Immunohistochemical localisation of caffeine in young tea (*Camellia sinensis* (L.) O. Kuntze) leaves.

By

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University of Pretoria
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SUMMARY:

The agricultural product using the top young shoots from the *Camellia sinensis* bush is known as tea. The leaves have many health benefits and contain high contents of antioxidants. The leaves contain caffeine, which is believed to be a phosphodiesterase inhibitor preventing the disposal of cyclic adenosine monophosphate, and thus when consumed, cellular responses are up regulated, and people experience unwanted effects such as being unable to fall asleep. Caffeine is currently believed to be synthesized within chloroplasts and stored within the vacuoles of parenchyma cells. It is hypothesized that the molecule acts as a chemical defence as well as an aid in the sequestration of catechins and polyphenols within the vacuoles of parenchyma cells. Different methods of decaffeination exist using solvents such as methylene chloride, supercritical CO_2, and using hot water. Each method has a specific disadvantage. Currently, the Swiss Water® Process is a 100 % chemically free method of decaffeination for coffee beans, and a similar process should be envisaged for the tea decaffeination industry as there are many advantages over the other current methods of decaffeination.

The anatomical localisation of caffeine within young *C. sinensis* leaves was investigated using immunohistochemical methods, and confocal scanning laser microscopy. Preliminary fixation experiments were conducted with young *C. sinensis* leaves to determine which fixation procedure retained caffeine the best as determined by high-performance liquid chromatography analysis. High pressure freezing, freeze substitution, and embedding in Lowicryl K4M resin was deemed the best protocol as it retained most of the caffeine, and allowed for the samples to be sectioned with ease. Immunohistochemical localisation with primary anti-caffeine antibodies, and conjugated secondary antibodies on leaf sections proved at the tissue level that caffeine was localised and accumulated within vascular bundles, mainly the precursor phloem. Immunocytochemical studies using a secondary antibody conjugated to gold were attempted but were inconclusive. With the use of a pressure bomb, xylem sap was analysed by thin-layer chromatography, and the presence of caffeine was determined to be present in a small amount. We hypothesize that caffeine is synthesized in the chloroplasts of photosynthetic cells and transported to vascular bundles where it acts as a chemical defence against various pathogens, and predators. Complex formation of caffeine with chlorogenic acid is also discussed as this may also help explain caffeine’s localisation.

Using the knowledge acquired from microscopy analysis, and thin-layer chromatography analysis of xylem sap we investigated three possible methods of decaffeination; vacuum, pressure, and a combination of both. All were based on using the intercellular air within the young leaves to our advantage as this might act as a natural way to ‘squeeze’ caffeine out of its localised areas. Other

possible methods of decaffeination were also attempted using hot water, and addition of an external PPO source for black tea production.

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Abbreviations:

- +C: (+)- Catechin
- AMP: Adenosine 5'-monophosphate
- ANOVA: Analysis of variance
- ATP: Adenosine 5'-triphosphate
- BSA: Bovine Serum Albumin
- c-AMP: Cyclic adenosine monophosphate
- CSLM: Confocal scanning laser microscopy
- CTC: Crush, tear and curl
- Cv.: Cultivar
- $DddH_2O$: deionised double distilled water
- DFT: Density functional theory calculations
- DIC: Differential interference contrast
- EC: (-)- Epicatechin
- ECg: (-)- Epicatechin gallate
- EGC: (-)- Epigallocatechin
- EGCg: (-)- Epigallocatechin gallate
- EtOH: Ethanol
- FD: Freeze drying
- FDA: U.S Food and Drug Administration
- FS: Freeze substitution
- FT: Fourier transform
- GC: (+)- Gallocatechin
- GCE: Green coffee extract
- GCg: (-)- Gallocatechin gallate
- GMP: Guanosine 5'-monophosphate
- HPF: High pressure freezing
- HPLC: High-performance liquid chromatoraphy
- IBMK: Isobutyl methyl ketone
- IMP: Inosine 5'-monophosphate
- L.A.S.E.R: Light Amplification by Stimulated Emission of Radiation
- LDL: Low-density lipoprotein
- NAD^+: Nicotinamide adenine dinucleotide
- Nd: YAG: Neodymium – doped yttrium aluminium garnet
- NIR: Near infrared

- NMR: Nuclear magnetic resonance
- PBS: Phosphate buffered saline
- PPO: Polyphenol Oxidase
- PVP: Polyvinylpyrrolidone
- Rf: Retention factor
- RGB: Red Green Blue
- SAM: S-Adenosyl methionine
- SD: Standard deviation
- S.E.M: Standard error of the mean
- TEM: Transmission electron microscopy
- TLC: Thin-layer chromatography
- XMP: Xanthosine 5’-monophosphate

Chapter 1: Introduction

1.1 Problem statement:

The localisation of caffeine within young *Camellia sinensis* leaves is unknown. Small molecule localisation by microscopy is extremely challenging due to the fact that current techniques cannot fix small molecules as many of these molecules are readily soluble in reagents used during sample preparation. By ensuring the retention of caffeine within *C. sinensis* leaves during sample preparation various methods such as immunolabeling can be used in localisation studies. This will shed light on the exact function of caffeine within young *C. sinensis* leaves from which various hypotheses may be confirmed or suggested. This information will also help in the development of new modern *C. sinensis* decaffeination processes. Current tea decaffeination technology has a number of flaws and is outdated. Three factory processes exist: hot water, solvent, and super critical CO_2 decaffeination. Hot water decaffeination removes the majority of caffeine from fresh leaves and a very small amount of catechins, but the process denatures the PPO enzyme preventing enzymatic oxidation and thus no black tea production can take place. Solvent decaffeination uses chemicals such as methylene chloride that may impose a harmful risk to the consumer, factory employees as well as the surrounding environment. The process also has a number of strict rules and guidelines to follow. Supercritical CO_2 uses specialised machinery in order for CO_2 to reach its critical point. Downtime of the equipment is common due to repairs, and the final product is normally more expensive than other decaffeinated products. The Swiss Water® process is a 100 % chemical-free decaffeination process used for green coffee beans. The beans are decaffeinated based on a principle of diffusion into green coffee extract free of caffeine in a temperature, and pressure controlled environment. The extract is also continually recycled to be used in more batches of coffee bean decaffeination. A similar tea decaffeination process is envisaged that is 100% chemically free, easy to perform, safe to both the consumer and factory employees, inexpensive, and that will allow for decaffeinated black tea production.

1.2 Tea in general:

The origin of the *C. sinensis* bush is assumed to be in the Yunnan district of South western China (Yamaguchi and Tanaka 1995). Chinese legend claims that tea consumption goes back as far as 2737 B.C. and that a legendary emperor known as the divine healer, Sheng Nung, consumed and used tea leaves for its health properties. This is considered an anthropological conclusion. On the other hand, archival documentation reveals a servant’s contract dated 59 B.C describing his duties of tea purchasing and brewing in China. However, it is very likely that tea has been consumed by

mountain tribes for thousands of years and today is an enjoyable beverage consumed worldwide (Hara 2001).

Tea is the agricultural product of *C. sinensis*, utilizing the top youngest leaves of the bush. The chief teas of commerce are (1) green tea; made from leaves withered, rolled and fired immediately to prevent oxidation, (2) black tea; made from leaves fermented and oxidized before firing as this allows for enzymatic oxidation of the catechins and (3) oolong; made from leaves that are partly oxidized before the firing process, i.e., semi-fermented (Willson and Clifford 1992). After water, tea is the second most consumed beverage around the world (Macfarlane and Macfarlane 2004).

Fig. 1.1: Image of a *C. sinensis* bud, first and second leaf (http://www.benefits-of-tea.com/silver-leaf-tea-company/).

C. sinensis can contain up to 30% catechins in dry weight with the principle compound being EGCg. Other major catechins that exist are +C, EC, EGC, ECg, GCg and GC. Catechins are very powerful polyphenolic antioxidants and provide many beneficial health properties after the consumption of tea. Some examples are the reduction of coronary heart disease due to the decrease in oxidation of LDL-cholesterol, slowing the growth of early cancer cells by decreasing the rate of cell duplication and enhancing the growth of beneficial bacteria, eliminating those with harmful attributes thus possibly modifying bacterial flora. Many other beneficial and nutritional effects after tea consumption exist (Hara 2001).

When tea leaves are mixed with hot water, caffeine (1,3,7-trimethylxanthine) that accounts for some 3 – 4 % w/w of the solid material is extracted. Methyl xanthines - caffeine, in particular - are generally held responsible for most of the consequences of drinking tea such as disturbances in sleep as well as caffeinism, despite the small contribution of caffeine to the mass of the dried extract (Willson and Clifford 1992).

1.3 Caffeine:

1.3.1 General chemistry:

The synonyms for caffeine are, 1,3,7-trimethylxanthine, 7-methyltheobromine and 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione. Caffeine has a formula of $C_8H_{10}N_4O_2$, a molecular mass of 194.19 g/mol and is known to have a density of 1.23 g/ml at 18°C. In its pure form, it is white, solid and odourless with a melting point of ± 238°C. One gram dissolves in 46 ml water, 5.5 ml water at 80°C, 1.5 ml hot water, 66 ml alcohol, 22 ml alcohol at 60°C, 50 ml acetone, 5.5 ml chloroform, 530 ml ether, 100 ml benzene, and 22 ml boiling benzene. Sublimation of the compound occurs at 178°C and the LD_{50} oral of caffeine in rats has been determined to be 200 mg/kg (The Merck Index 1976).

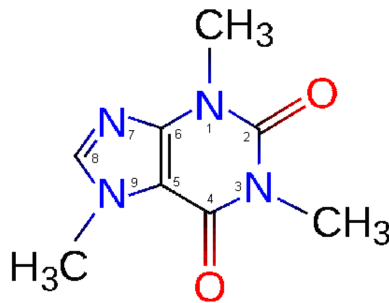


Fig. 1.2: The chemical structure of caffeine with atoms numbered (http://en.wikipedia.org/wiki/File:Caffeine.svg).

1.3.2 Caffeine biosynthesis within *C. sinensis*:

Caffeine is also known as a purine alkaloid, i.e., a xanthine (a purine base found in the tissues or fluids of many organisms) that is formed as a product part of purine degradation. In its biosynthesis (Fig. 1.3), the purine ring is gradually elaborated by using small components from primary metabolism. A C_2N unit is incorporated using glycine while the other carbon units come from formate by using N_{10}-formyl-tetrahydrofolate and bicarbonate. Two of the four nitrogens are supplied by glutamine, and a third is supplied by aspartic acid. AMP biosynthesis is via IMP and GMP biosynthesis is via XMP. IMP via the enzyme IMP dehydrogenase and NAD^+ forms XMP. If AMP is available, it can be used to form IMP via AMP deaminase. By branching off from XMP via 5'-nucleotidase and SAM, purine alkaloids can then be formed. If SAM is utilized in the methylation of XMP, N-methylation of the purine at nitrogen 7 occurs and a positive charge is transferred to the glycosylated nitrogen 9 forming 7-methyl XMP and loss of the phosphate group leads to 7-methylxanthosine. Hydrolysis of a phosphate ester forms xanthosine via 5'-nucleotidase. Seven-methylxanthosine can also be formed from xanthosine via xanthosine 7-*N*-methyltransferase and SAM. Seven-methylxanthosine then can form 7-

methylxanthine by S_N2 displacement of the purine-leaving group (D-ribose) by 7-methylxanthosine nucleosidase that utilizes H_2O to displace the purine group. Methylation on nitrogen 3 of 7-methylxanthine via the respective enzyme forms theobromine (3,7-dimethylxanthine), which has its nitrogen 1 methylated by the respective enzyme and thus finally forms caffeine (1,3,7-trimethylxanthine). Theophylline (1,3-dimethylxanthine) can also form from caffeine via a demethylation as part of a degradative pathway, i.e., removal of the methyl group on nitrogen 7. If a different methylation sequence is followed from xanthine, theophylline can also be formed (Dewick 2009).

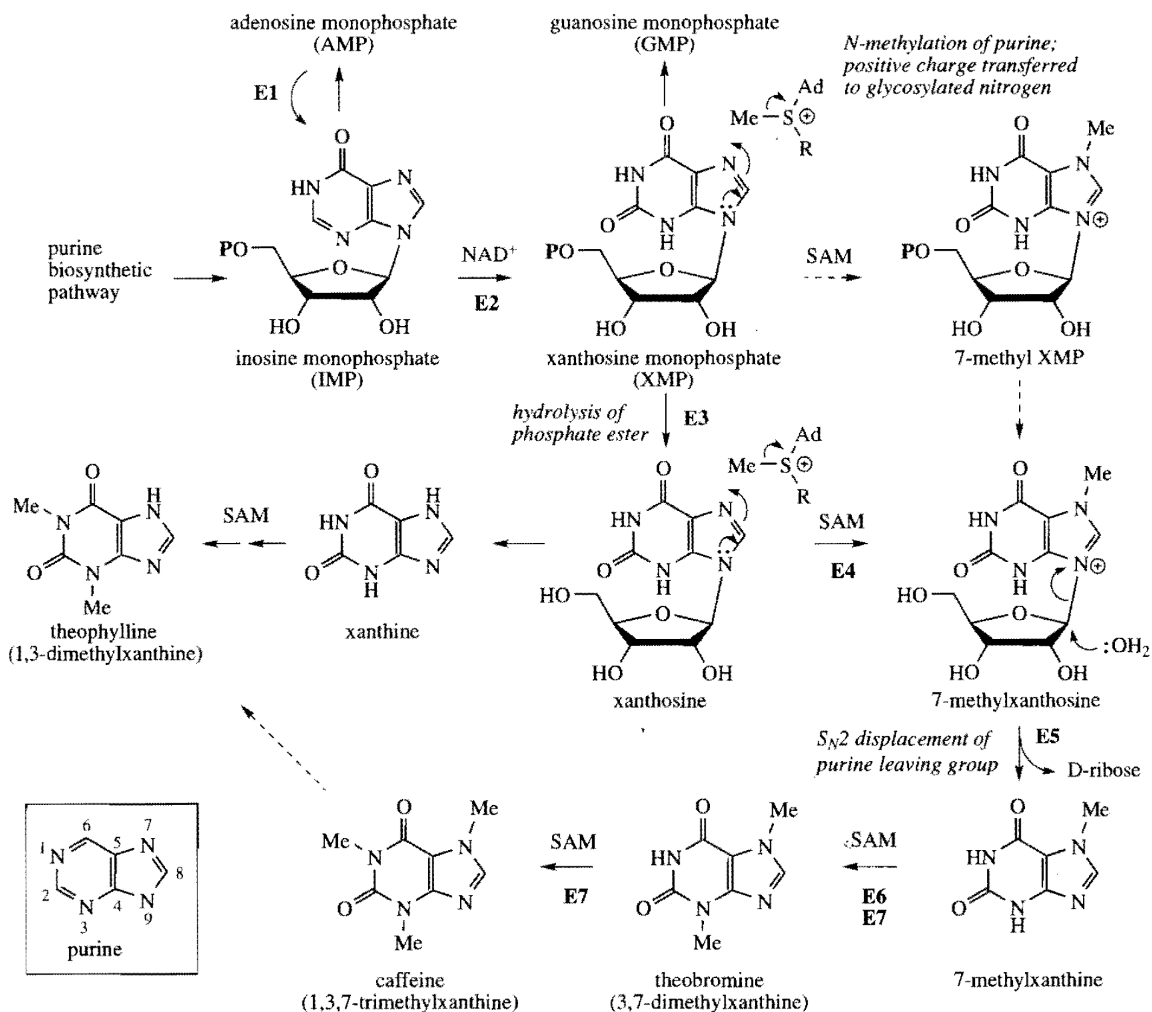


Fig. 1.3: The biosynthetic pathway of caffeine (1,3,7-trimethylxanthine) in *C.sinensis*. E1: AMP deaminase. E2: IMP dehydrogenase. E3: 5'-nucleotidase. E4: Xanthosine 7-*N*-methyltransferase. E5: 7-methylxanthosine nucleosidase. E6: 7-methylxanthine 3-*N*-methyltransferase (Theobromine synthase). E7: Theobromine 1-*N*-methyltransferase (caffeine synthase) (Dewick 2009).

1.3.3 General leaf anatomy and the localisation of caffeine synthase and catechins *C. sinensis* leaves:

General leaf anatomy can be seen in Fig. 1.4. An artist's impression is shown together with a surface view as well as a cross section.

The epidermis contains a compact arrangement of cells and the presence of a cuticle and stomata. The cuticle is a waxy layer that prevents significant loss of moisture from the leaf. Gases such as CO_2 and O_2 enter or exit via the stomata. In dicotyledons, the stomata are scattered and may be on the same level as the other epidermal cells. The term 'stoma' refers to the entire unit consisting of the stoma pore, two guard cells as well as two subsidiary cells. The guard cells contain chloroplasts that are known to produce starch. The stomatal pore is surrounded by two guard cells, which change in shape allowing for opening and closing of the aperture.

The mesophyll contains a large amount of chloroplasts and may be differentiated into palisade and spongy parenchyma cells. The palisade parenchyma are usually elongated perpendicular to the surface of the blade and are usually found on the adaxial (upper) side while the spongy parenchyma are located below the palisade cells and are usually on the abaxial (lower) side. The palisade parenchyma are densely packed, and since they are close to the surface of the leaf they aid in photosynthesis while the spongy parenchyma cells are irregular in shape and size with numerous intercellular spaces between adjacent cells that aid in the rapid diffusion of atmospheric gases. The spongy parenchyma cells generally have a horizontal continuity parallel with the surface of the leaf.

The vascular system is distributed throughout the blade and has a close spatial relation to the mesophyll. The veins of the leaf are called vascular bundles. In dicotyledons, the smaller veins are embedded in the mesophyll while the larger veins are enclosed in ground tissue that is not differentiated as mesophyll and has few chloroplasts. The vascular bundles (especially the smaller ones) in the mesophyll are enclosed by compactly arranged cells called the bundle sheath cells. These cells are generally parenchymatic but may be sclerenchymatic (cells lacking protoplasts and often have thick lignified cell walls) or a combination of both. Sometimes these cells connect with the epidermis and are then known as bundle sheath extensions. Veins are generally the starting point for the uptake of products of photosynthesis and their translocation out of the leaf.

Vascular bundles consist of xylem and phloem. The xylem is continuous throughout the plant body and is structurally and functionally complex. It contains cells responsible for water conduction, storage and support. It transports water and soluble mineral nutrients from the roots throughout the plant,

replacing lost moisture due to transpiration and photosynthesis. The principal water conducting cells are tracheids and vessel members (joined end to end). Parenchyma cells are responsible for storage arranged as vertical files and in the secondary xylem, also rays. The xylem also contains fibers and sclereids (at maturity). Phloem also occurs throughout the plant body and is responsible for food storage and support. The principal conducting cells are sieve cells and sieve – tube members (both typically enucleate at maturity). At maturity they contain very few organelles not to impede movement of fluids. Sieve – tube members are joined end to end by means of sieve plates into sieve tubes and are responsible for long – distance conduction of food materials. They are associated with companion cells (specialised parenchyma cells) that provide metabolic needs and are connected by plasmodesmata. The phloem also contains phloem parenchyma cells (storage and radial translocation of food substances) while supporting cells are fibers and sclereids. Fiber cells may be septate or nonseptate and may be living (serve as storage cells) or non living at maturity. Sclereids may be found alone or in combination with fibers and may be present in both axial and radial systems of the secondary phloem. They typically differentiate in older parts of the phloem as a result of sclerification of parenchyma cells which may or may not be preceded by intrusive growth of the cells. The intermediate cell types are fiber-sclereids. Other phloem parenchyma cells occur in vertical lines and secondary phloem contains parenchyma in the form of rays. The xylem is located on the adaxial side while the phloem is located on the abaxial side (Esau 1960).

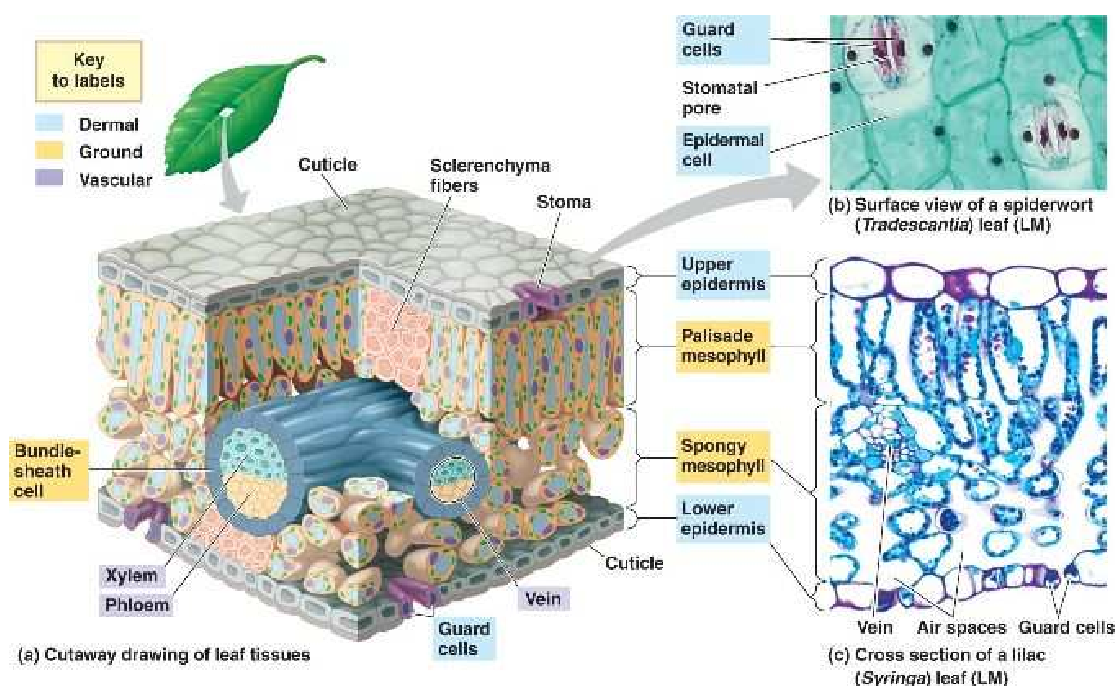


Fig. 1.4: General leaf anatomy: (a) an artist's impression, (b) surface view of a leaf and (c) cross section of a leaf (http://bio1152.nicerweb.com/Locked/media/ch35/35_18-LeafAnatomy-L.jpg).

It has been determined that caffeine synthase is likely to be an enzyme located in the chloroplasts of young tea leaves (Fig. 1.5) (Kato *et al.* 1998; Koshiishi *et al.* 2001) furthermore, it is likely to be located within the stroma of the chloroplasts (Kato *et al.* 1999). The caffeine synthase gene is highly expressed in the palisade parenchyma cells of the leaf (Li *et al.* 2007) and this makes sense due to the high chloroplast content of these cells.

The tissue distribution and intracellular localisation of catechins in tea leaves has been determined to be present in the palisade and spongy parenchyma cells, being restricted to regions within the central vacuoles. It has been proposed that after catechins are synthesized, they are incorporated into small vacuoles and transported to the large central vacuoles (Suzuki *et al.* 2003).

A hypothesis exists that describes alkaloids as helping to sequester catechins within vacuoles by forming co-aggregates (Matile 1976) and this has been previously determined for cocaine (a tropane alkaloid) within coca leaves (Ferreira *et al.* 1998). Mösli Waldhauser and Baumann (1996) also suggested that caffeine accumulates in cell vacuoles being mostly bound to chlorogenic acid. Thus is it very likely that caffeine is located within the vacuoles of mesophyll cells but in higher concentrations in the palisade parenchyma. However, it is possible that after synthesis, caffeine is transported and accumulated elsewhere within the leaf.

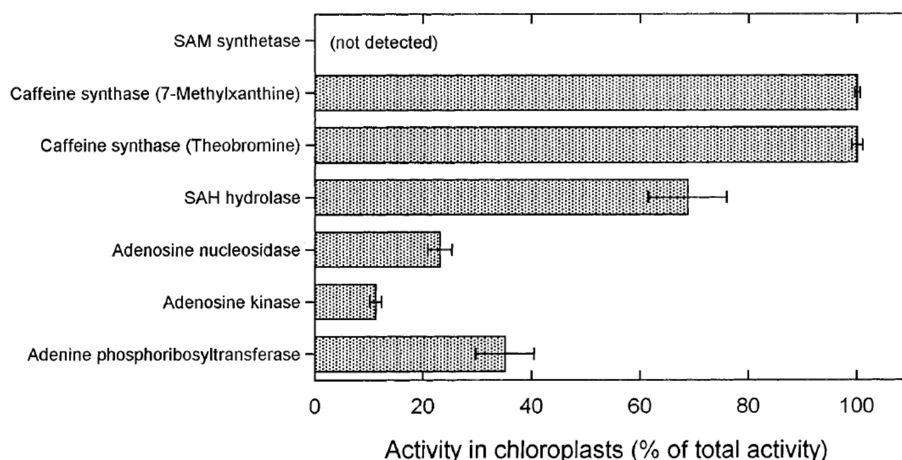


Fig. 1.5: Localisation of enzymes involved in the caffeine biosynthesis in chloroplasts of young tea leaves. Total enzymatic activity is shown as a percentage (Koshiishi *et al.* 2001).

1.3.4 The possible role of caffeine within *C. sinensis* leaves:

A theory that explains the possible role of caffeine in *C. sinensis* is the chemical defence theory. It explains that caffeine found in young leaves protects the soft tissue from predators such as larvae and

beetles from herbivory (Harborne 1994). Experiments where insects were fed caffeine show that it may either have a lethal effect, killing larvae of the tobacco hornworm, *Manduca Sexta,* at a dietary concentration of 0.3% (Nathanson 1984) or it may cause sterility as this was seen in the beetle *Callosobruchus chinensis* at a concentration of 1.5% (Rizvi *et al.* 1980). As the leaf ages it is less vulnerable to predators suggesting that caffeine biosynthesis is no longer required and a decrease in caffeine synthase activity has been determined (Li *et al.* 2007). The alkaloid is likely to be recycled as the nitrogen could be used in protein synthesis (Harborne 1994).

1.3.5 Pharmacokinetics, pharmacodynamics and effects of caffeine consumption:

Peak blood caffeine levels are reached within 30 – 60 min after ingestion on an empty stomach. The normal plasma half-life of caffeine is in the region of four to six hours in healthy individuals that are not pregnant and that do not smoke (Willson and Clifford 1992). The half-life is greatly increased in women during pregnancy and in patients with chronic liver disease (Aldridge *et al.* 1981). Caffeine is seen contained in breast milk of breast feeding mothers and this can be sufficient to keep the plasma levels of caffeine at a measurable level in the child. During pregnancy caffeine is also detected in the umbilical cord. This could lead to possible harmful effects on the infant but no records exist (Willson and Clifford 1992). When analysing urine in normal adults, around 60% of controlled and known caffeine consumption can be recovered as recognisable methyl xanthine metabolites (Scott *et al.* 1986).

The enzyme responsible for disposing of c-AMP is phosphodiesterase and caffeine has been well known to be a potent inhibitor of the intracellular enzyme. The best known intracellular second messenger of receptor-mediated biological functions is c-AMP. Since the enzyme responsible for disposing of c-AMP is inhibited by caffeine, c-AMP increases in concentration within the cells, thus exaggerating the stimulatory or inhibitory effects of such receptor interactions (Willson and Clifford 1992). This observation has however been challenged (Burg and Werner 1975). Activation of protein kinase A to begin the phosphorylation of certain enzymes is done by c-AMP. Protein kinase A is a tetramer consisting of two regulatory subunits and two catalytic subunits. When c-AMP binds to the regulatory dimmer subunits, release of the two catalytic subunits occurs. The active kinase phosphorylates a target enzyme or transcription factor. Fig. 1.6 shows that the binding of the hormone to the receptor leads to production of ATP and c-AMP. The reaction is mediated by a G protein (signalling molecule) and it is catalysed by adenylate cyclase. Once c-AMP is formed, it binds to the regulatory subunits of protein kinase and the active catalytic subunits are released and catalyse the phosphorylation of a target enzyme. The enzyme in turn elicits a response of the cell to the hormonal signal (Voet and Voet 2004). Thus, if c-AMP is not broken down; a cellular response will be up

regulated. The effects of the hormone will also be prolonged and this can lead to unwanted experiences after consumption. It is also believed that caffeine acts as a competitive receptor antagonist for adenosine cell surface receptors (Willson and Clifford 1992).

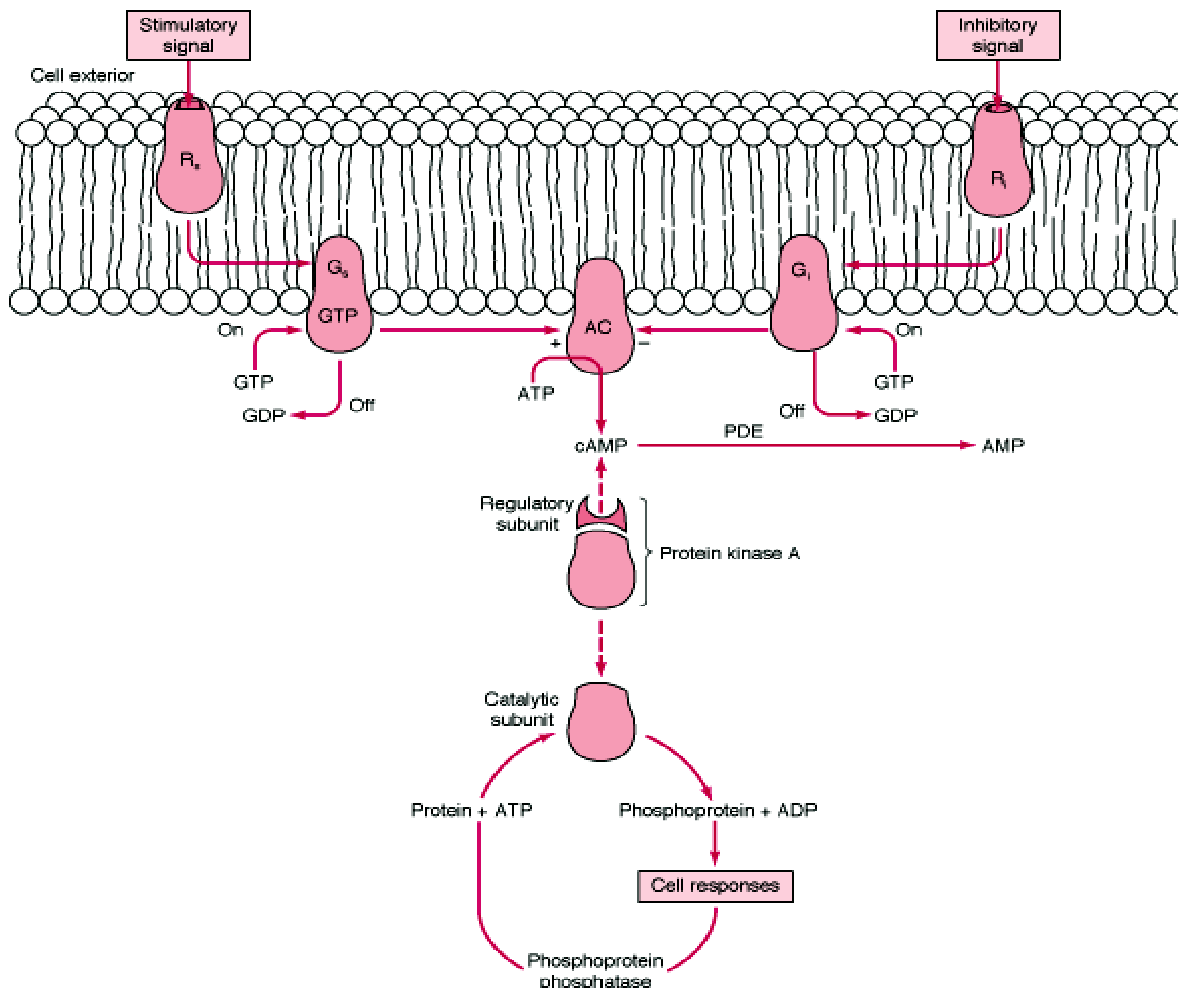


Fig. 1.6: Activation of adenylate cyclase by a hormone (e.g. epinephrine) to the receptor and the mode of action of c-AMP. AC = Adenylate cyclase (http://fig.cox.miami.edu/~lfarmer/BIL265/Image9.gif).

Caffeine's action on the cardiovascular system depends on the dose and also if short or long-term effects are considered. It also depends on the subject being studied i.e., if they have a high exposure to caffeine or don't really consume caffeine. There is a possibility that caffeine can lead to atrial or ventricular arrhythmias in predisposed subjects (Willson and Clifford 1992). A recent review suggests that patients with arrhythmias or suspected arrhythmias can tolerate caffeine in moderate doses (Pelchovitz and Goldberger 2011). Another critical review has suggested that caffeine increases blood pressure leading to certain cardiovascular complications (James 2004).

Caffeine is known to disturb sleep as consumption gives an increased sensation of wakefulness. There is evidence that ingestion of 100 mg (one cup of tea is considered to contain less than 50 mg)

of caffeine delays the onset of sleep (Willson and Clifford 1992; Landolt *et al.* 1995; Shilo *et al.* 2002). The effects differ from person to person as some people may be slow metabolisers that can achieve higher and possible toxic plasma levels with doses that are normally tolerated by people that are fast metabolisers (Willson and Clifford 1992). The P450 isoenzyme responsible for caffeine and that might classify people as slow or fast metabolisers is the CYP1A2 (Smith *et al.* 1997).

The symptoms of caffeinism can be restlessness, nervousness, excitement, insomnia, flushed face, diuresis, gastrointestinal complaints, muscle twitching, cardiac arrhythmia and psychomotor agitation (Bruce and Lader 1989). Withdrawal symptoms include headache, fatigue, apathy and drowsiness (Lorist *et al.* 2003; Nehlig *et al.* 1999).

1.4 Decaffeination:

1.4.1 Solvent, supercritical CO_2 and hot water decaffeination:

The main decaffeination processes that exist are the use of solvent, hot-water or supercritical carbon dioxide (Fig. 1.7).

Solvents such as methylene chloride and ethyl acetate are currently used to decaffeinate tea. This is known as the solvent extraction route. Use of a solvent such as methylene chloride has attracted large amounts of criticism. The compound is used in paint strippers, aerosols and dry cleaning solutions and is a hazard when inhaled in large concentrations. Ethyl acetate naturally occurs in tea leaves and is used as a solvent in the decaffeination process. So any residues might be seen as less risky when compared to methylene chloride. The solvent is not highly selective for caffeine and this may result in a weakly flavoured tea (Willson and Clifford 1992). After processing residual solvent remains on the leaf and thus regulations have been set by the Food and Drug Administration. A negative connotation with consumers exist when consuming decaffeinated products that used a solvent process and handling large quantities of solvent is risky as well as expensive (Dawson-Ekeland and Stringfield 1991). Another disadvantage that exists is that the method has to meet stringent environmental restriction when discarding the solvent and when this is not met; serious damage to the environment may occur (Gokulakrishnan *et al.* 2005).

When CO_2 is under extreme pressure at certain temperature it reaches its super critical state, liquefies and acts as a solvent. Leaves are moistened and placed in an extractor where the super critical CO_2 is circulated. After a few hours the CO_2 laden with caffeine is separated and the tea dried. The process is a physical one rather than a chemical. No chemical residues are left behind and no change in the

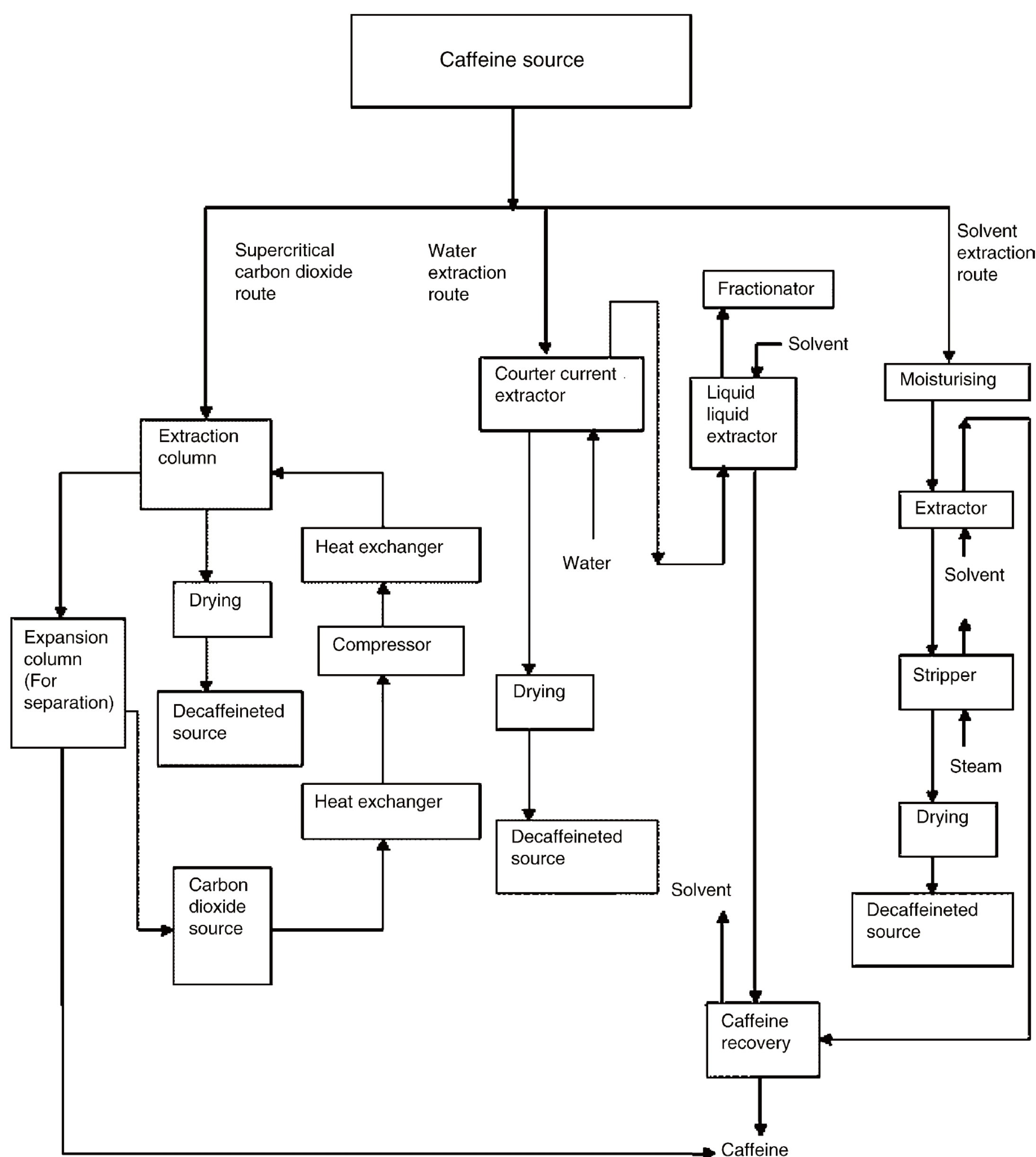


Fig. 1.7: A schematic diagram representing the different methods of caffeine removal i.e., supercritical carbon dioxide route, water extraction route and the solvent extraction route (Gokulakrishnan *et al.* 2005).

chemical composition of the leaf occurs except for the loss of caffeine (Willson and Clifford 1992). A decaffeinated tea product can then be obtained with caffeine content as little as 0.1 % dry w/w (Vitzthum and Hubert 1979). Since the method needs to be used under high pressure, it poses a possible safety hazard and thus, the machinery that is used is specialised and very expensive. This can affect the price of the decaffeinated product. Additionally, down time due to mechanical failure is also a problem because of the high pressures that need to be attained. Costs and downtime also come into effect when the machine needs to be maintained in order operate correctly and safely (Dawson-Ekeland and Stringfield 1991).

Decaffeination of tea can be done by exposing tea leaves to hot water. When a 1:20 w/v ratio of tea to water is used at boiling point for 3 - 5 min to decaffeinate tea, the caffeine concentration can be decreased by 83 % and only a 5 % decrease of total catechins occurs. Using the hot water treatment is considered a safe and inexpensive way for decaffeinating tea. Leaf damage in the extraction of caffeine plays an important role. Leaf damage can arise if rolling or drying of the leaves is done before the extraction process as this can damage the cells in the leaves and essentially cause the caffeine and other compounds to be squeezed out onto the surface of the leaf. This can make the extraction of caffeine easier but may also lead to loss of important compounds such as catechins. The rolled leaf is thus not suitable for decaffeination when just using hot water. The hot water treatment is not suitable for decaffeinated black tea production since black tea needs to be cut or rolled before being fermented, during which the tea catechins are oxidized by the enzyme PPO and a group of black tea pigments called theaflavins and thearubigins are formed. PPO is denatured by the hot water treatment and thus enzymatic oxidation leading to theaflavins and thearubigins cannot occur (Liang *et al.* 2006).

1.4.2 The Swiss Water® Process:

In this process (Fig. 1.8) the extraction of caffeine from the coffee beans takes place in water saturated with green coffee extract free of caffeine. Firstly, green unroasted beans are soaked and extracted in hot water. The extract is then passed through a patented activated carbon filter that selectively adsorbs caffeine. The resulting solution is known as green coffee extract (GCE). New green coffee beans containing caffeine can now be decaffeinated and are placed in the GCE. By the process of diffusion only caffeine will be removed from beans while other compounds that are needed will remain in the beans since the GCE is already saturated with all the soluble compounds except caffeine. The whole process is temperature and pressure regulated while the caffeine in the green coffee extract is cycled through the patented activated carbon filter that will selectively remove the caffeine so that the extract may be reused (Roy 1995). Drying to 10 % moisture affords a coffee bean with 0.04 % w/w caffeine content retaining nearly all its flavour compounds. The process is considered to be 100 % chemically free and environmentally friendly (http://www.swisswater.com; Ramalakshmi 1999).

The activated carbon that is laden with caffeine can easily be reactivated i.e., the caffeine is removed and the carbons active sites open up again and can be reused in another caffeine extraction step (Roy 1995).

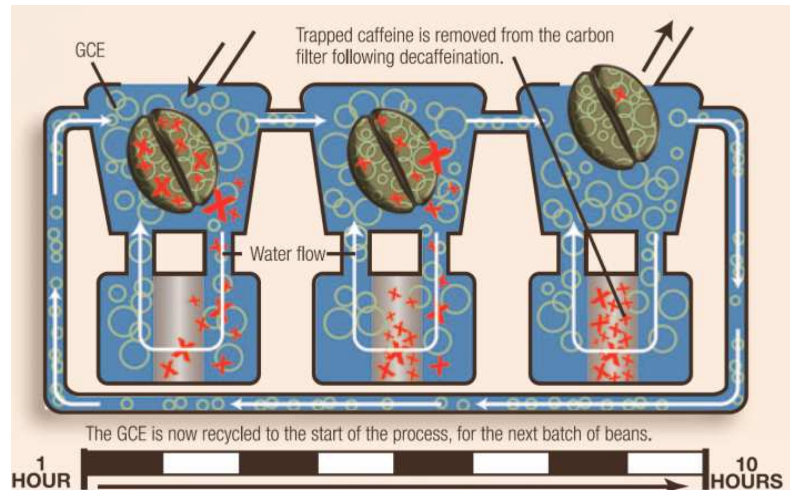


Fig. 1.8: The Swiss Water® Process over 10 hours indicating caffeine (red) removal from green coffee beans in green coffee extract to decaffeinate beans to 0.04 % caffeine content (http://www.swisswater.com).

1.4.3 The decaffeination market:

As early as the 1970's the need for a decaffeinated beverage has existed and the demand has climbed rapidly since then, for example decaf coffee sales in the U.S.A. had a 23 % share of the market estimated to be around 4 billion U.S Dollars (Ashihara and Crozier 2001). Tea can also be considered to be part of this market.

The FDA does not have its own set of guidelines but relies on the expertise of the Tea Association Technical committee (TATC) for decaffeination guidelines, which suggest that a decaffeinated tea should contain no more than 0.4 % caffeine based on dry weight. The Benelux Tea Law (a very old law in Belgium, Luxembourg and The Netherlands) indicates that decaffeinated tea should not contain more than 0.1 % caffeine in the dry product (Willson and Clifford 1992).

1.5 Black Tea:

The PPO enzyme has both monophenolase and diphenolase activity. During monophenolase activity, one atom of oxygen is incorporated into the aromatic ring (aromatic C-H bond) of the monophenol during the hydroxylation reaction. The phenol hydroxylation is carried out by the oxy form of PPO and the oxidation of catechols by both the met and oxy form (Granata *et al.* 2006). The diphenolase activity of PPO oxidizes *o*-diphenols to *o*-diquinones. The highly reactive quinones can react with amino acids and peptides (Pierpoint 1969) and also polymerise to form brown or blank pigments (Martinez and Whitaker 1995).

One of the most important stages during black tea production is the fermentation stage or enzymatic oxidation by the PPO enzyme. This is when the colourless catechins are converted to coloured theaflavins and thearubigins. Tannins and volatile oils (monoterpene alcohols and aromatic alcohols) provide the flavour and theaflavins are thought to act as antioxidants providing beneficial health benefits (Willson and Clifford 1992). Thus when comparing black tea levels of catechins to the levels in green tea they are generally lower (Cabrera *et al.* 2003).

Different types of theaflavins exist and are formed when catechins are oxidized and dimerized. The theaflavins that form are theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3'-gallate (TF_2B) and theaflavin-3,3'-digallate (TF_3) (Leung *et al.* 2001).

When the catechins that are present in the tea leaf polymerise they form thearubigins (Leung *et al.* 2001). Theaflavin and thearubigins are significant flavan-3-ols in tea (Peterson *et al.* 2005). These compounds are important in black tea as they are responsible for the characteristic taste and can depict the final quality of the black tea, thus it is important for both the catechins and PPO enzyme to be preserved after decaffeination. A method whereby the levels of theaflavins can be determined is known as the Flavognost method (Reeves *et al.* 1985; Robertson and Hall 1989). This level of theaflavins can correlate to the quality of the black tea (Robertson and Hall 1989).

1.6 Different microscopy techniques used to localise alkaloids within plant material:

Microscopic detection of small molecules such as alkaloids in leaf material is extremely difficult as has been stated in previous localisation studies (Ferreira *et al.* 1998). This is primarily due to standard fixatives only fixing proteins and lipids (Hayat 2000). At the same time standard fixation disrupts permeability barriers in cells allowing for the movement of small molecules (Hayat 2000; Coetzee and van der Merwe 1984). This information will serve as a review concerning the different techniques used to localise alkaloids using microscopes and where the different alkaloids were localised in plant material.

1.6.1 Histochemical localisation of alkaloids:

Histochemical methods (Yoder and Mahlberg 1976) were used to determine the sites of alkaloid accumulation within *Catharanthus roseus*. Ungerminated embryos, seedlings grown on moist filter paper and selected mature tissues were tested with Jeffrey reagent, Dragendorff reagent, iodine - potassium iodide and ceric ammonium sulphate (all alkaloid indicators). As a standard vindoline, an alkaloid known to accumulate in the plant was sprayed with the reagents on chromatographic paper

and colour as well precipitate reactions were recorded. Positive reactions were generally observed within laticifers cells of all parts in the mature plant (including the leaf, corolla, style and ovary of flowers) and in specialised parenchyma cells part of the cortex and pith regions. These cells also showed yellow – green primary fluorescence corresponding to exuded latex. There was an observed difference in positive reactions and the age (mature and immature) of the tissues as well as subterranean and aerial portions suggesting the uneven accumulation of alkaloids.

Furr and Mahlberg (1981) used alkaloid specific reagents (Wagner, Dittmar, Ellram, Dragendorff, Hager and chromic acid) to analyse fresh - cryostat preparations of *Cannabis sativa* laticifers, latex exudates, tissue components of fruit, seedlings, leaves and floral parts of pistillate and staminate axes. Reference standards of a variety of alkaloids were used as tests of alkaloid detecting solutions. It was determined that the accumulation of alkaloids took place in laticifers (regardless of maturity and organ) and capitate glandular trichomes. Positive responses were also detected in the exuded latex. Younger seedlings as well as embryos showed no reactions while positive responses were detected in the first true leaves of seedlings and various organs of the shoot. The pattern of alkaloid localisation was determined to be similar to that of cannabinoids and a variety of different alkaloids may accumulate in these areas such as hordenine, trigonelline and choline.

Idioblast oil cells were isolated from avocados (*Persea americana* vars. Hass and Fuerte) (Platt and Thomson 1992). These cells are present within the leaf, fruit, root and seed cotyledon. The idiobalst oil cells were successfully isolated from soft, ripe fruit as well as immature and mature unripe fruit. Dittmar and Wagner reagents were used as indicators and were applied directly to a suspension of isolated oil cells on a microscope slide and viewed using a light microscope. Positive reactions were observed but were however incomplete i.e., not all cells present stained the same. This was attributed to a variation in the types and amounts of alkaloids present or differences in the penetration of the stain as well as cell leakage. There was a variation observed between the immature, mature unripe and mature ripe fruit for Wagner reagent.

The close relationship between laticifer cells and the presence of alkaloids was further demonstrated by Sacchetti *et al.* (1999) in the plant *Vinca sardoa*. Fresh cross and longitudinal sections of the different organs were made by hand and histochemical (Ellram reagent, chloroauric acid, iodine - potassium iodide and phosphotungstic acid) analysis revealed positive reactions within laticifers throughout the roots, stems, petioles, leaves and flowers (corolla). The organs presented unbranched, articulated laticifers. Stem sections revealed laticifers with different alkaloid reactivity below the epidermis and close to the vascular bundles. Petiole sections had positive reactions in numerous laticifers below the epidermis and phloem parenchyma. Fresh and resin embedded leaf specimens

had laticifers cells close to the abaxial surface that reacted positively. Corolla sections had laticifers below the epidermis and in the parenchyma between vascular bundles showing positive reactions. Different degrees of alkaloid accumulation within the latex can explain the different alkaloid reactivities observed.

The secretory structures of the *Hypericum elodes* flowers were investigated by Bottega *et al.* (2004). Peduncles, sepals, petals, stamens and pistils were used for analysis. Two types of secretory canal structures were identified: one with a narrow lumen and another with a wide lumen. Different secretory structures exist such as glandular emergences, stipitate glandular pockets and the secretory canals. The different canals and stipitate glandular pockets were determined to be rich in alkaloids by use of Wagner's and Dittmar's reagent as well as iodo - iodinated solution.

Nikolakaki and Christodoulakis (2006) used Wagner's reagent, Dittmar's reagent and iodine – iodine solution in order to detect alkaloids in *Lavandula vera*. Mature leaves were detached from the plants and free hand sections of fresh tissue as well as cultured tissue were analysed. Histochemical investigations revealed that positive reactions are confined to the mesophyll cells (vacuoles) and glandular hairs (large peltate or small capitates). The mesophyll cell can be considered to be the secretory cell of the glandular hair. Positive reactions were also observed within the first and second generation callus being localised to the vacuole of cells. Corsi and Bottega (1999) also describe the localisation of alkaloids within glandular hairs of *Salvia officinalis*.

Alkaloids such as caffeine, theobromine and trigonelline were localised within *Coffea canephora* leaves using Dragendorff's reagent (Mondolot *et al.* 2006). Reactions were observed in the bundle sheath and phloem cells. Caffeoylquinic acids i.e., chlorogenic acids (intermediate in lignin biosynthesis) were determined to also be present within the same area as the alkaloids thus being in line with the hypothesis that caffeine is in a complex formation with chlorogenic acid (Mösli Waldhauser and Baumann 1996).

Histochemical analysis was used to investigate root cytology of two genotypes of *Senecio coronatus* (Thunb.) Harv. (Asteraceae) (Mesjasz – Przybyiowicz *et al.* 2007). The two genotypes have been identified as a Ni hyperaccumulator and a non-hyperaccumulator. Specialised cells with an organelle rich cytoplasm that produced a number of spherical bodies occurred in the inner cortical region of the hyperaccumulator. These were missing in the non-hyperaccumulator. Cortical cells here had a thin parietal cytoplasmic layer and produced fewer spherical bodies. In both genotypes the spherical structures were extruded from the cytoplasm into air spaces between cells where they formed amorphous deposits that were larger and more abundant in the hyperaccumulator. The spherical

deposits as well as the accumulated material within the intercellular air spaces were determined to contain alkaloids by using Dittmar and Wagner reagents on hand cut fresh root sections and observed with a light microscope. It is likely that pyrrolizidine alkaloids are stained and transported via the phloem from the roots to the shoots. Similar spherical deposits were seen in the endodermis, pericycle and phloem explaining their translocation.

Other examples of alkaloid localisation using histochemical techniques include the investigation of six *Simira* species (Rubiaceae). Dragendorff's reagent was used to stain freehand sections of mature leaves and localise general alkaloids to the vacuoles of mesophyll cells (Moraes *et al.* 2009). Wagner and Ellram reagents for alkaloids showed positive reactions in the glandular trichomes (types A, C and D with type D giving the strongest reaction) of *Lippia citriodora* fully developed leaves and calyces (Argyropoulou *et al.* 2010). The alkaloid sinomenine was detected in the walls of the lignified cells such as sclereid and pericyclic fibres (Cai *et al.* 1999). It was suggested by Liang *et al.* (2009) that the alkaloids of *Caulis sinomenii* should exist in the xylem of the cross section. Alkaloids were seen to accumulate in the mesophyll cells and most likely secreted by glandular trichomes in *Teucrium polium* fresh sections by use of Dragendorff's reagent (Christodoulakis *et al.* 2010).

1.6.2 Histochemical and radioactive isotopes use in localisation of alkaloids:

Histochemical and radioactive isotopes were used in parallel to determine the location of alkaloids within *Datura innoxia*, *Vinca minor* and *Valeriana officinalis* (Verzár - Petri 1975). Different positions of labelled ^{14}C precursors or marked alkaloids were used in parallel with histochemical reagents Dragendorff, Wagner - Mayer, picric acid and platinum chloride on fresh plant material. The material was prepared from tissues of seedlings, developed vegetative organs (*D. innoxia*) and individual organs (*V. minor* and *V. officinalis*). It was determined that major part of detection was in the intercellular spaces. Positive reactions within the laticiferous vessels of *V. minor* were also observed. In *D. innoxia* it was determined that alkaloids accumulate in crystalline form (glandular trichomes and vacuolar segregation). *V. officinalis* had positive reactions within the root cortex. It was determined that alkaloids are transported through the endoplasmic reticulum tubes from one cell to another and their formation was accompanied by radioactivity within the cell nucleus.

1.6.3 Histochemical and immunocytochemical localisation of tropane alkaloids:

Ferreira *et al.* (1998) investigated the localisation of tropane alkaloids i.e., cocaine within *Erythroxylum coca* var. *coca* and *Erythroxylum novogranatense* var. *novogranatense*. Sections of 30 – 40 µm thickness were made from fresh leaves, stems, roots and immature green, halfway mature orange and

mature red fruits embedded in Tissue Tek. Sectioning was performed at very low temperatures. Dragendorff's reagent was used as a histochemical marker and positive reactions were seen in the palisade and spongy mesophyll cells as well the vascular parenchyma and some cells in the collenchymas (abaxial isolated idioblasts). Positive reactions were also observed in the endosperm and embryos of orange and red fruits but not in green immature fruits as well as in brown stems (pith, cells among xylem rays and cortical parenchyma). As for flowers, alkaloids were present in the pedicels, petals, base filaments and pollen. No reaction occurred in the roots. Standard fixation and sectioning procedures were used for TEM as well as immunolabeling in order to perform cyotchemical investigations on leaves. For cytochemical investigations tannic acid was included in the fixative as it is known that alkaloids complex with phenolics. Anti-benzoylecgonine polyclonal antibodies (recognition of coca alkaloids) were used as primary antibodies while the secondary antibodies used were Protein A-gold. It was determined that the palisade and spongy mesophyll cells of young *E. coca* leaves contained vacuoles with small electron opaque spheres (0.5 µm). Addition of tannic acid revealed these small spheres being surrounded by particles that are more electron opaque. Immunocytochemical investigations revealed reactions to the periphery of these globular inclusions thus indicating that the vacuolar complex is composed of a core of phenolic compounds surrounded by cocaine and other related alkaloids aiding in their sequestration. In conclusion it was determined that cocaine was present primarily within the photosynthetic layer, within the vacuole of the photosynthetic tissue and *in vivo* complexed with phenols.

1.6.4 Histochemical and fluorescence localisation of alkaloids:

Camptothecin (a monoterpene indole alkaloid) is produced by the tree *Camptotheca acuminata*. The molecule autofluoresces when exposed to UV radiation and this characteristic was used to determine its cellular localisation. Fresh leaf, stem and root sections were used for analysis. Histochemical reagents (Jeffrey, Dittmar, Wagner and Dragendorff) were also used to observe the samples in brightfield although no positive reactions were observed. Camptothecin accumulation was determined to be within the palisade and spongy mesophyll (leaf), cortical and medullar (stem), cortical and phloematic (root). The results indicate accumulation occurs in segregator idioblasts localised to the parenchyma and epidermal tissues. Crystals of camptothecin were located in the vacuoles of the segregator idioblasts. Furthermore, the crystals were also localised to the glandular trichomes (unicellular as well as multicellular) of both the leaf and stem. The authors also observed the alkaloid within the lumen of xylematic vessels but suggested that it was a hydroxylated form (10-hydrossicamptothecin) which is a soluble thus allowing for its transport to various parts of the plant (Pasqua *et al.* 2004).

Other investigations of alkaloids within plant material by fluorescence microscopy include that of ancistroheynine A from the liana *Ancistrocladus heyneanus* (Bringmann *et al.* 1996), sanguinarine accumulation in elicitor – treated opium poppy cell cultures (Alcantara *et al.* 2005) and different alkaloids within four kinds of Chinese herbal medicines (Liang *et al.* 2009).

1.6.5 Immunofluorescence and immunocytochemical localisation of vindoline:

Brisson *et al.* (1992) investigated the localisation of vindoline within mesophyll protoplasts of *Catharanthus roseus*. The authors raised antivindoline antisera in rabbits and used this for immunofluorescence and immunogold labelling. Initially cryostat semi thin sections of fresh leaf material were used for immunofluorescence followed by isolated mesophyll protoplasts while cryotechniques were applied to isolated protoplasts for immunogold labelling in order to retain the antigen. Immunofluorescence labelling localised vindoline in the vacuoles of mesophyll protoplasts. Gold labelling lead the authors to suggest small vesicles labelled with gold are involved in the packaging or shuttling of vindoline toward the central vacuole. Labelling was observed within the vacuoles. Some labelling was also observed in the cytoplasm and chloroplasts but this was suggested to be cross-reactivity of the antibodies used.

1.6.6 Fluorescence and TEM localisation of indole alkaloids:

Indole alkaloid formation and storage within *C. roseus* was investigated by use of fluorescence and TEM by Neumann *et al.* (1983). Cell suspensions were used and bright blue fluorescence characteristic of indole alkaloids were observed in whole cells as well as the vacuoles of the cells. This was further investigated by TEM. Sample preparation was standard and OsO_4 was used. It is a known fact that certain alkaloids precipitate in the presence of OsO_4 (Hayat 2000). Alkaloid precipitation was observed within the vacuole of the cells and thus it was determined that this was the site of accumulation for alkaloids in *C. roseus* cells.

1.6.7 Fluorescence and FT – Raman localisation of naphthylisoquinoline alkaloids:

The unique potential of Raman microscopy was demonstrated by Frosch *et al.* (2006). A great advantage is that it is a non - invasive and a non - destructive technique. The authors localised naphthylisoquinoline alkaloids within the tropical liana *Triphyophyllum peltatum*. Fresh cross sections of 100 µm were investigated using an Nd: YAG laser with excitation wavelength of 1064 nm (NIR) to avoid high background fluorescence associated with plant material. By comparing the FT – Raman spectra of pure reference material of three structurally related naphthylisoquinolines, dioncophylline A

was identified in 10 μm large inclusions located within the cortex of the stem or beginning of the leaves, with the help of fluorescence microscopy and DFT calculations. The authors experienced problems in detecting low concentrations of the alkaloids outside the inclusions due to a necessary reduction in laser power to avoid sample damage.

1.6.8 FT – Raman localisation of ancistroheynine A and ancistrocladine:

Urlaub *et al.* (1998) investigated naphthylisoquinoline alkaloids within in the tropical liana *A. heyneanus* by FT – Raman microscopy, a study continued from fluorescence microscopy (Bringmann *et al.* 1996) and NMR microscopy (Meininger *et al.* 1997) investigations. The same type of laser as Frosch *et al.* (2006) was employed. A spectral range of 200 – 4000 cm^{-1} with a resolution of 4 cm^{-1} was used. The cells containing alkaloids were first localised visually by fluorescence microscopy according to Bringmann *et al.* 1996. The alkaloids ancistroheynine A and ancistrocladine were detected during analysis on hand cross sections made from fresh plant material. Ancistroheynine A was located in the tip of the shoot (pith region) as well as the leaf midrib (vascular tissue) while ancistrocladine was located to the bark region of the branch roots of the plant. The authors proved that the technique used was very sensitive when distinguishing between different alkaloids of similar structure thus making it highly specific.

1.6.9 UV Raman localisation of dioncophylline A and quinine:

UV Raman microscopy has been applied for the localisation of dioncophylline A (antiplasmodial naphthylisoquinoline alkaloid) in *T. peltatum* (Frosch *et al.* 2007a) and quinine in cinchona bark (Frosch *et al.* 2007b). The method proved to be advantageous over Raman microscopy in the NIR due to low fluorescence backgrounds and it is highly sensitive and specific due to the method resonantly enhancing vibrational modes of the molecule of interest allowing distinguishing between molecules of very similar structure as well as requiring minimal sample preparation and it is non – invasive and non - destructive. Mode assignments are completed by means of DFT calculations and wavelengths of 244 and 257 nm were applied for dioncophylline A while 244 nm was applied for quinine. For *T. peltatum*, fresh cross sections of the leaf, stem and root were analysed. This was a follow up study from Frosch *et al.* (2006) due to the lower sensitivity of NIR FT – Raman microscopy not being able to detect dioncophylline A outside of the inclusions in different parts of the plant because the laser power needed to be increased and this destroyed the sample. Dark coloured plant material such as roots also could not be investigated as well. UV Raman resonance sensitivity proved that the technique has the ability to bypass these problems and the alkaloid could be detected in the different parts of the plant. The same was observed for the localisation of quinine in cinchona bark in fresh cross sections.

1.6.10 NMR localisation of naphthylisoquinoline alkaloids:

NMR microscopy reviews exist explaining the technique in detail (Ishida *et al.* 2000). Naphthylisoquinoline alkaloids within the tropical liana *Ancistrocladus heyneanus* were investigated (Meininger *et al.* 1997). The technique was used to observe and localise the biosynthesis of the alkaloids as well as to identify the molecular and chemical state *in vivo*. The investigation included quantitative NMR microscopy and chemical shift imaging (detection of metabolites) as well as light and fluorescence microscopy to support their findings. Fresh materials from the plant were used for NMR studies. To identify and localise metabolites spatially resolved NMR spectroscopy, autofluorescence microscopy of plant tissue and high resolution NMR spectroscopy of tissue extracts was used. Ancistroheynine A was determined to be the main alkaloid within tissue extracts. An aromatic marker at 6.7 ppm was used as a reference and its strongest signal was seen in the cortex, pith with lower intensity in the xylem of the leaf. Autofluorescence of the ancistroheynine A was used to confirm a similar distribution to that of the aromatic marker.

1.6.11 Conclusion:

The site of alkaloid accumulation in plants differs in different species. Some plants accumulate alkaloids within the vacuoles of parenchyma cells (photosynthetic layer), idiobalsts, laticifers, others store and secrete them via glandular trichomes as a possible defensive mechanism against predators and some even transport them via the phloem or xylem (localised to the vascular areas) to other parts of the plant. Other areas of localisation have been mentioned and no two plants can be assumed to have similar alkaloid accumulations. When doing investigations using microscopy, it is imperative to ensure that minimal extraction of the molecule of interest occurs. This is either done by non-invasive techniques such as Raman microscopy or unconventional sample preparation such as cryotechniques to fix the molecule of interest in place within the sample. It is also important to make sure that the method of choice is specific and sensitive for the molecule that will be investigated, unless the investigation focuses on an entire class of compounds (such as alkaloids) instead of a single molecule within a class.

1.7 Aims of the study:

- To develop a method of sample fixation for microscopic analysis that will result in the minimal extraction of caffeine from leaf sections as shown by HPLC analysis.
- To anatomically determine the localisation of caffeine within *C. sinensis* leaf sections using different microscopy techniques.

- Using the knowledge of caffeine localisation within *C. sinensis*, develop a new unique method of tea decaffeination.

1.8 Hypotheses:

- H1: Caffeine is localised to vacuoles of parenchyma cells of young *C. sinensis* leaves.
- H2: There is a statistically significant difference in the percentage of decaffeination between the new method developed in this study and the hot water method of decaffeination at the 95 % level of confidence.

1.9 Null Hypotheses:

- $H1_0$: Caffeine is not localised to the vacuoles of parenchyma cells of young *C. sinensis* leaves but is accumulated elsewhere within the plant.
- $H2_0$: There is no statistically significant difference in the percentage of decaffeination between the new method and the hot water method of decaffeination at the 95 % level of confidence.

1.10 Research outputs:

Parts of this study have been presented as a poster and oral presentation at the following conferences respectively:

van Breda, S.V., van der Merwe, C.F. and Apostolides, Z. (2010). Differential extraction of caffeine and polyphenols from fresh leaves of *Camellia sinensis* by water. Microscopy of Southern Africa 48th annual conference, 25 – 28 October, Bela Bela, South Africa.

van Breda, S.V., van der Merwe, C.F., Robbertse, H. and Apostolides, Z. (2011). Immunohistochemical localisation of caffeine in young *Camellia sinensis* (tea) leaves. Microscopy of Southern Africa 49th annual conference, 5 – 9 December, Pretoria, South Africa.

Awards

Wirsam Scientific prize for best student paper submitted: **van Breda, S.V., van der Merwe, C.F., Robbertse, H. and Apostolides, Z.** (2011). Immunohistochemical localisation of caffeine in young *Camellia sinensis* (tea) leaves. Microscopy of Southern Africa 49th annual conference, 5 – 9 December, Pretoria, South Africa.

The following article has been accepted for publication at Planta (impact factor = 3 in 2011):

van Breda, S.V., van der Merwe, C.F., Robbertse, H. and Apostolides, Z. (2012). Immunohistochemical localization of caffeine in young *Camellia sinensis* (L.) O. Kuntze (tea) leaves. Planta, X, X – X. DOI: 10.1007/s00425-012-1804-x

The following patent has been awarded for part of the work:

van Breda, S.V. and Apostolides, Z. (2011). A method for decaffeinating tea. Claiming priority from S.A provisional patent 2011/05726, August 3 2011. Complete patent filed July 23 2012.

Chapter 2: localisation of caffeine within young *C. sinensis* (tea) leaves

2.1 Introduction:

Chemical fixation involves the use of formaldehyde (HCHO, one aldehyde group), glutaraldehyde ($HCO-(CH_2)_3-CHO$, two aldehyde groups) and the use of osmium tetroxide (OsO_4). Formaldehyde is a small molecule with rapid penetration into samples, adding to the side-chains of basic amino acids i.e., lysine and the amide nitrogen atoms of peptide linkages. The molecule can also form methylene bridges if two of these atoms are very close together thus anchoring soluble proteins to the cytoskeleton (Hayat 2000). Glutaraldehyde is a larger molecule taking longer to penetrate membranes. Cross-linking is greater due to two aldehyde groups, anchoring proteins in a similar manner as formaldehyde. Glutaraldehyde also exists as low polymers containing lots of free aldehyde groups after fixation. These groups can bind covalently with any free amino group such as those on antibodies, excluding it from immunolocalisation as this might lead to false positives. Autofluorescent complexes may also form, excluding glutaraldehyde's use from fluorescent microscopy. OsO_4 acts as a fixative and a stain that provides contrast to the image in electron microscopy by embedding the heavy metal into cellular membranes and the reader is referred to Hayat (2000) for more information on this topic. Fixation is normally followed by dehydration at room temperature, reducing the solubility of protein molecules, and disrupting hydrophobic interactions allowing for precipitation and aggregation of proteins. Dehydration also helps with resin infiltration as resins are generally water immiscible (Hayat 2000). Chemical fixation at room temperature releases permeability barriers on the sample causing shifting and extraction of molecules (Coetzee and van der Merwe 1984; Coetzee and van der Merwe 1986; Hayat 2000). Therefore this method is not suitable for localisation of small molecules.

Localisation of alkaloids (being small molecules) is extremely difficult and challenging due to problems associated with fixation procedures as well as disruption of permeability barriers leading to diffusion of small molecules. Another concern is caffeine's solubility in alcohol (1 g in 66 ml) (The Merck index 1976), thus likely making room temperature dehydration unfeasible. Preliminary embedding experiments using chemical fixation, HPF/ FS, and HPF/ FD were conducted using young *C. sinensis* leaf material in order to choose the most suitable method for caffeine retention within in the sample for microscopy analysis. The difference in molecule retention within a sample using various embedding procedures has been noted (Brisson *et al.* 1992) and once the molecule of interest can be retained throughout the embedding procedure, minimal extraction will occur from resin embedded sections during localisation studies (Brisson *et al.* 1992; Ferreira *et al.* 1998).

Cryotechniques have shown to be promising in the retention of small molecules (Brisson *et al.* 1992 and refs therein). HPF/ FS or FD are better methods than chemical fixation due to preservation of the internal structure as well preventing damage to the sample's ultrastructure (Steinbrecht and Zierold 1987; Bourett *et al.* 1999; Hawes *et al.* 2007). It must be noted that these techniques also use similar compounds as mentioned for chemical fixation but the extraction artefacts seen are far lower due to the very low temperatures used. Therefore, HPF/ FS or FD aid in the retention of the antigen (caffeine) as opposed to other techniques performed at room temperature. HPF is based on the principle of vitrification, preventing crystallisation of water and freeze damage to the sample. The process is a physical one and the reader is referred to Steinbrecht and Zierold (1987) for more theory on the topic. Instead of chemical fixation, samples are frozen rapidly at low temperatures, and at high pressure, by vitrified water (amorphous). HPF has a number of advantages over standard chemical fixation such as preventing the shifting and extraction effects of molecules that normally occur during chemical fixation (Somlyo *et al.* 1985; Zierold 1991), rapidly arresting physiological processes (Heuser *et al.* 1979; Knoll and Brdiczka 1983) and avoiding the use of harmful chemicals that will disrupt permeability barriers. After HPF, samples are normally FS or FD. Water is gently removed from the sample via these methods with the main prerequisite being good cryofixation and minimal freeze damage. FS normally involves the substitution of water by an organic solvent. The low temperature prevents formation of artefacts and secondary ice crystal growth. The temperature is raised gently over a period of time. FD involves the removal of frozen water by sublimation under vacuum at low temperatures for the same reason as FS. Once the samples are dry they can be infiltrated by a resin of choice at low temperature or room temperature (Steinbrecht and Zierold 1987).

Many different types of resins exist (Hayat 2000). In my experiments I decided that Lowicryl K4M and LR White resins (both known as water - miscible embedding media being able to polymerise in low water percentage) were the best. The resins have very low viscosities allowing easy penetration into the sample even at very low temperatures. Some resins polymerise only at 60°C. This high temperature could lead to antigen disruption, while my resins of choice have the ability of low temperature polymerisation that will help in antigen preservation, possibly minimal extraction, and shifting effects of molecules. These resins have also been shown to be optimal for immunolabeling as opposed to others (such epoxy and polyester resins) that require etching before labelling as the resin may chemically interact with the antigen. Solutions of hydrogen peroxide or EtOH are generally used to etch and, may extract the antigen of interest (such as a small molecule like caffeine). Lowicryl K4M (polar, hydrophilic, acrylate – methacrylate, aliphatic crosslinked) can be polymerised at low temperatures under UV light (initiates a free - radical reaction allowing crosslinking). Lowicryl K4M resin was developed to have properties that improve preservation of molecular structure including antigenicity, being more efficient in immunolocalisation furthermore reducing non-specific

immunolabeling background. This is mainly due to low temperature sample preparation and resin embedding. The low temperature also immobilises molecules due to decreased thermal vibrations during solvent exchange. LR White (polar, monomer polyhydroxylated aromatic acrylic) is capable of thermal or cold polymerisation. Sections of polymerised LR White are hydrophilic allowing easy access of an antibody to an antigen. For more information on the resins the reader is referred to Hayat (2000) and references within.

Histochemical analysis using stains such as Dragendorff's (potassium iodobismuthate) reagent are not specific for a small molecule such as caffeine but rather an entire class of molecules such as alkaloids. Furthermore these indicators are known to give false positives or side reactions (Pedersen 2006). The potassium iodobismuthate complex is anionic. This can form a complex with a cationic alkaloid causing a precipitation and colour reaction i.e., $(alkaloid^+)_m(BiI_4^-)_m$. Dragendorff's reagent reacts with many quaternary and tertiary amine groups so the risk of false positives is possible as it could cause a positive reaction with cumarines, hydroxyflavones and even proteins may give positive reactions (Pedersen 2006). Caffeine does not necessarily react directly with these histochemical reagents as during TLC analysis with Dragendorff's reagent it is suggested to apply a 20 % nitric acid solution and heating the TLC plate to 100°C before spraying with the reagent for a positive development. These stains were originally developed to be used for TLC and not staining tissue samples for microscopic analysis (Baerheim – Svendsen and Verpoorte 1983; Pedersen 2006). Thus a more specific method of small molecule localisation is required. Raman microscopy has this ability, and has the additional advantage of being non-invasive. However, Raman mapping of caffeine within green *C. sinensis* leaves has been attempted with no conclusive results (Baranska and Proniewicz 2008). A Nd:YAG laser emitting at 1064 nm and measurements using green *C. sinensis* leaves was used by Baranska and Proniewicz (2008). Unfortunately, there was much questionability about the actual results and a formal conclusion about the localisation of caffeine using the FT-Raman spectra obtained could not be made due to other plant components having similar spectral features. Localisation of caffeine could not be completed and thus the results were inconclusive. We attempted confocal Raman microscopy on our samples (young *C. sinensis* leaves) with help from WITec in Germany using an alpha 300 series microscope. Very similar results were obtained that were also inconclusive (data not shown). However a 1064 nm laser was never used on the samples and it was suggested a laser in the UV range should be explored. Other techniques such as UV Raman and NMR microscopy might be able to circumvent this issue, however accessibility to such equipment is difficult. Therefore, it was decided to use immunolabeling as a specific technique for localisation of caffeine within *C. sinensis* using confocal (immunofluorescence) and TEM (immunocytochemical) analysis (Immunogold labelling).

Albert Coons and his fellow colleagues were the first to label an antibody with a fluorescent dye and use it to identify an antigen in a tissue section (Coons *et al.* 1941, 1955; Coons and Kaplan 1950). Immunolabeling is extremely useful in localising small molecules (Brisson *et al.* 1992; Ferreira *et al.* 1998; Kim and Mahlberg 1997), as the technique is much more specific and sensitive compared to other techniques such as histochemical analysis. Polyclonal antibodies are a better choice over monoclonal antibodies for immunolocalisation since they are able to detect more than one epitope in the antigen. Small changes in the structure of the compound of interest due to fixation protocols usually won't affect binding affinity and they react with structurally related compounds compared to the compound being localised (Ferreira *et al.* 1998; Ibrahim 1990). Using a primary antibody specific for the antigen in question and using a secondary conjugated antibody (fluorophore or gold) to detect the primary antibody is known as the indirect method (Polak and Noorden 1987; Hibbs 2004). This method has a number of advantages over the direct method such as increasing signal intensity due to multiple secondary antibodies binding to a single primary antibody (Polak and Noorden 1987). Conjugating a secondary antibody to a fluorophore allows for visualisation using a confocal microscope. Confocal microscopy has a number of advantages over traditional fluorescence microscopes such as increased effective resolution, reduced blurring of the image from light scattering, improved signal-to-noise ratio, z-axis scanning, depth perception in z-sectioned images and electronic magnification adjustment. Another great advantage is excitation and emission of very specific wavelengths, thus signals obtained from analysis will be very specific for the fluorophore being used. It is imperative that an anti-photobleaching mounting media is included as photobleaching of the fluorophore conjugated to the secondary antibody bound to primary antibody within the sample will occur (Robinson 2001). Conjugating colloidal gold to a secondary antibody will allow for analysis using a TEM and has proved to be a very useful method at the ultrastructural level and allows for *in situ* localisation of molecules (Polak and Noorden 1987; Hayat 2000). Using colloidal gold has the additional advantage of quantification (Polak and Noorden 1987; Hayat 2000), morphometric analysis (Brisson *et al.* 1992) as well as statistical analysis (Mayhew 2011). Different texts are available that offer extensive reviews describing the techniques and problems involved in immunolabeling (Polak and Noorden 1987; Skepper 2000; Hayat 2000; Hibbs 2004).

I decided to use a pressure bomb, also known as a pressure chamber or Scholander bomb. Instead of using the instrument to determine water potential I decided to collect xylem sap for caffeine analysis. The instrument was originally designed to measure water potential of plant tissues. It contains a chamber in which a leaf attached to a stem is placed. A small part of the stem is exposed to atmospheric pressure while the rest of the sample is placed within the sealed chamber. As the pressure increases within the chamber at some point sap will be forced out the xylem and will become

visible at the cut end. From this visualisation and pressure readings water potential can be calculated (Scholander *et al.* 1965).

2.2 Method and materials:

2.2.1 Plant material:

C. sinensis (L.) O. Kuntze leaves of cv. PC105 were collected in the morning from the experimental farm at the University of Pretoria, Hatfield, South Africa. A plucking method used was to collect two leaves containing a bud although only the first and second leaves were used for all analysis. Mature *C. sinensis* leaves used for analysis (as a control group) were collected at the same time from the bottom of a cv. PC105 bush. *Hedera helix* L. (English ivy) leaves were collected from the University of Pretoria's gardens in the morning to be used as a control containing no caffeine.

2.2.2 Preparation of a decaffeinated tea leaves (hot water decaffeination):

As a control, some of the tea leaves were used to prepare decaffeinated tea leaf. The method used was according to Liang *et al.* (2006) where boiling young fresh tea leaves in water for 3 minutes removes ± 83 % of caffeine content and only ± 5 % of total catechins. The leaves prepared in this manner are referred to as decaffeinated *C. sinensis* leaves within the text.

2.2.3 Preliminary fixation experiments:

In order to determine which fixation method would retain the most caffeine, preliminary fixations were carried out. Young *C. sinensis* leaf material of 600 mg fresh weight was used and cut into pieces of 5 mm^2. For chemical fixation, the leaf material was placed in 2.5 % (v/v) glutaraldehyde, 2.5 % (v/v) formaldehyde in 0.15 M phosphate buffer (pH 7.4) for 2 hours. Samples were rinsed 3 x 5 minutes with phosphate buffer and then dehydrated in 30, 50, 70, 90 and 2 x 100 % EtOH (Saarchem, Gauteng, South Africa) for 5 minutes each. In each instance, 10 ml of solution was used. For HPF, samples were snap frozen in liquid nitrogen and then either prepared for FS or FD. For FS, 10 ml of 100 % EtOH (pre-cooled to -70°C) was added to the frozen samples, and kept at -70°C for 24 hours, then warmed to -20°C for 24 hours, followed by 4°C for 2 hours. For FD, samples were placed in a freeze drier at -50°C (VIRTIS, New York, USA) for 24 hours or until dry. Samples were then infiltrated with resin at 4°C over night. Once complete, samples were rinsed in ddH_2O (to remove excess resin that might damage any HPLC equipment), blot dried with paper towel and dried over night at 103°C for

HPLC analysis (caffeine). Duplicate experiments were carried out on the same day and two independent experiments were completed.

2.2.4 Moisture content determination:

Moisture content determination was according to ISO 1573. However, only 0.1 g ± 0.001 g of sample was used. The formula used was actual mass of sample (mg) = wet weight (mg) - (wet weight (mg) x % moisture content). Triplicate measurements were carried out and two independent experiments completed. Standards used were either Lipton black tea or Lipton green tea bought at a local supermarket.

2.2.5 Caffeine content determination by HPLC:

Two different methods were used to determine the caffeine content of samples by HPLC analysis. Method 1 was adopted from ISO/CD 14502-2 while method 2 was our own PVP treatment method. Both methods can determine related alkaloids theophylline and theobromine while method one has the additional advantage of determining polyphenols and catechins. All chemicals used were of HPLC grade and only 0.2 μm filtered $dddH_2O$ was used. After the dried samples were ground and their moisture content determined, they were placed into centrifuge tubes (method 1: 0.2 g ± 0.001 g and method 2: 0.1 g ± 0.001 g) for HPLC analysis. For both methods the extraction procedure was done according to ISO/CD 14502-2 with method 1 using 10 ml of an extraction mixture of methanol/ water 70 % (v/v) Chromasolv® methanol (Sigma–Aldrich, Steinheim, Germany) containing ethyl gallate (Fluka, Steinheim, Germany) (1 mg/ml) as an internal standard at 70°C, while for method 2 an extraction solvent of 10 ml $dddH_2O$ containing theophylline (Sigma, Steinheim, Germany) (1 mg/ml) as an internal standard was used at 90°C. Once samples were extracted they were treated as follows: For method 1, the samples were diluted five times in stabilizing solution containing 10 % (v/v) acetonitrile (Merck, Darmstadt, Germany) with 500 μg/ml EDTA (Sigma, Steinheim, Germany) and 500 μg/ml L-ascorbic acid (Sigma, Steinheim, Germany) while for method 2 the extracted tea liquors were added to 500 mg PVP (Sigma, Steinheim, Germany) in 50 ml tubes, mixed using a vortex and incubated at room temperature for 10 minutes. Caffeine (Merck, Darmstadt, Germany) was used as a standard at a concentration of 0.2 mg/ml dissolved in stabilising solution for method 1 while for method 2 a concentration of 1 mg/ml dissolved in $dddH_2O$ was used. These were also used in construction of a standard curve with $R^2 > 0.98$ for both methods. One ml of each sample was then filtered using 0.2 μm Acrodisc® PSF syringe filters (PALL life sciences, Michigan, USA) or MINISART RC4 (SartoriusStedim biotech, Goettingen, Germany) into vials and sealed with septa. Bubbles were removed using a vortex and the samples were placed in a 717 plus autosampler (Waters™,

Massachusetts, USA) at 4°C for method 1, or a 717 autosampler (Waters™, Massachusetts, USA) at room temperature for method 2. Mobile phases used for method 1 were (A1) 10 % (v/v) acetonitrile, 0.1 % (v/v) formic acid (Saarchem, Gauteng, South Africa) and (B1) 80 % (v/v) acetonitrile, 0.1 % (v/v) formic acid while for method 2 they were (A2) 1 % (v/v) methanol, 0.1 % formic acid and (B2) 80 % (v/v) methanol, 0.1 % (v/v) formic acid. Gradient conditions for method 1 were according to ISO/CD 14502-2 except that before resetting and end equilibration in 100 % A1, 100 % B1 was used for 10 min first then reset to 100 % A1 over 1 minute and equilibrated for 10 min. Gradient conditions for method 2 were 100 % A2 for 5 min, then over 20 min a linear gradient to 100 % B2 and this composition was held for 20 min, then over 3 min a linear gradient back to 100 % A2 which was held for 10 min before the next injection. For both methods, a flow rate of 1 ml/min was used and solvents were degassed using a sonicator while for method 1 the solvents were additionally sparged with helium gas at 30 ml/min during analysis. Method 1 used a G600 series controller (Waters™, Massachusetts, USA) and 600 pump (Waters™, Massachusetts, USA) while method 2 used a 510 binary pump (Waters™, Massachusetts, USA). The column used in both methods was a Luna 5 µm Phenyl-Hexyl column (Phenomenex®, California, USA) with dimensions 250 mm x 4.6 mm and filtered with a SecurityGuard™ Phenyl-Hexyl cartridge (Phenomenex®, California, USA) with dimensions 4 mm x 3 mm. For method 1, the column was maintained at a temperature of 35°C using a column thermostat (Merck, Darmstadt, Germany). Once the systems were primed and the column conditioned, 10 µl of each sample were injected for both methods. The detectors used were a 996 photodiode array detector (Waters™, Massachusetts, USA) with UV wavelengths set between 190 nm and 500 nm for method 1 and an 486 tunable absorbance detector (Waters™, Massachusetts, USA) set at 280 nm UV wavelength for method 2. After each batch of analysis in both cases systems were flushed using 50 % (v/v) acetonitrile and software used for data analysis was Empower™ 2 version 6.2 (Waters™, Massachusetts, USA). Lipton green tea was used as a standard and analysis was completed in duplicate on the day and two independent experiments were completed in each case.

2.2.6 HPF/ FS and embedding of samples:

Fresh leaf samples with a thickness of c. 200 µm were used for HPF/FS. From the leaf blade, discs of c. 1.2 mm in diameter were cut using a HPF punch. Using a syringe, samples were vacuum infiltrated with 1-hexadecene (Merck, Darmstadt, Germany). Once infiltrated and all air had been removed, samples were placed into the 200 µm depression of Leica gold HPF planchettes (Vienna, Austria) and coated with a 1 % w/v L-α-phosphatidylcholine type X-E (Sigma, Steinheim, Germany) solution. The samples were then placed in the sample holder of a Leica EM PACT2 (Vienna, Austria) and frozen under high pressure. Once frozen, samples were placed in FS sample holders and care was taken to ensure that the samples remained in liquid nitrogen at all times. Samples were then placed in a Leica

EM AFS 2 system (Vienna, Austria) at a temperature of -90°C. Once the liquid nitrogen had evaporated from the FS sample holders, 100 % EtOH (Merck, Gauteng, South Africa) pre-cooled to -90°C was added to the sample holders. Samples were FS in 100% EtOH at -90°C for 12 hours followed by warm-up at 5°C h^{-1} until -30°C was reached. Once at -30°C, samples were carefully dislodged from their planchettes and infiltrated with Lowicryl K4M resin (Polysciences Inc, Eppelheim, Germany) and EtOH with the following ratios of 1:1, 2:1 and 3:1 for 1 hour each at -30°C. A cross linker concentration of 18 % w/w was used to make the resin. This was followed by an infiltration with 100 % Lowicryl K4M resin at -30°C for 24 hours with two changes of the resin. Samples were then placed in embedding moulds with Lowicryl K4M and polymerised at -30°C for 72 hours under a UV light. Samples were embedded in triplicate over two independent embedding experiments. Samples were also embedded and polymerised in LR White™ (SPI Supplies, Philadelphia, USA) in the same procedure as above. In order to polymerise LR White resin at low temperatures under UV light, the initiator used to polymerise Lowicryl K4M resins was used in the same concentration. LR White embedded samples were used to make sections for TEM while Lowicryl K4M embedded samples were used to make sections for confocal microscopy.

2.2.7 CSLM analysis of samples:

Lowicryl K4M resin blocks were sectioned at 5 μm thickness with a dry glass knife using a Reichert Ultracut E Ultramicrotome (Vienna, Austria). Sections were transferred to slides with an eyelash brush and fixed using a slide warmer and ddH_2O. In all cases contact of the sections to water was kept to an absolute minimum to avoid caffeine extraction. Immunolabeling was then performed at room temperature. Once fixed to slides, samples were blocked for 1 hour in 2.5 % (v/v) donkey serum (Sigma, Steinheim, Germany) or 2 % (w/v) BSA (Merck, Dramstadt, Germany), dissolved in 1 x PBS (pH 7.4) followed by labelling with sheep polyclonal anti-caffeine Ig fraction (American Research Products, Inc, Massachusetts, USA) (cross – reactivity data displayed in Table 2.1) at a dilution of 1:200 (v/v) for 1 hour. Samples were then rinsed 3 x 3 min in blocking buffer and 3 x 3 min 1 x PBS (pH 7.4). Samples were secondary labelled with donkey polyclonal anti-sheep IgG conjugated to DyLight®488 (AbD Serotec, Oxford, United Kingdom) at a dilution of 1:200 (v/v) for 1 hour, after which samples were rinsed as described but with 1 x PBS (pH 7.4). Above antibodies were always diluted according to manufacturer's specification. Samples were then mounted in an anti-photobleaching media containing 0.1 M ethyl gallate (Fluka, Steinheim, Germany), 10 % (v/v) 0.15 M sodium phosphate buffer (pH 7.4) in glycerol (Sigma, Steinheim, Germany), adopted from Giloh and Sedat (1982) and then sealed with a cover slip and nail polish. Controls included samples labelled excluding primary antibody and labelled with primary antibody pre-adsorbed with caffeine. Other controls included the decaffeinated caffeine *C. sinensis* leaf, mature *C. sinensis* leaf and the *H. helix* leaf

described above. Samples were then viewed using a Zeiss CSLM 510 META microscope (Jena, Germany). Two channels were used for analysis: Ar/Kr 488 nm laser with a band pass between 505 and 530 nm (green) to detect the secondary antibody and a He/Ne 543 nm laser with a long pass from 560 nm (red) to detect autofluorescence in the samples. A pin hole size of ≈ 70 μm was also used when images were taken. Samples were viewed using a 40x water immersion lens and areas of interest were enlarged using the scan zoom (crop) function of Zeiss LSM 510 META software version 3.2 SP2 (Jena, Germany). A Z series stack of images was taken comprising of between 10 and 40 optical slices being ≈ 1 μm apart. DIC images were also taken of the areas of interest in the samples. Images of samples were taken with the following procedure: young fresh *C. sinensis* were scanned with the green laser and optimal settings for minimal background and autofluorescence with highest signal for the conjugated secondary antibody were determined mainly by gain manipulation and the palette function. The red laser was also optimally determined for autofluorescence. Once this was complete, the green and red laser settings were confirmed with controls in order to determine if the signal observed was from the labelled secondary antibody. The same settings were used for the decaffeinated *C. sinensis* leaf, mature *C. sinensis* leaf and the *H. helix* leaf except that only the red laser settings were manipulated for optimal settings and the green laser settings optimized for young *C. sinensis* were maintained throughout. Images were further edited using Image J (Abramoff *et al.* 2004).

Table 2.1: Cross – reactivity data of primary caffeine antibody with related alkaloids (http://www.arp1.com catalogue number: 13 - 2148)

Alkaloid	Relative activity (% of caffeine)
Caffeine	100
Theophylline	100
Theobromine	< 1

*Determined using polarisation fluoroimmunoassay

2.2.8 TEM analysis of samples:

LR White resin blocks were sectioned with a Diatome 35° diamond knife to a silver or pale gold thickness. Sections were collected using Cu/Pd 200 mesh grids coated in formvar. Samples embedded in LR White were used for TEM analysis. All buffers and washing solutions were filtered using 0.2 μm membrane disc MINISART RC4 (SartoriusStedim biotech, Goettingen, Germany) and in each case 30 μl of solution was used. Once the grids were dry, samples were blocked by immersion for 1 hour in 2.5 % (v/v) donkey serum (Sigma, Steinheim, Germany) or 2 % (w/v) BSA (Merck, Dramstadt, Germany) dissolved in 1 x PBS (pH 7.4) followed by labelling with sheep polyclonal anti-

caffeine Ig fraction (American Research Products, Inc, Massachusetts, USA) for 1 hour at a dilution of 1:50 (v/v) (cross – reactivity data displayed in Table 2.1). Samples were then rinsed 3 x 3 min in blocking buffer and 3 x 3 min in 1 x PBS (pH 7.4). Before the diluted secondary antibody was used, it was centrifuged to remove any micro-aggregates of gold that might have accumulated during storage. Samples were secondary labelled with the supernatant of donkey polyclonal anti-sheep IgG conjugated to 10 nm gold particles (Abcam, Massachusetts, USA) at a dilution of 1:20 (v/v) for 1 hour, after which samples were rinsed 5 x 3 min in 1 x PBS (pH 7.4). Once rinsed, the grids were drained using filter paper and left to dry. Grids were always floated section face down on all solutions and the above antibodies were always diluted according to manufacturer's specifications. Samples were viewed using a JEOL 2100 transmission electron microscope (JEOL, Tokyo, Japan). No counterstaining was performed.

2.2.9 Light microscopy of samples:

In order to identify the undifferentiated cells of the young fresh *C. sinensis* leaves, sections of the young as well as the mature leaves were made as described above. Samples were then stained with 0.2 % (w/v) Toluidine blue O in 0.5 % sodium bicarbonate. Sections were stained for 30 seconds at room temperature and sections were then rinsed with ddH_2O. Samples were then mounted in glycerol and viewed using a Nikon Optiphod transmitted light microscope attached with a Nikon DXM1200 digital camera and the software used was Nikon ACT-1 ver. 2 (Nikon Instech Co., Kanagawa, Japan). ImageJ was used for image processing.

2.2.10 Editing of images with ImageJ software:

ImageJ 1.43j 32-Bit was used for image processing for CSLM images. Images in .lsm format were opened and both green and red channels were merged. The image stack was then converted to RGB and extended depth of field (EDF) easy algorithm (Forster *et al.* 2004) was used. If needed, the transform function was used to rotate images or vertically flip them. A background subtraction for the images was done. Noise was reduced using the outlier function and colours were enhanced.

2.2.11 Pressure bomb and TLC experiments:

A pressure bomb instrument was used to extrude xylem sap from one or two *C. sinensis* shoots. Approximately 100 µl xylem sap was collected and spotted onto a silica gel 60 F254 TLC plate (Merck, Darmstadt, Germany). A caffeine (Merck, Darmstadt, Germany) standard concentration of 1 mg/ml was used and 10 µl was spotted. A mobile phase of methanol: ammonia solution (ammonium

hydroxide) with ratio 99:1 was used to develop the plate, and it was visualised under UV light. The TLC method was adopted from Rogers *et al.* (1993).

2.2.12 Statistical analysis:

All statistical analysis was completed using JMP® 9.0.0 software copyright© 2010 SAS institute. Standard deviations, standard errors of the mean and one way ANOVA's were calculated. Average dry weight of reference young *C. sinensis* leaves and reference mature *C. sinensis* leaves were determined. Reference leaves were used, as leaves of different sizes would naturally have different weights yielding varying amounts of caffeine. For caffeine content determination, caffeine content was determined per leaf then averaged.

2.3 Results:

2.3.1 Determination of caffeine content in leaf samples and preliminary fixation experiments:

Whole young leaves of *C. sinensis* contain 2.39 $\pm$ 0.05 % (w/w) caffeine (Table 2.2) based on a dry weight. When these young leaves were hot water decaffeinated, a caffeine content of 0.53 $\pm$ 0.04 % (w/w) was determined and thus a total reduction of 78 %. HPLC analysis also revealed that there was no significant loss of catechins such as epigallocatechin gallate (EGCg) in the decaffeinated *C. sinensis* leaf when compared to the young leaf (data not shown). HPLC analysis also revealed that theophylline and theobromine are present in the young leaf in a minimal concentration compared to caffeine. Theobromine was present at a concentration of 0.03 % (w/w) and theophylline at 0.44 % (w/w). The mature *C. sinensis* leaf contained 0.04 % w/w caffeine, 83 % lower than in the young leaves. This suggests they could serve as suitable control material low in caffeine but with fully differentiated cells and no damaging effects from boiling the sample to remove caffeine. *Hedera helix* was determined to contain no caffeine, confirming its usefulness as an independent control sample. An interesting observation was the fact that as the tea leaf matures, it has an increase of three times its original weight (dry) while its caffeine content decreases by 50 % (according to reference leaves used).

The best fixation technique determined was HPF and FD, since the young *C. sinensis* leaves retained 95 % of their caffeine content (Fig. 2.1) and, according to the one way ANOVA, it was not statistically different from the control sample. Traditional chemical fixation was the worst with only 23 % of caffeine being retained. The method of HPF and FS was determined to retain 49 % of its caffeine content throughout the fixation and infiltration process, making it an intermediate method of choice for fixation.

HPF/ FS was used as the method of choice for resin embedding since difficulty in producing adequate sections with HPF/ FD were continually encountered as it seemed that the samples were poorly infiltrated with resin.

Table 2.2: Caffeine and related alkaloid analysis of different samples

Leaf material	Caffeine content (% dry w/w) mean ± SD	Average dry weight (mg) of reference leaf	Caffeine (mg) per dry reference leaf
Young *C. sinensis* leaves	2.39 ± 0.05	84.23	2.01
Decaffeinated *C. sinensis* leaves	0.53 ± 0.04	-	-
Old *C. sinensis* leaves	0.40 ± 0.04	245.1	0.98
Young *H. helix* leaves.	0.00 ± 0.00	-	-

-, not determined (negligible for conclusions)

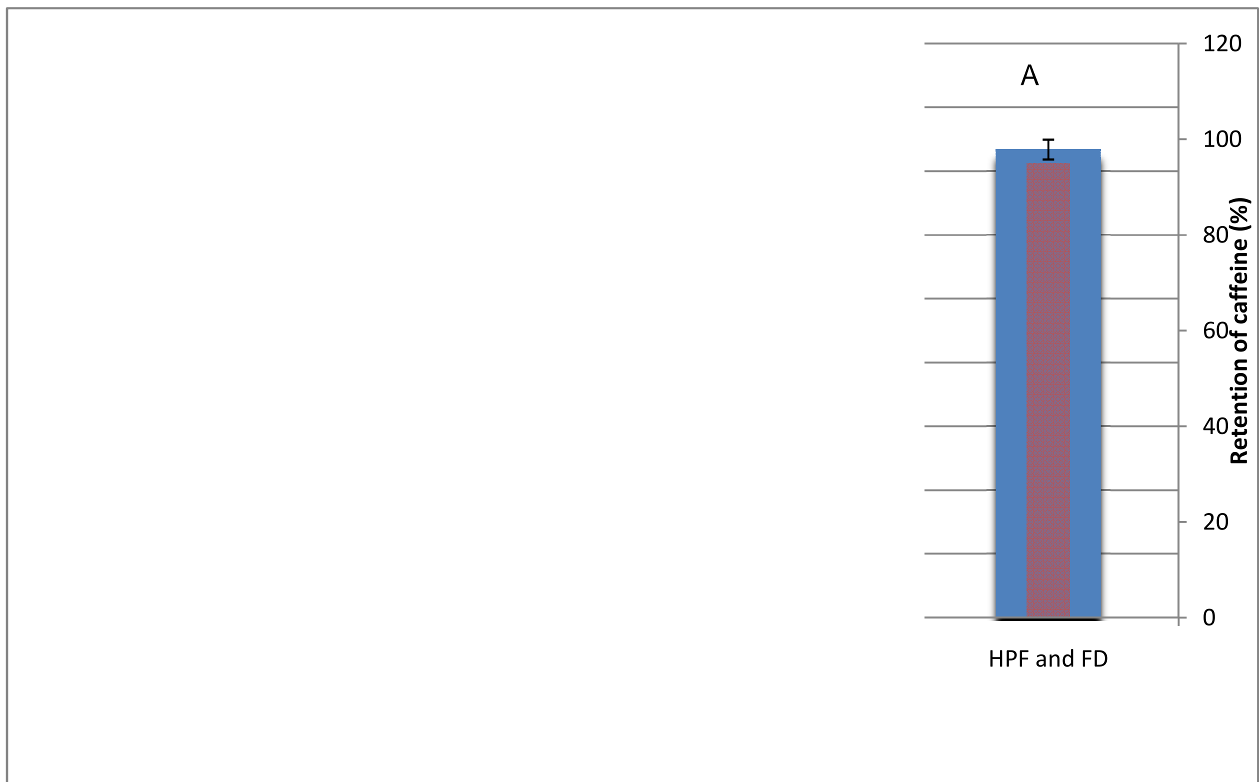


Fig. 2.1: Histogram displaying the caffeine content (% w/w) retained after each preliminary fixation experiment on the left y – axis (blue) and the retention (%) of caffeine is displayed on the right y – axis (maroon). Error bars display S.E.M. Letter groupings are according to one way ANOVA. Means annotated with different letters indicate statistically significant differences at the 95 % level of confidence.

Regrettably HPLC data was lost due to water damage to the computer where the results were stored and thus chromatograms cannot be reported here.

2.3.2 The localisation of caffeine within young *C. sinensis* leaves using CSLM and TEM analysis:

Dragendorff's reagent and another stain called chlroamine T (N – chloro – 4 – toluene sulphonamide sodium salt), a very specific stain for caffeine based on the murexide reaction (Kozuka *et al.* 1980) were tested to detect caffeine in fresh leaves, but no positive reactions were observed (data not shown). In contrast, immunolabeling with a secondary antibody conjugated to a fluorophore showed caffeine localised to the vascular area of young *C. sinensis* leaves (Fig. 2.2). The area that visually gave the highest level of fluorescence and thus likely the highest in concentration of caffeine were the vascular bundles. The parenchyma cells did not visually show characteristics of high caffeine content or any type of accumulation.

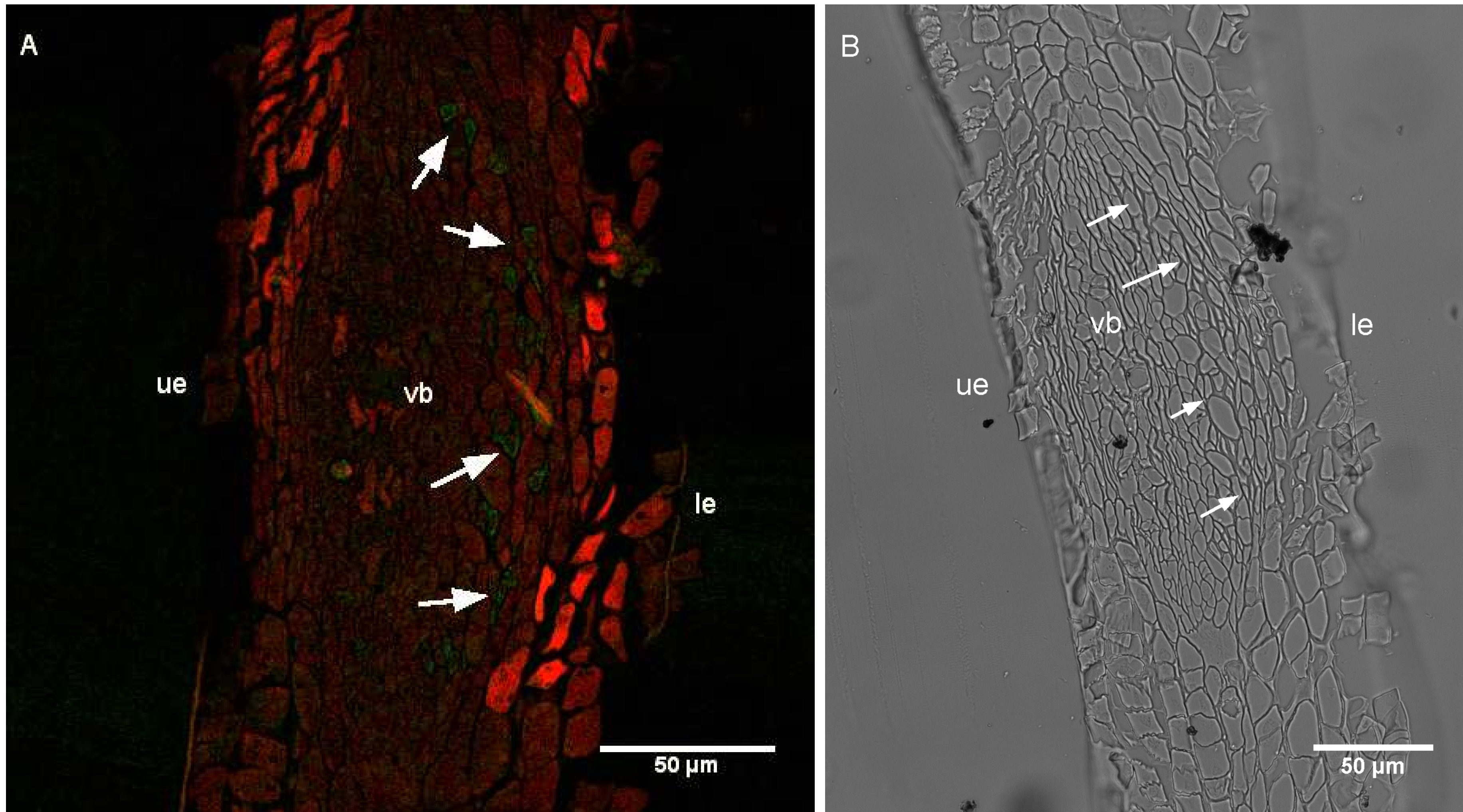


Fig. 2.2: Confocal (λ_{ex} 488, λ_{em} 505 – 530 (green), λ_{ex} 543, λ_{em} 560 (red)) and DIC micrographs of a young *C. sinensis* leaf cross section with indicated scale bars. (A) Micrograph taken with a 40x water immersion objective; (B) DIC micrograph of the image in A and (C) Scan zoom (crop) function of an area of cells indicating a positive reaction; White arrows indicate areas of green fluorescence at the bottom of the vascular bundle (vb) in the cross section and thus the localisation of caffeine using immunolabeling. Upper epidermis of the cross section is indicated (ue) and the lower epidermis is indicated (le).

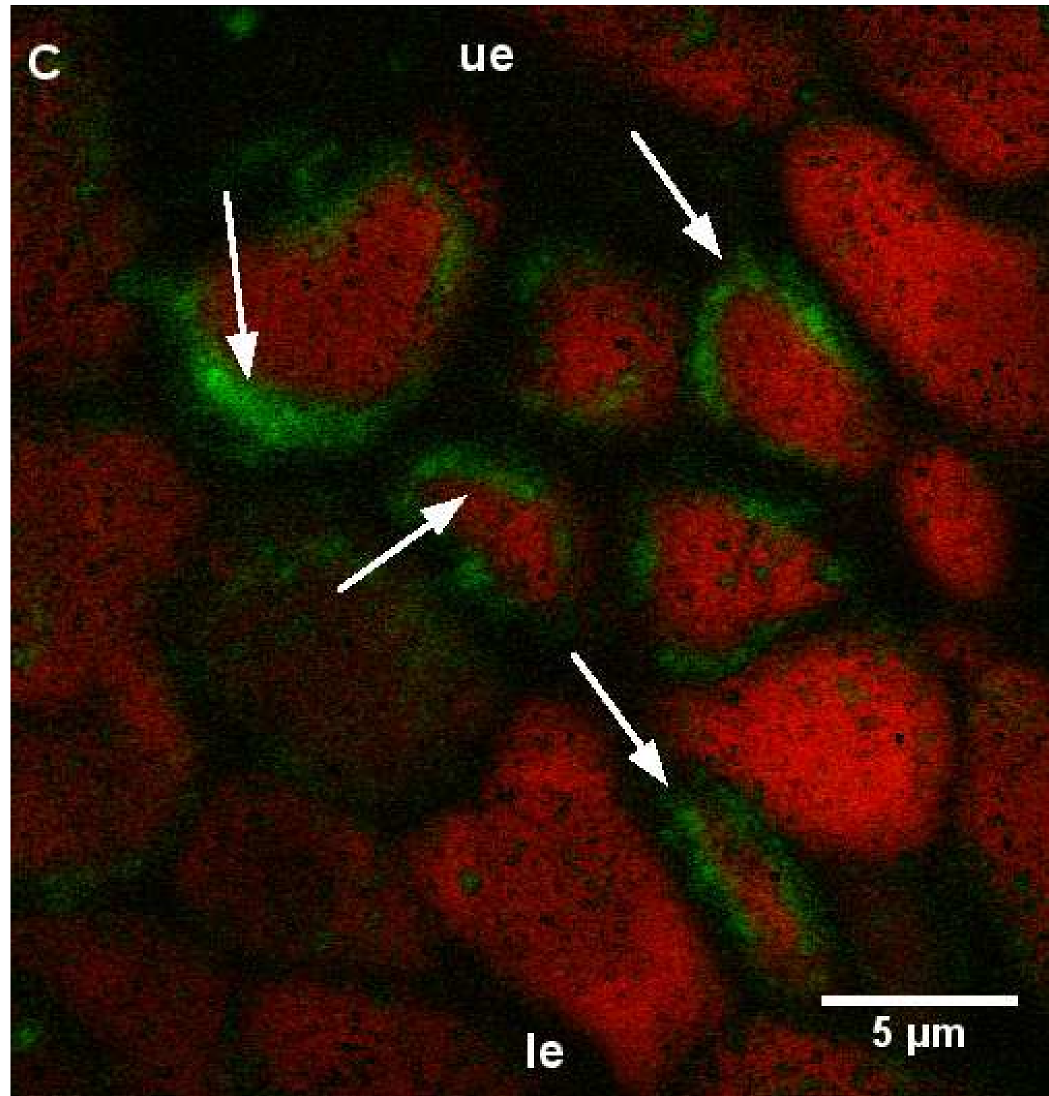


Fig. 2.2: Confocal (λ_{ex} 488, λ_{em} 505 – 530 (green), λ_{ex} 543, λ_{em} 560 (red)) and DIC micrographs of a young *C. sinensis* leaf cross section with indicated scale bars. (A) Micrograph taken with a 40x water immersion objective; (B) DIC micrograph of the image in A and (C) Scan zoom (crop) function of an area of cells indicating a positive reaction; White arrows indicate areas of green fluorescence at the bottom of the vascular bundle (vb) in the cross section and thus the localisation of caffeine using immunolabeling. Upper epidermis of the cross section is indicated (ue) and the lower epidermis is indicated (le).

The parenchyma cells did not show characteristics of high caffeine content or any type of accumulation. The vascular bundle cells of young *C. sinensis* leaves were not fully differentiated and made it difficult to determine the exact cells in which caffeine accumulates. The cells on the periphery of the vascular bundle (abaxial side) were seen to give strong levels of fluorescence specific for caffeine. When using the scan zoom (crop) function of an area of cells with the characteristic green fluorescence, it can be seen that the accumulation of caffeine was confined to the periphery of those cells with minimal fluorescence seen within the actual cell. The DIC image suggested that the cells of caffeine accumulation would be the precursor phloem. The 543 nm channel with long pass detection (red) was used to give a better overall visualisation of the cross sections as well as rule out any possibility of autofluorescence and thus possible false positives. As seen in Fig's. 2.2 A and C, there is no overlap of red and green fluorescence in the areas determined to accumulate caffeine. To ensure that the signals visualised were not from non-specific binding or background, negative controls treated with only secondary antibodies (i.e., the primary antibody being omitted from the diluting buffer) or the primary antibody pre-adsorbed with caffeine, were visualised with the same settings. The absence of any characteristic green fluorescence signal on the abaxial side of the vascular bundle viewed (Fig's. 2.3 A and B) when compared to the young *C. sinensis* leaves (Fig's. 2.2 A and C) eliminated the possibility of a false positive from autofluorescence in the areas viewed, as well as confirming the specificity of the primary and secondary antibody used.

An interesting observation that must be noted was that when using a blocking solution of higher concentration i.e., 5 % (w/v BSA or v/v donkey serum), green fluorescence from the secondary antibody was eliminated.

The decaffeinated (22 % caffeine), mature *C .sinensis* leaves (17 % caffeine) and the *H. helix* leaves (0 % caffeine) were used as controls (Fig. 2.4). Using the same settings as for the young *C. sinensis* leaf, no green fluorescence was detected throughout the cross sections. Comparing relative response intensities (fluorescence) and the HPLC data (Table 2.2), indicates that caffeine is present at an undetectable level. The cells in the decaffeinated *C. sinensis* leaf have a different appearance when compared to the young leaf, which can be attributed to the boiling process. There were problems encountered when trying to obtain a suitable level of red channel signal for the decaffeinated *C. sinensis* leaf and *H. helix* leaf, most probably due to an absence of autofluorescent compounds such as chlorophyll. No caffeine was detected in the cells on the abaxial side of the vascular area or forming an outline along these cell walls in the controls as seen in the young *C. sinensis* leaf.

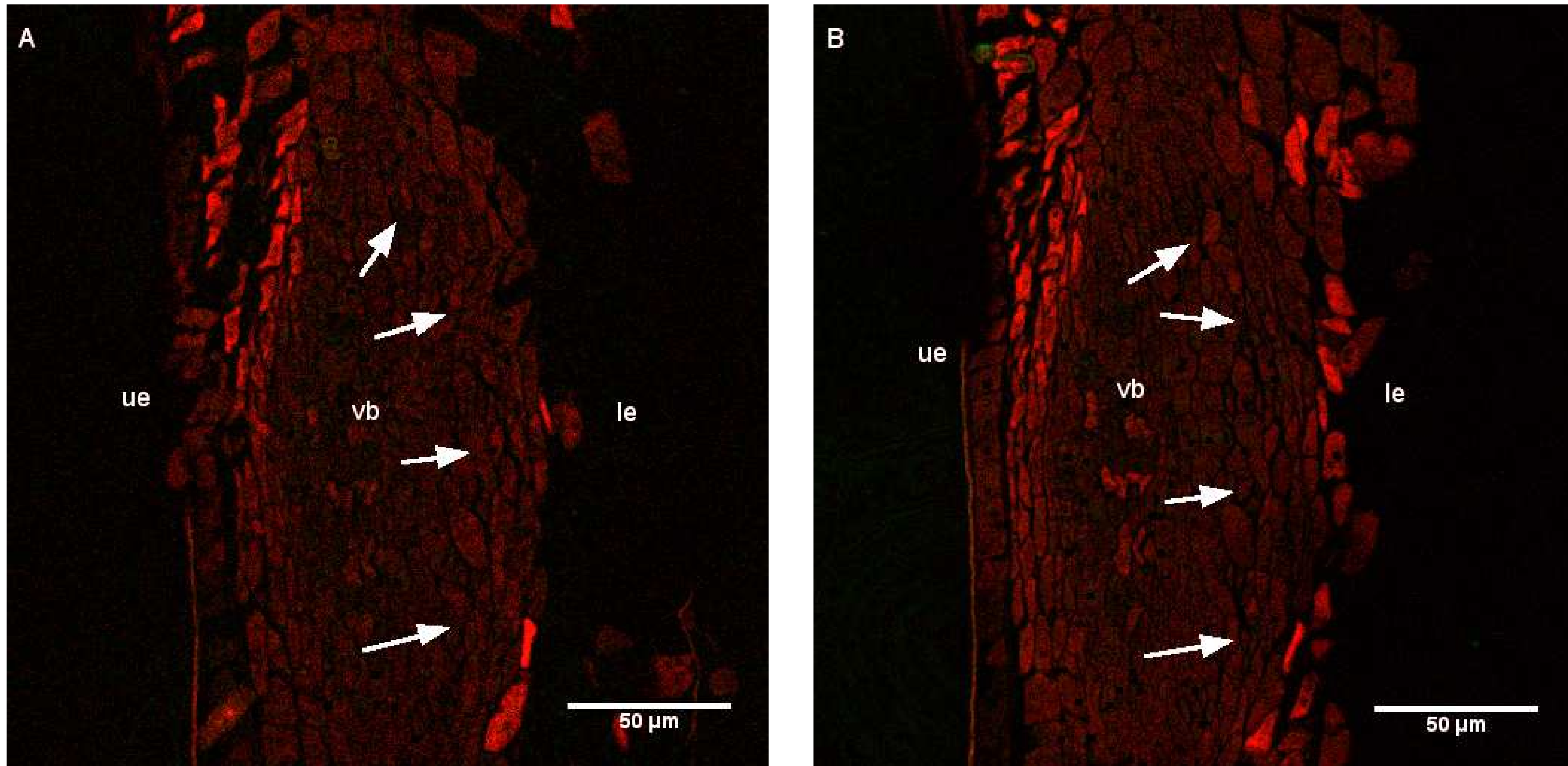
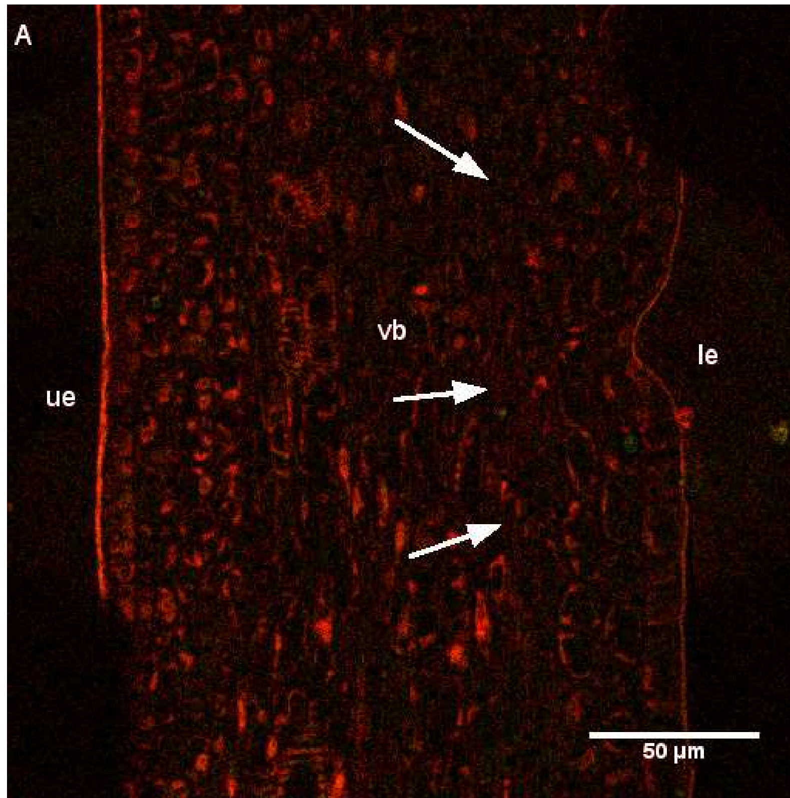


Fig. 2.3: Confocal (λ_{ex} 488, λ_{em} 505 – 530 (green), λ_{ex} 543, λ_{em} 560 (red)) micrographs of a control young *C. sinensis* leaf cross section with indicated scale bars taken with a 40x water immersion objective. (A) Control was labelled without secondary antibody; (B) Control was labelled with primary antibody pre-adsorbed with caffeine. White arrows indicate areas where green fluorescence was previously observed in Fig's. 2.2 A and C. Upper epidermis (ue), vascular bundle (vb) and lower epidermis (le).



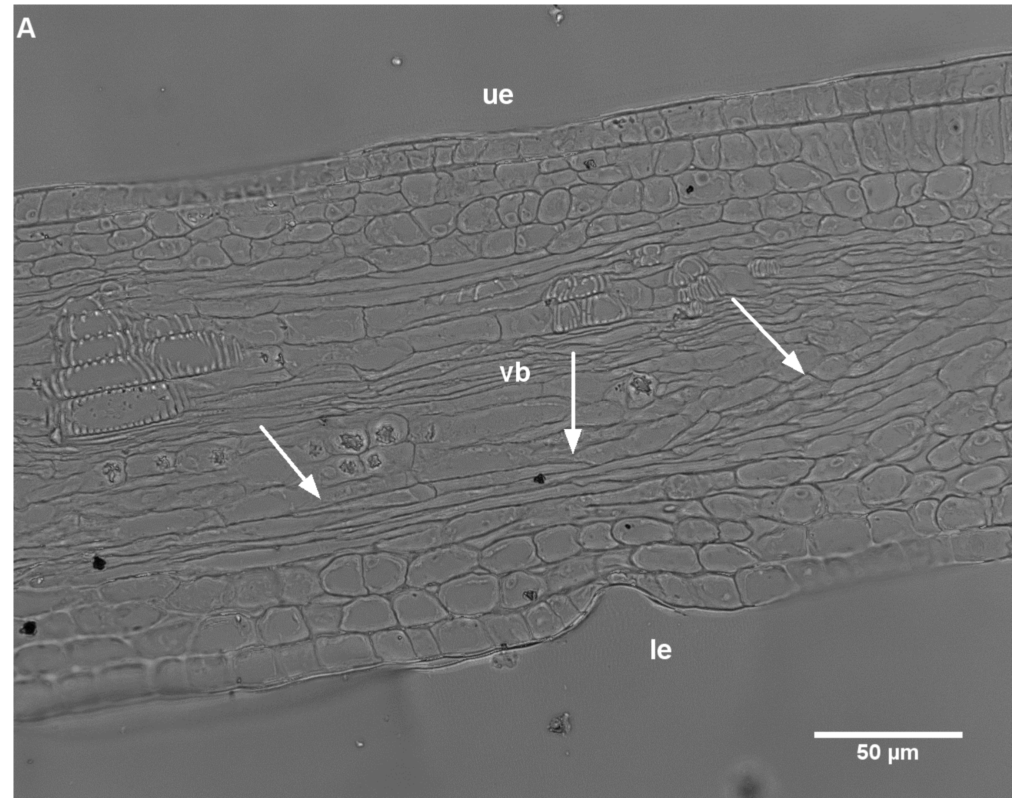
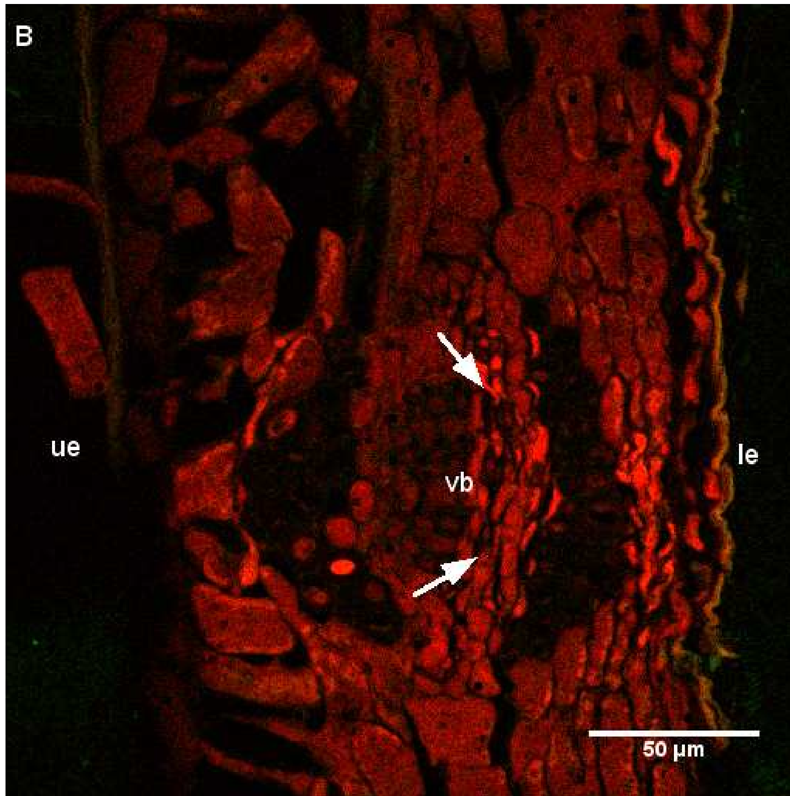


Fig. 2.4: Confocal (λ_{ex} 488, λ_{em} 505 – 530 (green), λ_{ex} 543, λ_{em} 560 (red)) micrographs of different leaf cross sections containing minimal or no caffeine with accompanying DIC images taken with a 40x water immersion objective. (A) Decaffeinated *C. sinensis* leaf; (B) Mature *C. sinensis* leaf and (C) Young *H. helix* leaf. White arrows indicate areas where green fluorescence was previously observed in the young *C. sinensis* leaf (Fig's. 2.2 A and C). Upper epidermis (ue), vascular bundle (vb) and lower epidermis (le).



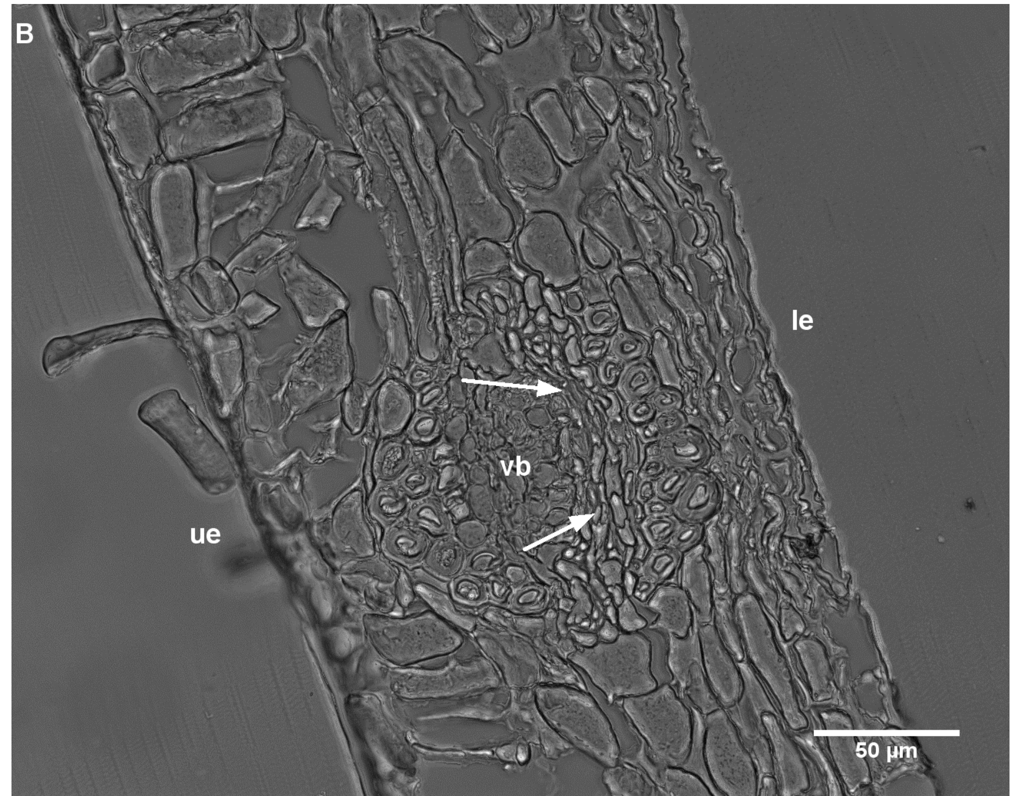
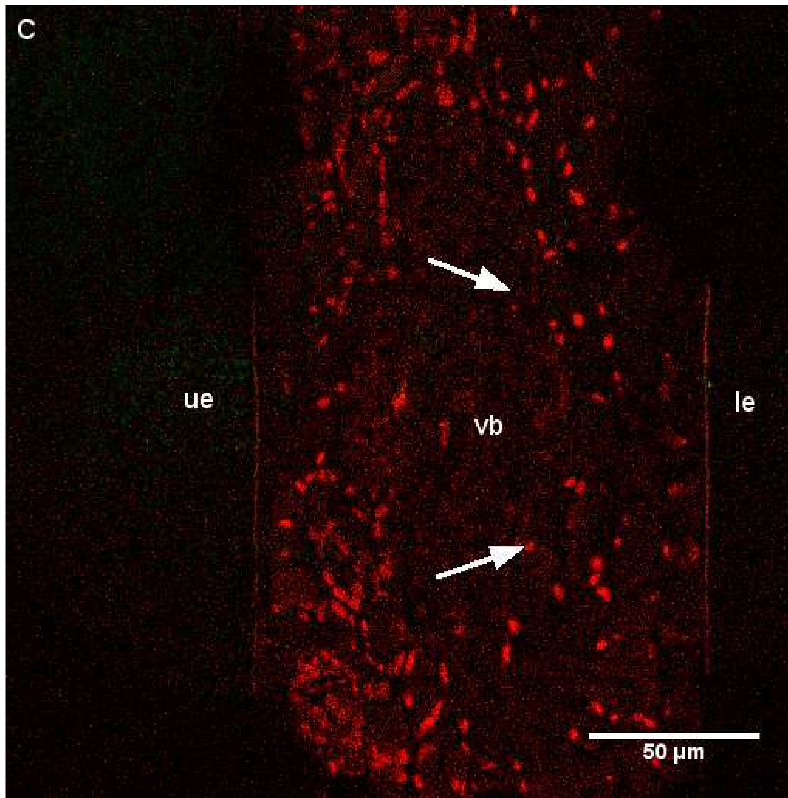


Fig. 2.4: Confocal (λ_{ex} 488, λ_{em} 505 – 530 (green), λ_{ex} 543, λ_{em} 560 (red)) micrographs of different leaf cross sections containing minimal or no caffeine with accompanying DIC images taken with a 40x water immersion objective. (A) Decaffeinated *C. sinensis* leaf; (B) Mature *C. sinensis* leaf and (C) Young *H. helix* leaf. White arrows indicate areas where green fluorescence was previously observed in the young *C. sinensis* leaf (Fig's. 2.2 A and C). Upper epidermis (ue), vascular bundle (vb) and lower epidermis (le).



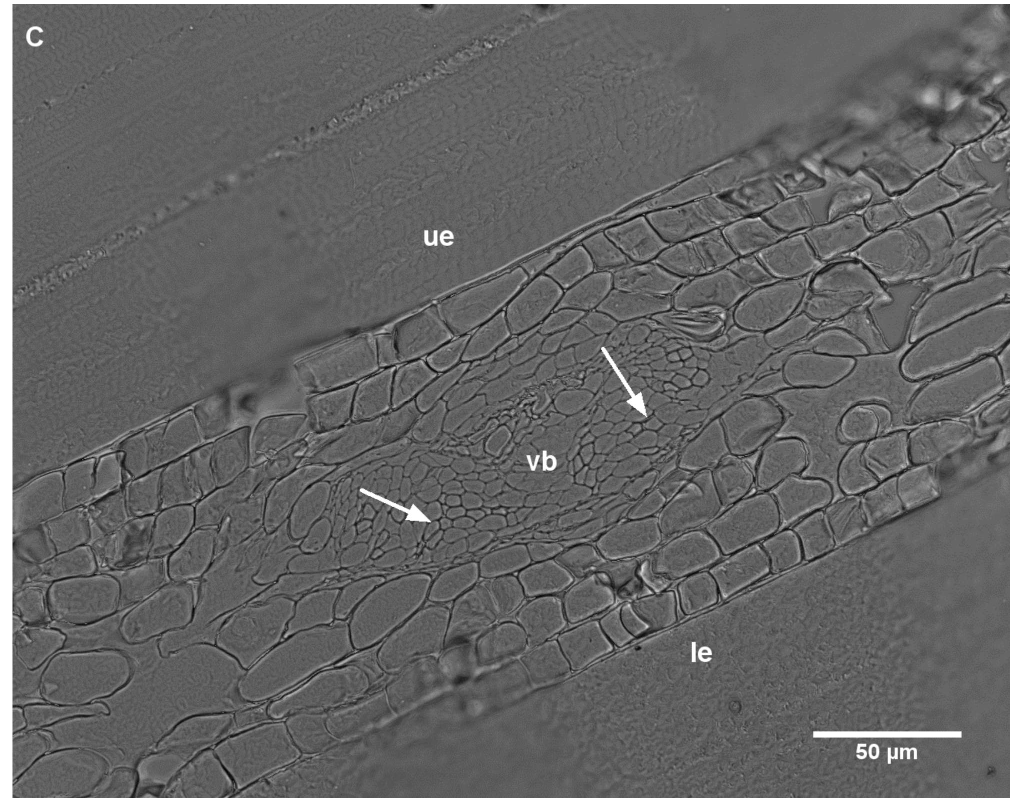


Fig. 2.4: Confocal (λ_{ex} 488, λ_{em} 505 – 530 (green), λ_{ex} 543, λ_{em} 560 (red)) micrographs of different leaf cross sections containing minimal or no caffeine with accompanying DIC images taken with a 40x water immersion objective. (A) Decaffeinated *C. sinensis* leaf; (B) Mature *C. sinensis* leaf and (C) Young *H. helix* leaf. White arrows indicate areas where green fluorescence was previously observed in the young *C. sinensis* leaf (Fig's. 2.2 A and C). Upper epidermis (ue), vascular bundle (vb) and lower epidermis (le).

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Gold labelling never revealed substantial evidence of positive caffeine labelling and no trend was observed. Immunocytochemical studies were performed using immunogold labelling and viewed using a TEM (Fig. 2.5). No caffeine was observed to accumulate in the xylem cells of young *C. sinensis* leaves using CSLM analysis (Fig. 2.2), but gold labelling was observed within these cells (Fig. 2.5 A). The xylem cells of the controls had similar labelling (Fig 2.5 B, C, D) that do not correspond to results obtained from CSLM analysis. The cells on the abaxial side of the vascular bundles i.e., precursor phloem, showed similar gold labelling in young *C. sinensis* leaf and the control samples containing low or no amount of caffeine (images not shown). Minimal or no gold labelling was observed when using antibody controls i.e., labelling without the primary antibody and labelling with the primary antibody pre-adsorbed with caffeine.

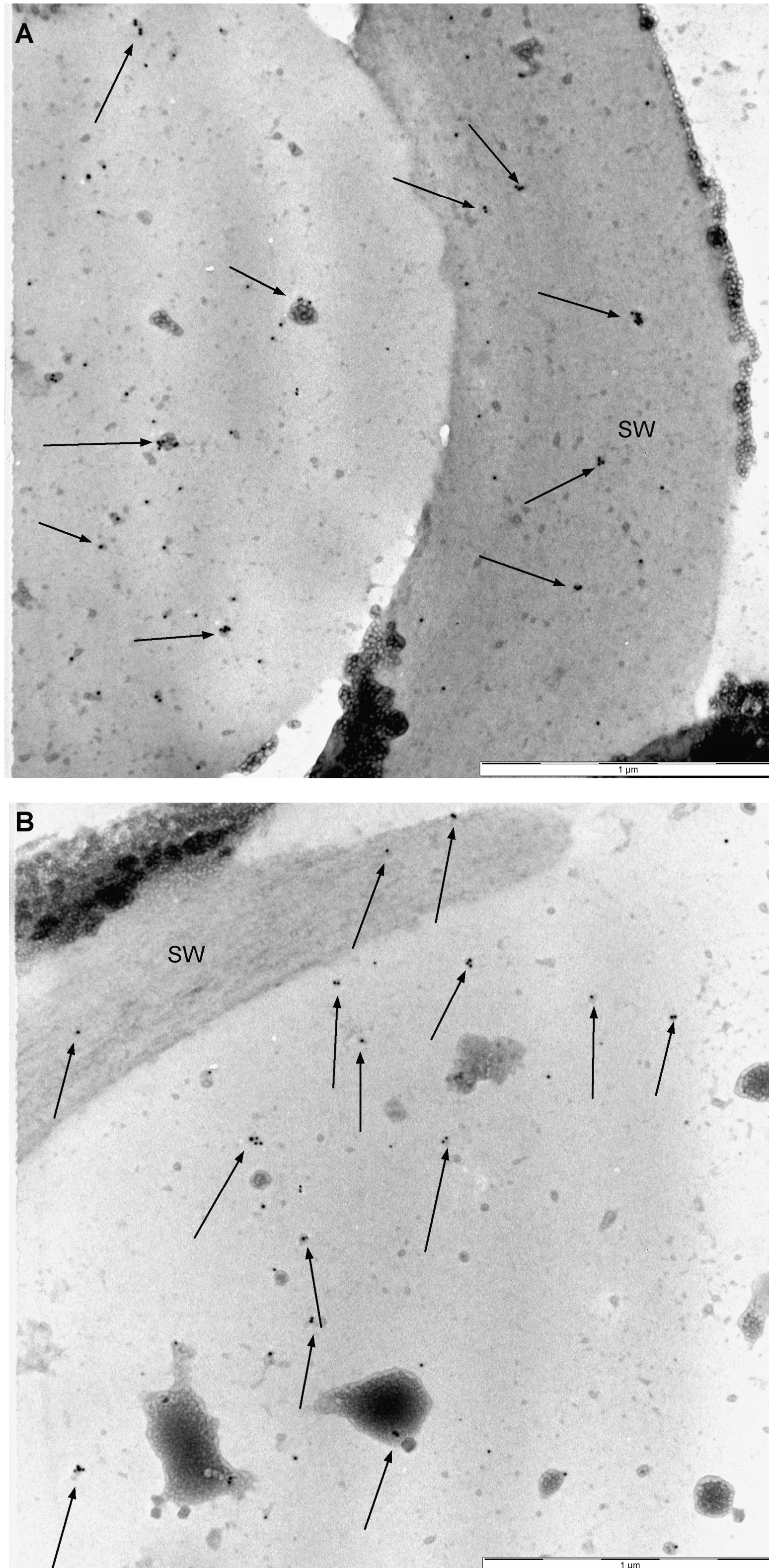

Fig. 2.5: TEM micrographs of different leaf cross sections that were immunolabelled for caffeine localisation focusing on xylem cells. (A) Young *C. sinensis* leaf; (B) Decaffeinated *C. sinensis* leaf; (C) Mature *C. sinensis* leaf and (D) Young *H. helix* leaf. Black arrows indicate gold labelling. Secondary cell wall thickenings of xylem (SW).

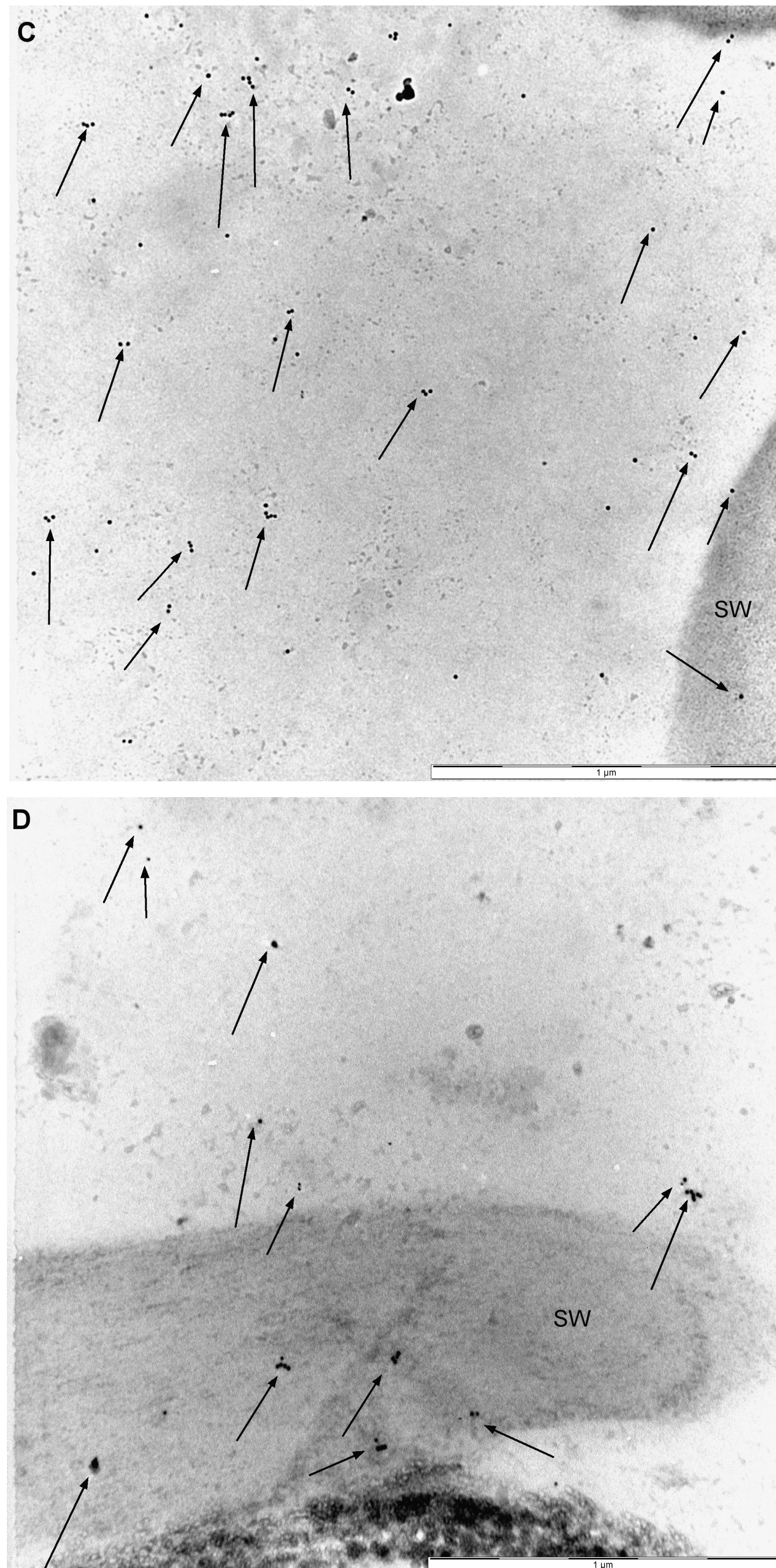

Fig. 2.5: TEM micrographs of different leaf cross sections that were immunolabelled for caffeine localisation focusing on xylem cells. (A) Young *C. sinensis* leaf; (B) Decaffeinated *C. sinensis* leaf; (C) Mature *C. sinensis* leaf and (D) Young *H. helix* leaf. Black arrows indicate gold labelling. Secondary cell wall thickenings of xylem (SW).

2.3.3 Undifferentiated young *C. sinensis* cells and differentiated mature *C. sinensis* cells:

A vascular bundle from a young *C. sinensis* leaf (Fig. 2.6 A) is indicated with arrows. Xylem cells are evident due to their secondary wall thickenings due to the age of the leaf. Cells on the adaxial side and abaxial side are not fully differentiated.

In the mature *C. sinensis* leaf (Fig. 2.6 B) it can be seen that the cells around the periphery of the vascular bundle have differentiated. Fiber cells have been characteristically stained with toluidine blue O as they contain highly lignified secondary cell walls.

There is a likely possibility that some of the cells surrounding the vascular bundle periphery i.e., the precursor xylem and phloem in the young *C. sinensis* leaves might develop into the fiber cells surrounding the vascular area of the mature *C. sinensis* leaf.

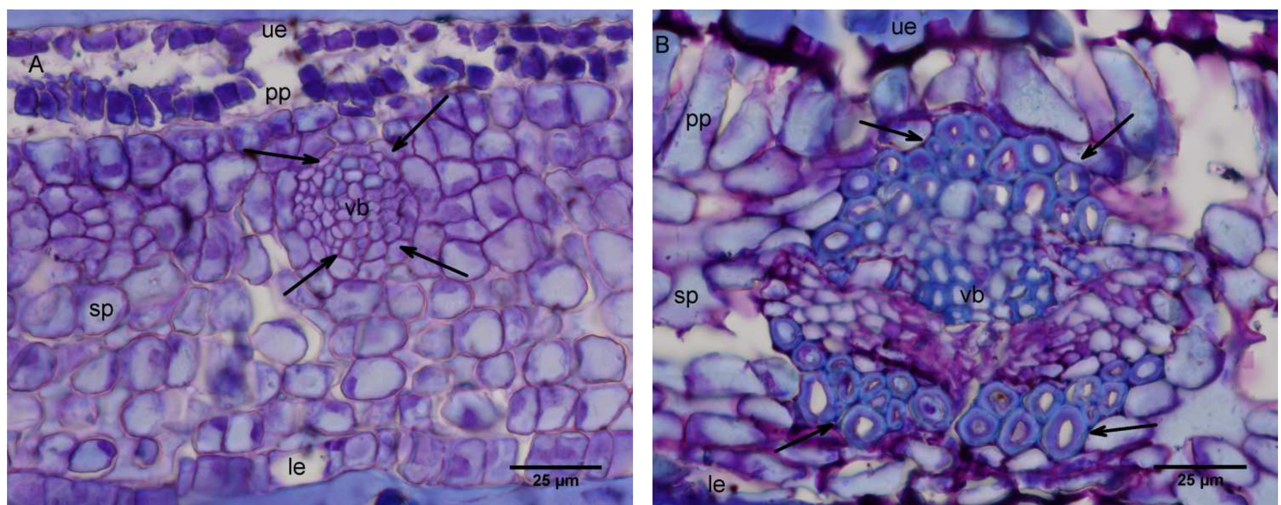

Fig. 2.6: Light micrographs of leaf cross sections stained with toluidine blue taken with a 40x oil immersion objective. (A) = young *C. sinensis* leaf, (B) = mature *C. sinensis* leaf. Arrows in (A) indicate vascular areas the periphery where the precursor xylem and phloem are located. Arrows in (B) indicate phloem fibers surrounding the vascular area. Upper epidermis (ue), palisade parenchyma (pp), vascular bundle (vb), spongy parenchyma (sp) and lower epidermis (le).

2.3.4 TLC analysis of xylem sap from young *C. sinensis* leaves:

The caffeine standard control had an Rf = 0.64 and the xylem sap that was spotted and developed also had a spot with an Rf = 0.64 indicating the presence of caffeine within the xylem of young leaves. There was a remarkable difference in the intensities of the relative spots. The control was very dark against the fluorescence background of the TLC plate while the spot from xylem sap considerably

fainter suggesting the xylem contains a very low amount of caffeine. Even though the spot was considerably fainter there was still a detectable amount of caffeine present within the xylem sap that was spotted and developed.

2.4 Discussion:

By using preliminary fixation experiments we were able to develop a method of fixation where the majority of caffeine is be retained. Chemical fixation was avoided as most caffeine was extracted. Previous work has shown that chemical fixation extracts compounds such as Ca, Mg, reducing sugars, and amino acids (Coetzee and van der Merwe 1984). Caffeine is also most likely to be extracted during fixation for a number of reasons, such as osmotic effects on plant tissues (Coetzee and van der Merwe 1985, 1986, 1987). At room temperature caffeine, also dissolves more easily during dehydration which, coupled with the effects of permeability disruption and unwanted osmotic effects from aldehyde fixation (Hayat 2000) makes chemical fixation unsuitable when localising small compounds such as caffeine. The methods of HPF and FS or FD are better, since the unwanted effects of room temperature aldehyde fixation are avoided, and in the case of FD alcoholic dehydration is avoided. HPF/ FS allows for minimal disruption of the permeability barrier, a better preservation of the internal structure (Steinbrecht and Zierold 1987) and overall a higher retention of caffeine. FD procedure removes only 5 % of caffeine due to the fact that the sample never comes into contact with alcoholic dehydration, but this makes resin infiltration extremely difficult so the use of EtOH was needed in order to adequately infiltrate the sample. Due to inadequate resin infiltration sample from FD, sectioning was extremely difficult, producing poor quality cross sections and this was continually encountered and hence FS. The difficulty in resin infiltration could be attributed to *C. sinensis* high polyphenol content, thus making it difficult to section. When FS takes place at a temperature below -30°C the solubility of caffeine in alcohol is reduced allowing for its retention within the sample and this dehydration method allowed for better infiltration of the resin into the sample, thus producing better cross sections for microscopic analysis. Lowicryl K4M resin was superior for sectioning thicker sections for light and confocal analysis but when it was attempted to make thin sections for electron microscopy analysis it was extremely difficult. LR White was substituted for Lowicryl K4M and suitbale sections of pale gold or silver were obtained, but it was necessary to use grids coated in Formvar to prevent sections from breaking during the labelling procedure.

Caffeine localisation in the confocal micrographs (Fig’s. 2.2 and 2.4) correspond well with the quantitative HPLC caffeine content analysis (Table 2.2) in the sense that samples with the highest level of fluorescence signal (caffeine content) to lowest are young *C. sinensis* leaves followed by decaffeinated *C. sinensis* leaves, mature *C. sinensis* leaves, and *H. helix* leaf (according to relative

response intensities). This observation together with the antibody controls used, i.e., primary antibody excluded and primary antibody pre-adsorbed with caffeine, emphasize the high specificity that immunohistochemistry will have for a specific antigen such as caffeine.

Unfortunately, inconclusive results were obtained when attempting immunocytochemical studies using immunogold labelling and TEM analysis. This can be due to a number of factors. The fact that controls excluding the primary antibody show minimal background labelling suggest that the secondary antibody is specific for the primary antibody. Furthermore, minimal background is seen when excluding the primary antibody or when it is pre-adsorbed with caffeine suggests that rinsing steps are adequate. Thus, it is possible the primary antibody is not specifically labelling the caffeine antigen. It is likely that the antibody is binding to the Formvar coat as the gold labelling is uniform throughout samples due to immersion of the grids. It might also be non-specifically binding to the LR White resin. Unfortunately this issue could not be resolved even when using different blocking buffers of different concentrations. One way of possibly solving the issue would be to pre-adsorb the primary antibody with a compound similar to the resin or formvar that will hopefully make it more specific. Gelatin has been suggested as a solution for this problem (Hayat 2000). Besides the non-specific background, there should have been differences in labelling of caffeine within the young *C. sinensis* leaf when compared to the controls, and there were no major differences. This suggests that caffeine had been removed or was in a considerable low concentration within the sample. The molecule might have dissolved when sectioning onto water, thus reducing its concentration or the molecule was in such a low concentration due to the sections being ± 50 times thinner i.e., 5 µm sections used for CSLM analysis vs 100 nm sections used for TEM analysis. These issues contribute to the low level of labelling and non-specific labelling within the young *C. sinensis* leaves and the controls. Unfortunately, it was difficult to rectify these issues and there are too many variables involved, so it is suggested a method such as Confocal Raman microscopy be explored to reveal any new information. However, it is recommended that a 1064 nm Nd:YAG laser is used or a laser within the UV range as to minimise any autofluorescence effects that will give inconclusive spectral analysis. It is also suggested that cross sections of leaves are analysed instead of whole leaves as this will give more information about localisation within the leaf as well as spectra for interpretation of results.

Using the young *C. sinensis* leaf for caffeine localisation, it was determined that accumulation was greatest in the vascular bundle, mainly in the cells around the periphery on the abaxial side. In some cases, labelling was seen on the adaxial side, i.e., precursor xylem but controls revealed a certain level of autofluoresece thus giving ambiguous results (data not shown). Therfore, another method was required to confirm the presence of caffeine within the xylem cells. By use of a pressure bomb and collecting the sap from the xylem, caffeine was determined to be present within these cells but in low

concentration. The concentration of caffeine within the precursor xylem cells seemed to be much lower than that of the precursor phloem cells as no detectable amount could be determined when using immunolabeling and CSLM analysis, as well as for the fact that labelling was sometimes present within the xylem at a low level. TLC analysis revealed very low levels of caffeine present within the xylem sap supporting its presence in low concentration. Photosynthetic cells, i.e., palisade and spongy parenchyma, may also contain caffeine but in a much lower concentration, i.e., amounts undetectable by immunolabeling and CSLM analysis. This result could be misleading, as the area of palisade and spongy parenchyma is much larger when compared to the vascular area. Thus, a large proportion of caffeine may be distributed over this area opposed to the smaller vascular area where caffeine is concentrated giving higher fluorescence signal. Another possibility could be the fact that half of caffeine is extracted during the fixation and embedding process, thus giving a lower concentration of the molecule within sections and producing undetectable amounts within the parenchyma cells. It is possible that the extraction might be uniform suggesting that the sites of detectable accumulation are within the vascular areas.

Since these parenchyma cells are photosynthetic and caffeine synthase has been located within the chloroplasts of young leaves (Kato *et al.* 1998; Koshiishi *et al.* 2001) it is expected that for caffeine to be present within these cells. The possibility of caffeine being present within photosynthetic cells might follow the pattern of many alkaloids being located within these cells (White and Spencer 1964; Brisson *et al.* 1992; Ferreira *et al.* 1998) (also see chapter 1). Furthermore, much work has been done with regards to alkaloids associating with phenolic-like compounds. For example a hypothesis exists that alkaloids help sequester phenolic compounds within vacuoles by forming co-aggregates (Matile 1976) and data obtained for the localisation of cocaine in coca leaves revealed vacuolar complex composed of a core of phenolic compounds surrounded by a layer of cocaine (Ferreira *et al.* 1998). As suggested by the authors, cocaine allows for phenolic compounds to be sequestered in the vacuoles until breached by predators when they can then act as a chemical defence. This is important as it might suggest caffeine to be present within the vacuoles of these parenchyma cells since catechins (or polyphenols) were localised to the mesophyll i.e., palisade and spongy parenchyma cells vacuoles (Suzuki *et al.* 2003). Thus, it is possible that caffeine is synthesized within the chloroplasts, (sequestered with polyphenols within the vacuoles of photosynthetic cells) and transported and accumulated in the cells of the vascular bundles. The cells of detectable accumulation are the precursor phloem cells using immunolabeling and CSLM while caffeine was detected within the xylem cells occasionally via this method but due to autofluoresence ambiguity this was confirmed analysing xylem sap. The presence of caffeine within vacuoles could not be shown here and remains a hypothesis. A more sensitive method than CSLM is required. TEM analysis using immunogold labelling may be sensitive enough, but as experiments in this study suggests a fair amount of

optimisation is required. Another method that might aid in confirming this is that use of confocal Raman microscopy as mentioned, however if caffeine is in a complex with phenolics or other compounds within the vacuole, conclusive caffeine Raman spectra might be difficult to obtain in order to confirm the molecule's localisation.

The mature *C. sinensis* leaf has a low caffeine content since the leaf has differentiated and become highly lignified and is thus no longer vulnerable to predators. It can be seen that the mature leaves have a very low caffeine content (Fig's. 2.4 and Table 2.2) and is approximately 3 times larger in mass than the young leaf and contains approximately half the caffeine (according to reference leaves collected as in nature leaves can be of varying sizes and masses). It is likely that caffeine synthesis has halted and the alkaloid is catabolised and recycled as the nitrogen could be used for *de novo* biosynthesis of caffeine in young leaves or in protein synthesis (Harborne 1994). Since the leaves contain a lower amount of caffeine and is much larger in size compared to the young leaf, there is a 'dilution' of caffeine over a larger area accounting for the reduction in signal.

I hypothesize that cells in the young vascular bundle where caffeine was shown to be located, differentiate into xylem and phloem cells surrounded by fiber cells (Fig. 2.6 B). As the leaf ages, and the fiber cells become fully developed, there is less need for caffeine as a chemical defence and thus its reduction in the mature leaves. These fiber cells may act as a protective barrier around the xylem and phloem cells preventing access of pathogens and predators such as aphids accessing nutrients within the plant, whereas in the young leaf such a physical defence system doesn't exist and hence the need for the presence of caffeine for a chemical defence. The compound forms a chemical defence barrier around the young vascular bundle. It is possible that caffeine may be transported in the precursor xylem and precursor phloem in response to abiotic and biotic stresses as proposed previously for *C. canephora* leaves (Mondolot *et al.* 2006). Caffeine in *C. canephora* leaves was suggested to be localised within the phloem cells (Mondolot *et al.* 2006), swainsonine was determined to be in the phloem of *Astragalus lentiginosus* (locoweed) (Dreyer *et al.* 1985) and with regards to the xylem, tropane alkaloids were localised in the xylem cells of roots of *Duboisia myoporoides* (Khanam *et al.* 2000) or that quinolizidine alkaloids were located in the xylem of the *Lupinus albus* (White Lupin) (Bäumel *et al.* 1995). This localisation of alkaloids probably functions as a chemical defence protecting the plant from sap sucking insects such as aphids (Dreyer *et al.* 1985). Caffeine may also protect against certain pathogens in the vascular area such as fungi as it has been determined that *Puccinia poarum* invades the vascular bundles of *Tussilago farfara* L. leaves (Coltsfoot) (Al Khesraji *et al.* 1980). Thus, it is less likely for caffeine to act as a defence against herbivores and more likely to act as a defence against sap sucking insects and pathogens that infiltrate via the vascular system.

Another possible explanation for caffeine's localisation in addition to acting as a chemical defence is its association with chlorogenic acid. Caffeine has been suggested to form complexes with chlorogenic acid (a caffeoylquinic acid) (Spencer *et al.* 1988; Mösli Waldhauser and Baumann 1996; Mondolot *et al.* 2006). Mondolot *et al.* (2006) determined that caffeine was present within phloem cells in association with caffeoylquinic acids and most likely chlorogenic acid due to the compound being in very high concentrations in young leaves. Mondolot's localisation of caffeine confirms our results, and it is most likely that caffeine is also in association with chlorogenic acid within the precursor phloem of young *C. sinensis* leaves. This needs to be confirmed, and might be confirmed by microscopy using Neu's reagent (Neu 1957), when viewed using UV light, and analyzed for a greenish white fluorescence (Mondolot-Cosson *et al.* 1997; Mondolot *et al.* 2006). Hypothesising that caffeine is in complex with chlorogenic acid within the vascular bundle, and using the suggestion from Mondolot *et al.* (2006), the transportation of caffeine from chloroplasts to vascular bundles may be explained. Caffeoylquinic acids synthesis was suggested to take place within chloroplasts (same as caffeine) and then accumulated within vacuoles. From the vacuoles, the compounds are transported to vascular bundles where caffeine may act as a chemical defence and chlorogenic acid acts as an intermediate in formation of highly lignified fiber cells. The complex formation of caffeine and chlorogenic acid may have aided in the compounds localisation as it possibly acts as a natural fixative for the molecule.

The transport of caffeine and cholorgenic acid from chloroplasts to the vascular area would require biochemical and enzymatic analyse. It might be possible to use *in situ* RNA hybridisation and immunocytochemistry to establish the cellular distribution of the purine alkaloid biosynthesis. This has previously been completed for monoterpenoid indole alkaloid biosynthesis in *C. roseus* (St-Pierre *et al.* 1999).

Since caffeine was visually determined to be on the periphery of cells, it might be possible that the molecule is bound or in complex with cell wall phenolics. During the immunolocalisation protocol, free caffeine and caffeine might be washed out easily. Thus, tissues such as leaf mesophyll that contain less cell wall phenolics will produce a weaker signal as they retain less caffeine. Determining the amount of caffeine retained after the immunolicalisation protocol will help to establish the total proportion of caffeine retained and thus visualised within the relevant localised tissues.

2.5 Conclusion:

In conclusion, using preliminary fixation experiments of chemical fixation, HPF/ FS and HPF/ FD were investigated using HPLC analysis and a method of sample embedding was developed where minimal extraction of caffeine may occur. HPF and FS was determined to be the best method to use as it

allowed for adequate sample sectioning due to a better resin infiltration and retained 49 % of the total caffeine content within the sample. It must be noted that there is a possibility that caffeine is extracted during the labelling protocol and the amount of caffeine left over in the final sample should be determined. Lowicryl K4M proved best for CSLM analysis and making thicker sections, but LR White and Formvar coated grids were required in order to make suitable sections for TEM. The anatomical localisation of caffeine within young *C. sinensis* leaves was investigated using immunohistochemical methods and CSLM. Immunocytochemical investigations using TEM analysis proved to be inconclusive due to a number of complications. CSLM revealed caffeine was localised in the precursor phloem and the use of various controls, i.e., primary anti-caffeine antibodies excluded from labelling or pre-adsorbed with caffeine proved their specificity as well as ruling out any autofluorescence issues. Other controls such as the decaffeinated *C. sinensis* leaf (78 % less caffeine), mature *C. sinensis* leaf (83 % less caffeine) and the *H. helix* leaf (no caffeine) were all negative, giving confidence in determining the results as positive. By using a pressure bomb instrument and TLC, caffeine was determined to be present within the precursor xylem cells but only in a low concentration. The Rf value obtained was 0.64, the same as the control indicating the presence of caffeine. It suggests here that presence of caffeine within these areas are most likely for the compound to act as chemical defence protecting the leaf against sap sucking insects and pathogens from getting access to vital nutrients, not necessarily protection from herbivory. As the leaf ages there is a decrease in caffeine due to the fact that xylem and phloem fibers develop protecting the vascular bundle from pathogens and sap sucking insects. The presence of caffeine within these areas might also be attributed to its complex formation with chlorogenic acid that will add in the development of highly lignified cell walls as seen in the mature *C. sinensis* leaf. The transportation of caffeine to the vascular bundle from the parenchyma cells might be explained using *in situ* RNA hybridisation and immunocytochemical studies specific for certain enzymes present in the biochemical pathway of caffeine synthesis. For the present observations, our original hypothesis is rejected and the null hypothesis accepted i.e., H1 (caffeine is localised to vacuoles of parenchyma cells of young *C. sinensis* leaves) is rejected and $H1_0$ is accepted (caffeine is not localised to the vacuoles of parenchyma cells of young *C. sinensis* leaves but is accumulated elsewhere within the plant).

Chapter 3: Decaffeination of young *C. sinensis* (tea) leaves

3.1 Introduction

In the present aspect of the study I aimed at producing a decaffeinated black tea from various possible methods that would have a number of advantages over current methods used for black tea decaffeination. Three aspects of this study are important, extracting as much caffeine as possible without losing too many polyphenols responsible for the health benefits of tea, retaining the activity of PPO enzyme that will convert the polyphenols to theaflavins, and producing a decaffeinated black tea with high theaflavin content.

From the data in chapter two, suggesting caffeine is localised within the vascular area, I investigated different methods of extracting this molecule, by placing the leaves under vacuum, pressure or cycling between vacuum and pressure. The reasoning behind this is the fact that young *C. sinensis* leaves, like most young leaves contain intercellular air pockets. Under vacuum or pressure, these air spaces would swell or shrink (respectively) and this could aid in squeezing out the caffeine from the leaf via vascular bundles and even stomata, thus it is possible to reduce the caffeine content of the leaves, producing a new method of tea decaffeination. By releasing vacuum or pressure, water acting as an solvent wherein the leaves are placed, may forcefully enter the leaves, dissolve caffeine and once vacuum and pressure is applied again the dissolved caffeine may exit the leaves selectively. As discussed in chapter one, the method would have the advantage over current methods of decaffeination mentioned, that only water will be used, i.e., no harsh or expensive solvents such as methylene chloride that pose harmful effects to the environment, possibly on the workers and even the consumer. No complicated machinery will be needed, as in the method of decaffeination by supercritical CO_2. Furthermore the process won't be susceptible to machinery down time as in the method of supercritical CO_2. This all will help reduce the price of the decaffeinated tea. The temperature of the solution will also remain below a critical temperature that will aid in the preservation of the PPO enzyme. As discussed in chapter one, the preservation of the PPO enzyme is extremely important as it is responsible for fermentation and production of theaflavins (from polyphenols), giving black tea its characteristic taste. Polyphenols need to be retained to provide the antioxidant and health benefits from tea as well as ensuring high theaflavin content during fermentation. The method of hot water decaffeination uses extreme temperatures that denature the enzyme and thus no black tea production can take place due to the PPO enzyme being denatured. Another method of decaffeination would be to use the boiling method of decaffeination and add an external source of PPO before black tea production. With the enzyme preserved there will be the possibility of producing a decaffeinated black tea without the use of complicated equipment or any solvents.

Different techniques exist for the extraction of compounds from plants, for example, ultra sound assisted extraction, microwave assisted extraction, supercritical fluid extraction and accelerated solvent extraction have all been developed for the extraction of nutraceuticals from plants (Wang and Weller 2006). New techniques are continually being invented, such as sonication assisted extraction and a critical review exists explaining current techniques and possible new developments (Wang and Weller 2006). Industries that extract compounds from plants try to invent methods that are more economical, not harmful to the environment, more selective and specific extraction of certain molecules and low temperature to prevent thermal degradation i.e., maintaining temperatures at an acceptable level during extraction.

Vacuum extraction of compounds from plant material seems to be a relatively new novel idea. A technique of pressure extraction of compounds that exists is known as pressurised liquid extraction (Benthin *et al.* 1999).

3.2 Method and materials

3.2.1 Plant material:

Young fresh *Camellia sinensis* (L.) O. Kuntze (tea) leaves were obtained from a farm in Tzaneen, South Africa. The plucking method used was two and a bud as described in chapter 2.2.1.

3.2.2 Preliminary decaffeination experiments using vacuum, pressure, vacuum together with pressure as possible methods:

First and second leaves were used, not the bud. The leaves were cut into ≈ 0.5 – 1 cm sections along their widths and mixed to get a homogenous sample for sub-sampling. Samples of the homogenous mixture were weighed, placed in beakers, and water was added (1:20 w/v). Using a hot plate the solution was heated to ≈ 30 - 35°C and it was ensured at all times that the leaves were submerged in the water. The beaker was placed in a freeze drier, although only the vacuum pump was used. The sample was placed under a ≈ 100 torr vacuum for 30 – 60 sec, after which the vacuum was released and the pressure returned to atmospheric conditions. This was repeated 10 times for each sample. Once complete, the temperature of the water was measured to be 20 - 25°C. The water was decanted and new warm water (≈ 30 - 35°C) was added to the tea leaves. This was done to ensure the temperature of the solution never dropped below room temperature which would hamper caffeine extraction. The entire process (10 x vacuum with release and change of water) was repeated 5 times. For pressure decaffeination, the pressure bomb was sealed and new samples processed as described

above were placed within the chamber. Pressure was applied and released ten times. Once complete the temperature of the water was measured to ensure it was at room temperature and was decanted and new warm water (≈ 30 - 35°C) was added. The entire process was also repeated 5 times. As for a combination of vacuum and pressure, the two processes were combined as described except for the fact that new warm water was added only after pressure was applied. Once complete the leaves were rinsed and prepared for HPLC analysis by drying them over night at 103°C and subsequent grinding of the samples into a powder with a mortar and pestle. Controls used were leaves of the same homogenous batch placed in water at room temperature but not under vacuum or pressure (Tzaneen leaf control). The experiment was repeated in duplicate on the day and two independent experiments were conducted on different days. The best of these methods would be selected to be upscaled and used in black tea production.

3.2.3 Preparation of a decaffeinated tea leaves (hot water decaffeination):

Please refer to section 2.2.2 for the preparation of the decaffeinated tea leaves. Three different external sources of PPO enzyme were added separately to the samples. Samples from section 3.2.2 never received an external source as it was assumed their PPO enzyme was still intact. One form of PPO was young fresh *C. sinensis* leaves in the ratio of 1:1, 1:5. 1:10 and 1:20. Another form of PPO was the addition of freeze dried young fresh *C. sinensis* leaves in the ratio of 1:5, 1:25, 1:50 and 1:100 (this was added in a dry form as sufficient moisture during maceration was attained to wet the freeze dried tea). These ratios are indicated below as hot water decaffeinated tea: fresh Tzaneen tea or hot water decaffeinated tea: freeze dried Tzaneen tea. A third method of adding PPO was Suberase™ from Novozymes® as a 10 % v/v sprayed ratio (dissolved in water).

3.2.4 Black tea production of different decaffeinated teas:

Once samples were decaffeinated, they were prepared for black tea production in the tea factory at the University of Pretoria, Faculty of Natural and Agricultural Sciences, Department of Biochemistry. Approximately 250 grams fresh weight of each sample was collected, and macerated using a CTC machine three times before collection of the dhool for black tea production. The samples were placed in a fermenter for quality development (fermentation producing theaflavins) i.e., black tea production for 60 minutes and wet bulb temperature was set at 29°C while dry bulb temperatures were set at 30°C to ensure constant humidity. Once fermentation was complete, excess moisture was removed by drying in a fluid bed dryer using heated air at 120°C until dry, then at 80°C for 5 min. Once dry, samples were sieved into smaller size fractions (which also removes any unwanted fibres) and ground to a powder. Controls included hot water decaffeinated tea with no addition of the PPO enzyme and

young fresh *C. sinensis* tea leaves not decaffeinated. Samples were analysed in duplicate on the same day and independent experiments were conducted on different days.

3.2.5 Moisture content determination:

Please refer to section 2.2.4

3.2.6 Caffeine content determination by HPLC:

Please refer to section 2.2.5. HPLC analysis was only done for section 3.2.2. Spectrophotometric methods were relied on for any other sample analysis.

3.2.7 Caffeine content determination spectrophotomerically:

Unfortunately we were not able to conduct HPLC analysis on all of our samples due to instrumentation being out of service. Thus an alternative method for caffeine quantification was used. The method used to determine the caffeine content spectrophotomerically was adopted from Yao *et al.* (2006) modified by Maliepaard (2007). The standard curve was made up exactly as described and then caffeine measurements in duplicate were conducted using lead acetate (Merck, Darmstadt, Germany) (0.5 g/ml in dddH_2O), hydrochloric acid (Saarchem, Muldersdrift, South Africa) (0.032 % in dddH_2O) and sulphuric acid (Saarchem, Muldersdrift, South Africa) (16.3 % in dddH_2O) solutions. The absorbance's of the samples were read at 274 nm using a UV/visible spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.8 Theaflavin quantification:

Theaflavin quantification was done according to the Flavognost method adopted from Reeves *et al.* (1985), Robertson and Hall (1989) and Maliepaard (2007). Sample extraction was the same as section 3.3.7 (Maliepaard 2007). In an Eppendorf tube, 600 µl IBMK (Merck, Darmstadt, Germany) was added to 600 µl of extracted tea liquors. Samples were vortex mixed four times for 30 sec each and in duplicate. The top (IBMK) layer was transferred to new Eppendorf tubes in 100 µl aliquots. A 1 % w/v flavognost (diphenylboric acid - 2 – aminoethyl ester) (Sigma - Aldrich, Steinheim, Germany) dissolved in 96 % EtOH in was added to the aliquots (200 µl). Samples were vortex mixed and incubated at 37 ± 2°C for 12 min. The absorbance was read at 620 nm using IBMK/EtOH (1:2 v/v) as a blank. The reader is referred to the references for information regarding calculations (Reeves *et al.* 1985; Robertson and Hall 1989; Maliepaard 2007).

3.2.9 Total polyphenols:

The method used to determine the polyphenol content spectrophotomerically was adopted from Maliepaard (2007). For the extraction procedure the reader is referred to Maliepaard (2007). A gallic acid (Sigma – Aldrich, Steinheim, Germany) dilution range of 1, 2, 3, 4 and 5 µg/ml was made up from a 0.1% (w/v) gallic acid stock solution i.e., 0.1 +/- 0.001 g gallic acid in 100 ml $dddH_2O$. For each sample, 200 µl ($dddH_2O$ acting as a blank) in duplicate were transferred to a 2 ml Eppendorf tube. One ml of Folin-Ciocalteu (Merck, Darmstadt, Germany) (1:10 (v/v)) was added to each sample and vortex mixed. The samples were allowed to stand for 5 minutes. After 5 minutes, 800µl of a 7.5% w/v sodium carbonate (Merck, Darmstadt, Germany) solution was added to each Eppendorf tube, vortex mixed and left to stand for 1 hour at room temperature. After 1 hour standing, the tubes were vortex mixed again and the absorbance was measured at 765 nm using a UNICO® spectrophotometer.

3.2.10 Statistical analysis:

Please refer to section 2.2.12. The Student's t-test was used to determine if differences between means were statistically significant at the 95 % level of confidence. When comparing caffeine loss (%) unequal variances were used.

3.3 Results

3.3.1 Preliminary vacuum, pressure, vacuum together with pressure extraction of caffeine:

After samples were treated by the three methods, HPLC analysis revealed the sample caffeine content (Fig. 3.1). The control sample that was untreated was determined to have a caffeine content of 4.39 $\pm$ 0.11 % w/w. When compared to the different methods of decaffeination, it was determined that vacuum treating the leaves removed 12.01 % of caffeine, while placing the samples under pressure only removed 0.40 %. Combining both methods of vacuum and pressure resulted 2.67 % of caffeine being removed.

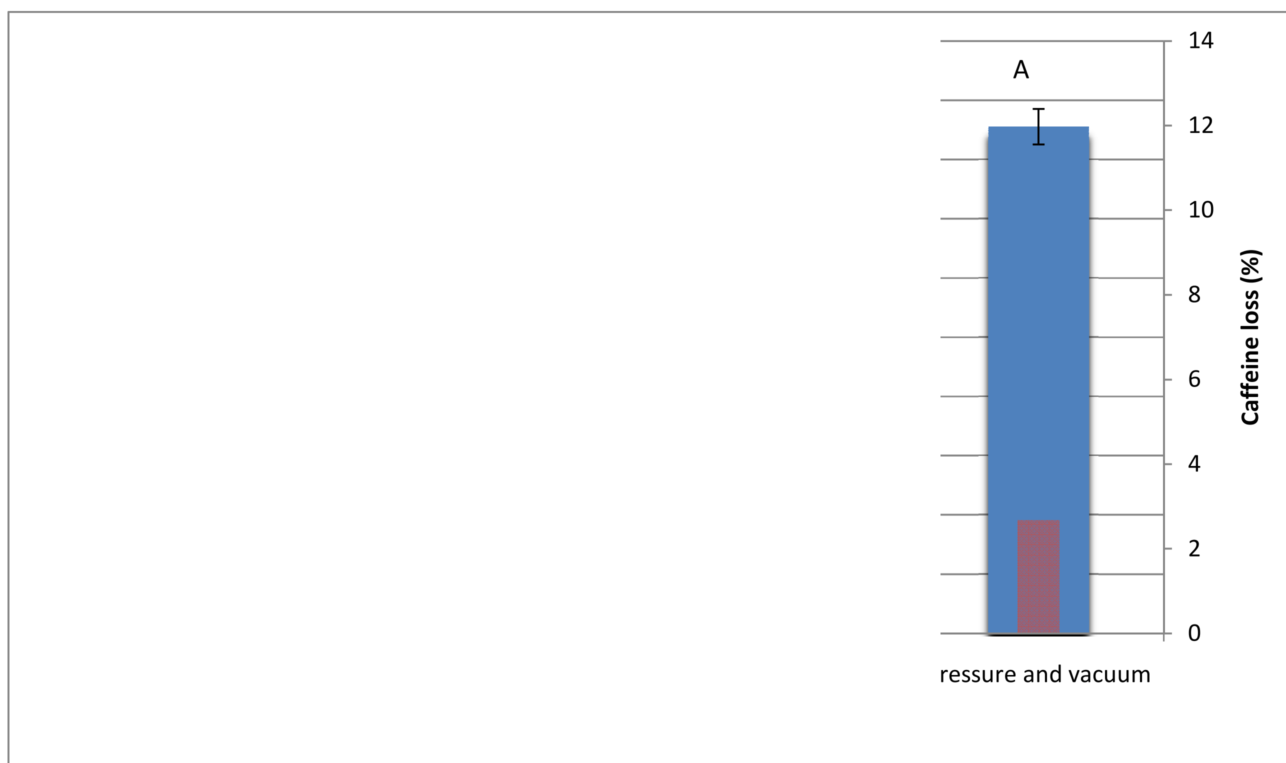

Fig. 3.1: Histogram displaying the caffeine content of leaves (% w/w) retained after each different method of decaffeination on the left y – axis (blue) and the caffeine loss (%) for each decaffeination method is displayed on the right y – axis (maroon). Error bars display S.E.M. Letter groupings are according to one way ANOVA. All means were compared to the Tzaneen control. Means annotated with different letters indicate statistically significant differences at the 95 % level of confidence.

In Figure 3.1 it can be seen that the best method of decaffeination of the different methods attempted was placing leaves in water under vacuum. One way ANOVA revealed that statistically there was a difference of the vacuum decaffeination method's caffeine content compared to the control and other methods of decaffeination (pressure, pressure and vacuum combined). The other methods, pressure and pressure together with vacuum revealed no statistical difference when compared to the Tzaneen control. Therefore, the best method of choice amongst the group of decaffeination methods was the method of vacuum decaffeination and this method was to be used in an upscale decaffeinated black tea production with the hot water decaffeinated method (external source of PPO added in different ratios).

3.3.2 Black tea production of different decaffeinated teas:

Table 3.1 displays the caffeine content, loss of caffeine, total polyphenol content, loss of polyphenols, theaflavin content, and loss of theaflavins after decaffeination, and black tea production for the different methods of tea decaffeination. Starting with theaflavin content, the fresh Tzaneen tea leaves,

with a letter grouping of A, had a 0 % loss since it was a control and had not been treated except with water. The other two methods that also had a letter grouping of A and theaflavin content loss of 0.61 % and 6.86 % was hot water decaffeinated tea: fresh Tzaneen tea (1:1) and vacuum decaffeinated tea respectively. When total polyphenols are compared, both of these methods (hot water decaffeinated tea: fresh Tzaneen tea (1:1) and vacuum decaffeinated tea) had a letter grouping of E, which was the same as the control group and total polyphenol losses of 13.32 % and 10.87 % respectively. Since these two methods of decaffeination gave the best decaffeinated black tea, taking theaflavin and total polyphenol content into consideration, their loss of caffeine content was compared to the hot water decaffeinated tea control. Hot water decaffeinated tea: fresh Tzaneen tea (1:1) had a total loss of 28.00 % caffeine content with a letter grouping of B. Vacuum decaffeinated tea had a total loss of 4.80 % caffeine content with a letter grouping of A. The letter grouping of A is comparable to the caffeine content of the fresh Tzaneen leaf control and thus, the latter method of decaffeination was used and compared to the hot water decaffeinated tea control (letter groupings of D and E) using the Student's t test (Table 3.2). The other methods of decaffeination had losses of theaflavin content that were too large to be considered to be used as a method of decaffeination for black tea production. For example, hot water decaffeinated tea: fresh Tzaneen tea (5:1) had a loss of 51.96 % theaflavin content with a letter grouping of B, making it undesirable for black tea production.

Hot water decaffeinated tea: fresh Tzaneen tea (1:1) was accepted to be used in the production of black tea, and had a total loss of 28.00 % caffeine content. The hot water decaffeinated tea control had a total loss of 57.20 % caffeine content. These losses were compared using a Student's t test for unequal variances (Table 3.2). A confidence level of 95 % was used and for unequal variances a Pr > t value of 0.0294 was obtained. These values thus suggest that the two methods of decaffeination are significantly different when comparing their loss of caffeine content (%). This indicates that there is much room for optimisation for the decaffeination method as the two methods should statistically be the same with regards to loss of caffeine.

1 **Table 3.1:** Different methods of decaffeination with added external source of PPO. Caffeine, total polyphenols and theaflavin contents/losses are displayed.

2 Letter groupings are according to a one way ANOVA.

Sample treatment	Caffeine content (% w/w) ± SD	Loss of caffeine (%)	Letter groupings (ANOVA)	Total polyphenol content (% w/w) ± SD	Loss of polyphenol (%)	Letter grouping (ANOVA)	Theaflavin content (µmol/g dry tea) ± SD	Loss of Theaflavin (%)	Letter grouping (ANOVA)
Fresh Tzaneen tea (control)	2.50 ± 0.07	0.00	A	11.41 ± 0.62	0.00	E	8.16 ± 0.58	0.00	A
Hot water decaffeinated tea (control)	1.07 ± 0.10	57.20	D,E	9.89 ± 0.16	13.32	A,B,C,D	0.02 ± 0.00	99.75	D
Hot water decaffeinated tea : Fresh Tzaneen tea (20:1)	1.17 ± 0.06	53.20	D,E	11.08 ± 0.29	2.89	A,B,C,D	0.77 ± 0.08	90.56	D
Hot water decaffeinated tea : Fresh Tzaneen tea (10:1)	1.24 ± 0.00	50.40	D,E	10.80 ± 0.27	5.35	A,B,C,D	2.25 ± 0.50	72.43	C
Hot water decaffeinated tea : Fresh Tzaneen tea (5:1)	1.40 ± 0.06	50.40	C,D,E	10.76 ± 0.09	5.70	A,B,C,D	3.92 ± 0.08	51.96	B
Hot water decaffeinated tea : Fresh Tzaneen tea (1:1)	1.80 ± 0.08	28.00	B	9.89 ± 0.41	13.32	C,D,E	8.11 ± 0.28	0.61	A
Hot water decaffeinated tea : Freeze dried tea (100:1)	1.20 ± 0.07	52.00	D,E	10.80 ± 0.59	5.35	B,C,D,E	0.65 ± 0.08	92.03	D
Hot water decaffeinated tea : Freeze dried tea (50:1)	1.29 ± 0.03	48.40	C,D,E	11.00 ± 0.78	3.59	A,B	0.77 ± 0.00	90.56	D
Hot water decaffeinated tea : Freeze dried tea (25:1)	1.41 ± 0.18	43.60	C,D	11.37 ± 0.24	0.35	A	0.98 ± 0.13	87.99	D
Hot water decaffeinated tea : Freeze dried tea (5:1)	1.62 ± 0.08	35.20	B,C	11.22 ± 0.31	1.67	A,B,C	3.06 ± 0.01	62.50	B,C
Hot water decaffeinated tea (10% v/v Suberase™ spray)	1.05 ± 0.00	57.60	E	9.58 ± 0.40	16.04	D,E	0.65 ± 0.16	92.03	D
Vacuum decaffeinated Tea	2.38 ± 0.50	4.80	A	10.17 ± 0.20	10.87	E	7.60 ± 1.12	6.86	A

Table 3.2: Student's t test comparing the decaffeinated method of tea with highest retention of theaflavins related to the decaffeinated tea control (Table 3.1).

Sample	Caffeine content (w/w) ± SD	Caffeine loss (%)	Variance	DF	Confidence (0.95) Pr > t
Hot water decaffeinated tea : Fresh Tzaneen tea (1:1)	1.80 ± 0.08	28.00	Unequal	1.10	0.0294

3.4 Discussion

Different methods of decaffeination were investigated in order to remove caffeine from young *C. sinensis* leaves. The methods were explored after microscopic analysis in chapter two revealed areas of caffeine accumulation to be within the vascular bundles of the young leaves. The methods investigated were vacuum, pressure and cycling between vacuum and pressure. They were compared to the control leaves and an established method of decaffeination i.e., hot water decaffeination. In Fig. 3.1 the vacuum method removed 12.01 % of caffeine, the pressure method removed 2.67 % and the combination of vacuum and pressure only removed 0.4 %. Using a one way ANOVA it was determined that the best method of removal was the vacuum method of decaffeination as it differed statistically from the other methods investigated in Fig. 3.1. We decided to use the vacuum decaffeination method in an upscale production of decaffeinated black tea with the other methods i.e., hot water decaffeinated tea containing different ratios of PPO enzyme.

It is hypothesised that the caffeine is removed from the sample due to intercellular air present within the young leaves. Once placed under vacuum, air expands, this can act as a squeezing effect within the young leaves forcing the caffeine out the vascular bundles i.e., xylem and phloem. There is also the possibility that the air can squeeze caffeine out of the parenchyma cells (this compound was hypothesised to be present in these cells in a low concentration) into the surrounding spaces. When the vacuum is broken these expanded air bubbles shrink, drawing fresh water into the vascular bundles, dissolving extracted caffeine. When vacuum is applied again, the remaining air squeezes out the water containing dissolved caffeine and thus decaffeinating the sample. The advantage of this method is the fact that the PPO enzyme should not be compromised in any way during the decaffeination process, allowing for fermentation to take place after decaffeination. A decaffeinated black tea can then be made without the use of chemicals and solvents. A limiting factor might be that the leaves become saturated with water and thus there is no more squeezing effect from the air within the leaves, hampering further extraction of any caffeine. A system specific for this method will need to

be developed that will allow for the sample to be put under vacuum, maintaining its temperature, with a constant release of vacuum for the optimal removal of caffeine from the sample.

Placing the leaves under pressure removes far less caffeine compared to the vacuum treatment. Under pressure the air within the sample compresses and it is possible that some water may enter the sample, but when pressure is released, the expansion of gas is not sufficient to remove the caffeine dissolved within the water. It is also possible that the leaves become saturated with water faster than using the vacuum treatment, thus reaching its limiting factor. It is also possible that higher pressures need to be attained to remove caffeine. The combination method removes the least amount of caffeine. This is most likely explained by the fact that caffeine is removed from the sample during the vacuum treatment, but when pressure is applied the water containing caffeine is forced back into the sample. Once the sample is saturated no caffeine can be removed again.

I then investigated other methods of decaffeination for the production of decaffeinated black tea. This included decaffeinating samples using hot water decaffeination and adding an external source of PPO in order to provide fermentation i.e., production of theaflavins from polyphenols retained within the sample. The external sources of PPO included fresh leaves from Tzaneen, freeze dried leaves and Suberase™ from Novozymes® as a sprayed solution. The reasoning for using fresh leaves was that during CTC maceration the leaves cells burst, releasing the PPO enzyme that would mix with contents of the burst decaffeinated tea cells. This then would provide an external source of PPO required for fermentation during black tea production. Freeze dried tea also has its PPO enzyme still active. I hypothesised that the PPO enzyme would also mix in a similar way providing fermentation possibilities. The external spray would also provide an external source of PPO for fermentation without the presence of additional caffeine, thus providing a tea very low in caffeine content. The vacuum method does not have its PPO compromised and there is no need for an external addition of the enzyme. From Table 3.1 it was determined that the hot water decaffeinated tea: fresh Tzaneen tea (1:1) along with the vacuum method of decaffeination, had the best production of theaflavins for black tea manufacturing. When compared to the fresh Tzaneen tea control they only had losses of 0.61 % and 6.86 % theaflavin content (all had letter groupings of A). The vacuum decaffeination method has more of a loss due to the enzyme being compromised during the process in some manner such as leakage via broken cells into the surrounding water solution. Unfortunately, the level of decaffeination for the method of vacuum decaffeination was far too low for it to be considered as a production method i.e., caffeine loss of 4.80 %. The other methods don't produce high enough levels of theaflavin. In the case of adding fresh tea during CTC maceration, the ratio of 1:1 provides enough PPO enzyme for production of theaflavins where as the lower ratios do not. For the freeze dried material, the tea is too dry to provide adequate mixing of the PPO enzyme with the content of the

decaffeinated tea, thus compromising the efficiency of fermentation. The spray solution of Suberase™ might be too low of a concentration to provide adequate PPO for theaflavin production and thus further optimisation of this method is required.

Since the hot water decaffeinated tea: fresh Tzaneen tea (1:1) was determined to be the best method of decaffeination when comparing theaflavin, total polyphenol and caffeine contents (Table 3.1) to the fresh Tzaneen control, the method of decaffeination was compared to the hot water decaffeination control. By using a Student's t test I determined these methods to be significantly different i.e., for unequal variances a Pr > t value of 0.0294 was obtained at a confidence level of 0.95. Thus H2 was accepted while $H2_0$ was rejected due to these results. Optimally, the method of decaffeination should not be statistically different from the hot water decaffeination method and thus more research and optimisation into the method is required.

The method of hot water decaffeinated tea: fresh Tzaneen tea (1:1) had the best production of theaflavins and one of the highest polyphenol contents and lowest caffeine contents when compared to the other methods of decaffeination used for black tea production. Even though the method is statistically different from the hot water decaffeinated method, a 28.00% of caffeine content was reduced. The method also has numerous advantages over current methods of decaffeination such as low cost, very low labour intensity, safety to the consumer and factory as well as being environmentally friendly. Furthermore the risk of down time due complicated machinery breaking is very low risk. There is also no need for complicated equipment, the production can be done with most of the machinery already found in a tea factory. All these factors will also contribute to a lower cost decaffeinated black tea.

3.5 Conclusion

By determining caffeine's accumulation within the vascular area of young *C. sinensis* leaves three methods of decaffeination were investigated i.e., vacuum, pressure and vacuum together with pressure. This was based on using the intercellular air as a mechanism to squeeze out the caffeine from these areas as well as dissolve the molecule within the leaves. The best method was vacuum decaffeination determined by an one way ANOVA. The initially the method was determined to remove 12.01% of the overall caffeine content. This method was then included in a larger scale black tea production with other methods decaffeinated using hot water and the external addition of the PPO enzyme required for black tea production in the form of fresh tea leaves, freeze dried tea leaves and a sprayed solution of Suberase™ from Novozymes®. The method of hot water decaffeinated tea: fresh Tzaneen tea (1:1) and the vacuum treatment had the best production of theaflavins i.e., losses of only

0.61% and 6.86% respectively. Their total polyphenol contents were also comparable to the fresh Tzaneen tea control. The vacuum method, however, had a caffeine content reduction of only 4.80% whereas the method of hot water decaffeinated tea: fresh Tzaneen tea (1:1) had a reduction of 28.00%. It was determined that the method of hot water decaffeinated tea: fresh Tzaneen tea (1:1) was statistically different when compared to the decaffeinated tea control and thus H2 (there is a statistically significant difference in the percentage of decaffeination between the new method developed in this study and the hot water method of decaffeination at the 95 % level of confidence) was accepted and $H2_0$ (there is no statistically significant difference in the percentage of decaffeination between the new method and the hot water method of decaffeination at the 95 % level of confidence) was rejected. The method of vacuum decaffeination needs to be optimised with a system specific for its use and this could possibly increase the amount of caffeine removed to more attractive levels. The method of hot water decaffeinated tea: fresh Tzaneen tea (1:1) will have an advantage over current market decaffeination as no chemicals and complicated machinery are used, the consumer and factory worker won't be harmed, and it is less labour intensive. Furthermore, the PPO enzyme required for fermentation in the production of black tea is added in a sufficient amount allowing for high levels of theaflavin production. Although only 28.00 % of caffeine was removed, there is still place for this method of decaffeination on the current markets.

Chapter 4: Concluding discussion

The aim of the study was to anatomically localise the caffeine molecule within young *C. sinensis* leaves revealing information about its function within the plant. A current hypotheses suggests that caffeine acts as a chemical defence within *C. sinensis* leaves, and that it is most likely localised within the vacuoles of photosynthetic cells where it aids in sequestration of catechins. Information from this study would be able to confirm these hypotheses or reject them and suggest new ones. Furthermore, a new method of tea decaffeination may possibly be developed that would have numerous advantages over current methods used around the world.

C. sinensis is farmed on a commercial basis, and the top young shoots are harvested for tea production. The beverage is considered to be the second most consumed around the world. Consumption leads to many beneficial compounds being ingested such as polyphenols that have shown to have many health properties such as the lowering of LDL – cholesterol. Caffeine, a xanthine alkaloid is also consumed along with these beneficial compounds and its consumption may lead to unwanted effects in certain individuals. The molecule is believed to be an inhibitor of the enzyme phosphodiesterase that is responsible for the disposal of cAMP. Thus without its disposal, phosphorylation of certain enzymes occur, up-regulating cellular responses and effects such as not being able to fall asleep are experienced. The enzyme responsible for caffeine synthesis is caffeine synthase and it has been localised to chloroplasts of photosynthetic cells in the leaves. The molecule is believed to be stored within the vacuoles of these cells where it forms complexes with polyphenols, and it has been suggested it forms complexes with chlorogenic acid within these vacuoles as well. Thus caffeine aids in the sequestration of polyphenols and chlorogenic acid. It has been previously hypothesised that the molecule acts as a chemical defence in the prevention of herbivory as it has been shown to be lethal in certain beetles and worms in previous experiments. Solvent extraction using methylene chloride, supercritical CO_2 extraction and hot water decaffeination are all current methods of tea decaffeination. Solvent extraction uses solvents in a process to extract caffeine from the *C. sinensis* leaves and it is out dated, harmful to the environment, possibly harmful to the consumer as well as factory workers and has to meet stringent FDA rules. Supercritical CO_2 can be considered to be better. The method uses very complicated machinery and down time due to repairs is common. The decaffeinated product is usually more expensive. Hot water decaffeination is a simple method and not as destructive to environment or harmful to the consumer but the PPO enzyme required for fermentation and black tea production is denatured during the process thus only decaffeinated green tea can be produced. In the coffee industry, a production method of decaffeination called the Swiss Water® process exists. The method is 100 % chemically free, using water saturated with green coffee extract free of caffeine and activated carbon filters specific for the

removal of caffeine. The method is not hazardous to the environment, economically viable, not harmful to the consumer. A similar method of decaffeination with the advantages should be envisaged for the tea decaffeination industry.

Many different microscopy techniques have been used for the localisation of alkaloids in plant material. The methods include histochemical analysis, fluorescence analysis, immunolabeling and localising the molecule on fluorescence or TEM microscopes, use of Raman microscopes and even NMR microscopes to name a few. In our study, immunolabeling was suggested as it is highly specific for the molecule of interest and the equipment needed for analysis was easily accessible.

In order to localise caffeine, a method of fixation and embedment needed to be developed in order to retain caffeine within the sample. Preliminary experiments of chemical fixation, HPF/ FD and HPF/ FS were investigated and analysed using HPLC. It was determined that the best method to use was HPF/ FS as it retained 49 % of caffeine. This method was a compromise in choice as it was easier to make sections of the sample for analysis compared to HPF/ FD due to difficulty in resin infiltration in the HPF/ FD (retained 95 % of caffeine) method. The difficulty in resin infiltration can be attributed to the leaves high tannin content. Lowicryl K4M resin was the best to use for CSLM analysis while LR White was the best for TEM analysis. Immunohistochemical localisation with primary anti-caffeine antibodies and conjugated secondary antibodies were conducted revealing caffeine to be accumulated within precursor phloem of the vascular bundles. Caffeine was confirmed to be present within the xylem sap by collection via a pressure bomb and analysis using TLC. Unfortunately, TEM analysis was inconclusive due to a number of reasons such as sectioning onto water that might have dissolved the molecule. The localisation of the molecule follows the pattern of other alkaloids and its presence within the phloem cells in complex with chlorogenic acid was suggested in a previous study. The molecule may be synthesised within the chloroplasts with chlorogenic acid and then transported to the vascular area where they are in complex with each other. Chlorogenic acid may further act as a natural fixative. It was suggested that caffeine acts as a chemical defence within the vascular area in response to biotic and abiotic stress. The molecule is most likely to prevent small insects such as aphids from attaining nutrients from the vascular area as well as preventing the access of certain pathogens. Thus it is unlikely to act as a defence against herbivory. Thus, overall H1 (caffeine is localised to vacuoles of parenchyma cells of young *C. sinensis* leaves) is rejected and $H1_0$ is accepted (caffeine is not localised to the vacuoles of parenchyma cells of young *C. sinensis* leaves but is accumulated elsewhere within the plant).

The knowledge obtained from microscopy analysis allowed me to investigate three methods of decaffeination i.e., vacuum, pressure and a combination of both. The methods were based on using

intercellular air to my advantage in order to squeeze caffeine out of the cells. The vacuum method of decaffeination was the best of the three methods as determined by a one way ANOVA. The method was determined to remove 12.01 % of caffeine from the sample analysed. This method was used in an upscale production of black tea with different methods of decaffeination. The different methods included the use of hot water decaffeination with the addition of an external source of PPO (required for fermentation during production). The different forms included the use of fresh tea, freeze dried tea and a sprayed version of Suberas™ from Novozymes®. The method of hot water decaffeinated tea: Fresh Tzaneen tea (1:1) and vacuum decaffeination had the best production of theaflavins i.e., losses of 0.61 % and 6.86 % respectively, with minimal losses of total polyphenols. This was due to sufficient addition and mixture of the PPO enzyme during maceration in the form of fresh tea leaves. The vacuum decaffeination did not have its PPO compromised to a large extent. The other methods of decaffeination did not produce enough theaflavins during fermentation due to inadequate mixing and low amounts of the enzyme present. The method of hot water decaffeinated tea: Fresh Tzaneen tea (1:1) had a reduction of 28.00 % in caffeine content while the vacuum method only had a reduction of 4.80 %. Thus the method of vacuum decaffeination needs more improvement and optimisation possibly in the form of equipment specifically designed for this use. The hot water decaffeinated tea: Fresh Tzaneen tea (1:1) method was then compared to the loss of caffeine obtained for the hot water decaffeination control using the Student's t test for both equal and unequal variances. The methods were determined to be statistically different and thus H2 (there is a statistically significant difference in the percentage of decaffeination between the new method developed in this study and the hot water method of decaffeination at the 95 % level of confidence) was accepted and $H2_0$ (there is no statistically significant difference in the percentage of decaffeination between the new method and the hot water method of decaffeination at the 95 % level of confidence) was rejected. This is mainly due to the addition of caffeine in the form fresh leaves, but without this addition, the possibility of manufacturing black tea after this method of decaffeination would not exist. The caffeine content can be further reduced if a method can be developed where by PPO can be added externally without the addition of caffeine. Hot water decaffeinated tea: Fresh Tzaneen tea (1:1) method still removed 28.00 % of caffeine and presents a number of advantages of current methods used such as no use of chemicals, safe to factory workers and consumers, environmentally friendly, not labour intensive, there is no need for complicated machinery and employment of specialised personal. This can also contribute to the reduction in cost of the production of the black tea low in caffeine. It is suggested that HPLC should be conducted on this product as well as tasting by a professional tea taster.

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