ROLE OF SPECIFIC ENZYMES IN BISENSOR FOR TEA ANALYSIS

Thesis

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by

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CERTIFICATE

I hereby certify that the thesis entitled "**ROLE OF SPECIFIC ENZYMES IN BIOSENSOR FOR TEA ANALYSIS**" submitted by **Mr. Sujith Kumar P.V.** for the degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE** for the result research work carried out by him at laboratories of Fermentation Technology and Bioengineering department, CFTRI, Mysore – 570020, India, under my guidance and supervision during the period 2003 – 2010.

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DECLARATION

I hereby declare that the thesis entitled *"***ROLE OF SPECIFIC ENZYMES IN BIOSENSOR FOR TEA ANALYSIS***"* submitted to **UNIVERSITY OF MYSORE** for the award of the degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY** is the work carried out by me at laboratories of Fermentation Technology & Bioengineering department under the guidance of Dr. M.S. Thakur, Senior Scientist, Fermentation Technology & Bioengineering department, CFTRI, Mysore – 570020, India, during the period 2003 – 2010. I further declare that the results are not submitted for the award of any other degree or fellowship.

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CERTIFICATE

I Sujith Kumar P. V., certify that this thesis is the result of research work done by me under the supervision of Dr. M. S. Thakur at Fermentation Technology and Bioengineering department, Central Food Technological Research Institute. I am submitting this thesis for possible award of Doctor of Philosophy (Ph.D) degree in Biotechnology of University of Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree/ diploma of this or any other university.

Signature of Doctoral candidate

Signed by me on ……(date)

Signature of Guide

Date:

Counter Signed by

Date:

Signature of Chairperson/Head of Department/

Institution with name and official seal.

Dedicated to my brother Unni……

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Sujith Kumar P V

Abstract

Polyphenol oxidase (PPO) from tea leaves, potato, brinjal, apple and mushroom were assessed for their selectivity towards PP (catechin-a specific PP present in tea leaves). PPO from tea leaves was purified to homogeneity with three isozymes. PPO from other sources also partially purified and compared with standard tyrosinase and laccase. Among all these enzymes tyrosinase found to have highest substrate specificity towards catechin. This was further confirmed by enzyme kinetics studies. In the immobilized condition PPO from tea leaves and tyrosinase were responded to catechin. For black tea sample analysis by tea biosensor, only tyrosinase was given measurable signals, but for green tea sample analysis both tyrosinase and PPO were found to be good.

A detector system comprising of two electrodes was fabricated in-house and used for the analysis of PP using enzyme based biosensor. It is programmed to detect at various concentrations of PP with good sensitivity.

Immobilized enzyme was stabilized by various PBSAs and lysozyme observed with greater stability and enhanced the number of analysis. Other physico-chemical parameters were optimized. With this conditioning biosensor could analyze about 100 samples by using single immobilized enzyme membrane. During PP analysis it was observed that immobilized enzyme membrane was inactivated due to fouling. This fouling was attributed to protein-PP interaction.

Protein-PP interactions were studied using various amino acids. Amino acid residues such as lysine, proline and serine observed with maximum affinity with PP. 10 mM concentration of PP found to be the optimum substrate concentration to minimize the fouling. Dissociation studies were carried out with various dissociating agents. Glycerol found to be better polyol to prevent the complete binding of PP with proteins.

Developed tea biosensor exhibited good robustness in a number of in lab and field trials. Commercial black tea samples and black tea grades were analyzed for their PP content to evaluate its quality.

Biosensor system was validated by using various conventional analytical techniques. Biosensor system analysis also validated by tea taster's score for various grades of tea. In all these studies biosensor has shown a good positive correlation with other conventional analytical techniques.

Tea biosensor developed as an outcome of this research work could be a potential tool to analyze tea quality based on PP content, hence major quality attributes of tea such as colour and astringency more or less derived from PP present in tea. This methodology is rapid, simple, user friendly and cost effective. It also helps in quality monitoring at different stages of processing (in-line monitoring) and could be adapted for the on field evaluation of quality of tea.

Synopsis

Recent advancements in the field of science and technology have lead to the development of fast, specific, accurate and reliable methods of analysis of food, pharmaceutical, chemical and environmental samples. Tea is a popular non-alcoholic healthy beverage consumed around the world. Tea contains many active biomolecules with lot of health significance and is known to impart strong detoxification effects in mammals. Tea leaves considered to be one of the richest source of natural polyphenols (PP) constitutes up to 30% of the dry weight. Quality of tea is depends on the preparation, where PP undergo biochemical reactions. In tea industry, quality of tea is mainly assessed and graded by tea tasters, based on color and astringency. These attributes emanate contents of PP. The human sensory methods to evaluate the tea quality, which is a subjective method of analysis, are prone to fluctuations with the physiological and psychological status of the taster. Conventional analytical methods are available to assess the total PP, but these techniques are time consuming, expensive and not portable for field application. Hence, there is a need to design an alternative technique which encompasses the drawback of available methods. Biosensors have emerged most preferable techniques in clinical, pharmaceutical, health, food and environment monitoring for their rapidity, specificity and amenability for field applications. Biosensor used in the present investigation was fabricated in-house and can be an alternative for the analysis of total PP.

Present study describes the development of an enzyme based amperometric biosensor system for PP analysis to assess the quality of tea.

Based on the background of cited literature on the above subject, the present work has been carried out and outcome is brought in the form of a thesis entitled “Role of Specific Enzymes in Biosensor for Tea Analysis”.

Objectives of the thesis are:

I. Isolation and purification of polyphenol oxidase from tea leaves
II. Application of enzymes in biosensor for tea analysis
III. Validation of enzyme based tea biosensor

The thesis have been organized and presented as different chapters and a brief of the chapters are summarized below.

Review of literature

Overviews a thorough review of relevant literature cited on the topic of investigation and summarizes all the research efforts that have been spent to biosensors for various applications in food and beverage analysis. The stability and reproducibility of the biosensor are also discussed. Further scope of the present investigation is emphasized.

Chapter I

Application of polyphenol oxidase (PPO) in biosensor for tea analysis

Section A. Purification of PPO

The chapter deals with the isolation and purification of PPO from tea leaves of various locations and application in biosensor. PPO enzyme from tea leaves was purified and partial characterization was carried out using various analytical methods. PPO from tea leaves was found to have different isoforms such as PPO-I, PPO-II and PPO-III. Molecular weight of purified proteins were estimated with 48 kilo Dalton (kDa) (PPO-I), 67 kDa (PPO-II) and 74 kDa (PPO-III). The enzyme activity of PPO-I, PPO-II and PPO-III were 145 U/mg, 5195 U/mg and 8451 U/mg respectively. PPOII and PPO–III were taken for further studies because of high specific activity. Isoelectric point (pI) was determined for PPO-II (5.7) and PPO-III (6.2). The isoenzymes have shown maximum activity at

pH of 6.8 in 100 mM of sodium phosphate buffer with an optimum temperature of 30±3˚C with a substrate concentration of 10 mM catechin.

PPO also extracted from different sources such as mushroom, fruits and different vegetable materials and enzyme kinetics studies were carried out. It was found that PPO obtained from the above sources exhibited different enzyme kinetics with catechin. The enzymes from tea leaves and mushroom tyrosinase showed good selectivity towards catechin as eluded by kinetic parameters (Km and Vmax). Hence, for further studies on biosensor application, tea isoenzyme PPO-II, PPO-III and mushroom-tyrosinase were selected. Along with these enzymes cross-linked enzymes crystals (CLEC) of laccase (gifted from National Institute for Interdisciplinary Science and Technology, NIIST, formerly Regional Research Laboratory, RRL, Thiruvanathapuram) and commercially available enzymes such as laccase and tyrosinase were taken as standard enzymes for studies on biosensor development.

Section B. Immobilization and stabilization of enzymes for biosensor applications

Immobilization is an essential step in the application of biomolecules in biosensor. Studies were carried out to use different PPO enzymes such as PPO-II and PPO-III from tea leaves, tyrosinase from mushroom and CLEC, along with commercial enzymes tyrosinase and laccase. These enzymes were immobilized on a dialysis membrane by cross linking with glutaraldehyde. Response to catechin by these enzymes was recorded separately using the detector system.

All immobilized enzyme preparations were assessed for their enzyme activities in a biosensor system with catechin substrate (10 mM). Commercial tyrosinase was found to give maximum response (1.0±0.028 V) followed by tyrosinase from mushroom (0.99±0.029 V), tea PPO-III (0.92±0.027 V), tea PPO-II (0.567±0.029 V), CLEC (0.094±0.003 V) and commercial laccase (0.058±0.003 V).

Stabilizing agents such as bovine serum albumin (BSA), gelatin and lysozyme were checked for their ability to enhance the stability of the immobilized enzyme for repeated use in biosensor application. Among these stabilizing agents used stability of immobilized enzymes were found to be high with lysozyme as it was possible to reuse an immobilized enzyme membrane for 80±5 analyses, whereas, BSA gave 55±3 and gelatin 50±5 numbers of repeated analyses of catechin containing samples.

Among the enzymes tested, commercial tyrosinase was found to be most efficient in catalyzing the catechin. Commercial enzyme is not very economical for biosensor development. Therefore, tyrosinase from mushroom and PPO-III were selected for the development of the biosensor. Various physicochemical parameters were standardized for biosensor analysis. The optimum pH, temperature and substrate concentration for biosensor application were found to be 6.8, 30˚C and 10 mM respectively.

With a single immobilized enzyme membrane at the least 100 numbers of catechin containing samples were analyzed.

Section C. Description of biosensor based detector system for the analysis of tea polyphenols

Biosensor system adopted for the study comprised of a biological recognition unit, a signal transducer and a signal processing system. The membrane assembly on the electrode consisted of two polymeric membranes, outer hydrophilic and inner hydrophobic with immobilized enzyme sandwiched in between. This electrode system constructed with a working electrode (gold) and a reference electrode (silver). A prototype of the detector system for the detection of PP in tea was developed in-house. The high precision operational amplifiers were used for the signal conditioning. The response from the analyte was measured by biosensor and given as volts (V). Biosensor was shown good sensitivity and linearity of detection in a range of 0.1 mM to 100 mM.

Section D. Studies on optimization of immobilized tyrosinase enzyme membrane for repeated use in biosensor application

Repeated use of enzyme membrane for PP analysis was limited by strong affinity of PP with protein to form complex which was found to reduce the enzyme activity. Thus attempts were made to prevent this phenomenon using different methods.

Various concentrations of catechin were used to assess the retained PP on immobilized enzyme membrane after 10 numbers of analyses. PP remained in the immobilized enzyme membrane was 5 mgEC/l (0.02 mM), 15 mgEC/l (0.05 mM) and 54.26 mgEC/l (0.19 mM) for 0.1 mM, 1 mM and 10 mM concentration of catechin respectively.

Various amino acids were studied for their affinity toward catechin. Among the various amino acids tested proline (29.00 mgEC/l, 0.09 mM), serine (26.86 mgEC/l, 0.09 mM) and lysine (28.57 mgEC/l, 0.1 mM) were found to have more affinity towards catechin and responsible for formation of protein- PP complexes.

Tyrosinase was immobilized on polymeric membranes having different molecular weight cut off (MWCO) range (3.5 to 14kDa) and membranes with MWCO range of 8 kilo Dalton (kDa) were found to have better suitability in biosensor applications.

Dissociation with methanol, ethanol, mild detergents were tried to remove the bound PP in enzyme membrane surface without affecting the enzyme activity. Co-immobilization with tannase was tried to break down the high molecular weight PP bound to the enzyme membrane during the analysis. These above mentioned methods were found to improve the membrane performance by 15-20%.

Chapter II

Analysis of tea samples and validation of tea biosensor system

Section A. Polyphenol determination in tea samples with tea biosensor

In tea industry only the made tea assessed for the quality. Tea processing involved distinct steps and quality monitoring at each stages will provide a better final product. Proposed system is a promising tool to assess the quality at critical points in tea manufacturing. Extractable total PP during conventional tea making was estimated. Tea samples were assessed for their PP content. Samples priced high in the market was incidentally found to contain more PP than low priced samples.

Section B. Validation of tea biosensor system for polyphenol analysis

Initially, validation of tea biosensor was done by frequent interactions with tea industry experts at Nilgiris, South India. Further tea samples collected from the auction centers and factory were analyzed to grade the tea quality based on PP content. In another study, tea biosensor was taken to the industry and samples were analyzed during the various stages of tea processing.

Tea biosensor was tested at several tea manufacturing units for various grades of tea and results were validated by experienced tea tasters and other standard analytical methods and it was observed in agreement with biosensor analysis.

Tea samples were analysed using the tea biosensor and the data were compared with HPLC, colour measurement system, sensory evaluation and Folin Ciocaltue (FC) assay method. The study indicates that gradation of tea based on the PP content can be a very efficient tool for quality monitoring. It is expected that the tea biosensor will have good commercial potential.

Summary and Conclusions

The results presented in the thesis describe the biosensor development and application of an enzyme based amperometric biosensor for the determination of quality of tea based on PP content.

Among the enzymes tested for their selectivity for biosensor development, tyrosinase enzyme responded well with black and green tea PP. Therefore, tyrosinase was chosen for tea biosensor development. It was observed that protein and PP interactions were a critical parameter for repeated use of enzyme membrane. Browning of enzyme membrane was prevented to a great extent by dissociation of bound PP. Various tea samples were analyzed with the developed amperometric biosensor system. It helps to distinguish various grades of tea based on PP content. Validation of the developed tea biosensor was done with various other available analytical techniques which were in accordance with standard methods of PP estimation.

Further, studies on molecular level of the protein – PP interactions would be useful in designing a more efficient commercial model of tea biosensor. Exploration of other potential enzymes for their substrates in tea and multienzyme biosensor may be an exciting development for tea evaluation. Miniaturization of biosensor may be useful, for field application for testing PP contents in different clones of tea plants, deciding the harvesting time of one bud and two leaves system and at various stages of tea processing for obtaining better quality of tea.

The thesis ends with a list of relevant references cited in the text is arranged in alphabetical order, which gives the details including title, journal year, volume and pagination.

The investigation carried out is compiled and documented in the form of a thesis for the Ph. D. degree and being submitted in the subject area of biotechnology to the University of Mysore, Mysore, India.

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List of Abbreviations

℃	Degree Celsius
ADC	Analogue to digital converter
Ag/AgCl	Silver/Silver cholride
BSA	Bovine serine albumin
C	Catechin
CBB	Coomassie brilliant blue
CFTRI	Central Food Technological Research Institute
CG	Catechin gallate
CLEC	Cross linked enzyme crystals
CMST	Centre for Multidisciplinary Science and Technology
CNBr	Cyanogen bromide
CTC	Cut-Tear-Curl
DEAE	Diethylaminoethane
DMSF	Dimethyl sulfoxide
EC	Epicatechin
ECDG	Epicatechin digallate
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCDG	Epigallocatechindigallate
EGCG	Epigallocatechin gallate
FET	Field effect transistors
GC	Gallocatechin
GCG	Gallocatechin gallate
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
ITC	International ***Tea*** Committee
IU	International unit
IUPAC	International Union of Pure and Applied Chemistry
KCl	Potassiumchloride
kDa	Kilodalton

Kgs	Kilograms
LCD	Liquid crystal display
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimoles
Mn - SOD	Manganese containing superoxide dismutase
mV	Millivolts
NaCl	Sodium cholride
NaPi	Sodium phosphate
nm	Nanometre
OD	Optical density
op-amp	Operational amplifier
PAGE	Polyacrylamide gel electrophoresis
PBSA	Protein based stabilizing agent
PCA	Principle component analysis
PDA	Photodiode array
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PIC	Peripheral interchange controller
PMSF	Phenylmethylsulfonylfluouride
PO	Peroxidase
PP	Polyphenols
PPO	Polyphenol Oxidase
PVPP	Polyvinyl polypyrrolidone
QDA	Quantitative Descriptive Analysis
RAM	Random Access Memory
RPM	Rotation per minute
SAW	Surface aquatic wave
SCB	Signal conditioning board
SDS	Sodium dodecyl sulphate-

TCA	Trichloro acetic acid
TEMED	Tetramethylethylenediamine
TF	Theaflavin(s)
TF3′G	Theaflavin 3′-gallate
TF3G	Theaflavin 3-gallate
TFA	Ttrifluoroacetic acid
TFDG	Theaflavin 3,3′-gallate
TR	Thearubigin(s)
USA	United States of America
UV	Ultraviolet
µL	microlitre
V	Volt(s)

1. Introduction

Tea is the most preferred beverage around the globe. Polyphenols (PP) constitutes about 30% of dry weight basis of tea leaves (Ho *et al.,* 1994; Hollman and Arts, 2000). Major quality attributes of tea are derived from PP. It is also gaining popularity due to its proven potential as an antioxidant, anti-cancerous and also known to reduce the low density lipoprotein in blood (Wei *et al.,* 2003; Luo *et al.,* 2006). Determination of PP is vital in assessing the quality and monitoring the efficiency of tea processing. Many analytical techniques are reported for the analysis of natural and synthetic phenols in food, agriculture and environmental samples (Kammerer *et al.*, 2004; Gu *et al.,* 2007; Sakakibara *et al.,* 2003).. Biosensors are becoming the most preferred techniques in clinical, pharmaceutical, health, food and environmental monitoring for their rapidity, specificity and amenability for field application.

This chapter overview a thorough review of relevant literature cited on the topic of investigation. The first part of this review of literature details a brief history and origin of tea, varieties of tea, tea processing, nutraceutical importance and its analysis, tea grades and tea tasting. Enzyme purification, application of enzymes, immobilization, stabilization of enzyme, the history and development of biosensor is described in the middle part of the review and the later part of the review deals with various conventional analytical methods, application of various enzyme based biosensors for PP analysis. Other topics discussed include protein-PP interactions and complex formation. Significance of biosensors in analysis and its market perspectives also reviewed.

2. History of tea

Tea originated in China (Dodd, 1994). Discovery of tea is traced back to nearly 5,000 years and was accidental, in 2737 B.C. by second emperor of China, Shen Nong (Chen and Sheng, 1981; Hara *et al*., 1995; Harbowy and Balentine, 1997). First written documentation about tea appeared in a Chinese dictionary as "Erh Ya" in 350 AD. Sooner or later people recognized tea as a medicinal beverage and a cultivation processes started during 400 - 600 AD.

China is the hometown of tea plantation, processing and drinking. There are some records suggesting that tea processing and making into a drinking became a part of daily life in China as early as 2200 years ago (Chen and Shen, 1981). China is also the second largest tea exporter of the world, exporting 252.3 thousand ton and earning 332 million US$ in 2002 (Wu, 2003). Tea cultivation in China progressively increased in last one decade.

The East India Company started the first tea plantations in Assam, India in 1835. By 1838, first tea obtained from Chinese tea cultivar from Indian soil, was sold in the Indian market. In 1856, tea is planted in and around Darjeeling, India. Twinings of England began to blend tea in 1870 for uniformity. By 1910 Sumatra, Indonesia becomes a cultivator and exporter of tea followed by Kenya and parts of Africa (Chen, 1981). Presently, it is cultivated in >30 countries around the world.

Today, tea is the most common beverage second only to water in worldwide consumption with per capita consumption of approximately 120 mL/day (Van der Wal, 2008; Katiyar and Mukhtar, 1996; Ahmad *et al*., 1998). Total world production of tea is 3721.16 M.Kgs. India has been leading producer of tea since 150 years but by 2006 China over took India in tea production (Table 1.1). There are as much as > 150 varieties of tea available in the market (Gulcubuk *et al.,* 2003).

Table 1. World tea production in M.Kgs (Adapted from Supplement to Annual Bulletin of Statistics-2008, ITC London)

Country	2008 (P)	2007	2006	2005
China	1160.00	1140.00	1028.06	934.86
India	980.82	986.43	981.81	945.97
Sri Lanka	317.70	304.61	310.82	345.82
Kenya	317.20	369.61	310.58	323.50
Turkey	155.00	178.00	142.00	135.00
Indonesia	148.31	149.51	140.05	156.27
Vietnam	144.00	148.27	142.50	133.35
Bangladesh	58.75	57.96	53.27	60.60
Malawi	41.64	48.14	45.01	37.98
Uganda	42.75	44.91	36.73	37.73
Tanzania	31.61	34.86	31.35	30.36
Others	323.38	340.64	350.48	344.77

P-projected

Tea plant is an evergreen perennial shrub (Bokuchava and Skobeleva, 1969). Tea plant come under Theaceae family and named as *Camellia sinensis,* Lin. (Jones, 1998; Hara *et al*., 1995). Scientific classification of tea is given in Table 1.2. *C. sinensis* is native to Southeast Asia. It can grow to heights up to 30 feet, but is usually pruned to 2-5 feet for the convenience of plucking of leaves. Tea plant cultivated and preferentially picked as young shoots.

Table 2. Classification of tea plant

Division	Angiospermae
Class	Dicotyledones
Order	Parietales
Family	Theaceae
Genus	Camellia
species	Sinensis

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Black tea in the classical sense refers to the tea that is processed from "the green leaves, buds or tender shoots of the tea bush and fermented"

(Schultert and Gunther, 1998). The quality of black tea is primarily determined by factors mainly consisting of the following four aspects. Firstly, genetic factors such as clones or varieties of the tea bushes (Millin, 1987). Secondly, there are environmental factors such as altitude, climate and soil in the growing region (Schultert and Gunther, 1998; Millin, 1987). Thirdly, a number of agronomic factors such as farm management, harvesting and fertilizer (Millin, 1987). Fourthly, factory practices (Schultert and Gunther, 1998; Millin, 1987; Roberts and Chandradasa, 1982; Cloughley and Ellis, 1980). Of these four factors, the first and the second are generally considered as uncontrollable factors for tea production, whereas the third and the fourth are controllable factors. Therefore, once the other quality factors (genetic, environmental and agronomic) have been more or less fixed, the processing or factory practice of tea production is considered a critical factor for producing high quality black tea.

3. Polyphenols

In plants, PP play a vital role in various physiological processes, which involves, in plant morphology, growth, reproduction and defense (Nahrstedt, 1989; Haslam, 1989; Mehansho *et al.,* 1987; Robbins *et al.,* 1987; Feeny, 1976, 1975, 1970; Feeny and Bostock, 1968). PPs are distributed in the leaves, bark and fruit of many higher plants (Harborne, 1993). When incision or rupture occurs in the plant tissue, PP undergoes biochemical reactions, rendering the tissues unpalatable and bitter. The production of polyphenolic constituents in the tea plant is assumed to be a means of chemical defense against insects, birds and animals, which would consume the plant as food (Beart *et al*., 1985). PP are described as astringent and it is generally held that their most important characteristic is their ability to form complexes with proteins, polysaccharides and alkaloids (e.g. caffeine) (Spencer *et al.,* 1988; McManus *et al.,* 1985; Hagerman and Butler, 1981). It is believed that the efficacy of many herbal and

folk medicines may be attributed to the astringent properties of the PPs that they contain (Haslam *et al.,* 1989).

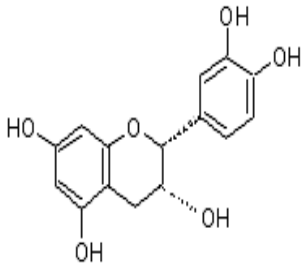
3.1. Tea polyphenols

The PP found in tea is more commonly known as flavanols or catechins. Flavonoids, which were previously called tea tannins (Bokuchava and Skobeleva, 1969) and also are referred to as tea PP (Harbowy and Balentine, 1997; Bravo, 1998; Xiao, 1994; Hertog, 1993; Graham, 1992; Baruah *et al*., 1986; Sanderson, 1972) are main natural phenolic compounds comprising 20-40% of dry matter in young shoots of tea plants. Naturally occurring flavonoids are generally classified into six classes according to their chemical structures (Peterson and Dwyer, 1998) including flavanones, flavones, isoflavonoids, flavans (flavanols), anthocyanins and flavonols. Flavonoids are a group of polyphenolic compounds, diverse in chemical structure and characteristics.

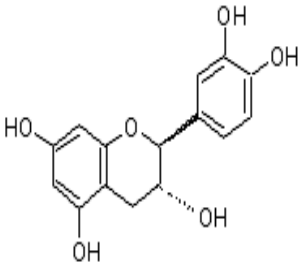
3.1.1. Green tea polyphenols

Green tea is made without enzymatic oxidation of PPs, as enzyme (Polyphenoloxidase; PPO) is inactivated by heat during the early stages of green tea processing (Hara *et al*., 1995). In a more specific sense, catechins found to be in tea leaves namely (+)-catechin (C), (-)- epicatechin (EC), (+)-gallocatechin (GC), (-)- epigallocatechin(EGC), while catechin gallates include epicatechin gallate (ECG), (-)-catechin gallate (CG), (-)- gallocatechin gallate (GCG) and (-)- epigallocatechin gallate (EGCG), (Figure 1.1) with the latter being the highest in concentration (Arts *et al*., 2000; Ahmad *et al.,* 1998; Katiyar and Mukhtar, 1996; Hara *et al*., 1995; Hilton *et al*., 1973; Forrest and Bendall, 1969). Two minor catechin digallates, epicatechin digallate (ECDG) and epigallocatechin digallate (EGCDG) (Hashimoto *et al*., 1987; Nonaka *et al*., 1983; Coxon *et al*., 1972) have also been considered as catechin gallates (Opie, 1992). The four most common catechins and catechin gallates are EGCG, EGC,

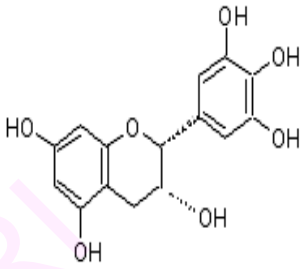
ECG, and EC. The methyl esters of ECG and EGCG were also identified in tea by Zeeb *et al*., (2000). The chemical constituents of PP in tea shoot were shown to be (% of total PP): C 0.4; EC 1.3; GC 2.0; EGC 12.0; ECG 18.1; EGCG 58.1; while the other PP were 6.67 % (Graham, 1992; Opie *et al*., 1988; Hilton, 1973; Bokuchava and Skobeleva, 1969). Seasonal variations in the content of PP in some jats (cultivars) and clones grown in north eastern India have been observed (Singh *et al*., 1999; Bhatia and Ullah, 1968). Towards the end of a plucking season, the total content of PP and the oxidase activity of Assam tea leaf tend to fall (Wood *et al*., 1964). An increase in the content of catechins and catechin gallates in raw tea leaf in summer months is due mostly to an active synthesis of EGCG and ECG (Bokuchava and Skobeleva, 1969). Other flavonoids include anthocyanins, flavonols (quercetin, kaemperol, myricetin) and their glycosides (Rio *et al*., 2004; Scharbert *et al*., 2004; Lakenbrink *et al.,* 2000; Engelhardt *et al*., 1993 and 1992; Finger *et al*., 1992 and 1991; McDowell *et al*., 1990), flavones (Kiehe and Engelhardt, 1996; Engelhardt *et al*., 1993; Chaboud *et al*., 1986), proanthocyanidins (Lakenbrink *et al*., 1999; Kiehne *et al*., 1997; Hashimoto *et al*.,1989; Nonaka *et al*., 1984, 1983) and phenolic acids (gallic acid, chlorogenic acid and theogallin) (Shao *et al*., 1995; Kuhr and Engelhardt, 1991; Bailey *et al*., 1990; Ullah and Jain, 1980; Roberts and Myers, 1958; Cartwright and Roberts, 1954).



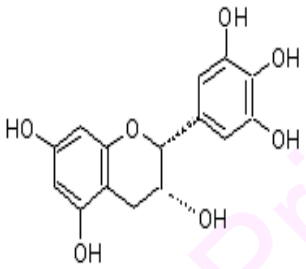
Catechin



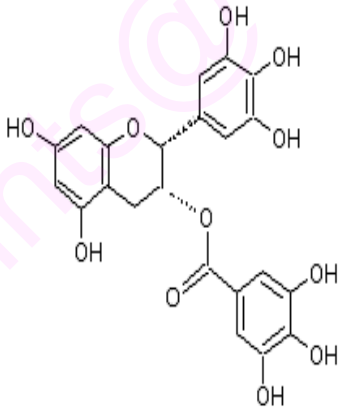
Epicatechin



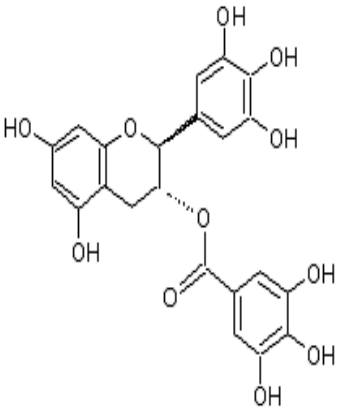
Gallocatechin



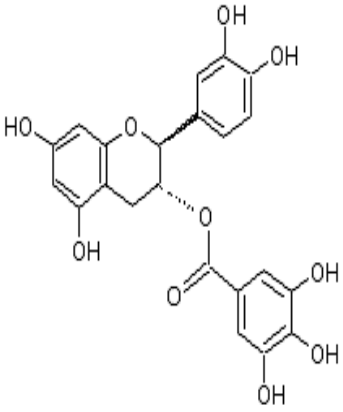
Epigallocatechin



Gallocatechin gallate



Epigallocatechin gallate



Epicatechin gallate

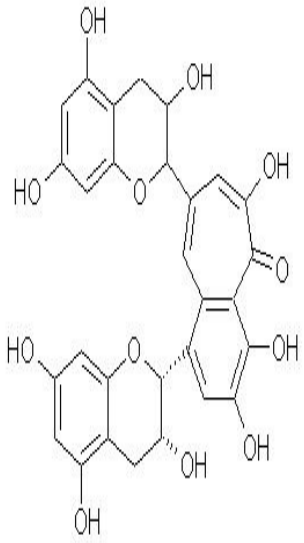
Figure 1. Green tea polyphenols

3.1.2. Black tea polyphenols

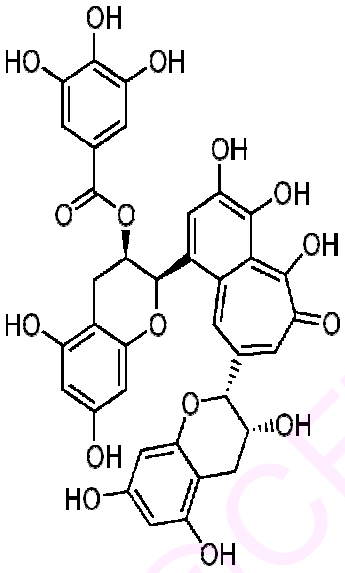
black tea is one of the major tea products, accounting for more than 75% of world tea production. Due to the key role of the polyphenol oxidase, the fermentation is designed to achieve optimal oxidation of the tea catechins, mainly epigallocatechin-3-gallate, epigallocatechin, epicatechin and epicatechin-3-gallate, yielding a diversity of reaction products which are believed to contribute to black tea quality (Roberts, 1958; Millin *et al.,* 1969). Among these fermentation products, the so-called theaflavins, consisting of a benzotropolone nucleus, are known to be formed by enzymatic oxidation and condensation of the di-hydroxylated B ring of a catechin and the tri-hydroxylated B ring of another flavan-3-ole molecule.

PPs occurring in black tea usually consist of residual green tea PP such as catechins (Ding *et al*., 1992; Bailey *et al*., 1990) flavonols (Bailey *et al*., 1990; McDowell *et al*., 1990) and oxidation products of green tea PP such as theaflavins (TF) and thearubigins (TR) (Subramanian, 1999; Harbowy and Balentine, 1997). Some catechins and catechin gallates may be epimerised or degallated during the processing of black tea; thus, it is possible to increase the levels of gallic acid and isomers of the catechins (Coggon *et al*., 1973).

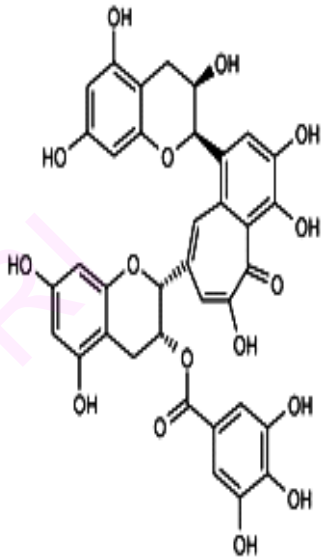
Green tea PPs are oxidized and dimerized during the manufacture of black tea and oolong tea to form orange-red pigments, TF, a mixture of theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3'-gallate (TF_2B) and theaflavin-3,3'-digallate (TF_3), Figure 1.2. TF contribute to the characteristic bright orange - red color of water extract of black tea (Balentine, 1992; Hertog, 1992).



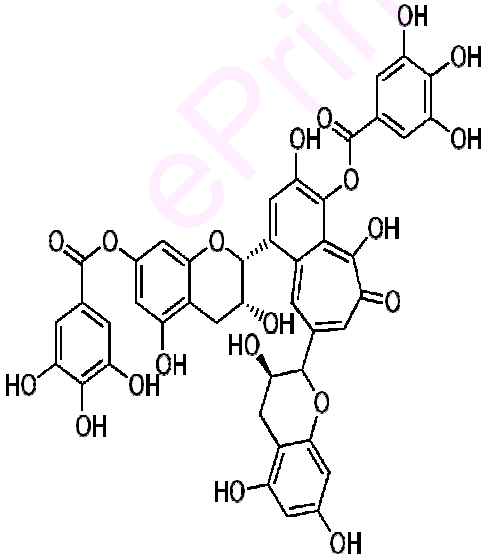
Theaflavin



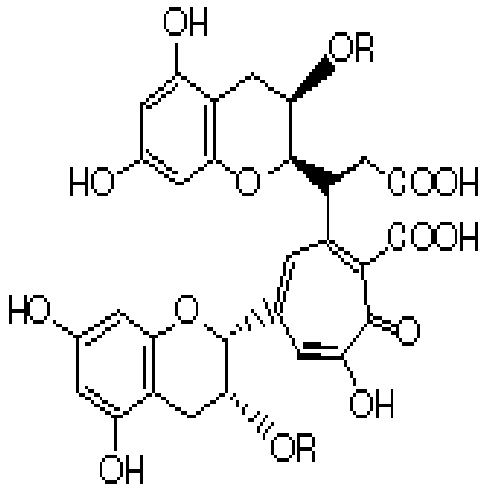
Theaflavin-3-gallate



Theaflavin-3'-gallate



Theaflavin-3-3'-digallate



R= Galloyl or other groups

Thearubigin

Figure 2. Black tea polyphenols

TR, the structures of which have not yet been fully elucidated, is a group of polymerized oxidation products of tea catechins and their gallates (Lakenbrink *et al*., 2000; Bailey *et al*., 1991; Roberts, 1962).

4. Tea processing

The types of tea are distinguished by the processing they undergo (Hara *et al*., 1995; Millin *et al*., 1987; Bhatia and Ullah, 1962). In its most general form, tea processing involves oxidizing the leaves, stopping the oxidation, forming the tea and drying it (Owuor and Langat, 1988).

Of these steps, the degree of oxidation plays a significant role of determining the final quality of the black tea, while curing and leaf breakage contributes to depreciation in the amount of flavor. Tea is consumed in different forms, namely, white, yellow, oolong, green, reprocessed and black tea depending on post - harvest treatment and chemical components (Hara *et al*., 1995). Of the total amount of tea produced and consumed in the world, 78% is black, 20% is green and <2% is oolong tea (ERF, 1985). Various kinds of tea based on their process methodology has briefed in Table 1.3.

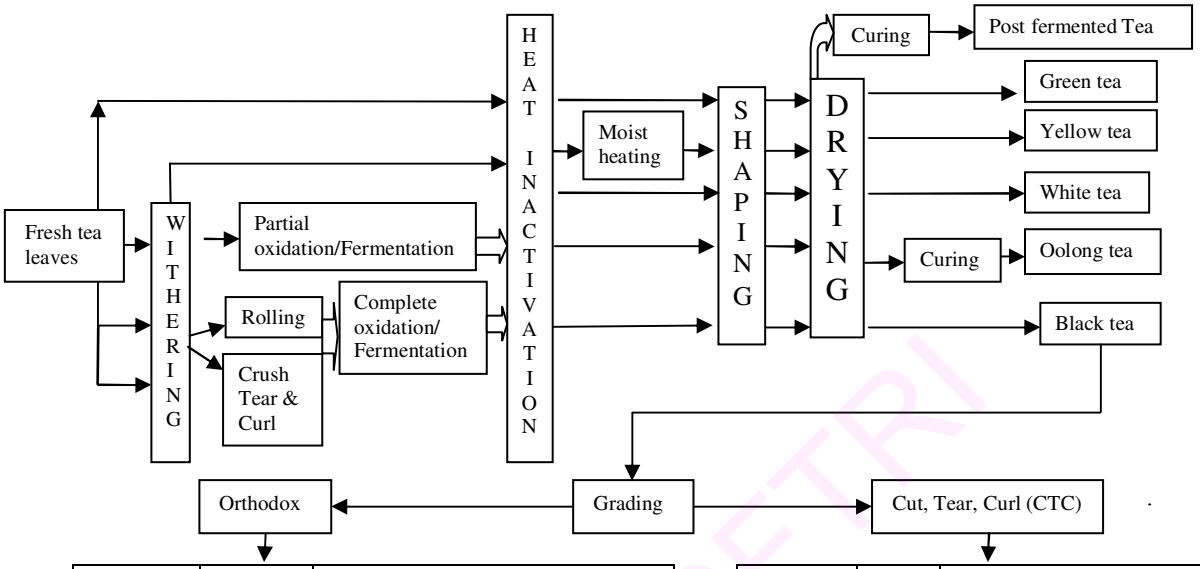
Table 3. Type specific processing of tea

White tea	Young leaves (new growth buds) that have undergone no oxidation; the buds may be shielded from sunlight to prevent formation of chlorophyll.
Green tea	The oxidation process is stopped after a minimal amount of oxidation by application of heat, either with steam or by dry cooking in hot pans.
Oolong Tea	Oxidation is stopped somewhere between the standards for green tea and black tea.
Black tea/Red tea	The tea leaves are allowed to completely oxidize. Black tea is the most common form of tea. Black tea is further classified as either *orthodox* or as *CTC.*

Post-fermented tea	Teas that undergo a second oxidation, such as Pu-erh, Liu'an, and Liubao, are collectively referred to as secondary or post-fermentation teas in English.
Yellow tea	Either used as a name of special tea processed similarly to green tea or high quality tea.

4.1. Steps of black tea processing

For the tea production the tea leaves undergo the different processes which are crucial for quality improvement (Yamanishi *et al.,* 1966). Schematic diagram of tea processing is given in Figure 3.



Whole leaf	FP	Flowery Pekoe
	FTGFOP	Fine Tippy Golden Orange Pekoe
	TGFOP	Tippy Golden Orange Pekoe
	TGFOP 1	Tippy Golden Orange Pekoe one
	GFOP	Golden Flowery Orange Pekoe
	FOP	Flowery Orange Pekoe
	OP	Orange Pekoe
Broken	BOP1	Broken Orange Pekoe one
	GFBOP	Golden Flowery Broken Orange Pekoe
	BPS	Broken Pekoe Souchong
	GBOP	Golden Broken Orange Pekoe
	FBOP	Flowery Broken Orange Pekoe
	BOP	Broken Orange Pekoe
Fanning's	GOF	Golden Orange Fanning's
	FOF	Flowery Orange Fanning's
	BOPF	Broken Orange Pekoe Fanning's
Dust	OPD	Orange Pekoe Dust
	OCD	Orange Churamani Dust
	BOPD	Broken Orange Pekoe Dust
	BOPFD	Broken Orange Pekoe Fine Dust
	FD	Fine Dust
	D - A	Dust - A
	S D	Special Dust
	G D	Golden Dust

Broken	PEK	Pekoe
	BP	Broken Pekoe
	BOP	Broken Orange Pekoe
	BPS	Broken Pekoe Souchong
	BP 1	Broken Pekoe 1
	FP 1	Flowery Pekoe
Fanning's	OF	Orange Fanning's
	PF	Pekoe Fanning's
	PF 1	Pekoe Fanning's One
	BOPF	Broken Orange Pekoe Fanning's
	PD	Pekoe Dust
	D	Dust
	CD	Churamani Dust
	PD 1	Pekoe Dust One
	D 1	Dust One
Dust	RD	Red Dust
	FD	Fine Dust
	SFD	Super Fine Dust
	RD 1	Red Dust
	GD	Golden Dust
	SRD	Super Red Dust

Figure 3. Steps in the processing of tea

4.1.1. Picking/plucking

Tea leaves or flush, which includes a terminal bud and two young leaves are plucked. Despite marked changes in the quantities of PP, the relative proportions of certain important PP, such as EGCG, ECG and EGC do not deviate much from their mean values (Bhatia, 1963). Thus, maintaining the plucking criteria for a particular type of tea would provide a consistent basis for making good quality tea.

4.1.2. Wilting/withering

Wilting is used to remove excess water from the leaves and allows a very light amount of oxidation (Owuor *et al.,* 1989). The primary role of withering is to reduce the moisture content of the fresh leaves, making them amenable to subsequent processing steps. In addition, diverse and important biochemical changes occur during withering (Hara *et al*., 1995). The level of total solids reduces slightly during the withering because of the respiration of green leaves (Wood *et al*., 1964). Some important compounds are developed during withering and these compounds may ultimately influence the flavour, aroma and/or the character of the tea brew (Hara *et al*., 1995; Opie, 1992; Takeo, 1984). Effect of withering was further detailed by physical and artificial withering (Owuor et al., 1987; Owuor and Orchard, 1989).

4.1.3. Bruising/Rolling or Cut-Tear-Curl (CTC)

During rolling of the tea leaves, the leaves are macerated and the cell structures are disrupted, which brings various enzymes into intimate contact with their substrates the PP. Rolling of tea leaves may be accomplished by orthodox rollers (e.g. rotorvanes), Lawrie Tea Processor (LTP) or CTC machines, a production method developed about 1932.

4.1.4. Oxidation/Fermentation

The principal reaction in fermentation is the oxidation of catechins and catechin gallates by the enzyme PPO, together with other enzymatic (e.g. peroxidase) and nonenzymatic reactions to form the unique character of black tea (Hara *et al*., 1995). Important reaction during this stage is the development of colour, strength and quality of tea brews from the production of non-volatile compounds through the enzymatic oxidation of catechins and their gallates (Owuor and Reeves, 1986). Thus, fermentation is the critical step in black tea processing and the chemical and biochemical reactions are the most complicated ones in tea processing, (Roberts, 1957; Roberts and Russell, 1957; Roberts *et al*., 1957). Fermentation time is an important parameter to determine the quality of black time (Owuor *et al.,* 1994).

4.1.5. Shaping

The damp tea leaves are then rolled to be formed into wrinkle strips.

4.1.6. Drying

Drying or firing of fermented tea leaves (dhool) is primarily intended to cause cessation of enzyme activity and reduce the moisture content to about 3% of the dry mass so that the tea can be stored (Hara *et al*., 1995). Endless chain pressure driers have been used by the industry for many years. Changes other than removal of moisture that occur during drying include a significant loss of volatile compounds, an increase in the levels of amino acids, the binding of PP to other tea components and an increase in carboxylic acids and Maillard reactions. Firing at an elevated temperature is necessary for the development of the taste, colour and aroma of black tea (Hara *et al*., 1995).

4.1.7. Curing

While not always required, some tea required additional aging, secondary-fermentation or baking to reach their drinking potential.

4.1.8. Sorting and grading of tea leaf

After drying tea contains different sized particle, which used sort to even sized by electrostatically charged rollers, which preferentially attract fibers and stalks (Hampton, 1992). This is an important stage for the marketing of tea, ensuring the correct particle size, shape and cleanliness. Different grades of the black tea are shown in Figure 3.

5. Blending

In most cases, tea is traded in bulk by tea traders in the consuming countries. The blenders then blend the tea to suit their market demand. Once bought by the blender, tea loses all identity as to its original source (Othieno and Owuor, 1984). The quality of the blend is dictated by two main factors: the market demand that prevails and the profit that the blender can maximize.

6. Nutritional and health benefits of tea

First authorized mention regarding the positive effects of drinking tea on human health and the benefits of growing tea plant made by Confucius (Kaptangil, 1993).

Health benefits of tea consumption have been intensively investigated and a fast growing body of scientific research in the last decade indicates a role for tea in the promotion of health and prevention of disease (Blumberg, 2003). The antioxidant, anticarcinogenic, antimutagenic and anti-microbial functions of tea have been repeatedly confirmed *in vitro* and in animal models and are

principally attributed to the rich contents of catechins or their oxidation derivatives TFs and TRs (Higdon and Frei, 2003; Lambert and Yang, 2003; McKay and Blumberg, 2002; Trevisanato and Kim, 2000; Yang and Landau, 2000; Rice-Evans *et al*., 1997). As a unique free amino acid in tea, theanine possesses human health promoting features such as improving immunity (Kamath *et al*., 2003) producing a relaxation effect, lowering blood pressure and improving memory and learning abilities (Juneja *et al*., 1999).

7. Enzymes involved in tea processing

Tea leaves constitutes various enzymes. Major enzymes present in tea leaves are mentioned below.

7.1. β-primeverosidase

β - primeverosidase present in tea (*Camellia sinensis*) plants is a unique disaccharide - specific glycosidase, which hydrolyzes aroma precursors of β - primeverosides (6 - *o* - β-d-xylopyranosyl- β - d - glucopyranosides) to liberate various aroma compounds and the enzyme is deeply concerned with the floral aroma formation in oolong tea and black tea during the manufacturing process (Mizutani *et al.,* 2002; Coggon *et al.,* 1973; Sanderson *et al.,* 1972).

7.2. β-glucosidases

These enzymes are important in the formation of floral tea aroma and the development of resistance against pathogens and herbivores in tea plants (Li *et al.,* 2005).

7.3. Polyphenoloxidase (PPO)

Enzyme present in tea leaves and is responsible for the formation of brown compounds known as TF and TR were studied in fermented or black tea (Halder *et al.,* 1998; Gregory and Bendall, 1966). PPO assisted processes result in the formation of black tea taste, liquor and flavor and they are connected with oxidative transformation of phenolics compounds (Goodsall *et al*., 2000). Phenol oxidase is the principle enzyme determining the rate and direction of these fermentative processes. The black tea producing technology is based on the action of this enzyme (Subramanian *et al*., 1999).

7.4. Peroxidase (PO)

Enhancing the polymerization of reactions during the tea processing and determine the antioxidant potential of tea (Kvaratskhelia *et al.,* 1997).

Other major enzymes found in tea leaves are cytochrome oxidase (Roberts, 1940), ascorbate peroxidase (Chen and Asada, 1989), β - galactosidase (Halder and Baduri, 1997) and manganese containing superoxide dismutase (Mn - SOD) (Vyas and Kumar, 2005).

8. Potential enzymes for tea biosensor development

8.1. Tyrosinase

Tyrosinase (E.C.1.14.18.1, monophenol monooxygenase and E.C. 1.10.3.1, *o*-diphenol oxidoreductase or catechol oxidase) is widely distributed throughout the phylogenetic scale from bacteria to mammals and even present different characteristics in different organs of the same organisms, such as in roots and leaves of higher plants. It is well known that tyrosinase catalyses two different oxygen - dependent reactions that occur consequently: the *o* -

hydroxylation of monophenols to yield *o* - diphenols (cresolase activity) and the subsequent oxidation of *o* - diphenols to *o* - quinones (catecholase activity) (Durán *et al*., 2002).

8.2. Laccase

Laccase (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) is a cupro - protein belonging to a small group of enzymes denominated blue oxidases. Laccase catalyzes the oxidation of various aromatic compounds; phenolic dyes, phenols, chlorophenols, lignin-related *p*-phenylenediamines, organophosphorus and non-phenolic beta-*o*-lignin model dimer with the concomitant reduction of oxygen to water (Durán *et al*, 2002). In a typical laccase reaction, a phenolic substrate is subjected to an electron oxidation giving rise to an oxyradical. This active species can be converted to a quinone in the second stage of oxidation. The quinone as well as the free radical product undergoes non-enzymatic coupling reactions leading to polymerization (Durán *et al*., 2002).

8.3. Tea polyphenol oxidase

PPO is characterized by the ability to act as a catalyst for two different reactions: monophenol hydroxylation into *o*-diphenols (hydroxylase activity) and dehydrogenation of *o*-dioxy substituted PP (catechol oxidase activity) (Fenoll *et al*., 2000; Rodriguez- Lopez *et al*., 2000; Mayer, 1987). The currently accepted enzyme nomenclature classifies hydroxylating phenol oxidase as monophenol monooxygenase (EC. 1.14.18.1) and *o*-diphenols oxidizing phenol oxidase as catechol oxidase (CO; EC 1.10. 3.1) (Rompel *et al*., 1999). Monophenol monooxygenase catalyzes hydroxylation of monophenols. Tsushida and Takeo, (1981) demonstrated that tea leaf phenol oxidase hydroxylizes monophenols into *o*-diphenols. Phenol oxidase displaying dehydrating activity (quinonizing) uses molecular oxygen for oxidation of *o*-diphenols with the formation of the corresponding *o*-quinones and water (Mayer, 1987).

PPO has been widely studied in various fruits and vegetables such as potato tuber (Matheis, 1983; Patil *et a*l., 1965), peach (Koca-cal-skan , 1987 and Jen, 1974), Apple (Oktay, 1995; Flurkey *et al.*, 1980), apricot (Janovitz-Klapp, 1990), banana (El-Tabey , 1949; Palmer, 1963; Kahn, 1985), grape (Galeazzi *et al.*, 1981; Cash, 1976), pear (Valero *et al.*, 1988; Rivas, 1973), green olive (Sapers, 1988), strawberry (Ben-Shalom, 1977), plum (Ebelling *et al.*, 1990), kiwi (Siddiq, *et al.*, 1992) and mango (Park *et al.*, 1985).

9. Immobilization of polyphenol oxidase

Immobilization of enzymes is very essential step in biosensor development. Several methods have been reported for enzyme immobilization such as, adsorption, ionic binding, covalent binding, cross-linking, matrix entrapment, membrane confinement or the combination of two or more of these methods can be used for immobilization of enzymes (Hartmeier, 1988).

10. Quality assessment of tea

10.1. Tea tasting

Conventionally tea tasters (Figure 4) are assessing the tea quality (Ellis and Nyirendra, 1995). Tea quality evaluation is done at first stage by its color, which is intimately connected to its PP content. Tea is sipped and held it in cheeks; given tea's taste.

Figure 4. Sipping of tea by tea taster for its evaluation

Tea is further moved back into the oral cavity and move it around a little; gives characteristics of its taste, including the sweetness, bitterness or astringency, fullness, lightness, liveliness and stimulatory characteristics, as well as how all of these combine to produce the overall effect. In assessment of tea quality or tea price, professional tea tasters mainly consider the tea liquor characteristics and astringency (Biswas and Biswas, 1971). Colour and astringency are due to PP contents in the tea.

10.2. Polyphenols and tea quality

PP compounds have long been regarded as one of the principal quality parameters or indicators of tea (Ding *et al.*, 1992; Davies, 1983; Owuor, 1982; Ellis and Cloughley, 1981; Deb and Ullah, 1968; Roberts and Smith, 1961). It is quite obvious that the constituents of PP are having great significance in quality aspects of tea from the green tea leaves to made (black) tea (Wright *et al.,* 2000; Obanda *et al.,* 1997; Cloughley, 1980; Hilton and Ellis, 1972; Roberts and Smith, 1963).

The quality of a tea is formed during the growth and development of the tea plant, when the compounds responsible for quality are synthesized (Bokuchava and Skobeleva, 1969). Regression analysis of tasters preferences

for black tea against green tea leaf chemical components showed positive and significant correlations for polyphenol contents (Obanda *et al*., 1997). Wright *et al*., (2000) showed that fresh leaf EC and ECG content together correlated well with the total score of black tea and the total TF content of black tea correlated significantly with the value of the tea. Liang and Xu, (2001) showed that TF makes a greater contribution to the brightness of black tea infusion than theaflavin gallate (TFG).

The quality index [(EGCG+ECG)/EGC] has been found to be directly related to the sensory properties of green tea (Yuan, 1962). Thus, this index has been used as an objective parameter for assisting the evaluation of green tea quality in China (Liang, 1990). A comparison of the polyphenolic profiles of different types of teas shows that, different dominant catechins and catechin gallates occur in green tea and different profiles of TF occur in fermented tea (Shao *et al*., 1995).

The content of TF is far less than that of TR in black tea, but TF are of primary importance to tea quality, since they impart the specific bright and vivid colour to the liquor and further, the ratio of TF to TR has been found to be responsible for the strength of the tea liquor (Bokuchava and Skobeleva, 1969). Good quality tea contains less high molecular weight PP compounds than those of inferior quality. For black tea, a high proportion of extractable PPs may indicate good quality liquor, with astringency and a bright reddish colour. As an aid to judging quality, during the cooling of black tea liquor is referred to by tea tasters as the “cream down” of tea (Bradfield, 1946). This cream consists largely of extractable PP such as TF and TR, and other flavonoids in combination with caffeine. Interactions between caffeine and the PP are primarily responsible for this cream (Collier *et al*., 1972).

Black tea produced from buds has the highest amount of polyphenols, whereas black tea produced from the internodes has the lowest, suggesting tea made from buds possesses higher quality (Stephen-Thanaraj and Seshadri,

1990). In Japan, levels of TF and TR especially their total contents showed high positive correlation with the evaluation of black tea quality (Takeo, 1974). Regardless of tasters and the methods of processing, the quality of north eastern Indian plains black tea depends mainly on briskness, with TF being the main factor for briskness (Biswas *et al*., 1973, 1971). After years of research, Eden (1976) concluded that the dynamics of the production of TF is the most potent single factor in promoting good quality in tea. In addition, Davies (1983) suggested that the content of TF and the percent of extractable solids in a black tea could be used as objective measures of the tea quality. Patterns or levels of phenolic compounds in black tea liquor have been used as means of predicting price and country of origin (McDowell *et al*., 1995, 1991). A good quality tea possessing brightness, briskness, good colour and body may possess a ratio of TF:TR of 1:10 (Deb and Ullah, 1968).

Thus, phenolic compounds, particularly the TF/TR, play an important role in determining the quality of black tea and the use of PP as quality indicators for black tea is discussed below. Roberts and Smith (1963) showed that polyphenols such as TF content is an important chemical compound in determining black tea quality. Hilton and Ellis (1972) and Cloughley (1980) confirmed that there was a close linear regressive relation between TF content and broker's valuation of Central African black tea.

In the quality assessment of black tea, TF analysis shows two important merits: "objective" and "quantitative." These properties of TF analysis enables the comparison and quality monitoring on the black tea produced in different places (e.g. countries) and at different times (e.g. years). Furthermore, Owuor, (1996) found that astringency is a better estimator of tea quality than aroma, with the four main theaflavins, TF_1, TF_2A, TF_2B and TF_3 being contributors to the astringency along with TR. Therefore, TF and TR may be the best choice as a quality indicator for black tea, both in the processing line and the end product.

10.3. Tea quality assessment with conventional methods

Traditional methods for the preparation and determination of PP from tea fresh shoots or manufactured tea have been described by several researchers (Roberts, 1962; Roberts and Smith, 1961; Roberts and Myer, 1958; Roberts *et al*., 1957; 1956) and widely used. The most commonly used methods are paper chromatography (Roberts and Myer, 1958; Roberts *et al*., 1957, 1956; Roberts and Wood, 1953, 1951; Oshima and Nakabayashi, 1953), column chromatography (Whitehead and Temple, 1992; Oshima and Nakabayashi, 1953) and colorimetric measurement (Oshima and Nakabayashi, 1953) or spectrophotometric analysis (Muralidharan, 1997; Takino *et al*., 1967; Roberts and Smith, 1961). All those methods are based on the oxidation and reduction properties of tea PP to form a system of colored mixtures. Other developed analytical techniques have been able to isolate, identify and determine individual PP compounds (Harbowy, 1997) such as high performance liquid chromatography-HPLC (Temple and Clifford, 1997; Bailey *et al*., 1991; Steinhaus and Engelhardt, 1989; Robertson and Bendall, 1983; Roberts *et al*., 1981; Wellum and Kirby, 1981). However, colorimetric methods are still the most practical in the determination of total phenolic compounds, TF and TR (Lakenbrink, 2000; Harbowy, 1997; Bhatia and Ullah, 1968; Bhatia, 1960).

Identification of tea PP was done using multi-wavelength detection and the ultraviolet/visible UV/VIS spectra reported in the literature (Opie *et al*., 1995, 1993, 1990, 1988; Powell *et al*., 1995, 1993; Bailey *et al*., 1994, 1993, 1992, 1991, 1990). The photodiode array (PDA) detector was used in the role of a coupled chromatographic-spectroscopic technique to obtain information about the complex tea liquor was analysed. The retention time and the spectrum of each peak were proved to be very strong evidence for the identity of an unknown compound (Bailey *et al*., 1993, 1990, 1992, 1991; Opie *et al*., 1995, 1993, 1990; Powell *et al*., 1995, 1993). Thus, the total PP content of fresh tea shoots has

been proposed as a reliable parameter for determining the quality of black tea (Obanda *et al*., 1997).

Methods for analyzing tea composition are important in assessing the quality of a tea (Crispin *et al*., 1968). Paper chromatography (Roberts, 1962) and column chromatography (Vuataz *et al*., 1959) were once the best choice for the separation of PP from tea leaf. As soon as HPLC was introduced into the analysis, these techniques were superseded by this much more powerful analytical technique. Normal-phase HPLC (Wedzicha *et al*., 1990; Wedzicha and Donovan, 1989) was used for the separation of TR but did not lead to their identification. However, the reversed-phase separation technique made the identification of TR possible (Bailey and Nursten, 1994). Based on HPLC analysis using the reversed-phase techniques, Bailey *et al*., (1991) classified the TR into three groups: Group I as anthocyanidins; Group II as TF and types I and II resolved TR, and Group III as unresolved TR. The resolved TR were analysed based on their absorption at 460 nm, where most of the pigments show strong absorption. Following on this study, a mixture of phenolic polymers was isolated from TR (Bailey *et al*., 1994, 1992). This brown polymeric mixture is free of protein, caffeine and flavonol glycosides, and is a flavanol polymer with various intermonomer linkages to proanthocyanidin polymers. This brown fraction of TR was designated as the theaflavin fraction (Bailey *et al*., 1994, 1992).

Chemical oxidations of catechins and catechin gallates formed the same resolved and unresolved pigments found in the black tea (Bailey *et al*., 1993). Model fermentation systems (Opie *et al*., 1995, 1993, 1990; Opie, 1992) have been used in combination with HPLC techniques for the classification of TR. The oxidation products were monitored at 450 nm (Powell *et al*., 1993). The proanthocyanidins from the TF fractions of TR were found to contain prodelphinidins, procyanidins and propelargonidins (Powell *et al*., 1995). Temple and Clifford (1997) found that the peaks of the TF and TR of the decaffeinated aqueous extract of black tea remained stable for up to 850 min, thus permitting

an automated HPLC analysis over a certain time. These researchers also found that the use of citric acid to acidify the chromatographic solvents did not produce superior separation to the use of acetic acid in the HPLC analysis, which is not in agreement with the result of Bailey *et al*., (1991) who recommended using citric acid solvents for the HPLC analysis of black tea liquor without being decaffeinated. There are many literatures reported to emphasize the methods for the detection of tea PP and HPLC is the routine method in practice (Brooner and Breecher, 1998).

Hoefler and Coggon (1976) first introduced the HPLC system for the analysis of both black and green tea components. A µBondapak C18 reversed phase packing (10 µm) column was used with an isocratic elution system. This system was improved by Robertson and Bendall (1983) who replaced the HPLC column with a 5 µm Hypersil ODS column. This replacement of column packing materials from a larger pore size (10 µm) to a much smaller size (5 µm) ensured the introduction of the sample close to the column surface and hence, maximum separation efficiency could be achieved (Robertson and Bendall, 1983). These researchers also used the isocratic elution system. Although this system provided good separation of the TF, the quantitative reproducibility was still very poor. The HPLC system provided a very powerful tool for studying black tea chemistry with the addition of photodiode array (PDA) detection. Since then, the system has been used to analyse the products from model *in vitro* fermentation systems used to study TR chemistry (Opie *et al*., 1995, 1993, 1990, 1988; Powell *et al*., 1995, 1993). Further, Bailey *et al.* (1994, 1993, 1992, 1991, 1990) successfully applied this HPLC system to the analysis of the chemical oxidation of green tea PP in a chemical fermentation model, including a comparison with the enzymatic oxidation products in black tea liquor. A method for the analysis was reported, Barroso and van de Werken (1999) for green and black tea composition by capillary electrophoresis wherein an analytical method is described for the analysis of black tea and green tea composition. It is a rapid

method but the sample preparation and expertise in analytical skill, which makes this method not user friendly. Those above mentioned methods are not feasible for the field applications and online monitoring of tea processing.

11. Biological sensors/Biosensor

Biosensor is an analytical device comprising a biological recognition element (e.g. enzyme, receptor, DNA, antibody or microorganism) in intimate contact with an electrochemical, optical, thermal or acoustic signal transducer that together permit analyses of chemical properties or quantities. Biosensors are opto-electronic devices which produce electronic/photonic signals as the result of biological interactions (Rechnitz, 1986). Basically, a biosensor includes a biological receptor linked to an opto-electronic transducer in such a way that biochemical activity is converted into electrical activity (Briggs, 1991).

In biologist's definition, a biosensor is "a device, which translates biological variables such as electric potentials, movement or chemical concentrations into electrical signals".

For chemists "a device that used specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compound, usually by electrical, thermal or optical signals".

According the physicists "a device, which detects, records and transmits information regarding a biological /physiological change or process".

In a general definition "an analytical device, which converts the concentration of the target substance, the analyte, into an electrical signal through a combination of a biological or biologically derived recognition system either integrated within or intimately associated with a suitable physico-chemical transducer" (Lowe and Golfinch, 1983).

"A device that uses specific biochemical reactions mediated by isolated enzymes, immune-systems, tissues, organelles or whole cells to detect chemical compounds, usually by electrical, thermal or optical signals (IUPAC, 1992).

A type of biomolecular probe that measures the presence or the concentration of biological molecules, biological structures, etc...

Biosensor = bioreceptor + transducer

11.1. Evolution of Biosensors (Killard and Smith, 2003)

First Generation: Physical entrapment of biological receptors, fixation of membranes to transducers.

Second Generation: Immobilization of biocompound onto the transducer.

Present Generation: Covalent binding of monolayers of recognition molecules directly to the surface of semiconductor devices

11.2. Biosensor combinations

Enzyme – Substrate: The function of the enzyme is providing selectivity by virtue of its biological affinity for a particular substrate molecule. For example, an enzyme is capable of catalysing a particular reaction of a given substrate even though other isomers of that substrate or similar substrates may be present. Typically, the progress of the enzyme reaction (which is related to the concentration of analyte) is monitored by the rate of formation of product or the disappearance of a reactant.

Receptor- Hormone: Communication of cells and biomolecules with each other established by specific biomolecular interactions. Cell surface contains receptors for this communication. It is facilitated by hormones in the living cells. This complex cell signaling pathways has being carried out by the many thousands of

cell receptors. These receptors are promising tools to understanding metabolic characteristics using in biosensor system as a biorecognition agent.

Antibody – Antigen: Proteins molecules that involved in defense mechanism against disease termed as antibodies. They have unique property of binding with specific molecules, antibodies, which can be a foreign body or an organism. In biosensor analysis, the property of very selective binding of antigen-antibody has been explored. Antigen – antibody biosensor is highly sensitive and they bind extremely strongly with antigen (McCormack, 1998).

DNA biosensor: DNA regulates the expression of genetic information. Study of the interactions of DNA gave information about the process controlling in gene expression and regulation. Nucleic acids are widely in use in bioanalytical systems.

11.3. Biosensor with various transducers

A transducer is converting the biorecognition event into a measurable signal (Figure 5). Typically, this is done by measuring the change that occurs in the bioreceptor reaction. Photometric, potentiometric (based on pH sensor), amperometric (based on Clark sensor) and piezoelectric measures change in mass eg. microbalance based sensors, surface aquatic wave (SAW) device based sensors, conductive: measures conductivity change, capacitive: Measures dielectric constant eg: antibody sensors, thermometric: measures temperature eg: enzyme thermistor, field effect transistors (FET) (Lowe, 1985).

Two types of biosensors are commonly used: enzyme-based metabolic biosensors and binding or bioaffinity biosensors. Enzymatic biosensors use enzymatic or metabolic processes to detect the product of the reaction which occurs between the incoming agent (substrate) and the immobilized receiver (e.g. an enzyme). Enzyme-based biosensors are best exemplified by enzyme

electrodes, which are devices which utilize standard electrodes able to detect dissolved gases (such as oxygen) or chemicals (such as urea) electronically. When enzymes attached to the electrodes, catalyze a reaction, a gas or chemical is produced. This chemical or gas is detected by a specific electrode, for example, oxygen or ammonia electrode. Perhaps the best known examples of enzyme electrodes are those which contain glucose oxidase or urease. They can be used to measure, respectively, glucose or urea, as well as to detect end products of multi-enzyme systems (for detection of other substrates). Such enzyme electrodes are well-defined and many are commercially available (Vadgama, 1981).

Bioaffinity sensors relay on biological binding events for detection of substances of interest (Taylor, 1986). The binding of the environmental substance (ligand) to the immobilized receptor produces a detectable change in the shape or conformation of the receptor and this produces an output signal. Detection of this change can utilize one of the methodologies, including optical (interference, refractive index and fluorescence), mechanical (mass or density) or temperature changes.

Until the present time, only antibodies or antigens have been used successfully for bioaffinity sensors. Thakur and Karanth (2003) and Turner (1988) have reviewed a variety of biosensors which have been successfully applied to industrial monitoring and control.

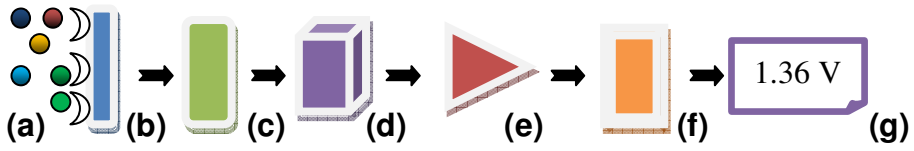


Figure 5. Schematic diagram of the working principle of biosensor

a) Analytes – natural, clinical or industrial
b) Bioreceptor- Enzyme, receptor, antibody, nucleic acid, cell
c) Measurand-Electron, heat, ions, mass, gas
d) Transducer-Electrochemical, piezoelectric, thermal, optical
e) Amplifier
f) Processor
g) Display

11.3.1. Electrochemical probes

Amperometric: Amperometry is the measurement of the diffusion boundary current at a constant potential between a polarized working electrode and reference electrode. Amperometric sensors use transference processes of ionic to electronic charges between electroactive species and electrode, produced in a redox reaction.

Potentiometric: Potentiometric biosensors are not as widely employed as their amperometric counterparts. Such biosensors must normally utilize the movement of small molecules such as gases or ions into or across permselective layers as was illustrated for the urea biosensor

11.4. Criteria of biosensor

1. Selection of a suitable bioreceptor molecule
2. Selection of a suitable immobilization method
3. Selection of a suitable transducer
4. Designing of biosensor considering measurement range, linearity, and minimization of interference
5. Packaging of biosensor

Points to design commercially successful biosensors

1. Relevance of output signal to measurement environment
2. Accuracy and repeatability
3. Sensitivity and resolution
4. Dynamic range
5. Speed of response
6. Insensitivity to temperature or temperature compensation
7. Insensitive to electrical and other environmental interference
8. Amenable to testing and calibration
9. Reliability and self-checking capability
10. Physical robustness
11. Service requirements
12. Capital cost
13. Running costs and life
14. Acceptability by user
15. Product safety-sample host system must not be contaminated by sensor

11.5. Enzyme based biosensors

The enzyme electrode is a combination of any electrochemical probe (amperometric, potentiometric or conductimetric) with a thin layer (10 – 200 µm) of immobilized enzyme. In these devices, the function of the enzyme is providing selectivity by virtue of its biological affinity for a particular substrate molecule. For example, an enzyme is capable of catalyzing a particular reaction of a given substrate even though other isomers of that substrate or similar substrates may be present. Typically, the progress of the enzyme reaction (which is related to the concentration of analyte) is monitored by the rate of formation of product or the disappearance of a reactant. If either the product or reactant is electro active, then the progress of the reaction can be monitored

directly using amperometry. In this technique, current flow is measured in response to an applied voltage (Marko - *Varga et al.,* 1995).

The final method of analysis used will ultimately depend on several properties of the enzyme. The main considerations are;

1. Does the enzyme contain redox active groups
2. Are the products of the biochemical reaction electroactive
3. Is one of the substrates or cofactors electroactive
4. What is the required speed of response
5. What will be the final application of the sensor

The answer to the first three criteria will depend largely on the system under investigation. The answer to the latter three depends on the requirements and application of the sensor under consideration. If the enzyme does not contain any redox groups, then the method of analysis will be restricted to monitoring either the release of products or the consumption of substrate by their reaction at the transducing electrode. The current produced can then be related to the concentration of analyte.

11.6. Polyphenol biosensor

The importance of using biosensors for environmental surveillance becomes more prevalent in literature with the emphasis to phenol determination and control (Rogers, 1995). Application of bioanalytical membrane electrodes for phenolic determination is extensively reviewed by Ciucu *et al.,* 1991. Many biosensors have been developed in the past using the catalytic activity of the redox enzymes for phenol determination. Biosensors are developed with enzymes such as tyrosinase, peroxidase, laccase, etc., (Duran and Esposito, 2000) using different electrode materials, flow systems and sample pretreatment techniques.

Vianello *et al.,* (2004) describes about a biosensor developed with laccases from various sources were tested and laccase from *Rigidoporus*

lignosus was found to be the most active towards syringaldazine and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), which are typical substrates of this class of enzymes and towards the phenols found in olive oil mill wastewaters. Cristea *et al.,* (2005) was constructed an organic phase enzyme electrode (OPEE) via PPO entrapment within a hydrophilic polypyrrole film electrogenerated on a new bispyrrolic derivative containing a long hydrophilic spacer. Horseradish peroxidase (HRP) is covalently immobilized on a self-assembled monolayer of mercaptopropionic acid on vapor-deposited gold electrode. The electrode allows the PP detection down to 2 mM with a linear relationship up to 25 mM (Imabayashi *et al.,* 2001). PP contents of the different wine samples examined were measured using a tyrosinase biosensor, (Campanella *et al*., 2004).

PPO was immobilized in several conducting copolymer matrices. Three different types of poly (methyl methacrylate-co-methyl thienyl methacrylate) matrices were used to obtain copolymers. Total amount of phenolic compounds in some Turkish red wines was analyzed using these electrodes (Yildiz *et al*., 2006).

11.6.1. Tea Biosensor

Tea processing is a highly complex and each step is so critical to determine its quality. The differences in chemical composition of young shoots result in corresponding quality differences in the processed tea (Hara *et al*., 1995; Nakagawa and Torri, 1964). Intervals of tea plucking affect the final quality of tea (Baruah *et al*., 1986). Thus, good quality black tea is produced from tea leaves from high PP and caffeine contents, relatively low protein content and an adequate amount of PPO (Bhatia, 1964). A biosensor for the determination of PP in tea, targeting the catechol present was developed, Ghindilis *et al*., (1992).

An amperometric principle-based biosensor containing immobilized tyrosinase has been used for the detection of PP in tea. The immobilized tyrosinase based biosensor could detect tea PP in the concentration range 10–80 mM. Immobilization of the enzyme by the crosslinking method gave good stable response to tea PP (Abhijith *et al.,* 2007).

One of the major problem associated with PP biosensors are the fouling of the immobilized enzyme membranes. PPs are having high affinity towards proteins which led to the formation of complexes, result in membrane fouling.

12. Protein-polyphenols interactions

Astringency of tea and wine is more or less attributed by binding/precipitation of PP/peptide complexes (Fishman *et al.,* 2002). PP has considerably high affinity towards the proteins. This affinity varies from one phenolic compound to other (Bartolomé *et al.,* 2000). Another interesting finding reported that, at molecular level amino acids exhibits different affinity towards the phenols (Abboud *et al.,* 2008). As the PP size increases, the number of binding sites is reduced at protein (Baxter *et al.,* 1997).

Polyphenolic metabolites of plants with a molecular weight larger than 500 has the ability to precipitate gelatin (Mehansho *et al.,* 1987). PP complexes formed during black tea manufacturing are of high molecular weight. The formation of these insoluble complexes is usually reversible and they may redissolve (Luck *et al.,* 1994).

The ability of tannins to precipitate different proteins varies considerably. Using a competitive assay, Hagerman and Butler (1981) found that the selective affinity of proteins for condensed tannin varied more than 1000-fold. The highest affinities were found for proteins, polypeptides and polymers with high proline content and the lowest affinities were obtained for small globular proteins such

as lysozyme. It is also interesting to note that different tannins show variations in interaction with a given protein. McManus *et al.,* (1985) observed that molecular size as well as flexibility affected the binding of tannin to protein. Potentially, such interaction could occur via covalent or ionic bonds, hydrophobic interaction, or hydrogen bonding. While PP are prone to oxidation and give rise to ortho-quinones which are highly reactive intermediates that potentially could result in tannin-protein covalent crosslinks (Haslam *et al.,* 1991). Several studies have presented evidence for a hydrophobic effect in tannin-protein interactions. Using condensed tannin, Oh *et al.,* (1980) found that the complex formation of tannin with gelatin increased with increasing temperature and ionic strength, indicating hydrophobic interaction and hydrogen bonds have also been shown to play a role in the formation of tannin-protein complexes (Hagerman and Butler, 1980) and it has been suggested that proline has an important role because the carbonyl oxygen next to the secondary amine in proline residues is a strong hydrogen bond acceptor (Hagerman and Klucher, 1986).

Thus, the stoichiometry and size of the PP - protein complexes depend on the concentrations of the reactants and the protein/PP ratio (McManus *et al.,* 1985). The multivalent nature of PP - protein interaction is also supported by the observations on haze formation reported by Siebert *et al.,* (1996). Similarly, Hagerman and Robbins (1987) found that by adding increasing amounts of albumin to a fixed amount of tannin, there was an optimal ratio of tannin to protein where maximal precipitation occurred.

Proteins are having high affinity to PP. During immobilization of enzyme for the preparation of enzyme membrane for application in biosensor, inert proteins such as lysozyme was used as a stabilizing agent. It will not only stabilizing the enzyme but also gave a uniform proteinacious support in the membrane surface. Usually the amount of PBSA used will be more than the

enzyme used for the immobilization in the membrane. So the interactions between these inert protein and PP are a primary requisite to understand and reduce the fouling of the immobilized main enzyme.

13. Validation

Any new technological/instrument development validation is a mandatory to establish its reliability as an analytical device or technique with the conventional or existing technology or device. A study was reported using HPLC/DAD, tyrosinase biosensor and differential pulse voltammetry (DPV) analyses to detect polyphenolic compounds in natural complex matrices. The analyses were applied to a series of both standards and natural extracts derived from grape, olives and green tea (Romani *et al.*, 2000).

14. Scope and future prospects of biosensors

Biosensors are analytical devices, which use biological interactions to provide either qualitative or quantitative results. They are extensively employed in many fields such as clinical diagnosis and biomedicine, military applications, anti-terrorism, farm, garden and veterinary analysis, process control, fermentation control and analysis, pharmaceutical and drug analyses, food and drink production and analysis, pollution control and monitoring, microbiology, bacterial and viral analysis, mining, industrial and toxic gases.

The biosensor market has significantly increased and will be mushrooming in the next decade. The total biosensor market is estimated to be $10.8 billion by 2007 (Bogue, 2005). The emerging biosensor market presents both opportunities and obstacles to start - up biosensor entrepreneurs. The major challenge and threat for these entrepreneurs is how to predict the biosensor market and how to convert promising biosensor technology into commercialized biosensors.

15. Scope and objectives of the study

With the above mentioned background in biosensor development and the literature review, the present work was carried out. Tea is an important beverage and India is one of the leading producers for last 150 year and continuing its supremacy. Assessing of tea quality acquainted with primordial importance, since the rules and regulation implemented by importing countries. To compel with the stringent quality measures and standards implemented around the world, it necessitates a thorough quality evaluation. Generally, tea quality is assessed with its sensory characters such as colour, astringency (mouth feel), body and flavor. Most of these attributes derived from the PP present in tea. PPs are the important constituent of tea having commercial and nutraceutical importance. An enzyme based amperometric biosensor system was developed for the assessment of tea PP concentration as the analyte of interest.

To achieve first objective as mentioned in synopsis, enzymes from various sources were purified and immobilized on the membrane for the PP analysis. Commercial enzymes were used for the standardization of enzyme performance.

Tea samples were analysed using the developed biosensor system for the evaluation of quality on the basis of PP content as the second objective.

Further tea biosensor system was evaluated by various conventional analytical methods for the fulfillment of final objective of the research work.

An enzyme based amperometric biosensor system was developed successfully, which can distinguish and grade various kinds of tea which include black and green tea samples.

A flow chart showing the major steps involved in the present investigation is given below (Figure 6).

For development of enzyme based biosensor system require a suitable enzyme for detection of tea polyphenols, thus in Chapter II deals with the isolation and purification of tea polyphenol oxidase from various sources. Enzymes were purified to homogeneity and three isozymes were found with PPO activity. Immobilization is an essential element in the application of enzymes and biomolecules for biosensor application. Section B of Chapter I deals with different immobilization and stabilization techniques for selected enzymes. Section C of Chapter 2 describes the fabrication of biosensor instrument system. Final section (D) of Chapter 2 is about the studies on protein-PP interactions and fouling of the enzyme membranes. Application for dissociation agents to remove the bound PP in enzyme membrane surface also discussed in this section.

Chapter 3 is having two sections. Section A is a compiled result of tea samples analysis with biosensor system. In this chapter experiments for extractable total polyphenols during conventional tea making were estimated. This section also shows the estimation of polyphenol content can be used as a quantifiable quality evaluation method for grading of tea. Section B of Chapter 3 details validation of developed biosensor for polyphenol analysis**.**

These chapters followed by summary, conclusions and bibliography.

Sample collection
(Tea Leaves)

Enzyme Extraction/Purification

Laccase | Polyphenoloxidase | Tyrosinase

Immobilization/Stabilization

Selection of
suitable enzyme

Development of
Biosensor

Analysis of Tea samples with
developed Biosensor

Validation of biosensor with
conventional techniques

Figure 6. Various Stages of Investigation on Role of Specific Enzymes in Biosensor for Tea Analysis

Section A. Purification of polyphenol oxidase

1A.1. Introduction

Polyphenols (PP) are the active components of tea which majorly determine the quality of tea in terms of colour and astringency. In order to assess the quality of tea, the determination of PP is essential. Polyphenol oxidase (PPO) is the enzyme of prime importance in black tea processing. PPO has a broad range of activities that include diphenol oxidase or catechol oxidase (EC 1.10.3.2), laccase (EC 1.10.3.1) and often monophenol mono-oxygenase or tyrosinase (EC 1.14.18.1) activities and catalyzes the oxygen dependent oxidation of phenols to quinones (Vamos-Vigyazo, 1981; Takeo and Baker, 1973). PPO can be suitable biorecognition element for the development of biosensor for tea PP analysis. This enzyme is widely distributed in various microorganisms, animals and plants. Heterogeneity in biochemical characteristics of plant PPO was reviewed by Mayer and Harel, 1991; Nicolas *et al.,* 1994. The physiological function of PPO in higher plants is yet to be fully determined, however it has been implicated in plant defense (Constabel, 1999), pigment formation (Vaughn and Duke, 1984) and scavenging molecular oxygen in the chloroplast (Vaughn *et al.,* 1988).

PPO activity is beneficial in many industries like chocolate, dates, grapes, tea and coffee as most of the time it relates to the quality determining factor of the final product. Tea manufacturing is a commercially important process where various enzymes are involved (Tanaka and Kouno, 2003; Subramanian *et al.,* 1999; Pradip *et al.,* 1993; Roberts *et al.,* 1952). Tea leaves consists of different enzymes such as β-glucosidase (Li *et al.,* 2005), superoxide dismutase (Vyas and Kumar, 2005), β-primeverosidase, (Mizutani *et al.,* 2002; Coggon *et al.,*1973; Sanderson *et al.,* 1972), PPO (Halder *et al.,* 1998; Gregory and Bendall, 1966), β-galactosidase (Halder and Baduri, 1997), catechol oxidase

(Coggon *et al.,* 1973), peroxidase (Kvaratskhelia *et al.,* 1997) and ascorbate peroxidase (Chen and Asada, 1989),. Among all these, PPO is most functionally important enzyme in tea manufacturing. PPO plays a key role in tea fermentation process. During tea leaves fermentation, the oxidation of simple phenolic substrates into complex characteristic PP ascribed to PPO activity. Tea leaves is rich in the PP known as catechins. Catechins are the major compounds present in the cytoplasmic vacuoles of palisade cells of tea leaves which undergo oxidation (Suzuki *et al.,* 2003; Forrest, 1969; Forrest and Bendall, 1969). Isolation and purification of PPO from tea leaves had been carried out by Halder *et al.,* (1998). But the study of the physicochemical aspects of PPO from south Indian tea leaves has not been done, where geo-climatic attributes were totally different.

PPs are the active biomolecules present in tea leaves which is a substrate for PPO. These PPs determine the quality of tea in terms of colour and astringency. In the present study, PPO was isolated from tea leaves and purified for its application in biosensor. The enzymes are purified using various chromatographic techniques and partially characterized for enzymatic properties. Apart from tea leaves, the PPO was isolated and partially purified from potato, brinjal, apple, banana and mushroom to estimate their biocatalytic activity against catechin.

1A.2. Materials

Triton X – 100 was procured from Rankem, India. Bovine serine albumin (BSA), Coomassie brilliant blue G-250, protease inhibitor cocktail, polyvinyl polypyrrolidone (PVPP) insoluble form procured from Sigma Aldrich, USA. Reagent-grade acetone, phenylmethylsulfonylfluouride (PMSF) and glycerol purchased from SRL, India. Sephadex G-100, DEAE Cellulose obtained from Pharmacia, USA. Dialysis tubing procured from Spectra/Por, Sweden. Amicon bioseparation Filter Unit with molecular weight cut off of 10 kDa, obtained from

Millipore, USA. The image analysis system for gel documentation procured from Vilber Lourmat, France.

All other chemicals were used of analytical grade. Buffer solutions were filtered through 0.4 µm filter prior to experiments.

1A.3. Methods

1A.3.1. Polyphenol oxidase extraction

Fresh tea leaves were collected from various localities of south India, which are the major tea cultivation zone, such as Nilgiris (Tamilnadu); Wayanad, (Kerala) and Madikeri (Karnataka). Plucking consists of only one bud and top two leaves of the tea plants. Tea of the highest quality has been found to be made from young shoots consisting of the tender bud and first two leaves (Baruah *et al*., 1986). The leaves transported with great care to the laboratory as soon as possible to avoid any crushing or other damage and stored at -20℃ till the time of processing. Rough handling may nullify the efforts of careful plucking. This is because any damage to leaf will initiate unexpected chemical and biochemical reactions at early stage (Hara *et al*., 1995). In addition, damage or injury to the tissues of the tea leaf may inactivate the coenzymes involved in carbohydrate oxidation (Deb and Roberts, 1940).

Fruits and vegetables were procured from the local market on the day of experimentation.

Enzyme extraction buffer was prepared with the method described by Halder *et al.,* (1998), which is modified slightly. Sodium phosphate (NaPi) buffers, of 100 mM with the pH 6.8 containing 100 mM NaCl, 0.2% Triton X-100, 1 mM PMSF, 30 µL protease inhibitor cocktail, 10 mM ascorbic acid and 10% glycerol in the final concentration. All purification steps were done at 4℃ unless otherwise mentioned.

Tea leaves and other materials were processed in the same manner. Each batch with 200 g of raw material was ground to slurry in waring blender for 30 sec with 100 mL of extraction buffer. The slurry was transferred to the funnel and immediately added two volumes of ice cold 80% aqueous acetone. Flask was connected to vacuum pump to facilitate faster transfer of the solvent through the filter. The slurry was mixed thoroughly to make the maximum contact area and faster removal of pigments. Aqueous acetone (80%) was added till the color pigments were removed from the homogenate and a clear solvent obtained through the slurry (Deepthi *et al.,* 2007). This step was followed by 100% acetone (chilled) to dehydrate the slurry. The powder collected as filtrate. Acetone powder kept in a solvent hood for drying at room temperature for overnight. The off white color powder collected from filter paper and stored at –20℃. Further the enzyme from the acetone powder was extracted in buffer and evaluated for yield and activity of PPO enzyme.

10 g of the acetone powder and 10 g of PVPP and 20 g of acid washed sand were measured for the extraction of enzyme. 100 mL of ice cold extraction buffer was added and homogenized and filtered through four layers of nylon cloth. The resulting homogenate was cleared by centrifugation (12,000 rpm, 30 min, 4℃) using a Kubota centrifuge.

Protein estimation of all samples was done according to Bradford method (Bradford, 1976). Standard curve was prepared by plotting the optical density (OD) at 595 nm against the concentration of BSA.

100 mM catechin solution was prepared in NaPi buffer. Catechin is sparingly soluble in buffer. Complete solubility achieved by slight warming of the sample.

PPO activity was determined by using the method of Sanchez-Ferrer *et al.,* (1989), by measuring the rate of increase in absorbance at 420 nm at 25℃ in an UV–Visible spectrophotometer (Shimadzu, Model/l601). The reaction mixture contained 990 µL of 10 mM catechin solution, 10 µL of the enzyme

solution. The reference cuvette contained only substrate solution without any enzyme. The straight line section of the activity was used to express the enzyme activity (U/mg). One unit of enzyme activity is defined as the amount of enzyme that caused a change in absorbance of 0.001/ min, under the conditions of the assay.

Crude PPO enzyme was fractionated (0-80%) with ammonium sulphate. The precipitate obtained was redissolved in a minimal volume of the NaPi buffer. Ammonium sulphate precipitate of 30-40%, 50-60% and 70-80% saturation were separately dialyzed against 100 mM NaPi buffer of pH 6.8 with several changes of dialyzing buffer.

Fractions of each ammonium sulphate precipitation obtained at different saturation levels were analysed for its enzyme (PPO) catalytic activity against catechin. Protein concentration of each fraction was estimated. Catechin was used as standard phenolics substrate for PPO enzyme (extract). Further enzyme kinetic parameters such as Km and Vm were estimated for each PPO extract against catechin.

1A.3.2. Purification of polyphenol oxidase

The active fractions of crude PPO obtained from ammonium sulphate saturations were further subjected to ion exchange column chromatography using DEAE cellulose. In ion-exchange chromatography one can choose whether to bind the substances of interest, or to adsorb the contaminants and allow the substance of interest to pass through the column. Schematic diagram of major steps of PPO purification is given in Figure 1.1.1.

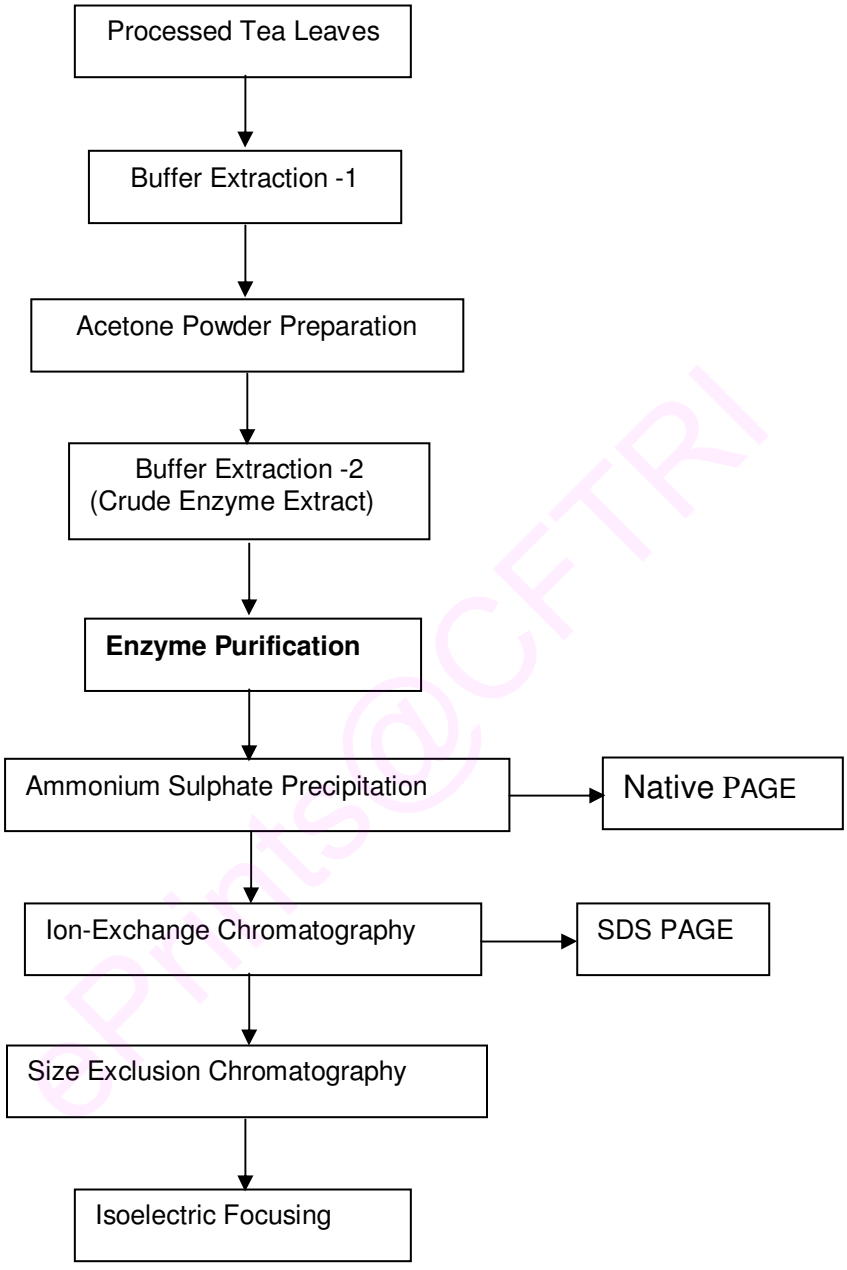


Figure 1.1.1. The steps involved in purification of PPO from tea leaves

1A.3.1.1. Ion exchange chromatography

Prior to use in chromatography, 20 g of DEAE-cellulose dry resin was kept for swelling overnight then washed with 500 mL of 0.25 M NaOH-0.25 M NaCl and then with 500 mL of deionized water. The latter step was repeated four times. It was then washed once with 500 mL of 0.25 M HCl and suspended in a 1-liter graduated cylinder filled with deionized water for removal of fine particles. After removal of fine particles and adjusting the pH to 6.8, the DEAE-cellulose was washed five times with 100 mM phosphate buffer at pH 6.8. The column was equilibrated with 250 mL of 100 mM phosphate buffer, pH 6.8, in a cold room (4°C) before use.

After concentrating the dialyzed enzyme, active enzyme fractions were loaded (10% v/v) onto DEAE-Cellulose column (4X20 cm) with a column bed volumes of 251 cm^3 previously equilibrated with extraction buffer (100 mM NaPi buffer containing 10% glycerol), and washed with the same buffer to remove unbound proteins (Yoruk and Marshall*.,* 2003), with a flow rate of 30 mL/hr. Fractions were collected in test tubes and each fraction volume was 5 mL. Stepwise gradient of salt ranging from 0 – 0.3 M NaCl was used to elute the proteins. The protein was eluted from the column by the same buffer with step wise increasing concentrations of NaCl (Wisseman, 1985; Wong, 1971).

Enzyme activity and protein concentration were measured at intervals of one fraction (5 mL / tube) (Khatun *et al.,* 2001). Fractions were further concentrated by Amicon bioseparation Filter Unit with molecular weight cut off of 10 kDa and stored at -20℃ prior to further purification (Constabel, 1995; Hrmova, 1993).

1A.3.2.2. Size exclusion chromatography

Enzyme was further purified by size exclusion chromatography. Sephadex G -100 of particle size of 40-120 µm, which gives a bed volume of 15-

20 mL /g of swollen gel was used. The exclusion limit of Sephadex G-100 is 4 kDa-150 kDa for globular proteins. 10 g of Sephadex G-100 dry powder was allowed to swell in 500 mL of 100 mM NaPi buffer of pH 6.8 for overnight. The slurry was packed into a glass column (60 cm x 1.1 cm) at a flow rate of 18 mL/h. Packed column was equilibrated with NaPi buffer 50 mM, pH 6.8. Void volume was measured with blue dextran of 2000 kDa. The protein fractions were eluted in the same buffer at the flow rate of 10 mL/hr. Sephadex G-100 column was used as the final step in the purification of PPO from tea leaves. The column was stored in buffer containing 0.03% sodium azide.

1A.3.3. Electrophoresis

Vertical slab gel electrophoresis was carried out on a Bio-Rad mini slab gel electrophoresis unit at room temperature.

1A.3.3.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of polyphenol oxidase from tea leaves

SDS-PAGE was carried out as described by Angleton and Flurkey (1984) in a discontinuous buffer system. The sample was centrifuged and dissolved in 20% glycerol (v/v), 0.1% bromophenol blue (w/v), 10% β-mercaptoethanol (v/v) and 10% SDS (w/v) as the sample buffer before being applied to 10% polyacrylamide gels. The solution of enzyme protein and molecular weight markers were heated separately in sample buffer in a water-bath at 100℃ for 3 min. A Bio-Rad Mini Gel with 1.5-mm thick spacers was used for electrophoresis. The 10 µL of enzyme samples of various stages of purification was loaded in to the wells and the solution of molecular weight markers (10 µL) were placed in separate wells. The gel was run at 50 mA at 25℃ until the dye had reached the end of the gel. The gel was immersed in Coomassie Brilliant Blue staining solution for 30 min, after which it was left overnight in 40% aqueous acetic acid–60% methanol as destaining solution.

1A.3.3.2. Native PAGE of polyphenol oxidase from tea leaves

The native PAGE was carried out as described above without the addition of SDS on 10% gel. The presence of PPO in active form in the gel was identified by assaying the activity. After electrophoresis the gel was stained with 100 mL of 10 mM NaPi buffer (pH 6.8) containing 10 mM catechin for PPO activity.

1A.3.4. Isoelectric focusing

Isoelectric focusing was done as the procedure described by Righetti (1989), which was modified accordingly. Gel casting was done on a hybond sheet. It was placed in isoelectric focusing unit by hydrophilic end towards the gel and hydrophobic end towards the plate. The isoelectric pH was determined in native conditions. The ampholyte used has a pH of 3.0 -10.0. The electrodes were connected to a power supply and the current was maintained at 200 V for 1.5 h and at 400 V for 1.5 h. The gels were stained for PPO activity in 100 mL of 10 mM NaPi buffer (pH 6.8) containing 10 mM catechin.

Electrode solution: Ortho phosphoric acid (18.1 M) 0.6 mL and made up to 10 mL with distilled water. Ampholyte used with a pH 3 to 9. Details of the composition of gel for isoelectric focusing are given in Table 1.1.1.

Table 1.1.1. Composition of gel

Preparation of Gel	Volume of Gel 25 mL
pH range of ampholyte	-
3-5	0.312
3-5	0.156
5-8	0.156
7-9	0.156
Water	17.695
Acrylamide stock 30%	6.250
TEMED	0.025
APS 10%	0.025
TOTAL	25.0

10% trichloro acetic acid (TCA) was used to remove the ampholytes. Further gel was washed with water and isoelectric focusing was done with 10% of acetic acid, 50% of methanol and 2% of formaldehyde (ampholyte buffer) for 2 hours. The pI of the PPO II and III were calculated by the image analysis system (Vilber Lourmat, France).

1A.3.5. Optimization of physicochemical parameters

1A.3.5.1. Effect of pH on polyphenol oxidase

PPO activity as a function of pH was determined using 10 mM of catechin, as a substrate. The buffer used was NaPi (pH 5 - 8.0, 10 mM) at 25℃. The effect of pH on the tea PPO was determined by the enzyme activity at various pH. The pH at which the enzyme had maximum activity was determined as optimum pH.

1A.3.5.2. Effect of temperature on polyphenol oxidase

The optimum temperature of the PPO reaction was determined at various temperatures ranging from 25-80℃, using 10 mM catechin as the substrate in 10 mM NaPi buffer, pH 6.8.

1A.4. Result and Discussions

1A.4.1. Extraction of polyphenol oxidase enzyme

Isolation and purification of enzymes are very important step in biosensor development.

First extraction of PPO from tea leaves with buffer found to have an activity 550 U/mL, which was further reduced to 50 U/mL during storage at 4℃, after two days. Activity reduction may be due to the native PP extracted along with the protein leading to irreversible binding with the enzyme. It is evident in fruit juices that the haze formed by protein – PP interactions (Silbert and Linn, 2000). Interactions of PP with BSA were reported by Papadopoulou *et al.,* 2005. Crude enzyme extract obtained from acetone powder precipitate (crude enzyme powder) having extremely low PP content, which showed an approximate activity of 2000 U/mg. It was also observed that when acetone powder was stored at 4℃ retained its activity for longer period up to 6 months.

Further ammonium sulphate precipitation of acetone powder precipitate extract yielded of proteins devoid of other impurities. The maximum enzyme activity (4800 U/mg) and protein content (7 mg/mL) was obtained at 60 % of saturation (Figure 1.1.2). The 0-40% precipitate contained 14% of the total activity but the specific activity was four fold less than the crude enzyme extracts (acetone powder extract). The 40-60% precipitate contained 65% of the total activity with a 2.5 fold increase in specific activity whereas the 60-90% precipitate contained 19.7% of the total activity with a 1 fold decrease in the

specific activity. No activity was detected in the supernatant after 90% saturation level. Hence a 40-60% ammonium sulfate fractionation was used as the next step of purification.

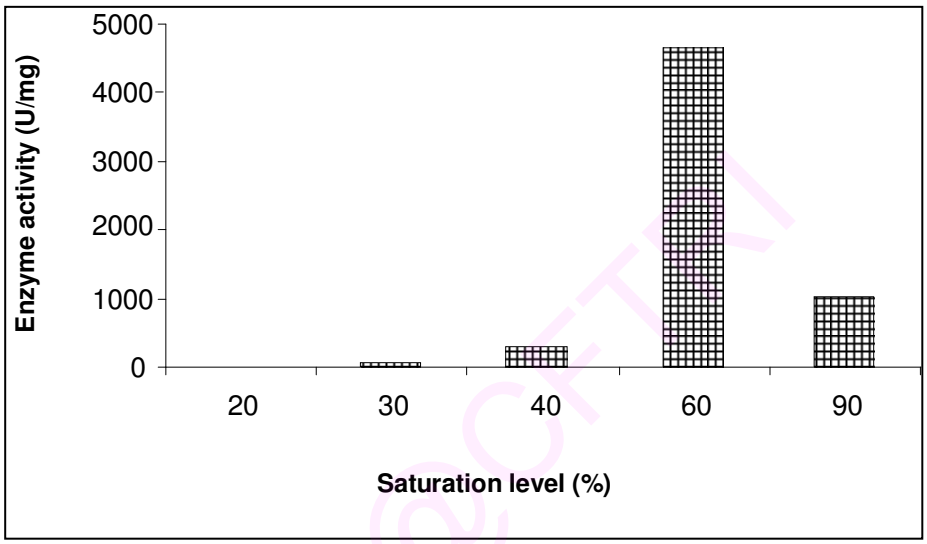


Figure 1.1.2. Optimization of enzyme precipitation with ammonium sulphate was carried out. Maximum protein precipitation and enzyme activity obtained at 60% saturation level.

Tea leaves from various locations were showed significant difference in the PPO activity (Figure 1.1.3). Except acetone powder extraction enzyme extract from Nilgiris tea leaves showed better activity during all the stages of enzyme purification. This may be due to favourable geoclimatic parameters and clonal selections at Nilgiris.

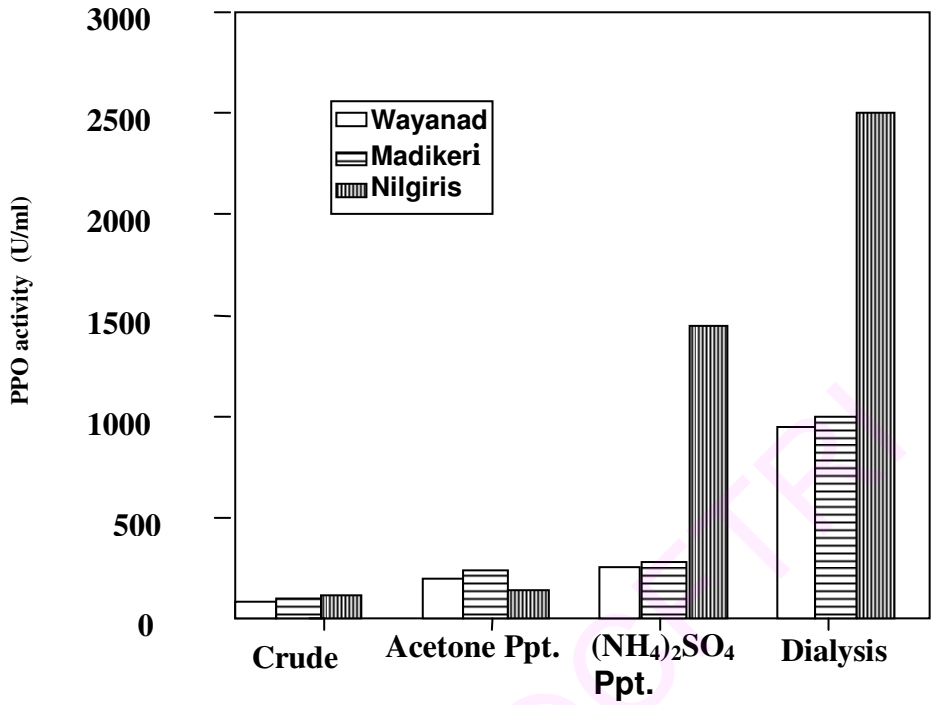
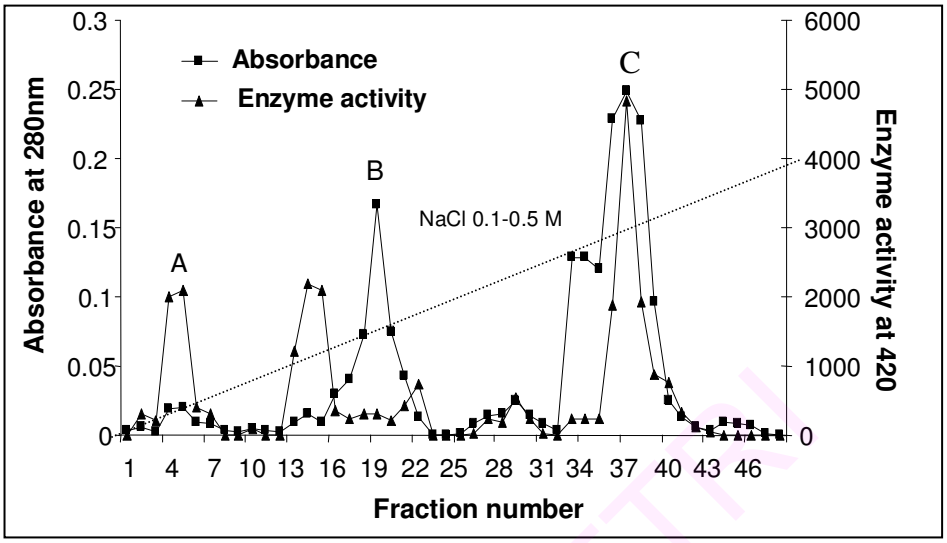


Figure 1.1.3. Comparative enzyme activities of PPO extracted from tea leaves from different locations. PPO extracted from Nilgiris regions showed better activity during the various steps of enzyme purification.

1A.4.2. Purification of polyphenol oxidase

1A.4.1.1. Ion exchange chromatography

The enzyme fractions eluted out between 0.1 to 0.2 M NaCl, 0.2 M NaCl and 0.3 M NaCl are shown in Figure 1.1.4 as peaks A, B and C, named as PPO-I, PPO-II and PPO-III respectively. PPO II and PPO III which were having maximum absorbance (0.11 and 0.25 at 280 nm respectively) and enzyme activity (5000 and 3800 U/mL respectively) compared to PPO I (0.03 and 2200 U/mL) were taken for further purification with size exclusion chromatography.



A-PPO-I, B-PPO-II, C-PPO-III

Figure 1.1.4. DEAE Cellulose chromatography of PPO enzyme after dialysis against 50 mM NaPi buffer, pH 6.8. The loaded column was washed with 50 mM NaPi buffer and eluted with a 0 - 0.5 M NaCl gradient. Absorbance at 280 nm and enzyme activity were monitored at 420 nm. Each fractions collected was of 5 mL volume. Tubes 3-8, 14-22 and 33-42 constitute PPO-I, PPO-II and PPO-III respectively.

The yield as fold purification PPO I, PPO II and PPO III were given in Table 1.1.2. The results indicate that the yield of PPO III was more compared to the other enzyme fractions. Fraction II and III were given good activity; 900 and 1700 U activity respectively.

Table. 1.1.2. Purification steps of polyphenol oxidase from tea leaves

Purification step	Total protein (mg)	Total activity	Purification (fold)	Specific activity (U/mg)	Yield (%)
Crude extract	206	17880	1	86.79	100
Ammonium sulphate fraction	41	12766	3.59	311.37	71.4
DEAE Cellulose					
PPO I	0.71	139	2.56	195.77	0.7
PPO II	1.32	1191	10.40	902.27	6.66
PPO III	2.64	4684	20.44	1774.24	26.19
Sephadex G-100*					
PPO - II	0.15	774	59.86	5195.01	4.32
PPO - III	0.35	2958	97.37	8451.42	16.54

*only PPO-II and PPO III was taken for size exclusion chromatography
PPO enzyme was found in three active isoforms as PPO-I, PPO-II and PPO-III. PPO-I was found comparatively low activity and yield. PPO-II and PPO-III were purified to homogeneity with 60 and 97 fold of purification and with a yield of 4.32 % and 16.54 % respectively.

Two major and a minor fraction were isolated and purified from tea leaves (Halder *et al.,* 1998). One of the fractions came out in unabsorbed fraction and other two fractions with gradient elution. In the present study there was no fractions obtained as unbound fractions. All three fractions were eluted with gradient elution. The active fractions obtained at different gradient of salt concentration are of three different isozymes of PPO. This is further confirmed by native PAGE and isoelectric focusing.

Four isozymes were isolated from cligstone peach (*Prunus persica*) using DEAE cellulose column chromatography with varying activity was reported by Wong *et al.,* (1971).

1A.4.2.2. Size exclusion chromatography

Collected fractions from size exclusion chromatography were assayed for the protein content and enzyme activity (Figure 1.1.5). It was observed that fractions Nos. 10 to 20 and 35 to 45 were found to have PPO activity. The

enzyme fractions obtained after size exclusion chromatography had a 97-fold purification for PPO-III and 60-fold purification for PPO-III (Table 1.1. 2).

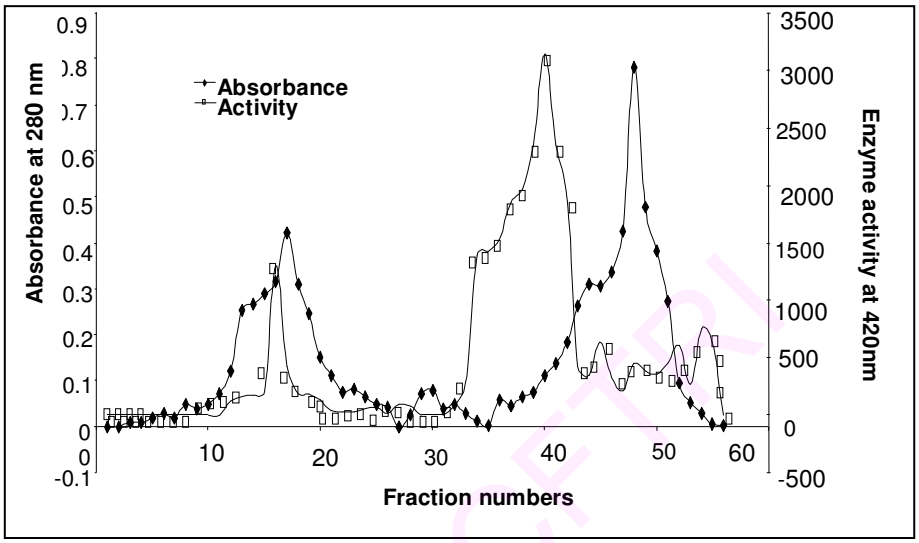


Figure 1.1.5. Size exclusion chromatography – G-100 of PPO-II and PPO-III. The samples were concentrated by ultrafiltration, applied to an equilibrated G-100 column and eluted with 50 mM NaPi buffer, pH 6.8, and 0.2 M NaCl. The protein content and PPO activity were monitored at 280 nm and 420 nm respectively for each 3 mL fraction.

1A.4.3. Homogeneity and molecular mass determination of purified tea polyphenol oxidase

SDS-PAGE gel of crude enzyme indicated that crude protein preparation had different molecular weight ranging from 30 kDa to 140 kDa. As seen from the Figure 1.1.6, the molecular weight found to be 68 kDa (PPO-III), 74 kDa (PPO-II) and PPO-I with relatively lower molecular weight (45 kDa). It was observed that single band of isoenzyme appeared in SDS PAGE (Figure 1.1.6) showed that the purification step yielding a homogenous protein. It was reported electrophoresis on 7.5% SDS-PAGE of the final purified enzyme of Indian tea leaves showed a single band of 72 kDa (Halder *et al.,* 1998). Further PPO activity was confirmed by a native gel (Figure 1.1.7) of active isoenzyme was demonstrated with along with the native structure of enzyme.

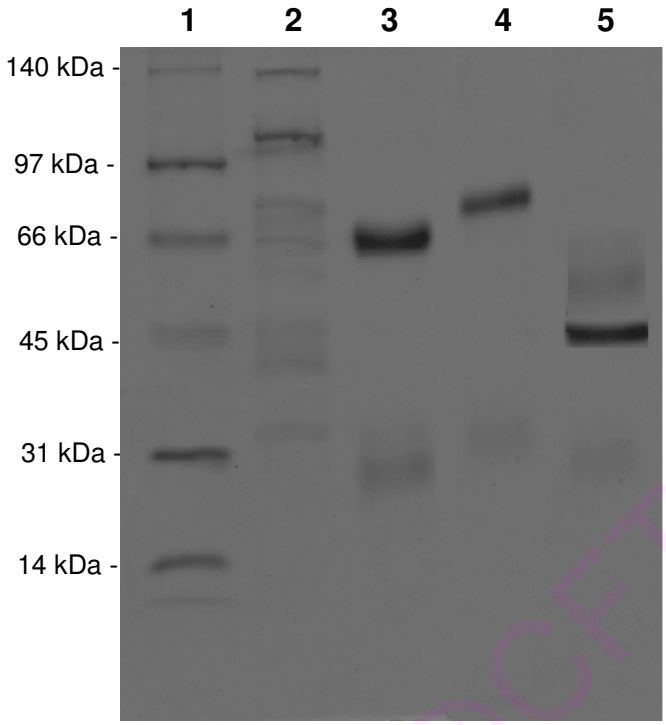


Figure 1.1.6. SDS-PAGE of the purified PPO. Lane 1. Mol.wt marker, Lane 2. Crude PPO, Lane 3. Purified PPO III, Lane 4 PPO II and Lane 5 PPO I.

1A.4.4. Native PAGE for activity staining of polyphenol oxidase

In the native PAGE sample was observed for their activity after the several steps of purification as mentioned in flow chart (Figure 1.1.1). The gel was later subjected for activity staining in presence of 10 mM catechin, which is a substrate for PPO that gave yellowish orange colour of bands (Figure 1.1.7) due to substrate catechin and enzyme activity. The results confirmed the different isoforms of the PPO enzyme were present in tea leaves. Major bands of isozymes having high mobility and are separated from each other.

Figure 1.1.7. Activity staining of purified PPO enzyme (native gel). Three distinguish bands shown activity and formed a bright orange colour. Tea leaves PPO are shown three active isozymes.

1A.4.5. Isoelectric focusing of polyphenol oxidase

The pI of PPO-II and PPO-III were 5.7 and 6.2 respectively (Figure 1.1.8).

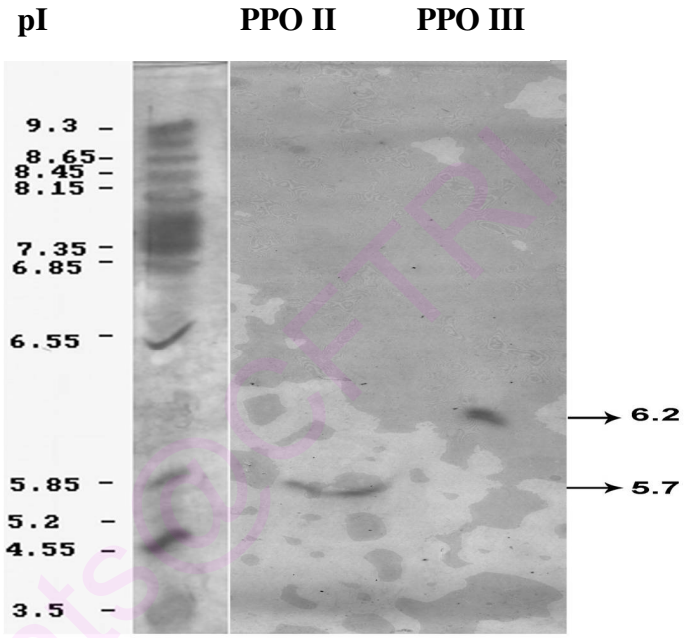


Figure 1.1.8. Isoelectric focusing of PPO II and PPO III of tea leaves on polyacrylamide gel containing ampholytes with a range of pH values between 3.0 and 10.0. Staining was done with 10 mM catechin in 10 mM NaPi buffer (pH 6.8). The lane contained 5 µg of enzymes purified from above mentioned methods.

1A.4.6. Optimization of parameters for the polyphenol oxidase activity

1A.4.6.1. Effect of pH on polyphenol oxidase activity

For this study partially purified enzyme (after ammonium sulphate precipitated and dialyzed) was used. The effect of pH on PPO activity was carried out to optimize the activity for its application in biosensor. The activity

profile of PPO is shown in Figure 1.1.9. The enzyme was active in a pH range of 5.0-8.0 and maximum activity 1150 U/mg was observed at pH 6.8.

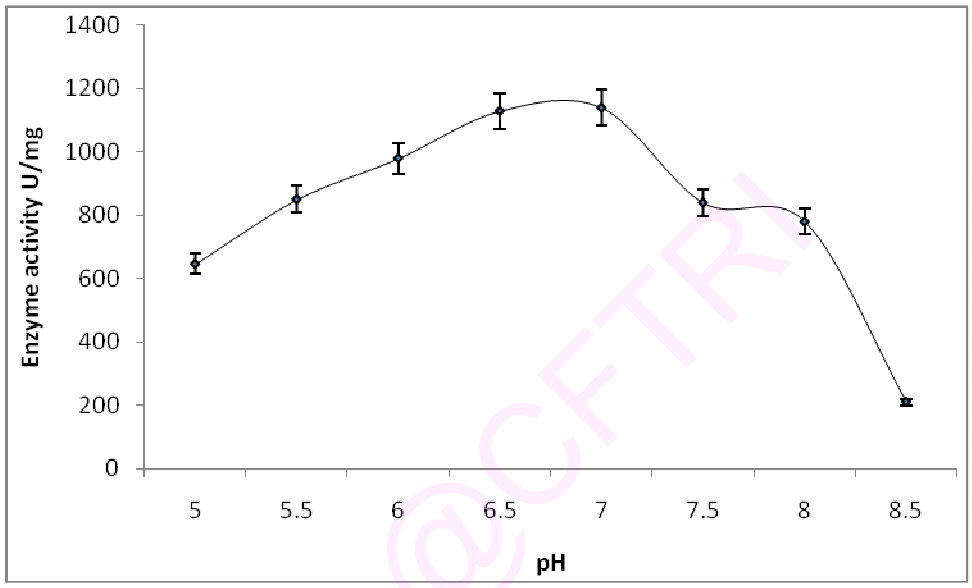


Figure 1.1.9. Effect of pH for purified PPO. Maximum activity was found to 6.8 Enzyme activity increased gradually from pH 5.0, reaching a maximum at pH 6.8 for purified PPO. Thereafter, activity decreased gradually at pH 8.5 for PPO.

1A.4.6.2. Effect of temperature on polyphenol oxidase activity

For this study partially purified enzyme (after ammonium sulphate precipitated and dialyzed) was used. Enzyme was assayed in a temperature range of 25-80℃. The enzyme activity was increased gradually from 30℃ (1400 U/mg) and reached a highest activity at 40℃ (4950 U/mg). The activity got reduced above 40℃.Gradual reduction of activity was observed with the increase in temperature (Figure 1.1.10). The enzyme turned to almost inactive at 80℃. PPO was given maximum activity at 40±3℃.

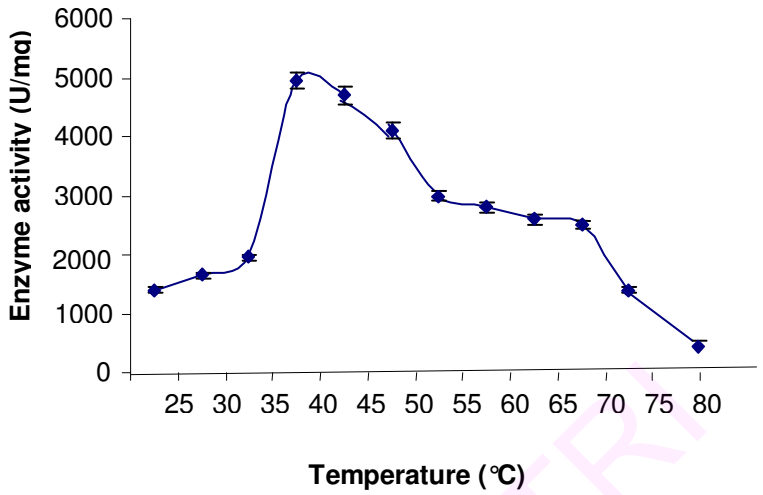


Figure 1.1.10. Effect of temperature on enzyme activity of PPO. Enzyme was active in a temperature range of 25-80°C. Maximum activity was observed at 40±3°C. Activity was reduced further increase of temperature. Enzyme activity gradually reduced at 60-70°C and sharply declined at 75°C and became completely inactive at 80°C.

1A.4.7. Extraction of polyphenol oxidase from fruits and vegetables sources

Enzyme was extracted from fruits and vegetables and the protein content of each extract, specific activity and details of enzyme kinetics (Km and Vmax) was estimated which is shown in Table 1.1.3. Protein content was found in brinjal extract - 1.2 mg/mL, mushroom extract 1.38 mg/mL, tea leaves extract 1.1 mg/mL, potato extract 0.82 mg/mL, banana 0.78 mg/mL, apple 0.2 mg/mL. The enzyme activity was found to be 10,159 U/mg (mushroom), 8,436 U/mg (tea leaves), 6,622 U/mg (brinjal), 4,226 U/mg (potato), 2575 U/mg (banana) and 1110 U/mg (apple).

Among the PPO from fruits and vegetable sources, mushroom tyrosinase is having minimum Km (0.8) and maximum Vmax (5.61). The results clearly indicated that mushroom is having high affinity to catechin as a substrate (Table

1.1.3). PPO from tea leaves also shown high Vmax (4.16), but the Km value (1.37) is comparatively high. PPO from banana also gave high Vm (4.0), with a high Km (2.85) value.

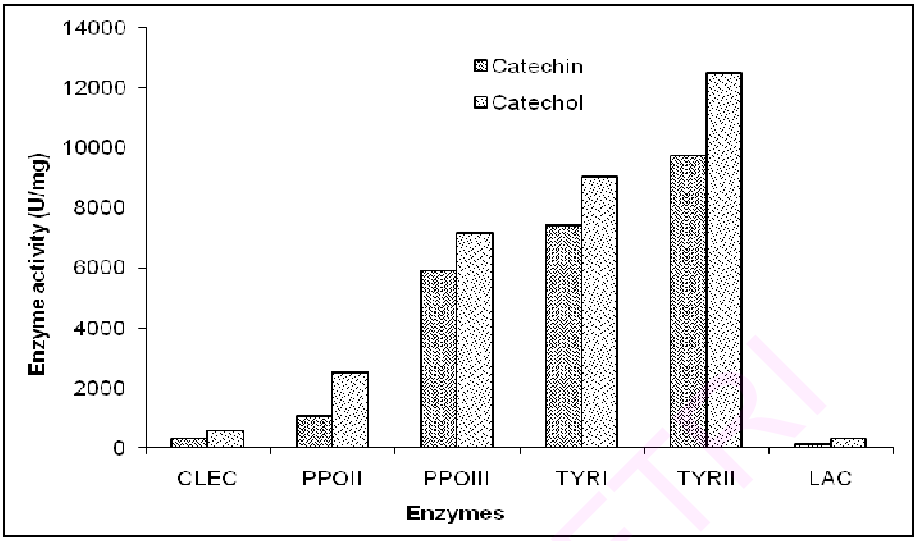
Table 1.1.3. Vmax and Km values of various polyphenol oxidase with catechin as a substrate

PPO Sources	U/mL/min	Protein mg/mL	Specific activity	Km(mM)	Vm(x 10^{-3})
Mushroom	14,020	1.38	10159	0.8	5.61
Tea Leaves	9280	1.1	8436	1.37	4.16
Brinjal	7947	1.2	6622	1.92	2.63
Potato	7049	0.82	4226	2.38	1.33
Banana	3086	0.78	2575	2.85	4.0
Apple	675	0.2	1110	2	1.88

Mushroom was shown maximum specific activity, followed by tea leaves brinjal, potato, banana and apple. Enzyme with low Km and high Vmax is considered to best for the development of a biosensor.

1A.4.8. Comparison of enzymes for their activity using catechin and catechol as substrate

Above selected enzymes were studied for their response to catechin and catechol separately with 10 mM concentration. PPO-II and PPO-III isolated from tea leaves have shown good activity with catechin (950 and 5900 U/mg) and catechol (2200 and 6900 U/mg) in comparison with laccase (80 and 150 U/mg). Among the laccase enzyme tested, only CLEC of laccase shown good response (250 and 560 U/mg) to the substrates catechin and catechol respectively (Figure 1.1.11). Among the enzymes tested tyrosinase (TYRI), gave maximum response for both catechin (7950 U/mg) and catechol (9100 U/mg). Tyrosinase commercially (TYRII) available gave a better activity (9600 and 12700 U/mg), than extracted and purified tyrosinase in the present work for catechin and catechol respectively.



CLEC- Laccase Cross-linked enzyme crystals, PPOII and III- Polyphenol oxidase II and III, TYRI and TYRII-Tyrosinase purified and commercial, LAC- laccase Commercial

Figure 1.1.11. Comparison of enzyme activity of various enzymes with catechin and catechol. All enzymes were given comparatively high response to catechol than catechin.

1A.5. Conclusion

During studies to select suitable enzyme for biosensor application several PPO related enzymes were extracted from tea leaves, mushroom, apple, brinjal and potato along with commercially available laccase and tyrosinase. Attempts were made to purify the enzymes from tea leaves. PPO enzyme from tea leaves were purified and obtained three active isozymes named as PPO-I, PPO-II and PPO-III. Among these PPO-II and PPO-III found to have higher activity when compared to PPO-I. Hence PPO-II and PPO-III were taken for further studies on biosensor development. PPO enzymes from other sources were also studied for their reaction kinetics against catechin. Among them mushroom tyrosinase gave low Km and high Vmax for catechin substrate.

Physicochemical parameters were optimized for better performance of enzyme in catalysis.

Standard enzyme such as laccase, tyrosinase were used to compare the purified enzyme. Among these enzymes tried, a better response in term of enzyme activity with catechin was found in tyrosinase than the purified PPO enzyme obtained from tea leaves.

PPO also extracted and partially purified from various fruits and vegetables to estimate the specificity towards the catechin. Highest substrate affinity was shown by mushroom tyrosinase followed by tea leaves.

For the further studies on the biosensor tea leaves PPO and tyrosinase were selected for analysis of tea PP.

Section B. Immobilization and stabilization of enzymes for biosensor applications

1B.1. Introduction

Immobilization is the process where physical or chemical fixation of biocatalysts on pre-existing supports, preserving enzymatic activity, especially under environment that typically render enzymes inactive (Hartmier, 1998). Immobilization of biocatalysts helps in their reuse and in the development of continuous bioprocesses cost effective. Immobilization often stabilizes structure of the enzymes and allow longer duration of activity of enzymes (long 1/2-life), thereby allowing their applications even under harsh environmental conditions like pH, temperature and organic solvents. Immobilized enzymes enhance process robustness; re-use of same enzyme in many reaction cycles, henceforth lowering the total production cost of enzyme mediated reactions (Buchholz *et al.,* 2005; Kallenberg *et al.,* 2005; Lalonde and Margolin, 2002). Thus these characteristics allow immobilized enzyme to be used in biosensor application.

Fixation of enzymes on porous supports prevents them from aggregation, proteolysis, interaction with hydrophobic interfaces, etc. Immobilization of the biorecognition element is the key to the development of biosensors (Tampion and Tampion, 1987; Mattiasson, 1983).

Gel entrapment is one of the methods widely used for enzyme immobilization. The entrapment method of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. It is done in such a way as to retain protein while allowing penetration of substrate. Different entrapment methods for the immobilization of tyrosinase were reported by Sharma *et al.,* (2003) for the monitoring phenolic constituents of industrial and environmental samples.

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. Generally, cross-linking is used in conjunction with one of the other methods. It is used mostly as a means of stabilizing adsorbed enzymes and also for preventing leakage from the matrix. During immobilization with this process the enzyme is covalently linked to the support matrix, therefore avert leakage of enzyme (Isgrove *et al.,* 2001; Marshall, 1973).

Different immobilization procedures were compared for the development of PPO-based electrochemical biosensor for on line monitoring of phenols in waste water (Canofeni *et al.,* 1994).

The most common reagent used for cross-linking is glutaraldehyde. Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity. It is possible to increase operational stability of enzyme with immobilization using protein based stabilizing agent (PBSA). PBSA minimizes the deleterious effect caused by glutaraldehyde (Gouda *et al.,* 2002). Being inert proteins they enhance the intermolecular linkages.

Besombes *et al*., (1997) immobilized PPO in poly (amphiphilic pyrole) enzyme electrodes *via* the incorporation of synthetic laponite-clay-nanoparticles, where the presence of incorporated laponite particles within the electrogenated polymer induced a strong improvement of the analytical performances of amperometric biosensors based on PPO. Boshoff *et al*., (1998) applied the combination of cross-linking, adsorption and membrane confinement methods for PPO. Commercial PPO was immobilized on the nylon membrane by using glutaraldehyde as cross-linking agent and on the polyethersulfone membrane by adsorption. The intermediate product of the PPO reaction, 4-methyl catechol, was detected when the enzyme was immobilized on the nylon membranes, but

they detected only the *o*-quinone final product when polyethersulfone was used as the immobilization matrix.

In this section, an investigation on various immobilization methods used for the immobilization of PPO and related enzymes and its stabilization for the application in biosensor is detailed.

1B.2. Materials

Standard laccase (*Tremetes versicolor)* and tyrosinase (*Agaricus bisporus),* PBSAs such as gelatin, lysozyme and bovine serum albumin (BSA), glycerol, glutaraldehyde and catechin were procured from Sigma Aldrich,U.S.A. PPO was purified from tea leaves and tyrosinase also isolated from mushroom. Cross-linked laccase crystals were obtained from Centre for Multidisciplinary Science and Technology (CMST) previously known as RRL, Trivandrum. Dialysis membrane was purchased from Specta/Por, USA and oxygen-permeable Teflon membrane was procured from WTW (Germany). Tea biosensor used for the studies was fabricated in-house which is described in next section (Section C). All other chemicals and reagents were of analytical grade and were procured from Qualigens (India).

1B.3. Methods

1B.3.1. Immobilization of enzymes

1B.3.1.1. Entrapment

PPO was immobilized on different matrices such as sodium alginate, kappa carrageen, agar, gelatin and polyacrylamide (Martinsen *et al.,* 1989). Immobilized gel beads were sliced. This slice was placed on a membrane and evenly spread to get a thin layer as much as possible. This membrane was attached to the electrode and tested for its activity against PP standards such as catechin and catechol.

Sodium alginate: 3 g of sodium alginate in 100 mL of distilled water, boiled till the powder completely dissolving to give a clear solution. When it becomes mild heat, crude PPO enzyme was added and stirred thoroughly. A solution of 0.2 M calcium chloride prepared and bought to ice cold temperature. Using a dropper slowly drop the alginate solution to the cooled calcium chloride solution. The beads kept for primary curing at 4℃ for 24 h and secondary curing was done with 0.02M calcium chloride for 48 h at 4℃. Beads were washed with distilled water preserved at 4℃ till the time of experimentation.

Agar: 3 g of agar in 100 mL of distilled water maintained at 80℃ to dissolve completely and cooling it to 40℃. Add the enzyme and mix thoroughly and before solidifying the preparation was dropped to cooled water (4℃). It formed solid gel beads containing entrapped enzyme.

Kappa carageen: 3 g of kappa carrageenan in 100 mL water was dissolved by heating at 60℃. Kept for cooling and at 40℃ crude PPO enzyme is added and mixed thoroughly to obtain a homogenous suspension. Drop this suspension slowly into an aqueous solution of 2% KCl at 4℃ using a dropper.

Gelatin: 3 g of solid gelatin powder in 100 mL water was heated at 60℃. Kept for cooling and at 30℃ crude PPO enzyme is added and mixed thoroughly to obtain a homogenous suspension. Drop this suspension slowly into an aqueous solution of 1.5% v/v glutaraldehyde at 4℃ using a dropper.

Polyacrylamide: 18 mL of Acrylamide (30%) and 2 mL bis- acrylamide were taken in beaker followed by enzyme solution, into which ammonium per sulphate and TEMED where added. It will initiate the polymerization to form the gel. Enzyme entrapped gel was sliced accordingly for the application in biosensor.

1B.3.1.2. Cross-linking

PPO (150 IU), Tyrosinase (150 IU) and laccase (150 IU) were immobilized by cross-linking method as described by Gouda *et al.,* (2002), with slight

modification. Each enzyme was dissolved in 1 mL NaPi buffer (pH 6.8). Lysozyme (30 mg) as stabilizer was dissolved in 1 mL NaPi buffer (pH 6.8). 4% v/v Glutaraldehyde solution was prepared by appropriately diluting stock solution of 70% glutaraldehyde in water. Dialysis membrane was washed thoroughly with distilled water. After draining, appropriate volumes of each enzyme were added on the surface of the membrane and enzyme was stabilized by the addition of 30 µL of (3%) lysozyme solution and mixed thoroughly so that enzyme and stabilizing agent were distributed evenly over the membrane. Finally cross-linking of the enzyme was done using 30 µL of glutaraldehyde (4%) solution. Enzyme membranes were incubated for 1 hr at room temperature; washed three times with 100 mM NaPi buffer (pH-6.8) to remove excess glutaraldehyde.

After immobilization, all the immobilized enzymes were assessed for their retention of activity respectively. The activity given by immobilized enzyme after immobilization is termed as retained activity in comparison with native form of enzyme. PPO activity was assayed according to method described by Sanchez-Ferrer *et al.,* (1989) with some modifications. The retained activity of respective enzyme was estimated using common substrate catechin (10 mM). Free and immobilized enzymes were incubated for five minutes in a glass tube, containing the 2 mL of above substrates. Activity of each free enzyme was arrested by adding 1 M sodium hydroxide (NaOH) solution in to the enzyme containing samples. The immobilized enzymes were physically removed and assayed for their retained activity. Activity was estimated by measuring absorbance at 420 nm. Control used was respective substrates in buffer. The difference in optical density by 0.001 is considered as enzyme activity and expressed in U/mL.

1B.3.2. Optimization of various parameters for immobilized enzymes

The physiochemical parameters such as pH and substrate concentrations were optimized for the immobilized enzyme for biosensor application. For pH

and temperature optimization, 10 mM catechin is used as standard phenolic substrate.

Range of pH used for laccase enzyme activity is 3.5 – 7.5. PPO II and PPO III were assessed with a pH range of 5.0 – 9.0. For tyrosinase I and II pH range was used for the optimization of pH is 5.5 – 8.0. All experiments were carried out in triplicate and mean values are reported with percentage error ±3%.

1B.3.3. Application of immobilized enzymes for biosensor analysis of polyphenols

The three enzymes viz. Tyrosinase, Laccase and Tea PPO were used for biosensor studies. Immobilized membrane was secured to the Clark's electrode with 'O' ring. Standard catechin (10 mM) was used as substrate for all the three enzymes. Response was recorded using the fabricated detector system. Initially for all biosensor experiments NaPi pH 6.8, 10 mM was used for three enzymes.

This enzyme membrane with electrode was connected to detector system fabricated at CFTRI. Electrode was dipped in a sample cell containing 2 mL phosphate buffer having different pH for respective enzyme. Schematic representation of biosensor operation is given in Figure 1.2.1. Different parameters such as optimum pH and substrate concentrations were studied.

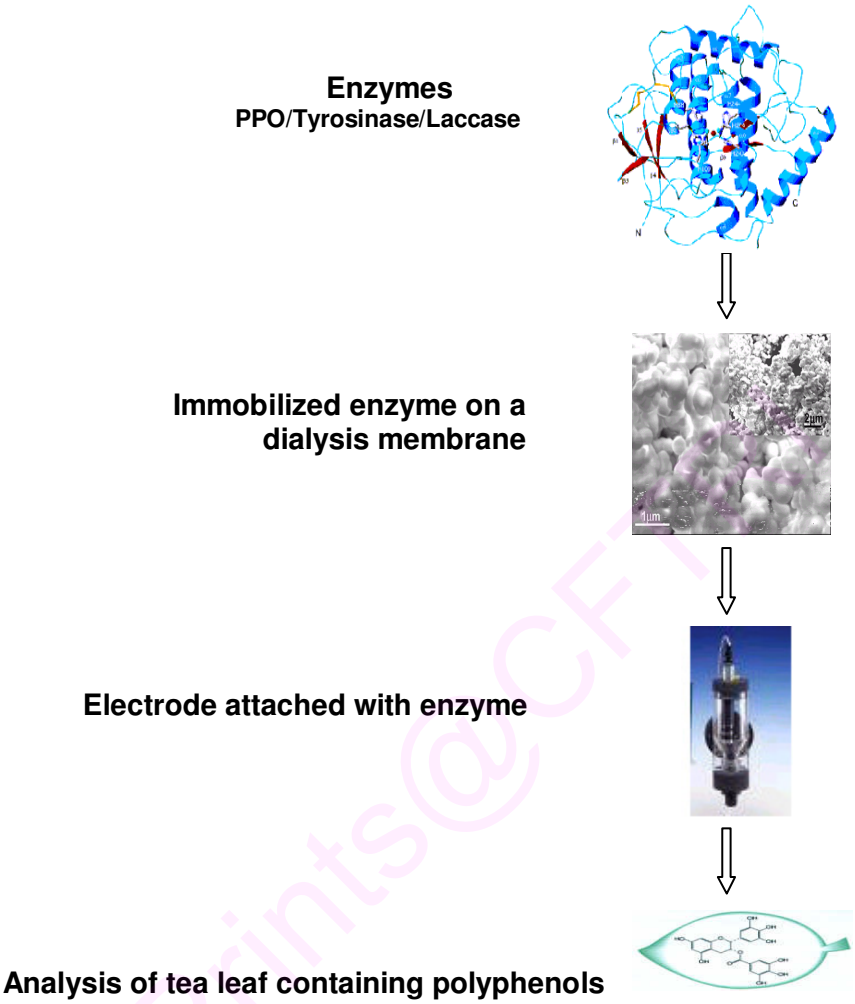


Figure 1.2.1. Schematic diagram of enzyme immobilization for biosensor applications

1B.4. Result and Discussions

1B.4.1. Immobilization of enzymes

1B.4.1.1. Entrapment

All the enzyme membranes were responded to the standard substrate catechin 100 mM (initially used). Maximum enzyme response (1.36 V) was observed for sodium alginate beads with catechin as a substrate (Table 1.2.1).

But it was observed that response time for each analysis was long (> 10 min) for reaching a steady state baseline. The reason for this phenomenon may be due to the diffusion time required for the substrate to reach out the active site of the enzyme and also taken much time to reach steady state response with catechin. It was not desirable for biosensor application where the time of analysis should be minimal.

Table 1.2.1: Comparison of immobilized PPO in various gel entrapment materials for biosensor analysis of polyphenols

Gel material	Max. enzyme response in V	No. of analysis (till ½ of the initial activity)
Sodium alginate	1.36±0.013	58±1.16
Kappa carrageenan	0.72±0.011	39±1.17
Agar	1.11±0.085	52±1.32
Gelatin	0.88±0.016	48±1.81
Polyacrylamide	0.94±0.013	55±1.56

This is also indicated that the immobilized enzyme with initial activity with high response (in voltage) used to give more number of analyses with the sample (Table 1.2.1).

1B.4.1.2. Cross-linking

It was observed that the enzyme retained its 60% of initial activity. In cross linking method as compared to the entrapment method more enzymes could be loaded to the membrane and which gives an expected activity of a particular membrane since the approximate retention of activity of a particular cross-linking method is known.

Tyrosinase enzyme gave maximum enzyme activity in its native free form (14,020 IU), (while in immobilized condition the activity reduced by almost 40%) (8,436 IU) and henceforth the percentage retention activity remained only 62.62. PPO also gave similar activity retention after immobilization, *i.e*. 57.6% (Table 1.2.2). Whereas in immobilized laccase enzyme activity retention was only 38% from its initial activity (free native form).

Table 1.2.2. Retained enzyme activity of immobilized enzymes

Enzymes	**Activity IU (native) in 1mg/mL protein with 10 mM catechin as substrate**	**Activity IU (Immobilized) initial loading 1mg/mL with 10 mM catechin as substrate**	**% of retained activity**
Tyrosinase	14020	8779	62.62
PPO	8436	4859	57.6
Laccase	760	290	38.16

Immobilized tyrosinase (Tyr- I and Tyr-II) responded well to the catechin substrate (10 mM 50 µL). The response was around 1.0 V and repeatable (TABLE 1.2.3). Immobilized PPO-III has given response to catechin (10 mM 50 µL) of approximately 0.8 V. Laccase enzyme showed little response, 0.05 V to the catechin substrate (10 mM 50 µL). The response with immobilized Laccase membrane was minuscule in comparison with other immobilized enzyme membranes. Analysis cycle time reduced down significantly (5 min), when comparing with gel entrapment methods where it took >10 min. for each analysis. Preparation time for cross linked enzyme membrane is minimal (approx. 1 h), then storage and transport of the cross linked enzyme membranes were easy.

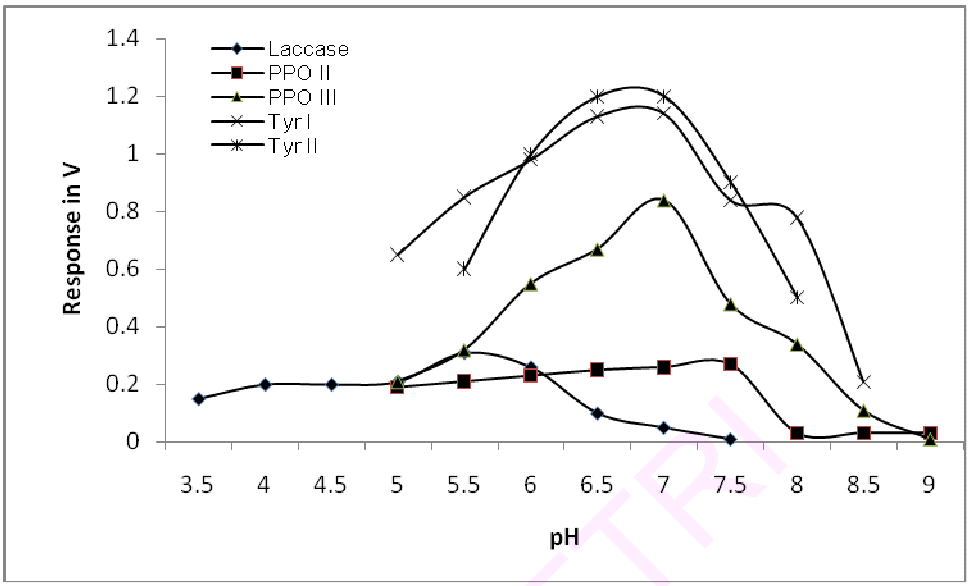
Table: 1.2.3. Comparison of biosensor performance of each enzyme with standard catechin

Enzymes	Biosensor response in V
Laccase	0.058±0.002
PPO	0.865±0.029
Tyrosinase	1.0±0.0.029

1B.4.2. Stabilization studies on different parameters for optimum performance of enzyme based biosensor application

Various physiochemical parameters like, pH, and substrate concentration were optimized for better performance of immobilized enzyme with more life of the membrane for the development of biosensor system.

For immobilized laccase enzyme, initially the activity of the enzyme was enhanced at pH 3.5 to 4.0 (0.15 V). Further the activity was dip down at pH 5.0 as shown in the Figure 1.2.2. Further the activity increased steeply to almost double the initial value at pH 5.5 (0.31V) followed by a sudden decline afterwards. To conclude the enzyme activity and its stability was seen maximum at pH 5.5 but no activity was observed at 7.



Tyr I – purified tyrosinase, Tyr II – Commercial tyrosinase

Figure 1.2.2. pH optimization of immobilized enzymes; 3.0 - 7.5 p H range for laccase, 5.0–9.0 for PPO II and PPO III and 5.0 – 8.5 for tyrosinase I and II

For the pH studies with PPO-III and PPO-II NaPi buffer with pH in the range (5.5–9.0) was used. Maximum response for PPO III enzyme from tea leaves was at pH 7.0.

In a given pH range (5.0 – 8.5), tyrosinase immobilized biosensor system gave maximum response at pH 7.0. At optimum pH tyrosinase enzyme responded maximally than any other immobilized enzyme membranes used for PP analysis.

Tyrosinase (Tyr-I and Tyr-II) gave much higher response (in voltage) with substrate, which is very essential for the maximum numbers of analysis using single membrane. When the fresh immobilized enzyme gave higher response the count of analysis also will increase.

Biosensor response of immobilized tyrosinase enzyme membrane to various concentrations of catechin 1 - 10 mM was done. The calibration graph gave good linearity (R^2 = 0.9991) within range of 1 – 10 mM of catechin (Figure 1.2.3a).

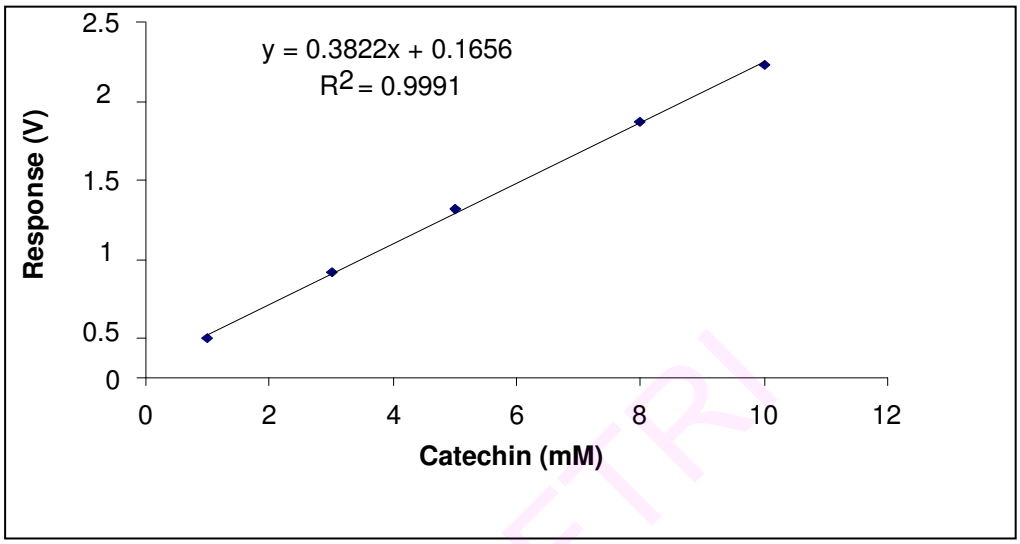


Figure 1.2.3a. Response for catechin using immobilized tyrosinase based biosensor.

Purified PPO–II from tea leaves responded well with the different concentration of catechin (Figure 1.2.3b). Maximum response was seen for 8 mM concentration of catechin. Where as higher concentrations above 10 mM of catechin did not give good linear response and finally signal above 9 mM was saturated. Also signal response for these catechin concentrations was low as compare to immobilized tyrosinase response.

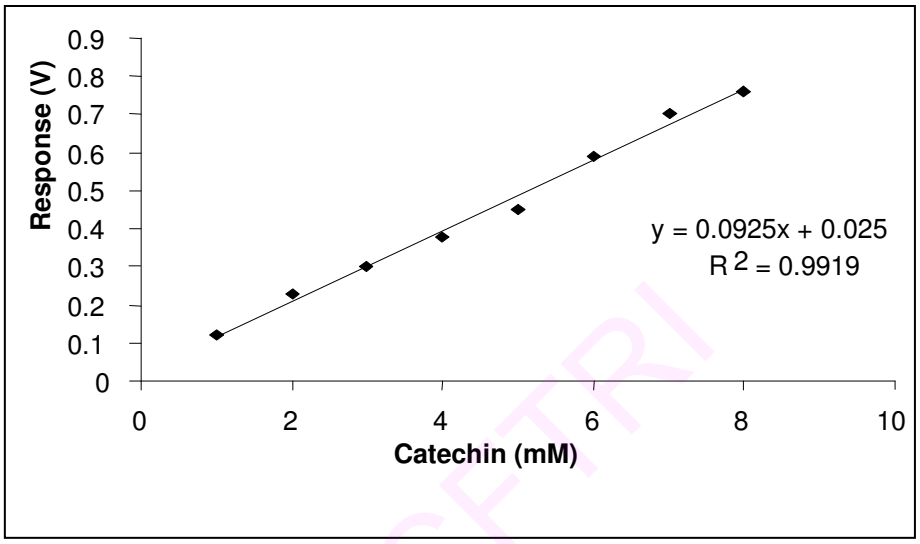


Figure 1.2.3b. Response curve of PPO -II.

Tea leaves PPO-II with concentration range of 1 - 8 mM, gave good linearity with a R^2 value of 0.9919 (Figure 1.2.3b), the maximum response for 8mM was found to be only 0.75 volts. PPO–III enzyme responded well with higher concentration of catechin and it was found that PPO-III responded well with 1-10 mM catechin concentration having a regression of 0.9936 (Figure 1.2.3c). It was interesting to note that this enzyme fraction from tea leaves gave better response but slightly less than the tyrosinase enzyme. It was also observed and discussed in the later section regarding the response of tyrosinase and PPO-III for black tea extract/decoction.

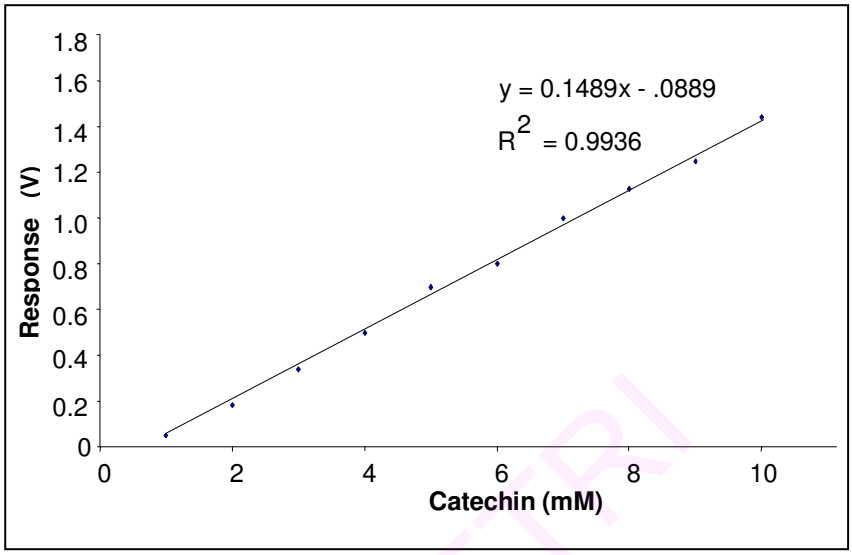


Figure 1.2.3c. Response curve for PPO III.

Laccase immobilized enzyme showed a regression of 0.9887, for the concentration of catechin used was 0.1 to 0.5mM (Figure 1.2.3d), CLEC also gave good linearity with regression value of 0.9916 and for a concentration of catechin used was 0.1-1 mM (Figure 1.2.3e).

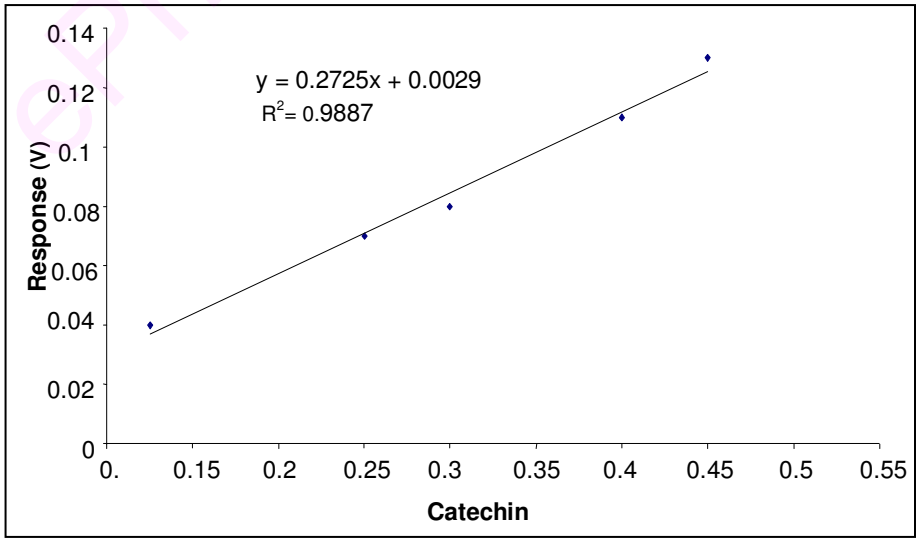


Figure 1.2.3d. Response of catechin as substrate by immobilized laccase.

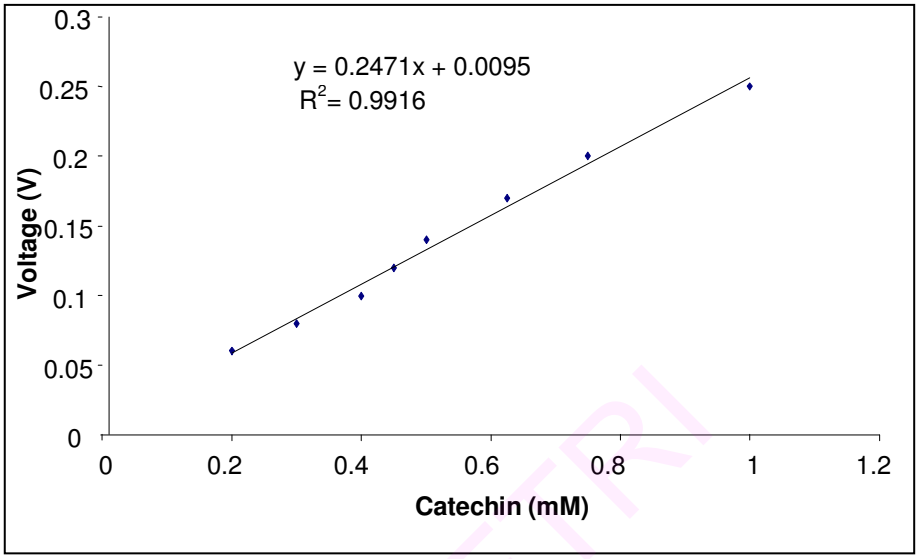


Figure 1.2.3e. Response using CLEC laccase with catechin as substrate.

1B.4.3. Application of immobilized enzymes for biosensor analysis of polyphenols

The response given by immobilized tyrosinase is 2.3 V is better among the three enzymes tried. The biosensor response of immobilized PPO isozymes (PPO-III) was also considerably high (1.4 V) but found second only to tyrosinase.

Laccase was found to be unsuitable as it gave low response with catechin in Figure 1.2.4. The major observation that laccase is active at highly acidic pH i.e. about 5.5, which is not suitable for the analysis of tea samples using biosensor. Both the PPO isozymes were immobilized and used for the PP analysis. It was observed that PPO-II is not stable after immobilization and retained activity was very low after the immobilization. Apart from this, during the cycles of analysis activity of enzyme was reduced considerably. So, only PPO-III was selected for analysis of PP and was compared with the tyrosinase standard and tyrosinase isolated from the mushroom.

PPO-III from tea leaves was thought to be suitable for biosensor application when catechin was used as a substrate, but surprisingly biosensor response with black tea was very low. During fermentation of green tea, all catechins get polymerized to TFs and TRs and their gallats in presence of sufficient ambient oxygen. It was interesting to note that green tea leaves catechins gave excellent response with biosensors based on PPO III where as it did not give good response with black tea PP. It was concluded that during black tea fermentation PPO-III is responsible to convert all green tea catechins to black tea PP. Converted black tea PP seems to be not suitable substrate for PPO-III based biosensor system. It was found that, both commercial and purified tyrosinases were superior to the purified PPO enzyme for black tea analysis. It was also observed that tyrosinase has wide substrate specificity in terms of green and black tea catechins. Therefore for further work on the biosensor development tyrosinase enzyme was used for the analysis of black and green tea.

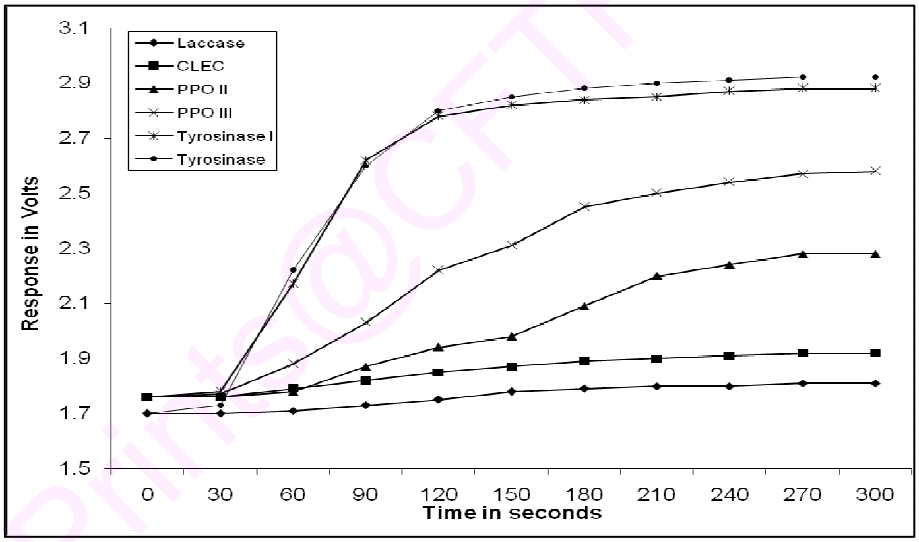
Among the enzymes studied for biosensor application, it was clearly indicated that tyrosinase was having better performance both in the native form and in the immobilized condition. Apart from this the specificity and affinity towards the catechins substrate was high for tyrosinase enzyme.

A preliminary experiment was carried out to see the performance of immobilized PPO-III and TYR-I for the analysis of PP in prepared (decoction) black tea. It was observed that the tyrosinase has given a better response with black tea samples than the PPO enzyme (Table 1.2.4). In the kinetic study (Section A Table 2.1.2) it was found that tyrosinase gave low Km and high Vmax with catechin and PPO was having high Km and comparatively low Vmax. Available substrate concentration to initiate the catalytic reaction may not be present in black tea for PPO since natural substrates may reduce during tea processing, whereas tyrosinase could give response with available PPs such as TF and TR present in black tea. Further it was concluded that tyrosinase is the

better enzyme for the black tea analysis. Therefore further work was carried out with tyrosinase enzyme for biosensor application.

Table 1.2.4. Preliminary analysis of black tea with PPO and tyrosinase immobilized biosensor.

Enzyme	Response(V)
Tyrosinase	2.3
PPO	0.8



CLEC – cross linked enzyme crystals of laccase, PPO II and III extracted from tea leaves, Tyrosinase – Standard enzyme, Tyrosinase I – Enzyme extracted from mushroom

Figure 1.2.4. Comparative analysis of various enzymes on biosensor for polyphenol analysis using 10 mM (100 µL) catechin substrate

A general problem for many biosensors is lack of necessary operational and storage stability. The tyrosinase immobilized membranes were stored up to 30 days and the activity checked daily by injecting 50 µL 10 mM catechin and recording the response as drift in steady state voltage response on each day. The enzyme membrane retained 50% of its initial activity after 10 days and

60 analyses. Membranes stored in NaPi buffer (pH 6.8, 10 mM) at 4℃ showed better stability than the membranes store as dry and at room temperature in NaPi buffer.

1B.4.3.1. Stabilization studies of enzyme for repeated use in biosensor

Tyrosinase was immobilized with 2% lysozyme, BSA, and gelatin. Analyses were carried out with 10 mM standard catechin until the membrane lost 50% of its initial activity. Among the three PBSA used, lysozyme was found to be the best, followed by BSA and gelatin. For tyrosinase immobilized with lysozyme it was possible to analyse 100 samples. The response gradually decreased with number of analyses, reaching 50% of the initial activity after 70 analyses. This may be due to accumulation of products formed, causing membrane fouling and can probably be attributed to the strong interaction between PP and the enzyme (protein) which has been reported to be irreversible (Papadapoulou and Frazier, 2004). Protein–PP binding forms a strong complex over the membrane surface, which in turn decreases the diffusion rate results in the reduction in the response of the biosensor. With BSA and gelatin it was possible to analyse 60 and 50 samples, respectively. Another significant observation from Figure 1.2.5 is that tyrosinase immobilized with gelatin and BSA lost its activity rapidly when compared to tyrosinase immobilized with lysozyme.

In this work, it was found that the incorporation of lysozyme during immobilization of PPO contributes to the long run operational stability of the immobilized enzyme for biosensor application for tea. Though all the three PBSA enhanced the stability of immobilized enzyme, lysozyme was found to be the best compared to BSA and gelatin. Therefore, for further studies lysozyme was taken and co-immobilized with tyrosinase.

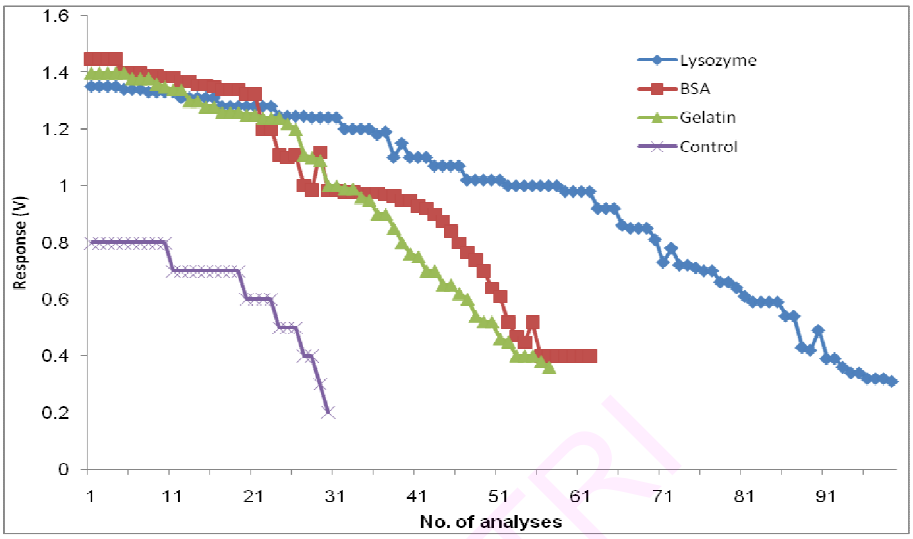


Figure 1.2.5. Operational stability of the enzyme membrane using different protein - based stabilizing agents

To check reusability, standard samples were analysed with the immobilized tyrosinase-based biosensor over a period (Figure 1.2.6). The response to catechin (10 mM) was measured until 50% of the initial activity was lost.

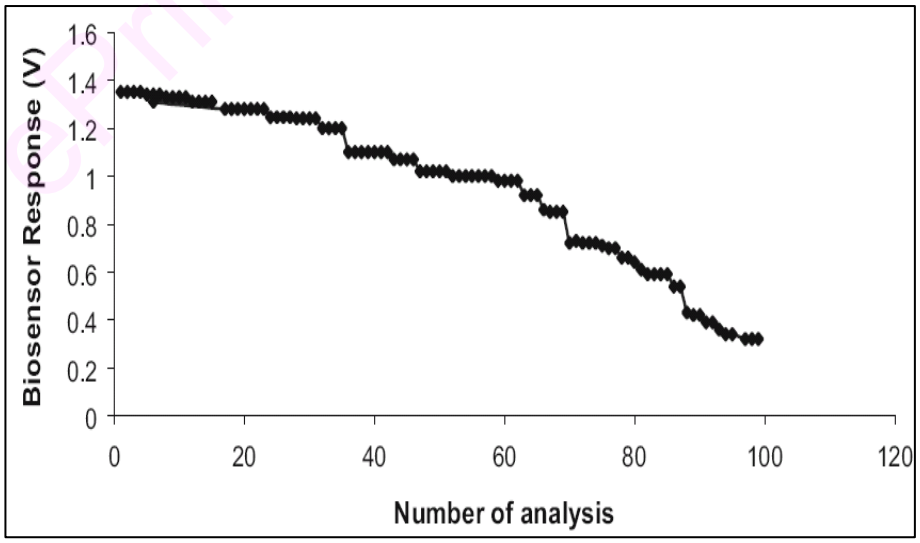


Figure 1.2.6. Reusability of the immobilized tyrosinase enzyme membrane. One hundred analyses were carried out over a 10-day period with 50 µL, 10 mM catechin.

The samples were analysed with the membrane continuously for evaluating the life of the membrane. Tyrosinase enzyme membrane was proved to be the best membrane for PP analysis. However, there were some major shortfalls with the tyrosinase immobilized membrane which has to be rectified. Getting the base line back in the detector was a problem with every sampling as the PP accumulation increases on the membrane. At the initial stage with 100 mM catechin enzyme would lost more than 50% of activity within 24 h just over 25 sample analysis. To overcome this reduced the sample concentration to 10 mM (catechin) with injection volume as before (50 µL). This decrease in the substrate concentration helped to reduce down fouling of the tyrosinase immobilized membrane and further improve the life of the membrane. On an average 10 mM concentration was preferred for the analysis. With 10 mM catechin it could able to go for around 100 analysis spanning 10 days on a single immobilized tyrosinase membrane. The voltage change was 0.15 V for all analysis with 5% (mV) fluctuation. The protein - PP interaction which led to the complex formation is supposed to be very strong and irreversible which hinder the enzyme activity to a great extent. The PP complex forms a thick layer over the immobilized enzyme over a number of analyses.

1B.5. Conclusion

Specific activity studies were shown that PPO having more affinity towards the indigenous substrate catechin. It was observed that all enzymes responded to PP. Tyrosinase found to be better among in all enzymes with highest specific activity and better performance with immobilized condition. Tyrosinase enzyme was chosen for all other experiments in tea biosensor for PP.

Section C. Description of biosensor based detector system for the analysis of tea polyphenols

1C.1. Introduction

Among the biosensors, amperometric sensors are widely used for the analysis of samples of origin from clinical, agricultural and environmental and having some advantages: such as simple to use, portability, intrinsic specificity and low costs (Turner *et al.,* 1987). Amperometry is the measurement of the diffusion boundary current at a constant potential between a polarized working electrode and reference electrode. Amperometric sensors use transference processes of ionic to electronic charges between electroactive species and electrode, produced in a redox reaction. An electric current generated during biochemical/chemical reactions passed through the electrode device immersed in a solution which is directly related to the analyte concentration. Current measurement was done using a two-electrode arrangement (working and reference electrodes).

The electrical signal from the transducer is often low and superimposed upon a relatively high and noisy (*i.e.* containing a high frequency signal component of an apparently random nature, due to electrical interference or generated within the electronic components of the transducer) baseline. The signal processing normally involves subtracting a 'reference' baseline signal, derived from a similar transducer without any biocatalytic membrane, from the sample signal, amplifying the resultant signal difference and electronically filtering (smoothing) out the unwanted signal noise. The analogue signal produced at this stage converted to a digital signal and passed to a microprocessor where the data is processed, converted to concentration units and output to a display device or data store.

In the present study, a detector system was fabricated for the analysis of PPs for the quality assessment of tea based on PP contents and details of the device developed is given below.

1C.1.1. Principle of amperometric biosensor operation

The principle of operation of the tea biosensor is the enzymatic transformation of PP with consumption of oxygen in the reaction. The change in the dissolved oxygen concentration correlates with the concentration of catechins, which is one of the important measurements of tea quality in terms of PP content. The biochemical reaction is represented by:

$$QH_2 + 1/2O_2 = Q + H_2O$$

where QH_2 and Q are the reduced and oxidized forms of phenols. In terms of electron transfer, the reaction can be written as

$$O_2 + 4H^+ + 4e^- = 2H_2O$$

The depletion of oxygen at the electrode caused by the biochemical reaction also involves consumption of electrons, resulting in an electrochemical signal which is proportional to the concentration of PP in the sample. This signal is conditioned, amplified and monitored by use of an amperometric detector system as discussed above.

1C.2. Materials

Clark-type electrode (Amperometric type 2 electrode system) procured from Century Instruments, Chandigarh, India. Signal-conditioning unit, current to voltage converter, amplifier circuit, data processing unit, parallel printer interface

and display and all other electronic components for the fabrication of the detector system purchased from local vendors.

1C.3. Methods

1C.3.1. Design of the instrument prototype

A prototype of the detector system was developed for the detection of PP in tea. The biosensor device shown in Figure 1.3.1 comprises a Clark type amperometric electrode, air pump, an immobilized enzyme membrane system consisting of hydrophobic polymer (teflon membrane) and a hydrophilic polymer (dialysis membrane) with immobilized enzyme sandwiched in between. A signal conditioning board (SCB) consisting of a current to voltage converter circuit was used for processing of the electrode signal which was digitized using an analogue to digital converter and a peripheral interchange controller (PIC) microcontroller unit for data processing with a keyboard and liquid crystal display (LCD). The microcontroller unit has a serial interface, keyboard, display, printer interface and a random access memory (RAM), with software for data-acquisition, processing, calibration and report generation.

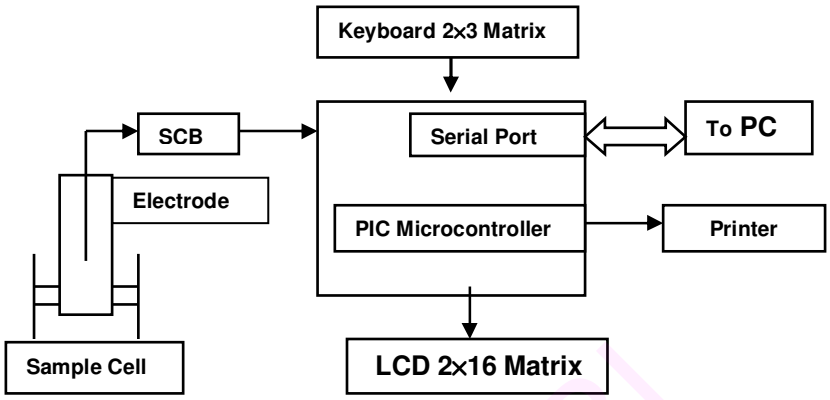


Figure 1.3.1. Schematic representation of electronic components of a biosensor system

When the substrate was introduced into the reaction cell using a sample injector, enzyme reaction proceeds, resulting in depletion of oxygen in the vicinity of the enzyme membrane thereby yielding the electrochemical signals. These signals were suitably conditioned using the signal-conditioning unit which was digitized using an analogue to digital converter (ADC) and further processed using the microcontroller for quantification of the PP. The acquisition and processing parameters were programmable. The system requires a two point calibration for quantification of PP.

The immobilized enzyme-based electrode used for construction of the biosensor for PP is shown in Figure 1.3.2.

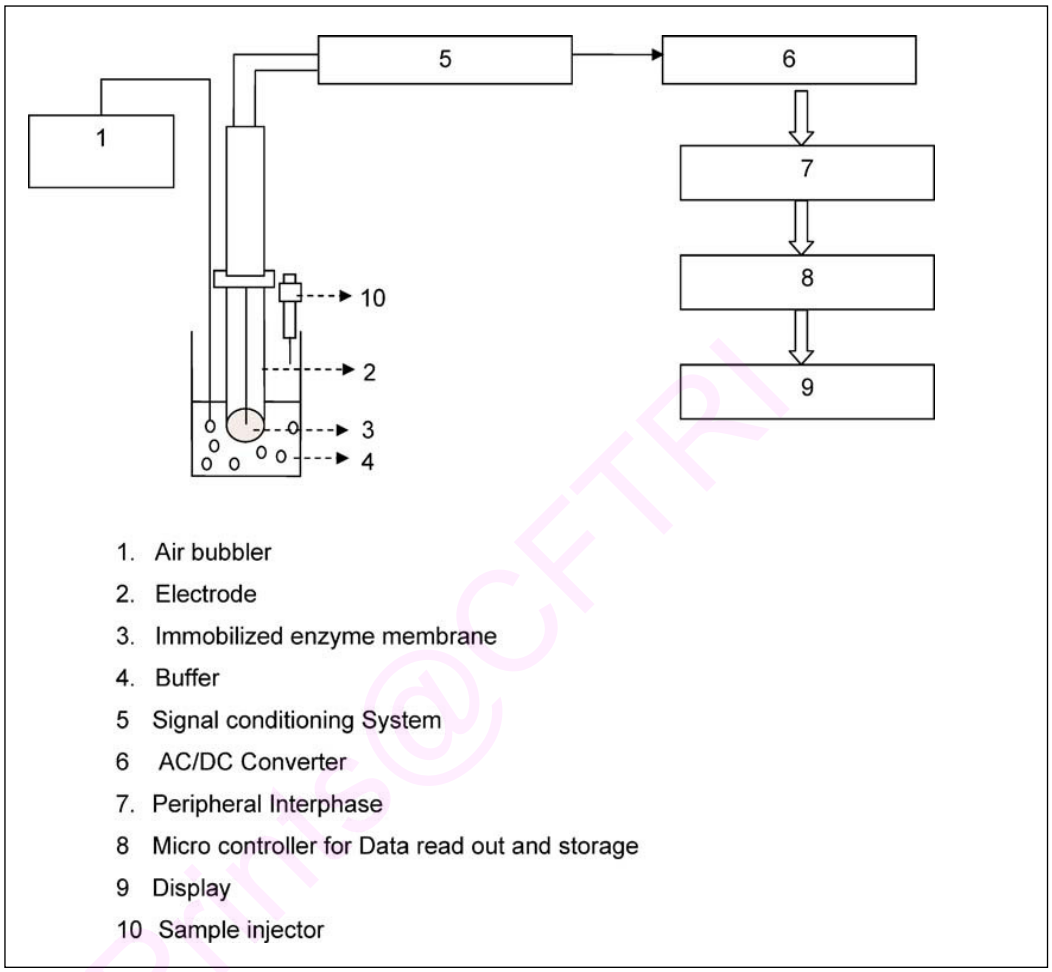


Figure 1.3.2. Schematic diagram of the biosensor system for PP detection

1C.3.2. Enzyme electrode construction and operation

The enzyme electrode was constructed as described in an earlier paper published by our group (Abhijith *et al.,* 2007, Roy *et al.,* 2005;). Clarke type electrode consists of a gold cathode and a reference Ag/AgCl electrode immersed with saturated KCl electrolyte enclosed by a teflon membrane (Membrane 1, Figure 1.3.3). Immobilized enzyme was sandwich between teflon (membrane 1) and cellophane membrane (Membrane 2). The

immobilized enzyme membrane was secured to the electrode using an "O" ring. The electrode containing the enzyme membrane was dipped in a sample cell containing 2 mL, 6.8 pH, 10 mM NaPi. The sample cell was continuously saturated with oxygen using an aerator. The electrode was connected to the detector system developed by the Central Food Technological Research Institute (CFTRI), Mysore, India. During biochemical reactions, O_2 is consumed, which was monitored as a decrease in current. This decrease in current was converted into a voltage by the system and the response in voltage, which is directly proportional to the concentration of the analyte, was displayed.

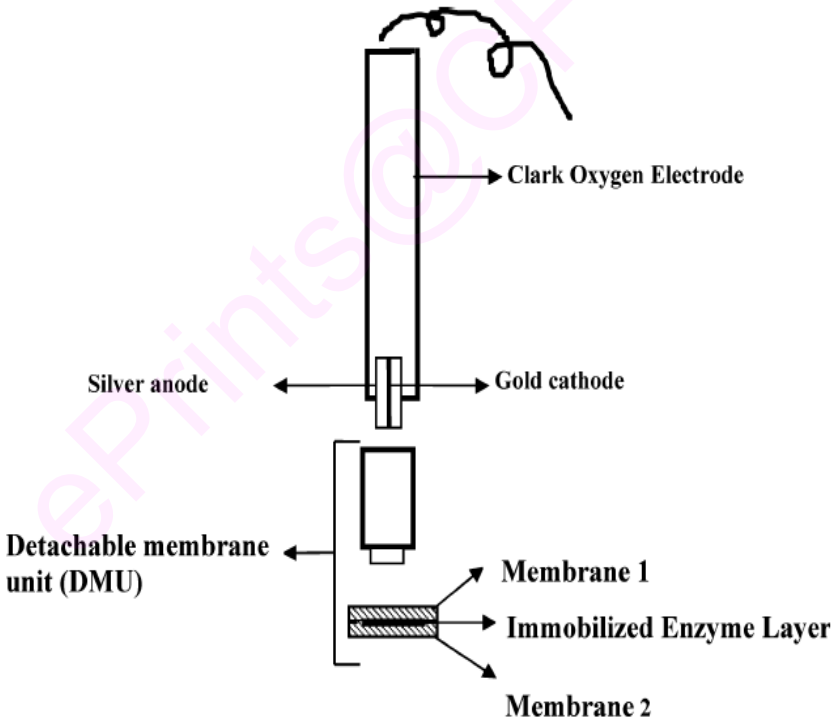


Figure 1.3.3. Schematic diagram of the immobilized enzyme based electrode used for the construction of the biosensor

In this system the controlled potential technique is used. In controlled potential maintained constant with respect to a reference electrode (Silver electrode). The potential of the working electrode controls the degree of completion of an electrolytic process. Hence the potentiostat with large output current and voltage capabilities is to provide constant voltage to working electrode.

Then the low current output from this system is conditioned to get the desired output using the signal-conditioning unit shown in figure 1.3.4.

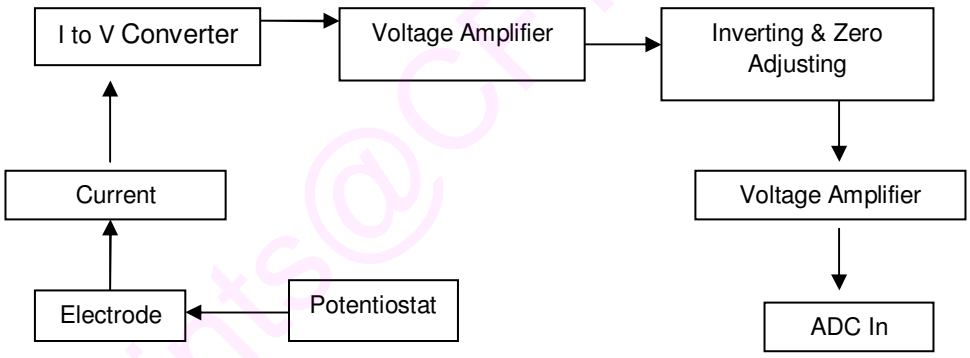


Figure 1.3.4. Schematic diagram of signal conditioning board for 2-electrode system

The high precision operational amplifier (op-amp) was used for the signal conditioning hence minimization of noise in the low current measurements is necessary. Due to usage of this op-amp avoids the any offset adjustment and also provides the greater stability with a 10 mV fluctuation.

In the biosensor instrument system, a microcontroller from Cypress Microsystems was used for processing of the data obtained from signal conditioning unit. Heart of this microcontroller was a high performance microprocessor, thus programming of parameters became easy.

The potentiostat provides specific potential ranging between -650 to + 800 mV to the working electrode with respect to reference electrode. Initially the output current from the electrode is amplified and then it is converted to voltage, which is proportional to the oxygen consumed during the enzymatic transformation, which is in turn related to the concentration of the catechin (PP) present in sample.

1C.3.3. Prototype instrument unit

The designed detector system (Figure 1.3.5) that is already explained in materials and methods above was used for the detection of tea PP.

Display menu of the developed detector system as explained below

Menus

1. *Set Zero*

   A timer of 1Hr is set here. Continues voltage will be displayed here

2. *Calibration*

   2.1. Concentration LOW (here we are going to set lower concentrations 1 - 10 mM)

   Set Zero

   2.2. Inject LOW

   Concentration HIGH (here we are going to set higher concentrations 10 - 100 mM)

   Set Zero

   2.3. Inject HIGH

   2.4. Constants (here constants m and c is displayed)

   Note: to skip from any menu and return to previous one enter **ESC**

3. *Start Analysis*

   3.1. Continue with previous calibration results?

Set zero

3.2 Inject unknown sample

3.4 Concentration

3.4 print? (Here press print key to generate report and print)

Exit

Returns to Main menu

Figure 1.3.5. Prototype of fabricated biosensor system for tea polyphenol analysis

1C.4. Conclusion

A biosensor based instrument was fabricated, which can detect signals at a nA scale, which was amplified and displayed as volts (V). Biosensor system programmed for a response, at various concentration of PP. Instrument gave steady baseline reading and recurring back to base line with little margin of error. It also has better signal to noise ratio. Instrument employs a user friendly programmed menu for its application in analysis. In order to assess and evaluate the biosensor performance biological recognition unit (enzyme) was required. For this immobilization of enzyme is necessary. Detailed description of immobilization and stabilization of enzymes for analysis of PP using this instrument was detailed in the previous chapter.

Section D. Studies on optimization of immobilized tyrosinase enzyme membrane for repeated use in biosensor application

1D.1. Introduction

Immobilized tyrosinase enzyme used for the detection of various phenols (Rajesh *et al.,* 2004) and PP in tea (Abhijith *et al.,* 2007). There are few bottlenecks to be resorted to establish a commercial model of biosensors for the detection of PP. One of the predominant problems in the tyrosinase was the complex formations on the enzyme membrane due to protein–PP interactions. Few reports are available on the protein-phenol interactions (Mozzicafreddo *et al.,* 2009; Codorniu-Herna´ndez *et al.,* 2007; Koval *et al.,* 2006; Guan *et al.,* 2005). Protein–PP interactions were evident during the making of milk tea. The coagulation and complex formed between PP in tea and protein present in milk (Liang *et al.,* 2002).

Astringency of tea and wine is more or less attributed by binding/precipitation of PP/peptide complexes. Intermolecular binding between protein and PP facilitated by stacking of polyphenolic rings onto planar hydrophobic surfaces and further strengthened by multiple cooperative binding of polyphenolic rings (Fishman *et al.,* 2002). Complex formation of protein-PP is commonly referred as fouling. Fouling of the membrane is due to formation of complex between protein and phenol (Nistor *et al.,* 1999). PPs are having very high affinity towards the proteins. This affinity varies from one phenolic compound to other (Bartolomé *et al.,* 2000). Another interesting finding reported that, at molecular level amino acids exhibits different affinity towards the phenols (Abboud *et al.,* 2008). Salivary proline-rich proteins (PRPs), which are secreted into the oral cavity, form complexes with and precipitate dietary PP and thus, they constitute the primary mammalian defense directed against ingested tannins. As the PP size increases, the number of binding sites is reduced at protein (Baxter *et al.,* 1997).

In present study, immobilization of tyrosinase was done on polymeric membrane and the rate of fouling was estimated with standard catechin. The same fouled membrane set up was further used for analysis for another substrate, catechol which is having more affinity towards the enzyme. Study was also carried out of the affinity of PP towards various amino acids. Fouling effect of different concentration of standard catechin along with various tea samples were carried out.

1D.2. Materials

Lysozyme, catechin, catechol, glutaraldehyde and amino acids were obtained from Sigma Aldrich, USA. Dialysis membranes were purchased from Spectra/Por, USA. Teflon membranes procured from WTW, Germany. Clark's electrode obtained from Century Instruments India Ltd. Detection unit assembled in-house. All other chemicals used were of analytical grade.

1D.3. Methods

1D.3.1. Estimation of total polyphenol

Tea samples and other experimental samples were prepared as described below and total PP content was estimated (Folin and Ciocalteu, 1927). A sample (0.1 mL) was made up to 5 mL with distilled water. To this solution, 0.2 mL Folin–Ciocalteau reagent and 0.5 mL saturated sodium carbonate was added. The volume was finally made up to 10 mL. This final mixture was incubated in dark for 60 min and samples were then analyzed by spectrophotometry at 765 nm. A standard graph was pareapred to calculate PP content in the sample.

1D.3.2. Effect of fouling on immobilized enzyme membrane

Enzyme tyrosinase was immobilized as described in Chapter I, section 1B3.1.2.

1D.3.2.1. Preparation of amino acid samples

1 mg of each amino acid was weighed out and made the volume to 1 mL using the NaPi buffer. From the amino acid preparation 100 µL was added to the membrane, along with the lysozyme (as PBSA) and the cross linker (glutaraldehyde) was added and mixed thoroughly. Various amino acids were prepared at 1mg/mL stock and co-immobilized with lysozyme, using glutaraldehyde as a cross-linker on the membrane. Effect of amino acids composition in the binding affinity of PP to immobilized membrane was determined keeping lysozyme cross linked with glutaraldehyde as the control. Membranes were treated with catechin 10 mM concentration for 2 hours. Enzyme membrane was removed and dissolved in 50% methanol. The total bound/ retained PP was estimated using FC method.

1D.3.2.2. Preparation of catechin samples

Catechin standards were prepared in NaPi buffer. 2 mL of 10 mM catechin solution was introduced in the sample cell on to the enzyme membrane surface. Cycle time was 5 minutes for all the analysis. Experimental set up was continuously aerated with a bubbler. Analysis was continued till the enzyme lost 80% of its initial activity. To the same membrane (which gave only 20% response to catechin) was introduced with 10 mM concentration of catechol.

1D.3.2.3. Preparation of tea samples

Tea samples were prepared using the method by Degenhardt *et al.,* 2000. In brief, 1.4 g of tea was extracted for 5 min with 100 mL of boiling water. Green tea and black (both CTC and orthodox) tea samples were prepared with same method.

1D.3.3. Affinity of enzyme membrane to polyphenols

This experiment was carried out to determine the affinity of PP to immobilized enzyme membrane. Stock solution was prepared of 100 mM concentration. Further stock was diluted to get concentrations of 1.0 mM, 10.0 mM and stock preparation as such used for the affinity studies of the PP with the immobilized membrane.

The enzyme membrane secured to the electrode (described in chapter I, section C and subsection 1C.3.2) was dipped in PP (catechin) having different concentrations such as 1, 10 and 100 mM. This facilitates the PP to come in contact with the immobilized tyrosinase membrane.

The enzyme membrane was in contact with the substrate for two hour with continuous aeration. Same membranes were washed thoroughly to remove unbound phenols from the surface. A control was kept in buffer. The bound PP (catechin) with enzyme membrane was extracted with 50% ethanol. These removed PP samples were analysed for the total PP content using FC method (Folin and Ciocalteu, 1927).

1D.3.4. Dissociation/prevention of protein-polyphenol interaction from complex formation

The main constraint for the immobilized tyrosinase based biosensor is the protein-PP interaction. The binding of the PP to the enzyme is irreversible. This is one of the main reasons for darkening of the enzyme membrane, which was resulted in the reduction in number of analyses of PP containing samples with single immobilized enzyme membrane. To overcome this problem, some dissociating agents were tried at different concentrations; they were dimethyl sulfoxide (DMSF), cyanogen bromide (CNBr), polyethyleneimine (PEI), methanol, ethanol, glycine, glycerol, polyethylene glycol (PEG). Analysis of sample was done with all dissociation agents along with free enzyme and immobilized tyrosinase.

1D.3.5. Studies on the dissociation of polyphenol with lysozyme

Studies were carried out on the dissociation of PP from the protein. Experiments were then conducted by addition of different polyols like glycerol, polyethylene glycol and other additives like polyethyleneimine (PEI) and sucrose along with lysozyme (as a model protein) and catechin (as a model PP) to see the effectiveness of these compounds on the prevention/dissociation/binding of the protein-PP complexes using Shimadzu HPLC system. Experiments were carried out by incubating catechin with lysozyme and the formation of lysozyme-catechin complex was performed using HPLC analysis with C-18 column. Binary gradient elution was used. Mobile phase A contained a mixture of acetonitrile, water and trifluoroacetic acid (TFA) (80:920:0.6, v/v). Mobile phase B contained methanol, acetonitrile, water and TFA (30:270:700:0.6, v/v), (Chu *et al.,* 2004).

1D.4. Result and Discussions

1D.4.1. Estimation of total PP content

A standard graph was prepared using catechin (Figure 1.4.1). Total PP was calculated and expressed as mg of catechin equivalent present in one litre of sample (mgEC/L).

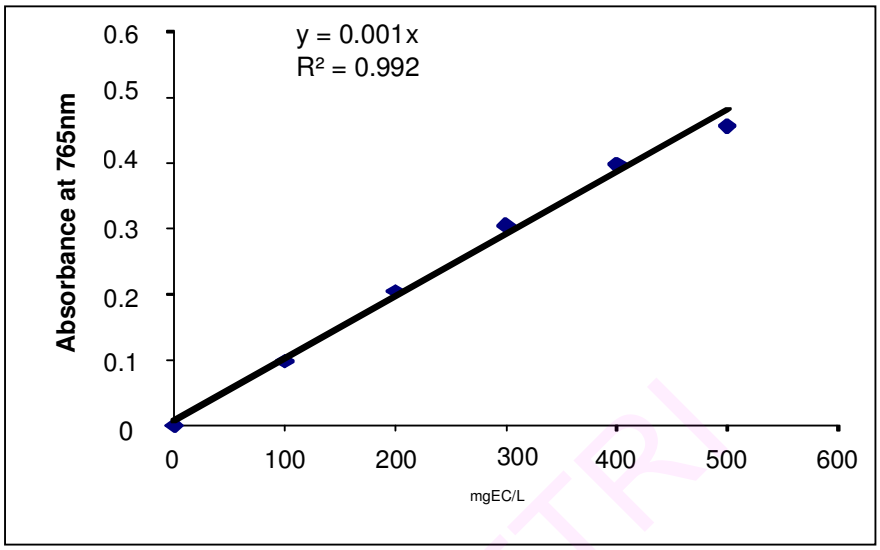
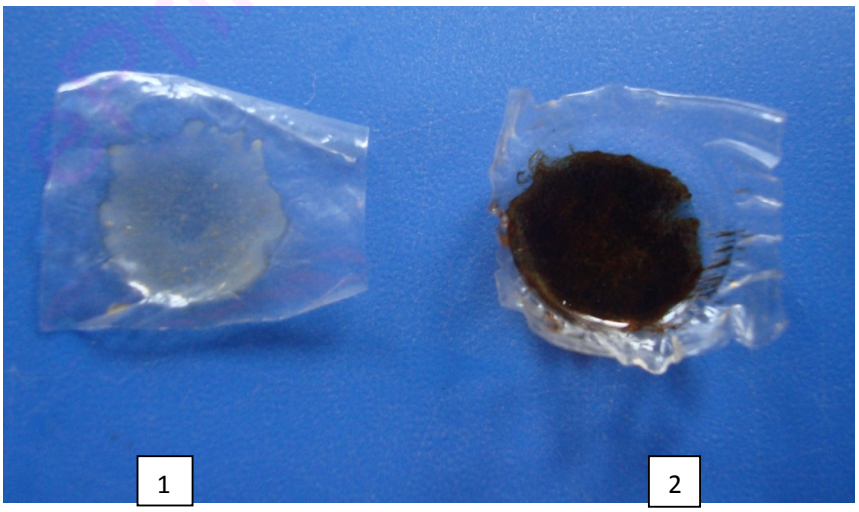


Figure 1.4.1. Standard graph for total PP estimation (FC method)

1D.4.2. Effect of fouling on membrane

With a single membrane about 100 analyses of PP (catechin 10 mM) were done. The membrane after 100 analyses was observed with fouling, which was visible by the dark colour formation, Fig. 1.4.2.



1. Immobilized enzyme membrane before analysis 2. Immobilized enzyme membrane after analysis

Figure 1.4.2. Immobilized enzyme membrane turned to brownish black after PP analysis over a period of time

Initial baseline was 2.9 V and initial response for catechin (10mM) was 0.77 V. The response for the first forty set of analyses with same concentration of catechin (10 mM) was of 0.7 V. Next forty analyses gave 0.6 V responses. After this point there was a sudden fall in the response and base line voltage reached down to 0.2 V.

It was noteworthy to mention that catechol (10mM) gave 0.9 V which was higher than that of the initial response given by catechin with fresh enzyme membrane. About 20 analyses for catechol was carried with fouled membrane after which membrane lost more than 80% of activity against catechin (Figure 1.4.3). This indicates the membrane fouling is not affecting the active site of the enzyme. Only reduction in diffusion is acting as a rate limiting factor for catechin analysis. It is interesting to note that catechol diffuses in to active site even after the enzyme membrane fouling and thus better biosensor response was obtained. But the response with catechin was low after 80 analyses. It was concluded that low response of biosensor was due to PP fouling/protein-PP interations (Figure 1.4.3). PP containing *o*-dihydroxy groups led to hydrogen bonds between the phenolic hydroxyl groups and the receptors of the proteins such as primary amine and carboxylic groups were involved in phenolic-protein interactions (Hagerman, 1989). Compounds containing *o*-dihydroxyphenolic groups can bind protein via a bidentate hydrogen bond, which is stronger than the hydrogen bond with an isolated hydroxyphenolic group (McManus *et al.,* 1981).

Figure 1.4.3. Effect of substrate on immobilized enzyme response, when catechin was followed by catechol.

Among the amino acids maximum binding was observed with proline and lysine (Table 1.4.1). Affinity binding was observed minimum with phenylalanine. This investigation also provided vital information about the significance of amino acid composition of various proteins involved in the process of immobilization. Among the PBSA used, BSA has more open structures with more lysine residue in the outer surface, which may enhance the PP-protein interactions led to enhanced rate of fouling, meanwhile reducing the number of analyses and retard the life of immobilized enzyme membrane. Lysozyme is a compact globular protein which may not provide much intermolecular linkage between PP and protein. It was also supported by previous experiment, where lysozyme co-immobilized membranes could perform more number of analyses for PP than the other PBSAs.

Several authors suggest that both hydrogen bonding and covalent linkages between oxidized phenolics and nucleophilic amino acid side chains, such as lysine or cysteine, were involved in the binding PP to proteins (Sastry

and Rao, 1990; Saeed and Cheryan, 1989; Prasad 1988; Sabir *et al.,* 1974). Although oxidation of polyphenols leads to compounds that interact more strongly with proteins (Siebert, 1999), there is still no evidence of covalent bonding implied in phenolic-protein interactions. Interactions between phenolics and proline-containing peptides may involve formation of bound complexes (Bianco *et al.,* 1997).

Table 1.4.1. Total PP bound to the various amino acids

Amino acids	**Total bound PP Estimated mgEC/L**	**Amino acids**	**Total bound PP Estimated mgEC/L**
Alanine	1.71	Leusine	9.29
Arginine	1.42	Lysine	28.57
Aspartate	3.142	Methionine	2.29
Aspergine	1.57	Phenylalanine	1.14
Cysteine	21.57	Proline	29.00
Glutamate	1.71	Serine	26.86
Glutamic acid	10.57	Threonine	14.71
Glycine	7.71	Tryptophan	6.71
Histidine	12.57	Tyrosine	15.29
Isoleusine	19.00	Valine	8.71

1D.4.3. Binding of PP on enzyme membrane

Binding studies of PP with different concentrations on immobilized enzyme revealed the binding in accordance with the concentration (Table 1.4.2). As expected maximum bound PP (54.25 mgEC/L) observed with 100 mM catechin treated membrane. Bound PP for 10 mM and 1 mM PP treated membranes were 15 and 5 mgEC/L respectively.

It is to be noted that above experiment was done to estimate bound PP in the immobilized tyrosinase enzyme using FC method. With this experiment it was concluded that the sample concentration for the analysis of PP using biosensor preferably below 10 mM to minimize fouling of the

immobilized enzyme for repeated use. The same concentration of catechin was taken for further experiments with biosensor.

Table 1.4.2. Estimation of total polyphenol bound to the enzyme membrane surface using FC method

Catechin (mM)	Total polyphenols (mgEC/L)
1	5
10	15
100	54.257

1D.4.4. Dissociation/prevention of protein-polyphenol interaction

This study helped to find out a suitable dissociation agent to increase the reusability of the enzyme membrane but the complete dissociation could not be achieved. The agents used for dissociation studies and the results were tabulated below (Table 1.4.3). Addition of dimethyl sulphoxide (DMSO), cyanogens bromide (CNBr) and polyethylene imines (PEI) did not help in reusing the immobilized membrane, where as glycerol containing buffer with tyrosinase co-immobilized with lysozyme gave better performance in reusing the enzyme membrane.

Table 1.4.3. Dissociation studies using different reagents

Reagent	**Method**	**No. of analyses possible with single membrane**
Dimethyl sulfoxide (0.125%)	Membrane treated	10
Cyanogen Bromide (0.1%)	Membrane treated overnight	10
Polyethyleneimine (1%)	Added to the buffer	00
Methanol (20%)	Added to the buffer	30
Ethanol (5-20%)	Added to the buffer	30
Glycine (1%)	Treated overnight	30
Glycerol (5%)	Added to the buffer	65
Polyethylene glycol (1%)	Added to the buffer	35
Free enzyme	No additives	1
Immobilized tyrosinase	No additives	25

The PPs have the property to bind strongly with the proteins, due to this property the fouling of membrane occurs causing decrease in the number of analyses with single enzyme membrane. To increase the reusability of the enzyme membrane, effect of different solvents such as methanol, ethanol, acetone and ethyl acetate along with the working buffer at varying concentration were tried. The response was unsatisfactory with all these solvents. Whereas, the addition of glycerol (5%) with the working buffer the number of analysis has increased significantly from 25 to 60. Reusability of an immobilized tyrosinase membrane retained half of its activity for about 60 analyses.

1D.4.5. Dissociation studies with lysozyme

The HPLC profiles showed the positive influence of polyols on minimizing protein-PP interaction. Figure 1.4.4a shows the HPLC analysis of

catechin standard (1 mg/mL) showed a peak at a retention time (RT) of 2.63 and absorption maximum set at 297nm.

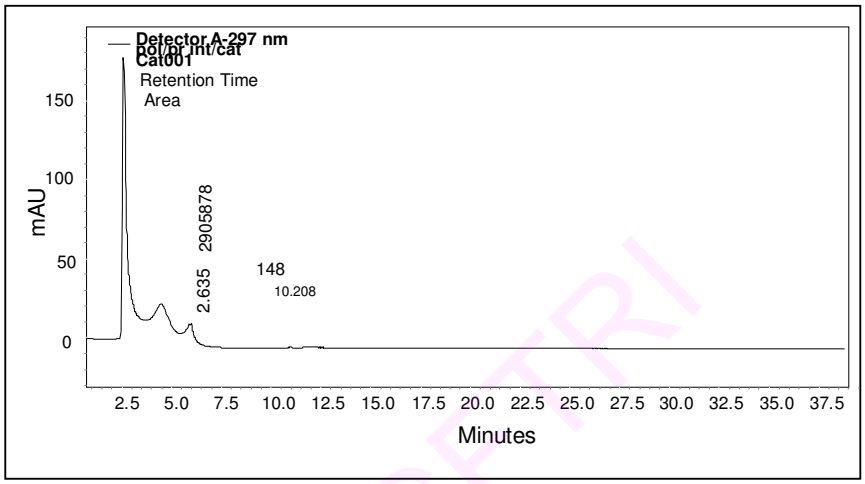


Figure 1.4.4a. HPLC profile of standard catechin (1 mM)

As seen in figure 1.4.4b the peak for model protein lysozyme (1 mg/mL) eluted at a RT of 70.83 min.

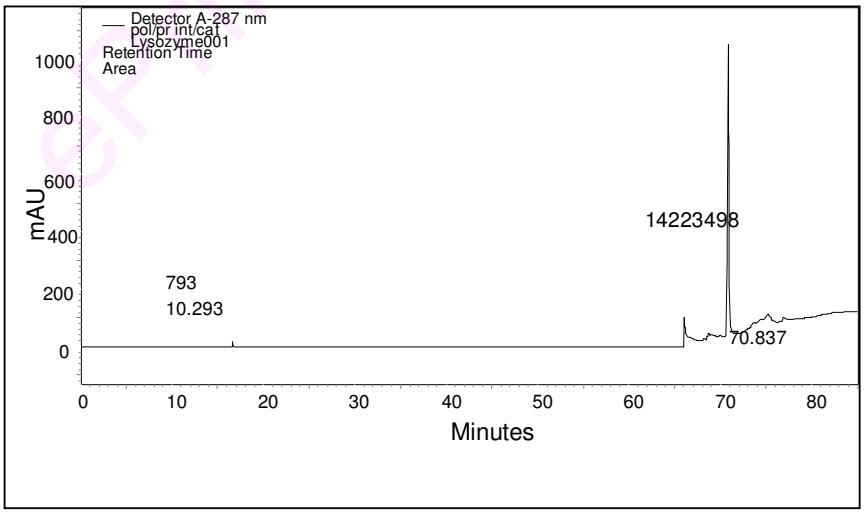


Figure 1.4.4b. HPLC analysis of lysozyme used as a protein model for dissociation studies

Figure 1.4.4c shows the protein-PP binding, where the sample was a mixture of catechin and lysozyme as the chromatogram clearly showing the absence of catechin peak and the retention time for the protein slightly extended (RT 80.10 min).

Lysozyme was used for immobilization for tea biosensor application. There is shift in the retention when catechin treated with lysozyme. This clearly demonstrated that there is a binding occurred between catechin and lysozyme. It was found that catechin was completely bound to the protein in the absence of any polyols (Figure 1.4.4c).

Using a competitive assay, Hagerman and Butler (1981) found that the selective affinity of proteins for condensed tannin varied more than 1000-fold. The highest affinities were found for proteins, polypeptides and polymers with high proline content and the lowest affinities were obtained for small globular proteins such as lysozyme. It is also interesting to note that different tannins show variations in interaction with a given protein. McManus *et al.,* (1985) observed that molecular size as well as flexibility affected the binding of PP to protein.

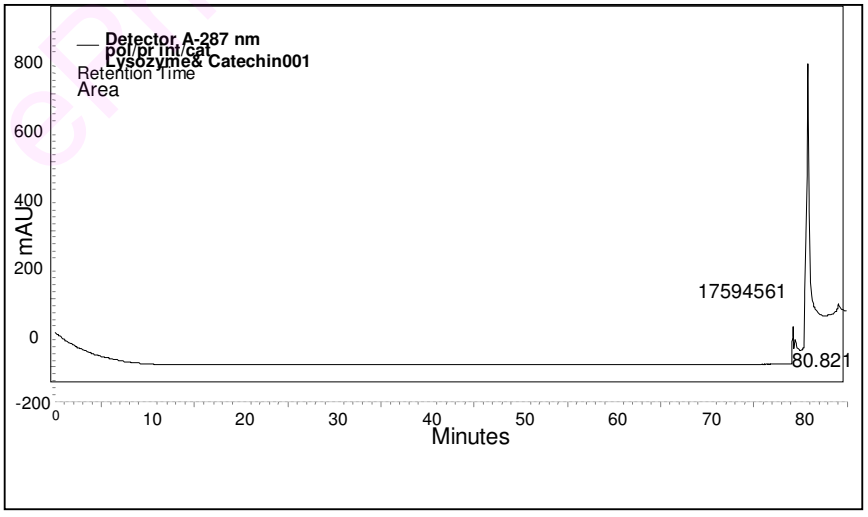


Figure 1.4.4c. HPLC analysis of mixture of catechins and protein (lysozyme) in absence of polyols

All the three polyols tested did not have any dissociating effect. However they were found to prevent the complete binding of the catechins to lysozyme (Figure 1.4.4d).

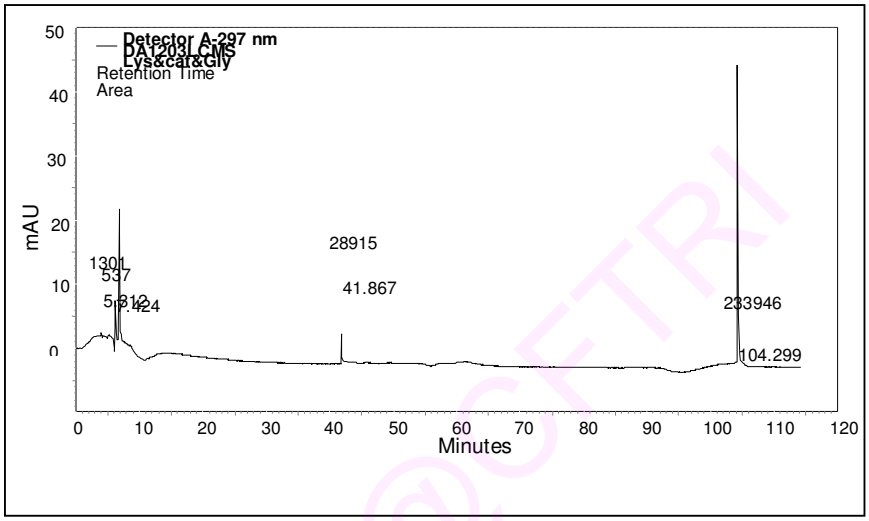


Figure 1.4.4d. HPLC analysis of mixture of catechins and protein (lysozyme) in presence of polyol

The effectiveness of the polyols in biosensor was tested and it was found that glycerol was more effective with more than 60 analyses possible, whereas sucrose and PEI inhibited the enzyme partially. Reduction in binding and the protein-PP interaction was proved with HPLC.

When catechin was incubated with mixture of lysozyme and 5% glycerol and analysed by HPLC the chromatogram showed appearance of two peaks (both catechin and lysozyme peaks), but the catechin area was smaller than the pure catechin peak as shown in Figure 1.4.4d. This clearly indicates that the addition of glycerol had an effect on the lysozyme-catechin binding though it could not able to dissociate completely protein from PP. Further studies are required to dissociate completely the catechin from the enzyme for several repeated use of the enzyme membrane.

1D.5. Conclusions

Studies illustrated that, by optimizing the conditions for the enzyme membrane and sample conditions the overall performance of the enzyme membrane can be improved. Here, a novel and simple method used to understand the complex mechanism of binding/fouling. This hypothesis is also interesting from a biological perspective.

In order to minimize the fouling PP concentrations below 10 mM is preferable to enhance the life of the membrane and biosensor performance. Glycerol 5% had proven as a better stabilizer increase the number of analysis. This investigation results were also proven that the incorporation of polyols assisted in the dissociation or preventing the complete binding of PP with protein.

Major outcome of this work lead to understand, at molecular level, the phenomena that could be at the origin of food astringency, which remains a matter of interest in future research. Further studies on this complex formation will give vital information about the nutritional effects of tea and other PP which is almost same as an in - vivo study reveals.

Section A. Polyphenol determination in tea samples with tea biosensor

2A.1. Introduction

Tea is one of the beverages which are rich in antioxidants whose health benefits have been actively pursued (Iwasa, 1977). Tea leaves contains many PP in which catechins being particularly prolific. The catechin content of tea leaves goes up to 30% of the dry weight (Hertog, 1993; Graham, 1992). During tea leaves fermentation, in the presence of the PPO, catechins are oxidized to form polymers called TFs and TRs (Takino and Imagava, 1963). Biosensors have been reported for the phenolic analysis of industrial and environmental samples (Deng *et al*., 1996, Puig and Barcelo, 1996). There is a report on the determination of antioxidant capacity of different PP containing foodstuffs using a tyrosinase based superoxide dismutase biosensor (Campanella *et al.,* 1999). Amperometric sensor based on electrical reduction of *o*-quinones liberated by the enzyme catalytic reaction has been reported (Kulys and Schmid, 1990).

In assessment of tea quality or tea price, professional tea tasters mainly consider the tea liquor characteristics such as colour and astringency (Biswas and Biswas, 1971), which is majorly depend on the PP content in tea. Quality of tea relies on the PP content (Hilton and Ellis, 1972; Roberts and Smith, 1963).There is a need for rapid detection and quantification of PP for quality assessment mainly based on PPs contents in tea samples.

Conventional analytical methods such as spectrophotometry and HPLC are laborious and time consuming for the PP analysis in tea. Traditionally in tea industries tea tasters are appointed for grading the tea which is based on colour (cuppage) and mouth feel (astringency). This evaluation has some disadvantages, as the analyses are subjective and may also be biased, so there

is a chance of discretion. Therefore, it is necessary to develop objective methods to identify the tea quality. The fabricated biosensor for detection of tea PP content in black and green tea was used in present study.

2A.2. Materials

Tyrosinase, lysozyme, glutaraldehyde, catechin and catechol, were procured from Sigma (USA). A cellophane membrane with a molecular weight cut off of 6–8 kDa obtained from Spectra/por, USA. An oxygen-permeable Teflon membrane purchased from WTW, Germany. Whatman No.1 filter paper procured from Millipore, USA. UV–visible spectrophotometer-1601 procured from Shimadzu, Japan. All biosensor studies were carried out using a detector system fabricated at CFTRI (Mysore, India). A two-electrode system procured from Century Electrodes (India) was used for construction of the amperometric probe. Description of the system is given in Chapter II Section C subsection 2C.3.2. All other chemicals and reagents were of analytical grade and were procured from Qualigens (India).

Black tea and green tea commercial brands were procured from local market. Several Tea samples (grades) of orthodox and CTC (cut, tear and curl) were collected from the auction centre at Coonoor, Nilgiris, India.

2A.3. Methods

2A.3.1. Determination of PP in black, green and grade tea samples

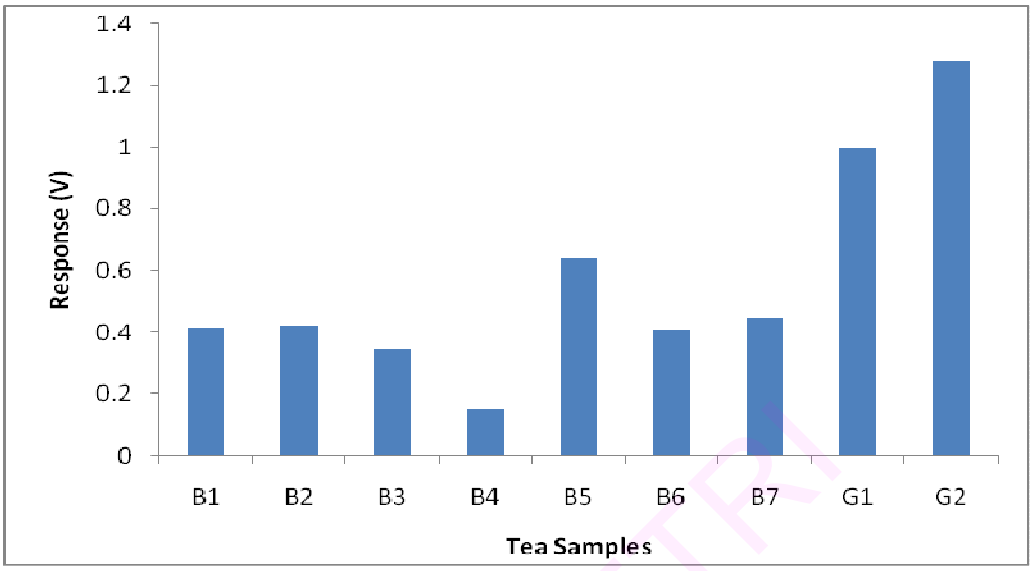
Extraction of PP was performed according to method described by Degenhardt *et al.,* 2000. The tea (2.4 g) was infused in 100 mL and kept at 90°C for 5 min. The infusion was filtered through Whatman No.1 filter paper and the filtrate (100 µL) was injected into sample cell where the enzyme electrode containing the immobilized tyrosinase membrane was immersed in 2 mL working

buffer, NaPi 10 mM with pH 6.8 (detail of biosensor system is given in chapter 2 section C). Green tea extract also prepared in the same manner, but further diluted 10 times before introducing to the immobilized enzyme based biosensor system. The response was measured in volts (V). The same tea samples were also analyzed for total PP by the FC reagent method (spectrophotometrically).

2A.4. Result and Discussions

2A.4.1. Tea sample analysis with tea biosensor

Figure 2.1.1 shows the biosensor response to different commercial black tea samples (designated as B1-B7) along with two leading brands of green tea (designated as G1 and G2). Among the various black tea samples analyzed, sample B5 given maximum response and mM concentrations as (0.65 V, 1.55 mM) followed by B7 (0.43 V, 0.92 mM), B1 and B2 (0.41 V, 0.9 mM), B6 (0.39 mV, 0.85 mM), B3 (0.37 V, 0.8 mM) and B4 (0.14 V, 0.2 mM). As expected green tea samples (G1 and G2) were showed higher activity 1.0 V and 1.28 V corresponds to 2.45 mM, 2.18 mM concentrations. Natural PP present tea given higher response with the tea biosensor in green tea, whereas in black condensed and polymerized catechins such as TFs and TRs were given a comparatively lesser response. Good quality black tea in market made out of blending. Proper blending yields higher PP yields in the extract. One of the reasons for low response in black tea may be due under fermented tea or black tea made without proper blending or colour adulteration.



B – black tea samples, G – green tea samples

Figure 2.1.1. Analysis of commercial black and green tea samples using the immobilized tyrosinase-based biosensor

During the green tea making it is an important criterion to keep PP intact to avoid oxidation for manufacturing good quality of green tea, whereas in the making black tea from tea leaves, oxidation is a very essential step. In the process of making black tea most of the free catechins such as catechin and its derivatives get polymerized in the presence of oxygen and colour of tea leaves gets turned from green to brownish-black. It is interesting to note that green tea responded almost two fold more than the black tea which possesses maximum free PPs. It is also concluded that these free PP get polymerized in black tea manufacturing. This polymerized PP did not give higher response as compared to green tea PP. Using tea biosensor it was possible to quantify the PP contents, and it was also possible to assess the quality of green tea and black tea independently.

2A.4.2. Analysis of tea grades and commercial black tea samples with tea biosensor

In another experiments black tea samples were analyzed for their respective PP content by the tea biosensor and the same is shown in mM concentration (Figure 2.1.2). The commercial black tea samples (Figure 2.1.2 DART, TART, PT, RTF, RTL, KD, 3R, DD and TT, kindly note these are short forms of tea brand and their name is not disclosed) were analysed with biosensor and it was found that leading brands gave better response than local branded black tea (DART, RTF, PT RTL and TART). Whereas, it was also observed that tea grades at tea manufacturing centers/industries have shown difference in the PP content. This is because; the black tea samples in market undergo blending, for its uniformity before coming to market. This gave an advantage to make tea of better quality by proper blending ratios of various grades of tea. Only tea experts can predict the market demand and prepare black tea according to that.

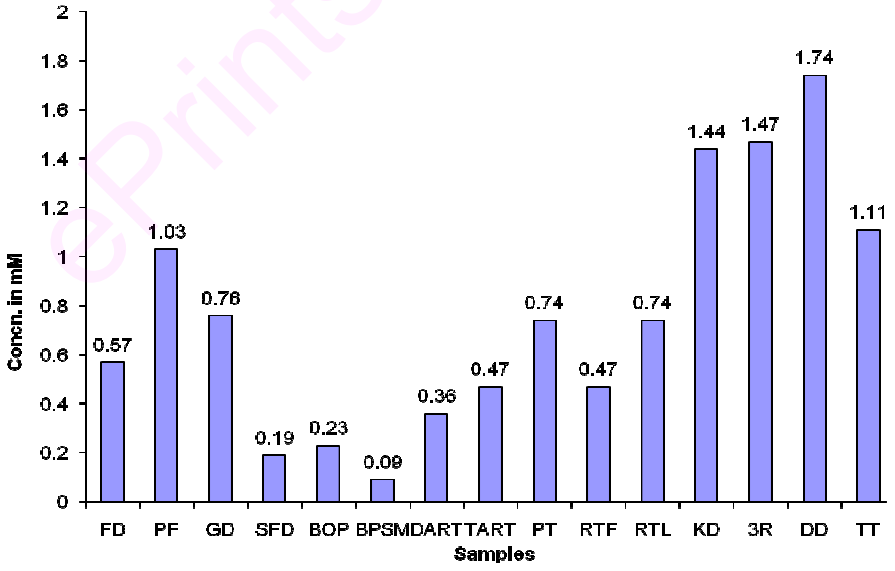


Figure 2.1.2. Analysis of black tea samples from market and auction yards. Data labels were showing the mM concentration of respective brands and grades.

Traditionally black tea at manufacturing level is graded as orthodox and CTC. Orthodox and CTC black tea samples were analyzed separately for their PP content. It was observed in initial studies that there is a lot of difference in the PP content between CTC and orthodox tea (Table 2.1.1 and 2.1.2). Orthodox tea processing yields whole leaf (FOP, FP and OP), broken (BOP, FBOP and BOPF) and dust (OPD, BOPD, FD and GD) grades. Among orthodox whole leaf grades of tea, FOP was found to have highest PP content than FP and OP which may correlates with the quality of leaf where leaf consists of younger leaves. Whole leaf which contains more amount of PP where as in broken leaf grades such as BOP and BP the PP has been polymerized which reduces the availability of PP substrate to react with biosensor and thus gives lesser response. Further the dust grades were given comparatively lesser response with the biosensor because of the reduced amount of PP present in them, Table 2.1.1.

Table 2.1.1. Orthodox black tea grading based on polyphenol content

Orthodox Grades	FC analysis of PP (Catechin eq. in mM)	Biosensor response (Catechin eq. in mM)
FP	20-28	24-28
FOP	28-41	33-40
OP	20-28	25-29
BOP	10-17	8-15
FBOP	14-20	12-18
BOPF	7-17	8-14
OPD	7-14	6-12
BOPD	3-10	4-9
FD	3-7	5-8
GD	3-7	3-7

F – Flowery, P – Pekoe, O – Orange, B – Broken, D – Dust

In CTC tea sorted to two major grades of tea such as dust (PD, D, RD, SRD and SFD) and broken (BP, BOP and BOPF). Dust grades of tea shown

maximum PP content than broken grades, Table 2.1.2. In orthodox highest PP content was found in whole leaf grades whereas in CTC dust grades given the maximum response with the biosensor for PPs. Even though available substrates for biosensor is less in CTC processing, but the extractability of the available PP is high in the dust grades as dust grades have more surface area for more extractability.

` CTC whole process undergoing rapid changes which used to end in 45 minutes to 1 h, whereas in orthodox tea process used to take 4 - 6 h (Owuor and Langat, 1988). In orthodox tea product composition of original PP, intermediate PP and complex PP in a different proportion (Hara *et al*., 1995; Millin *et al*., 1987; Bhatia and Ullah, 1962). In CTC processing tea leaves PP will turn to final product within a short span of time due facilitated oxidation. In orthodox the natural fermentation time was high. The conversion rate also slows in progress. From the decoction it is quite visible that the colour of orthodox is brighter (less black) than the CTC varieties. The complex polymerized PP such as TFs and TRs are reported to be high in CTC (Owour et al., 1986).

Table 2.1.2. Comparative analysis of CTC grades of black tea

CTC Grades	Polyphenol range (Catechin eq. in mM)	Biosensor response (Catechin eq. in mM)
BP	7-14	8-12
BOP	0.6-4	0.3-4
BOPF	4-7	4-8
FP	4-14	2.5-12
PF	0.2-3	0.8-3.5
PD	7-17	8-14
D	6-10	6-12
RD	3-10	4-9
SRD	14-20	15-18
SFD	10-17	13-17

B – Broken, O – Orange, P – Pekoe, F – Flowery, D – Dust, S – Super, R - Red

While analyzing the black tea it is necessary to have the knowledge of the tea whether it is orthodox or CTC and then follow the range of response for each grades to evaluate its quality. Each tea extract analysed with tea biosensor for PP also, measured with FC method. Biosensor response showed good correlation with spectrophotometer measurement of PP with a regression value of 0.995.

2A.5. Conclusions

Among the commercial black tea samples analysed with the tea biosensor system, PP content was determined and it was found that branded tea have more PP content than the local brands. Whereas grades of black tea shown a lot of difference in their PP content. It was also affected by the process they undergo. Orthodox grades and CTC grades were having different PP content. On the other hand biosensor response for green tea was high, showing the presence of high natural PP.

PP plays a very important role in the quality of tea. Therefore tyrosinase based biosensor reported in this work provides a very good alternative to conventional methods. It can also be used for grading tea on the basis of the PP content, which can be a very efficient tool for pricing of tea in the market. Next chapter describes validation of biosensor system with conventional analytical techniques.

Section B. Validation of tea biosensor system for polyphenol analysis

2B.1. Introduction

Generally, tea manufacturers required to know the PP content in black tea and green tea (Hara, 2001). Conventionally, tea quality is assessed by tea tasters (Ellis and Nyirendra, 1995). There are several analytical techniques are reported based on spectrophotometry (Wright, 2000), HPLC (Whitehead and Temple, 1992), Colorimetry (Oshima and Nakabayashi, 1953) and sensory evaluation (Ellis and Cloughley, 1981) for the estimation of total PP, TF and TR in tea. Advanced methods such as capillary electrophoresis, electronic tongue and lipid membrane taste sensor have been applied to tea quality estimation (Ivarsson *et al.,* 2001; Horie and Kohata, 1998; Legin *et al.,* 1997). All these methods have limitations in terms of applicability in the tea manufacturing. Tea manufacturing industries are located in the remote area in India, it was felt that field applicable and easy to use system will be useful to tea manufacturing industries. Therefore, attempts were made to develop system for detection of PP in green and black tea and validated with other conventional analytical methods.

Aim of this work is the development of one single technique that would be rapid and convenient, yield accurate results and also be comparatively cost effective. Validation of the tea biosensor system was done with other established and conventionally practiced methods for PP analysis such as spectrophotometric, colour measurement system and conventional sensory evaluation method along with principal component analysis. For further testing biosensor system was taken to tea plantations and tea factory in various locations in India. During the experiments, robustness of the instrument also assessed such as portability, ability to with stand various environmental condition – moisture, temperature etc.,

performing along side of various heavy machineries where lot of electromagnetic inductions may be occurred.

2B.2. Materials

Tyrosinase, lysozyme, glutaraldehyde, catechin, theaflavin, epicatechin, epigallocatechin gallate and gallocatechin were procured from Sigma, USA. Clark's electrode procured from Century Instruments, India. CIE Color measurement system with Minolta CM-3500d spectrophotometer procured from Konika-Minolta, USA, Spectrophotometer and HPLC were purchased from Shimadzu, Japan. all other chemical used were analytical grade

2B.3. Methods

2B.3.1. Total PP analysis with spectrophotometric method (Roberts, 1962).

Preparation of the tea solution: 200 mL boiling water was added to 2 g of leaf tea in a 250 mL conical flask and stirred by a magnetic bar on a heated (~90 ℃) hot plate for 10 minutes. After filtration, the tea solution was allowed to cool down to room temperature and then made up to 250 mL with distilled water.

Tartrate solution: 1g $FeSO_4$ and 5g potassium sodium tartrate ($KNaC_4H_4O_6$) were dissolved in distilled water and made up to 1000 mL.

Buffer solution: 23.377 g Na_2HPO_4 was dissolved in distilled water to 1000 mL. 9.078 g KH_2PO_4 was dissolved in distilled water to 1000 mL. 85 % (v/v) Na_2HPO_4 solution and 15 % (v/v) KH_2PO_4 solution were mixed as the buffer solution.

Measurement: 1 mL tea solution, 4 mL water and 5 mL tartrate solution were added in a volumetric flask. The buffer was added to make up the mixture to 25 mL. The mixture was measured using a Shimadzu 1601 UV/visible spectrophotometer at 540 nm.

Calculation: Total polyphenols were calculated as follows:

Polyphenols (%) = 3.914 E / 1000 x V0 / V1 / W x 100

Where E is the reading of the spectrophotometer; V_0 is the total volume of the tea solution (250 mL); V_1 is the volume used for the measurement (1 mL) and W is the dry weight of the tea sample.

2B.3.2. Analysis of Theaflavin (TF) and Thearubigin (TR) by spectrophotometry (Roberts and Smith, 1961; Roberts, 1962)

For the analysis of TF and TR, four solutions **Sol. A**, **Sol. B**, **Sol. C** and **Sol. D** were prepared as presented below

(I) 10 mL black tea extract + 10 mL ethyl acetate (EA)

Sol. A	**Sol. B**	**Sol. D**
4 mL organic layer (EA)+ 21 mL methanol	2 mL aqueous layer + 10 mL distilled water + 13 mL methanol	2 mL aqueous layer+ 2 mL oxalic acid + 6 mL distilled water+ 15 mL methanol

(II) 10 mL black tea extract + 10 mL ethyl acetate

↓

10 mL organic layer + 10 mL 2.5% $NaHCO_3$

↓

Sol. C

4 mL organic layer + 21 mL methanol

The optical densities of **solutions A, B, C and D** (E_A, E_B, E_C and E_D) were measured respectively in a 1 cm quartz cuvette in a Shimadzu UV-1601 spectrophotometer.

The percentage of TF and TR was calculated by measuring the tea infusions at an optical density (OD) 460nm and by using the formula (Roberts, 1961).

Calculations

TF % = 2.25 x Ec / (1 - M)

TR % = 7.06 x (2Ea + 2Ed – 2Eb – Ec) / (1 - M)

Ea, Eb, Ec and Ed are the corresponding readings from the spectrophotometer of the above solutions, while M is the moisture content of the tea sample. If the sample is not 3 g, the calculations should be multiplied by 3 and then divided by the practical weight of sample analysed because this empirical formula was originated from a 3 g basis. The percentage of each compound was calculated on a dry weight basis. (Please note, this method is adopted from Roberts and Smith, 1962, and it is practiced in tea industries regularly).

2B.3.3. Statistical analysis through Principal component analysis (PCA) for tea quality analysis through sensory evaluation.

PCA was performed using the sensory scores of each attribute over samples to find the underlying interrelationships of the attributes for quality of made tea.

The tea infusions were subjected to sensory profiling according to Quantitative Descriptive Analysis (QDA) method (Stone *et al.,* 1974). Trained panelists (12 members) participated in the evaluation at Sensory Science Department of CFTRI to evaluate the quality of the black tea. The descriptors were derived after initial discussion, where the panelists were provided with evaluation charts and were asked to describe the samples with descriptive terms suitable for the samples. The common descriptors chosen by more than one third of the panelists were used for development of a 'score card', which consisted of all attributes on a 15 cm scale each. Evaluation was carried out in a sensory laboratory with individual booths under fluorescent lighting similar to daylight.

Samples of tea infusion were kept in hot water bath and served at 40℃ in three digit coded glass containers. Panelists were asked to indicate the perceived intensities of the attributes by drawing a vertical line on the scale and writing the code number. Distilled water was used for rinsing and puffed rice for cleansing the palate in between evaluations.

2B.3.4. Color measurement system analysis for black tea

The samples were analysed for color using the CIE color measuring system (Chrisment, 1998; Committee, 1974; Ramanamurthy *et al.,* 1993) using a Minolta CM-3500d spectrophotometer at 380 and 460 nm.

The measurement of color and the assessment of color differences are essential in helping to meet the specifications agreeable between the customer and the supplier. In the CIE system the total color difference (∆E*) combines the difference of three independent variables, namely: ***L*, a*, b* method: In rectangular coordinates:***

i) The lightness difference on the L* axis, expressed as ∆ L*

ii) The red-green color difference on the a* axis expressed by ∆a*

ii) The yellow-blue color difference on the b* axis, expressed by ∆ b*

2B.3.5. Tea biosensor analysis for PP

PP analysis of black tea samples were done with tea biosensor. 100 µL of black tea extract (as described above section 2B.3.1) was injected to the sample cell containing 2 mL NaPi buffer of pH 6.8 and the response to each black tea samples were recorded.

Black tea samples were also prepared by the method described by Degenhardt *et al.,* (2000) to compare with spectrophotometrc analysis for its validation in total PP estimation.

2B.3.6. Field trials of tea biosensor

Biosensor system was taken to various tea growing regions in India. Initial studies were conducted at Carrolyn estate, Gudalur and Parry Agro, Attikunnu, Tamilnadu, India. Further work was done at TANTEA and other factories in the Nilgiris locality such as Silver cloud and Five Star at Gudalur, Tamilnadu, India. Field trials were conducted at the estates of TANTEA at Naduvattam, Nilgiris, India. Apart from the factory trial and field trials, tea biosensor system was taken to various auction yards and grading centers at Coonoor, Nilgiris.

To check the tea biosensor performance and to analyze the tea samples at different stages of tea processing, the biosensor had been taken to Pandalur tea factory in Tamilnadu.

At final stage of the trial tea instrument was taken to Kolkata. Instrument was demonstrated in front of an assembly of tea experts and tea analysis report was presented in front of the experts. Biosensor analytical reports were interpreted by professional tea tasters.

2B.3.7. Remarks of tea taster for the validation of grades

Tea tasters analyze tea on the basis of colour and astringency (mouth feel). Only a few grades of tea samples were selected from the auction yards and factory for the PP analysis. Results were compared with the conventional grades of tea taster. According to the evaluation of tea taster, tea is broadly classified in very good, good, average, poor and very poor. This scale may vary with regions and grades.

2B.4. RESULTS AND DISCUSSIONS

2B.4.1. Spectrophotometric analysis of polyphenols

Total PP in tea samples were determined. Commercial black tea samples were designated as brands A to H. It was observed that the maximum PP content present in sample A (230 mg %) and C, F and H have shown little difference among them in total PP content (Figure 2.2.1). Minimum PP content was observed in brand B. This method is usually practiced in the tea industries to estimate PP content in tea samples (Roberts *et al*., 1956, 1957; Roberts & Myer, 1958; Roberts & Smith, 1961; Roberts, 1962).

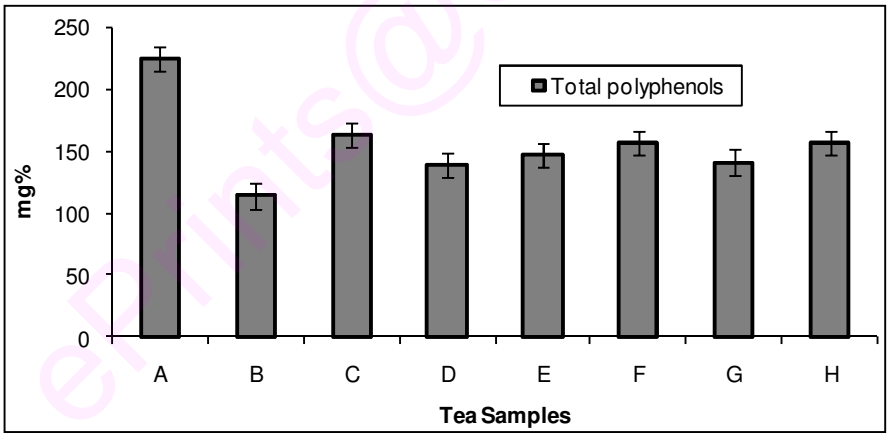


Figure 2.2.1. Spectrophotometric analysis of black tea samples

2B.4.2. Analysis of Theaflavin (TF) and Thearubigin (TR) by spectrophotometry.

Spectrophotometric analysis of TF was done according to Roberts and Smith, (1962). In TF analysis of tea there is a lot of difference found among the

various brands. But brand A shown maximum TF content (0.4mg %), followed by brand B, C and D (Figure 2.2.2). Brand G found with minimum TF (0.05).

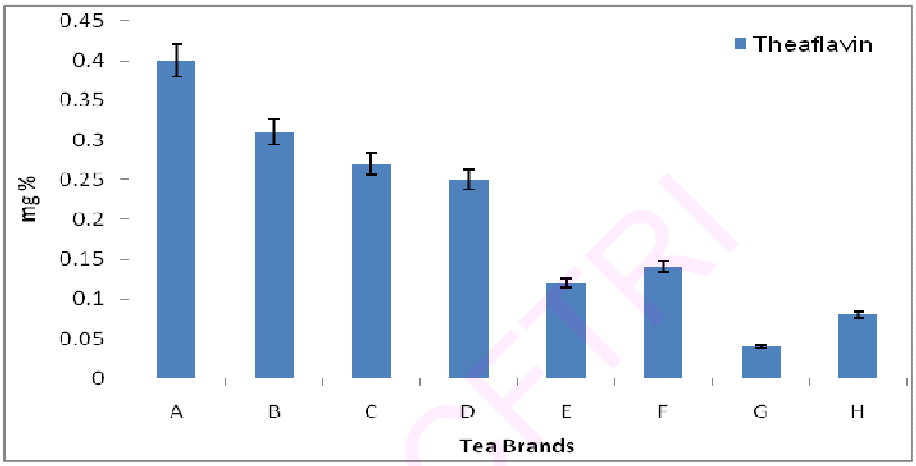


Figure 2.2.2. Specctophotometric estimation of theaflavin in black tea samples

As the equation (Roberts & Smith, 1961; Roberts, 1962) establishes in general where the TR content is low in tea extract where TF found to be high (please note this is empirical relation). Brands C, D and F are comparatively higher TR content (approx. 25 mg %) as compared to other brand tested (Figure 2.2.3). Brand E observed to have minimum TR content (13 mg %).

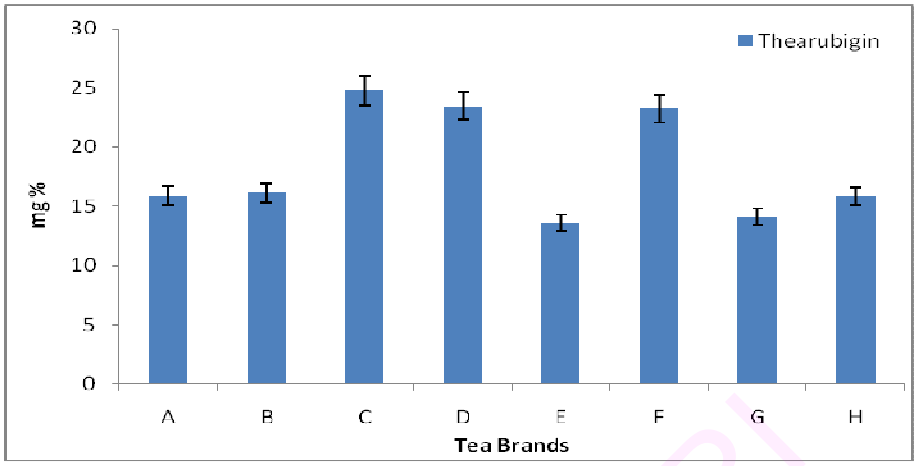


Figure 2.2.3. Spectrophotometric estimation of thearubigins

2B.4.3. TF and TR ratio of black tea

Calculation was done according to formula which is given in materials and methods (Roberts and Smith, 1962). This formula is still practiced by tea industries in India.

TF and TR ratio is critical parameter in made tea. Conventional grading in tea industry is done according to the tea taster's evaluation. Along with this occasionally the analysis of TF/TR analysis is done to assess and confirmation on the grades of tea. Levels of TFs and TR, especially their total contents showed high positive correlation with the evaluation of black tea quality (Takeo, 1974).

Any factor for converting optical density into a percentage of TF or TR will be somewhat arbitrary as dealing with a rather complex mixture of substances, none of which have been isolated in a pure state. Consequently little error will be introduced by expressing total TFs as TF gallate as the latter is always considerably more than the former in tea (Roberts, 1961). The optical densities, E_C at 380 nm and 460 nm are converted into a percentage of anhydrous TF gallate by multiplying with the factors 2.25 and 6.69 respectively. TF ratio was obtained from the ratio of optical densities at 380 and 460 nm and similarly for TR

ratio. The TF ratio of optical densities at 380 and 460 nm should be 2.98 – 1 to indicate the complete removal of TR during TF extraction. The TR ratio is generally found to be between 3.6-8.2. A high ratio implies relatively light color and a low ratio a correspondingly deeper color.

There is a high TF ratio for brand G with 2.4 followed by brand E. All other brands were given more or less same values for TF ratio (Figure 2.2.4a).

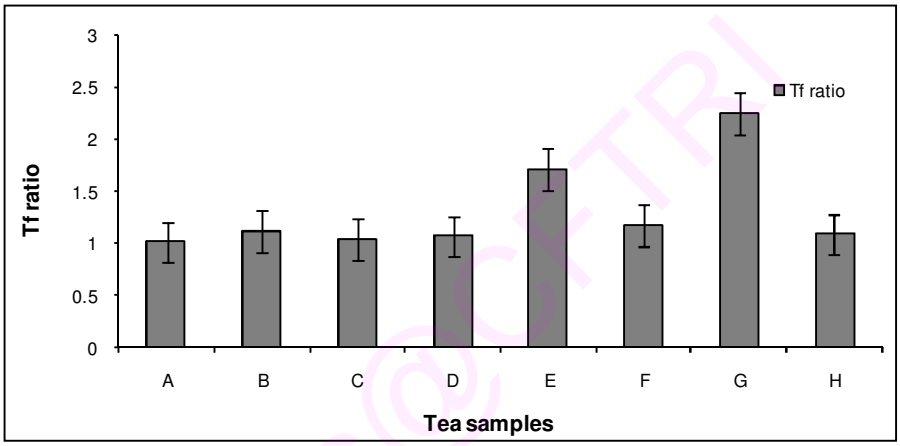


Figure 2.2.4a. TF ratio of black tea samples

Among the black tea samples, TR value was high with the brand F (Figure 2.2.4b). Tea with high TR will be high in astringency.

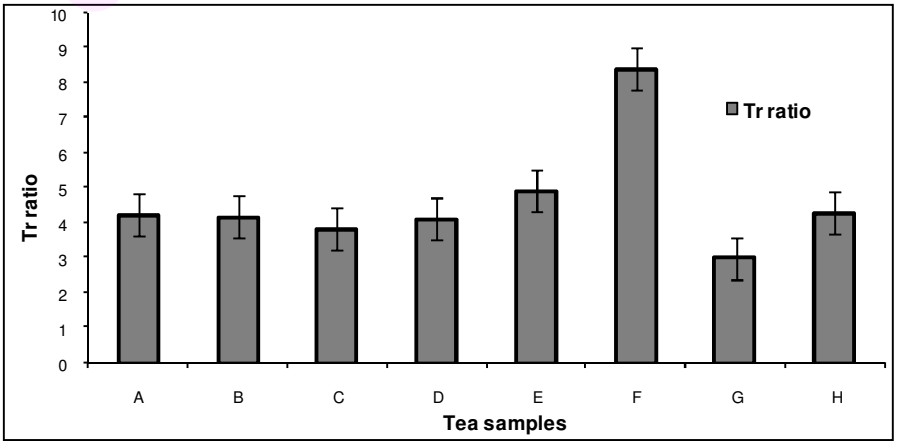
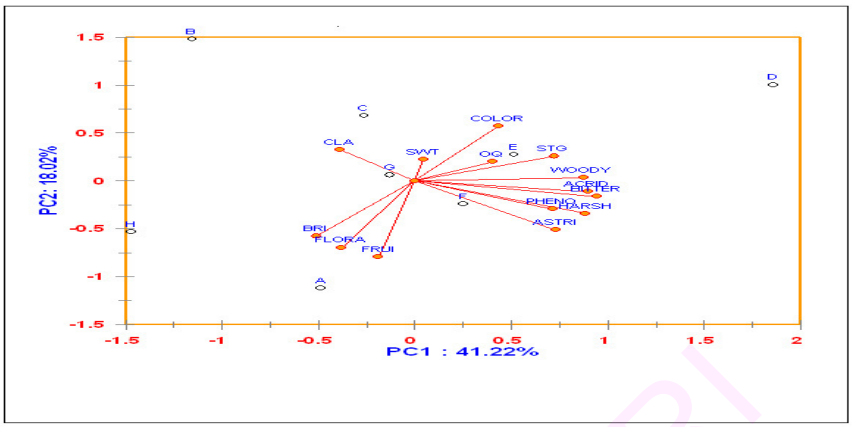


Figure 2.2.4b. TR ratio of tea samples

2B.4.4. Principal component analysis (PCA) of tea samples

Figure 2.2.5 depicts the PCA biplot of sensory attributes of tea samples. The variance accounted by the principal component axis 1 (PC1) was 41.22% while PC 2 accounted 18.02% and together almost 60% of the variance of the sensory data. The most desirable attributes like body, strength, colour and overall quality were associated with sample D followed by sample E that are coincidentally two well known brands of the same company. Some of the undesirable attributes in tea quality assessment like phenolic; harshness and astringency were associated with sample F that is a locally available brand. Samples B, C and G were characterized more with sweetness and clarity. In the third quadrant, sample A closely followed by sample H was clearly associated with attributes like brilliant, floral and fruity. Figures 2.2.5-7 represent the results obtained by sensory evaluation of properties such as color and the principal component analysis of color and various sensory parameters such as flavor, odor, taste, clarity etc. Comparing Figures 2.2.5-7, it was observed that the samples lying in the III quadrant (Figure 2.2.7) of the PCA plot showed good response in the tyrosinase based biosensor. Interestingly only sample G was found to be the closest to the origin in Figure 2.2.5, implying low score in sensory attributes due to adulteration and low TF content by spectrophotometry (Figure 2.2.2) confirming the low response in biosensor analysis, PC analysis from the above observations is found to be effective in characterizing the sensory parameters of tea samples.

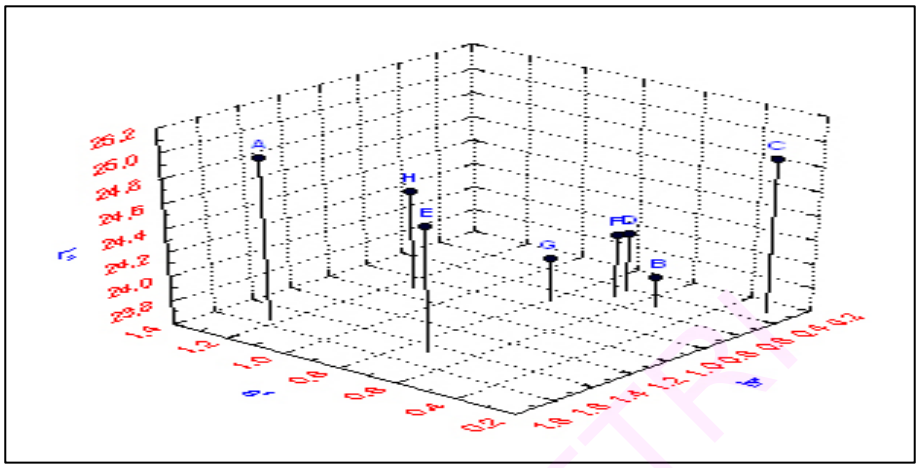


Astri - astringency; Harsh - harsh; Pheno - phenolic; Acrid - acrid; Bitter - bitter; Wood - woody flavor; Stg - strong; Oq - opaque; Color - color; Swt - sweetness; Cla - clarity; Bri - brightness; Flor - floral; Frui - fruity; A-H - Different Tea samples

Figure 2.2.5. Principal component analysis of sensory parameters of tea samples

2B.4.5. Colour measurement system

The positive values of a* and b* obtained (Figure 2.2.6) by the CIE color measuring system suggest that the black tea infusions were red-yellow in color which is also due to TF and TR and the biosensor response showed maximum values for samples with high content of the above two compounds which contribute much to the color of tea and thereby improving its acceptability.

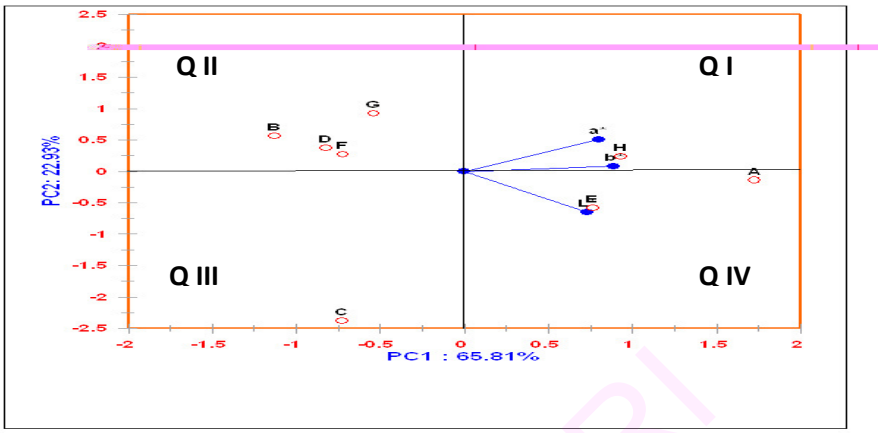


L- lightness, a-red to green color, b- yellow-blue color

Figure 2.2.6. Color measurement of tea samples by CIE color measuring system

The method described by Ullah (1972) for measuring TF and TR also is used to measure total colour and percentage brightness. This uses the ratio of the reading at 380 and 460 nm. The spectrophotometric reading at 380 nm is used to calculate the TF, so is comparable to the reading at 520 nm from fermentation monitoring described by Whitehead and Muhime, (1989). The drop in the ratio of readings at 520 and 460 nm at the optimum fermentation time can be compared to the drop in % brightness.

The instrumental color parameters, L*, a* and b* were subjected to Principal Component Analysis (PCA) obtained with tea samples. The results showed discrimination between samples. Principal component axis 1 (PC 1) accounted for 65.81% while PC 2 accounted for 22.93% of the variance of the data (Figure 2.2.7). They together accounted for more than 88% variance in the data.



L-lightness, a-red to green color, b- yellow-blue color
Q I-IV: Quadrant I-IV; A - H different Tea samples

Figure 2.2.7. Principal component analysis of Color measurement of different tea samples

The analysis indicates that all the three-color parameters are highly loaded on the positive side of the PC1 axis. It is clear from the plot that sample H prepared in laboratory was distributed evenly between the reddish and yellow components 'a' and 'b' respectively while sample E was lighter in color. On the other hand, sample A appeared inclined towards the yellowish component 'b' and other samples like B, D, G and F forming a group on the second quadrant were negatively correlated with lightness component 'L'. All these samples gave low response in a biosensor (Figure 2.2.8) in comparison to sample A. Sample G though placed in the II quadrant, appeared red (visually) and showed high b* values, showed less TF and gave very low response in biosensor. Therefore, the apparent red color is due to adulteration with artificial colors which had shown less response with immobilized tyrosinase used in the biosensor confirming the absence of required tea PP. Apart from these samples, sample C was clearly separated from rest of the samples and was located on the third quadrant. It was negatively correlated with reddish component 'a'. It is clear from the biplot that PCA was effective in discriminating color parameters with tea samples.

2B.4.6. Tea biosensor analysis for PP

Tea biosensors could distinguish between each samples based on the PP content. Response was measured in V. Brand A given maximum response and least in grade G (Figure 2.2.8).

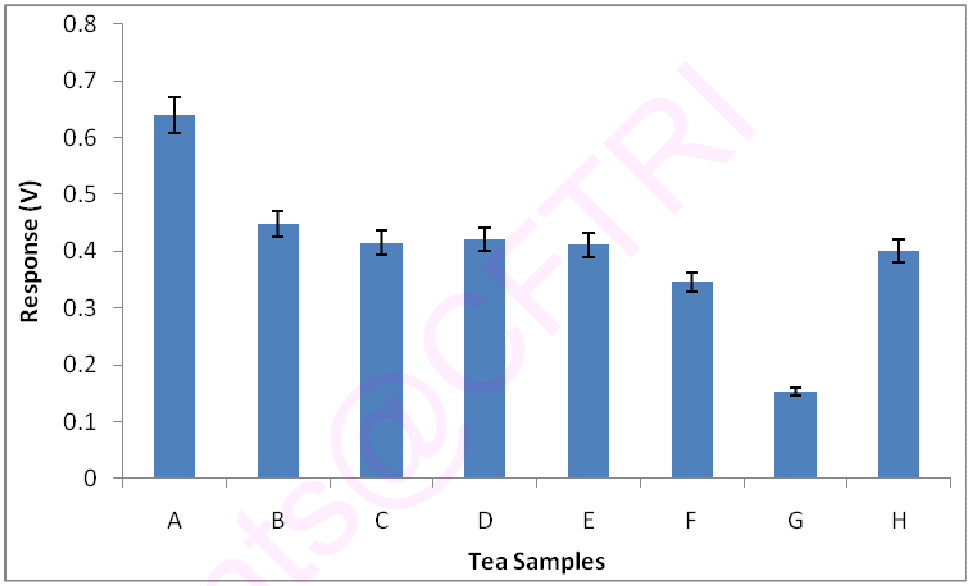


Figure 2.2.8. Polyphenol analysis of black tea samples with tea biosensor

Figure 2.2.8 represents the biosensor studies conducted on the tea samples in comparison with the above-mentioned methodologies. Sample A showed high total PP content (Figure 2.2.1) and high response with biosensor (in terms of voltage) (Figure 2.2.8). The same sample showed good values of TF by spectrophotometry (Figure 2.2.2) and was also graded one of the best through color estimation (Figure 2.2.6 and 2.2.7). The TR content of tea is always considerably higher than the TF content, but as the TFs are much more intensely colored, their contribution to total color is a decidedly significant one (Roberts, 1961). It was observed that the samples containing higher TF percentage showed better response with biosensor. Samples C, D and F showed comparatively high

content of TR (Figure 2.2.3). Interestingly it was found that a local brand, sample G having low amount of TF (Figure 2.2.2) showed low response in biosensor (Figure 2.2.8). This was also confirmed by obtaining the TF ratio (Figure 2.2.4a) which was high and was due to low TF content and incomplete removal of TR (Roberts, 1961 and 1958) indicating that the dark color of tea infusions of sample G (Figure 2.2.3) was due to apparently synthetic color additives during manufacturing process. The TF ratio of 1 - 2.9 indicates bioconversion, but a ratio higher than this range indicates the incomplete removal of TR during extraction of TF as observed. A value lying below of this range indicates that greater TF content in the sample. Similarly sample A gave a low TF ratio indicating high TF content and therefore gave a good response in biosensor and was also graded best by the sensory panelists.

2B.4.7. Validation of biosensor response with other analytical methods

Conventional analytical methods tried for PP analysis and the results were compared with the biosensor response.

2B.4.7.1. Biosensor Vs Spectrophotometer

The biosensor response is in agreement with the results of spectrophotometer (FC method). The value of spectrophotometer was marginally high for all the samples (Figure 2.2.9a). This may be due the interference caused by the protein present in the tea extract.

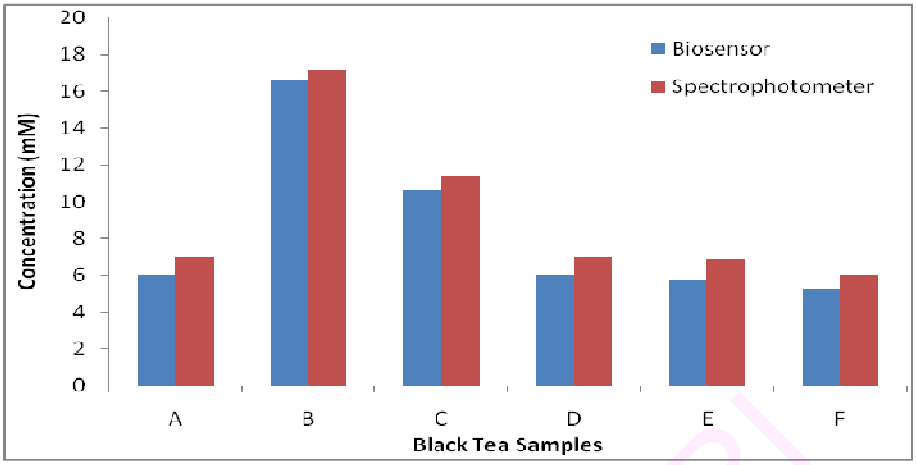


Figure 2.2.9a. Comparison of biosensor response for tea samples with spectrophotometer results for PP

Both the methods were shown good linearity (Figure 2.2.9b). For spectrophotometer regression value of 0.9701 and for biosensor it is 0.9962 were observed.

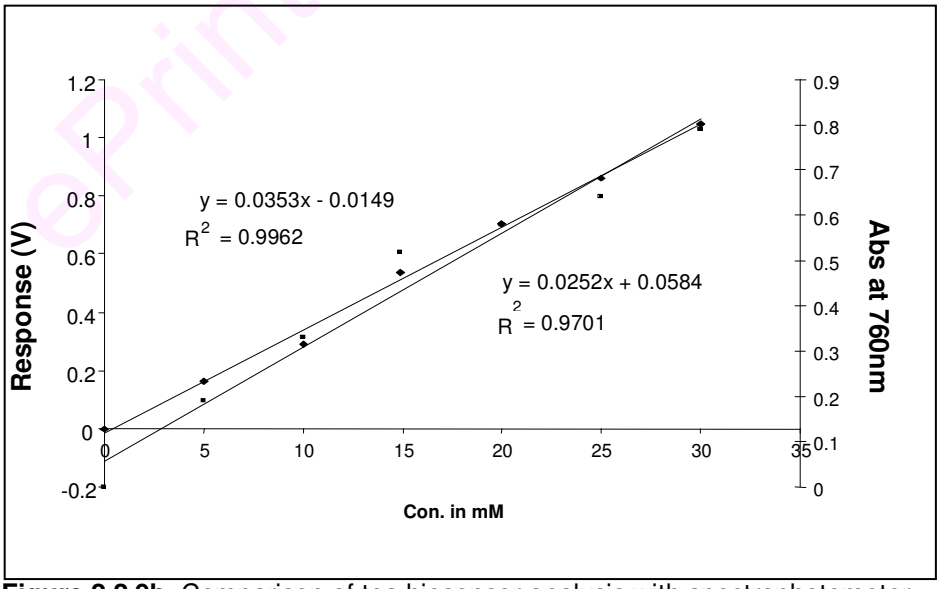


Figure 2.2.9b. Comparison of tea biosensor analysis with spectrophotometer analysis using catechin standard

10 black tea samples were graded based on their respective PP content using tea biosensor and the same was compared with FC method, Table 2.2.1. It was concluded that biosensor is more sensitive than FC method. Enzymes are highly specific with the substrate, so there is no interference occurred by the presence of other components. Enzyme catalytic activity is even triggered by the nanoscale presence of its substrate. These properties gave an enzyme based alalysis of substrate more sensitive over any other analytical methodology. In FC method PP content observed with more or less same 1.17-1.48 mM, where as biosensor distinguished a range of PP content of 0.36-1.74 mM. Biosensor showed a correlation with FC method in PP content, where PP content was high, where as low PP content was not detected by FC method and was not able to detect at low pp content.

Table 2.2.1. Comparative analysis of various methods for tea polyphenol analysis

Grading	Tea Samples	Biosensor (mM)	FC Method (mM)
1	BT 2	1.74	1.48
2	BT 3	1.48	1.41
3	BT 4	1.44	1.42
4	BT 7	1.30	1.46
5	BT 10	1.20	1.26
6	BT 1	0.5	1.27
7	BT 8	0.36	1.24
8	BT 9	0.24	1.17
9	BT 6	0.19	1.05
10	BT 5	0.09	1.03

2B.4.8. Field trials of tea biosensor

2B.4.8.1. Testing of biosensor at different stages of tea processing

Tea biosensor system was shown a great stability throughout the trials. It was observed that the fresh young leaves showed the highest PP content where as the PP content reduced at the final stage of tea processing called firing (Table

2.2.3). This may be due to the bioconversion of most of the catechins into TFs and TRs by native tea leaf PPO activity to form polymerized complex PP. The same samples were analysed in the laboratory by spectrophotometric method for total PP content to validate the biosensor results. The results were tabulated below

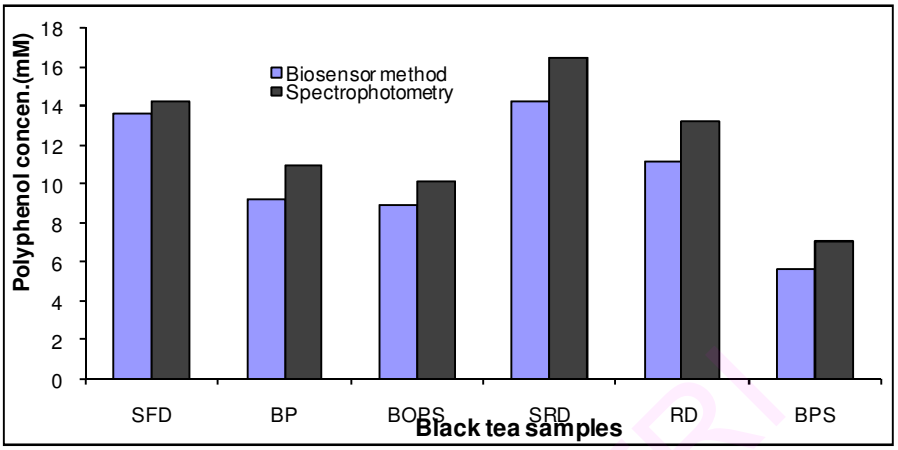
Table 2.2.2. Analysis of tea samples at different stages of tea processing (CTC)

Samples	Total polyphenol content in mM	
	Biosensor method	Spectrophotometry
Young leaves	30.6	33.52
Withering	24.2	27.6
Cut	25.0	28.9
Tear	22.2	26.6
Curl	22.4	25.9
Fermentation	19.6	22.4
Firing	17.2	20.7

CTC tea samples were assessed at different processing stages (leaf, weathering etc., as given in table 2.2.2) with biosensor system. The difference in the level of PP during the process is lesser in orthodox, where the final orthodox tea was given a higher response with biosensor in comparison with CTC. Please see section A of Chapter 3.

2B.4.8.2. Total polyphenol content of various grades of black tea samples

Black tea samples were tested for their PP content using tea biosensor (Figure 2.2.10). It was observed that tea grades SRD and SFD shown maximum PP content. Minimum PP content was in BPS. In this evaluation one exclusive phenomenon was came in to attention, that, the content of PP found in processed tea (CTC) is that in an ascending order from dust to pekoe grades. One more sensitive parameter that the grades of black tea should be categorize and evaluate separately as orthodox and CTC.



SRD: small Red dust, **BP:** Broken Pekoe, **BOPS:** Broken Orange Pekoe Small
BPS: Broken Pekoe Small, **SFD:** Super Fine Dust, **RD:** Red Dust
Figure 2.2.10. Total polyphenol content of various grades of black tea samples

In general, orthodox teas have high TFs contents while CTC teas have high contents of TRs. Differences in TF and TR are also produced by the geographical location of tea production (Owuor *et al*., 1986).

2B.4.9. Tea taster's evaluation

Tea samples were analysed by professional tea taster at factory level and results were compared with the biosensor (Table 2.2.4). Results were shown good correlation between biosensor and tasters score.

Table 2.2.3. Comparison of tea biosensor with tea tasters score

Tea Grades	Tea tasters remarks	Biosensor(mV)/ mM
BOP	Good	1400/3.5
RD	Good	1200/2.9
PO	Medium	890/2.1
PF	Medium	770/1.8

When tea taster referred to his sensory evaluation techniques based on colour astringency, cuppage, body and mouth feel it was in accordance with

biosensor analytical results. Here major components like astringency, body and cuppage are having direct correlation with the PP content in it. All these components are the attributes of PP content present in it. In this widely practiced subjective method tea biosensor could be objective method to provide a check point in the determination of tea quality.

2B.5. Conclusion

Generally astringency and color are the two most commonly tested parameters of black tea, which are contributed mainly by TF and TR. In this present work it was attempted for the first time to establish an association between the content of the two most important PP oxidized products (TF/TR) in different tea samples and their effect on subjective analysis by sensory evaluators and also their response in a use a tyrosinase based biosensor. Sensory evaluation of a large number of samples at a time could lead to dubious results and correct evaluation of tea is may not be performed. Though the results obtained from spectrophotometric analysis, color estimations and sensory evaluation help in establishing a relationship between the various attributes involved in quality assessment of tea as discussed above, these methods are time consuming, slow and required highly trained personnel and are not cost effective. A biosensor helps in achieving a correlation between the varietal differences among different brands of tea can perform multiple analyses accurately, does not require highly skilled hands, is rapid and is cost effective as it can be used at the factory level and in auction yards. A tyrosinase based biosensor system used in the present study gave good response for the commercial tea samples that was also rated the best according to traditional methods of quality assessment which were adopted in the present study. The biosensor response varied proportionally to the TF content in the tea sample. The total content of polyphenols is a major determinant of tea quality.

Summary and Conclusions

Polyphenols (PPs) play a vital role in tea quality. Tea PPs is well known for their antioxidant properties. In present study attempts were made to detect tea polyphenols using enzyme based biosensor system. PPO and related enzymes extracted from various sources such as tea leaves, mushroom, apple, brinjal and potato along with commercially available laccase and tyrosinase were explored for their potential application in biosensor system. Major find outs of the investigations are summarized as below

- PPO enzyme from various sources was assessed for their activity towards catechin (PP).
- PPO enzymes from tea leaves were purified to homogeneity and obtained three active isozymes named as PPO-I, PPO-II and PPO-III.
- Among these, PPO-II and PPO-III found to have higher activity than PPO-I.
- Tyrosinase enzyme from mushroom also partially purified using the same protocol.
- Among these, highest substrate response for tea polyphenol was shown by mushroom tyrosinase followed by tea leaves.
- All the enzymes were studied for their reaction kinetics against catechin. Among PPO enzymes from various sources, mushroom tyrosinase gave low Km and high Vmax for catechin substrate. This found to be suitable enzyme for biosensor application.
- Physicochemical parameters were optimized for better performance of tyrosinase. Optimum conditions for enzyme performance were observed with pH 6.8 and at a temperature of 30°C

- Standard enzymes such as laccase, tyrosinase along with the CLEC of laccase were used and compared with purified enzyme for suitability for biosensor application.

- Studies were carried out using different immobilization methods for immobilization of enzymes obtained from tea leaves and tyrosinase for the application in biosensor. Cross-linking method was observed to be better method than entrapment method.

- PBSAs such as gelatin, BSA and lysozyme were tried for the stabilization of the immobilized enzyme. Lysozyme found to be the suitable PBSA for tyrosinase immobilization.

- Among these enzymes tried, a better response in terms of enzyme activity with catechin was found with tyrosinase than the purified PPO enzyme obtained from tea leaves.

- A biosensor instrument system was fabricated which gave steady and good response with catechin, green and black tea extract. It also has better signal to noise ratio.

- Catechin standard was tried to assess the response of immobilized PPO and tyrosinase with biosensor system. Both PPO and tyrosinase enzymes responded well to a catechin concentration range of 10-100 mM.

- In preliminary studies with black tea infusions PPO gave a poor repose in comparison with tyrosinase, whereas for green tea both the enzymes were given good response.

- Tyrosinase enzyme was selected and used for all further analysis for PP in tea.

- Protein-PP interactions were studied to understand fouling mechanism. Glycerol 5% was found to be better dissociating agent, further confirmed by HPLC, using catechin and lysozyme as model PP and model protein.

- Developed tea biosensor was used for grading tea on the basis of the PP content, which can be a very efficient tool for quality assessment of tea. Tea biosensor analytical results fell in agreement with tea taster's analysis and other conventional analytical techniques. Field trials of developed tea biosensor gave steady and reproducible results.

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M.Madhava Naidu, S.R.Sampathu, B.Raghavan, V Prakash, **Sujith Kumar Pokka Vayalil**. An improved process for the preparation of natural vanilla extract. {WO2009031160(A1)-2009-03-12}.