Specialty Wood Products Research Partnership

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

Developing GC methods for analysing the foliar chemistry of durable eucalypts: a potential pest-tolerance screening tool

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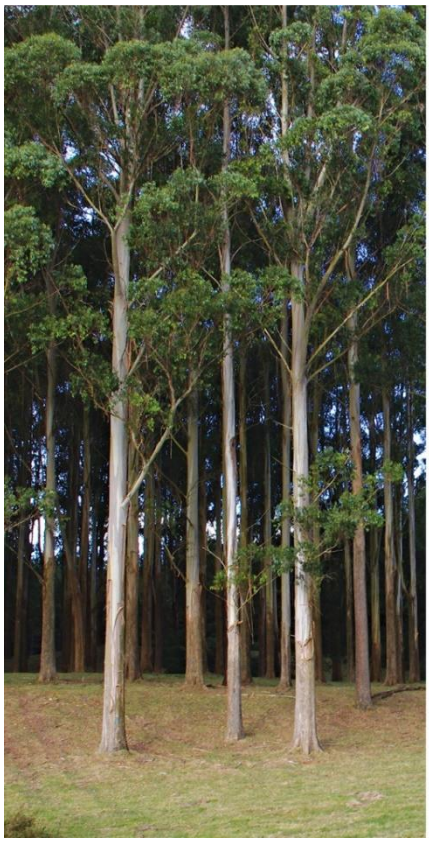

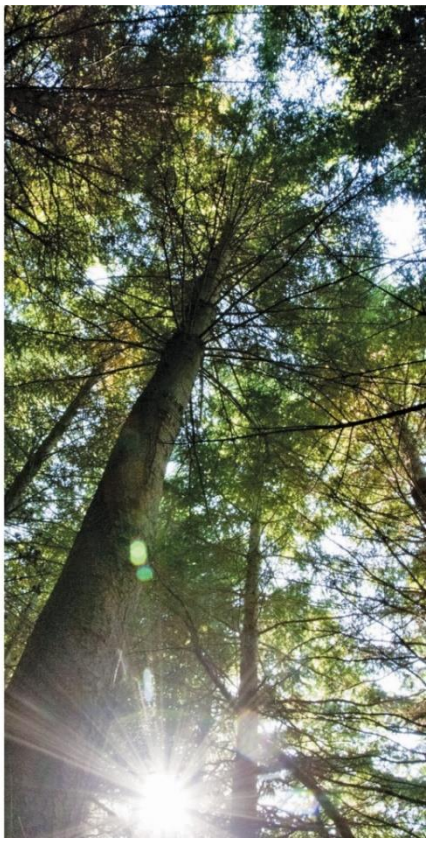

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INTRODUCTION

Background

A key component of the durable eucalypt insect pest management programme at UC is to develop tools for the rapid assessment of the breeding populations with respect to insect pest susceptibility. Historically this has required time-intensive visual assessments of defoliation levels and defoliation impacts on trees in the field during spring and summer when insects are actively feeding. We aim to determine if foliar chemistry can be used to predict and screen for insect susceptibility in a more quantifiable and rapid manner. Eucalypt foliage has a number of characteristics (toughness / sclerophylly, low nitrogen value, high in protein-binding tannins that limit nitrogen availability, high levels of toxic plant secondary metabolites (PSM)) that make it unpalatable to most herbivores (Cooper 2001). As such, insects that do feed on eucalypts foliage tend to be highly specific to the genus and often show preferences for individual species, trees or even individual branches within trees (e.g. Padovan et al. 2012) and use PSMs, among other chemical and physical foliar characteristics, to select oviposition and feed sites.

The numerous PSMs found in eucalypt leaves, such as terpenes and phenols, are considered to play a key role in defence against both mammalian (e.g. Lawler et al. 1998, Lawler et al. 2000, O'Reilly-Wapstra et al. 2004, McArthur et al. 2010b) and insect (Edwards et al. 1993, Bustos-Segura et al. 2015) herbivores, and show marked quantitative and qualitative variation both within and between species (Moore et al. 2014). If foliar 'chemotypes' (qualitative chemical differences within a plant species that remain constant through most life stages of a particular tissue (Bustos-Segura et al. 2015)) can be identified that are 1) under genetic control and 2) indicative of susceptibility to the suite of eucalypt insect pests established in New Zealand, this may provide a means of quickly screening breeding trails to 'weed out' genotypes that are likely to suffer high levels of defoliation and maintain those which are relatively resistant to herbivores. Chemotypes are considered relatively common in the Myrtaceae (Keszei et al. 2008), including *Eucalyptus*, and examples have been definitively identified within NZDFI interest species *E. tricarpa* and *E. camaldulensis* (Andrew et al. 2013, Bustos-Segura et al. 2017).

Here we aim to develop a sampling protocol (leaf collection and preparation), chemical extraction method and gas chromatography (GC) method to assess and compare chemical profiles of eucalypt foliage. The protocol developed will then be field tested on at least one species (*E. bosistoana*) and if successful the resulting methods will be available to a future PhD student (position currently being advertised), allowing that student to rapidly commence an assessment of foliar chemistry across species and sites with respect to their observed and expected susceptibility to insect attack. This will fast track the new PhD student's work programme allowing them to apply the methodology as part of the selection process for insect resistance in the durable eucalypt breeding programme. The expression of heritable chemical and physical leaf traits that effect defoliation by insect herbivores are also likely to be influenced by the environment (e.g. McArthur et al. 2010a, McKiernan et al. 2014, Bustos-Segura et al. 2015), and this will be a focus of later work.

GC for analyse of foliar chemical composition

Gas chromatography is a well-established analytical tool used to separate and analyse chemical compounds. The basic principal of GC is that the chemical and physical properties of the different compounds in a sample determine the time they take (retention time) to pass along a column within the gas chromatograph under specific conditions. Each compound is detected as it is 'eluted' (i.e. reaches the end of the column), resulting in a unique GC profile.

The retention times, and therefore separation, of chemical compounds in a sample are influenced by numerous variables which need to be optimised and standardised in order to produce high quality repeatable data. This process is known as 'Method Development' and involves column selection (stationary phase and dimensions: column id, length, and film thickness), carrier gas selection

(nitrogen, helium, flow rate), temperature programing (initial temperature, initial hold, ramp rate, final temperature, and final hold), injector temperature and detector temperature (Singh, et al. 2013).

Steps involved in Method development are (Charde et al. 2014);

- Understanding the Physicochemical properties of sample
- Selection of chromatographic conditions
- Developing the approach of analysis
- Sample preparation
- Method optimisation
- Method validation

Temperature programming as part of the chromatographic conditions is particularly important as temperature influences the volatility of the analytes, as such the column at the heart of the GC is placed in a thermostatically controlled oven (Grob & Barry, 2004).

Objective

Develop and test methods (foliage preparation and GC Method Development) for assessing the foliar chemistry of durable eucalypt leaves to determine if foliar chemical analysis can be used in the rapid assessment of eucalypt susceptibility to insect pests as part of the durable eucalypt breeding programme.

EXTRACTION METHOD DEVELOPMENT

For initial method development foliage was obtained from potted *Eucalyptus bosistoana* grown from seed by Proseed nursery. Seed material was originally obtained from Australia as part of the NZDFI breeding programme and as such the trees used are representative of some seed lots included in the NZDFI planting trails at Harewood nursery (Christchurch) and field trials established in Marlborough. Leaves were collected from 4 individual *E. bosistoana* trees exhibiting variable leaf morphology (Fig. 1).

Figure 1: Potted *Eucalyptus bosistoana*, with highly variable foliar morphology, used for leaf extraction and GC method development trials.

Methods

Two solvents were tested (Table 1) for obtaining foliar extracts based on methods previously used for GC analysis of *E. camaldulensis* and *E. globulus* foliage. (1) Following Bustos-Segura et al. (2017) approximately 0.5g (fresh weight) of leaf material, was cut into small pieces, placed in 5ml EtOH (500ml EtOH+25µl n-hexadecane as internal standard[1]) and refrigerated for 7 days. (2) Following Troncoso et al. (2011), two leaves were cut into small pieces, placed in 50ml n-hexane (no internal standard) and kept in the fridge for 2 days. A variation of (2) was also included where approximately 0.5g of leaf material was placed in 50ml n-hexane (no internal standard) and kept in the fridge for 7 days to allow a more direct comparison with the EtOH method.

Table 1: Fresh weight (g) of leaf material placed in either EtOH or n-hexane to extract chemical compounds for GC analysis.

Sample	Solvent	Fresh weight (g)	Refrigeration (days)
Plant 1	5ml EtOH	0.53	7
Plant 2	5ml EtOH	0.52	7
Plant 3	5ml EtOH	0.54	7
Plant 4	5ml EtOH	0.54	7
Plant 1	50 ml n-Hexane	0.57	7
Plant 1	50 ml n-Hexane	1.26	2

[1] 500ml was an error; 100ml+25µl n-hexadecane should have been used and was subsequently used in the GC Method Development where it proved to be a much more appropriate volume producing better results.

The resulting analytes were assessed using gas chromatography following the operation procedures as outlined in Bustos-Segura et al. (2017) and Troncoso et al. (2011) respectively:

EtOH analysis: Injector temperature 250°C, splitless, DB-5 Agilent column (30m), oven temperature: 100°C for 4 min, ramping to 180°C at 20°/min, held for 10 min, ramp up again at 20°C/min and held at 250°C for 4.5 min, total elution time 26 min, detector temperature 350°C.

n-Hexane analysis: Injector temperature 275°C, splitless, DB-5 Agilent column (30m), oven temperature: 60°C for 5 min, ramping to 275°C at 10°/min, held for 15 min, detector temperature 350°C.

Results

Both solvents trialled gave peaks, however stronger and more clearly separated peaks were detected using the EtOH solvent (Fig. 2). The EtOH solvent resulted in a double peak at 1.735 and 1.993 minutes (Fig. 2) indicating the method as outlined in the literature is not well optimised for this particular analysis and does require further Method Development. When the EtOH extraction method was repeated for samples of all 4 plants, peaks were clearly detected in all cases, indicating the extraction method is performing consistently (Fig. 3). Despite being the same species, the four plants sampled all showed clear differences in GC profiles, indicating, as expected, quantitative and qualitative variation in PSMs.

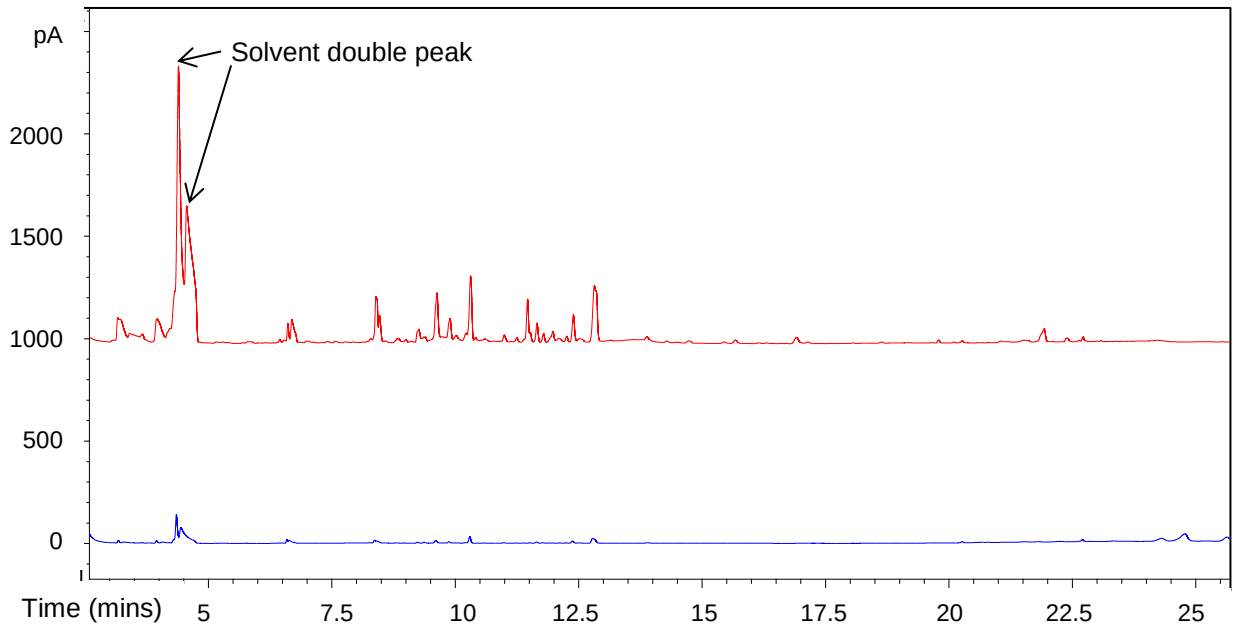

Figure 2: Comparison of GC profiles detected for plant 1 foliage using ethanol (red) vs. hexane (blue) as a solvent with a 7 day extraction period.

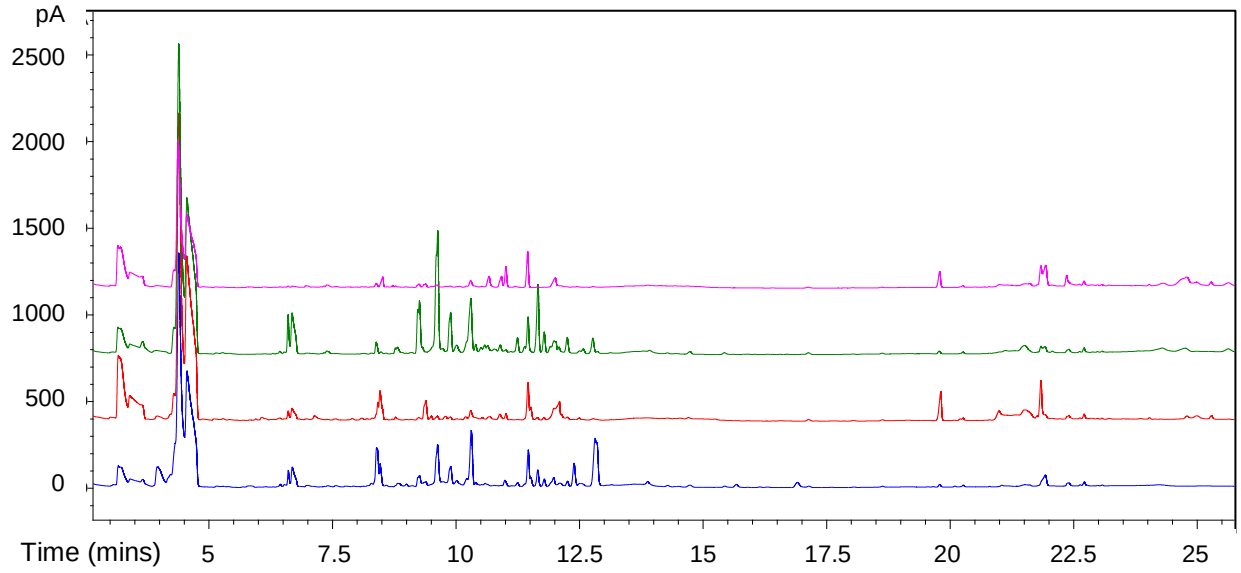

Figure 3: GC profiles for *E. bosistoana* Plants 1-4 (blue = 1, red = 2, green = 3, pink = 4) following extraction with EtOH as solvent.

Using the Hexane solvent, extraction over just 2 days (following the methods of Troncoso et al. (2011)), resulted in lower concentrations of foliar chemical compounds with peaks barely visible, while those extracted over 7 days were more concentrated (Fig. 4).

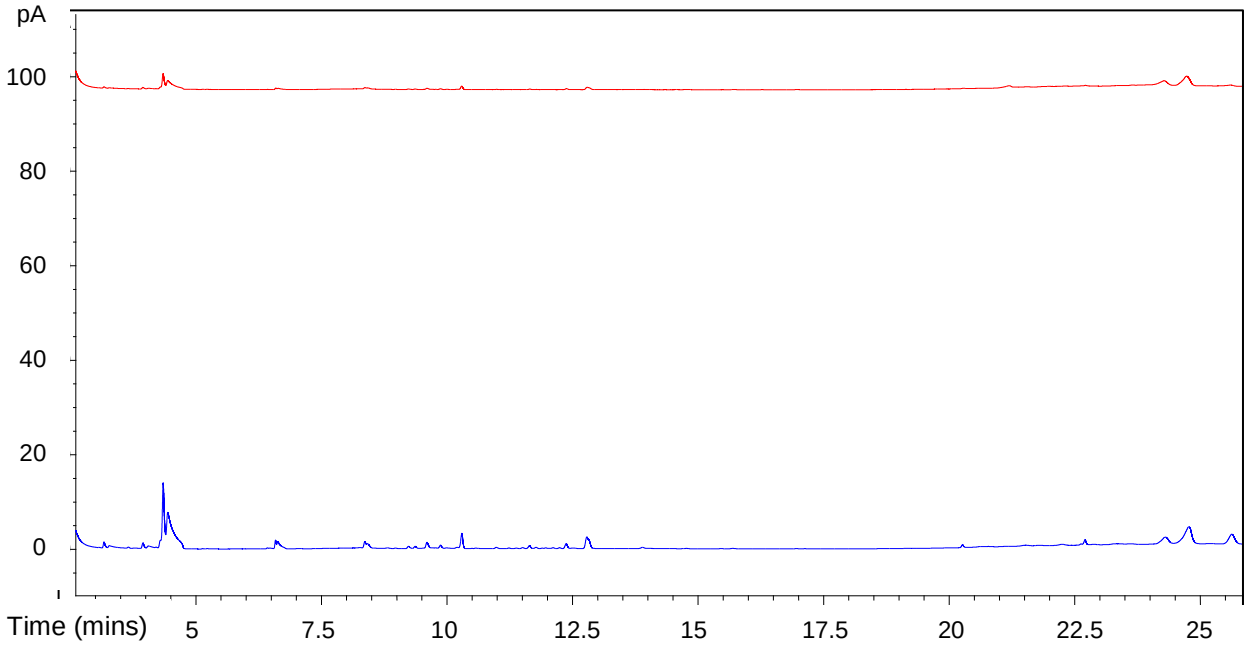

Figure 4: GC profiles following n-Hexane extraction for 2 (red) vs. 7 (blue) days.

GAS CHROMATOGRAPHY METHOD DEVELOPMENT

Methods

For development of the GC method preparation of leaf material was standardised for all trials. *Eucalyptus bosistoana* leaves were cut into small pieces and foliar chemical compounds extracted from 0.5g samples with EtOH (100ml EtOH+25µl n-hexadecane) over a refrigeration period of 7 days. An initial trial was run with 2 separate replicates of each of the 4 plants to determine if one leaf per sample was sufficient to obtain repeatable results. GC profiles indicated multiple leaves per sample were more appropriate, therefore multiple leaves of individual plants were used in the preparation of samples for all subsequent tests.

A control run was conducted to identify the retention time of the standard (Fig. 5).

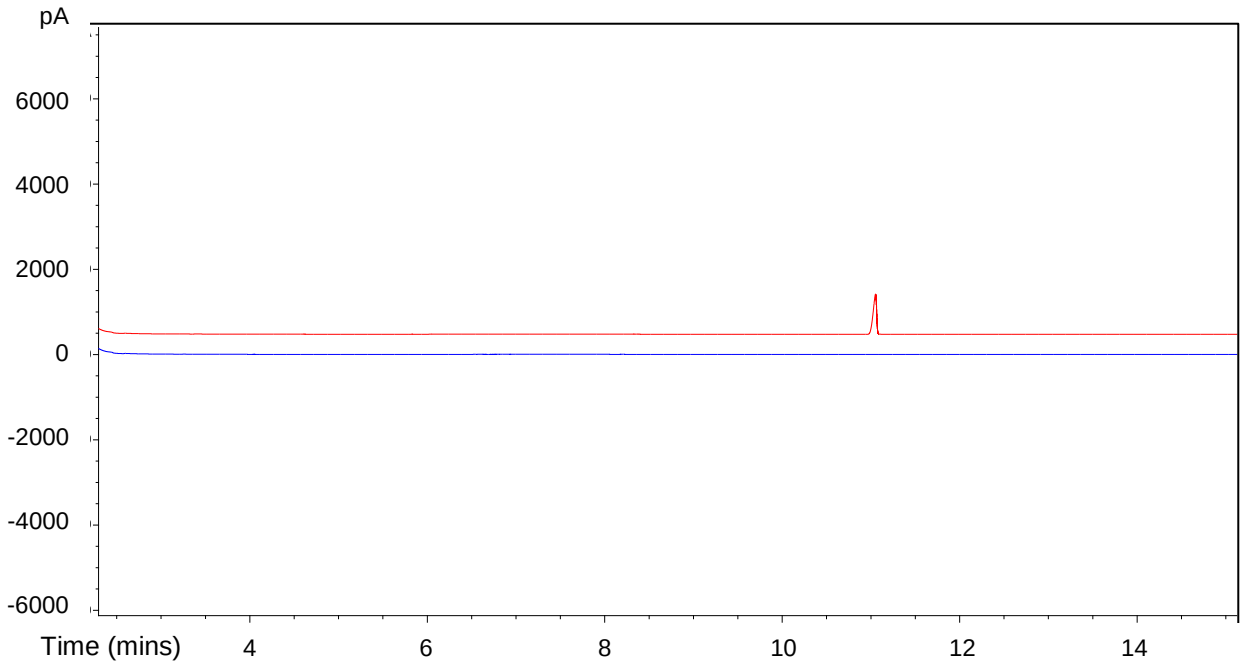

Figure 5: GC profile for pure ethanol (blue) vs. ethanol + standard (red: 100ml Ethanol + 25 µl n-hexadecane). The standard exhibited a retention time of 11.052 min, 2599pA.

Method development (temperature regime)

To improve on the performance of the original method a series of 14 method iterations were run with each successively varying a single parameter at a time (Table 2).

Table 2: Chromatographic conditions used in 14 successive trails showing progressive variation in temperature, ramp rate and hold time for GC Method Development.

	Rate °C/min	Value °C	Hold time (min)
Test 1	Initial	100	4
	20	180	10
	20	250	4.5
Test 2	Initial	120	4
	20	180	10
	20	250	4.5
Test 3	Initial	120	4
	10	180	10
	20	250	4.5
Test 4	Initial	100	4
	10	180	10
	20	250	4.5
Test 5	Initial	100	4
	10	180	5
	20	250	4.5
Test 6	Initial	100	4
	10	180	5
	30	250	4.5
Test 7	Initial	50	4
	20	180	10
	20	250	4.5
Test 8	Initial	50	0.5
	20	180	5
	30	300	5
Test 9	Initial	50	0.5
	20	180	0.5
	50	300	5
Test 10	Initial	50	0.5
	50	100	4
	10	180	5
	30	250	4.5
Test 11	Initial	50	0.5
	50	100	4
	10	180	5
	30	300	4.5
Test 12	Initial	50	0.5
	50	100	4
	10	180	5
	50	300	4.5
Test 13	Initial	50	0.5
	50	100	2
	10	180	3
	50	300	4.5
Test 14	Initial	50	0.5
	50	100	0.5
	10	180	0.5
	50	300	4.5

Results

The double solvent peak observed in the extraction method development was present in tests 1-6 (Fig 6, 7) but was eliminated by changing the initial temperature to 50°C in test 7 (Fig. 7). The number of compounds separated and detected was increased under conditions applied in test 10-12. A comparison of test 10 with 11 and 12 indicated an end temperature of 300°C was required to get all compounds off the column (Fig. 8) however total duration was still long. Shortening the second and third hold times to 2 and 3 minutes respectively was found to reduce duration (test 13, Fig. 9) however this resulted in the standard peak being overlaid with another peak. Test 14 (1st, 2nd and 3rd hold times reduced to 0.5 mins) eliminated the overlay and was an improvement with regard to separation of the peaks before the standard. A comparison of test 14 with the original method (Fig. 9) showed the overall improvement in the separation of peaks detected as result of increasing the end temperature from 250ºC to 300ºC, and the reduction in total duration required to get all compounds off the column.

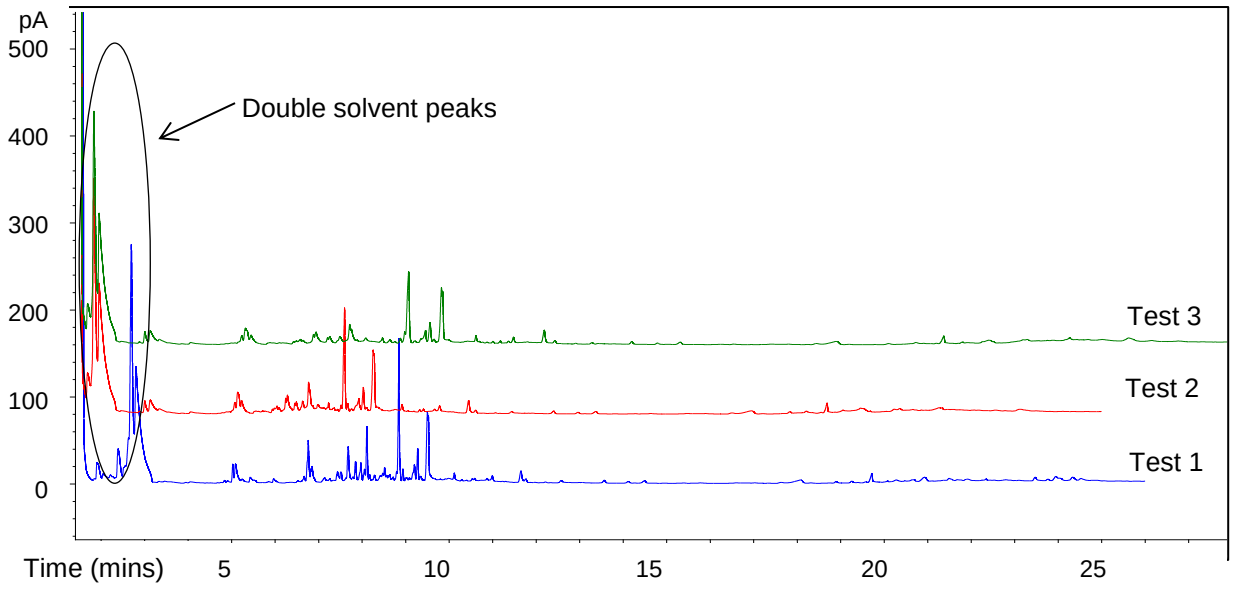

Figure 6: GC profiles comparing Tests 1 (blue), 2 (red) and 3 (green) showing retention of a double solvent peak.

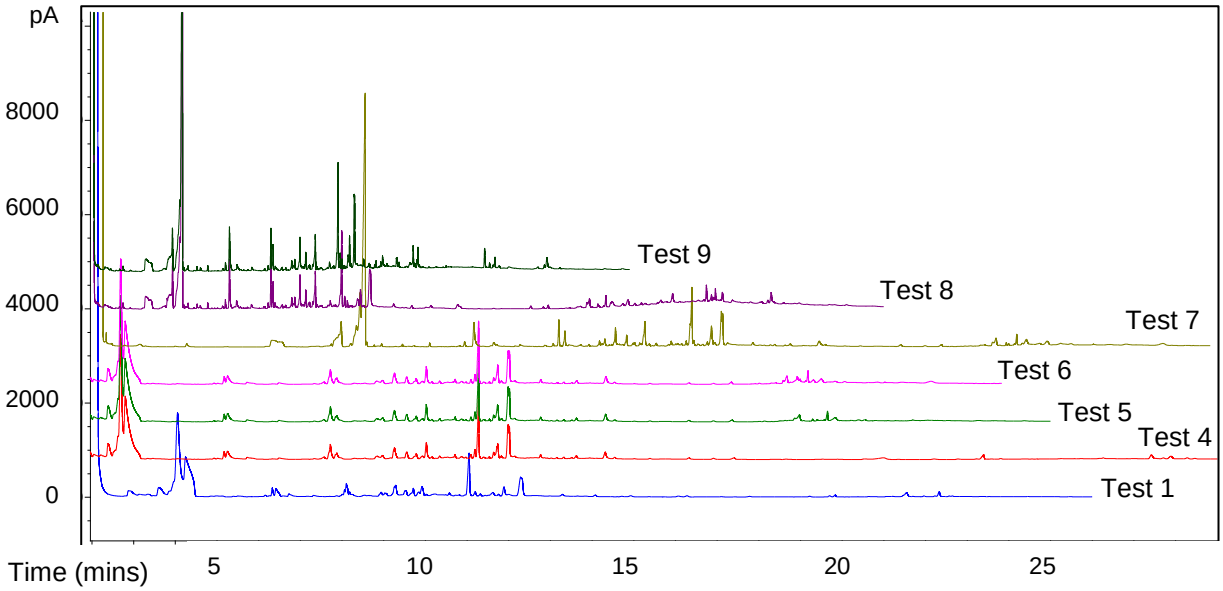

Figure 7: GC profiles comparing test 1 with tests 4-9 showing elimination of double solvent peat in tests 7, 8, 9 by reducing initial temperature to 50ºC.

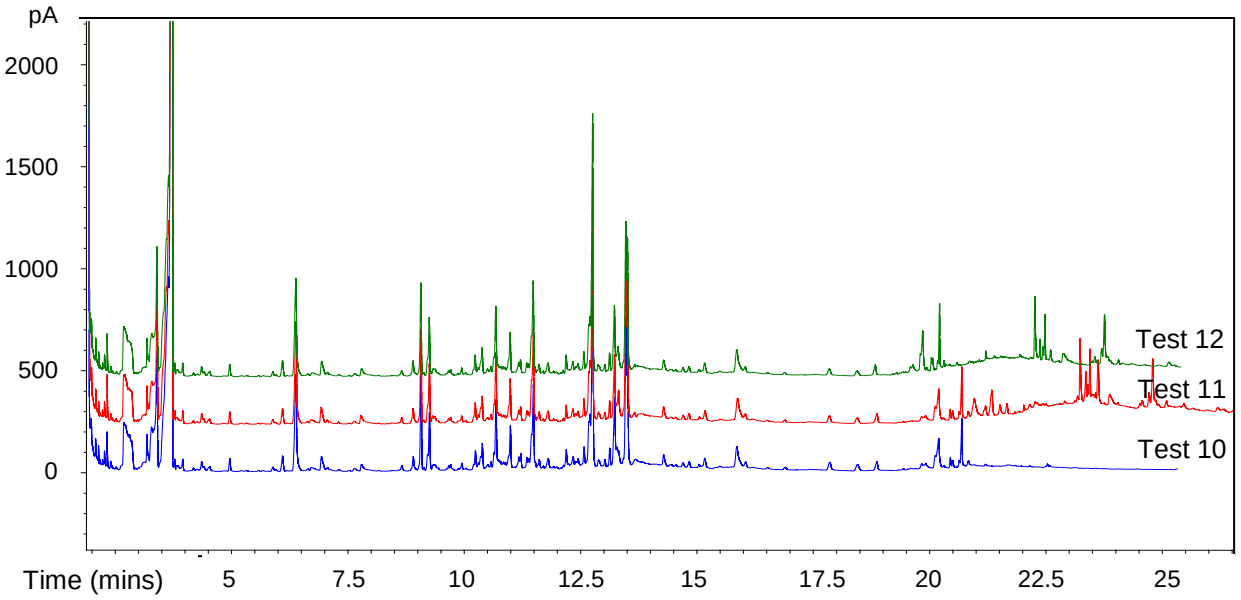

Figure 8: GC profiles comparing tests 10 (blue), 11 (red) and 12 (green). Test 10 has lower end temperature (250°C vs. 300°C) preventing all compounds from coming off the column.

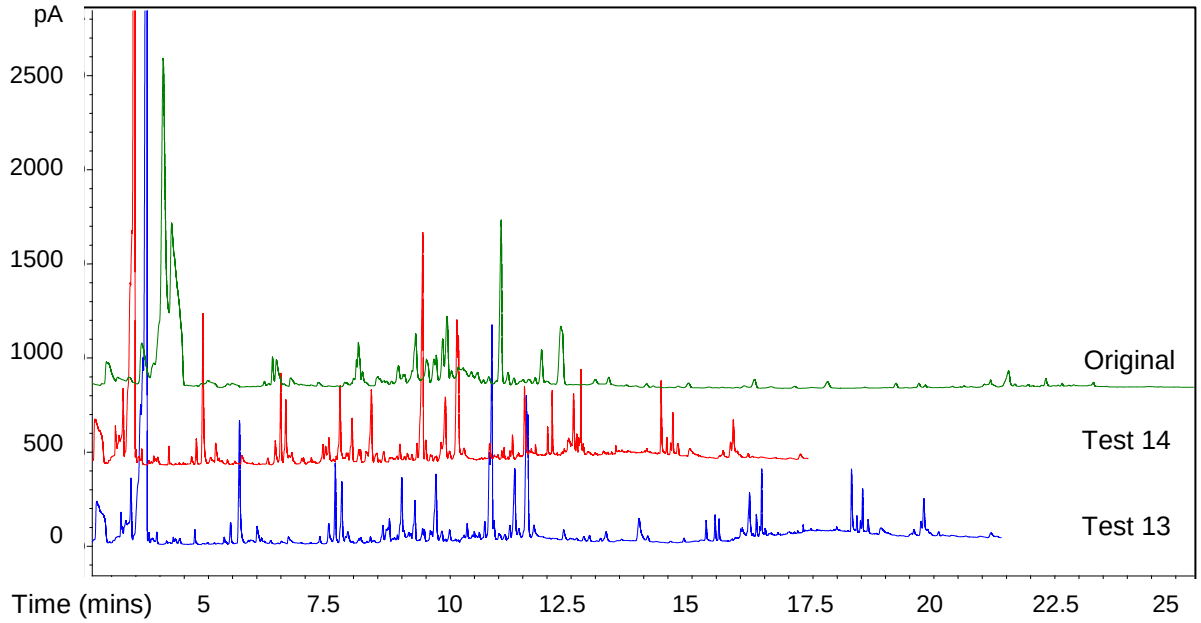

Figure 9: GC profiles comparing tests 13 (blue, standard = 10.86 min retention time) and 14 (red, standard = 9.43 min retention time) with the original method (green, standard = 11.05 min retention time) showing overall improvement in the separation and number of peaks detected.

CONCLUSIONS

Ethanol was found to be a better solvent for use with *E. bosistoana* leaf material in terms of the number and concentrations of compounds able to be extracted and subsequently detected using GC analysis. The initial method applied here, based on Bustos-Segura et al. (2017), was found to require substantial optimisation. A series of Method Development trials removed the double solvent peak observed using the original method and resulted in a much clearer separation of compounds, larger clearer peaks, elution of more compounds and reduced total duration.

Original method:
Injector temperature 250°C, splitless, DB-5 Agilent column (30m), oven temperature: 100°C for 4 min, ramping to 180°C at 20°/min, held for 10 min, ramp up again at 20°C/min and held at 250°C for 4.5 min, total elution time 26 min, detector temperature 350°C.

Improved method:
Injector temperature 250°C, splitless, DB-5 Agilent column (30m), oven temperature: 50°C for 0.5 min, ramping to 100°C at 50°/min, held for 0.5 min, ramp up again at 10°C/min and held at 180°C for 4.5 min, total elution time 26 min, detector temperature 300°C.

NEXT STEPS

Further refinement and validation of the GC method using the EtOH solvent will continue to reduce analysis error and variation to ensure high quality, repeatable results. Investigations are required with regard to foliage collection and storage prior to analysis. We are currently assessing the influence of using young vs. older foliage and how many leaves should be used per sample. We will develop a standardised collection protocol detailing, for example, number and mixing of leaves per sample, from which part of crown, which season, collection and processing fresh material directly into ethanol in the field vs. freezing with dry ice of liquid nitrogen for subsequent processing in the lab.

Once we are confident with the foliar preparation and analysis methods larger trials will be run to establish if GC profiles can be used effectively to detect different foliar chemotypes between and within eucalypt species planted as part of the NZDFI programme. Foliage for this analysis will be collected from 1) Harewood nursery (specie comparisons) and 2) North Canterbury and Marlborough breeding trials (intraspecific comparisons). If the method proves useful we aim to link chemical profiles with observed defoliation and oviposition in field trials as part of a future PhD programme (currently advertising for a suitable student). We hope to eventually using the protocol developed to screen large numbers of individual trees from across multi-species breeding trials in different geographical locations.

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