.

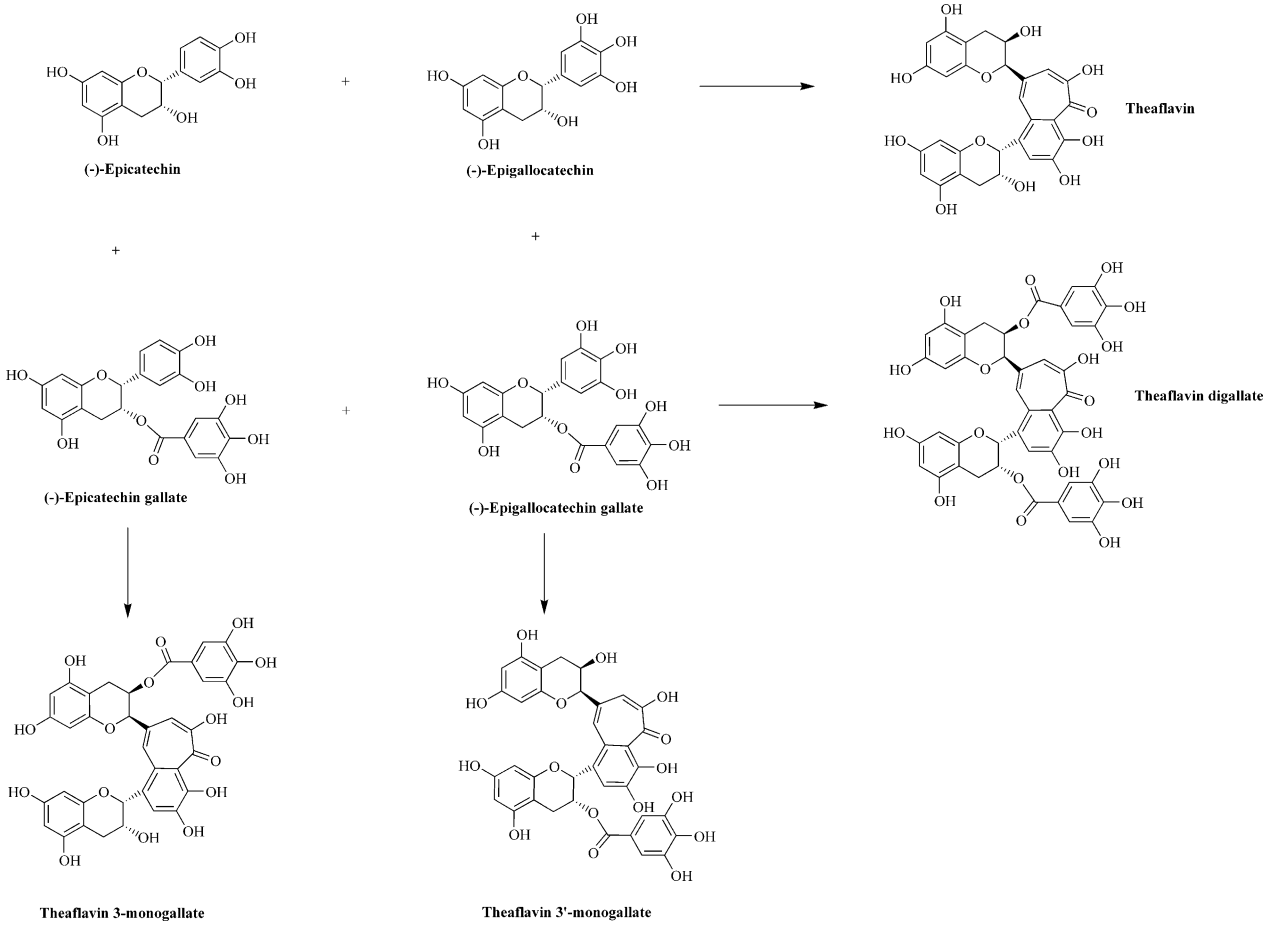

Fig. 6. Reactions involved in theaflavins formation.

or after the formation of the theaflavin digallate, constitutes another possible pathway of theaflavin-3′-monogallate formation. Oxidative degallation, generated by PPO, has already been demonstrated under aerobic conditions, similar to those described in this study (Coggon et al., 1973). Such a chemical transformation would explain why neither EGC nor theaflavin were found at any stage of the reaction. It is interesting to note that the gallic acid generated progressively disappears. The maximum gallic acid content was reached after 30 min and, after 7 h of reaction, 70% of the gallic acid generated had disappeared. Since gallic acid is not a substrate for PPO (Coggon et al., 1973), its disappearance was brought about by chemical reaction in the medium.

These results indicate that the model oxidation system quite accurately simulates the chemical and enzymatic changes that occur during the fermentation step of the black tea process. The results obtained with this model oxidation system are consistent with the biochemistry and the known chemistry of flavonoid oxidation and theaflavin formation.

Fig. 7 shows a comparison of ^{1}H NMR fingerprints of a standard theaflavin mixture and a product of a 1 h PPO oxidation of the ethyl acetate extract of green tea. Many similarities between the two ^{1}H NMR fingerprints can be seen. The specific resonances of the benzotropolone group can be seen in both spectra. Signals characteristic of the A and C ring of theaflavin can also be seen (confirming the HPLC-DAD findings). Finally, thearubigin resonances are present in the spectrum, suggesting that, during the generation of theaflavins, thearubigins are also formed from theaflavin and/or from flavan-3-ol oxidation. It is noteworthy that interferences of non-theaflavin or non-thearubigin species are negligible in the sample generated by the model oxidation, thus simplifying the interpretation of the ^{1}H NMR spectra.

To further investigate the relevance of the model oxidation system to the study of black tea processing, the ^{1}H NMR spectrum of a methanol extract of black tea was compared with the spectrum of a product of 24 h PPO oxidation of the ethyl acetate fraction of green tea (Fig. 8). In both spectra, similar signals for the A, B and C rings of the oxidised flavan-3-ols are present. This indicates that the theaflavin and thearubigin oxidation products generated in the model system are analogous to those found in black tea. Furthermore, the absence of

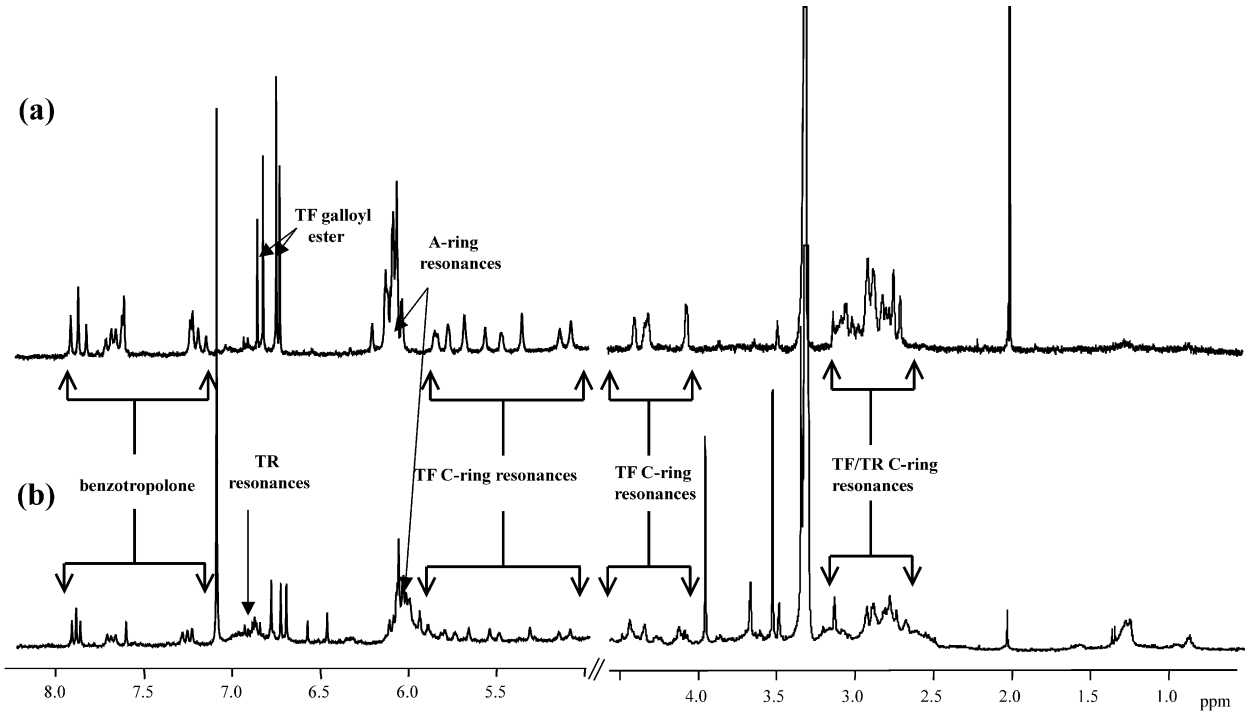

Fig. 7. (a) ^{1}H NMR fingerprints of a standard theaflavins mixture and (b) a 1 h PPO oxidation of the ethyl acetate fraction of green tea.

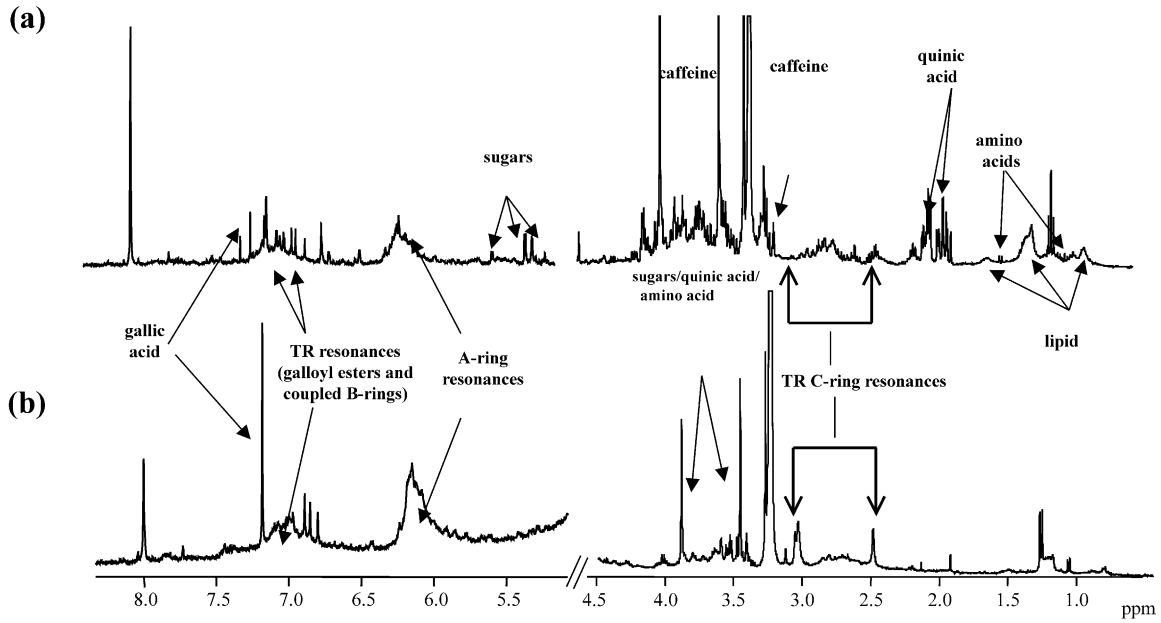

Fig. 8. (a) ^{1}H NMR fingerprints of a methanol/water (70/30, v/v) extract of black tea and (b) a 24 h PPO oxidation of the ethyl acetate fraction of green tea.

interfering impurities in the ^{1}H NMR spectra obtained with the model system made it much easier to interpret. Whilst lipid, amino acid, caffeine and sugar signals are detected in the black tea methanol extract, none of these compounds are present in the sample generated with the model oxidation system.

4. Conclusion

A new model oxidation system has been set up using tea leaves. Removal of the flavonoids without affecting PPO and POD activity of the tea leaves has been achieved. The resulting heterogeneous catalysis system

has significant benefits over other systems recorded in the literature. It can be used to prepare products with a high level of polyphenols and low levels of interfering impurities and requires no buffering.

The model has been validated by the HPLC-UV monitoring of the PPO oxidation of flavan-3-ols extracted from fresh leaves (Fig. 4). Disappearance of each of the flavan-3-ols and appearance of different theaflavin species were monitored (Fig. 5). The gallated flavan-3-ols are oxidised faster than the non-gallated species. Formation of the different theaflavin species is consistent with the known enzyme-catalysed pathways. Some chemical rearrangements have also been identified. The validity of this model oxidation system has been tested using ^{1}H NMR spectral comparisons (Figs. 7 and 8). These showed that the products generated by the model oxidation system are similar to a black tea extract.

Acknowledgements

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