Automation of Solid-Phase Microextraction–Gas Chromatography–Mass Spectrometry Extraction of Eucalyptus Volatiles

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Abstract

Solid-phase microextraction (SPME) coupled with gas chromatography (GC)–ion-trap mass spectrometry (ITMS) is employed to analyze fragrance compounds from different species of eucalyptus trees: *Eucalyptus dunnii*, *Eucalyptus saligna*, *Eucalyptus grandis*, and hybrids of other species. The analyses are performed using an automated system for preincubation, extraction, injection, and analysis of samples. The autosampler used is a CombiPAL and has much flexibility for the development of SPME methods and accommodates a variety of vial sizes. For automated fragrance analysis the 10- and 20-mL vials are the most appropriate. The chromatographic separation and identification of the analytes are performed with a Varian Saturn 4D GC–ITMS using an HP-5MS capillary column. Several compounds of eucalyptus volatiles are identified, with good reproducibility for both the peak areas and retention times. Equilibrium extraction provides maximal sensitivity but requires additional consideration for the effect of carryover. Preequilibrium extraction allows good sensitivity with minimal carryover.

Introduction

The volatile and semivolatile substances, other than CO and CO_2, produced by plants and other living species are collectively known as biogenic volatile organic compounds (BVOCs). They are comprised of a large number of organic substances, which include isoprene and terpenoid compounds, alkanes, alkenes, carbonyl compounds, alcohols, and esters (1) and are typically present in the atmosphere at concentrations in the range of parts per trillion to parts per billion. Isoprene and terpenoid compounds are the principal components found (2,3). These volatile compounds are responsible for multiple interactions between plants and other organisms (4) such as pollinators (5) and herbivore predators (6). Also, some BVOCs are produced and emitted by plants as a defense against attack by herbivores (7).

Analysis of BVOCs may be conducted using macerated or cut parts of a plant or in situ. The volatile fraction obtained by both methods may be different and bring complementary information about the plant (8). Methods employed for the in vitro analysis of volatile compounds from fresh or dried parts of plants are distillation (steam or hydro), distillation with solvent (9,10), vacuum distillation (11), headspace (12), microwave-assisted liquid–liquid extraction (13), solvent (14), supercritical or subcritical fluid extraction (9), sorbent traps (8), or solid-phase microextraction (SPME) (15,16).

SPME is a fast, simple, and sensitive preconcentration and extraction technique introduced in 1990 (17). It has been applied to a wide variety of in vitro analyses of metabolites and similar compounds (18). Using short extraction times and porous-polymer-coated fibers a semiquantitative approach has been applied to monitor the behavior of BVOCs emitted in situ by *E. citriodora* over nine days (19), and six other *Eucalyptus* species have had the headspace of stems extracted by a polydimethylsiloxane (PDMS) fiber in situ (20). Several other studies have been conducted using SPME to extract volatile compounds from aromatic and medicinal plants. The majority have used dried parts of the plant (15,16,21), and only a few have used chopped or powdered fresh parts of the plant (22,23). The goal of this project was to develop an automated extraction method to be used for the chemometric differentiation of plants. Experiments using headspace SPME to extract the volatile compounds of chopped leaves of *Eucalyptus dunnii* (24) were the starting point to develop a method using powdered leaves for chemometric analysis.

Determination of the chemical composition of the BVOC mix is essential for studies of the biological processes involved in the production, emission, and effects of such substances. The pattern of volatiles emitted by different species of related plants

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(or different hybrids within a species) may also be used as a type of chemical signature. Correlations with other physical characteristics of the plant may be used to identify plants with desired characteristics without the direct measurement of those characteristics. The goal of this project was to develop an SPME method for the automated characterization of eucalyptus volatiles and thus support the genetic improvement of the forest. It was hoped that eventually a correlation will be identified between a specific pattern (or patterns) of eucalyptus volatiles in seedlings and the quality of pulpwood in mature trees. Brazilian eucalyptus is used primarily as a source of pulpwood, and conventional genetic improvement requires the analysis of mature wood. If promising hybrids could be identified very early (6 mo) by the chemometric analysis of volatiles rather than waiting for mature wood formation (7 years), significant savings in time and cost could be realized. In this work, an automated method using SPME–gas chromatography (GC)–ion-trap mass spectrometry (ITMS) was developed to be used for the chemometric differentiation of eucalyptus species. Mature trees were employed in this study according to the availability of the pulp and paper industry; however, this first step opens the perspective of using seedlings in the future.

In this study, we report on the development of a method for the sample preparation and analysis of eucalyptus volatiles and the suitability of the method for routine chemometric analysis.

Experimental

For SPME analysis experiments, freshly picked leaves were sampled randomly around the tree canopies of 12 eucalyptus trees (different species and hybrids) at the Barba Negra tree farm (Guaíba, RS, Brazil) and transported to Canada in Styrofoam boxes containing ice packs. Leaves of different sizes and ages as well as different locations on the trees were selected in an attempt to account for intraplant variation in volatile emissions. Because of the proprietary nature of these plants, their specific identities are not described in detail. Immediately upon arrival, the leaves were placed in dry ice (solid CO_2) until extraction and analysis were performed. During sample preparation, leaves were first placed in liquid nitrogen. When frozen, the leaves were partially ground in a stainless steel mortar and pestle, after which dry ice was added. Leaves were further ground into a powder, during which time the nitrogen evaporated but the solid CO_2 remained. The mixture of powdered leaf and solid CO_2 was sieved through a 50-mesh (U.S.A. series) stainless steel sieve. Immediately after the CO_2 had evaporated, powdered leaf (0.05 g) was weighed into 10-mL vials stored on dry ice. Phosphate buffer was added to each vial (2 mL, pH 7.0, 50mM), whereupon it immediately froze. The temperature was kept low during sample preparation (< 4°C) in order to avoid loss of volatile compounds. Once thawed, vials were capped and placed on the temperature-controlled autosampler tray (30°C) and analysis was initiated. For these analyses, SPME and chromatography were as follows. Sample vials were preheated to 30°C for at least 24 h. The SPME fiber (7-µm PDMS) was placed in the vial headspace, and volatiles were extracted for 30 min. It was previously determined that this was sufficient for the equilibrium extraction of all compounds. Analysis was performed on a Varian (Walnut Creek, CA) 3400 GC Saturn 4D ITMS equipped with an SPI injector and an HP5-MS column (Agilent, Wilmington, DE) (30-m × 0.25-mm, 0.25 df). The fiber was desorbed in the injector (250°C) for 30 min. Column oven programming was set initially at 60°C and then ramped at 5°C/min to 250°C. Helium was used as the carrier gas at 1.2 mL/min. The transfer line was held at 250°C and the ion trap at 150°C. Data collection was handled with Saturn Version 5.0 software, and data analysis was handled with Varian Saturn GC/MS Workstation Version 5.4 software. Automation of the extraction and injection was achieved with a CombiPAL autosampler (CTC Analytics, Basel, Switzerland).

Although the use of internal standards would have been desirable to correct for variation in the detector response, it was not possible to include internal standards in these preliminary investigations because it was necessary to first determine the types of compounds present in the different cases in order to make a reasonable selection of the internal standard. Also, the technical difficulties in introducing internal standards to a matrix such as the one used in this study requires additional research to be conducted in order to ensure that the inclusion of internal standards will not itself add more error to the analysis than it is eliminating. However, prior to commencing these experiments, the linearity of the detector was verified for several representative compounds over a range of concentrations, and operational parameters for the instrument were monitored throughout the experiments to ensure that there was no variation in instrument performance.

The procedure described for the analysis of the powdered leaf with the 7-µm fiber produced acceptable precision and sensitivity, although the leaf preparation required quite a lot of time and a number of fiber bleed peaks were identified. A simplified sample preparation and SPME analysis was conducted and evaluated for sensitivity, precision, carryover, and the presence of fiber bleed peaks. In the method a *Eucalyptus dunnii* leaf (approximately 0.38 g) was cut into approximately 0.5-cm^2 pieces with scissors and placed in a 10-mL sample vial. Vials were capped immediately and placed on the sample tray at room temperature. The extraction method was similar to that described previously, except that a 30-µm PDMS fiber was used with a 10-min extraction at 30°C and a 1-min desorption at 250°C. This was a preequilibrium extraction time for most compounds. The carrier gas flow was 1.0 mL/min. The shorter extraction time was intended to provide reduced carryover, and the resulting lower sensitivity was partially compensated by the thicker film. The short desorption time was intended to allow for reduced fiber bleed.

Hydrodistillation was performed in a Clevenger modified apparatus for 5 h, according to the method of Guenter et al. (25). Approximately 300 g of fresh leaves was used in 1 L of deionized water. The refrigeration system was maintained between –4°C and 4°C using a mixture of water and ethylene glycol to avoid losses of volatile compounds. The hydrodistilled oil was dried with sodium sulfate.

For all of the chromatographic results, background peaks were identified and ignored for the analysis, and preliminary identification was attempted for compounds arising from the sample. Those compounds that appeared to be eucalyptus volatiles (based on MS analysis) were identified by retention time and tracked. Positive identification was not possible for all compounds tracked.

Results and Discussion

Comparison of hydrodistillation and SPME analysis for the characterization of eucalyptus volatiles

The conventional method for the analysis of volatile fragrance compounds from plant material is hydrodistillation. Validation of the SPME method presented was carried out by comparison of compounds extracted by hydrodistillation and SPME. A comparison of the chromatographic profiles obtained by the two methods has shown that the major compounds were extracted by both methods. Chromatographic results (GC–flame ionization detection) are presented in Figure 1. The most obvious difference in the two extraction methods was the absence of the solvent peak from the SPME chromatogram. For compounds eluting after approximately 7 min, the two methods appeared to have equivalent sensitivities. For early eluting peaks, SPME appeared to have lower sensitivity, although this may be because of the very thin (7 µm) extraction film used on the SPME fiber. This fiber is primarily used for high partition coefficient compounds for which longer extraction times are required, but overloading of the column would be a problem with thicker films. Thicker film fibers are typically used for low partition coefficient compounds in which sensitivity is more of a concern. If early eluting compounds are of primary concern, a thicker film fiber would be more appropriate.

Setup of automated SPME analysis

The autosampler used in this study was highly flexible and the setup was user-determined. Because of this, the configuration of this unit for general fragrance analysis was not trivial. Details of the different components that were evaluated are included in order to assist others in developing similar methods. The SPME-configured CombiPAL setup used in these experiments is shown in Figure 2. CombiPAL programming was performed using the hand-held unit mounted on the left side of the autosampler. This was sufficient for the straightforward extractions performed for these analyses. For more-complex extractions, computer control of the CombiPAL and additional programming flexibility were provided by Cycle Composer software. For extractions and injections, the SPME fiber was mounted in the robotic head unit, which also performed sample transport. Samples in 10-mL vials were loaded on the temperature-controlled sample tray (30°C) mounted to the autosampler. The unit used was similar to the one shown in Figure 2 but with shorter arms. For volatile analysis a larger headspace is often required, and this can be accommodated with 20-mL vials using a tray holder with longer arms because it is important that vial tops are aligned below the position of the head unit. For thermally labile analytes in 20-mL vials, it was found that the Peltier cooled tray shown was more appropriate. It should be noted that the extension on the top of the sample tray allowed for the cooling of the entire vial. This item and the longer arms for the Peltier cooled tray had to be obtained by custom order. The sample trays were constructed of solid alu-

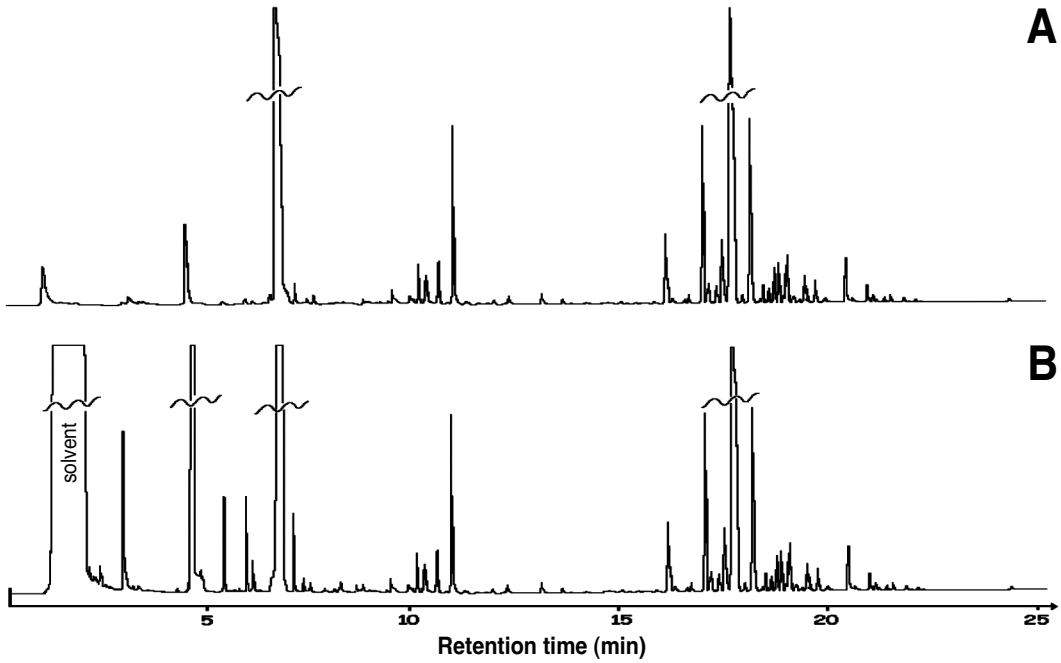

Figure 1. Chromatograms of volatiles from *Eucalyptus dunnii:* (A) SPME extraction and injection of powdered leaves in buffer and (B) injection of oil extracted by hydrodistillation.

minum to further facilitate efficient cooling. Temperature control and sample agitation during extraction were performed in the agitation unit. Vials were transported between the sample trays and the agitation/thermal control unit by means of a magnetic holder on the robotic head and metal crimp seal caps. For volatile fragrance analysis, it is imperative that the crimp seal caps are sealed as tightly as possible. The two vial sizes and two SPME fiber assemblies (it should be noted that the springs are absent) are also shown in Figure 2. Injections were performed immediately after the extraction had completed, after which the robotic head moved the sample vial back to the sample tray and extraction of the next sample was initiated.

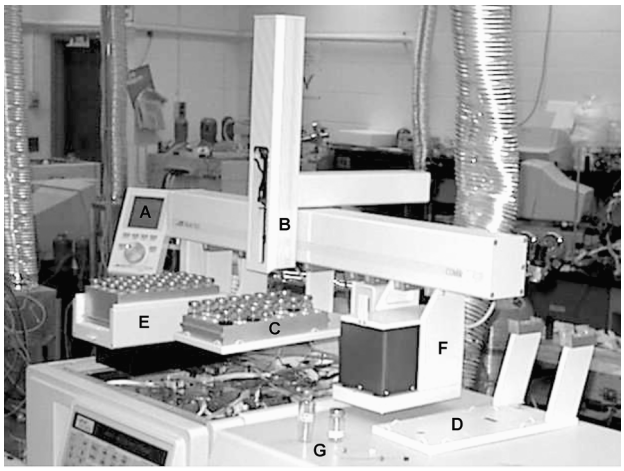

Figure 2. Setup of the CombiPAL autosampler for SPME extraction and injection of eucalyptus volatiles: (A) hand-held programming unit, (B) robotic head unit, (C), 10-mL vial tray at room temperature, (D) tray holder for 20-mL vials, (E) Peltier cooled tray holder, (F) extraction temperature control and agitation unit, (G) 10-mL and 20-mL extraction vials for volatiles and two SPME fiber assemblies (it should be noted that springs are absent).

Evaluation of the method

For chemometric analysis, the reproducibility of peak areas and retention times as well as reduction or elimination of carryover are essential. These parameters have been assessed for the method developed and will be discussed in greater detail.

Analysis of carryover and fiber bleed

With the 7-µm fiber extraction regime, sensitivity was optimized for a broad range of analytes, and carryover was minimal (within experimental error) primarily because of the long desorption time. Bleed from the fiber was observed however, and several fiber 'bleed' peaks were detected in the total ion chromatograms (as shown in Figures 3 and 4B). Although these peaks were typically small, a shorter desorption time would address the issue of bleed peaks and possibly extend fiber life.

CTC Analytics has recently introduced a fiber desorption unit for this autosampler; therefore, fiber carryover may now be eliminated in some applications by off-line thermal conditioning. This may also help to reduce the appearance of fiber bleed peaks in the traces. This unit was not available during the experiments described, but in our subsequent evaluations it has proven useful for reducing or eliminating carryover, depending on the nature of the analytes and the specific fiber used. We have not evaluated its impact on fiber bleed.

In order to analyze the impact of a shorter desorption time on carryover and bleed peaks, an alternate extraction regime was tested using a thicker phase fiber (30-µm PDMS) and shorter extraction and desorption times. With this regime, carryover was not significant and bleed peaks were minimal as compared with the extraction with the 7-µm fiber (data not shown). Sensitivity for some of the larger sesquiterpenes appeared lower; however, because the samples for these experiments were different (chopped fresh leaves in air rather than powdered, meshed leaves in buffer), a direct comparison of

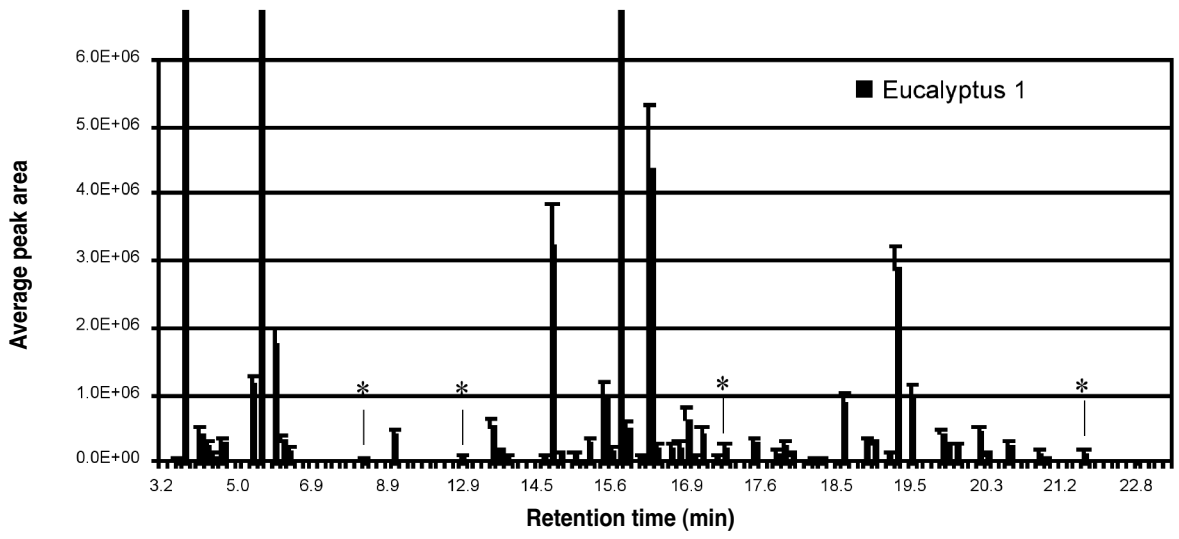

Figure 3. Histogram of average peak areas from seven replicate extractions of *Eucalyptus dunnii* 1. All chromatograms were searched for the presence of 136 compounds. The *x*-axis indicates peak retention time, but because of the histogram format each peak was assigned equal width thus the *x*-axis scale is not linear. Four bleed peaks were identified in most chromatograms, and these are indicated on the figure by asterisks. Bars for α-pinene (3.5 min), eucalyptol–limonene (5.3 min), and aromadendrene (15.7 min) are off-scale in order to enable visualization of the smaller peaks.

peak heights may not be valid. It appeared however that the proportions of different compounds between the two traces were different in that the chromatogram from the 7-µm fiber indicated that the sesquiterpenes were present in a larger proportion. Although the impact of the different sampling methods could not be ruled out as a cause of this discrepancy, it was not unexpected because the 7-µm extraction was performed at equilibrium conditions for all compounds (therefore, the overall extraction of the higher partition coefficient compounds was expected to be higher).

Reproducibility of peak areas

Peak areas for 19 compounds were recorded for seven replicate SPME injections of powdered *Eucalyptus dunnii*. Peak-area averages and standard deviations were calculated and are presented in Table I. The rows in Table I are arranged in order of peak area, but no correlation between the peak area and peak-area standard deviation was observed. Thus, there was not a systematic integration error producing a larger relative standard deviation (RSD) for smaller peak areas. Histograms of the average chromatographic results for seven replicate injections of four of the 12 eucalyptuses tested are also presented in Figure 4. Peak-area standard deviations are shown as vertical error bars in Figure 4. Peak-area precisions for the other eucalyptuses were similar to those shown in Table I, with most compounds producing an RSD percentage of less than 20%. This is considered acceptable for a biological samples analysis.

Peak areas were tracked for 20 of the major compounds in the *Eucalyptus dunnii* chromatogram using the simplified regime (chopped leaves rather than leaf powder, a thicker phase fiber, and shorter extraction and desorption times). In separate analyses of seven different leaves from one tree, peak-area standard deviation was observed to be much larger for the simplified regime than for the powdered leaf regime. In the simplified regime, the RSD percentage varied in the range of 3% to 140% with a median RSD percentage of 27% (data not shown). As is seen in Table I, for the powdered leaf regime the RSD percentage varied in the range of 3% to 30% with a median of 14%. Clearly, the simplified regime had the advantages of simplicity, minimal carryover, and fiber bleed and would be useful for a quick survey of sam-

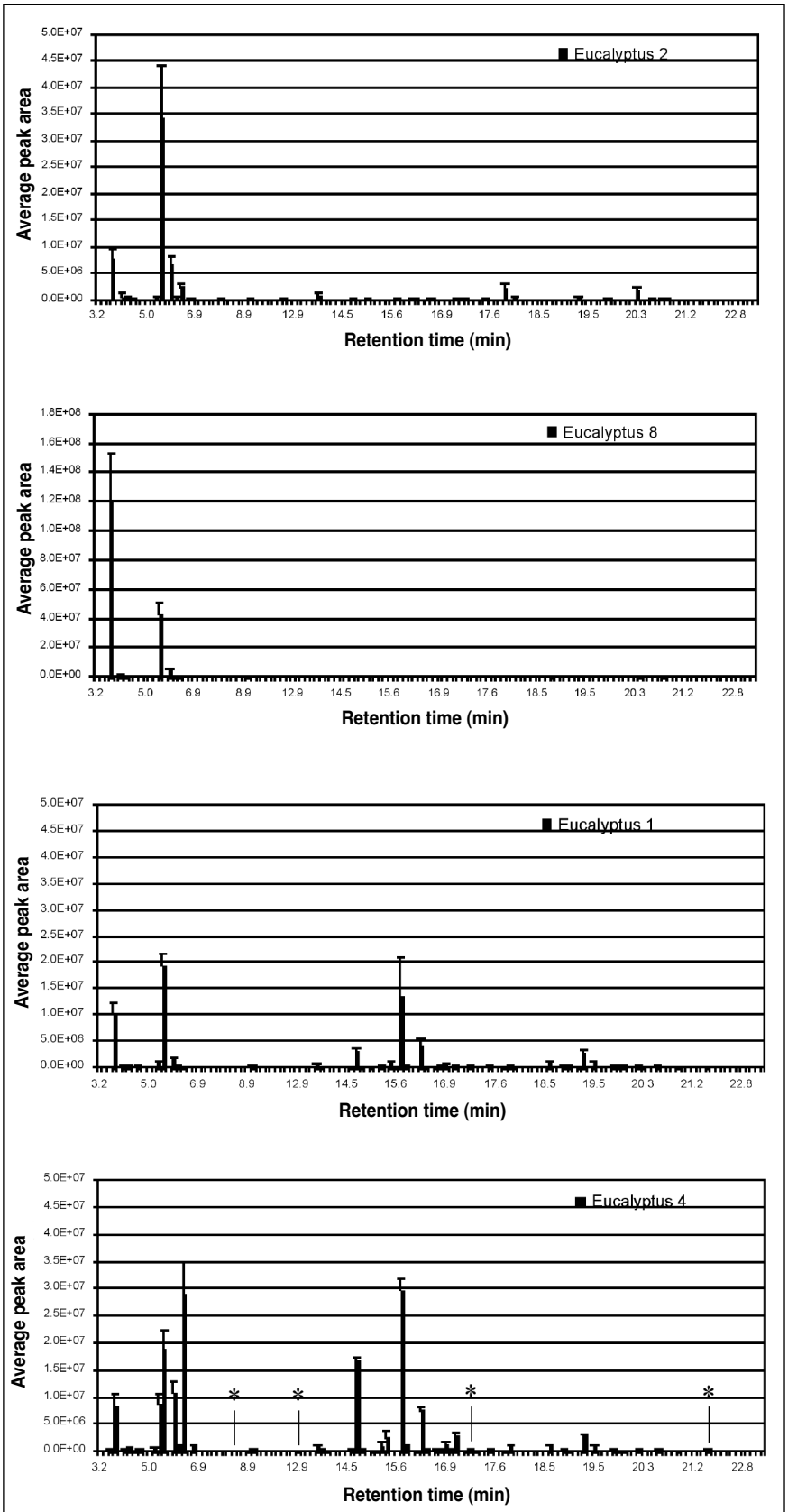

Figure 4. Histograms of average peak areas from seven replicate extractions of eucalyptus. Eucalyptus 2 and 8 show primarily monoterpenes, and those of eucalyptus 1 and 4 (*Eucalyptus dunnii*) show both mono- and sesquiterpenes. All histograms are plotted on the same *y*-axis except eucalyptus 8. Asterisks in the histogram for eucalyptus 4 indicate the positions of bleed peaks.

Table I. Average Peak Areas and RSD Percentage for Seven Replicate Extractions of 19 Compounds Extracted from *Eucalyptus dunnii* (Leaf Powder)*

Retention time (min)	Average peak area	%RSD
16.21	262,387	13.7
15.16	277,751	10.4
20.62	306,887	8.5
18.00	350,607	20.4
20.80	362,616	16.9
14.92	394,366	9.2
4.25	428,861	15.1
5.68	464,422	20.6
20.87	523,026	2.7
19.26	565,971	8.5
4.49	710,223	28.0
14.04	1,001,559	19.0
20.29	1,684,669	11.4
17.79	3,840,704	11.5
5.27	9,233,150	29.7
13.37	11,826,351	13.9
3.54	14,095,345	25.2
5.46	15,046,217	18.8
5.34	27,627,439	14.0

* The magnitude and range of errors shown are representative of those seen for all 136 compounds and 12 eucalyptuses. The data are arranged in order of peak area to demonstrate that there is no systematic skewing of standard deviations with peak area.

Table II. Average Retention Times, Ranges, Standard Deviations, and RSD Percentages for Seven Replicate Extractions of 19 Compounds from *Eucalyptus dunnii* (Leaf Powder)*

Average retention time (min)	Range (min)	Standard deviation (s)	%RSD
3.54	3.52–3.58	1.24	0.59
4.25	4.24–4.29	1.18	0.46
4.49	4.47–4.52	1.05	0.39
5.27	5.26–5.31	1.12	0.35
5.34	5.32–5.37	0.97	0.30
5.46	5.44–5.49	1.08	0.33
5.68	5.66–5.71	1.06	0.31
13.37	13.35–13.39	0.91	0.11
14.04	14.02–14.06	0.92	0.11
14.92	14.90–14.94	0.85	0.10
15.16	15.15–15.19	0.94	0.10
16.21	16.19–16.24	0.96	0.10
17.79	17.77–17.81	1.00	0.09
18.00	17.98–18.03	1.10	0.10
19.26	19.25–19.29	0.96	0.08
20.29	20.27–20.32	0.92	0.08
20.62	20.61–20.65	0.92	0.07
20.80	20.78–20.83	1.00	0.08
20.87	20.85–20.89	0.95	0.08

* These data were obtained early in the method development, and the retention time standard deviations for the analysis of the 12 eucalyptuses were slightly better (< 1.0 s).

ples. However, for acceptable chemometric data the more-rigorous sample preparation is required.

Reproducibility of retention times

Retention times for each of the 19 different compounds were recorded for seven SPME injections of powdered *Eucalyptus dunnii*. Retention time averages and standard deviations were calculated and are presented in Table II. For the seven replicate injections of the 12 eucalyptuses, the standard deviations ranged from 0.5 to 1 s, which was slightly better than for *Eucalyptus dunnii* (mostly approximately 1.0 s). This discrepancy likely occurred because the *Eucalyptus dunnii* experiments were performed early on in the method development with a less-optimal GC setup. In either case, the variation in retention times was acceptable for a chemometric analysis of the data.

Chromatographic data in support of chemometric analysis

A typical chromatogram for the analysis of *Eucalyptus* volatiles (7-µm fiber) from *Eucalyptus dunnii* powdered leaves is shown in Figure 5 with several of the main components identified. As stated previously, a positive identification of all 136 compounds tracked was not possible.

Leaf powder was analyzed for each of the 12 eucalyptus trees with seven replicate samples analyzed for each plant. The averaged peak areas are shown in Figure 4 as histogram plots with the retention time on the *x*-axis. For each eucalyptus, 136 components were analyzed. These peaks are not all evident in Figure 4, because several compounds dominate in terms of peak area. If the *y*-axis is expanded, the additional compounds may be observed (shown in Figure 3). The error bars represent one standard deviation calculated for the peak areas. Data for only four of the 12 eucalyptus trees are shown because of space limitations. This set of 12 trees was chosen because of the variety of its pulp and paper properties, which includes eucalyptus trees that are considered good, average, and poor for pulp production. From the analysis of the 12 eucalyptus trees, it was noted that plants fell into two broad categories, those with both mono- and sesquiterpenes (*Eucalyptus dunnii*) and those with the predominantly earlier-eluting monoterpenes (other eucalyptuses). As can be seen in Figure 4, *Eucalyptus dunnii* (1 and 4) have both mono- and sesquiterpenes, and eucalyptus 2 and 8 have predominantly monoterpenes. It is known that pulp properties vary even between two individuals of the same species (e.g., *Eucalyptus dunnii*), thus it was important to note the difference in the volatile profiles for this species (seen in Figure 4B). For Eucalyptus 2 and 8, α-pinene and eucalyptol dominated the histogram (the difference in the *y*-axis scale should be noted). Even when the *y*-axis was expanded, sesquiterpene peaks were quite minimal (data not shown).

Conclusion

The automation of SPME extraction for eucalyptus volatiles has been demonstrated with sufficient precision in order to allow for meaningful chemometric analysis of volatile variations between different plants. While 136 com-

pounds were monitored for each of the plants tested, it was observed that the differences in the chromatographic profiles between the different plants were significant enough that the monitoring of all these compounds may not be necessary. A statistical evaluation of the data is underway and a full description of the chemometric analysis will appear in a separate publication.

Acknowledgments

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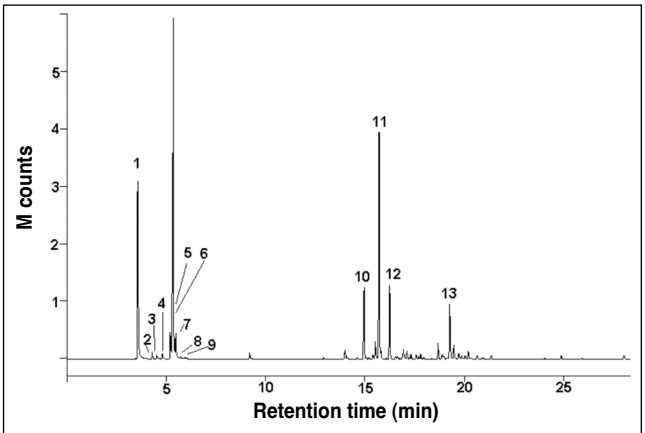

Figure 5. Typical chromatogram for the analysis of volatiles from *Eucalyptus dunnii* 1: α-pinene, 1; β-pinene, 2; β-myrcene, 3; α-phellandrene, 4; limonene, 5; eucalyptol, 6; β-cis-*o*-cimene, 7; β-trans-*o*-cimene, 8; γ-terpinene, 9; α-gurjunene, 10; aromadendrene, 11; *allo*-aromadendrene, 12; and globulol, 13.

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