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Sweetgum (*Liquidambar styraciflua* L.): Extraction of Shikimic Acid Coupled to Dilute Acid Pretreatment

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Abstract *Liquidambar styraciflua* L., also known as sweetgum, is an understory hardwood species that has widespread distribution in the southeast USA, especially in pine plantations. In addition to being a possible biorefinery feedstock, sweetgum contains shikimic acid, which is a precursor for the drug Tamiflu®. Sweetgum bark was extracted with 65 °C water and yielded 1.7 mg/g of shikimic acid, while sweetgum de-barked wood yielded 0.2 mg/g of shikimic acid. Because shikimic acid can be extracted with water, the coupling of the phytochemical hot water extraction with dilute acid pretreatment was examined. The addition of a 65 °C shikimic acid extraction step coupled to pretreatment with 0.98% H_2SO_4 at 130 °C for 50 min resulted in 21% and 17% increases in xylose percent recovery from bark and de-barked wood, respectively. These results indicate that, in addition to recovering a high value product, the 65 °C wash step also increases xylose recovery.

Keywords Extraction · Pretreatment · Shikimic acid · Water · Dilute acid · Sweetgum

Introduction

The economic competitiveness of cellulosic ethanol production is highly dependent on feedstock cost, which ranges from 35–50% of the total ethanol production costs [1]. In addition to a $30–36 per dry ton payment to the producer, harvesting, storage and

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transportation costs are estimated at $40–48 per dry ton of biomass, depending if the feedstock is harvested as a bale, a loaf or ensiled [2].

The extraction of co-products from feedstock, prior to biofuel conversion, could present options for increased revenue. For co-product extraction to be economically feasible, the extraction should be on site, or in close proximity, to the biorefinery operations that carry out the biochemical/thermochemical conversion of the feedstock to biofuel. In addition, the co-product extraction process must be carried out with a solvent that is compatible with existing biorefinery technology. Interestingly, water can be such a solvent.

As a hardwood feedstock, sweetgum (*Liquidambar styraciflua* L.) is an understory species that has widespread distribution throughout the southeast USA. The species is of interest due to its co-existence in forest management areas that are logged for softwoods, such as pine. Although sweetgum is not commercially used in logging, it has potential as a sustainable biomass that can be harvested prior to the logging of the forests' softwood and used in biochemical or thermochemical conversion to biofuels [3].

Although the major use for sweetgum is in its potential for conversion to biofuels, scientific and anecdotal evidence indicate that *L. styraciflua* could also serve as a source of high value phytochemical co-products. Sweetgum sap derivatives have been used historically for medicinal purposes [4]. Water extractions (65 °C) of sweetgum contain shikimic acid [5], which is a precursor for the manufacturing of the important drug, oseltamivir phosphate or, as it is widely known, Tamiflu®. The most noteworthy plant source for shikimic acid extraction is the seed from star anise [6–9]. Because of this rather limited source of shikimic acid, the cost is more than $50/g [10]. Other avenues of shikimic acid production, using certain bacterial strains of *Escherichia coli*, are currently being utilized for mass production of Tamiflu® [11, 12]. However, even with these increased efforts, Tamiflu® remains in short supply. Therefore, we must utilize not only the predominant alternative sources, such as star anise, but to also search for other potential sources, including sweetgum.

Depending on the variety, researchers have reported shikimic acid yields from sweetgum seed in the range of 32 mg/g [13]. Although shikimic acid yields from sweetgum seed are comparable to star anise seed, their harvesting would be somewhat difficult because of low biomass yields combined with energy intensive seed extraction operations. On the other hand, if sweetgum is to be used as a biorefinery feedstock, copious amounts of wood will pass through the doors of the biorefinery on a daily basis. In this context, a lower yield shikimic acid extraction step, could be economically integrated into the biorefinery technology because the entire plant biomass is extracted and not just the seed.

This paper will report on the identification and quantification of shikimic acid from *L. styraciflua* bark and de-barked wood using water as the extracting solvent. The effect of interfacing this water extraction step on the release of xylose in a dilute acid 130 °C pretreatment will also be discussed.

Materials and Methods

Plant Material

Chipped wood and bark from mature and juvenile sweetgum trees were obtained from Dr. Matthew Pelkki and Dr. Philip Tappe, School of Forest Resources, University of Arkansas, Monticello, AR. Star anise was purchased from a local retail store in Fayetteville, AR. Star anise seeds were separated from the seed pods and finely ground using a household coffee grinder. Sweetgum bark and wood chips were stored at 4 °C before being ground to a

particle size of 4 mm [14] using a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ) as described by Torget et al. [15].

Reference Compounds

Shikimic acid was purchased from Sigma-Aldrich Inc (St. Louis, MO). Shikimic acid standards were prepared at concentrations of 0.5, 0.25, 0.125, 0.0625 mg/mL in methanol. D-(−) arabinose, D-(+) cellobiose, D-(−) fructose, D-(+) galactose, D-(+) glucose, D-(+) mannose and D-(+) xylose were purchased either from Fluka Analytical (Buchs), Sigma-Aldrich or Alfa Aesar (Heysham, Lancashire). Sugar standards were prepared in sterile deionized water at 1 mg/mL.

Pretreatment

Soxhlet Extraction of Shikimic Acid

Two gram ground samples of either star anise seeds, sweetgum bark or sweetgum wood were added to a Whatman Thimble (Maidstone, UK), placed in a Soxhlet apparatus and extracted with 200 mL of methanol for 7 h. The final volume of solvent was recorded. Fifty milliliters from the final volume was reduced using a rotary evaporator (Yamato, BM 200 Yamoto Scientific, Japan). The residue volume was recorded. Aliquots of the residue were analyzed by HPLC as described below.

Water Extraction of Shikimic Acid

Two grams of ground or milled material was extracted with 50 mL of water at 65 or 85 °C by shaking the mixture for 12 h in 250 mL amber bottles in a Precision shaking water bath (Winchester, VA) at 100 RPM as described by Vaughn et al. [16]. The extractions were filtered through #1 Whatman filter paper with vacuum in a Buchner funnel. Twenty-five milliliters of each sample were freeze-dried in a Freezone 18 lyophilizer (Labconco, Kansas City, MO) and re-suspended in 5 mL of methanol. Aliquots of these suspensions were analyzed by HPLC as described.

HPLC Analytical Method for Shikimic Acid Extraction

Sample extractions for shikimic acid determination were analyzed with a Waters 2690 Alliance Separations Module equipped with a 4.6×250 mm Symmetry column, operated isocratically with 0.02 M H_2SO_4 as the eluent [5]. The flow rate was 0.5 mL/min. Detection of shikimic acid was obtained with a Waters 996 Photodiode Array Detector (Milford, MA) set at 225 nm. The retention time of the major peak, shikimic acid, was on average 6.4 min.

Dilute Acid Pretreatment

Mature wood and bark were pretreated in a 2 L Parr reactor (Moline, IL). A solids loading of 10% w/w was used, resulting in 20 g of bark or wood in 200 mL of 0.98% H_2SO_4. The pretreatment was conducted at 130 °C and 60 psig (pressurized using nitrogen) for 50 min. The agitation rate was set at 144 RPM. Pretreatment was initiated as the time when the reactor reached 130 °C (approximately 10 min); this procedure was adapted from Lloyd and

Wyman [17] and Esteghlalian et al. [18]. Once the extraction cycle was completed, the pressure was allowed to return to atmospheric, and the extractor contents were filtered through Miracloth (Calbiochem, San Diego, CA). The pH of the exudate was adjusted to a pH of 5.2 by adding $CaCO_3$ [19]. The carbohydrate content was analyzed as described below.

Water Wash Combined with Dilute Acid Pretreatment

The wood and bark were extracted with 65 °C water prior to pretreatment, where 20 g of material were placed in 200 mL of water in the Parr reactor. The material was extracted for 2 h at atmospheric pressure. After the 2 h, 65 °C extraction cycle, the material was filtered through Miracloth and the collected solids were placed back into the Parr reactor for a 130 °C (60 psig), 50 min extraction cycle. Upon completion of the extraction cycle, the contents were filtered through Miracloth, and the pH of the exudate was adjusted to a pH of 5.2 [19]. Carbohydrates and shikimic acid were analyzed as described below.

HPLC Analytical Method for Carbohydrate Extraction

Samples were analyzed basically as described by NREL LAP/TP-510-42623 [19], with an increased flow rate. The instrument used was a Waters 2690 Alliance Separation Module (Milford, MA) equipped with a Shodex pre-column (SP-G, 8 µm, 6×50 mm, Waters) and a Shodex column (SP0810, 8 µm, 8×300 mm, Waters). The column was heated to 85 °C, and water, flowing at 1 mL/min, was the eluent. The carbohydrates were detected with a Waters 2414 Refractive Index Detector (Milford, MA).

Statistical Analysis

Percent extraction yields of carbohydrates from bark and wood was analyzed using analysis of variance (ANOVA) with the Fit Least Squares Model and LSMeans Differences Student's *t* test procedure of JMP (SAS Institute, Cary, NC). Significance was determined at $P<0.05$. Pretreatment experiments at 130 °C were conducted in duplicate. Extractions in the water bath or Soxhlet were conducted in triplicate. Each HPLC analysis, either for shikimic acid or for carbohydrates, was at least conducted in duplicate.

Results and Discussion

Shikimic Acid in *L. styraciflua*

In light of the fact that our laboratory did not have expertise to detect and quantify shikimic acid, we proceeded to extract this phytochemical in a material that was reported to contain this primary metabolite, star anise [8]. The extractions were performed in a Soxhlet apparatus with methanol. Figure 1a, b present the HPLC trace of Soxhlet-extracted star anise (left panel) and the extract enriched with the pure reference compound (right panel). Both panels show that the compounds eluted at identical retention times, confirming the presence of shikimic acid in the star anise extract. Confirmation was also based on comparison of their UV spectra.

Having established the methodology to detect shikimic acid in star anise, we proceeded to extract *L. styraciflua* bark and de-barked wood. Figure 1c–f present the HPLC traces of the *L. styraciflua* bark and de-barked wood extracts and the extracts supplemented with

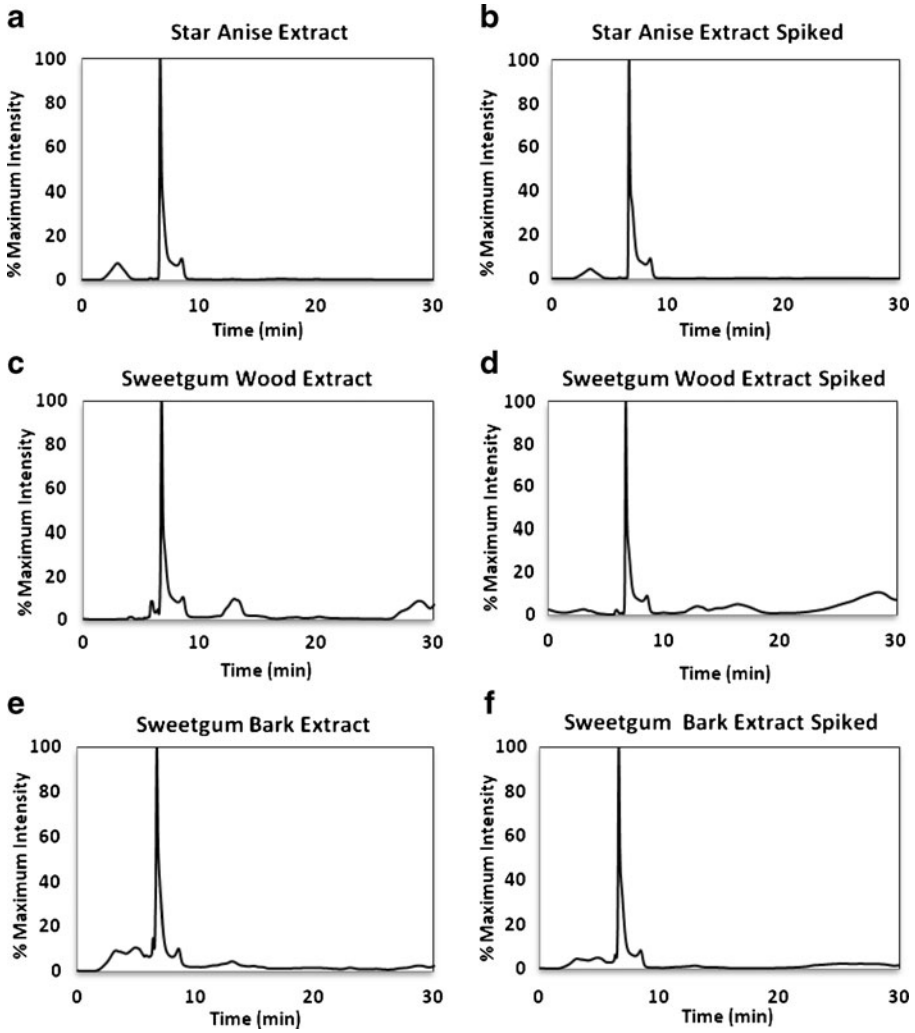

Fig. 1 Chromatograms from HPLC analysis of Soxhlet extractions of star anise seeds, sweetgum wood and bark. Retention time of major peak=6.4 min, detected at 225 nm. **a** Star anise seed extract, **b** star anise seed extract spiked 1:1 with 0.5 mg/mL shikimic acid standard. **c** Sweetgum wood extract, **d** sweetgum wood extract spiked 2:1 with 0.125 mg/mL shikimic acid standard, **e** Sweetgum bark extract, **f** sweetgum bark extract spiked 1:1 with 0.125 mg/mL shikimic acid standard

pure reference compounds, respectively. Similar to Fig. 1b, no additional peaks were observed in Fig. 1d, f, confirming the presence of shikimic acid in *L. styraciflua* bark and de-barked wood.

Table 1 displays the peak areas for the shikimic acid test samples (star anise, sweetgum wood and sweetgum bark) extracted by Soxhlet (methanol), and in 65 °C and 85 °C water. Theoretical and actual peak areas are shown. Co-chromatography verified the presence of shikimic acid content in test samples.

Soxhlet extractions with methanol were used to quantify shikimic acid and the results are presented in Table 2. The star anise seeds resulted in a shikimic acid yield of 8.7 mg/g,

Table 1 HPLC results displaying peak areas for shikimic acid standards and shikimic acid extractions of star anise, sweetgum (s. gum) wood and bark from test samples, using methanol (Soxhlet method) and water 65, 85 °C.

HPLC samples	Star anise peak areas	S. gum wood peak areas	S. gum bark peak areas
Soxhlet extractions	35705727	805000	1520891
Spiked (mg/mL)	(spiked 1:1 w/ 0.5)	(spiked 2:1 w/0.125)	(spiked 1:1 w/0.125)
(Theoretical)	27429992	1703111	2061057
(Actual)	28236518	2038678	1841370
Shikimic acid standards	(0.5 mg/mL) 19154258	(0.125 mg/mL) 2601223	(0.125 mg/mL) 2601223

which was on the lower end of previously reported yields, [8, 20]. Shikimic acid concentrations from extractions vary between *Illicium* species as shown by Avula et al. [6], who reported that the lowest and highest concentrations were in *Illicium lancealatum* and *Illicium religiosum* species, respectively. The lower star anise shikimic acid yield reported in this work could be due to the use of unspecified species samples that were purchased from a local food store. Star anise species used by other researchers [8] were freshly harvested at optimum seasonal times in specific growing regions in China, known for producing star anise species which contains high percentages of shikimic acid.

Quantification of shikimic acid in *L. styraciflua* bark and de-barked wood was performed in methanol-Soxhlet extractions, also shown in Table 2. Soxhlet methanol extractions of bark and de-barked wood yielded 1.4 and 0.2 mg/g of shikimic acid, respectively, which is lower than what is reported for seeds and foliage. Autumn-harvested excised *L. styraciflua* seeds were reported to contain between 24 and 37 mg/g of shikimic acid [5]. Additionally, Li et al. [13] reported shikimic acid yields from *L. styraciflua* of 37 mg/g for seeds, and 33 and 57 mg/g for green and yellow leaves, respectively.

Organic solvent-based extractions could be difficult to integrate into bioprocessing conversion schemes that involve dilute acid or hot water pretreatments. Hence, a water-based biomass wash step could easily be integrated with dilute acid or hot water pretreatments. Consequently, water-based *L. styraciflua* bark and de-barked wood extractions were conducted. Table 2 shows that 65 and 85 °C water was effective in extracting shikimic acid from *L. styraciflua* bark and de-barked wood. Bark, extracted with 65 °C water, gave the highest yield, 1.7 mg/g, which was higher than the yield obtained from methanol Soxhlet extracts. It is important to note that results presented in Table 2 are preliminary results, and extraction parameters, such as solids loading, extraction temperature and duration, as well as product recovery, need to be better defined. Nonetheless, the results presented in Table 2 indicate that it could be possible to extract this valuable phytochemical with a 65 °C water wash, which then could be integrated into existing dilute acid or hot water pretreatment technology.

Table 2 Extraction yields of shikimic acid from star anise, sweetgum bark and sweetgum wood using either methanol (Soxhlet method) or water at 65 and 85 °C (in a shaking water bath).

	Soxhlet methanol	Water 65 °C	Water 85 °C
Star anise	8.73 mg/g ±2.18	7.27 mg/g ±0.21	6.37 mg/g ±0.88
Sweetgum bark	1.37 mg/g ±0.21	1.68 mg/g ±0.12	1.16 mg/g ±0.14
Sweetgum wood	0.18 mg/g ±0.10	0.15 mg/g ±0.02	0.20 mg/g ±0.01

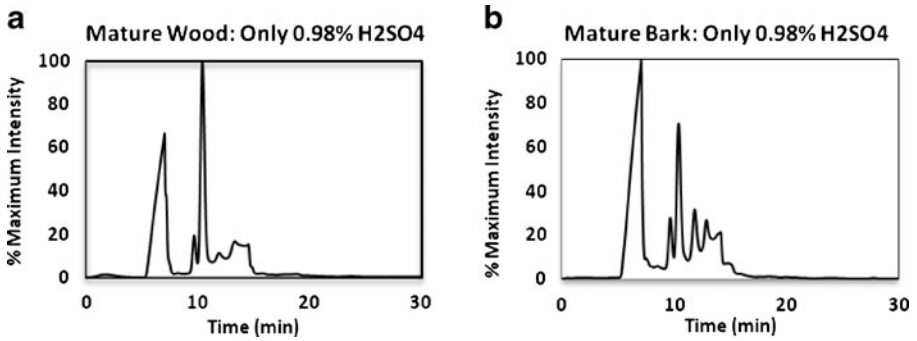

Fig. 2 HPLC-RI using method of 130 °C for 50 min at 60 psig in 0.98% H_2SO_4. Retention times for major peaks: glucose=9.6 min, xylose=10.4 min, arabinose=11.8 min, mannose (only in de-barked wood) = 12.8 min. **a** HPLC-RI of sweetgum mature wood, **b** HPLC-RI of sweetgum mature bark

Dilute Acid Pretreatment of *L. styraciflua*

Dilute acid pretreatment was chosen in this study because of its high reaction rates, which are advantageous in a large scale refinery [17]. The recovery of xylose is especially important because xylose fermentation has the potential to increase the ethanol yield by up to 50% with little additional cost, provided that an organism capable of utilizing C_5 sugars is readily available [21]. Moreover, xylose is the major compositional sugar of hardwood hemicelluloses [22]. Dilute acid pretreatments can be carried out at temperatures up to 180 °C [16, 17]. Unfortunately, due to equipment limitations, this work reports solely on pretreatments carried out at a maximum temperature of 130 °C.

Figure 2 presents the obtained carbohydrate profiles of bark and wood pretreated in 0.98% H_2SO_4 at 130 °C for 50 min. Using the compositional data provided by Torget et al. [23] for bark, a 130 °C pretreatment enabled the recovery of 37% of the bark xylose. Similarly, using a wood compositional analysis provided by Torget et al. [24], 54% of the xylose in de-barked wood was obtained. A higher xylose recovery from de-barked wood than from bark has previously been reported in sweetgum by Torget et al. [23, 24], who recovered a maximum of 98% of the xylose in wood and 88% of the xylose in bark, respectively, both using a pretreatment of 160 °C for 5 min. Due to the equipment

Table 3 Cumulative extraction yields of carbohydrates for sweetgum bark and wood, loaded at 10%, and pretreated at 130 °C in 0.98% dilute sulfuric acid for 50 min, when combined with a 65 °C wash step; eeference comparisons listed below (P=0.0001).

Treatments	Tissue	Xylose % recovered	Glucose	Arabinose	Mannose
130 °C for 50 min at 60 psi in 0.98% H_2SO_4	Bark	37.26±2.76	2.51±0.10	44.22±5.41	N/A
	Wood	53.68±11.37	2.89±0.22	16.28±17.78	299.73±271.19
65 °C for 2 h in H_2O, then 130 °C for 50 min at 60 psi in 0.98% H2SO4	Bark	56.15±1.37	6.11±0.12	53.41±4.80	N/A
	Wood	74.32±15.50	3.49±1.23	21.56±18.76	324.29±154.67
Composition		% dry wt			
Torget et al. [23]	Bark	6.6	23.8	3.8	N/A
Torget et al. [24]	Wood	17.5	49.5	0.4	0.4

limitations mentioned previously, pretreatment at 160 °C could not be carried out in this work which accounts for the lower xylose percent yields compared to Torget et al. [23, 24]. It is important to note that the tested material in our laboratory may contain higher concentration of mannose that that reported by Torget et al. [23, 24], as seen by the greater than 300% mannose recovery.

Feedstock conditioning before the treatment, *per se*, is currently being incorporated into the biochemical processing of biomass into biofuels. Because shikimic acid could be extracted with 65 °C water, as shown in Table 2, the idea of coupling a feedstock wash to pretreatment was examined. Preliminary experiments conducted with 65 °C and 60 psig of nitrogen showed that longer extraction times enabled a higher shikimic acid recovery. Additional experiments showed that higher shikimic acid yields were recovered when extracting material at 65 °C, but at atmospheric pressure. Thus, the wash step was conducted at 65 °C for 2 h at atmospheric pressure.

Results presented in Table 3 show that the addition of a wash step allows for higher sugar recovery, be it for xylose, glucose, arabinose, or mannose. With the addition of the 65 °C wash coupled to pretreatment, 56% of the xylose was recovered from the bark, instead of 37% with the dilute acid pretreatment alone. This resulted in a 21% increase in xylose percent recovery, which is significantly different. Similarly, the wash step increased xylose recovery from de-barked wood by 17%, also significantly different.

The shikimic acid recovered from the 2 h, 65 °C wash step was 0.8 and 0.1 mg/g for bark and de-barked wood, respectively. These results are lower than what was presented in Table 1 which may be due to changes in the length of extraction time and extraction vessel geometries. Future experiments will need to be carried out to determine the optimal extraction parameters that do not hinder the pretreatment step. However, the work presented in this report shows that a co-product can be extracted from the feedstock prior to the pretreatment step. As this is a preliminary study, there is most likely room for co-product extraction yield improvements in terms of temperature, agitation, duration, solids loading, and reactor geometries. In addition to producing a high value product, this work shows that this added wash step increases xylose recovery in a 130 °C dilute acid pretreatment.

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