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RAPID COMMUNICATIONS

Low-Temperature Treatment of Soybean (*Glycine max*) Isoflavonoid Aglycon Extracts Improves Gas Chromatographic Resolution

Keywords: *Gas chromatography; isoflavonoid aglycons; temperature; Glycine max*

High-performance liquid chromatography (HPLC) has long been the choice for analysis of isoflavonoids from plant sources. With HPLC, both free and conjugated moieties can be determined (Coward et al., 1993; Farmakalidis and Murphy, 1984; Nicollier and Thompson, 1982; Wang et al., 1990; Wang and Murphy, 1994). Over the years gas chromatography (GC) and, more recently, capillary gas chromatography have gained acceptance as viable methods for analyzing flavonoid and isoflavonoid aglycons (Bankova et al., 1992; Creaser et al., 1989; Furuya, 1965; Schmidt et al., 1994).

Techniques for isolating isoflavonoids rely upon some form of cleanup procedure to minimize interfering substances during instrumental analyses. These can be time-consuming and costly and can limit the numbers of samples analyzed over a period of time. Wang et al. (1990) described a simple, rapid method for isolation and HPLC analysis of total free isoflavonoids from soybeans without cleanup. However, it was not determined if the procedure was suitable for GC analysis using flame ionization detection (FID). It is likely that contaminants in the isolates may be masked during HPLC analyses resulting from differences in absorbance for the contaminants and isoflavonoids. However, these components may be prominent on GC-FID chromatograms.

This paper describes an efficient, cost effective method for the analysis of large quantities of soybean total isoflavonoid aglycons by gas chromatography.

MATERIALS AND METHODS

Extraction and isolation of total isoflavonoid aglycons from soybean seeds was performed according to the method of Wang (1990). Briefly, 200 mg of finely ground soybean meal was extracted for 2 h with 2.4 mL of 1 M HCl at 98−100 °C in a steam bath. Three sets of three replicates were analyzed over two experiments. After extraction, samples were spiked with a known quantity of quercetin as a quantitative standard to determine extraction efficiency. Samples were allowed to cool, 9.6 mL of acetonitrile was added, and the tubes were shaken for 1 min. After centrifugation to pellet particulate material, the replicates were treated as follows: (a) stored overnight at −8 to −10 °C; (b) stored overnight at room temperature; (c) analyzed immediately. After the overnight low-temperature treatment, samples were allowed to thoroughly warm to room temperature before processing for GC analysis as described below. In separate trials, 1 mL of chloroform was added to 2 mL aliquots from another set of samples either on the same day of extraction or after an overnight storage at room temperature. The mixture was shaken and then centrifuged to allow phase separation. The organic phase was recovered and the aqueous phase discarded.

One milliliter aliquots were removed from each sample and dried under nitrogen. Samples were converted to trimethylsilyl ether (TMSE) derivatives with 0.1 mL each of pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 60 °C for 30 min. Derivatized samples were analyzed directly on a Varian 3500 gas chromatograph equipped with FID, and data analyses were conducted using Varian Workstar software. The column was a 30 m × 0.32 mm i.d., 0.25 μm SPB-1 fused silica capillary column (Supelco Inc., Bellefonte, PA) operated under the following conditions: oven temperature, 240 °C; injector, 300 °C; detector, 300 °C; split ratio, 20:1; flow rate (He), 1.5 mL min^{-1} (average linear carrier gas velocity 36.5 cm s^{-1}); 15 psi column head pressure. Isoflavonoids were identified by comparison of retention times to those of authentic daidzein and genistein (Sigma Chemical Co., St. Louis, MO). Isoflavonoids were quantified from GC response factors produced by injecting 1 μg μL^{-1} genistein, which was used as an external GC standard.

RESULTS AND DISCUSSION

The method employed for the isolation of total isoflavonoid aglycons for subsequent HPLC analysis by Wang et al. (1990) is amenable to GC analysis. However, as expected, additional precautions must be taken to account for the differences in detection between the two chromatographic systems. Figure 1 shows a chromatogram of an aliquot taken directly from an extract and

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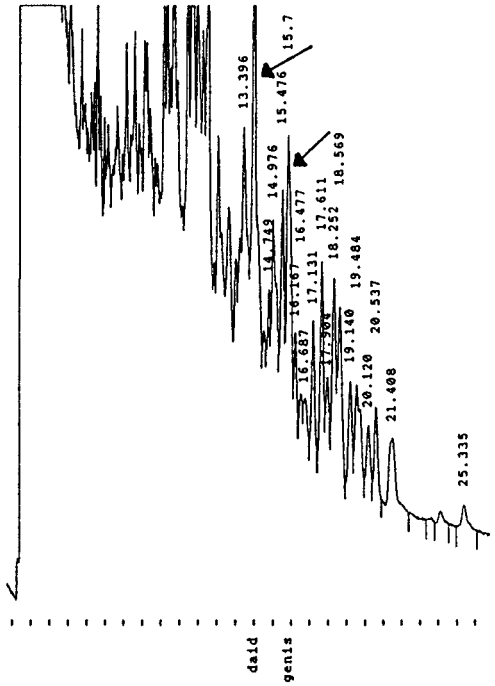

Figure 1. GC chromatogram of isoflavonoid aglycons sampled directly from the soybean seed extract (daid, daidzein; genis, genistein). Arrows denote peaks for daidzein and genistein, respectively. Retention time for quercetin (not shown) = 35.16 min.

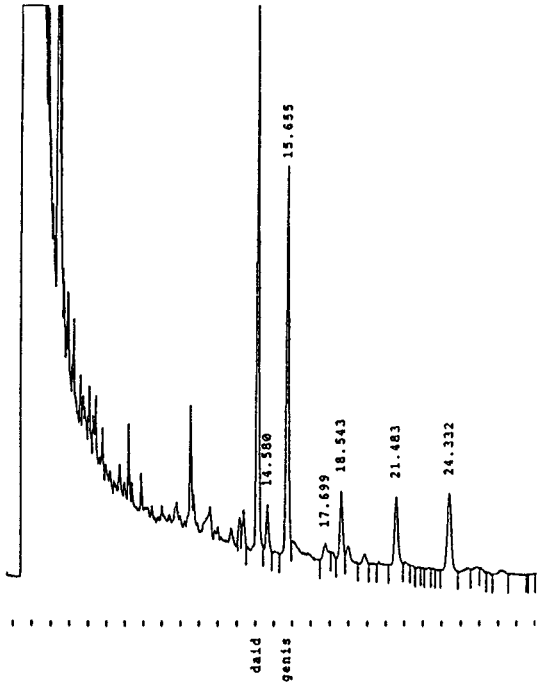

Figure 2. GC chromatogram of soybean seed isoflavonoid aglycons after overnight storage of extracts at −8 to −10 °C (daid, daidzein; genis, genistein). Retention time for quercetin (not shown) = 35.16 min.

prepared as described. What is most noticeable are the large numbers of extraneous peaks (whose identities were not determined for this paper) in addition to the major soybean isoflavonoids daidzein and genistein. The results were consistent regardless of whether the samples were analyzed on the same day of extraction or the next day.

Fortuitously, it was discovered that when the extract (or aliquots thereof) was placed under freezing conditions, the contaminants precipitate from solution. Figure 2 shows a chromatogram following overnight storage at freezing temperatures. A comparison of Figures 1 and 2 illustrates the striking effect of the low-temperature treatment.

In another attempt to remove the contaminants, chloroform was used to partition the extract with mixed results. Isoflavonoids partitioned into the organic phase, but they appeared to do so unevenly, resulting in a smaller peak area for daidzein than genistein as compared to the other chromatograms (Figure 3). An analysis of the aqueous phase showed that a portion of the daidzein remained in that fraction. However, as the figure shows, chloroform was effective in removing the contaminants. These results were also consistent regardless of whether the samples were partitioned with chloroform on the day of extraction or the next day. Though effective in removing the contaminants, chloroform partitioning may be unsuitable since it removes part of the compound of interest.

Quantitatively, the linearity of the detector response for daidzein and genistein was 0.96 with a linear range up to 4.5 μg μL^{-1} injection volume under the GC conditions employed. The lower detection limit was 1 ng μL^{-1} injection volume. For quercetin, the extraction standard, the amount recovered seemed to be enhanced by the low-temperature treatment. From samples that were stored at room temperature only 60−70% of the quercetin was recovered compared to 98−100% for those

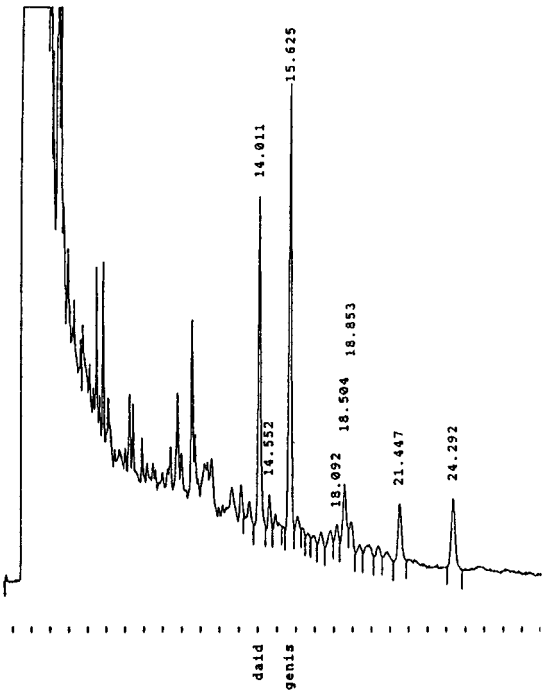

Figure 3. GC chromatogram of isoflavonoid aglycons after soybean seed extracts were partitioned with chloroform (daid, daidzein; genis, genistein). Retention time for quercetin (not shown) = 35.16 min.

stored under freezing conditions. Data for samples partitioned against chloroform showed the percentages of quercetin recovered to be similar to those from the low-temperature-treated samples. A direct comparison of quantitative data from the low-temperature treatment vs direct sampling was precluded by the extraneous peaks on chromatograms of samples analyzed directly. However, quantitative data for daidzein and genistein from low-temperature storage showed there to be 834.3 and 807.6 μg g^{-1} seed tissue, respectively. Though soybeans vary in their isoflavonoid content,

these values are within the ranges for isoflavonoid content reported elsewhere (Anderson and Wolf, 1995; Wang et al., 1990; Wang and Murphy, 1994). Additionally, when extracted particulate seed material was rewashed with additional acetonitrile and re-examined by GC analysis, there was no additional recovery of isoflavonoids. Thus, on the basis of the quercetin data, it seems that an apparent interaction that takes place between the isoflavonoids and other isolates is overcome by both low temperatures and chloroform partitioning. Glycitein, another soybean isoflavonoid, accounts for 10% of the total isoflavonoids. However, it was not accounted for during these analyses since the objective of the study was to devise a GC method to efficiently categorize soybeans on the basis of quantitative data for the two major isoflavonoid components.

The method described in this paper allows for the rapid analysis of soybean isoflavonoid aglycons by gas chromatography. Though the need to subject the extract to low-temperature delays analyses, it is still less costly and time-consuming than other cleanup methods. Whether this technique is effective on extracts from other plants or plant parts was not determined. The technique described in this paper does not supplant HPLC methodologies whose advantages have been discussed previously. Nonetheless, the reduced costs for solvents and solvent disposal associated with HPLC make this GC method seem attractive when attempting to analyze total aglycons from large numbers of soybean seed samples. This would be especially beneficial when performed in conjunction with screening programs to provide rapid data to soybean breeders attempting to enhance soybean germplasms, including isoflavonoid content.

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