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The Rapid Determination of Sideroxylonals in *Eucalyptus* Foliage by Extraction with Sonication followed by HPLC

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A rapid method is described for the quantification of sideroxylonals, a group of formylated phloroglucinol compounds found in some eucalypts. Samples of dry, ground foliage were extracted by sonication with 20% methanol in acetonitrile, 7% water in acetonitrile or 40% water in acetonitrile and the extracts analysed by reversed phase HPLC. The extracts from the two water–acetonitrile extractions were stable for at least 48 h. All three sonication methods recovered more sideroxylonals than did the Soxhlet extraction with petroleum spirit and acetone. Adding 0.1% trifluoracetic acid to the water–acetonitrile extraction solvents led to even higher recoveries of sideroxylonals. Soaking the sample in extracting solvent for 5 min recovered 70% of the sideroxylonals, whilst sonicating the suspension for 1 min recovered the remainder. The developed method involving sonication of the sample for 5 min in 7% water in acetonitrile with 0.1% trifluoroacetic acid is fast and requires minimal equipment and solvents compared with the traditional methods. With an autosampler it is possible to prepare and run 100 samples a day. More importantly, the technique is ideal for the analysis of small samples, e.g. individual leaves, which is essential when studying the evolutionary ecology of eucalypts.

Keywords: Formylated phloroglucinol compound; eucalypt; folivorous; marsupial; sonication.

INTRODUCTION

The sideroxylonals (Fig. 1) are a group of formylated phloroglucinol compounds (FPCs) found in many species of the genus *Eucalyptus*. Sideroxylonal-A is the dominant form, followed by sideroxylonal-C, while only traces of sideroxylonal-B occur. Amongst the wide range of biological effects of the sideroxylonals (Ghisalberti, 1996), the suppression of feeding on *Eucalyptus* leaves by koalas and other folivorous marsupials and insects (e.g. Lawler *et al*., 1998; Lawler and Foley, 2002; Wallis *et al*., 2002) is of particular interest as this partly moulds the chemical ecology of eucalypt forests.

Sideroxylonals occur patchily in *Eucalyptus* and there is considerable intraspecific variation in their concentration. For example, Lawler *et al*. (2000) reported that in *Eucalyptus polyanthemos* the foliar sideroxylonal concentration varied from 0 to 13 mg/g of dry matter (DM). Foliage of *E. melliodora* contains higher concentrations (0–52 mg/g DM; Wallis *et al*., 2002), while a Western Australian mallee species, *E. loxophleba ssp lissophloia*, may contain up to 100 mg/g DM (Wallis, I.R. and Foley, W.J., unpublished results).

It was the important ecological role of sideroxylonals in Australian forests that led Wallis *et al.* (2003) to develop a quantification method for this class of compounds. Briefly, the method involved Soxhlet extraction of dry, ground leaf with petroleum spirit and acetone,

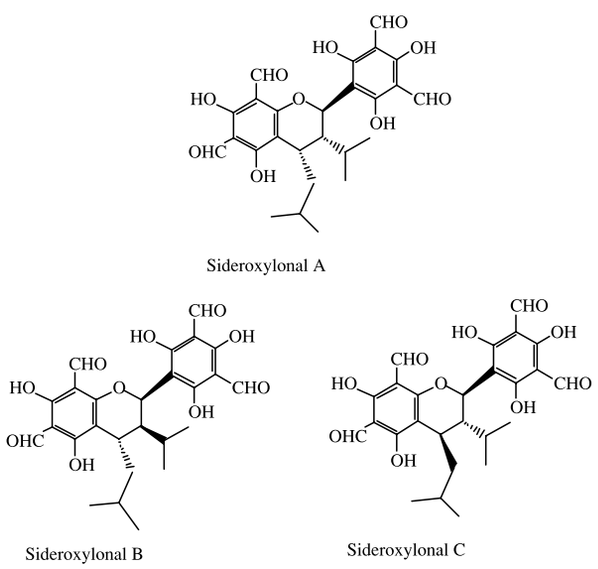

Figure 1. The structures of sideroxylonal-A, -B and -C.

evaporation to dryness and transfer of the crude extract to a pre-weighed vial with dichloromethane and methanol. This extract was air-dried, reweighed, a precise amount was weighed into a vial, diluted with solvent containing an internal standard and filtered into an HPLC vial. This simple analysis can be carried out with a coefficient of variation between duplicates of less than 5%, but the method is costly in terms of time, equipment and solvents, and each of the many steps presumably increases the analytical error. This problem can be largely circumvented by using the analysis in conjunction with near infrared reflectance spectroscopy (NIRS) to predict

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sideroxylonal concentrations (Foley *et al*., 1998). Even so, this still precludes measuring sideroxylonal concentrations in small samples, e.g. individual leaves, and hence exploring many interesting ecological questions, particularly in plant-herbivore interactions. Thus, a rapid assay for the sideroxylonals would be of great value, but it was presumed that the compounds were tightly bound even in dry, ground plant tissue and would be difficult to remove with a simple extraction.

Our view on this matter changed following the work by Benson *et al*. (2001), in which a rapid method was developed for the isolation of terpenoid aldehydes from cotton samples by ultrasonication in HPLC running solvent and direct injection of the extract onto an HPLC column. The present paper reports the development of a similar successful rapid extraction of sideroxylonals from dry, ground eucalypt leaf. It also provides information on sonication solvents, sonication time, the influence of trifluoroacetic acid on the extraction efficiency, and the deterioration of sideroxylonals upon storing leaf extracts in mobile phase.

EXPERIMENTAL

Chemicals. Petroleum spirit (40–60°C boiling point), acetone, dichloromethane, methanol, trifluoroacetic acid and 2-ethylphenol were all of analytical grade. HPLC-grade acetonitrile and methanol and Milli-Q® water were used for sample preparation and in mobile phases.

Chromatographic analyses. The chromatographic separations were carried out on a SGE (Ringwood, Australia) GL Wakosil II 3C18RS column (250 × 4.0 mm; 3 μM) connected to a Waters (Milford, MA, USA) Alliance HPLC system which consisted of a model 2690 separation module equipped with an autosampler fitted with a 250 μL syringe and a 100 μL sample loop, and a model 996 diode array detector. The optimal separation of the sideroxylonals was obtained following isocratic elution using 7% water in acetonitrile, with 0.1% trifluoroacetic acid at a flow rate of 0.75 mL/min and a column temperature of 40°C. Samples in the autosampler carousels were maintained at 15°C. The typical operating pressure was 120 bar with a pressure ripple of 1 bar, and the run time was 14 min. Waters Millennium 3.2 software was used for both data collection and integration. Sideroxylonals-A and -C were detected at 275 nm and eluted at 8.70 and 9.12 min, respectively, and quantified by comparison with standards prepared in our laboratory (Eschler and Foley, 1999). Determinations were made for sideroxylonal-A, C and -A+C; sideroxylonal -B was not detected in any of the extracts. Unless stated otherwise all analyses were carried out in duplicate.

Plant materials. Samples of leaves were collected from *Eucalyptus tricarpa* trees which were being grown in conjunction with other research examining the heritability of sideroxylonals. The trees were roughly 2.5 m tall so it was possible to strip the leaves directly from the branches. About 75 ***g*** of leaves were placed in plastic bags on ice in the field and then stored at –20°C in the laboratory for 1–2 weeks before drying. Leaf samples were freeze-dried and then ground to pass through a 1 mm sieve in a Cyclotec 1093 Mill (Tecator, Hoganas, Sweden). The resulting powder was stored in the dark in clear, plastic 50 mL specimen containers. The 14 samples used in this study were selected by NIRS to provide a range of sideroxylonal concentrations from about 3 to 50 mg/g DM (Table 1).

Normal (Soxhlet) extraction. In order to obtain a sideroxylonal-rich extract by the Soxhlet method (Wallis *et al*., 2003), 1.5500 ± 0.0500 ***g*** of dried, ground leaf was weighed into a cellulose extraction thimble (80 × 20 mm; Whatman, Maidstone, UK) and refluxed with 100 mL of 20% acetone in light petroleum spirit in a Soxhlet

Table 1. The mean concentrations of sideroxylonals-A+C (mg/g DM) and their coefficients of variation (CV) in 14 samples of *Eucalyptus tricarpa*. The samples were analysed by the normal (Soxhlet) extraction method using 20% acetone in petroleum spirit or by sonicating for 10 min in 20% methanol in acetonitrile (20:80S), 7% water in acetonitrile (7:93S) or 40% water in acetonitrile (40:60S); the last two solvents contained 0.1% trifluoroacetic acid

Sample	Normal method[a,b]	20:80S method[a]	7:93S method[a]	40:60S method[a]
1[c]	47.7 (5.4)	50.1 (0.9)	53.6 (1.5)	53.9 (0.1)
5	36.2 (2.9)	35.0 (5.0)	36.4 (0.9)	37.5 (0.4)
7	48.5 (2.6)	48.2 (2.7)	52.2 (1.8)	53.9 (3.5)
16	3.0 (0.8)	3.4 (6.6)	3.6 (3.7)	3.4 (1.7)
38[c]	34.7 (3.8)	34.2 (3.2)	35.3 (3.0)	34.8 (4.8)
42	28.6 (2.9)	28.9 (1.3)	29.9 (0.9)	30.5 (2.4)
44[c]	25.8 (2.6)	25.0 (1.0)	26.9 (3.1)	27.8 (0.01)
54	4.0 (0.9)	4.3 (5.9)	4.4 (9.9)	4.9 (1.0)
55[c]	7.4 (10.9)	7.9 (6.5)	8.0 (1.6)	8.1 (3.3)
80	26.0 (1.7)	27.6 (0.3)	30.4 (3.3)	29.8 (1.1)
95	30.4 (5.2)	34.8 (0.2)	36.3 (0.01)	36.8 (1.9)
107	35.6 (2.7)	38.5 (2.5)	40.2 (1.2)	41.2 (2.0)
108[c]	41.4 (1.6)	43.0 (0.01)	42.8 (0.3)	44.6 (0.5)
113	24.7 (1.8)	25.9 (1.0)	25.2 (3.6)	27.4 (2.4)
Mean	28.26 (3.67)	29.06 (2.63)	30.40 (2.48)	31.02 (1.80)

[a] Mean concentrations (mg/g DM) and CV values.
[b] The extracts of several samples were reanalysed by HPLC because they had unacceptably high CVs (>5%). The CVs of samples 1, 55 and 95 are still high, so these samples would normally be re-extracted from ground leaf. None of the sonicated samples were reanalysed.
[c] Samples also used for the sonication time and the trifluoroacetic acid studies. $n = 14$.

extractor (40 mL siphoning volume) connected to a 250 mL round-bottom flask, heated on a water bath (85°C) for 4 h. The solvent was removed by rotary evaporation at 50°C and the resulting crude extract was transferred quantitatively into a pre-weighed 20 mL glass vial with 20% methanol in dichloromethane. The vial was dried under a stream of air for 24 h and then left exposed to air in a fume hood for a further 48 h. It was then reweighed before the extract was scraped from the walls and bottom of the vial. Finally, a known mass (ca. 8 ***g***) of standard diluent (20% methanol in acetonitrile containing 0.300 mg/mL of 2-ethylphenol as internal standard) was added to a known amount of extract (10 ± 1 mg). This suspension was placed in an ultrasonic bath (model FX12P, Unisonics, Sydney, Australia) until dissolved (ca. 2 min) and then filtered (0.45 μM) into an autosampler vial (2 mL).

Rapid extraction. The rapid extraction was designed to eliminate Soxhlet extraction and all steps that follow it. As a starting point, 100 ± 1.0 mg of dried ground leaf were weighed into a glass vial to which was added a weighed amount (ca. 8 ***g***) of standard diluent. The samples were allowed to soak for 15 min, sonicated for 5 min, soaked for 5 min and then sonicated again for 5 min. They were then filtered into autosampler vials and analysed (injection volume 10 μL) together with standard samples in random order by the HPLC. The results from this preliminary study indicated that the mean sideroxylonal concentrations over all samples determined by the two methods (normal and rapid extraction) were not significantly different (27.3 and 28.0 mg/g, respectively).

Extraction method and solvents. Three solvents were tested for their suitability for the sonication procedure: (1) the standard diluent, 20% methanol in acetonitrile; (2) 7% water in acetonitrile; and (3) 40% water in acetonitrile. All solvents contained 2-ethylphenol as internal standard (0.300 mg/mL) while the latter two also contained 0.1% v/v trifluoracetic acid. Solvent 2 was that used for the HPLC separation of sideroxylonals, while solvent 3 was the starting solvent used in an 80 min gradient to separate the multitude of FPCs, including sideroxylonals, which some eucalypts contain (Eschler *et al*., 2000). Depending on the concentration of sideroxylonals, between 25 and 75 mg of dried, ground leaf material was weighed into a glass vial (20 mL), and a known mass (ca. 10 ***g***) of solvent was added using an autodispenser and an analytical balance (±0.1 mg). After adding the solvent to the 14 samples (ca. 5 min), they were allowed to stand for 5 min before being sonicated for 10 min. They were then filtered directly into autosampler vials. For comparative purposes, fresh dilutions of the crude extract from the original standard extractions were made and all samples were analysed within 10 h of preparation in a random sequence on the HPLC.

Sonication time. Five of the 14 samples (containing ca. 8, 28, 35, 43 and 54 mg/g sideroxylonals; see Table 1) were selected in order to measure the effect on the analysed sideroxylonal concentration of varying the sonication time in a factorial design. Six sub-samples of each were weighed as described above. The solvent (7% water in acetonitrile with TFA and 2-ethylphenol) was added to the vials in random order before sonicating them for 0, 1, 2, 4, 8 or 16 min (only one vial of each sample for each sonication time). The samples were filtered immediately and analysed by HPLC within 6 h.

The potential deterioration of sideroxylonals in mobile phase. Samples from the rapid extractions with 7 and 40% water in acetonitrile were retained in their autosampler vials, stored refrigerated (4°C) and analysed again by HPLC at 42, 56, and 152 h (7% water in acetonitrile) and 55, 164 and 216 h (40% water in acetonitrile) to measure any changes in sideroxylonal concentration. The assumption was that minimal deterioration occurred before the samples were analysed initially (time zero). The fate of decomposed sideroxylonals was not investigated.

The effect of trifluoroacetic acid on the measured concentrations of sideroxylonals. In the comparison of solvents, those made with water and acetonitrile extracted the most sideroxylonals. However, these were also solvents that contained trifluoracetic acid, thus potentially confounding the interpretation of the results. In order to separate these effects, duplicate 10 min sonication extractions were made of the five samples used for the sonication time study using 7 and 40% water in acetonitrile either with or without trifluoracetic acid but each containing internal standard (0.300 g/L).

Statistical analysis. All data were analysed using the analysis of variance algorithm in Genstat 5 release 6.2 (Numerical Algorithms Group, Oxford, UK). In most cases mean concentration data were used, but for the sonication time study the data were standardised by expressing them as percentages of the highest concentration measured for that sample. In some cases there were insufficient degrees of freedom to include a sample × method interaction term in the analysis of variance. This was tested separately by grouping samples according to total sideroxylonal concentration (<10, 20–30, 30–40 and >40 mg/g DM) and testing the model:

$$\text{sideroxylonal (mg/g DM)} = \text{method} + \text{concentration category} + \text{method} \times \text{concentration category}$$

The data from the trifluoracetic acid study were analysed by regarding the samples as blocks and the solvent and trifluoracetic acid as treatment factors. Thus, the statistical model tested was:

$$\text{sideroxylonal (mg/g DM)} = \text{solvent} + \text{trifluoracetic acid} + \text{solvent} \times \text{trifluoracetic acid}$$

All models were tested to ensure that residual values were normally distributed and randomly distributed against fitted values. Means were assumed to be statistically different at $p < 0.05$.

RESULTS AND DISCUSSION

The effect of extraction method and solvent on the yield of sideroxylonals from *Eucalyptus* leaf

The extraction method significantly influenced the determined sideroxylonal concentration ($F_{3,39} = 18.2$; $p < 0.001$). All three sonication methods resulted in significantly higher sideroxylonal concentrations than

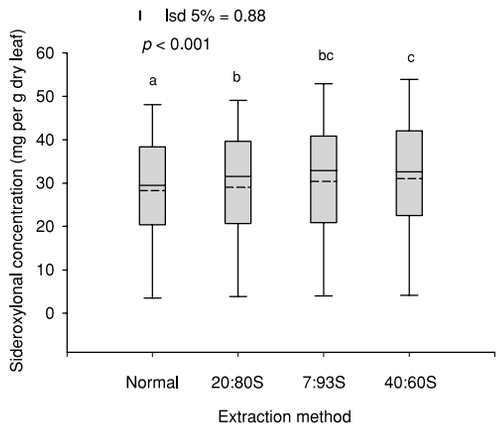

Figure 2. The effect on the measured concentration of sideroxylonal-A+C of extracting by the normal (Soxhlet) method or by sonicating for 10 min in 20% methanol in acetonitrile (20:80S), 7% water in acetonitrile or 40% water in acetonitrile; the latter two solvents contained 0.1% trifluoroacetic acid. Data are represented as box and whisker plots where the box incorporates the 25–75% quartiles, the median (solid line) and the mean (dashed line). The whiskers are the 10 and 90% quartiles.

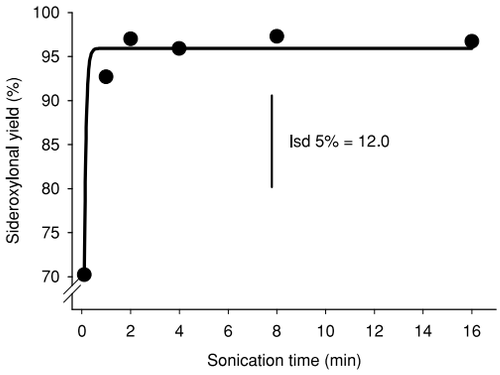

Figure 3. The effect on sideroxylonal yield (%) of extracting dry, ground *Eucalyptus tricarpa* foliage by sonication with 7% water in acetonitrile for 0, 1, 2, 4, 8 or 16 min. Results are expressed as percentage yield of sideroxylonals relative to the highest yield.

were obtained using normal (Soxhlet) extraction (Table 1; Fig. 2). Among the three sonication methods, the use of 40% water in acetonitrile resulted in higher determined sideroxylonal concentrations (mean of all samples = 31.0 mg/g DM) than could be obtained using the standard solvent (20% methanol in acetonitrile; mean = 28.3 mg/g DM). Other comparisons were not significantly different.

More importantly, the sonication assays showed minimal variation between duplicate samples. The overall coefficient of variation (CV) for the normal assay was 3.7%, which was calculated after repeated HPLC runs for six of the 14 samples. In contrast, the corresponding CV values for the sonication extractions were all under 2.7% without any repeat runs. The method with the lowest CV (1.8%) was the sonication treatment using 40% water in acetonitrile. Although the numerous steps in the normal extraction method undoubtedly increase variation, it is the weighing of the crude extract that causes most problems. Samples are often very sticky or very dry and both are difficult to weigh, the latter because of problems caused by static electricity in the dry climate of the ACT (Australia). Thus, repeating an analysis from the crude extract stage as described earlier frequently reduces the CV.

It is difficult to recommend a particular sample size and volume of extraction solvent. In the present study, a known mass of solvent (ca. 8 ***g***) was added to between 25 and 75 mg DM, depending on sideroxylonal content. These amounts are ideal in terms of container size and weighing error but also give acceptably sized peaks on chromatograms. The increased sample size made filtering more difficult but there was no indication that sample size influenced the coefficient of variation.

Sonication time

Sonication time had a highly significant effect on the measured concentration of sideroxylonals-A+C ($F_{5,20}$ = 7.3; $p < 0.001$). However, closer examination showed that the only difference was between samples that were not sonicated and those that were (Fig. 3). Repeating the statistical analysis with the non-sonicated samples excluded showed that there was no significant effect of sonication time and that the experimental variance was greatly reduced. On average, 70% of the sideroxylonals were extracted from the dried, ground leaf simply by soaking it for a few minutes in extraction solvent, in this case 7% water in acetonitrile with trifluoroacetic acid. Sonicating this suspension for as little as 1 min released the remaining sideroxylonals. Such short sonication times are not practical and may risk poor recovery of sideroxylonals from certain samples. Thus, a 5 min sonication is recommended. This is convenient because it is about the time required to add extraction solvent to another batch of 10–12 samples.

The deterioration of sideroxylonals when extracts are stored in mobile phase

Wallis *et al*. (2003) showed that sideroxylonals decompose when the crude extract is stored in HPLC mobile phase. However, the losses were relatively small, being ca. 7% in the first 4 days. The deterioration was much slower in this study (Fig. 4). Those samples stored in 7% water in acetonitrile [Fig. 4(A)] did not deteriorate significantly in >150 h ($F_{3,34}$ = 0.56; p = 0.65), whilst those stored in 40% water in acetonitrile [Fig. 4(B)] contained 3% less sideroxylonals after 164 h ($F_{3,39}$ = 10.5; $p < 0.001$), but there was no further deterioration at 216 h. It is difficult to explain why the present samples were better preserved than those in the earlier study, but one obvious explanation lies in the presence of trifluoroacetic acid. These data suggest that it is acceptable to analyse samples within 48 h of preparation.

The effect of adding trifluoroacetic acid to the extraction solvent on the yield of sideroxylonals from *Eucalyptus* leaf

The addition of trifluoroacetic acid to the extraction solvent significantly increased the determined concentrations of sideroxylonals-C ($F_{1,32}$ = 46.98; $p < 0.001$) and sideroxylonals-A+C ($p < 0.001$; Fig. 5), while there was a strong trend towards an increase in the determined

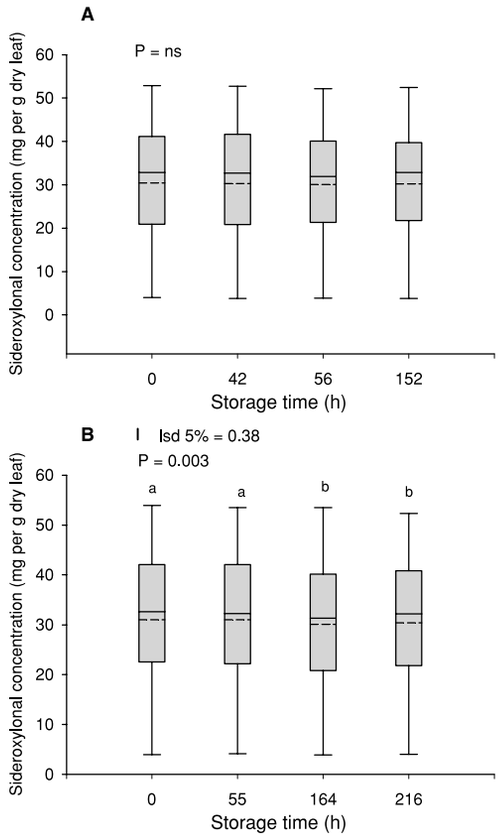

Figure 4. The effect on sideroxylonal yield of storing samples in (A) 7% water in acetonitrile, and (B) 40% water in acetonitrile (both solvents containing also trifluoroacetic acid). Data are represented as box and whisker plots where the box incorporates the 25–75% quartiles, the median (solid line) and the mean (dashed line). The whiskers are the 10 and 90% quartiles.

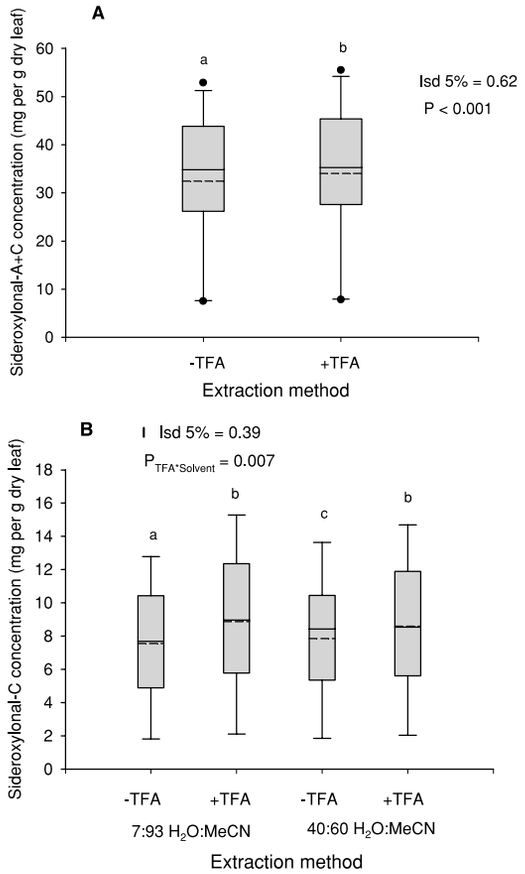

Figure 5. The effect on the concentrations of (A) sideroxylonal-C, and (B) sideroxylonal-A+C (of sonicating dry, ground *Eucalyptus tricarpa* foliage in 7 or 40% water in acetonitrile with or without trifluoracetic acid). Data are represented as box plots where the box incorporates the 25–75% quartiles, the median (solid line) and the mean (dashed line). The whiskers are the 10 and 90% quartiles and the dots are the minima and maxima.

concentration of sideroxylonal-A ($F_{1,32} = 3.78$; $p = 0.061$). There is no simple explanation for trifluoroacetic acid appearing to act differently on these two structurally similar molecules. However, the difference appears to be a real extraction phase effect because the retention times of the sideroxylonals did not change when the extraction solvent was altered. Extracting with 7 or 40% water in acetonitrile did not alter the determined concentrations of sideroxylonals ($p > 0.3$). However, there was a strong interaction between solvent and trifluoroacetic acid, with trifluoroacetic acid having a larger effect when added to 7% water in acetonitrile compared to its effect with 40% water in acetonitrile. This interaction was significant for sideroxylonal-C ($F_{1,32} = 8.44$; $p = 0.007$) but not for sideroxylonal-A ($p = 0.47$). This caused a trend towards an interaction between solvent and trifluoroacetic acid for the combined concentration of the sideroxylonals ($F_{1,32} = 3.26$; $p = 0.08$). One noticeable effect of using trifluoroacetic acid was that the samples extracted with 40% water in acetonitrile were much easier to filter. Thus, adding 0.1% trifluoroacetic acid to the extraction solvent is recommended.

In conclusion, the procedure recommended for the extraction of sideroxylonals is to sonicate 25–50 mg of freeze-dried, ground foliage in a known mass (ca. 8 ***g***) of 7% water in acetonitrile (with 0.1% trifluoroacetic acid and 0.3000 g/L of the internal standard, 2-ethyl phenol) for 5 min and then filter (0.45 μM) the extract directly into an autosampler vial. The samples should be analysed isocratically on an HPLC using 7% water in acetonitrile with 0.1% trifluoroacetic acid as described previously (Wallis *et al*., 2003). This method provides a simple, extremely fast and accurate method of analysis with an average CV between samples of less than 2%. Using the former method, 14 samples could be prepared in duplicate per day, by running the Soxhlet apparatus overnight. Even so, 3 days were needed to prepare a sample for HPLC analysis. By comparison, with the sonication method it is possible to prepare this many samples in about 1 h. Unlike the previous method, this rapid analysis saves large volumes of solvent and does not require various other pieces of equipment such as a Soxhlet extraction apparatus and a rotary evaporator. Furthermore, in order to save water, our Soxhlet apparatus and rotary evaporator are fitted with re-circulating cooling devices which are clearly no longer needed. Perhaps the greatest advantage of the new method is that it is ideally suited to duplicate analyses on small samples, for instance individual eucalypt leaves that may weigh anything between 0.1 and 1.5 ***g*** when dry. This opens the way for examining interesting ecological and evolutionary topics that have previously been beyond reach, such as the variation in sideroxylonal content among leaves from an individual tree and the change in sideroxylonal content as a seedling matures.

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