Inhibition of Xanthine Oxidase by catechins from tea (*Camellia sinensis*)

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List of abbreviations

ACE	Angiotensin converting enzyme
AIDS	Acquired Immunodeficiency Syndrome
AOR	Aldehyde oxido-reductase
BGE	Back ground electrolyte
C	(+)-catechin
CE	Capillary elctrophoresis
CMC	critical micellar concentration
CTAB	Cetylttrimethylammoniun boromide
CTC	cut, tear and curl
CYP	Cytochrome P-450 gene notation
CZE	Capillary zone electrophoresis
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EC	(-)-epicatechin
E.C.	Enzyme Commission
ECg	(-)-epicatechin gallate
EGC	(-)-epigallocatechin
EGCg	(-)-epigallocatechin gallate
EGF	Epidermal growth factor
EOF	Electro-osmotic flow
FAD	Flavin adenine dinucleotide
GC	(+)-gallocatechin
GC	Gas chromatography
GCg	(-)-gallocatechin gallate
GTP	Green tea polyphenols
GSH	Reduced glutathione
HETE	hydroxyeicosatetraenoic acid
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HIV	Human immunodeficiency virus
HPCCC	High performance centrifugal counter current chromatography
HPLC	High performance liquid chromatography

HRMS	High resolution mass spectrometry
i.g.	intragastrically
IMP	inosine monophosphate
ITP	Isotachophoresis
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MAPK	Mitogen activated protein kinase
MEKC	Micellar electrokinetic chromatography
MMP	Matrix-metallo proteinases
Moco	molybdenum cofactor
mRNA	messenger ribonucleic acid
MT	Migration time
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
ORM	Overlapping resolution mapping
P450	Cytochrome P-450
pBR	phosphorylated retinoblastom protein
PPRP	phosphoribosylpyrophosphate
PKC	Protein kinase C
pNP	*p*-nitrophenol
RP-HPLC	Reversed phase high performance liquid chromatography
RSD	Relative standard deviation
RNA	ribonucleic acid
SC	Sodium cholate
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	standard error of the mean
TEAC	Trolox-Equivalent Antioxidant Capacity
TNFα	Tumor necrosis factor α

TPA	12-O-tetradecanoylphorbol-13-acetate
TPM	tea polyphenol mix
UV	Ultraviolet
UVB	Ultraviolet B radiation
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
γ-CD	gamma-cyclodextrin

Chapter 1

Introduction

Natural products are organic and inorganic compounds that occur in plants (leaves, needles, bark, roots, flowers and seeds) and microbial organisms found in highly diverse and sometimes extreme conditions. Natural products fall into several different categories: steroids from marine animal, plant and fungal sources, pyrimidines and purines from microbes and proteins, amino acids and antibiotics from microbes. Other compound classes such as polyphenols and coumarins from plants, alkaloids from plants and some bacteria, pigments from microbes and plants and terpenes, carbohydrates, fats and macromolecular products from all sources are also important natural products. A wide spectrum of natural products is created by combining different classes of compounds e.g. polyphenols and sugars to produce flavonoid glycosides. Substitutions with hydroxyl or ketones and additions with methyl or prenyl groups also increase variety. The majority of natural products are secondary metabolites, produced by microorganisms and plants for the purpose of protection, procreation and survival in general. It is thus not surprising that some natural products can have toxic effects in humans and animals.

1 The importance of natural products

The natural products that occur in food contribute to the organoleptic properties of the food products. The polyphenols and organic acids content may determine the quality of the food products. To regulate the quality of consumables, natural compounds must be analyzed in the food industry. The Quality Control must prevent off-flavors, toxins and other harmful compounds, produced by microorganisms, from reaching the market place. Many natural products have bioactivities that may be harmful to

humans. Aflatoxins produced by *Aspergillus flavus* are of the most potent liver carcinogens and phalloidins from the mushroom, *Amianita phalloides,* causes liver damage. It is important to determine sources of toxins and the presence thereof in food destined for human consumption to prevent food poisoning.

Natural products first gained prominence through the isolation of antibiotics from microbes. An example is the family of β-lactam antibiotics (cephalosporins) that are produced by the mold *Cephalosporium acremonium*. Today natural products have a variety of medicinal uses. They are used as immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigrane agents, herbicides, antiparasitic agents and bio-insecticides. The pharmaceutical industry isolates natural products as lead compounds that may potentially, after chemical modification, give rise to a new class of compounds with a certain bioactivity. The majority of the lead compounds originate from micro-organisms while the rest originate from plants (Borris, 1996). Although isolating natural products from plants is more challenging, it is important because micro-organisms and plants do not produce the same secondary metabolites. This maximizes the chemical diversity that is evaluated in pharmaceutical screening programs. Plants produce a whole range of flavonoids and flavonoid glycosides that have pharmaceutical or nutritional value. Ephedrine alkaloids from Chinese herbal preparations have medicinal value and coumarins found in several plant families have blood anti-coagulant activities. Quinoline, quinazoline and acridone alkaloids have been extracted from plants and tested as anti-malarial drugs (Michael, 1997). Similarly artemisinin drugs isolated from the plant *Artemisia annua* have been shown to have anti-malarial properties (van Agtmael, 1999). Pharmaceutical industries are exploring nature's libraries of natural products

for novel lead compounds that after combinatorial chemical modifications may result in active drugs against diseases such as cancer and AIDS.

It is important to study the metabolic pathways that synthesize these secondary metabolites, as well as their efficacy and toxicity. The separation, detection and quantification of bioactive secondary metabolites are essential in conducting these studies.

2 Natural products contributing to quality of tea

Catechins are the most abundant polyphenols in the leaves of tea (*Camellia sinensis*). They may constitute up to 30% (w/w) of the dry weight of young tea leaves. The major catechins in tea are (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg). (+)-Gallocatechin, (GC) and (+)-gallocatechin gallate (GCg) also occur in tea, but only in low concentrations (Wilson, 1992). The total catechin content is highest in young leaves and decreases significantly with leaf aging. Catechins contribute to the astringency of the tea brew. The quality of the tea correlates with its catechin content and composition.

Quercetin, kaempferol, myricetin and their glycosylated derivatives are also found in tea. Bisflavanols or theasinensis are dimers formed from the catechin monomers. These flavonoids occur at low concentrations in tea and probably do not contribute to the quality of tea. During the oxidation process required for the production of black tea, the catechins are polymerized. The major polyphenols in black tea are theaflavins (catechin dimers) and thearubigens (catechin polymers). A positive correlation has

been found between the quality of made black tea and the amount of gallated catechins in fresh leaves of Kenyan cultivars (Obanda, 1997). The gallated monomers produce gallated theaflavins that contribute more to quality than ungallated theaflavins.

Caffeine is another prominent compound of tea and may constitute 3 to 4% (w/w) of the dry weight (Wilson, 1992). It contributes to the bitterness of the tea liquor. Theophylline and theobromine are two methylxanthine precursors of caffeine also found in tea. They only occur in low concentrations and probably have very little influence on the quality.

Theanine (5-N-ethyl-glutamine/ γ-ethyl-glutamine) is unique to tea and the most abundant free amino acid, constituting between 1 and 2% (w/w) of the dry weight of tea leaves. Theanine is equally abundant in all types of tea (i.e. green, oolong or black). It contributes to the savory sweet taste of tea (Wilson, 1992), (Ekborg-Ott, 1997). Aspartic and glutamic acid are the other two important amino acids in tea, but their levels are usually less than 0.001% by weight. The monosodium glutamate contributes to the brothy taste of tea (Horie, 1998 (a)).

Free gallic acid is not found in fresh tea leaves. About two thirds of the catechins in fresh tea leaves are esterified at the 3-OH position with gallic acid forming complex catechins (Obanda, 1997). Some of the complex catechins are de-esterified during the manufacturing process to yield free gallic acid in the made tea. Gallic acid influences the pH and color of made black tea. Oxalic, citric, malic and quinic acids are other organic acids that are found in green tea. The organic anions chelate with aluminum,

calcium, iron and other metals, hence influencing the bioavailability of metals (Horie, 1998 (a)).

Free ascorbic acid occurs in very low concentration in tea and has no effect on the quality. Bottled ice tea is fortified with ascorbic acid for preservation and nutrition. It contributes to the quality of bottled tea in an indirect manner, making it important to quantify ascorbic acid in bottled ice teas.

3 Bioactive natural products in tea

3.1 Identification of the bioactive tea compounds

Plants have been the source of a variety of bioactive natural products. In the mid 1980's several initial studies indicated that water-extracts of green tea contained substances with antimutagenic effects (Kada, 1985), (Cheng, 1986). In several cases plant phenols have been shown to have prophylactic properties (Boone, 1990), (Das, 1987), (Mukhtar, 1988). It was therefore very likely that the prophylactic properties observed from green tea extracts were manifested through its large polyphenol contents. Green tea polyphenols (GTP) were extracted from green tea leaves with ethylacetate. The main constituents of the GTP extract were EGC, EC, EGCg, ECg and caffeine (Mukhtar, 1992). From the initial studies it could be seen that GTP's antimutagenicity was a result of at least two different mechanisms. 1) Preventing the conversion of pro-carcinogens to DNA-adduct forming carcinogens via modulation of the activity of CYP 450 systems both *in vitro* (Wang, 1989 (a)) and *in vivo* (Wang, 1989 (b)) and 2) the ability of the polyphenols to scavenge and neutralize oxygen and other genotoxic radicals (Perchellet, 1989).

3.2 Intrinsic properties of polyphenols

The polyphenolic flavonoids have the diphenylpropane ($C_6C_3C_6$) skeleton. The flavonoids constitute a large class of compounds, ubiquitous in plants, containing a number of phenolic hydroxyl groups attached to the aromatic ring structures. Various methoxylation, sulfation and glycosylation patterns also exist. This family includes monomeric subgroups such as flavanols, flavanones, anthocyanidins, flavones and flavonols, as well as isoflavonoids, neoflavonoids and flavylium salts. The differences between the sub-groups are due to variation in number and arrangement of hydroxyl groups, unsaturated and saturated bonds (Rice-Evans, 1996). The inter-relationships between the most important flavonoid sub-groups are shown in Fig. 1.1. Plant polyphenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants, singlet oxygen quenchers and metal chelators. The chemical property of polyphenols in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers predicts their antioxidant activities.

For a polyphenol to be defined as an antioxidant, it must satisfy two basic conditions: first, when present in a low concentration relative to the substrate to be oxidized, it can delay, retard or prevent the auto-oxidation, or free radical mediated oxidation (Halliwell, 1990). Second the resulting radical formed after scavenging must be stable through intramolecular hydrogen bonding after further oxidation (Shihadi, 1992). The chemistry of the flavonoids is predictive of their free radical scavenging activity, because the reduction potentials of flavonoid radicals are lower than those of alkyl peroxyl radicals and the superoxide radicals. This means that the flavonoids may inactivate these oxyl species.

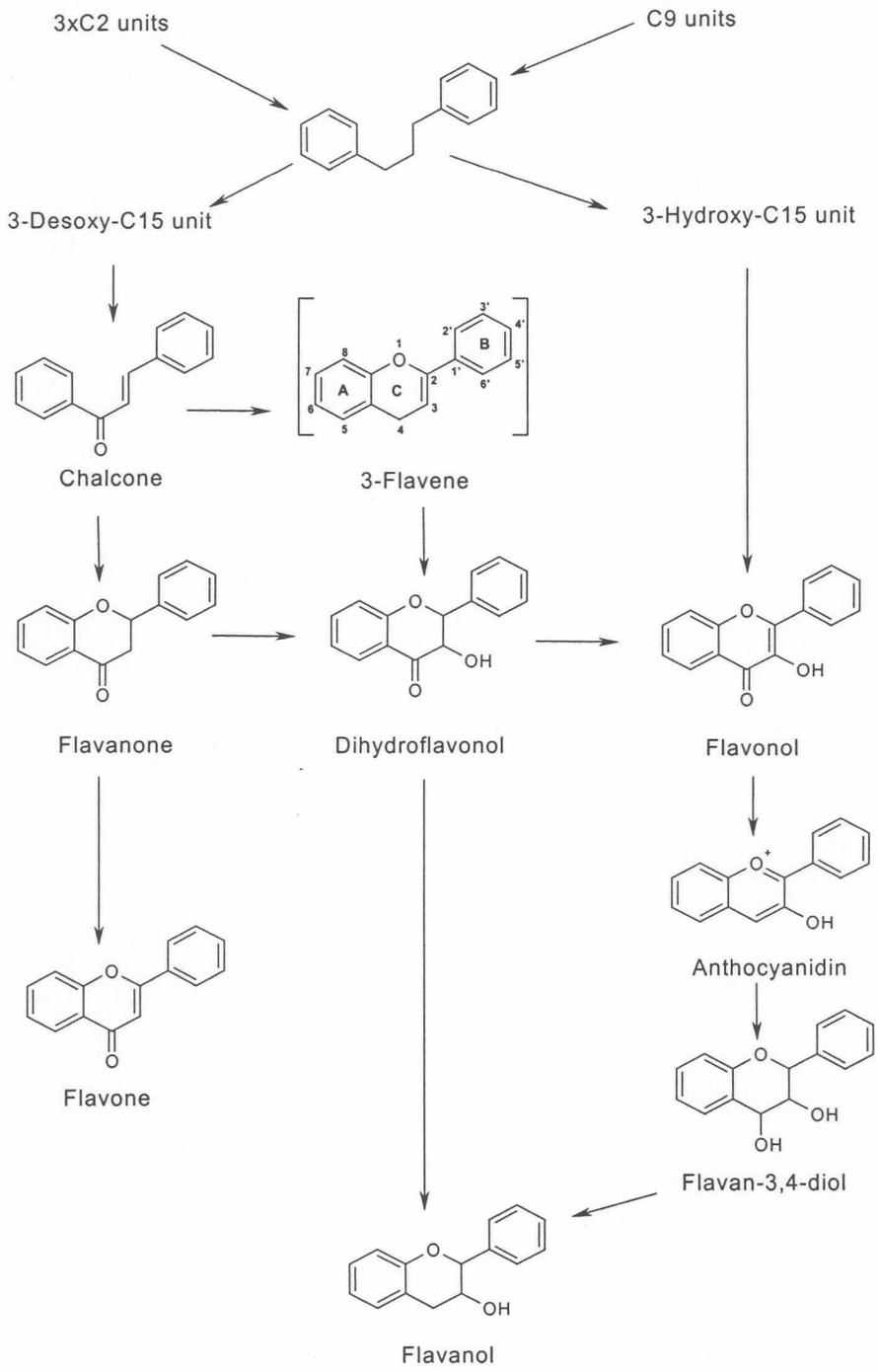
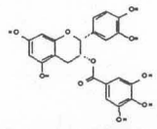
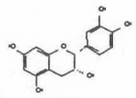
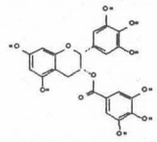
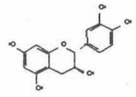
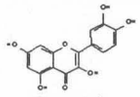
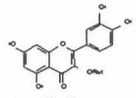
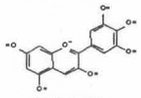
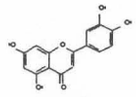
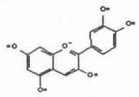
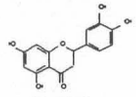
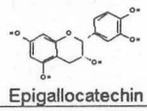
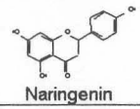
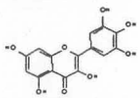
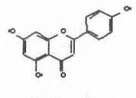
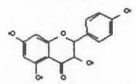
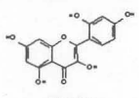
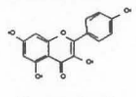


Figure 1.1 A schematic representation of the inter-relationship between the most important flavonoid sub-groups (Adapted from (Rice-Evans, 1996)).

3.3 Structural Principles for Radical Scavenging by Flavonoids

The glycosylation of flavonoids reduce their antioxidant activity when compared to the corresponding aglycones. The antioxidant values of polyphenols are expressed as Trolox-Equivalent Antioxidant Capacity values (TEAC). This value indicates the concentration of a Trolox solution in mM units with equivalent antioxidant potential as a 1 mM solution of the polyphenol of interest. A high TEAC value for a polyphenol indicates that it has good radical scavenging properties. Different flavonoids, their hydroxylation patterns and TEAC values are shown in Table 1.1. Blocking the C3-hydroxyl group in the C ring of quercetin with rutinose forming rutin (quercetin rutinoside) results in a decrease the compound antioxidant activity. Removal of the C3-hydroxyl group as in luteolin also results in decreased antioxidant activity. Maximal effectiveness for radical scavenging apparently requires the C3-hydroxyl group attached to the C2,C3-double bond and adjacent to the C4-carbonyl in the C ring. Removal of the C2,C3 double bond in the C ring, eliminates the means of delocalization of radicals from the aryloxyl radical on the B ring to the A ring as in taxifolin. The comparison of quercetin with luteolin and rutin demonstrates the influence of the C3-hydroxyl in combination with the adjacent double bond in the C ring. If one is dispensed with, the other apparently loses its impact on the antioxidant activity. The reduction of the C2,C3 unsaturated bond in the C ring of kaempferol forming dihydrokaempferol has no influence on the total antioxidant activity. This substantiates the notion of the lack of effect of the specific structural activities in the C ring on the total antioxidant activity in the absence of the orthodiphenolic structure in the B ring. The single hydroxyl group in the B ring makes little contribution to the antioxidant potential, despite the double bond conjugation and the C3-hydroxyl group.

Table 1.1 A list of selected polyphenols with their free hydroxyl group distribution and antioxidant potential (Adapted from Rice-Evans, 1996)

Compound	Free OH-Substituents	TEAC (mM)	Family	Compound	Free OH-Substituents	Glycosylation position	TEAC (mM)	Family
 Epicatechin gallate	3,5,7,3',4',3'',4'',5''	4.9 ± 0.02	Flavanol	 Epicatechin	3,5,7,3',4'		2.5 ± 0.02	Flavanol
 Epigallocatechin gallate	3,5,7,3',4',5',3'',4'',5''	4.8 ± 0.06	Flavanol	 Catechin	3,5,7,3',4'		2.4 ± 0.05	Flavanol
 Quercetin	3,5,7,3',4'	4.7 ± 0.10	Flavonol	 Rutin	5,7,3',4'	3-rutinose	2.4 ± 0.06	Flavonol
 Delpinidin	3,5,7,3',4',5'	4.44 ± 0.11	Anthocyanidin	 Luteolin	5,7,3',4'		2.1 ± 0.05	Flavone
 Cyanidin	3,5,7,3',4'	4.4 ± 0.12	Anthocyanidin	 Taxifolin	3,5,7,3',4'		1.9 ± 0.03	Flavanone
 Epigallocatechin	3,5,7,3',4',5'	3.8 ± 0.06	Flavanol	 Naringenin	5,7,4'		1.53 ± 0.05	Flavone
 Myricetin	3,5,7,3',4',5'	3.1 ± 0.30	Flavonol	 Apigenin	5,7,4'		1.45 ± 0.08	Flavone
 Gallic acid	3,4,5	3.01 ± 0.05	Hydroxybenzoate	 Dihydrokaempferol	3,5,7,4'		1.39 ± 0.02	Flavonol
 Morin	3,5,7,2',4'	2.55 ± 0.02	Flavonol	 Kaempferol	3,5,7,4'		1.34 ± 0.08	Dihydroxyflavonol

The C2,C3 double bond apparently contributes very little to the hydrogen-donating ability without the diphenolic structure in the B ring, for naringenin with a saturated heterocyclic ring and no C3'-hydroxyl has approximately the same antioxidant potential as apigenin.

Quercetin with two hydroxyl groups in the *o*-dihydroxy arrangement in the B ring has a higher antioxidant activity than morin with the two hydroxyl groups in the meta arrangement. Kaempferol with a lone C4' hydroxyl group in the B ring has 27% less antioxidant activity than quercetin with the additional C3' hydroxyl group. Presumably the C2,C3 double bond is not so relevant when the B ring lacks the *o*-hydroxyl arrangement because the monophenolic ring is not as an effective hydrogen donor. The presence of a third hydroxyl group in the B ring does not enhance the effectiveness against aqueous phase radicals as in myricetin compared with quercetin and anthocyanidin delphinidin compared with cyanidin.

It can be concluded that there are three structural groups that are important in determining the radical scavenging and/or antioxidative potential. a) The *o*-dihydroxy (catechol) structure in the B ring, which is the obvious radical target site for all flavonoids with a C2,C3-saturated bond (flavan-3-ols, flavanones and cyanidine chloride). The catechol moiety does not confer stability to aroxyl radicals and participates in electron delocalization. b) The C2,C3-double bond in conjugation with a C4-oxo function that is responsible for electron delocalization from the B ring to the A ring. c) The additional presence of both the C3- and C5- hydroxyl groups for maximal radical-scavenging potential and stronger radical absorption.

3.4 Antioxidant activity of the catechins and catechin gallate esters

Catechins are the most important antioxidant compounds in tea. Catechins include epicatechins, gallocatechins (with the three hydroxyl groups in the B ring) and catechin gallates with a gallic acid esterified to the C3-OH group in the C ring.

There is no electron delocalization between the A and the B rings due to the saturation of the hetrocyclic C ring. The antioxidant activity responds broadly to the tenet that the structures with the most hydroxyl groups exert the greatest antioxidant activity. Quercetin has an identical number of hydroxyl groups in the same positions as catechin, but also contains the C2,C3-double bond in the C ring and the C4-oxo group. The catechin structure can be modified to enhance its antioxidant potential by incorporating the C2,C3-double bond and C4-oxo function or by forming epigallocatechin gallate. Both the modifications result in approximately the same antioxidant potential. The antioxidant potential of the tea catechins on a molar basis, against the radicals generated, in the aqueous phase are, in order of decreasing effectiveness: ECg ≈ EGCg > EGC > Gallic acid > epicatechin > catechin.

A green tea preparation at 1000 ppm (0.1% w/v) gave a TEAC value of 3.78. A black tea preparation of the same concentration gave a similar TEAC value of 3.49. A total green tea polyphenol extract (44% w/w of the dry weight of the green tea preparation) shows a total antioxidant activity of 3.36. The total polyphenol content of a black tea extract is similar to that of green tea (44.94% w/w), but only 6.9% by weight is comprised of catechin and catechin gallate components. The rest of the polyphenols includes theaflavins, thearubigens and undefined polymeric polyphenols formed during the oxidation process. The antioxidant activity of black tea extract is 3.49

which is similar to that of green tea (Salah, 1995). The above results suggest that theaflavins and thearubigens have similar antioxidant capacity as their catechin precursors.

3.5 Properties of methylxanthines

Theophylline, theobromine and caffeine are three closely related alkaloids from the family of methylated xanthines. Beverages containing methylxanthines have been popular since antiquity. One such beverage is tea. The basic belief is that beverages containing caffeine have stimulatory effects that elevate mood, decrease fatigue and increase capacity for work (Gilman, 1985). The solubility of the methylxanthines is low and is enhanced by the formation of complexes with a wide variety of more soluble compounds. In black tea the methylxanthines complex with gallated theaflavins to increase their solubility. At low temperatures these complexes aggregate to form "cream". This is a problem with bottled iced tea. "Cream" formation must be prevented to be able to produce and deliver a product with freshly made and appetizing appearances. To prevent the formation of "cream" the tea must be decaffeinated or the gallated theaflavins must be hydrolyzed to simple theaflavins with tannin acylhydrolase enzymes [E.C. 3.1.1.20].

4 Bioactivities documented for tea components

4.1 Tea polyphenols and cancer

The antimutagenic and anticarcinogenic properties of tea polyphenols are poorly understood and are being investigated extensively. From the evidence currently available it is certain that there are several mechanisms of action by which polyphenols manifest their prophylactic properties.

Several studies have shown that tea polyphenols prevent tumor initiation. Tumor initiation is usually the result of permanent covalent DNA modification. Oxygen radicals oxidize the nucleotide bases thus causing alterations of the genetic code. This may lead to tumorigenesis. The antioxidant activities of polyphenols reduce tumor initiation occurring in this manner (Steele, 1985), (Nagabhushan, 1988), (Bu-Abbas, 1994 (a)).

Activated chemical compounds, known as carcinogens, can form adducts with DNA resulting in erroneous transcription or replication of DNA also altering the genetic code. Polyphenols may act as alternative targets for the activated chemical compounds, thus sparing the DNA. Many carcinogens enter the body as inactive pro-carcinogens that do not have the ability to bind to DNA. Cytochrome P-450's (P450), phase I detoxification enzymes, convert pro-carcinogens to electrophillic carcinogens through oxidation. The P450 is a superfamily of isoenzymes with different substrate specificities and reactivities. Different isoenzymes may convert the same xenobiotic to different products of which one may be carcinogenic and the other may be harmless. It was shown that EGCg inhibited the activities of P450 1A, 2B1 and 2E1 from liver microsomes, preventing the activation of NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) a tobacco-specific carcinogen (Shi, 1994). Quercetin, kaempferol and other polyphenols are known to be selective inhibitors of P450 isoenzymes (Yang, 1994). Tea polyphenols are able to selectively induce P450 1A2 and 4A1 (Bu-Abbas, 1994 (b)), (Sohn, 1994). This is an indication that it may not only prevent pro-carcinogens from being converted to carcinogens, but may also be

able to induce alternative options for converting pro-carcinogens to harmless compounds.

From the literature it is evident that phase II detoxification enzymes are modulated in a similar manner by tea polyphenols as with the phase I enzymes. Tea treatment enhanced the UDP-glucuronosyl transferase activity, resulting that tobacco-specific carcinogens were conjugated more readily with glucuronic acid (Bu-Abbas, 1995). This renders the carcinogen inactive and increases the excretion of the carcinogens. It was found that tea polyphenols inhibited the glucuronidation of estradiol and estrone (endogenous compounds) which shows that there is some sort of selectivity in the induction/inhibition process (Zhu, 1998). Glutathione-S-transferase, another phase II enzyme, is also inhibited by several plant polyphenols, including morin, quercetin and apigenin (Zhang, 1994).

Polyphenols have been shown to be able to prevent or reduce tumor promotion (Mukhtar, 1992). Recent research presents evidence that tea polyphenols are modulators of various cell growth regulators. Tea polyphenols have been shown to inhibit lipoxygenase from soybeans (Wang, 1990). It has been shown that lipoxygenase plays an important role in tumor promotion, since the metabolic products (HETE) of this pathway via the arachidonic acid cascade are involved in the process of tumor promotion (Nakadate, 1989).

Tea polyphenols inhibit one or several signal transduction pathways. It was shown that tea polyphenols prevent tumor promotion induced by either epidermal growth factor (EGF) or phorbol esters (Mukhtar, 1992), (Conney, 1992). Phorbol esters

mimic 1,2-diacylglycerol activation of protein kinase C (PKC) and EGF activate PKC via a receptor mediated pathway. It was shown that EGCg and theaflavin inhibits EGF and phorbol esters induced phosphorylation of the protein c-jun (Dong, 1997). The c-jun NH_2 terminal kinase is downstream of PKC in the signal transduction pathway, and is required to form an active AP-1 complex. AP-1 is a transcription factor that is important in tumor promoter-induced neoplastic transformation (Angel, 1991).

Recently it was also shown that EGCg inhibits UVB-induced AP-1 activity (Barthelman, 1998). UVB-induced signal transduction pathways are normally PKC independent, indicating that EGCg also modulates other signal transduction enzymes. Evidence was presented showing that EGCg inhibits the phosphorylation of p38 MAPK resulting in the reduced expression of the c-fos gene (Chen, 1999). The c-fos protein is required to form the active AP-1 heterodimer complex. GTP was shown to inhibit UVB-induced ornithine decarboxylase and cyclooxygenase and restore UVB-induced catalase, GSH and glutathione peroxidase activity in epidermal cells. Additional evidence shows the inhibition of UVB-induced TNFα, known to be released by keratinocytes subsequent to UVB irradiation. EGCg, EGC and theaflavin-3,3'-digallate inhibited cell growth in Ha-ras transformed cells. The Ha-ras activated AP-1 pathway is the major growth stimulant in Ha-ras transformed cells (Yang, 1999).

Green tea polyphenols have an inhibitory effect on the growth of transformed cell lines and tumors transplanted into mice, indicating it may play a role in regulating cell cycle progression (Conney, 1999). Tea polyphenols are able to inhibit the phosphorylation of retinoblastoma protein (pBR). pBR binds transcription factors

such as the E2F family required for the progress of the cell cycle from G1 to the S phase (Khafif, 1998). It has been demonstrated that EGCg can induce apoptosis and cell cycle arrest in human epidermoid carcinoma cells A431. The apoptotic response was specific to cancer cells.

It was shown that green tea inhibits *in vivo* metastasis and *in vitro* invasion of mouse lung carcinoma cells (Sazuka, 1995). It was shown that EGCg, ECg, theaflavin and theaflavin digallate inhibited the type IV collagenase activity of MMP-2 and MMP-9 matrix-metallo proteinases *in vitro* (Sazuka, 1997) and stabilizes collagen against collagenase activity (Wauters, 1986). With computer modeling it was suggested that EGCg might well serve as an inhibitor of urokinase, one of the enzymes that are most frequently expressed in human cancers (Jankum, 1997).

4.2 Inhibition of other enzymes

In vitro studies with tea extracts and pure compounds have shown inhibition of salivary α-amylase (Hara, 1990), small intestine sucrase (Welsch, 1989), liver NADH-cytochrome c reductase (Wanmg, 1988), glucosyltransferase from *Streptococcus mutans* (Otake, 1991) as well as HIV reverse transcriptase and cellular DNA and RNA polymerases (Nakane, 1990). The tea polyphenols do not only display selective inhibition with the P450 isoenzymes. Specific inhibition of type 1 but not type 2 steroid 5-reductase is seen (Liao 1995) and strong inhibition of angiotensin converting enzyme (ACE) is found, while only weak inhibition is found for mechanistically similar carboxypeptidase A (Hara, 1989).

4.3 Prophylactic properties of tea methylxanthines

Some of the beneficial health properties in animal studies can be produced with caffeine in the drinking water (Chung, 1998). Caffeine induces hepatic CYP 1A2 in rats just as polyphenols, although the induction is somewhat weaker (Ayalogu, 1995). Consumed carcinogens are probably converted to innocuous compounds by the induced P450 isoenzymes, or the increased biotransformation leads to increased excretion of the toxin. Theophylline and theobromine and caffeine share common pharmacological actions. They stimulate the central nervous system, act on the kidney to produce diuresis, stimulate cardiac muscle, and relax smooth muscle. Theophylline is used as therapy for bronchial asthma. High doses of caffeine or theophylline may have adverse effects on the central nervous system (Gilman, 1985).

5 Analytical methods in natural product chemistry

In the search and identification of novel natural products several analytical techniques are employed. These include high performance liquid chromatography (HPLC), high performance centrifugal counter current chromatography (HPCCC), capillary electrophoresis (CE) and high resolution mass spectrometry (HRMS). Analytical HPLC has always been the favorite and most popular analytical tool in natural product and clinical chemistry. Since the development of capillary electrophoresis many papers on the topic of CE have been catalogued. The pioneer days of trial and error method development have passed. This work has been integrated forming trends and concepts that enable the design of systematic strategies for the development of new analytical methods. The technique has matured to such an extent that similar CE methods exist for almost all HPLC methods, particularly in the fields of natural product and clinical chemistry.

CE has become the preferred technique because it has several advantages over HPLC. The separation efficiency of CE is much higher than that of HPLC and comparable to that of GC. The electro-osmotic flow causes less increase in the peak width than conventional laminar flow, which reduce the width of analyte peaks and increase the plate numbers. Plate numbers for CE are typically in the region of 10^5 to 10^6 m^{-1}, while platenumbers for HPLC are generally in the region of 10^4 m^{-1}. Uncoated CE columns are generally rinsed with acid or base for regeneration taking between 2 and five minutes while HPLC columns require long rinse times for column equilibration. This simplifies CE methods considerably, making automation a lot easier and increase the sample throughput. The amounts of solvents and in particular organic solvents are reduced to milliliters compared to the 1-2 liter organic solvents per day that require disposal with HPLC. Since uncoated capillaries can be regenerated with harsh acid or base conditions, extensive sample pretreatment is not as important as with HPLC where regeneration conditions are much milder (Issaq, 1997). The different modes of CE enable it to be a very powerful and versatile technique. CE has been used to analyze proteins, peptides, amino acids and a whole variety of drugs in serum. CE is ideal for therapeutic drug monitoring and testing for intoxication and drug abuse since small volumes of sample is required and the method automation is possible. The analysis of the low volumes of serum and urine collected for time dependent therapeutic monitoring and the huge sample throughput required can be achieved with CE.

All specialized techniques have limitations and CE is no exception. Analytes should not be highly volatile and have to be soluble in polar solvents to some extent. Analyte

concentrations in the order of 1mg/l (1ppm) is a necessity to avoid sample pre-concentration steps. On-column detection restricts the limit of detection (LOD) to between 0.1 and 1.0 mg/l, which is not as good as obtained with HPLC (0.02–0.1 mg/l) but still comparable. Flow-cell designs, different detection window geometries, stacking procedures as well as electrochemical and fluorescence detection can improve the LOD (Albin, 1993). The maximum peak capacity with CE is 10 peaks/min and more complex samples should undergo sample pretreatment. The low volume of buffer and sample used in CE restricts it to analytical tasks while preparative work should preferably be done with LC.

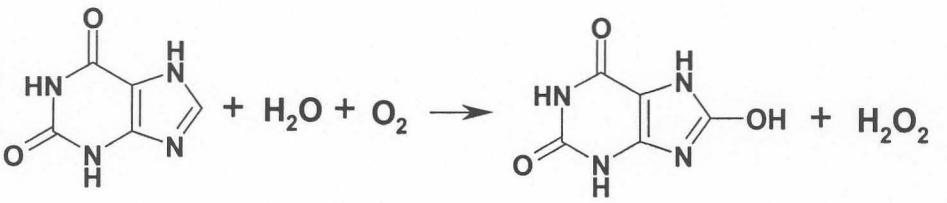
6 Objectives of the study

6.1 The detection and quantification of important constituents in tea leaf extracts

Plant extracts normally contain a large range of closely related secondary metabolites such as flavonoids, flavonoid glycosides, alkaloids or coumarins. Tea leaf extracts have an array of closely related catechins. CE with its high resolution and column efficiency is ideal for analysis of complex plant extracts without performing any sample clean-up procedures. Differences of secondary metabolite types and distribution patterns between cultivars or species can be detected easily with CE. The ability of MEKC to separate both charged and neutral analytes in one run makes this mode of CE an ideal analytical method to separate and quantify all the pharmacologically and organoleptically important tea constituents in one run.

6.2 To study the inhibition of Xanthine Oxidase by tea polyphenols

Xanthine oxidase (XO) belongs to the oxomolybdenum group of enzymes. This group contains both oxidase and reductase enzymes. Oxomolybdenum enzymes differ from other oxidase enzymes by utilizing water instead of dioxygen as the source of the oxygen molecule destined to be incorporated into the products. They also produce reducing equivalents instead of consuming them (Fig. 1.2). Xanthine oxidase catalyzes the oxidation of a broad spectrum of aromatic heterocycles and simple aliphatic aldehydes.



Xanthine **Uric acid**

Figure 1.2 Xanthine oxidase catalyzes an oxidation-reduction reaction. It obtains its reducing equivalents from water. In the process xanthine is oxidized to uric acid and the reducing equivalents are donated to molecular oxygen to produce hydrogen peroxide.

Physiologically XO catalyzes the irreversible formation of uric acid from xanthine and therefore it is one of the enzymes that regulate the levels of uric acid in body fluids. Uric acid levels of more than 7 mg/dl usually result in a condition known as hyperuricemia. This may lead to the formation of monosodium urate crystals in the kidneys (kidney stones) and joints (gout). Inhibition of XO alleviates hyperuricemia, reducing the occurrence of kidney stones and gout. Oxygen radicals are the byproduct in the reaction which may result in increased oxidative stress that lead to diseases such as cancer and atherosclerosis. The inhibition of radical formation may reduce oxidative stress and increase the antioxidant potential in body fluids. Inhibition of uric acid formation may compromise the antioxidant potential of the serum, since urate

also serves as antioxidant that contributes to the total antioxidant status of the serum (Rice-Evans, 1994).

Allopurinol is the most widely used drug for the treatment of hyperuricemia. It is a substrate analogue of hypoxanthine. It causes hypersensitivity reactions as well as debilitating side-effects such as nausea, headaches and drowsiness. Many other synthetic inhibitors of XO have been identified. These inhibitors include several anti-allergy drugs with aromatic heterocycles (White, 1981) and several derivatives of allopurinol (Okamoto, 1995), (Springer, 1976).

Different natural compounds also inhibit XO. These include coumarins (esculetin and 7-hydroxycoumarin) (Chang, 1995 (a)), phenolic carboxylic acids (2,3,4 hydroxybenzoic acid and β-resorcylic acid) (Chang, 1995 (b)), caffeic acid and its analogues such as *m*-coumaric acid (Chang, 1995 (c)) and flavonoids such as quercetin, baicalein and hesperidin (Chang, 1993). Polyphenol (anthocyanidins) extracts from berries are capable of inhibiting XO and scavenge the superoxide radicals that have formed (Costantino, 1992). Polyphenols are the most potent of all the natural product inhibitors. Several polyphenols have K_i values that are similar to that of allopurinol. Many of the allopurinol analogues have been tested *in vivo* to determine their potency and selectivity and evaluate the possibilities of replacing allopurinol as drug of choice. No such studies have been conducted for the polyphenols (*in vivo*).

The different tea polyphenols are likely to inhibit XO *in vitro*. This needs confirmation before *in vivo* studies can be conducted. Most studies on the anti-

carcinogenic and anti-mutagenic properties of tea polyphenols were conducted in rat models. The inhibition of XO *in vivo* may reduce the amount of superoxide radicals, making it a significant mechanism whereby tea polyphenols may prevent tumorigenesis. A rat model was selected for the *in vivo* inhibition studies even though little data is available on the pharmacokinetic behavior and bioavailability of tea polyphenols. Evidence available suggested that this model would not be inappropriate for this type of study. In male Wistar rats that were orally fed, catechins were absorbed from the intestinal tract and were carried to the liver by the rat portal vein (Okushio, 1996). According to (Chen, 1997) the absorption and elimination of EGCg fits a one compartment model for the intragastrically (i.g.) route and a two compartment model for the intravenously route in male Sprague-Dawley rats. Glucuronide and glucuronide-sulfate conjugates of catechins are found in serum (Da Silva, 1998). This indicates that the catechins do reach the liver, which is the target organ for the inhibition of XO. EGCg reaches levels of 0.1-0.5 μg/ml in rat and 2-4 μg/ml in human plasma. CE will be used to quantify the amounts of xanthine and uric acid in the serum and urine of the rats. The analyte levels will be an indication of the amount of inhibition obtained with the polyphenols.

7 Aims of study

a) Develop a MEKC method that is suitable for the analysis of the important constituents in tea.

b) Determine whether tea polyphenols inhibit XO.

c) Identify an enzyme inhibition model and predict structure-activity relationships.

d) Identify a suitable method for analyzing rat urine and serum for XO substrates and products and implement it.

e) Determine the ability of the EGCg and a polyphenol extract as *in vivo* inhibitors of XO in a rat model and compare them with a positive control (allopurinol).

8 Hypotheses

a) MEKC will be a useful method for the separation and quantification of the quality contributing components in tea.

b) Tea polyphenols are a potential novel class of XO inhibitors.

Chapter 2

Development of a MEKC method for analyzing tea compounds

1.1 Introduction

HPLC is the most popular technique used to detect and quantify natural compounds that occur in green and black tea. Several HPLC methods exist for the analyses of different groups of tea compounds. The type of samples, extraction methods of catechins and the modes of detection vary between methods. Of importance is the analysis time required for HPLC analytical methods. The equilibration required between runs is generally 10 min long. With column equilibration all methods have an analysis time of 30 minutes or longer. The long duration of the methods limits the amount of samples that can be analyzed. A HPLC method was developed to analyze eight catechins as well as caffeine from green tea extracts (Goto, 1996). The analysis time was 20 minutes excluding column equilibration. A HPLC method was developed to analyze the five major catechins in green, oolong and black tea liquors (Khokhar, 1997). Eight catechins were analyzed in human plasma and saliva with a HPLC method developed by (Tsuchiya, 1997), (Tsuchiya, 1998). They made use of complex formation between diphenylborate and the vicinal hydroxyl groups of the catechins to achieve organic phase extraction and concentration of the polyphenols. This method significantly improved the LOD for catechins in samples. Without column equilibration the analysis time is 18 minutes. Five catechins could be detected in human plasma with a HPLC method developed by (Maiani, 1997). Sample treatment entailed HCl-methanol hydrolysis followed with ethyl acetate extraction. The method failed to separate caffeine from background analytes. Analysis of caffeine may be important in some bio-

medical applications, since both tea catechins and caffeine are substrates and regulators of P450 iso-enzymes (Ayalogu, 1995). The analysis time without column equilibration is 20 minutes. A HPLC method using chemiluminescence detection was developed to determine only EGCg in rat and human plasma (Nakagawa, 1997(a)). It is the most sensitive method to date, detecting EGCg in picomole quantities. All the methods above used reverse phase columns and gradients of increasing organic solvent concentrations to shorten the retention times of the more hydrophobic catechins. All the methods used UV detection except for the use of chemiluminescence (Nakagawa, 1997), electrochemical detection (Lee, 1995) and spectrofluorometry (Tsuchiya, 1998). In a study where fluorescence detection and normal UV detection were compared it was found that fluorescence improved the LOD more than 10 times without losing any specificity (Arts, 1998). Catechin concentrations could be detected in the ng/l range. Only one method exists for the determination of the total and enantiomeric (D/L forms) of theanine (Ekborg-Ott, 1997). The separation of D-L theanine was achieved with a γ-CD column used in the polar organic mode.

In both RP-HPLC and MEKC separation is achieved by the selective distribution of analytes between a long aliphatic carbon chain stationary/pseudo-stationary phase and a mobile aqueous/organic phase. Both techniques have the ability to separate neutral analytes. RP-HPLC does not have the ability to separate the charged molecules (anions, cations and zwitterions) since all of them elute on the void. We proposed that these charged molecules could be separated from each other with MEKC based on their different charge/mass ratios.

When we set out to develop a MEKC method to separate the different catechins, no other CE method separating tea compounds was available. During the course of the investigation several publications appeared where different tea components were analyzed with CE methods. At that time only several CE methods existed for the analysis of different flavonoids and flavonoid-O-glycosides with isotachophoresis (ITP) (Seitz, 1991), capillary zone electrophoresis (CZE) or micellar electrokinetic chromatography (MEKC). In all the publications the authors focused on the separation of a group of polyphenols and not a mixture of analytes with vastly different properties as found in tea extracts.

Closer scrutiny of the methods indicated four distinct groups of conditions used to separate the polyphenols (Table 2.1). For MEKC three different pH ranges have been used namely pH lower than 8 (group 1), pH 8-9 (group 2) and pH higher than 9 (group 3). For CZE only buffers with a pH higher than 9 (group 4) have been used. In group 1, low to moderate concentration of borate was mixed with phosphate when SDS or CTAB was used as the surfactants. These methods relied mainly on the hydrophobic interaction with the surfactants for selectivity. Borate complex formation and weakly anionic properties contributed selectivity to a lesser extent. Phosphate, cholate and taurine systems have also been used where the chiral taurine adds selectivity as well. In group 2, moderate to high concentrations of borate were used with SDS as surfactant. These methods relied mainly on borate complex formation and the anionic properties of the polyphenols. Hydrophobic interactions were not as important as can be seen from the use of low SDS concentrations used in these methods. In group 3 low concentrations of borate, phosphate, SDS and SC were used. The negative charges on the polyphenols were the major selectivity factor. Borate complex

Table 2.1 The importance of pH on the selection of MEKC and CZE buffer conditions for flavonoids and flavonoid glycosides separation methods.

pH	Buffer Conditions	Surfactant	Reference
Group 1			
6.97	5mM Borate, 15mM Phosphate, 40%acetonitrile	50 mM SC	(Li, 1995)
7.5	50mM Borate, 50mM Phosphate	42mM SDS	(Ng, 1992)
7.0	12mM Borate, 20mM Phosphate, 4%1-propanol 100mM Phosphate500mM Taurine, 6% 1-propanol	40mM CTAB 35mM cholate	(Bjergegaard, 1993)
Group 2			
8.3	20mM Borate	50mM SDS	(Pietta, 1994)
8.0	200mM Borate	50mM SDS	(Ferreres, 1994)
8.0	20mM Borate, 20% Methanol	25mM SDS	(Morin, 1997)
8.0	200mM Borate, 10% Methanol 100mM Borate, 10% Methanol 200mM Borate, 10% Acetonitrile	50mM SDS 30mMSDS 50mMSDS	(Gil, 1995)
8.3	20mM Borate	50mM SDS	(Pietta, 1991)
8.5	20mM Borate, 1mM 1,3-diaminopropane	48mM SDS	(Liang, 1996)
Group 3			
9.7	12.5mM Borate, 10mM Phosphate	20mM SDS	(Liu, 1994)
9.35	12.5 mM Borate, 10 mM Phosohate	18mM SDS, 2mM SC	(Sheu, 1995)
Group 4			
9.5	25 mM Ammonium acetate	None	(Aramendia, 1995)
9.5	25mM Borate	None	(McGhie, 1993)
10.0	150 mM Borate	None	(Seitz, 1992)
9.6	100 mM Boric Acid	None	(de Simón, 1995)

formation and hydrophobic interaction added selectivity to a lesser extent. In group 4 only borate in moderate to high concentrations has been used. The charge to mass ratio differences results in separation in the CZE mode. Charges originate from the deprotonated hydroxyl groups as well as the borate complex formation that takes place.

Several properties of analytes can be exploited to achieve separation with MEKC. The analytes with hydrophobic moieties undergo hydrophobic interactions with the surfactants. Changes in the surfactant concentration will change the distribution of the hydrophobic analytes between the surfactant and aqueous phase. The addition of organic solvents will make hydrophobic analytes more soluble in the aqueous phase, thus changing the distribution pattern. The selectivity can also be manipulated when chiral selectors are added to the aqueous phase. These chiral selectors form complexes with the analytes in the aqueous phase. This changes the distribution between the surfactant and aqueous phase. Borate is one chiral selector normally used to obtain separation of polyphenols. It complexes only with vicinal hydroxyl groups on sugars or aromatic rings. The negatively charged borate then results that the analyte is more negatively charged. This prevents the analyte from entering the surfactant phase. It also changes the charge/mass ratio and migration properties of the analyte. Changes in the pH of the aqueous phase, especially near the pKa values of analytes are useful in changing the migration properties for achieving separation.

Our aim was to develop one method to analyze tea catechins (weakly anionic), caffeine (neutral), theanine (zwitterionic) and ascorbic acid and gallic acid (anionic). Caffeine was found to co-migrate with the electro-osmotic flow (EOF) in a buffer

lacking surfactants (CZE), with pH 5-8 being tested (Lee, 1992). In a buffer lacking surfactant the zwitterionic theanine will also co-migrate with the EOF. The lack of selectivity by ITP and CZE for neutral and zwitterionic compounds ruled them out as options for method development. MEKC was deemed the best CE mode to separate the weakly anionic, anionic, neutral and zwitterionic compounds of interest. Since the majority of the publications used MEKC successfully for polyphenol separations, it seemed likely that success would be possible with this mode of CE.

Besides our MEKC method, eight other CE methods exist to analyze different tea polyphenols with, one CZE method for the analysis of organic anions in tea (Horie, 1998 (a)) and one cLC/ESI-MS method for the detection of catechins in tea and in human plasma (Dalluge, 1997). Of the eight methods on catechins, two of them are CZE methods. Boric acid was used to separate the five major catechins, quercetin, gallic acid, caffeic acid, theophylline and adenine (Arce, 1998). Borate was used to separate the five major catechins as well as caffeine, theanine and ascorbic acid (Horie, 1997). The conditions used in this method lead to significant peak tailing of all the analytes, resulting in inaccurate quantification. This is probably why the same author developed MEKC methods to separate the same analytes (Horie, 1998 (b)), (Horie, 1999). Some of the catechins still showed significant peak tailing. Two other methods were developed that used the combination of SDS, sodium tetraborate and sodium phosphate to separate the tea catechins (Watanabe, 1998), (Barroso, 1999). A MEKC method using borate, SDS and β-cyclodextrin was developed by (Nelson, 1998) to separate six tea catechins. A MEKC method was developed that used borate complexing and acetonitrile organic phase partitioning at pH 6.0 (Larger, 1998). This method was used to analyze catechins, flavonoid glycosides and chlorogenic acid ob-

tained with aqueous, ethyl acetate or methanol extraction procedures. Their results give an appreciation for the complexity of tea samples. It is unlikely that one single set of analytical conditions will be sufficient to analyze all the diverse compounds in tea infusions e.g. catechins, theaflavins, thearubigens, flavonoid glycosides, carboxylic acids, amino acids and carotenoids.

2 Materials and Methods

2.1 Strategy for optimization of a MEKC method

Many molecules can behave as acids or bases. The pH of the buffer has a major influence on the selectivity. The pH conditions should be considered well, since pH influences the EOF and determine the charge to mass ratio of the analytes. For the analysis of small molecules it is best to select the pH conditions in the range of the pKa values of the analytes, since the most selectivity can be achieved then (Wätzig, 1998). From Table 2.1 it is clear that pH is the most important parameter in this situation even though a MEKC method (with predominantly hydrophobic interactions) is being developed. The pH value determines the type of buffer and the physiochemical properties that will be exploited to achieve separation. Several preliminary studies were conducted. The pH was tested in the range of 7-8 and it was found that the pH should be kept below 7.6 to prevent peak shape distortion. This restriction places the method in group 1 with borate/phosphate mixtures and SDS or CTAB as surfactants. Another preliminary study showed that the borate caused severe peak tailing whereas phosphate provides more symmetrical peaks. It was decided to optimize a method with phosphate and SDS in the pH range of 6.5 to 7.6. Methods can be developed with either the overlapping resolution mapping (ORM) procedure or with the simple uni-

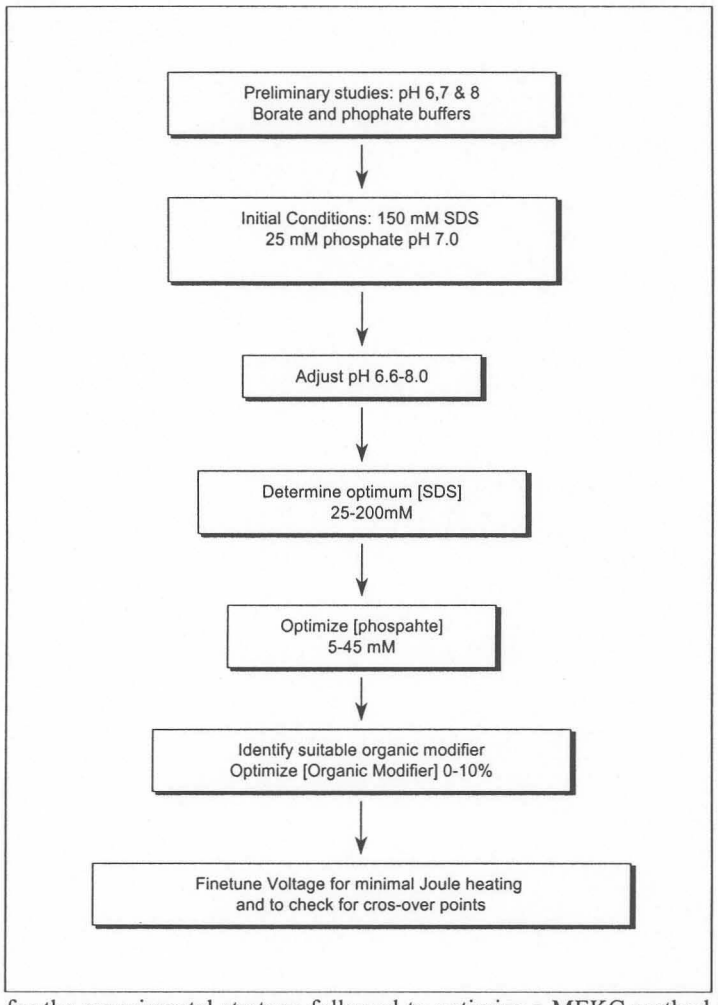


Figure 2.1 Scheme for the experimental strategy followed to optimize a MEKC method to analyze tea catechins.

variate procedure. With the ORM procedure two or three parameters can be varied at the same time (Ng, 1992). Polynomial equations are then derived to define the resolution between neighboring peaks. A global optimum condition is then derived. This procedure results in optimization with typically 9 runs or less. Unfortunately the migration properties of the analytes are defined theoretically and knowledge of precise crossover regions and deviations from the theoretical models are not available. We opted for the univariate procedure where only one parameter is varied at a time. This enabled us to define the migration behaviors of all the analytes and select conditions far from regions that may result in inconsistent separations. The univariate

procedure also allows easier fine-tuning of the method to reduce analysis times to a minimum. A flow diagram of the optimization procedure is depicted in Fig. 2.1.

2.2 Instrumentation

Electrophoresis was carried out using the Beckman P/ACE 2100 (Beckman Instruments, Fullerton, CA, USA) with on-column detection. An uncoated fused silica capillary column from Beckman with an internal diameter of 50 μm and total length of 57 cm was used. The effective separation length was 50 cm. All samples were injected pneumatically (0.5 psi) for 2 sec. The operating temperature was 25°C. Detection was effected by measurement of UV absorbance at 200 nm.

2.3 Reagents

The five catechin standards (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg) as well as theanine were gifts from Mutsui Norin (Tokyo, Japan). The gallic acid and ascorbic acid were obtained from Sigma Chemical Company (St Louis, MO, USA). Caffeine and sodiumdodecyl sulphate (SDS) were obtained from Merck (Darmstadt, Germany). The SDS was of analytical grade suitable for electrophoresis. All buffer salts were of analytical grade. All solutions were prepared with distilled water that was deionized with a Milli-Q system (Millipore Corp., Bedford, MA., USA).

Methanol and *p*-nitrophenol were obtained from BDH Laboratory Supplies (Poole, England) and Sudan III from SAARChem Pty. Ltd. (Muldersdrift, RSA). The metha-

nol and Sudan III were used for the capacity factor (k') calculations of each analyte. *p*-Nitrophenol was used both as reference peak and internal standard.

2.4 Analytical conditions

Standards and samples were analyzed with the anode on the inlet side and a running buffer of 100 mM SDS, 25 mM phosphate, 6% (v/v) methanol pH 7.0. The buffer pH was adjusted by mixing monosodium and disodium phosphate (25 mM) solutions. Samples of fresh tea leaves as well as green and black tea were analyzed with an applied voltage of 14 kV. Bottled tea samples were analyzed with the running buffer containing only 5% (v/v) methanol and an applied voltage of 14 kV.

At the beginning of each day the capillary was regenerated by rinsing for 5 min with H_2O, 10 min with 0.1M HCl, 5 min with H_2O, 5 min with 1.0M NaOH, 5 min with H_2O, 10 min with 0.1M NaOH and finally 2 min with H_2O. The column was equilibrated by repeating 5 analyses of the standards after the regeneration. Before each analysis the capillary was rinsed with running buffer for 2 min. After each analysis the capillary was rinsed for 30 sec with H_2O, 2 min with 0.1 M NaOH and 2 min H_2O.

2.5 Preparation of samples and standard

Fresh tea leaves were obtained from the Grenshoek tea estate in the district of Tzaneen in South Africa. Black tea was prepared in our laboratory. Briefly the leaves were withered overnight at room temperature, macerated by three passes through a cut, tear and curl (CTC) machine and left at room temperature for 60 minutes to oxidized. The oxidized leaves were dried in a fluid bed drier. The dried tea was sifted

in a series of sieves and only particles between 500 and 1700 µm were selected for further work. Green tea was prepared in the same manner with omission of the oxidation step. All samples were stored in airtight aluminium lined paper bags at 4°C.

Fresh tea leaves were steamed for 30 seconds and oven dried at 90°C overnight. Bottled ice teas were obtained from a local supermarket. Samples were not filtered with membrane or cartridge filters as some polyphenols absorb to the filter material.

Black and green tea liquors were prepared from made tea samples. Tea liquors were prepared as 1% (w/v) tea solutions (1 g dry, fine ground tea leaves in 100 ml H_2O). The water extracts of tea leaves were made by shaking the tea leaves for 10 min in boiling hot H_2O in a thermal flask. The extracts were then filtered through Schleicher & Schüll No. 595 filter paper (Germany) to remove particulate matter. This is the concentration of tea normally consumed by the general public. Professional tea tasters use a 2% (w/v) concentration for easier detection of off-notes and deleterious flavours. Filtered black tea samples were diluted to 0.5% (w/v), while filtered green tea samples were diluted to 0.1% and 0.5% (w/v) with 0.1mg/mL pNP dissolved in H_20 before injection. pNP was added to bottled tea at a concentration of 0.1mg/ml before analysis.

All standards were prepared by dissolving individually in H_2O. EC and ECg were prepared at 0.5 mg/ml, theanine and ascorbic acid at 10 mg/ml, pNP at 5 mg/ml and all the other analytes at 1 mg/ml.

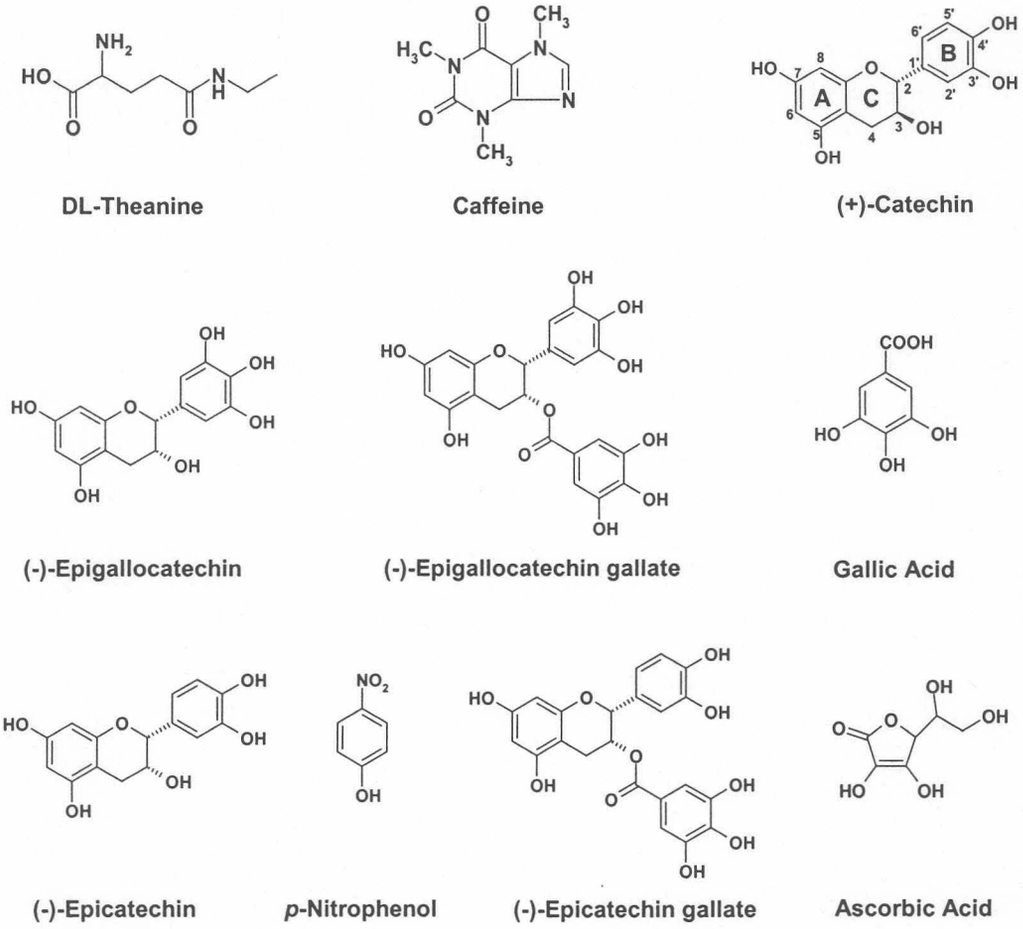


Figure 2.2 Chemical structures of organoleptically important compounds in tea and the pNP internal standard.

3 Results and Discussion

The compounds that have a large influence on the quality of tea were used in this investigation (Fig. 2.2). The influence of pH, surfactant concentration, background electrolyte, organic modifier and voltage on their resolution, peak shape and capacity ratios were determined. Near optimum conditions were selected from our experience. Each variable was changed individually over the range of interest while all other variables were kept constant. All optimization analyses were done at an applied voltage of 14 kV.

Capacity factors (k') were calculated with equation 1 and used as a convenient way of expressing the results. All migration times were measured in minutes.

$$k' = \frac{t_r - t_0}{t_0(1 - \frac{t_r}{t_{mc}})} \qquad (1)$$

k' = capacity factor

t_0 = retention time for unretained compound

t_r = retention time of analyte

t_{mc} = retention time of completely retained compound

The values t_o and t_{mc} were determined with methanol and Sudan III respectively. These values are indicative of the electro-osmotic flow (t_o) and surfactant phase (t_{mc}) velocities.

The column efficiency (N) was calculated with equation 2 and the resolution (R_s) between peaks with equation 3. In equation 3 t_{r1} and t_{r2} are the migration times of two neighboring peaks, where t_{r2} has the longer migration time. The symbols w_1 and w_2 represent the peak widths at baseline that correspond with t_{r1} and t_{r2} respectively.

$$N = \frac{(t_r)^2}{\left(\frac{w}{4}\right)^2} \qquad (2)$$

$$R_s = \frac{(t_{r2} - t_{r1})}{\frac{1}{2}(w_1 + w_2)} \qquad (3)$$

3.1 Influence of pH

The pKa values of the catechins are not listed in the literature. The pKa values of the pyrocatechol and pyrogallol subunits are 9.37 and 9.28 respectively (Nelson, 1998) The presence of the electron-withdrawing aromatic rings will tend to decrease the pKa values further. It is safe to assume that the polyphenols have pKa values between 8 and 10. This causes them to be neutral to partially anionic in the tested pH range 6.6 to 8.0. A running buffer with 150 mM SDS and 25 mM sodium phosphate was used for the pH optimum study. The pH was adjusted by changing the ratio of mono- and disodium phosphate in the buffer. The EOF did not change significantly in this pH range. All changes in the separation of the analytes are due to the changes in their anionic charges. This caused changes in the distribution coefficient of the analytes between the bulk and micellar phases. The decrease in pH had the most significant effect on the capacity factors (k') of the more hydrophobic ECg and changed the k' of less hydrophobic C, EC, EGC and EGCg only slightly (Fig. 2.3). No significant changes in k' of caffeine, ascorbic acid, gallic acid, theanine and pNP were found in this pH range. This is because none of these analytes have pKa values in the tested range.

The peak shapes of EGC, EGCg, ECg were affected significantly by increase in pH. The polyphenols showed extensive peak tailing and distortion above pH 7.6 (results not shown). There can be several reasons for this behavior. This could be the result of

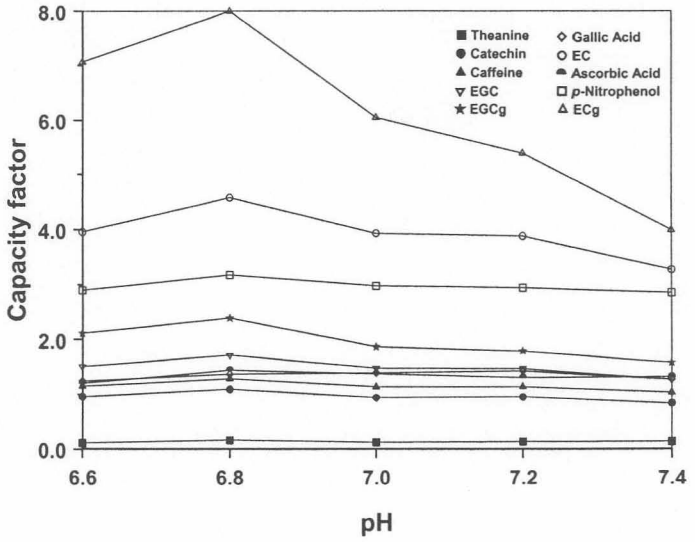


Figure 2.3 Influence of pH on the capacity factors of compounds. Conditions: 150 mM SDS, 25 mM phosphate buffer, 0% (v/v) methanol. Separation with 14 kV.

auto-oxidation of the catechins in an alkaline environment. Alternatively the polyphenols become more negatively charged, resulting in unwanted interactions with the column or the surfactant. Ascorbic acid showed an increased leading front with a decrease in pH. The peak shape of gallic acid, caffeine, theanine and pNP were unaffected by pH changes in this range. The optimum pH was chosen as pH 7.0 because peak shapes did not improve with further decrease in pH. Lower pH only increased the analysis time and therefore the capacity factors but not the resolution of the analytes. Generally capacity factors between 0.5 and 5 are required for good separation. With the running buffer conditions as mentioned above, only gallic acid and ascorbic acid co-migrates at pH 7.0 (Fig. 2.3).

3.2 Influence of SDS concentration

An increase in surfactant concentration, and thus micellar-phase volume, showed an increase in the k' of all analytes except for theanine, gallic acid and ascorbic acid. These compounds were not influenced significantly (Fig. 2.4). They had a higher affinity for the bulk phase than for the micellar phase and their ionic properties influenced their separation. The negatively charged ascorbic acid and gallic acid are the least soluble in the negatively charged SDS pseudo-stationary phase due to electro-

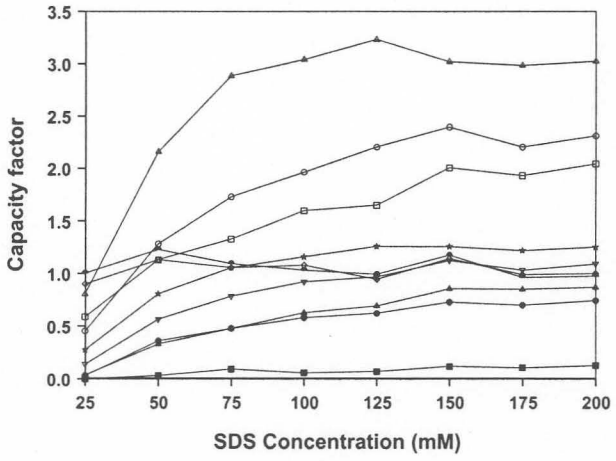


Figure 2.4 Influence of SDS concentration on the capacity factors of compounds. Symbols as in Fig. 2.3. Conditions: 25 mM phosphate pH 7.0, 0% (v/v) methanol. Separation with 14 kV.

static repulsion, yet their $k' \approx 1$. This is because these organic acid anions have a negative migration mobility against the EOF resulting in a longer migration time than t_0. Theanine, a zwitterion with an overall charge of zero, elutes very early with $k' \approx$ 0.1. This is probably because theanine is retained only slightly when the hydrocarbon side-chain and the α-amino group form weak interactions with the surfactant. The hydrophobicity of ECg caused its k' to increase drastically with increasing SDS concentration. The linear relation of k' vs. [SDS] for the catechins and caffeine (Fig. 2.5)

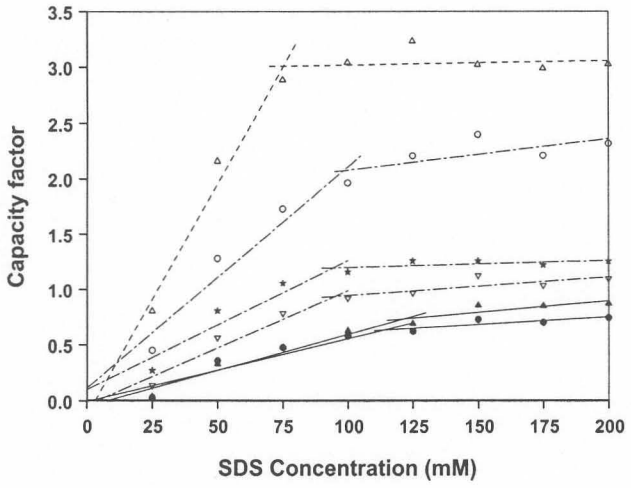


Figure 2.5 Linear dependence of analyte capacity factors on the surfactant concentration. The symbols represent the same analytes as in Fig. 2.3 and the conditions are the same as in Fig. 2.4.

is an indication that there are no significant charges on the catechins (Terabe, 1985). The least hydrophobic analytes (caffeine and catechin) display a first order linearity up to 125 mM, the more hydrophobic EGC, EGCg and EC display a first order linear dependence up to 100 mM and he most hydrophobic catechin, ECg, only up to 75 mM SDS. After the first order linear region follows a zero order linear region indicating that an equilibration or saturation point is reached where the catechins do not show an increased uptake into the micelles. All of them intercept in the vicinity of the origin, indicating their dependence on the surfactant concentration.

An increase in the SDS concentration extended the retention window (Fig. 2.6) and helped to resolve the different analytes. The migration window did not become larger with increase in SDS concentration above 150 mM. The concentration of 100 mM SDS provided good resolution of almost all the compounds (Fig. 2.4). Fig. 2.7 shows the effect of increased SDS concentration on the platenumbers for caffeine, EC and pNP. The platenumbers increase up to 100 mM SDS, as the effects of micelle poly-

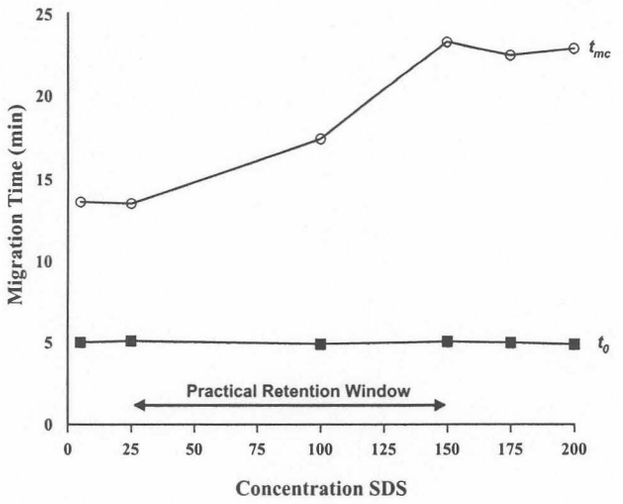


Figure 2.6 The effect of the surfactant concentration on the retention window required for separation of analytes. The increase in SDS concentration had no effect on the migration time of the EOF (t_0) (■). The migration time of the surfactant phase (t_{mc})(O) showed a first order linear relationship with increase of SDS from 5 mM-150 mM. There was a zero order linear relationship between the surfactant phase migration time and the SDS concentration between 150 mM and 200 mM.

dispersity are reduced. An increase in surfactant concentration reduces polydispersity. Polydispersity is the variation in aggregation number of the micelles, resulting in micellar heterogeneity. As the surfactant concentration increases, the intermicelle mass transfer and monomer/micelle exchange rates increase. This results in less variation in the micellar aggregation number and ensures more homogenous micellar structures. The maximum in platenumbers is achieved in the concentration range of 100-125 mM SDS. A further increase in surfactant concentration results in decreased platenumbers due to thermal band dispersion. The thermal band dispersion is a result of the high conductivity and excessive Joule heating.

Black tea contains a diverse group of catechin dimers (bisflavanols and theaflavins) and polymers (thearubigens). The rather high SDS concentration results in slower

migration times for these more hydrophobic multimeric forms than for the monomers. A clean and stable baseline is obtained for the region of importance, resulting in easier detection of the catechins. After taking all the data into consideration it was decided to use 100 mM SDS in the following steps of the univariate procedure.

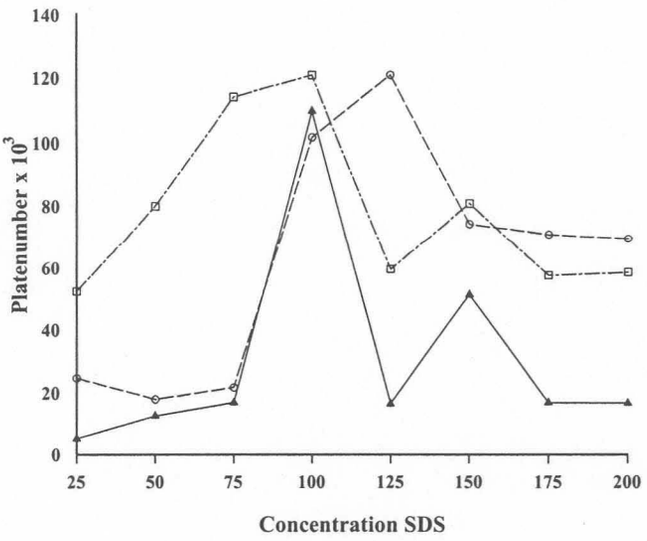


Figure 2.7 The effects of increased surfactant concentration on the column efficiency (N) as calculated for the caffeine (▲), EC (O), and pNP (□) peaks.

3.3 Influence of phosphate concentration

An increase in the concentration of the background electrolyte (BGE) resulted in a decrease in the EOF. This is consistent with results found by other researchers who found a linear relationship between the mobility of the EOF and the natural logarithm of the concentration anions in the running buffer (VanOrman, 1991). The *k'* decreased with an increase in the BGE concentration (Fig. 2.8). The increased ion concentration resulted in higher conductivity and Joule heating. The temperature of the running buffer could increase with excessive Joule heating. An increase in temperature can shift the phase-partitioning coefficients more to the aqueous phase with the hydro-

phobic compounds becoming more soluble in the water. This could decrease the capacity factors despite of a decrease in the EOF. Increased BGE concentration resulted

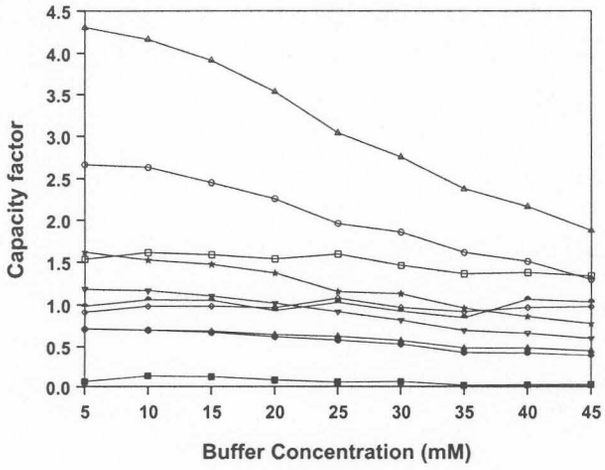


Figure 2.8 Influence of background electrolyte concentration on the capacity factors of compounds. Symbols as in Fig. 2.3. Conditions: 100 mM SDS, pH 7.0, 0% (v/v) methanol. Separation with 14 kV.

in a significant increase in the sharpness and resolution of the peaks. This is the result of stacking, which occurs when the buffer zone has a higher conductivity than the sample zone. The field strength is higher in the sample zone causing a concentration effect of the charged analytes (Chien, 1992). The phosphate and sodium ions in the running buffer bind to the silica column. When these ions shield the hydroxyl groups of the column it is called screening. By screening the column the zeta potential of the inner surface is reduced and thus also the EOF. At high ion concentrations this screening becomes less reproducible and fluctuation in the EOF and analyte migration times occur more frequently. The phosphate ions do not affect the micellar phase-bulk phase partitioning, thus having no contribution to the selectivity of the catechins. A concentration of 25 mM phosphate was considered as optimal. The peaks were sharp, the migration times were reproducible and the Joule heating was within the limits when separation was done at 14 kV.

3.4 Influence of organic modifier concentration

The effects that acetonitrile, methanol and 1-propanol have on the separation of the catechins were determined. The organic modifiers were tested in the range of 0-10% (v/v). Methanol was selected as organic modifier, since it increased the selectivity of the buffer, separating the analytes well. The effects of the methanol concentration on the capacity factors are shown in Fig. 2.9. An increase in methanol increased the t_0

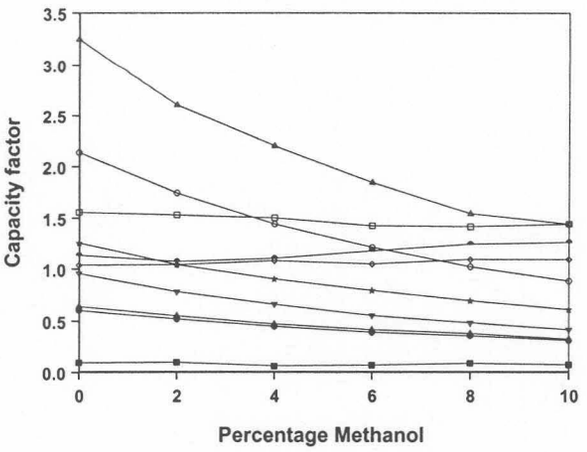


Figure 2.9 Influence of organic modifier on the capacity factors of compounds. Symbols as in Fig. 2.3. Conditions: 100 mM SDS, 25 mM phosphate, pH 7.0. Separation with 14 kV.

and t_{mc} values. A linear quantitative relationship between $1/k'$ and the concentration organic modifier was derived by (Chen, 1995 (a)). The linear relationship is only observed when no significant change occurs on micellar structure upon the addition of the organic modifier. When the organic modifier concentration is to high, deviations from the straight line is observed since the ionic conditions and the aggregation number of the micelles change. The relationship between $1/k'$ and percentage methanol was linear (Fig. 2.10A), indicating that there was no change in micellar structure upon addition of the organic modifier. The plot of mobility ratio vs. organic modifier (Fig. 2.10B) gives the same conclusion. The mobility ratio mainly characterizes the surface

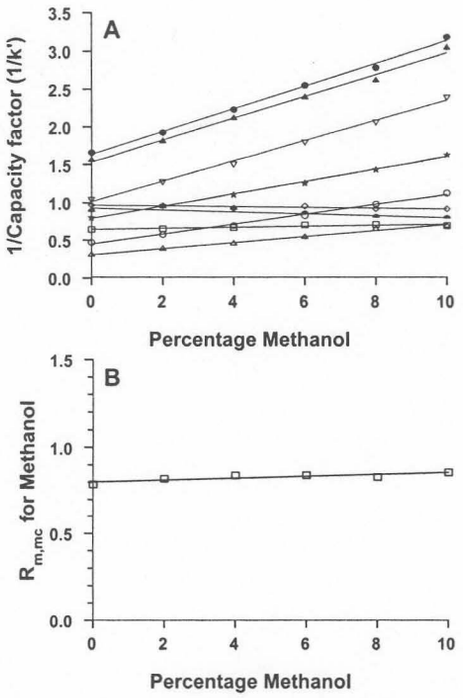


Figure 2.10 The linear quantitative relationships found for **A**:1/*k'* vs. methanol concentration and **B**: $R_{m,mc}$ vs. methanol concentration. This indicates that the methanol had no effects on the micellar structure or surface charges in the range tested. The symbols for Fig 2.10A is the same as for Fig 2.3 and the conditions are the same as in Fig. 2.9.

charge density of the micelles and is given by the equation $R_{m,mc} = 1 - {}^{t_o}/_{t_{mc}}$. It was found to be a good parameter in the investigation of the effect of organic modifier on the micelles. A linear relationship can generally be found between $R_{m,mc}$ and low concentrations of organic modifier where the CMC of the surfactant is not influenced (Chen, 1995 (b)). From this it can be concluded that all changes in *k'* could be contributed to changes in the bulk phase-surfactant phase distribution coefficients. The *k'* of the relatively more hydrophobic compounds (EC, EGCg and ECg) decreased most significantly with an increase in percentage methanol. This is an indication that the compounds become more soluble in the bulk phase because they are more soluble in methanol. The methanol does not alter the solubility of the ionic

analytes (theanine, gallic acid and ascorbic acid) in the bulk phase resulting in no changes in their *k'* values. A 6% (v/v) methanol concentration was considered optimal

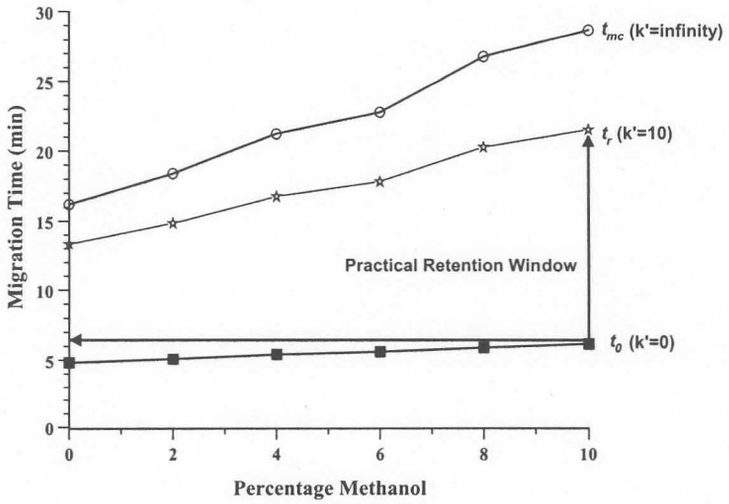


Figure 2.11 The effects of the methanol concentration on the retention window required for separating the analytes. The increase in methanol concentration increased the migration time EOF (■) slightly. The migration time of the surfactant phase (O) showed a first order linear relationship with increase of methanol from 0%-10% (v/v).

for the analysis of green and black tea samples because it provided the shortest analysis time with good resolution. Bottled tea samples required an optimal concentration of 5% (v/v) methanol to provide good resolution between ascorbic acid and EC. Methanol concentrations of 5 and 6% (v/v) provide a window of more than 15 minutes (between the EOF and t_{mc}) for all the 9 analytes to elute in (Fig. 2.11). Since the micellar phase is moving, compounds with a $k' = \infty$ will also move through the column. This results that analytes with *k'* between 10 and ∞ are crammed together in the last part of the electropherogram. From a practical point of view it is best if the analyte migrating last has a *k'* of no more than 10. This ensures that the analyte peaks are evenly spaced. When taking this into consideration the retention window is reduced to 12.20 min.

The tables of organic solvent properties (Snyder, 1978) show that the dipole strength decrease in the order acetonitrile > methanol > 1-propanol. From this it can be concluded that acetonitrile is the most polar and 1-propanol the least polar of the three solvents. The table for the solvent strengths indicate that 1-propanol is the strongest solvent, while methanol and acetonitrile are approximately equally strong. Fig. 2.12 shows the effects of the concentration 1-propanol on the migration times of the analytes. The 1-propanol dissolves the hydrophobic catechins (EC, ECg and EGCg) more readily in the aqueous phase resulting that their phase equilibrium shifts more to

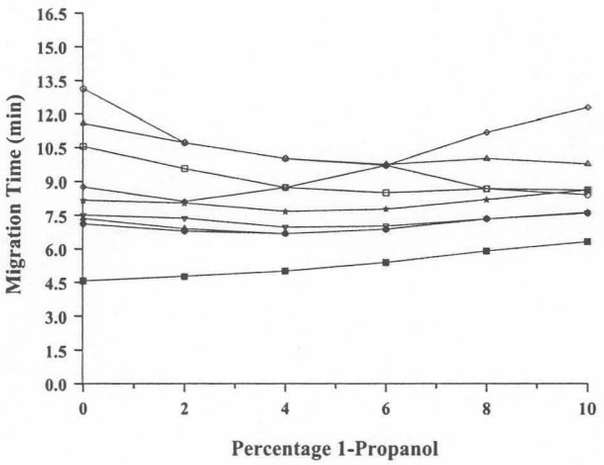


Figure 2.12 The influence of the 1-propanol concentration on the migration times of the analytes. Symbols as in Fig. 2.3. Conditions: 25 mM phosphate pH 7.2, 150 mM SDS. Separation with 14 kV.

the bulk (aqueous) phase. This explains why the more hydrophobic analytes (EC, ECg and EGCg) start to co-migrate. All conditions being the same, except for a pH of 7.2, 6% (v/v) acetonitrile did not provide the resolution obtained with 6% (v/v) methanol (Fig. 2.13). The acetonitrile is more polar than the methanol and does not effect as large a change in the phase equilibrium of EC, ECg and EGCg. The longer migration time of ECg with acetonitrile ($t_r \approx 15$ min) than with methanol ($t_r \approx 11$ min) is an indication of the effect the organic solvents have on the phase equilibrium.

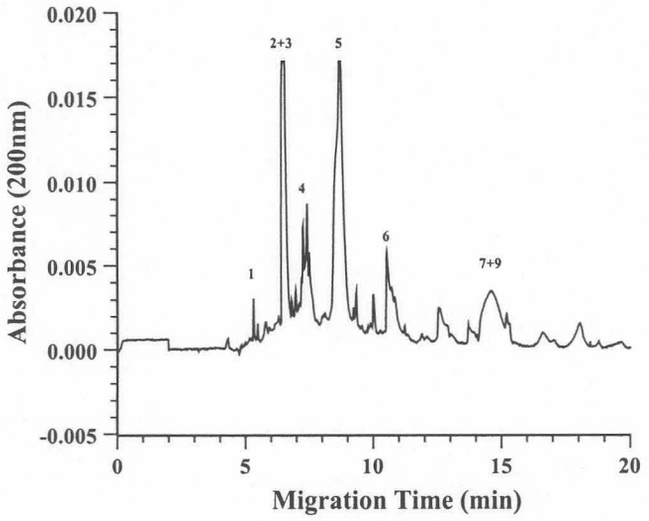


Figure 2.13 The analysis of a 2% (w/v) water extract of green tea with acetonitrile as organic modifier. Conditions: 25 mM phosphate pH 7.2, 100 mM SDS, 6% (v/v) acetonitrile. Separation 14 kV. The peaks are numbered the same as for the standards in Fig. 2.15A.

3.5 Influence of applied voltage

The applied voltage was varied from 10-20 kV. The influence of kV on *k'* is shown in Fig. 2.14. The *k'* of the zwitterion, theanine, and the anions, gallic and ascorbic acid, were effected very slightly with higher voltages. The *k'* of the caffeine, pNP and all the catechins decreased with higher voltage. The more hydrophobic the analyte is, the more extensive was the decrease in the *k'*. The increase in voltage increased the Joule heating. The increased Joule heating could increase the effective temperature in the column, thus reducing the viscosity of the buffer. This causes the micelles to move more rapidly through the column and could explain why the hydrophobic analytes were affected the most. The peaks became narrower with increasing kV, due to shorter migration times and less diffusion as can be expected. The increased Joule

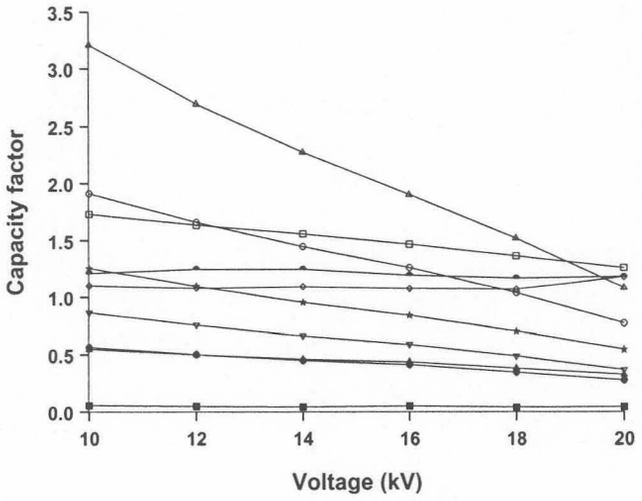


Figure 2.14 The influence of applied voltage on the capacity ratios of the analytes. Symbols as in Fig. 2.3. Conditions: 100 mM SDS, 25 mM phosphate, pH 7.0, methanol 5% (v/v).

heating results in practical problems, such as fluctuating migration times and peaks changing positions near the crossover points. The optimal voltage was selected as 14 kV because it was away from any cross-over points (near 12 and 17 kV) and the Joule heating was within the limits for the type and length of capillary that was used.

3.6 Suitability of internal standard

p-Nitrophenol (pNP) seems to have been a good selection as internal standard. The k' of pNP was not influenced significantly by changes in pH, phosphate, methanol and voltage conditions over the ranges investigated here. pNP never occurs in tea. It was used as internal standard to compensate for variation in injection volume. pNP was also used as a migration time reference compound for automated peak identification by the data system software. It migrates far from adjacent catechins, permitting a wide window definition.

3.7 Regression Analysis

The LOD's were determined and are shown in Table 2.2. The limits of quantification (LOQ) are taken as the concentrations that result in 5% or less relative standard deviation (RSD) upon quantification and meet the requirements for analysis of fresh leaf, green and black tea liquor as well as bottled ice teas. The upper limit of linearity for calibration curves were determined and calibration curve correlation coefficients (r) were better than 0.994. All results are shown in Table 2.2.

Table 2.2 Limits of detection and quantification values, linear limits and regression coefficients of all analytes.

Compound	LOD (µg/ml)	LOQ (µg/ml)	Linear Limit (µg/ml)	r
Theanine	4.0	10.0	600.0	0.995
Caffeine	2.0	5.0	75.0	0.998
Catechin	1.0	2.0	55.0	0.999
EGC	2.0	4.0	55.0	0.997
EGCg	6.0	11.0	110.0	0.994
Gallic Acid	2.5	6.0	120.0	0.998
EC	1.0	2.0	135.0	0.996
pNP	4.0	8.0	160.0	0.998
ECg	3.0	10.0	100.0	0.994
Ascorbic Acid	20.0	50.0	500.0	0.994

3.8 Validation of analytical procedure

Precision tests were done to determine both intra-day and inter-day variation in migration times. Calculations are based on the analysis of green tea samples. The gallic and ascorbic acid levels in green tea are below the LOD and therefore no data is available for gallic and ascorbic acids. Table 2.3 summarizes the statistical evaluation

of data from 32 consecutive runs for the intra-day precision test and data from 9 days with a minimum of 10 runs per day for the inter-day precision test. The compounds with the longest migration time showed the largest standard deviation (S.D.) and %RSD. From this one would expect gallic acid and ascorbic acid to have %RSD values higher than that of EGCg and less than that of EC.

Table 2.3 Statistical evaluation of intra-day and inter-day data to determine the variation in the migration times. (MT = migration times in minutes). The analytical conditions are the same as in Fig 2.15.

Compound	Intra-day MT n=32		Inter-day MT n=9	
	MT	%RSD	MT	%RSD
Theanine	6.29	2.10	6.33	4.70
Catechin	7.29	2.24	7.37	3.65
Caffeine	7.43	2.15	7.49	3.28
EGC	7.84	2.38	8.00	3.33
EGCg	8.67	2.52	8.79	3.81
Gallic Acid	-	-	-	-
Ascorbic Acid	-	-	-	-
EC	9.84	2.67	10.03	3.91
pNP	10.74	2.47	10.84	3.72
ECg	11.39	3.02	11.59	4.22

Repetitive runs were done with green, black and bottled teas to determine the repro-ducibility of both the extraction and analysis of the samples. The same green or black tea was extracted five separate times and each infusion was analyzed in five-fold. An electropherogram of the standards is shown in Fig. 2.15A while green and black tea profiles are shown in Fig. 2.15B and 2.15C respectively. Five spiked solutions were

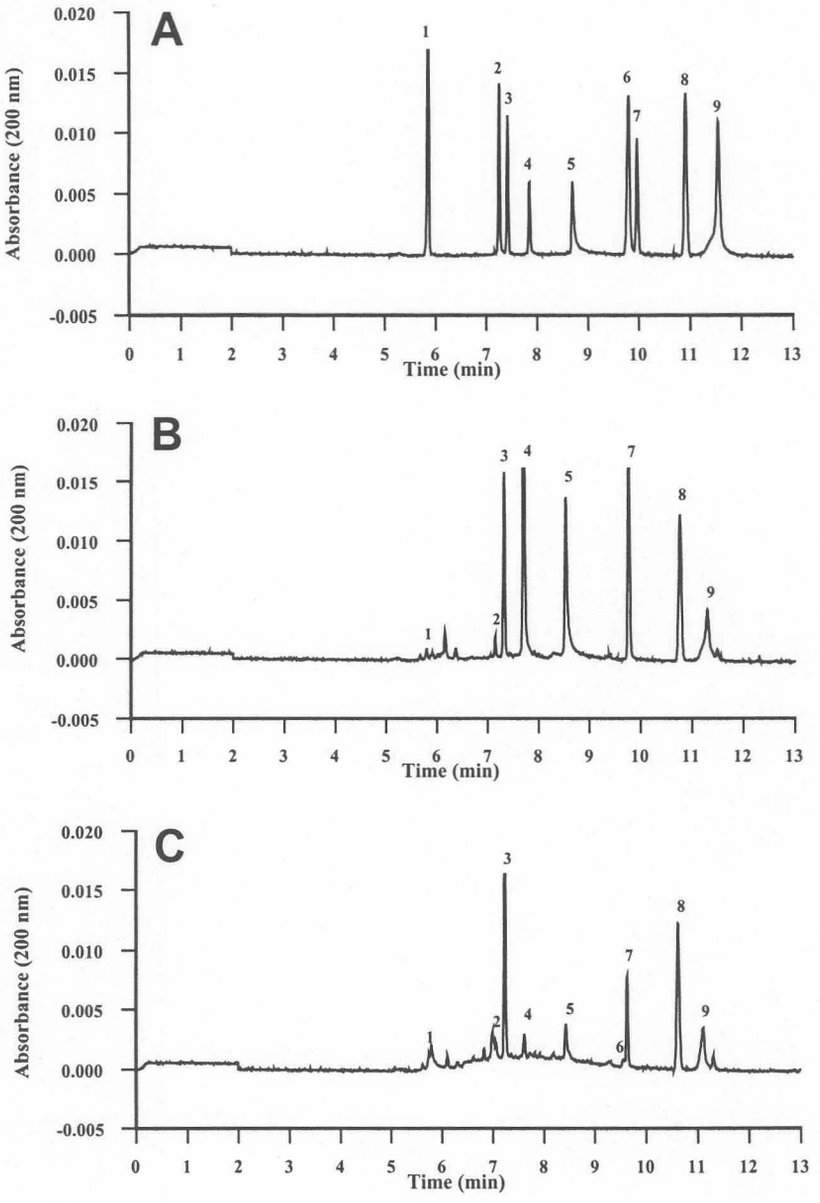


Figure 2.15 **A**: Separation of standard mix. The peaks are: 1 theanine, 2 catechin, 3 caffeine, 4 EGC, 5 EGCg, 6 gallic acid, 7 EC, 8 pNP, 9 ECg. **B**: Separation of fresh leaf sample 1% (w/v) solution 5 times diluted. **C**: Separation of black tea sample 1% (w/v) solution 3 times diluted. Conditions: 100 mM SDS, 25 mM phosphate, 6% (v/v) methanol pH 7.0. Separation was done with 14 kV. Detection at 200 nm.

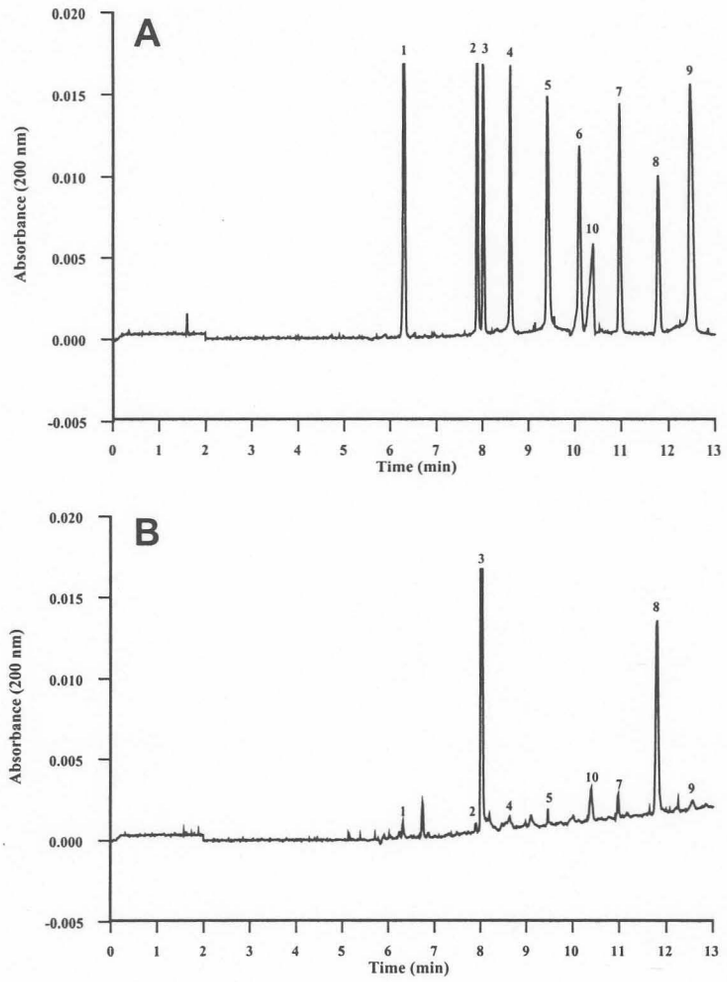


Figure 2.16 **A**: Separation of standard mix. All peaks are the same as in Fig. 2.15A and peak 10 is ascorbic acid. **B**: Separation of undiluted canned tea sample. Conditions: 100 mM SDS, 25 mM phosphate, pH 7.0, 5% (v/v) methanol. Separation was done with 14 kV. Detection at 200 nm.

prepared from one sample of bottled tea and analyzed in five-fold. The results are shown in Table 2.4. Typical electropherograms of the standards (including ascorbic acid) and a sample of bottled black iced tea are shown in Fig. 2.16A and 2.16B respectively.

Table 2.4 Typical concentrations of analytes found in dried fresh leaf (green tea), black tea (mg/g dry weight) and bottled tea (μg/ml). A typical cup of tea (black or green) is brewed as a 1% (w/v) solution.

Compound	Dried Fresh Tea Leaf		Black Tea		Bottled Black Tea	
	mg/g	%RSD	mg/g	%RSD	μg/mL	%RSD
Theanine	11.15	5.45	7.27	4.42	< LOQ	-
Caffeine	37.93	1.18	20.59	3.80	100.33	4.07
Ascorbic Acid	< LOD	-	< LOD	-	224.98	4.60
Gallic Acid	< LOQ	-	2.29	2.33	< LOD	-
Catechin	0.87	3.71	1.21	4.92	< LOQ	-
EGC	25.68	1.75	1.14	2.46	< LOQ	-
EGCg	78.77	2.85	10.62	4.56	< LOQ	-
EC	6.73	1.60	2.17	3.31	2.51	4.96
ECg	15.18	2.87	7.51	1.40	< LOQ	-
Total Catechins	127.23	-	22.65	-	2.51	-

The dried fresh leaves (green tea) have about five fold more free catechins than the black tea. When the green tea leaves are fermented to form black tea the catechin monomers polymerize to form theaflavins and thearubigens leaving less free catechins in black tea. The catechin content of bottled black tea is between 5 and 10 times less than that of black tea 1% (w/v) liquor. This is based on the content of EC that has the lowest level of detection and thus easily quantified in the bottled black tea. The caffeine content is about half of that found in 1% (w/v) black tea. In the analyzed commercial brand of bottled tea most catechins are below their LOQ's. If we assume that no caffeine was added, these results indicate that the catechins are less stable than the caffeine during the bottling process. Previously it was shown that tea catechins undergo epimerization during heat sterilization (Seto, 1997), (Komatsu, 1991). The extra peaks in the iced tea electropherogram (Fig. 2.16B) could be epimers such as (-)-GCg which may have formed from (-)-EGCg. At pH higher than 6 the catechins dis-

appear due to oxidation and polymerization (browning) (Komatsu, 1991). These polymers may cause the upward baseline drift found in black iced tea samples (Fig. 2.16B).

For green tea analysis a 0.1% (w/v) infusion is sufficient to analyze all the compounds except for theanine and catechin which is below the LOQ. To quantify theanine and catechin, a 0.5% (w/v) infusion is required. At this concentration caffeine, EGCg and EGC exceed the upper limits of linearity, necessitating two electrophoretic runs for quantification of all the compounds. All the compounds in black tea can be quantified from a 0.5% (w/v) infusion in a single run.

For MEKC, with the pseudo-stationary phase, the resolution (R_s) can be expressed as in equation 5.

$$R_s = \frac{\sqrt{N}}{4} \bullet \frac{\alpha - 1}{\alpha} \bullet f(k') \qquad (5)$$

The function $f(k')$ is defined as shown in equation 6. It is logical that the resolution will be the best when $f(k')$ reaches a maximum. The optimum k' is defined as the k' where $f(k')$ is at its maximum The optimum k' for a given set of conditions is calculated according to the migration times for the t_0 and the t_{mc}. Equation 5 (Terabe, 1985) and equation 6 (Wätzig, 1998) can be used for the calculation of the optimum k' for our conditions.

$$f(k') = \left(\frac{k'}{1+k'}\right)\left(\frac{1 - t_0 / t_{mc}}{1 + (t_o / t_{mc})k'}\right) \qquad (6)$$

The dependence of the function $f(k')$ on k' is shown in Figure 2.17 where t_0=5.86 min and t_{mc}=36.81 min. The maximum of $f(k')$ is normally attained at intermediate k' values. Since $f(k')$ is determined by t_0 and t_{mc}, the optimal k' (k'_{opt}) can be defined as

in equation 7. The optimum k' is an indication of the k' region where the peaks should elute to obtain the maximum possible resolution.

$$k'_{opt} = \sqrt{\frac{t_{mc}}{t_0}} \qquad (7)$$

The optimum k' under our optimum conditions is calculated as 2.5 and all the capacity factors of the optimum conditions range between 0.5 and 2.5 except for theanine with a k' of 0.2.

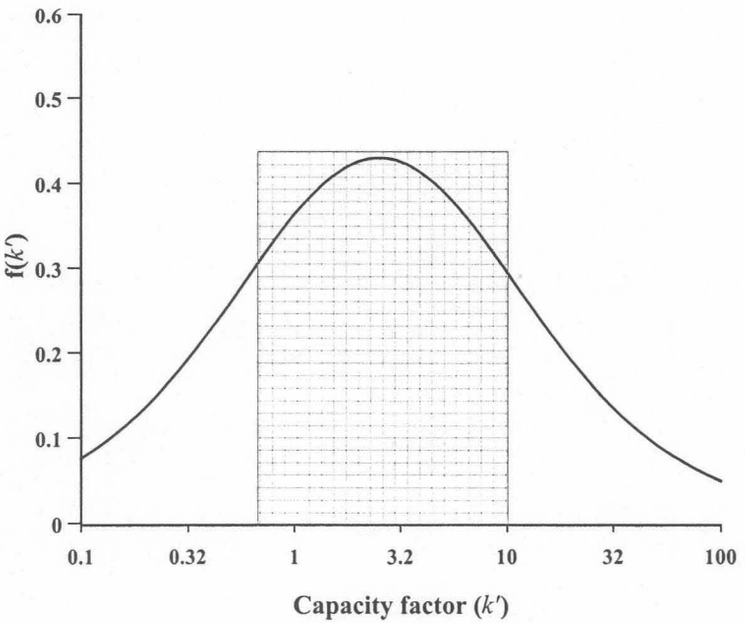


Figure 2.17 The determination of the optimum capacity factor. The region where good separation should be achieved under our conditions is shown with the grid. All the analytes with a k' of 0.5 to 10 can theoretically be separated with a good resolution.

3.9 Optimum condition

We have found that 100 mM SDS, 25 mM phosphate, 6% (v/v) methanol at pH 7.0 provided adequate resolution, consistent migration times and speedy analysis for all tea samples. The best resolution was found at 14 kV. The optimum conditions for

analysis of bottled ice tea samples are 100 mM SDS, 25 mM phosphate, 5% (v/v) methanol at pH 7.0, 14 kV applied voltage on a 50 cm effective length uncoated fused silica capillary column.

4 Conclusion

A new MEKC method has been developed for analyzing tea samples. The method was used for analysis of the catechins, gallic acid, caffeine and theanine content in fresh leaf extracts as well as green and black tea liquors. A slight modification to this method was used to analyze bottled ice tea fortified with ascorbic acid.

The five most abundant tea catechins, theanine, ascorbic acid, caffeine and gallic acid have been separated and quantified with one analysis of approximately 13 min. A few small unidentified peaks have been observed in the green and black tea samples. It is possible that other minor catechins form these unidentified peaks. The equilibration time is 6.5 min extending the time between injections to under 20 minutes. The effect of the chromatographic variables on the resolution and capacity factors presented here may enable other workers to select conditions suitable for their applications. This MEKC method is convenient and precise. Thus it can be used for routine analysis of tea liquors and bottled teas. The separation order is similar to that reported for other MEKC (Horie, 1997), (Horie, 1998 (b)), (Larger, 1998) and reverse phase HPLC methods (Goto, 1996) where the gallated catechins elute later than their ungallated counterparts. This method may possibly be improved in the future to detect other organoleptically important compounds such as aspartic and glutamic acid in tea and dehydro-ascorbic acid in bottled tea. This method was published recently (Aucamp, 2000).

Chapter 3

In vitro and *in silico* inhibition studies on Xanthine Oxidase with tea polyphenols

1 Introduction

Molybdenum containing enzymes can be grouped into two classes, namely nitrogenases and hydroxylases (oxo-transferases). This classification is based on the cofactor metal constituents as well as on other cofactors that are required in the electron acceptor domain (Romão, 1998). Nitrogenases catalyze the reduction of molecular nitrogen to ammonia. The electron acceptor domain contains a hetero-metal cofactor (Fe-Mo) and no pterin cofactor. All the molybdenum hydroxylase enzymes possess only Mo as metal ion and a pterin cofactor that consists of a tetrahydropterin core with a dithiolene sidechain and a phosphate group. This pterin is called a molybde-

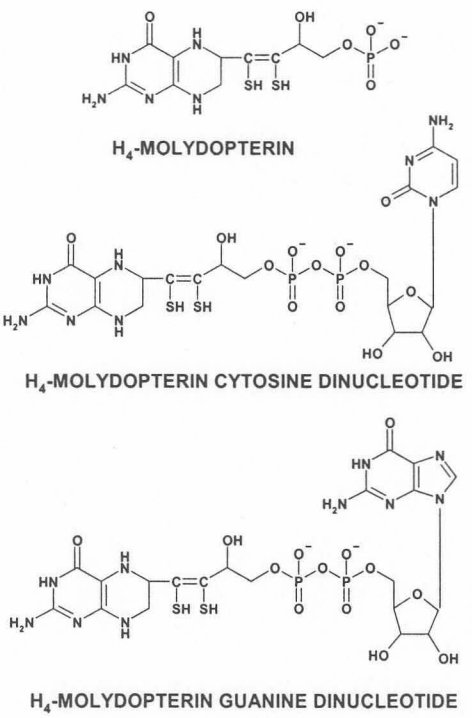


Figure 3.1 Structures of the variant forms of molydopterin. The structures are shown in the tetrahydro state, but they may also occur in the dihydro state *in vivo*.

num cofactor (Moco) and different variations on the cofactor structure do exist. The variant with a guanine linked to the phosphate of the Moco occurs mainly in eukaryotes, while the variant with a cytosine occurs mainly in prokaryotes (Fig. 3.1) (Rajagopalan, 1991). XO isolated from bovine milk has the monophosphate Moco variant.

The molybdenum hydroxylases promotes a variety of two-electron oxidation-reduction reactions whereby the oxygen atom from H_2O is transferred to a substrate at the electron acceptor domain. The remaining reducing equivalents obtained from the H_2O molecule are transferred to an electron acceptor at the electron donating domain (Fig. 3.2). These enzymes are classified into three families on the basis of the reactions they catalyze, as well as the basis of the characteristics of the molybdenum centers. They are (1) the xanthine oxidase family, (2) the sulfite oxidase and nitrate reductase family and (3) the DMSO reductase family. This classification is supported by the amino acid homologies within the protein families (Romão, 1998).

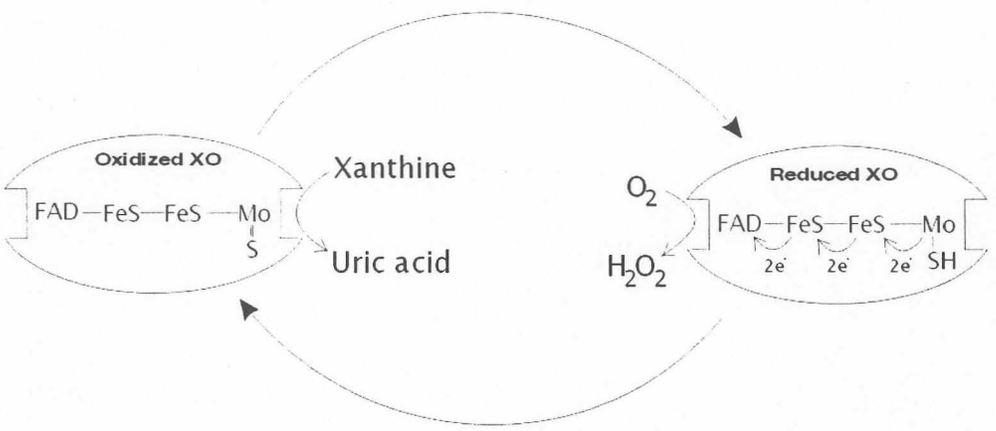


Figure 3.2 A schematic presentation of the ping-pong catalytic action of xanthine oxidase.

The large xanthine oxidase family of enzymes may be considered one of the true hydroxylases. In the electron acceptor domain the Mo is coordinated to an oxygen

(oxo), a sulfur (sulfido) and a water molecule as well as to the substructure dithiolene via the two sulfur atoms. In the complex the $MoOS(H_2O)$ is coordinated in the *fac* orientation. The two sulfurs of the dithiolene forms the equatorial plane of the penta-coordinated square pyramidal complex (Fig. 3.3) and the sulfido forms the apical ligand of the complex. Members of this family have been found broadly distributed within eukaryotes, prokaryotes and archaea. They catalyze the oxidative hydroxy-lation of aldehydes and aromatic hetrocycles in reactions involving C-H bond cleavage.

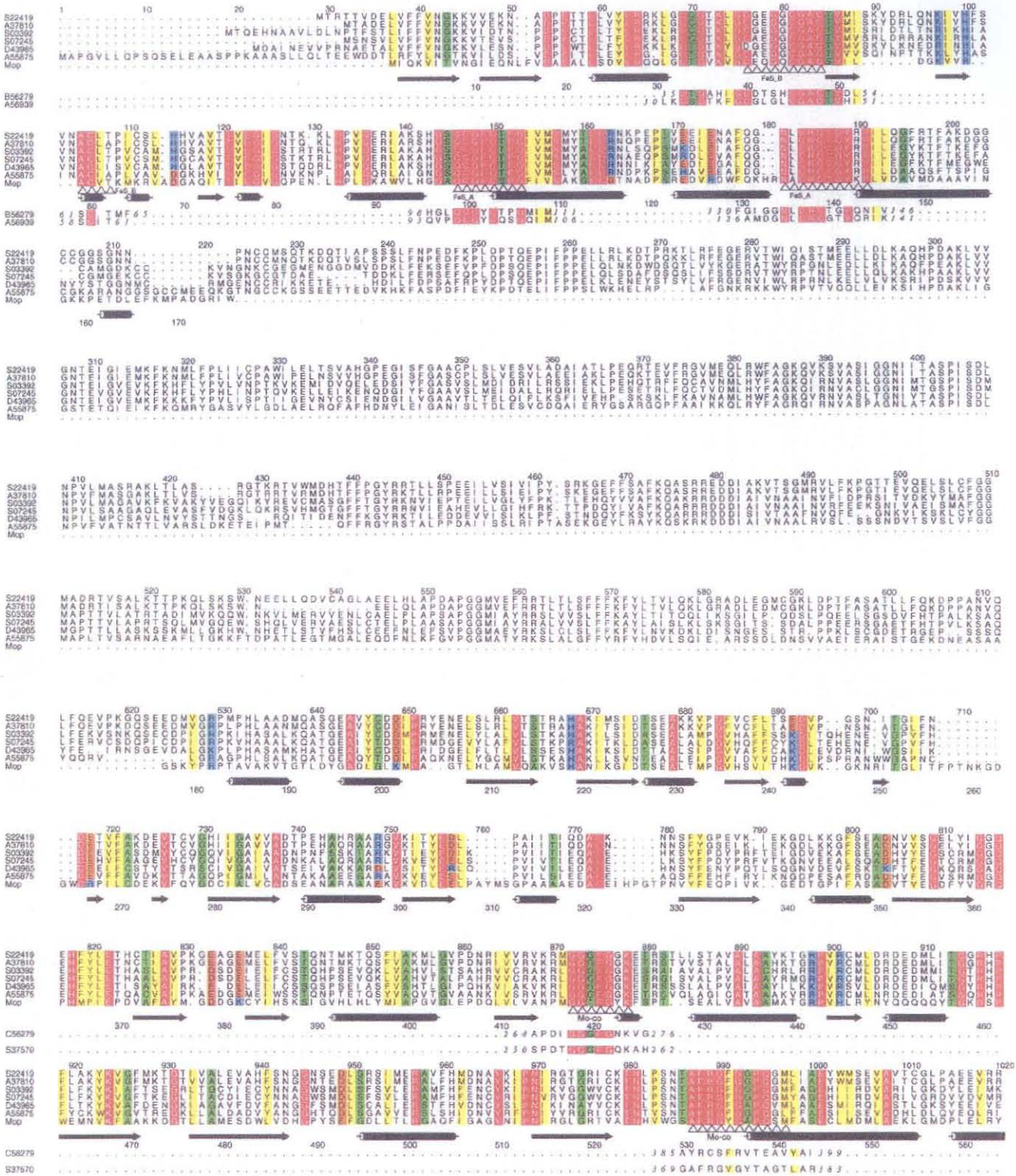
oxidized enzyme (Mo^{VI}) reduced enzyme (Mo^{IV})

Figure 3.3 The structure of the molybdenum coordination complex. The Mo^{VI} is penta-coordinated in the square pyramidal geometry. The equatorial plane is defined by the two dithiolene sulfurs, oxido and water ligands. The sulfido is in the apical position.

XO is named so because xanthine was thought to be its physiological substrate. Later it was found that XO has a low substrate specificity and it oxidizes many aromatic heterocycles, aldehydes and alcohols. XO has a molecular mass of approximately 300 kDa, and is a homodimer. Besides a molybdenum center and Moco it also possesses two iron-sulfur centers of the 2Fe/2S variety and a flavin adenine dinucleotide (FAD) per subunit (Hille, 1994). Physiologically the enzyme uses NAD^+ as electron acceptor and the enzyme is known as xanthine dehydrogenase (XDH). Under stress conditions it is converted to XO and uses O_2 as electron acceptor. The difference between XDH

and XO is with the electron donator domain and not the xanthine binding electron acceptor domain.

The aldehyde oxido-reductase (AOR) from *Desulfovibro gigas* is the first representative of the xanthine oxidase type enzymes for which the three-dimensional structure is available (Romão, 1995). Although there are differences between XO and AOR, a wealth of information can be extrapolated from AOR about the structure and enzyme reactions of XO. Multiple alignment with the family of XO's from mammalian, insect and fungal sources showed that the AOR amino acid sequence is highly conserved with *ca.* 52% homology and 25% identity, suggesting a close structural relationship (Fig. 3.4) (Romão, 1998). The homology is particularly high in those segments associated with binding of the redox cofactors as well as within residues of the substrate-binding pocket, and for the residues of the substrate tunnel. The segment binding to the cytosine dinucleotide substructure is not conserved because XO, isolated form bovine milk, has the monophosphate variant of the Moco. In Fig. 3.4 it can be seen that the binding segments of the cofactors are well conserved with a high proportion of invariant residues and secondary structures. Interruptions by deletions and insertions occur only in loop regions and at the N- and C-termini. The long deletion of about 400 residues in the AOR structure between residues 176 and 177, corresponds to the FAD (electron donating) domain in the xanthine dehydrogenases and is absent in AOR. This additional domain must be placed, in the XO family of enzymes, somewhere along the extended connecting segment (white part in Fig. 3.5). The FAD binding domain has been tentatively assigned on the basis of mutant studies. A mutation that has received considerable attention, was the Y395F xanthine dehydrogenase from *Drosophila melanogaster*, which has shown to be enzymatically inactive.


S22419
A37810
S03392
S07245
D43965
A55875
Mop
B56279
A56939
C56279
S37570
C58279
S37570

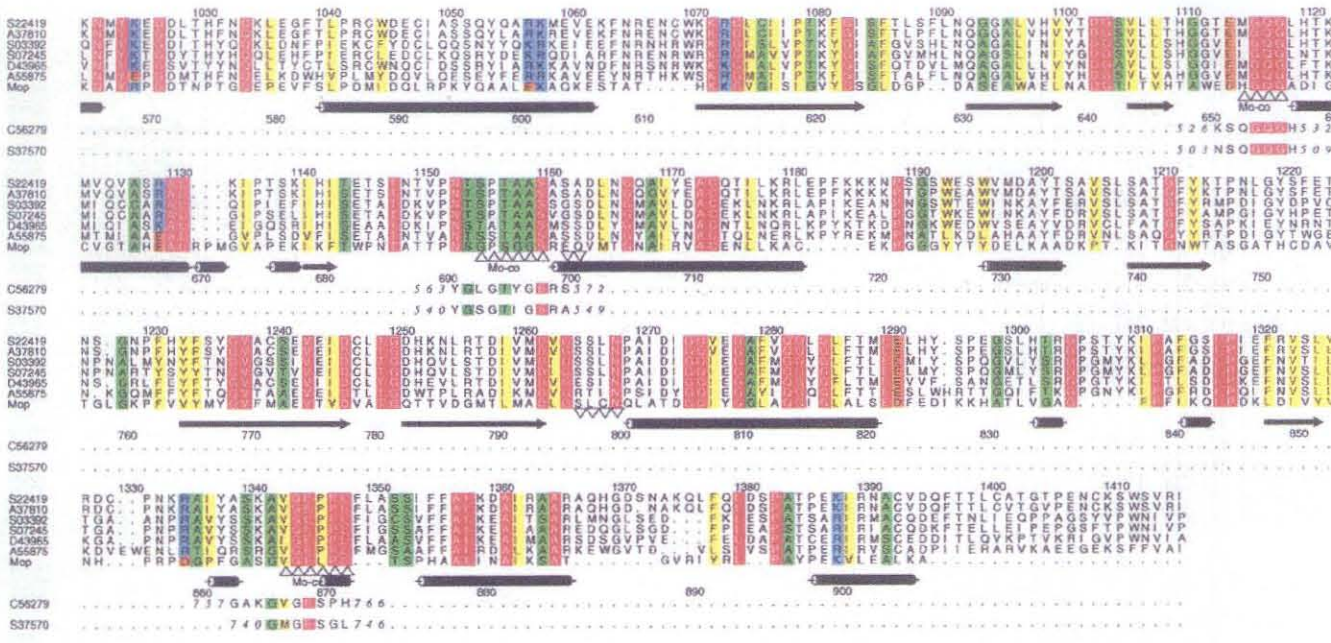


Figure 3.4 Amino acid sequence alignment of Mop (Aldehyde Oxido-reductase) with Xanthine Dehydrogenases. The sequences were taken from the PIR data bank. S22419: mouse xanthine dehydrogenase; A37810: rat xanthine dehydrogenase; SO3392: *Calliphora vicinia* xanthine dehydrogenase; SO7245: *Drosophila melangoster* xanthine dehydrogenase; D43965: *Bombyx mori* xanthine dehydrogenase; A55875: *Ernericillia nidulans* xanthine dehydrogenase. Also included are the partial sequences around the cofactor binding sites of carbon monoxide dehydrogenase from *Pseudomonas carboxydovorans*, B56279, C56279, isoquinoline-1-oxidoreductase from *Pseudomonas diminuta*, A56939 and nicotine dehydrogenase from *Arthrobacter nicotinovorans*, S37570. Alignments were performed with the program PILEUP and ALSCRIPT within The GCG package. Invariant residues are colored red, conserved hydrophobic residues yellow, conserved small neutral residues green, and conserved charged residues brown (negatively charged) and blue (positively charged). The secondary structural elements of β-strands (⟨) and helices (■) and the segments contacting the iron-sulfur clusters and the molydopterin (ΔΔΔΔΔΔΔ), and the cytosine (∇∇∇∇∇∇) are defined as in (Romão, 1995) and marked as shown. (Adapted from (Romão, 1998))

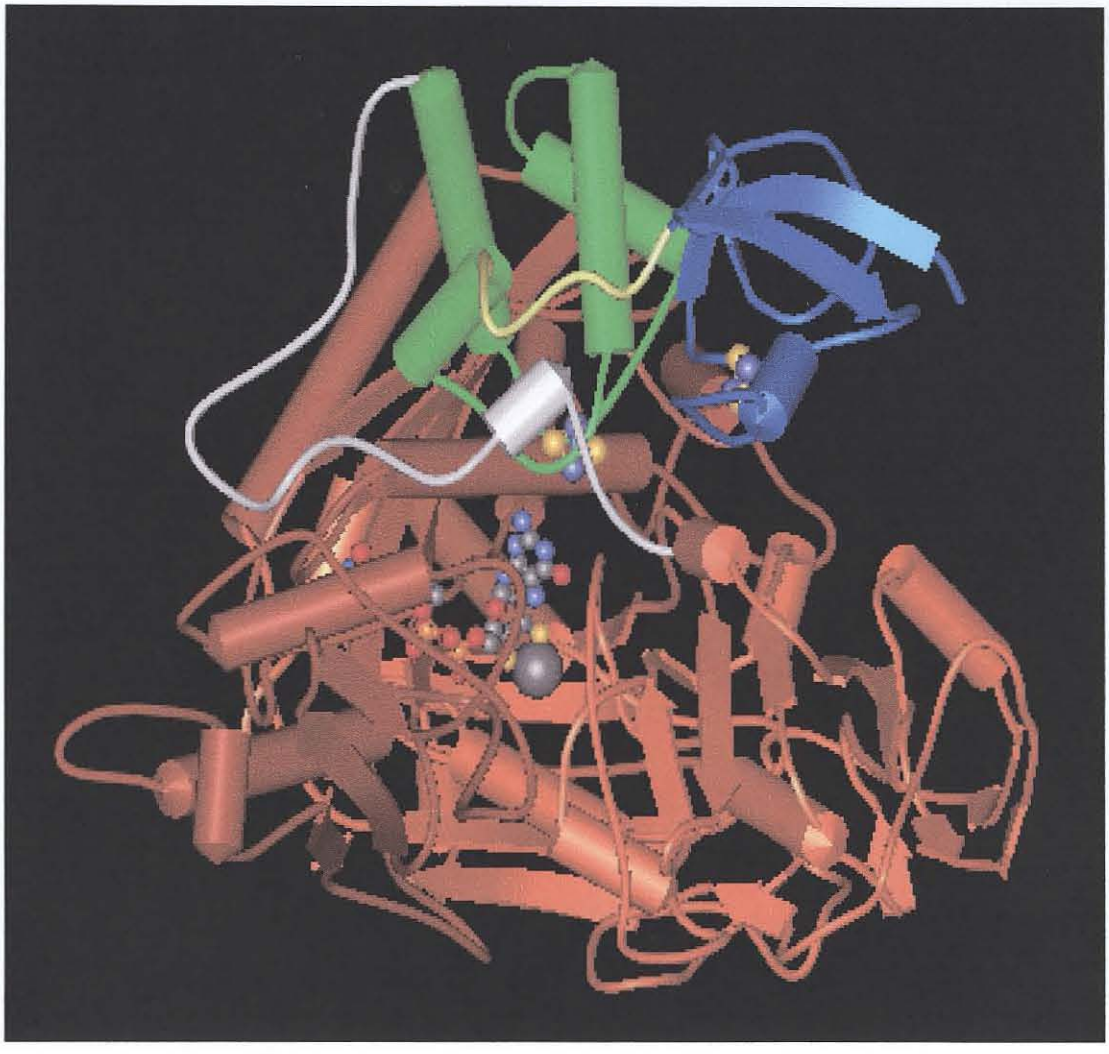


Figure 3.5 Molecular structure of aldehyde oxido-reductase and ligands. The helical and β-sheet secondary structures are drawn as rods and arrows with WebLab ViewerLite. The sulfurs of the cofactors are yellow, and the metal atoms are silver. The substrate binding tunnel is in the middle lower third of the molecule. The view is onto the molybdenum from the approaching substrate. The blue and green domains are the first [2Fe-2S] and second [2Fe-2S] domains respectively. The white domain is the connecting domain where the FAD site of XO and XDH is presumably inserted. The light brown and dark brown domains are the Mo1 and Mo2 domains respectively. The molydopterin cofactor is bound in between the Mo1 and Mo2 domains, seen as a ball and stick model.

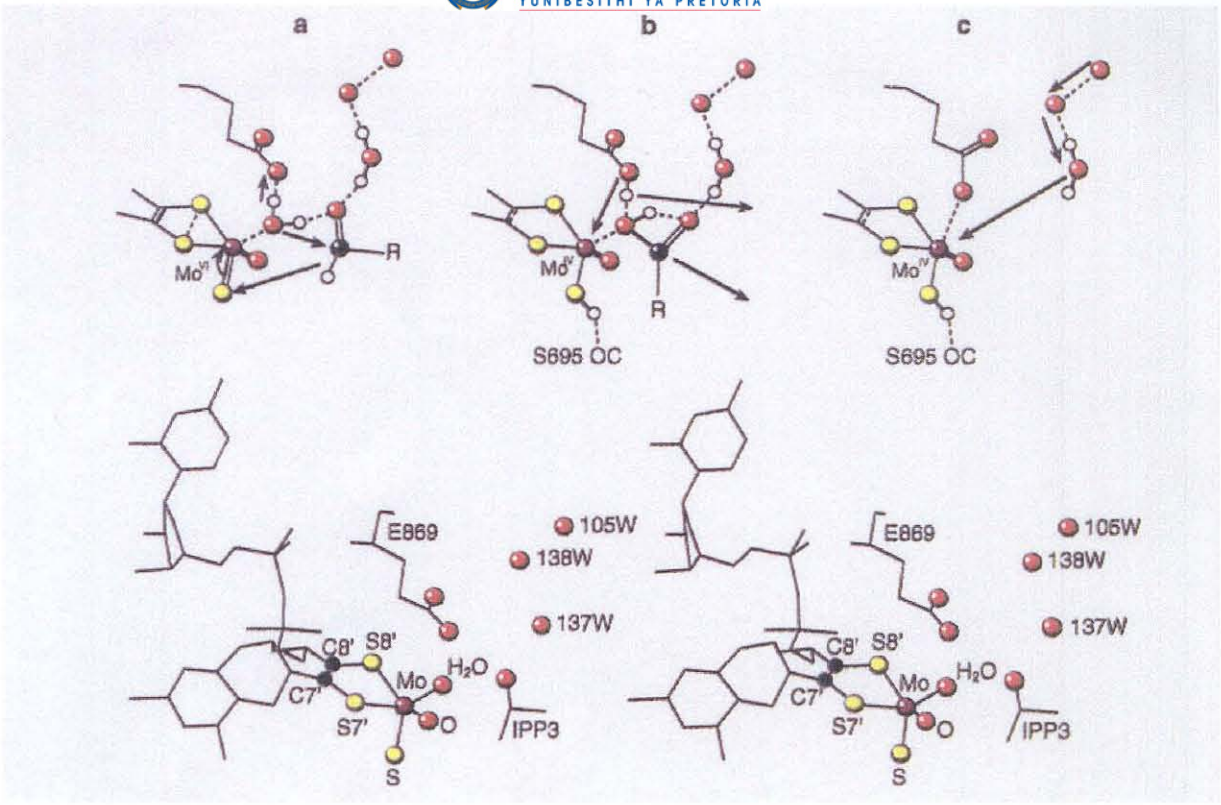


Figure 3.6 The reductive half-cycle of the hydroxylation reaction of AOR and XO.

The close structural relationship and ability of XO to utilize a wide range of aldehydes implies a common mechanism of action for AOR and XO. Furthermore, studies of the structural and spectroscopic properties of the metal cofactors of both enzymes suggest a close similarity. Of particularly significance are the similar electron paramagnetic resonance (EPR) properties of the molybdenum centers of both enzymes. This justifies the interpretation of results obtained from XO on the basis of the structural data for the AOR enzyme.

The reaction of XO follows a typical ping-pong bi-bi mechanism. The ping-pong mechanism for the steady-state formation of uric acid from xanthine can be presented as two independent half-reactions, shown in equations 1 and 2 (Hille, 1994).

reductive half-reaction

$$(XO)_{ox} + Xan + H_2O \rightleftharpoons (XO)_{ox}\bullet Xan \longrightarrow (XO)_{red} + urate \quad (1)$$

oxidative half-reaction

$$(XO)_{red} + O_2 \rightleftharpoons (XO)_{red}\bullet O_2 \longrightarrow (XO)_{ox} + H_2O_2 \text{ (or } O_2^-) \quad (2)$$

Although the mechanism for the reductive half cycle is not entirely certain, the structural data obtained from the three-dimensional structure and spectroscopic data of AOR and XO suggests a mechanism as outlined in Fig. 3.6. The Phe425 of AOR has been exchanged with the homologous Glu in XO (not shown in Fig.3.6). The glutamate is well positioned to bind to the N3, N9 imidium substructures of purines. Water 137 is suggested to be the specific recognition site for N7. The Glu869 serves as proton abstracting base, taking a proton from the water ligand bound to Mo. This provides a metal activated OH- that launches a nucleophillic attack on the C8 of a purine. The C8 of the purine is co-planar with the sulfido group, Mo and the coordinated H_2O such that the educt may be viewed as an open six-membered ring orientated perpendicular to the Mo=O bond. In the next step a hydride from C8 is transferred to the sulfido group, reducing the Mo^{VI} to Mo^{IV} and protonating the sulfido to a sulfhydryl group. The metal bound H_2O is transferred to the C8 upon which Glu869 abstracts the proton leaving a hydroxylated C8. The release of the product from the molybdenum may be facilitated by transient binding of Glu869 to the metal to maintain the penta-coordination. The molybdenum water coordination site may then be refilled from the chain of internal water molecules. At the end of the reductive cycle the Mo^{VI} is reduced to Mo^{IV}.

During the oxidative cycle the reducing equivalents obtained by the Mo-center are transferred to dioxygen at the flavin to form either peroxide or superoxide. These electrons are transferred via the Moco and iron-sulfur centers to the FAD site where it is transferred to molecular oxygen. The reducing equivalents are transferred through the partially conjugated system of the pterin and a hydrogen bond to the first Fe/S center. Electron transfer then proceeds via several covalent bonds and one hydrogen bond towards the second Fe/S center. From here the electrons will flow to FAD as an electron acceptor probably in a similar manner through covalent bonds and conjugated systems. The electron transfers are made possible by redox potential differences and reorganization energies. At the end of the oxidative cycle the Mo^{IV} is oxidized back to Mo^{VI}.

Allopurinol, a structural analogue of xanthine is the best known inhibitor of XO. Allopurinol is converted to oxypurinol in the catalytic center of XO. Both compounds compete with the physiological substrates for the active center of the enzyme. Once oxypurinol has formed a complex with the enzyme, it dissociates very slowly from the active center of the enzyme. Oxypurinol is an efficient tight binding inhibitor of XO and exerts non-competitive type inhibition (Massey, 1970).

The majority of XO inhibitors are heterocyclic aromatic compounds and aldehydes or alcohols that mimic purine and aldehyde substrates of XO. Most of the inhibitors exert non-competitive or mixed type inhibition. Uric acid and 8-bromoxanthine are two compounds that show uncompetitive type inhibition (Hille, 1984), (Radi, 1992). Natural plant products such as polyphenols and coumarins have also been shown to

inhibit XO (Chang, 1995 (c)). Although the types of inhibition for these inhibitors are known, the exact mode of action has not been investigated.

2 Materials and Method

2.1 Materials

XO Grade III from buttermilk (1.2 units/mg), quercetin, kaempferol and allopurinol were purchased from Sigma Chemical Company (St Louis, MO, USA). (+)-C, (-)-EC, (-)-EGC, (-)-ECg and (-)-EGCg were gifts from Mutsui Norin (Tokyo, Japan). All buffer salts and solvents needed for assay or running buffers were of analytical grade.

2.2 Xanthine Oxidase assay

All assays were done aerobically at 20°C. The enzyme activity was measured spectrophotometrically by determining uric acid formation at 295 nm with xanthine as substrate (Kalckar, 1947). The XO assay consisted of 200 µl reaction mixture containing phosphate buffer pH 7.4, 0.05 M; 0.004 U/ml XO and xanthine substrate. The assays that were conducted without inhibitors, were started by addition of the enzyme to the reaction mixture. The reference cuvette was identical and only enzyme was absent. The assay mixture was incubated for 3 minutes and absorbency readings were taken every 20 seconds.

The inhibitor concentrations were selected by assaying at a substrate concentration equal to Km (2.5 µM) (White, 1981). Three inhibitor concentrations were chosen by pre-trails to give approximately 50%, more than 30% and less than 70% inhibition at a substrate concentration equal to Km. All inhibitors were pre-incubated with enzyme for 10 minutes. The reaction was started by addition of the substrate. A substrate

range of 1.25 μM (0.5 Km) to 10 μM (4 Km) was used to avoid substrate inhibition, which was evident above 25 μM xanthine in our assay (Aucamp, 1997). EDTA was added to the assay mixture (33.3 μM for EGC, ECg and EGCg, since color changes were noticed on standing at room temperature or on ice in the dark.

2.3 Molecular modeling of XO inhibitors

Various inhibitors of XO were taken from the literature and modeled in the CORINA software package (http://www2.ccc.uni-erlangen.de/software/corina/free_struct). CORINA generates a low energy conformation structure for each inhibitor or substrate. The 3D structures of the inhibitors were compared in pursuit of identifying a structure-activity relationship and to evaluate the possibilities of designing other XO inhibitors.

3 Results and Discussion

3.1 Inhibition of XO with tea polyphenols

The compounds tested are shown in Table 3.1 together with their K_i, K_I and apparent K_i values and types of inhibition displayed. The Lineweaver-Burk method was used to confirm the K_m of xanthine, the type of inhibition and apparent K_i value of each inhibitor. The K_m value for xanthine was found to be 1.25 μM, (Fig. 3.7). For mixed type inhibition the secondary plots of $1/V_{max}$ vs [I] and slope vs [I] were used to determine K_i and K_I respectively. The secondary plots of the mixed type inhibitor (-)-EC are shown in Fig. 3.8. Linear secondary plots indicate the formation of dead-end complexes and parabolic non-linear plots indicate that more than one inhibitor molecule binds per enzyme. Non-linear secondary plots were only observed for quercetin and kaempferol. The secondary plots of the mixed type inhibitor kaempferol are shown in

Fig. 3.9. The inhibitory constants were estimated from non-linear secondary (plot slope vs [I]). These results will be interpreted after the inhibition model is discussed for better understanding.

Table 3.1 Summary of the kinetic data of the tested polyphenols.

Inhibitor	Type of Inhibition	Apparent K_I (μM)	K_I (μM)	K_i (μM)	K_i/K_I
Allopurinol	Mixed type	0.30	0.24	0.93	3.88
Quercetin	Mixed type	0.25	0.20	0.53	2.65
Kaempferol	Mixed type	0.33	0.17	0.82	4.85
(+)-Catechin	Uncompetitive	303.95	-	303.95	0
(-)-Epicatechin	Mixed type	20.48	24.62	241.02	9.8
(-)-Epigallocatechin	Mixed type	10.66	14.44	25.02	1.73
(-)-Epicatechin gallate	Mixed type	2.86	1.83	28.45	15.55
(-)-Epigallocatechin gallate	Competitive	0.76	0.76	-	∞

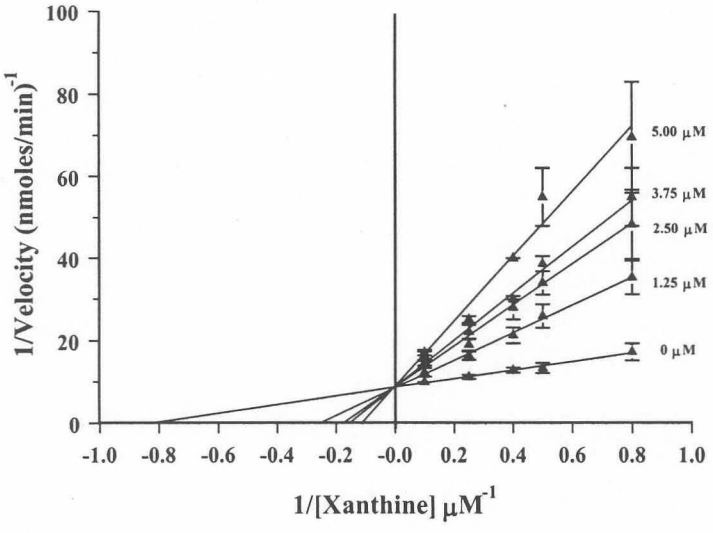


Figure 3.7 The Lineweaver-Burk plot for the inhibition of xanthine oxidase by (-)-EGCg with xanthine as substrate.

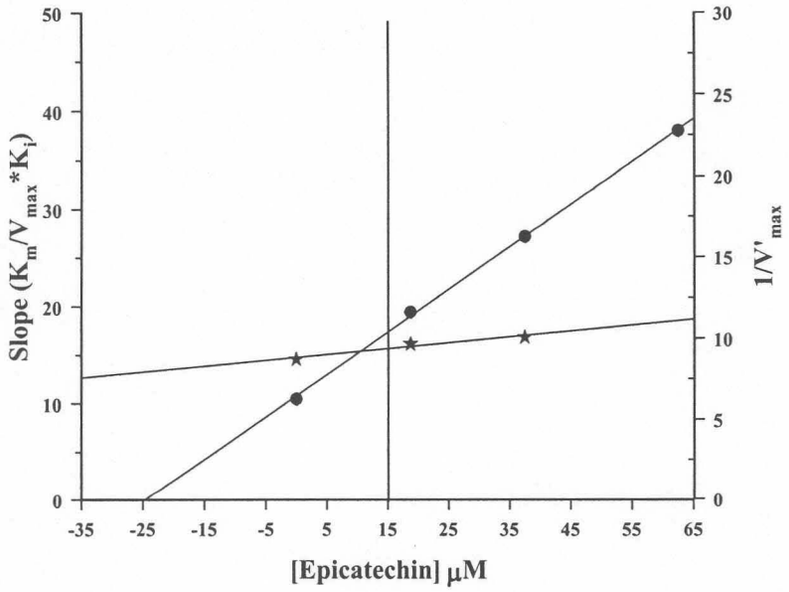


Figure 3.8 The linear secondary plots of the inhibitor (-)-EC. The 1/Vmax vs. [I] plot is represented by the (★) and the Slope vs.[I] plot is represented by (●).

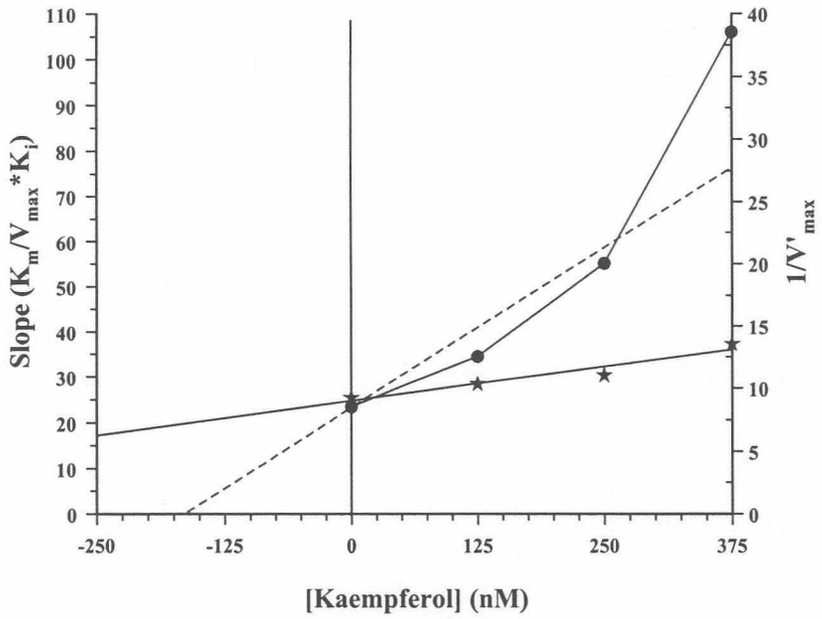


Figure 3.9 The secondary inhibition plots of the inhibitor kaempferol. The 1/Vmax vs. [I] plot is represented by the (★) and the Slope vs.[I] plot is represented by (●).

3.2 Model for structurally similar XO inhibitors

The first requirement for determining a structure-activity relationship is to obtain a model that can explain all the observed activities in a rational manner. Since XO has a ping-pong bi-bi catalytic mechanism, it is unlikely that normal single-substrate equations such as Michaelis-Menten (even under pseudo-single substrate conditions) will give adequate explanation for the inhibition results. This statement can be substantiated with the results obtained with the XO inhibitors, alloxanthine (oxypurinol) and 8-bromoxanthine (Fig. 3.10). The two inhibitors are structurally similar and both have been shown to bind to the electron acceptor domain of XO. Yet alloxanthine shows mixed type inhibition (Massey, 1970) and 8-bromoxanthine uncompetitive inhibition (Hille, 1984). According to single substrate inhibition models, both inhibitors have to bind to the ES complex to exert mixed-type or uncompetitive inhibition. This will

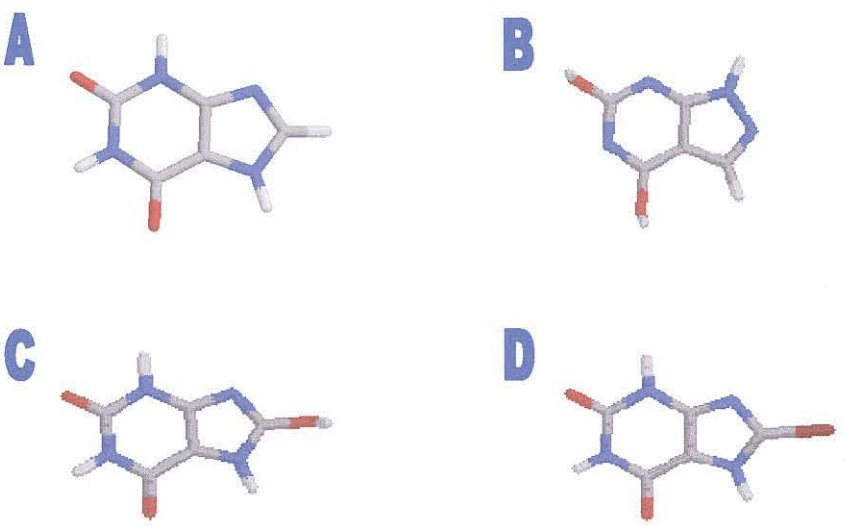


Figure 3.10 The three dimensional structures of the XO substrates, products and their inhibitory analogues. A: Xanthine (substrate), B: alloxanthine / oxypurinol (substrate analogue), C: uric acid (product), D: 8-bromoxanthine (product analogue).

form an ESI enzyme complex, but since both S and I binds to the electron acceptor domain it is impossible to form an ESI complex. Thus single-substrate Michaelis-Menten kinetics is not a suitable model for explaining this inhibition.

In explaining this inhibition behavior, the inhibitors should be seen as competing with the substrate for the electron acceptor domain of the enzyme. The inhibitors simulate "alternative substrates" for the enzyme. This allows the implementation of the mathematical procedures used for explaining the effects of alternative substrates on the enzyme activity for physiological substrates (Huang, 1979). From the schematic presentation of the XO ping-pong bi-bi reaction, it can be seen that there is an oxidized (E_{ox}) and a reduced (E_{red}) form of the enzyme to which competitive inhibitors can bind (Fig. 3.11).

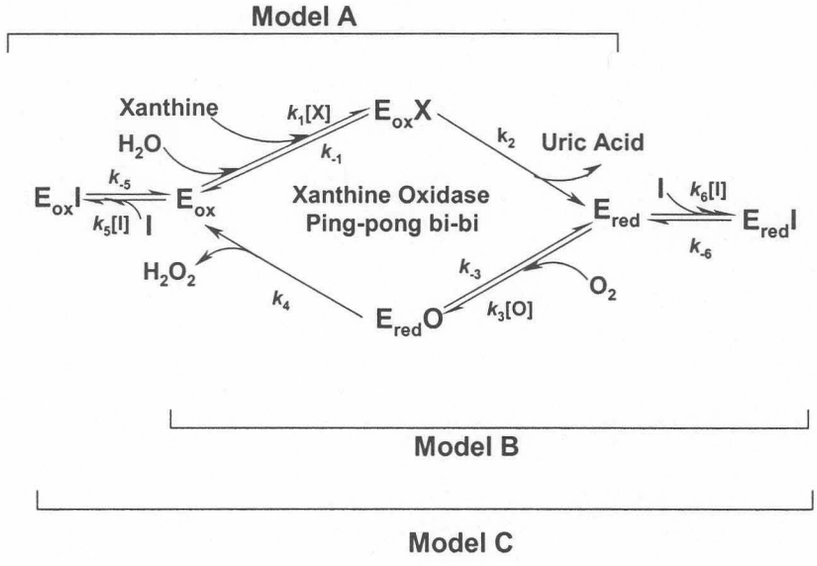


Figure 3.11 A possible model by which the tea polyphenols as well as XO substrate and product analogues exert their inhibitory effects.

Three-dimensional structures obtained from X-ray crystallography data of oxidized, reduced and partially reduced *Desulfovibrio gigas* AOR indicated that the major differences are in the structures of the Mo coordination complex and the Moco. The Mo^{VI} is reduced to Mo^{IV}, a substantial bond lengthening is observed for the apical sulfur ligand and the Mo deviates from the equatorial plane with 0.4 - 0.7 Å towards the apical ligand. In the reduced form the dithiolene sulfurs are also wider apart which results in a considerable puckering of the dithiolene molybdenum ring. Other structural differences are far from the molybdenum and presumably insignificant for the enzymatic reaction. There are no structural differences in the spatial arrangement of the amino acids in the substrate binding domains of the oxidized and reduced enzyme forms. Reduction of the Fe/S cofactors also resulted in no structural differences. Since the only major structural change in the active center involves the Mo^{IV}, it is most probably the factor that permits binding of the inhibitor to the E_{red} enzyme form. This is a reasonable assumption since 8-bromoxanthine has been shown to bind only to the E_{red} form.

When an inhibitor binds to the E_{ox} form of the enzyme it is clear that it competes with the xanthine for binding to the electron acceptor domain. With the inhibitor binding to the E_{red} form of the enzyme, it seems as if it is competing with the oxygen for a binding site. Oxygen is reduced at the FAD cofactor binding domain (electron donating) which is far from the electron acceptor domain. XO can potentially accommodate six reducing equivalents under anaerobic conditions and is thus able to complete three catalytic turnovers in the absence of oxygen. This means that xanthine is able to bind to the enzyme even if the Fe/S cofactors are in the reduced or partially reduced forms. It is possible that the same inhibitor can also compete with

xanthine for the electron acceptor domain of E_{red}. Alternatively it is possible that the binding of the inhibitor to E_{red} prevents the reducing equivalents from being transferred to oxygen.

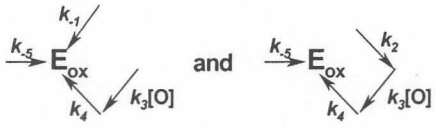
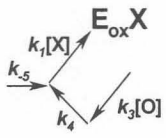
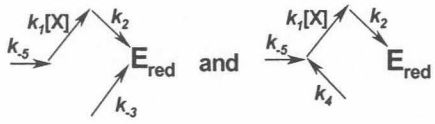
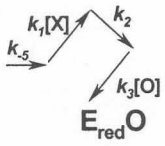
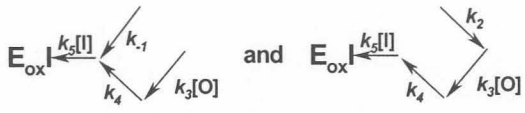
Since polyphenols are structurally similar it is possible that they all might bind to the same binding site with varying affinities. Circumstantial evidence suggests that the polyphenols may bind to the electron acceptor domain. The XO enzyme is not very substrate specific and the basic structural requirements for substrates are the presence of aromatic heterocycles containing nitrogen or oxygen or a carbonyl group in the position α to the carbon that will be oxidized. The polyphenol EGCg exerts competitive inhibition with varying xanthine concentration. The secondary plots indicate linear competitive inhibition with the formation of a dead-end complex. All these results are indications that EGCg may bind to the electron acceptor domain of XO.

Models are proposed where the polyphenols and other structurally related inhibitors bind to the electron acceptor domain of either E_{ox} (Model A) or E_{red} (Model B) or to both forms of the enzyme (Model C) (Fig. 3.11). These models represent the initial reaction conditions where no products are present in the system. Under these conditions the product formation steps are far from equilibrium and essentially unidirectional. The reverse reaction where xanthine is produced from uric acid is also extremely slow and virtually non-existent. The water molecule that takes part in the reaction is ignored in the theoretical model. The reaction takes place in an aqueous medium where the concentration of water is not a rate-limiting factor. Two inhibitor constants K_I and K_i are used to define the affinity of the inhibitor for the oxidized or reduced forms of the enzyme respectively. The King-Altman procedure has been used

to derive the Alberty equation for each model. The inhibitory Alberty equation for each model will be derived mathematically.

King and Altman developed a schematic method for determining the rate laws for enzyme catalyzed reactions. For a scheme containing n enzyme forms *n* different steady-state equations are derived. The concentration of each enzyme form is defined as the sum of all the products of (*n-1*) rate constants and substrate concentrations (King, 1956).

Model A

Enzyme form	Paths forming enzyme form	Sum of *kappa* products
E_{ox}		$k_{-1}k_3k_4k_{-5}[O]+k_2k_3k_4k_{-5}[O]$ $=(k_{-1}+k_2)k_3k_4k_{-5}[O]$
$E_{ox}X$		$k_1k_3k_4k_{-5}[X][O]$
E_{red}		$k_1k_2k_{-3}k_{-5}[X]+k_1k_2k_4k_{-5}[X]$ $=(k_{-3}+k_4)k_1k_2k_{-5}[X]$
$E_{red}O$		$k_1k_2k_3k_{-5}[X][O]$
$E_{ox}I$		$k_{-1}k_3k_4k_5[O][I]+k_2k_3k_4k_5[O][I]$ $=(k_{-1}+k_2)k_3k_4k_5[O][I]$

$$E_{ox}X + E_{red}O = (k_2 + k_4)k_1k_3k_{-5}[X][O]$$

The enzyme reaction is monitored relative to uric aid formation. The reaction rate is defined by the equation: $v_0 = k_2[E_{ox}X]$

$$\frac{[E_{ox}X]}{[E_0]} = \frac{k_1k_3k_4k_{-5}[X][O]}{(k_{-3}+k_4)k_1k_2k_{-5}[X]+(k_{-1}+k_2)k_3k_4k_{-5}[O]+(k_{-1}+k_2)k_3k_4k_5[O][I]+(k_2+k_4)k_1k_3k_{-5}[X][O]}$$

$$v_0 = \frac{k_1k_2k_3k_4k_{-5}[E_0][X][O]}{(k_{-3}+k_4)k_1k_2k_{-5}[X]+(k_{-1}+k_2)(k_{-5}+k_5[I])k_3k_4[O]+(k_2+k_4)k_1k_3k_{-5}[X][O]}$$

$$v_0 = \frac{\left(\frac{k_2k_4}{k_2+k_4}\right)[E_0]k_1k_3k_{-5}[X][O]}{\left(\frac{k_{-3}+k_4}{k_2+k_4}\right)k_1k_2k_{-5}[X]+\left(\frac{k_{-1}+k_2}{k_2+k_4}\right)(k_{-5}+k_5[I])k_3k_4[O]+\left(\frac{k_2+k_4}{k_2+k_4}\right)k_1k_3k_{-5}[X][O]}$$

$$v_0 = \frac{\left(\frac{k_2k_4}{k_2+k_4}\right)[E_0][X][O]}{\frac{k_2}{k_3}\left(\frac{k_{-3}+k_4}{k_2+k_4}\right)[X]+\frac{k_4}{k_1}\left(\frac{k_{-1}+k_2}{k_2+k_4}\right)\left(\frac{k_{-5}+k_5[I]}{k_{-5}}\right)[O]+[X][O]}$$

For the ping-pong bi-bi system:

$$V_{max} = \left(\frac{k_2k_4}{k_2+k_4}\right)[E_0] \qquad K_m^X = \frac{k_4}{k_1}\left(\frac{k_{-1}+k_2}{k_2+k_4}\right)$$

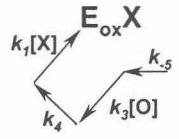
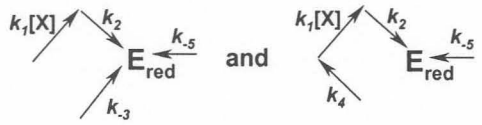
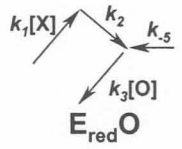
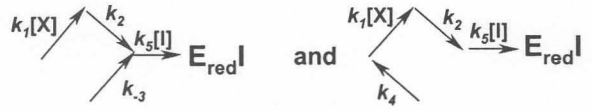
$$K_m^{O_2} = \frac{k_2}{k_3}\left(\frac{k_{-3}+k_4}{k_2+k_4}\right) \qquad K_I = \frac{k_{-5}}{k_5}$$

$$v_0 = \frac{V_{max}[X][O]}{K_m^{O_2}[X]+K_m^X\left(1+\frac{[I]}{K_I}\right)[O]+[X][O]}$$

Under saturating O_2 conditions the Alberty equation can be reduced to a pseudo-single substrate Michaelis-Menten equation. This equation shows that inhibitors binding to the E_{ox} form (Model A) exhibits only competitive inhibition where V_{max} is unaffected and K_m is reduced.

$$v_0 = \frac{V_{max}[X]}{[X] + K_m^X\left(1 + \frac{[I]}{K_I}\right)}$$

Model B

Enzyme form	Paths forming enzyme form	Sum of *kappa* products
E_{ox}		$k_{-1}k_3k_4k_{-5}[O] + k_2k_3k_4k_{-5}[O] = (k_{-1} + k_2)k_3k_4k_{-5}[O]$
$E_{ox}X$		$k_1k_3k_4k_{-5}[X][O]$
E_{red}		$k_1k_2k_{-3}k_{-5}[X] + k_1k_2k_4k_{-5}[X] = (k_{-3} + k_4)k_1k_2k_{-5}[X]$
$E_{red}O$		$k_1k_2k_3k_{-5}[X][O]$
$E_{red}I$		$k_1k_2k_{-3}k_5[X][I] + k_1k_2k_4k_5[X][I] = (k_{-3} + k_4)k_1k_2k_5[X][I]$

$$E_{ox}X + E_{red}O = (k_2 + k_4)k_1k_3k_{-5}[X][O]$$

$$\frac{[E_{ox}X]}{[E_0]} = \frac{k_1k_3k_4k_{-5}[X][O]}{(k_{-3}+k_4)k_1k_2k_{-5}[X]+(k_{-3}+k_4)k_1k_2k_5[X][I]+(k_{-1}+k_2)k_3k_4k_{-5}[O]+(k_2+k_4)k_1k_3k_{-5}[X][O]}$$

$$v_0 = \frac{k_1k_2k_3k_4k_{-5}[E_0][X][O]}{(k_{-3}+k_4)(k_{-5}+k_5[I])k_1k_2[X]+(k_{-1}+k_2)k_3k_4k_{-5}[O]+(k_2+k_4)k_1k_3k_{-5}[X][O]}$$

$$v_0 = \frac{\left(\frac{k_2k_4}{k_2+k_4}\right)[E_0]k_1k_3k_{-5}[X][O]}{\left(\frac{k_{-3}+k_4}{k_2+k_4}\right)(k_{-5}+k_5[I])k_1k_2[X]+\left(\frac{k_{-1}+k_2}{k_2+k_4}\right)k_3k_4k_{-5}[O]+\left(\frac{k_2+k_4}{k_2+k_4}\right)k_1k_3k_{-5}[X][O]}$$

$$v_0 = \frac{\left(\frac{k_2k_4}{k_2+k_4}\right)[E_0][X][O]}{\frac{k_2}{k_3}\left(\frac{k_{-3}+k_4}{k_2+k_4}\right)\left(\frac{k_{-5}+k_5[I]}{k_{-5}}\right)[X]+\frac{k_4}{k_1}\left(\frac{k_{-1}+k_2}{k_2+k_4}\right)[O]+[X][O]}$$

For model B the Alberty and pseudo-single substrate Michaelis-Menten equations indicate that inhibitor binding to the E_{red} form changes both K_m and V_{max} thus result in uncompetitive inhibition.

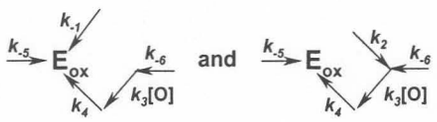
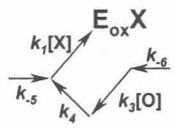
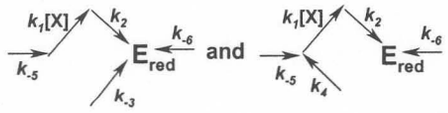
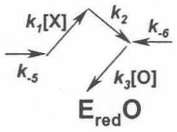
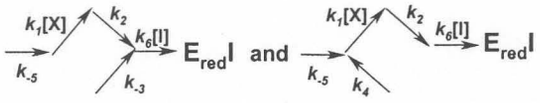
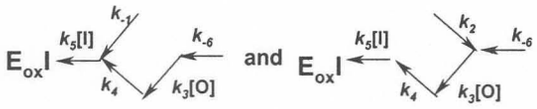
$$v_0 = \frac{\frac{V_{max}}{\left(1+\frac{[I]}{K_i}\right)}[X][O]}{K_m^O[X]+\frac{K_m^X}{\left(1+\frac{[I]}{K_i}\right)}[O]+[X][O]}$$

Alberty equation (Model B)

$$v_0 = \frac{\frac{V_{max}}{\left(1+\frac{[I]}{K_i}\right)}[X]}{[X]+\frac{K_m^X}{\left(1+\frac{[I]}{K_i}\right)}}$$

Michaelis-Menten equation (Model B)

Model C

Enzyme form	Paths forming enzyme form	Sum of *kappa* products
E_{ox}		$k_{-1}k_3k_4k_{-5}k_{-6}[O]+k_2k_3k_4k_{-5}k_{-6}[O]$ $=(k_{-1}+k_2)k_3k_4k_{-5}k_{-6}[O]$
$E_{ox}X$		$k_1k_3k_4k_{-5}k_{-6}[X][O]$
E_{red}		$k_1k_2k_{-3}k_{-5}k_{-6}[X]+k_1k_2k_4k_{-5}k_{-6}[X]$ $=(k_{-3}+k_4)k_1k_2k_{-5}k_{-6}[X]$
$E_{red}O$		$k_1k_2k_3k_{-5}k_{-6}[X][O]$
$E_{red}I$		$k_1k_2k_{-3}k_{-5}k_6[X][I]+k_1k_2k_4k_{-5}k_6[X][I]$ $=(k_{-3}+k_4)k_1k_2k_{-5}k_6[X][I]$
$E_{ox}I$		$k_{-1}k_3k_4k_5k_{-6}[O][I]+k_2k_3k_4k_5k_{-6}[O][I]$ $=(k_{-1}+k_2)k_3k_4k_5k_{-6}[O][I]$

$$\frac{[E_{ox}X]}{[E_0]}=\frac{k_1k_3k_4k_{-5}k_{-6}[X][O]}{(k_{-3}+k_4)(k_{-6}+k_6[I])k_1k_2k_{-5}[X]+(k_{-1}+k_2)(k_{-5}+k_5[I])k_3k_4k_{-6}[O]+(k_2+k_4)k_1k_3k_{-5}k_{-6}[X][O]}$$

$$v_0=\frac{k_1k_2k_3k_4k_{-5}k_{-6}[E_0][X][O]}{(k_{-3}+k_4)(k_{-6}+k_6[I])k_1k_2k_{-5}[X]+(k_{-1}+k_2)(k_{-5}+k_5[I])k_3k_4k_{-6}[O]+(k_2+k_4)k_1k_3k_{-5}k_{-6}[X][O]}$$

$$v_0=\frac{\left(\frac{k_2k_4}{k_2+k_4}\right)[E_0][X][O]}{\frac{k_2}{k_3}\left(\frac{k_{-3}+k_4}{k_2+k_4}\right)\left(\frac{k_{-6}+k_6[I]}{k_{-6}}\right)[X]+\frac{k_4}{k_1}\left(\frac{k_{-1}+k_2}{k_2+k_4}\right)\left(\frac{k_{-5}+k_5[I]}{k_{-5}}\right)[O]+[X][O]}$$

The Alberty and pseudo-single substrate Michaelis-Menten equation of Model C represent the non-competitive and mixed inhibition types. Two different inhibition constants are used for the defining the affinity of the inhibitor for E_{ox} and E_{red}.

$$v_0 = \frac{V_{max}[X][O]}{K_m^O[X]\left(1+\frac{[I]}{K_I}\right)+K_m^X[O]\left(1+\frac{[I]}{K_i}\right)+[X][O]}$$

Alberty equation (Model C)

$$v_0 = \frac{\frac{V_{max}}{\left(1+\frac{[I]}{K_I}\right)}[X]}{[X]+\frac{K_m^X\left(1+\frac{[I]}{K_i}\right)}{\left(1+\frac{[I]}{K_I}\right)}}$$

Michaelis-Menten equation (Model C)

The ratio K_i/K_I will be used to assess structure-affinity relationships of mixed type inhibitors. For $K_i/K_I < 1$ the inhibitor has a higher affinity for the E_{red} form than for the E_{ox} form of the enzyme. For $K_i/K_I = 1$ the inhibitor is a non-competitive inhibitor with equal affinity for both enzyme forms. For $K_i/K_I > 1$ the inhibitor has a higher affinity for the E_{ox} than E_{red} enzyme form.

3.3 Factors influencing apparent Ki values

The region in polyphenols that mimic the structures of other XO substrates will be named the primary binding region in polyphenols (Fig. 3.12). This region is defined as the A-ring together with the heterocyclic oxygen atom in the C-ring. Groups capable of forming hydrogen or coordination bonds are required in this region of the molecule. The orientation in itself does not determine whether the inhibitor binds to the E_{ox} or E_{red} enzyme forms preferentially. It does seem that at least one hydrogen bond forming group should be present on the positions C5-C7 on the A ring. Substi-

tution with methoxy or glycosyl groups in these positions reduces the affinity of the inhibitor for the enzyme. The affinity is increased when the heterocyclic oxygen atom is replaced with a nitrogen atom as found with purines.

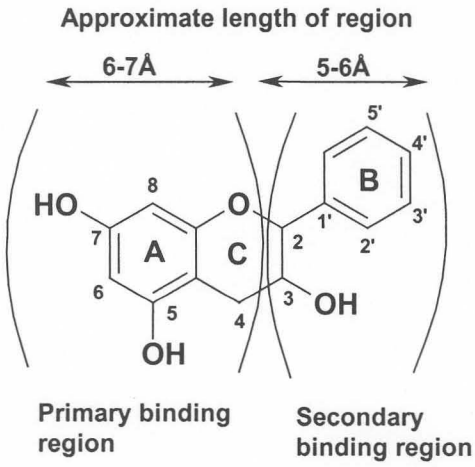


Figure 3.12 Polyphenols may have two regions that are important in defining their inhibitory effects on XO. The two regions are designated the primary and secondary binding regions.

The secondary binding region is defined as the rest of the polyphenol molecule. The function of this region is to enhance the inhibitor affinity and to modulate the ratio by which the inhibitor binds to the E_{ox} and E_{red} enzyme forms.

When comparing the inhibition constants (K_i) of synthetic pyrazolo-pyrimidine derivatives (Fig. 3.13A-C) (Springer, 1976), it was found that large aromatic groups as secondary binding regions increase the affinity of the enzyme for both forms of the enzyme. A similar pattern was observed for EC and ECG as well as for EGC and EGCg. (Fig. 3.13E-H). The extra aromatic gallate group increased the interaction of the secondary binding region with the enzyme resulting in increased affinities. The electron acceptor domains of AOR and XO are conserved with differences at only two residues.

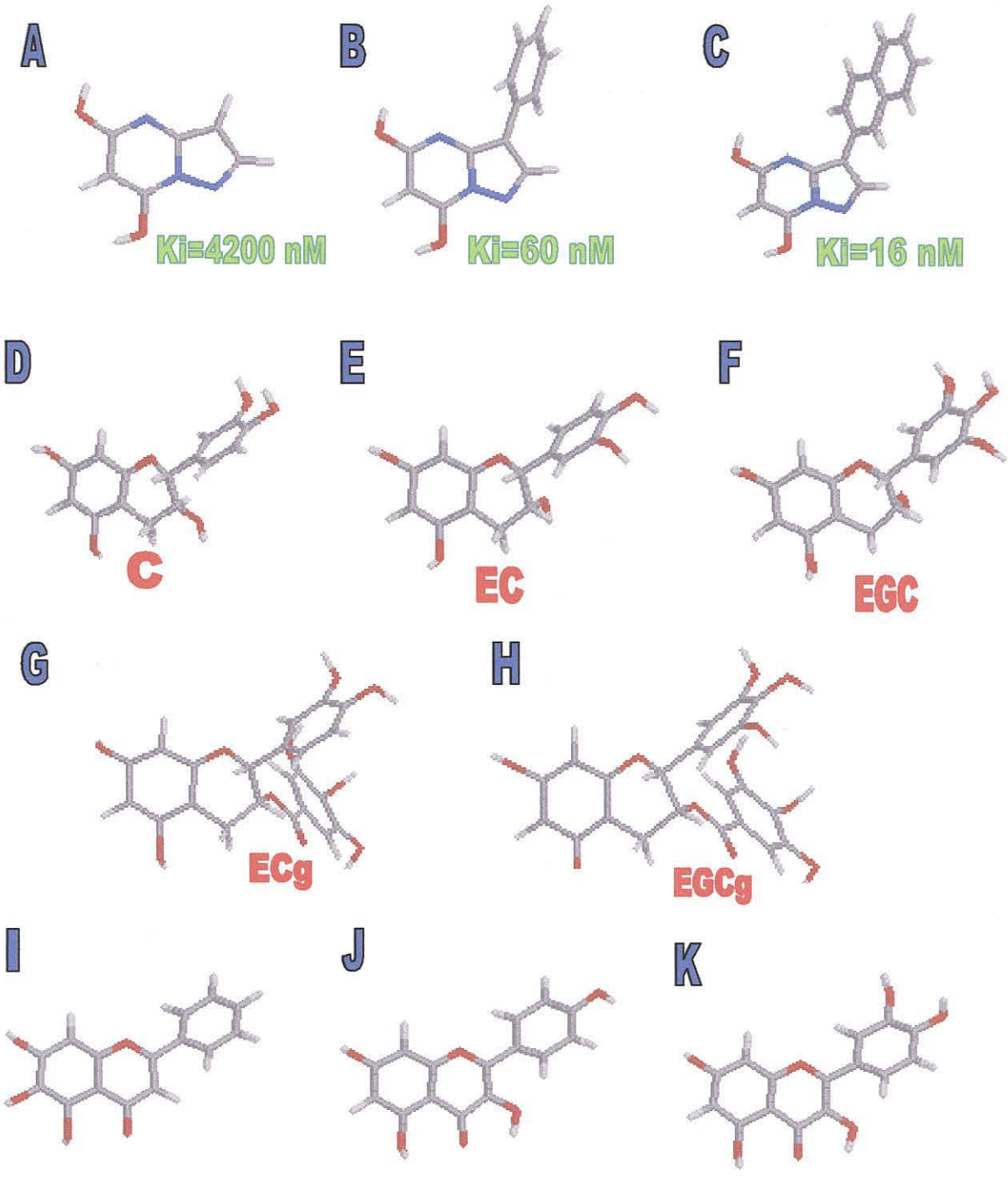


Figure 3.13 Structural similarities between synthetic pyrazolo-pyrimidines XO inhibitors and tea polyphenols. A structural pattern is observed between synthetic pyrazolo-pyrimidines (A-C) and tea polyphenols (E-H) that may explain the increase in affinity of inhibitor for the XO enzyme. Bulky secondary binding regions increase the affinity of the inhibitors for the enzyme. The pyrazolo-purine

inhibitors increase in potency from A to C. Likewise the gallated catechins, ECg (G) and EGCg (H), are also more potent inhibitors than the ungallated catechins, C (D), EC (E) and EGC (F). The three dimensional structures of bacalein (I), kaempferol (J) and quercetin (K) are also depicted. These inhibitors display the different structural properties that modulate the selective affinities of the inhibitors for the E_{ox} and E_{red} enzyme forms as discussed.

When studying the substrate binding or electron acceptor domain of AOR, (which is theoretically very similar to that of XO) it can be seen that it is funnel-shaped, about 15 Å deep and lined with hydrophobic moieties (Fig. 3.14). This explains why inhibitors with voluminous hydrophobic secondary binding regions have higher affinities (lower K_i). The primary binding region binds to the normal substrate binding residues near the Mo center at the base of the funnel. The secondary binding region of the inhibitor can then interact with the hydrophobic residues in the wider part of the substrate binding domain of XO. Larger secondary binding regions may fit more snugly in this cavity, allowing a larger surface for hydrophobic interaction and result in a higher affinity of the inhibitor for the enzyme. A schematic presentation is shown in Fig. 3.15.

The oxo-group on the C4 of bacalein, kaempferol and quercetin (Fig. 3.13I-K) also seems to bind to both forms of the enzyme since the affinity is significantly increased when this group is present. Bacalein is the uncompetitive inhibitor with the lowest K_I (2.48 μM) (Chang, 1993). The 1/Vmax vs [I] secondary plot of quercetin and kaempferol is parabolic non-linear. From this plot the K_I value is obtained indicating the affinity for the E_{ox} enzyme form. Parabolic non-linear secondary plots are observed when more than one inhibitor molecule binds per enzyme molecule. It is possible that polyphenols with an oxo group on C4 may bind to an additional site on the XO

enzyme. The secondary plot used for determining K_i is linear. This indicates that as with the other polyphenols a dead-end complex is formed with the E_{red} enzyme form.

3.4 Structural requirements for modulating K_i/K_I ratios

The K_i/K_I ratio is an indication of the affinity of the inhibitor for the E_{red} form relative to the E_{ox} form. For competitive inhibitors $K_i/K_I = \infty$ and for uncompetitive inhibitors $K_i/K_I = 0$. For true non-competitive inhibitors $K_i/K_I = 1$. Two regions seem to be important in altering the K_i/K_I ratios or XO inhibitors. The properties and orientations of the substituents on the C3 of the C-ring play an important role. A hydroxyl must be present and orientated in the plane of the C-ring or behind the plane in the (-)-configuration (epicatechin derivatives) (Fig. 3.13E-H) to allow the inhibitor to bind to the E_{ox} enzyme forms. A lack of a hydroxyl or a hydroxyl oriented towards the front of the C-ring plane leading to (+)-configuration (catechin) (Fig. 3.13D) only allows binding to the E_{red} enzyme form. When the hydroxyl in the (-)-configuration is substituted with a gallate, the K_i/K_I ratios increase significantly indicating that the inhibitor binds preferentially to the E_{ox} enzyme form. When EC is gallated to give ECg the K_i/K_I ratio jumps from 9.8 to 15.55 and when EGC with a ratio of 1.73 was changed to EGCg it was found to bind to the E_{ox} enzyme form only. Similarly, uric acid and 8-bromoxanthine (Fig. 3.10C and 3.10D) also have strong electronegative groups in the same region as the C3 hydroxyl group of the polyphenols. Both these two molecules only bind to the E_{red} enzyme form (uncompetitive inhibition). Binding studies of 8-bromoxanthine with XO showed that it binds only to the E_{red} enzyme form. The Mo is in the Mo^{IV} state and the bromine atom is 4 Å away from the molybdenum (Hille, 1984). The bromine atom is too far away from the Mo to form a direct bond and the nature of the interaction or the influence of bromine on the Mo center is

not understood. Xanthine and alloxanthine (oxypurinol) (Fig. 3.9A and 3.9B) lack hydroxyl groups in this region and bind to both forms of the enzyme.

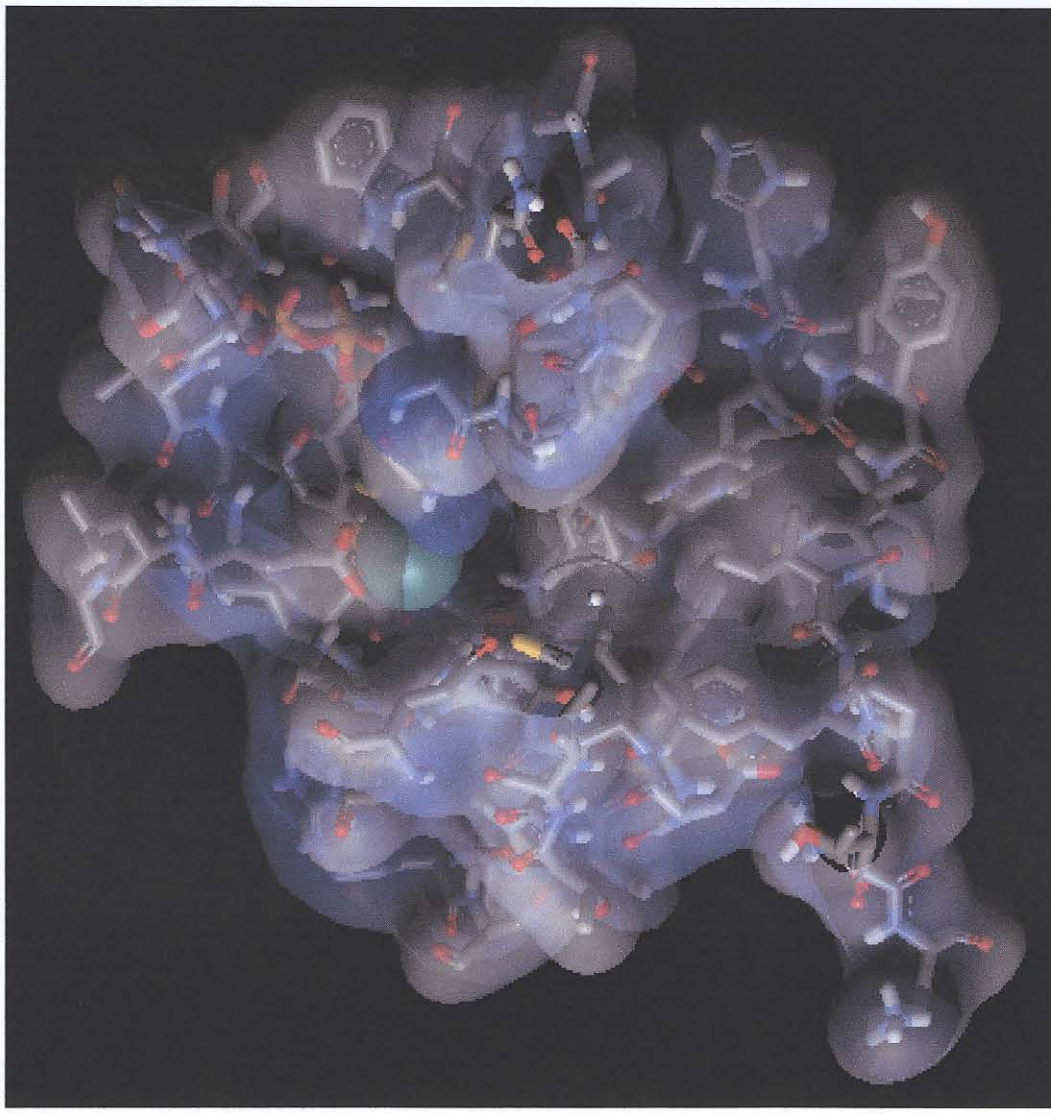


Figure 3.14 The substrate binding cavity of aldehyde oxido-reductase. The figure shows a cross-sectional view of the substrate binding domain with the mouth of the enzyme facing to the right. The substrate binding site is wide near the surface of the protein and gets narrower deeper into the enzyme core. Inhibitors with bulky secondary binding regions would presumably fit more tightly into the funnel shaped cavity.

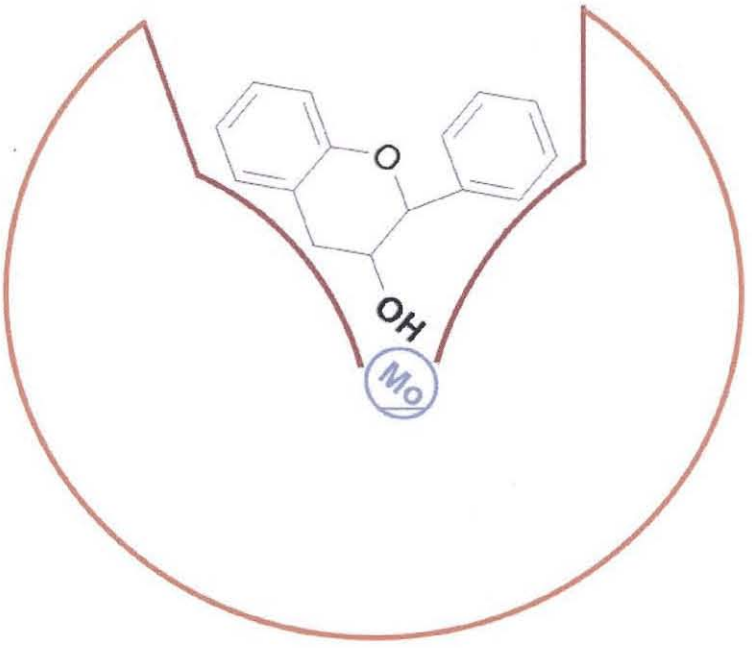


Figure 3.15 A schematic presentation of one way how the polyphenol may fit into the funnel-shaped active center of XO. The primary binding region of the inhibitor binds to the normal substrate recognition amino acids on one side. The secondary binding region interacts with the funnel wall and increases the affinity. The C3' OH is in close proximity of the Mo, resulting in an interaction that differentiates into competitive or uncompetitive inhibition.

The amount of hydroxylation on the B-ring also influences the selectivity of binding. The mono-hydroxylated kaempferol binds more selectively to the E_{ox} form (K_i/K_I = 4.85) than the di-hydroxylated quercetin (K_i/K_I = 2.65). Similarly the di-hydroxylated EC binds more selectively to the E_{ox} form (K_i/K_I = 9.8) than the tri-hydroxylated EGC (K_i/K_I = 1.73). Bacalein (Fig. 3.12I) with no hydroxyl groups on the B-ring does not bind to E_{ox} (uncompetitive) and tri-hydroxylated EGCg only binds to the E_{ox} form (competitive). Bacalein has no hydroxyl group on the C3 (consistent with uncompetitive inhibition) and EGCg has a gallated group on the C3 (resulting in preferential

binding to E_{ox}). This is a possible indication that the properties of the C3 are more important in determining the selectivity of binding than the hydroxylation pattern of the B-ring.

4 Conclusion

The tea polyphenols are good inhibitors of XO. The most potent inhibitor is EGCg and it compares well with allopurinol. Quercetin and kaempferol are two other polyphenols also showing inhibition of XO with similar potency as EGCg. Polyphenols and anthocyanidin extracts from other plants showed inhibition of XO (Costantino, 1992). This confirms our results of XO inhibition by the polyphenols. What is surprising is that compounds that are structurally similar can result in vastly different types of inhibition. The hypothetical model that is proposed is by no means the only possible explanation for the results obtained. The mathematical model and theory is sound and are commonly employed to elucidate multi-substrate mechanisms. It also agrees with experimental data obtained for the inhibition of XO with allopurinol and 8-bromoxanthine. Although it is understood that 8-bromoxanthine binds preferentially to the E_{red} enzyme form, it is not understood what interaction takes place between the bromine and the Mo. Similarly the interaction between the C8 OH of uric acid and the Mo center is unknown. Further studies of the interactions of the catechins with the XO enzyme may shed some light on the issue. It would be interesting to see whether catechin also binds in close proximity to the molybdenum center. This would prove conclusively whether catechin has a similar inhibitory mechanism as 8-bromoxanthine. This would place the hypothesis on firmer scientific grounds. The dimensions of the active centers of XO and AOR will most likely be very similar. Three-dimensional simulations of protein-ligand interactions with molecular modeling software

could also generate information of the interaction between XO and the catechins. One question that remains is whether polyphenols and coumarins can serve as substrates for XO or are they just molecules that have the ability to modulate the activity of the enzyme. Literature searches provided no published scientific evidence that polyphenols or coumarins are hydroxylated by XO.

A parallel can be drawn between XO and another liver enzyme, the cytochrome P450. The family of P450 mono-oxygenase enzymes catalyze hydroxylation reactions and also show low substrate specificity. Polyphenols have been shown to be able to modulate the activities of some of the P450 iso-enzymes by acting as inhibitors or allosteric activators (positive heterotropic cooperativity). In doing this, the polyphenols modulated the ability of the P450 enzymes to convert pro-carcinogens to carcinogenic or innocuous compounds. Inhibition of XO *in vivo* would lead to a reduced oxidative stress and this could explain some of the beneficial effects of tea on cancer and atherosclerosis that have been widely documented (Aucamp, 1997). If the *in vitro* results shown here could be repeated *in vivo*, tea polyphenols may become a novel treatment for gout.

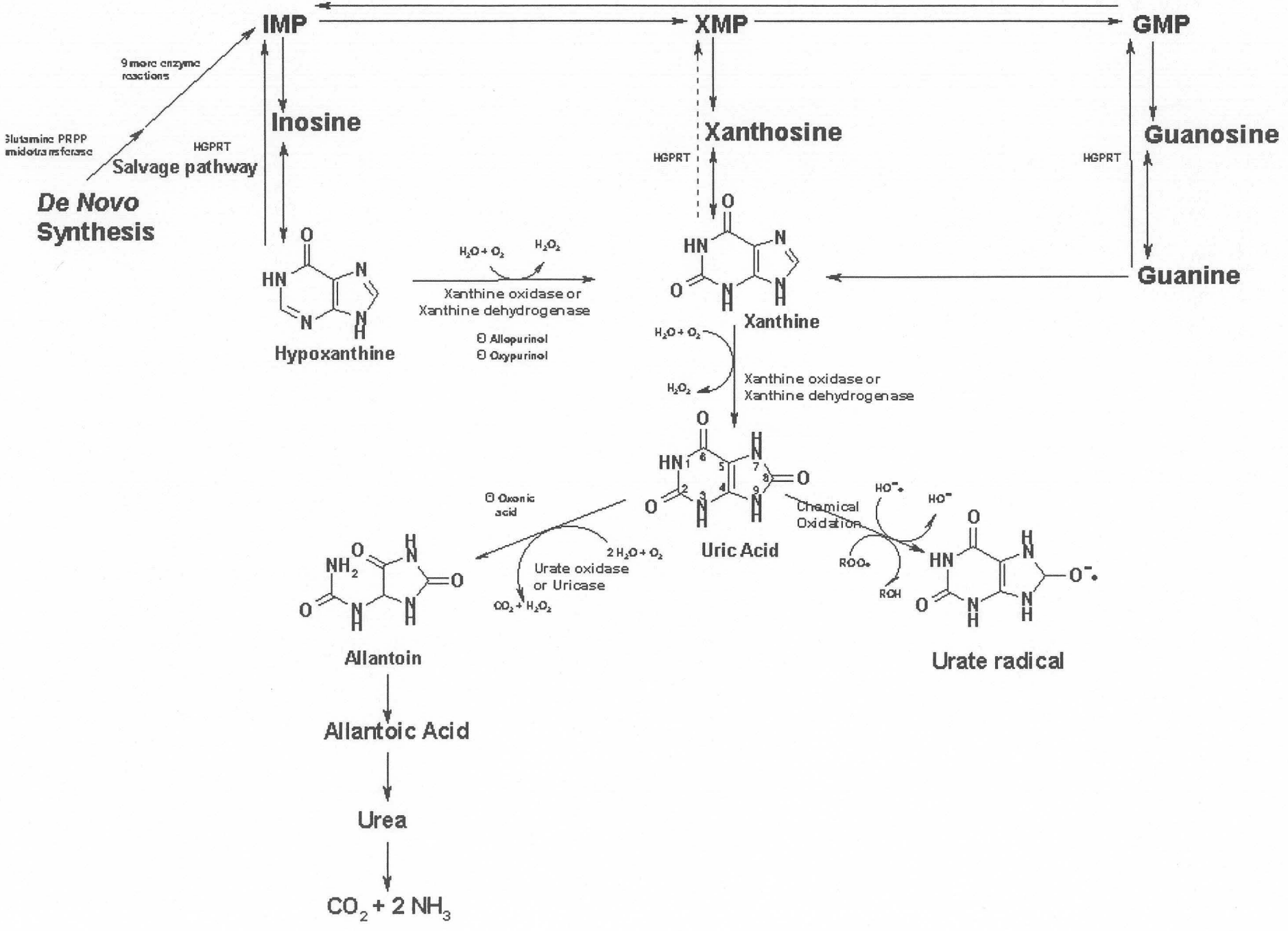
Chapter 4

In vivo analysis of Xanthine Oxidase substrates and products with CE

1 Introduction

Uric acid is derived solely from adenine and guanine–based purine compounds. Purines are essential compounds in animal cells and continuous metabolic turnover of these compounds occurs. In humans and higher primates, enzymatic catabolism of purines proceeds only as far as uric acid. Most mammals however excrete allantoin or urea as the major nitrogen containing enzymatic purine degradation products.

The concentration of uric acid levels in extracellular fluids is determined by: (1) normal metabolic turnover and obligatory nitrogen loss, (2) purine intake and (3) efficiency of excretion of uric acid. There are three important enzymes in the biochemical pathway of uric acid production: (1) pyrophosphate-ribose-P-amidotransferase (PPRP amidotransferase), (2) hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and (3) xanthine oxidase (Fig. 4.1). PPRP amidotransferase catalyses the first reaction in the purine biosynthetic pathway and is the primary rate-limiting step in the pathway. It is stimulated by increased levels of PPRP and inhibited by purine ribonucleotides. HGPRT is the enzyme that catalyses the reutilization of hypoxanthine ("salvage pathway") to inosine, a product that exerts negative feedback inhibition on PPRP amidotransferase. Xanthine oxidase is the third enzyme involved in the regulation of the uric acid levels. Physiologically the enzyme uses NAD^+ as electron acceptor and is known as xanthine dehydrogenase (XDH), but under stress conditions it is converted to xanthine oxidase (XO). XDH occurs mainly in the heart muscle and intestines while XO occurs mainly in the liver. The conver-


IMP
XMP
GMP
9 more enzyme reactions
Glutamine PRPP amidotransferase
De Novo Synthesis
Salvage pathway
HGPRT
Inosine
Xanthosine
Guanosine
Guanine
Hypoxanthine
Xanthine
Xanthine oxidase or Xanthine dehydrogenase
Allopurinol
Oxypurinol
Uric Acid
Oxonic acid
Urate oxidase or Uricase
Allantoin
Allantoic Acid
Urea
CO2 + 2 NH3
Chemical Oxidation
Urate radical

sion process was investigated by (Corte, 1972) and it was concluded that two factors were of essence. Proteolysis and changes in the enzyme cysteine thiol groups converted the enzyme from a NAD^+ reducing dehydrogenase to an oxygen reducing oxidase. The thiol groups are in close proximity to the FAD and may be essential for coupling with the NAD^+ or binding of NAD^+. Oxidation of the thiol groups may result in the loss of the binding site or a weakening in the coupling. This could be caused by increased steric hindrances that result from conformational changes in the protein structure. Proteolysis alters the protein conformation, moving the thiol away from the vicinity of the FAD. Isolated XO treated with dithioerythritol is restored to XDH. XO pre-treated with proteolytic enzymes before reduction with dithioerythritol did not regain the XDH activity. Only XO and XDH can produce uric acid.

In clinical studies, hyperuricemia is defined as uric acid levels of 2 S.D. above the population mean (40-50 µg/ml) (Beck, 1981). In pathophysiological terms hyperuricemia occurs when the uric acid concentration reaches saturation levels in the serum (60-70 µg/ml). There are three main causes of hyperuricemia (Wyngaarden, 1974). 1) A partial deficiency in the enzyme HGPRT results in insufficient salvaging of hypoxanthine so that more hypoxanthine is converted to uric acid. 2) An increase in the activity of the enzyme PPRP-synthetase leading to increased purine biosynthesis. 3) The renal tubulars are defective in handling uric acid, resulting in increased reabsorption or decreased excretion of uric acid. Potent diuretics and drugs such as salicylates and antituberculous drugs may also cause hyperuricemia.

Hyperuricemia can be treated with uricosuric agents or with xanthine oxidase inhibitors. Uricosuric agents increase the excretion of uric acid lowering the serum

urate levels. Allopurinol is the drug of first choice for treatment of gout. Allopurinol and its major metabolite oxypurinol (Fig. 4.2A) are potent inhibitors of xanthine oxidase, thereby decreasing the uric acid production from hypoxanthine and xanthine. The accumulated hypoxanthine is "salvaged" by HGPRT forming inosine mono-phosphate (IMP). IMP is a potent inhibitor of PPRP amidotransferase and causes the entire *de novo* purine synthesis pathway to slow down.

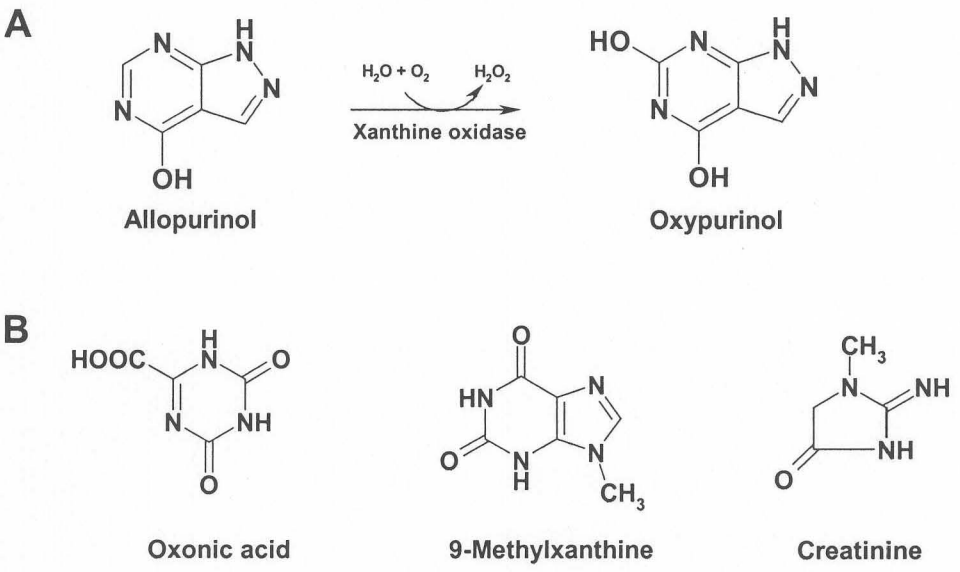


Figure 4.2 A: Xanthine oxidase catalyzes the conversion of allopurinol to oxypurinol. Both compounds serve as inhibitors of XO. B: The chemical structures of other compounds of interest in the analysis of rat serum.

The levels of serum uric acid in most mammals are 10% of that found in humans. The distinction arises from the active uricase (urate oxidase) enzyme responsible for trans-forming uric acid to allantoin. The gene required for producing this enzyme is apparently still present in the human genome but is no longer expressed (Lee, 1988), (Motojima, 1988), (Yeldandi, 1992). There is no certainty whether uric acid offers humans any physiological advantage or if evolutionary processes merely designated uric acid as the waste product of purine catabolism in higher primates. Serum uric

acid is freely filtered at the glomerules but undergoes extensive reabsorption (up to 98%). Uric acid secretion in the kidneys takes place through an anionic transport system and also the secreted uric acid undergoes extensive reabsorption (Diamond, 1973).

It would seem as if the body goes to great lengths to reabsorb this "waste product" which is notoriously known for causing gout and nephrolithiasis. Once hypoxanthine is converted to xanthine and uric acid it cannot be salvaged for reuse as purines. Purines have to be synthesized *de novo* at great energy expenditure to maintain the purine levels. Despite this huge energy wastage, all the purines taken up in the diet are converted to uric acid in the gastrointestinal tract by the mucosal XDH. Only uric acid appears in the circulatory system after ingestion of purines.

Uric acid has the ability to undergo non-enzymatic oxidation. One-electron oxidation by strong oxidants such as hydroxyl or peroxyl radicals produces an urate radical anion in neutral solution (Simic, 1989) (Fig. 4.1). An interspecies comparison of primates yields a positive correlation between plasma uric acid concentrations and longevity, with man coming out on the forefront (Cutler, 1984). Because cellular aging in general, and life-limiting diseases such as cancer and cardiovascular disorders may well be caused in part by oxidants and radicals, the positive relationship has been ascribed to the antioxidant capacity of uric acid. It has been estimated that uric acid comprises 30-65% of the peroxyl-radical (Wayner, 1987) and 10-15% of the hydroxyl scavenging capacity of blood plasma (Thomas, 1992). The concentration of uric acid is generally high in comparison to other non-enzymatic antioxidants such as ascorbate (6 µg/ml), tocopherols (9-19 µg/ml), methionine (3-6

µg/ml) (Cole, 1998), (van Guldner, 1999) and glutathione (7-10 µg/ml) (Orter, 1982), (Halliwell, 1989). Uric acid has the capacity to interrupt radical chain reactions and prevent the formation of peroxides. It also plays a role in the attenuation of lipid auto-oxidation in plasma. Uric acid has access to all extracellular fluid compartments and is not compartmentalized as is the case with the enzymatic scavengers superoxide dismutase, catalase and glutathione peroxidase (Halliwell, 1989).

Ironically the production of one mole uric acid by XO from xanthine results in the production of two moles of peroxide or superoxide radicals. The production of uric acid from hypoxanthine produces double the amount of oxygen radicals when compared to xanthine. This contributes to the serum radical contents that may result in the onset of carcinogenesis, arteriosclerosis and other diseases linked to radicals.

The *in vivo* inhibition of XO activity is discussed in Chapter 5. For now suffice it to say that the inhibition of XO *in vivo* results in a decrease in uric acid levels and an increase in xanthine levels in both serum and urine samples. Inhibition of XO and therefore uric acid production with polyphenols may not compromise the total anti-oxidant status of the serum. From a clinical point of view there are many reasons for the determination of uric acid and its precursors as can be deduced from the occur-rence of metabolic disorders and the possible physiological effects.

The aim was to identify and implement an appropriate method to analyze the purines, allopurinol, oxypurinol and allantoin with the internal standard 9-methylxanthine. Several CZE and MEKC methods existed for similar applications. A CZE method was developed by (Shihabi, 1995) to analyze hypoxanthine, xanthine, uric acid, allo-

purinol and oxypurinol in serum samples. This method required that acetonitrile be added to the serum sample for deprotonation and for on-column stacking purposes. A CZE method developed by (Xu, 1997) used amperometric detection for high sensitivity to quantify uric acid in human urine and serum. A MEKC method was developed by (Atamna, 1991) to separate several different methylxanthines and methyluric acid derivatives. Another MEKC method was developed by (Thormann, 1992) to analyze methyl-substituted xanthines in body fluids. The samples were either injected directly onto the column without any pre-treatment taking place, or solid-phase or liquid-liquid extraction was done. Direct injection made this method an attractive prospect, since time would be saved if no sample pre-treatment was required.

2 Materials and Methods

2.1 Experimental strategy

It was decided that the CZE method of (Shihabi, 1995) and the MEKC method of (Thormann, 1992) will be compared with each other using standards. The best method will then be used to analyze rat serum and urine samples to determine its applicability. The method of Shihabi was selected because it already separates the required metabolites in serum. The method of Thormann was selected because direct injection would be less laborious. Both methods were set up and the LOD's and column efficiencies obtained with each method were compared.

2.2 Instrumentation

Electrophoresis was carried out using the HP 3^{D}CE instrument (Hewlett Packard, Waldbronn, Germany) with on-column detection. For the MEKC method a 50 μm ID

uncoated fused silica capillary column with an extended optical window from HP was used. The total length was 64.5 cm and the effective separation length was 56 cm. The detection window was a bubble window with a pathlength of 150 μm. All samples were injected pneumatically (50 mbar) for 2 sec. The operating temperature was 40°C. For the CZE method an uncoated fused silica capillary column from HP with an internal diameter of 50 μm and total length of 64.5 cm was used. The effective separation length was 56 cm and the detection window had a pathlength of 50 μm. All samples were injected pneumatically (50 mbar) for 20 sec. The operating temperature was 35°C. Detection for both methods was effected by measurement of UV absorbency at 200 and 280 nm. Analyte identification was done based on migration times and UV spectrum analysis.

2.3 Reagents

The creatinine, allopurinol, allantoin, oxypurinol, xanthine, hypoxanthine and uric acid were obtained from Sigma Chemical Company (St Louis, MO, USA). The internal standard 9-methyl-xanthine was obtained from ICN Pharmaceutical (CA). Sodium dodecyl sulfate (SDS) was obtained from E. Merck (Darmstadt, Germany). The SDS was of analytical grade suitable for electrophoresis. All buffer salts were of analytical grade. All solutions were prepared with distilled water that was deionized with a Milli-Q system (Millipore Corp., Bedford, MA. USA).

2.4 Analytical conditions

Standards and samples were analyzed with the anode on the inlet side and a running buffer of 75 mM SDS, 6 mM disodium tetraborate and 10 mM disodium phosphate

for the MEKC method. The buffer pH was adjusted to 9.5 by 0.1M NaOH. A few serum and urine samples were analyzed with an applied voltage of 30 kV. For the CZE method a running buffer with 0.7% (w/v) boric acid and 0.7% (w/v) sodium carbonate was used. The pH was adjusted to 9.5 with 0.1N NaOH. Samples were analyzed with an applied voltage of 27 kV.

At the beginning of each day the capillary was regenerated by rinsing for 5 min with H_2O, 10 min with 0.1M HCl, 5 min with H_2O, 5 min with 1.0M NaOH, 5 min with H_2O, 10 min with 0.1M NaOH and finally 2 min with H_2O. Before each analysis the capillary was rinsed with running buffer for 2 min. After each analysis the capillary was rinsed for 30 sec with H_2O, 2 min with 0.1 M NaOH and 2 min H_2O.

2.5 Preparation of samples and standard

MEKC:

Serum samples were filtered with 0.2 µm syringe filters before they were injected into the capillary (Thormann, 1992). All standards were prepared by dissolving individually in 0.01 N NaOH. Creatinine and allantoin were prepared at 2.0 mg/ml, hypoxanthine at 1.5 mg/ml, uric acid at 0.6 mg/ml, allopurinol at 0.35 mg/ml and oxypurinol and xanthine at 0.1 mg/ml.

CZE:

The serum sample (50 µl) was first spiked with 3 µl of 1 mg/ml 9-methylxanthine before it was deproteinated with 97 µl cold (-10°C) acetonitrile. The final concentration of 9-methylxanthine was 20 µg/ml. All standards were prepared by dissolving

individually in 0.01 N NaOH. Allantoin was prepared at 2.0 mg/ml, hypoxanthine, uric acid, allopurinol, oxypurinol and xanthine at 1.0 mg/ml and 9-methylxanthine at 0.88 mg/ml.

2.6 Preliminary inhibition experiments.

Allopurinol is the classical inhibitor of xanthine oxidase and potassium oxonate is a known inhibitor of the uricase enzyme (Fig. 4.2A). Preliminary studies were done to determine whether changes in the levels of the xanthine and uric acid could be detected in rat urine and serum upon inhibition of these enzymes. This is important to evaluate the applicability of the analytical method. This was only done with the CZE since it was found to be the better of the two methods.

Blood and urine were collected from rats at t=0h before any treatment. The blood was obtained by making an incision in the tail and collecting from the abdominal tail vein. Three rats were then injected 250 mg/kg potassium oxonate subcutaneously and three were orally fed 20 mg/kg allopurinol. Blood and urine were again collected two hours later at t=2h. Urine samples were marked and immediately snap-freezed in liquid nitrogen. Blood samples were centrifuged at 11000g for five minutes and the serum collected. The serum was then snap-freezed. All samples were stored at –20°C for later analysis.

3 Results and Discussion

All the compounds of interest are shown in Fig. 4.1, 4.2A and 4.2B. The level of creatinine is an indication of the metabolic size of individual animals and was therefore included as an analyte that could potentially be detected. In the end however it

was not possible to quantify this metabolite with capillary zone electrophoresis, since it had no charge in the working pH range.

3.1 Comparison of the two methods

In both methods the migration sequence of the analytes were the same (Fig. 4.3A & 4.3B). This is an indication that the surfactant did not add selectivity to the analysis in the MEKC method. The method was originally intended for methylxanthines where the surfactant might have exploited the hydrophobicity changes better than with hydroxypurines. The MEKC method still provided good resolution of all the metabolites of interest. The sequence of elution can easily be explained when the migration times (from the CZE method) are compared to the mass to charge ratios of the analytes at pH 9.5 (Table 4.1, Fig. 4.4). The ionic charges on the analytes were calculated according to Equation 1 with the pKa values shown in Table 4.1. When a pKa value is two or more units below the pH value, the specific group is seen as fully dissociated and will have a charge of –1 (providing dissociation takes place as for a weak acid). The apparent electrophoretic mobility (μ_{app}) is the vectorial sum of the electrophoretic mobility (μ_{ep}) and the electro-osmotic mobility (μ_{eo}) (Equation 2).

$$\alpha = \frac{1}{10^{(pKa-pH)}+1} \qquad (1)$$

$$\mu_{app} = \mu_{ep} + \mu_{eo} \qquad (2)$$

The mobility is calculated with Equation 3.

$$\mu = \frac{L_d / t_M}{V / L_t} \qquad (3)$$

Where L_d is the column length to the detector, L_t is the total length of the column and

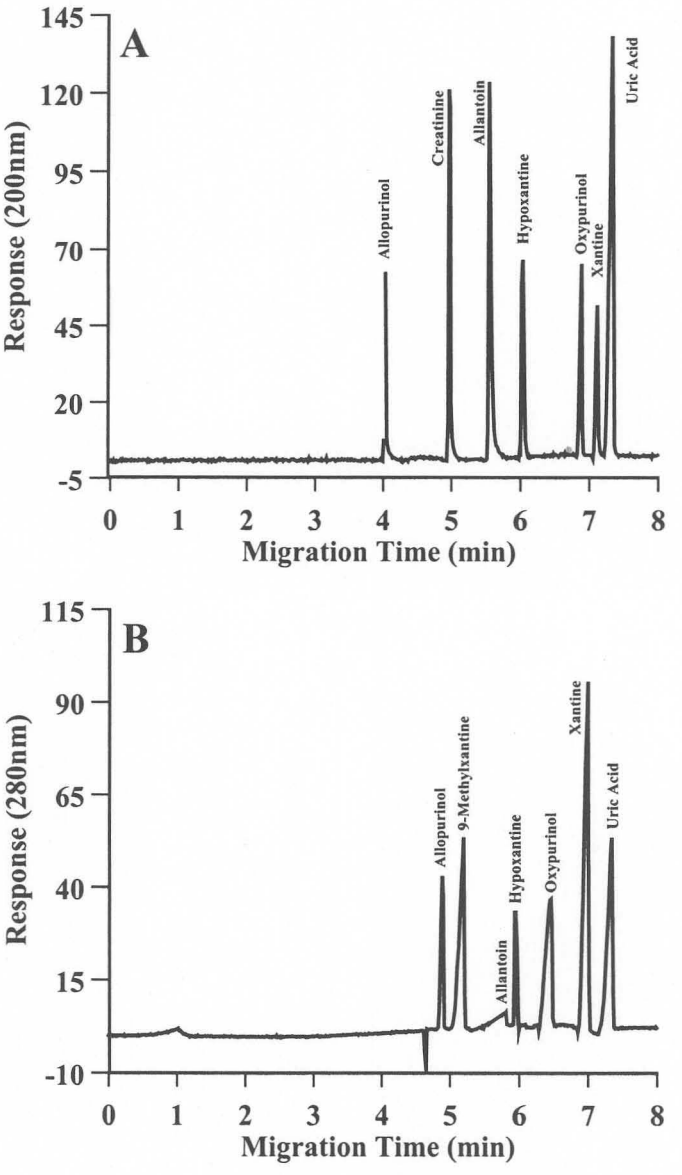


Figure 4.3 The separation of XO substrates, products and inhibitors. A: The MEKC method Conditions: 75 mM SDS, 6 mM $Na_2B_2O_7$, 10 mM Na_2HPO_4 pH 9.5. Separation with 30 kV. B: The CZE method Conditions: 0.7% (w/v) H_3BO_4, 0.7% (w/v) $NaHCO_3$ pH 9.5. Separation with 27 kV.

V the applied voltage. When calculating μ_{app}, the t_M equals the migration time of the analyte and for μ_{eo}, the t_M is equal to t_0 which is the migration time of the EOF. A larger negative charge on the analyte will result in a larger negative electrophoretic mobility and a smaller apparent electrophoretic mobility. The smaller the apparent electrophoretic mobility the longer the analyte needs to pass the detection window resulting in a longer migration time.

Table 4.1 The calculated charges and mass:charge ratios from pKa values as well as the calculated electrophoretic mobilities for the analytes using the CZE method.

Analyte	pKa	Charge at pH 9.5	Mass g/mol	Charge/Mass Ratio	Mobility $m^2.V^{-1}.s^{-1}$
Allopurinol	10.20	-0.166	136.11	-0.00122	-0.00966
9-MX	6.30	-1.000	165.14	-0.00606	-0.02388
Allantoin	8.96	-0.776	157.12	-0.00494	-0.04054
Hypoxanthine	1.98, 8.94, 12.10	-0.784	135.11	-0.00580	-0.04414
Oxypurinol	7.74	-0.983	151.11	-0.00651	-0.05651
Xanthine	0.80, 7.44, 11.12	-1.017	151.11	-0.00673	-0.06009
Uric acid	5.40, 10.30	-1.137	167.11	-0.00680	-0.06773
Creatinine	3.55	0	0	0	0

With the MEKC method creatinine could be separated in addition to all the other compounds for it separated solely on hydrophobic interactions at pH 9.5 where it is neutral. Unfortunately the LOD's were very poor and the MEKC method could not be used. Even with solid phase extraction the detection limits remained poor. Only uric acid could be detected in urine and the baseline level in serum was too low to be detected.

With the CZE method the LOD was improved with a factor of between two and ten. The enhanced sensitivity was achieved by means of stacking. With stacking the

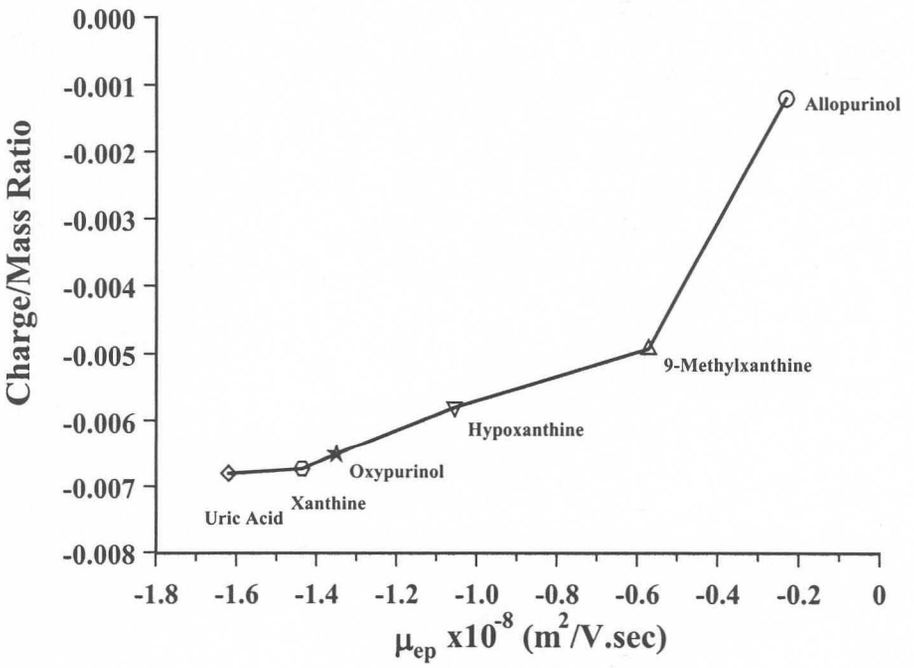


Figure 4.4 The linear relationship between the mobilities and charge/mass ratios of the different analytes in CZE mode.

sample solution has a lower conductivity than the running buffer. This is achieved by preparing the sample in water or adding a high concentration organic solvent. A long plug of sample is introduced pneumatically into the capillary and a separation voltage applied across the column. Because the solution in the sample plug has a low concentration of ions, the resistivity in the sample plug region will be higher than the resistivity in the rest of the column. Consequently, a high electric field is set up in this region. As a result, the ions will migrate rapidly under this high field toward the steady-state boundary between the lower concentration plug and the running buffer. Once the ions pass the concentration boundary between the sample plug and the rest of the column, they immediately experience a lower electric field and slow down, thus causing a narrow zone of analyte to be formed in the running buffer region (Burgi,

1991). For optimum stacking the conductivity difference between the sample solution and the running buffer must be between 10 (Vinther, 1991); (Burgi, 1991) and 100 fold (Mikkers 1979). The sample plug should not fill more than 10% of the column (Roder, 1995). These restrictions are necessary to limit the occurrence of laminar flow that causes peak broadening. The laminar low is generated due to mismatch EOF between the sample solution and running buffer. The EOF pressure difference is alleviated by laminar flow that occurs as the sample plug pushes the running buffer forwards (Chien, 1992). The field-amplified sample injection (FASI) method could increase the loading efficiency even further but the loading is performed electrokinetically resulting in bias loading, for analytes with a large negative charges are loaded preferentially due to higher mobilities.

High concentrations of organic solvent also precipitate the serum proteins resulting in sample clean-up. In a study conducted, the protein precipitation efficacy of acetonitrile, acetone, ethanol and methanol was determined (Blanchard, 1981). It was found that when two volumes of acetonitrile at room temperature were added to one volume serum 99.7% of the proteins precipitated out. For the other organic solvents the efficacy ratings were acetone (99.4%), ethanol (98.3%) and methanol (98.7%). In this method two volumes acetonitrile were added to one volume sample resulting the precipitation of serum proteins and a reduction in the conductivity of the sample solvent. It had an added advantage in that it had the highest boiling point of the four organic solvents, resulting in less evaporation during automation and more reliable results. The creatinine co-eluted with the EOF and could not be analyzed with this method. The efficiency of the stacking that occurred is underlined by the column efficiency that was obtained. The initial peak width (upon injection) for the CZE method

was ten times more than for the MEKC method, yet the column efficiency obtained with CZE compared well with that of the MEKC method (Table 4.2).

Table 4.2 Comparison of the MEKC and CZE methods, focusing on the column efficiency, peak symmetries and limits of detection for the respective methods.

Analyte	MEKC			CZE		
	Plate-number	Peak symmetry	LOD (200nm) µg/ml	Plate-number	Peak symmetry	LOD (280 nm) µg/ml
Creatinine	263508	0.656	100.0			-
Allopurinol	216132	0.786	17.5	132184	3.979	5
9-Methyxanthine				81070	7.784	<5
Hypoxanthine	121887	0.232	75.0	153906	0.886	5
Allantoin	165893	0.333	125.0	9228	10.034	8.33
Oxypurinol	215077	1.766	5.0	41478	2.865	2.5
Xanthine	162243	0.983	5	142823	2.514	0.5
Uric acid	92554	3.46	30	81031	6.628	2

The pH condition of 9.2 used by (Shihabi, 1995) did not resolve the xanthine and uric acid well in our system. The charge difference between xanthine (-1.01) and uric acid (-1.07) is a mere 0.06 at pH9.2 compared to a difference of 0.114 at pH 9.5. A pH optimum study was done to determine conditions that resolve the analytes completely. (Fig. 4.4) It was found that a pH of 9.5-9.6 resolved the peaks completely with a slight increase in the analysis time. We selected to work at pH 9.5 even though allopurinol and 9-methylxanthine migrated close together. Both the allopurinol and 9-methylxanthine peaks were very sharp, resulting in good resolution even when migrating near each other. The total analysis time was slightly shorter when working at pH 9.5 rather than pH 9.6. The MEKC method also required a pH of 9.5 to separate the peaks properly, instead of the pH 9.0 used by (Thormann, 1992). The pH curve indicates an

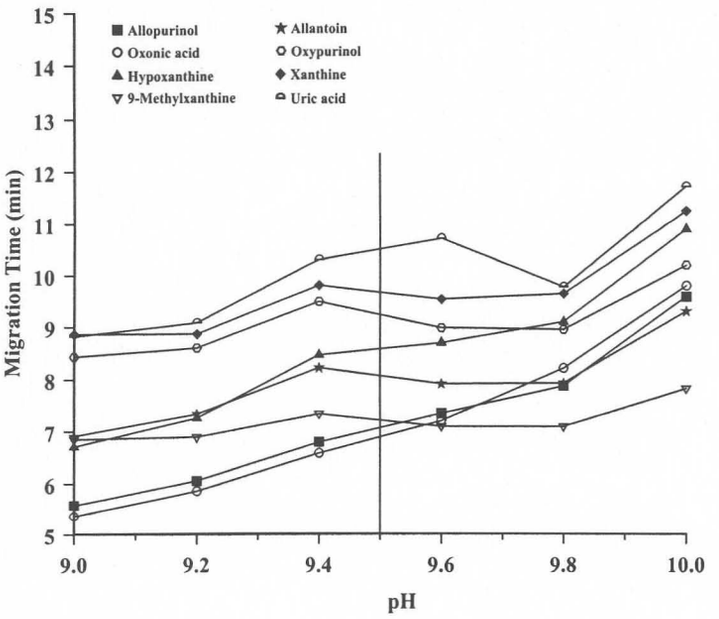


Figure 4.5 The influence of pH on the migration times of the analytes in CZE mode.

Table 4.3 A summary of the calibration curve data for the analytes with the CZE method.

Analyte	Linear range µg/ml	r	Linear equation for calibration curve	%RSD
Allopurinol	5-1000	0.999	y = 3.07e-3*x	2.40
Hypoxanthine	5-1000	0.999	y = 1.68e-3*x	1.29
Allantoin	8.33-2000	0.999	y = 1.10e-2*x	9.58
Xanthine	0.5-500	1.000	y= 9.62e-3*x	0.56
Uric Acid	2-1000	0.999	y = 8.30e-3*x	3.39
Oxypurinol	5-100	0.998	y = 5.91e-3*x	1.76
9-Methylanthine	5-500	0.996	y = 2.41e-3*x	6.58

increase in the anionic properties of the metabolites with an increase in the pH. As the analytes became more anionic their negative electrophoretic mobility increased and therefore the migration times became longer. The fact that the compounds separate solely on their charge to mass ratio's can be seen in the peak symmetry values that were obtained. Most of the analytes had either leading fronts or lagging tails which is

an indication of differences between the charge to mass ratio's of the analytes and that of the surrounding running buffer ions. Both with CZE and MEKC the peak symmetry was poor. Calibration curves were setup for the analytes in the CZE method and all correlation coefficients were better than 0.995 (Table 4.3)

3.2 Serum and Urine Samples

The suitability of the CZE was determined by its ability to detect changes in the levels of hypoxanthine, xanthine and uric acid in serum and urine samples of rats. Rats were treated with allopurinol or potassium oxonate. Upon treatment with allopurinol the levels of xanthine and oxypurinol increased in both urine (Fig. 4.6A&B) and serum (Fig. 4.7A&B) samples. There was a concurrent decreased in the levels of uric acid, as was expected. The levels of uric acid increased significantly in serum (Fig. 4.8A&B) and urine (Fig. 4.9A&B) samples taken from animals treated with potassium oxonate. No accumulation of hypoxanthine or xanthine occurred. The reverse reaction where xanthine is produced from uric acid is to slow to produce any xanthine. Although uric acid is capable of inhibiting XO, the K_i is very high (250 μM) and the serum uric acid levels are probably still to low to have a significant inhibitory effect on XO. In the urine samples a lowering in the allantoin level at 280 nm was difficult to detect. The LOD for allantoin at 280 nm is very poor. Detection at 230 or 200 nm was also very difficult due to noise and the presence of other peaks that are not well resolved from allantoin.

The baseline levels of hypoxanthine and xanthine were not always detectable in serum. Significant increases, as expected with the inhibition studies, would however be easily detectable. The compounds were identified mainly on their UV spectrums,

since the migration times varied by up to 2 minutes. The UV spectrums of 9-methylxanthine, uric acid, oxypurinol and xanthine are compared to that of the pure

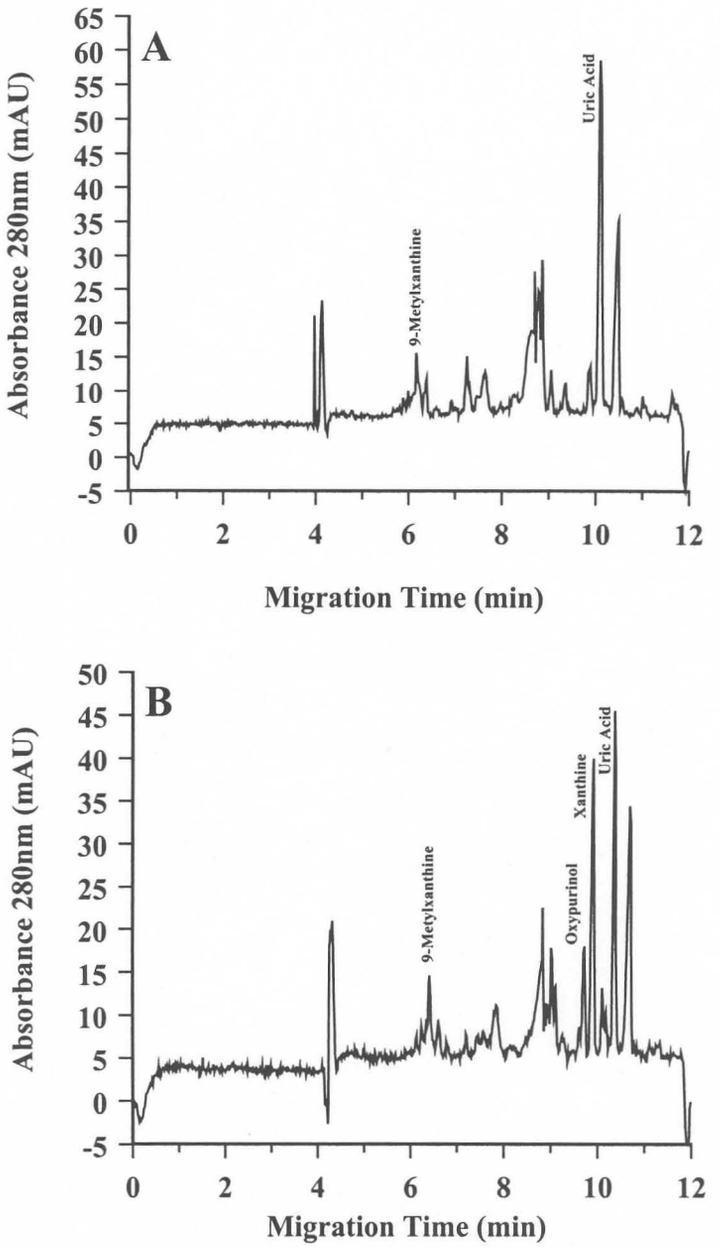


Figure 4.6 A: Analysis of control rat urine (untreated) separated with the CZE conditions as in Fig 4.3. **B**: Analysis of rat urine from rats orally dosed 20 mg/kg allopurinol with the same analytical conditions.

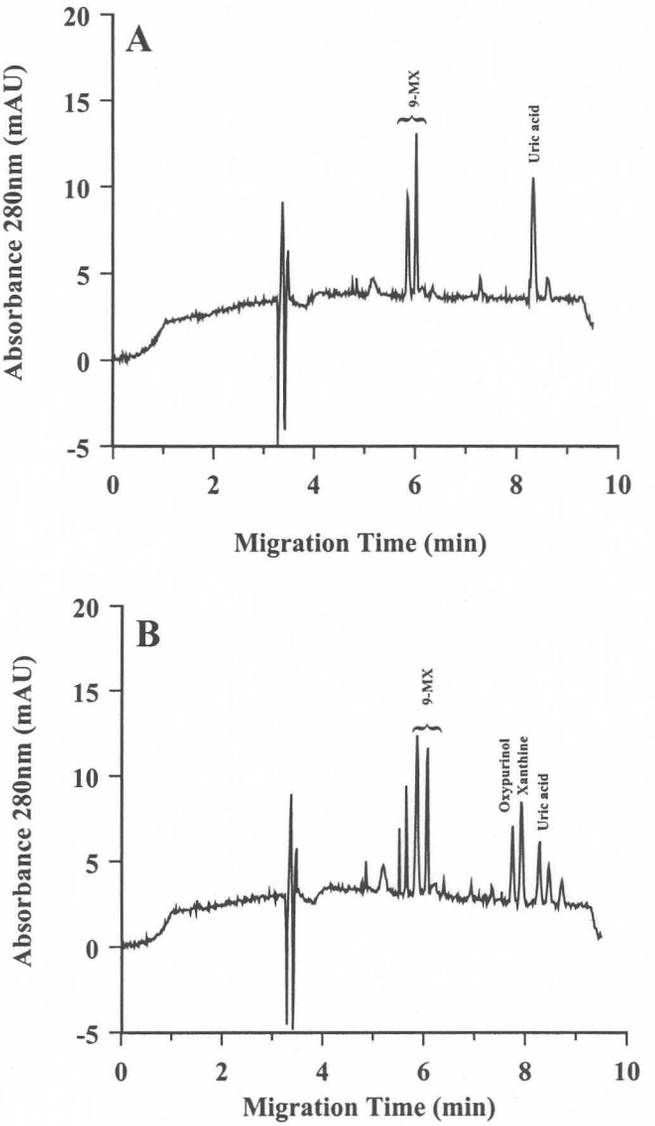


Figure 4.7 **A**: Analysis of control rat serum (untreated) with same conditions as in Fig. 4.6. **B**: Analysis of rat serum from rats orally dosed 20 mg/kg allopurinol with same analytical conditions.

standards (Fig. 4.10A-D). It was found that the buffer composition had a huge influence in the repeatability of separations. When repeated runs are done without buffer renewal in the anodic buffer vial changes can be observed in the relative migration order and migration times. Continuous analysis with large buffer vials

(5ml) showed less variance than the same analyses with small buffer vials (2ml) (Schmutz, 1994). In this study the buffer vials have a volume capacity of only 0.8 ml. This caused small inter-run variation due to buffer property changes of the anodic vial. A significant deterioration in electropherograms were found if more than six runs were done without replenishing the anodic vial.

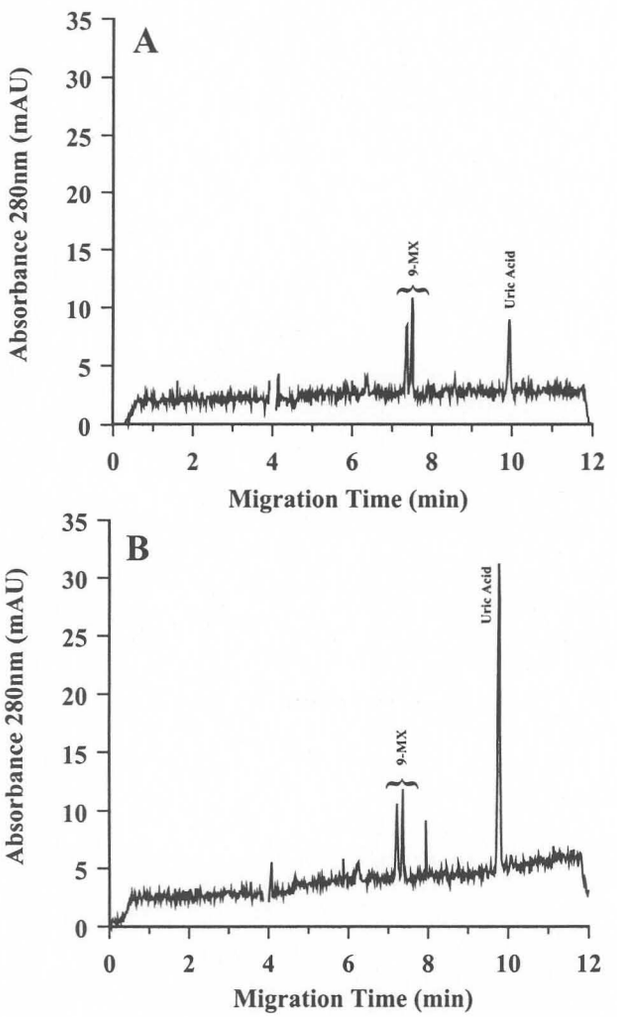


Figure 4.8 **A**: Analysis of control rat serum (untreated) with same conditions as in Fig 4.6. **B**: Analysis of rat serum from rats subcutaneously dosed 250 mg/kg potassium oxonate with same analytical conditions.

The sample matrix composition played a significant role on the migration time of the analytes. Analytes from urine or serum samples migrated slower than the standards of the same analyses. Analytes from urine samples migrate slower than the same analytes from serum samples. This variation might be the result of protein-analyte interactions or differences in the conductivites of the samples. Double peaks were

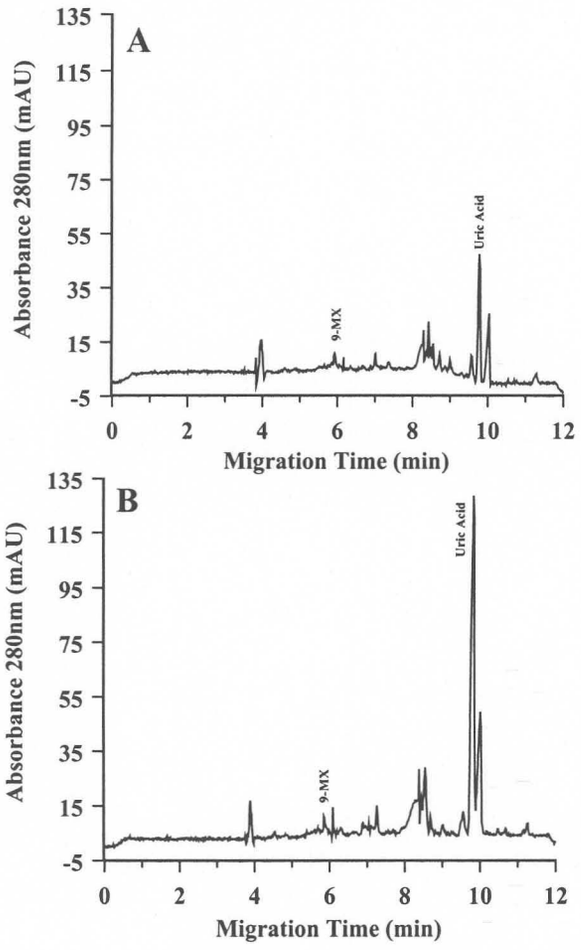


Figure 4.9 **A**: Analysis of control rat urine (untreated) separated with the CZE conditions as in Fig. 4.6. **B**: Analysis of rat urine from rats subcutaneously dosed 250 mg/kg potassium oxonate with the same analytical conditions.

observed for some of the analytes at low concentrations. The 9-methylxanthine displayed two well separated peaks in serum samples. Both peaks had the characteristic UV spectrum of 9-methylxanthine and co-integration of the two peaks added up to the amount that the samples were spiked with. This phenomena was previously reported by authors who tried direct serum injection with MEKC ((Schmutz, 1994)

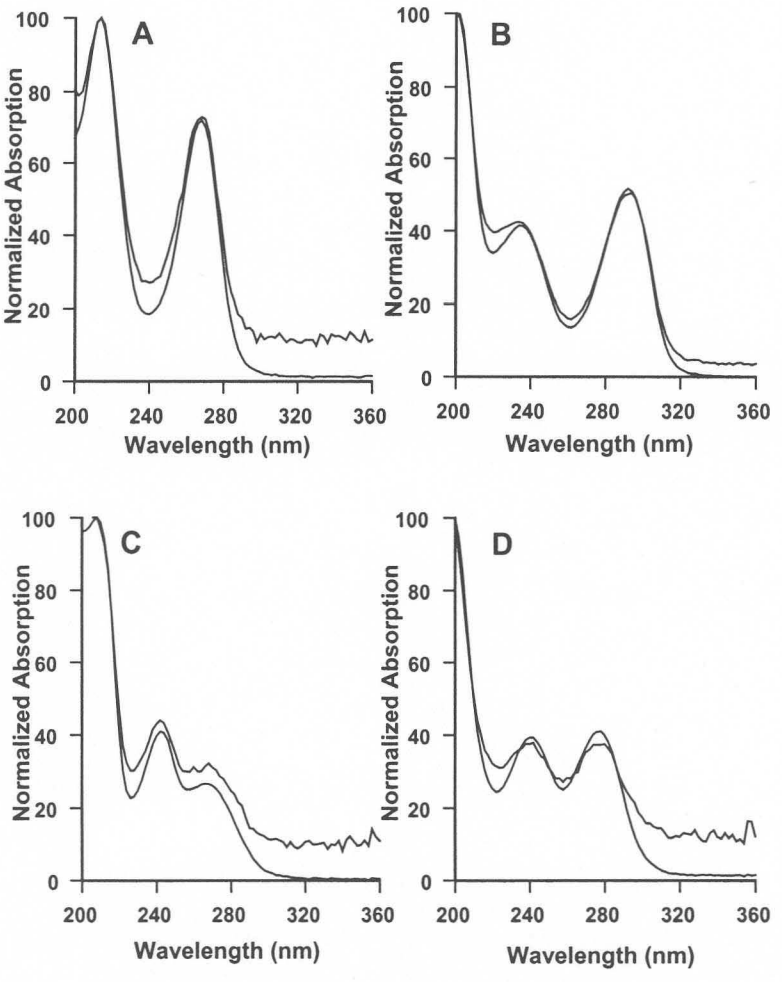


Figure 4.10 Comparison of UV spectra of analytes (**A**: 9-methylxanthine, **B**: uric acid, **C**: oxypurinol, and **D**: xanthine) from rat serum or urine with that of pure standards.

and with micellar liquid chromatography (Haginaka, 1987), (Palmisano, 1989). It was suggested that the analytes interacted with proteins and resulted in two closely related migration times. It may be that low amounts of analytes can interact with residual proteins still left in the sample, causing the double peaks.

Inconsistent migration times can also be due to the high ion concentration in the running buffer required for the stacking process. The positive sodium ions can cause variable screening of the negative silica hydroxyl charges on the column resulting in an erratic EOF. High Joule heating also occurred due to high voltage and current, causing variation in the buffer viscosity. Unfortunately it was impossible to change the buffer, for the high ion concentration required for sample stacking (and better LOD's) decreased the stability of the method due to the reasons mentioned above. In the trade-off it was decided that the LOD's were more important than reproducible migration times since peak identity could be confirmed with the UV spectra.

4 Conclusion

Two different CE modes were compared to assess which one will serve the purpose of analyzing purine metabolites in urine and serum the best. A direct serum injection MEKC method was compared to a CZE method utilizing the principles of sample stacking. Both methods were capable of separating the analytes of interest, but only the CZE method could reach the desired limits of detection. In the end only hypoxanthine, xanthine, uric acid, oxypurinol and 9-methylxanthine were analyzed. Only pH as electrophoretic variable was tested to determine the optimum pH conditions. The CZE method was suitable to detect and monitor drug induced changes in the uric acid and xanthine levels in both rat serum and urine. The total analysis time

varied according to the type of samples analyzed, with urine samples taking the longest. The separation time for the urine samples was 12 minutes with 7 minutes column conditioning. The analytical method and limits of detection may be enhanced when sample pre-concentration or different injection procedures are implemented.

In the end the CZE method was reliable enough to do automated sample analysis of both serum and urine samples from rats treated with drugs altering the purine metabolism. No standard runs were required in between the automated runs since all analytes were identified based on their UV spectrums. A total of more than 50 runs could be done in a 24 hour period.

Chapter 5

In vivo Xanthine Oxidase inhibition studies

1 Introduction

Few studies have been undertaken to collect data of the pharmacological effects of tea polyphenols in animal models. The majority of studies focused on the anticarcinogenic and antimutagenic activities of tea polyphenols. The anticarcinogenic activities of tea polyphenols are evaluated by their ability to a) prevent the biotransformation of a pro-carcinogen to a carcinogen as measured in urine (Dashwood, 1999), b) prevent DNA-adduct formation, c) reduce the amounts and sizes of tumors in lungs and the colon (Narisawa, 1993) and d) induce the activities of specific phase I and phase II detoxification enzyme (Sohn, 1994). The antimutagenic properties were tested in animal models where mutations were induced with UV radiation or oral administration of 12-O-tetradecanoylphorbol-13-acetate (TPA). The prevention of mutagenesis is evaluated by a) determining the amounts and sizes of the induced tumors (Conney, 1999), (Landau, 1998) and b) measuring mRNA levels of tumor-promotion marker molecules such as interleukin-1α (Katiyar, 1995).

Metabolic studies are normally conducted with rats as the animal model. No specific strain was preferred for the pro-carcinogen biotransformation studies. Previous studies have used several strains e.g. CDF (F344), Cl/Br, Sprague Dawley, BDVI and Wistar rats. In all the studies young adult (6-8 weeks) or adult (10 weeks) male rats were used. Female mice were used as animal model where the antimutagenic activities were evaluated. The SENCAR and A/J strains were used frequently.

XO inhibition studies were conducted in both rat (male Sprague-Dawley) and mice (male ICR) models (Osada, 1993), although rat model seemed to be used more predominantly. Again there was no preference towards the strains that were used. Studies have been conducted with Sprague-Dawley and Wistar rats. From a practical point of view, adult male rats are more suitable. Their large body weight makes it possible to collect serum and urine in larger volumes or at more frequent time intervals.

The dosage of tea, in the drinking water, used for treatment in animal models varied between 0.002% (w/v) and 6.25% (w/v). In all studies tea was available *ad libitum* to the groups that were treated with it. If it is assumed that a full grown rat of 275 gram drinks approximately 20 ml of fluid per day and the tea extracts contain 10% polyphenols (Wiseman, 1997), then the doses can be converted to 0.145 mg/kg – 453 mg/kg body weight. The dosage of XO inhibitors, such as allopurinol and TEI-6720 (Osada, 1993) are generally in the range of 5 – 50 mg/kg body weight. In all the studies the control groups had only water to drink.

2 Materials and Method

2.1 Experimental Strategy

Unlike humans, rats catabolize uric acid further to allantoin. This results in allantoin being the major excretion product of purines in the urine as opposed to uric acid in humans. Rats were treated with potassium oxonate to inhibit the enzyme uricase and prevent uric acid from being turned into allantoin. This effectively renders the rats hyperuricemic, simulating hyperuricemia in humans. For each hyperuricemic group of rats there was a control group.

The best *in vitro* inhibitor was selected for *in vivo* inhibition experiments in a Sprague Dawley rat model. Three different inhibitors were tested namely allopurinol, EGCg, and a tea polyphenol mix (TPM). The polyphenolic content of TPM is listed in Table 5.1. TPM was included in the study to examine the possibility of synergistic inhibition of XO by several catechins.

Table 1. The catechin contents in Polyphenon-70S (TPM in this study). Caffeine content is less than 0.5%

Compound	Weight %	Molecular weight
(-)-EC	18.3	290.28
(-)-EGC	8.6	306.27
(-)-EGCg	35.9	458.38
(-)-GCg	3.5	458.38
(-)-ECg	11.2	442.38
(-)-Cg	0.0	442.38
Total	77.5	

2.2 Animals

Inbred male Sprague-Dawley strain rats of 4-6 weeks of age were obtained from South African Vaccine Producers (Modderfontein, RSA). They were kept in single cages for one week to acclimatize to the environment. They were transferred to metabolic cages to enable urine collection after treatment commenced. All animals were kept in an air-conditioned room and given standard chow and water *ad libitum* for the duration of the study.

2.3 Materials

The (-)-EGCg and Polyphenon-70S (TPM) were gifts from Mutsui Norin (Tokyo, Japan). The allopurinol, oxypurinol, xanthine, hypoxanthine, uric acid, potassium oxonate and Gum Arabic were obtained from Sigma Chemical Company (St Louis, MO, USA). The internal standard 9-methylxanthine was obtained from ICN Pharmaceutical (CA). All buffer salts and solvents needed for running buffers were of analytical grade. All solutions were prepared with distilled water that was deionized with a Milli-Q system (Millipore Corp., Bedford, MA. USA).

2.4 Test drugs

Allopurinol was tested at three concentrations, namely 5, 20 and 50 mg/kg. The EGCg and TPM were tested at 50 and 100 mg/kg. The inhibitors were prepared as suspensions in water with 5% Gum Arabic at concentrations of 5, 20 and 50 mg/ml for allopurinol and 50 and 100 mg/ml for both EGCg and TPM. Control groups were also included for both the normal and hyperuricemic rats. Control animals were treated with carrier only.

2.5 Treatment protocol

The rats were randomly divided into groups of six. The groups of normal rats were given a single dose of the test compound orally at t=0h. Blood and urine samples of t=0h were collected the day before the actual experiment. Samples for t=2h-8h were collected every two hours up to eight hours after the treatment. Groups of hyperuricemic rats were injected with 250 mg/kg potassium oxonate at t=-2h and dosed with a single dose of test compound at t=0h. Blood and urine samples for t=-2h were also collected on the day before the actual treatment. The samples for t=0-8h

were collected every two hours afterwards up to eight hours. The control samples of t=0h for normal rats and t=-2h for hyperuricemic rats were collected the previous day to familiarize the animals to being handled, reduce stress on the animals and to be able to collect larger volumes of blood over all the intervals. Blood samples (0.5-1.0 ml) were centrifuged immediately at 11000g for five minutes before the serum supernatants were collected. The serum (100-400 µl) and urine samples were snap-freezed in liquid nitrogen and all samples were marked and stored away at –20°C until they were analyzed. The samples of each animal was analyzed individually.

2.6 Measurement of analytes in urine and serum samples

Samples were spiked and deproteinated. The levels of uric acid, xanthine, hypoxanthine, allopurinol and oxypurinol were quantified with the CZE method as described in Chapter 4.

2.7 Statistical analysis

The data from both *in vitro* and *in vivo* experiments are presented as means ± SEM. The *in vivo* results were statistically analyzed using the Student's t-test (unequal variance).

3 Results

3.1 Hypouricemic effects of inhibitors on hyperuricemic rats.

Upon treatment with potassium oxonate a significant increase in the uric acid levels could be detected in both serum and urine (Fig. 5.1). The uric acid levels were elevated between 3 and 5 fold in both serum and urine after 2h and it took longer than 8h for them to return to normal levels (Fig 5.2). With progression of time the uric acid

levels decreased since the inhibition effect of the potassium oxonate wore of due to excretion. The oxonate did not interfere with the analysis, for it could not be detected with the CZE method at 280 nm with the concentrations used.

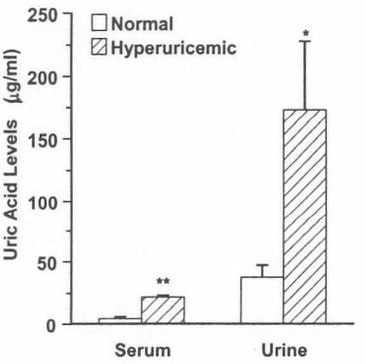


Figure 5.1 The hyperuricemic effect of 250 mg/kg potassium oxonate on serum and urine urate levels of normal rats. The data presents the mean with SEM of 6 rats. The serum and urine results for normal rats t=–2h (control) and t=0h (2 h after potassium oxonate treatment of normal rats) were compared. The results were statistically significant with * representing $P < 0.05$ and ** representing $P < 0.0001$.

Treatment with allopurinol reduced the elevated uric acid levels (Fig. 5.3). The serum uric acid levels decreased faster in allopurinol treated rats than in the control rats (Fig 5.2). The levels of uric acid were decreased in a dose-dependant manner by treatment with allopurinol. Both the 20 and 50 mg/kg allopurinol reduced the uric acid levels to below the levels expected in normal rats. The 50 mg/kg EGCg treatment decreased the uric acid levels but no statistically significant change could be detected in the uric acid levels with the 100 mg/kg EGCg treatment. Both the TPM doses had no significant effect on the uric acid levels. The serum uric acid levels decreased in a similar manner the control animals and the animals treated with EGCg or TPM (Fig.

5.2). None of the polyphenol inhibitors inhibited XO since no increases in xanthine levels were detected. With allopurinol treatment, the levels of xanthine increased in a dose-dependent manner.

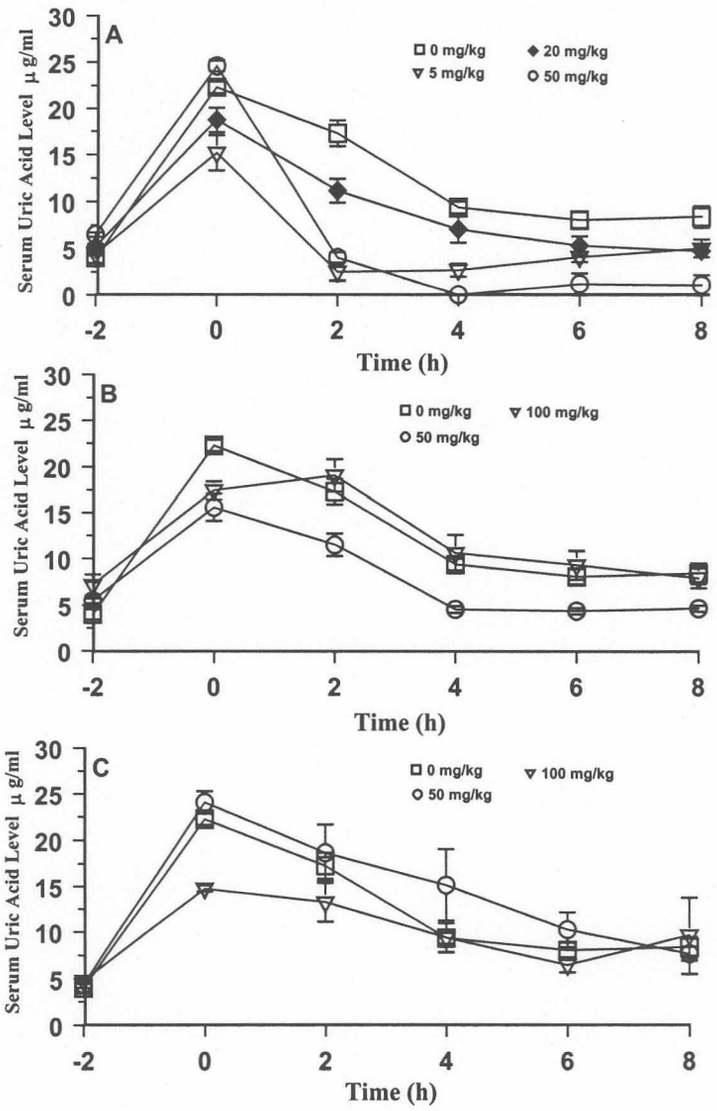


Figure 5.2 A Time course of the effect of allopurinol on serum uric acid levels of hyperuricemic rats. (□) Square, (∇) triangle, (◆) diamond and (○) circle represents doses of 0, 5, 20 and 50 mg/kg respectively. **B**: Time course of the effect of EGCg on serum uric acid levels. (□) Square, (○) circle and (∇) triangle represents doses of 0, 50 and 100 mg/kg respectively. **C**: Time course of the effect of TPM on serum uric acid levels. (□) Square, (○) circle and (∇) triangle represents doses of 0, 50 and 100 mg/kg respectively. The data represents the means and SEM for 6 rats.

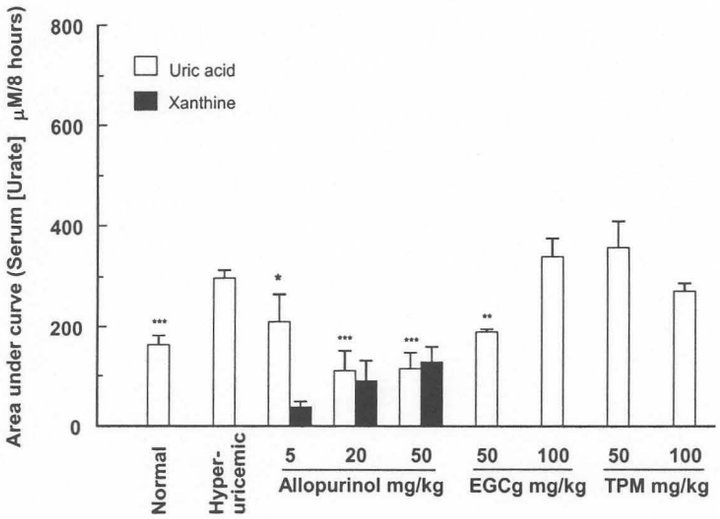


Figure 5.3 The effect of different doses allopurinol, (-)-EGCg and TPM on the serum urate and xanthine levels in hyperuricemic rats. The results were shown as the area under curve from 0-8h for both urate and xanthine concentrations (µM). The data represents the mean with the SEM for 6 rats. Statistical analysis shows significant difference between the treated groups and the hyperuricemic control with * $P < 0.02$, ** $P < 0.005$ and *** $P < 0.0002$.

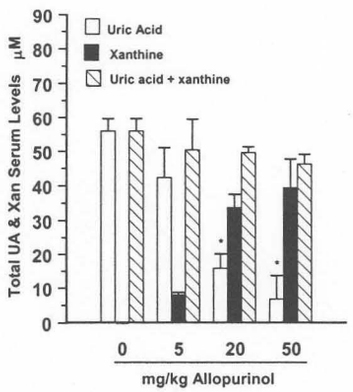


Figure 5.4 The total amount of xanthine and uric acid 4h after the treatment of hyperuricemic animals was conserved. The graph shows that with an increase in allopurinol dosage the concentration xanthine increase (solid bar) and the uric acid concentration decrease. The total amount of uric acid and xanthine (hashed bar) is nearly equal indicating that the 1 µM xanthine is detected for every 1 µM uric acid that has not been formed. The data represents the mean and SEM from 6 rats. The decrease in uric acid concentrations were statistically significant with * representing $P < 0.001$.

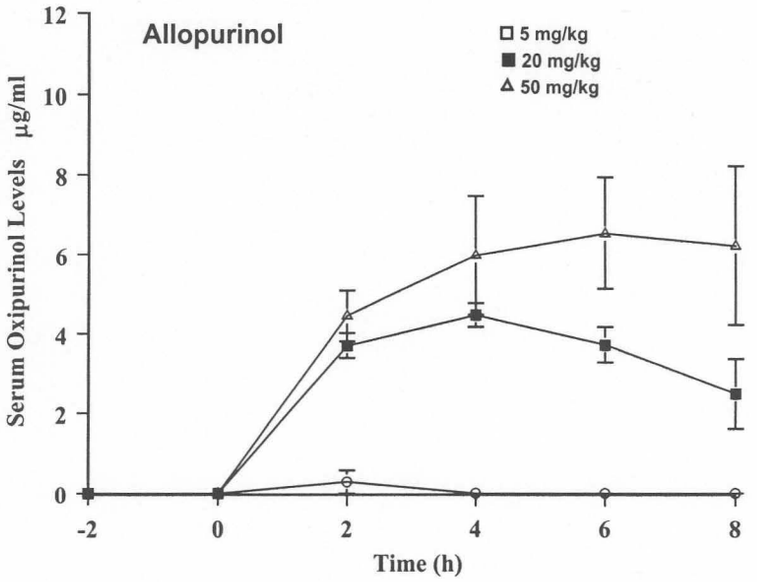


Figure 5.5 The serum levels of oxypurinol increased with an increase in the allopurinol dosage concentration. The (□), (■) and (▲) represent 5, 20 and 50 mg/kg respectively. For low levels of oxypurinol (5 mg/kg) the maximum serum level is reached 2h after dosage and for higher dosages (20 and 50 mg/kg) the maximum serum levels are reached 4-6h after treatment. The data represent the mean ± SEM for 6 rats.

The total concentrations of uric acid and xanthine were conserved as shown in Fig. 5.4. High levels of oxypurinol could be detected in body fluids 2 hours after allopurinol treatment. Very little or no allopurinol was detected after 2 hours. The oxypurinol levels stayed elevated throughout the 8 hours of monitoring (Fig. 5.5). In agreement with literature, allopurinol is converted to oxypurinol very rapidly and the the *in vivo* inhibition is the result of oxypurinol and not allopurinol (Massey, 1970).

3.2 Hypouricemic effects of inhibitors in normal rats

From Fig. 5.6A it can be seen that allopurinol reduced the uric acid levels in normal rats in a dose-dependent manner. The biggest decrease in uric acid levels occurred at

2 hours after treatment, but maximal inhibition only occurred at 4 hours for the two higher doses. With the 5mg/kg dose the uric acid levels returned to normal after 4 hours. With the 20 mg/kg dose the uric acid levels have not returned to normal even after 8 hours, and with the 50 mg/kg dose uric acid levels were still undetectable after 8 hours. As in the hyperuricemic group, the xanthine levels increased with higher doses of allopurinol, indicating that XO was inhibited. From Fig. 5.6B and C it can be seen that EGCg and TPM caused a slight but statistical significant reduction in the uric acid levels. In the *in vivo* system, inhibition is observed as an increase in the concentration of xanthine in urine and serum. Although EGCg is a competitive inhibitor of XO with a relative high affinity for the enzyme *in vitro*, it failed to effect an increase in xanthine concentration in the serum or urine of treated rats. It seems that concentrations of up to 100 mg/kg EGCg had no XO inhibitory effect *in vivo* in a rat model.

3.3 Bioavailability of catechins

In pharmacokinetic studies it has been found that less than 1μM (0.67 mg/l) EGCg occurred in the serum of rats, after an intragastrical (i.g.) dose of 500 mg/kg body weight (Yang, 1999), (Nakagawa, 1997(b)). Less than 1% of the total administered EGCg became bioavailable. A large amount of the EGCg was found in the feces, suggesting a lack of absorption. After repeated doses, the highest catechin concentrations were found in the tissue of the esophagus, large intestines, kidneys, bladder, lungs and prostate. Some of these organs are in direct contact with the catechins as they are consumed and move through the digestive tract to be excreted. This may explain the catechin accumulation in the tissue of these organs. The fact that catechins are found in the lungs, kidneys and prostate is an indication that they are

absorbed and do have some organ specificity to some extent. Very little catechins

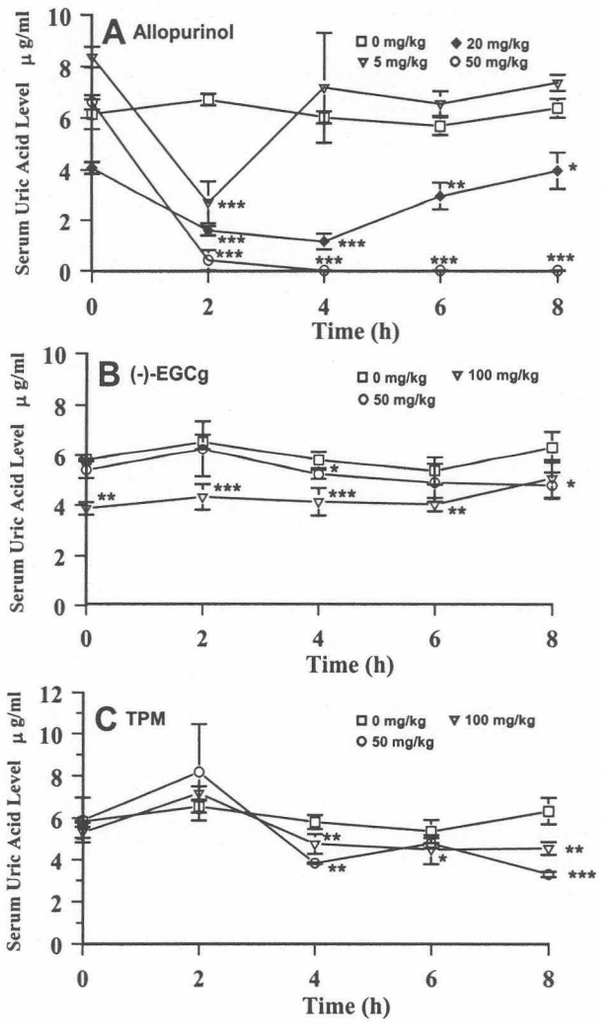


Figure 5.6 A Time course of the effect of allopurinol on serum uric acid levels of normal rats. (□) Square, (▽) triangle, (♦) diamond and (○) circle represents doses of 0, 5, 20 and 50 mg/kg respectively. **B**: Time course of the effect of EGCg on serum uric acid levels. (□) Square, (○) circle and (▽) triangle represents doses of 0, 50 and 100 mg/kg respectively. **C**: Time course of the effect of TPM on serum uric acid levels. (□) Square, (○) circle and (▽) triangle represents doses of 0, 50 and 100 mg/kg respectively. The data represents the means and SEM for 6 rats. Statistical significant differences are indicated with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

accumulated in the spleen, liver and thyroid. The distribution studies in Sprague-Dawley rats showed that only 48 ng/ml EGCg occurred in the liver 60 min after a single i.g. dose of 500 mg/kg body weight. The absorption of the different catechins also varied. After a single i.g. dose, only 14% of the EGC and 31% of the EC became bioavailable, as compared to only 1% for the EGCg. This is an important clue in the designing of catechin-like drugs with an increased systemic absorption in rats.

Bioavailability of catechins

In a pharmacokinetic study with radiolabeled EGCg in CD1 mice, it was found that EGCg was absorbed into almost all the organs. The blood and liver contained the most radioactivity after 1h. The amount of radioactivity in the serum converted to approximately 1.2 mg/l EGCg, although it is not certain how much of the EGCg has been biotransformed (Suganuma, 1998).

Few catechin bioavailability determination studies have been conducted with humans. From the available results, it is clear that the bioavailability of the catechins depends heavily on the doses that the subjects receive. In one study human subjects ingested 1.2 g of decaffeinated tea in warm water. Blood EGCg levels reached 0.046-0.27 mg/l (0.1-0.6 μM) after 1h. The majority of these catechins were already conjugated with glucuronic acid or sulfate (Lee, 1995). In another study human subjects drank a tea infusion with approximately 400 mg of total catechins. Both EGCg and ECg were detected in plasma at a maximum concentration of 2 μM after 2h (Pietta, 1998). In the most recent study humans were dosed i.g. with different amounts of EGCg, the highest being 525 mg. The plasma EGCg reached a maximum of 4.4 μM after 90 min (Nakagawa, 1997 (c)).

As far as the bioavailability is concerned, a mouse or human model may be more appropriate than a rat model. Humans may be able to absorb up to 4 times more and mice up to two times more EGCg than rats. The catechin levels in mice and humans are still in the low micromolar range.

Although the EGCg serum level is in the order of 1μM, this should be sufficient to inhibit XO, since EGCg has an inhibitory binding constant (K_i) of 0.76 μM. Despite of this, inhibition was not seen. Several reasons exist why this is not the case. Polyphenols are known to interact with proteins. Proanthocyanidins are phenolic polymers that have the ability to bind to proteins and even precipitate proteins (Hagerman, 1981). Proteins are precipitated the easiest by proanthocyanidins at pH's near their iso-electric points (pI). In accordance with this, proteins with acidic pI's such as bovine serum albumin have a higher affinity for polyphenols at pH 4.9 than at pH 7.8 and basic proteins such as lysozyme have higher affinities at the higher pH. The relative affinities of proteins and polypeptides for polyphenols are influenced by the size of the polymer. Proteins with a molecular mass of less than 20 kDa have a rather low affinity for polyphenols. Proline-rich proteins, such as some salivary proteins, have a particularly high affinity for polyphenols (Murray, 1994). Proteins with compact globular structures such as cytochrome c and myoglobin have lower affinities for proanthocyanidins and polyphenols than loosely structured globular proteins such as bovine serum albumin and histone F. Hydrogen bonding between phenolic hydroxyl and peptide carbonyl is a major force stabilizing proanthocyanidin-protein complexes. With loosely structured proteins there is an increased accessibility to the peptide backbone, hence a higher affinity of proanthocyanidin for such proteins. The main binding proteins in plasma are albumin and α_1-glycoprotein. Albumin

consists of a single polypeptide chain and is present in plasma at a concentration of 35-45 g/l in normal healthy individuals. Despite its large molecular weight (68.5 kDa), albumin is not retained exclusively in the plasma compartment, but is distributed extravascularly. Albumin binds both acidic and basic drugs, while α_1-glycoprotein binds only basic drugs (D'Arcy, 1996). No catechin-albumin binding studies have been conducted, but it is very likely that a large part of the serum EGCg will bind to the albumin, influencing its availability, distribution and elimination.

The biotransformation of catechins reduces the bioavailability even further. One pharmacokinetic study showed that the biotransformation of EC administered to male Wistar rats involves glucuronidation, sulfation and methylation (Piskula, 1998). UDP-glucuronosyl transferase activity was found in the liver and the intestinal mucosa. The highest glucuronosyl transferase activity was found in the intestinal mucosa, suggesting that the EGCg is biotransformed as absorption takes place. The highest phenolsulfo-transferase activity occurred in the liver. Catechol-O-methyl transferase activity was found in the liver and kidneys. Polyphenols and phenol-like compounds have been shown to be biotransformed by phenolsulfotransferases and glucoronosyl transferases in rats (*in vivo*) and by rat liver subcellular fractions (i*n vitro*) (Mulder, 1974). The majority of the conjugates that are formed in the liver and involve polyphenols are sulfo-conjugates. These conjugated polyphenols are eliminated predominantly via the biliary excretion path. The low levels of EGCg in the liver of rats could be a result of efficient biotransformation and excretion with the bile. The majority of the catechins in the plasma are in the conjugated forms only one hour after administration. The rapid conjugation of the polyphenols may be an indication that they have a high affinity for phase II enzymes. The polyphenols may have a higher

affinity for the active centers of the phase II detoxification enzymes than for the active center of XO. Since the catechins undergo extensive conjugation, it seems unlikely that their *in vitro* XO inhibition activity is retained *in vivo* in the rat model.

Intestinal bacteria also influence the bioavailability of catechins. In an *in vitro* study it was shown that human intestinal bacteria degrade both simple and gallated catechins. Simple catechins are degraded almost twice as efficiently as gallated catechins. Rat intestinal bacteria could degrade simple catechins, but not gallated catechins (Meselhy, 1997). Sulfated catechins are also formed in the intestines by intestinal bacteria arylsulfotransferase enzymes (Koizumi, 1991). It is not clear whether sulfated polyphenols are more readily absorbed from the intestines than the unmodified catechins.

The uric acid levels in normal rats treated with EGCg (Fig. 5.5B) and TPM (Fig. 5.5C) were slightly lower, but no xanthine substrate accumulation has been detected. EGCg may be a weak uricosuric agent, but this can only be confirmed when glomerular filtration studies are conducted.

4 Conclusion

Both hyperuricemia and hypouricemia could be induced with oxonic acid and allopurinol respectively. These controls indicated that the dosing procedure of the animals was successful and the analysis procedure could determine changes in the serum and urine xanthine and uric aid concentrations. The hypouricemic effect of allopurinol was dose-dependant as found with previous studies from other authors (Osada, 1993).

Treatment with EGCg and TPM did not result in xanthine accumulation in the urine or serum, therefore no inhibition of XO occurred. The tea catechins did cause a slight decrease in the serum uric acid levels of normal rats. Only the 50 mg/kg EGCg treatment showed a statistically significant decrease in the serum uric acid levels of hyperuricemic rats. No statistically significant changes in the serum uric acid levels of hyperuricemic rats could be observed with the 100 mg/kg EGCg and both TPM doses. It is possible that the tea catechins may have a weak uricosuric action. The total amount of uric acid excreted could not be determined to verify this, since the bladders of the rats were not cleared completely at each time interval.

Several factors may be responsible for the lack of inhibition. The low systemic uptake of EGCg, protein binding of polyphenols and the biotransformation of polyphenols must be overcome in order to produce a successful *in vivo* polyphenol inhibitor of XO. A better understanding of the absorption and distribution of the polyphenols may enable the sensible designing of novel polyphenol like inhibitors of XO.

Chapter 6

Concluding discussion

According to Chinese tradition, Emperor Shennong was the first to drink tea. He was one of the mythical emperors, the one the Chinese worshiped since ancient times as the deity of medicine and agriculture. It is since then, approximately 3000 BC that tea has been used as a medicinal drink by the Chinese. By the end of the sixth century AD they began to consume it as a beverage. The western nations were introduced to it at the beginning of the seventeenth century. Tea polyphenols contribute to both the pharmacological and quality properties of tea. A family of flavanols, known as catechins, has been identified as the bioactive compounds in tea. The catechins have been shown to have both anti-carcinogenic and anti-mutagenic activities. Tea polyphenols are antioxidants and scavenge oxygen radicals that can cause DNA mutations and result in disease such as atherosclerosis. They also modulate the activities of phase I and phase II detoxification enzymes. This reduces the amount of pro-carcinogens that are converted to DNA-mutating carcinogens. The catechins have also been shown to inhibit enzymes in the signal transduction pathways that are important in regulating transcription and cell-signaling.

Polyphenols and coumarins from other plant sources have been shown to inhibit XO *in vitro*. XO is a liver enzyme that converts hypoxanthine to xanthine and xanthine to uric acid. It has a low substrate specificity for aromatic heterocyclic compounds, making it possible that xenobiotics may also be hydroxylated by this enzyme. XO also produces reactive oxygen radicals (superoxides or peroxides) during the catalytic process. Oxygen

radicals have been linked to carcinogenesis. Since natural plant products inhibit the production of a potential cancer-causing oxygen radicals, it is a possible way by which tea polyphenols may prevent carcinogenesis. This has not been tested before.

Capillary electrophoresis is a very powerful technique and is becoming more and more popular in investigations regarding natural product chemistry and pharmacology. Several different modes of capillary electrophoresis exist which include micellar electrokinetic chromatography and capillary zone electrophoresis. This makes it a versatile analytical technique that can analyze ionized and neutral analytes simultaneously.

The first aim of this study was to develop a MEKC method that is suitable for the analysis of the five major tea catechins, as well as theanine, caffeine, gallic acid and ascorbic acid. This method would be used for studies related to quality determination. The second aim was to determine the *in vitro* inhibitory effects of tea polyphenols on XO and to present a model explaining the structure activity relationship for the observed results. Finally the *in vivo* inhibitory effects were tested in a rat model. CZE was used to detect the levels of XO substrate, product and inhibitor levels in rat urine and serum.

A univariate optimization approach was used to develop a MEKC method that could analyze some of the organoleptically important compounds in tea. The influence of pH, surfactant concentration, background electrolyte concentration, organic modifier concentration and applied voltage was determined and optimal conditions were selected for each parameter. The pH parameter was the most important factor influencing the separation.

From the literature a correlation could be drawn between the pH, the type of buffer that was used and the physiochemical properties that were exploited to achieve separation. In our study the pH was varied in the range of pH 6.8 to 8.0. A pH of 7.0 was selected as the optimum condition. At this pH the polyphenols are usually only slightly negatively charged. The separation is achieved mainly with the differential hydrophobic interaction that the polyphenols have with the surfactant in the buffer. The interaction with the surfactant was almost sufficient to resolve all the analytes. Organic modifiers were added to try and increase the selectivity of buffer and obtain better separations. Acetonitrile, 2-propanol and methanol were tested as organic modifiers. Organic modifiers alter the distribution of the polyphenols between the aqueous phase and the surfactant phase. Increased organic solvent concentrations increase the solubility of the polyphenols in the aqueous phase. The addition of organic modifiers has an additional benefit by reducing the migration time of hydrophobic analytes. Methanol was found to increase the selectivity of the buffer the most. A concentration of 6% (v/v) methanol in the running buffer was considered as optimal for analysis of green and black tea samples. A methanol concentration of 5% (v/v) in the running buffer was used when iced tea samples fortified with ascorbic acid were analyzed. The optimal conditions for the analysis of all tea samples were 100 mM SDS, 25 mM phosphate and 6% (v/v) methanol at pH 7.0. An uncoated open fused silica column with an effective length of 50 cm was used. An applied voltage of 14 kV was found to provide good resolution and speedy analysis without resulting in excess Joule heating.

XO inhibition studies were conducted *in vitro* and an attempt was made to explain the results with kinetic models and *in silico* models. Since polyphenols have previously been shown to inhibit XO, it was not surprising to find that catechins also inhibit the enzyme. All the catechins exerted an inhibitory effect. The most potent inhibitor was EGCg which had a K_i value of 0.76 μM comparable to that of allopurinol (0.30 μM). The most intriguing aspect was the vastly different modes of inhibition that was displayed by the structurally related catechins. Competitive, uncompetitive and mixed-type inhibitions were observed. Similar inhibition results have been observed before with other classes of polyphenols as well as coumarins.

A model is proposed based on the ping-pong bi-bi catalytic action of the XO. The enzyme cycles between an oxidized form (E_{ox}) and a reduced form (E_{red}). According to three-dimensional data obtained from the X-ray crystallography structures of AOR there are no significant structural differences in the spatial arrangement of the amino acids in the active center or anywhere else on the two enzyme forms. The only significant difference is in the Mo coordination complex where the bond length of the apical sulfur atom has increased upon conversion from the E_{ox} to E_{red} enzyme forms.

The catechins are structurally very similar and it is likely that they all bind to the same region on the enzyme. Since EGCg showed competitive inhibition it is possible that they may bind to the active center of the enzyme. In the XO ping-pong catalytic cycle the inhibitors can bind to the active centers of either the E_{ox} or E_{red} enzyme forms. The inhibitors simulate "alternative substrates" for the enzymes. When the steady-state

kinetics equations are derived it can be seen that an inhibitor binding only to the active center of the E_{ox} enzyme form results in competitive inhibition and an inhibitor binding only to the E_{red} enzyme form results in uncompetitive inhibition. An inhibitor that has the ability to bind to both forms of the enzyme with different affinities results in mixed type inhibition. K_I is defined as the inhibitory constant of an inhibitor for the E_{ox} enzyme form. K_i is the inhibitory constant of an inhibitor for the E_{red} enzyme form. A competitive inhibitor only has a K_I value and an uncompetitive inhibitor has only have a K_i value. Mixed type inhibitors have both K_I and K_i values. For mixed type inhibitors the ratio of K_i/K_I will be used to assess their structure-activity relationships.

When comparing the structures of different xanthine oxidase inhibitors, two different regions can be distinguished. The first is called the primary binding region and mimics the structure of xanthine oxidase substrates. It requires a heterocyclic ring and hydrogen bond forming groups. The secondary binding region is responsible for enhancing the affinity and to modulate the selectivity of the inhibitor for the different enzyme forms. Large hydrophobic moieties increase the overall affinity of the inhibitor for the enzyme. In polyphenols the C3 of the C-ring is important in modulating the K_i/K_I ratio. A hydroxyl present and orientated in the plane or behind the plane in the (-)-configuration allow the binding to the E_{ox} enzyme form. A lack of a hydroxyl or a hydroxyl oriented towards the front of the C-ring plane in the (+)-orientation only allows binding to the E_{red} enzyme form. The amount of hydroxylation of the B-ring also influences the K_i/K_I ratio. Binding to the E_{ox} enzyme form is favored with a decrease in hydroxylation on the B-ring.

Since the tea catechins showed promise as inhibitors of xanthine oxidase *in vitro*, the next logical step was to test them *in vivo* in an animal model. A rat model was selected, since rats are best suited for metabolic studies. A CZE analytical method was used to quantify the *in vivo* concentrations of xanthine, uric acid, oxypurinol and allopurinol. The principle of stacking was used to increase the sensitivity of the method. The method was sensitive enough to detect the basal uric acid levels in the rat serum. It was not sensitive enough to detect the basal levels of xanthine in all the rat serum samples. Upon inhibition of XO, xanthine accumulates in the serum. Even with weak inhibition, levels of 1 μg/ml xanthine is to be expected. The limit of detection for xanthine was 0.5 μg/ml, making the method sufficiently sensitive to detect the inhibition of xanthine oxidase in a rat model.

In humans uric acid is the excretion product of purine degradation. In rat however uric acid is converted to allantoin by the uricase enzyme. This enzyme is inhibited with oxonic acid treatment. The uric acid accumulates in the serum, rendering the treated rats hyperuricemic. Hyperuricemia is defined as uric acid levels of 2 S.D. above the population mean. In humans the mean uric acid levels are 40-50 μg/ml and hyperuricemia occurs at saturation levels of uric acid which is 60-70 μg/ml. The normal rats had a mean serum uric acid concentration of 5-8 μg/ml and the oxonic acid treated rats had a mean serum uric acid concentration of 25 μg/ml. The uric acid levels are not as high as in humans, but increased between 3 and 8 fold above the norm for rats. This ensures that the oxonic acid treated rats are hyperuricemic according to the definition.

Treatment with allopurinol served as positive control for the *in vivo* inhibition experiment. Treatment with allopurinol decreased the uric acid concentration in both the serum and urine of normal and hyperuricemic rats. An equimolar accumulation of xanthine was observed for hyperuricemic rats. The inhibition with allopurinol also occurred in a dose-dependant manner. These results indicated that the treatment protocol and the analytical procedures were successful.

Treatment of the hyperuricemic group with 50 mg/kg (-)-EGCg resulted in a low but statistical significant decrease in serum uric acid concentration. The 100 mg/kg (-)-EGCg dose and both TPM doses did not result in any statistically significant changes of the serum uric acid levels of hyperuricemic rats. No accumulation of xanthine was observed for any of the catechin treatments. The treatment of normal rats with either (-)-EGCg or TPM at both the 50 and 100 mg/kg doses resulted in a low but statistically significant decrease in serum uric acid concentration. No accumulation of xanthine was observed, indicating that XO was not inhibited. The decrease in uric acid levels did not seem to be dose-dependant. Since the change is small, the lack of dose-dependence may be due to variance in the animal model and analytical method. The tea polyphenols may have a weak uricosuric effect on the normal rat groups or an enzyme further up-stream of XO in the purine degradation pathway may be inhibited.

The *in vitro* studies showed that allopurinol and (-)-EGCg have similar K_i values. Yet in the i*n vivo* model only allopurinol inhibited the XO enzyme. The systemic absorption and bioavailability of (-)-EGCg in rats are very low. According to a pharmacological study

only 1% of the (-)-EGCg became bioavailable and the serum concentration was in the order of 1μM. Since the K_i of (-)-EGCg is 0.76 μM, one would still expect to see at least 50% inhibition with a competitive inhibitor. There are however other factors that influences the amount of free (-)-EGCg that are available. Polyphenols have been shown to bind to proteins. The aromatic hydroxyl groups form hydrogen bonds with the peptide carbonyl groups. This interaction is not very selective, resulting that polyphenols can easily bind to any serum proteins. Polyphenols bind more readily to globular proteins with a loose tertiary structure such as albumin. Albumin is present in plasma at concentrations of 35-45 g/l (600 μM). It is very likely that a large percentage of the absorbed EGCg can bind to serum albumin. This will reduce the effective amount of free EGCg significantly. The biotransformation of polyphenols occurs via UDP-glucuronosyl transferase and phenolsulfotransferase. Both these enzymes are liver enzymes and have some affinity for EGCg. If the affinity of EGCg is higher for the detoxification enzymes (cytochrome P 450) than for XO, it is unlikely that the 1 μM EGCg that reaches the liver will even bind to the XO (Yang, 1999). It is also unlikely the biotransformed EGCg will be a good inhibitor of the XO enzyme.

In the future a novel class of XO inhibitors with a polyphenol-like structure could be developed. Research studies can be conducted to investigate and improve the absorption and specificity. Studies will have to be conducted to elucidate the molecular mechanism of the inhibitory actions. Modeling software may be used to predict enzyme-inhibitor interactions. Several spectroscopic methods may be used to determine whether the inhibitor binds in close proximity of the Mo complex. Once the molecular basis of the

action is known, the inhibitor structure can be altered to improve the affinity and selectivity. When new inhibitors are designed it should be taken into consideration that the bioavailibility and overall bioavailability (affinity for proteins, affinity for detoxification enzymes and excretion) should also be improved. Software packages are available that can help predict the absorption and organ selectivity properties of drugs. This software can be used and *in vivo* studies preformed to determine the absorption and bioavailability of drugs. Since tea polyphenols have no adverse effects a slightly modified tea polyphenol inhibitor may be a better therapeutic drug for gout than allopurinol and colchicine.

Summary

Catechins and catechin derivatives are the most abundant polyphenols in tea. They contribute to the quality of tea and have several prophylactic properties, which include antimutagenic and anticarcinogenic effects. In the tea industry it is important to quantify and control the quality of tea. Chemical analytical methods that are able to separate and quantify the catechins can assist in the Quality Control process.

It is also important to identify all possible mechanisms by which tea polyphenols exert their prophylactic properties. It was thought that one such mechanism might be the inhibition of oxygen radical producing enzymes such as xanthine oxidase. Xanthine oxidase is also important in the occurrence of gout since it forms uric acid from xanthine. Effective inhibition of xanthine oxidase with tea polyphenols may lead to new natural product drugs with less adverse effects than current gout therapy drugs such as colchicine and allopurinol.

The first aim of this research was to develop and implement an analytical method suitable for analyzing tea liquors from green and black tea as well as from fresh tea leaves. The second aim of this research was to evaluate tea catechins as xanthine oxidase inhibitors both *in vitro* and *in vivo*.

Micellar electrokinetic chromatography was deemed the most suitable analytical technique for the separation and quantification of the major compounds influencing the quality of tea. A micellar electrokinetic chromatography method was developed to analyze the five major catechins, caffeine, theanine, gallic acid and ascorbic acid in green, black and bottled iced teas. The pH and the concentration of organic modifier

had the largest influence on the separation of the analytes. The optimal separation conditions used to analyze green and black tea samples were 25 mM phosphate, 100 mM sodium dodecyl sulfate, 6% (v/v) methanol at pH 7.0. For the analysis of iced tea samples the conditions were kept the same, except the methanol concentration was reduced to 5% (v/v). This allowed the added separation of ascorbic acid, which is added to bottled iced tea as fortification.

Seven different tea polyphenols were tested as *in vitro* inhibitors of xanthine oxidase. It was found that (-)-epigallocatechin gallate, the most abundant tea catechin, was one of the more potent inhibitors that were tested. The majority of the tea catechins showed mixed type inhibition. Catechin showed uncompetitive inhibition and (-)-epigallocatechin gallate showed competitive inhibition.

(-)-Epigallocatechin gallate was tested as a xanthine oxidase inhibitor in a rat model. The tests were done in normal and hyperuricemia induced Sprague-Dawley rats. The positive control was allopurinol that is currently used as gout therapeutic drug. A capillary zone electrophoresis method was implemented and used to detect and quantify changes in hypoxanthine, xanthine, uric acid, allopurinol and oxypurinol levels in the serum and urine of treated animals.

Allopurinol prevented the production of uric acid in a dose-dependent manner in both normal and hyperuricemic rats and resulted in the equimolar increase of the xanthine levels. The (-)-epigallocatechin gallate reduced the levels in normal rats slightly, but no xanthine accumulation was observed. This suggests that (-)-epigallocatechin gallate might be a weak uricosuric agent, but did not inhibit xanthine oxidase at the

levels tested. (-)-epigallocatechin gallate also did not inhibit xanthine oxidase activity in hyperuricemic rats. In hyperuricemic rats, low (-)-epigallocatechin gallate levels reduced serum uric acid levels and high (-)-epigallocatechin gallate doses increased the serum uric acid levels. These indifferent results cannot be explained.

The three dimensional molecular structures of the tested inhibitors as well as other synthetic inhibitors from the literature were created. A model is proposed where the polyphenols bind to the active center of the xanthine oxidase enzyme. The polyphenol binds to either the E_{ox} or E_{red} or both forms of the enzyme in the ping-pong catalytic cycle. Inhibitors binding solely to the E_{ox} form show competitive inhibition, while inhibitors binding solely to the E_{red} form show uncompetitive inhibition. When an inhibitor can bind to both forms of the enzyme it exhibits mixed type inhibition. Primary and secondary binding regions are identified on the polyphenol molecules. The primary binding regions mimic the xanthine oxidase substrates and products, causing them to bind to the active center of the enzyme. The secondary binding region enhances the affinity of the inhibitor for the enzyme. It also modulates the selectivity for the E_{ox} and E_{red} forms of the enzyme respectively.

The proposed enzyme inhibition model is a starting point for determining the exact inhibitory mechanism of action that the tea polyphenols have on xanthine oxidase. Molecular modeling and ligand docking studies may confirm the model and also assist in modifying polyphenols into xanthine oxidase inhibitors that are more efficacious *in vivo*.

The analytical capillary electrophoresis method development creates a firm base for future research on other important compounds in tea. capillary electrophoresis may be used to study the polymerized polyphenols (theaflavins, thearubigens and bisflavanols) found in black tea. Very little is known of these compounds' contribution to quality and prophylaxis.

Opsomming

Tee polifenole dra nie net tot die kwaliteit van die tee by nie, maar besit ook gesondheidseienskappe. Katechien verbindings (catechins) is die polifenole wat die volopste in tee voorkom. Analitiese metodes wat die verskillende derivate kan skei en kwantifiseer kan gebruik word in die kwaliteits beheer proses.

Al die meganismes waarmee polifenole hul gesondheids eienskappe manifesteer is nog nie bekend nie. Een moontlike meganisme is die inhibisie van ensieme wat suurstof-radikale vorm. Xantienoksidase is 'n voorbeeld van so 'n ensiem. Xantienoksidase is ook betrokke by die ontstaan van jig aangesien dit uriensuur produseer. Die effektiewe inhibisie van xantienoksidase met tee polifenole kan aanleiding gee tot die tot-standkoming van 'n nuwe reeks natuurlike inhibitore. Die inhibitore kan minder newe-effekte hê as die huidige middels teen jig (allopurinol en kolgisien).

Die eerste doel van die navorsingsprojek was om 'n analitiese metode te ontwikkel wat gebruik kan word om groen- en swarttee ekstrakte te analiseer. Die tweede doel van die navorsing was om katechien verbindings te evalueer as inhibitore van xantienoksidase, beide *in vitro* en *in vivo*.

Misellêre elektrokinetiese chromatografie was as die mees gepaste tegniek geag vir die skeiding en kwantifisering van die verbindings in tee. 'n Misellêre elektrokinetiese chro-matografie metode is ontwikkel om die skeiding van die vyf belangrikste katechien

verbindings, kaffeïen, teanien, gallussuur en askorbiensuur te bewerkstellig in groen-, swart- en gebottelde tee. Die pH van die buffer en die konsentrasie organiese oplosmidel in die buffer het die grootste invloed op die skeiding gehad. Vir die analisering van groen- en swarttee was die optimum kondisies 25 mM fosfaat, 100 mM natrium dodekiel sulfaat, 6% (v/v) metanol by pH 7.0. Gebottelde tee is geanaliseer met dieselfde kondisies, bewalwe vir die metanol konsentrasie wat na 5% (v/v) verminder is. Die verandering verseker dat askorbiensuur ook geanaliseer kan word.

Sewe tee polifenole is *in vitro* as inhibitore van xantienoksidase getoets. Epigallo-katechiegallaat was die beste inhibitor. Die meerderheid van die katechien verbindings het gemengde tipe inhibisie getoon. Katechien was 'n onkompeterende inhibitor en epigallokatechiengallaat 'n kompeterende inhibitor.

'n Rot model was gebruik om epigallokatechiengallaat *in vivo* as xantienoksidase inhibitor te toets. Dit is gedoen in normale rotte sowel as rotte waarin hiperurisemia geïnduseer is. Allopurinol is gebruik as positiewe kontrole. 'n Kapillêre sone elektroforese metode is gebruik om die konsentrasies van hipoxantien, xantien, uriensuur, allopurinol en oksipurinol in rot serum en uriene te bepaal.

Allopurinol het die produksie van uriensuur op 'n dosis afhanklike wyse verlaag in beide die normale en hiperurisemiese rot groepe. Epigallokatechiengallaat het die konsentrasie van uriensuur in normale rotte effens verlaag, maar geen xantien het gevorm nie. Epigallokatechiengallaat inhibeer nie xantienoksidase *in vivo* nie, maar mag dalk 'n swak

urikosuriese (uricosuric) middel wees. 'n Lae epigallokatechiengallaat dosis het die serum konsentrasie van uriensuur in hiperurisemiese rotte verlaag. Hoër epigallokatechiengallaat dosisse het geen effek gehad op die uriensuur konsentrasies nie. Die verskynsel kan nie verklaar word nie.

Drie-dimensionele strukture is gegenereer vir al die inhibitore wat getoets was, sowel as vir sintetiese inhibitore wat vanuit die literatuur verkry was. 'n Model is voorgestel waar die polifenole aan die aktiewe sentrum van xantienoksidase bind. Die polifenol bind aan die E_{ox} of E_{red} vorm van die ensiem in die ping-pong katalitiese siklus. Inhibitore wat aan die E_{ox} vorm bind vertoon kompeterende inhibisie. Inhibitore wat aan die E_{red} vorm bind vertoon onkompeterende inhibisie. As 'n inhibitor aan beide vorme bind vertoon dit gemengde tipe inhibisie. Primêre en sekondêre bindings gebiede is gedefinieer op die polifenol molekule. Die primêre bindings gebied simuleer die xantienoksidase substrate en produkte wat verseker dat die inhibitor aan die aktiewe sentrum bind. Die sekondêre bindings gebiede verhoog die affiniteit van die inhibitor vir die ensiem. Dit moduleer ook die selektiwiteit van die inhibitor vir die twee vorme van die ensiem.

Die voorgestelde model is 'n goeie beginpunt om die presiese meganisme van inhibisie te bepaal. Molekulêre modelering en ligandbinding simulasies kan dalk die model bevestig. Dit kan ook help om beter polifenol gebaseerde inhibitore te ontwikkel. Die analitiese kapillêre elektroforese metodes skep 'n stewige basis waarop toekomstige navorsing kan voortbou. Die gepolimeriseerde katechien verbindings wat in swart tee voorkom kan dalk met die metode geskei en gekwantifiseer word.

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