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A drought induced modulation of the *Camellia sinensis* metabolome

by

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Pretoria

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April, 2016.

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I declare that the dissertation, which I hereby submit for the degree *Magister Scientiae* in Biochemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Summary

Climate change is causing droughts affecting crop production on a global scale. Classical breeding and selection strategies for drought tolerant cultivars will help prevent crop losses. Plant breeders, for all crops, need a simple and reliable method to identify drought-tolerant cultivars, but such a method is missing. Plant metabolism is often disrupted by abiotic stress conditions. To survive drought, plants reconfigure their metabolic pathways. Studies have documented the importance of metabolic regulation, i.e. osmolyte accumulation such as polyols and sugars (mannitol, sorbitol); amino acids (proline) during drought. This study identified and quantified metabolites in drought tolerant and drought susceptible *Camellia sinensis* cultivars under wet and drought stress conditions. For analyses, GC-MS and LC-MS were employed for metabolomics analysis. %RWC results show how the two drought tolerant and two drought susceptible cultivars differed significantly ($p \leq 0.05$) from one another; the drought susceptible exhibited rapid water loss compared to the drought tolerant. There was a significant variation ($p < 0.05$) in metabolite content (amino acid, sugars) between drought tolerant and drought susceptible tea cultivars after short-time withering conditions. These metabolite changes were similar to those seen in other plant species under drought conditions, thus validating this method. The Short-time Withering Assessment of Probability for Drought Tolerance (SWAPDT) method presented here provides an easy method to identify drought tolerant tea cultivars that will mitigate the effects of drought due to climate change on crop losses.

Some of the results presented in this dissertation have been published in the Journal of plant physiology in April 2016. The article is found in Appendix A.

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Acronyms and abbreviation

ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionisation
AtTPS1	*Arabidopsis* trehalose-6-phosphate synthase 1
BEH	Bridged ethylene hybrid
CEC	Capillary electro-chromatography
CoA	Coenzyme A
DIMS	Direct injection mass spectrometry
DoE	Design of experiments
d-OAT	d-ornithine aminotransferase
DS	Drought susceptible
DT	Drought tolerant
EC	Epicatechin
ECG	Epicatechin-3-gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
ELSD	Evaporative light scattering detector
ESI	Electrospray ionisation
FTIR	Fourier transform infrared spectroscopy
GABA	γ-amino butyrate
GR	Glutathione reductase
GSA	Glutamate-semialdehyde

HIV	Human immunodeficiency virus
mtlD	Mannitol dehydrogenase gene
NetCDF	Network Common Data Form
NMR	Nuclear magnetic resonance
P5C	Pyrroline-5-carboxylate
P5CS	Pyrroline-5-caboxylate synthetase
PCA	principal component analysis
PDA	Photo-diode array
PPO	Polyphenol oxidase
RWC	Relative water content
SRM	Selected reaction monitoring
TF	Theaflavins
TPC	Total polyphenol content
TR	Thearubigins
TRFCA	Tea Research Foundation of Central Africa
TRFK	Tea Research Foundation of Kenya
UPLC-MS	Ultra-perfomance liquid chromatogtaphy-Mass spectrometry

CHAPTER ONE

(Introduction)

Camellia sinensis

Tea made from the leaves of *Camellia sinensis*, as green or black tea, has been drunk as a mild stimulant due to the caffeine content, since time immemorial! Tea consumption has increased in recent years, due to the health promoting effects associated with its rich polyphenol content (Tong, Taylor, Giles, Wittert, & Shi, 2014). Tea is one of the popular non-alcoholic beverages consumed worldwide (L. Chen, Zhou, & Yang, 2007). Tea producers demand new cultivars which are drought tolerant, to reduce crop losses. Readily quantifiable markers for drought tolerance will help tea breeders identify and select tea cultivars suitable for drought prone environments. Three main types of tea exist, green, oolong and black tea, these being determined by the fermentation concentration of their flavan-3-ols. *Camellia sinensis* is cultivated in over 52 countries around the world and is consumed in the form of black (78%), green (20%) or oolong (2%) tea. Green tea is however a favourite in several countries in North Africa and the Middle East, whereas black tea is mostly consumed in Western countries (Cooper, Morré, & Morré, 2005). Green tea has traditionally been evaluated based on the quality of its leaf appearance (colour, colour intensity and clarity), aroma (floral, sweet, grassy, etc.), and taste (astringency, bitterness and sweetness). Caffeine is responsible for giving it its characteristic astringent, bitter taste but the other compounds that influence its taste and flavour include catechins, polyphenols, sugars, organic acids and amino acids. Amino acids, especially theanine, which constitutes two-thirds of the total amino acids content in tea leaves, are responsible for its sweet, brothy taste. *C. sinensis* consist of polyphenols and phenolic acids in large quantities, responsible for its therapeutic properties. However, the chemical constituents influencing tea quality differs between the green and black tea. The quality of black tea is dependent on theaflavin, thearubigen, catechin and caffeine levels, whereas green tea quality depends on amino acid (especially theanine), catechin and caffeine contents (Le Gall, Colquhoun, & Defernez, 2004). An estimated 3.92 metric tonnes of tea is produced annually, with black and green tea representing 60% and 30% respectively of the produced tea (Meeting & Organization, 2010). However, the production of green tea, whose consumption is immensely limited to Japan and China (Sajilata, Bajaj, & Singhal, 2008),

is predicted to have a considerable increase in comparison to black tea (Meeting & Organization, 2010). Green and black teas are produced in Japan and China from the leaves of *C. sinensis* and *C. assamica* respectively (Chu & Juneja, 1997), though black tea production is on the rise in countries all over Asia, Africa and Latin America (Meeting & Organization, 2010).

Flavonoids in Tea

Camellia sinensis leaves are a major source of flavonoids in human nutrition. Flavan-3-ols make up 25–30% young tea leaf dry weight (Singh, Ravindranath, & Singh, 1999). Flavonoids are a diverse group of plant natural products synthesised from phenylpropanoids and acetate attained from carbohydrate metabolism as shown in Figure 1. Several different types of flavonoids exist. The most important are the dietary flavonoids, which are categorised into six major groups namely flavanols, flavonols, anthocyanidins, flavones, flavonones and isoflavonoids (Yilmaz, 2006). Flavonoids are vital in plant growth and development, serving also a defensive role against microorganisms and pests. Flavonoids are involved in the generation of phytoalexins, which serve as insect repellents and interfere with plant microbe interactions (Lattanzio, Lattanzio, & Cardinali, 2006). *C. sinensis*, like all other vascular plants, utilises carbon derived from tryptophan, tyrosine and phenylalanine metabolism to biosynthesise of flavonoids, which possess a 15 carbon skeleton formed via condensation and decarboxylation of phenylpropanoid derivatives (Cuendet, Potterat, & Hostettmann, 2001). Through the pentose pathway, *Camellia sinensis* links carbohydrate metabolism to the shikimate pathway, which in turn is responsible for the biosynthesis of phenylpropanoids. Phenylpropanoid biosynthesis begins with the deamination phenylalanine by the enzyme phenylalanine ammonia lyase which converts phenylalanine to cinnamic acid, which is then modified by the enzyme hydroxylase and O-methyltransferases. The enzyme 4-Coumaryl CoA ligase is responsible for catalysing the reactions which generates CoA esters of cinnamic acid. The resultant intermediates then function in the biosynthesis of flavonoid compounds via specific branch pathways (Cuendet et al., 2001). The phenylpropanoids from the shikimate pathway are in turn involved in carbohydrate metabolism via the pentose phosphate pathway (Häusler,

Ludewig, & Krueger, 2014). The metabolic pathways are dependent on photosynthesis, which is affected by water deprivation due to drought stress. Gallic acid, which attaches to the flavan-3-ols via an ester bond, is also derived from the shikimate pathway, (Heleno, Martins, Queiroz, & Ferreira, 2015). The phenolic metabolites synthesised are water soluble and combine with sugars to form glycosides, stored in the vacuole.

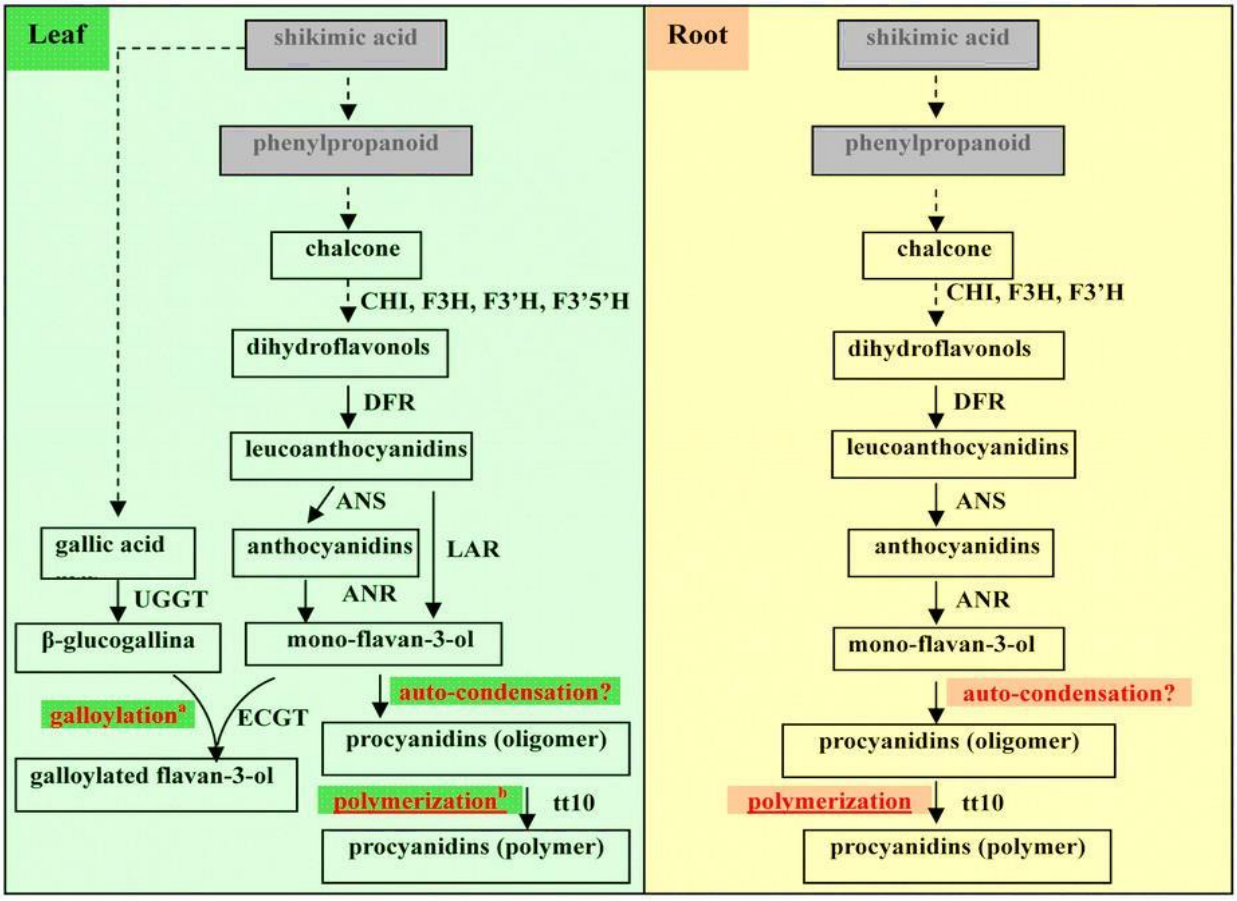


Figure 1: The biosynthetic pathways of flavan-3-ols and their derivatives in leaves and roots of tea plants (Jiang et al., 2015).

Tea polyphenols

Most osmolytes are secondary metabolites, and tea contains high polyphenol amounts (Cheruiyot, Mumera, NG'ETICH, Hassanali, & Wachira, 2007). In this study it was documented that the total polyphenol content in tea plants used was influenced by the soil water content. A decrease in the water content resulted in a reduction in the shoot polyphenol content in both tolerant and susceptible cultivars. The response degree

varied between the two classes, with overall higher polyphenol content in the tolerant varieties as compared to the susceptible. Polyphenols are compounds made up of joined benzene rings, each containing several hydroxyl groups. Although more than 90% of polyphenols found in tea are flavonoids, polyphenols can either be flavonoids or non-flavonoids (Sumpio, Cordova, Berke-Schlessel, Qin, & Chen, 2006). Polyphenols, like most other osmolytes, are secondary metabolites and are derived from the condensation reactions involving cinnamic acid and three malonyl-CoA groups. Tannins are large polyphenol molecules, making up 90% of active compounds found in tea.

Health benefits of tea

Both green and black tea, have been documented for their efficacious health promoting qualities, enhancing human health especially in dealing with protection against cancer, cardiovascular disease (Bahorun et al., 2012), diabetes, obesity (Uchiyama, Taniguchi, Saka, Yoshida, & Yajima, 2011) and metabolic syndrome (Thielecke & Boschmann, 2009). The major catechins in tea are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). Research has shown that catechins have anti-mutagenic, anti-tumorgenic, antioxidant, anti-hypertension and anti-inflammatory properties while theanine induces relaxation and enhances cognitive abilities (Haskell, Kennedy, Milne, Wesnes, & Scholey, 2008; Lisman et al., 2008). Further investigation has shown that the co-administration of drugs and catechins EC, EGCG and tannic acid results in the inhibition of glucoronidation and sulfation reactions of orally administered drugs. This increases the drugs bioavailability in the body (Suganuma, Saha, & Fujiki, 2011). Consumption of tea results in relaxation due to the presence of theanine. Theanine can translocate through the blood brain barrier within 30 minutes after consumption in a dose-dependent manner (Terashima, Takido, & Yokogoshi, 1999). Physiology studies on anxiety and stress have documented the effects of theanine, showing that it has relaxing effects (Kimura, Ozeki, Juneja, & Ohira, 2007). Theanine has also been shown to act as an antagonist to the stimulatory effects of caffeine (Rogers, Smith, Heatherley, & Pleydell-Pearce, 2008), displaying anti-hypertensive properties (Yokogoshi & Kobayashi, 1998). Theanine also appears effective in preventing liver damage due to alcohol consumption (Sadzuka et al., 2005).

These pharmacological polyphenol properties have led to several tests on tea extracts as prophylactics or drugs. An example is polyphenon E, which underwent preclinical studies for lung cancer chemoprevention in 2005 (Lambert, Hong, Yang, Liao, & Yang, 2005). In addition to this, EGCG has also been documented as an antagonist of human immunodeficiency virus reverse transcriptase (Nance & Shearer, 2003). HIV transcription is suppressed by theaflavins in the cell while the gallic acid moiety of theaflavins enhances suppressive activity (Gramza, Korczak, & Amarowicz, 2005). Several studies have shown that catechins possess antioxidative properties which can be attributed to their high scavenging affinity for both reactive oxygen and nitrogen species (Figure 2). Catechins have a 3,4 dihydroxy catechol structure or a 3,4,5 trihydroxyl group in the B ring, an esterified gallate group at position three on the C ring and hydroxyl groups at positions 5 and 7 on the A ring (Figure 3) (Frei & Higdon, 2003). The antioxidant activity of catechins is dependent on the number and the location of hydroxyl groups on the flavonoid rings (Lien, Ren, Bui, & Wang, 1999). It has been documented that catechins are capable of suppressing the growth of cancerous cells by blocking angiogenesis. Catechins are also capable of suppressing glucose uptake through the inhibition of the sodium dependant transporter SGLT1 mechanism, in turn prevents diabetes (Khan & Mukhtar, 2007). As a result tea has ceased to just be a beverage consumed and enjoyed for its pleasant aroma and taste and has found therapeutic applications (A. Zhang, Sun, Wang, Han, & Wang, 2012).

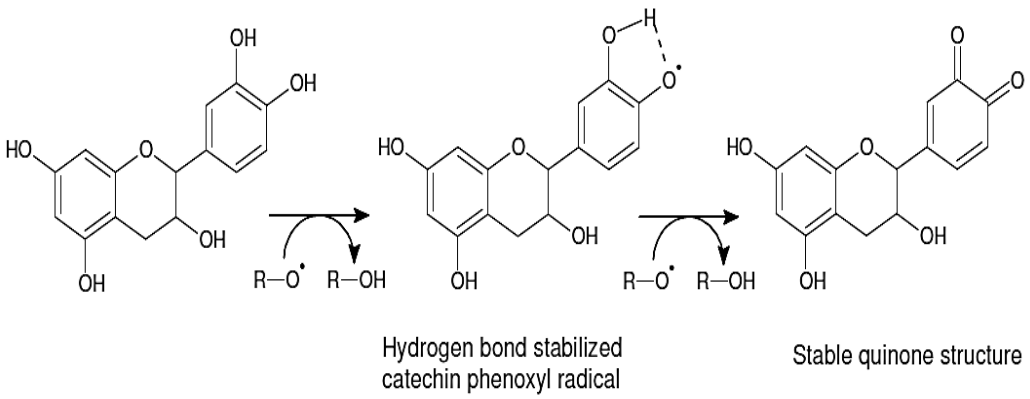


Figure 2: Catechin antioxidant mechanism of action (Amic et al., 2007).

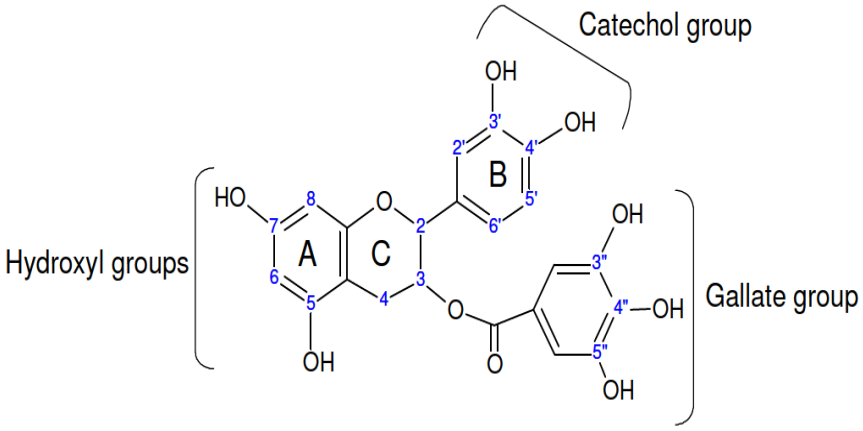


Figure 3: Functional groups responsible for catechin antioxidant properties e.g. ECg. (Frei and Higdon, 2003).

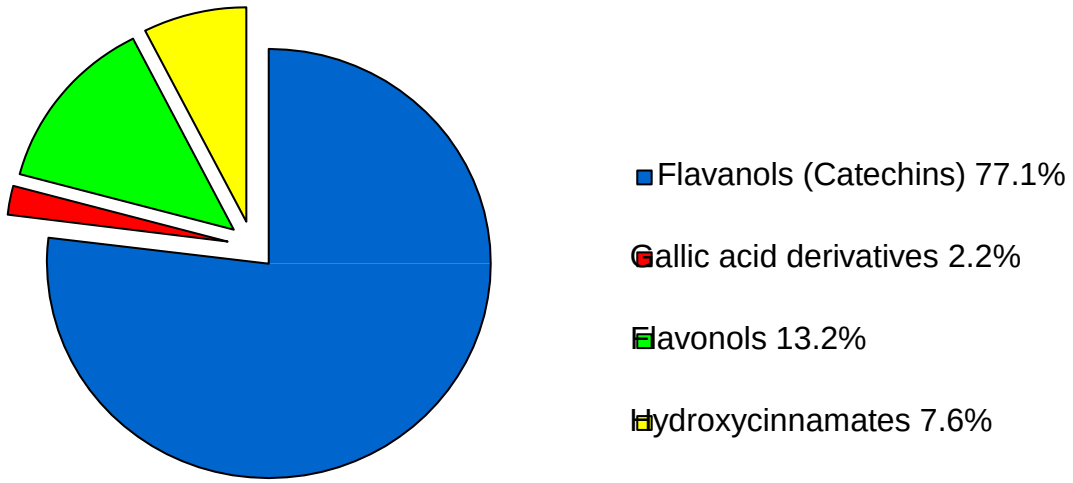


Figure 4: Green tea phenolic composition (Del Rio et al., 2004).

Tea is classified into three general categories: non-fermented green tea, semi-fermented oolong tea and fully fermented black tea. This beverage is manufactured using the tender top *Camellia sinensis* (L.) O. Kuntze shrub leaves. Fresh, green tea leaves contain approximately 40% (w/w) polyphenols, with catechins being the most abundant (Figure 4) (Del Rio et al., 2004). Throughout the tea leaves processing, the enzyme polyphenol oxidase (PPO) is released and it in turn oxidises the catechins to

theaflavins and thearubigins. This process is referred to as fermentation. During green tea manufacture, PPO is inhibited by steaming, roasting, parching and or oven heating fresh leaves (Gulati, Rawat, Singh, & Ravindranath, 2003) to prevent catechin oxidation, responsible for its flavour and astringent taste. PPO inhibition also serves to retain the leaves green colour, a quality determining factor, and retains almost all the polyphenols in the leaves. Oolong tea undergoes limited fermentation. This results in a mixture of catechins, theaflavins and thearubigens. Lastly, black tea undergoes full fermentation with almost all the catechins being converted to theaflavins and thearubigens, giving it its distinct aroma, colour and taste. Figure 5 below shows several structures of catechins found in green tea.

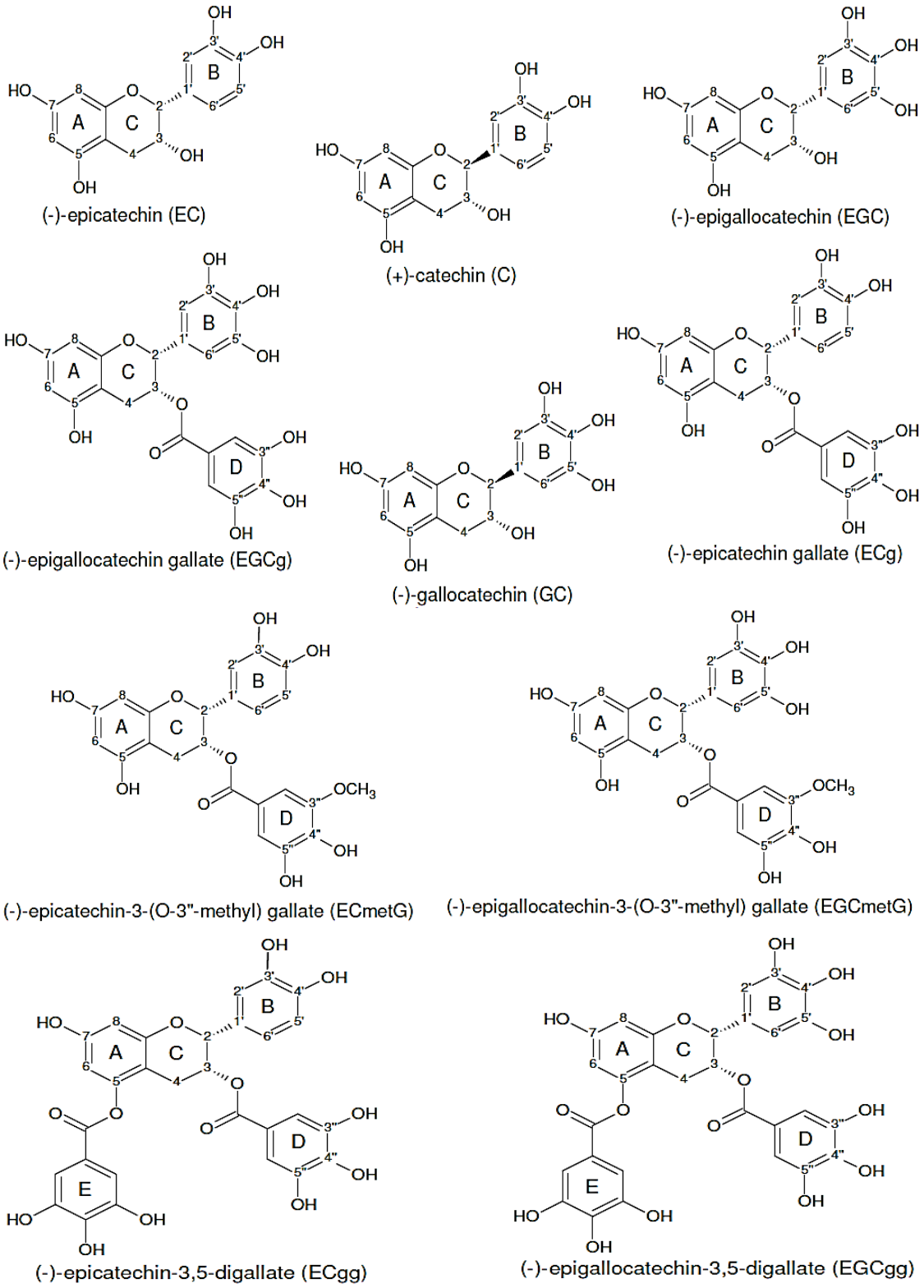


Figure 5: The structures of catechins found in green tea.

Deprivation of water affects many physiological and biochemical pathways, though it varies from plant species and the degree and duration of water stress and the stage of plant growth. Being a perennial crop, *C. sinensis* experiences both abiotic and biotic stresses which affect its growth and yield. Biotic stress is documented to reduce total yield by 20% whereas abiotic stress is estimated to reduce it by 65% (Waheed et al., 2012). The main climate changes that influence *C. sinensis* growth and yield are temperature, atmospheric pressure and of course soil moisture content. Plant response to stress is manifested by physiological and metabolomic responses (Maritim et al., 2015). This enables the screening and selection of tea cultivars resistant to drought stress, through organic osmolytes accumulation. No metabolites have been investigated in *C. sinensis* in relation to drought. However, several metabolites have been documented in literature relative to drought stress in other plant species. The current study focuses on polyphenols, flavonoids, amino acids and sugars.

Plant metabolomics

When plants are subjected to abiotic stress conditions, metabolic enzyme inhibition or substrate shortage, etc. disrupts plant metabolism resulting in metabolic pathway reconfiguration, ensuring plant survival (Hamanishi, Barchet, Dauwe, Mansfield, & Campbell, 2015). Plants have established innumerable strategies in response to drought (Ogbaga, Stepien, & Johnson, 2014). A common leaf response to drought stress involves both physical and morphological changes. Several hypotheses have been postulated in an attempt to account for these leaf changes in stressed plants. An example of such morphological changes includes the rolling or folding, that is observed in leaves as a result of drought stress, reducing transpiration (Engelbrecht & Kursar, 2003). In a separate study, drought susceptible indices of drought tolerant (DT) and drought susceptible (DS) pearl millet cultivars were exposed to drought stress. The initial visual response of drought stress was the withering of the lower part of the leaves, with the DT withering at a slower rate than the DS; resultant leaf death after continued stress exposure was observed. From the 18 samples used, leaves from the DT (IP8210) consistently demonstrated high tolerance to drought stress in all experiments while the DS (IP8949) was most prone to drought stress. Also, because of an increased

rate of leaf rolling observed in the DS cultivars, there will be an expected decrease in photosynthesis resulting in less carbohydrate biosynthesis (Kusaka, Ohta, & Fujimura, 2005). This inevitably means DT produce more sugars than DS plants resulting in their increased drought tolerance. Studies on leaves in connection with drought tolerance have been documented in different plants but not in *Camellia sinensis*. Crop breeders are keen to understand the biological mechanisms responsible for crop survival in drought stricken and salinised environments. These stress response mechanisms involve regulatory changes that activate multiple genes and their subsequent pathways. Several studies have been conducted on plants subjected to drought stress, showing the importance of metabolic regulation, i.e. accumulation of osmolytes in a response to drought stress (Slama, Abdelly, Bouchereau, Flowers, & Savouré, 2015). Hyperosmotic stress produces osmolytes which include polyols and sugars, e.g. mannitol, sorbitol and trehalose; amino acids, such as proline and betaine (Weckwerth, Wenzel, & Fiehn, 2004) and are water soluble and non-toxic at high concentrations. They stabilise protein structure while decreasing protein-solvent interactions during water deficit, repair damaged tissues and support growth (Ruan & Teixeira da Silva, 2011). Glucosinolates are nitrogen and sulphur rich metabolites synthesised from aliphatic amino acids e.g. methionine, valine and or leucine; aromatic amino acids phenylalanine or tyrosine and tryptophan, an indolic glucosinolate amino acid (Arbona, Manzi, Ollas, & Gómez-Cadenas, 2013). Biosynthesis is by cytochromes (CYP79F1/F2 and CYP79B2/B3) (Zandalinas, Vives-Peris, Gómez-Cadenas, & Arbona, 2012). Bound to the side chains of glucosinolate compounds are a hydroxyaminosulphate group and a β-thioglucosyl residue, which are dependent on the amino acid precursor. They have been linked to the plant's defence mechanisms against abiotic stress, i.e. drought. To function, the β-thioglycosidic bond on the β-thioglucosyl residue is cleaved by the enzyme myrosinase to give isothiocyanates, thiocyanates, and nitriles (Zandalinas et al., 2012), which become conjugated with intracellular glutathione (Keum, Jeong, & Kong, 2005) to give the biological activity of glucosinolates. In drought stressed *Arabidopsis thaliana* studies, CYP79 converts tryptophan to indole-3-acetaldoxime, a primary indolic glucosinolate precursor. During drought stress, plants induce osmolyte syntheses for turgor maintenance through osmotic adjustment (Arbona et al., 2013). Among the amino

acids, proline is responsible for approximately 50% of the osmotic adjustment, as seen in maize (Nishizawa, Yabuta, & Shigeoka, 2008). Metabolomic changes in *Arabidopsis* leaves under drought conditions show that proline, raffinose, γ-amino butyrate (GABA) and Krebs cycle intermediates accumulate in response to drought stress (Urano et al., 2009). Transcriptome analysis showed that GABA-dependent transcriptional regulation is responsible for the activation of branched chain amino acid, polyamine and proline biosynthesis. GABA shunt and saccharopin metabolic pathways are also activated when the plant undergoes dehydration (Skirycz et al., 2011).

Proline accumulation is integral for a cell's adaptation to hyperosmotic stress; it decreases water potential resulting in osmotic adjustment and maintaining of cell turgor. A proline accumulation in the roots of DT plants is detected in the early stages of drought and only synthesised and accumulate in the leaves at a later stage. Maritim et al., (2015) documented how water stress induces proline accumulation. The results showed that after four days of water stress the increase in proline content was insignificant but after day 12, a six to seven fold increase was observed in the drought stressed leaves. A large number of plant species accumulate proline in response to osmotic stress. Do Thu Hien in 2002, attributed proline accumulation to an increase in its biosynthesis, triggered by osmotic stress. Proline biosynthesis is activated under dehydration conditions with pyrroline-5-caboxylate synthetase (P5CS) as the target enzyme. Under drought stress its biosynthesis in the cytosol or chloroplast is from glutamic acid. The enzyme P5CS reduces it to glutamate-semialdehyde (GSA), which is then converted to pyrroline-5-carboxylate (P5C) and further reduced to proline by P5C reductase (P5CR) (Szabados & Savouré, 2010). Plants subjected to osmotic stress use glutamate pathway to ease stressful conditions (Delauney & Verma, 1993). Over the years, it has been proven that the ability to synthesise and accumulate proline is a trait found in DT varieties of plant species. To prove this, a DS citrus plant was transformed with the P5CS gene. The result was an increased ability for osmotic adjustment and subsequent tolerance to drought (Arbona, Flors, Jacas, García-Agustín, & Gómez-Cadenas, 2003) because of an increased biosynthesis of proline. In a different study, the resultant P5CS transgenic DS tobacco cultivars showed an increase in the

expression of proline. Upon comparison with the wild type they were found to have a better stress response to water deficits (Cvikrová, Gemperlová, Martincová, & Vanková, 2013). Alternative pathways responsible for proline up-regulation under drought stress include the pentose phosphate pathway. Proline biosynthesis also regulates cytosolic pH and $NADP^+$ synthesis, which are key in stimulating the pentose phosphate pathway (Hare & Cress, 1997). High levels of proline in tea samples are also a result of mitochondrial P5C produced by d-ornithine aminotransferase (d-OAT) enzyme transaminates ornithine to GSA and then P5C and later converted to proline. A study conducted by Miller et al., (2009) showed that expressing *Arabidopsis* d-OAT enhances proline levels resulting in an increase rice and tobacco drought stress tolerance. In a study conducted by Kumar and Yadav, (2008) entitled "Proline and betaine provide protection to antioxidant and methylglyoxal detoxification systems during cold stress in *C. sinensis* (L.) O. Kuntze", proline was documented to enhance glutathione-S-transferase and glutathione reductase (GR) activity during drought stress. A follow up study was conducted regarding the effects of proline and betaine on the glyoxalase pathway enzymes. Both showed protective effects on glyoxalase I and activating effects on glyoxalase II during water stress in tea buds. Levels of aspartic acid decrease under drought stress while there is an increase in proline levels, owing to the significant decrease in the activity of aspartate amino-transferase during stress imposition.

Considerable evidence indicates that drought stress also affects the metabolism of soluble carbohydrates, which have been shown to increase as a result of water stress (Sircelj, Tausz, Grill, & Batic, 2005). Glucose and fructose levels increase in apple trees subjected to drought conditions while starch levels decrease (Ayaz, Kadioglu, & Turgut, 2000). This suggests that both sugar alcohols and monosaccharaides play a key role in osmotic adjustment (Pandey, Agarwal, Jeevaratnam, & Sharma, 2004). The decrease in starch concentration can be attributed to the fact that drought stress reduces the rate of photosynthesis. Water stress has also been documented to inhibit the photosynthetic rate in grapevines (Rodríguez-Pérez, Riaño, Carlisle, Ustin, & Smart, 2007). The reduction in the rate of photosynthesis as a result of stomata closure due to an accumulation of ABA restricts CO_2 intake resulting in a reduction in the plant's capacity

to synthesise starch and sucrose. Carbon dioxide assimilation impairment due to stress affects metabolite concentration in plant tissue. This has adverse effects on the regulatory networks dependent on said metabolites (Krasensky & Jonak, 2012; Valerio et al., 2011). Carbohydrate metabolism is linked to photosynthesis, making it pivotal in the stress tolerance. Monosaccharaides such as glucose and fructose represent 38% (w/w) and sucrose 62% (w/w) of the total soluble sugars (daily average) found in watered plants, and 53% (w/w) and 47% (w/w) respectively in drought subjected plants (Rodrigues et al., 1993).

Sucrose is a major transport sugar in many plants, which accumulates under stress conditions (Rolland, Baena-Gonzalez, & Sheen, 2006). Sucrose is the second most abundant sugar in DT Selaginella species (Yobi et al., 2012). Sucrose and raffinose form intracellular glasses, which protect the vegetative tissues from damage by replacing water molecules, which prevents membrane fusion during drying (Sakurai et al., 2008). As drought exposure is prolonged, a reduction in the abundance of the two sugars occurs because they are increasingly being converted into protective sugars (Farrant, Lehner, Cooper, & Wiswedel, 2009). Ferns and angiosperms with DT traits have been documented to amass sugars such as raffinose, uninvolved with energy provision in the presence of stress (Oliver et al., 2011). Such metabolites have been documented to reduce oxidative damage to cell membranes and ROS scavenging (Arbona et al., 2013). Davies et al., (1999) documented mannitol as a drought tolerance marker when mannitol dehydrogenase gene (mtlD) was introduced into wheat resulting in a substantial increase in drought stress tolerance. In a separate study, an mtlD gene obtained from *E. coli* was used in the transformation of drought prone sorghum, resulting in the enhancement of tolerance to both water deficit and salinity (Maheswari et al., 2010). *Arabidopsis* trehalose-6-phosphate synthase 1(AtTPS1) plays a role in sugar and ABA signalling, enhancing drought tolerance (Kolukisaoglu, Weinl, Blazevic, Batistic, & Kudla, 2004). Over expression of this gene in rice results in increased production of glucose and fructose, improving drought stress response (Ge et al., 2008). This is a clear indication that the modulation of enzymes involved in the biosynthesis of osmoprotectants confers drought tolerance on otherwise susceptible plants. Plants

subjected to drought stress have been shown to accumulate phenolic compounds, while decreasing in total plant biomass. A water deficit results in an increased biosynthesis of secondary metabolites by reallocating the carbon assimilated through photosynthesis, which is progressively reduced. The chloroplasts' exposure to excess excitation energy and the increased ROS formation results in stomatal closure. This causes a reduction in CO_2 uptake. *C. sinensis* is rich in polyphenols. The increased synthesis of polyphenols serves to scavenge ROS (de Abreu & Mazzafera, 2005).

Current drought tolerance assessment

Recurring droughts and future climate change necessitate the selection of DT tea cultivars for a sustainable tea industry. The current method for drought tolerance assessment in *Camellia sinensis* is visual assessment of leaf wilting and scoring on a five-point scale. This is done under field conditions, during natural droughts that occur every 3-7 years. This method is subjective and poorly reproducible due to environmental conditions. An accurate and reproducible method is required to help tea breeders classify new cultivars as DT or DS. The new method should be independent of natural droughts and subjective evaluations.

Factory withering

Withering is the first stage in a tea factory. Freshly harvested tea leaves are placed in withering troughs and air is pumped through the leaves to remove moisture. The fresh tea leaf has a moisture content of about 80% RWC. This must be reduced to 70% RWC, for the second process, called rolling, in the tea factory. This process takes about 12-24 hours, depending on the weather and the tea cultivar. Discussions with tea factory managers revealed that the DT cultivars withered slower than DS cultivars in the tea factory. Although slow withering cultivars are the bane of tea factory managers during wet years, they are the darlings of tea estate managers during the dry years. This inspired us to develop a short-time withering method and objective measurement of RWC, as a surrogate marker for calculating the probability of drought tolerance of new tea cultivars. This method is based on leaf RWC by mass balance as described

below. The modulation of leaf metabolites (amino acids, sugars and flavonoids) between wet and drought conditions, has been determined in several plant species (as described above), but never in tea. Thus, modulation of tea leaf metabolites will be measured to validate the new method. We anticipate that the metabolite changes found in other plant species, under prolonged drought conditions, will occur in plucked tea shoots during the new short-time withering method.

Phytometabolomics

Phytometabolomics is the science that deals with metabolic profiling of plants, qualitatively and quantitatively analysing metabolites to determine the metabolic profile of the cells or organs. This profile is then used to understand metabolic responses to biotic and abiotic stress conditions (Schauer & Fernie, 2006). Metabolite profiling was first used diagnostically in determining the mode of action of several herbicides. Since then metabolomics has been used in ascertaining differences between genetically altered and conventional crops as well as genotypically classifying them to discover novel genes (Hagel & Facchini, 2008). Metabolomics also characterises metabolic responses to both biotic and abiotic stresses and identifies genetic determinants of biochemical composition (Hall, Brouwer, & Fitzgerald, 2008). Understanding the dehydration response metabolome aids in determining the final steps in signal transduction pathways (Urano et al., 2009). A typical metabolomics workflow is shown below in Figure 6.

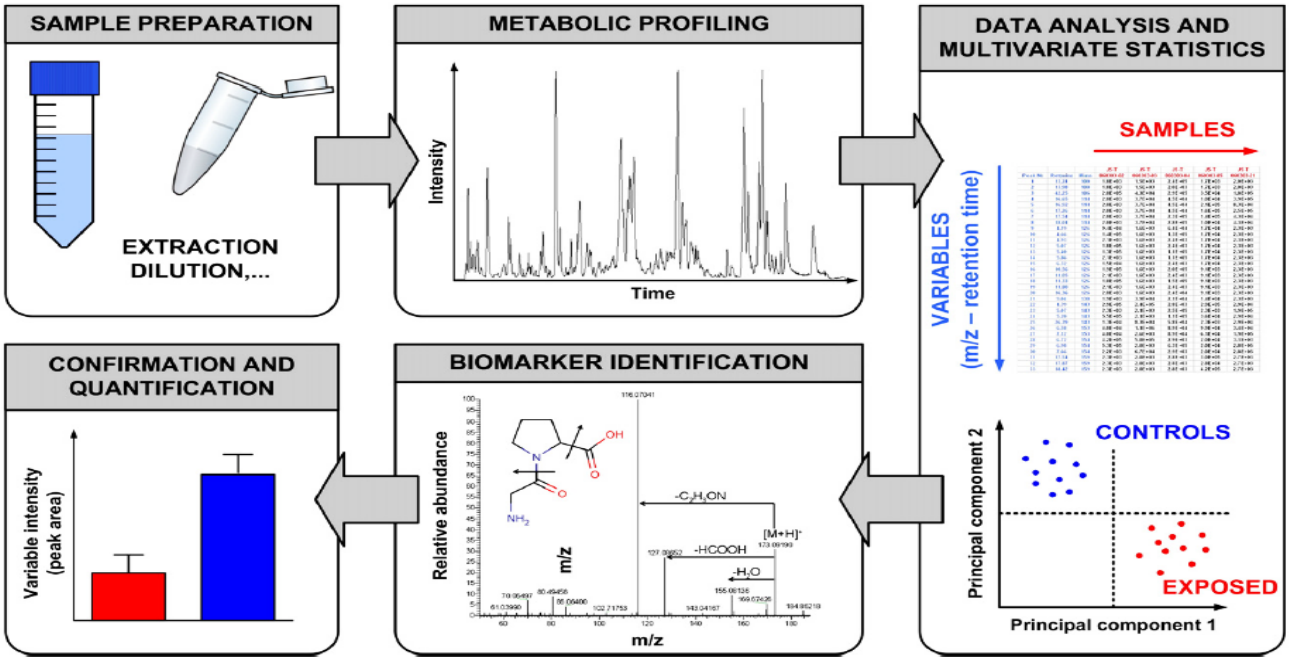


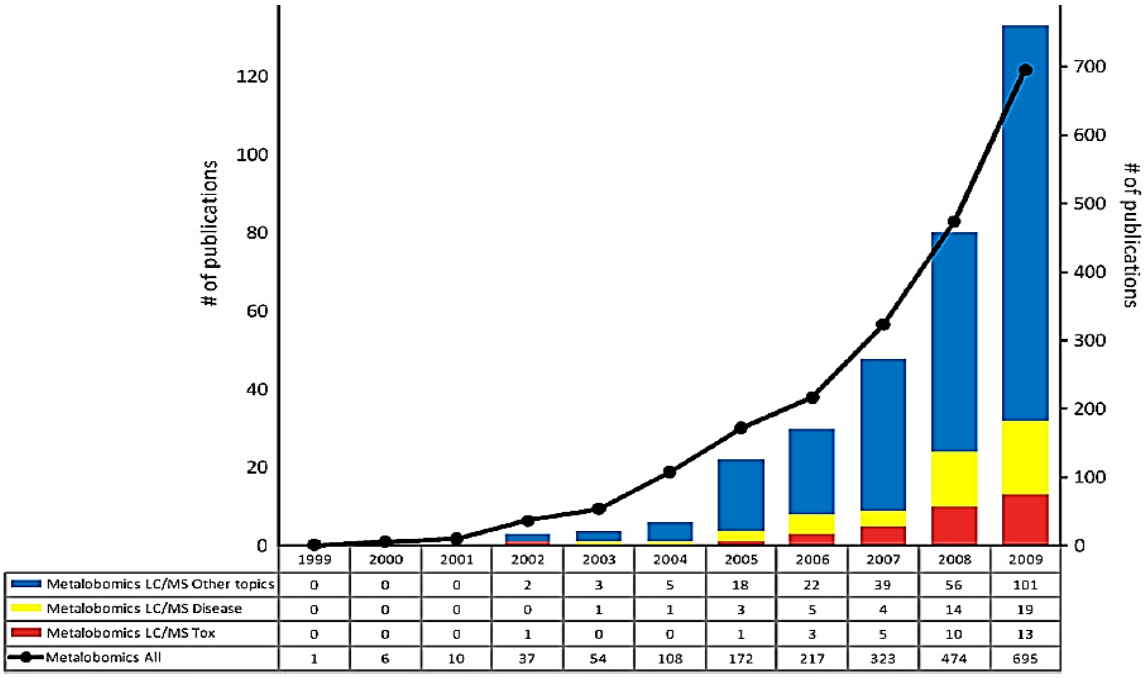
Figure 6: A metabolomics workflow showing the different steps involved while conducting metabolomics studies from sample preparation to the confirmation and quantification of analytes.

The key to metabolomic studies is the use of analytic tools for the comprehensive analyses of metabolites. Holistic metabolic profiles from complex samples, e.g. biological fluids or tissue extracts are obtained using spectroscopic technologies, which are powerful, high resolution, information-rich analytical methods. Mass spectrometry coupled to liquid chromatography (LC-MS), because of its advances within the field, has become a fundamental part of metabolomic research (Theodoridis, Gika, Want, & Wilson, 2012) and is increasingly being used in differential profiling to explain observed phenotypes and identify biomarkers (Khan & Mukhtar, 2007). Figure 7 shows the increased use of LC-MS in metabolomics over the past ten years. Currently, nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR), and direct injection mass spectrometry (DIMS), are employed as metabolomics techniques in plant studies, though NMR and MS are the predominate strategies. Gas chromatography (GC)-MS is also a technique that is utilised in plant metabolomics for the analysis of terpenoids (F. Chen, Duran, Blount, Sumner, & Dixon, 2003) and other volatiles (Tikunov et al., 2005). At first, GC was the only separation technique that could be

linked to MS. The problem with GC however, was that it was restricted to a small set of biological molecules namely the volatile compounds. This meant that biological molecules with a higher molecular weight such as proteins were excluded. LC-MS has enjoyed a growth in popularity over the years, as an instrument for metabolomic studies. This is because of its high throughput, soft ionisation, and vast coverage of metabolites (Zhou, Xiao, Tuli, & Ressom, 2012).

The introduction of LC combined with MS, exhibiting high sensitivity, dynamic range and versatility was the solution to the problem (Roux, Lison, Junot, & Heilier, 2011). However, LC-MS has proven best suited to the targeted profiling of plant compounds displaying similar ionising behaviour e.g. alkaloids (Halket et al., 2005). Metabolic analyses can be categorised into targeted and untargeted. In the targeted approach, the objective is to identify and quantify selected metabolites i.e. enzyme substrates or reaction products, metabolites involved in a metabolic pathway. When employing a targeted approach, the chemical properties of the compounds under investigation are known. Sample preparation for analysis is in such a way that there is minimum interference with the sample by the techniques employed. A targeted approach is usually hypothesis-driven, whereas untargeted analysis results in the generation of a new hypothesis involving the measuring of all the metabolites within a biological system (Zhou et al., 2012). By combining LC-MS and GC-MS data, a better and more complete picture of a plant metabolome is obtained. Due to recent plant metabolomic technique developments, it is now possible to, in a non-targeted manner, rapidly measure hundreds of metabolites allowing for the detection of a wide range of metabolites, which will in turn give a much broader insight into the chemical composition of samples, e.g. tea (Fraser et al., 2012). LC-MS-based metabolomics is dependent on multiple experimental, analytical, and computational steps (Zhou et al., 2012). When LC–MS is employed for analysis it generates mass spectral peak lists, which when aligned with respective samples and linked with multivariate statistics, results in the identification of spectral features. This enables the differentiation between samples, i.e. DT and susceptible cultivars. Non-targeted analyses on *Camellia sinensis* include work done by Fukusaki in 2008, employing LC-MS to predict tea quality based on sample metabolite composition.

Seeing as no single technique is adequate for detecting and identifying all metabolites, a combination of techniques is required to encompass most of them. A hybrid of chromatography, MS and NMR has been proven to be the best metabolite profiling technique (Ward, Baker, & Beale, 2007). UPLC provides narrower peaks indicating an increase in peak capacity and increased sensitivity. UPLC is therefore superior to LC and is increasingly being used due to its higher stability chromatographic system and separation supremacy (Lenz & Wilson, 2007). It is because of this that 20% of UPLC applications are related to metabolomics. This is documented in UPLC–ToF-MS metabolomic analyses of human urine (Gika, Theodoridis, & Wilson, 2008) and blood serum (Zelena et al., 2009) and plasma (Michopoulos, Lai, Gika, Theodoridis, & Wilson, 2009). Metabolomics-based studies are increasingly employing UPLC, exact mass MS, and MarkerLynx Software data processing for multivariate statistical analysis. The UPLC-MS system combines the benefits of UPLC and high resolution exact mass MS in the rapid generation and interpretation of information-rich data, enabling rapid and knowledgeable decision making. Both systems will now be looked at in depth.



	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Metalobomics LC/MS Other topics	0	0	0	2	3	5	18	22	39	56	101
Metalobomics LC/MS Disease	0	0	0	0	1	1	3	5	4	14	19
Metalobomics LC/MS Tox	0	0	0	1	0	0	1	3	5	10	13
Metalobomics All	1	6	10	37	54	108	172	217	323	474	695

Figure 7: Number of publications from various fields where LC-MS was used in metabolomics from 1999 to 2009 (left axis) versus all metabolomics related publications (right axis). This shows how over the years LC-MS has become a popular technique (Roux et al., 2010).

Gas chromatography

Literature studies show the increase in popularity for the use of GC-MS with quadrupole mass analyser, tandem mass spectrometry (MS–MS). GC has over the years been coupled to high resolution instruments, but during the last decade a growing interest in coupling it with time-of-flight (ToF) and Orbitrap mass analysers has been documented. This coupling as a result means a wider array of tasks such as pre- and post-target analysis, retrospective analysis, discovery of metabolite and transformation products, as well as non-targeted analysis can be performed (Hernández et al., 2012). GC-MS produces more accurate results as compared to LC-MS, especially for the analyses of plant extracts. The analysis of non-volatile compounds by GC-MS presupposes their conversion into volatile derivatives through chemical derivatisation. (Proestos, Sereli, & Komaitis, 2006). A demand for simultaneous analysis of non-volatiles such as amino acids and sugars, has led to the development of GC-MS techniques which employ derivatisation steps (Molnár-Perl, 1999), silylation being a favoured procedure. Silylation is a nucleophilic substitution reaction in which nucleophilic attack on the silicon atom of the silyl donor produces a bimolecular transition state. In the reaction, an active hydrogen from -OH, -COOH, -NH, or -SH is replaced by a trimethylsilyl group. By comparison, TMS derivatives are more volatile than their parent compounds (Proestos et al., 2006). Derivatisation of analytes is conducted prior to analysis. This ensures a reduction in their polarity, and effectively facilitates chromatographic separation on a low polarity column, characteristic of metabolic profiling. This technique is used in quantitative analysis as it provides for and offers an increased tolerance to fluctuations in experimental conditions.

Conventional GC-MS makes use of electron ionisation. The energy that responsible for inducing parent ion fragmentation to give the mass spectrum is kept at a constant 70 eV. This enables and ensures reproducibility, and as such, spectral libraries can be made commercially available for use by fellow investigators. (Halket et al., 2005). Mass spectral libraries and GC retention data are crucial in metabolomics work. As a result, GC-MS has become a recognised robust and widely employed technique, which combines high sensitivity and specificity (Niessen, 2001).

Ultra performance liquid chromatography (UPLC)

As alluded to previously, mass spectrometry is widely accepted as an analytical tool for both qualitative and quantitative analyses of metabolites. MS detection is significantly enhanced by UPLC. Unger et al., (2008) demonstrated that higher chromatographic efficiency, resulting from employing UPLC, translated into better resolution with higher peak capacity (number of peaks resolved per unit time). The use of UPLC in separation procedures has been shown to detect up to 20% more compounds as compared to HPLC, which was previously used (Unger, Skudas, & Schulte, 2008). A crucial parameter affecting the number of compounds being detected in chromatographic separations is the column length. Moreover, UPLC as compared to HPLC displays better retention time reproducibility and signal-to-noise ratios for samples, making it a more suitable technology for use in non-targeted metabolomics (Swartz, 2005).

The ACQUITY UPLC BEH (bridged ethylsiloxane hybrid) 1.7 µm C18 columns have been termed the universal columns of choice for UPLC separations. They provide the widest pH range by incorporating trifunctional ligand bonding chemistries, which give forth low pH stability (Swartz, 2005). The low and high pH stability that comes with the BEH particles provides a wide pH range. Before the use of BEH particles, silica based particles were employed as stationary phase particles in the HPLC as they had good mechanical strength. These, however, suffered from numerous disadvantages, the main one being pH range limitation. Polymeric columns were then introduced to circumvent this problem, but they too had their own issues, namely low efficiencies and limited capacities. To eliminate these problems and provide the enhanced mechanical stability required for UPLC, the BEH technology was developed. The use of sub-2 µm particles and mobile phases at higher linear velocities and operating at higher pressures than could be handled by the HPLC system allows for the use of flow rates of up to five ml/min. This also results in better resolution (separation efficiency), faster chromatography and an increase sensitivity, as a result of sharper (narrower) and higher peaks. The sensitivity increase in UPLC detection is 2–3 times higher than that observed in HPLC separations.

Mass spectrometry

Mass spectrometry has established itself as a useful tool for metabolomics analysis for its capability to measure compounds present at very low levels and at the same time provides structural information. Mass spectrometric analyses have an advantage over other techniques as they provide useful characterising information and the ability detect and measure a wide range of compounds, significantly increasing the amount of information that can be generated from a sample. MS can be defined as an analytical technique that separates ionized molecules by utilising the differences in their mass/charge ratio (m/z). This tool functions to quantify molecules and determine their molecular weights, also providing their chemical and structural data (Griffiths et al., 2010). A mass spectrometer consists of an ion source, a mass-selective analyser, and an ion detector (Steinmann & Ganzera, 2011). MS systems have over the years been coupled to chromatographic systems i.e. LC-MS. The molecules are charged up using one of two ways namely electron impact ionisation (EI) and chemical ionisation (CI) (Kind & Fiehn, 2010). In EI, the molecules are subjected to a “stream” of electrons fired at 70 electron volts (eV) within a high vacuum. It is here that these molecules become charged, producing a molecular ion and fragments (McLafferty & Turecek, 1997). CI, on the other hand, is a soft ionisation method which involves colliding sample molecules with charged “reagent” ions. This results in the transferring of charges to the target molecule (Munson & Field, 1966). Though this method brings about minimum sample fragmentation, it produces more intense molecular ions, enabling better molecular weight determination. The mass analysers employed in this study include the triple quadrupole (Q), and ToF.

Triple Quadrupole-MS

The triple quadrupole mass spectrometer (Figure 8) was among the first mass detector variations used in metabolomics research, in conjunction with LC (LC-MS) and GC (GC-MS) (Allwood & Goodacre, 2010). The triple quadrupole functions as a mass filter, permitting the passage of only a narrow range of mass ions to the detector at any one time (Hopfgartner et al., 2004). Three quadrupole cells are linearly oriented with respect

to one another as shown in Fig. 5B below. The 1st quadrupole is a single quadrupole analyser whose function is to filter ions or ranges of ions with a definite mass. The 2nd quadrupole functions as a collision cell, which permits the passage of a stream of inert collision gas e.g. Nitrogen, Argon, Helium through the trapped sample mass ion/ ions at a pressure of 10 Torr and collision energy of 30 eV. This collision between the ions and the inert gas results in the formation of ion fragments and this process is known as collision-induced decomposition. The 3rd and final quadrupole filters and detects the daughter ion fragments produced by collision-induced decomposition. The triple quadrupole mass analyser is regarded as a “tandem-MS in space analyser” as it represents a form of MS/MS making it ideal and well suited for targeted MS analysis. In addition to product ion monitoring, the triple quadrupole also has a precursor ion monitoring function in which the 2nd analyser selects a product ion (Hopfgartner et al., 2004). The 1st analyser scans all mass ions in a process known as neutral loss monitoring after which the 2nd analyser, based on the neutral mass of interest, is given an off set to identify mass ions that have lost their specific neutral moiety (Wen, Ma, Nelson, & Zhu, 2008). Also, selected reaction monitoring (SRM) is employed where both mass analysers scan for specific precursors and product mass ion transition pair. This improves the sensitivity over conventional product ion monitoring (Lisec, Schauer, Kopka, Willmitzer, & Fernie, 2006).

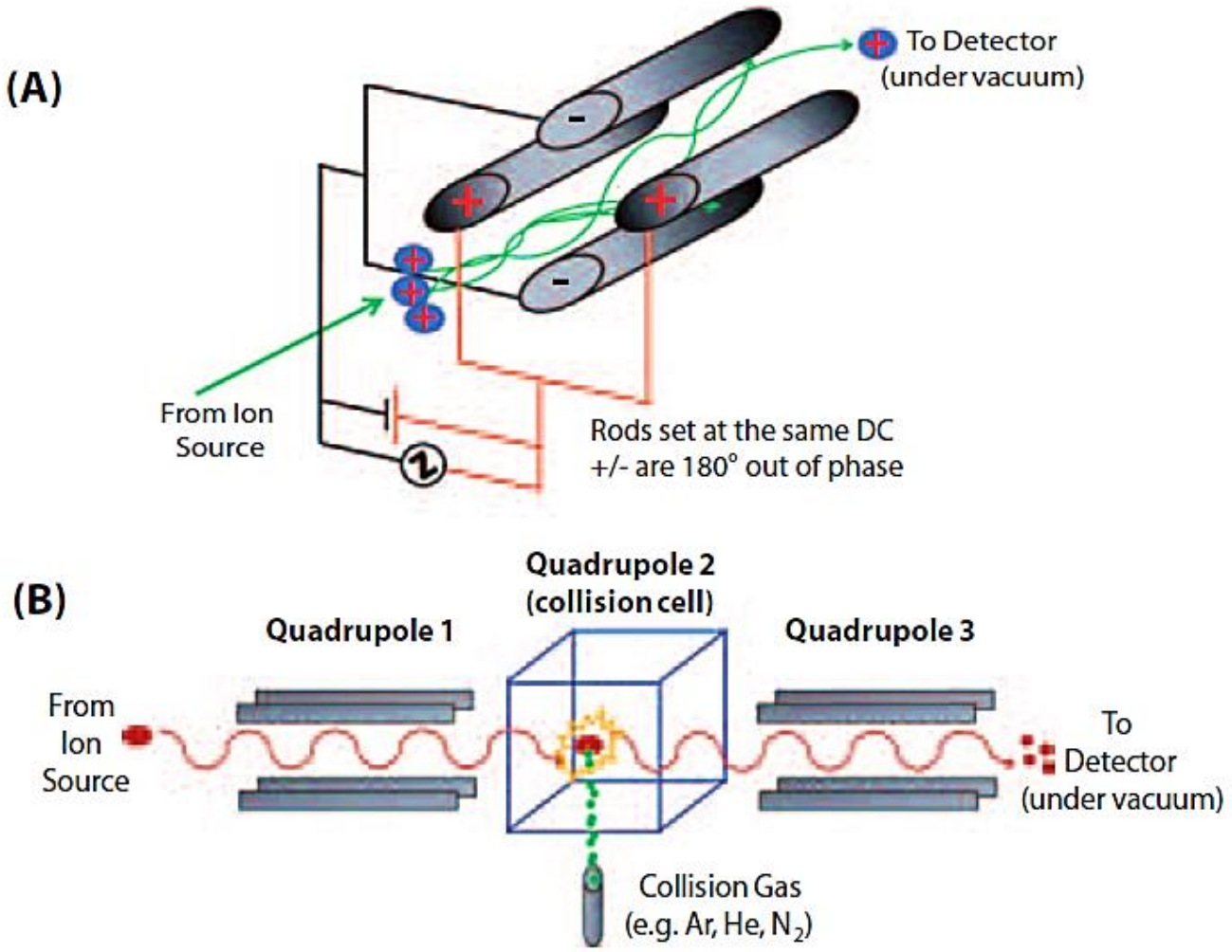


Figure 8: Instrument schematics to illustrate the quadrupole (A) and the triple quadrupole (QQQ) arrangement (B) (Allwood, Ellis, & Goodacre, 2008).

Time of flight-MS (tof)

Introduced 13 years ago on a commercial level, quadrupole–ToF mass spectrometers have rapidly become known as a powerful and robust instrument in the analytical community. These systems combine the high performance of time of-flight analysis with widely used techniques such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) (Mirivel, Riffault, & Galloo, 2010). One of the MS systems used in this project was the SYNAPT G2 system, which employs a new hybrid quadrupole ion mobility separation (IMS) orthogonal acceleration time of flight (oa-tof) MS. This provides a new level of high resolution exact MS performance. The system

makes use of innovative QuanTof technology, which functions by combining high field pusher and dual stage reflectron designs. These incorporate high transmission parallel wire grids, which bring about a reduction in ion turnaround times because of the pre-push kinetic energy generated and spread, this resulting in improved focusing of high energy ions respectively. Quantof delivers exact mass accuracy with high precision across LC peaks, providing high selectivity for the detection and identification of analytes in complex mixtures. The ion detection system employed combines an ultra-fast electron multiplier with a hybrid analog-to-digital conversion (ADC) detector to give increased sensitivity, resolution, exact mass measurement, accurate isotope rations, and comprehensive MS data. All this takes place at acquisition rates highly compatible with ACQUITY UPLC separations. This new tof system makes SYNAPT G2 ideal for metabolomics studies. Figure 9 below shows the components in the SYNAPT G2 system.

UPLC-MS

As already mentioned, MS is increasingly being used as an analytical tool for the qualitative and quantitative analyses of various types of compounds. MS detection is significantly improved by using UPLC. An increase in the peak concentrations is observed together with reduction in chromatographic dispersion at lower flow rates, promoting increased source ionisation efficiencies (Xu, Fan, Rieser, & El-Shourbagy, 2007). This enhanced resolution enables the MS data to be interpreted, since most of the MS peaks consist of single compounds. An investigation making use of UPLC-MS for analysing the metabolites as well as for metabolic profiling. The results obtained illustrate how the use of the UPLC gave extra resolution in both specificity and spectral quality. This also revealed new information which minimised the risk of failing to detect vital metabolites (Plumb et al., 2006). HPLC-MS was first employed in global metabolite profiling by van der Greef in 1981. This technique has thus been shown to provide complementary data to NMR spectroscopy, allowing for the detection and identification of biomarkers. MSE is another LC/MS data acquisition method, which utilises an intelligent approach in which parallel alternating scans are obtained through either low collision within a collision cell so as to get precursor ion data, or at high collisions to

obtain accurate mass fragment, precursor ion and neutral loss information. The UPLC-MSE technique promises to provide outstanding chromatographic and MS results to be used in structure elucidation of complex mixtures.

GC separation provides higher chromatographic resolution as compared to the best LC columns which at the time was HPLC. However, the introduction of sub 2 µm particles in the UPLC has brought about much improved chromatographic resolution for LC. The smaller particles provide more theoretical plates as compared to the 5 µm particles, providing better resolution (Nguyen, Guillarme, Rudaz, & Veuthey, 2006). Several studies have using LC-MS, documented a lot of compounds found in tea. Del Rio et al., (2004) published a highly cited paper where LC-MS was used to identify and quantify the phenolic compounds and purine alkaloids found in tea. Tea quality has in previous years been assessed by specialists evaluating the products quality on based on leaf appearance, taste and aroma of the brew. However, the use of analytical tools such as LC-MS to correlate the results obtained by the specialists is becoming more and more favoured. In another study UPLC-ToF-MS was employed in the comprehensive analysis of Japanese green tea with the intent of relating the obtained metabolite profile results to tea quality. The study showed that both high and low grade tea produced the same chromatographic peaks but differed in intensities of their components (Pongsuwan et al., 2008).

Evaporative Light Scattering Detector: Alternative method of detection (ELSD)

Evaporative light scattering detector (ELSD) in principle measures the amount of light scattered by particles after drying of the mobile phase through evaporation (Ganzera & Stuppner, 2005). There are three steps in the operation of ELS detectors, namely nebulisation, evaporation and detection. The 1st step involves nebulization of the mobile phase is into small droplets through the use of a nebulizing gas, either air or nitrogen. Following this, the droplets then pass through a heated drift tube, where the volatile components evaporate leaving particulate residues containing the analyte molecules. The particles are passed through the detection section, where they scatter light from a lamp, resulting in varying amounts of the light reaching a photo-detector. Three light

scattering mechanisms are known to occur depending on the ratio of the particle diameter (D) to the wavelength (λ) of the light source. Rayleigh scattering occurs when $D/\lambda < 0.1$, Mie scattering when $0.1 < D/\lambda < 10$ and reflection-refraction occurs when $D/\lambda > 10$ (Van der Meeren, Vanderdeelen, & Baert, 1992). These components are shown in Figure 9 below.

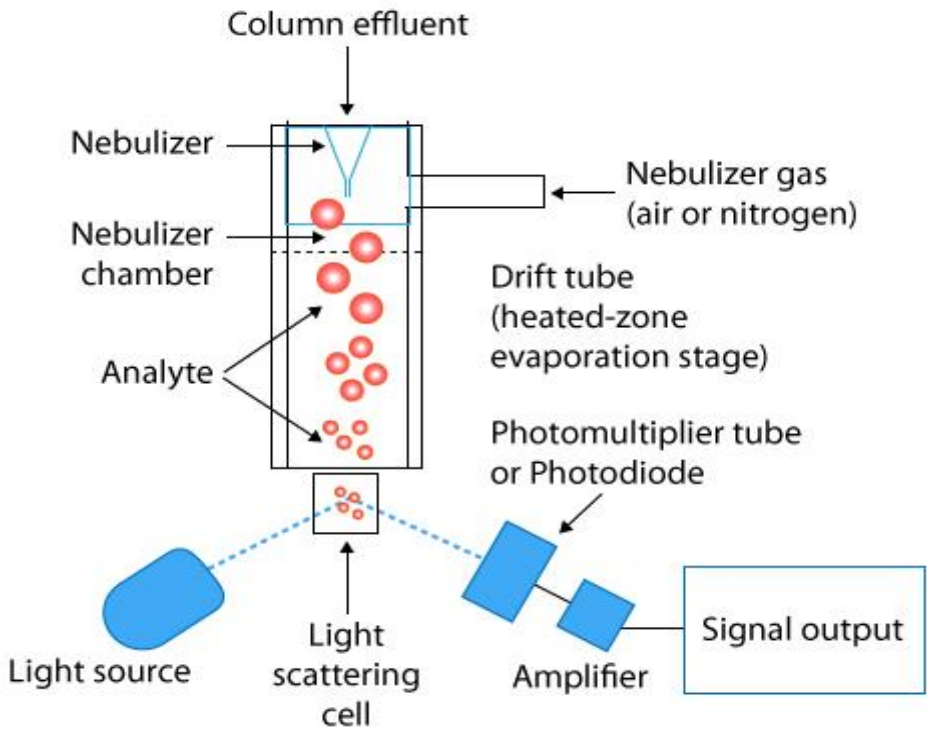


Figure 9: Foundation of ELSD.

ELSD offers a complementary mode of detection capable of detecting almost all the analytes eluted under either isocratic or gradient conditions. ELSD is sensitive enough for most applications, offering limits of detection as low as the hundreds of picograms on column (Ganzera & Stuppner, 2005). Over the last decade, ELSD has steadily evolved into a reliable, economic and versatile mode of detection, especially with carbohydrates, which have usually weak chromophores making them ideal targets. ELSD is increasingly being employed in LC–MS detection applications. The reason for this is that ELSD and MS detectors both have an evaporative stage. This makes the eluent used in MS compatible with ELSD. As a result of this commonality, ELS detectors can function as surrogate MS detectors in, for example, method development

activities, preventing the damage of the more expensive MS detectors. The resultant method can be used in MS detection. Water and acetonitrile are common mobile phase eluents used in gradient elution. A modifier (ethanolamine or ethylenediamine) is added to the mobile phase. This interacts with the silanol groups of the stationary phase resulting in a pH increase. The employment of PDA and ELSDs is documented as advantageous in that this detection method responds to all eluted compounds, both chromophoric and non-chromophoric.

Method development for LC

As alluded to previously, UPLC-MS has become a popular analytical technique for analysing metabolites. For effective UPLC-MS analysis, method development for UPLC is essential. This can be done using computer based software, which saves time, effort and money. Statistically speaking, the probability of adequately separating a complex mixture rapidly decreases with an increase in the number (n) of analytes. It has been documented that with $n > 20$, it becomes increasingly difficult to separate all analytes in a single chromatographic run. Where such a situation arises i.e. $n > 20$, it can be overcome by conducting multi-variable optimisation, which improves selectivity and band spacing, increasing the number of experiments (Dolan, Snyder, Djordjevic, Hill, & Waeghe, 1999). It is impractical to attempt separating a sample containing a large number of components whose bands are crowded together using a single chromatographic method. Separation is possible by employing an approach in which the 1^{st} separation of the sample is into fractions. Each fraction is then resolved by a 2^{nd} separation which offers different selectivity (Dolan, Snyder, Wolcott, et al., 1999) . Another option involves column-switching to obtain similar results, without any required manual intervention (Opiteck, Ramirez, Jorgenson, & Moseley III, 1998). The employment of capillary electro-chromatography (CEC) for the separation of complex samples is also an option, which results in an increase in column efficiency (N) and peak capacity (Beltran, Ferrer, & Guiteras, 1998). Separating a sample two or more times while varying chromatographic conditions may result in the separation of each analyte component of the sample. Total sample analyses is then obtained by combining results from the two separations.

A reduction in the time required to understand the analyte composition of a mixture results in an increase in the generation of results. Over the last 30 years, scientists have been developing chromatography modelling software packages for calculating resolution and capacity factors to visually modelling chromatograms to test peak movements with varying conditions. It is for this reason that DryLab, a computer based simulation software package which enables the prediction of isocratic separation from two gradient runs varying only $t_{G,}$ was developed. This computerised chromatography method development began in 1985 with the introduction of the IBM PC, the first "Personal Computer". In 1988, the first version of the DryLab software was developed, allowing modelling of band spreading, during optimisation of isocratic %B solvent. Subsequent years later saw the introduction of isocratic multi-parameter software, "DryLab Imp", which allowed the user to make changes in pH, temperature (*T)*, ionic strength, ternary eluent composition and ion-pair chromatography and gradient elution, which proved more difficult to model with other factors. This as a result led to the development of the 2-dimensional modelling of time-gradient (t_G) and *T*, the "t_G-*T*-model". Conducting a Design of Experiments (DoE) by running e.g. a t_G-*T* model with four runs, the resultant chromatograms will differ, which thus enables the chromatographer gain insight on how the analyte peaks move. To understand peak movements resultant from changes in experimental parameters, the chromatographer should keep all variables constant except one i.e. %B, t_G or pH. By doing so this helps with the understanding of how separation changes. Figure 10 below explains the principles of DoE.

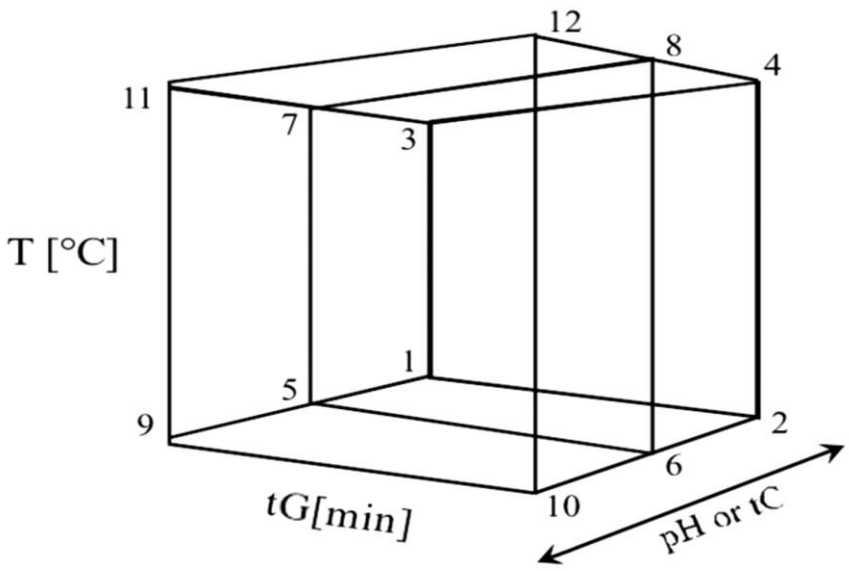


Figure 10: DoE simultaneously optimises t_G, *T* and pH or ternary composition (tC) of the eluent. The pH is changed by altering the ratios of the two aqueous eluents. The short t_G are at points 1, 5, 9, 3, 7 and 11, while the long t_G are at 2, 6, 10, 4, 8, and 12. The low *T* experiments are: 1, 2, 5, 6, 9 and 10, the high *T* runs are at 3, 4, 7, 8, 11 and 12. (Molnár et al., 2013).

To date, the most successful 2-dimensional model is the t_G-*T* model, especially when combined with ternary gradient elution technique (Euerby, Scannapieco, Rieger, & Molnar, 2006). In a study by Dolan et al., (1999), their focus was directed at better understanding the effects of varying column *T* and t_G between runs. They demonstrated how *T* and t_G could effectively be used as separation variables for separating samples with $n < 20$. The study also suggests that *T* and t_G are effectively similar for use in solvent optimisation for resolving complex samples. Reverse-phase LC separation is capable of being predicted as a function of T and t_G, column size and flow rate, where four experiments are 1st conducted at temperatures T1 and *T* and gradient times t_G1 and t_G2, with the column size, flow-rate and other conditions fixed (Zhu et al., 1996). In a follow up study Zhu et al., (1996) showed that a simultaneous variation of *T* and t_G results in a considerably significant change in the selectivity of the sample, where most samples had $n < 20$. The determination of the "best" values of T, t_G, and any other conditions can be made possible by utilising computer simulations, which are capable of providing resolution maps (*R*s) as a function of *T* and t_G. An example of such map is shown in Figure 11 below:

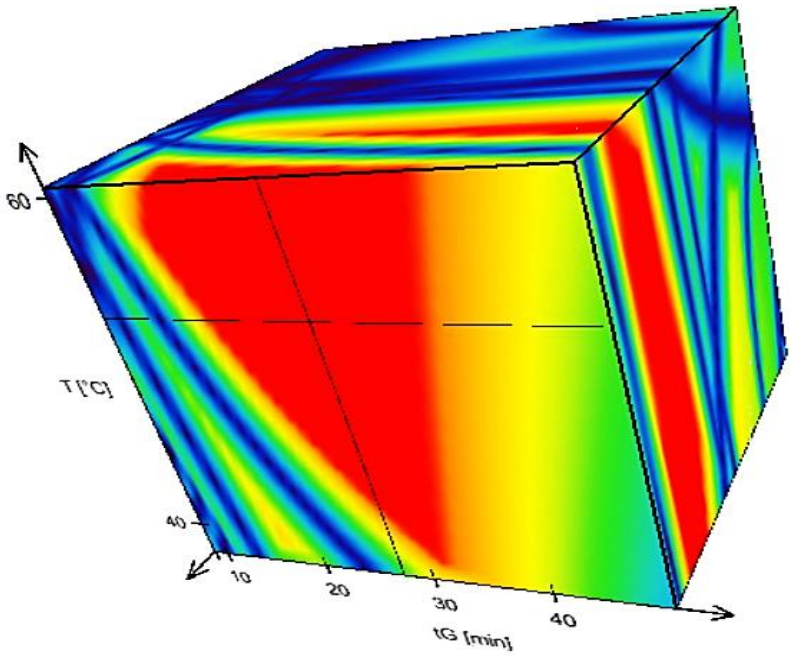


Figure 11: DryLab 4 Laboratory screen with 2-D and 3-D Resolution space. Red areas show baseline separation of all peaks (where the method is robust, i.e., Rs,crit > 1.5.) Blue areas represent peak overlaps. The cube assists by saving time and reducing the experimental work load required by determining the working points in advance.

Other factors such as column length, ID, particle size (dp), flow rate, dwell volume, gradient %Bstart and %Bend, and up to ten gradient steps can be calculated (Imre Molnár, Rieger, & Kormány, 2013). Sergej Galushko et al., (1996) developed similar software, which enabled the prediction of an analytes retention time based on its molecular structure. This software later found importance in drug design. The DryLab software is based on the “Solvophobic Theory”. The fundamental concept of this theory is based on the enforcement of water as a retarding component in the mobile phase, in retention on the reversed-phase column. As such the dissolution of any nonpolar compounds in the water would necessitate high amounts of energy; the capacity factor is proportional to the energy required (Imre Molnár et al., 2013). As such, a typical approach employed in RPC method development involves running a scouting gradient on a C18 column, resolving about 95% of the analyte peaks. Gradient elution begins with water or water-rich eluents. Following sample injection into the mobile phase, interaction between the water and the hydrophobic constituents of the sample result in

these analytes being forced onto the C18 column where interaction with silanol groups would occur. The capacity factors of such organic molecules are higher in water than in acetonitrile or methanol (Molnar, 2002). Increasing the %B, the organic eluent, results in a reduction of eluent surface tension as the force from water becomes weaker. Gradient elution allows for the precise calculation of analytes retention. Using just two gradient runs, it is possible to calculate isocratic conditions and determine how the k-values decrease as %B increases in the mobile phase. Retention time and the peak area measurements are the basis on which reversed-phase separations are modelled (I Molnár, Rieger, & Monks, 2010). The other scientific reasons behind the development of this tool are simply to develop a much more reliable method, which produces reliable results. Another reason, a more economical one, was to save time and money.

In South Africa, 2/10 tea plantations are currently financially viable, these being Ntingwe in KwaZulu-Natal (KZN) and Tshivhase in Limpopo. These estates are different from others in that they made use of new cultivars obtained from the Tea Research Foundation of Central Africa (TRFCA) in Malawi. The closure of the other eight plantations resulted in a loss of approximately 10,000 jobs. This was also detrimental to the economy as closure resulted in a drop in forex obtained from exporting tea to neighbouring countries. Several interventions are needed to resuscitate these estates and use of high quality high yielding cultivars is one of the potential solutions. This is where the current project intends to play a key role. Identification of DT cultivars will aid in the resuscitation of these estates which will in turn benefit tea producing countries, e.g. Zimbabwe, Malawi and Kenya. The employment of new techniques, i.e. DNA sequencing can aid in the selection of new cultivars that are DT, these having been identified by looking at their metabolite regulation. This has successfully been employed with rice, wheat and soya.

In this study, an efficient and sensitive method based on the above notion was developed and validated, using UPLC-MS for simultaneous identification and quantification of amino acids and carbohydrates in *Camellia sinensis* affected by drought stress.

Null Hypothesis

$H1_0$: There will be no statistically significant difference between the metabolite content of the DT and DS *Camellia sinensis* cultivars under wet conditions at the 95% level of confidence.

$H2_0$: There will be no statistically significant difference between the metabolite content of the DT and DS *Camellia sinensis* cultivars after the SWAPDT method at the 95% level of confidence.

Aims

- Identify as many metabolites as possible affected by drought in tea cultivars using UPLC-MS
- Establish global metabolic profile for both the DT and susceptible cultivars
- Determine the concentration of metabolites significantly affected by drought
- Linking affected metabolites to specific biochemical pathways

CHAPTER TWO (Materials and Methods)

Materials and methods

The Folin-Ciocalteu phenol reagent (Merck Chemicals, South Africa) was used for the determination of the total phenolic content. Gallic acid and anhydrous sodium carbonate with 99% purity were obtained from Sigma Aldrich (South Africa). Amino acid and carbohydrate standards were purchased from Sigma Aldrich (South Africa). The purity of each compound was > 98%. Acetonitrile and Methanol were UPLC-grade from Merck (Darmstadt, Germany). Other reagent solutions, such as ammonium formate and formic acid, were of analytical grade. Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Deionized water (H_2O) was purified by a purification system (ELGA PURELAB Ultra, Labotec). Minisart® RC4 Syringe Filters (Sartorious). Mettler Toledo analytical balance from Microcep (South Africa). Microplate Reader Thermo Multiskan Ascent. Waters Acquity UPLC system with a quaternary pump solvent management system, an online degasser, and an autosampler. A Waters Synapt G2 quadrupole ToF mass spectrometer (Milford, MA, USA), fitted with a Waters Acquity UPLC and photo-diode array detector (PDA). Trace 1300 (Thermo scientific) coupled to a Thermo TSQ 8000 GC-MS/MS with TriLusRSH autosampler and a nonpolar (95% dimethylpolysiloxane) capillary column Restek –Rxi ®-5Sil MS w/Intrega-Guard ® (15 m, 0.25 mm ID, 0.25 µm film thickness) was used.

Five biological replicates from each of the two DT cultivars (PC168 and PC153) and five biological repeats each from the two DS cultivars (PC105 and PC165) developed at the Tea Research Foundation for Central Africa in Malawi grown in pots under shade net at the University of Pretoria experimental farm in Hatfield, Pretoria and four DT cultivars (SFS 150, TN 14-3, 301/4 and 303/577) and four DS cultivars (AHP S15/10, TRFK 371/8, SC12/28 and Ejulu) grown at the Tea Research Institute in Kenya were used in this study.

Evaporative Light Scattering Detection (ELSD) and Dry lab optimisation

The detection of analytes was preliminarily conducted using ELSD and UV-Vis detector before employing MS to determine carbohydrate and amino acid concentrations. The ELSD operating parameters such as the flow rate of nebulizing gas, drift tube

temperature and pressure, in pounds per square inch (PSI), were optimized to improve the sensitivity of the detector and the resultant signal-to-noise ratio. Firstly, a design of experiment (DoE), which simultaneously optimises operating parameters, was conducted using JMP Pro 11 software package. DoE was employed because important factors were known, allowing the use of response surface experiments (RSE). The objective of RSE is to generate a predictive model of the relationship between factors and the response. This in turn would enable determination of operating conditions before conducting wet lab experiments. After inputting factors and obtaining prediction variance profile, UPLC-ELSD was set up. The chromatographic runs were performed at drift tube (DT) temperatures of 30, 40, 50, 60 and 100 °C. The optimum temperature was selected based on the limit of detection (LOD) of the threitol analyte, which is the least abundant metabolite of interest in drought stricken plants. It is recommended that DT temperature is set to the lowest temperature yielding an acceptable low-noise baseline response, revealing all analytes of interest. The nebuliser (Neb) was set at 30, 45, 60 and 100 %. Lastly, pressure was set at 20, 30, 40 and 50 psi. The resultant areas obtained were inputted into the JMP software, and a response surface plot obtained enabling the determination of the best conditions of DT and Neb temperatures and PSI.

Polyphenol extraction and content determination

Before extractions, fresh leaves from each cultivar growing under a shade net were microwave dried for five min, which in the process deactivated the oxidizing enzyme polyphenol oxidase. A coffee grinder was used to grind the dried leaves and sieved through a 355 µm stainless-steel sieve and stored stored at 4 °C in plastic zip-lock bags prior to extracting polyphenols. International Organization for Standardization (ISO) extraction method was used as is described in the ISO document 14502-1: 2005. Briefly, 0.200 ± 0.001 g of each sample was weighed out and transferred into a glass extraction tube. A five ml volume of 70:30 methanol: water (v/v) at 70 °C was added to each extraction tube, stoppered and vortex mixed for approximately five seconds before placing into a water bath set at 70°C. The extraction mixture was vortex mixed after five min and again at ten min when tubes were removed from water bath. After cooling at

room temperature with the stopper off for an additional five min, the extracts were centrifuged at 2000 X g for ten min, with the resultant supernatant decanted into a ten ml measuring cylinder. The extraction step was repeated twice. Both extracts were pooled, and the volume adjusted to ten ml with cold 70:30 methanol: water (v/v).

A volume of one ml of the extract was diluted with water to 100 ml. The total polyphenol content (TPC) was determined according to ISO 14502-1: 2005 procedure, with gallic acid as standard. From the 1:100 ml extract sample dilution, a one ml volume was transferred in duplicate into separate glass tubes. Five ml of a 1/10 dilution of Folin-Ciocalteu reagent in water was pipetted into each tube and mixed. After five min, four ml of sodium carbonate solution (7.5% w/v) was added to each tube, stoppered and mixed before being allowed to stand at room temperature for 60 min. The absorbance was measured at 765 nm against water. Gallic acid standards were used for quantification and the results were expressed as % Gallic acid equivalents in g/100 g dry weight plant material. The gallic acid standard curve which was linear from 10 to 50 µg/ml in the assay was used to measure the polyphenol content in each of the samples. TPC, expressed as a % (w/w) by mass on a sample dry matter basis, is given by the formula:

$$\%TPC = \frac{(OD_{sample} - OD_{intercept}) \times V \times d \times 100}{Slope_{std} \times M_{sample} \times 10\,000 \times DM}$$

where OD_{sample} is optical density obtained for the sample, $OD_{intercept}$ is optical density at the point the best fit linear regression line intercept the y-axis (c), $Slope_{std}$ is slope obtained from best fit linear regression (m), M_{sample} is mass of sample (mg), V is extraction volume (ml), d is dilution factor used prior to the colorimetric determination (one ml to 100 ml = 100X), DM is the dry matter content expressed as a mass fraction of test sample and 10 000 is a dilution factor.

The SWAPDT method

The rate of RWC loss between the DT and DS cultivars was evaluated as described by Yobi et al., (2012) in a comparative metabolic profiling study between DT and DS *Selaginella* species. Three shoots with two leaves and a bud from a single bush of each of the cultivars were immersed in 20 ml of distilled water at room temperature and weighed hourly for five hours until the leaves reached constant weight. The hydrated (turgid) leaves were then removed from respective solutions, blot dried to remove surface water and weighed (t = 0). After the initial weighing, the leaves were oven dried at 37°C and weighed at 60 min intervals for five hours, until their RWC was approximately 50% (based on prior explorative experiments). The leaves were again placed in water, with the leaves above water and petiole in the water (Figure 12), and left for 24 hours with the weights noted hourly for the first five hours. The leaves were weighed after 24 hours and oven dried at 105°C for 24 hours to obtain each leaf's dry weight. The % RWC 0...5 hours was then calculated using the formula:

$$\% \text{ RWC } 0...5 = (F_{wt} - D_{wt})/(FT_{wt} - D_{wt}) * 100$$

where F_{wt} 0...5 is the hourly weight while drying at 37°C for five hours, D_{wt} is the dry weight after 24 hours in 105°C oven and FT_{wt} is the weight after 24 hour rehydration. The final % RWC was normalised with respect to the first value, making all values relative to t = 0. The % RWCs at t = 0 and t = 5 were chosen for the comparative metabolite composition study between the two types of cultivars. Figure 13 shows a diagrammatic representation of the experimental procedure.

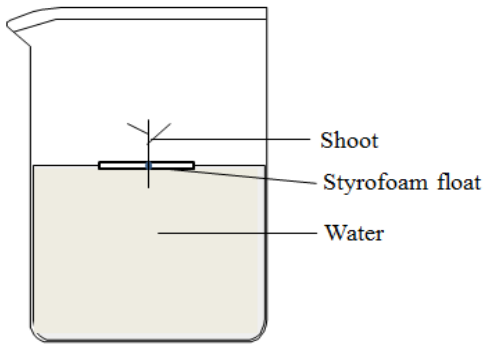


Figure 12: Shows the placement of the shoot in water; the petiole is submerged in distilled water.

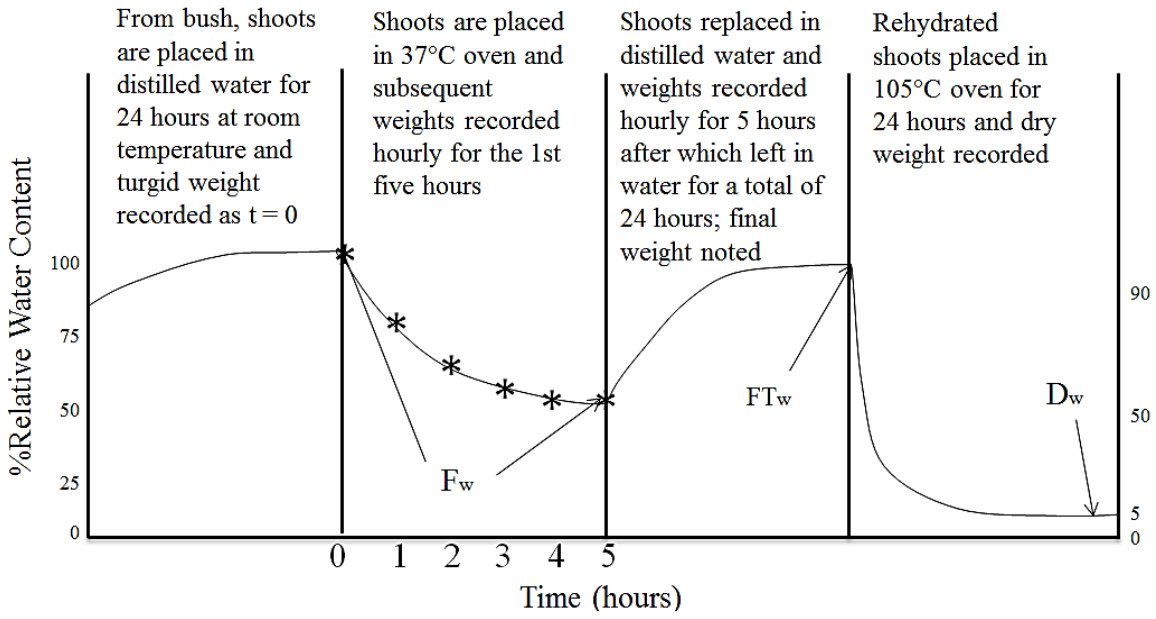


Figure 13: Diagrammatic representation of the experimental procedure.

Chromatography

To practice the interpretation of MS chromatograms and identification of analytes, a cocktail of metabolites found in *C.sinensis* and drug standards was prepared and analysed. The separation was carried out on a reverse phase Luna 5 µm C18 column (250 x 4.6 mm) from Phenomenex® (Torrance, CA, USA), protected by a Phenomenex® SecurityGuard™ guard column containing C18 cartridges. The mobile phase consisted of acetonitrile/acetic acid/water. The flow rate was one ml/min with column temperature set at 40 °C and an injection volume of 50 µl/ sample. Neochlorogenic acid (NCHA) (*m/z 354*) will be used as an example to show how the spectra were used to identify each of the compounds based on their fragmentation peaks. Below is a typical Retro-Diels-Alder (RDA) fragmentation reaction of the non-variable portion of the catechin ring (Figure 14). This is a chemical reaction between a conjugated diene and a substituted alkene, giving rise to a cyclohexene system. Selective ion monitoring was used to detect specific fragment ions. Using MassLynx, the fragmentation patterns were obtained for each compound and from these, we were able to identify each. Analysis was conducted on both positive and negative ion modes.

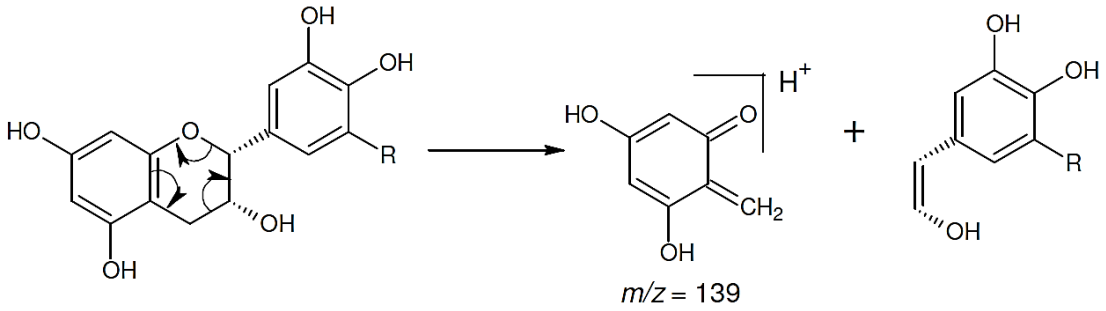


Figure 14: The Retro-Diels-Alder fragmentation of the non-variable portion of the catechin ring (Kraka, Wu, & Cremer, 2003). A characteristic m/z 139 product ion is observed when performing mass spectrometry under positive ion mode for non-gallated catechins. This fragment ion is also observed as a minor fragment component of mono-gallated catechins.

The resultant chromatograms obtained were then interpreted to identify the standards in the cocktail. Once this learning phase was complete two working stock solutions were prepared. One consisted of amino acids (valine, leucine, isoleucine, glycine, glutamic acid, aspartic acid, asparagine, phenylalanine, proline and lysine) and the carbohydrates (glucose, fructose, mannitol and citrate) standards dissolved in 50:50 methanol: water (v/v) at one mg/ml. The second cocktail consisted of phenolic compounds (trans-cinnamic acid, vanillic acid, coumaric acid, gallic acid, caffeic acid and protocatechuic acid) at one mg/ml in 50:50 methanol: water (v/v). These functioned as internal standards for both GC-MS and LC-MS analysis. An injection volume of one µl was used to give a standard final concentration of one ug/injection. The standard solutions were filtered through a 0.2 µm Minisart® RC4 syringe filters with hydrophilic, solvent-resistant regenerated cellulose membranes prior to injection. All extracts, stock and internal standard solutions were stored at 4°C.

Targeted metabolomics

GC-MS sample preparation and analysis

Fresh tea leaves were picked from the twenty individual tea plants at 06h00, placed in labelled plastic bags and kept on ice. These samples were couriered overnight to the Central Analytical Facility of the University of Stellenbosch where the GC-MS and LC-MS analysis were done as described below.

The fresh leaves were ground to a fine powder in liquid nitrogen. The powder samples were then weighed and extracted with one ml 70:30 methanol:water (v/v). After extraction, 100 µl of ribitol was added as an internal standard after which the samples were kept overnight at 70°C. The overnight mixture was allowed to cool before centrifugation at 17 000g for five min. Two hundred microliters of the extracts was transferred into a clean Eppendorf tube and dried on a Savant DNA 110 Speed vac. The concentrator of the speed vac setting was switched on with the drying rate set at low. The low drying rate was used to preserve the metabolites, preventing amino acids break down. The dried extracts were reconstituted in 100µl (2.5%) methoxyamine hydrochloride in 50:50 pyridine: acetonitrile (v/v) and left for two hours at 40°C. Fifty µl of BSTFA with 1% TMCS was added and the mixture derivatised at 60°C for 30 min. The samples were then cooled and vortexed for a few seconds before being transferred into a GC vial with an insert. A Trace 1300 coupled to a Thermo TSQ 8000 GC-MS/MS (Thermo scientific) with a TriLusRSH autosampler and a non-polar (95% dimethylpolysiloxane) capillary column Restek –Rxi ®-5Sil MS w/Intrega-Guard ® (15 m, 0.25 mm ID, 0.25 µm film thickness) was used for targeted metabolite profiling. The initial oven temperature was maintained at 100°C for four min, and then ramped at 6°C/min to 180°C, held for two min and ramped at 15°C/min to 300°C and then held for five min. Helium was used as the carrier gas at a flow rate of one ml/min with the injector temperature maintained at 280°C, operated in a split less mode. The mass spectral data was recorded on a TSQ operated in a single ion monitoring mode. Both the ion source and transfer line temperatures were set at 240°C.

LC-MS analysis

The samples were ground to a fine powder and extracted as described for the GC analyses. An injection volume of three µl was used. A Waters Synapt G2 quadrupole ToF mass spectrometer, fitted with a Waters Acquity UPLC and PDA, was used for LC-MS analysis. Separation was achieved on a Waters Acquity HSS T3 column (1.8 µm, 2.1 x 150 mm). Solvent A consisted of water with 0.1% formic acid while solvent B was made up of 0.1% formic acid in acetonitrile. The gradient employed consisted of a flow rate 0.32 ml/min, starting with 0% B to 5.0% B over four min, then to 40% B over 11 min, followed by a linear gradient to 100% B over the next one min and kept constant for one min during column wash in 100% B. This was followed by re-equilibration to initial conditions over three min for a total runtime of 20 minutes. Electrospray ionisation was applied in the positive mode, with a capillary voltage of 2.5 kV, a cone voltage of 15 V, desolvation temperature of 275°C and desolvation gas (N_2) flow of 650 $L.h^{-1}$. The source temperature was set to 120°C. The rest of the MS settings were optimised for best sensitivity. Data was acquired in MSE mode, consisting of a scan using low collision energy of 6 eV and a scan using a collision energy ramp from 25 to 60 V. Sodium formate was used to calibrate the instrument and leucine encephalin was used for the lock spray for accurate mass determination. The PDA detector was set to scan over the range 230–700 nm. The raw LC-MS data was obtained from the Central Analytical Facility (CAF) on an external drive.

Data Processing and Statistical Analysis

All the data from the samples was 0 normalised and 1 standardised to minimise systematic variation within the data, before multivariate analysis. Data acquisition and processing was conducted using MassLynx 4.1 software. The raw data obtained from LC-MS was converted into Network Common Data Form (NetCDF) format using the Databridge software application manager from Waters Corp, Milford, MA. GC-MS results were analysed directly. SIMCA-P 14.0 (Umea, Sweden) and JMP Pro 12 software were used to conduct multivariate statistical analysis to identify key metabolites. JMP Pro 12 was used to perform one way analysis of variance (ANOVA). Both the Student's t-test, with the alpha level set to 0.05 and ANOVA were conducted to

determine the significance of the up or down regulation of each metabolite. High-dimensional and complex data sets are generated whenever metabolomic studies are conducted. The analysis and interpretation of such data sets proves impossible just by visual inspection or univariate statistical analysis. As a result, multivariate statistical data analysis mathematical modelling approaches, namely PCA was employed to enable accurate extraction and interpretation of large empirical data sets. Logistic regression models were developed for classifying *C. sinensis* cultivars into DT and DS categories, based on specific metabolites. The data was transformed to adjust for leaf weight and moisture content at five hours, after which a stepwise logistic regression was done. Two models were developed at t = 0 and t = 5 for different variables/metabolites. Due to the small number (20) metabolites, it was decided to use the two-variable model with the variables appearing frequently in other higher order models at the t = 0 and t = 5 levels.

CHAPTER THREE

(Results)

ELSD and Dry lab optimisation

The drift tube temperatures for ELSD are selected based on the LOD of the threitol analyte as well as the boiling point of the mobile phase used, ensuring minimal disintergration of the metabolite being analysed. This also rids the possibility of interference by unevaporated mobile phase during detection. The gain and drift tube temperature combinations enable the ideal nebuliser temperature (%) and nebuliser gas pressure (psi) to be determined. From this we established ideal conditions as DT of 100 °C, nebuliser at 100 % and pressure of 40 PSI as shown in Figure 15 below. However, when the cocktail of amino acid and carbohydrate standards was analysed, the ELSD detection method did not work as it was unable to detect and identify the analytes in the cocktail.

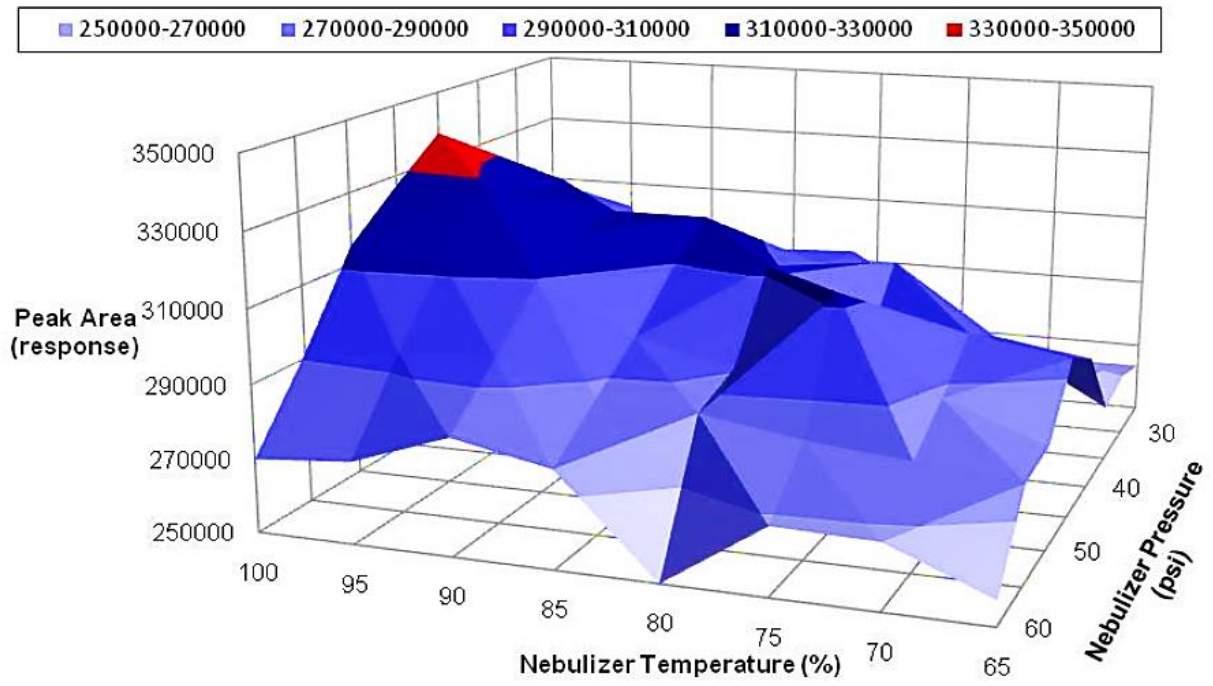


Figure 15: The optimum conditions for threitol detection. The ideal conditions were set at DT of 100 °C, nebuliser at 100 % and pressure of 40 PSI.

Chromatography optimisation

An optimisation approach for UPLC from an HPLC method was initially performed using a standard cocktail consisting of amino acids and carbohydrates to establish the ideal chromatographic separation within the constraints of a practical run time and the suitability of solvents for MS. Firstly, because the method being employed in the ISO method was specific for HPLC, the ACQUITY UPLC column calculator software was used to transfer the HPLC method into a UPLC method, calculating new column dimensions, flow rate, particle size and gradient conditions to enable the same chromatographic results on the new instrument. The mobile phase composition was optimised by investigating the effects of ammonium formate and formic acid and acetonitrile, with and without formic acid on metabolite retention times, resolution and peak shape. The resultant aqueous mobile phase employed contained ammonium formate while the organic mobile phase was acetonitrile containing formic acid. These generated lower pressure and faster column equilibrations, good peak shapes and resolution, enabling better separation and the detection of all standards in the cocktail. The ammonium formate and formic acid concentrations that were used were those that were also suitable for ESI so as to avoid corona discharge in the ion source. Also, green and black tea samples were prepared at varying dilutions to check for ion suppression effects.

MS detection was initially conducted using SYNAPT G2 system with a mobile phase containing triethylamine, which was specific for separating carbohydrates and amino acids on the BEH amide column. However, we later discovered that TEA was not compatible with MS analysis as it contaminated the system and proved difficult to flush out. Literature showed that this was a common error made by researchers working with MS as was addressed by a letter to the editor in chief of the journal “Rapid communications in mass spectrometry”: 14, 122–123 (2000). The letter states the problems that arose when mobile phase containing TEA was used for analysis of low molecular weight analytes within the range m/z 50–650 in the positive ion mode. TEA made analysis impossible by giving a very intense signal at m/z 102, [M + H]. In our

case, the exact same thing happened, and this problem wasn't only restricted to suppression of ionisation of the analytes in our sample but also severely increased detection limits. This was because the ion-trap which collects a limited number of ions and is incapable of excluding a single m/z species and as such was filled with only triethylammonium ions. This as a result hindered the detection of the compounds prepared at approximately one mg/ml. Further literature studies document that the use of TEA in analyses using ion-trap LC/MS must be refrained from. An alternative mobile phase must instead be used for analysing low molecular weight analytes in positive ion mode. This led to the use of the mobile system mentioned in previous chapter. The amide column used had been contaminated. Several cleaning cycles, which involved flushing of the column with 50:50 MeCN/H_2O for 72 hours commenced. The buffer pipes were replaced due to contamination. This, however, did not lead to a significant reduction in the intensity of the TEA signal. In the end, it was determined that the system has sustained severe contamination, and needed servicing. A new AQUITY BEH Amide column was purchased after which concomitant analysis was conducted on a Xevo triple quadrupole-MS system.

The MS system was then changed to a reverse phase Luna 5 µm C18 column (250 x 4.6 mm) from Phenomenex® (Torrance, CA, USA), protected by a Phenomenex® SecurityGuard™ guard column containing C18 cartridges. The mobile phase consisted of acetonitrile/acetic acid/water. The flow rate was one ml/min with column temperature set at 40 °C and an injection volume of 50 µl/ sample. The cocktail was analysed on this and the resultant chromatogram shown in figure 16 was obtained.

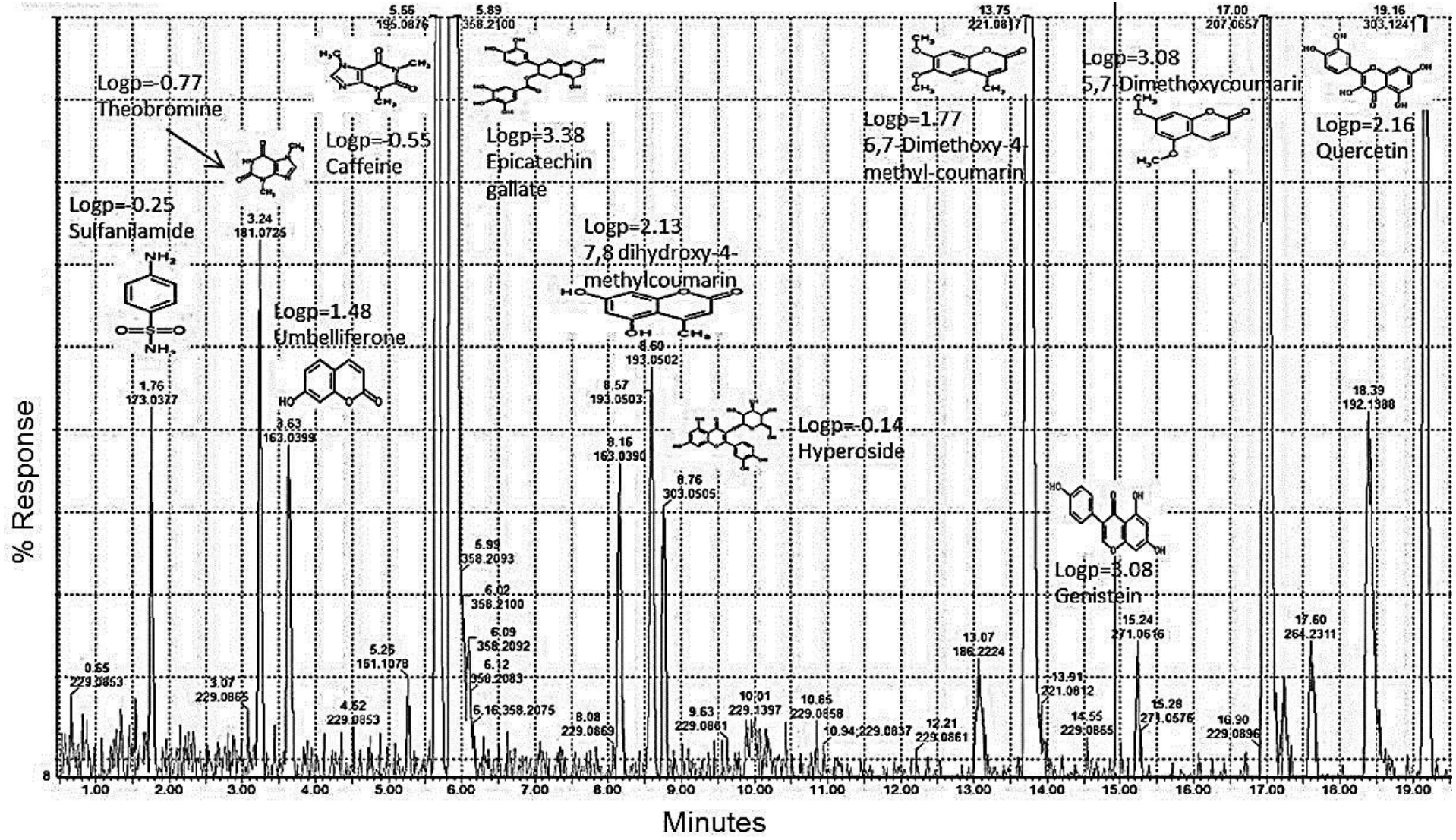


Figure 16: A UPLC-MS chromatogram of green tea metabolites and drug standards. The logP and structures of each metabolite are given along with their monoisotopic masses and retention times.

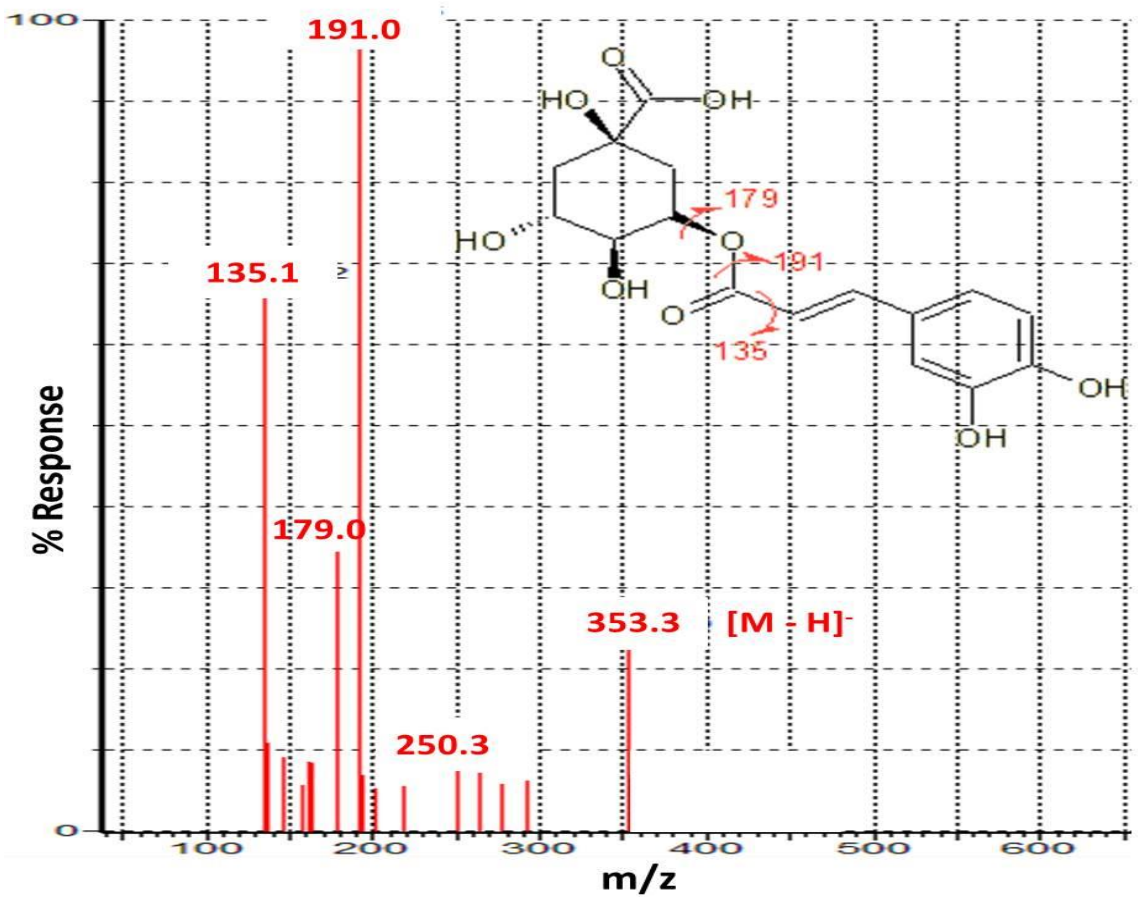


Figure 17: Fragmentation mass spectrum for NCHA ([M - H]$^-$ 353 m/z).

When looking at an MS chromatogram, the ion with the greatest m/z value is the molecular ion. However, some compounds have mass spectra lacking a molecular ion peak. The reason for this is that the molecular ion may have fragmentend and as such will not be detected. For example, in the mass spectrum of NCHA, the heaviest ion has an m/z value of 353. The fragment ions have m/z values of 135,179 and 191. From the structure of NCHA provided in Figure 17, the different bonds that are broken to give rise to the daughter ions are shown.

Short-term wither method

The sPC105 and sPC165 had a higher rate of dehydration than the DT tPC153 and tPC168 cultivars, with sPC165 losing more water than sPC105. After SWAPDT, the DT cultivars tPC153 and tPC168 had 75 and 65% RWC respectively, while sPC105 and sPC165 had 50 and 48% RWC respectively (Figure 18).

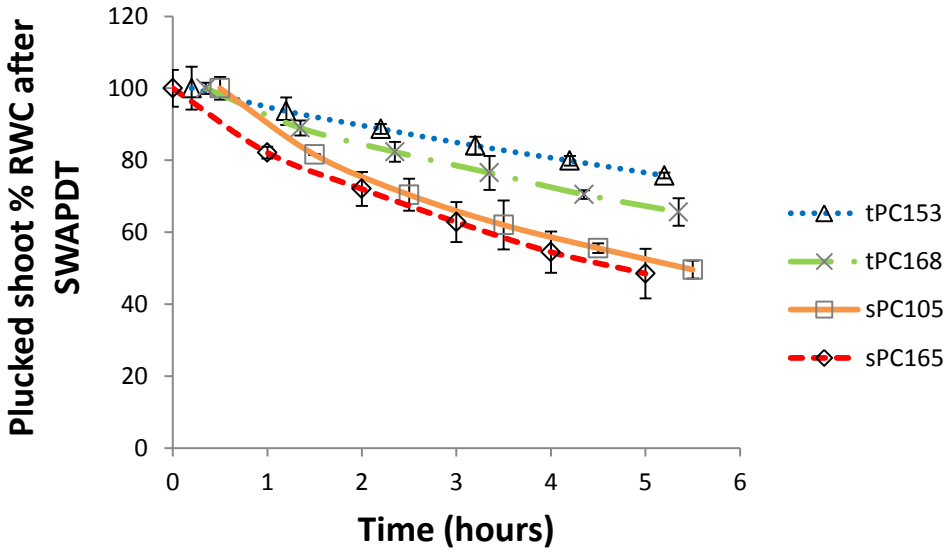


Figure 18: RWC (%) of *C. sinensis* DT (tPC168 and tPC153) and DS (sPC105 and sPC165) cultivars. The error bars are representative of S.E.M with n = 5.

Further work was done on four DT cultivars (SFS 150, TN 14-3, 301/4 and 303/577) and four DS cultivars (AHP S15/10, TRFK 371/8, SC12/28 and Ejulu) grown at the Tea Research Institute in Kenya to validate the SWAPDT method and those results are shown in Figure 19.

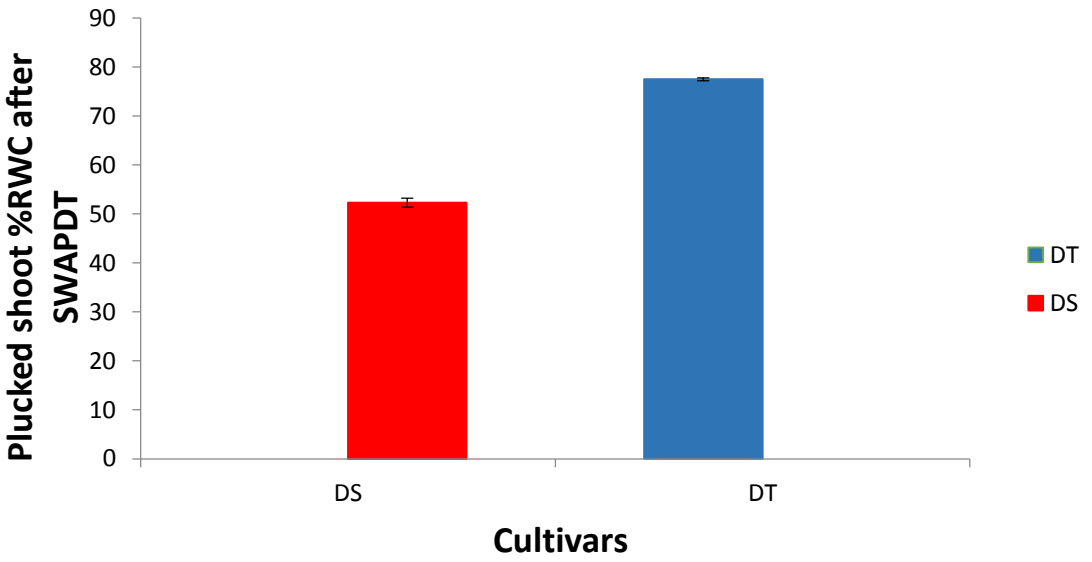
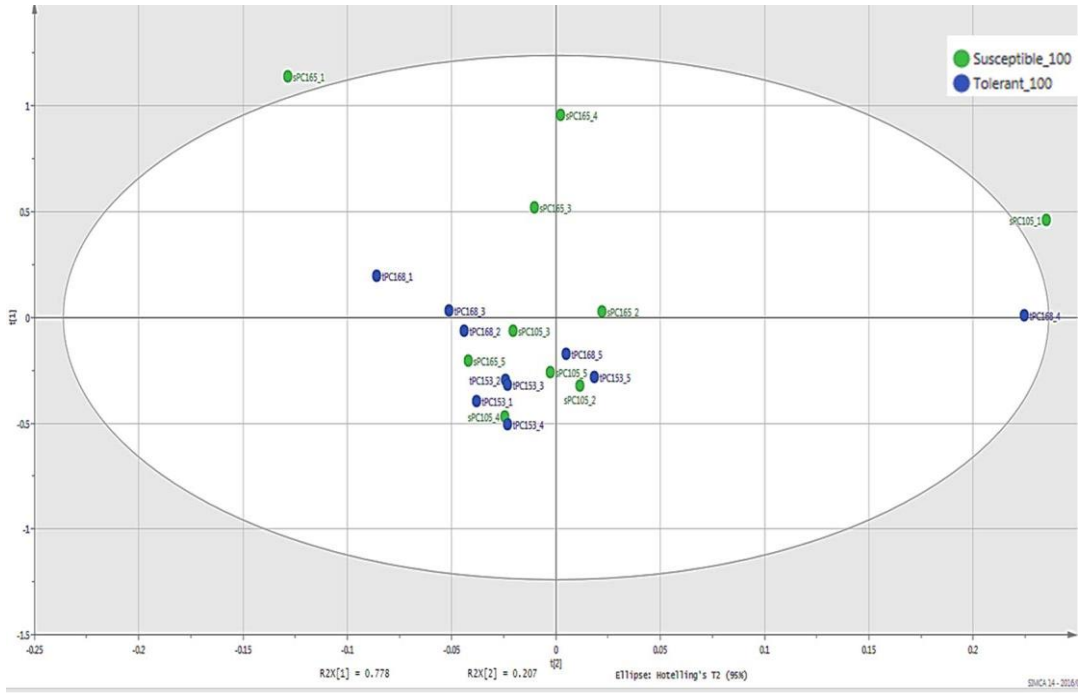


Figure 19: % RWC of plucked tea shoots of *C. sinensis* DS (AHP S15/10, TRFK 371/8, SC12/28 and Ejulu) and DT (SFS 150, TN 14-3, 301/4 and 303/577) after SWAPDT in cultivars classified as DS or DT after many years of field observations. The two groups are statistically significant with p = 0.0008. The error bars represent S.E.M with n = 4.

The metabolomic results confirmed that the changes in amino acids, flavonoids and carbohydrates, during this 5-hour wither in tea, are similar to the metabolomic changes found in other plant species, over longer times.

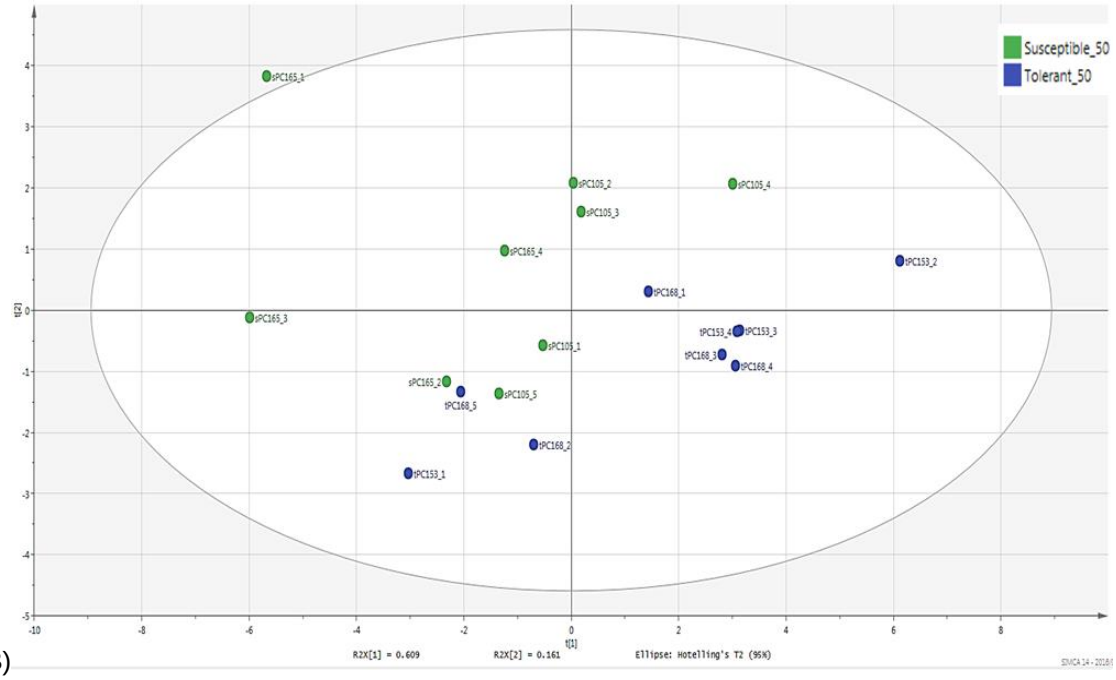
Data Processing and Statistical Analysis

From the 20 metabolites investigated, a few key metabolites were responsible for causing clustering between the tolerant and the susceptible cultivars. The trend observed (Figure 20) is the same that documented for other plants that have been exposed to drought stress over longer times (Engelbrecht & Kursar, 2003). The cross-validation results show that the model developed on the t = 5 data works equally well on the t = 0 and t = 5 data, namely 85% and 83% correct classification respectively. However, the model built on the t = 0 data did not work equally well on the t = 0 and t = 5 data, namely 65% and 83% correct classification respectively (Table 3). This means that targeted metabolomics of fresh leaves (t = 0) cannot be used to classify tea cultivars as DT or DS.



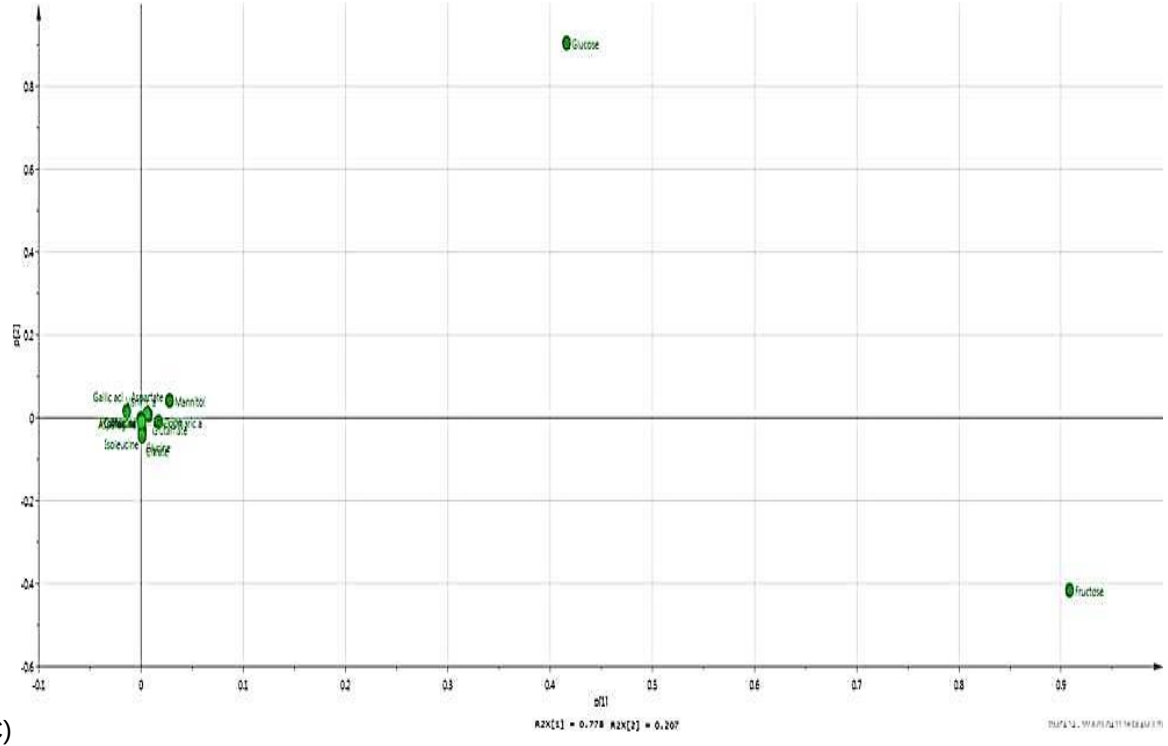
A)

Figure 20: A) Shows components one and two of a PCA clustering of *C. sinensis* cultivars at t = 0, with n = 5 for each cultivar. SIMCA-P 14 was used to obtain the plot.



B)

Figure 20: B) Shows components one and two of a PCA clustering of *C. sinensis* cultivars at t = 5, with n = 5 for each cultivar. SIMCA-P 14 was used to obtain the plot.



C)

Figure 20: C) Loadings plots showing the metabolites (Glucose and Fructose) responsible for the clustering of *C. sinensis* cultivars at t = 5 observed in the PCA scores plot along components one and two.

Table 1 below shows the variables used to create the logistic regression model at t = 0.

Table 1: Logistic regression model at t = 0 as a single model.

Regression Models Selected by Score Criterion at t = 0		
Number of Variables	Score Chi-Square	Variables Included in Model
1	8.9934	Glu
1	5.8461	Val
1	5.0152	Leu
2	12.4037	Val Glut
2	11.8386	Leu Glut

2	10.9995	Val Man
3	15.2223	Val Glut Man
3	14.9354	Leu Glut Man
3	14.7392	Val Glut Glu
4	16.3191	Leu Glyc Glut Glu
4	16.2382	Val Glut Prot Glu
4	16.2245	Leu Glut Prot Glu

Table 2 below shows the variables used to create the logistic regression model at t = 5.

Table 2: Logistic regression models at t = 5 as a single model.

Regression Models Selected by Score Criterion at t = 5		
Number of Variables	Score Chi-Square	Variables Included in Model
1	6.0708	Gal
1	4.9392	Asp
1	4.4514	Prot
2	11.6893	Val Asp
2	11.0604	Leu Asp
2	10.6539	Glut Asp
3	14.4470	Val Asp Citric
3	13.7405	Leu Asp Citric

3	12.7857	Val Asp TCin
4	15.3486	Val Asp Asn Van
4	15.1651	Val TCin Asn Prot
4	14.8718	Val Glyc TCin Asn

$$For\ the\ Model\ t = 0;$$

$$p = 1/(1 + e - (2192.90674170605\text{Val-}759.258878377219\text{Glut})$$

$$For\ the\ Model\ t = 5;$$

$$p = 1/(1 + e - (439.122693812145Val - 683.855963516112Asp)$$

Table 3: The cross-validation results showing how the developed model based on the t = 5 data works equally well on the t = 0 and t = 5 data (85% and 83%) but the model based on the t = 0 data did not work equally well (65% and 83%). These models show that targeted metabolomics of fresh leaves (t = 0) cannot be used to classify tea cultivars as DT or DS.

Model	t = 0	t = 5	Total
t = 5	15/18 = 83%	17/20 = 85%	32/38 = 84%
t = 0	15/18 = 83%	13/20 = 65%	28/38 = 74%

Targeted metabolomics

Amino acid metabolism

The amino acid data obtained from the GC-MS analysis showed that nine amino acids were detected from the ten that were analysed. Glycine, isoleucine and proline were significantly ($p < 0.05$) higher in the DT cultivars, after five hours at 37^{o}C than in the DS cultivars. Aspartic acid was significantly ($p < 0.004$) lower in the DT cultivars than the DS cultivars at this time (Figure 21).

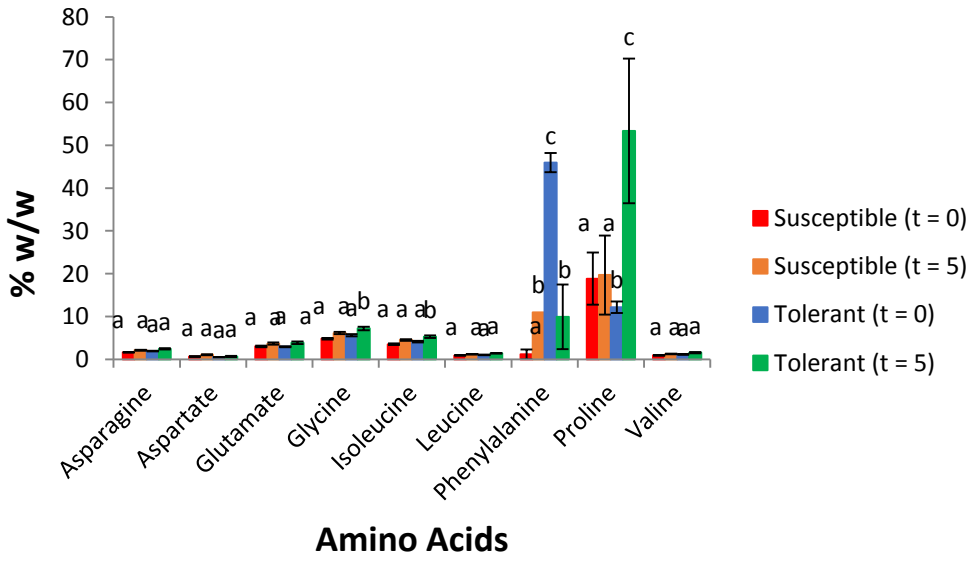


Figure 21: Differences in amino acid abundances between DT and DS at t = 0 and t = 5. The means are based on five independent growing plants of two DT or two DS cultivars. Error bars are representative of S.E.M. with n = 5.

Carbohydrates metabolism

Carbohydrates are among the most studied metabolites with regards to their accumulation in the vegetative tissues of plants in response to drought stress. Unlike what was observed with the amino acids, remarkable differences were apparent between the DT and DS cultivars. There was a significant down regulation of three carbohydrates noted in the DT cultivars as compared to the DS cultivars (Figure 22)., viz glucose, fructose and mannitol, whereas citrate showed a significant difference between DT and DS cultivars.

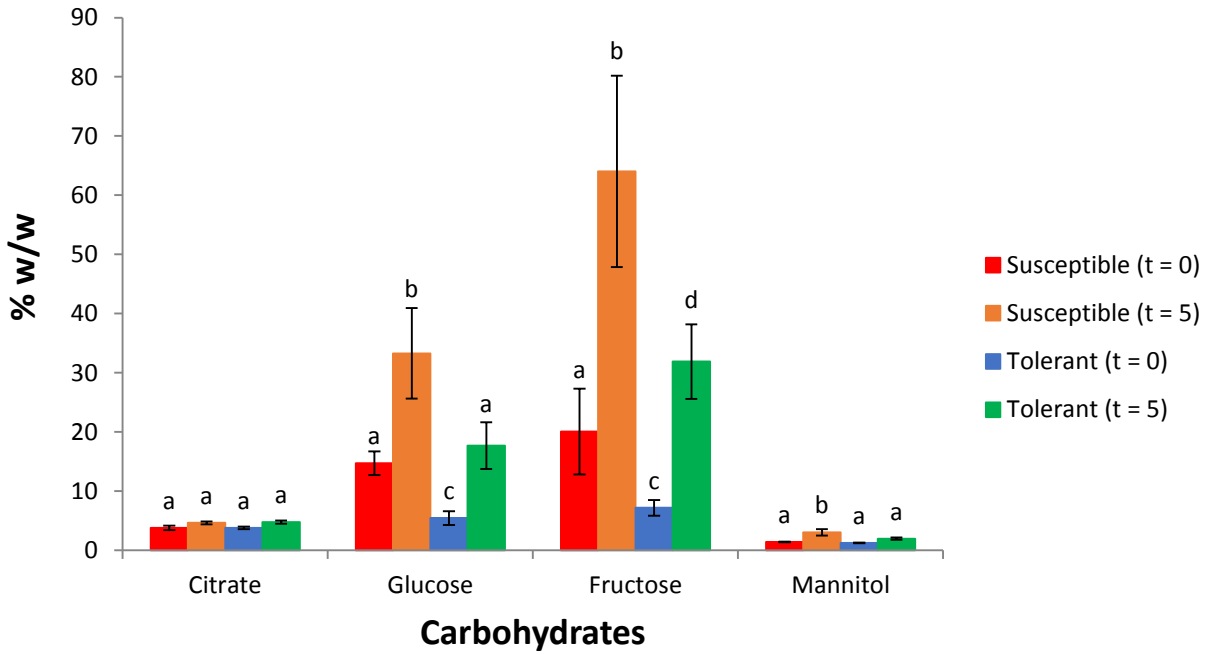


Figure 22: Differences in carbohydrate abundances between DT and DS at t = 0 and t = 5. The means are based on five independent growing plants of two DT or two DS cultivars. Error bars are representative of S.E.M. with n = 5.

Flavonoid metabolism

Protocatechuic acid, gallic acid and caffeic acid were significantly ($p > 0.05$) higher in the DT compared to the DS cultivars after five-hour wither. Coumaric acid, though not statistically significant, had a lower abundance in DT compared with DS cultivars after five-hour wither (Figure 23). These results indicate that the DT cultivars use flavonoids at a higher rate than DS, which enables them to cope with drought stress. Many plants use phenylpropanoids to respond to and mitigate stress through the shikimate pathway, which produces phenylalanine (Tounekti, Joubert, Hernández, & Munné-Bosch, 2013).

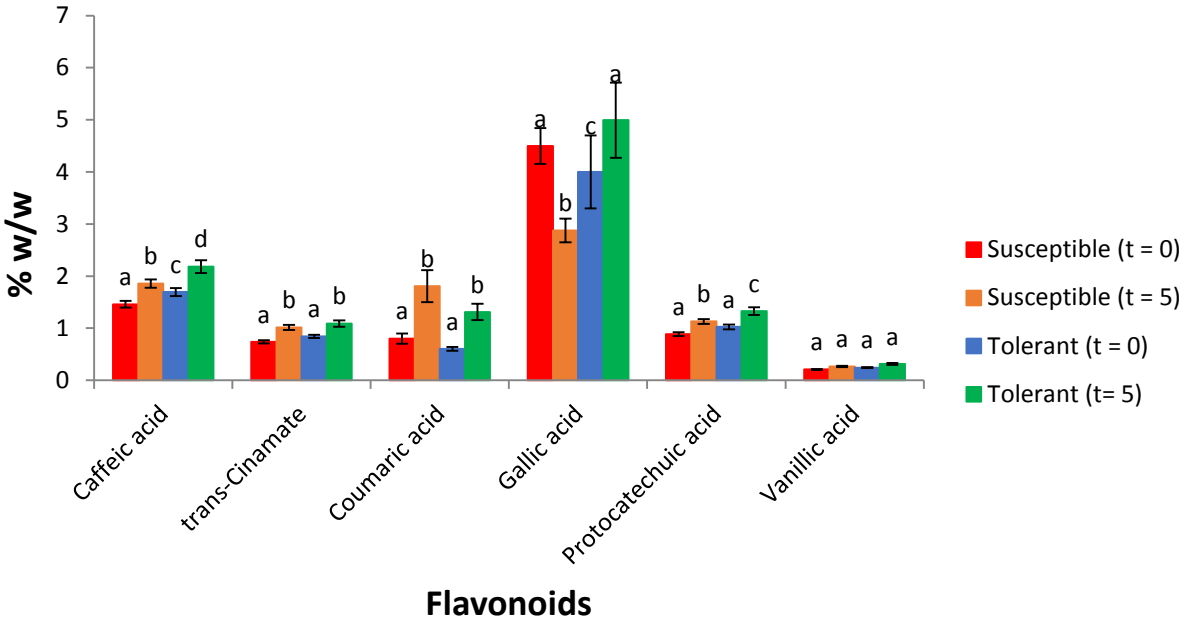


Figure 23: Differences in flavonoid abundances between DT and DS at t = 0 and t = 5. The means are based on five independent growing plants of two DT or two DS cultivars. Error bars are representative of S.E.M with n = 5.

Total polyphenol content (TPC)

Figure 24 shows the standard curve for gallic acid used to determine the TPC of cultivars.

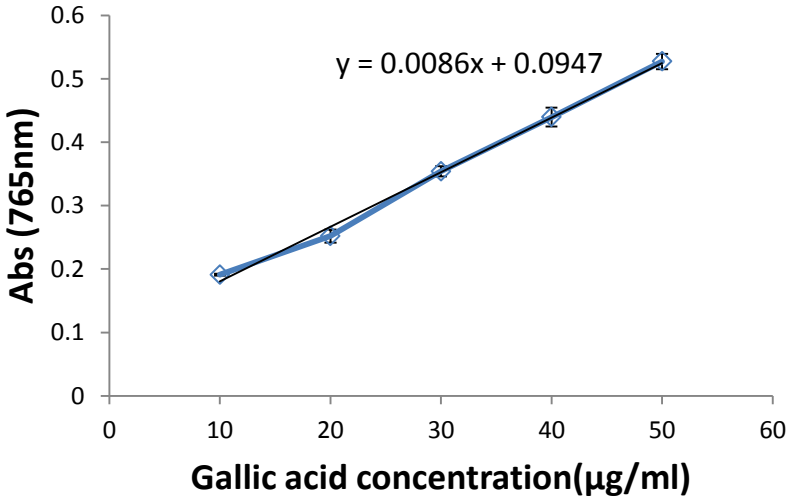


Figure 24: Gallic acid standard curve.

Table 4 below shows the weights of the cultivars used in the extraction process.

Table 4: Weight of leaf samples used for extraction.

Cultivar	Rep. 1	Rep. 2	Rep. 3	Average
PC105	0.2025	0.2053	0.2085	0.205433
PC165	0.2001	0.204	0.204	0.2027
PC153	0.2035	0.204	0.2043	0.203933
PC168	0.207	0.2064	0.2003	0.204567

Table 5 below shows the absorbance values of the cultivars used.

Table 5: Absorbance readings for different cultivars.

Cultivar	OD. 1	OD. 2	OD. 3	Avg. OD	SD	S.E.M.
PC105	0.370	0.336	0.387	0.364	0.026	0.015
PC165	0.301	0.316	0.341	0.319	0.021	0.011
PC153	0.314	0.254	0.259	0.276	0.033	0.019
PC168	0.357	0.364	0.292	0.338	0.039	0.023

The moisture content was calculated using the formula:

$$\% MC = [(W2-W3)*100/(W2-W1)]$$

where W1 is the weight of the empty tray, W2 is the weight of the tray + sample before drying and W3 is the weight of the tray + sample after drying.

Table 6 below shows the % DM of each of the cultivars used to calculate the % TPC.

Table 6: Shows the % DM of each of the cultivars. These values were used to calculate the % TPC.

Cultivar	W1	W2	W3	DM	%DM
PC105	45.75	47.76	47.69	3.4825	96.52
PC165	49.22	51.23	51.14	4.4776	95.52
PC153	49.44	51.45	51.38	3.4826	96.52
PC168	48.44	50.45	50.36	4.4776	95.52

Using the equation of the line y = 0.086x + 0.0947 obtained from the Gallic acid standard curve (Figure 24), the average TPC of PC105 was calculated and is shown below as an example:

$$\% \text{ TPC} = \frac{(OD_{sample} - OD_{intercept}) \times V \times d \times 100}{Slope_{std} \times M_{sample} \times 10\,000 \times DM}$$

$$= \frac{(0.364 - 0.0947) \times 10 \times 100 \times 100}{0.0086 \times 0.2054 \times 10\,000 \times 96.52}$$

$$= 15.8$$

The average %TPC for: PC165 = 13.5

PC153 = 10.7

PC168 = 14.5

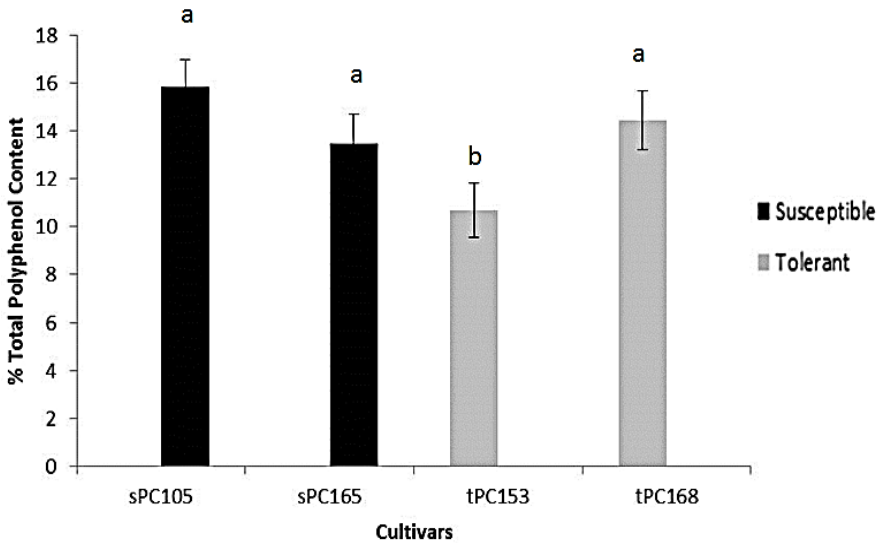


Figure 25: Differences in %TPC abundances between DT (tPC153 and tPC168) and DS (sPC105 and sPC165). The means are based on five independent growing plants of two DT or two DS cultivars. Error bars are representative of S.E.M. with n = 5.

RWC

The %RWC values (Table 7) plotted for the Malawi cultivars in the logistic regression plot (Figure 26) were obtained by averaging the means of three biological repeats, each with three technical repeats. The values plotted for the Kenyan cultivars were obtained by averaging the means of two biological repeats, each with two technical repeats. From this plot, logistics probability formulas were generated as shown below:

Cum [Susceptible] = 1/(1 + Exp (-356.927 + 5.535 * % RWC))

P (tolerant) = 1 - Cum [Susceptible]

where % RWC = the RWC at five hours after SWAPDT

Using this formula, the % RWC after five-hour withering, can be used to calculate a new cultivars probability for DT. The cultivars with P (tolerant) > 0.5 can be classified as DT. The closer P (tolerant) is to 1, the higher the probability that the cultivar will be DT. The probability of drought tolerance will be higher than 90%, when the RWC > 62% in the SWAPDT method. This method is objective, reproducible and practical because it is based only on a mass balance and a drying oven set at 37^{o}C.

Table 7: The different DT and DS cultivars and their resepective % RWC 5 hours after SWAPDT method.

Cultivar Name	Cultivar Type	% RWC 5 hours after SWAPDT
sEjulu	Susceptible	55.1
sEjulu	Susceptible	59.7
sPC105	Susceptible	30.7
sPC105	Susceptible	39.2
sPC105	Susceptible	43.2
sPC165	Susceptible	45.8
sPC105	Susceptible	49.4
sPC165	Susceptible	50.9
sPC165	Susceptible	53.5
sPC165	Susceptible	55.3
sPC165	Susceptible	55.8
sPC105	Susceptible	59.9
sTRFK 6/8	Susceptible	55.1
sTRFK 6/8	Susceptible	57.9
sAHP S15/10	Susceptible	51.3
sAHP S15/10	Susceptible	54.7
sSC12/28	Susceptible	41.8
sSC12/28	Susceptible	43.2
tSFS 150	Tolerant	72.4
t303/577	Tolerant	74.7
tSFS 150	Tolerant	74.6
t303/577	Tolerant	77.1
tPC168	Tolerant	56.3
tPC168	Tolerant	56.5
tPC168	Tolerant	62.1
tPC168	Tolerant	62.4
tPC153	Tolerant	63.1
tPC168	Tolerant	64.2
tPC153	Tolerant	69.3
tPC153	Tolerant	69.1
tPC153	Tolerant	72.2
tPC153	Tolerant	76.6
t301/4	Tolerant	77.2
t301/4	Tolerant	82.7
tTN 14-3	Tolerant	79.1
tTN 14-3	Tolerant	82.6

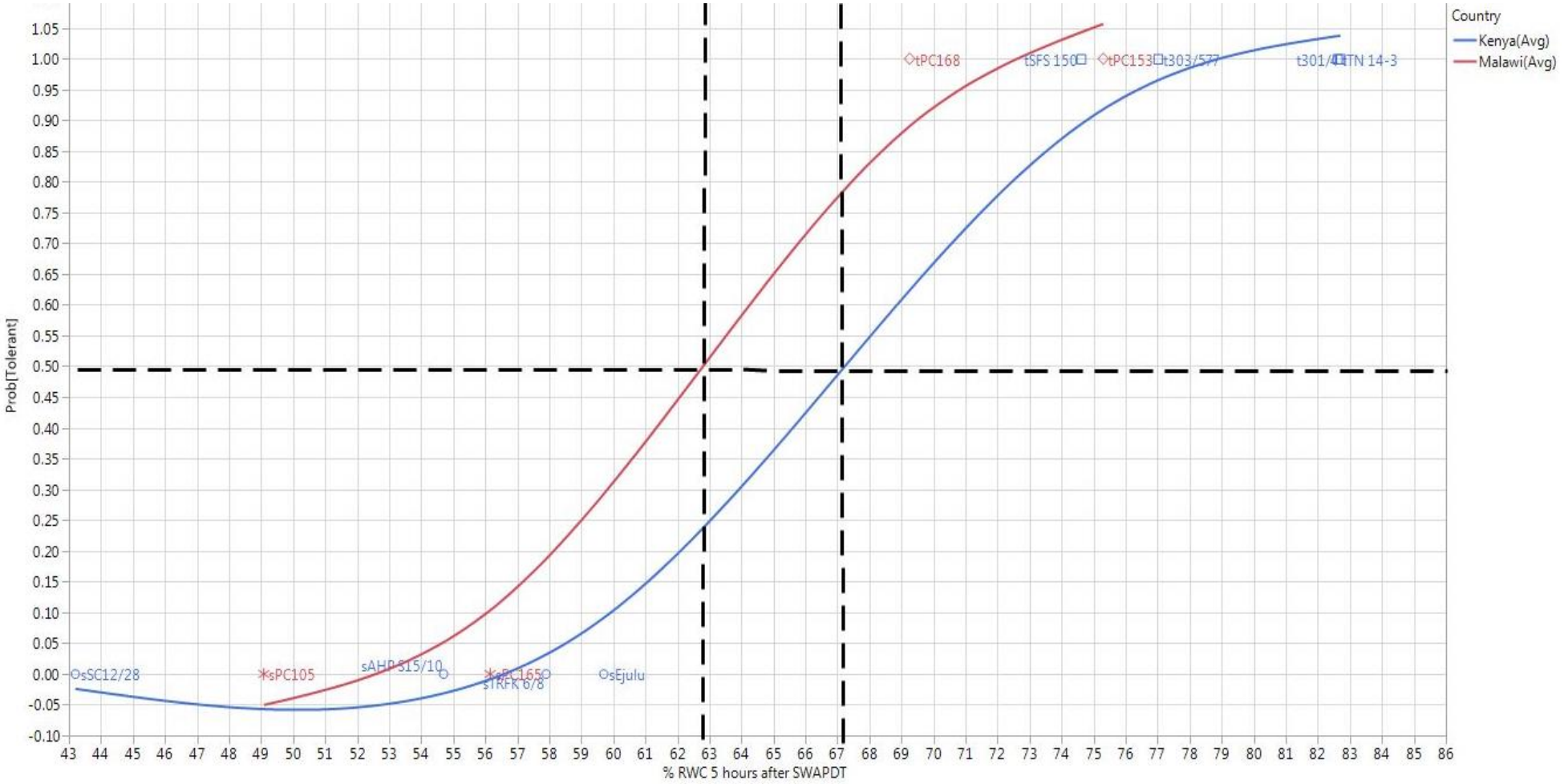


Figure 26: Logistic regression cumulative fit probability for drought tolerance versus % RWC after SWAPDT.

CHAPTER FOUR

(Discussion)

Short-term wither method

Although all the cultivars used i.e. DT (tPC153 and tPC168) and DS (sPC105 and sPC165) share similar ancestral and anatomical properties, they have been classified as DT or DS based on field observations over many years. The dehydration curves of the two DT and two DS cultivars show small differences between cultivars within the same class; surprisingly they show large differences between the classes. The sPC105 and sPC165 exhibited rapid water loss as compared to the DT tPC153 and tPC168 cultivars. sPC165 had a more drastic water loss rate than sPC105. The DT cultivars tPC153 and tPC168 had 75 and 65% RWC respectively, after five hours, while sPC105 and sPC165 had 50 and 48% RWC respectively. The %RWC differed significantly (non-overlapping S.E.M. error bars) between DT and DS cultivars, after two hours. The difference continued to increase up to five hours. The two classes showed differences in the rate at which they rehydrated, with the DT rehydrating faster than the DS. The tPC153 and tPC168 cultivars attained full rehydration within five hours, while sPC105 and sPC165 were rehydrated after six hours (results not shown). Even though the results documented in Figure 18 are over five hours of water stress, they are comparable and correlate with the results obtained by Yobi et al., (2012) who conducted similar studies on ferns over 24 hours

Targeted metabolomics

Amino acid metabolism

The amino acids concentrations in the stressed leaves display an accumulation pattern similar to that found in related studies. This substantiates their role as osmolytes (Rontein, Dieuaide-Noubhani, Dufourc, Raymond, & Rolin, 2002). Drought stress affects plant metabolism, also hindering protein synthesis. The elevated levels of amino acids obtained in this study are attributed to a reduction in protein synthesis and an increase in the breakdown of current proteins. In this study, there was an up-regulation of valine, leucine and isoleucine in the DT cultivars as compared to the DS cultivars (Figure 21). This result coincides with results by Arbona et al., (2013) who investigated the accumulation of glucosinolates in *Arabidopsis* plants subjected to drought stress. The levels of proline in the current study were significantly ($p < 0.04$) higher in DT as compared to DS cultivars. This is attributed to the P5CS gene, which

is highly expressed in tolerant than susceptible varieties under drought stress resulting in an accumulation of proline in Rapeseed (Janská, Maršík, Zelenková, & Ovesna, 2010). The results also showed an increase in the levels of isoleucine in the DT cultivars. A study on the drought response of Poplar trees found that isoleucine had the highest fold increase in DT Poplar trees as compared to the DS under drought stress (Hamanishi et al., 2015). This is in agreement with the results obtained in this study.

Carbohydrates metabolism

The photosynthetic pathway is the most affected when plants are subjected to abiotic stresses such as drought (Kerchev, Fenton, Foyer, & Hancock, 2012). This negatively impacts on primary metabolism, affecting metabolites such as sugars, sugar alcohols and amino acids. Most plants use fructose as an energy source when subjected to stress (Kaplan & Guy, 2004). This explains the increase the levels of in fructose concentration observed in the DS tea cultivars. The lower fructose levels in the DT cultivars are because DT cultivars use fructose at a faster rate than the DS, ensuring their survival under drought stress. Under water deficit glucose concentrations have also been documented to increase in DS plant varieties (Iordachescu & Imai, 2008), which is agreement with results obtained in this study. An accumulation of glucose also results in the biosynthesis of trehalose, which is a disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose residues. Trehalose is a sugar reserve, supplying the plant with energy to cope with stress, and stress protectant. This carbohydrate is also responsible for protein and membrane stabilisation (Yoshida & Sakamoto, 2009). Furthermore, a significant increase in the concentrations of polyols i.e. mannitol was observed. This observation is consistent with results obtained in a study which showed polyols can be synthesised from their analogue sugars under reducing conditions (Pavli, Vlachos, Kalloniati, Flemetakis, & Skaracis, 2013). The accumulation pattern observed in this study agrees with the findings by other researchers that polyols function in osmoregulation under drought stress (Rizhsky et al., 2004). Mannitol confers stress tolerance through actively scavenging hydroxyl radicals and is found in lower concentrations in DT cultivars than in DS wheat crops (Abebe, Guenzi, Martin, & Cushman, 2003). Similar results were obtained in the reported study where

the levels of mannitol were lower in the DT than DS cultivars. Carbohydrates have been documented to affect ABA - dependent metabolic pathways, crucial for drought modulation in plants (J. Zhang, Jia, Yang, & Ismail, 2006). In addition, carbohydrates also affect the biosynthesis of other metabolites that generate energy, alter gene expression regulation and signal transduction (Hoekstra, Golovina, & Buitink, 2001). The accumulation of sugars in this study was accompanied by an increase in the concentrations of the organic acid citrate, though there was no statistically significant difference ($p > 0.74$) between the DT and DS cultivars. An increase in citrate leads to an increase in other Krebs cycle metabolites. This is supported by a study which showed how fluoroacetate initially increased citrate levels without significantly increasing the levels of the other substrates. When citrate levels increased up to three fold, a significant increase in the levels of the other Krebs cycle intermediates occurred (Goldberg, Passonneau, & Lowry, 1966). This result obtained in our study also corroborates the result obtained in another study, which documented higher levels of Krebs cycle intermediates in DT *Sullu* variety as compared to the DS *Negra Ojosa* variety (Vasquez-Robinet et al., 2008).

Flavonoid metabolism

The levels of phenylalanine (Figure 21) obtained in this study showed no statistically significant difference ($p = 0.08$) between DT and DS cultivars. However, the levels were higher in the DT cultivars at $t = 0$ explaining the subsequent rise in the levels of caffeic acid. This is in agreement with results obtained in a study where higher concentrations of caffeoylquinic acid and phenylalanine were detected in DT species while cinnamic acid and quercetin were higher in the DS species (Lugan et al., 2009). Coumaric acid levels, though not significantly different, were lower in DT than in DS cultivars. This correlates with the results obtained in a study by Hu et al., (1999) where coumaric acid levels significantly increased in the xylem sap of DS maize plants over a 12 day period. Coumaric acid was identified as an intermediate in lignin biosynthesis in water stressed maize, which explains an increase in coumaric acid (Hu et al., 1999) to prevent water loss. The increase in amino acids content i.e. phenylalanine, triggers the biosynthesis of phenolic acids through the cinnamic acid pathway. This results in lignin synthesis. Gallic acid, caffeic acid and

Protocatechuic acid were higher in the DT cultivars (Figure 23) and this is due to an increase in amino acids biosynthesis due to drought stress.

The above metabolite results confirm that the five-hour withering of tea leaves has similar metabolite modulation patterns as seen in other plant species over longer times 5-12 days growing in soil (Engelbrecht & Kursar, 2003). This may be due to the absence of roots in the tea shoots that cause the metabolites to change in a short-time. Thus we believe that the SWAPDT method may be a valid method for predicting drought tolerance in tea.

Total polyphenol content

In the current study, it was shown that sPC105, sPC165 and tPC168 had higher TPC than the tPC153 cultivar (Figure 25). This was the expected result because water is one of the raw materials used in photosynthesis; its lack thereof would have a negative impact on the synthesis of primary and secondary metabolites. This could also be because of several mechanisms for modulating drought by DT cultivars. Polyphenols have antioxidant properties which play a key role in scavenging free radicals produced under stress conditions in plants (Lien et al., 1999). As a result DS cultivars depend on both high concentrations of carbohydrates such as fructose, and high TPC to compensate for the lack of other stress combating mechanisms. A study on the flavonoid content demonstrated that a variety with higher TPC was more tolerant to both light and water stress unlike the susceptible varieties with lower flavonoid levels (Yaginuma, Shiraishi, & Igarashi, 2003). This differs from our results. The DT cultivars had a lower TPC with their levels ranging from 9.41% to 15.88% as compared to the DS cultivars, which ranged from 12.49% to 16.89%, respectively. The PC105 cultivar had the highest TPC of 16.89% while PC165 had 14.69%. The lowest TPC was recorded in the PC153 cultivar with 9.4%. The TPC of the PC168 was 11.99%, higher than PC153. The variation of TPC obtained in this study however coincides with results in a study aimed at analysing the influence of shade on flavonoid biosynthesis in relation to flavonoid pathway gene expression in tea leaves. Shade notably reduces flavonoid concentration (catechins and *O*-glycosylated flavonols) in tea leaves, with *O*-glycosylated flavonols compounds decreasing up to 43.26% in shade grown tea plants compared to field grown plants (Wang et al., 2012) explaining why PC153 has a low TPC. There is however no

explanation as to why PC168 did not drop in TPC as this particular cultivar has been classified as tolerant from field studies over several years (Mphangwe et al., 2013). Although these studies serve to further confirm that TPC can be used as an indicator for drought tolerance in *C. sinensis* (Cheruiyot et al., 2007), it must be noted that TPC cannot be used to classify tea cultivars as DT or DS when the plants are grown under shade, as shade has been documented to influence flavonoid biosynthesis. Thus, TPC is considered unreliable because we need to assess new cultivars at an early stage, while growing in the nursery, under shade.

Several documented factors influence TPC and these include genotype, geographical origin, soil composition, harvesting time, post-harvest treatment and physical structure of the leaves (Lin et al., 2003). The results from this study may vary with seasonal changes therefore a follow up study to investigate the effects of these seasonal variations in TPC biosynthesis will have to be conducted. This study may however prove difficult, owing to the polyphenol derivatives generated from the several polyphenols contained in tea, most of which are unstable.

CHAPTER FIVE

(Conclusion)

An inexpensive and practical method, named SWAPDT, has been developed for predicting the drought tolerance of tea cultivars. The metabolite changes for amino acids, flavonoids and sugars, were similar in this five-hour withering in plucked tea shoots, as found in other plant species with whole plants growing in pots or in the field over longer periods of drought (Cramer et al., 2007). This method relies only on the %RWC of tea leaves after the five-hour withering method. The metabolite profiles obtained from this study show that DT tea cultivars differ from DS tea cultivars. The metabolomes indicate that the DT and DS cultivars have an accumulation of either primary sugar, amino acid or citric acid metabolites. There is a significant variation in metabolite content (amino acid, sugars) in DT and DS tea cultivars between t=0 and t=5. The resultant variation in %RWC observed in each of the cultivars provides a basis for the selection, improvement and management of the cultivars to ensure better yields and quality. Although the metabolomes are variable among DT and DS cultivars, further studies into the relationship among metabolites showed some metabolites had a high degree of similarity in their abundance profiles in DT and DS tea cultivars. Several metabolites with significant differences in accumulation under drought-like conditions in both DT and DS tea cultivars exhibited intraspecific variation in metabolite accumulation. The results presented herein demonstrate that future experiments aimed at the comprehension of the complexities of drought stress responses in tea plant must take into consideration the intraspecific variation in genotypes. Based on results obtained in this study we accept $H1_{,}$ which states that there will be no statistically significant difference between the metabolite content of the DT and DS *Camellia sinensis* cultivars under wet conditions at the 95% level of confidence. The basis of this is shown in both the PCA plots at 100% RWC where there is no distinction between DT and DS cultivars. This was validated by the stepwise logistic regression. We as a result reject $H1_0$ which states that there will be no statistically significant difference between the metabolite content of the drought tolerant and DS *Camellia sinensis* cultivars after the SWAPDT method at the 95% level of confidence because from both PCA plots and logistic regression the DT cultivars are distinguishable from the DS cultivars. As a result of the many misclassifications from the targeted metabolomic studies and the fact that to conduct e.g. amino acid analysis for each sample is too expensive, and also because of the difficulties faced with plucking of shoots and ensuring they are kept cold until analysis, targeted metabolomics can in this case be considered an unreliable

method of distinguishing DT from DS cultivars. From the methods employed i.e. SWAPDT, targeted metabolomics and TPC and the subsequent statistical analysis results, it can be seen and thus concluded that the SWAPDT method shows an excellent distinction between the DT and DS cultivars.

The SWAPDT method provides a basis for selection of new DT tea cultivars that may lead to improvement of crop productivity, amidst challenges imposed by drought due to climate change.

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Appendix A

Ms. Ref. No.: JPLPH-D-15-00628R2
Title: SWAPDT: A method for Short-time Withering Assessment of Probability for Drought Tolerance in *Camellia sinensis* validated by targeted metabolomics
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SWAPDT: A method for **S**hort-time **W**ithering **A**ssessment of **P**robability for **D**rought **T**olerance in *Camellia sinensis* validated by targeted metabolomics

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Abbreviations

- DS Drought susceptible
- DT Drought tolerant
- GC Gas chromatography
- LC Liquid chromatography
- PCA Principal component analysis
- P5CS Pyrroline-5-caboxylate synthetase
- GR Glutathione reductase
- GSA Glutamate-semialdehyde
- RWC Relative water content
- TPC Total polyphenol content

Abstract

Climate change is causing droughts affecting crop production on a global scale. Classical breeding and selection strategies for drought-tolerant cultivars will help prevent crop losses. Plant breeders, for all crops, need a simple and reliable method to identify drought-tolerant cultivars, but such a method is missing. Plant metabolism is often disrupted by abiotic stress conditions. To survive drought, plants reconfigure their metabolic pathways. Studies have documented the importance of metabolic regulation, i.e. osmolyte accumulation such as polyols and sugars (mannitol, sorbitol); amino acids (proline) during drought. This study identified and quantified metabolites in drought tolerant and drought susceptible *Camellia sinensis* cultivars under wet and drought stress conditions. For analyses, GC-MS and LC-MS were employed for metabolomics analysis. %RWC results show how the two drought tolerant and two drought susceptible cultivars differed significantly ($p \leq 0.05$) from one another; the drought susceptible exhibited rapid water loss compared to the drought tolerant. There was a significant variation ($p < 0.05$) in metabolite content (amino acid, sugars) between drought tolerant and drought susceptible tea cultivars after short-time withering conditions. These metabolite changes were similar to those seen in other plant species under drought conditions, thus validating this method. The Short-time Withering Assessment of Probability for Drought Tolerance (SWAPDT) method presented here provides an easy method to identify drought tolerant tea cultivars that will mitigate the effects of drought due to climate change on crop losses.

Keywords

Abiotic stress; amino acids; *Camellia sinensis*; carbohydrates; drought tolerance; GC-MS; LC-MS; metabolite profiling; short-time withering; targeted metabolomics.

Introduction

Camellia sinensis

Tea made from the leaves of *Camellia sinensis*, as green or black tea, has been drunk as a mild stimulant due to the caffeine content, since time immemorial! Tea consumption has been increasing in recent years, due to the health promoting effects associated with its rich polyphenol content (Tong et al., 2014). Tea producers demand new cultivars that are drought tolerant, to reduce crop losses. Readily quantifiable markers for drought tolerance will help tea breeders identify and select tea cultivars suitable for drought-prone environments. Plant response to stress is manifested by physiological and metabolomic responses (Maritim et al., 2015). This enables the screening and selection of tea cultivars resistant to drought stress, through organic osmolytes accumulation. Most osmolytes are secondary metabolites, and tea contains high polyphenol amounts (Cheruiyot et al., 2007). *C. sinensis*, like all other vascular plants, utilises carbon derived from aromatic amino acid metabolism to synthesise phenylpropanoids through the shikimate pathway. Phenylpropanoid biosynthesis begins with the deamination phenylalanine by the enzyme phenylalanine ammonia lyase (PAL) which converts phenylalanine to cinnamic acid. The phenylpropanoids from the shikimate pathway are in turn involved in carbohydrate metabolism via the pentose phosphate pathway (Häusler et al., 2014). The metabolic pathways are dependent on photosynthesis, which is affected by water deprivation due to drought stress. Gallic acid biosynthesis is also derived from the shikimate pathway (Heleno et al., 2015). No metabolites have been investigated in *C. sinensis* in relation to drought. However, several metabolites have been documented in literature relative to drought stress in other plant species. This study focuses on polyphenols, flavonoids, amino acids and sugars.

Plant metabolomics

When plants are subjected to abiotic stress conditions, metabolic enzyme inhibition or substrate shortage, etc. disrupts plant metabolism resulting in metabolic pathway reconfiguration, ensuring plant survival (Hamanishi et al., 2015). Plants have established innumerable strategies in response to drought (Ogbaga et al., 2014). A common leaf response to drought stress involves both physical and morphological changes. Several hypotheses have been postulated in an attempt to account for

these leaf changes in stressed plants. An example of such morphological changes includes the rolling or folding, that is observed in leaves as a result of drought stress, reducing transpiration (Engelbrecht & Kursar, 2003). In a separate study, drought susceptible indices of drought tolerant (DT) and drought susceptible (DS) pearl millet cultivars were exposed to drought stress. The initial visual response of drought stress was the withering of the lower part of the leaves, with the DT withering at a slower rate than the DS; resultant leaf death after continued stress exposure was observed. From the 18 samples used, leaves from the DT (IP8210) consistently demonstrated high tolerance to drought stress in all experiments while the DS (IP8949) was most prone to drought stress. Also, because of an increased rate of leaf rolling observed in the DS cultivars, there will be an expected decrease in photosynthesis resulting in less carbohydrate biosynthesis (Kusaka et al., 2005). This inevitably means DT produce more sugars than DS plants resulting in their increased drought tolerance. Studies on leaves in connection with drought tolerance have been documented in different plants but not in *Camellia sinensis*. Several studies have been conducted on plants subjected to drought stress, showing the importance of metabolic regulation, i.e. accumulation of osmolytes in a response to drought stress (Slama et al., 2015). Hyperosmotic stress produces osmolytes which include polyols and sugars (mannitol, sorbitol and trehalose) and amino acids (proline and betaine) (Weckwerth et al., 2004). These compounds are water soluble and non-toxic at high concentrations. They stabilise protein structure while decreasing protein-solvent interactions during water deficit, repair damaged tissues and support growth (Ruan & Teixeira da Silva, 2011). During drought stress, plants induce osmolyte synthesis for turgor maintenance through osmotic adjustment (Arbona et al., 2013). Among the amino acids, proline is responsible for approximately 50% of the osmotic adjustment, as seen in maize (Nishizawa et al., 2008). Metabolomic changes in *Arabidopsis* leaves under drought conditions show that proline, raffinose, γ-amino butyrate (GABA) and Krebs cycle intermediates accumulate in response to drought stress (Urano et al., 2009).

Proline accumulation is integral for a cell's adaptation to hyperosmotic stress. It decreases water potential resulting in osmotic adjustment and maintaining of cell turgor. A proline accumulation in the roots of drought tolerant plants is detected in the early stages of drought and only synthesised and accumulate in the leaves at a later stage. Maritim et al., (2015) documented how water stress induces proline accumulation. The results showed that after four days of water stress the increase in proline content was insignificant but after day 12, a six to seven fold increase was observed in the drought stressed leaves. A large number of plant species accumulate proline in response to osmotic stress. Proline biosynthesis is activated under dehydration conditions with pyrroline-5-caboxylate synthetase (P5CS) as the target enzyme. Plants subjected to osmotic stress use glutamate pathway to ease stressful conditions (Delauney & Verma, 1993). Alternative pathways responsible for proline upregulation under drought stress include the pentose phosphate pathway. Proline biosynthesis also regulates cytosolic pH and $NADP^+$ synthesis, which are key in stimulating the pentose phosphate pathway (Hare & Cress, 1997). High levels of proline in tea samples are also a result of mitochondrial P5C produced by d-ornithine aminotransferase (d-OAT). This enzyme transaminates ornithine to GSA and then P5C and later converted to proline. A study conducted by Miller et al., (2009) showed that expressing *Arabidopsis* d-OAT enhances proline levels resulting in an increase rice and tobacco drought stress tolerance.

Considerable evidence indicates that drought stress also affects the metabolism of soluble carbohydrates, which increase under water stress (Sircelj et al., 2005). Glucose and fructose levels increase in apple trees subjected to drought conditions while starch levels decrease (Ayaz et al., 2000). This suggests that both sugar alcohols and monosaccharaides play a key role in osmotic adjustment (Pandey et al., 2004). The decrease in starch concentration can be attributed to the fact that drought stress reduces the rate of photosynthesis. The inhibition of the photosynthetic rate in grapevines by water stress was showed by Rodriguez et al., (1993). The reduction in the rate of photosynthesis as a result of stomata closure due to an accumulation of ABA restricts CO_2 intake. This results in a reduction in the plant's capacity to synthesise starch and sucrose. Carbohydrate metabolism is linked to photosynthesis, making it pivotal in the stress tolerance. Monosaccharaides such as glucose and fructose represent 38% (w/w) and sucrose 62% (w/w) of the total

soluble sugars (daily average) found in watered plants, and 53% (w/w) and 47% (w/w) respectively in drought subjected plants (Rodrigues et al., 1993). Sucrose is a major transport sugar in many plants, which accumulates under stress conditions (Rolland et al., 2006). Sucrose and raffinose form intracellular glasses, which protect the vegetative tissues from damage. They do so by replacing water molecules, which prevents membrane fusion during drying (Sakurai et al., 2008). As drought exposure prolongs, a reduction in the abundance of the two sugars occurs because they are increasingly being converted into protective sugars (Farrant et al., 2009).

Current drought tolerance assessment

Recurring droughts and future climate change necessitate the selection of DT tea cultivars for a sustainable tea industry. The current method for drought tolerance assessment in *Camellia sinensis* is visual assessment of leaf wilting and scoring on a five-point scale. This is done under field conditions, during natural droughts that occur every 3-7 years. This method is subjective and poorly reproducible due to environmental conditions. An accurate and reproducible method is required to help tea breeders classify new cultivars as DT or DS. The new method should be independent of natural droughts and subjective evaluations.

Factory withering

Withering is the first stage in a tea factory. Freshly harvested tea leaves are placed in withering troughs and air is pumped through the leaves to remove moisture. The fresh tea leaf has a moisture content of about 80% relative water content (RWC). This must be reduced to 70% RWC, for the second process, called rolling, in the tea factory. This process takes about 12-24 hours, depending on the weather and the tea cultivar. Discussions with tea factory managers revealed that the DT cultivars withered slower than DS cultivars in the tea factory. Although slow withering cultivars are the bane of tea factory managers during wet years, they are the darlings of tea estate managers during the dry years. This inspired us to develop a short-time withering method and objective measurement of RWC, as a surrogate marker for calculating the probability of drought tolerance of new tea cultivars. This method is based on leaf RWC by mass balance as described below. The modulation of leaf metabolites (amino acids, sugars and flavonoids) between wet and drought conditions, have been determined in various plant species (as described above), but

never in tea. Thus, modulation of tea leaf metabolites will be measured to validate the new method. We anticipate that the metabolite changes found in other plant species, under prolonged drought conditions, will occur in plucked tea shoots during the new short-time withering method. The main focus throughout this study was to identify, quantify and validate the metabolites in DT and DS tea (*C. sinensis*) cultivars affected by drought stress using the Short-time Withering Assessment of Probability for Drought Tolerance (SWAPDT) method.

Materials and methods

Five biological replicates from each of the two drought tolerant cultivars (PC168 and PC153) and five biological repeats each from the two drought susceptible cultivars (PC105 and PC165) developed at the Tea Research Foundation for Central Africa in Malawi grown in pots under shade net at the University of Pretoria experimental farm in Hatfield, Pretoria and four DT cultivars (SFS 150, TN 14-3, 301/4 and 303/577) and four DS cultivars (AHP S15/10, TRFK 371/8, SC12/28 and Ejulu) grown at the Tea Research Institute in Kenya were used in this study. The Folin-Ciocalteu phenol reagent (Merck Chemicals, South Africa) was used for the determination of the total phenolic content. Gallic acid and anhydrous sodium carbonate with 99% purity were obtained from Sigma-Aldrich (South Africa). Amino acid and carbohydrate standards were purchased from Sigma Aldrich (South Africa). The purity of each compound was > 98%. Acetonitrile and Methanol were UPLC-grade from Merck (Darmstadt, Germany). Other reagent solutions, such as ammonium formate and formic acid, were of analytical grade. Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Deionized water (H_2O) was purified by a purification system (ELGA PURELAB Ultra, Labotec). Minisart® RC4 Syringe Filters (Sartorious). Mettler Toledo analytical balance from Microcep (South Africa). Microplate Reader Thermo Multiskan Ascent. Waters Acquity UPLC system with a quaternary pump solvent management system, an online degasser, and an autosampler. A Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Milford, MA, USA), fitted with a Waters Acquity UPLC and photo-diode array detector (PDA). Trace 1300 (Thermo scientific) coupled to a Thermo TSQ 8000 GC-MS/MS with TriLusRSH autosampler and a non-polar (95% dimethylpolysiloxane) capillary column Restek – Rxi ®-5Sil MS w/Intrega-Guard ® (15 m, 0.25 mm ID, 0.25 µm film thickness) was used.

Polyphenol extraction and content determination

Before extractions, fresh leaves from each cultivar growing under a shade net were microwave dried for five min, which in the process deactivated the oxidizing enzyme polyphenol oxidase. A coffee grinder was used to grind the dried leaves and sieved through a 355 µm stainless-steel sieve and stored at 4 °C in plastic zip-lock bags prior to extracting polyphenols. International Organization for Standardization (ISO) extraction method was used as is described in the ISO document 14502-1: 2005. Briefly, 0.200 ± 0.001 g of each sample was weighed out and transferred into a glass extraction tube. A five ml volume of 70:30 methanol: water (v/v) at 70 °C was added to each extraction tube, stoppered and vortex mixed for approximately five seconds before placing into a water bath set at 70°C. The extraction mixture was vortex mixed after five min and again at ten min when tubes were removed from water bath. After cooling at room temperature with the stopper off for an additional five min, the extracts were centrifuged at 2000 X g for ten min, with the resultant supernatant decanted into a ten ml measuring cylinder. The extraction step was repeated twice. Both extracts were pooled, and the volume adjusted to ten ml with cold 70:30 methanol: water (v/v).

A volume of one ml of the extract was diluted with water to 100 ml. The total polyphenol content (TPC) was determined according to ISO 14502-1: 2005 procedure, with Gallic acid as standard. From the 1/100 ml extract sample dilution, a one ml volume was transferred in duplicate into separate glass tubes. Five ml of a 1/10 dilution of Folin-Ciocalteu reagent in water was pipetted into each tube and mixed. After five min, four ml of sodium carbonate solution (7.5% w/v) was added to each tube, stoppered and mixed before being allowed to stand at room temperature for 60 min. The absorbance was measured at 765 nm against water. Gallic acid standards were used for quantification and the results were expressed as % Gallic acid equivalents (GAE) in g/100 g dry weight plant material. The Gallic acid standard curve which was linear from 10 to 50 µg/ml in the assay was used to measure the polyphenol content in each of the samples. TPC, expressed as a % (w/w) by mass on a sample dry matter basis, is given by the formula:

$$\%TPC = \frac{(OD_{sample} - OD_{intercept}) \times V \times d \times 100}{Slope_{std} \times M_{sample} \times 10\,000 \times DM}$$

where OD_{sample} is optical density obtained for the sample, $OD_{intercept}$ is optical density at the point the best fit linear regression line intercept the y-axis (c), $Slope_{std}$ is slope obtained from best fit linear regression (m), M_{sample} is mass of sample (mg), V is extraction volume (ml), d is dilution factor used prior to the colorimetric determination (one ml to 100 ml = 100X), DM is the dry matter content expressed as a mass fraction of test sample and 10 000 is a dilution factor.

The SWAPDT method

The rate of RWC loss between the DT and DS cultivars was evaluated as described by Yobi et al., (2012) in a comparative metabolic profiling study between DT and DS *Selaginella* species. Three shoots with two leaves and a bud from a single bush of each of the cultivars were immersed in 20 ml of distilled water at room temperature and weighed hourly for five hours until the leaves reached constant weight. The hydrated (turgid) leaves were then removed from respective solutions, blot dried to remove surface water and weighed (t = 0). After the initial weighing, the leaves were oven dried at 37°C and weighed at 60 min intervals for five hours, until their RWC was approximately 50% (based on prior explorative experiments). The leaves were again placed in water, with the leaves above water and petiole in the water (Figure 1), and left for 24 hours with the weights noted hourly for the first five hours. The leaves were weighed after 24 hours and oven dried at 105°C for 24 hours to obtain each leaf's dry weight. The % RWC 0...5 hours was then calculated using the formula:

$$\%RWC\ 0...5 = (F_{wt} - D_{wt})/(FT_{wt} - D_{wt}) * 100$$

where F_{wt} 0...5 is the hourly weight while drying at 37°C for five hours, D_{wt} is the dry weight after 24 hours in 105°C oven and FT_{wt} is the weight after 24 hour rehydration. The final % RWC was normalised with respect to the first value, making all values relative to t = 0. The %RWCs at t = 0 and t = 5 were chosen for the comparative metabolite composition study between the two types of cultivars. Figure 2 shows a diagrammatic representation of the experimental procedure.

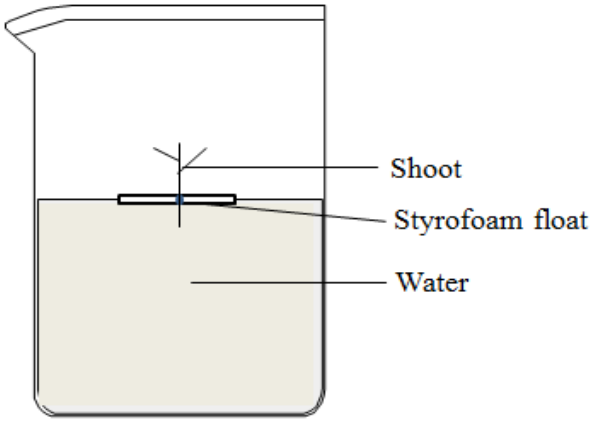

Figure 1: Shows the placement of the shoot in water; the petiole is submerged in distilled water.

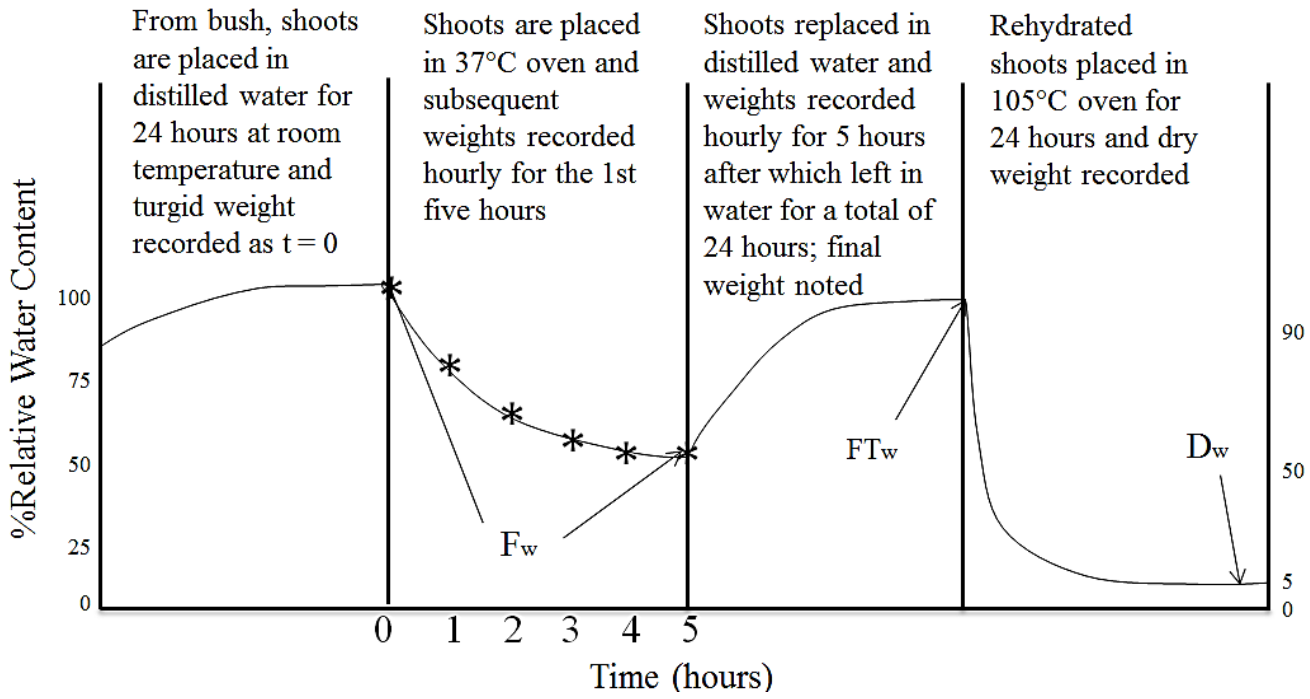

Figure 2: Diagrammatic representation of the experimental procedure.

Chromatography

Two working stock solutions were prepared. One consisted of amino acids (valine, leucine, isoleucine, glycine, glutamic acid, aspartic acid, asparagine, phenylalanine, proline and lysine) and the carbohydrates (glucose, fructose, mannitol and citrate) standards dissolved in 50:50 methanol: water (v/v) at one mg/ml. The second cocktail consisted of phenolic compounds (trans-cinnamic acid, vanillic acid, coumaric acid, gallic acid, caffeic acid and protocatechuic acid) at one mg/ml in 50:50 methanol: water (v/v). These functioned as internal standards for both GC-MS

and LC-MS analysis. An injection volume of one µl was used to give a standard final concentration of one ug/injection. The standard solutions were filtered through a 0.2 µm Minisart® RC4 syringe filters with hydrophilic, solvent-resistant regenerated cellulose membranes prior to injection. All extracts, stock and internal standard solutions were stored at 4°C.

Targeted metabolomics

GC-MS sample preparation and analysis

Fresh tea leaves were picked from the 20 individual tea plants at 06h00, placed in labelled plastic bags and kept on ice. These samples were couriered overnight to the Central Analytical Facility of the University of Stellenbosch were the GC-MS and LC-MS analysis were done as described below.

The fresh leaves were ground to a fine powder in liquid nitrogen. The powder samples were then weighed and extracted with one ml 70:30 methanol: water (v/v). After extraction, 100 µl of ribitol was added as an internal standard after which the samples were kept overnight at 70°C. The overnight mixture was allowed to cool before centrifugation at 17 000g for five min. Two hundred microliters of the extracts was transferred into a clean Eppendorf tube and dried on a Savant DNA 110 Speed vac. The concentrator of the speed vac setting was switched on with the drying rate set at low. The low drying rate was used to preserve the metabolites, preventing amino acids break down. The dried extracts were reconstituted in 100µl (2.5%) methoxyamine hydrochloride in 50:50 pyridine: acetonitrile (v/v) and left for two hours at 40°C. Fifty µl of BSTFA with 1% TMCS was added and the mixture derivatised at 60°C for 30 min. The samples were then cooled and vortexed for a few seconds before being transferred into a GC vial with an insert. A Trace 1300 coupled to a Thermo TSQ 8000 GC-MS/MS (Thermo scientific) with a TriLusRSH auto-sampler and a non-polar (95% dimethylpolysiloxane) capillary column Restek –Rxi ®-5Sil MS w/Intrega-Guard ® (15 m, 0.25 mm ID, 0.25 µm film thickness) was used for targeted metabolite profiling. The initial oven temperature was maintained at 100°C for four min, and then ramped at 6°C/min to 180°C, held for two min and ramped at 15°C/min to 300°C and then held for five min. Helium was used as the carrier gas at a flow rate of one ml/min with the injector temperature maintained at 280°C, operated in a split less mode. The mass spectral data was recorded on a

TSQ operated in a single ion monitoring (SIM) mode. Both the ion source and transfer line temperatures were set at 240°C.

LC-MS analysis

The samples were ground to a fine powder and extracted as described for the GC analyses. An injection volume of three µl was used. A Waters Synapt G2 quadrupole time-of-flight mass spectrometer fitted with a Waters Acquity UPLC and photo-diode array detector (PDA), was used for LC-MS analysis. Separation was achieved on a Waters Acquity HSS T3 column (1.8 µm, 2.1 x 150 mm). Solvent A consisted of water with 0.1% formic acid while solvent B was made up of 0.1% formic acid in acetonitrile. The gradient employed consisted of a flow rate 0.32 ml/min, starting with 0% B to 5.0% B over four min, then to 40% B over 11 min, followed by a linear gradient to 100% B over the next one min and kept constant for one min during column wash in 100% B. This was followed by re-equilibration to initial conditions over three min for a total runtime of 20 minutes. Electrospray ionisation was applied in the positive mode, with a capillary voltage of 2.5 kV, a cone voltage of 15 V, desolvation temperature of 275°C and desolvation gas (N_2) flow of 650 $L.h^{-1}$. The source temperature was set to 120°C. The rest of the MS settings were optimised for best sensitivity. Data was acquired in MSE mode, consisting of a scan using low collision energy of 6 eV and a scan using a collision energy ramp from 25 to 60 V. Sodium formate was used to calibrate the instrument and leucine encephalin was used for the lock spray for accurate mass determination. The PDA detector was set to scan over the range 230–700 nm. The raw LC-MS data was obtained from the CAF on an external drive.

Data Processing and Statistical Analysis

All the data from the samples was 0 normalised and 1 standardised to minimise systematic variation within the data, before multivariate analysis. Data acquisition and processing was conducted using MassLynx 4.1 software. The raw data obtained from LC-MS was converted into Network Common Data Form (NetCDF) format using the Databridge software application manager from Waters Corp, Milford, MA. GC-MS results were analysed directly. SIMCA-P 14.0 (Umea, Sweden) and JMP pro 12 software were used to conduct multivariate statistical analysis to identify key metabolites. JMP Pro 12 was used to perform one way analysis of variance

(ANOVA). Both the Student's t-test, with the alpha level set to 0.05 and ANOVA were conducted to determine the significance of the up or down regulation of each metabolite. High-dimensional and complex data sets are generated whenever metabolomic studies are conducted. The analysis and interpretation of such data sets proves impossible just by visual inspection or univariate statistical analysis. As a result, multivariate statistical data analysis mathematical modelling approaches, namely PCA (Figure 5) was employed to enable accurate extraction and interpretation of large empirical data sets. Logistic regression models were developed for classifying *C. sinensis* cultivars into DT and DS categories, based on specific metabolites. The data was transformed to adjust for leaf weight and moisture content at five hours, after which a stepwise logistic regression was done. Two models were developed at t = 0 and t = 5 for different variables/metabolites (Table 1 and 2). Due to the small number (20) metabolites, it was decided to use the two-variable model with the variables appearing frequently in other higher order models at the t = 0 and t = 5 levels.

Results and Discussion

Short-term wither method

Although all the cultivars used i.e. DT (tPC153 and tPC168) and DS (sPC105 and sPC165) share similar ancestral and anatomical properties, they have been classified as DT or DS based on field observations over many years. The dehydration curves of the two DT and two DS cultivars show small differences between cultivars within the same class, they surprisingly show large differences between the classes. The sPC105 and sPC165 exhibited rapid water loss as compared to the DT tPC153 and tPC168 cultivars. sPC165 had a more drastic water loss rate than sPC105. The DT cultivars tPC153 and tPC168 had 75 and 65% RWC respectively, after five hours, while sPC105 and sPC165 had 50 and 48% RWC respectively (Figure 3). The %RWC differed significantly (non-overlapping SEM error bars) between DT and DS cultivars, after two hours. The difference continued to increase up to five hours. Even though the results documented in Figure 3 are over five hours of water stress, they are comparable and correlate with the results obtained by Yobi et al., (2012) who conducted similar studies on ferns over 24 hours.

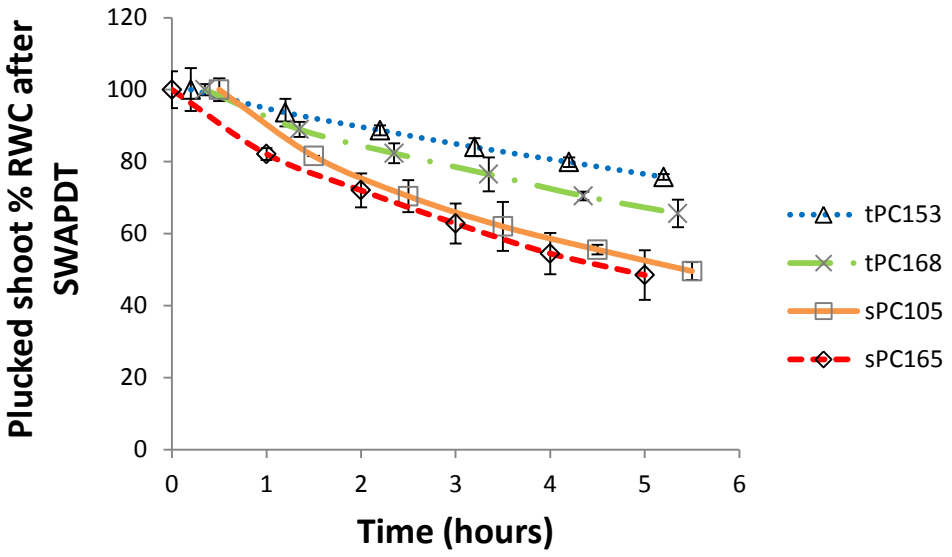

Figure 3: RWC (%) of *C. sinensis* DT (tPC168 and tPC153) and DS (sPC105 and sPC165) cultivars. The error bars are representative of S.E.M with n = 5.

Further work was done to on four DT cultivars (SFS 150, TN 14-3, 301/4 and 303/577) and four DS cultivars (AHP S15/10, TRFK 371/8, SC12/28 and Ejulu) grown at the Tea Research Institute in Kenya to validate the SWAPDT method and those results are shown in figure 4:

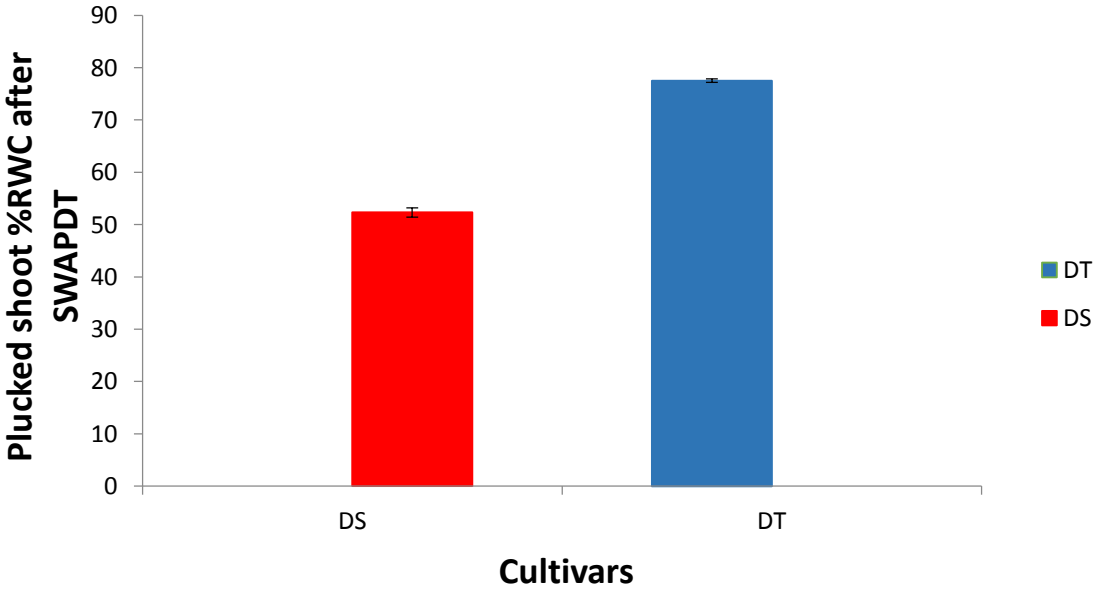

Figure 4: % RWC of plucked tea shoots of *C. sinensis* DS (AHP S15/10, TRFK 371/8, SC12/28 and Ejulu) and DT (SFS 150, TN 14-3, 301/4 and 303/577) after SWAPDT in cultivars classified as DS or DT after many years of field observations. The two groups are statistically significant with p = 0.0008. The error bars represent S.E.M with n = 4.

The metabolomic results confirmed that the changes in amino acids, flavonoids and carbohydrates, during this five-hour wither in tea, are similar to the metabolomic changes found in other plant species, over longer times.

Data Processing and Statistical Analysis

From the 20 metabolites investigated, a few key metabolites were responsible for causing clustering between the tolerant and the susceptible cultivars. The trend observed (Figure 5) is the same as what has been documented in other plants that have been exposed to drought stress over longer times (Engelbrecht & Kursar, 2003). The cross validation results show that the model developed on the t = 5 data works equally well on the t = 0 and t = 5 data, namely 85% and 83% correct classification respectively. However, the model built on the t = 0 data did not work equally well on the t = 0 and t = 5 data, namely 65% and 83% correct classification respectively. This means that targeted metabolomics of fresh leaves (t = 0) cannot be used to classify tea cultivars as DT or DS.

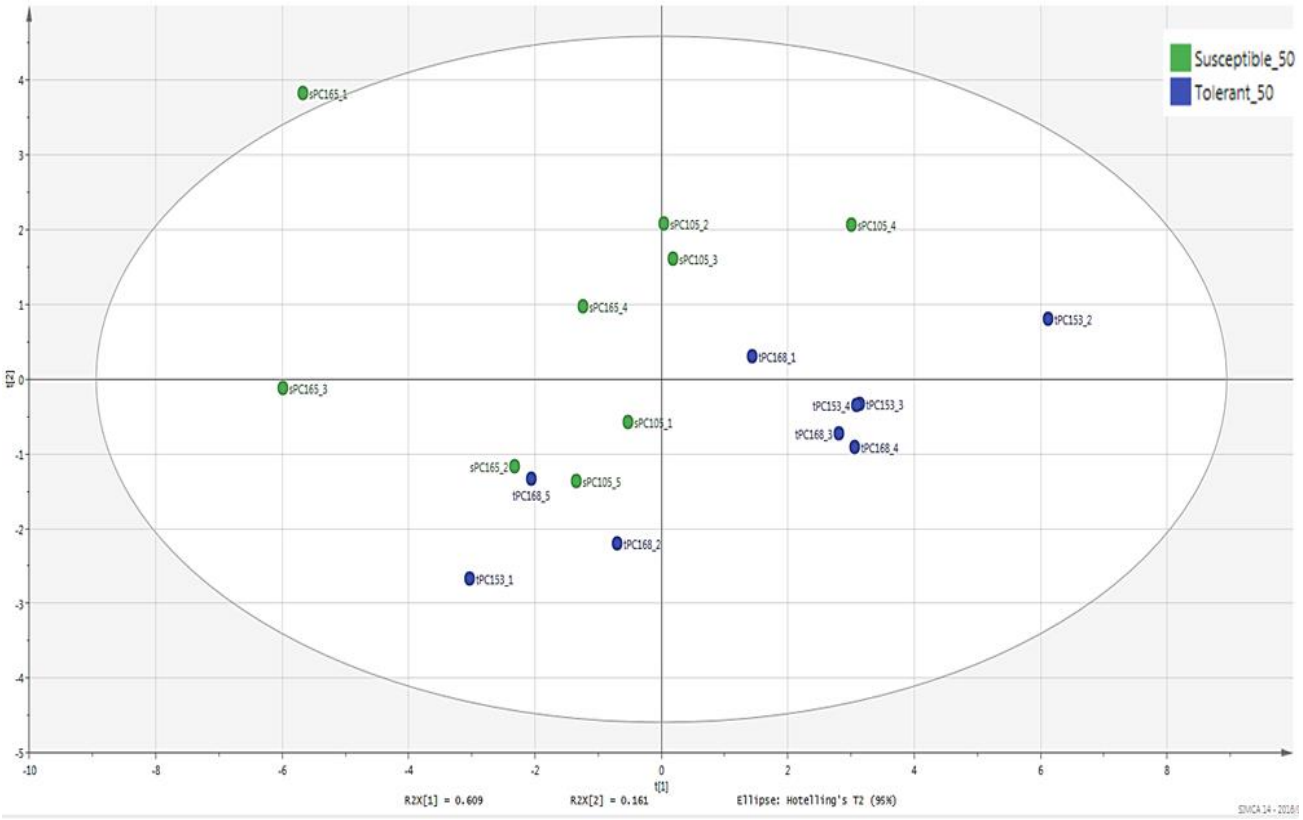

Figure 5: Shows components one and two of a PCA clustering of *C. sinensis* cultivars at t = 5, with n = 5 for each cultivar. SIMCA-P 14 was used to obtain the plot.

Table 1: Logistic regression model at t = 0 as a single model.

Regression Models Selected by Score Criterion at t = 0		
Number of Variables	Score Chi-Square	Variables Included in Model
1	8.9934	Glu
1	5.8461	Val
1	5.0152	Leu
2	12.4037	Val Glut
2	11.8386	Leu Glut
2	10.9995	Val Man
3	15.2223	Val Glut Man
3	14.9354	Leu Glut Man
3	14.7392	Val Glut Glu
4	16.3191	Leu Glyc Glut Glu
4	16.2382	Val Glut Prot Glu
4	16.2245	Leu Glut Prot Glu

Table 2: Logistic regression models at t = 5 as a single model.

Regression Models Selected by Score Criterion at t = 5		
Number of Variables	Score Chi-Square	Variables Included in Model
1	6.0708	Gal
1	4.9392	Asp

1	4.4514	Prot
2	11.6893	Val Asp
2	11.0604	Leu Asp
2	10.6539	Glut Asp
3	14.4470	Val Asp Citric
3	13.7405	Leu Asp Citric
3	12.7857	Val Asp TCin
4	15.3486	Val Asp Asn Van
4	15.1651	Val TCin Asn Prot
4	14.8718	Val Glyc TCin Asn

$$For\ the\ Model\ t = 0;$$

$$p = 1/(1 + e - (2192.90674170605\text{Val-}759.258878377219\text{Glut})$$

$$For\ the\ Model\ t = 5;$$

$$p = 1/(1 + e - (439.122693812145Val - 683.855963516112Asp)$$

Table 3: The cross-validation results showing how the developed model based on the t = 5 data works equally well on the t = 0 and t = 5 data (85% and 83%) but the model based on the t = 0 data did not work equally well (65% and 83%). These models show that targeted metabolomics of fresh leaves (t = 0) cannot be used to classify tea cultivars as DT or DS.

Model	t = 0	t = 5	Total
t = 5	15/18 = 83%	17/20 = 85%	32/38 = 84%
t = 0	15/18 = 83%	13/20 = 65%	28/38 = 74%

Targeted metabolomics

Amino acid metabolism

The amino acid data obtained from the GC-MS analysis showed that eight amino acids were detected from the 10 that were analysed. Asparagine, glycine, valine, isoleucine, proline and leucine were significantly ($p < 0.05$) higher in the DT cultivars, after five hours at 37^{o}C than in the DS cultivars. Aspartic acid was significantly ($p < 0.004$) lower in the DT cultivars than the DS cultivars at this time.

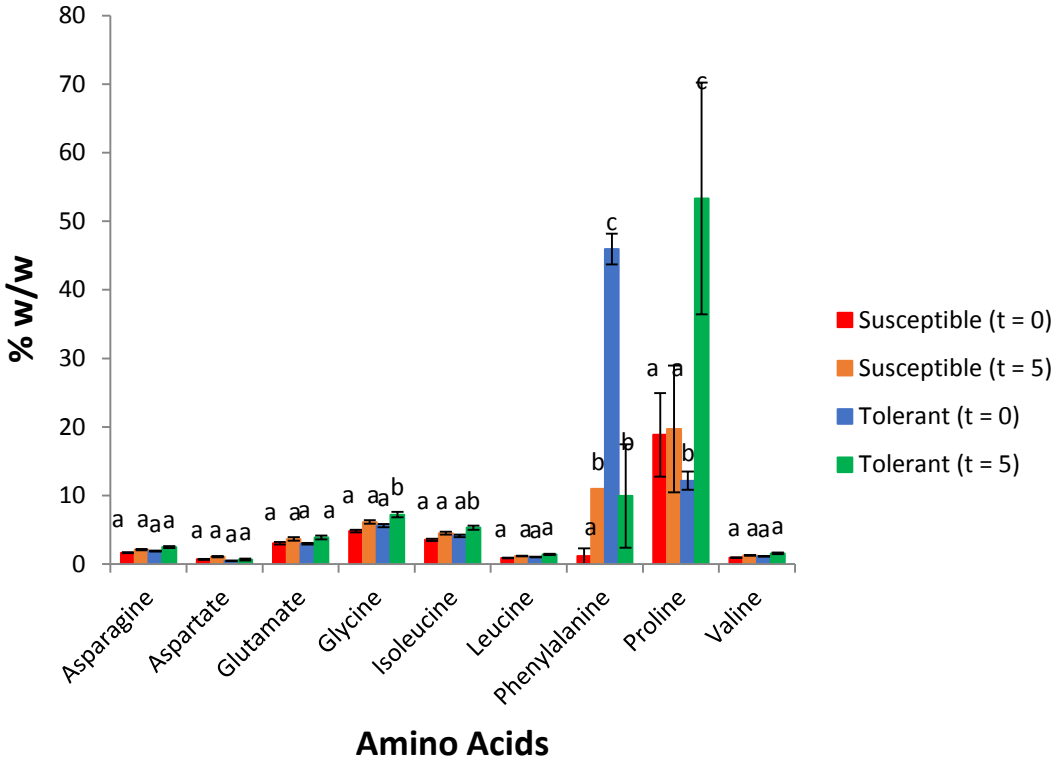

Figure 6: Differences in amino acid abundances between DT and DS at t = 0 and t = 5. The means are based on five independent growing plants of two DT or two DS cultivars. Error bars are representative of S.E.M. with n = 5.

The amino acid concentrations in the stressed leaves display an accumulation pattern similar to that found in a study which substantiates their role as osmolytes (Rontein et al., 2002). Drought stress affects plant metabolism, also hindering protein synthesis. The elevated levels of amino acids obtained in this study are attributed to a reduction in protein synthesis and an increase in the breakdown of current proteins. In this study, there was an up-regulation of valine, leucine and isoleucine in the DT cultivars as compared to the DS cultivars (Figure 6). This result coincides with results by Arbona et al., (2013) who investigated the accumulation of

glucosinolates in *Arabidopsis* plants subjected to drought stress. The levels of proline in the current study were significantly ($p < 0.04$) higher in DT as compared to DS cultivars. This is attributed to the P5CS gene, which is highly expressed in tolerant than susceptible varieties under drought stress resulting in an accumulation of proline in Rapeseed (Janská et al., 2010). The results also showed an increase in the levels of isoleucine in the DT cultivars while aspartic acid levels were lower in the DT. A study on the drought response of Poplar trees found that isoleucine had the highest fold increase in DT Poplar trees as compared to the DS under drought stress (Hamanishi et al., 2015). This is in agreement with the results obtained in this study.

Carbohydrates metabolism

Carbohydrates are among the most studied metabolites with regards to their accumulation in the vegetative tissues of plants in response to drought stress (Iglesias, Lliso, Tadeo, & Talon, 2002). Unlike what was observed with the amino acids, remarkable differences were apparent between the DT and DS cultivars. There was a significant down regulation of the carbohydrate metabolites noted in the DT cultivars as compared to the DS cultivars (Figure 7).

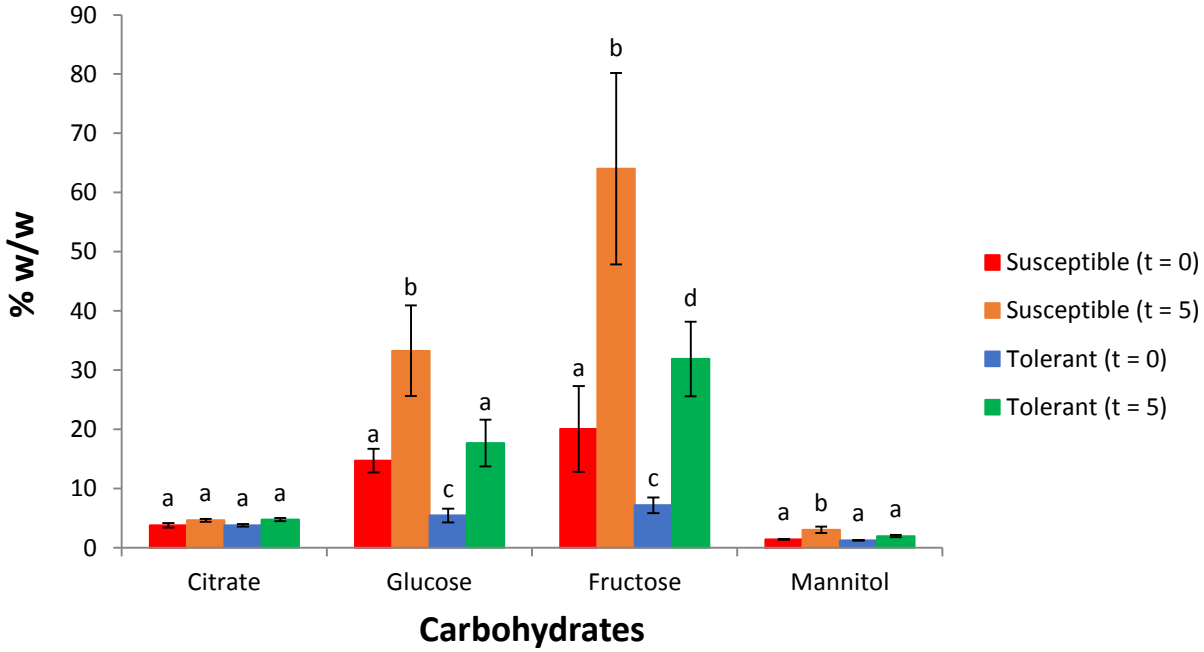

Figure 7: Differences in carbohydrate abundances between DT and DS at t = 0 and t = 5. The means are based on five independent growing plants of two DT or two DS cultivars. Error bars are representative of S.E.M. with n = 5.

The photosynthetic pathway is the most affected when plants are subjected to abiotic stresses such as drought (Kerchev et al., 2012). This negatively impacts on primary metabolism, affecting metabolites such as sugars, sugar alcohols and amino acids. Most plants use fructose as an energy source when subjected to stress (Kaplan & Guy, 2004). This explains the increase in fructose concentration observed in the DS tea cultivars. The lower fructose levels in the DT cultivars are because DT cultivars utilise fructose at a faster rate than the DS, ensuring their survival under drought stress. Under water deficit glucose concentrations have also been documented to increase in DS plant varieties (Iordachescu & Imai, 2008), which is agreement with results obtained in this study. An accumulation of glucose also results in the biosynthesis of trehalose, which is a disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose residues. Trehalose is a sugar reserve, supplying the plant with energy to cope with stress, as well as a stress protectant. This carbohydrate is also responsible for protein and membrane stabilisation (Yoshida & Sakamoto, 2009). Furthermore, a significant increase in the concentrations of polyols i.e. mannitol was observed. This observation is consistent with results obtained in a study which showed polyols can be synthesised from their analogue sugars under reducing conditions (Pavli et al., 2013). The accumulation pattern observed in this study agrees with the findings by other researchers that polyols function in osmoregulation under drought stress (Rizhsky et al., 2004). Mannitol confers stress tolerance through actively scavenging hydroxyl radicals and is found in lower concentrations in DT cultivars than in DS wheat crops (Abebe et al., 2003). Similar results were obtained in the reported study where the levels of mannitol were lower in the DT than DS cultivars. Carbohydrates have been documented to affect ABA - dependent metabolic pathways, crucial for drought modulation in plants (J. Zhang et al., 2006). In addition, carbohydrates also affect the biosynthesis of other metabolites that generate energy, alter gene expression regulation and signal transduction (Hoekstra et al., 2001). The accumulation of sugars in this study was accompanied by an increase in the concentrations of the organic acid citrate, though there was no statistically significant difference ($p > 0.74$) between the DT and DS cultivars. An increase in citrate leads to an increase in other Krebs cycle metabolites. This is supported by a study which showed how fluoroacetate initially increased citrate levels without significantly increasing the levels of the other substrates. When citrate levels increased up to three fold, a significant increase in the levels of the other

Krebs cycle intermediates occurred (Goldberg et al., 1966). This result obtained in our study also corroborates the result by Vasquez-Robinet et al., (2008) who documented higher levels of Krebs cycle intermediates in DT *Sullu* variety as compared to the DS *Negra Ojosa* variety.

Flavonoid metabolism

Vanillic acid, protocatechuic acid, gallic acid, caffeic acid and trans-cinnamic acid were significantly ($p > 0.05$) higher in the DT compared to the DS cultivars after five-hour wither. Coumaric acid had a lower abundance in DT compared with DS cultivars after five-hour wither (Figure 8). These results indicate that the DT cultivars use flavonoids at a higher rate than DS, which enables them to cope with drought stress. Many plants use phenylpropanoids to respond to and mitigate stress through the shikimate pathway, which produces phenylalanine (Tounekti et al., 2013).

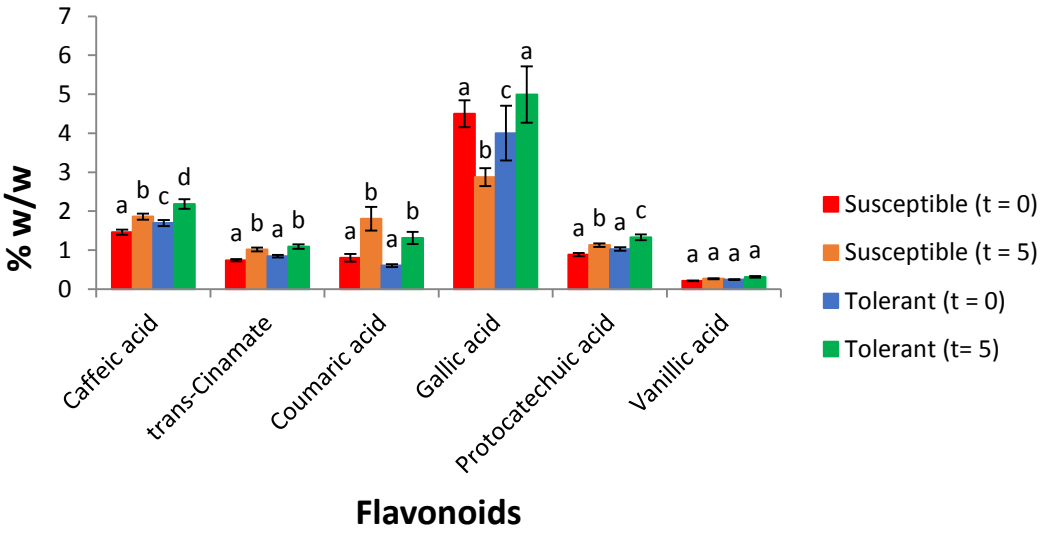

Figure 8: Differences in flavonoid abundances between DT and DS at t = 0 and t = 5. The means are based on five independent growing plants of two DT or two DS cultivars. Error bars are representative of S.E.M with n = 5.

The levels of phenylalanine (Figure 6) obtained in this study showed no statistically significant difference ($p = 0.08$) between DT and DS cultivars. Although not significantly different, the levels were higher in the DT cultivars and this in turn resulted in a subsequent rise in the levels of caffeic acid that was significantly different between the DT and DS cultivars, also conferring drought tolerance on the Dt cultivars. This is in agreement with results obtained in a study where higher concentrations of caffeoylquinic acid and phenylalanine were detected in DT species

while cinnamic acid and quercetin were higher in the DS species (Lugan et al., 2009). Coumaric acid levels were lower in DT than in DS cultivars. This correlates with the results obtained in a study by Hu et al. (1999) where coumaric acid levels significantly increased in the xylem sap of DS maize plants over a 12 day period. Coumaric acid was identified as an intermediate in lignin biosynthesis in water stressed maize, which explains an increase in coumaric acid (Hu et al., 1999) to prevent water loss. The increase in amino acids content i.e. phenylalanine, triggers the biosynthesis of phenolic acids through the cinnamic acid pathway. This results in lignin synthesis. Gallic acid, caffeic acid and trans-cinnamic acid were higher in the DT cultivars (Figure 8) and this is due to an increase in amino acids biosynthesis due to drought stress.

The above metabolite results confirm that the five-hour withering of tea leaves has similar metabolite modulation patterns as seen in other plant species over longer times 5-12 days growing in soil (Engelbrecht & Kursar, 2003). This may be due to the absence of roots in the tea shoots that cause the metabolites to change in a short time. Thus we believe that the SWAPDT method may be a valid method for predicting drought tolerance in tea.

Total polyphenol content

In the current study, it was shown that both sPC105 and sPC165 had higher total polyphenol content (TPC) than the tPC153 and tPC168 cultivars, Figure 9.

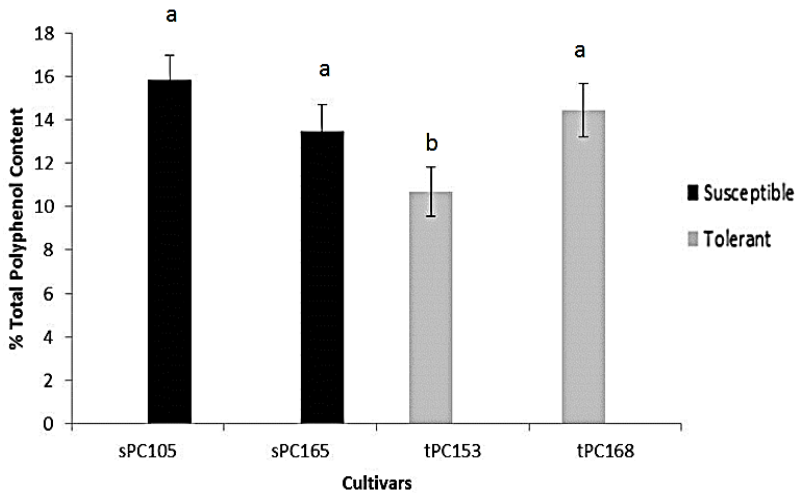

Figure 9: Differences in %TPC abundances between DT (tPC153 and tPC168) and DS (sPC105 and sPC165). The means are based on five independent growing plants of two DT or two DS cultivars. Error bars are representative of S.E.M. with n = 5.

This was the expected result because water is one of the raw materials used in photosynthesis; its lack thereof would have a negative impact on the synthesis of primary and secondary metabolites. This could also be because of several mechanisms for modulating drought by DT cultivars. Polyphenols have antioxidant properties which play a key role in scavenging free radicals produced under stress conditions in plants (Lien et al., 1999). As a result DS cultivars depend on both high concentrations of carbohydrates such as fructose, and high TPC to compensate for the lack of other stress combating mechanisms. A study on the flavonoid content demonstrated that a tea variety with higher TPC was more tolerant to both light and water stress unlike the susceptible varieties with lower flavonoid levels (Yaginuma et al., 2003). This differs from our results. The variation of TPC obtained in the reported study however coincides with results in a study aimed at analysing the influence of shade on flavonoid biosynthesis in relation to flavonoid pathway gene expression in tea leaves. Shade notably reduces flavonoid concentration (catechins and *O*-glycosylated flavonols) in tea leaves, with *O*-glycosylated flavonols compounds decreasing up to 43.26% in shade grown tea plants compared to field grown plants (Wang et al., 2012), explaining why PC153 has a low TPC. There is however no explanation as to why PC168 did not drop in TPC as this particular cultivar has been classified as tolerant from field studies over several years (Mphangwe et al., 2013). Although these studies serve to further confirm that TPC can be used as an indicator for drought tolerance in *C. sinensis* (Cheruiyot et al., 2007), it must be noted that TPC cannot be used to classify tea cultivars for drought tolerance when the plants are grown under shade, as shade has been documented to influence flavonoid biosynthesis. Thus, TPC is considered unreliable because we need to assess new cultivars at an early stage, while they are growing in the nursery, under shade.

RWC

A logistic regression plot (Figure 10) was created from the RWC data obtained at the five hour mark for each sample in the SWAPDT method for known DT and DS cultivars. This plot separates the DT from the DS cultivars. The plot shows that after five-hour wither in the SWAPDT method, the DT cultivars have > 57% RWC. Only four of the 36 plants were misclassified, based on this 59% threshold value. From this plot, logistics probability formulas were generated as shown below:

P (tolerant) = 1 - Cum [Susceptible]

where Cum [Susceptible] = 1 + Exp (-31.434 + 0.5314 * % RWC at five hours after SWAPDT. Using this formula, the % RWC after five-hour withering, can be used to calculate a new cultivars probability for DT. The cultivars with P (tolerant) > 0.5 can be classified as DT. The closer P (tolerant) is to 1, the higher the probability that the cultivar will be DT. The probability of drought tolerance will be higher than 90%, when the RWC > 62% in the SWAPDT method. This method is objective, reproducible and practical because it is based only on a mass balance and a drying oven set at 37^{o}C.

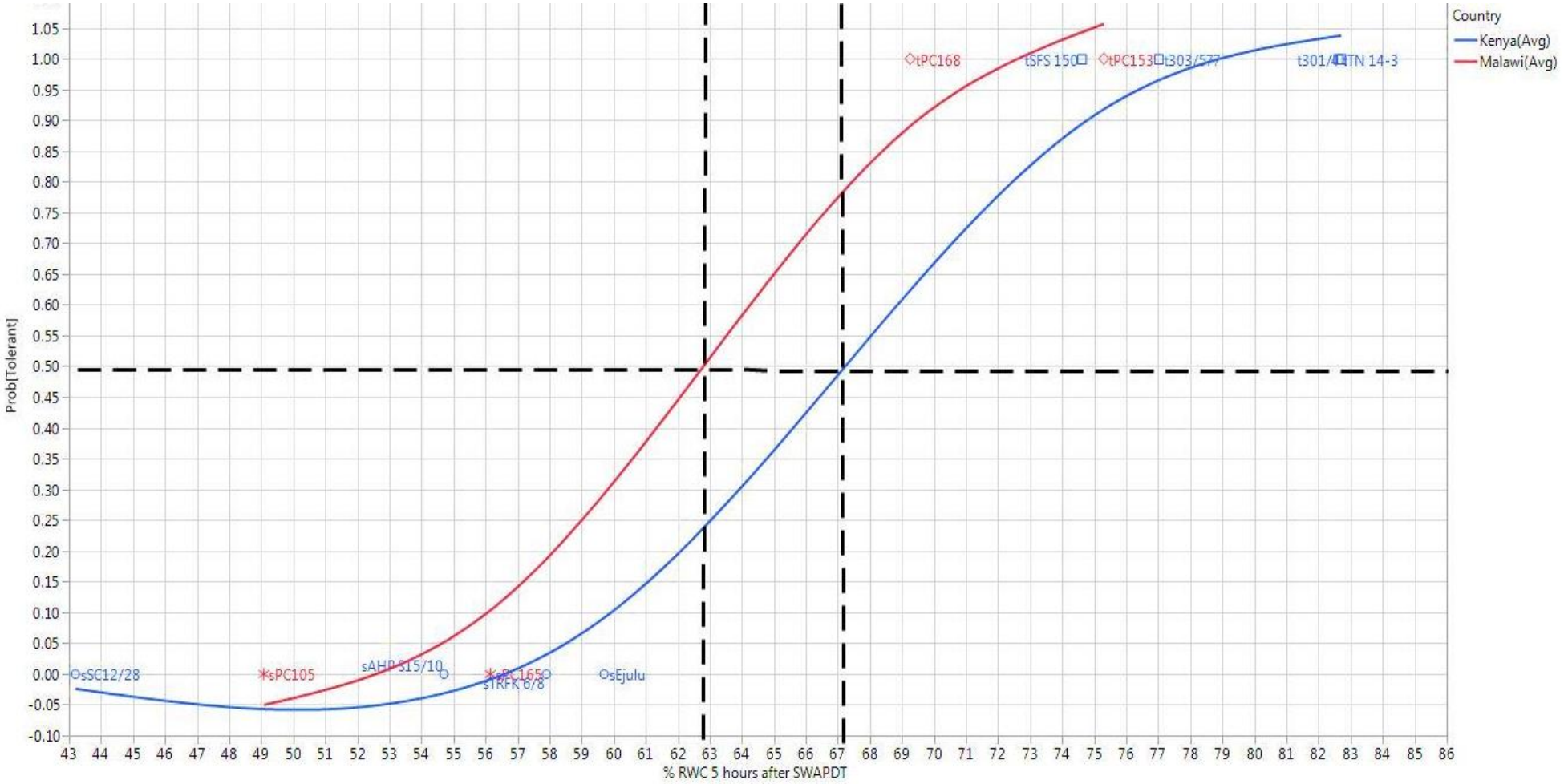

Figure 10: Logistic regression cumulative fit probability for drought tolerance versus % RWC after SWAPDT.

Conclusion

An inexpensive and practical method, named SWAPDT, has been developed for predicting the drought tolerance of tea cultivars. The metabolite changes for amino acids, flavonoids and sugars, were similar in this five-hour withering in plucked tea shoots, as found in other plant species with whole plants growing in pots or in the field over longer periods of drought (Cramer et al., 2007). This method relies only on the %RWC of tea leaves after the five-hour withering method. The metabolite profiles obtained from this study show that DT tea cultivars differ from DS tea cultivars. The metabolomes indicate that the DT and DS cultivars have an accumulation of either primary sugar, amino acid or citric acid metabolites. There is a significant variation in metabolite content (amino acid, sugars) in DT and DS tea cultivars between t=0 and t=5. The resultant variation in %RWC observed in each of the cultivars provides a basis for the selection, improvement and management of the cultivars to ensure better yields and quality. Although the metabolomes are variable among DT and DS cultivars, further studies into the relationship amongst metabolites showed some metabolites had a high degree of similarity in their abundance profiles in DT and DS tea cultivars. Several metabolites with significant differences in accumulation under drought-like conditions in both DT and DS tea cultivars exhibited intraspecific variation in metabolite accumulation. The results presented herein demonstrate that future experiments aimed at the comprehension of the complexities of drought stress responses in tea plant must take into consideration the intraspecific variation in genotypes. The SWAPDT method provides a basis for selection of new drought tolerant tea cultivars that may lead to improvement of crop productivity, amidst challenges imposed by drought due to climate change.

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