0273-2289/04/114/0569–0583/$25.00

Extraction of Antioxidant Compounds from Energy Crops

Ching S. Lau,[1] Danielle Julie Carrier,[2] Luke R. Howard,[3] Jackson O. Lay Jr.,[4] Jean A. Archambault,[5] and Edgar C. Clausen*,[1]

[1]Department of Chemical Engineering, University of Arkansas, 3202 Bell Engineering Center, Fayetteville, AR 72701, E-mail: eclause@engr.uark.edu; [2]Department of Biological and Agricultural Engineering, University of Arkansas, 203 Engineering Hall, Fayetteville, AR 72701; [3]Department of Food Science, University of Arkansas, 2650 N. Young Avenue, Fayetteville, AR 72704; [4]Department of Chemistry and Biochemistry, University of Arkansas, 115 Chemistry Building, Fayetteville, AR 72701; and [5]Phytobiotech Inc., 525 des Prairies Boulevard, Laval, Québec, Canada, H7V 1B7

Abstract

Energy crops offer enormous opportunities for increasing the sustain ability of agriculture and energy production in the United States. Nevertheless, opportunities for sustaining biomass energy production may well hinge on producing energy and extracting high-value products from the same crop. Seven potential energy crops (mimosa, sericea, kudzu, arunzo, switchgrass, velvet bean, and castor) were extracted and assayed for the presence of potentially high-value antioxidant compounds. Of these crops, mimosa and sericea had the highest antioxidant potential and were selected for further study. High-performance liquid chromatography (ultraviolet) and liquid chromatography/mass spectrometry techniques were then utilized to help identify the compounds with high antioxidant potential using extract fractionation, and total phenolics and oxygen radical absorbance capability assays as a guide. These analyses indicate that methanol extracts of mimosa foliage most likely contain quercetin, a flavonol that has been associated with cardioprotection. Future work will concentrate on quantifying the quercetin content of mimosa (likely parts-per-million levels), as well as identifying and quantifying other antioxidants found in energy crops.

Index Entries: Mimosa; sericea; energy crops; quercetin; antioxidant.

*Author to whom all correspondence and reprint requests should be addressed.

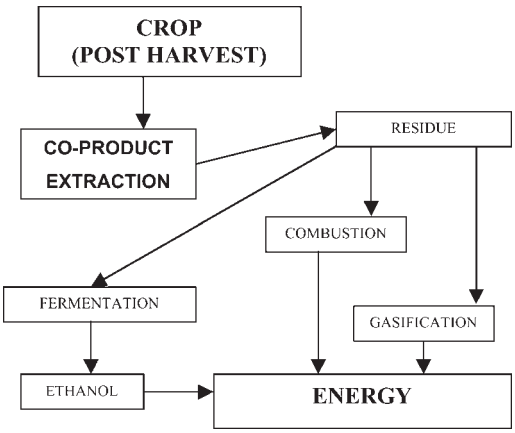

Fig. 1. Possible scenario for coupling extraction of coproducts and energy production.

Introduction

Energy crops offer enormous opportunities for increasing the sustainability of agriculture and energy production in the United States. This is particularly true in the Southeast, where 1.59 quads of biomass is used in energy production, or 56% of the total used nationally. To date, energy crops have not been economically competitive with fossil fuels. At $50/t of biomass, energy crops cost $3.57 per million BTU, whereas coal typically costs only $1.50–$1.80 per million BTU. However, provisions in the pending energy bill will help to change this situation by providing incentives for incorporating biomass into energy production.

Although these developments will certainly launch the commercialization of energy crops, opportunities for sustaining biomass energy production for power and liquid fuel production may well hinge on producing energy and extracting high-value-products from the same crop. In this scenario, shown diagrammatically in Fig. 1, high-value products such as antioxidants and antimicrobials are first extracted from the biomass using water or other solvents. Depending on the extraction technique employed, extraction may either replace or enhance pretreatment prior to enzymatic hydrolysis and fermentation during liquid fuel production. Because the concentrations of the extractables are likely to be at parts-per-million levels, the extracted biomass is available for combustion, gasification, or hydrolysis/fermentation without a detectable decrease in energy yield. A further benefit of extraction coupled with energy production is the potential increase in income to the limited resource farmer who grows the biomass. Since the total value of products obtained from biomass increases, the income per acre for the farmer will likely increase as well.

David Bransby (personal communication, October, 2002) recommended seven potential energy crops (mimosa, sericea, kudzu, arunzo, switchgrass, velvet bean, and castor) as candidates for the extraction of high-value products based on crop yields and largely anecdotal medicinal information.

Of these crops, mimosa and sericea showed the most promise based on preliminary analysis of antioxidant potential and thus were the focus of the present study. Mimosa (*Albizia juribrissin*) and sericea (*Sericea lespedeza*) are two leguminous species that have shown high potential as energy crops in the Auburn University energy crop research program over the last 10 yr *(1)*. Mimosa shows high forage yields (4.7 dry t/acre over a 5-yr test); high forage quality that decreases very little with age; a long growing season; no need for nitrogen (since it is a legume), potassium, and phosphorus fertilizer; extremely good drought tolerance; a relatively low-cost establishment by seed; no need for treatment with insecticides; and excellent longevity. Sericea is a leguminous forage that has undergone genetic improvement at Auburn University for more than three decades. It is a low-cost perennial plant that is better adapted to acidic soils than most legumes; is established from seed; and, in energy crop tests conducted in Alabama, has recorded yields (5–10 t/acre·yr) similar to those obtained from switchgrass (*Panicum virgatum*).

In addition to using mimosa and sericea as energy crops, it is possible that they could yield valuable compounds that could be used in the health arena. From a health benefit perspective, living cells are constantly battling free-radical invasions. However, the cell fights off this invasion by internal antioxidant mechanisms. Detoxification occurs by converting the invaders into electrophilic water-soluble compounds, which will then be conjugated to molecules, such as glutathione or uridine 5'-diphosphate-glucoronosyl, before being excreted. Mimosa has documented medicinal uses in the Orient (David Bransby; personal communication, October, 2002). Kinjo et al. *(2)* reported isolation of the saponins julibrosides A_1, A_2, A_3, A_4, B_1, and C_1 from the dried stem bark of mimosa, which would have uterotinic activity. Flavonoids and saponins have been documented to have antimicrobial activity against, among others, *Pseudomonas*, *Xanthomonas*, *Staphylococcus*, and *Streptococcus* strains *(3)*. Sericea and mimosa most likely contain flavonoids and saponins.

In this article, we present results from the extraction of potentially high-value antioxidant compounds from energy crops. Extract fractions were examined for antioxidant activity, and the results from high-value compound identification efforts are presented. These identification studies are a major first step in making these energy crops economically sustainable.

Materials and Methods

Extraction Experiments

Samples of dried and ground (0.3 mm) energy crops (mimosa, sericea, kudzu, arunzo, switchgrass, velvet bean, and castor) were kindly supplied by Dr. David Bransby of the Department of Agronomy and Soils at Auburn University. To perform an extraction, 2 g of the selected energy crop was mixed with 60 mL of solvent (either water, 60% methanol in water, or 60% ethanol in water) and blended in a standard household blender for 1 min

at 40ºC. The resulting mixture of solvent and solids was centrifuged at 12,000*g* for 30 min to separate the supernatant from the solids. The supernatant was then filtered through a 0.45-µm syringe filter. The solids-free crude extracts were collected and stored at 4°C for subsequent fractionation and analysis. These extractions should not have removed significant quantities of sugars or protein to hinder subsequent biomass conversion, but this assumption needs to be demonstrated in later biomass hydrolysis studies.

Compound Fractionation

Fractionation of the crude extracts was done in 2-mL disposable Symmetry® (Waters, Milford, MA) C_{18} Sep-Pak columns. The columns were preconditioned by first injecting 10 mL of methanol through the column, followed by the injection of 10 mL of HCl at pH 2.0. To produce fractions from the crude extracts, 0.25 mL of filtered crude extract was injected into the column. Then, 2 mL of 20% methanol (or ethanol) solution was injected and collected as the first fraction. This was followed by the sequential injection of first 60% and then 100% methanol (or ethanol) solutions, and the collection of the second and third fractions. The three fractions were dried using a speed vacuum (Savant, Holbrook, NY) without heat. After drying, the samples were dissolved in 2 mL of methanol.

Antioxidant Analysis

Preliminary assessments of the antioxidant potential of each of the energy crop extracts, fractionated crude extracts and high-performance liquid chromatography (HPLC) fractions (described later) were obtained by two different methods: (1) a modified Folin-Ciocalteu (FC) assay, which yields the concentration of the total phenolic compounds *(4)*, and (2) a modified oxygen radical absorbance capability (ORAC) assay, which measures the degree to which a sample inhibits the action of an oxidizing agent and how long it takes to do so *(5)*.

Dilutions of a 400-ppm chlorogenic acid solution were used to obtain a calibration curve for the FC assay. One milliliter of 0.25 *N* Folin reagent was added to 1 mL of the diluted chlorogenic acid solution. After mixing, the solution was allowed to stand for 3 min. One milliliter of sodium carbonate (1 *N*) was then added, mixed, and allowed to stand for another 7 min. Subsequently, 7 mL of deionized water was added, mixed, and allowed to stand for 2 h. The absorbance of the mixture was read on a spectrophotometer (HP 8452A Diode Array) at a wavelength of 726 nm and plotted as a function of the chlorogenic acid concentration. Samples of crude and fractionated plant extracts were analyzed in a similar manner.

Fluorescein (FL) (3',6'-dihydroxyspiro[isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one) was used for the ORAC assay. Excitation and emission filters of 490 and 520 nm, respectively, were used. A stock solution of 1.2 m*M* FL was diluted to a working solution of 94 n*M* made up in 0.75 m*M* phosphate buffer (pH 7.4). Two pipettors, which were part of the microplate

reader (FLUORstar Optima; BMG, Durham, NC), added FL solution (400 µL) on cycle 2 and AAPH (150 µL of 0.137 g/16 mL of phosphate buffer) on cycle 4. The assay was run at 37ºC with a total of 28 reading cycles of 197 s duration. The duration of the reading cycle could vary depending on the number of wells assayed. Trolox (40 µL of 6.25, 12.5, 25, and 50 µ*M* solutions) was used as standard.

HPLC Analyses for Separation of Antioxidant Compounds

Initial separation of compounds for subsequent identification was performed with a Symmetry (Waters) C_{18} column (250 × 4.6 mm). A 100-mL sample volume was injected. Solvent A contained 5% formic acid in water, and solvent B consisted of HPLC-grade methanol. The gradient program was initiated with 98:2 solvent A:solvent B and linearly decreased to 40:60 solvent A:solvent B over 60 min. Compounds were monitored at 280, 320, 360, and 510 nm.

Crude extract was also separated and collected on another Waters system, which consisted of a 600 pump, a 2996 Photodiode Array Detector, and a 2767 fraction collector. The detection wavelength was set in the ultraviolet (UV) between 190 and 400 nm. The column used was a 150 × 21 mm long ACE AQ with 10-mm particles (Advanced Chromatography Technologies, Aberdeen, UK). The system was operated at room temperature. The injection volume was 1500 µL. The mobile phase consisted of 1:3 acetonitrile:water with 0.01% trifluoroacetic acid, which was flowing at a rate of 10 mL/min. The system was operated in the isocratic mode. Fractions of 1.25 mL were collected every 7.5 s.

Identification of Compound

The Sep-Pak fractionated compounds were analyzed using liquid chromatography/mass spectrometry (LC/MS) with a quadrupole ion-trap MS (Bruker Esquire LC/MS, Billerica, MA). The column used was a Symmetry (Waters) C_{18} column (250 × 4.6 mm). A 25-mL sample volume was injected using the system's autosampler. Solvent A contained 5% formic acid in water, and solvent B consisted of HPLC-grade methanol. The gradient program was initiated immediately by starting with a ratio of 98:2 A:B, and B was increased to 60% over 60 min. The flow rate was set to 0.7 mL/min. The UV response during LC/MS was monitored at 360 nm, the highest absorbance wavelength for each set of components as determined from prior HPLC studies. The LC/MS was operated in the positive-ion mode using the electrospray ionization (ESI) source and the manufacturer's recommended operating conditions.

Results and Discussion

Extraction Experiments

Extraction experiments were performed to evaluate the antioxidant capacity of selected energy crops. Table 1 presents the total phenolics con-

Table 1
Total Phenolics and ORAC Values
of Crude Methanol/Water Extracts

Energy crop	Phenolics value [a]	ORAC value [b]
Mimosa foliage	68,000	470
Sericea	65,000	330
Velvet bean foliage	63,000	300
Mimosa seed	1200	16
Kudzu	17,000	190
Arunzo	25,000	170
Switchgrass	11,000	110
Spinach	6300	16
Castor foliage	48,000	150

[a] Milligrams of chlorogenic acid equivalents per gram of dry weight.
[b] Micromoles of Trolox equivalents per gram of dry weight.

Table 2
Total Phenolics Values of Crude Extracts in Different Solvents

	Total phenolics value [a]		
Energy crop	Methanol:water (60:40)	Ethanol:water (60:40)	Water
Mimosa foliage	68,000	60,000	9400
Sericea	65,000	65,000	3600

[a] Milligrams of chlorogenic acid equivalents per gram of dry weight.

tent and ORAC values of crude extracts of selected energy crops in 60% methanol. Also shown for comparison are values for spinach, a crop with known antioxidant properties. As is noted, mimosa foliage, sericea, and velvet bean foliage showed the highest antioxidant potential of the selected energy crops, even exceeding the total phenolics and ORAC values of spinach. Although a high antioxidant potential does not ensure the presence of useful antioxidants for commercial use owing to possible toxicity, high antioxidant potential does indicate that compounds with high antioxidant content are present. The presence of total phenolics also implies the presence of flavonoid compounds, of which antimicrobial properties have been documented *(3)*. Mimosa foliage and sericea were also extracted in 60% ethanol and water (*see* Table 2). The alcohol solutions were found to be better extracting solvents than water.

Fractionation of Compound

LC/MS analyses of both crude extracts showed the presence of many peaks, making identification of compounds very difficult. See, e.g., Figs. 2 and 3, where crude sericea extracts prepared in 60% methanol and water

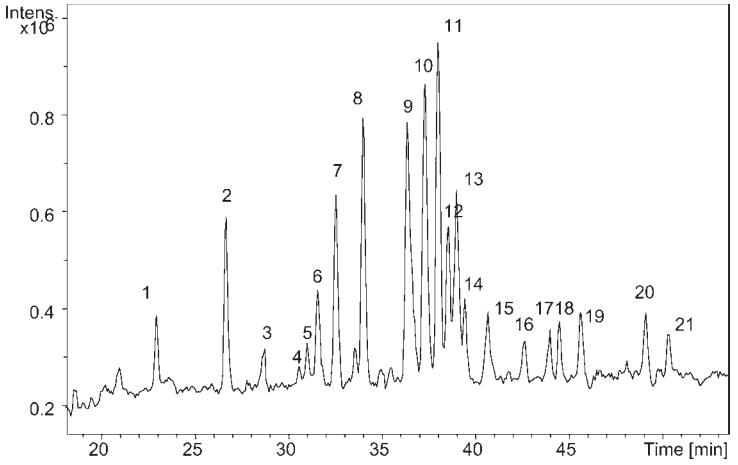

Fig. 2. LC/MS (total ion current [TIC]) analysis of crude sericea extracted with 100% water.

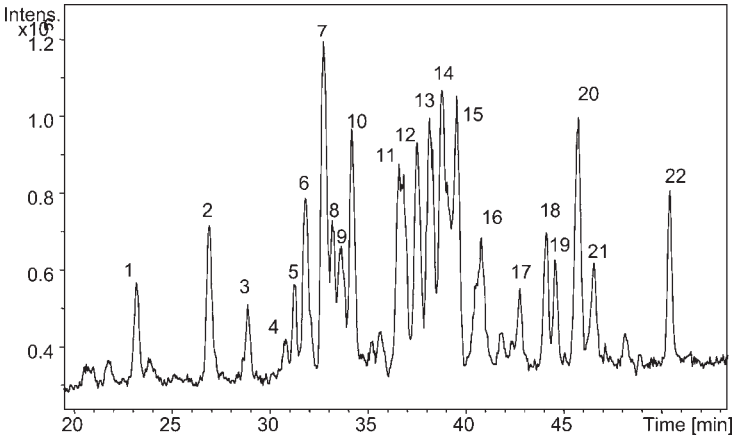

Fig. 3. LC/MS (TIC) analysis of crude sericea extracted with 60% methanol.

are presented. Each of the peaks may represent one or more compounds present in the crude extract. Typically chromatographically separated peaks would contain a single major component by ESI giving two ionmasses corresponding to the protonated molecules for a substituted flavonoid (e.g., sugar attached) and the aglycone component (parent flavonoid). All of the peaks have molecular masses >490 (data not shown), which indicates that the flavonoids present are most likely conjugated with carbohydrate or phenolic acid moieties. Although the flavonoid and substituent masses give some indication of the specific compound, identification of a specific flavonoid required a reference standard and tandem MS as noted later. At a retention time of about 40 min, the two chromatograms differ somewhat, with the methanol extract showing the presence of a few additional compounds.

Table 3
Phenolic and ORAC Values for Sep-Pak Fractionation of Crude Methanol Extracts

	Total phenolics[a]			
Energy crop	20% Methanol in water	60% Methanol in water	100% Methanol	Total phenolics value[b]
Mimosa foliage	23,000 (140)	31,000 (290)	14,000 (92)	68,000
Sericea	11,000 (71)	26,000 (224)	11,000 (60)	48,000

[a] Milligrams of chlorogenic acid equivalents per gram of dry weight. ORAC value, micromoles of Trolox equivalents per gram of dry weight, are given in parenthesis.
[b] Sum of the three fractionates' phenolics value.

Sep-Pak fractionation of the crude extracts prior to analysis helped to reduce the number of peaks per sample, thus aiding LC/MS analysis. In performing total phenolics and ORAC tests on the three fractions, the 60% methanol fraction was found to yield the highest antioxidant values for both crops, as shown in Table 3. As expected, HPLC analyses of the 60% methanol fractions at wavelengths of 280, 320, 360 and 510 nm showed tremendously reduced and more distinct peaks (*see* Figs. 4A, 5A). Neither mimosa foliage nor sericea showed much evidence of the presence of anthocyanins, based on their low absorbances at 510 nm. However, both of the extracts showed high absorbance at 360 nm, a wavelength characteristic of the presence of flavonols.

The 60% methanol sep-pak fractions from the mimosa foliage and sericea extracts were analyzed by LC/MS. The mimosa foliage fraction had nearly the same profiles by both LC/MS (Fig. 4B) and HPLC (Fig. 4A), as indicated by the three major peaks. The LC/MS profile showed that the mimosa foliage fraction had major peaks at 42.5 (significant ions at m/z 303 and 633), 49.0 (m/z 303 and 487), and 53.5 (m/z 303 and 471), min. The molecular weights of compounds were inferred from the positive-ion mass spectra (results not shown). On the other hand, the chromatograms for the sericea extracts (Fig. 5A, 5B) are quite different. The LC/MS profile for sericea in Fig. 5B shows major peaks at 47.0 (major ions at m/z 433 and 545) and 48.5 (m/z 517 only) min.

The same 60% methanol Sep-Pak fraction from the mimosa foliage extract was fractionated into 10 subfractions by HPLC (a different HPLC was used from the Fig. 4 results) and collected for subsequent analysis (*see* Fig. 6). In analyzing the ORAC value of these sub-fractions, it was found that the sixth sub-fraction contained nearly 90% of the total ORAC value (*see* Table 4). Initial HPLC and LC/MS tests on this sixth subfraction indicated the presence of a single peak giving strong ions at m/z 303 and 487 (*see* Fig. 7A, 7B). The m/z 303 ion in this component was subsequently confirmed as protonated quercetin (*see* Fig. 8 for the structure of querce-

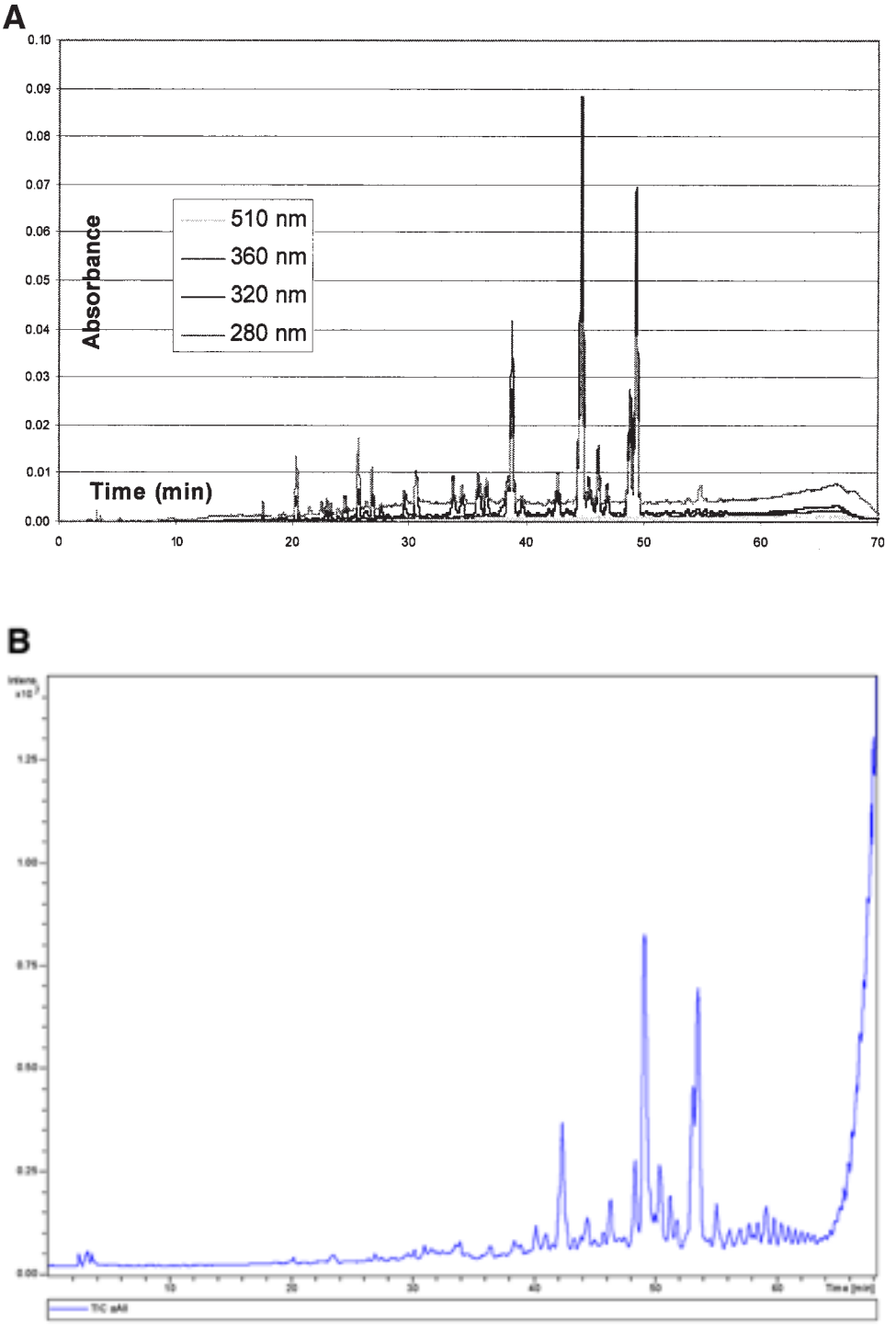

Fig. 4. Analyses of mimosa foliage crude extract fractionated with 60% methanol in Sep-Pak column. **(A)** HPLC (UV); **(B)** LC/MS.

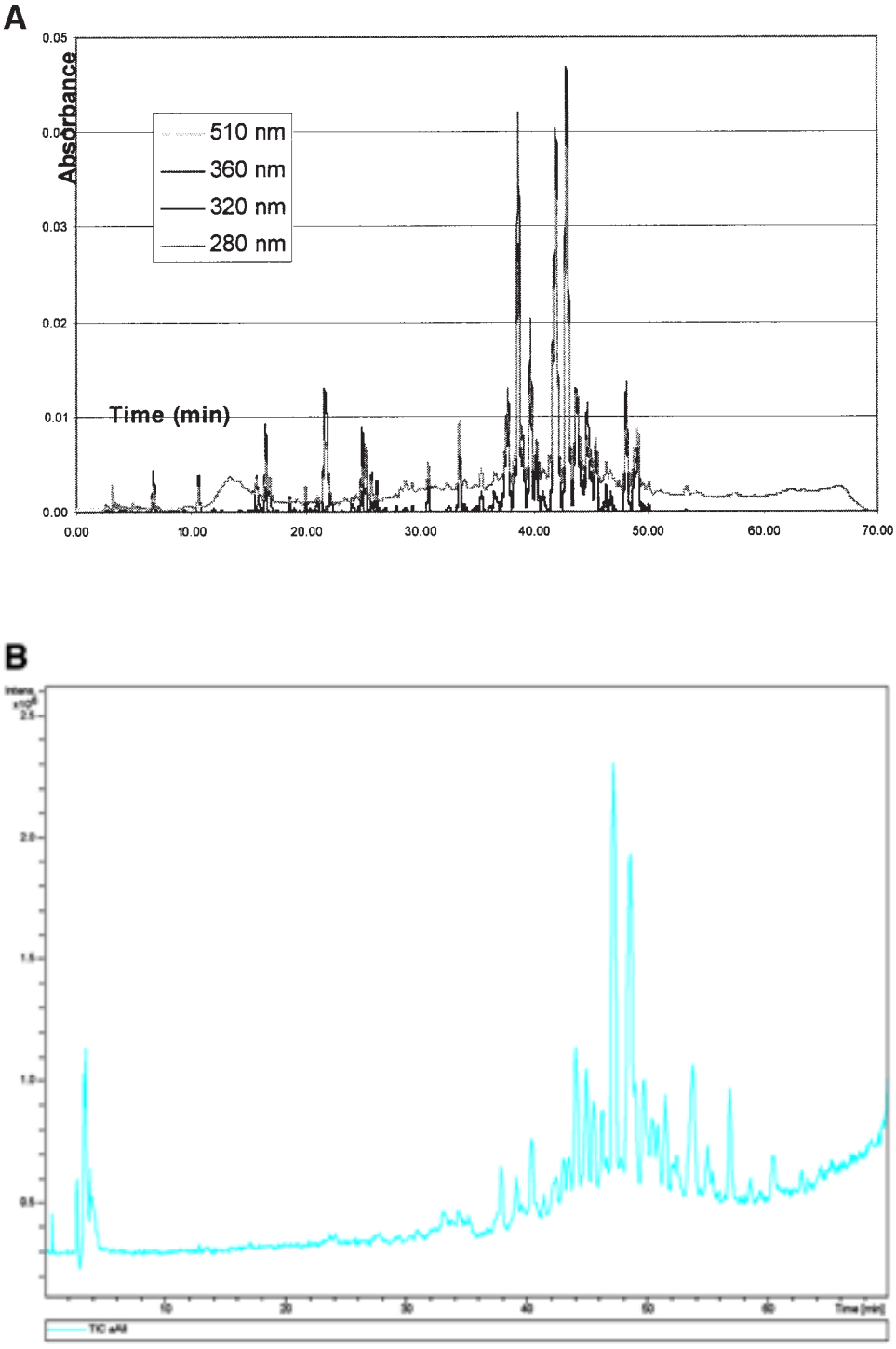

Fig. 5. Analyses of crude sericea extract, fractionated with 60% methanol in Sep-Pak column. **(A)** HPLC (UV); **(B)** LC/MS.

Table 4
ORAC Values of 10 Subfractions from 60% Methanol Extract of Mimosa Foliage

Subfraction	ORAC value [a]
1	0.22
2	0.55
3	0.78
4	1.58
5	0.02
6	38.41
7	0.55
8	0.39
9	0.40
10	0.00
Total	42.90

[a] Micromoles of Trolox equivalents per gram of dry weight.

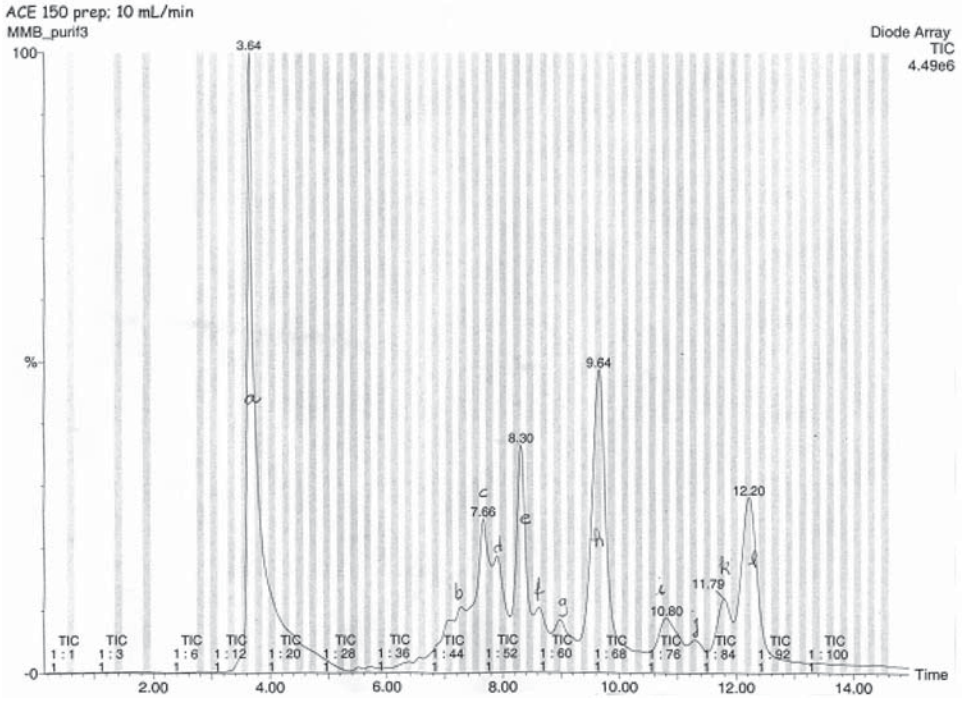

Fig. 6. Second HPLC analysis of mimosa foliage extract, fractionated with 60% methanol in Sep-Pak column.

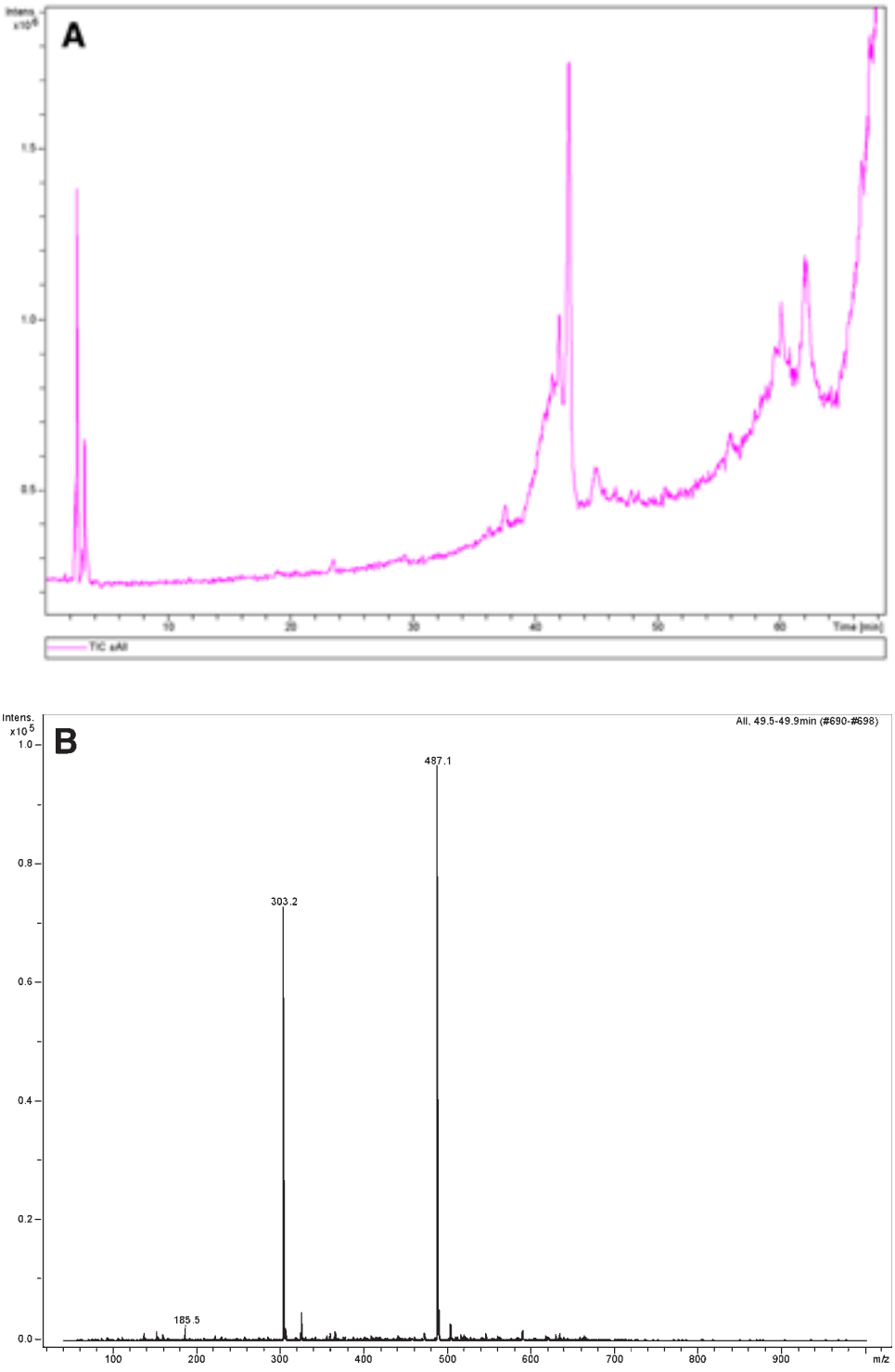

Fig. 7. HPLC/UV analyses of fraction 6 from 60 % methanol mimosa foliage extract. **(A)** HPLC (UV); **(B)** LC/MS.

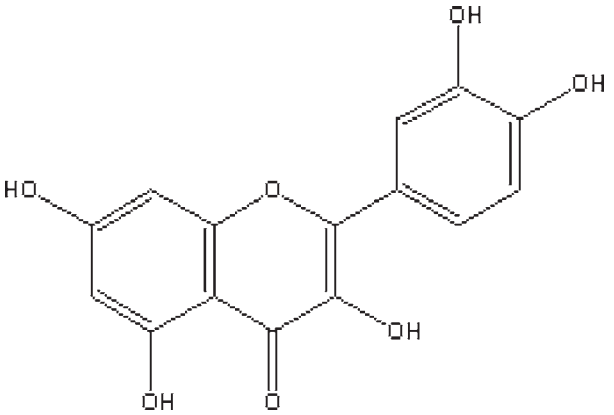

Fig. 8. Molecular structure of quercetin ($C_{15}H_{10}O_7$), mol wt = 302.

tin) based on a comparison between the MS/MS product-ion spectrum obtained during the HPLC separation and a reference spectrum from reagent-grade quercetin (*see* Fig. 9A, 9B). The difference in mass between m/z 487 and 303 in the full-scan spectrum for this component indicated the presence of a sugar or other moiety attached to the quercetin. Fragmentation and loss of this moiety to yield the parent flavonol protonated molecule is typical in positive-ion ESI analyses of this class of compounds. The position and identification of this substitutent are currently under investigation. However, it can be inferred that its mass is about 184 Daltons.

Quercetin is a flavonol, which belongs to a group of naturally occurring compounds that are usually present in the plant as glycosides and are colorless or light yellow *(6)*. It is found in plants such as onions, apples, and berries, as well as many seeds, nuts, flowers, bark, and leaves. It is also found in medicinal botanicals including *Ginkgo biloba*, *Hypericum perforatum* (St. John's Wort), and *Sambucus canadensis* (elder). Quercetin has been associated with cardioprotective phenomenon *(7–10)*. The mechanism of protection has been speculated to involve the action of the quercetin as an antioxidant, which attenuates tissue injury that results from the production of proinflammatory oxidants such as hypochlorous acid (HOCl). HOCl is generated by neutrophils through the enzyme myeloperoxidase (MPO), which catalyzes the oxidation of chloride anion (Cl^-) by H_2O_2 to yield HOCl. The production of HOCl is an integral part of the nonspecific host defense mechanism triggered by opsonized bacteria or activated complement components, but under certain conditions it can also destroy healthy tissues. The ability of quercetin to effectively inhibit MPO activity as well as directly scavenge HOCl may limit the vascular injury associated with inflammatory reactions *(9)*.

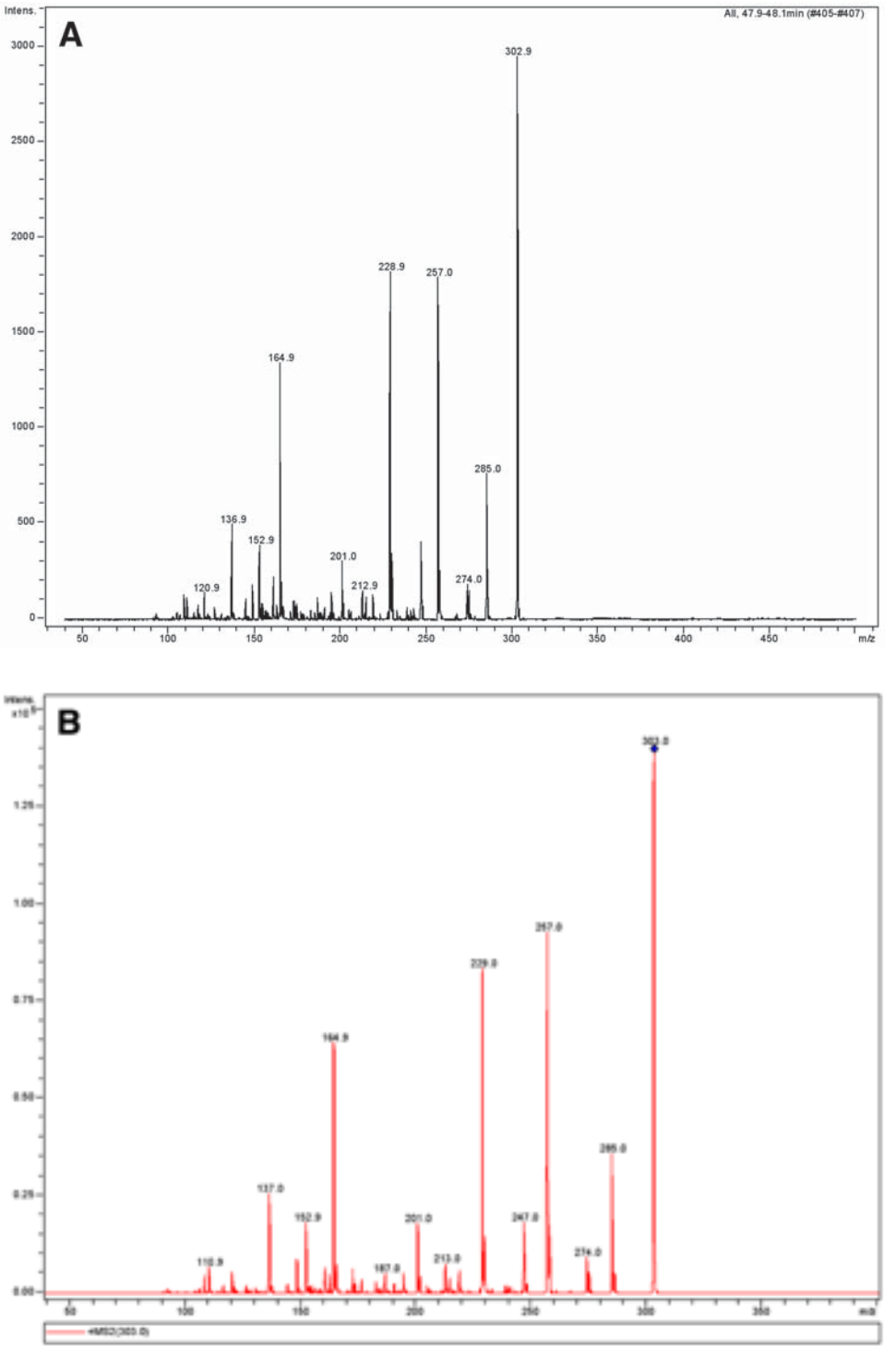

Fig. 9. MS/MS spectrum of m/z 303 of **(A)** fraction 6 from a 60 % methanol mimosa foliage extract and **(B)** protonated quercetin.

Conclusion

Seven possible energy crops (mimosa, sericea, kudzu, arunzo, switchgrass, velvet bean, and castor) were extracted and assayed for the presence of potentially high-value antioxidant compounds. Of these crops, mimosa foliage and sericea showed the highest antioxidant potential. HPLC (UV) and LC/MS techniques were utilized to help identify the compounds with high antioxidant potential using extract fractionation, and total phenolics and ORAC assays as a guide. These analyses indicate that methanol extracts of mimosa foliage most likely contain parts-per-million levels of quercetin, a flavonol that has been associated with cardioprotection. Future work will concentrate on quantifying the quercetin content of mimosa, as well as identifying and quantifying other antioxidants found in energy crops.

Acknowledgment

This project was supported by the Southeastern Regional Biomass Energy Program and administered by the Southern States Energy Board for the United States Department of Energy.

References

1. Sladden, S., Bransby, B., Aiken, G., and Rose, P. (1992), *Highlights Agric. Res*. **39,** 4.
2. Kinjo J., Araki, K., Fujui, K., Higuchi, H., Ikeda, T., Nohara, T., Ida, Y., Takemoto, N., Miyakoshi, M., and Shoji, J. (1992), *Chem. Pharm. Bull.* **40**, 3269–3273.
3. Naidu A. (2000), *Natural Food Antimicrobial Systems*, CRC Press, Boca Raton, FL.
4. Singleton, V. L. and Rossi, J. A. (1965), *Am. J. Enol. Vitic.* **16,** 144–158.
5. Cao, G. H. and Prior, R. L. (1999), *Methods Enzymol.* **299,** 49–62.
6. Van der Sluis, A. A., Dekker, M., Skrede, G., and Jongen, W. M. F. (2002), *J. Agric. Food Chem.* **50,** 7211–7219.
7. Meyer, A. S., Heinonen, M., and Frankel, E. N. (1998), *Food Chem.* **61,** 71–75.
8. Chan, M. M-Y., Mattiacci, J. A., Hwang, H. S., Shah, A., and Fong, D. (2000), *Biochem. Pharmacol*. **60,** 1539–1548.
9. Binsack, R., Boersma, B. J., Patel, R. P., Kirk, M., White, C. R., Darley-Usmar, V., Barnes, S., Zhou, F., and Parks, D. A. (2001). *Alcohol. Clin. Exp. Res. Online* **25,** 434–443 (Website: http://fn.cfs.purdue.edu/bot/Downloads/Publications/Binsack_quercetin_ACER_2001.pdf).
10. Careri, M., Corradini, C., Elviri, L., Nicoletti, I., and Zagnoni, I. (2003), *J. Agric. Food Chem.* **51,** 5226–5231.