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Extraction of Hyperoside and Quercitrin From Mimosa (*Albizia julibrissin*) Foliage

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Abstract

Mimosa, an excellent energy crop candidate because of its high growth yield, also contains, on a dry basis, 0.83% hyperoside and 0.90% quercitrin. Hyperoside has been documented as having anti-inflammatory and diuretic properties, whereas quercitrin may play a role in intestinal repair following chronic mucosal injury. Thus, mimosa might first be extracted for important antioxidant compounds and then used as a feedstock for energy production. This article presents results from studies aimed at determining the effect of three extraction parameters (temperature, solvent composition, and time) on the yield of these important quercetin compounds. Conditions are sought which maximize yield and concentration, whereas complementing subsequent biomass pretreatment, hydrolysis and fermentation.

Index Entries: Biomass pretreatment; energy crops; hyperoside; mimosa; quercitrin; value-added compounds.

Introduction

Although the US produced a record 3.4 billion gal (1.3×10^{10} L) of ethanol from grain in 2004 (*1*), it is estimated that 150 billion gal (5.7×10^{11} L) of liquid fuel are required annually to make this nation energy independent. Lignocellulosic feedstocks, including crop and forestry wastes, municipal solid waste, and energy crops, are the logical feedstock alternative which could help to fill the gap. Depending on the conversion technology, $10–25 \times 10^6$ t of dry biomass are required to produce one billion gal of ethanol (2.4–6.0 kg/L). When this large feedstock requirement is coupled with the regional aspects and low bulk density of biomass, which makes shipping difficult and expensive, it seems prudent to utilize a host of biomass feedstock alternatives. In the southeastern United States, energy crops are a

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viable source of lignocellulosic material because they can often be cultivated on marginal land, and thus serve as a source of additional revenue to farmers. Switchgrass is one such energy crop, with biomass yields of 6–9 dry t/acre/yr (1.3–2.0 kg/m^2/yr) (**2**). A number of other energy crops have been proposed, including hybrid poplar, sericea lespedeza, arundo, and mimosa.

There are inherent difficulties in developing energy crops as biomass feedstocks for ethanol production. In addition to transportation costs, the farmer must receive compensation for producing and harvesting the energy crop, a fact, which increases the cost of the raw material for conversion. Lynd et al. *(3)* note that feedstock costs (at $40/t) account for 52% of the cost of producing sugar from hybrid poplar and up to 75% of the cost of ethanol. One way that feedstock costs can be offset is to select energy crops, which also contain compounds, which may be used in pharmaceutical and health care products. One such crop is mimosa, which showed forage yields of 6–7.5 dry t/acre/yr (1.3–1.7 kg/m^2/yr) in Alabama over a 5-yr test. Recent studies performed at the University of Arkansas showed that two documented antioxidant flavonoids, hyperoside (quercetin-3-*O*-galactoside) and quercitrin (quercetin-3-*O*-rhamnoside) constitute 2.3% of dry mimosa foliage *(4)*. Hyperoside has been documented as having anti-inflammatory and diuretic properties, whereas quercitrin may play a role in intestinal repair following chronic mucosal injury. Worldwide, about 50% of all drugs in clinical use are derived from natural products, and at least 25% of all prescription drugs contain ingredients extracted from higher plants *(5)*. These products can be very profitable. Each new drug is estimated to be worth an average of $94 million to a private drug company and $449 million to society as a whole *(6)*. Furthermore, pharmaceutical chemicals including conventional drugs and medicinals, vitamins, minerals, and herbal extracts constitute an $11.4 billion market in the United States and a $9.3 billion market in Europe *(7)*.

One key to effectively and economically extracting high value compounds from energy crops is to maximize the selectivity of the target compound in order to simplify downstream purification requirements. Another key is the ability to couple extraction with biomass conversion to ethanol, the major focus of biomass conversion. Although a number of technologies exist for the conversion of biomass to sugars (hydrolysis with dilute acid or concentrated acid, enzymatic hydrolysis), biomass pretreatment with either dilute acid or steam explosion followed by enzymatic hydrolysis is receiving the most attention because of its ability to develop a highly fermentable sugar stream. If high value products could be effectively extracted from energy crops prior to hydrolysis, a simple unit operation could be added to the existing biomass conversion technology to generate revenue from compounds that could be used in human and animal health care.

The purpose of this article is to investigate the feasibility of coupling antioxidant extraction and biomass conversion to ethanol by employing dilute acid, dilute base, or water as nonspecific extraction solvents. Simple

batch extraction experiments were performed to measure hyperoside and quercitrin yields as a function of solvent composition and temperature. Most often flavonoids are extracted with acidic ethanol or methanol solutions (8), and indeed, methanol is a very effective solvent for the extraction of hyperoside and quercitrin from mimosa (9). However, an aqueous based extraction process would be more desirable, since the use of organic solvents for flavonoid extraction would require solvent recycle and residues containing organic solvent would not interface well with existing biomass conversion technologies.

Materials and Methods

Plant Material

Samples of fresh mimosa (*A. julibrissin*) foliage, ranging from 1 to 90 d in age, were harvested by hand in August, 2004, at Auburn, Alabama. The foliage included the petiole, branches and leaflets of the entire compound leaf. Foliage samples were placed in a forced air oven at 65°C within 1 h of harvesting, dried to constant weight, ground to a 0.32 mm particle size (as measured by ANSI/ASAE S319.3) and stored in sealed plastic bags at room temperature. A voucher specimen has been deposited at the Department of Chemical Engineering, University of Arkansas (Fayetteville, AR).

Chemicals

Hyperoside, quercitrin, and rutin (quercetin-3-*O*-rutinoside) were purchased from Indofine Chemical Company, Inc (Somerville, NJ). HPLC grade acetonitrile and methanol were obtained from EMD Chemicals Inc. (Gibbstown, NJ), formic acid was purchased from EM Science (Gibbstown, NJ), and H_2SO_4 and NaOH were purchased from VWR International (West Chester, PA).

Extraction of Mimosa Foliage

In extracting mimosa foliage, 2 g of dried biomass were extracted with 60 mL of solvent (either 100% methanol, 60% aqueous methanol, water, 0.1% NaOH, or 0.1% H_2SO_4) at 50, 70, and 85°C by blending the mixture in an insulated household blender for 10 min or by shaking the mixture in 280 mL amber bottles in a Precision shaking water bath (Winchester, VA) at 150 rpm. All experiments were performed at least in triplicate. Samples of biomass/solvent were removed with time in the shaking bath experiments. To separate the supernatant from the solids, the resulting mixture of solvent and solids was filtered through cheesecloth and then centrifuged at 12,000*g* for 30 min in an induction drive centrifuge (Beckman Coulter, Fullerton, CA). The crude extracts were collected and stored at 4°C for subsequent analysis. Aliquots (1 mL) of the extractions were dried under vacuum using a SpeedVac Plus (Savant Instruments, Holbrook, NY) without

heat. After drying, the samples were redissolved in 1 mL of methanol for subsequent flavonoids analysis. The samples were then filtered through a 0.45 μm syringe filter (VWR International, West Chester, PA).

Quantitative Flavonoids Analysis by HPLC

The high-performance liquid chromatography (HPLC) analysis of flavonoids was conducted on a Waters Instrument, equipped with a 2996 photodiode array detector and a 2695 separations module controlled with Empower software. A 10 μL sample was injected into the Symmetry® C_{18} (150 mm × 4.6 mm) column (Waters, Milford, MA). The mobile phases used for the gradient consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The gradient program was initiated with 85:15 solvent A: solvent B, and maintained for 5 min. The gradient was linearly increased to 80:20 solvent A: solvent B over 20 min, and then increased to 20:80 solvent A: solvent B over 1 min. The gradient was then increased to 10:90 solvent A: solvent B over 10 min, and finally decreased to 85:15 solvent A: solvent B over 1 min, and then maintained for 3 min. This process was followed by a re-equilibration of the column at the final operating condition for 10 min. The flow rate was 0.75 mL/min, and the column temperature was 30°C. Each of the compounds was detected by the photodiode array detector at 360 nm. The authenticity of the peaks was verified as described by Lau et al. *(4)*.

A typical HPLC chromatogram of a mimosa extract is shown in Fig. 1. Rutin was added as an internal standard (two parts sample to 1 part rutin, at a concentration of 1.0 mg/mL in methanol) and elutes at 13.1 min. Quercitrin elutes at 14.3 min and hyperoside elutes at 21.6 min. Quercetin-rhamnosylgalactoside, a third flavonoid found in mimosa extracts that has antioxidant activity *(4)*, elutes at 9.9 min. Other compounds found in the extract have not been identified at this time.

Results and Discussion

Blender Experiments

An initial set of 10 min experiments was carried out with five solvents at three temperatures in the household blender to quickly evaluate the suitability of the solvents in extracting hyperoside, quercitrin, and quercetin-rhamnosylgalactoside from mimosa foliage. The household blender has the advantage of very good contact between solid and liquid in a short period of time, but suffers from the problem of maintaining constant temperature, despite the addition of insulation around the blender and restricting the experiment duration to 10 min. Results from these initial experiments are shown in Table 1, whereas the yields of flavonoids in ppm (mg flavonoid per kg of dried mimosa foliage) are shown for the five extraction solvents at three extraction temperatures. No standards are available for quercetin-rhamnosylgalactoside, so the yields of this quercetin

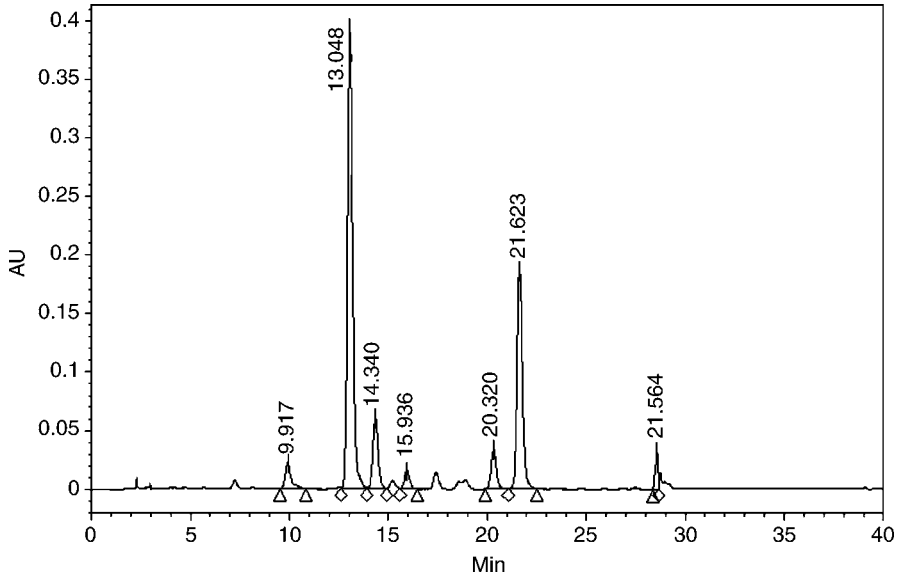

Fig. 1. HPLC chromatogram of mimosa foliage extracted with 50°C water for 10 min in a household blender. The retention times of quercetin-rhamnosylgalactoside, rutin (internal standard), quercitrin and hyperoside are 9.9, 13.1, 14.3, and 21.6 min, respectively.

Table 1
Extraction of Hyperoside and Quercitrin From Mimosa Foliage in a Household Blender Using Various Solvents

Temperature (°C)	Solvent				
	100% Methanol	60% Methanol	Water	0.1% NaOH	0.1% H_2SO_4
	ppm Hyperoside				
50	1896 ± 268	2551 ± 95	1701 ± 160	615 ± 109	2036 ± 36
70	N.A.	1903 ± 35	1708 ± 212	943 ± 288	1258 ± 308
85	N.A.	2542 ± 44	1596 ± 153	N.A.	1683 ± 38
	ppm, Quercitrin				
50	5431 ± 744	7520 ± 256	4954 ± 310	2853 ± 543	5589 ± 158
70	N.A.	5916 ± 95	5480 ± 608	4213 ± 1142	3709 ± 881
85	N.A.	7753 ± 123	4227 ± 1443	N.A.	4882 ± 11
	Estimated ppm Quercetin-rhamnosylgalactoside[a]				
50	552 ± 90	824 ± 15	645 ± 59	180 ± 37	742 ± 15
70	N.A.	655 ± 15	711 ± 58	367 ± 175	475 ± 128
85	N.A.	856 ± 19	624 ± 94	N.A.	638 ± 4

Not applicable, data were not taken at this condition.
[a]Estimated from the quercetrin calibration curve, because standards are no available.

compound were *estimated* using the calibration curve for quercitrin, a compound having a similar structure.

In analyzing the data, it is seen that 60% methanol is the best solvent for extracting mimosa flavonoids, followed by 0.1% H_2SO_4, 100% methanol and water. 0.1% NaOH was ineffective in flavonol extraction. In fact, 0.1% H_2SO_4 appears to be a very effective alternative to 60% methanol. The use of 60% methanol was far superior to 100% methanol, such that experiments at 70 and 85°C were not run for 100% methanol, since neither 100% methanol or 60% methanol would be a good candidate for linking extraction to biomass conversion to energy. Yields were generally expected to increase or remain constant with increasing temperature (little compound degradation was expected at these low extraction temperatures), but temperature variation in the blender during extraction may have affected the yields. Although the yields in water were lower than those obtained from 0.1% H_2SO_4 and 60% methanol, water could still be an interesting and effective solvent system when it is realized that the yield in extracting a high-value, relatively low demand compound from a high volume energy crop is relatively unimportant.

NaOH was not a good extraction solvent. The yields of hyperoside and quercitrin with 0.1% NaOH were one third to one half the yields from the other solvents. Furthermore, whereas the ratio of extracted hyperoside to extracted quercitrin was 0.31–0.38 for all of the other solvents, the hyperoside to quercitrin ratio was only 0.22 for NaOH. These negative extraction results with NaOH were somewhat expected, at least in a qualitative sense, since acidified solvents have been shown to be more effective in extracting flavonoids *(8)*.

Water Bath Experiments

Because of the inherent problems of maintaining a constant temperature in the blender experiments, time-course extraction experiments were carried out in a shaking water bath with 0.1% H_2SO_4 and water as the extraction solvents at 50 and 85°C. Thus, in these experiments, extraction efficiency was sacrificed for the ability to maintain a constant extraction temperature. Once again, at least three replicate experiments were performed at each extraction condition. Extraction times were expected to be longer in the water bath experiments with agitation at 150 rpm, so the first experiment in each set was carried out for 2–3 hr, whereas, after viewing the initial extraction results, subsequent experiments had a maximum extraction time of 60 min.

Water Extraction

Figure 2 shows the extraction yields (mg flavonoid per kg of dried mimosa foliage) of hyperoside, quercitrin, and quercetin-rhamnosylgalactoside from mimosa foliage in the water bath with water as the extraction solvent at 50 and 85°C. Once again, since standards are not available for quercetin-rhamnosylgalactoside, the yields of this quercetin compound

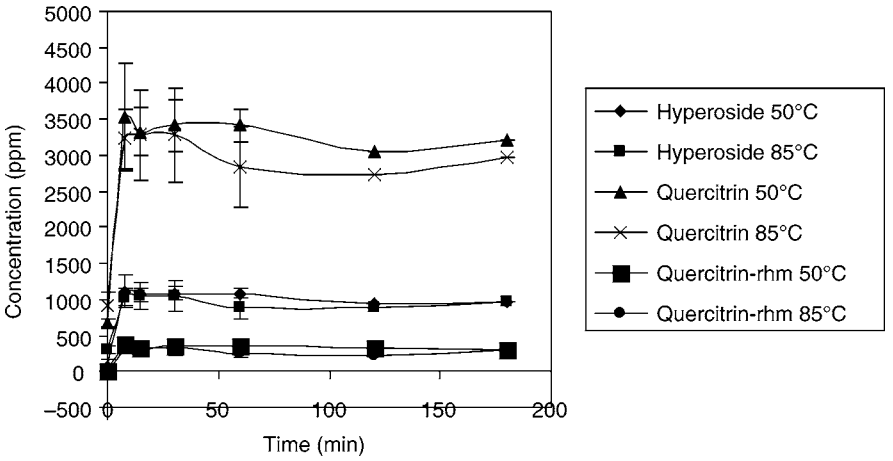

Fig. 2. Extraction of mimosa foliage with water at 50 and 85°C in a shaker bath to yield hyperoside, quercitrin, and quercetin-rhamnosylgalactoside.

were estimated using the calibration curve for quercitrin. As is noted in the figure, the maximum yield of each of the flavonoid compounds was attained in about 10 min, with perhaps a small but detectable decrease in yield observed after 30–60 min, possibly owing to compound degradation. Temperature had a minimal effect on extraction, with slightly higher yields at 50°C. The maximum yields of hyperoside, quercitrin and quercetin-rhamnosylgalactoside at 50°C were about 1100, 3500, and 400 mg/kg of dried mimosa foliage, respectively. Thus, the ratio of hyperoside to quercitrin after 10 min of extraction was 0.31 and the ratio of hyperoside to quercetin-rhamnosylgalactoside was 2.75, both quite similar to the values obtained in the blender experiments. Finally, the yields in the water bath experiments were only 60–80% of the yields in the blender (even at long extraction times), indicating that the flavonoids may be bound inside the mimosa cell matrix and are thus not accessible with water as the solvent without further breaking of the finely ground mimosa.

Extraction With 0.1% H_2SO_4

The extraction of mimosa foliage with 0.1% H_2SO_4 gave similar results, as presented in Fig. 3. Once again, the maximum yield of each of the flavonoid compounds was attained in about 10 min, and then leveled off. In contrast to the experiments with water, the maximum yields of the primary flavonoids with 0.1% H_2SO_4 (1500 mg hyperoside and 4500 mg quercitrin per kg of dried mimosa foliage) were obtained at 85°C. The ratio of hyperoside to quercitrin after 10 min of extraction at 85°C was 0.33 and the ratio of hyperoside to quercetin-rhamnosylgalactoside was 3.7, once again quite similar to the values obtained in the blender experiments. Finally, the yields in the water bath at 85°C were essentially the same as obtained in the blender experiments.

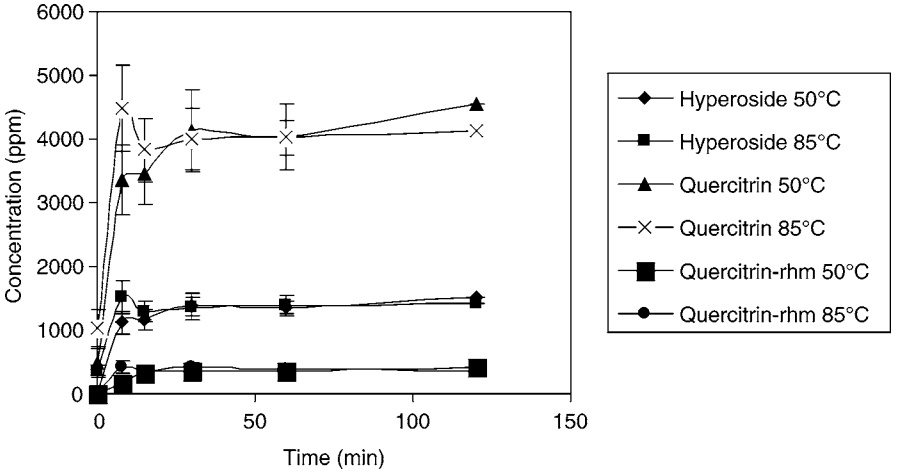

Fig. 3. Extraction of mimosa foliage with 0.1% H_2SO_4 at 50 and 85°C in a shaker bath to yield hyperoside, quercitrin and quercetin-rhamnosylgalactoside.

Conceptual Design

Figure 4 shows a conceptual design for coupling flavonoid extraction with biomass conversion to ethanol using enzymatic hydrolysis and fermentation. Milled biomass (mimosa or similar energy crop) is sent to extraction in which dilute acid (likely 0.1% H_2SO_4 or less) or water is used to extract flavonoids at a temperature of less than 100°C. Although experimental data have not yet been obtained for flavonoid extraction from mimosa foliage at temperatures higher than 85°C, there is likely an upper temperature limit in which compound degradation will become a problem. As an example of the potential for degradation at increased temperature, Duan et al. *(10)* saw significant silymarin degradation in extracting milk thistle with subcritical water at temperatures at or above 100°C. The dilute acid or water will be recycled in a continuous extraction process to maximize the flavonoid concentrations in the extract. It should be remembered that the large quantity of biomass required for ethanol production will generate an abundance of flavonoids so that only a portion of the biomass stream may be used in extraction. After extraction, the concentrated flavonoid stream is sent to flavonoid recovery to produce either an extract (solid mixture of mixed flavonoids) or the individual flavonoid compounds, which may be used as health beneficial compounds for humans or other animals.

Dilute acid is used to pretreat the biomass at 160–200°C before enzymatic hydrolysis and fermentation. Schell et al. *(11)*, for example, used 0.5–1.41% H_2SO_4 at 165–183°C for the pretreatment of corn stover in NREL pilot studies, and others have effectively used even lower acid concentrations. Fresh acid may be used for pretreatment, although an opportunity exists to use dilute acid from flavonoid extraction. Following pretreatment, the pretreated solids are available for subsequent hydrolysis and fermentation.

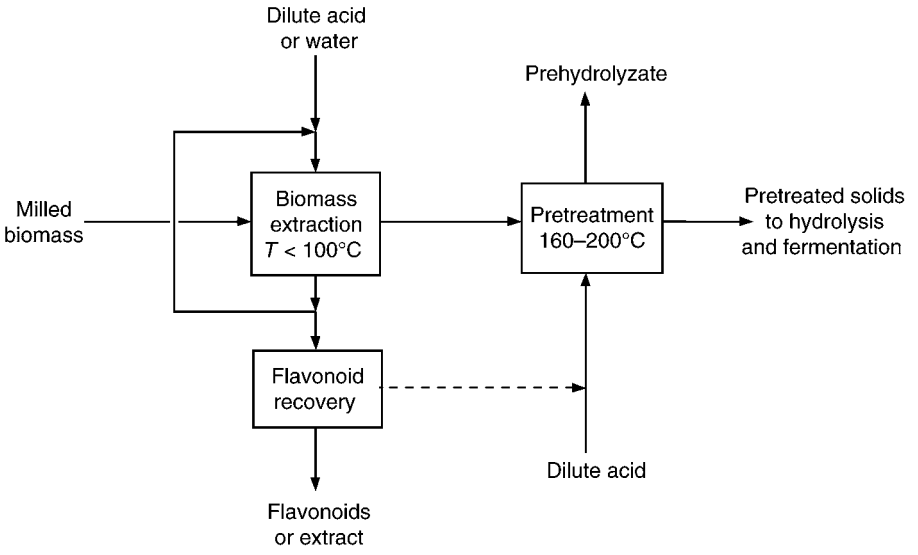

Fig. 4. Conceptual design for coupling flavonoid extraction with biomass pretreatment, hydrolysis and fermentation.

Conclusions

Water and 0.1% H_2SO_4 are very good solvents for the extraction of the flavonoids hyperoside, quercitrin and quercetin-rhamnosylgalactoside from mimosa foliage. By contrast, 0.1% NaOH was not effective as a solvent. When extracting mimosa foliage with 0.1% H_2SO_4 at 50°C in a household blender, flavonoid yields were 70–80% of the yields obtained when extracting with 60% methanol, the preferred solvent for flavonoid extraction. In using water as the extraction solvent, the yields fell to 60–70%, but water is still judged to be an effective solvent in extracting a high-value, relatively low demand compound from a high volume energy crop. Time-course experiments performed in a shaking water bath showed that the flavonoids could be extracted in about 10 min, and were not particularly sensitive to temperature between 50 and 85°C. A conceptual design shows that flavonoid extraction could be easily coupled with biomass pretreatment prior to enzymatic hydrolysis and fermentation.

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