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A SIMPLE METHOD TO EXTRACT ESSENTIAL OILS FROM TISSUE SAMPLES BY USING MICROWAVE RADIATION

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Abstract—A microwave protocol to extract lipophilic substances from tissue was modified to extract essential oils (EOs) from plant tissue and insect feculae. The material, in a solvent transparent to microwave radiation, is exposed for a short time to steam in a microwave oven. EO extracts are analyzed directly by GC or GC-MS when plant material is fresh and terpenes contained in glandular structures on leaf surfaces are readily released into the solvent. For dried material or insect feculae, mechanical means are utilized first to break up tissue; however, the complete procedure is carried out inside the same vial to reduce losses. Statistical analysis shows that the reproducibility of the modified method is high. Several samples can be run within an hour with this method.

Key Words—Microwave, essential oil, *Cordia curassavica*, Chrysomelidae, Boraginaceae, lower terpenes, extraction method, chamomile.

INTRODUCTION

Terpenoids have been studied for different reasons. Leaf terpenoids have been evaluated for chemosystematic and geographic variability (Salgueiro et al., 1997; Fahlen et al., 1997; Souto et al., 1997), biosynthetic studies (Gershenzon, 1994; Clark et al., 1997), herbivore–host-plant relationships (Steinbauer et al., 1998; Gómez et al., 1999), bioassays (Lis et al., 1998), proof of authenticity (Casabianca et al., 1998), and aroma chemistry (Pallado et al., 1997).

A number of methods for obtaining essential oils (EOs) from plant foliage have been published (Ruberto et al., 1999; Simandi et al., 1999; Lis et al., 1998;

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Anitescu et al., 1997; Laenger et al., 1997; Muzika et al., 1990). However, many of these techniques require gram amounts of samples and long extraction times. In contrast, methods to extract EOs from insect wastes appear to have received little attention.

A common problem encountered by chemical ecologists during their assessments is the small amount of sample material available for chemical analysis, especially when that material is insect physiological wastes and discharges, e.g., feculae and regurgitates. Similar situations are encountered with plant material, for instance, when herbivory causes defoliation, especially of young leaves (Gómez, 1997; Coley and Kursar, 1996), or conversely, when considerable quantities of plant tissue are needed for studies of geographical distributions of EOs.

We developed a method based on the work of Pare (1994, 1995) and modified by Clark et al. (1997) to quantitatively extract EOs from plant tissue and insect feculae. Here we present data on the reproducibility of that method by quantifying EOs in plant material and insect feculae with known and unknown terpenoid levels and EOs. We also compare our results with those obtained from steam distillation.

METHODS AND MATERIALS

Plant Material. We tested five samples (40 mg) from *Matricaria chamomilla* L. from the company Caolo (30.06.95, Ch. B. 53618395), with 0.63% EO to assess the efficacy of the modified method (see below). Five (40-mg) samples from *Mentha piperita* L. and *Salvia triloba* L., with unknown terpenoid levels, were also examined. Foliage of *Cordia curassavica* (Jacq.) Roem. & Schult. (field plants from Central Panama) also were examined. Branches of *C. curassavica* were collected in the field and transported to the laboratory in plastic bags. Young leaves (newly expanded at nodes 1 and 2) were excised, pooled, dried at room temperature, and then coarsely ground before weighing and extracting. Vouchers of *C. curassavica* were deposited in the Herbarium of the Smithsonian Tropical Research Institute.

Insect Feculae. Larval feculae came from *Eurypedus nigrosignata* Boh. (Coleoptera: Chrysomelidae), a beetle that feeds on *Cordia curassavica* in Central Panama. Feculae were collected from third instars that fed on plants with differing compositional profiles of EOs (Gómez et al., 1999). Larvae of *E. nigrosignata* produce elaborate dorsal structures, by using feculae and cast skins, that can be detached easily from their bodies with forceps. The size and weight of dorsal structures vary with larval age, and their chemical composition varies depending on plant terpenoid content (Gómez et al., 1999). Three groups of feculae (40 mg) were examined. Feculae for batch 1 ($N = 5$) and batch 2 ($N = 4$) came from larvae feeding on *C. curassavica* shrubs carrying the α-pinene profile, and

batch 3 ($N = 5$) came from larvae raised on *C. curassavica* cultivated in a green house (Gómez, 1997) and carrying the β-terpinene profile. Replicates in each batch contained several dorsal structures. Only quantifiable monoterpenoids and caryophyllene were considered for total concentration.

Steam Distillation. Distillation was performed in a steam-distillation apparatus as described in the German Pharmacopoeia (DAB10, 1991). Samples (1 g, $N = 2$) of dried material from *C. curassavica* (see above) were placed in a flask, 200 ml distilled water was added, and the distillation carried out for 5 hr. The distillate was collected in 0.75-ml *n*-hexane. *n*-Octadecane was added to the samples to arrive at a final concentration of 20 μg/mg. Only major terpenoids were used for comparison among methods.

Microwave Method. A method to extract lipophilic substances from fresh material as developed by Pare (1994, 1995) and modified by Clark et al. (1997) was adjusted further to extract EOs from small amounts of fresh and dried samples. The material was extracted with 0.75 ml *n*-hexane (p.a.) containing 50 μg/ml of *n*-octadecane in a vial (2.0-ml, conical, polypropylene Twist Cap vials, with screw caps with an O-ring seal). Two glass beads (4 mm diam.) were added to the sample vial, and shaken in a Vortex at medium speed for approximately 120 sec (or until tissue was crushed). A polypropylene lid with five to six radially symmetrical holes served as a vial holder (Clark et al., 1997). The lid was placed on a 250-ml glass beaker containing 50 ml water at room temperature. Sample vials were then heated in a microwave oven at full power (800 W) for 60 sec. After the microwave exposure, the vials with lids were cooled in a polypropylene jar of cold water and centrifuged at 14,000 rpm for 3 min. The clear supernatant was transferred and kept in 2.0-ml glass vials (screw cap with Teflon lining) at -18°C until GC and GC-MS were carried out (see below). When fresh plant material was used, a clear extract was obtained without the need for maceration or centrifugation. The total EO concentration, and the concentration of the major terpenoids was determined for each sample (see below). Only major terpenoids were considered for comparison of methods.

GC and GC-MS. Separation, quantification, and identification of terpenoids were carried out as described in Gómez et al. (1999).

Scanning Electron Microscopy (SEM). The surface of young *C. curassavica* leaves was viewed directly before and after microwave extraction, by using backscattered electrons at low vacuum, and they were photographed in a Jeol model 5300LV scanning electron microscope.

Statistical Analysis. The reproducibility of the microwave method was tested by comparing coefficients of variation (CV) (Muzika et al., 1990; SYSTAT, 1992), and the intraclass correlation coefficient (ICC) within groups (Sokal and Rohlf, 1995; SYSTAT, 1992). Yields obtained by the microwave method and the distillation method were compared by linear regression (LR) analysis, and the adjusted

squared multiple R was utilized to compensate for unequal sample sizes. In one case, a LR was made by considering the mean values of the distillation method as the independent variable. If the microwave method extracts a higher quantity of terpenoids than the distillation method, then the slope of the LR equation should be larger than unity. A second LR was made by considering the mean values of the microwave method as the independent variable. Correlation coefficients were used to compare the fit and the variance explained by the method.

RESULTS

Plant Material. The EO concentration of commercial *M. chamomilla* samples determined by the microwave method was $0.65 \pm 0.05\%$ (Table 1), the same as the reported one of 0.63%. Terpenoids detected in the yellowish extract of chamomile were β-farnesene, α-bisabolol oxide B, α-bisabolone oxide, α-bisabolol, bisabolol oxide A, and spiroether. Plant material (40 mg) of *M. piperita*, *S. triloba*, and *C. curassavica* (wild plants) with unknown levels of EO also were examined (Table 1). The CV varied from 5% to 15% for the total concentration, and from 3% to 16% for the amount of the major terpenoid.

The ICC was used to evaluate the reproducibility of the microwave method and showed 9% variation for the total concentration within the groups, and 11% for the main component.

Comparison of Extraction Methods. The first LR demonstrates that the microwave method was more efficient in obtaining higher concentrations of components (slope $= 1.729$; $p < 0.001$). The microwave method produced less variable yields ($R^2 = 0.900$) than the distillation method ($R^2 = 0.787$).

SEM. After exposure to hot hexane, glandular trichomes on the surface of fresh leaves collapsed (Figure 1A, B).

Insect Material. Three different batches of insect feculae were utilized to evaluate this method. CVs varied from 14% to 21% for the total concentration of EOs and from 17% to 20% for the main component, depending on fecula origin

TABLE 1. EO CONCENTRATION OF DIFFERENT PLANT SPECIES SAMPLES WITH MICROWAVE METHOD[a]

Plant species	Total concentration	%CV	Major terpenoid concentration	%CV
Matricaria chamomilla	6.59 ± 0.43	7	3.66 ± 0.11^a	3
Mentha piperita	5.30 ± 0.29	5	2.14 ± 0.19^b	8
Salvia triloba	13.50 ± 2.20	15	6.89 ± 1.25^c	16
Cordia curassavica	12.66 ± 0.90	7	3.74 ± 0.54^d	16

[a] $N = 5$; concentration (mean $\pm$ SD) in mg/g, dry weight; %CV, coefficient of variation in percentage; a, bisabololoxide A; b, menthol; c, 1,8-cineol; d, β-terpinene.

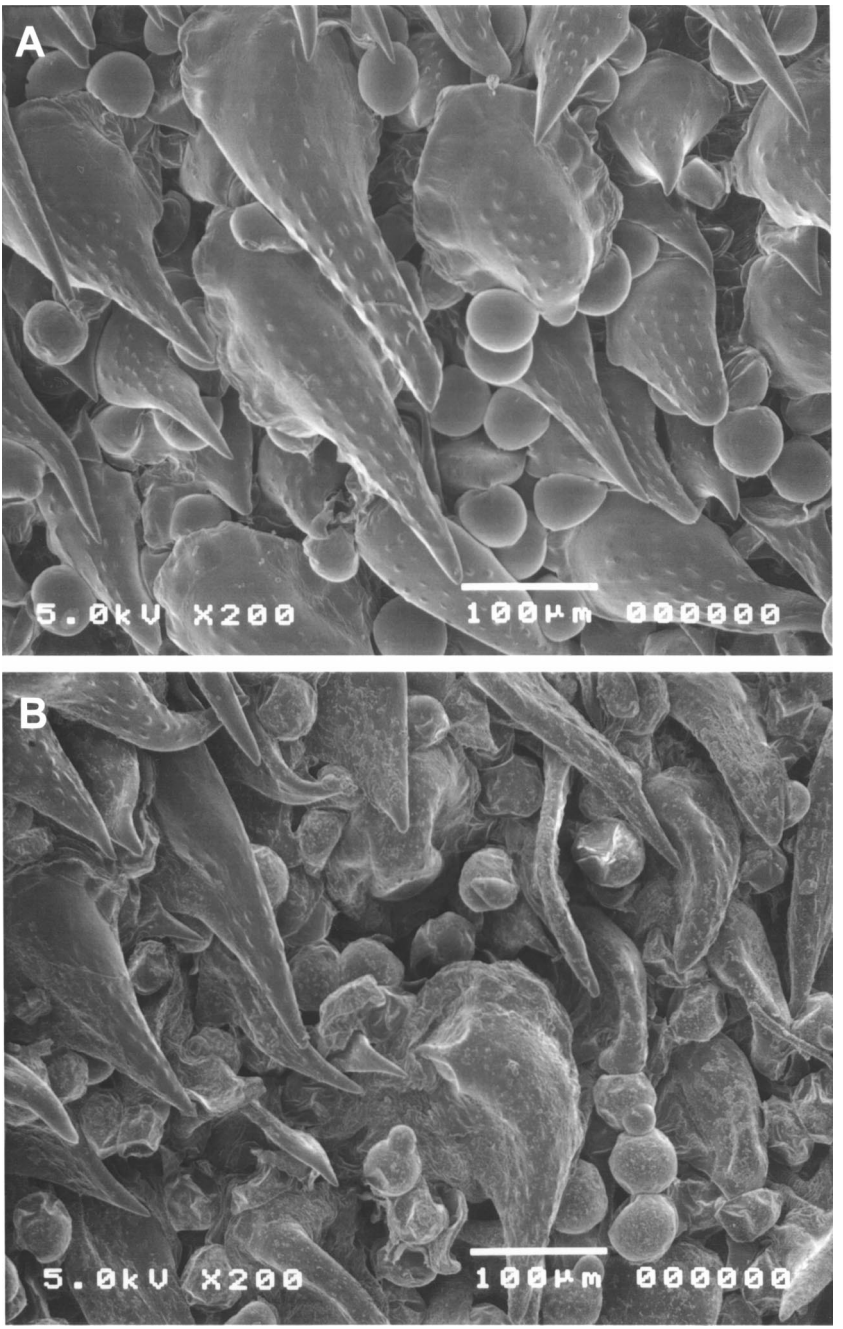

FIG. 1. Adaxial surface of *C. curassavica* showing glandular round trichomes (A) before and (B) after exposure to hot hexane.

TABLE 2. EO CONCENTRATION FOR THREE FECULA BATCHES COLLECTED FROM THIRD INSTARS OF *Eurypedus nigrosignata*[a]

Batch	Avg. weight (mg) (±SD)	Avg. number of dorsal structure per replicate (±SD)	Total concentration (mean ±SD)	%CV	Major terpenoid concentration (mean ± SD)	%CV
Wild larvae ($N = 5$)[b]	40.9 ± 1.2	6.6 ± 0.5	2.88 ± 0.61	21	1.12 ± 0.22	20
Wild larvae ($N = 4$)	39.9 ± 2.6	7.3 ± 0.8	4.99 ± 0.92	18	2.57 ± 0.46	18
Greenhouse larvae ($N = 5$)[c]	37.8 ± 3.2	7.0 ± 1.4	2.67 ± 0.39	14	1.58 ± 0.26	17

[a] Concentration in μg/mg, dry weight; %CV, coefficient of variation in percentage.
[b] α-Pinene profile.
[c] β-Terpinene profile.

(Table 2). Feculae from animals feeding on field plants registered higher CVs than those from animals raised on greenhouse plants.

The ICC was calculated also to evaluate the microwave method for insect samples. The ICC percentage variation within groups was 23% for the total concentration, and 17% for the major component.

DISCUSSION

For fresh plant material containing EOs in glands, the hot solvent was efficient at collapsing glandular structures and extracting terpenes (Clark et al., 1997) without need of maceration. Indeed, treatment of fresh plant material with glass beads in these trials did not cause tissue destruction. Destruction of plant tissue other than that containing EOs may lead to undesirable plant contaminants in the extract. By extracting fresh plant material as described above, the clear extract obtained can be analyzed directly by GC.

In the case of insect material, we found that the use of glass beads to macerate both fresh and dried insect feculae before microwave exposure, in order to destroy the matrix holding EOs, facilitates their release into the solvent. Maceration with glass beads was a good alternative to pulverization with a mill or maceration with a mortar and pestle, because these procedures lead to significant sample loss, particularly when dealing with small samples containing volatile substances. This step usually produced fine particles that were separated by centrifugation. Losses of volatiles can be reduced further by carrying the procedure to completion in the same extraction vial and by avoiding rotary evaporation to eliminate the solvent, because it may lead to the losses of volatile compounds.

The coefficient of variation (CV) has been used as an index of reproducibility of five techniques for extracting volatile compounds from needles of two conifer

species (Muzika et al., 1990). The CV was developed to compare the relative amounts of variation in populations having different means (Sokal and Rohlf, 1995, p. 58). CV analysis indicates that the modified microwave method is reproducible both for total terpenoid concentration and for individual terpenoids. The intraclass correlation coefficient (ICC) measures the similarity of individuals within a group, relative to the degree of difference found among the groups (Sokal and Rohlf, 1995, p. 213). A low value for ICC indicates that there is little or no variance within groups. The ICC showed low variation within groups for both plant and insect materials, supporting the reproducibility of the modified method.

Higher EO yields were attained with the microwave method compared to yields obtained by steam distillation. In addition, this method yields less variable results. Several authors have reported that steam distillation produces lower extraction yields (Simandi et al., 1999; Tuan and Ilangantileke, 1997; Stashenko et al., 1997), contributes to thermolysis of sensitive terpenes (Ruberto et al., 1999; Ammann et al., 1999; Eikani et al., 1999), and takes longer than other techniques (Muzika et al., 1990). With the modified method, we can not rule out the possibility that some thermal degradation may occur. However, we were not able to detect this in our samples. On the other hand, comparison of various methods that have been used to extract EOs from conifer needles has shown that circular steam distillation of 10-g samples for 8 hr yielded higher quantities (Muzika et al., 1990) than did solvent extraction, liquid carbon dioxide extraction, or rapid steam distillation.

The tremendous natural variation in leaf terpenoids (Langenheim, 1994; Gershenzon, 1994) may also influence the composition of physiological discharges from phytophagous insects, e.g., many insects prefer to eat young leaves (Coley and Kursar, 1996). We suggest collecting and extracting newly expanded leaves (assuming that they are small enough to fit inside a vial) and/or removing a predetermined leaf area from more mature leaves. Analysis of fresh material results in higher yields of secondary metabolites (Gómez et al., 1999; Coley and Kursar, 1996) and prevents the loss of volatiles due to drying (Ross and ElSohly, 1996; Yen and McGaw, 1996).

When analyzing insect material with the microwave method, one should consider that variation due to the nature of the sample rather than the method itself may occur. Our particular plant–insect system is under the strong chemical influence of a particular host plant as well as the larval age (Gómez et al., 1999).

Specific Considerations. Because hexane is transparent to microwave radiation, water steam is the heating source. A solvent not transparent to microwave radiation would itself boil, making water steam unnecessary. However, solvent polarity may be important, depending on the chromatographic analysis chosen. Additionally, an explosion-proof oven should be used, in case extraction vials have leaks or if open containers are used. It is advisable to use high-quality, seal-proven extraction vials to assure that solvent fumes (and volatile substances) are not released into the oven chamber. The watt output of the microwave oven

may vary depending on brand, country of manufacture, and local electrical characteristics, so that the exposure time may need adjustment. We found that dried leaflets longer than half of the vial height were difficult to grind with two glass beads. In such a case, smaller beads especially designed for tissue analysis may be needed. Alternatively, the leaf can be broken manually.

Chemical ecologists usually are confronted with a limited sample of insect waste material for analysis. Factors such as insect age and insect preferences for specific plant organs at specific developmental stages are important considerations during the analysis of EOs.

In summary, the microwave method is reproducible, easy to run, allows simultaneous analysis of many samples, and offers an excellent alternative to methods that utilize gram amounts of material, i.e., steam distillation and solvent extraction. Researchers engaged in evaluating the role of EOs in plant–insect interactions, terpenoid differences in individual plants, and biogeographical studies of EOs, may profit greatly from using this modified method.

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