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Calibration of sap flow techniques in citrus using the stem perfusion method

by

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DECLARATION

I hereby certify that this thesis is my own work, except where duly acknowledged. I also certify that no plagiarism was committed in writing this thesis.

Signed _____
(Mpaballeng Catherine Sam)

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LIST OF ABBREVIATIONS

Symbol/ Abbreviation	Explanation	units
c_{dw}	Specific heat capacity of oven-dry wood	g
CHP	Compensation heat pulse	-
c_s	Specific heat capacity of sap	J g^{-1} K^{-1}
c_{sm}	Specific heat capacity of sap plus the woody matrix	J g^{-1} K^{-1}
HFD	Heat field deformation	-
HPV	Heat pulse velocity	-
HR	Heat ratio	-
m_c	Moisture content of the sapwood	g cm^{-3}
SFD	Sap flux densities	$cm^3cm^{-2}s^1$
SHB	Stem heat balance	-
t_c	Time	s
TDP	Thermal dissipation probe	-
v_1	Temperature increase at downstream needle	°C
v_2	Temperature increase at upstream needle	°C
$\mathbf{v_z}$	heat pulse velocity	cm/h
X	Axial distance	cm
x_{down}	Distance downstream of the heater	cm
x_{up}	Distance upstream of the heater	cm
ΔT	Temperature difference	k
ΔT_o	Temperature difference during zero flow	k
ρ_b	Dry wood density	g cm^{-3}
ρ_s	Density of the sap	g cm^{-3}
ρ_{sm}	Density of sap plus the woody matrix	g cm^{-3}
CV	Coefficient of Variation	%
WRC	Water Research Commision	-

ABSTRACT

The aim of this study was to calibrate and decide on the most appropriate sap flow technique for citrus species in the laboratory by pushing water through cut branches. Various sap flux density techniques, including heat pulse techniques (heat ratio and compensation heat pulse methods) and the heat dissipation technique were calibrated in four citrus species, namely *Citrus sinensis* (Oranges), *Citrus reticulata* (Soft citrus), *Citrus paradise* (Grapefruit) and *Citrus limon* (Lemons). Sap flux density, determined by these three techniques, was compared to that determined gravimetrically, which was calculated as the rate of change in the mass of water passing through the stem segment divided by the area of conducting wood. Results showed that the sap flux density was consistently underestimated by all techniques and across all citrus species/varieties. However, fairly good correlations (R^2>0.7) between sap flux densities determined by a sap flow technique and gravimetric determinations were found for all techniques in some stems. Despite the good correlations found in the study, a single calibration factor for each technique could not be found for citrus using the stem perfusion method. Calibration factors were determined as the inverse of the slope of the linear relationship between sap flux density determined with a sap flow technique and that determined gravimetrically. These correction factors varied between citrus species and even within stems of the same species.

Vessel dimensions (lumen diameter) and distance between groups of xylem vessels in citrus species was determined in order to try and explain the underestimation of sap flux density and the large variations in the calibration factors obtained during the calibration of the various sap flow techniques. The results revealed that the variation and underestimation were caused by contact of the probes with inactive xylem and due to differences in the nature of sapwood. The xylem vessels were unevenly distributed throughout the sapwood with large distance between the vessels, meaning that the sapwood of the studied species was considered inhomogeneous and therefore departed from the idealised theory of heat pulse measurements. The theory needs to be adapted to account for such sapwood and because of the large variation in the sapwood

properties between different citrus species, calibration of these techniques is probably necessary for each new species and orchard in which measurements are to be made. Our analysis of the performance of sap flow techniques showed that the HR method should perhaps be considered before the CHP and TD methods.

CHAPTER 1: GENERAL INTRODUCTION

Irrigated agriculture plays a vital role in food production and more than two-thirds of all freshwater withdrawals are used to irrigate crops. It is expected that agricultural water use will increase in the near future due to higher food demands and the Food and Agriculture Organization (FAO) estimate a net expansion of 45 million hectares of irrigated land in 93 developing countries by 2030 (FAO, 2004). Competition for this scarce resource is, however, on the increase due to population growth and the expansion of the industrial and energy sectors and World Water Day (2007) states that the amount of water used by these sectors will increase by 60 – 90% by 2050. These challenges in fresh water competition have focused attention on water management in agriculture, with the goal of producing food with less water. In South Africa, water demand for irrigation is high and water scarcity has resulted in a reduction in food production (Farmers' Weekly, 2016). This clearly illustrates that further improvements of water use in agriculture need to be considered in order to ensure that industries, like citrus, remain sustainable.

Citrus is an important woody perennial crop worldwide, covering over 5.4 million ha, of which 4.1 million ha are planted with oranges (Carr, 2012). The citrus industry in South Africa is ranked as one of the world's leading exporters and covers approximately 64 202 ha of land in Limpopo, Mpumalanga, KwaZulu-Natal, Eastern Cape and Western Cape provinces (CGA, 2012). The citrus industry is labour intensive and it is estimated to employ more than 600 000 people, which support more than a million households (DAFF, 2012)

South Africa produces a wide range of citrus which includes, sweet oranges (Valencias, Navels and Midseason), Grapefruit and Pummelos, Soft citrus, Lemons and limes. In Figure 1.1 information on the area planted with citrus in South Africa is presented (CGA, 2012). The largest area planted is Valencias (25 398 ha), followed by Navels (14 832 ha), Grapefruit (9 477 ha) and Soft citrus (5 200 ha) (CGA, 2012).

Variety	(ha)
Valencia	25 398
Navel	14 832
Grapefruit	9 477
Soft Citrus	5 200
Lemon	4 726
Midseason	636
Pummelo	86
Grand Total	**60 355**

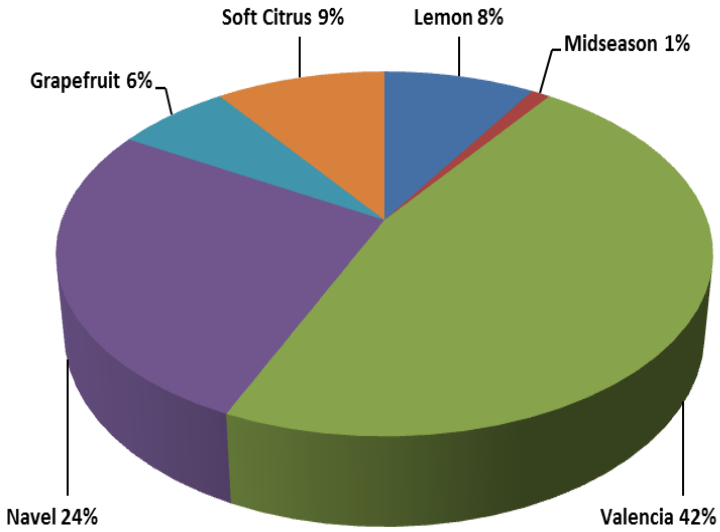

Figure 1.1 Hectares of citrus planted per variety in South Africa (CGA, 2012)

Citrus trees are evergreen and therefore require water all year round (Carr, 2012). However, water use varies depending on the tree age, tree size, citrus species, climate, soil type and the type of the irrigation system. In a previous Water Research Commission (WRC) project mature citrus orchards transpired approximately 680 mm per annum (Taylor et al. 2015). Water stress in citrus can affect all development processes, as well as fruit quality, which can occur even prior to a tree showing symptoms of water stress (Falivene et al., 2006). It is therefore important that citrus trees receive sufficient water and that irrigation is scheduled according to the needs of the plants. Irrigation scheduling entails determining how much water should be applied to a crop and when this water should be applied (Annandale, 1999). Proper irrigation scheduling is the main component of water management and it requires knowledge of the daily water use of the crop (i.e. the amount of water transpired per plant), the soil water-holding capacity and lower limit of soil water for each crop and the amount of water applied to the field (Rousseaux et al., 2009). To achieve this, accurate estimates of transpiration under non-limiting conditions are required in order to determine the upper limit of water use (transpiration only) by the crop (Villalobos et al., 2013). Sap flow

techniques are among the most widely accepted and appropriate techniques for determining transpiration in woody plants (Bleby et al., 2004). The techniques include heat pulse velocity techniques, heat dissipation techniques (also known as the Granier method) and the stem steady state heat energy balance method (Lascano et al., 1992). The principle governing each method differs, but common to all is the use of heat as a tracer. They differ in their operating principles in terms of installation and calculations and they also have different benefits and drawbacks. However, measurements using these techniques are generally considered to be reliable and robust (Smith and Allen, 1996). Heat pulse techniques are most preferred due to the simplicity of their instrumentation and low power requirements (Smith and Allen, 1996). They are inexpensive, easy to install and suited to automation in terms of collecting data and storage. In addition, heat pulse techniques allow the measurement of sap flow through the use of thermocouples at different depths taking into account the variability of sap flow in the xylem across the cross sectional area of the stem (Green et al., 2003).

It is, however, widely acknowledged that the methods often tend to over or underestimate sap flow and consequently, many authors agree that it is necessary to perform species-specific calibration when using these techniques, due to associated limitations in measurements. The calibration can be accomplished by comparing the rate of sap flow determined by sap flow techniques with that measured using an independent method. This can be performed using the cut stem or potometer experiment in the field, in a greenhouse on lysimeters or in the laboratory by forcing water through cut branches or stems, using the stem perfusion method (Fernández et al., 2006, Green and Clothier, 1988, Smith and Allen, 1996, Steppe et al., 2010). For instance, studies by Fernández et al. (2006) on orange (*Citrus sinensis* (L.) Osbeck. ‘Cadenero’) trees, revealed that the heat pulse velocity technique underestimated sap flow by 12%, but this has yet to be done on other citrus species. In addition, sapwood structure of some species does not adhere to the original definition of a homogenous and porous material (Marshall, 1958). If the distance between the xylem vessels is too large, the time required for thermal equilibrium between sap and woody matric becomes significant and affects the transmission and measurement of the heat pulse (Smith and

Allen, 1996). As citrus has diffuse porous sapwood, meaning that it has narrower vessels which are packed more densely per unit wood area (Taneda and Sperry, 2008), this may need to be taken into account when using these techniques. The study therefore aims to calibrate and test various sap flow techniques in various citrus species using the stem perfusion method.

1.1 Hypotheses

- The calibration of sap flow techniques in citrus is necessary as the sapwood structure is diffuse porous and therefore does not adhere to the idealised theory of heat pulse measurements.
- The stem perfusion method will allow the accurate and robust calibration of sap flow techniques for citrus trees, because the method allows direct comparison of sap flux density determined by the sap flow technique with that determined gravimetrically.
- The calibration factor for the various sap flow techniques for citrus will be consistent across cultivars, but will differ for different citrus species, as a result of differences in xylem anatomy between species, relating to vessel size and spacing.
- Heat pulse techniques are more accurate and robust than the thermal dissipation method.
- The heat ratio (HR) method has the potential to capture low flow rates better than the compensation heat pulse method (CHP), but the CHP method will capture high flow rates better than the HR method.

1.2 Aims and objectives

The overall aim of this study is to calibrate and test the most appropriate sap flow technique for citrus using the stem perfusion method.

Specific objectives

- To measure sap flux density in stem segments of 'Delta' Valencia, 'Bahaininha' Navel, 'Nadorcott' Mandarin, 'Star Ruby' Grapefruit and 'Eureka' Lemon using sap flow techniques, whilst determining the rate of water moving through the stem gravimetrically.
- To conduct measurements under a wide range of sap flow densities in a number of cut stems.
- To examine the potential and adequacy of each technique in different citrus species.
- To calculate the correction factors for each citrus variety/cultivar.
- To determine if xylem anatomy of citrus is the same across cultivars and species and if this impacts the empirical calibration.

Outline of the dissertation

The outline of the chapters for this dissertation is as follows: Chapter 1 presents the general introduction for the study and includes the hypotheses, aims and objectives. Chapter 2 is a review of the different sap flow techniques and different independent methods used to calibrate sap flow techniques. Chapter 3 details the laboratory methodology for both calibration of the three sap flow techniques performed using the stem perfusion method and for the analysis of citrus sapwood anatomy. Chapter 4 presents the results from the calibration of sap flow techniques, whilst Chapter 5 presents the results of the citrus sapwood anatomy analyses. Chapter 6 is the discussion of the results and includes conclusions from the study. A list of the references used in all chapters is presented in the final chapter. The appendix includes details of the statistical analyses.

CHAPTER 2: LITERATURE REVIEW

2.1 Sap flow techniques

Sap flow techniques are among the most widely accepted and appropriate methods for determining transpiration in woody plants. These systems are easy to automate, the data is fairly easily interpreted, they do not alter the microclimate of the plant and they can estimate transpiration over extended periods of time (Smith and Allen, 1996). Whilst a number of sap flow techniques are available, a distinction needs to be made between methods which measure sap flow rates (g h^{-1}) and methods which measure sap flux density (cm^3 cm^{-2} h^{-1}, also expressed as cm h^{-1}) (Vandegehuchte and Steppe 2013). The first method measures total sap flow in a plant stem or stem section and is very useful for determining whole plant water use, whilst sap flux density measurements estimate the amount of sap flowing through a certain surface per time and are well suited to investigate the variation of sap flow within plants. Whilst the theory between the various techniques differs, they all have one thing in common, i.e. they use heat as a tracer to estimate sap flow. Methods which measure sap flow rates include the stem heat balance technique (Vieweg and Ziegler, 1960). For the purposes of this study, where sap flow systems are to be calibrated for mature citrus orchards, the stem heat balance technique will not be discussed further. This is due to the fact that the method is not readily applicable to large stems of >15 cm diameter, different stem sizes require specific sensors; it is expensive and has high power usage (Smith and Allen, 1996). Only sap flux density methods will be used in this study and will be discussed further.

Sap flux density methods are divided into three major categories, viz. those which use a pulse of heat and include the heat ratio (HR), compensation heat pulse (CHP), Tmax, calibrated average gradient and Sap flow+ methods. Those that apply continuous heat include the thermal dissipation (TD) or Granier method and heat field deformation (HFD) (Vandegehuchte and Steppe, 2013). Three techniques (HR, CHP and TD) that have been used successfully in a number of studies within the stem, and even within the branches, of woody plants will be the focus of this study. Each method has associated

advantages and disadvantages related to accuracy, required parameters, ease of installation and use, ease of data interpretation, cost, power requirements, systematic error and available expertise (Table 2.1). The major disadvantage of these methods is the underestimation of sap flow, which occurs as a result of wounding caused by the implantation of sensors into the sapwood. It is therefore important to understand how each technique works under different circumstances and to test and calibrate the various sap flow techniques prior to the measurement phase, as it is imperative that accurate transpiration rates are captured. This chapter will provide the basic theory of the various sap flow techniques and how these techniques can be calibrated using other independent methods, such as stem perfusion, cut tree and weighing lysimeters. The calibration can be conducted on either tree stems (Bush et al., 2010, Hultine et al., 2010, Fernández et al., 2001) or branches (Clearwater et al., 1999, Cohen et al., 1981, Paudel et al., 2013, Burgess and Dawson, 2008, Nadezhdina et al., 2007).

Table 2.1 Advantages and disadvantages of sap flow techniques and the parameters required to determine sap flux density.

Method	Advantages	Disadvantages	Parameters to be measured
Heat ratio method	Measures low flow rates and even reverse sap flow Has a simple function to describe wound effects Low power requirements Simple instrumentation The data is produced in the form of an electrical signal, suitable for further processing or storage on data loggers Less susceptible to natural temperature gradients within the sapwood	Performs poorly at high flow rates (>45 cm^3 cm^{-2} h^1) Damage occurs during the drilling of holes for sensor installation, which must be accounted for by wounding correction coefficients Accuracy depends on the correct spacing during sensor installation Requires species-specific calibration	Sapwood density Sapwood water content Wound width Area of sapwood Axial thermal diffusivity of wood Specific heat capacity of the woody matrix Specific heat capacity of the sap
Compensation heat pulse	Low power requirements Simple instrumentation The data is produced in the form of an electrical signal, suitable for further processing or storage on data loggers Less susceptible to natural	Incapable of resolving reverse, low or very high flux densities (<5 cm^3 cm^{-2} h^{-1} and >100 cm^3 cm^{-2} h^{-1}) Damage occurs during the drilling of holes for sensor installation, which must be accounted for by wounding correction coefficients	Sapwood density Sapwood water content Wound width Area of sapwood Specific heat capacity of the woody matrix Specific heat capacity of the sap

	temperature gradients within the sapwood Independent of thermal diffusivity	Accuracy depends on correct spacing during sensor installation Requires species-specific calibration	
Thermal dissipation method	Can be used in large diameter trees Allows estimation of low, average and high sap flux densities (0-80 cm^3 cm^{-2} h^{-1}) Simple and reliable method Low cost Accuracy does not depend on probe spacing, provided the reference probe is not influenced by the heated probe	Damage occurs during the drilling of holes for sensor installation Natural temperature gradients in the sapwood impact measurement accuracy Difficult to detect zero flux High power requirement Not capable of detecting reverse flow Requires species-specific calibration	Zero flow rate is required for calculations Natural temperature gradients Proportion of the length of the heater probe in contact with the sapwood and inactive xylem

2.2 Heat pulse velocity methods

Heat was first used as a tracer to determine sap flow by Huber and colleagues in the 1930's (Huber, 1932). This technique has been refined over the intervening years and a number of heat pulse velocity (HPV) methods have been developed, which are appropriate for estimating transpiration in orchards and forest trees. It is recommended that these sensors are only applied to woody stems larger than about 40 mm in diameter, due to the fact that they must be inserted into the stem (Allen et al., 2011).

Heat-pulse techniques are preferable most of the time due to their simplicity of instrumentation, and low power requirements (Smith and Allen, 1996). They are inexpensive, easy to install and suited to automation in terms of collecting data and storage. These techniques allow the measurement of sap flow through the use of thermocouples at different depths, which take into account the variability of sap flow in the xylem across the cross sectional area of the stem (Green et al., 2003, Poblete-Echeverría et al., 2012). The greatest disadvantage of this method is the damage that occurs during the drilling of holes for sensor installation, which disrupts the natural flow of sap in the stem (Swanson and Whitfield, 1981a). This problem, however, applies to all probe-based sap flow techniques (Clearwater et al., 1999). Wounding results from the removal of wood by the drill bit and from mechanical interruption of vessels at the edge of the drilled hole, which causes blockage and discolouration of the xylem vessels (Barrett et al., 1995).

As a result of wounding, correction factors are required for all of these techniques in order to correct the heat pulse measurements for the wound effect resulting from probe insertion. Initially, Marshall's idealized theory assumed that the probes have no influence on the sap flow measurements. However, the study by Green et al., (2003) and Swanson and Whitfield (1981) showed that the choice of probe material can also influence sap flow measurements, although it is not as influential as wound width. From the study of Green et al. (2003), wound width was shown to have a great influence on

sap flow measurements and was dependent on the probe diameter. Figure 2.1 shows that when using a small probe (1.6 mm) under flow rates of 40 cm h^{-1}, the uncorrected measurements are approximately 50% of the actual sap flow, whilst under higher flow rates of 80 cm h^{-1}, the uncorrected sap flow measurements for the large probe (3.2 mm) was approximately 25% of the actual flow.

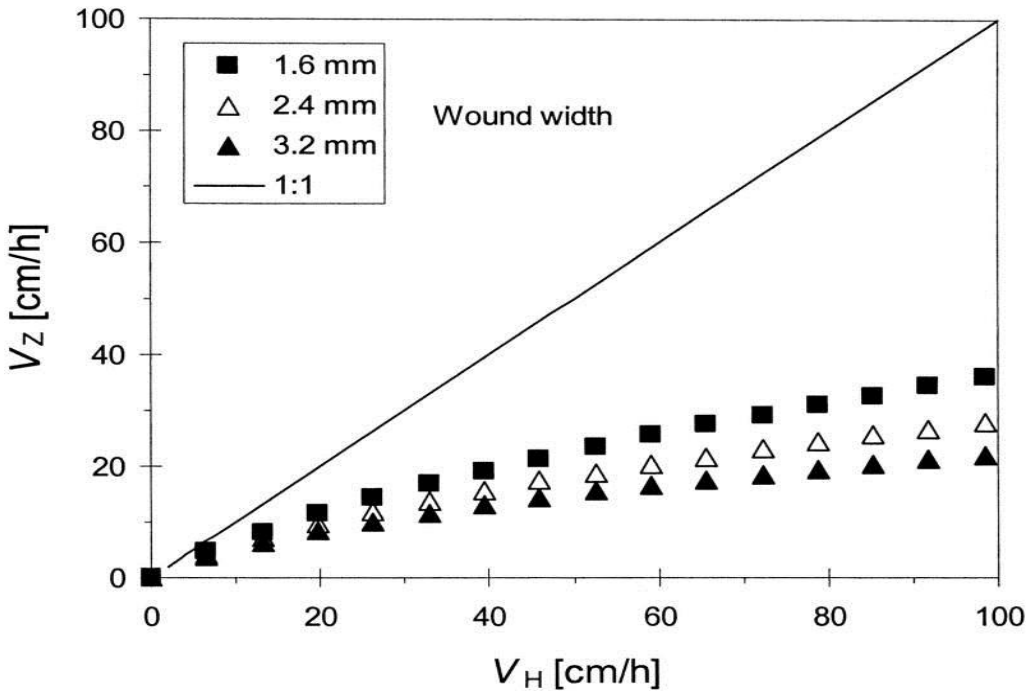

Figure 2.1 The influence of wound width on relationship between the actual flow (V_h) and heat pulse velocity (V_Z) for the compensation heat pulse method (Green et al., 2003).

Therefore, in order to obtain stable measurements using heat pulse methods, it is important to have an accurate estimate of wound width and a single calibration for each wound width is considered adequate (Green et al., 2003). Also, care needs to be taken when inserting the probes and a good alignment of the probes is required for the calculation of the heat pulse velocity (Burgess et al., 2001).

It is not only the above mentioned limitations that lead to underestimation or errors associated with sap flow measurements. There is also the assumption made in the HPV theory, that the stem is infinitely homogenous, allowing heat exchange between the water in the conducting tissue and the matrix of the xylem to take place without delay (Green and Clothier, 1988, Marshall, 1958, Swanson and Whitfield, 1981a). This assumption only applies to those species, such as apples, with small and fairly uniform vessels (Green and Clothier, 1988). However, in some species, such as kiwifruit and citrus, the sapwood does not adhere to the original definition of a homogenous and porous material, as the sapwood possesses few, large and wide vessels and this affects the transmission and measurement of the heat pulse (Hall et al., 1998, Green and Clothier, 1988).

2.2.1 Compensation heat-pulse (CHP) method

The CHP method was initially developed by Huber and Schmidt (1937) and was later improved by Marshall (1958). This method performs well under high flow rates, but has the limitation of not being able to resolve sap flux densities lower than 2-10 cm^3 cm^{-2} h^{-1}. It is also incapable of resolving zero or reverse flow rates (Edwards and Warwick, 1984).

When conducting CHP measurements, the sensors are inserted radially into the xylem at the position where the sap flow will be estimated (Fernández et al., 2006). The heater is inserted into a central hole and temperature probes are inserted in upstream and downstream holes (Figure 2.2). The upstream probe is placed 0.5 cm from the heater probe, whilst the downstream probe is placed 1 cm from the heater probe. Following the release of a pulse of heat (pulse length can be varied depending on sapwood and environmental conditions) the temperature increases more at the closer upstream probe than at the downstream probe due to conduction of heat. However, the heat which is carried by the moving sap, together with conduction, rapidly warms the downstream probe so that after a certain time (t_c) the temperature of the two probes is equal. This is the time it takes for the peak of the heat pulse to move midway between the two temperature sensors. As this time decreases the sap velocity increases.

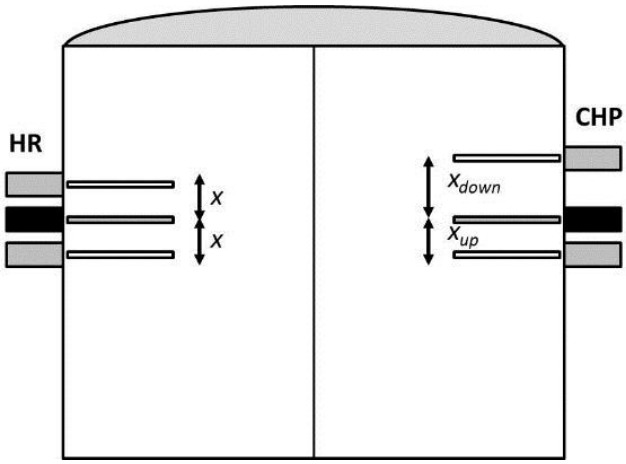

Figure 2.2 Schematic representation of the two most common heat pulse techniques, with heater probes in black and thermocouples in grey (HR – heat ratio method; CHP – compensation heat pulse method) (Steppe et al., 2010).

The heat pulse velocity (V_h) can then be calculated as:

$$V_h = \frac{x_{down} - x_{up}}{2t_c} \quad (1)$$

Where, t_c (s) is the time to thermal equilibrium of the downstream and upstream thermocouples after application of the heat pulse and x_{down} and x_{up} are the distances (cm) between the downstream and upstream thermocouples and the heater probe. The time multiplier (3600 for an hour in this instance) varies according to the logging interval.

Due to the disturbance of the natural xylem conditions caused by the installation of probes, V_h can be corrected (V_h', cm h^{-1}) for the effect of wounding and to account for the influence of materials used to construct the heater and sensor probes. The wound coefficients are calculated according to the numerical model of Swanson and Whitfield (1981) as follows:

$$V_h' = a + bV_h + cV_h^2 \quad (2)$$

where a, b and c are the correction coefficients corresponding to wound width (cm) (*z*):

$$a = -11.744z^2 + 14.596z - 1.6424 \quad (3)$$

$$b = 7.2088z^2 - 6.4412z + 2.2024 \quad (4)$$

$$c = 2.3935z^2 - 0.3194z + 0.0259 \quad (5)$$

The corrected heat pulse velocities can then be converted to sap flux densities (*SFD*, $cm^3\ cm^{-2}\ s^{-1}$) using the following equation:

$$SFD = \frac{\rho_{sm} c_{sm}}{\rho_s c_s} V_h' = \frac{\rho_b}{\rho_s}\left(m_c + \frac{c_{dw}}{c_s}\right) V_h' \quad (6)$$

where ρ_{sm} is the density (g cm^{-3}) of sap plus the woody matrix (including gas), c_{sm} is the specific heat capacity (J $g^{-1}\ K^{-1}$) of sap plus the woody matrix (including gas), ρ_s is the density of sap assumed equal to water (=1.0 g cm^{-3}), c_s is the specific heat capacity of sap, assumed to equal water (= 4.186 J $g^{-1}\ K^{-1}$), ρ_b is the dry wood density (g cm^{-3}), m_c is the water content of the sapwood, c_{dw} is the specific heat capacity of oven-dry wood and c_{dw}/c_s is the normalized specific heat capacity of dry wood, often assumed constant at 0.33 (=1.380/4.186) (Dunlap 1912; Edwards and Warwick 1984). Volumetric sap flow is subsequently calculated from the product of sap velocity and conducting sapwood area. Gross wood cross-sectional area is calculated from under bark radius and heartwood is discounted by observing visual discolouration of wood or staining conducting sapwood with safranin (Fernández et al., 2006).

2.2.2 Heat ratio (HR) method

The HR method was developed by Burgess et al. (2001) and differs from the CHP method in that the temperature sensors are placed at equal distances (0.5 cm) above and below a heater probe (Figure 2.2). The HR method allows the determination of low flow rates (0-10 $cm^3\ cm^{-2}\ h^{-1}$) and even reverse flow to be measured, but it performs poorly at high flow rates (>45 $cm^3\ cm^{-2}\ h^{-1}$) (Vandegehuchte and Steppe, 2013). As compared to the CHP method, the HR method has an improved measurement range

and resolution, procedures to correct for physical and thermal errors in sensor placement, and simple functions to describe wound effects (Burgess et al., 2001). The V_h is calculated according to (Marshall, 1958) as

$$V_h = \frac{k}{x} ln\left(\frac{v_1}{v_2}\right) 3600 \quad (7)$$

where *k* is the thermal diffusivity of green (fresh) wood (assigned a nominal value of 2.5 x 10^{-3} cm^2 s^{-1} (Marshall, 1958), *x* is distance in cm between the heater and either the upper or lower thermocouple, v_1 and v_2 are increases in temperature after the heat pulse is released (from initial temperatures), as measured by the upstream and downstream thermocouples and 3600 converts seconds to hours. The coefficients used in CHP method to account for wounding do not pass through the origin, and the resulting corrections do not hold at low and reverse rates of sap flow. Burgess et al. (2001) therefore, developed a new numerical model for the HR method as follows:

$$V_h' = bV_h + cV_h^2 + dV_h^3 \quad (8)$$

where V'_h is the corrected heat pulse velocity and the correction coefficients corresponding to wound width (z) are calculated as:

$$b = 6.6155z^2 + 3.332z + 0.9236 \quad (9)$$

$$c = -0.149z^2 + 0.0381z - 0.0036 \quad (10)$$

$$d = 0.0335z^2 - 0.0095z + 0.0008 \quad (11)$$

Sap flux density (*SFD*) can then be calculated as follows (Burgess et al. 2001):

$$SFD = \frac{V_h' \rho_b (c_w + m_c c_s)}{\rho_s c_s} \quad (12)$$

where c_w is the specific heat capacity of the wood matrix (1200 J kg^{-1} $°C^{-1}$ at 20°C (Becker and Edwards 1999). The rest of the abbreviations are the same as are described for the CHP method (eq. 6). Volumetric flow can then be derived as the

product of SFD and cross-sectional area of conducting sapwood, as described in section 2.2.1.

2.3 Thermal dissipation (TD) method

The TD method is an empirical method for measuring sap flux density that was developed by (Granier ,1987) and is commonly used for measuring water use of whole trees (Lu et al., 2004), including those with a large diameters (Allen et al., 2011). The method is simple, low cost and reliable, provided it is used properly. Limitations, such as the effect of changes in natural temperature gradients (NTG) in the sapwood, must be considered. The major difference between the heat pulse methods and the TD method is that with the TD method heat is applied constantly and not as a pulse (Thomsen et al., 2008).

The TD method is based on the temperature difference (ΔT) between a constantly heated needle and an unheated needle inserted in the xylem approximately 10 cm below (upstream) the heated needle (Figure 2.3). As sap flow increases the heat is dissipated more rapidly from the heated needle and ΔT decreases. Zero flow conditions are, however, required for calculations and reverse flows cannot be detected with this method. It is also necessary to calibrate this technique for different species (Vandegehuchte and Steppe, 2013). Natural temperature gradients within the sapwood, which are often attributed to thermal heat storage in the soil, stem and root tissues, result in temperature differences between the sap and plant tissues. Whilst insulation of the probes and shielding them from direct radiation minimizes these gradients, the effects of NTG cannot be excluded completely and can result in errors of more than 100% if corrections are not made (Lu et al. 2004; Reyes-Acosta et al. 2012).

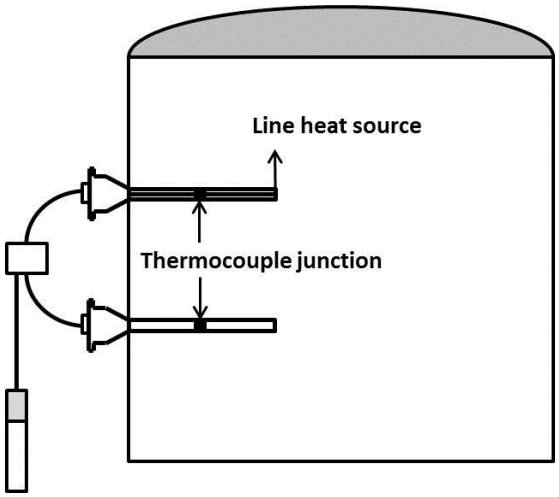

Figure 2.3 Schematic representation of the thermal dissipation technique (Steppe et al., 2010).

Sap flux density is calculated from an empirical equation developed by Granier (1985).

$$SFD = 0.000119\left(\frac{\Delta T_o - \Delta T}{\Delta T}\right)^{1.231} \quad (13)$$

where ΔT_o is the temperature difference (ΔT) assessed during a period of zero flow (i.e. the maximum temperature difference between the two needles). Although the original goal of the TD method was to develop a species independent empirical formula (Granier, 1987), which did not need to take wounding into account (Lu et al., 2004), it is now evident that species-specific calibration is required (Steppe et al., 2010, Vandegehuchte and Steppe, 2013), as underestimations have been found in a number of species when using this technique (Lundblad et al., 2001, Bovard et al., 2005, Iida and Tanaka, 2010, Silva et al., 2006). The empirically determined coefficients (0.000119 and 1.231) therefore do not apply under all conditions and for all species.

2.4 Calibration of sap flow techniques

Both the HPV and TD methods are intrusive, as they involve the physical implantation of measuring probes into the tree. A number of authors (Cohen and Fuchs, 1989b, Green and Clothier, 1988, Smith and Allen, 1996) have advised that species–specific calibration coefficients need to be determined for these methods. Some techniques work better in certain species compared to others, which is possibly due to differences in the xylem anatomy of the sapwood. Fernández et al. (2006) conducted a study on the relationship between xylem anatomy and the calibration of the CHP method in olive, plum and orange. Results showed that xylem anatomy varied between the three species. Orange trees had larger and fewer xylem vessels, as compared to plum and olive trees, whilst plum had the greatest total lumen vessel area per transverse section of the three species (Table 2.2). These authors concluded that these differences could have contributed to the different calibration results found for the three species. Green and Clothier (1988) concluded that vessel lumen diameter and the distance between vessels affects the thermal homogeneity of the sapwood, which impacts the heat transmission through the sapwood, and therefore the heat pulse measurement. The importance of xylem anatomical difference in determining calibration coefficients for HPV techniques underlines the necessity for doing species-specific calibration and the reason for the current study.

The calibration of HPV and TD techniques is performed by testing the sap flux density method against an independent and reliable measure of sap flux density or transpiration. There are a number of independent methods that can be used to assess the accuracy of sap flow techniques. These methods include weighing lysimetry, cut-tree methods, stem perfusion and micrometeorological methods (Poblete- cheverria et al. 2012). Table 2.3 provides a summary of literature on the calibration of sap flow techniques in various species. The calibration of sap flow techniques using micrometeorological methods will not be discussed further in this dissertation.

Table 2.2 Differences in xylem vessel anatomy of olive, plum and orange trees (Fernández et al. 2006)

Species	Depth (mm)	Lumen diameter (µm)	Distance between groups vessels (µm)
Olive	5	40.3 ±4.5	102.9±33.1
	25	36.9 ±4.6	126.9±36.8
Plum	5	45.4±4.9	81.9±21.5
	25	44.8 ±2.9	80.1±20.3
Oranges	5	83.9 ± 13.6	280.1±60.3
	25	79.5 ±11.4	340.7±133.8

Table 2.3 Examples of the calibration of sap flow systems (citrus examples are indicated in bold text). CHP – compensation heat pulse method; HFD – heat field deformation; HPV – heat pulse velocity; HR – heat ratio method; SHB – stem heat balance; TD – thermal dissipation

Reference	Technique	Calibration method	Species
Cohen et al. (1981)	T -max	Stem perfusion	*Populus alba,* **Citrus**: *Platanus orientalis*
Green and Clothier (1988)	CHP	Cut tree/potometer	Apple, kiwifruit
Olbrich (1991)	CHP	Cut tree/potometer	*Eucalyptus*
Dugas et al. (1992)	SHB	Weighing lysimeter	*Prosopis glandulosa*
Shackel et al. (1992)	SHB	Weighing lysimeter	Peach
Caspari et al. (1993)	CHP	Drainage lysimeter	Asian pear
Barrett et al.(1995)	CHP	Cut tree/potometer	*Eucalyptus maculate, Doryphora sassafras, Ceratopetalum apetalum*
Hatton et al. (1995)	CHP	Cut tree/potometer	*Eucalyptus populnea*
Teskey and Sheriff (1996)	CHP	Cut tree/potometer	*Pinus radiate*
Marshall et al. (1997)	CHP	Stem perfusion	*Eucalyptus camaldulensis*

Reference	Technique	Calibration method	Species
Hall et al. (1998)	CHP	Stem heat balance	*Populus trichocarpaxdeltiodes*
Lu and Chacko (1998)	TD	Weighing lysimeter	Mango
Braun and Schmid (1999)	TD	Stem perfusion and weighing lysimeter	Grapevine
Burgess et al. (2000)	HR	Weighing lysimeter	*Eucalyptus marginata*
Burgess et al. (2001)	HR	Weighing lysimeter	*Eucalyptus marginata*
Fernández et al. (2001)	CHP	Stem perfusion and cut tree/potometer	Olive
Green et al. (2003b)	CHP	Weighing lysimeter	Willow
	T-max	Weighing lysimeter	Poplar
Zreik et al. (2003)	CHP	Weighing lysimeter	Plum,.mandarin
Bleby et al. (2004)	CHP, HR	Weighing lysimeter	*Eucalyptus marginata*
Williams et al. (2004)	HR	Eddy covariance	Olive
Alarcón et al. (2005)	CHP	Weighing lysimeter	**Lemon**
Dragoni et al. (2005)	CHP	Whole-tree gas-exchange	Apple
de Oliveira Reis et al. (2006)	TD	Stem perfusion	Papaya
Fernández et al. (2006)	CHP	Cut tree/potometer	Olive, plum, **orange**
McCulloh et al. (2007)	TD	Pallet truck scale and potted tree	*Pseudobombax septenatum, Calophyllum longifolium*
Conceição and Ferreira (2008)	TD	Eddy covariance and microlysimeters	Pear
Nortes et al. (2008)	CHP	Weighing lysimeter	Almond
Pernice et al. (2008)	CHP	Whole-tree gas-exchange	Olive
Taneda and Sperry (2008)	TD	Stem perfusion	*Quercus gambelii,, Acer grandidentatum*
Uddling et al. (2009)	TD	Cut tree/potometer	*Populus tremuloides, Betula papyrifera*
Bush et al. (2010)	TD	Stem perfusion	*Elaeagnus angustifolia, Gleditsia triacanthos,*

Reference	Technique	Calibration method	Species
			Quercus gambelii, Sophora japonica, Populus fremontii, Tilia cordata
Hultine et al. (2010)	TD	Stem perfusion	*Tamarix* spp.
Ayutthaya et al. (2010)	TD	Cut tree/potometer	*Hevea brasiliensis*, *Mangifera indica*, ***Citrus maxima***
Steppe et al. (2010)	CHP, TD, HFD	Stem perfusion	*Fagus grandifolia*
Poblete-Echeverría et al. (2012)	CHP	Eddy covariance and microlysimeters	Grapevine
Paudel et al. (2013)	TD	Weighing lysmeter	Persimmon, Apple
	TD	Stem perfusion	Apple, Nectarine

2.4.1 Weighing lysimetry

Weighing lysimeters are considered the most accurate method for crop evapotranspiration estimations, as a result of the measurement being simple and direct, without injury to the plant (Aboukhaled et al., 1982). Transpiration can also be determined provided evaporation from the soil surface can be eliminated by using a soil cover. They are, however, expensive to install and maintain and have therefore not been used extensively for crop water use measurements, especially tree crops. This should, however, be considered the “gold standard” for tree transpiration, to which sap flow measurements should be compared, as it is a measure of whole plant transpiration that does not incur injury to the plant or embolisms in the xylem tissue. The calibration of sap flow systems with weighing lysimetry is often performed in potted trees on smaller balances or specially constructed lysimeters with load cells (Alarcón et al., 2005, Burgess et al., 2000).

2.4.2 Cut tree method or potometer

This method of calibration is usually conducted under field conditions and it includes the use of a whole tree. The cut tree method is intrusive and is likely to cause changes

in leaf water potential and stomatal conductance which may affect the measurements (Wullschleger et al., 1998). An additional disadvantage is that the rate of sap flow changes when cutting the stem, because a portion of the xylem vessels become occluded during cutting (Marshall, 1958). Some trees are also very large and it can be difficult to handle them and may require specialized equipment to keep the tree upright. Following cutting the tree under water, the bottom of the stem should be shaved to remove possible embolisms and then placed in a container with water, covered with a plastic cover to prevent evaporation (Figure 2.4). The container can either be placed on a balance to record mass loss or the volume of water transpired can be recorded following refilling to a pre-determined level (Knight et al., 1981). The advantage of this method is that it provides a direct measure of transpiration for the whole tree and a dye can be added to the water for the determination of conducting sapwood area (Hatton et al., 1995). In addition calibration can be achieved in the same tree in which the measurements were made. It is, however, destructive and in some instances the tree may only survive for 24-48 hours following cutting.

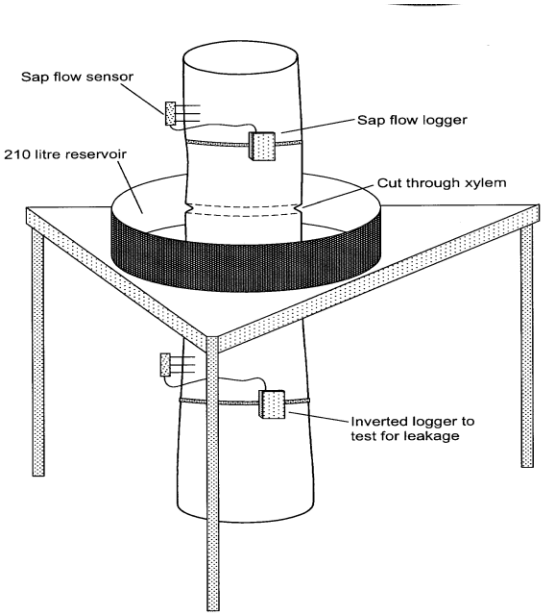

Figure 2.4 The cut tree method for calibrating sap flow techniques (Vertessy et al., 1997).

2.4.3 Stem perfusion method

The stem perfusion method is performed in a laboratory and involves the forcing of water through a section of a stem or branch in which probes are inserted, as shown in Figure 2.5 (Fernández et al., 2001, Steppe et al., 2010). The stem is attached to a cylinder and the sap that moves through the stem is collected in a beaker and weighed using an electronic balance. Pressure is applied and maintained during the measurements to achieve the desired flow rates, which are comparable to the flow rates obtained in the field. The gravimetric measurements made using the electronic balance are compared with those measured with sap flow sensors, following normalisation for conducting sapwood area.

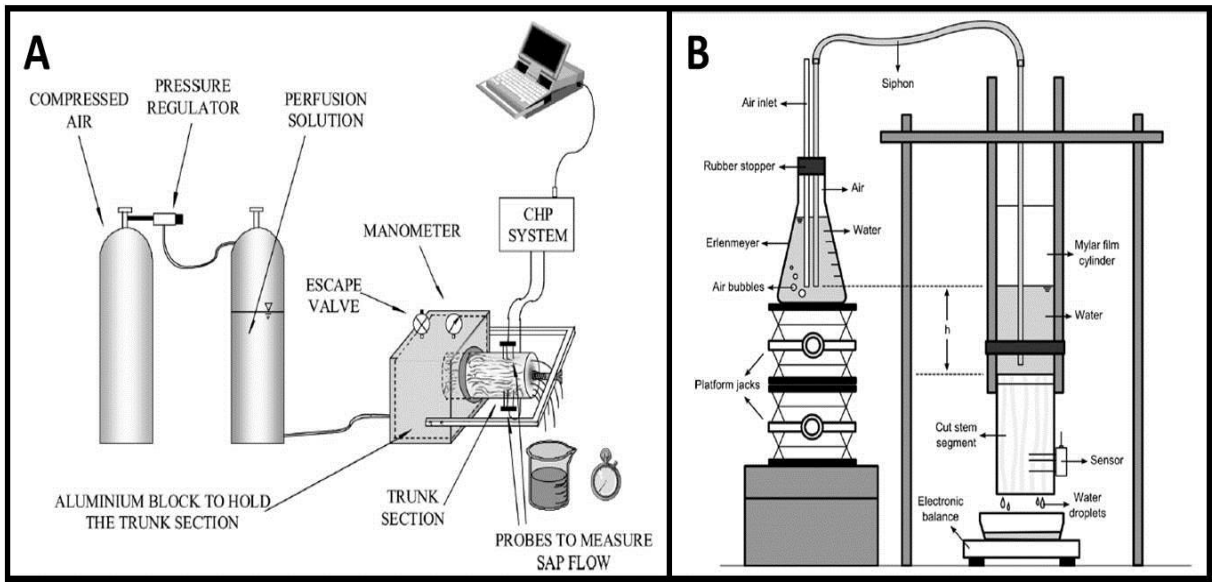

Figure 2.5 Experimental set-up for calibrating sap flow systems using the stem perfusion assay. A) The pressurised system described by Fernández et al. (2001) and B) the Mariotte's bottle principle described by Steppe et al. (2010).

The advantage of this method is that it allows the direct comparison of sap flux densities and calibration can be performed in a number of species, using excised branches, fairly quickly. The disadvantage with this method is the same as for the cut tree method, as cutting of the stem or branch can result in some xylem vessels becoming occluded during cutting. The stem characteristics such as sapwood conducting area, wood density and stem water content which are required for calculation of sap flux density can be easily obtained from the cut stem or branch segments.

2.5 Calibration of sap flow techniques using independent methods in various species

Calibration results from various authors using various techniques and species are quite variable. Most authors have demonstrated that the techniques are accurate and reliable when used for estimation of transpiration (Alarcón et al., 2005, Barrett et al., 1995, Braun and Schmid, 1999, Burgess et al., 2000, Burgess et al., 2001, Dugas et al., 1992, Fernández et al., 2001, Green et al., 2003, Olbrich, 1991, Teskey and Sheriff, 1996). Accuracy did, however, depend on the accurate estimation of wound widths and accurate probe spacing (Alarcón et al., 2005, Fernández et al., 2001, Green and Clothier, 1988, Green et al., 2003, Hall et al., 1998, Olbrich, 1991, Pernice et al., 2008, Shackel et al., 1992). In contrast, some authors have found that sap flow techniques tend to underestimate transpiration (Bleby et al., 2004, Cohen et al., 1981, Green and Clothier, 1988, Hall et al., 1998, Hatton et al., 1995, Hultine et al., 2010, Lu and Chacko, 1998, McCulloh et al., 2007, Paudel et al., 2013, Poblete-Echeverría et al., 2012, Steppe et al., 2010). Some authors have even found overestimations (Caspari et al., 1993, Fernández et al., 2006, Poblete-Echeverría et al., 2012). The underestimation of transpiration has largely been attributed to thermal inhomogeneity of the sapwood, resulting from large distances between the xylem vessels in the sapwood (Ayutthaya et al., 2010, Cohen et al., 1981, de Oliveira Reis et al., 2006, Fernández et al., 2006, Green and Clothier, 1988, Steppe et al., 2010). and due to the contact of the sensors with inactive sapwood (Clearwater et al., 1999, Paudel et al.,

2013). The calibration of the TD method was assumed to be valid for all types of species (Lundblad et al., 2001). However the original calibration was found not to be applicable to all species (Bush et al., 2010). As a result it is suggested that calibration be performed for each new species in which the technique is to be used (Steppe et al., 2010). The need for calibration of sap flow techniques has been reported in various citrus species (Ayutthaya et al., 2010, Cohen et al., 1981, Fernández et al., 2006), which emphasises the importance of this study to calibrate various techniques in various species of citrus.

2.6 Conclusions

Knowledge concerning the application of sap flow techniques in orchards is required in order to provide accurate measurements of transpiration and a reliable calibration is necessary to relate the estimated sap flow to the actual transpiration. Several studies have attempted to calibrate sap flow techniques, both in the laboratory and the field for different species. Differences exist between techniques, which results in them performing differently in different species under different circumstances and it may also depend on the aims and objectives of the particular study. For instance the CHP method is unable to resolve low sap flux densities, whilst the HR method accurately resolves low sap flux densities.

The accuracy of each method depends on a number of factors, including the amount of care taken during probe insertion. Plant sapwood structure and the shape of the tree stem also influences accuracy. For example, Avilés (1995) found the estimation of transpiration in avocado trees difficult as trunk shape made it difficult to accurately insert the probes and this had an influence on sap flow measurements.

Other limiting factors that may lead to the underestimation of sap flow measurements also need to be taken into consideration. These factors include the insertion of probes in non-conductive sapwood, heterogeneous flow density in the sapwood and

inadequate probe positioning (Ayutthaya et al., 2010). Therefore proper attention needs to be paid and taking the necessary precautions are crucial in order to achieve accurate results and to eliminate experimental errors, which can lead to under- or overestimation and affect the measurement of tree transpiration. It is also important to take more measurements in each stem by inserting a number of sensors in the stem at different depths.

Despite the limitations and errors associated with the use of sap flow techniques, many studies consider sap flow techniques to be useful and reliable tools for the estimation of plant water use. The technique also has the ability to be used for long term measurements. However, as the accuracy of these techniques is at times questionable, there is the need for species specific calibration. Therefore it is critical to test and calibrate these techniques on various citrus species prior to their deployment in the field for the determination of orchard scale transpiration. The stem perfusion technique represents an important method to test the accuracy of and calibrate the various sap flow techniques in a number of citrus species and varieties. The major advantage of this method is that it allows the direct comparison of sap flux density determined by the sap flow technique with that determined gravimetrically. Some sap flow techniques require a period of zero flow (e.g. TD method) and the stem perfusion stem method gives control over the flow rate, as it can be physically increased and decreased. All parameters (sapwood density, sapwood water content, area of sapwood, specific heat capacity of the woody matrix, specific heat capacity of the sap, sapwood moisture content) are also measurable using this system. Finally, it gives an opportunity of estimating sap flux densities from a wide range of different species of citrus, in a relatively short period of time.

CHAPTER 3: MATERIALS AND METHODS

Selection of plant material

Evaluation and calibration of two heat pulse velocity techniques and a constant heat technique were conducted in the Environmental Biophysics laboratory of the Department of Plant and Soil Sciences at the University of Pretoria. Calibration of the heat ratio (HR), compensation heat pulse (CHP) and thermal dissipation (TD) methods was done with the stem perfusion method for four different species of citrus trees, namely *Citrus sinensis* (Orange), *Citrus reticulata* (Soft citrus), *Citrus paradise* (Grapefruit) and *Citrus limon* (Lemon). Branch samples were collected from different orchards, i.e. 'Delta' Valencia and 'Star Ruby' Grapefruit were collected from Golden Frontiers Farm in Hectorspruit, 'Bahianinha' Navel from Dirishana and 'Nadorcott' Mandarin were collected from Naranja Farm, both in Burgersfort, all situated in the Mpumalanga Province. 'Eureka' Lemon was collected from the Hatfield Experimental Farm at the University of Pretoria. These are the most widely grown cultivars in South Africa, which have been selected for their good quality and wide adaptation to different climatic conditions of different areas. They are also species that were selected for a Water Research Commission (WRC) project (K5/2275/4) for field measurements of citrus water use. The trees were between 7 and 11 years old and the characteristics of the branches are given in Table 3.1 and Table 3.2. Branches that were approximately 20 cm in circumference and without any visible damage were selected in various orchards. The branches were cut at the base close to the main axis of the tree. The samples were marked to indicate direction of sap flow before they were immediately submersed into water to maintain moisture and to prevent embolism formation. The samples were then taken back to the laboratory and the experiment was immediately started.

Five different species of citrus (treatments) and a minimum of two branches of each cultivar (replicates) were used to calibrate the three sap flow techniques (Table 3.1 and Table 3.2).

Table 0.1 Stem characteristics of different citrus species used for calibration of both the HR and CHP methods with the stem perfusion technique.

Species	Replicate	Length (cm)	Circumferences (cm)	Cross sectional area (cm^2)	% Area of conducting sapwood	Area of conducting sapwood (cm^2)
***Citrus sinensis* 'Delta'**	1	28.0	15	17.9	10.5%	1.8
	2	26.0	14	15.6	31.6%	4.9
	3	25.0	14	15.6	16.2%	2.5
***Citrus paradise* 'Star Ruby'**	1	29.0	16.5	21.6	28.9%	6.2
	2	29.0	15	17.9	32.5%	5.8
	3	24.5	15	17.9	38.7%	6.9
***Citrus reticulate* 'Nadorcott'**	1	37.0	18	25.7	12.8%	3.3
	2	36.0	17.5	24.3	8.8%	2.1
***Citrus sinensis* 'Bahianinha'**	1	34.0	15	17.9	39.8%	7.1
	2	30.0	14.5	16.7	4.8%	0.8
***Citrus limon* 'Eureka'**	1	33.0	14.5	16.7	62.2%	12.8
	2	33.5	15.0	17.9	47.4%	8.7
	3	35.0	15.0	17.9	48.0%	9.7
	4	33.5	14.0	15.6	34.4%	7.8

Table 0.2 Stem characteristics of different citrus species used for calibration with the stem perfusion technique for the TD method.

Species	Replicate	Length (cm)	Circumferences (cm)	Cross sectional area (cm^2)	% Area of conducting sapwood	Area of conducting sapwood (cm^2)
***Citrus sinensis* 'Delta'**	1	28.0	15	20.3	12.3%	**2.5**
	2	26.0	14	16.7	14.3%	2.3
	3	25.0	14	15.6	8.6%	1.3
***Citrus paradise* 'Star Ruby'**	1	29.0	16.5	21.6	18.9%	4.0
	2	29.0	15	16.7	7.5%	1.2
	3	24.5	15	23.0	14.3%	3.2
***Citrus limon* 'Eureka'**	1	25.0	16.1	17.9	46.6%	8.3
	2	25.0	15.2	20.3	36.6%	7.4
	3	25.0	16	20.3	32.1%	6.5

2.7 Experimental setup

2.7.1 Heat pulse velocity

Each probe set consisted of two type-T copper-constantan thermocouples embedded in polytetrafluoroethene tubing with an outside diameter (OD) of 2 mm placed upstream and downstream of a 1.8 mm OD stainless steel line-heater probe. The probes were inserted to different depths within the xylem of each branch to cater for the radial variation in sap flux within the conducting sapwood. Two probe sets were inserted per method per stem at a depth of 1.0 cm and 1.5 cm below the bark. Depths were selected based on the size of the stem and to ensure that each probe set represented a similar percentage area of conducting sapwood. Holes were carefully drilled using a drill jig to ensure that the probes were correctly spaced and the holes drilled parallel to each other. For the HR method, thermocouples were placed equidistant (0.5 cm) upstream and downstream of the heater probe, whilst for the CHP method the upstream thermocouple was placed 0.5 cm from the heater probe and the downstream thermocouple 1 cm from the heater probe**.** The heater heated for 0.4 seconds. Heat pulse velocities were measured and logged at 10 min intervals using a CR1000 data logger and an AM16/32B multiplexer (Campbell Scientific Inc., Logan, Utah, USA). The thermocouples, heater probes and relay control modules were manufactured locally (Andrew Everson, Pietermaritzburg).

2.7.2 Thermal dissipation

For the TD method, the temperature difference ΔT (K), was measured between a constantly heated needle and an unheated needle located approximately 40 mm from each other. According to Vandegehuchte and Steppe (2013) exact spacing is less important in this method, as long as the reference probe is not influenced by the heated probe. The TD probe set (model TDP30, Dynamax Inc., Houston, TX, USA) consisted of two 30 mm long stainless steel needles with an OD of 1.2 mm. Holes were drilled into the branch using a drill guide supplied by the manufacturer. The probes were attached to a Dynamax FLGS-TDP XM1000 sap velocity system (Dynamax Inc., Houston, TX, USA), which consisted of a CR1000 logger, an AM16/32B multiplexer

and an adjustable voltage regulator (AVRD) that was set at 3V for the two TDP30 probes. Data was logged every 5 min.

2.7.3 Stem perfusion calibration system

A modified Mariotte-based verification system (Figure 3.1) was used for the calibration of the various sap flow techniques using cut citrus stems and/or branches, according to the method described by Steppe et al. (2010). Different designs were tested before finding a design able to achieve the desirable head of water pressure on the stems. For the initial design, an approximately 30 cm high cylinder made of plastic film was fixed directly to the stem with a polymer and double sided adhesive tape to ensure a good tight fit and to avoid any leakage (Figure 3.1). A 4 L flask was filled with water and sealed with a stopper in which two glass tubes were installed, with one acting as an air inlet and the other, attached to a third glass rod by flexible tubing, acting as a siphon. By adjusting the height of the flask, the siphon maintained the flow of water to the column, which allowed a constant head of water on the stem segment, regardless of flow rate through the stem. Although the authors (Steppe et al., 2010) found good flow rates without the use of complicated equipment in their experiment, high flow rates were not achieved in the citrus branches and significant leakage occurred from the plastic column during the initial stages of this study.

Therefore, the design was modified with the aim of achieving high flow rates by applying pressure on a stem segment. A plastic tube 0.8 m in length, with taps fitted at both ends, was used and a bicycle tyre pump was used to pump air into the tube to increase the pressure on the column of water (Figure 3.2). With this design, a maximum pressure of approximately 100 kPa was achieved. This design also allowed for the preparation of stems and calibration to be completed within 12 h, which also significantly reduced any damage and blockage to the xylem that would have prevented high sap flow rates as a result of embolism formation. Later it was realised that the pressure applied using the bicycle type pump was not constant and this had a great impact on the sap flow measurements. The design was therefore changed again

and a CO_2 regulator was installed to achieve a constant pressure with reasonably constant flow rates.

Figure 0.1 Initial experimental set-up for the calibration of sap flow techniques using the stem perfusion method

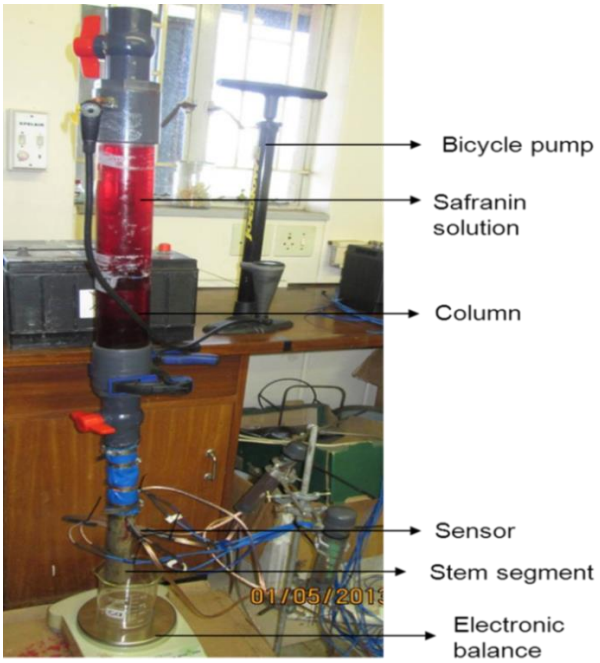

Figure 0.2 The modified experimental set-up for the calibration of sap flow using the stem perfusion method

Segments of the samples taken from the orchards were removed at both ends with a circular saw until a final sample length of 35 cm was achieved. Prior to attaching the stem to the column they were prepared for probe insertion. Holes for probe insertion were drilled at 90° angles to each other around the stem i.e. two probe sets of the HR method, two probes sets of the CHP method inserted simultaneously in one stem. The TD method was inserted into separate stems. A drill guide was used to ensure that the holes were spaced correctly vertically and parallel and to avoid any misalignment. For each method the probes were inserted opposite each other at 180°, with each technique separated vertically by 2 cm but not vertically in line to avoid disturbance by wounding between the probe sets.

A sharp blade was then used to re-open any closed xylem vessels resulting from sawing. Both sides of the branch sample were covered with a wet cloth during stem preparation to avoid dehydration and the formation of embolisms. A bark section of about 4 cm was removed from the branch to ensure that water flowed through the xylem and not the phloem and then attached with a rubber hose to the plastic tube containing the water. All joints were then checked for leaks.

The probes were coated with petroleum jelly to ease probe insertion and to ensure good thermal contact between the probes and the xylem tissue. Each probe set was thermally insulated with foam. Prior to forcing water through the branch, data was logged from the installed sensors for approximately 1 hour to determine zero flow conditions. The stem was then flushed with distilled water for one hour and once the readings had stabilized, different flow rates were achieved by applying different pressures, using the CO_2 regulator. Each flow rate was maintained for at least 45 min, before changing to another flow rate. Water passing through the stem segment was collected in a glass beaker and measured every 10 min for the HR and CHP methods and 5 min for the TD method using an electronic balance (Mettler Toledo model PB3002-S or Precisa model 800M both manufactured in Switzerland).

At the end of each experiment Safranin O dye was added to the water in the column and pressure was increased so that sap flow occurred, resulting in the colouration of

active xylem of the branch (Figure 3.3). The branches were then cut at the point where the probes were inserted into the stem. Photographs were taken of the cross section and digitally analysed using Adobe® Photoshop® CS5 Extended (Version 12.0x32). Using the marquee tool the total area of the stem was selected and the total number of pixels in this area was determined. The colour range tool was then used to select the colour corresponding to the dye. Using the colour range menu the number of pixels corresponding to that particular RGB range was determined, as shown in Figure 3.3. The % conducting sapwood was then calculated as the total number of pixels representing the staining divided by the total number of pixels in the stem section multiplied by 100.

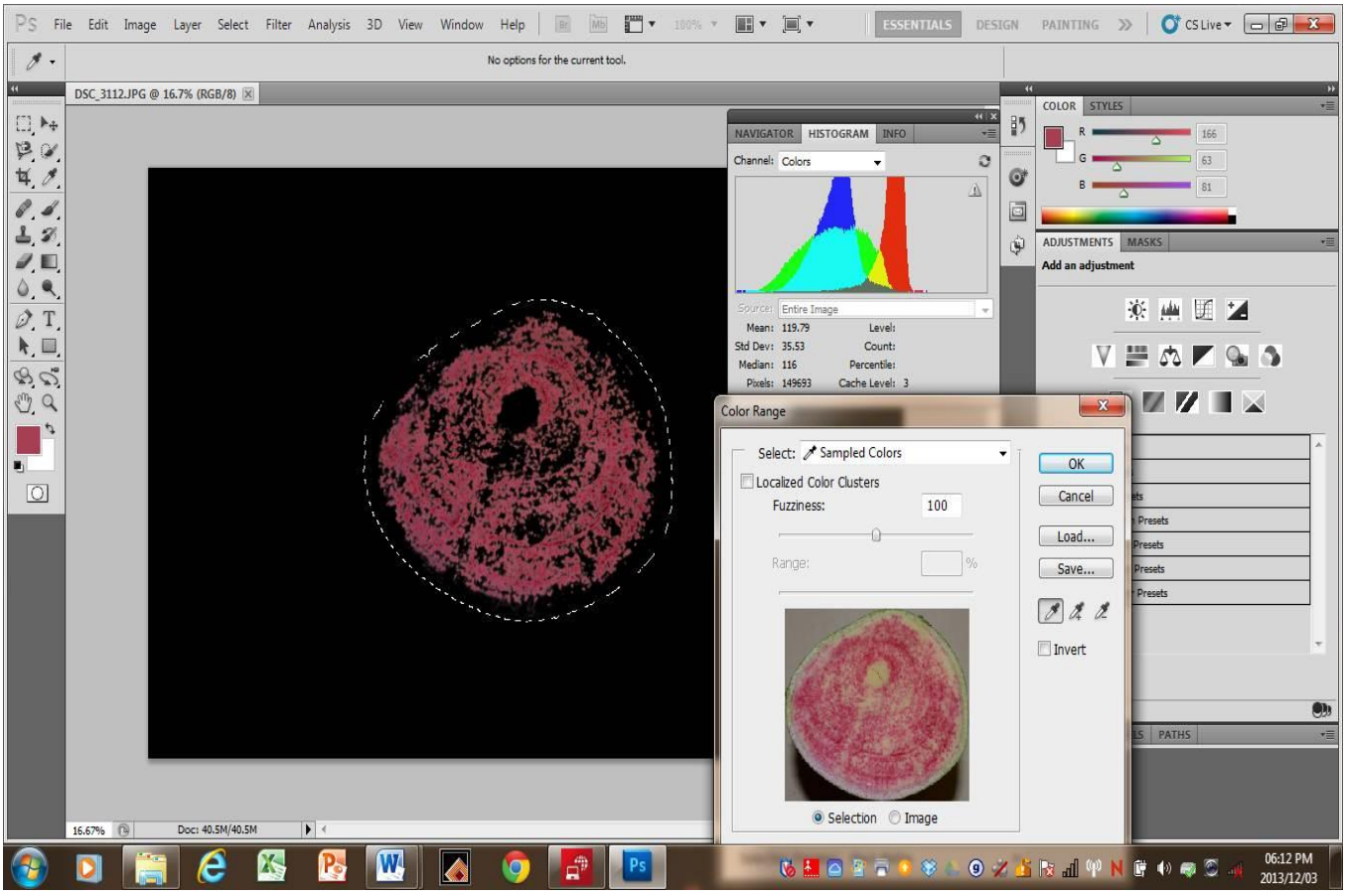

Figure 0.3 Determination of sapwood conducting area using pixel analysis in Adobe® Photoshop®

Gravimetric sap flux density was calculated as the rate of mass water passing through the stem segment divided by the area of conducting sapwood. Sap flux density calculations for the different sap flow techniques are described in Chapter 2. The calculations for the CHP method are outlined in section 2.2.1, the HR method in section 2.2.2 and the TD method in section 2.3. The wound width for the heat pulse velocity techniques was considered to be 0.2 cm, which is the diameter of the thermocouples. It was assumed that the wound would not extend beyond this distance, as calibration was completed within 5 h of probe insertion. No evidence of wounding beyond 0.2 cm could be observed with the naked eye. The temperature difference during a period of zero flow (ΔT_o), required for the TD method calculations, was determined prior to the start of calibration when no water was forced through the stem for at least 1 h.

The linear relationship between SFD determined gravimetrically and the sap flow techniques was evaluated using correlation and regression analyses. The correction factor was then calculated as the reciprocal of the slope of this relationship, as performed by Steppe et al. (2010). Sap flow determined with the sap flow technique was then corrected using this correction factor to assess the ability of a single correction factor to provide accurate estimates of sap flow.

2.7.4 Thermal interference

The possible thermal inference between probe sets installed together on the same branch was evaluated in order to ensure that good quality measurements were obtained and the heating of one probe set was not interfering with another. This was achieved by installing two probe sets of the HR method and 6 additional thermocouples in one stem according to the layout in Figure 3.4. Probe 1U, 1L, 3 and 4 were placed at 0.5 cm from the heater probe, whilst probe 1 and 2 were placed at 1 cm from the heater probe and probe 5 and 6 were placed at 2 cm. The two HR probe sets were placed opposite each other in the stem. It was then possible to determine how far the heat from the heater moves in the sapwood. Temperatures were recorded for 60 s after

the release of the heat pulse and the change in temperature was also evaluated as a result of different lengths of the heat pulse (0.3 to 0.8 s).

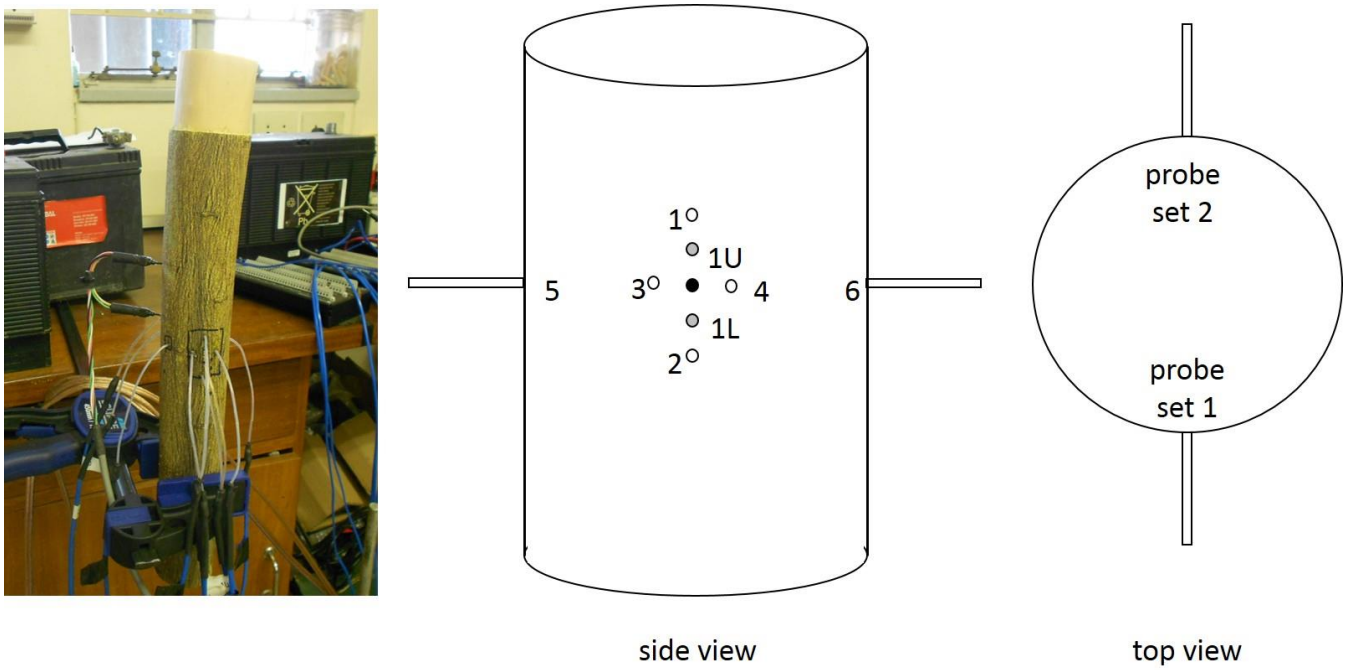

Figure 0.4 The positioning of sensors in stem to determine the possible thermal interference between the different techniques. The grey dots indicate the positioning of thermocouples for the HR method for probe set 1, whilst the black dot indicates the positioning of the heater. The white dots indicate additional thermocouples used to determine the distance the heat pulse travels in the sapwood.

The results show that the heat pulse was sensed by the upper and lower thermocouples of the probe sets (1U and 1L, together with 2U and 2L), and the thermocouple to the left, 0.5 cm away from the heater (TC 3) (Figure 3.5). The increase in temperature measured with thermocouple 3 (Figure 3.5) is probably a reflection of the positioning closer to the heater probe than intended, as a result of the angle at which the hole was drilled (Figure 3.5). However, no change in temperature was registered by the other thermocouples (4, 5 and 6) (Figure 3.5)

regardless of the heat pulse duration. Therefore, inserting the probe sets for the HR and CHP method at 90° from each other will not result in any interference between the methods.

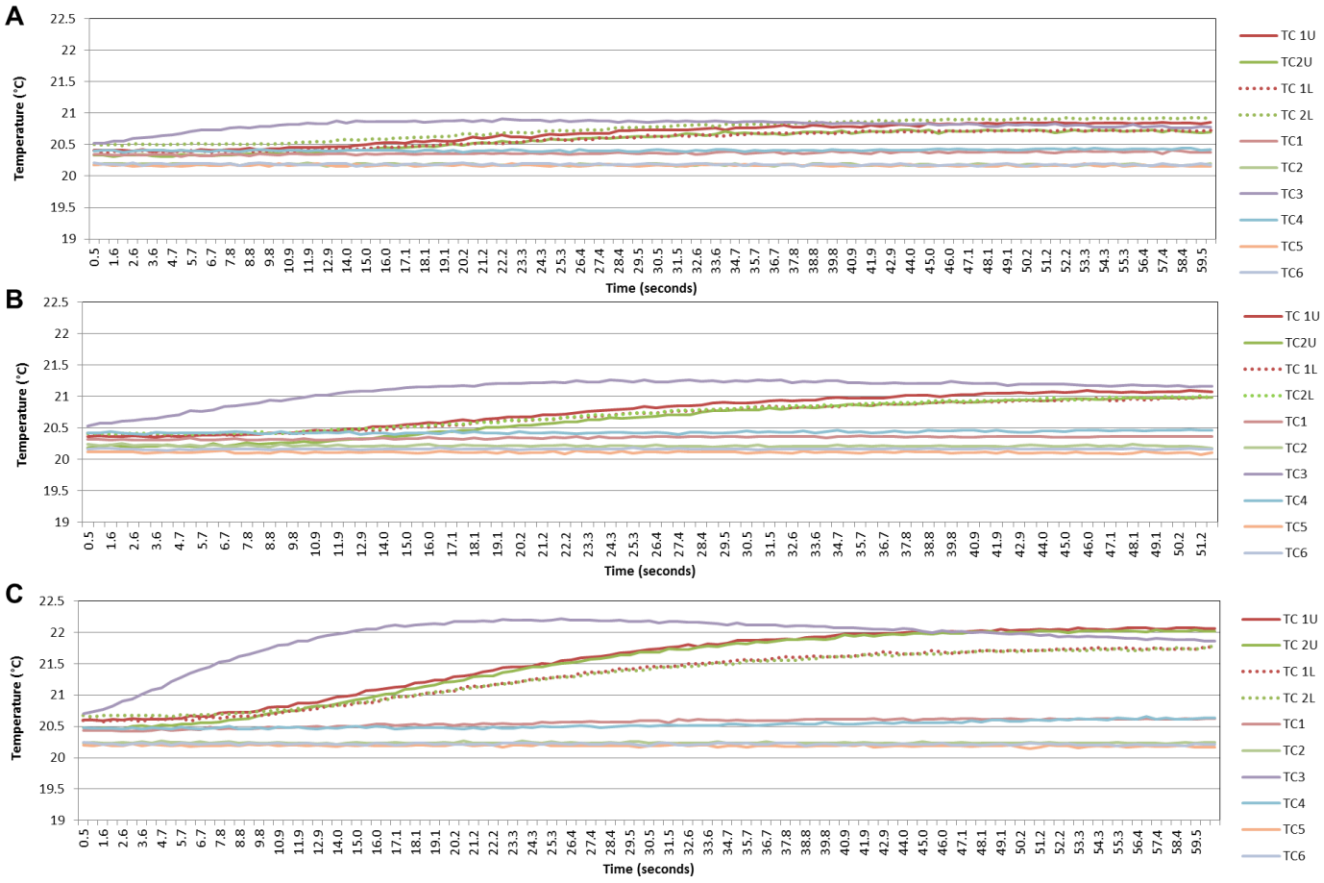

Figure 0.5 Temperature of the thermocouples at different positions in the stem following the release of a A) 0.3 s, b) 0.5 s and C) 0.8 s heat pulse. Thermocouple positions are as illustrated in Figure 3.4.

2.8 Sapwood properties

2.8.1 Physical properties

Sapwood characteristics are important parameters required for the calculation of sap flux density. Sapwood density and sapwood water content were determined following the procedures outlined by Steppe et al. (2010). Bark was removed from small pieces of wood and the fresh mass was determined. The samples were then submerged in

water for at least 30 minutes to ensure adequate swelling, removed from the water, and submerged into a beaker on a balance. This was done carefully without touching the bottom or side walls of the beaker and the mass was recorded immediately. This allowed the estimation of the volume of the sapwood sample. Thereafter the samples were oven dried at 60^{o}C. Samples were removed periodically and weighed until there was no further loss in mass. This was the dry mass of the sample. The volume of sapwood and fresh and dry mass were used to determine sapwood density and water content. Sapwood density (p_b) was calculated as:

$$p_b = \frac{w_d}{v_f} \quad (1)$$

where w_d (g) is the oven dry mass and v_f is the volume of a freshly excised section of wood.

The sapwood moisture (m_c) content was calculated as:

$$m_c = \frac{w_f - w_d}{w_d} \quad (2)$$

where w_f (g) is the fresh mass.

In Table 3.3 the sapwood moisture content and sapwood density are given for different species of citrus, which were used for calculation of sap flux density in this study and the broader project. The average sapwood moisture content was 0.61 ± 0.08 and the wood density was 0.74 ± 0.04 g cm^{-3}. This shows that these parameters are fairly conservative for citrus species.

Table 0.3 Sapwood densities and moisture content for citrus samples collected from Mpumalanga, Gauteng and the Western Cape

Cultivar	fresh mass (g)	dry mass (g)	sapwood volume (cm^3)	sapwood density ($g\ cm^{-3}$)	Sapwood moisture content
'Delta'	28.664	19.766	25.164	0.79	0.45
'Delta'	17.225	11.563	15.610	0.74	0.49
'Nadorcott'	31.662	20.847	27.022	0.77	0.52
'Nadorcott'	41.706	26.878	35.146	0.76	0.55
'Star Ruby'	12.881	8.026	11.054	0.73	0.61
'Star Ruby'	17.776	11.062	15.522	0.71	0.61
'Bahianinha'	13.165	9.605	12.648	0.75	0.43
'Eureka'	29.500	18.230	25.94	0.70	0.62
'Eureka'	26.300	15.290	23.73	0.64	0.72
'Eureka'	19.710	12.510	16.53	0.76	0.58
'Eureka'	25.910	16.210	21.85	0.74	0.60
'Eureka'	25.303	15.110	21.71	0.70	0.68
'Eureka'	20.500	12.430	17.32	0.72	0.65
'Bahianinha'	17.335	10.819	14.105	0.77	0.60
'Bahianinha'	20.966	13.418	17.572	0.76	0.56
'Midknight'	35.092	21.477	28.81	0.75	0.63
'Midknight'	26.807	16.843	20.348	0.83	0.59
'Midknight'	7.876	4.773	6.364	0.75	0.65
'Midknight'	8.324	4.635	6.081	0.76	0.80
			Average	**0.74**	**0.61**
			StdDev	**0.04**	**0.08**

2.9 Anatomical analyses

2.9.1 Plant material and sample preparation

Xylem anatomy was assessed in stems and branches of the different varieties of citrus used in this study namely: 'Delta' Valencia, 'Star Ruby' Grapefruit 'Bahianinha' Navel, 'Nadorcott' Mandarin and 'Eureka' Lemons. Samples were taken from the branches used for calibration of the sap flow methods and additional samples were collected from Patrysberg Farm in Citrusdal situated in the Western Cape. Core samples from stem and branches were collected from three trees per cultivar, using a 5.15 mm

diameter incremental borer (Haglöf Sweden AB, Långsele, Sweden). The position of the cambium and inner sapwood were carefully marked on each core. The cores were sealed in air tight Ziploc bags for transportation to the laboratory. In the laboratory the cores were removed from the Ziploc bags and preserved in formalin: acetic acid: ethanol alcohol solution (FAA, 1:1:1.8 v/v) for a few days. The samples were subsequently removed from the solution and rinsed with water. Cross-sections of the core samples were then made using a sliding microtome (Figure 3.6). Three sections per sample were taken from both the inner and outer part of the sapwood. The sections were placed on a microscope slide with water and glycerine to avoid drying out.

To assist with better contrast and tissue identification, the sections were dehydrated in a sequential series of 30%, 50%, 70% and 100% ethanol and then stained with Safranin solution for 1h. Following this, the sections were stained again with fast green and rinsed with a sequential series of 30%, 50%, 70% and 100% xylene and mounted in DPX fixative on microscopic slides. The sections were then observed and analysed under a Wild Leitz GMBH light microscope (Abbott Laboratories, Illinois, USA). The images were obtained with a Power Shot A630 digital camera (Canon Inc., USA) mounted on the microscope. Images were subsequently merged together using the Microsoft Image Composite Editor.

Lumen diameter of xylem vessels and distance between groups of vessels from both spring flush and summer flush wood of the inner and outer part of the sapwood were measured using the UTHCA Image Tool for Windows version 3.00. The xylem vessels were chosen by randomly selecting one xylem vessel and measuring the distances around that selected vessel to the nearest vessels, as indicated in Figure 3.7. The lumen diameter of these vessels was also measured. The sampling of each stem and branch from the inner and outer part was replicated three times.

Figure 0.6 Cutting of sections using the sliding microtome

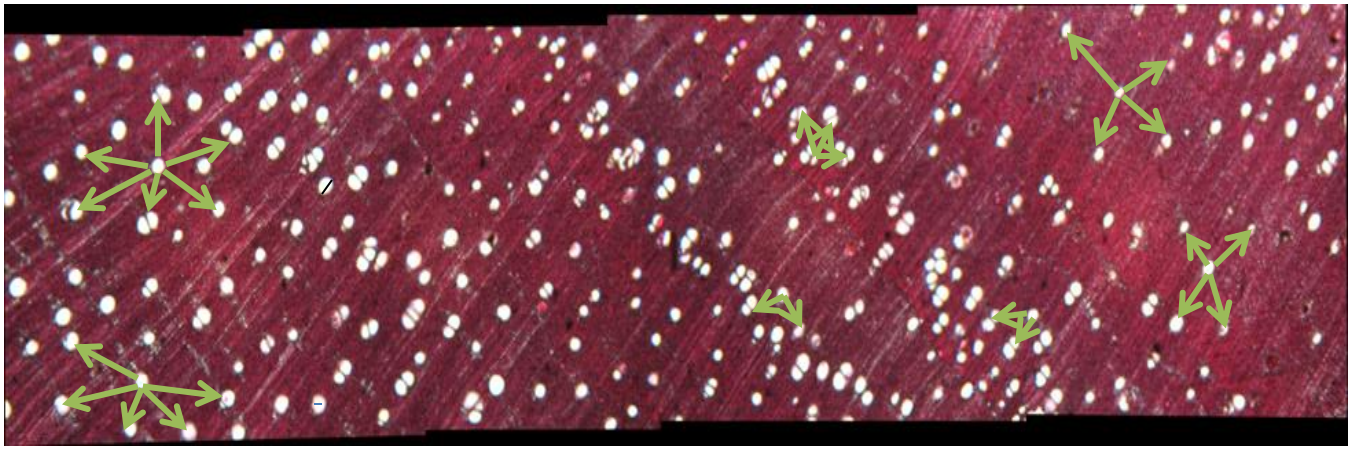

Figure 0.7 Cross sectional area of a core from a citrus tree showing how the distance between the xylem vessels was measured, as indicated by the arrows.

2.10 Statistical analysis

The performance of the sap flow techniques was evaluated with the aid of statistical parameters, such as the coefficient of determination (R^2), mean absolute error (MAE), root of the mean square error (RMSE) and Willmott index of agreement (D) (Willmott, 1982). The performance of each sap flow technique was considered accurate when R^2 >0.8, MAE <20% and D >0.8 according to (De Jager, 1994). The SAS program version 9.3 was used to determine the P values for the correlations between the sap flux density determined by sap flow techniques and that determined gravimetrically for each stem of each variety.

CHAPTER 4: RESULTS – CALIBRATION OF SAP FLOW TECHNIQUES

3.1 Introduction

The calibration of various sap flux density (SFD) techniques in different species is crucial for accurate measurements of transpiration rates in woody plants, as it establishes the reliability and accuracy of the method (Chen et al., 2012). Sap flow techniques have been calibrated in some citrus species, including young lemon (Alarcón et al., 2005, Ortuño et al., 2006) and orange trees (Fernández et al., 2006), both using the CHP method, and in *Citrus maxima* Merr (Ayutthaya et al., 2010), using the TD method. However, not all methods have been evaluated in all the Citrus species commercially cultivated. It is important to know if Citrus species need to be treated individually or if a single calibration of these techniques will apply to all Citrus species. Calibration is necessary as underestimations have often been noted, which are largely attributed to the wounding associated with probe insertion and the structure of the functional wood of some species, which does not adhere to the original definition in heat pulse theory of a homogenous and porous material (Marshall, 1958). Uncertainties regarding the area of conducting tissue and wood physical properties can also affect the accuracy of these techniques (Cohen and Fuchs, 1989a).

In this study it was hypothesised that: 1) the stem perfusion method will allow the accurate and robust calibration of sap flow techniques for citrus trees, because the method allows direct comparison of sap flux density determined by the sap flow technique with that determined gravimetrically, and 2) the calibration factor for the various sap flow techniques for citrus will be consistent across cultivars, but will differ for different citrus species, as a result of differences in xylem anatomy between species, relating to vessel size and spacing. Detailed materials and methods are presented in Chapter 3.1.

3.2 Calibration of sap flow techniques

3.2.1 Comparison of the methods over time

Sap flux density measured by the HR and CHP methods was compared to that determined gravimetrically, by recording the mass of water passing through the stem segment using an electronic balance (Figure 3.1). Wounding was taken into account using equation (2) and (8) in section 2.2.Importantly, all three measurements followed the same trend in this example, but it is evident that a constant flow could not be achieved, as explained in Chapter 3.3. In this particular stem, the HR method was able to resolve SFDs of approximately 30 $cm^3\ cm^{-2}\ h^{-1}$, whilst the CHP method was able to resolve SFDs up to 75 $cm^3\ cm^{-2}\ h^{-1}$ (Figure 3.1). Throughout the study the highest SFD measured by the HR method was approximately 45 $cm^3\ cm^{-2}\ h^{-1}$, whilst for the CHP method it was 77 $cm^3\ cm^{-2}\ h^{-1}$.

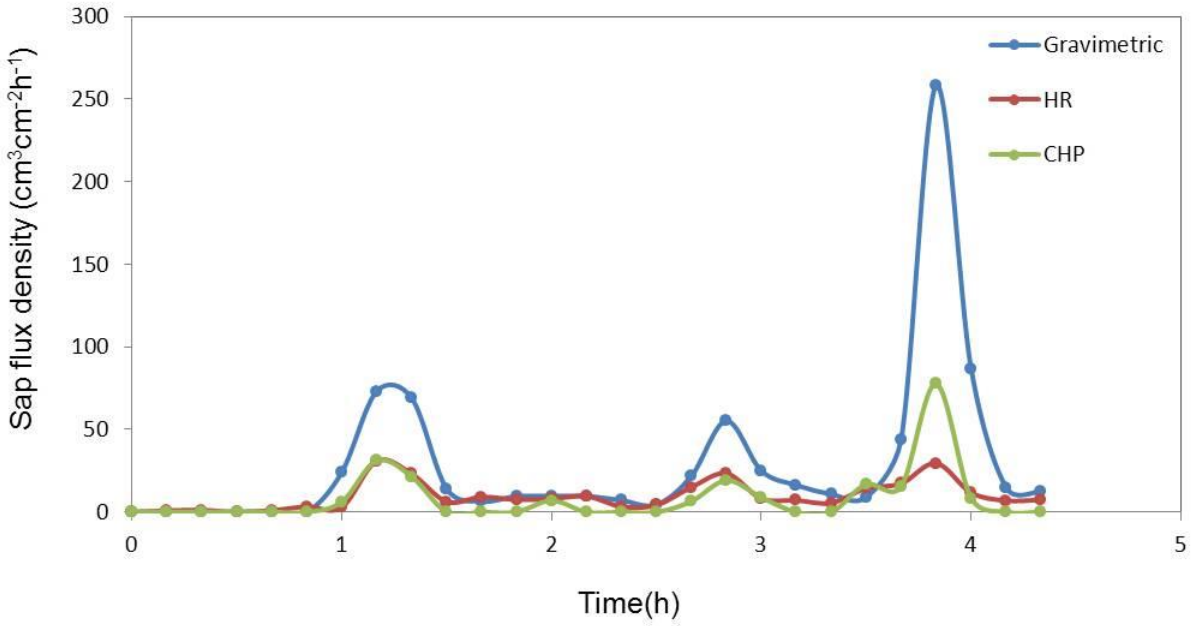

Figure 3.1 Comparison of sap flux density determined using the two heat pulse methods (HR and CHP methods) with that determined gravimetrically for a 'Star Ruby' Grapefruit sample (stem 2 for HR and stem 3 for CHP) over the course of the calibration period. The peaks in sap flux density indicate periods when pressure was applied to the column of water.

The TD method was calibrated separately from the heat pulse methods, to avoid the constant heat from the TD probe interfering with the HPV measurements in the relatively small branches. Sap flux density determined by the TD method and that determined gravimetrical for a 'Eureka' lemon sample followed the same trend, but there was a large difference in the magnitude of the SFD measurements between the two methods (Figure 3.2), with the TD method severely underestimating the SFD determined gravimetrically. The TD method was able to measure SFD up to a maximum of approximately 45 $cm^3\,cm^{-2}\,h^{-1}$ in all stems, whilst over 300 $cm^3\,cm^{-2}\,h^{-1}$ was determined gravimetrically.

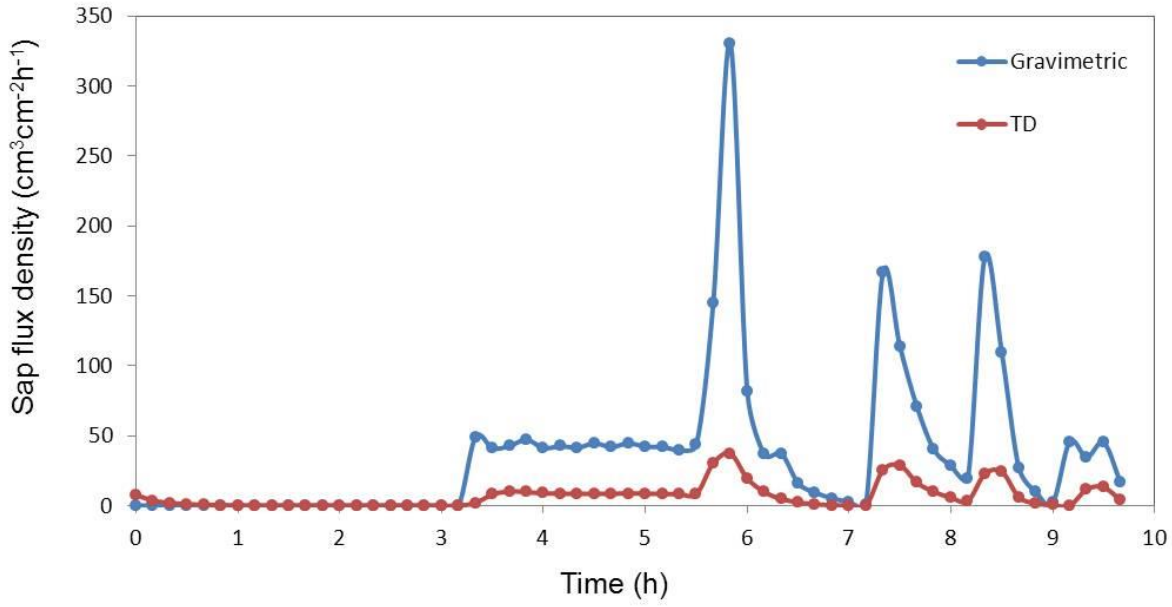

Figure 3.2 Comparison of sap flux density determined using the thermal dissipation method with that determined gravimetrically in a 'Eureka' Lemon sample over the course of the calibration period. The peaks in sap flux density indicate periods when pressure was applied to the column of water.

From Figure 3.1 and Figure 3.2 it is evident that the sudden increases in flow rate, as a result of increasing the pressure using the CO_2 regulator, resulted in very high values of SFD measured gravimetrically and that these flow rates were not captured by the sap flow techniques. Literature confirms that such high flow rates cannot be resolved by the various techniques and therefore SFDs above 250 $cm^3\ cm^{-2}\ h^{-1}$ for the HR, 300 $cm^3\ cm^{-2}\ h^{-1}$ for CHP and 200 $cm^3\ cm^{-2}\ h^{-1}$ for TD method were not considered in the calibration studies. These flow rates were also far higher than anything that has been observed in orchards during the WRC project and were therefore probably unrealistically high for citrus.

3.2.2 Calibration of the heat ratio method

Sap flux density determined by the HR method and that determined gravimetrically are presented in Figure 3.3 and Figure 3.4. Sap flux densities were underestimated in all the species and cultivars, except for 'Eureka' Lemon stem 3 (Figure 3.3). However, good correlations (R^2>0.7) were observed in some stems of 'Star Ruby' Grapefruit, 'Delta' Valencia, 'Bahianinha' Navel and 'Eureka' Lemon, but poor correlations (R^2 = 0.19) were found for 'Nadorcott' Mandarin (Table 4.1). Plotting this data on a single set of axes (Figure 3.4) revealed that the slope of the regression equations were inconsistent from stem to stem within the same cultivar and also between species and cultivars, with the slope of the regression curves ranging from 0.012 to 1.153 (Table 3.1). This indicates that a single correction factor for the HR method for citrus could not be obtained with this calibration technique.

Table 3.1 The coefficients of the linear regression equations (slopes and intercepts) between sap flux density measured by the heat ratio method (y-axis) and that determined gravimetrically (x-axis). The coefficient of determination (R^2) is also provided. CV of the slope = 104%

Species	Rep	Slope	Intercept	R^2	P values
***Citrus paradise* 'Star Ruby'**	1	0.522	0.445	0.94	<.0001
	2	0.669	2.630	0.76	<.0001
	3	0.128	6.035	0.55	<.01
***Citrus sinensis* 'Delta'**	1	0.032	1.915	0.60	<.0001
	2	0.012	4.037	0.75	<.0001
***Citrus sinensis* 'Bahianinha'**	1	0.081	-0.109	0.67	<.0001
	2	0.408	3.903	0.83	<.0001
***Citrus reticulate* 'Nadorcott'**	1	0.047	2.217	0.35	<.01
	2	0.026	2.765	0.19	n.s.
***Citrus limon* 'Eureka'**	1	0.419	1.431	0.63	<.0001
	2	0.530	4.058	0.79	<.0001
	3	1.153	0.476	0.94	<.0001

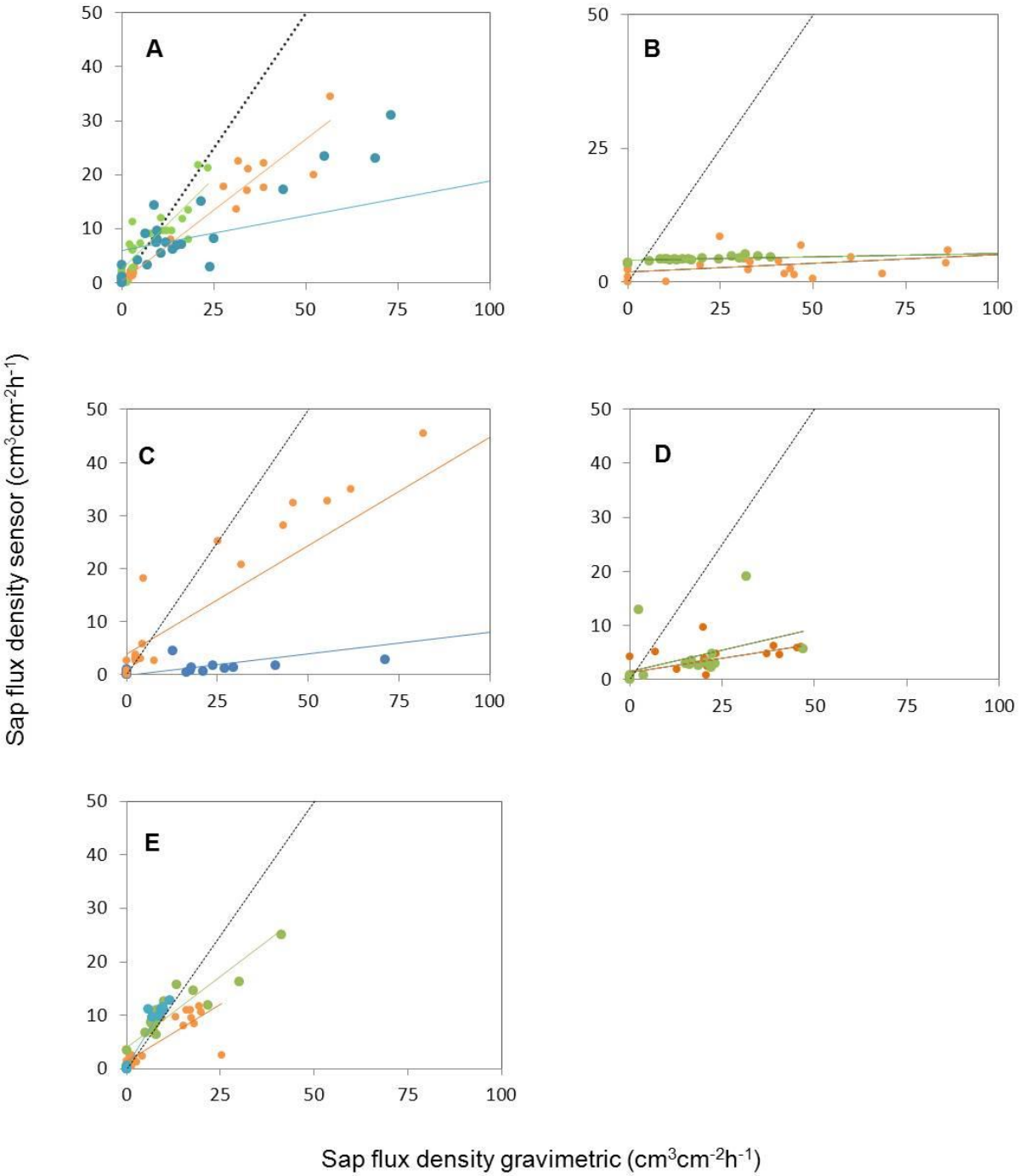

Figure 3.3 Relationship between the sap flux densities measured with the heat ratio method and actual sap flux density determined gravimetrically for A) 'Star Ruby' Grapefruit, B) 'Delta' Valencia, C) 'Bahianinha' Navel, D) 'Nadorcott' Mandarin and E) 'Eureka' Lemon. The 1:1 line is indicated by the dotted line. Each colour represents a separate stem.

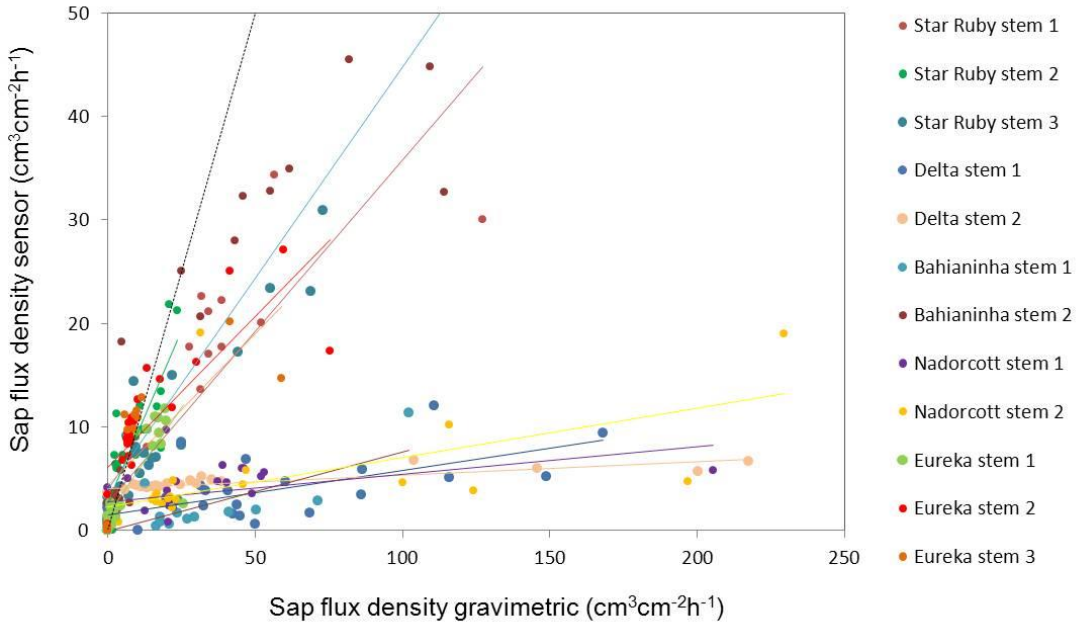

Figure 3.4 The relationship between the sap flux density measured using the heat ratio method for five citrus species and actual sap flux density determined gravimetrically. The 1:1 line is indicated by the dotted line.

3.2.3 Calibration of the compensation heat pulse method

As with the HR method, the sap flux density determined by the CHP method was also underestimated in all species, as compared to that determined gravimetrically. Good correlations were observed in some stems of 'Star Ruby' Grapefruit, 'Delta' Valencia, and 'Eureka' Lemon (R^2 >0.7), but once again, poor correlations were found for 'Nadorcott' Mandarin (R^2= 0.27) when using the CHP method (Table 3.2 and Figure 3.5). When all the species are plotted on the same axes (Figure 3.6) it was evident that the correlations differed from stem to stem of the same cultivar, however, the slope of the best fit lines was more consistent between the five species than was observed with the HR method (Figure 3.4). The slope of the regression curves ranged from 0.015 to 0.538.

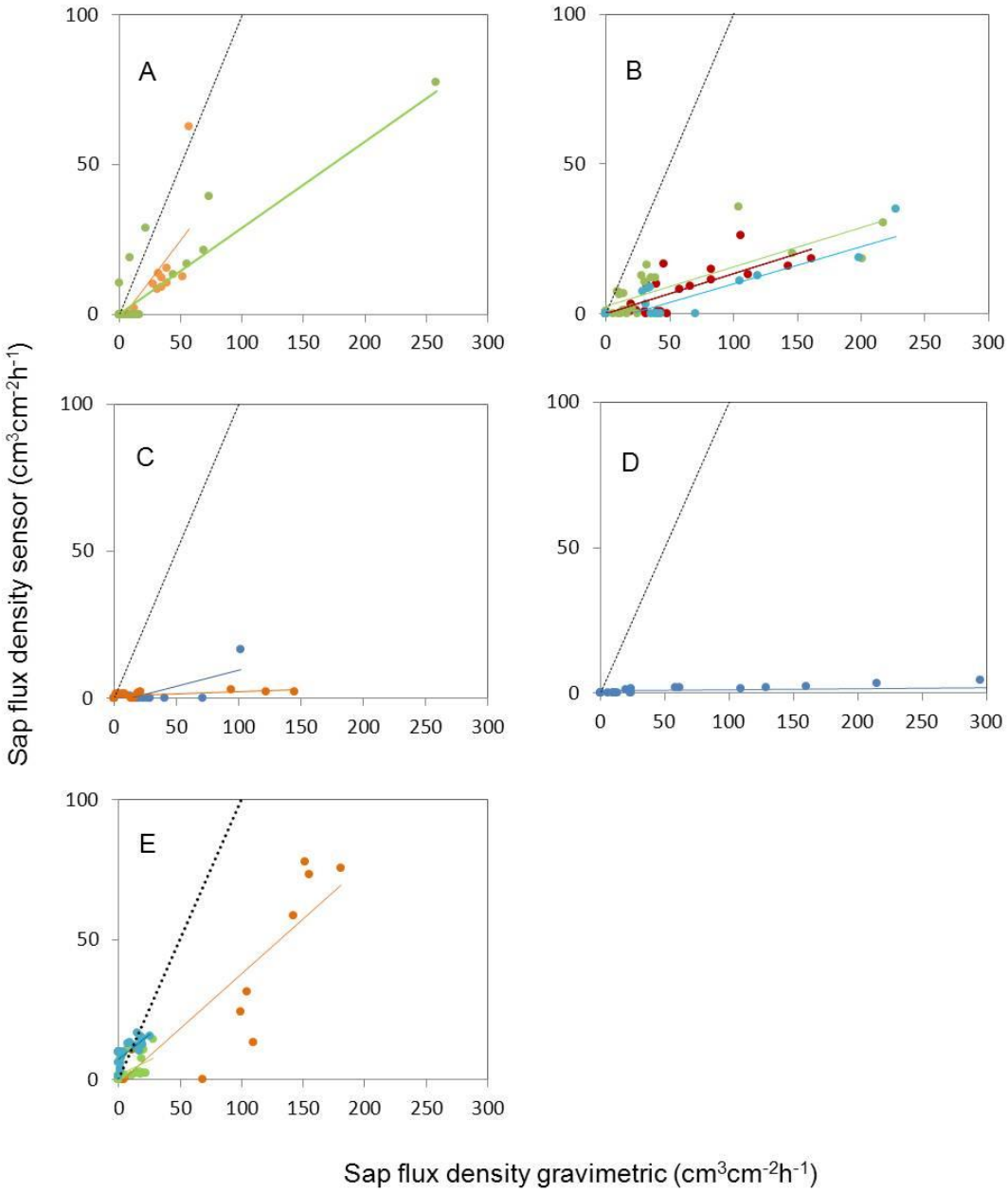

Figure 3.5 Relationship between the sap flux densities measured with the CHP method and actual sap flux density determined gravimetrically for A) 'Star Ruby' Grapefruit, B) 'Delta' Valencia, and C) 'Bahianinha 'Navel, D) 'Nadorcott 'Mandarin and E) 'Eureka' Lemon. The 1:1 line is indicated by the dotted line. Each colour represents a separate stem.

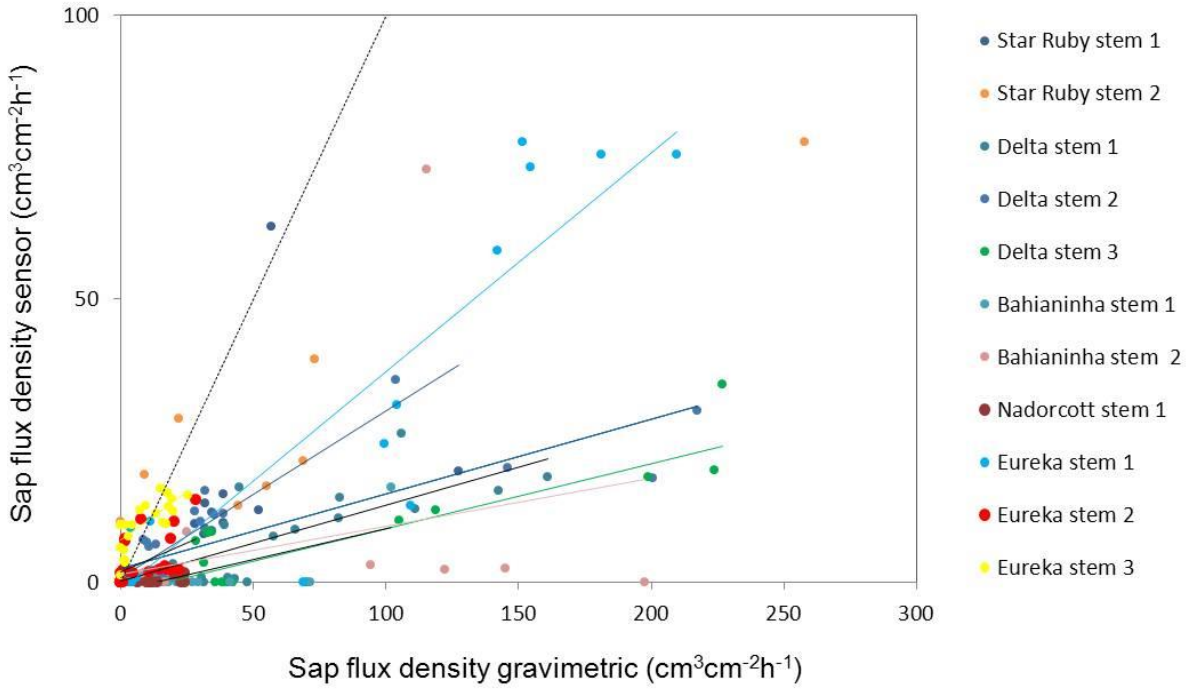

Figure 3.6 The relationship between the sap flux densities measured using the CHP method for five citrus species and actual sap flux density determined gravimetrically. The 1:1 line is indicated by the dotted line.

Table 3.2 The coefficients of the linear regression equations (slopes and intercepts) between sap flux density measured by the compensation heat pulse method (y-axis) and that determined gravimetrically (x-axis). The coefficient of determination (R^2) is also provided. CV of the slope = 77%

Species	Rep	Slope	Intercept	R^2	P values
***Citrus paradise* 'Star Ruby'**	1	0.538	-2.130	0.60	<.0001
	2	0.288	-0.032	0.73	<.0001
***Citrus sinensis* 'Delta'**	1	0.134	0.110	0.61	<.0001
	2	0.132	2.414	0.64	<.0001
	3	0.124	-2.405	0.73	<.0001
***Citrus sinensis* 'Bahianinha'**	1	0.107	-1.380	0.54	n.s.
	2	0.015	0.408	0.40	<.01
***Citrus reticulate* 'Nadorcott'**	1	0.027	0.089	0.27	<.01
***Citrus limon* 'Eureka'**	1	0.393	-1.566	0.84	<.0001
	2	0.206	1.730	0.23	n.s.
	3	0.343	7.379	0.50	<.0001

3.2.4 Calibration of the thermal dissipation method

The relationship between SFD measured with the TD method and actual SFD determined gravimetrically is presented in Figure 3.7, Figure 3.8 and Table 3.3. As was observed with the HR and CHP methods, SFD was also underestimated using the TD method. Despite the observed underestimation, good correlations were found for some stems of 'Star Ruby' Grapefruit, 'Delta' Valencia and 'Eureka' Lemon (R^2 > 0.7). Although there was some variation in the slope of the regression equations, (0.019 to 0.194), within stems of the same species (Figure 3.7) and between species/cultivars (Figure 3.8), the variation within the same species was less than that observed for the HR method (CV 78%).

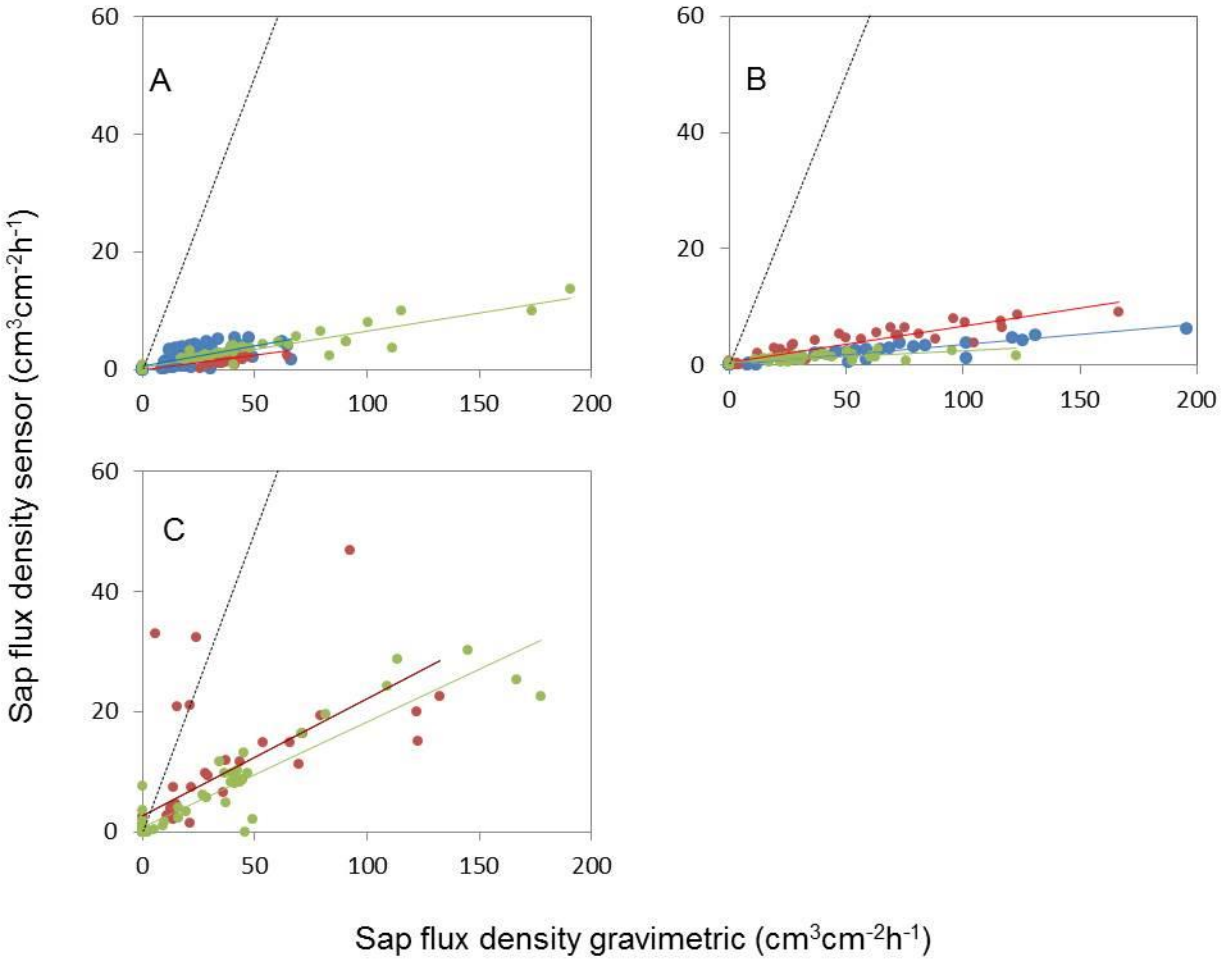

Figure 3.7 Relationship between the sap flux density measured with the thermal dissipation method and actual sap flux density determined gravimetrically for A) 'Star Ruby' Grapefruit, B) 'Delta' Valencia and C) 'Eureka' Lemon. The 1:1 line is indicated by the dotted line. Each colour represents a separate stem.

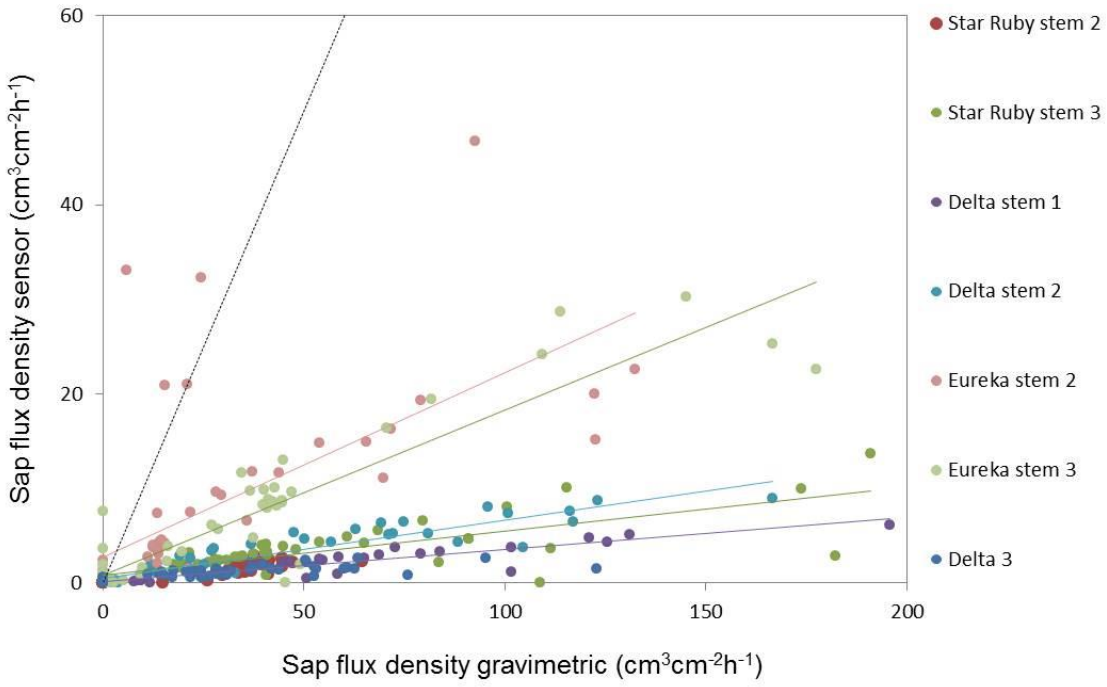

Figure 3.8 The relationship between the sap flux density measured using the thermal dissipation method for three citrus species and actual sap flux density determined gravimetrically. The 1:1 line is indicated by the dotted line.

3.2.5 Corrected sap flux density

There was large variation in the percentage underestimation of SFD for each method in each citrus variety (Table 3.4). For the HR and CHP methods, the highest percentage underestimation was found for ‘Nadorcott’ Mandarin, 89% and 99% respectively, as compared to the rest of the citrus species. The lowest percentage underestimation was for ‘Eureka’ Lemon (50% for the HR method and 27% for the CHP method) compared to other species. The TD method was only assessed in ‘Star Ruby’ Grapefruit, ‘Delta’ Valencia and ‘Eureka’ Lemon and once again SFD was underestimated by 94 % for ‘Star Ruby’ Grapefruit, 72% for ‘Delta’ Valencia and 78% for ‘Eureka’ Lemon.

Table 3.3 The coefficients of the linear regression equations (slopes and intercepts) between sap flux density measured by the thermal dissipation method (y-axis) and that determined gravimetrically (x-axis). The coefficient of determination (R^2) is also provided. CV of the slope = 78%.

Species	Rep	Slope	Intercept	R^2	P values
***Citrus paradise* 'Star Ruby'**	1	0.069	0.525	0.45	<.01
	2	0.048	0.036	0.79	<.0001
	3	0.061	0.452	0.85	<.0001
***Citrus sinensis* 'Delta'**	1	0.033	0.167	0.86	<.0001
	2	0.061	0.480	0.87	<.0001
	3	0.019	0.443	0.54	<.0001
***Citrus limon* 'Eureka'**	1	0.194	2.801	0.43	<.01
	2	0.174	0.859	0.85	<.0001

Table 3.4 The average percentage underestimation of the actual sap flux density determined gravimetrically by the various sap flow techniques for the different citrus varieties

Species	Underestimation %		
	HR method	CHP method	TD method
'Star Ruby' Grapefruit	59	68	94
'Delta' Valencia	60	64	72
'Bahianinha' Navel	70	91	ND*
'Nadorcott' Mandarin	89	99	ND*
'Eureka Lemon'	50	27	78
CV%	37	32	8

ND*TD method was not tested in these species.

Correction factors for each method were calculated as the reciprocal of the slope of each regression line of each variety, when forced through the origin (resulting in a zero

intercept). These correction factors were averaged to determine a single correction factor for each citrus variety/cultivar. Only stems where R^2>0.5 were considered when determining the average correction factor. There was large variation in correction factors between the species for each method (Table 3.5), confirming that the correction factor is species dependent and a single correction factor for citrus could therefore not be determined in this study. Correction factors were not determined in 'Nadorcott' Mandarin, as reliable correlations between SFD determined using the sap flow sensors and that determined gravimetrically were not achieved.

Table 3.5 The average correction factors ± the standard error determined for the various citrus species for each of the three sap flux density methods

Species	Correction factors		
	HR method	CHP method	TD method
'Star Ruby' Grapefruit	1.72 ± 0.52	2.66 ± 1.13	17.52 ± 3.66
'Delta' Valencia	8.77 ± 3.42	7.69 ± 0.32	21.40 ± 9.22
'Bahianinha' Navel	7.22 ± 7.15	11.30 ± 2.86	ND*
'Nadorcott' Mandarin	-	-	ND*
'Eureka' Lemons	2.18 ± 0.21	3.43 ± 1.23	5.73 ± 3.94

ND*TD method was not tested in these species.

Figure 3.9, Figure 3.10 and Figure 3.11 present the relationship between the SFD determined by sap flow techniques and that determined gravimetrically, after the SFD for each technique was corrected using the average correction factors shown in Table 4.5. In some species/cultivar ('Star Ruby' Grapefruit and 'Eureka' Lemon) a single correction factor resulted in good agreement between SFD determined by each technique with that determined gravimetrically for that particular species/cultivar, whilst in others ('Delta' Valencia and 'Bahianinha' Navel) a single correction factor resulted in poor agreement between the two methods. Surprisingly, applying the derived correction factors resulted in the consistent overestimation of SFD in all species using all techniques. The performance of the techniques as determined by the statistical

parameters are presented in Table 4.6, Table 4.7 and Table 4.8. Overall, the attempted calibration was not good, as the mean absolute error (MAE) was higher than 20% in all species using all techniques, however, Willmott index of agreement (D) was satisfactory (>0.80) in most species for each technique.

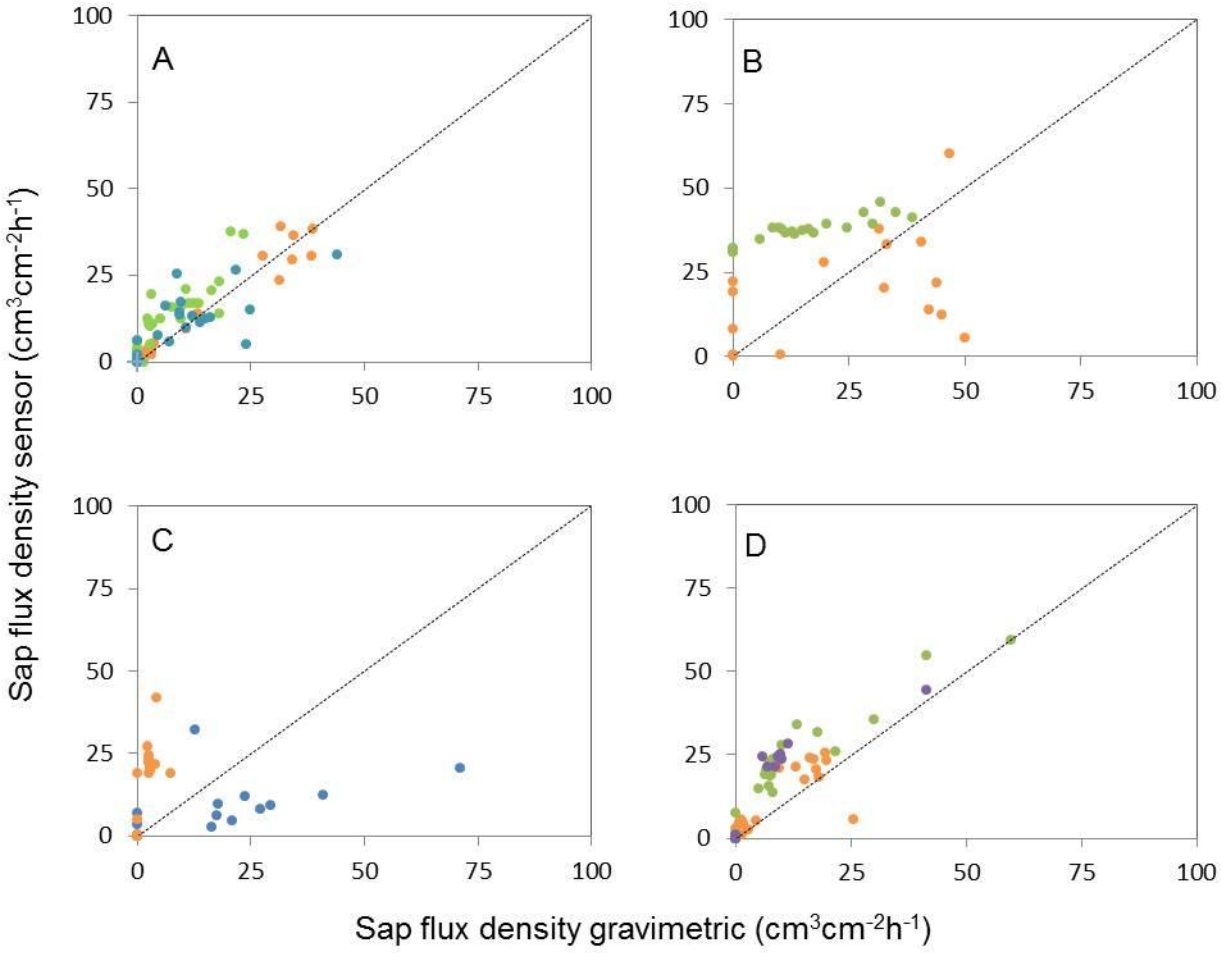

Figure 3.9 Corrected sap flux density measured with the heat ratio method as compared to that determined gravimetrically. Correction factors were calculated as the average of the reciprocal of the slope of each regression line for A) 'Star Ruby' Grapefruit, B) 'Delta' Valencia, C) 'Bahianinha' Navel, D) and 'Eureka' Lemon. The 1:1 line is indicated by the dotted line. Different coloured markers represent different stems.

Table 3.6 Performance of the corrected heat ratio method in various citrus species as indicated by the coefficient of determination (R^2), Willmott index of agreement (D), root of the mean square error (RMSE) and mean absolute error (MAE).

Species	Rep	R^2	D	RMSE ($cm^3\ cm^{-2}\ h^{-1}$)	MAE (%)
Citrus paradise 'Star Ruby'	1	0.94	0.98	6.58	49
	2	0.85	0.90	7.9	118
	3	0.59	0.86	16.5	120
Citrus sinensis 'Delta'	1	0.67	0.90	18.5	67
	2	0.76	0.78	22	142
Citrus sinensis 'Bahianinha'	1	0.74	0.89	22	86
	2	0.84	0.58	141	593
Citrus limon 'Eureka'	1	0.91	0.93	6	88
	2	0.82	0.93	7	84
	3	0.54	0.81	14	105

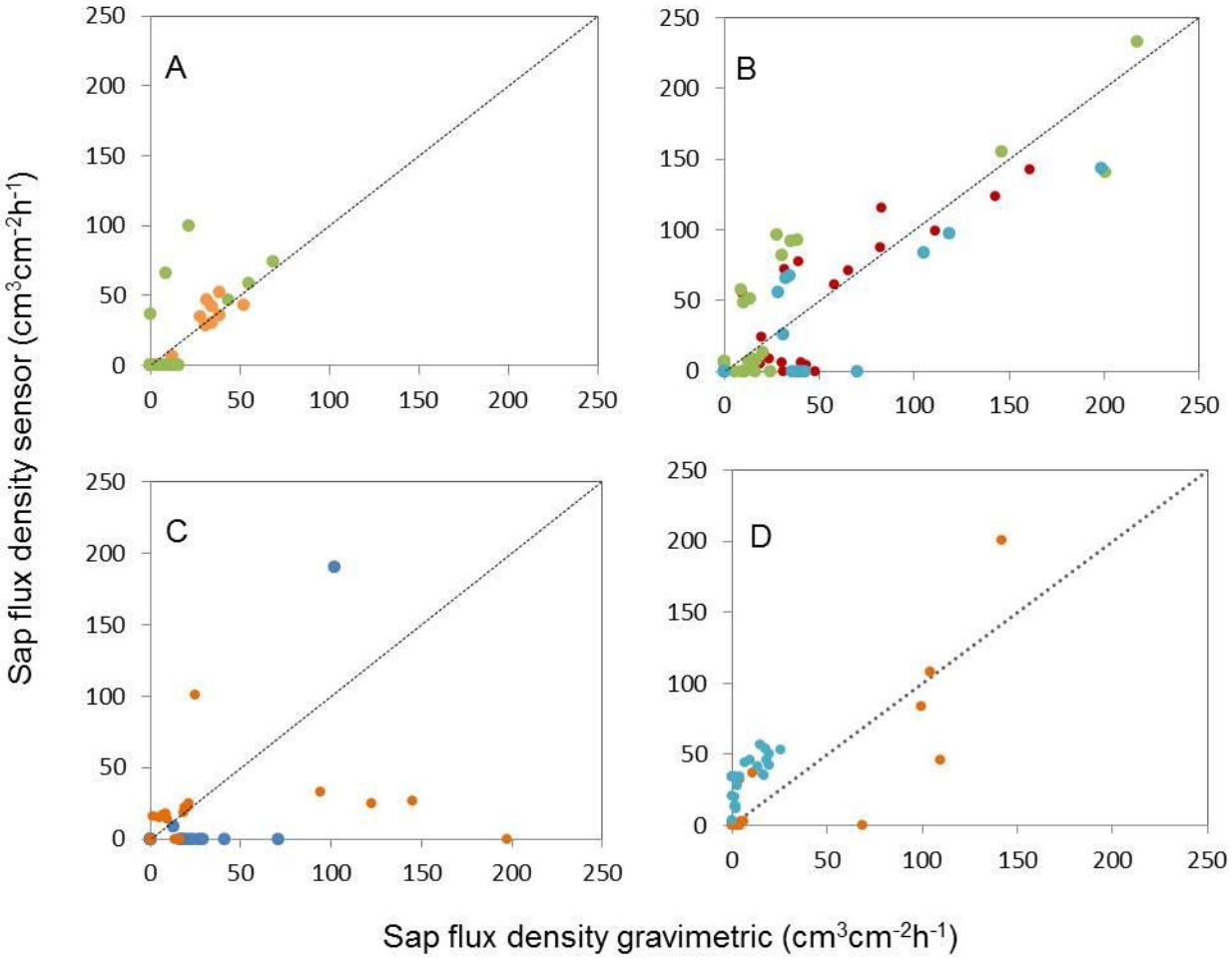

Figure 3.10 Corrected sap flux density measured with compensation heat pulse method as compared to that determined gravimetrically. Correction factors were calculated as the average of the reciprocal of the slope of each regression line for A) 'Star Ruby' Grapefruit, B) 'Delta' Valencia, C) 'Bahianinha' Navel and D) 'Eureka' Lemon. The 1:1 line is indicated by the dotted line. Different coloured markers represent different stems.

Table 3.7 Performance of the corrected compensation heat pulse method in various citrus species as indicated by the coefficient of determination (R^2), Willmott index of agreement (D), root of the mean square error (RMSE) and mean absolute error (MAE).

Species	Rep	R^2	D	RMSE ($cm^3\ cm^{-2}\ h^{-1}$)	MAE (%)
Citrus paradise 'Star Ruby'	1	0.92	0.96	8	55
	2	0.74	0.91	41	142
Citrus sinensis 'Delta'	1	0.82	0.92	62	82
	2	0.68	0.87	69	158
	3	0.77	0.89	59	136
Citrus sinensis 'Bahianinha'	1	0.02	0.61	40	148
	2	0.25	0.33	226	198
Citrus limon 'Eureka'	1	0.78	0.93	82	84
	2	0.106	0.50	26	250
	3	0.50	0.44	42	733

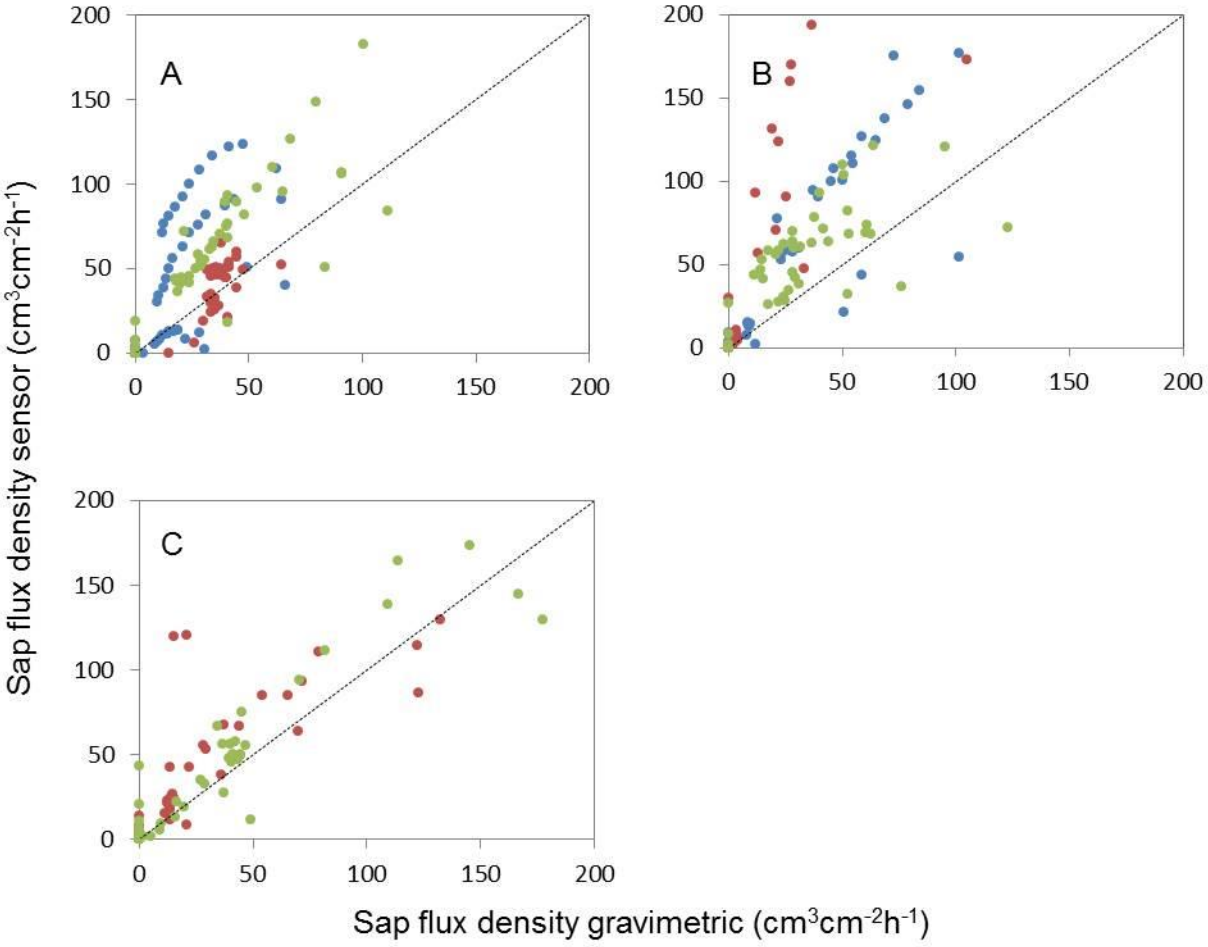

Figure 3.11 Corrected sap flux density measured with thermal dissipation method as compared to that determined gravimetrically. Correction factors were calculated as the average of the reciprocal of the slope of each regression line for A) 'Star Ruby' Grapefruit, B) 'Delta' Valencia and C) 'Eureka' Lemon. The 1:1 line is indicated by the dotted line. Different coloured markers represent different stems.

Table 3.8 Performance of the corrected thermal dissipation method in various citrus species as indicated by the coefficient of determination (R2), Willmott index of agreement (D), root of the mean square error (RMSE) and mean absolute error (MAE).

Species	Rep	R^2	D	RMSE ($cm^3\ cm^{-2}\ h^{-1}$)	MAE (%)
Citrus paradise 'Star Ruby'	1	0.46	0.63	70	523
	2	0.79	0.93	18	81
	3	0.81	0.92	44	168
Citrus sinensis 'Delta'	1	0.88	0.90	69	236
	2	0.87	0.94	38	91
	3	0.25	0.64	94	308
Citrus limon 'Eureka'	1	0.63	0.84	52	240
	2	0.87	0.96	38	149

3.2.6 Variation in conducting sapwood

The variations in conducting sapwood in various citrus species, which were used for the calibration of different sap flow techniques, are illustrated in Figure 3.12. Details are also provided in Chapter 3 in Table 3.1. Staining of the sapwood was not uniform in all species, and the percentage area of conducting sapwood varied from stem to stem of the same cultivar and between different species/cultivars. A large proportion of the sapwood in the 'Nadorcott' Mandarin sample was not stained (Figure 3.12B) and following analysis it was found that only 8% the area of sapwood was able to conduct water. On average, for both 'Nadorcott' Mandarin stems only 10.8% of the sapwood was able to conduct water. In addition, it was evident that one of the probes for one of the stems, for both the HR and CHP methods, was inserted into non-conducting sapwood (Figure 3.12B). This could have contributed to the very poor correlations between the actual SFD determined gravimetrically with that determined by the sap flow sensors.

Figure 3.12 Cross sections of stem segments A) 'Star Ruby' Grapefruit, B) 'Nadorcott' Mandarin, C) 'Delta' Valencia, D) 'Bahianinha' Navel, and E) 'Eureka' Lemon used for sap flow calibration. The stems were stained with safranin dye to indicate the conducting sapwood as indicated by the pink colour.

Clear evidence of the impact of the implantation of probes on the movement of water is shown in Figure 3.13. Stain is clearly evident on the upstream side of the drilled hole, but no stain is evident on the downstream side of the drilled hole. This clearly demonstrates the drilling of holes for probe insertion breaks xylem vessels and further movement of water in these vessels. Therefore it is necessary to take wounding into account when calculating the SFD. In this instance the wound width can be taken as the width of the thermocouple, i.e. 0.2 cm.

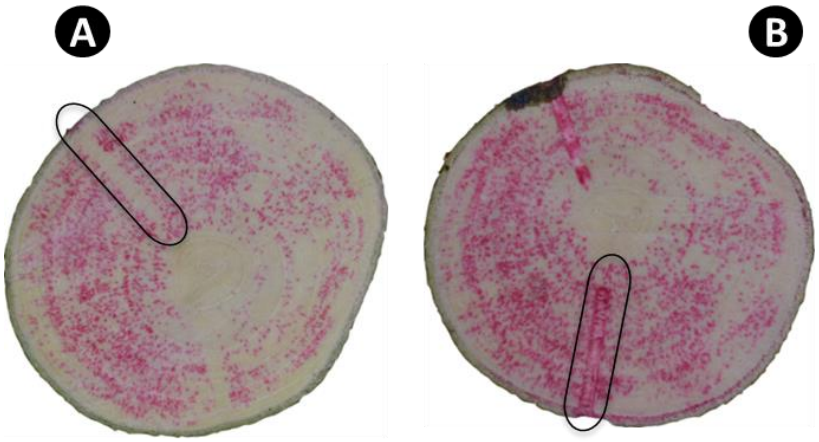

Figure 3.13 Cross section of stems indicating A) no water movement on the downstream xylem where the thermocouple was inserted and B) staining in the upstream drilled hole where the thermocouple was inserted, indicating direct water movement to the thermocouple, but not beyond.

CHAPTER 5: RESULTS – CITRUS SAPWOOD ANATOMY

4.1 Introduction

The theory for heat pulse velocity (HPV) techniques includes the assumption that the stem is a homogenous and porous material (Swanson and Whitfield, 1981). However, the sapwood of some species does not adhere to this assumption. Steppe et al. (2010) and Vandegehuche et al (2013) highlighted that although the assumption of thermal heterogeneity is most often applied to the HPV methods, there is a possibility that it also applies to thermal dissipation (TD) measurements. Sap flow techniques are generally assumed to perform well in trees where xylem vessel size is uniform and the vessels are evenly distributed throughout the sapwood, with less than 400 µm between vessels (Swanson, 1994). On the other hand, Green and Clothier (1988) concluded that diffuse porous wood, with few large and widely distributed vessels in the sapwood, will result in departure from the idealised theory of transport of heat in the woody matrix. Generally, sap flow measurements from such sapwood tend to be underestimated (Marshall, 1958). Therefore, the aim of this study was to determine the vessel dimensions (lumen diameter) and distance between groups of xylem vessels in citrus species. This was done in order to determine if this could explain the underestimation of sap flux density and the large variations in the calibration factors obtained during the calibration of the various sap flow techniques. It was also hypothesised that the calibration of sap flow techniques in citrus is necessary as the sapwood structure is diffuse porous and therefore does not adhere to the idealised theory of heat pulse measurements.

Within the stem of a tree the properties of the wood change systematically. Typically, properties of the wood cells produced in spring (early wood) often differ to the wood cells produced in autumn (late wood). However, in this dissertation the terms mature leaf wood and flush leaf wood will be used instead of spring or early wood and autumn or late wood, as in many deciduous species. This is because in evergreen citrus trees flush and mature leaf wood form (i.e. radial expansion of the sapwood) more than once

in the season, due to a number of distinct flushes of vegetative growth (Carr 2012). The expansion of sapwood therefore occurs at different times. The flush leaf wood is produced during periods when the new flush leaves are still immature, whilst mature leaf wood is produced after the last flush leaves have matured. The density of the wood produced during a flush differs to that produced when the leaves are mature. Mature leaf wood is less dense with wider vessels and thinner walls, whilst flush leaf wood has narrower vessels with thicker walls (Raven et al., 1992). Measurements were taken from the inner part and outer part of the sapwood, where the inner part is the area towards the centre of the sapwood and the outer part is closest to the bark.

4.2 Citrus sapwood anatomy

Cross sections of ‘Delta’ Valencia, ‘Star Ruby’ Grapefruit, ‘Bahaininha’ Navel, ‘Nadorcott’ Mandarin and ‘Eureka’ Lemon sapwood indicating the xylem vessels in the mature leaf wood and flush leaf wood are presented in Figure 4.1. The xylem vessels in mature leaf wood (MLW), the wood produced early in the season, are widely distributed, with much larger distances between the vessels, whilst in the flush leaf wood (FLW), xylem vessels are closely distributed with shorter distance between the vessels (Figure 4.1D). In addition, most vessels in the flush leaf wood occur in groups of two to three. Mature and flush leaf wood was evident in sapwood samples taken from ‘Delta’ Valencia, ‘Star Ruby’ Grapefruit and ‘Eureka’ Lemon, whilst only mature wood was found in sapwood samples from ‘Bahaininha’ Navel and ‘Nadorcott’ Mandarin (Figure 4.1B and N).

Mean distances between the xylem vessels in mature leaf wood from both the inner and outer part of the stem and branch are shown in Table 5.1. In general, in the mature leaf wood vessels were more widely spaced in ‘Delta’ Valencia samples (554 µm in stems and 474 µm in branches), whilst they were most closely spaced in ‘Nadorcott’ Mandarin sapwood (415 µm in stems and 396 µm in branches). Although differences between species were not always significant (p<0.05), in all stems the distance between vessels exceeded the limit determined by Swanson (1983) for thermal

homogeneity of 400 µm. The same applied for branches, except for branches of 'Nadorcott' Mandarin.

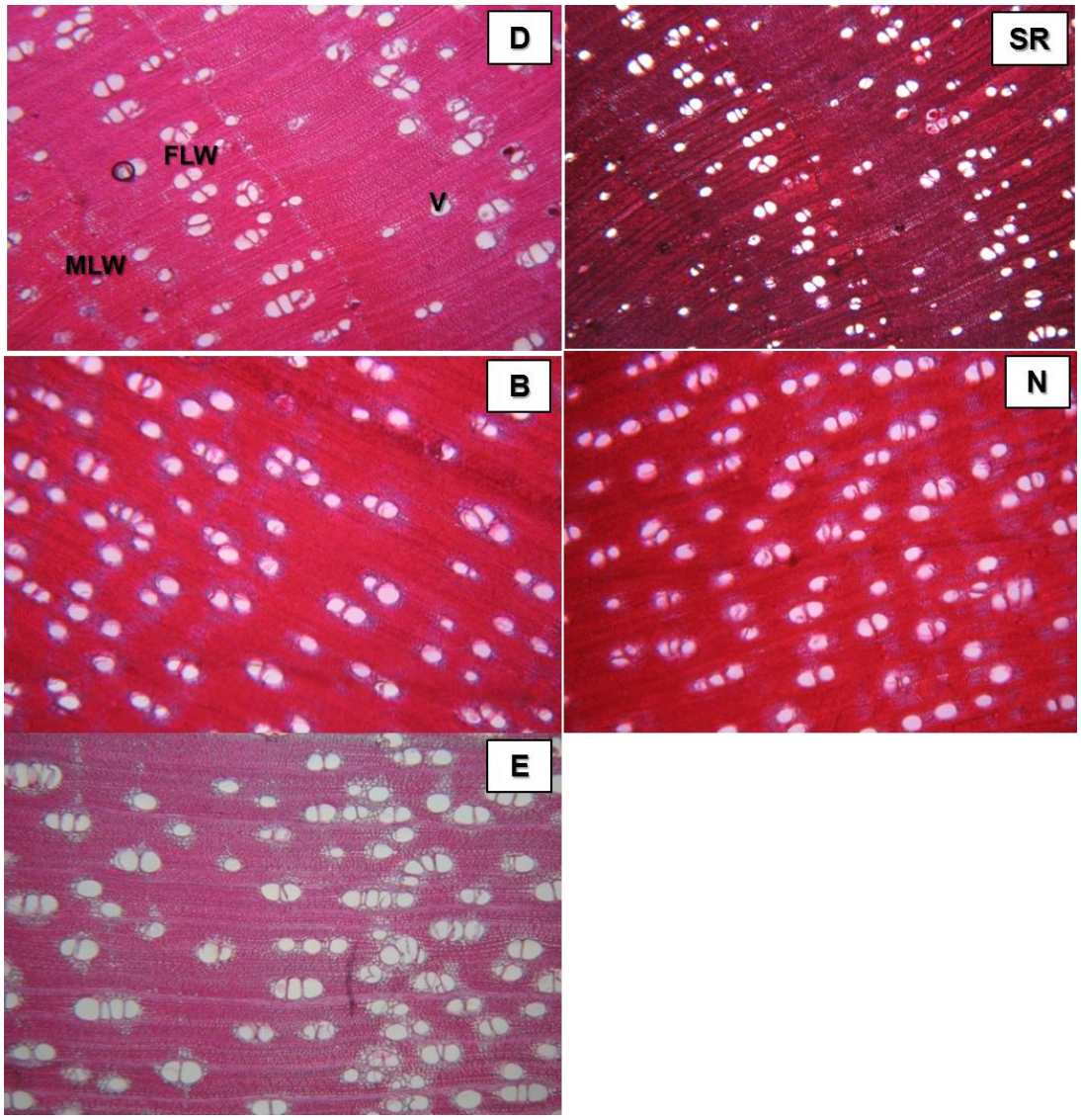

Figure 4.1 Cross sectional images of the wood anatomy for 'Delta' Valencia (D), 'Star Ruby' Grapefruit (SR), 'Bahaininha' Navel (B), 'Nadorcott' Mandarin (N), and 'Eureka' Lemon (E) illustrating both mature leaf wood (MLW) and flush leaf wood (FLW) and xylem vessels (V).

Table 4.1 Distance between the xylem vessels in branches and stems of various citrus species in mature wood. Values are means ± standard error

Species	Stem (µm)		Branch (µm)	
	Inner part	Outer part	Inner part	Outer part
***Citrus sinensis* 'Delta'**	566.8 ± 49.9[a]	542.2 ± 46.6[a]	468.1 ± 26.1[ab]	479.7 ± 65.5[ab]
***Citrus paradise* 'Star Ruby'**	474.0 ± 29.9[b]	539.1 ± 31.1[a]	407.5 ± 33.5[b]	436.5 ± 24.0[ab]
***Citrus limon* 'Eureka'**	458.5 ± 13.7[b]	432.0 ± 6.7[b]	425.7 ± 9.6[ab]	427.4 ± 11.9[ab]
***Citrus sinensis* 'Bahianinha'**	460.4 ± 15.4[b]	481.8 ± 16.8[ab]	518.0 ± 16.9[a]	454.2 ± 18.4[a]
***Citrus reticulate* 'Nadorcott'**	434.6 ± 21.1[b]	396.0 ± 14.5[b]	406.5 ± 23.2[b]	385.1 ± 21.0[b]

In each column, values followed by the same letter are not significantly different at ($p<0.05$).

In the inner part of the stem, the distance between vessels in 'Delta' Valencia was significantly larger than all the other species, which were not significantly different from each other. However, in the outer part of the stem, vessels were spaced significantly ($p<0.05$) wider apart in 'Delta' Valencia and 'Star Ruby' Grapefruit, as compared with 'Eureka' Lemon and 'Nadorcott' Mandarin. Vessel spacing in the branches differed from spacing in the stems, when comparing the different species. In the inner part of the branch, the distances between vessels were significantly greater in 'Bahianinha' Navel, as compared to 'Star Ruby' Grapefruit and 'Nadorcott' Mandarin. In the outer part of the branch, the vessels were more widely spaced in 'Bahaininha' Navel as compared to 'Nadorcott' Mandarin. In general there was very little difference between vessel spacing between the inner and outer sapwood in both stems and branches.

Measurements in the flush leaf wood were only performed for 'Delta' Valencia, 'Star Ruby' Grapefruit and 'Eureka' Lemon, as flush leaf wood was not readily evident in 'Bahaininha' Navel and 'Nadorcott' Mandarin. It was immediately evident that vessels

were spaced much closer together in the flush leaf wood than in the mature leaf wood. For example, whilst xylem vessels were on average spaced 554 µm apart in the mature leaf wood of stems of 'Delta' Valencia, vessels were spaced on average 209 µm apart in the flush leaf wood of these stems. In the flush leaf wood the distance between vessels fell within the limit determined by Swanson (1983) for thermal homogeneity of 400 µm.

Table 4.2 Distance between the xylem vessels in branches and stems of various citrus species in flush leaf wood. Values are means ± standard error.

Species	**Stem (µm)**		**Branch (µm)**	
	Inner part	Outer part	Inner part	Outer part
***Citrus sinensis* 'Delta'**	186.8 ± 8[b]	232.2 ± 35[a]	200.0 ±11[a]	336.7± 132[a]
***Citrus paradise* 'Star Ruby'**	238.3 ± 9[a]	229.4 ±*[a]	197.6 ± 4[a]	169.8 ±15[ab]
***Citrus limon* 'Eureka'**	151.4 ± 8[c]	159.2 ±12[a]	127.3 ± 13[a]	138.3 ± 5[b]
***Citrus sinensis* 'Bahianinha'**	*	*	*	*
***Citrus reticulate* 'Nadorcott'**	*	*	*	*

In each column, Values followed by the same letter are not significantly different at ($p<0.05$).*no flush wood observed in these species

There were no significant differences between species in terms of outer part of the stem and the inner part of the branch. However, in the inner part of the stem there were significant differences between all three species with 'Star Ruby' Grapefruit having the most widely spaced vessels and 'Eureka' Lemon the most closely spaced vessels. In the outer part of the branch the vessels were significantly wider apart in 'Delta' Valencia than the other two species.

The homogeneity of the sapwood is not only dependent on the number of vessels or the distances between the vessels, but also on the size of the vessels (Steppe and Lemeur, 2007). Lumen diameter was therefore also determined in the various citrus species in both stems and branches selected randomly from both mature and flush leaf wood (Table 5.3). 'Bahianinha' Navel tended to have wider vessels (114 µm) on average, whilst 'Nadorcott' Mandarin tended to have narrower vessels (92 µm). Vessel lumen diameter was significantly greater in 'Bahianinha' Navel than in 'Nadorcott' Mandarin in the inner and outer part of the stem, but was not significantly different in samples from branches. In the outer part of the stem 'Star Ruby' Grapefruit also had vessels with a significantly larger diameter than 'Nadorcott' Mandarin, but in the inner part of the branch the vessels from 'Star Ruby' Grapefruit were significantly smaller than those from 'Bahaininha' Navel.

Table 4.3 Diameter of the xylem vessels in branches and stem of various citrus species. Values are means ± standard error.

Species	**Stem (µm)**		**Branch (µm)**	
	Inner part	Outer part	Inner part	Outer part
***Citrus sinensis* 'Delta'**	103.7±11.2^{ab}	112.8± 10.4^{ab}	93.37 ± 6.5^{ab}	94.8 ± 8.5^{a}
***Citrus paradise* 'Star Ruby'**	94.0 ± 4.3^{ab}	121.7 ± 7.7^{a}	89.33 ±8.4^{b}	109.0 ± 18.0^{a}
***Citrus limon* 'Eureka'**	94.1 ± 3.2^{ab}	106.1 ± 1.6^{ab}	106.80 ±3.0^{ab}	116.7 ± 11.2^{a}
***Citrus sinensis* 'Bihaininah'**	106.9 ± 6.6^{a}	128.6 ± 3.1^{a}	109.32 ±5.0^{a}	110.4 ± 3.5^{a}
***Citrus reticulate* 'Nadorcott'**	83.9 ± 4.5^{b}	94.6 ± 2.7^{b}	92.36 ±2.6^{ab}	95.8 ± 1.7^{a}

In each column, values followed by the same letter are not significantly different at ($p<0.05$).

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

5.1 Calibration results

Sap flow techniques provide direct measurements of transpiration rates and have been successfully used to determine xylem sap flow and transpiration rates of woody plants (de Oliveira Reis et al., 2006). However, Smith and Allen (1996) advised that the techniques should be calibrated for each species in which they are to be used, due to uncertainties associated with the use of the techniques. The accuracy of these techniques are critically dependent on assumptions which do not apply to all species, and the determination of parameters that are required for the upscaling of heat pulse velocities to sap flux densities and transpiration. Accurate estimations of transpiration are critical as transpiration plays an important role in physiological processes in plants, affecting their growth and productivity by influencing water relations (Villalobos et al., 2013). Accurate measurements of transpiration rates are important not only for research purposes, but also for irrigation scheduling and improving water use efficiency (Alarcón et al., 2005). It is therefore important that calibration of these techniques occurs before actual measurements in the field (Chen et al., 2012).

In this study the calibration of sap flow techniques was conducted in a laboratory using the stem perfusion method. A similar method to that described by Steppe et al. (2010) was used to obtain independent measurements of sap flux density, which allowed a direct comparison of sap flux density measured by the sap flow techniques to that measured gravimetrically. It was also possible to achieve a range of desirable flow rates and the testing of a wide range of citrus varieties within a short period of time. The stem perfusion method does, however, have some disadvantages. The method is associated with the cutting of the stem, which damages the tissue and creates embolisms in xylem vessels. This can affect the sap flux density measurements, as flow through the stem may not be comparable with that achieved whilst still part of the tree. The problem of tissue damage caused by cutting stems was highlighted by Bleby et al. (2004) and was the reason why these authors choose to conduct their experiment under controlled and natural conditions in a greenhouse using weighing lysimeters.

However, potted citrus trees of a number of varieties and of sufficient size for the insertion of probes are not readily available for calibration of the sap flux density methods using weighing lysimetry.

Calibration of the heat ratio (HR), compensation heat pulse (CHP) and thermal dissipation (TD) methods in citrus species conducted in the laboratory yielded large variations between species, as indicated by different slopes of the linear regression equations in Table 4.1, 4.2 and 4.3, between sap flux density determined gravimetrically and that determined using sap flow sensors. Furthermore, the slope and interception of the regression equations were inconsistent between stems of the same variety. However, significant correlations (R^2>0.7) were attained in some stems of each variety, indicating a good relationship between independent measurements. The performance of heat pulse techniques in our study is similar to reports by Pearsall (2011) who found that even though there were good correlations between the CHP and lysimeter measurements in grapevines, the nature of the relationship was inconsistent ($y = 0.33x + 0.45$, $R^2 = 0.92$ and $y = 1.47x – 1.00$, $R^2 = 0.95$). Similarly, Bleby et al. (2004) found significantly different slopes and intercepts in the relationship between the CHP method and the gravimetric method in *Eucalyptus marginata*. Large variations in sap flux density measured with the CHP method in stems of *Fagus grandifolia* were also observed, although the authors obtained a reasonably good relationship (Steppe et al., 2010).

Measured values of sap flux density across citrus species were higher overall with the TD and CHP methods, than the HR method. As was expected, the HRM performed well under low flow rates and this is one advantage the method has over the CHP method (Burgess et al., 2001). However, unlike the HR method, the CHP method performed better under high flow rates as shown in Figure 4.1 as previously reported by Becker (1998). The good performance of the HR method at low flow rates, as compared to the good performance of the CHP method at high flows rates was also previously observed by Bleby et al. (2004).

All three methods evaluated in this study consistently underestimated the sap flux densities, with large variation in percentage underestimation between techniques, species and individual stems. Previously it was not thought that calibration was necessary for the TD method. It was calibrated in five woody species and in sawdust and this was assumed to be valid for all species (Lundblad et al., 2001). However, other studies have demonstrated the necessity of species specific calibration, in order to obtain accurate measurements (Vandegehuchte and Steppe, 2013). In this study the TD method underestimated sap flux density by between 50 to 90% in the three species evaluated. Similar underestimations were found by Steppe et al. (2010) in *Fagus grandifolia*, where a 60% underestimation of actual sap flow was observed, Hultine et al. (2010) in excised branches of *Tamarix ramosissima* × *chinesis,* where a 50% underestimation was noted and Taneda and Sperry (2008) in *Quercus gambelii* and *Acer grandidentatum*, where underestimations of >50% were recorded. In the study by Paudel et al. (2013) underestimations of 70% were found for apple in greenhouse trees and cut branches, 55% for peltophorum cut branches, 60% for a persimmon orchard tree and 60% for nectarine cut branches.

When comparing our results using the heat pulse methods, the underestimation percentages are higher (50-90% for the HR method and 27-99% for the CHP method) than what was found in previous reports by Cohen et al. (1981) in field and laboratory calibrations of the T-max for *Citrus sinensis* L.*, Pseudotsuga menziesii*, *Platanus orientalis L*. and *Populus alba L*., where the HPVs were underestimated by 45%. Steppe et al. (2010) found that transpiration was underestimated in *Fagus grandifolia* by 35% when using the CHP method. The results were quite similar to the findings of Green and Clothier (1988) in kiwifruit where the CHP method underestimated transpiration by 62% (Sorensen et al., 1999). Smith and Allen (2006) also found serious underestimation of flow through branches of *Azdirachta indica* A. Juss using a heat pulse technique and these authors attributed the underestimation to the diffuse porous nature of the sapwood.

The inconsistency in the regression coefficients and large variation in the percentage of underestimation of the sap flux density obtained from each sap flow method in each species in this study was likely due to the nature of the sapwood, which is reported to be very influential in determining the accuracy of the techniques (Fernández et al., 2006, Green and Clothier, 1988, Swanson and Whitfield, 1981). An additional source of error is also attributed to the contact of the probes with inactive xylem. There are a number of studies which found that large variation in their measurement was mostly due the same problems as observed in this study (Clearwater et al., 1999, Paudel et al., 2013, Pearsall, 2011).

The manner in which flow was increased through the stem segments in this study was also a possible source of error. During this study very high flow rates (>300 cm^3 cm^{-2} h^{-1}) were observed when calibrating all the methods, which was due to the sudden increases in pressure as a result of increasing flow rates using a CO_2 regulator. Whilst the HPV techniques involve a measurement at a single point in time, every 10 min in this study, the TD method is a constant heat method where averages in temperature difference (ΔT) were recorded every 5 min. It was therefore expected that the TDP method would be better able to capture these sudden changes in flow rates. However, although R^2 values of >0.8 were frequently observed in some stems, the TD method greatly underestimated the flow determined gravimetrically, with most slopes of the regression equations being <0.1. The sudden increases in pressure which occurred when trying to increase the flow rate also resulted in water coming out of the holes where the probes were inserted. This could have influenced the measurements of heat pulse velocities and may even have caused the sensors to shift, resulting in probe misalignment. Therefore, in future, when using the stem perfusion method it is suggested that the method used to achieve different flow rates be improved, as these sudden changes in flow are registered by the gravimetric readings but are not necessarily taken into account by the sap flow techniques.

5.2 Sapwood properties

The estimation of sap flux density, both gravimetrically and using sap flow techniques, requires accurate estimation of the cross-sectional area of xylem actively transporting water. In the laboratory calibration this was accomplished by forcing dye solution through each citrus stem to stain the actively conducting area. The distribution of conducting sapwood may explain the variation in measured sap flow between stems, as probes may be placed inadvertently in non-conducting tissue or in areas were conductance is low. This has important implications for studies in whole trees, as it is almost impossible to determine this variation prior to probe insertion, but attempts should be made at the end of field studies to make accurate estimates of conducting tissue relative to probe placement. Nadezhdina et al. (2002) demonstrated that errors of between 90 and 300% result from the assumption of uniform flow at all sapwood depths, which is dependent on the species being evaluated. In the current study, the largest variation in conducting sapwood within a stem was found in all 'Nadorcott' Mandarin samples. Only sapwood towards the outside of the stem was stained with safranin dye, whilst the rest of the sapwood was not stained (Figure 4.12). This could have accounted for the low R^2 values for this species for both the HR and CHP methods. The solution to this problem when conducting field measurements could be to increase the number of replicates per stem to account for the greater variability. In addition, measurements should also focus on the outer sapwood where conductance is higher. A survey of the variability in conducting sapwood area using core samples should also be done prior to probe insertion in order to gain an understanding of this variability in each orchard. This is important as the underestimation of sap flow due to inactive xylem in the profile increases with sap flux density, and it has been found that if 20% of the xylem is inactive, the error in the estimation of sap flux densities can exceed 50% (Paudel et al., 2013).

The reasons for the large areas of inactive xylem within some stems are not clear, but could be attributable to the propensity for greater xylem embolism in some species than others (Eilmann and Rigling, 2012). Paudel et al. (2013) also encountered the problem of continuous inactive xylem and attributed it to the fact that in hot and dry

regions, climate extremes often challenge xylem integrity, and as a result inactive xylem is more common than in other places. This is, however, typically a problem in species with large vessel diameters (Taneda and Sperry, 2008) and as 'Nadorcott' Mandarin samples had smaller vessels than most of the other samples, embolism formation may not have been the major contributing factor. Disease such as citrus blight and gumming can also contribute to the inactive xylem (Newbanks et al., 1983, Vasconcellos and Castle, 1994).

Parameters required for up scaling HPV to sap flux densities and then transpiration, also influence the accuracy of measurements. The conversion of heat pulse velocity to sap velocity is very sensitive to the sapwood moisture content and sapwood density (Green et al., 2003, Steppe et al., 2010, Swanson and Whitfield, 1981b). Steppe et al. (2010) pointed out the importance of these measurements and suggested that samples should be taken at regular intervals. Sapwood moisture content and sapwood density did not exhibit much variation between a wide range of citrus varieties collected from a number of locations. These values can therefore be used with confidence for the determination of transpiration in citrus trees and this is unlikely to be a major source of error in the calculation of sap flux density for the HR and CHP methods.

The arrangement and spacing of xylem vessels within the sapwood can also influence sap flux density measurements. Smith and Allen (1996) suggest that heat pulse techniques can be used in species with thermally homogeneous sapwood without the need for calibration. A thermally homogenous wood is described as having distances between vessels of less than 400 µm (Swanson, 1994), in order for time taken for thermal equilibrium between sapwood and woody matrix to be considered negligible. However, if this distance is exceeded, errors in HPV calculations are expected and calibration of sap flow techniques for such species should be considered. In this study the distances between the xylem vessels in mature leaf wood from both the inner part and outer part of the sapwood for the stems and branches of 'Delta' Valencia, 'Star Ruby' Grapefruit, 'Eureka' Lemon and 'Bahaininha' Navel exceeded 400 µm. As a

result the sapwood of these cultivars can be described as thermally inhomogeneous. The one exception was ‘Nadorcott’ Mandarin, where the distance between the vessels was less than or very close to 400 µm. The sapwood of this cultivar could therefore be considered thermally homogeneous. However, the variation and underestimation of sap flux density observed in ‘Nadorcott’ Mandarin in this study was due to the insertion of probe in non-conducting tissue, as mentioned above. According to Green and Clothier (1988), the lack of thermal homogeneity in the sapwood affects the transmission and the measurements of heat pulse and results in underestimations of sap flux density. Empirical corrections factors are therefore required for these species, to match measured sap flux density to actual sap flux density. Alternatively Green and Clothier (1998) suggest that the theory needs to be adapted to account for heat transport in this type of sapwood.

Importantly, the distribution of vessels within the sapwood of the ‘Delta’ Valencia, ‘Star Ruby’ Grapefruit and ‘Eureka’ Lemon sapwood samples was not consistent and marked differences between mature leaf wood and flush leaf wood were observed. Differences between mature leaf and flush leaf wood were, however, not observed in samples from ‘Bahianinha’ Navel and ‘Nadorcott’ Mandarin, but the techniques did not perform any better in these stems. The distances between vessels in the flush wood was completely different to distances measured in mature leaf wood, with distance between vessels of less than 400 µm (ranging from 127 to 337 µm), which means it did not depart from the definition of a homogenous, porous material. As a result heat is expected to move uniformly through this part of the sapwood and inserting probes into this part of the sapwood is therefore likely to result in accurate measurements, which do not require empirical adjustment. The non-uniform nature of the sapwood in some citrus species could potentially complicate the determination of a conservative calibration for citrus, as it will depend heavily on probe placement i.e. whether probes are placed in mature or flush leaf wood. Zreik et al. (2003) found considerable variation in calibration factors between citrus trees (CV 20%) and warned that the calibration of sap flux density determined by the CHP method cannot be very accurate due to the inhomogeneous nature of the xylem vessel distribution in this tree species. Similar

variation in calibration factors between stems of the same specie was found in this study. One of the contributing factors to this variation was most probably the large variation in sapwood structure between mature leaf and flush leaf wood. Steppe and Lemeur (2007) also noted that differences in wood anatomy between beech and oak trees caused differences in the calibrated values of the hydraulic parameters of the stem.

Underestimation of transpiration by sap flux density techniques has also largely been attributed to the mechanical damage to the sapwood as a result of probe insertion and wounding as a result of intermittent heating (Swanson, 1994). Swanson and Whitfield (1981b) and Burgess et al. (2001) have both derived numerical solutions which allow the estimation of the impact of wounding on the measured heat pulse velocities. Whilst, the determination of wounding can be difficult in whole trees and may even vary with time, in cut stems it is easier to determine by including a stain in the perfusion solution. In addition, as calibration is complete within a few hours, the wound was not expected to develop and using the width of the probe (2 mm) was probably sufficient to account for wounding. Longer term calibration experiments are therefore recommended to account for the impact of wounding on estimates of sap flux density.

Finally, branches were used in this study as opposed to stems, as these could be collected from commercial orchards without felling the entire tree. Whilst, previous studies have also used branches to calibrate sap flux techniques (Burgess and Dawson, 2008, Clearwater et al., 1999, Cohen et al., 1981, Nadezhdina et al., 2007, Paudel et al., 2013, Taneda and Sperry, 2008), it should be noted that the sapwood anatomy differs between stems and branches. Compression or tension wood in branches may have contributed to the variability in sap flow observed in this study.

5.3 Conclusions

In this study sap flux density was consistently underestimated by all techniques and across all citrus species/varieties. Across all varieties, the underestimation of sap flux

density by the HR method was less as compared to other two methods (Table 4.2). Whilst, fairly good correlations between sap flux densities using all techniques and that determined gravimetrically were obtained, indicating that the underestimation was consistent under a range of flow rates, this was not consistent between stems. As a result a single empirical calibration factor was not sufficient to correct the sap flow estimated using sap flux density techniques to that determined gravimetrically. The difficulty in determining a single correction factor and the consistent underestimation of sap flux density by the three techniques suggests that calibration of these techniques may be necessary in each new species and orchard in which measurements are to be made.

The large variation in calibration factors between stems of an individual species and between species was attributed to a number of factors. Firstly, it was noted that on a number of occasions the inserted probes were placed in non-conducting tissue. Large variations in conducting sapwood were noted in some stems, which was impossible to identify prior to the insertion of probes. Secondly, according to the definition of Swanson (1983) the mature sapwood of citrus branches in this study were not thermally homogenous and therefore deviated from the ideal heat pulse velocity theory (Smith and Allen 1996). However, the flush leaf wood adhered to the definition of a thermally homogenous medium, but was not found in all species. Once again it was impossible to determine in which section of sapwood the probes were inserted. Finally, the sudden transient increases in pressure, caused by the use of a CO_2 regulator, could have resulted in flow rates which the sap flow techniques were unable to capture. This could have caused discrepancies between the sap flow determined gravimetrically and that determined with the sap flow sensors. Therefore the most reliable independent method to calibrate sap flow techniques remains weighing lysimetry.

The main aim of this study was to calibrate the most appropriate sap flow technique for citrus. The data showed that all three methods can potentially be used to estimate water use in citrus, provided corrections are made for the departure of the sapwood from the definition of thermal homogeneity and that the area of conducting sapwood is

accurately determined. Our analysis of the performance of sap flow techniques showed that the HR method should perhaps be considered before the CHP and TD methods, as the underestimations of sap flux density were less with this method. Additional support for this technique is provided by Burgess et al. (2001) who suggests that it is important to measure whole plant water use both during the night and when transpiration rates are low, as these are essential for the water balance in seasonally dry environments. Capturing low flow rates is one of the strengths of the HR method (Burgess et al. 2001). From this study it is clear that the xylem anatomy of the stem will have a large influence on measurements and this must be taken into account in each new species in which measurements are to be made and most probably each new orchard.

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APPENDIX A

The GLM Procedure

t Tests (LSD) for stemDisInMw

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	6063.026
Critical Value of t	2.22814
Least Significant Difference	81.786

Means with the same letter are not significantly different.

t Grouping	Mean	N	cult
A	566.85	9	delta
B	474.04	9	StarRuby
B			
B	460.40	9	Biahanin
B			
B	458.56	9	Lemon
B			
B	434.64	9	Nadorcot

The GLM Procedure

t Tests (LSD) for stemDisOutMw

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	8425.718
Critical Value of t	2.22814
Least Significant Difference	96.414

Means with the same letter are not significantly different.

t Grouping	Mean	N	cult
A	542.20	9	delta
A			
A	539.13	9	StarRuby
A			
B A	481.82	9	Biahanin

B			
B	432.03	9	Lemon
B			
B	396.00	9	Nadorcot

The GLM Procedure

t Tests (LSD) for brnchDisInMw

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	10447
Critical Value of t	2.22814
Least Significant Difference	107.36

Means with the same letter are not significantly different.

t Grouping	Mean	N	cult
A	517.95	9	Biahanin
A			
B A	468.07	9	delta
B A			
B A	425.67	9	Lemon
B			
B	407.53	9	StarRuby
B			
B	406.53	9	Nadorcot

The GLM Procedure

t Tests (LSD) for brnchDisOutMw

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	5678.201
Critical Value of t	2.22814
Least Significant Difference	79.148

Means with the same letter are not significantly different.

t Grouping	Mean	N	cult
A	479.75	9	delta
A			
B A	454.21	9	Biahanin
B A			
B A	436.53	9	StarRuby
B A			
B A	427.44	9	Lemon
B			
B	385.15	9	Nadorcot

The GLM Procedure

t Tests (LSD) for stemDiamIn

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	418.0763
Critical Value of t	2.22814
Least Significant Difference	21.477

Means with the same letter are not significantly different.

t Grouping		Mean	N	cult
	A	106.936	9	Biahanin
	A			
B	A	103.770	9	delta
B	A			
B	A	94.107	9	Lemon
B	A			
B	A	94.027	9	StarRuby
B				
B		83.977	9	Nadorcot

The GLM Procedure

t Tests (LSD) for stemDiamOut

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	502.5935
Critical Value of t	2.22814
Least Significant Difference	23.547

Means with the same letter are not significantly different.

t Grouping		Mean	N	cult
	A	128.64	9	Biahanin
	A			
	A	121.66	9	StarRuby
	A			
B	A	112.79	9	delta
B	A			
B	A	106.14	9	Lemon
B				
B		94.64	9	Nadorcot

The GLM Procedure

t Tests (LSD) for brnchDiamIn

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	332.5637
Critical Value of t	2.22814
Least Significant Difference	19.155

Means with the same letter are not significantly different.

t Grouping	Mean	N	cult
A	109.317	9	Biahanin
A			
B A	106.805	9	Lemon
B A			
B A	93.370	9	delta
B A			
B A	92.363	9	Nadorcot
B			
B	89.329	9	StarRuby

The GLM Procedure

t Tests (LSD) for brnchDiamOut

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	892.5371
Critical Value of t	2.22814
Least Significant Difference	31.38

Means with the same letter are not significantly different.

t Grouping	Mean	N	cult
A	116.72	9	Lemon
A			
A	113.13	9	Biahanin
A			
A	109.02	9	StarRuby
A			
A	95.84	9	Nadorcot
A			
A	94.78	9	delta