Assessment of Dietary Isoflavone Intake among Middle-Aged Chinese Men

Sang-Ah Lee,1,2 Wanqing Wen,2 Yong-Bing Xiang,3 Stephen Barnes,4 Dake Liu,3 Qiuyin Cai,2 Wei Zheng,2 and Xiao Ou Shu2*

1Department of Health Promotion and Disease Prevention, Shanghai Jiao Tong University School of Public Health, Shanghai, China; 2Department of Medicine, Vanderbilt Epidemiology Center, Vanderbilt University School of Medicine, and Vanderbilt-Ingram Cancer Center, Nashville, TN 37232-8300; 3Department of Epidemiology, Shanghai Cancer Institute, Shanghai 200032, P. R. China; and 4Department of Pharmacology and Toxicology, University of Alabama, Birmingham, AL 35294

Abstract

We evaluated the reproducibility and validity of the FFQ used in the Shanghai Men’s Health Study (SMHS) for assessing dietary isoflavone intake, using multiple 24-h dietary recalls (24-HDR) and urinary isoflavones as the reference criteria, with data from the dietary validation study of the SMHS. A total of 196 study subjects completed the 24-HDR and 2 FFQ and donated a quarterly spot urine sample during the 1-y study period. Levels of urinary isoflavones were measured in a random sample of 48 study participants. The correlation coefficient between the 2 FFQ administered 1 y apart was 0.50 for soy protein intake and ranged from 0.50 to 0.51 for isoflavone intake. The correlations of isoflavone intake from the second FFQ with those from the multiple 24-HDR ranged from 0.38 (genistein) to 0.44 (glycitein), and the correlations with urinary isoflavone levels were 0.48 for total isoflavones, 0.44 for daidzein, 0.42 for genistein, and 0.54 for glycitein. The intraclass correlation coefficients for the 4 spot urine samples were 0.36, 0.42, and 0.40 for daidzein, genistein, and glycitein, respectively, and 0.62, 0.68, and 0.55 for their metabolic products equol, dihydrodaidzein, and O-desmethylangolensin, respectively. These results suggest that the SMHS FFQ can reliably and accurately measure usual intake of isoflavones, and that the levels of isoflavones in urine samples are relatively stable among men in Shanghai.


Introduction

Experimental studies have shown that isoflavones have antiviral, antiangiogenic, and antioxidant properties (1). Because the chemical structure of isoflavones is similar to estrogen, and because isoflavones can bind to estrogen receptors, it has been suggested that isoflavone intake may protect against estrogen-sensitive cancers (2–4). In addition, isoflavones can inhibit steroid metabolizing enzymes, including 5α-reductase and aromatase, and also may alter cell-signaling by the inhibition of tyrosine-specific protein kinases (1). Dietary soy is a rich source of isoflavones, and it has been suggested that high intake of soy food alters the risk for many diseases, such as breast cancer, prostate cancer, and cardiovascular disease and affects bone health (5–7). However, the evidence for these claims is not entirely consistent (8). One reason for the inconsistency may be the challenge in epidemiologic studies to accurately estimate isoflavone intake over a long period of time. The FFQ is the most commonly used instrument in the investigation of the relationship between dietary factors and chronic disease in large-scale population studies. Validating a FFQ often poses a challenge because of the lack of a true standard reference measurement and correlated random errors between the reference measurement and the FFQ (9,10). Two or more valid reference measurements, each of which has a different source of error, are an effective means of addressing this issue (11).

In this article, we describe the validity and reproducibility of the FFQ used in the Shanghai Men’s Health Study (SMHS)5 for assessing dietary isoflavone intake. We also describe the usefulness of a spot urine measurement as a biomarker for future research on isoflavones and disease risk.

Methods

Subjects. The parent study, the SMHS, is a population-based cohort study. All men who resided in the 8 study communities and were between the ages of 40 and 74 y were eligible for the study. Trained interviewers visited the homes of 83,107 eligible men and 74.1% (n = 61,582) were recruited into the study. Reasons for nonparticipation were refusals (21.1%), absence during the study period (3.1%), and other miscellaneous reasons including poor health or hearing problems (1.7%). The study protocol was approved by the Institutional Review Boards of the Vanderbilt University Medical Center and the Shanghai Cancer Institute., and all participants provided written, informed consent.

Between April 2003 and May 2004, we carried out a dietary validation study among SMHS participants. Participants were randomly

1 This work was supported by USPHS grant R01CA82729 from the National Cancer Institute. Funds for the mass spectrometer were provided by an NIH/NCRR Shared Instrument Grant (S10 RR 19231), and operation of the UAB mass spectrometry shared facility came from a Cancer Center Core Support grant (P30 CA13148; E. Partridge, PI) and the UAB Center for Nutrient-Gene Interaction (U54 CA100949, S. Barnes, PI).
2 To whom correspondence should be addressed. E-mail: xiao-ou.shu@vanderbilt.edu.
3 Abbreviations used: 24-HDR, mean of multiple 24-h dietary recalls; ICC, intraclass correlation coefficients; JPHC, Japan Public Health Center; O-DMA, O-desmethylangolensin; SMHS, Shanghai Men’s Health Study.
selected from SMHS rosters for 2 of the 8 study communities. Fifteen
trained interviewers were hired to conduct unannounced, in-person
interviews, and 17 primary and 68 alternate candidate subjects were
selected for each interviewer to target for possible recruitment. A total of
214 subjects were recruited into the study, 69.3% of whom were primary
candidates. The FFQ, which assessed usual dietary intake over the
previous 12 mo, was administered twice as part of the in-person
interview over an ~12-mo period (the mean interval was 1.2 y and
ranged from 0.9 to 2.1 y). Of the 214 recruited subjects who completed
the first FFQ, 196 subjects (91.6%) completed the second FFQ interview,
and at least 24-h dietary recalls (24-HDR). These subjects constitute
the study sample for this analysis. A peripheral blood sample and spot
urine sample were collected from the study participants every 3 mo (up
to 3 years), and a completed FFQ was sent every 6 mo. In addition, the
interval between the last meal and the collection of the samples was
documented. The biological samples were placed in an ice box (4°C),
processed within 6 h, and stored at −70°C.

Estimation of isoflavone intake from 24-HDR and FFQ. The refer-
cence instrument, a 24-HDR, was administered once a month over the
course of 1 y for the 196 subjects. At each administration, the study
subjects were asked to record the names and amounts of the foods that
they had consumed over the preceding 24 h. The days that the 24-HDR
were administered were chosen to ensure a balanced representation of
weekdays and weekend days. All 196 subjects completed at least 10 24-HDR.

The SMHS FFQ includes 81 foods and food groups that cover >88%
of the commonly consumed foods in urban Shanghai (12). The FFQ
includes the following 8 soy and isoflavone-rich food items or groups:
soy milk, bean curd, fried bean curd, dried soybeans, mung beans,
soybean sprouts, peanuts, and fresh beans (baby soy beans, fresh peas,
fresh broad beans, and yard long beans). For each food item or food
group, subjects were asked about the frequency (daily, weekly, monthly,
yearly, or never) and the amount in liang (liang

The soy isoflavone intake levels derived from the FFQ and 24-HDR were
operated in the negative mode with a source temperature of 400
operating mass spectrometry analysis was performed after injection of 5
ul. The concentration of B in the mobile phase was increased at 5% per min
to 70% B over a 0–10 min period. Then, the concentration of B was decreased at 50% per
min for 1 min and then held isocratically for a further 4 min. The total cycle time
was 15 min per sample. The mobile phase flow rate was 0.2 mL/min. The
column eluate was passed into the chemical ionization interface of a MDS-
Sciex 4000 Qtrap triple quadrupole mass spectrometer. The interface was
operated in the negative mode with a source temperature of 400°C and a
nebulizing current of ~3 amps. Each isoflavone and metabolite ion was
detected by selecting their molecular ion [M-H]− in the first quadrupole,
fragmenting it with collision gas, and measuring a selected, specific frag-
ment ion in the third quadrupole. Quantification was performed using
peak areas after adjustment for internal standard recovery (chrysin, phe-
nolphthalein glucuronide, and 4-methylumbelliferyl sulfate). Variation
in the assays was concentration dependent. The lowest variation (3–6%)
occurred for the highest concentrations (>1000 nmol/L), intermediate
variation (4–10%) for concentrations between 100 and 1000 nmol/L,
and the highest variation (10–30%) for concentrations <100 nmol/L. The low 23
percentile cutpoints for isoflavone levels among men in our validation
study were 189 nmol/L for equol, 3,095 nmol/L for glycine, 5,955 nmol/L
for genistein, 20,105 nmol/L for daidzein. We adjusted the amount of urine
sample to be tested in this study to achieve optimal assay perfor-
mance conditions.

All samples were independently worked up and analyzed in duplicate.
The assays were performed in Dr. Stephen Barnes’ laboratory at the
University of Alabama at Birmingham. Urinary creatinine level was mea-
sured using a VITROS CREA slide method on a VISTOS 250 Analyzer.

Statistics. Means and percentages of selected demographic character-
istics of the study subjects were compared with the parent cohort, using a Z-test or 1-way Chi-square test. The reproducibility of the FFQ was
evaluated by comparing the medians of the soy and isoflavone intakes
derived from the 2 FFQ and calculating the Spearman correlation coef-
ficients between them. Partial correlation was estimated with adjustment
for age and energy intake.

The validity of the FFQ was evaluated by comparing the second FFQ
with 2 reference measurements: the 24-HDR and urinary isoflavone
levels. The reason for choosing the second FFQ is because it covered the
same period of time (the previous 12 mo) as that of the 24-HDR and the
urine samples. The Spearman correlation coefficients between the FFQ
and the 24-HDR were estimated and adjusted for age and total energy
intake. The correlation coefficients between the FFQ and urine samples
were additionally adjusted for the interval between the time of the most
recent meal and the time of urine collection, medicine use status, and
number of cigarettes smoked during the previous 24 h. The compared
continuous variables were also categorized into quartiles. The percent-
ages of agreement for same quartile (in complete agreement), adjacent
quartiles (in partial agreement), skip 1 quartiles (in partial disagree-
ment), and opposite quartiles (in disagreement) were calculated.

To determine the stability and usefulness of a single spot urine
measurement as a biomarker for long-term exposure to soy intake, we
evaluated the Spearman correlation coefficients between the individual
measurement of seasonal samples and the mean measurement of the 4
urine samples from each subject. In addition, we randomly selected 1 of
the 4 urine measurements from each of the 48 subjects, and the correlation
coefficients between those randomly selected single measurements and
the mean of 4 measurements were calculated using the bootstrap method.
We repeated the bootstrap process 2,000 times to derive the point
estimates and 95% CI for the correlation coefficients. Finally, the intraclass corre-
lation coefficients (ICC) were estimated to evaluate the within-person
seasonal variability of urinary isoflavones. In the analysis of urinary iso-
flavones, smoking and medication use were adjusted for, because smoking
is known to influence estrogen metabolism (17), and medication use,
particularly antibiotic use, can affect phytoestrogen metabolism through
changing the micro-flora conditions in the human intestine (18).

All statistical analyses were completed with SAS, version 9.1 (SAS
Institute).

Results

The 196 study participants who completed 2 FFQ and the 24-
HDR and the 48 study participants in the urinary isoflavone
study were comparable to participants in the parent cohort for
basic demographic characteristics, selected lifestyle factors, and
total energy, soy, and isoflavone intake measured by the baseline
FFQ (Table 1). The only significant difference was that the
1 Spearman Correlation Coefficients adjusted for age and the total energy intake

TABLE 2

<table>
<thead>
<tr>
<th>Component</th>
<th>Median intake (unit/d)</th>
<th>1st quartile</th>
<th>2nd quartile</th>
<th>Same quartile</th>
<th>Adjacent quartile</th>
<th>Skip 1 quartile</th>
<th>Opposite quartile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kJ</td>
<td>8018.4</td>
<td>7895.9</td>
<td>0.50</td>
<td>44</td>
<td>41</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Soy protein, g</td>
<td>11.85</td>
<td>10.50</td>
<td>0.50</td>
<td>44</td>
<td>44</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>Isoflavone, mg</td>
<td>33.87</td>
<td>30.39</td>
<td>0.51</td>
<td>41</td>
<td>37</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Daidzein, mg</td>
<td>13.72</td>
<td>12.25</td>
<td>0.50</td>
<td>42</td>
<td>35</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>Genistein, mg</td>
<td>18.58</td>
<td>16.40</td>
<td>0.50</td>
<td>41</td>
<td>38</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>Glycitein, mg</td>
<td>2.77</td>
<td>2.45</td>
<td>0.50</td>
<td>40</td>
<td>37</td>
<td>21</td>
<td>2</td>
</tr>
</tbody>
</table>

1 Spearman Correlation Coefficients derived from the SMHS FFQ, were comparable to those reported

Discussion

The SMHS FFQ was designed to capture usual intake of soy food, as well as main nutrient and food intake, among men in urban Shanghai. The validity of the questionnaire for capturing nutrient intake has been described elsewhere (12). In this study, we found that the FFQ has reasonable reproducibility and validity for capturing major isoflavone intake among participants of the SMHS.

Levels of isoflavone intake in our study population, as determined by the SMHS FFQ, were comparable to those reported valid for capturing major isoflavone intake among participants of the SMHS. When isoflavone intake was categorized by quartile, complete or partial agreement (in the same or adjacent quartiles) ranged from 72 to 81% for isoflavones, and disagreement was from 3 to 6%. The Spearman correlation coefficients between isoflavone intake from the second FFQ and urinary isoflavone levels varied from 0.42 to 0.54 (P < 0.05), and complete or partial agreement by quartiles was 66 to 83%.

We evaluated the stability and usefulness of a single spot urinary isoflavone measurement as an objective and stable biomarker for assessing the association of isoflavones and health risks (Table 4). Mean urinary isoflavone levels were similar across the 4 seasons. The correlation between individual measurements and the mean measurement across 4 seasons was reasonably high, with the Spearman correlation coefficients ranging from 0.72 to 0.89 (P < 0.05). The correlation estimated by the bootstrap method between the randomly chosen single spot measurement and the mean measurements were also reasonably high; Spearman correlation coefficients ranged from 0.72 to 0.87 (P < 0.05). The ICC of the isoflavone levels from the 4 spot urine samples across 4 seasons ranged between 0.36 (daidzein) and 0.68 (equol).
The validity and reproducibility in our study is lower than that of the Japan Public Health Center (JPHC)-based study FFQ, the only FFQ for which information on the validity and reproducibility of dietary isoflavones \( (r = 0.57 \text{ and } r = 0.75, \text{ respectively}) \) is available (24). The validity and reproducibility of the JPHC study were examined among a group of male \((n = 102)\) and female \((n = 113)\) volunteers who were recruited from health clinics. People who visit health clinics regularly may pay closer attention to their dietary intake than the general population, which may explain the higher reproducibility found in that study. However, we cannot exclude the possibility that the consumption of soy food in the Japanese population may be more stable than in Chinese men.

One of the most challenging tasks in assessing the validity of a dietary instrument is to find a standard for measuring long-term dietary intake. In our study, we chose monthly 24-HDR and urinary isoflavone measurements over a 1-y period as the reference criteria for assessing usual dietary isoflavone intake. We found that the correlation for total isoflavones between the FFQ and 24-HDR was 0.4, lower than that reported in a US study of Caucasians (25) and the JPHC (24). The US study compared isoflavone intake estimated from an FFQ with dietary recalls \((r = 0.5) \) (25), whereas the JPHC used four 7-d dietary records \((r = 0.6) \) for comparison (24). Because a large proportion of Caucasian Americans do not eat soy food [only 9.55% have soy food more than once a day, according to reports from the 2001–2002 NHANES (27)], the high correlation between the FFQ and the reference criterion in the American population is not unexpected. In our study population, most men consumed soy food regularly (almost 100%). Thus, one would expect the variation of dietary soy intake to be smaller than that in the study of Caucasian Americans. For the JPHC validation study, in addition to the above-mentioned limitations relating to the nature of recruiting participants from health clinics and including both genders, the validity of the JPHC FFQ may also have been overestimated because the reference method used in the study (four 7-d dietary records) required weighing or measuring the foods eaten, as well as documenting the method of food preparation—practices that may result in a training effect (28). Similarly, the multiple 24-HDR used in our study could also have sensitized the study participants. We tried to limit the effect of sensitization by using biomarkers as the reference method in the validation study. In our study, the correlation between isoflavone levels derived from the FFQ and the mean measurement of the 4 spot urine samples was

### Table 3: Spearman correlation and agreement in quartile distribution of nutrient intake between second FFQ and mean of 24-HDR or urinary isoflavonoid measurements among 196 men, SMHS, 2002–2006

<table>
<thead>
<tr>
<th></th>
<th>Same quartile</th>
<th>Adjacent quartile</th>
<th>Skip 1 quartile</th>
<th>Opposite quartile</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd FFQ vs. 24-HDR, ( n = 196 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy</td>
<td>0.61</td>
<td>43</td>
<td>44</td>
<td>9</td>
</tr>
<tr>
<td>Soy protein</td>
<td>0.38</td>
<td>37</td>
<td>39</td>
<td>19</td>
</tr>
<tr>
<td>Isoflavone</td>
<td>0.39</td>
<td>32</td>
<td>40</td>
<td>22</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.40</td>
<td>33</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.38</td>
<td>33</td>
<td>41</td>
<td>22</td>
</tr>
<tr>
<td>Glycitein</td>
<td>0.44</td>
<td>40</td>
<td>41</td>
<td>15</td>
</tr>
<tr>
<td>2nd FFQ vs. urinary isoflavone using mean of 4 urine measurements, ( n = 48 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoflavone</td>
<td>0.48</td>
<td>38</td>
<td>42</td>
<td>18</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.44</td>
<td>31</td>
<td>52</td>
<td>13</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.42</td>
<td>46</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Glycitein</td>
<td>0.54</td>
<td>31</td>
<td>35</td>
<td>30</td>
</tr>
</tbody>
</table>

1 Spearman Correlation Coefficients adjusted for age and total energy intake except for energy.
2 Spearman Correlation Coefficients adjusted for the interval between the time of the most recent meal and the time of urine collection, medicine use status, number of cigarettes smoked during the previous 24 h, and total energy intake.

In other Asian studies, including Japanese (19–22) and Chinese populations (23), but they were much higher than levels reported for Caucasians in the USA (24). For example, the daidzein intake level in this study \((16.0 \text{ mg/d})\) was similar to levels reported for a Chinese population in Singapore \((16.6 \text{ mg/d})\) (23) and a Japanese population \((18.3 \text{ mg/d})\) (24), but higher than those from a study in Los Angeles \((7.6 \text{ mg/d}; \text{ Caucasian}, 0.74 \text{ mg/d})\) (25). Genistein intake estimates \((21.7 \text{ mg/d})\) were also similar to those from Asian reports \((\text{ ranging from } 10.8 \text{ to } 31.4 \text{ mg/d})\) (25). Genistein intake estimates \((21.7 \text{ mg/d})\) were also similar to those from Asian reports \((\text{ ranging from } 10.8 \text{ to } 31.4 \text{ mg/d})\) (25). Genistein intake estimates \((21.7 \text{ mg/d})\) were also similar to those from Asian reports \((\text{ ranging from } 10.8 \text{ to } 31.4 \text{ mg/d})\) (25). Genistein intake estimates \((21.7 \text{ mg/d})\) were also similar to those from Asian reports \((\text{ ranging from } 10.8 \text{ to } 31.4 \text{ mg/d})\) (25).

### Table 4: Medians of urinary isoflavone levels by season and stability of a single spot urinary isoflavonoids using bootstrap and ICC\(^1\,\,\,^2\)

<table>
<thead>
<tr>
<th></th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzein</td>
<td>44.65</td>
<td>42.20</td>
<td>41.23</td>
<td>49.91</td>
<td>44.45</td>
</tr>
<tr>
<td>Dihydrodaidzein</td>
<td>4.21</td>
<td>5.00</td>
<td>5.32</td>
<td>7.70</td>
<td>4.91</td>
</tr>
<tr>
<td>Equol</td>
<td>0.51</td>
<td>0.53</td>
<td>0.64</td>
<td>0.57</td>
<td>0.54</td>
</tr>
<tr>
<td>Genistein</td>
<td>20.29</td>
<td>21.12</td>
<td>18.00</td>
<td>19.58</td>
<td>19.50</td>
</tr>
<tr>
<td>Glycitein</td>
<td>15.49</td>
<td>11.22</td>
<td>13.99</td>
<td>27.66</td>
<td>12.28</td>
</tr>
<tr>
<td>O-DMA</td>
<td>6.21</td>
<td>4.57</td>
<td>4.57</td>
<td>9.02</td>
<td>5.38</td>
</tr>
<tr>
<td>Total isoflavones</td>
<td>122.29</td>
<td>106.76</td>
<td>92.16</td>
<td>114.61</td>
<td>109.50</td>
</tr>
</tbody>
</table>

1 Values are medians, \( n = 48 \) men who participated in the SMHS dietary validation study.
2 Data were log-transformed.
3 Detection limits: 5 nmol/L for daidzein and equol; and <5 nmol/L for the other isoflavonoids. The concentration levels of isoflavones for all of our study samples are higher than detection limits.
4 Spearman correlation coefficient between individual urinary isoflavones and the mean of the 4 urinary isoflavones.
5 Correlations between a randomly chosen individual measurement and averaged measurements, estimated using the bootstrap method.
and excreted within 12–24 h [24] may be a better measurement of isoflavone intake for the assessment of health for epidemiological studies.

Useful tool for the investigation of the effect of soy intake on mating isoflavone intake in the study population and provides a useful tool for the investigation of the effect of soy intake on health for epidemiological studies.

Urinary isoflavone levels are an aggregate measurement of the intake, absorption, and metabolism of isoflavonoids, and thus may be a better measurement of isoflavone intake for the assessment of health risks. Because isoflavonoids are water soluble and excreted within 12–24 h (~80% of isoflavonoids are excreted within 12 h [31,32]), there is concern that a single spot urine sample may not be able to capture usual exposure. In a US study conducted by Lampe et al. in 1999 [33], the correlations of dietary (derived from 5-d diet records) and urinary (derived from 24-h urine samples) isoflavonoids were 0.20 for daidzein, 0.25 for genistein, and 0.25 for total genistein + daidzein. In our study, although based on spot urine samples, the correlations between dietary and urinary isoflavonoids are much higher than those observed in the US population, suggesting that urinary isoflavones may be better biomarkers for measuring the effect of soy consumption on health in populations where soy consumption is common. This is particularly true for metabolites of isoflavonoids, such as equol, dihydrodaidzein, and O-DMA, which have lower within-person variability than their parent compounds. Furthermore, we found the correlation between the measurement of a single spot urine sample and the mean of 4 seasonal samples was relatively high, suggesting that a single spot urine sample can provide a reasonable measure of the long-term isoflavone exposure in a study population.

In conclusion, in this representative sample of the SMHS, using both multiple 24-HDR and urinary tests as the reference criteria, we have shown that the SMHS FFQ can provide a reasonably good measurement of dietary soy intake among middle-aged and elderly men in Shanghai. We have also shown that in a population with consistent consumption of soy foods, urinary isoflavone levels are relatively stable and levels of isoflavonoids measured in a single spot urine sample, particularly the metabolites of isoflavonoids, may serve as a valuable biomarker for research on the effects of isoflavonoids on health.

Acknowledgments
We thank Drs. Hui Cai and Qing Wang for their contributions in statistical support and in managing the biological samples, Mr. Ray Moore and Mr. Ali Arabshahi for their analysis of the urinary isoflavonoids, and Ms. Brandy Sue Bentley and Ms. Bethanie Hull for their assistance in the preparation of this manuscript.

Literature Cited


