Improved methods for extraction and quantification of resin and rubber from guayule

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ABSTRACT

Guayule, a shrub native to the Chihuahuan desert, is a natural source of high quality, hypoallergenic rubber. Unlike rubber trees that produce rubber in laticifers, the rubber in guayule is produced in parenchyma cells of the bark tissue of stems and roots. Consequently, guayule tissue must be mechanically broken before the rubber can be extracted and analyzed. Since rubber extraction and analysis is time-consuming, progress towards increasing the rubber content of guayule through breeding or better cultivation practices has been limited by the slow rate of sample processing. To address the need for faster and more efficient sample throughput, conditions were optimized for automated extraction of dried guayule tissue using accelerated solvent extraction (ASE) and rapid methods were developed to replace gravimetric determination of resin and rubber content. For resin analysis, ultraviolet absorbance was used to determine resin concentration after ASE of the tissue with acetone or acetonitrile. For rubber analysis, evaporative light scattering (ELS) was used to determine the amount of rubber recovered after ASE of the tissue with cyclohexane. Extraction of guayule tissue with high latex rubber content verified that the amounts of resin and rubber determined by these methods were similar to the amounts determined gravimetrically. Since these methods automate extraction and increase the speed of resin and rubber quantification, they could be used in combination with ASE to increase the throughput and efficiency of guayule evaluation in germplasm enhancement and agronomic improvement programs.

1. Introduction

Natural rubber is an economically important biopolymer produced by plants. Alternatives to natural rubber can be synthesized from petroleum products, but the properties of synthetic rubber are inferior to those of natural rubber for many applications (Beilen and Poirer, 2007). Almost all of the world’s natural rubber comes from the Para rubber tree (Hevea brasiliensis Muell. Arg). This tree is cultivated in tropical regions, with the Asian nations of Malaysia, Thailand and Indonesia accounting for over 90% of the world’s production of natural rubber. The importance of natural rubber for the global economy and concerns about the spread of South American Leaf Blight to Asia and Type I latex allergy have spurred a renewed interest in developing alternative sources of natural rubber (Beilen and Poirer, 2007).

Guayule, a desert shrub native to the Chihuahuan desert, has long been regarded as a promising alternative to the rubber tree for production of natural rubber (Hammond and Polhamus, 1965; Whitworth and Whitehead, 1991). In fact, considerable effort was expended during the Second World War towards the development of guayule as a domestic (US) source of rubber. That effort ended after the war, was temporarily revived during the oil embargo of the 1970s and 1980s, and has only recently been renewed with the commercialization of guayule as a source of hypoallergenic latex by Yulex Corporation (Cornish et al., 2001). While a number of barriers to successful widespread commercialization remain, the most significant biological hurdle is the development of high yielding, stress-tolerant germplasm (Estilai and Ray, 1991; Ray et al., 2005). In the past, efforts to improve guayule germplasm through classical breeding and selection were complicated by the ability of guayule to reproduce both sexually and by apomixis. However, with the advent of molecular approaches, including transgenics (Veatch et al., 2005), marker-assisted breeding and ploidy analysis by flow cytometry, new tools are now available for improving guayule germplasm (Estilai and Ray, 1991; Ray et al., 2005; Veatch et al., 2005).

Evaluation of guayule germplasm for industrial product production requires high throughput methods for determining the resin and rubber content of the shrub. While NIR methods have been proposed for measurement of the rubber content in intact plants

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(Cornish et al., 2004), these methods have not been refined sufficiently for reliable use. Consequently, measurement of resin and rubber content in guayule tissue requires destructive methods that extract the tissue with either an aqueous solvent (i.e., for latex rubber) or organic solvents (i.e., for resin and rubber). Quantification of rubber in latex has been successfully employed (Cornish et al., 1999, 2000, 2005) but accurate quantification is dependent upon stringent post-harvest storage conditions and analysis within two weeks to ensure that the rubber does not coagulate, but remains as an emulsion in the latex. In contrast, organic solvent extraction can be used with dried tissue samples, and provides information on both rubber and resin amount. Extraction of dried and ground tissue with a soxhlet apparatus or a homogenizer has been used in past studies with guayule (Spence and Caldwell, 1933; Black et al., 1983; Hammond and Polhamus, 1965; Jasso de Rodriguez and Kuruvadi, 1991; Coffelt et al., 2009a,b). Once extracted, resin and rubber are usually determined gravimetrically, although photometric methods based on sample turbidity have been developed (Traub, 1946; Hammond and Polhamus, 1965; Sundar and Reddy, 2001).

In recent years, instruments for accelerated solvent extraction (ASE), also called pressurized liquid extraction, like the Dionex ASE 200 (Dionex Corp., Bannockburn, IL), have become available, providing a means for automating the sequential extraction of guayule tissue (Thurbide and Hughes, 2000). An important advantage of ASE systems over traditional extraction methods is reduced handling of organic solvents. In addition, ASE instruments increase the speed and efficiency of extraction by using high pressure, alone or in combination with high temperature, for solvent extraction (Thurbide and Hughes, 2000).

In the present study, we describe the development of protocols for ASE of guayule tissue and for instrumental analysis of resin and rubber content. These methods can be used to increase the throughput of samples in screening programs designed to evaluate guayule germplasm.

2. Materials and methods

2.1. Plant material

Two-year old guayule (Parthenium argentatum Gray, line AZ-2) plants were harvested in April, 2008 from a commercial field near Eloy, AZ. Plants were defoliated, chipped and screened through a 3.8 cm mesh. The chipped material was dried by either lyophilization for 24 h after freezing overnight at −80 °C, oven drying at 60 °C for 2 days, or air-drying for 4 days at 23 °C. The dried material was stored at room temperature in a desiccator. After four weeks of storage, the dried material was pulverized in a ball mill (SPEX Centriprep, Metuchen, NJ) by shaking for 3 min in a 30 ml hardened steel cup containing two 0.6 cm stainless steel ball bearings. The ground material, equivalent to <1.7 mm, was stored at room temperature for one to two weeks in a sealed vial at room temperature before extraction.

For some experiments, guayule plants were grown in growth chambers under controlled conditions of temperature and photoperiod to induce or suppress vegetative growth and subsequent rubber synthesis (Bonner, 1943; Sundar and Reddy, 2001). To stimulate vegetative growth and flowering and suppress rubber synthesis, plants were grown under a 14 h, 30 °C light/10 h, 7 °C dark photoperiod. To suppress vegetative growth and flowering and stimulate rubber synthesis, plants were grown under a 9 h, 16 °C light/15 h, 7 °C dark photoperiod. Plant material was harvested and representative samples of the stem material were either freeze-dried or immediately extracted for latex rubber (Cornish et al., 1999). Freeze-dried material from plants with either high (9.6%) or low (<1%) latex rubber content was pulverized and the ground material was stored for six months at room temperature.

2.2. Accelerated solvent extraction (ASE) of guayule tissue

Guayule stem tissue was extracted sequentially with organic solvent using a Dionex ASE 200 (Bannockburn, IL). One gram of guayule tissue was mixed with Ottawa sand and loaded into an 11 ml extraction cell. Samples were generally extracted with acetone and then cyclohexane using two static extractions with each solvent and a volume equivalent to 150% of the residual cell volume of about 7 ml. However, for some experiments, acetonitrile was used in place of acetone for the first extraction. Extractions were carried out at either 24 or 100 °C and for the durations indicated in the text using a pressure of 1500 psi. Twenty eight to thirty milliliters of extract volume was recovered from the two static extractions plus flush and analyzed for resin (acetone- or acetonitrile-soluble) or rubber (cyclohexane-soluble) as described below. For some experiments, additional extraction cycles using the same static conditions of temperature, pressure and duration were performed with each solvent.

2.3. Gravimetric determination of resin and rubber content

Four ml aliquots of the acetone or cyclohexane extract were transferred in triplicate to pre-weighed borosilicate glass vials (17 mm × 58 mm). The liquid was taken to dryness under vacuum at 65 °C in a Savant speed-vac centrifugal concentrator (Thermo Scientific, Waltham, MA) and the vials were re-weighed to determine the dry weight of the extract.

2.4. Determination of resin content by UV absorption

To determine the maximum wavelength and extinction coefficient of un fractionated guayule resin, resin samples representing the acetone-soluble extracts from different plant samples were co-evaporated with and dissolved in HPLC-grade acetonitrile. The UV spectrum of the material was determined in acetonitrile and an extinction coefficient at 272 nm was developed for both the UV–vis spectrophotometer (Cary 100, Varian Instruments, Inc., Palo Alto, CA) and a Synergy HT microtiter plate reader (Biotek Instruments, Inc., Winooski, VT).

For routine determination of resin by absorbance at 272 nm, 1 ml of the acetone extract from ASE was transferred to a 1.5 ml centrifuge tube and centrifuged at 24 °C for 5 min at 10,000 × g. Aliquots of the supernatant (0.1 ml) were transferred to second tubes containing 0.9 ml HPLC-grade acetonitrile. The acetone and acetonitrile were co-evaporated to near dryness (0.1 ml) under vacuum for 25 min using a speed-vac at 65 °C. Co-evaporation was repeated following the addition of a second 0.9 ml of acetonitrile. Following evaporation, acetonitrile was added to the residual liquid to a final volume of 1 ml and the solution was mixed using a vortex mixer. The absorbance of the solution at 272 nm was determined by transferring 0.2 ml aliquots to a 96-well UV-transparent microtiter plate and immediately measuring the absorbance with the microtiter plate reader. The data presented are the means ± SEM of at least two separate ASE extractions for each sample and treatment. Duplicate aliquots of the acetone extract were co-evaporated for each ASE sample and the UV absorption of each aliquot was measured in triplicate.

2.5. Analysis of rubber content by evaporative light scattering (ELS)

Rubber content in the cyclohexane extracts was determined using an evaporative light scattering detector (ELSD). The outlet of an HPLC pump was plumbed directly into a Varex MKIII ELSD (Alltech Associates, Inc., Deerfield, IL). A manual injection valve with a 10 μl loop was inserted in-line to introduce the sample. For
measurements, cyclohexane was pumped at 0.5 ml min$^{-1}$ through the ELSD, using a drift tube temperature of 98 °C and a flow rate of 1.61 pm for the nitrogen gas stream. Triplicate 10 μl aliquots from each extraction were injected manually into the flow stream and solute particles were detected by the ELS detector. The analog signal from the ELSD was converted to digital using a Chromtech (Alltech Associates, Inc.) A/D converter and associated software. The data presented are the means ± SEM of at least two separate ASE extractions for each sample and treatment.

3. Results

3.1. Accelerated solvent extraction

Stem tissue from guayule plants with low and high latex rubber content, determined by aqueous extraction (Cornish et al., 1999), was used to refine the conditions for ASE. Multiple extraction cycles of dried stem tissue from plants with a high latex rubber content using the same solvent established that >90% of the acetone-soluble material and >88% of the cyclohexane-soluble material were extracted at room temperature with a single extraction cycle, i.e., two 20 min static extractions at 150% cell volume (Fig. 1). Increasing the temperature used for the cyclohexane extraction from 24 to 100 °C increased the efficiency of the first extraction cycle. Increasing the extraction temperature from 24 to 100 °C also increased the total amount of rubber (cyclohexane-soluble material) recovered after three extraction cycles by about 10%. When 140 °C was used for cyclohexane extraction, a precipitant was visible in the extracts and the amount of rubber in the soluble portion of the extract was about 10% lower than after extraction at 100 °C (data not shown).

Gravimetric determination of the cyclohexane-soluble material extracted from tissue with high and low latex rubber content showed large differences between the amounts of rubber extracted from the two types of tissue (Table 1). Increasing the temperature of the acetone extraction from 24 to 100 °C greatly increased the amount of material extracted from tissue with a high latex rubber content. However, a portion of this material precipitated from solution once the acetone had cooled to room temperature (see below). The white precipitate was partially, but not completely, soluble in water and 100% cyclohexane at 25 °C, indicating that it was not resin. The amount of rubber extracted by the ASE system decreased markedly when high latex tissue was first extracted with acetone at 100 compared with 24 °C. Taken together, the data indicate that extraction of guayule tissue with hot acetone either dissolves and/or degrades the rubber or makes the rubber unrecoverable during the subsequent extraction with cyclohexane.

### Table 1

<table>
<thead>
<tr>
<th>Latex content</th>
<th>Temperature (°C)</th>
<th>Resin (%)</th>
<th>Rubber (%)</th>
</tr>
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<tbody>
<tr>
<td>Low</td>
<td>24</td>
<td>6.2 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>High</td>
<td>24</td>
<td>9.1 ± 0.6</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>High</td>
<td>100</td>
<td>16.2 ± 0.8</td>
<td>1.5 ± 0.7</td>
</tr>
</tbody>
</table>

The resin from guayule is a complex mixture of terpenoid compounds that have a strong absorbance in the UV (Schloman et al., 1983; Sidhu et al., 1995). This property can be used to estimate the amount of resin recovered from guayule tissue after ASE extraction with acetone. However, because of the high UV cut-off of acetone, it was necessary to evaporate the acetone and to determine the spectra in acetonitrile, a solvent with a polarity index of 5.8, similar to acetone at 5.1, but with a much lower UV cut-off. Fig. 2 shows the absorption spectra in acetonitrile of five different samples of resin from the guayule bagasse remaining after aqueous latex extraction. The samples were partially purified by repeated extraction and concentration in acetone, followed by co-evaporation with and dilution in acetonitrile. Also, shown is the UV spectrum of the acetone-soluble material from an ASE of two different guayule stem samples also in acetonitrile. For all samples, acetone-soluble material from guayule had a pronounced peak of absorbance at 250–300 nm, with maximum absorbance near 272 nm.

A relationship between the $A_{272}$ in acetonitrile and the amount of acetone-soluble material extracted by ASE was developed in order to determine extinction coefficients for the crude resin fraction from ASE of guayule tissue (Fig. 3). The relationship between the $A_{272}$ and the amount of acetone-soluble material determined gravimetrically was linear with both a spectrophotometer and a microtitrate plate reader, consistent with Beer’s–Lambert Law. The extinction coefficient ($ε$) for the crude resin fraction from the ASE was 8.81 cm$^{-1}$ (mg ml$^{-1}$)$^{-1}$. With the volumes and geometry of the microtitrate plate reader, the absorbance readings with this instrument were exactly half the values determined with the UV–vis spectrophotometer. Consequently, the $ε$ value of the crude resin fraction in acetonitrile was 4.45 (mg ml$^{-1}$)$^{-1}$ or about 50% of the...
value determined with the spectrophotometer. The $\varepsilon$ for resin from the partially purified preparations of resin was 5.13, only slightly higher than the value for the crude acetone extract from the ASE.

3.3. Rubber analysis by ELS

Rubber can be extracted from dried guayule tissue using non-polar solvents like hexane, benzene, petroleum ether or cyclohexane. Once in solution, the dissolved rubber can be detected using ELS, which detects the scattering of light caused by particles released into a heated drift tube by nebulization and evaporation of the mobile phase. Fig. 4 shows the relationship between the area of the ELS peak and rubber concentration for a sample of guayule rubber dissolved in cyclohexane. The data show that the light scattering signal was linear over rubber concentrations from 0 to 2 mg/ml and departed slightly from linearity between 2 and 5 mg/ml.

The cyclohexane extract from plants with high and low latex rubber content (Table 1) was analyzed by ELS to determine the effectiveness of ELS in detecting differences in the amount of cyclohexane-soluble material with plant samples extracted by ASE. Fig. 5 shows the raw data from this analysis, i.e., the detector response for a series of injections. Samples with high (>4% dry weight) and low (<1% dry weight) latex were easily differentiated. The ELS response of high latex samples that were extracted with acetone at 100 °C resembled the response of samples with low latex, consistent with the results based on gravimetric determination (Table 1). When ELSD and gravimetric determinations of rubber content were compared directly, a linear relationship was established with a slope of 1.15 (Fig. 6).

3.4. Verification of the methods for resin and rubber determination

Guayule shrubs with a latex rubber content of 5.3% were analyzed for resin and rubber using UV absorption and ELS and the results compared with those determined gravimetrically (Table 2). Whereas the plant material used in the experiments described above was stored as a powder for six months at room temperature prior to analysis, the material used here was analyzed within two weeks of pulverizing. The use of fresher tissue provided a more...
and rubber contents were determined gravimetrically and by UV absorption at 272 nm (resin) and ELS (rubber).

Table 2

<table>
<thead>
<tr>
<th>Acetone temperature</th>
<th>Resin (%)</th>
<th>Rubber (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Gravimetric</td>
<td>UV</td>
</tr>
<tr>
<td>24 °C</td>
<td>8.3 ± 0.5</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>100 °C</td>
<td>17.0 ± 0.6</td>
<td>4.8 ± 0.09</td>
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*a After centrifugation to remove insoluble material.

Table 3

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<thead>
<tr>
<th>Drying conditions</th>
<th>Resin (%)</th>
<th>Rubber (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gravimetric</td>
<td>UV</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>8.2 ± 0.9</td>
<td>8.4 ± 1.3</td>
</tr>
<tr>
<td>Air-dried</td>
<td>8.8 ± 0.5</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>Oven-dried</td>
<td>7.4 ± 0.1</td>
<td>6.8 ± 0.3</td>
</tr>
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</table>

Table 4

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<tr>
<th>Primary solvent</th>
<th>Resin (%)</th>
<th>Rubber (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>6.6 ± 0.2</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>6.2 ± 0.1</td>
<td>8.4 ± 0.3</td>
</tr>
</tbody>
</table>

reliable estimate of rubber recovery from samples of known latex rubber content.

Under standard extraction conditions, i.e., acetone at 24 °C and cyclohexane at 100 °C, the amounts of resin and rubber determined using UV absorption for resin and ELS for rubber were similar to the amounts determined gravimetrically. The amount of rubber recovered from the dried tissue was 7.7%, which was higher than the latex rubber content measured in a separate aliquot of this tissue, i.e., 5.3%, indicating that some of the latex rubber had coagulated to solid rubber in the plant before or after harvest. Gravimetric determination showed that increasing the temperature of the acetone extraction to 100 °C doubled the amount of material extracted with acetone, but decreased the amount of material recovered after extraction with cyclohexane. As discussed above, a significant portion of the material extracted with hot acetone was no longer soluble when the acetone cooled to room temperature. When insoluble material was removed by centrifugation, the amount of acetone-soluble material was only slightly greater when hot acetone was used for extraction. Pre-extraction of dried guayule tissue with water at 100 °C, reduced the amount of resin and rubber recovered from the tissue by about 20 to 25% (data not shown).

3.5. Effect of drying method, extraction time and acetonitrile extraction on resin and rubber determination

Using the high latex rubber content shrub described in the previous section and the standard method of extraction, the effect of various drying conditions on the amounts of resin and rubber were determined both gravimetrically and by UV absorption and detection by ELS (Table 3). The results showed that the three methods for drying guayule stem, prior to pulverizing, gave comparable results. Again, the results for resin and rubber content determined by UV absorption and ELS, respectively, were similar to those determined gravimetrically. The only difference among the drying methods that was consistent with both methods of analysis was resin content after oven-drying. The analysis showed that oven-drying at 60 °C caused a small, but significant decrease in the amount of resin extracted compared with the other two methods of drying.

To determine if shorter extraction times could be used to increase throughput, the same high latex-containing tissue used above was extracted for different lengths of time and the amount of resin and rubber determined by UV absorption and ELS, respectively (Fig. 7). The results showed that two 5 min static extractions with acetone at 24 °C were sufficient for complete extraction of resin. In contrast, increasing the extraction time with cyclohexane at 100 °C from 5 to 20 min increased the amount of rubber recovered by almost 30%. This result was consistent with the results from multiple extractions with the same solvent (Fig. 1) which showed that a second round of two 20 min static extractions with cyclohexane recovered nearly 12% more rubber.

The main drawback of the UV absorption method for high throughput analysis of resin is the requirement for co-evaporation with acetonitrile to remove the acetone. To obviate this problem, the possibility of replacing acetone with acetonitrile for resin extraction was investigated. When guayule tissue was extracted with acetonitrile via ASE at 24 °C, the absorption spectrum of the extract was nearly identical to the spectrum of the acetone extract after co-evaporation with acetonitrile (Fig. 8). This result indicates that acetonitrile extracts the same suite of UV absorbing compounds from guayule tissue as acetone. Determination of resin content by UV absorption showed that the amount of resin extracted was similar when acetonitrile was substituted for acetone in the primary ASE extraction (Table 4).
After extraction with acetonitrile, the cyclohexane extract sometimes contained a small amount of insoluble material whereas this fraction was usually clear after prior extraction with acetone. The cyclohexane-insoluble material was soluble in acetonitrile, suggesting that it was a solute that carried over into the cyclohexane fraction with the small amount of residual acetonitrile that was visible in the collection vial. That this material was not in the cyclohexane fraction after acetone extraction is probably related to the lower amount of residual solvent carried over into the cyclohexane fraction after flushing with nitrogen. Because of the difference in boiling points, the volume of solvent carried over from the primary extraction was much less with acetone than with acetonitrile. After removing this cyclohexane-insoluble material by centrifugation, the rubber content was similar in acetone- and acetonitrile-extracted tissue.

4. Discussion

Widespread commercialization of guayule as an alternative source of natural rubber and resin for the bioproducts industry will require the development of improved germplasm with increased rubber and resin content (Estilai and Ray, 1991; Ray et al., 2005). Germplasm enhancement, whether by traditional breeding and selection or through molecular approaches to trait improvement, requires rapid and reliable methods for determining rubber and resin content in large numbers of plant samples. Unfortunately, existing methods for processing and extracting the plant tissue and for analysis of the extracted material are extremely time-consuming, thus limiting the number of samples that can be screened. In addition, the extraction methods require considerable handling of organic solvents, subjecting workers to organic solvents that are potentially dangerous to their health.

4.1. Solvent extraction of guayule tissue

In most early studies (Spence and Caldwell, 1933) and even more recently (Jasso de Rodriguez and Kuruwadi, 1991), resin and rubber were sequentially extracted from dried guayule tissue with polar (i.e., acetone or ethanol) and non-polar solvents (i.e., benzene, petroleum ether, hexane or cyclohexane), respectively, using a soxhlet apparatus. In some of the early studies, the plant material was first extracted with hot water to remove compounds that purportedly interfere with the subsequent extraction of resin and rubber (Spence and Caldwell, 1933; Hammond and Polhamus, 1965). The soxhlet extraction method is extremely time-consuming, requiring up to 12 h of extraction in each solvent and considerable handling of organic solvents. In addition, the soxhlet method does not easily lend itself to automation. To increase the speed and efficiency of the extraction process, several investigators have replaced the soxhlet extraction with homogenization in blenders or polytrons either in organic solvents or in ethyl alcohol (Hammond and Polhamus, 1965; Black et al., 1983; Coffelt et al., 2009a,b). However, exposure to solvents can be even greater with homogenization methods, especially if the residue is re-extracted with fresh solvent. In addition, homogenization methods, like the soxhlet method, are difficult to automate. Homogenization of fresh tissue in a mixture of water and ethyl alcohol to recover solid rubber is less toxic but requires considerable manipulation, including determination of moisture content and deresination of the solid rubber in acetone (Hammond and Polhamus, 1965).

Measurement of rubber in latex after extraction in aqueous solution at high pH avoids the problems with organic solvents, but the extraction requires fresh material and is still time-consuming and difficult to automate (Cornish et al., 1999). In general, the content of rubber in latex should be the same or lower than the total rubber content. For example, when considerable coagulation occurs, e.g., in tissue with low water content, the latex rubber content greatly underestimates the total amount of rubber in the tissue. In addition, extraction of tissue for latex rubber does not provide a measure of the amount of resin in the tissue since resin determination is usually made on a separate dried sample.

In contrast to the homogenization or soxhlet methods, methods based on solvent extraction systems like ASE provide a means for automating the extraction of plant tissue with multiple solvents (Thuribe and Hughes, 2000). The use of high pressure alone or in combination with high temperature increases the efficiency of solvent extraction, thus shortening extraction time. In addition, worker safety is improved since handling of organic solvents during extraction is essentially eliminated because extraction with each solvent is fully automated. The results from multiple extractions showed that most (88–95%) of the acetone- and cyclohexane-soluble material was eluted with just a single extraction regime for each solvent consisting of two static extractions at 150% cell volume. For resin, the static acetone extractions could be as brief as 5 min, while longer cyclohexane extraction times (i.e., 20 min) were needed for rubber. Pre-extraction of dried guayule tissue with water at 100 °C did not aid in recovery of either resin or rubber. Instead, recovery of both were reduced when tissue was initially extracted with water at 100 °C, suggesting that the combination of the high temperature and pressure generated during aqueous extraction with the ASE caused degradation of both the resin and rubber.

Temperature can be easily controlled with ASE, facilitating the use of high temperatures and well as high pressures to increase the efficiency of extraction. Extracting guayule tissue with hot (100 °C) acetone caused considerable loss of rubber in samples that were known to contain high rubber based on latex rubber determinations made with fresh tissue. This effect was noted in early studies and was attributed to breakdown of rubber into lower molecular weight components (Meeks et al., 1950; Meeks and Feustel, 1951). Thus, key to quantitative extraction of rubber was the use of low temperature for extraction of the resin with acetone. Using the two-solvent ASE method, 13 samples can be extracted into the 26-vial carousel. Since the entire extraction process is fully automated, solvent handling is minimal during extraction.

4.2. Analysis of resin and rubber

Gravimetric methods have generally been used for determining resin and rubber content after extraction in suitable solvents (Black et al., 1983). These methods, while not specific for either compound, are generally acceptable because of the high content of these...
compounds in guayule tissue. Gravimetric determination requires evaporation of the solvent. Although equipment has been developed for this purpose, this step is still time-consuming when a high boiling point solvent like cyclohexane is used to extract the rubber. Chromatographic separation provides a more accurate measure of resin and rubber content (Proksch et al., 1981; Kang et al., 2000), but these methods can be even more time-consuming, limiting the rate of throughput.

In the present study, resin content was determined from the absorbance of the resinous solution at 272 nm and an extinction coefficient was determined empirically from the absorbance of known amounts of resin extracted from guayule plants. Although the UV absorption method requires repeated co-evaporation of the sample with acetonitrile when acetone is used as the solvent, a large number of samples can be co-evaporated simultaneously using a vacuum concentrator. Also, measurement of UV absorbance does not require precipitation of the resin from solution, making it more amenable for microtiter plate analysis than photometric analysis of sample turbidity (Traub, 1946). Finally, the UV method is more specific for resin than gravimetric methods since the predominant resin components in guayule, guayulin A and B and the argentatins (Proksch et al., 1981; Schloman et al., 1983; Sidhu et al., 1995) are UV absorbing whereas non-resinous, but acetone-soluble compounds like fatty acid triglycerides and low molecular weight rubber are not.

Because of the low volatility of rubber compared with solvent, ELS has been used to detect rubber during separation by gel permeation HPLC (Kang et al., 2000). In the present study, a columnless system was used to provide rapid throughput for rubber analysis. Cyclohexane extracts can be analyzed immediately after extraction with ASE and the response of ELSD signal to rubber concentration is linear from 0 to 2 mg ml$^{-1}$ of rubber. Extraction of 1 g of guayule tissue in an 11 ml cell with two 20 min static extractions of 150% cell volume produced about 30 ml of rubber extract that could be taken directly from the ASE vial and manually injected into the ELSD with no concentration. For samples with rubber content greater than about 7%, a simple 1:1 (v:v) dilution with cyclohexane kept readings within the linear detection range. Sampling rates can be as fast as 1 per 2.5 min, allowing 24 samples to be analyzed per hour.

4.3. Notes and recommendations about the methods

Processing of the guayule tissue to determine resin and rubber content involved drying freshly chipped shrub, pulverizing the dried material in a ball mill and extracting the dried material in an ASE system. Good yields of rubber were obtained from dried tissue (i.e., stem pieces) stored for four weeks at room temperature prior to pulverizing, whereas significant losses occurred with material that was pulverized and then stored for six months at room temperature (data not shown). Based on these findings, we recommend that dried tissue be extracted soon after pulverizing.

A single extraction sequence of two static extractions at 150% cell volume extracted most of the resin and rubber. Thus, to increase throughput, a single extraction sequence using a single collection vial should be used for each solvent. The results of the present study showed that two 5 min static extractions with acetone at 24°C were necessary for good resin and rubber recovery. After extraction, the resin and rubber can be separated by columnless ASE using a 20% acetone in cyclohexane mobile phase. The resin and rubber fractions can be analyzed directly with UV and ELS detectors, respectively. The flowchart in Fig. 9 illustrates the methods for extraction and analysis of resin and rubber from guayule, optimized for the maximum rate of throughput.
sufficient for extraction of resin, but longer extraction times, i.e., 20 min, are required for extraction of the rubber with cyclohexane at 100 °C. Thus, ASE of guayule was still a relatively time-consuming process, requiring nearly 1 h for extraction of each sample with the two solvents. However, by automating the extraction process, the rate of throughput is increased since the time normally devoted to extraction can be used to process plant material or conduct analysis of the extracts.

Resin analysis by absorption at 272 nm and rubber analysis by ELSD gave comparable results to gravimetric methods. Thus, gravimetric determination of resin and rubber content can be substituted for these more sophisticated methods in laboratories without one or more of the specialized instrumentation described here, i.e., speed-vac, UV spectrophotometer or microtiter plate reader and ELSD. Instrumental analysis of resin and rubber content greatly reduced the time required for analysis by eliminating the need to pre-weigh vials and caps before extraction and by not requiring complete sample dry-down.

By adapting resin analysis to a microtiter plate format, 96 samples can be analyzed for resin concentration within a few minutes. The only drawback is the requirement for co-evaporation to eliminate acetone. By substituting acetone for acetonitrile it was possible to eliminate the co-evaporation step and measure resin directly in the extract after a simple 1:10 dilution of the ASE vial contents. Similarly, rubber analysis using ELSD required no additional preparation, except when acetone was used for the first solvent, in which case centrifugation or settling of the insoluble material in the cyclohexane extract is required. By introducing the cyclohexane extract directly into the detector withoutGPC separation, 24 samples can be analyzed per h. The method could be automated by addition of an autosampler, however this would require an additional step; i.e., transferring sample from the ASE vials to autosampler vials. A robotic liquid handling system would allow the process to be fully automated, thus facilitating screening of large numbers of individual plants for rubber content. A flow chart of the methods described here for the extraction and analysis of resin and rubber in guayule tissue, optimized for the maximum rate of throughput, is shown in Fig. 9.

5. Conclusions

A protocol was developed for resin and rubber extraction using the ASE that involved (1) grinding the dried sample in a ball mill; (2) extracting the resin with acetone or acetonitrile; and (3) extracting the rubber with cyclohexane. Rapid and highly accurate methods were developed for quantification of both the resin and the rubber components that elute with each solvent. For resin analysis, ultraviolet absorbance was used to determine the resin content of the tissue in acetone or acetonitrile extracts. For rubber analysis, evaporative light scattering was used to determine the rubber content of the tissue after extraction with cyclohexane. Extraction of guayule tissue known to contain a high latex rubber content verified that the amounts of resin and rubber determined by these methods were similar to the amounts determined gravimetrically. Since these methods increase the speed of resin and rubber analysis, they could be used in combination with ASE to increase the throughput of guayule evaluation in germplasm enhancement programs, agronomic studies, and other experiments involving the quantification of resin and rubber in large numbers of samples.

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