Specialty Wood Products Research Partnership

UC UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

Quantifying compounds in heartwood extractives of durable eucalypts

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TABLE OF CONTENTS

INTRODUCTION

Many tree species deposit a wide range of compounds into heartwood tissue (Hillis, 1987; Rowe, 1989). Some of these compounds are coloured or have bioactive properties. These compounds can also interfere with processing, e.g. if they interact with adhesives or pulping chemicals. Therefore, heartwood extractives determine the value of these timbers.

Not much is known about the heartwood extractives of the durable eucalypts considered in the SWP programme. Some information is available for other durable eucalypts not subject to the SWP programme (Hillis, 1991). Table 1 lists compounds which have been reported in the heartwood of durable eucalypts. It is likely that some of these compounds will also be present in the species considered in the SWP programme.

The majority of eucalyptus plantations are grown for the pulp industry, featuring sapwood of non-durable species. It can be expected that the compounds reported for these differ to those in the heartwood of durable eucalypts.

Table 1: Extractives present in some durable eucalyptus species

Species	Compounds	Tissue	Reference
Eucalyptus wandoo	• 3,5,4'-trihydroxystilbene • 3,5,4'-trihydroxystilbene-3β-D-glucoside	Heartwood	(Hathway and Seakins, 1959)
Eucalyptus microcorys	• cycloeucalenol	Heartwood	(Da Costa and Rudman, 1958)
Eucalyptus sideroxylon	• 3,5,4'-trihydroxystilbene (resveratrol) (and cis isomer) • 3,5,4'-trihydroxystilbene-3β D glucoside (and cis isomer) • 3,3'-di-o-methylegallic acid • 3,3'-di-o-methylegallic acid glucoside • 3,3',4-tri-o-methylegallic acid • 3,3',4-tri-o-methylegallic acid glucoside • gallic acid • catechin • egallic acid • polymerised leucocyanidin	Heartwood	(Hart and Hillis, 1974; Hillis et al., 1974; Hillis and Isoi, 1965)
Eucalyptus marginata	• leucoanthocyanins	Heartwood	(Hillis, 1956)
Eucalyptus citridora	• *trans*-calamenene • T-muurolol • α-cadinol • β-hydroxy-a-cadinol • 4-hydroxy-3,5-dimethoxybenzaldehyde • 4-hydroxy-3,5-dimethoxybenzoic acid • linoleic acid • squalene • α-tocopherol • erythrodiol • morolic acid • betulonic acid • cycloeucalenol • cycloeucalenol vernolitate • β-sitosterol	Heartwood	(Lee and Chang, 2000)

	• β-sitosteryl-β-D-glucopyranoside • β-sitostenone • yangambin • sesamin		
Eucalyptus astrigens	• catechin • gallic acid • 3,5,4'-trihydroxystilbene (resveratrol) • 3,5,4'-trihydroxystilbene (resveratrol) glucoside • chlorogenic acid • polymerised ellagitannins • ellagic acid	Heartwood	(Hillis and Carle, 1962)
Eucalyptus camaldulensis	• gallic acid • vanillin • syringaldehyde sinapaldehyde • ellagic acid • naringenin • proanthocyanidin	Branch, mostly sapwood	(Cadahia et al., 1997; Conde et al., 1995)

It is known that the relative amounts of heartwood compounds differ between trees of the same species (Fries et al., 2000; Haupt et al., 2003; Partanen et al., 2011). This within species variation implies different wood quality, i.e. durability, colour or processing parameters. The nature of the chemicals, the variation in their abundance within a species, the genetic control of their abundance as well as their biological or chemical properties are unknown for the durable eucalypts in the SWP programme. Being able to quantify the individual extractive compounds in the heartwood of these trees can be useful in several aspects:

1. It will be possible to determine the variation in the chemical composition of the heartwood extracts within a species. In conjunction with our breeding trials this will allow us to quantify genetic parameters and get an idea on the variability of the product.
2. It will be possible to identify the most fungicidal compounds in conjunction with bioactivity tests.
3. Currently the breeding programmes for heartwood quality focuses on high extractive content (Li and Altaner, 2016). Knowledge of the chemical composition and the bioactivity of the compounds will allow to select those individuals with not only a high but also the most potent extractives in the heartwood – a second generation improvement of heartwood quality.
4. Separating the individual compounds is a first step towards identifying their chemical structure.
5. Some difficulties in the processing of durable heartwood, e.g. gluing, has been contributed to heartwood extractives. Processing can be optimised with the knowledge of the nature of the heartwood compounds.
6. Individual heartwood compounds might have some value themselves. Consequently they could provide an additional revenue stream for growers of durable eucalypts.
7. Understanding the nature of key heartwood components will give deeper insight into the biochemical processes of heartwood formation.

METHODS

Chromatography is typically used for the separation and quantification of heartwood extractives. We report here on the use of gas chromatography (GC) and reverse-phase high pressure liquid chromatography (RP-HPLC) to analyse ethanol extracts of *E. bosistoana* and *E. globoidea* heartwood.

Extraction

Milled heartwood was extracted with ethanol using an Accelerated Solvent Extractor (Thermo). Extraction conditions were 70 ºC, 15 min static time and 2 cycles. The ethanol extract was chosen as growth of wood decaying fungi were retarded the most by the ethanol soluble fraction of the heartwood extracts of *E. bosistoana* (Van Lierde, 2013).

Gas Chromatography (GC)

Ethanol extracts were dried. From the dry extracts 10 mg of dry exact were dissolved in 0.1 ml pyridine. A 0.5% (w/v) solution of betulin in pyridine was used as internal standard (IS). 10 µl of IS were added to 90 µl of the extract solution. The samples were then silylated by adding 50 µl BSTFA (N,O-bis-trimethylsilyl-trifluoroacetamide) to 15 µl of the pyridine solution at room temperature for at least 30 min.

1 µl of the silylated sample was injected into a GC (Agilent 7820A) equipped with an Agilent HP-5 (30 m x 320 µm x 0.25 µm) column and a FID detector. GC settings were:

- injection temperature: 300°C
- initial oven temperature: 116°C
- final oven temperature: 280°C
- temperature ramp: 7°C/min
- holding time: 20 min
- detector temperature: 300°C,
- H_2 flow rate: 30 ml/min
- air flow rate: 400 ml/min
- make-up gas flow rate: 25 ml/min

Reverse-phase high pressure liquid chromatography (RP-HPLC)

Ethanol extracts were filtered and injected into a Dionex HPLC system equipped with a Phenomenex Luna C18 column and a UV detector. The elution gradient was from a 9:1 water: acetonitrile mixture to pure acetonitrile over 60 min. The flow rate was set to 1 ml/min and the column oven temperature to 40°C.

Data management

Agilent's Chemstation software package was used to automatically integrate the peaks in the chromatograms. The peak tables were imported into R (R Core Team, 2014) and the GCalignR package (Ottensmann et al., 2017) was used to align the elution times of the compounds in the individual chromatograms for further analysis.

RESULTS

Gas Chromatography (GC)

Numerous (~75) compounds could be separated by GC in the silylated ethanol heartwood extracts of *E. bosistoana* Figure 1. Chromatograms of silylated *E. globoidea* ethanol heartwood extracts revealed that the majority of these compounds were identical to those of *E. bosistoana* (data not shown). The objective of this work was not to investigate the differences in heartwood extractive composition between species and within species but to develop a method able to quantify heartwood compounds for future investigations.

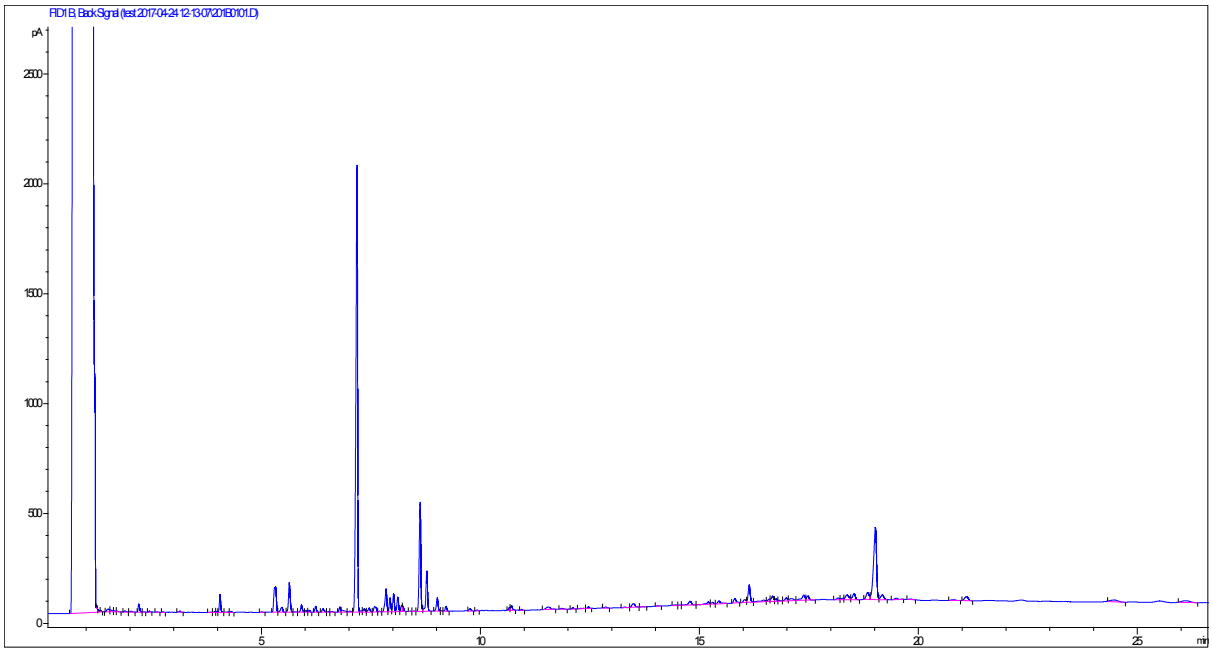

Figure 1: Gas chromatogram of an ethanol extract of *E. bosistoana* heartwood after silylation.

The repeatability of the analytical method was investigated by repeatedly (7 times) analysing an *E. globoidea* ethanol extract. Of the 75 signals only 35 were found in all runs. The signals which were not reliably detectable, i.e. too small quantities, were not included on the further analysis. Between seven repeated measurements of the *E. globoidea* ethanol extract 30 of the 35 reliably detectable signals had a CV of less than 10% (average 6%). Generally a coefficient of variation (CV) of 5% is achievable for quantification of compounds by GC (Hübschmann, 2015).

Some compounds showed a CV of up to 44% between the runs. A closer investigation showed that this was either caused by incomplete separation of two compounds (peak overlap) and associated difficulties with setting integration limits or an instability of the silylation product (Figure 2).

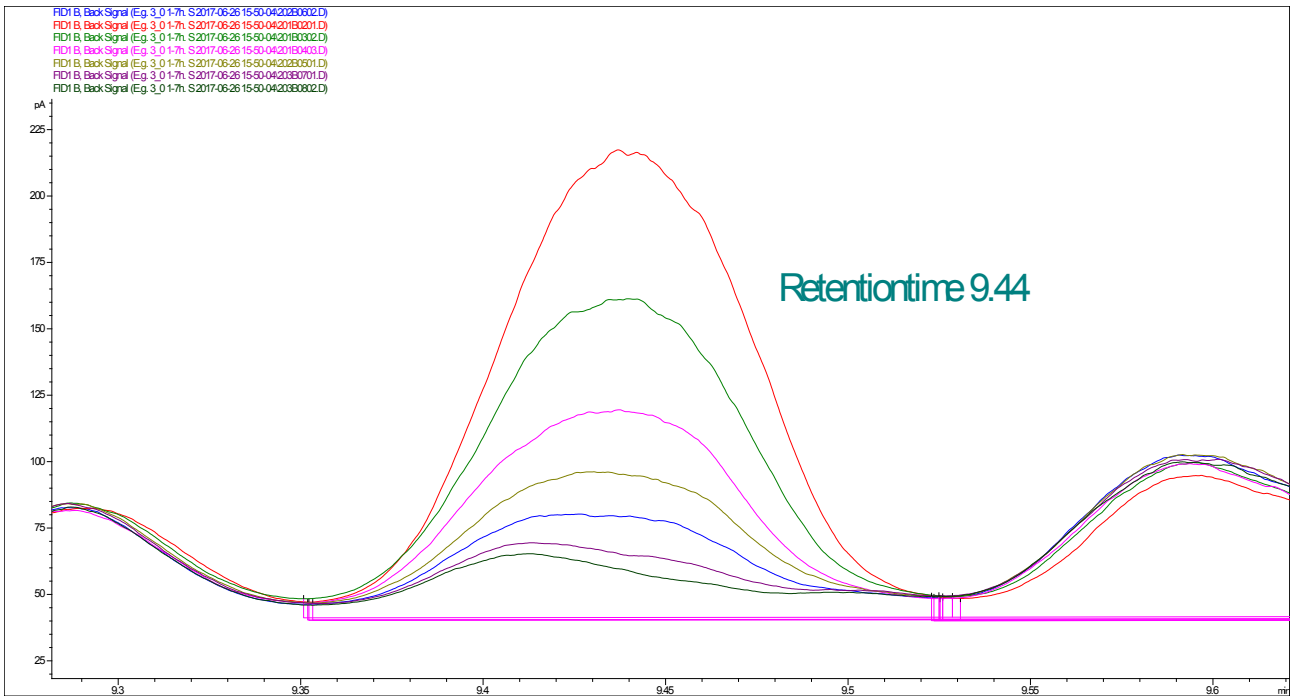

Figure 2: Example of an unstable silylation product of a compound in an ethanol extract of *E. globoidea* heartwood. The time between the runs was ~1h (the 3rd last number of the file name encods the sequence) Note: the good repeatability of the neighbouring signals.

Internal Standard (IS)

Seven compounds were tested for their suitability to act as an internal standard (IS) for the durable eucalypt ethanol heartwood extracts. These were vanillin, betulin, quercetin, flavone, gallic acid, trans-stilbene and syringic acid. Betulin was judged to be suitable as it did not co-eluate with extractive compounds (Figure *3*), had a stable silylation product and a linear detector response (Figure *4*).

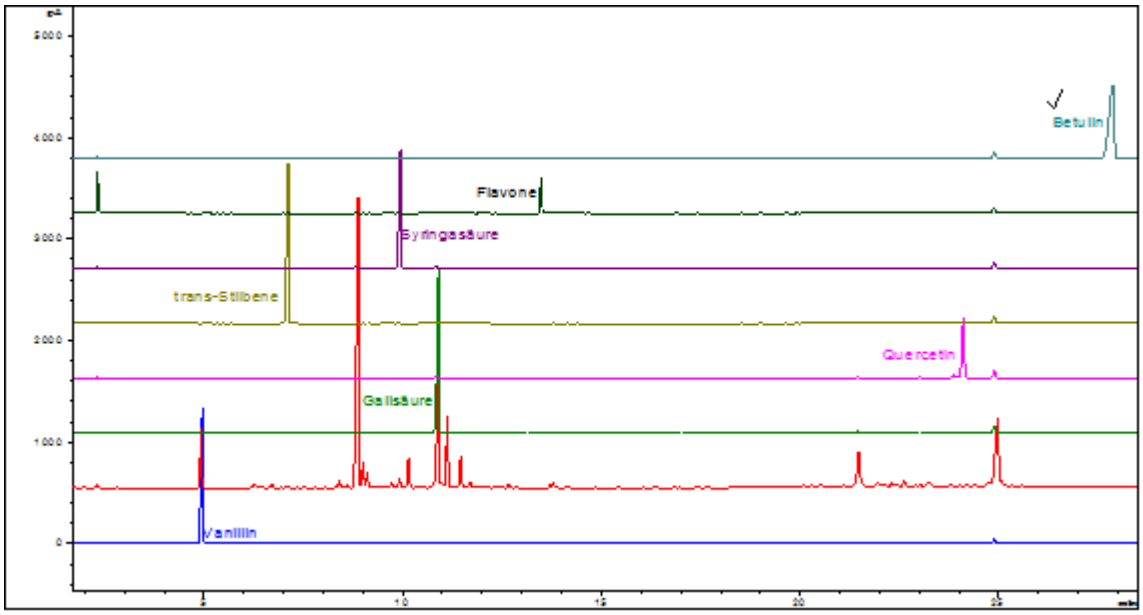

Figure 3: Elution times of compounds considered as internal standard (IS) as well as an *E. bosistoana* heartwood ethanol extract (red).

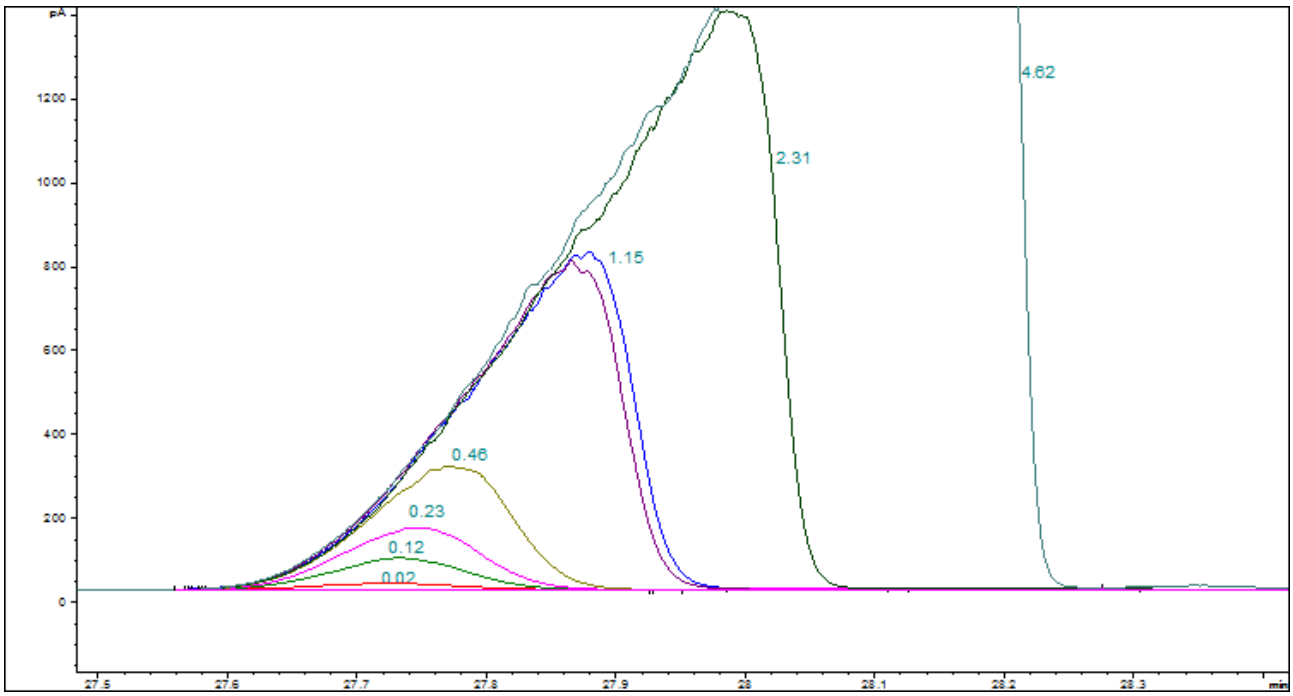

Figure 4: Concentration series of betulin. Note: the blue chromatogram is a repeat measurement of the betulin stock solution after several weeks.

Reverse-phase High Pressure Liquid Chromatography (RP-HPLC)

Numerous (~15) UV active peaks (compounds) can be separated by RP-HPLC in the ethanol extract of *E. bosistoana* heartwood (Figure 5). Most peaks had a very similar UV spectrum, indicating a common molecular core structure. The chromatogram also showed that the analysis time can be approximately halved as no compounds were detected after 30 min. As GC separated more compounds compared to RP-HPLC, it was decided not to further pursue work to optimise the RP-HPLC method at this stage. However, the technique might be useful in future for isolating compounds or if derivatisation is not acceptable.

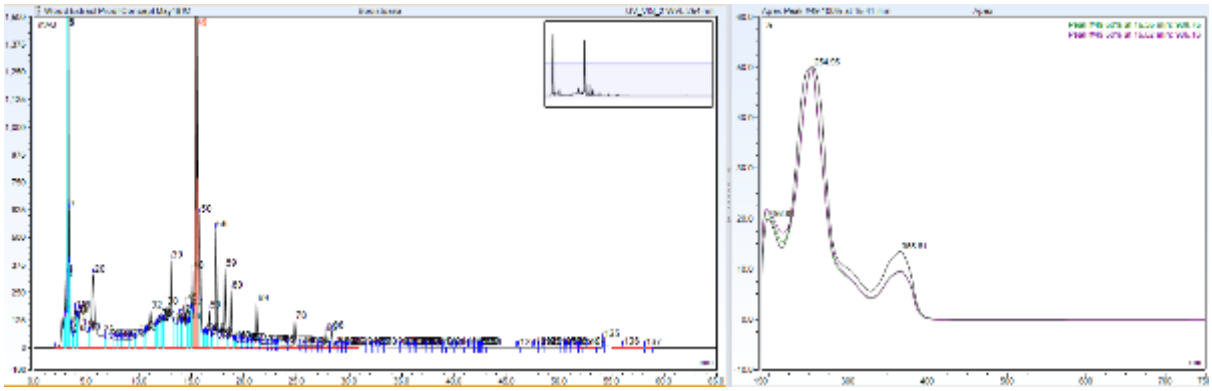
Figure 5: RP-HPLC chromatogram of an ethanol extract of *E. bosistoana* heartwood (left). UC spectra of two signals (right).

CONCLUSION

Gas chromatography (GC) of silylated ethanol extracts was found to be able to quantify 30 compounds in the heartwood of *E. bosistoana* and *E. globoidea*. A suitable internal standard was betulin. The variation in the composition of heartwood extracts can be investigated with this method.

Reverse-phase liquid chromatography (RP-HPLC) has potential to analyse the chemical composition of ethanol heartwood extracts without derivatisation. However, fewer compounds can be separated with RP-HPLC compared to GC.

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