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EXTRACTION OF PHENOLIC COMPOUNDS FROM FRESH LEAVES: A COMPARISON OF METHODS

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Abstract—The conventional sonicator/shaker bath method for phenolic extraction was compared with a less traditional one using a homogenizer. The homogenizer proved to be both more efficient and consistent in extracting phenolics from tender, as well as tough, leaves. We propose that adoption of the homogenizer technique will increase phenolic yield and efficiency.

Key Words—Extraction, phenolics, homogenizer, *Ouratea lucens, Acomastylis rossii*, tannin, sonicator.

INTRODUCTION

Phenolics are carbon-based compounds present in many plants. They are of general interest because of their wide-ranging ecological effects from the organism to ecosystem level (Appel, 1993). They are perhaps most noted for their ability to bind to proteins in vitro, forming soluble and insoluble complexes (Goldstein and Swain, 1965; Feeny, 1976; Hagerman and Butler, 1980; McManus et al., 1981; Hagerman and Klucher, 1986; Hagerman and Robbins, 1987). These phenolic–protein interactions are thought to be, in part, responsible for the putative function of phenolics as plant defense compounds (Feeny, 1976; Rhoades and Cates, 1976; Coley, 1983; Mole and Waterman, 1987).

To explore any of the several hypotheses in which phenolics play a prominent role, e.g., plant defense theory, phenolic quantification is often necessary. Numerous researchers have investigated various phenolic assays (Peri and Pom-

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pei, 1971; Hagerman and Butler, 1980, 1989; Mole and Waterman, 1987; Wisdom et al., 1987; Mole et al., 1989), appropriate standards (Hagerman and Butler, 1989) and extraction solvents (Hagerman, 1988). To our knowledge, no studies have systematically investigated the extraction technique itself.

Conventional methods typically involve reducing the plant material into smaller particles using liquid nitrogen and a mortar and pestle. The pulverized plant material is then extracted in a solvent, and the extraction is enhanced by using a sonicator or shaker bath. This step is repeated several times (Hagerman, 1988; Hagerman and Butler, 1989). Our extraction method differs from the conventional method in the use of a homogenizer to facilitate the release of phenolics instead of several sonicator/shaker bath extractions. We propose that the homogenizer method is superior to that of the sonicator/shaker bath technique in both accuracy and efficiency of extraction. Moreover, we predict that this difference will be most pronounced in plants with tough, fibrous leaves, as the thicker cell walls should further hinder extraction by conventional methods.

METHODS AND MATERIALS

We chose two species of plants that differ in leaf toughness: *Acomastylis* (*Geum* Ser.) *rossii* (R.Br.) Greene (Rosaceae), a temperate alpine plant with relatively tender leaves, and *Ouratea lucens* Engl. (Ochnaceae), a tropical species with tough leaves. Toughness was estimated from the amount of mass required to force a 5-mm-diameter rod through leaf material and is correlated with fiber content (Coley, 1983). Toughness was measured directly on *O. lucens*, and is 500 g (Kursar, unpublished data). It was impossible to directly measure toughness on *A. rossii* leaves because they are highly dissected. However, we calculated *A. rossii*'s toughness from its acid detergent fiber (ADF) content (Dearing, unpublished data), using an equation obtained by Coley (1983), who regressed toughness on ADF content for 46 species. Her equation is $y = 11.95x + 77.5$, where y is toughness in grams and x is percent ADF content ($r^2 = 0.32$, $P < 0.01$). The estimated toughness of *A. rossii* is 220 g, which is representative of a very nontough species.

A. rossii leaves were harvested in August 1993 and kept at −70°C until the day of the experiment. *O. lucens* was freshly harvested from the University of Utah greenhouse and immediately put on Dry Ice.

General Leaf Preparation. Major veins were removed with a razor and the leaf fragments were cut into small pieces. The leaf fragments were further ground under liquid nitrogen using a mortar and pestle. Grinding in liquid nitrogen was necessary for both techniques. A subsample was weighed, dried at 50°C, and reweighed to obtain the dry weight/wet weight ratio.

Extraction. Samples of ground leaves were simultaneously subjected to both extraction techniques three separate times (trials 1–3). Wet weight samples of powdered leaves, approximating 0.1 g dry weight, were extracted first with 25 ml of 85% MeOH (extract A). Each sample was extracted three successive times with 10 ml 85% MeOH (extracts B, C, and D).

Phenolics were extracted by using either a Di Sontegrator (Ultrasonic Industries Inc.) sonicating bath followed by shaking in a standard shaker bath, or a Brinkmann Homogenizer Polytron 10/35, equipped with a PTA 10 TS generator with saw teeth (Brinkmann Instruments, Inc.). For the conventional extraction, test tubes with ground leaves and solvent were placed in a sonicator at 4°C. Tubes were removed after 30 min and centrifuged at 16,000*g* for 15 min at 4°C. The supernatant volume was measured, and 1 ml was saved for phenolic analysis (extract A). The pellet was resuspended in 10 ml solvent, vortexed and placed in a shaker bath at 4°C at high speed for 30 min. The sample was centrifuged as described above, the supernatant was measured, and again 1 ml was saved (extract B). This was repeated two more times, for a total of four extracts (A–D). Extracts were stored in a freezer (−5°C) in the dark until the phenolics analyses were conducted.

The plant material was extracted by using the homogenizer for 60 sec at maximum speed. Homogenization for 60 sec slightly warms but does not greatly raise the temperature of the solvent. The test tubes were then centrifuged at 16,000*g* for 15 min at 4°C, after which the supernatant was removed, measured, and 1 ml was saved for phenolic assaying (extract A). For the subsequent extracts (B–D), the pellet was resuspended by vortexing with 10 ml of 85% MeOH. The samples were centrifuged between extractions as described above.

To determine whether the homogenization extraction procedure was exhaustive, we passed samples through a French press (Aminco, SLM Instruments Inc.). Because the plant material must be ground extremely fine for use in the French press, more than that which is possible with a mortar and pestle, we first extracted the plant material by homogenization as described above. After the four extractions, the pellet was then resuspended in 15 ml of 85% MeOH and passed once through the French press at 21 MPa. This machine imparts a force of 21 MPa on sample material, which causes the rupture of all cells (Milner et al., 1950). The French press extracts were then analyzed for phenolics.

Phenolics Assay. Extracts were assayed within one week of extraction. Sonicator/shaker bath and homogenizer samples that had been extracted on the same day were assayed at the same time. Extracts A–D were assayed individually for total phenolics using the Folin-Ciocalteu method (Singleton and Rossi, 1965). The amount for each extract was combined (A + B + C + D) for an absolute quantity of phenolics/sample. Tannic acid was used as the standard (Sigma lot #1764 KCNT).

RESULTS

The homogenizer extracted significantly more phenolics for all extracts combined (A–D) than did the sonicator/shaker bath method. This difference was consistent for both plant species tested (Randomized complete block, blocking on trial: *A. rossii*, method F = 94.91, $P < 0.0001$, $df = 1, 20$; block F = 14.43, $P < 0.0001$, $df = 2, 20$; *O. lucens*, method $F = 30.53$, $P < 0.0001$, $df = 1, 20$; block $F = 8.61$; $P < 0.002$, $df = 2, 20$; Figure 1). The sonicator/shaker bath technique extracted only three quarters of the total amount of phenolics extracted by the homogenizer (*A. rossii*, 74.6% ± 5.2 and *O. lucens*, 68.0% ± 9.0, Figure 2). In addition, for both species we found three to four times less variability among samples using the homogenizer technique (Table 1).

Results from the analysis for absolute phenolic quantity in samples extracted after using the French press show zero phenolics for one sample of *A. rossii*, negligible phenolics for the other sample (0.06% of total), and no remaining phenolics for the two *O. lucens* samples. Hence, we are confident that the higher yields extracted using the homogenizer represent virtually all extractible plant phenolics detectable by the Folin-Ciocalteu assay.

Interfraction Comparison: Extracts A–D. The percent of the total extracted per extraction (A–D) for the two methods was compared. Because the homogenizer extracted all phenolics, this amount is used as the denominator for the sonicator extracts. We found that for both species, the homogenizer removed significantly more phenolics in the first extraction than did the sonicator and that this difference diminished, and even reversed, in successive extractions (Figure 2). The homogenizer extracted virtually all phenolics with only one extraction, while even with four extractions, the sonicator had not always extracted a comparable amount of phenolics. In the initial extraction of *A. rossii* leaves, the homogenizer extracted 93.8% of the total extractible phenolics, whereas the sonicator/shaker bath extracted only 38.7% (Figure 2). The difference in the extraction efficiency of the two techniques was similar in *O. lucens*; the homogenizer extracted 99.2% of the total phenolics in the initial extract and then negligible amounts in subsequent extractions. The sonicator/shaker bath extracted only 45.5% of the total in the initial extract. In both species, the homogenizer pellet additionally released only small amounts of phenolics in subsequent extractions (B–D), while the sonicator/shaker bath consistently yielded more phenolics in these extracts (Figure 2).

DISCUSSION

Our results show that the homogenizer technique extracts totally more phenolics, with less variability, than the sonicator/shaker bath technique. Moreover, extraction time for the same number of samples (up to eight) with four extrac-

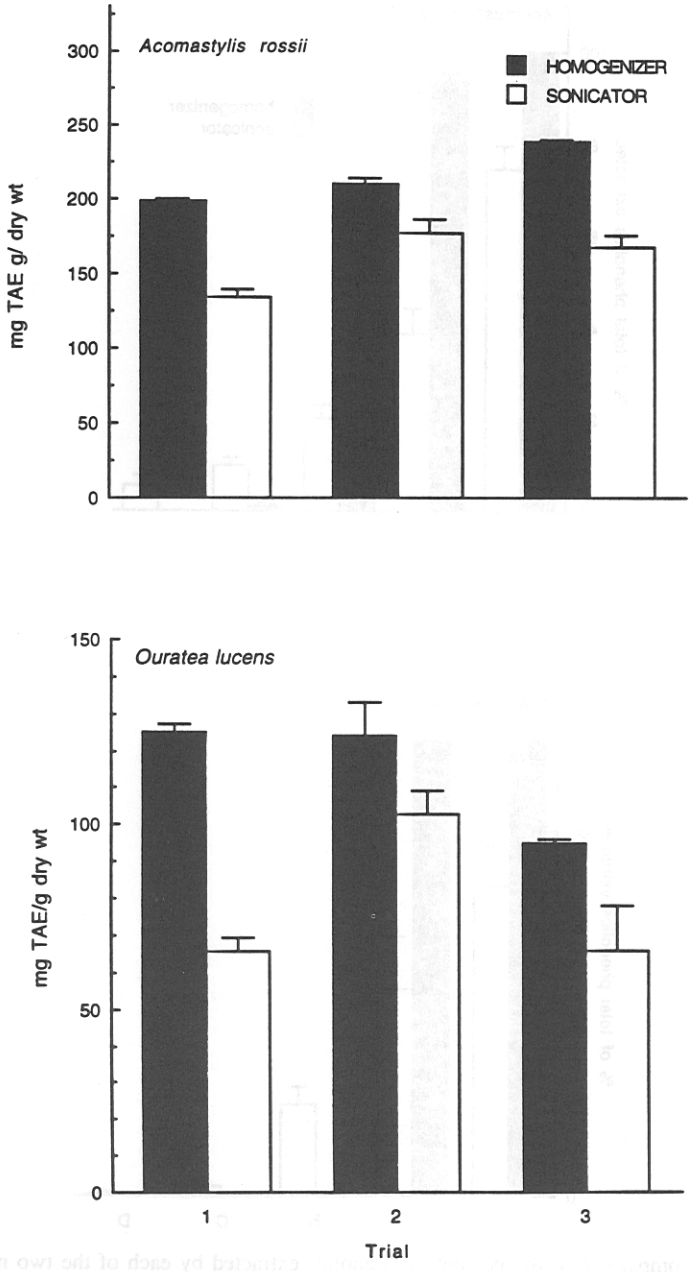

FIG. 1. Comparison of total phenolics extracted by the two methods. Bars represent means for each trial ($N = 3$) plus 1 standard error.

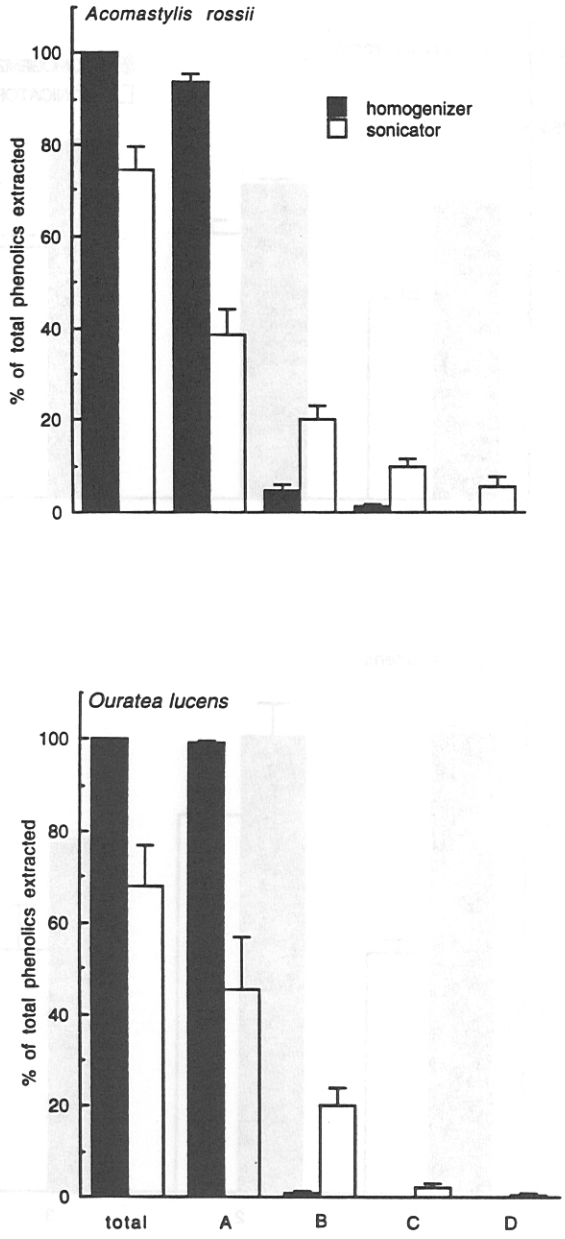

FIG. 2. Comparison of the percent of phenolics extracted by each of the two methods: overall and for each extraction (A–D). Bars are means for each trial (N = 3) plus 1 standard error.

TABLE 1. COEFFICIENTS OF VARIATION FOR TENDER AND TOUGH LEAVES EXTRACTED BY TWO METHODS[a]

	A. rossii (tender leaves)	*O. lucens* (tough leaves)
Homogenizer	1.9 (0.9)	6.7 (4.1)
Sonicator	9.2 (3.5)	20.4 (8.4)

[a] Average coefficient of variation for three trials plus one standard error.

tions per sample is much shorter using the homogenizer technique: 1 hr 10 min for the homogenizer vs. 3 hr for the sonicator/shaker bath. The number of extractions for the homogenizer technique can be reduced further to two extractions, with no decline in accuracy, as the third and fourth extractions are superfluous, yielding negligible phenolics. Thus, total extraction time using the homogenizer requires approximately 40 min, which represents a considerable time savings over the 3-hr sonicator/shaker bath technique.

Those familiar with the sonication method may initially conclude that the homogenizer is less efficient for large numbers of samples than the sonicator. It is important to note that the number of samples that can be processed using the sonication method is not infinite. The sonication method may be limited by either the sonicator or centrifuge size. Furthermore, even if neither the sonicator nor the centrifuge is restricting, measuring the supernatant after centrifugation can become tedious with sample sizes greater than 20. We suggest grinding be done in subsamples as numerous as the centrifuge will allow and that other subsamples are ground while the first set of subsamples are spinning in the centrifuge. Thus, large sample sizes, i.e., 20, can be managed using the homogenizer with little increase in total time.

The dramatic differences between the two techniques can be partly attributed to the different modes of action of the machines used in the extractions. Several compounding factors contribute to the variability and inefficiency of a sonicating bath. First, ultrasonic waves are effective at emulsifying lipids and are typically employed to break up the lipid membrane surrounding bacteria and animal cells. However, the lipid membrane of a plant cell is encased within a rigid, fibrous cell wall that is probably more resilient to ultrasonic waves. Thus, sonication effectiveness is likely to be dependent on the extent of cell wall damage during mortar and pestle grinding. Moreover, the ultrasonic waves in a sonicating bath are typically not focused or uniform; thus, some samples may receive more sonication than others. In practice, sonicating probes, not baths, are employed to disrupt cells, as probes better focus the sound waves within the sample, while sonicating baths are typically used for cleaning surfaces. Given these considerations, the appropriateness of the sonicating bath for the purpose

of rupturing plant cells is questionable. Cork and Krockenberger (1991) found no difference in extraction yield when a shaker bath was substituted for a sonicator. In addition to these technical drawbacks, the long extraction period (3 hr) may further reduce yield due to phenolic oxidation during the extraction itself.

The superiority of the homogenizer is partially due to its efficacy in breaking down cell walls. The joint action of the partial vacuum created by the homogenizer and the tearing by the saw tooth generator serve to break the cell walls of plant leaves in a way in which a sonicating bath cannot. This difference is obvious in the appearance of the ground leaves. The initial extractions prepared in the homogenizer are bright green in color, while first extractions from the sonicator are much lighter green.

Contrary to our prediction, there was no major difference in extraction efficiency with respect to leaf toughness. The sonicator/shaker bath extracted approximately 75% of all phenolics, regardless of leaf toughness. The homogenizer consistently extracted all detectable phenolics in both tender and tough leaves. Furthermore, the pattern of extraction was similar with both fresh and frozen leaves.

CONCLUSION

We propose that the efficiency and accuracy of phenolic extraction can be enhanced by the utilization of the homogenizer method. The adoption of this device may be beneficial for extractions of other types of phenolics as well and should facilitate investigations in the area of plant defense.

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