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HPLC Determination of Isoflavone Levels in Osage Orange from the Midwest and Southern United States

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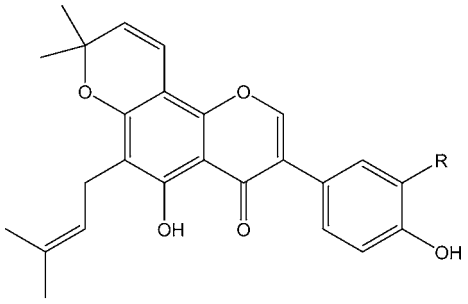
ABSTRACT: The fruit of the *Maclura pomifera* tree is a sustainable source for the pharmacologically interesting isoflavones, osajin and pomiferin. A reversed-phase HPLC method was developed to identify osage orange samples with high isoflavone content and to determine the optimum conditions for sample preparation. Analytical run time was 8 min at a flow rate of 1 mL/min using a gradient of acetonitrile in H_2O (0.1% formic acid) and UV peak detection at 274 nm. The method was validated for specificity, accuracy, precision, and limits of detection and quantitation (LOD/LOQ). The method was applied to determine the levels of osajin and pomiferin in extracts prepared from different samples of osage orange growing in the Midwest and southern United States. Results demonstrated the effect of different variables, such as sample preparation, geographical location, and growth stage, on the levels of osajin and pomiferin in analyzed samples.

KEYWORDS: *horse apple, osage orange, Maclura pomifera, isoflavones, osajin, pomiferin, HPLC, phytochemical analysis*

INTRODUCTION

Maclura pomifera (Raf.) Schneid. (family Moraceae) is a tree that grows throughout the United States and parts of southeastern Canada.[1] The Osage Indians used *Maclura* wood to make bows and clubs because of its durability and resistance to decay.[2] The tree is also grown as hedges surrounding homes and farmlands.[2,3] The fruit of *M. pomifera* has different common names related to its shape, source, traditional uses, and functions. Such names include osage orange, hedge apple, horse apple, and road apple. Fruits grow to their full size (ca. 500 g) every fall, and each fruit can bear up to 300 seeds per fruit.[2] Osage orange has traditionally been used as an insect repellent and as a home remedy for pest control.[1] Fruit extracts and extracts of the bark, seeds, leaves, and roots, as well as the two major isoflavone constituents of the fruit, osajin and pomiferin (Figure 1), were



Osajin, R = H
Pomiferin, R = OH

Figure 1. Chemical structures of the major isoflavones of Osage orange.

reported to possess a number of biological activities. Some of the reported activities include insect repellant,[4] antimicrobial,[5] anti-inflammatory/antinociceptive,[6] antitumor,[7−9] cardioprotective,[10] and cholinesterase inhibitory activities.[11] Osage orange isoflavones, especially pomiferin, also have marked antioxidant activity and have been shown to inhibit lipid peroxidation and to reduce free radicals, reactive oxygen species (ROS), and other unstable molecules.[1,3,12] At present, there are no osage orange-based dietary supplements available on the market, but its potential has been suggested.[9] Biological evaluation of semisynthetic osajin and pomiferin analogues, iso-osajin and iso-pomiferin, has also been attempted by Orhan et al.[11] Being edible by squirrels, horses, and other animals suggests that osage orange is safe.[13] Nevertheless, the toxicities of the different extracts have not been fully established.[2]

Few chromatographic methods have been reported for the determination of osajin and pomiferin in *M. pomifera* fruit and other organs of the plant. The most recent is a validated LC-DAD-MS method developed by Kartal et al. to determine isoflavone levels in different tissues of the fruit. The method utilized a reversed-phase (RP) C8 column and a gradient of acetonitrile in water (40 mM formic acid).[14] Whaley et al. described an isocratic (methanol-acetonitrile, 9:1, as eluent) HPLC-UV method, using an RP C16-amide column, to determine the concentration of the two isoflavones as part of an extended undergraduate laboratory experiment to demonstrate compound isolation and structure elucidation in drug discovery.[15] Tsao and co-workers quantified osajin and pomiferin in osage orange by gradient RP-HPLC-DAD (acetonitrile in 2% aqueous acetic acid) as part of an investigation of the antioxidant activities of these compounds.[1] The methods reported by Whaley and Tsao were only partially validated as they were not the main focus of their projects.

Because of the growing interest in osage orange as a renewable source for osajin and pomiferin as lead compounds for drug discovery and to guide decision making on the optimum procedures leading to rich extracts and/or better isolation yields

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of these two compounds, the need for a fast, simple, and economical analytical method became obvious. Thus, we hereby describe the development, validation, and application of a new method for the detection and quantification of osajin and pomiferin in osage orange fruit using RP-HPLC with UV detection. Validation parameters included linearity, precision, accuracy, range, specificity, and limits of detection and quantitation following established guidelines.[16] The method was used to determine the levels of osajin and pomiferin in fresh and dry osage orange samples collected from the Midwest and southern United States. The effects of such factors as sample preparation, geographical location, and stage of ripeness on the levels of the two isoflavones were investigated. This method can also be used for the quality control of osage orange-based products that may become available in the future as herbal dietary supplements.

MATERIALS AND METHODS

Chemicals, Samples, and Solvents. Reference osajin and pomiferin were isolated from horse apple by an automated normal-phase flash chromatography method developed in our laboratory. Compound identity and purity were established by 1D and 2D NMR spectroscopy. The NMR data for both compounds were in agreement with published reports.[12,17] Osage orange fruits were gathered from two areas southwest of Chicago, Illinois (Frankfort and Thornton) and from northern Mississippi (Oxford) between August and December, 2011. Samples were coded as follows: Frankfort, IL, August, 2011 (Fr08, unripe); October, 2011 (Fr10); December, 2011 (Fr12); Thornton, IL, December, 2011 (Th12); Oxford, MS, December, 2011 (Ox12). Voucher specimens of collected samples are preserved at the College of Pharmacy, Chicago State University. All standard solutions, calibrators, quality controls, and unknowns were prepared using HPLC quality solvents (Fisher Scientific, Pittsburgh, PA).

Instrumentation and Chromatographic Conditions. An ultrasonic bath (model FS30D, Fisher Scientific, Pittsburgh, PA) was used to facilitate dissolution and extraction of standards and samples. A 5810 R model centrifuge (Eppendorf, Hamburg, Germany) was used for phase separation in sample extraction. An LC-2010 HPLC liquid chromatography system (Shimadzu, Kyoto, Japan) consisting of of a UV detector, pump, and autoinjector, and equipped with a HyPURITY C18 column (150 × 4.6 mm, 3 μm, Thermo Scientific, Waltham, MA) and an Econosphere C18 guard column (7.5 × 4.6 mm, Grace, Boca Raton, FL) was employed. The method used a mobile phase of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 1.0 mL/min with a linear gradient programmed as follows for solvent B (in A): 0−0.5 min 80%; 0.5−3.5 min 80−100%; 3.5−4.5 min 100%; 4.51−8.0 min 80%. Peak detection was performed at 274 nm. Chromatographic data were processed with LCSolutions software (Shimadzu, Kyoto, Japan) running under Windows XP. Data compilation and statistical analysis was performed by Prism, version 3.0 (GraphPad Software, La Jolla, CA)

Standard Solutions. *Standard Stock Solution A (For the Calibration Curve).* Osajin and pomiferin (5.0 mg each) were transferred to a 10-mL volumetric flask. Methanol (ca. 8 mL) was added, and the solution was ultrasonicated for 5 min to dissolve the two compounds. The volume was completed to mark with methanol.

Standard Stock Solution B (For Accuracy Determination). The same procedure described above was followed using 4.5 mg of each of osajin and pomiferin in 10 mL of methanol.

Method Validation. *Calibration Curve.* Standard stock solution A was used to prepare five concentrations (125.0−7.8 μg/mL) of osajin and pomiferin by serial dilution with methanol. Each concentration was injected in triplicate. A five-level calibration curve was automatically generated by linear regression of peak areas corresponding to the injected standard concentrations. The regression equation and coefficient (R) for each standard were automatically calculated and stored by the LCsolution software as part of the analytical method.

Limits of Detection (LOD) and Quantitation (LOQ). Three additional concentrations (3.9−0.98 μg/mL) were prepared as described above. The lowest concentration at which a peak was detected but was not accurately quantified was selected as LOD. The lowest concentration of the calibration curve (7.8 μg/mL) was selected as LOQ for both compounds.

Accuracy. To validate calibration curve accuracy, four quality control samples were prepared from standard stock solution B by serial dilution with methanol to obtain a range of 4 concentrations from 112.5−14.1 μg/mL. Each sample was analyzed three times after which its mean determined concentration was compared to the actual concentration and reported as percent accuracy for that level. For extraction efficiency, two 25-mg portions of a dry exhausted sample (a thoroughly extracted sample whose residue was dried and used as a matrix for spiking) were weighed and each placed in a 15-mL falcon tube. To the first tube, 2 mL of standard stock solution B (900.0 μg/25 mg sample or 90.0 μg/mL in final solution) was added followed by 1 mL of MeOH. To the second tube, 400 μL of standard stock solution B (180.0 μg/25 mg sample or 18.0 μg/mL in final solution) was added followed by 2.6 mL of MeOH. Both tubes were ultrasonicated in a water bath for 15 min followed by centrifugation at 2500 rpm for 5 min. The supernatant in each tube was decanted into a 10-mL volumetric flask. Each residue was subjected to two additional rounds of extractions using 3 mL of methanol per round. After 3 rounds of extraction, the combined extract in each flask was completed to volume with MeOH. About 1 mL of each solution was filtered into a separate HPLC vial using a glass syringe equipped with a 0.45 μm nylon filter. Both samples were analyzed in triplicate. The concentration of each sample was determined and calculated as percent recovery of the spiked concentration.

Precision. The relative standard deviation (RSD%) was calculated for each mean of the determined isoflavones in both validation and analytical runs to determine intraday repeatability. Interday precision was validated by calculating RSD % for the means of measurements (n = 3) obtained in three consecutive days at two concentration levels, 18.0 and 90.0 μg/mL, of stock solution B.

Sample Preparation and Analysis. For each collection, one-half of a fresh whole fruit was cut into smaller pieces and homogenized into a puree using a hand blender. Part of this puree was packed into a 50 mL falcon tube, labeled as fresh (by adding FP to the original sample label, e.g., Fr12FP), and stored in the freezer at 0−4 °C. The remaining puree was used to prepare the air-dried puree (AP) and oven-dried puree (OP) samples. The air-dried puree samples were prepared by placing the fresh puree in a weigh boat under the hood for approximately 4 days until a brittle mass with a fixed weight was obtained. The remaining fresh puree was dried in an oven at 80 °C for 48 h. Once dried, both sample types were ground into a fine powder using a coffee grinder and transferred into a capped plastic bottle and labeled (by adding AP and OP to the original sample labels, respectively, e.g., Fr12AP and Fr12OP). The remaining half of each fruit was cut into 2−3 mm-thick slices and dried in an oven at 80 °C for 48 h (fixed weight). The dried slices were ground to a fine powder in a coffee blender, transferred to capped plastic bottles, and labeled as oven-dried slices (by adding OS to the existing label, e.g., Fr12OS). The total number of samples generated was 20 samples corresponding to the 5 horse apple collections at different times and/or locations (4 different preparations per osage orange collection).

Dry samples were extracted by transferring 25 mg of the finely ground powder of each sample to a 15-mL falcon tube followed by the addition of 3 mL of methanol. Each suspension was ultrasonicated for 15 min followed by centrifugation at 2500 rpm for 5 min. The clear

Table 1. Chromatographic and Calibration Curve Properties of Osajin and Pomiferin

parameter	osajin		pomiferin
retention time (min)	5.63 ± 0.03		4.75 ± 0.03
resolution factor		4.5	
range (μg/mL)		7.8−125.0	
regression equation	$y = 1.917 \times 10^{-5}x - 2.14$		$y = 1.417 \times 10^{-5}x - 2.06$
regression coefficient (R)		0.999	
LOQ (μg/mL)		7.8	
LOD (μg/mL)		1.9	

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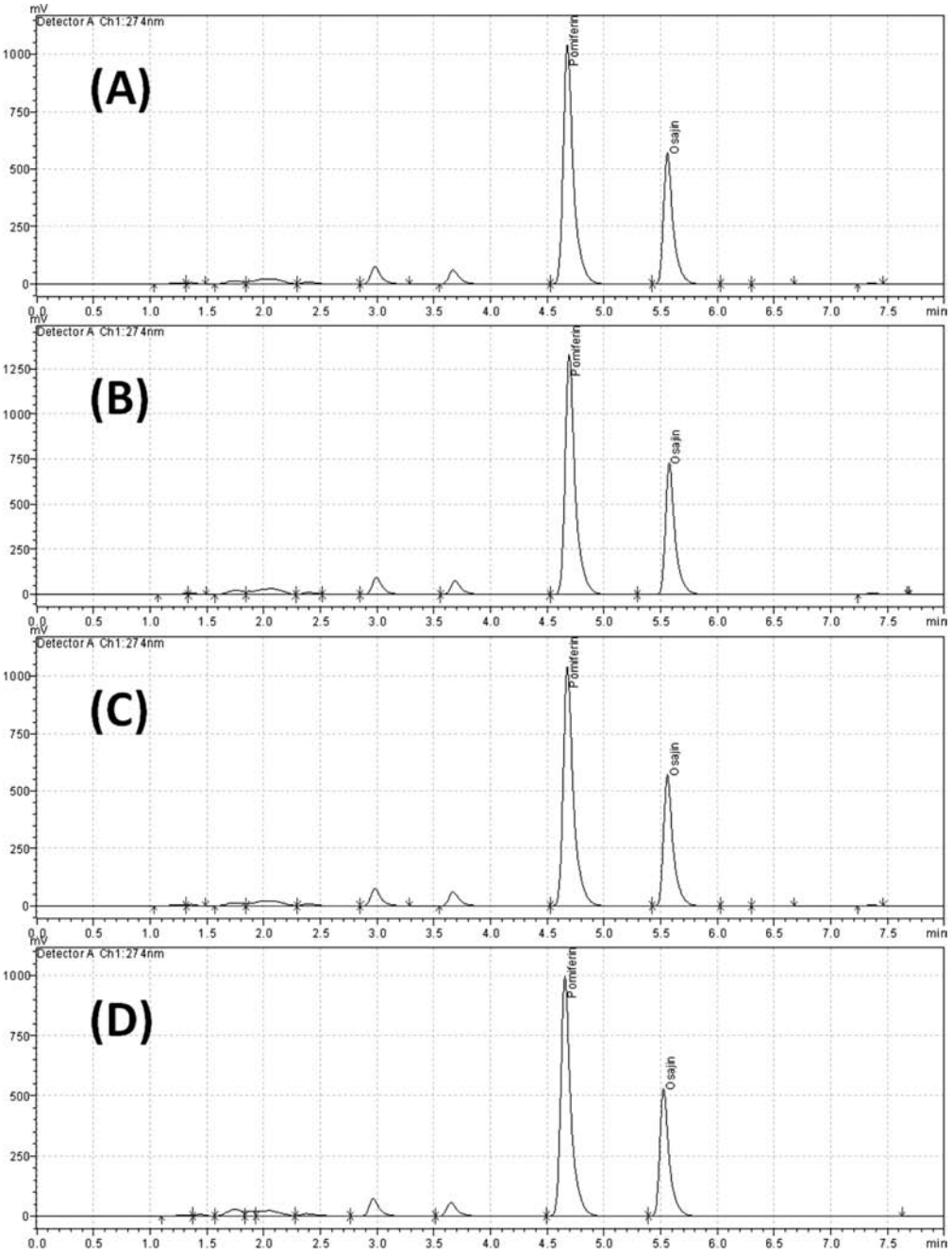

Figure 2. Representative HPLC chromatograms of Osage orange samples collected from Thornton, IL and prepared from (A) fresh puree, (B) air-dried puree, (C) oven-dried puree, and (D) oven-dried slices.

supernatant from each tube was poured into a labeled 10 mL volumetric flask. This procedure was run in triplicate, and the decanted solution in each volumetric flask was brought to volume by adding a sufficient amount of methanol (ca. 1 mL). Fresh samples were extracted by weighing 100 mg of each sample and following the same ultrasonication and centrifugation procedure described above for the dried samples. An aliquot of each sample (1−2 mL) was transferred to an HPLC vial using a glass syringe equipped with a 0.45 μm nylon filter. The concentration of each sample was calculated as the mean of three injections (10 μL per injection).

■ RESULTS AND DISCUSSION

Table 1 summarizes validation results related to specificity, calibration curve, LOD, and LOQ for each isoflavone. Method specificity was apparent in the baseline chromatographic separation of the two isoflavones (Figure 2) with a resolution factor of more than 4. The calibration curves of both compounds were linear in the specified range with correlation coefficients of 0.999 for their respective linear regression equations. Since quantitation was not accurate below the lowest level of the calibration curve, that level (7.8 μg/mL) was determined to be the LOQ for both compounds. The LOD was determined to be 1.9 μg/mL because it was the lowest concentration at which a peak could be detected. Peaks below 1.9 μg/mL were neither detectable nor quantifiable.

Results of method accuracy and precision are shown in Tables 2 and 3. Validation of accuracy was achieved by two methods. Calibration curve accuracy was validated by analyzing four quality control samples prepared from a different standard stock solution than the one used to generate the calibration

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Table 2. Validation of HPLC Method Accuracy and Intraday Precision for Osajin and Pomiferin[a]

marker		actual concn (μg/mL)	determined concn (μg/mL)	precision (RSD %)	accuracy (%)
osajin	(A) quality controls	14.1	13.8 ± 0.4	2.9	97.9
		28.1	28.6 ± 0.1	0.4	101.8
		56.3	57.8 ± 0.2	0.3	102.7
		112.5	110.8 ± 0.1	0.1	98.5
	(B) extraction efficiency	18.0	17.1 ± 0.3	1.8	95.0
		90.0	87.7 ± 0.3	0.3	97.4
pomiferin	(A) quality controls	14.1	14.1 ± 0.3	2.1	100.0
		28.1	28.7 ± 0.1	0.3	102.1
		56.3	57.8 ± 0.1	0.2	102.7
		112.5	112.0 ± 0.1	0.1	99.6
	(B) extraction efficiency	18.0	17.2 ± 0.2	1.2	95.6
		90.0	88.5 ± 0.3	0.3	98.3

[a]On the basis of $n = 3$.

Table 3. Validation of Interday Precision for Osajin and Pomiferin[a]

marker	actual concn (μg/mL)	determined concn (μg/mL)	precision (RSD %)	accuracy (%)
osajin	18.0	17.7 ± 0.1	0.6	98.3
	90.0	86.9 ± 0.6	0.7	96.6
pomiferin	18.0	18.2 ± 0.1	0.5	101.1
	90.0	89.7 ± 0.7	0.8	99.7

[a]For 3 consecutive days and based on $n = 3$ per day.

curve (to minimize quantitation bias). As seen in Table 2, the determined concentrations were 98.1−102.7% and 99.6−102.6% for osajin and pomiferin, respectively. Spiking and recovery from an inert matrix (exhausted sample) was performed at two concentration levels to further validate method accuracy under the described sample preparation procedure. Overall extraction efficiency of spiked samples ranged from 95.0 to 98.3% with a comparable range for both isoflavones (Table 2). Values were within range for both intraday (Table 2) and interday (Table 3) precision with RSD % of less than 5%.

Application of the developed method provided reliable results that not only confirmed previous findings but also added to our current knowledge about horse apple growing near Chicago and in northern Mississippi. The average ratio between osajin and pomiferin was about 1:2, which is similar to the ratio reported by Kartal et al. for the exocarp content of the two isoflavones in horse apple cultivated in Ankara.[14] This ratio was generally not affected by the geographical location, developmental stage, or sample preparation method. Our findings revealed that samples collected from Thornton, IL had the highest total content of both isoflavones (6.2%, dry samples), while those collected from Frankfort, IL around the same time had the lowest concentration (4.3%, dry samples) (Figure 3A). These observed levels further confirm that osage orange is a rich natural source of both osajin and pomiferin irrespective of geographical location. Since the fruiting season starts in August and ends in December, with fruits reaching their maximum size and weight of up to 500 g, we compared the levels of both isoflavones in three samples collected from Frankfort, IL at early, mid, and late season (Figure 3A). Interestingly, the levels of osajin gradually increased from early to late season, while those of pomiferin were relatively constant during the same period with a slight increase around mid season. To investigate the effect of sample preparation conditions on isoflavone levels, each sample was prepared by four different methods (described above). At an

Table 4. Levels of Osajin and Pomiferin in Collected Osage Orange Samples[a]

	osajin		pomiferin	
sample	found (μg/mL ± SD)	content % (mg/g)	found (μg/mL ± SD)	content % (mg/g)
Fr08FP	13.0 ± 0.1	**0.1 (1.3)**	35.5 ± 0.0	0.4 (3.6)
Fr08OS	14.4 ± 0.1	**0.6 (5.8)**	40.2 ± 0.1	1.6 (16.1)
Fr08OP	24.6 ± 0.4	**1.0 (9.8)**	55.7 ± 0.1	2.2 (22.3)
Fr08AP	23.8 ± 0.3	**1.0 (9.5)**	58.2 ± 0.4	2.3 (23.3)
mean[b]		0.8 (8.4)		2.1 (20.6)
Fr10FP	23.8 ± 0.6	**0.2 (2.4)**	58.4 ± 0.2	0.6 (5.8)
Fr10OS	28.6 ± 0.0	**1.1 (11.4)**	69.4 ± 0.1	2.8 (27.8)
Fr10OP	27.9 ± 0.2	**1.1 (11.1)**	64.3 ± 0.2	2.6 (25.7)
Fr10AP	28.2 ± 0.1	**1.1 (11.3)**	67.5 ± 0.3	2.7 (27.0)
mean[b]		1.1 (11.3)		2.7 (26.8)
Fr12FP	30.1 ± 0.3	**0.3 (3.0)**	38.7 ± 0.1	0.4 (3.9)
Fr12OS	47.8 ± 0.5	**1.9 (19.1)**	58.9 ± 0.0	2.4 (23.6)
Fr12OP	50.6 ± 0.1	**2.0 (20.3)**	60.1 ± 0.2	2.4 (24.1)
Fr12AP	47.0 ± 0.1	**1.9 (18.8)**	59.6 ± 0.3	2.4 (23.8)
mean[b]		1.9 (19.4)		2.4 (23.8)
Th12FP	37.8 ± 0.1	**0.4 (3.8)**	90.3 ± 0.2	0.9 (9.0)
Th12OS	34.7 ± 0.4	**1.4 (13.9)**	84.7 ± 0.1	3.4 (33.9)
Th12OP	53.5 ± 0.7	**2.1 (21.4)**	124.4 ± 0.0	5.0 (49.8)
Th12AP	49.8 ± 0.1	**2.0 (19.9)**	114.0 ± 0.1	4.6 (45.6)
mean[b]		1.8 (18.4)		4.3 (43.1)
Ox12FP	44.5 ± 0.3	**0.5 (4.5)**	86.7 ± 0.2	0.9 (8.7)
Ox12OS	38.9 ± 0.4	**1.6 (15.5)**	80.1 ± 0.3	3.2 (32.0)
Ox12OP	46.6 ± 0.0	**1.9 (18.6)**	88.5 ± 0.0	3.5 (35.4)
Ox12AP	52.0 ± 0.4	**2.1 (20.8)**	100.7 ± 0.0	4.0 (40.3)
mean[b]		1.8 (18.3)		3.6 (35.9)

[a]Geographical locations and sample preparation methods are described in the text. [b]Dry samples only (FP samples are excluded due to the diluting effect of water content).

average of 0.9%, the levels of both isoflavones were significantly lower in all fresh samples compared to dry ones (average of 4.5%) (Figure 3B). This finding is consistent with the fact that all fresh samples lost 75−80% of their weight upon drying. Thus, the ratio of isoflavone levels between fresh and dry samples (20%) correlates well with the dilution effect of water in the fresh fruits, and it also demonstrates that both isoflavones were stable to the applied drying conditions, both at room temperature and at 80 °C. Drying temperature had no significant effect on isoflavone levels as Table 4 shows for the oven-dried and air-dried purees (OP and AP, respectively) of each set of collected samples. The concentration of

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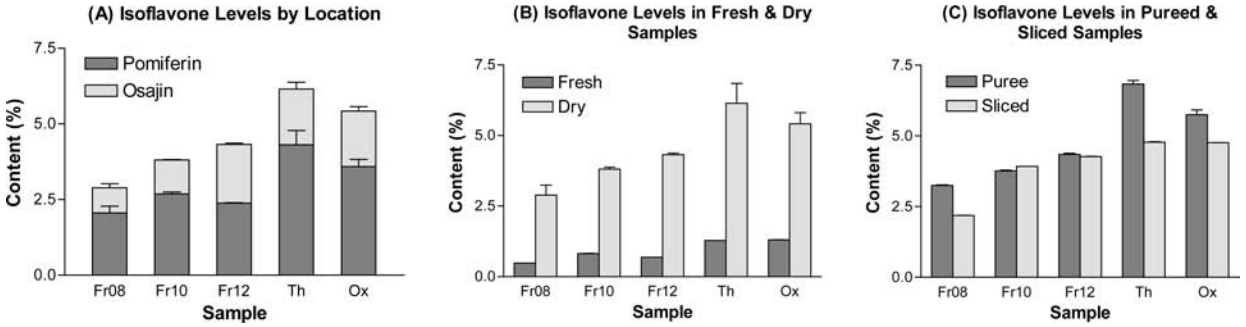

Figure 3. Effect of various conditions on the levels of osajin and pomiferin in osage orange. (A) Geographical location and growth stage; (B) fresh and dry samples; (C) freshly homogenized and sliced samples.

both isoflavones was also found to be consistent with results published by Tsao et al. for fresh samples from Ontario, Canada.[1] The effect of sample homogenization was studied by comparing isoflavone levels in sliced and pureed samples that were powdered after drying. The average isoflavone content in pureed samples (4.8%) was approximately 20% higher than that of sliced samples (4.0%), but considering the variation in individual sample data, it may be hard to generalize this observation (Figure 3C). Our collective data suggests that variation in preparation methods had no significant effects on isoflavone stability and levels in the analyzed osage orange samples. Therefore, any method may be adopted as appropriate.

In conclusion, a simple analytical HPLC method was developed and applied to identify/recommend rich *M. pomifera* sources of the isoflavones osajin and pomiferin and to recommend efficient procedures for their preparation from osage orange. With an analytical run time of 8 min, the developed method is the fastest and most economical among all those reported so far. Results obtained from the analytical runs showed a range of 4−6% of total isoflavones in full grown dry samples and minimal variation in such content under different preparation methods, which further confirms that osage orange is a rich and sustainable source of osajin and pomiferin, irrespective of geographical location. Pure isoflavones can be easily isolated from the fruits for further biological evaluation, chemical modification, or utilization as reference standards. Also, based on the growing body of evidence, the fruit extract or the dried powdered fruit may be considered for development as a potential antioxidant herbal dietary supplement.

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Notes

The authors declare no competing financial interest.

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