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Reproducibility of the high-performance liquid chromatographic fingerprints obtained from two soybean cultivars and a selected progeny

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Abstract

Roots from soybean cultivars Williams 82 and Hartwig along with one of their progeny 14a, were extracted with non-polar, moderately polar, and highly polar solvent systems. Extracts were compared by thin-layer chromatography and by HPLC. Methanol extractions conducted at ambient temperature coupled with analysis by reversed-phase HPLC using UV detection provided the most representative sets of reproducible fingerprints. Further optimization of the overall protocol should allow for the profiling of different soybean cultivars when their roots are exposed to various environments and insults during early growth.

Keywords: Soybean; Roots; Phytoalexins

1. Introduction

The profile of proteins [1–3] and natural product compounds within a soybean plant (SB plant) at any given moment depends upon the nature of the plant's past and present environment and how the plant's genotype has been and is being expressed up to that precise point in time. For example, developmental and temperature influences can be extremely important during the early growth of a SB root [4,5]. As part of a program to profile SB plants, we are working toward establishing an extraction and chromatographic assay protocol that can be used to compare representative chemical constituent fingerprints within SB roots exposed to different biotic and abiotic stresses. However, the topic of chromatographic fingerprinting is itself complex and it can sometimes be confusing, especially when dealing with matrices that have several components [6], contain volatile materials [7], or contain materials that are subject to chemical transformation during extraction/analysis either spontaneously (e.g., autooxidation [8]) or by some solvent dependent process (e.g., pH dependent hydrolyses [9,10]). Since SB extracts contain several undefined components, some of which could be volatile or subject to

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chemical transformation, our strategy has been to address this situation in a stepwise manner according to the following sets of sequential but interdependent experiments:

(i) Address the fundamental question of whether or not it is even possible to obtain reproducible chromatographic fingerprints for a given SB cultivar root when the latter is repeatedly grown under well-controlled, non-stressed conditions and, if so, to further define the inherent variability in a statistical manner.

(ii) Establish similar background chromatograms for each stress factor, particularly the biotic types, and conduct preliminary stress experiments to ascertain if the general parameters of the extraction/analysis protocol deployed during the first set of experiments can also discern at least some type of gross differences among stress treated SB roots.

(iii) Optimize the protocol and conduct parallel runs of untreated controls and stress treated SB root experiments at repetitions high enough to allow for comparisons of subtle differences to be accomplished in a statistically relevant manner.

Follow-up studies to characterize materials deemed to be of interest at each step will deploy liquid chromatography–tandem mass spectrometry (LC–MS–MS). Ultimately, it is anticipated that the comparison of fingerprints and characterization of relevant materials from roots grown under normal conditions versus roots challenged by biotic and abiotic stresses will prove to be a useful tool for the selection of specific, desirable traits associated with SB roots. Toward this end, the studies reported herein specifically address the fundamental questions raised in steps (i) and (ii).

Previous chemical examinations of SB root have been conducted by others to define parameters associated with the production of a specific family of phytoalexins exemplified by the glyceollins, daidzein and genistein (Fig. 1), the latter being of particular interest due to their purported anticancer properties [11]. These types of compounds can be readily extracted from SB materials by using common alcoholic solvents. Reported silica gel thin-layer chromatography (TLC) developing systems include simple mixtures of organic solvents [12], sophisticated mixtures of organic solvents also containing an aqueous base [13], and elaborate multi-development strategies that deploy formamide-impregnated plates [14,15]. Several high-performance liquid chromatography (HPLC) assays have also been reported [16–20] as well as a few gas chromatography (GC)–MS methods [13,21]. Most of the HPLC methods have utilized a reversed-phase column with a gradient mobile phase consisting of an alcoholic component and an acidic aqueous buffer.

Since we wanted to purview a broad range of chemical constituents while establishing an extrac-

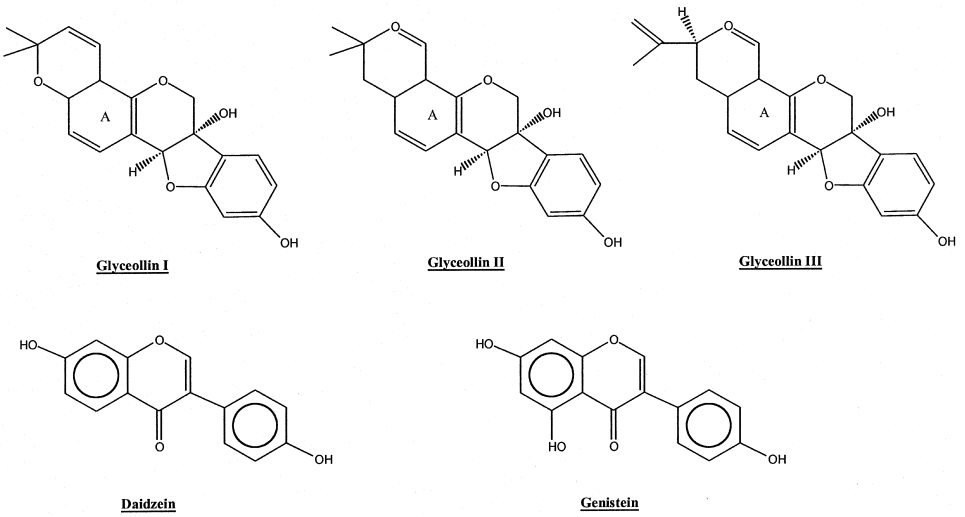

Fig. 1. Representative structures within soybean's key family of phytoalexins.

tion procedure that would ultimately provide for a representative, multi-component fingerprint rather than a fingerprint specifically geared toward certain pre-selected materials, a variety of extraction protocols were first examined in a quick manner by TLC deploying a relatively non-polar, moderately polar, and a highly polar TLC development system. These studies also provided a quick assessment about the volatility and stability of the materials being fingerprinted. The results from our initial studies were then used to guide the development of a generally representative extraction procedure that was coupled with an appropriate HPLC method such that the fingerprints for several different lots of single cultivar SB roots grown under well-controlled, identical conditions could be compared in a quantitative manner amenable to statistical scrutiny.

2. Experimental

2.1. Reagents and standards

All organic solvents were of HPLC grade and were purchased from either J.T. Baker (Phillipsburg, NJ, USA) or Fisher Scientific (Fairlawn, NJ, USA). HPLC-quality water was generated from a Milli-Q deionization system purchased from Millipore (Bedford, MA, USA). Ammonium acetate (NH_4OAc; HPLC/certified ACS grade), formic acid (HCO_2H; 88%; certified ACS grade), hydrochloric acid (HCl; trace metal grade), potassium phosphate, monobasic (KH_2PO_4; HPLC/certified ACS grade) and triethylamine (Et_3N; HPLC grade) were purchased from Fisher Scientific.

The standards daidzein (lot 38H4073) and genistein (lot 87H0782) having HPLC purities of 95% or higher were purchased from Sigma (St. Louis, MO, USA). A mixture of glyceollins I, II and III (internal lot 9/20/98) suitable for assigning HPLC retention times was kindly supplied as a gift by Dr. Hans VanEtten at the Department of Plant Pathology, University of Arizona (Tucson, AZ, USA).

2.2. Materials

Seeds from Williams 82 and Hartwig SB cultivars along with seeds from their progeny 14a (ca. 80% Williams 82 and 20% Hartwig DNA content) [22] were grown at Purdue University (West Lafayette, IN, USA) under controlled environmental conditions. Seedlings were grown in 50-ml test tubes in a water bath at 24°C under 16 h of light. Approximately 50 roots of each cultivar were harvested 15 days after planting, rinsed with water, patted dry on paper towels, dried at 50°C for 3 days and then lightly ground by mortar and pestle to achieve a nearly uniform mesh size of about 20 to 100. The dried powders, which weighed approximately 1.75 g in each case, were placed in plastic envelopes and mailed by overnight delivery to the Center for Drug Design and Development (CD3) at the University of Toledo (Toledo, OH, USA). Materials received by the CD3 were logged-in and immediately stored at 1 to 2°C until subjected to extraction. Extractions were conducted within 2 days of receipt of each material. A total of six replicates were produced over the course of 18 months.

Seeds deployed in the preliminary biotic stress study were grown in exactly the same manner except that their roots were inoculated on day 11 with 3000 eggs/juveniles from an SB cyst nematode inbred strain that is maintained at Purdue University. Roots were harvested at 48 and at 96 h after inoculation, treated as described above and delivered to the CD3 for assay within 2 days. Two replicates were produced over the course of 6 months.

2.3. Extraction procedures

2.3.1. Preliminary studies

Seven 100 mg samples of each of the three cultivars were separately extracted with 3 ml of each of the following solvents for 4 h at 22°C: (i) hexane; (ii) ethyl acetate; (iii) dichloromethane; (iv) methanol; (v) acetonitrile–water (1:1); (vi) acetonitrile–0.1 *M* sodium hydroxide (1:1); and (vii) acetonitrile–0.1 *M* HCl (1:1). Using a pipette, 2 ml of solution was withdrawn from each extract and examined by TLC according to assay methods A, B and C. Aliquots (i) to (iv) were then gently warmed with a heat gun until they began to reflux while (v) to (vii) were placed in a warming bath set at 60±2°C. All heat exposures were conducted for 5±0.5 min. The heated aliquots were then immediately re-examined by TLC according to assay methods A, B and C.

2.3.2. Procedure A

Day 1: 1.00 g of dried root powder was extracted with 250 ml of hexane under reflux for 2 h. The mixture was filtered and the resulting root powder air-dried for 15 min. The hexane filtrate was placed in a sealed flask and stored overnight at 1 to 2°C (eventually to become sample PA-1). The air-dried root powder was then extracted with 250 ml of methanol under reflux for 2 h. The mixture was filtered and the root powder discarded. The methanolic extract was evaporated under reduced pressure (water aspirator vacuum) on a rotary evaporator for 30±5 min during which time the water bath was maintained at a temperature of 40±2°C to provide a clear oil. The clear oil was taken up in 10 ml methanol and a 1 ml aliquot was removed, after which both portions were again evaporated according to the conditions described above. The evaporated 1 ml portion was saved (sample PA-2) while the clear oil remaining after evaporation of the 9 ml portion was partitioned between 50 ml water and 50 ml dichloromethane. The layers were separated and evaporated on a rotary evaporator according to the conditions noted above with the aqueous phase also being periodically spiked with toluene in order to allow for its easier removal as the lower boiling azeotrope. The evaporated dichloromethane phase provided only trace residues for all three of the cultivars (samples PA-3) while the evaporated aqueous phase provided a clearly visible residue for each cultivar (samples PA-4). Samples PA-2 to 4 were taken up in 1 ml of warm methanol and assessed by TLC methods A–C and by HPLC method D, as described in Section 2.5.

Day 2: The stored hexane extracts were evaporated on a rotary evaporator according to the conditions noted above. The resulting trace residues (sample PA-1) were taken up in 1 ml hexane and assessed by TLC methods A–C and by HPLC method E.

2.3.3. Procedure B

Day 1: 0.50 g of dried root powder for each cultivar was extracted with 250 ml of methanol while stirring at ambient temperature (ca. 22°C) for 4 h. The mixture was filtered and the root powder discarded. The methanolic extract was evaporated on a rotary evaporator according to the conditions noted above. The resulting clear oil (sample PB) was taken up in 1 ml of methanol and assessed by TLC methods A, B and C, and by HPLC method D.

Day 2: Sample PB was assessed by HPLC method E.

2.3.4. Procedure C

Day 1: 0.50 g of dried root powder for each cultivar was extracted with 250 ml of methanol under reflux for 2 h. The mixture was filtered and the root powder discarded. The methanolic extract was evaporated on a rotary evaporator according to the conditions noted above. The resulting clear oil (sample PC) was taken up in 1 ml of methanol and assessed by TLC methods A, B and C, and by HPLC method D.

Day 2: Sample PC was assessed by HPLC method E.

2.4. Equipment

TLC studies were conducted on EM Science precoated silica gel 60 F_{254} (250 μm) 20×10 cm glass plates purchased from Aldrich (Milwaukee, WI, USA). Visualization was effected by quenching of the silica-impregnated fluorophore when irradiated at 254 nm within an ultraviolet viewing cabinet mounted with a Model UVGL-58 Mineral Light Lamp purchased from UVP (San Gabriel, CA, USA) and by placement in an I_2 vapor chamber made by placing I_2 crystals within an empty 20×10 cm TLC development tank. Three solvent systems (methods A, B and C) were used to develop the thin-layer chromatograms.

HPLC studies were conducted on a Waters HPLC system (Milford, MA, USA) consisting of two Model 510 pumps, a WISP 710B auto-sampler with Model U6K manual injector option, a Model 486 ultraviolet detector and a dedicated NEC PowerMate PC (Boxborough, MA, USA) running Waters Baseline data collection software. Reversed-phase conditions (assay method D) utilized a Nova-Pak C_{18} 150×3.9 mm column having 4 μm packing (lot W82241) and a UV detection system set at 220 nm. Normal-phase conditions (assay method E) utilized UV detection set at 254 nm and a Hypersil Si 150×4.6 mm column having 5 μm packing (Ser. 5634172971H01)

purchased from Phase Separations (Franklin, MA, USA).

2.5. Assay conditions

TLC studies were conducted in duplicate by spotting samples 2 cm from the bottom of each plate and then running 16 cm within pre-equilibrated development chambers whose rear walls contained a moistened filter paper to provide for a more uniform vapor environment. Three solvent systems were separately deployed for development such that each sample was measured in duplicate within three different assay methods. Method A used hexane–ethyl acetate (1:1). Method B used acetonitrile–isopropanol (1:1). Method C used toluene–methanol (1:4). Plates were air-dried and then visualized by UV irradiation followed by placement within an I_2 chamber.

HPLC studies were conducted in duplicate or triplicate by injecting 50 μl of each sample series two or three times in staggered sequence arrangements using an auto-sampler. Two different HPLC methods were used depending upon the nature of the sample and the information being sought. Method D deployed the C_{18} column described above and used a two-component gradient mobile phase in which component A was methanol and component B was an aqueous buffer made from 0.01 *M* KH_2PO_4 (pH adjusted to 2.4 with HCl) and 0.1% Et_3N (final pH about 2.46). The gradient consisted of: component A increased from 5% to 55% over 50 min; component A maintained at 55% for 10 min; and, component A then returned to 5% over 5 min. Each sample run was followed by a 15 min re-equilibration period. A flow-rate of 1.0 ml/min and a detector setting at 220 nm was used throughout. Method E deployed the Si column described above and used a single component mobile phase consisting of hexane. Each sample run lasted 60 min. A flow-rate of 0.8 ml/min and a detector setting at 254 nm was used throughout.

HPLC fingerprints for duplicate and triplicate samples injected initially and at the end of an overnight autosampling run showed no evidence of deterioration (22°C, 15 h). Likewise, residual sample materials were found to provide similar chromatograms (i.e., relative peak areas were still within the normal variation as discussed later) upon re-analysis after as long as 5 days of storage when materials were kept at 1 to 2°C.

2.6. Qualitative and quantitative determinations

TLC was utilized initially to assist in quickly surveying a range of possible extraction procedures. Only gross, qualitative comparisons were made among the two soybean parents and their progeny.

HPLC fingerprinting was done both qualitatively and quantitatively by comparing the calculated ratios between peaks in one chromatogram versus the analogous peak ratios as present within another chromatogram. Two levels of concentration and HPLC detection sensitivity were obtained for each type of sample. In one set of runs, the samples were diluted to 25% (v/v) original concentration and the detection sensitivity was not enhanced. Thus all peaks within a given chromatogram could be kept on scale. For these runs, the area, height, area percent and height percent of individual peaks could all be observed and read directly from their respective chromatograms and numerical print-outs. Ratios between eight clearly discernible peaks of interest could be readily determined at this level of sensitivity. For the higher set of concentrations, samples were not further diluted from the extraction procedures described above and the instrument sensitivity was increased in order to provide a higher level of scrutiny along the baseline of each chromatogram. For these sample runs, several of the larger peaks went off-scale. Quantitative comparisons between on-scale peaks at both levels of sensitivity were done by comparing all of such peaks within a given spectrum to an on-scale peak that was common within all samples under consideration. Selection of a common peak for both the low concentration/normal sensitivity studies and for the high concentration/high sensitivity studies was accomplished after inspection of several runs of each different type of sample obtained according to procedures A and B and assayed by method D in each case. Criteria for selection included consistent retention times and peak areas within duplicate and triplicate runs, within different extraction runs of the same cultivar and, finally, between runs of different cultivars. The largest peak at retention time (t_R) 40.6±0.2 min was

selected as a common standard peak for the normal sensitivity peak ratio determinations while the peak at t_R 44.7±0.2 min was used to compare the baseline-associated peaks observed during high sensitivity runs. Mean values for each peak's area percent relative to the common standard peak's area (set at 100) were calculated from the various runs in each case (n=4 to 6 separate experiments). Two standard deviations (95% confidence limits) were also calculated for each peak area percent. Peaks having the same retention time that appeared to have different normalized area percentages between any two sets of different cultivar data were compared by a one-tailed t-test at $P<0.05$ (95% confidence).

3. Results and discussion

3.1. TLC and initial extraction procedures

While procedures useful for the selective extraction/isolation of specific phytoalexins such as the glyceollins are known, our initial extraction experiments sought to uncover a more generally representative range of materials. Thus, we first examined several solvent systems that spanned from non-polar to highly polar media. We did not attempt to trap any highly volatile materials (e.g., b.p.<30°C) that might be extracted because unlike the above-ground parts of a plant, there is little precedent for highly volatile natural products to be important within root systems. The solvents noted in the experimental section traverse a range of moderate volatility (e.g., b.p. ca. 40 to 100°C). Thus, in order to retain any natural products having boiling points in this range, all solvent extractions were initially conducted at ambient temperature (ca. 22°C) and directly assayed by methods A, B and C without any type of concentration (elevated temperature and/or reduced pressure) step. These same extracts were then warmed and re-examined to see if any gross changes occurred within their fingerprints. The overall results from these preliminary studies indicated that some highly non-polar materials can be extracted by hexane, separated by TLC method A and visualized by I_2 chamber (R_F values near 0.5 and 0.7). However, it was clear that the most chemically rich extracts were provided by the polar solvents wherein the method C TLC chromatograms exhibited numerous spots by both UV and I_2 chamber visualization (R_F values running from 0.1 to 0.8 with the heaviest densities in the range 0.6 to 0.8). Importantly, the materials present in the non-polar extracts were also present in the more densely populated polar extracts. A simple methanol extraction procedure proved to be the easiest to manipulate while also providing the most representative variety of materials in what appeared to be the most reproducible fashion across all of the samples. The warming studies did not reveal any significant changes in the TLC fingerprints derived from the non-protic solvent systems. Alternatively, the methanolic and aqueous systems appeared to have a subtle diminution in one of their spot intensities (see later discussion).

3.2. Finalization of extraction procedures and preliminary HPLC studies

Based upon the preliminary results described above and upon the earlier work reported by others who used refluxing ethanol to extract soybean roots (e.g., Refs. [16,17]), a two-tiered extraction protocol was examined next in order to assess the possibility that even more reproducibly discernible HPLC chromatograms might be derived from samples subjected to sequential extractions with a non-polar and a polar solvent system. As outlined in procedure A in Section 2.3.2, cultivar samples were first extracted with refluxing hexane and then with refluxing methanol. To further assist in the assessment of the methanolic extract, it was evaporated and its residue partitioned between water and dichloromethane as a prelude to HPLC fingerprinting. Concurrent to this protocol, an independent methanolic extraction was conducted on a separate root sample using methanol at ambient temperature (procedure B). Finally, procedure C was utilized to see if abundant, yet reproducibly discernible, fingerprints could be obtained by extracting fresh samples with refluxing methanol followed directly by HPLC assay.

In at least one case for the same lot of each of the three cultivars, the mass balance obtained during each of the extraction protocols was calculated as a percent of the initial material mass. The average residue mass percentages after evaporation of the four different phase samples associated with pro-

cedure A were as follows: hexane=1.1±2%; methanol (reflux)=20.9±6%; water=17.5±5%; and dichloromethane=2.8±2%. Average mass percentages for the single samples obtained from procedures B and C were 25.5±6% and 26.0±6%, respectively. It is interesting to note that although the extractions for the Williams 82 and Hartwig cultivars appeared to remain quite similar across all of the various fractions, the 14a progeny appeared to provide a higher yield from the methanolic extractions (ca. 35±4%) when compared to either parent (ca. 25±3% in each case) under either ambient temperature or reflux conditions.

Most of the prior HPLC methods in this area have utilized a reversed-phase column and a gradient mobile phase consisting of an alcoholic component and an acidic aqueous buffer. As described in the Experimental section, assay method D employs such components and it was utilized to assess all of the polar extracts generated according to procedures A, B and C. Alternatively, a normal-phase column eluted with a single, non-polar mobile phase (assay method E) was utilized to assess the initial hexane extract obtained in procedure A as well as a portion of the methanolic extracts afforded by procedures B and C. The results from the initial HPLC studies associated with development of a final extraction protocol are summarized below.

In line with the low yield obtained from the hexane extraction, deployment of HPLC method E failed to produce more useful chromatograms than the considerably detailed fingerprints obtainable via method D. Likewise, other than providing for an enhanced relative extraction of a material having t_R 45.7 min, the chromatograms obtained from the dichloromethane partition appeared to be only very dilute versions of those produced by the water partition. Furthermore, the water–dichloromethane partition did little to clarify the methanolic extract fingerprints that proved to be quite resolvable by HPLC despite their numerous peaks. Comparison of the chromatograms obtained from ambient versus refluxing methanol proved to be extremely interesting, particularly in view of the wide use of refluxing alcoholic conditions for the extraction of SB materials. Fig. 2 exemplifies some of the differences between peak ratios that can occur. Specific data is provided for Hartwig when extracted with methanol

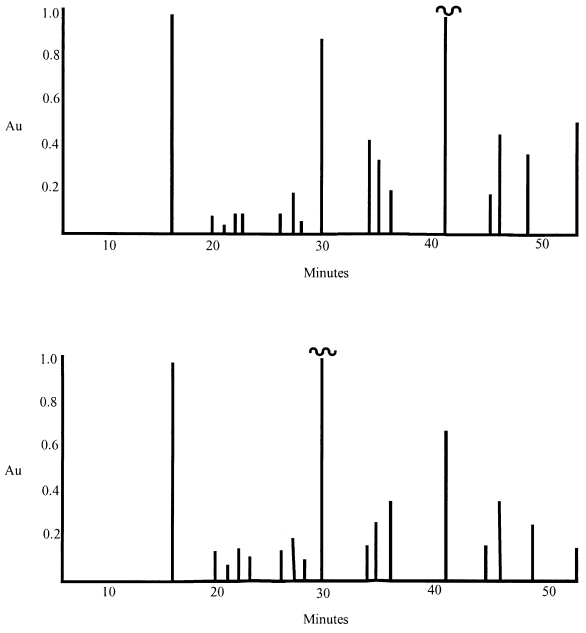

Fig. 2. Comparison of HPLC method D fingerprints obtained methanolic extracts of the Hartwig soybean cultivar when extractions are conducted at room temperature (upper chromatogram) and at reflux (lower chromatogram) according to procedures B and C, respectively. Wavy horizontal line indicates peak has surpassed detection scale.

at ambient (ca. 22°C) versus refluxing conditions (65°C). The relative loss of material associated with t_R 40.6 min under refluxing conditions is apparent. A loss of material at t_R 53 min is also noteworthy, along with the relative change of the three materials around t_R 35 min. Since similar alterations were not observed when studying the various non-protic solvents, it is unlikely that the noted decreases result from these particular materials being volatile near 65°C. Alternatively, an increase in solvent temperature will normally enhance a non-volatile solute's solubility such that extraction of substances from a dried solid setting should be improved with higher temperature. Thus, if the extraction efficiency were improved for all of the materials except those at t_R 41.0 and 53 min, their observed relative peak height reductions could be explained in this manner. However, considering the magnitude of the observed decreases relative to all of the other peak relationships, which did not appear to be necessarily enhanced, this second possibility also does not seem to

be operative. A third possibility would be that the observed losses are due to the conversion of the t_R 40.6 and 53 min materials to some other materials because of the higher thermal energy encountered during the refluxing methanol extraction process. That the net mass of the two types of extracts remains about the same, as evidenced during the aforementioned mass balance studies, supports this hypothesis. A candidate chemical conversion applicable to both situations and in line with SB phytochemistry would be the alcoholysis/hydrolysis of a glycosylated version of a phenolic-containing natural product such as the flavone/isoflavone natural products depicted in Fig. 1. That the noted alterations appeared to be of most concern for the protic solvent extraction systems further supports this overall scenario.

It should also be appreciated, however, that the vast majority of peaks are still nicely displayed and are clearly represented within both types of temperature extracts such that comparisons between lots or between different cultivars using either fingerprinting technique would be acceptable as long as they are done in a consistent manner. In this regard, the temperature studies indicate that the precise duration of time spent on a rotavap while at a specified elevated temperature during any solvent concentration step should be closely monitored to insure that such activities occur in an identical fashion from run to run. Alternatively, the use of overnight autosampling where methanolic injection samples wait at room temperature, or even the storage of such samples for up to 5 days when at refrigerator temperatures, did not appear to alter the reproducibility of the HPLC fingerprints. Based upon the preliminary results, procedure B (methanol, ambient temperature, 4 h) was selected as the finalized set of extraction parameters to be utilized for the quantitative comparisons of the HPLC fingerprints generated by using assay method D.

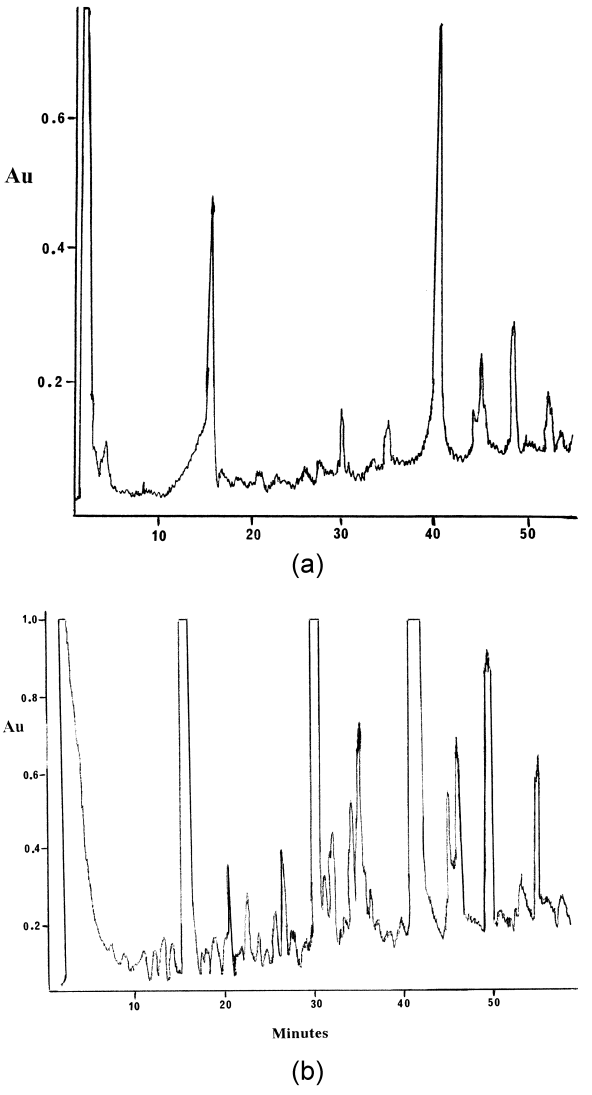

Fig. 3. Comparison of HPLC method D full chromatograms obtained from ambient methanolic extracts of the Williams 82 soybean cultivar conducted at standard concentration and sensitivity (upper chromatogram) and at high concentration and enhanced sensitivity (lower chromatogram).

3.3. Comparison of HPLC fingerprints

Comparisons of the two parental cultivars and their progeny were considered at two levels of scrutiny. Fig. 3 displays an illustrative set of direct print-out, HPLC chromatograms obtained for Williams 82 at low sample concentration and normal detection sensitivity (upper scan), and at high sample concentration and enhanced detection sensitivity (lower scan). The normal or standard run produces a typical chromatogram with all peaks on scale. Alternatively, the enhanced sensitivity run, even though it loses the scaling for several of the larger peaks, affords a chromatogram that emphasizes the interest-

ing array of materials that rise across the entire horizon of the spectrum's baseline. Note that complete spectra have been provided in this case so that the relative baseline noise levels can be fully appreciated. All other chromatographic figures utilize schematic representations in order to more readily focus upon just the most significant peaks.

Fig. 4 provides the comparative fingerprints for Williams 82, Hartwig and 14a progeny obtained at a low concentration and standard detection sensitivity, where all peaks have been kept on scale. These fingerprints contain several peaks, of which eight lend themselves to ready quantitative comparison of their relative ratios. All of the peak areas within each of these chromatograms were first normalized relative to the common peak located at t_R 40.6 min that was assigned a value of 100. The results from this type of calculation for duplicate or triplicate injections of the same cultivar lot and extraction run were extremely tight, i.e., their range varying by less than $\pm 2\%$ of their average value in all cases except for the peak located at t_R 14.5 min. The latter represents the first material off the column after the injection spike and the range for its variance was found to be much broader, approaching about $\pm 15\%$. The precision for the various peaks calculated for the same cultivar lots in different extraction replications was also very consistent, with all peaks other than the one at t_R 14.5 min still varying by a range of less than $\pm 5\%$. Comparison of peaks across different lots of the same cultivar did not cause the precision to change significantly, their peak ranges still being less than $\pm 5\%$. The latter suggests that seasonal and harvest-to-harvest considerations did not have a dramatic impact upon the overall chemical constituent nature of the HPLC extracts, at least when SB cultivars are grown under tightly controlled, identical environmental conditions. Thus, all of the results obtained in this manner were combined for each peak calculation within a given cultivar. These final results are summarized in Table 1 and allow for ready comparison between the normalized and so-averaged HPLC fingerprints of each cultivar. Although the parents, Williams 82 and Hartwig cultivars, have extremely similar HPLC fingerprints, there does appear to be a small but statistically significant decrease in the Hartwig's chemical constituent appearing at t_R 49 min (peak 12). Alternatively, there appears to be a statistically relevant increase in the material located at t_R 53 min (peak 13). The progeny's overall chromatogram is also very similar to those for the Williams 82 and Hartwig cultivars (Table 1). However, 14a appears

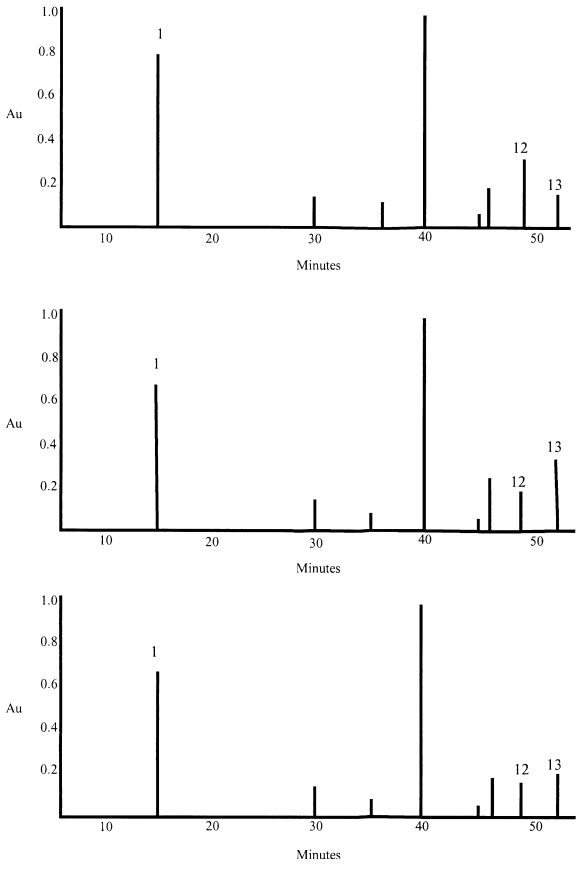

Fig. 4. Comparison of HPLC fingerprints for the Williams 82 (upper), Hartwig (middle) and their progeny (lower) soybean cultivars obtained by assay method D after performing methanolic extractions at ambient temperature (procedure B) and the using standard concentrations and detection sensitivity. Because of the considerable variance associated with the peak area at t_R 14.5 min, the responses observed at peak 1 should not be regarded as being different in any of the chromatograms. The response for peak 12 (middle chromatogram) is significantly less than that in the upper chromatogram while the response for peak 13 is significantly more. The response for peak 12 in the lower chromatogram is significantly less than that of the upper chromatogram but is not significantly different than half of 12 in the middle chromatogram. Likewise, the response for peak 13 is significantly more than that of the upper chromatogram but not more than 13 in the middle chromatogram. Also see Table 1 for quantitative comparisons.

Table 1
Quantitative comparison of HPLC fingerprints for the W, H and 14a soybean cultivars at standard concentration and detection sensitivity

Peak	W		H		14a	
	t_R	Area	t_R	Area	t_R	Area
1	14.6	76±14	14.3	66±12	14.2	68±10
5	30.2	17±4	30.1	19±2	30.1	20±2
8	35.4	12±4	35.3	10±2	35.3	12±1
9	40.7	100	40.6	100	40.6	100
10	44.7	6±1	44.7	7±1	44.7	8±1
11	45.7	21±3	45.7	26±2	45.7	22±3
12	49.0	28±3	49.0	20±3*	49.0	14±3*
13	53.1	20±2	53.0	37±2*	53.1	34±2*

Data represent mean values with 95% confidence limits for at least four determinations in each case. Peak numbering has been done so as to be consistent with the larger data set shown in Table 2. Retention times (t_R) are recorded in min and are within ±0.2 in all cases except for peak 1 which was within ±0.4. Peak areas are in percents relative to that of peak 9 which was set at 100%. Asterisk denotes peak area that is significantly different when assessed by a one-tailed *t*-test at $P<0.05$ during comparison to the corresponding W cultivar peak.

to have even less of the component at t_R 49 min although it is not statistically different from the decrease also observed for this peak in Hartwig. Likewise, 14a exhibits an increase in the peak 13 area over that of Williams 82 that is very similar to what was observed for Hartwig. Interestingly, since the peak 13 materials are significantly diminished by refluxing alcohol in all of the cultivar cases, this particular difference between the cultivars' chemical constitutions would have been completely missed by relying only upon a refluxing alcohol-type of extraction protocol. These results suggest that even though differences are rather subtle within the context of their overall chromatograms, 14a may have certain HPLC-detectable chemical features that are closer to those in the Hartwig parent than to those in the Williams 82 parent.

Fig. 5 provides the comparative fingerprints for the three lines (Williams 82, Hartwig and 14a) obtained at higher concentration and at a high level of detection sensitivity. This allows for some of the small peaks near the baseline to be more observable even though many of the larger peaks go off scale. Since the peak that was used as the common, normalizing standard for the lower sensitivity experiment now runs off scale, an alternate peak was

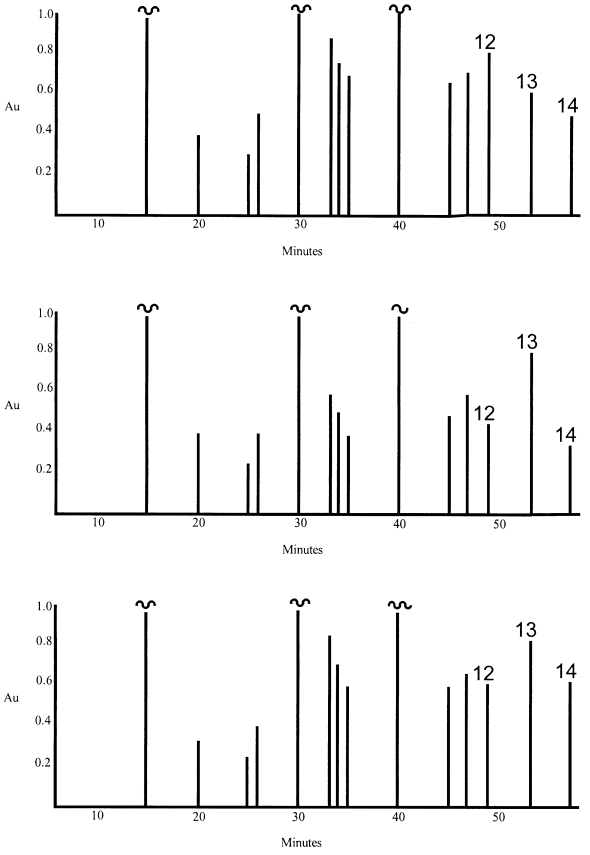

Fig. 5. Comparison of HPLC fingerprints for the Williams 82 (upper), Hartwig (middle) and their progeny (lower) soybean cultivars obtained by assay method D after performing methanolic extractions at ambient temperature (procedure B) and then using high concentration and detection sensitivity. Wavy horizontal line indicates peak has surpassed detection scale. The response for peak 12 (middle chromatogram) is significantly less than that in the upper chromatogram while the response for peak13 is significantly more. The response for peak 12 in the lower chromatogram is significantly less than that of the upper chromatogram but is not significantly different than that of 12 in the middle chromatogram. Responses for peaks 13 and 14 in the lower chromatogram are significantly more than that in the upper chromatogram but are not significantly different that their corresponding peaks in the middle chromatogram. Also see Table 2 for quantitative comparisons.

selected for normalizing the relationships within each of the high sensitivity chromatograms. A consistent peak proved to be the one at t_R 44.7 min (identical to peak 10 in Table 1). Setting this peak area at 100, all of the other relative peak areas were computed within a given chromatogram. These values were

then averaged for all replications of a given line. At least six additional and distinct experiments from those conducted at standard detection sensitivity were, again, run with at least duplicate HPLC injections for each replication. By using this approach, 14 on scale peaks of potential interest could be compared in a quantitative manner across the three SB lines' chromatograms. It should be noted, however, that the variability associated with the reading of a given peak's area from one chromatogram to another also became amplified, the range of variance approaching ±10% when the smaller peaks were recorded at higher instrument sensitivity. The results from these calculations are summarized in Table 2. These results further support the earlier conclusions. While the overall chromatograms continue to look very similar, the subtle differences in the peaks located at t_R 49 and 53 min are retained. The former decreasing and the latter increasing as one goes from Williams 82 to Hartwig to 14a, with 14a looking more like Hartwig than Williams 82. In addition, a smaller baseline peak located at t_R 57 min appears to be significantly increased in the 14a relative to Williams 82.

Table 2
Quantitative comparison of HPLC fingerprints for the W, H and 14a soybean cultivars at high concentration and detection sensitivity

Peak	t_R	Area		
		W	H	14a
1	15.1	OS	OS	OS
2	20.6	74±6	81±4	75±4
3	25.3	65±6	72±3	70±3
4	26.8	82±5	87±4	81±3
5	30.6	OS	OS	OS
6	33.3	112±4	110±5	114±4
7	34.2	105±6	99±6	109±5
8	35.5	101±6	90±5	98±4
9	40.5	OS	OS	OS
10	44.7	100	100	100
11	45.7	101±6	108±5	106±5
12	49.1	119±6	103±5*	107±4*
13	53.0	100±5	128±4*	132±3*
14	57.2	89±7	96±6	105±5*

Data represent mean values with 95% confidence limits for at least six determinations in each case. Retention times (t_R) are recorded in min and are within ±0.4 in all cases. Peak areas are in percents relative to that of peak 10 which was set at 100%. OS indicates peak was off-scale. Asterisk denotes peak area that is significantly different when assessed by a one-tailed *t*-test at $P<0.05$ in comparison to the corresponding W cultivar peak.

3.4. Consideration of standard materials

The subtle differences between the Williams, Hartwig and 14a cultivars noted above occur at t_R values that suggest involvement of the natural products associated with SB flavone/isoflavone biosynthesis. Some of the most prominent natural products associated with this key phytochemical pathway are illustrated in Fig. 1. Using method D the following t_R values (in min) were obtained for these materials when injected as standards: daidzein=44.6; genistein=49.9; and glyceollins I, II, III as a three peak cluster located between 58.8 to 60.3. This relative sequence is similar to that obtained by others who used a reversed-phase/aqueous methanol elution HPLC protocol [18]. The standard t_R values were the same when the materials were combined and injected as a single cocktail but were found to vary within ±1.5 min when the cocktail was subsequently used to spike the various cultivar extraction samples. These preliminary spiking experiments suggest that peaks 10 and 12 located at t_R 44.7 and 49.0 min within the on-scale chromatogram runs (Fig. 4 and Table 1) could potentially be daidzein and genestein, respectively, or at least some type of structures closely related to these materials in terms of physicochemical and chromatographic properties. Likewise, the spiking results suggest that peak 14 located at t_R 57.2 min within the enhanced detection chromatogram runs (Fig. 5 and Table 2) could potentially represent a portion of the glyceollins fraction, or at least some closely related physicochemical materials. More definitive assignments for these peaks as well as for some of the other significant peaks within each cultivar's chromatograms are currently being pursued via LC–MS–MS.

3.5. Preliminary biotic stress studies

As a prelude to further optimization of the overall extraction/assay method, the protocol was deployed in its present stage of development to see if it could discern at least some type of gross differences among stressed SB roots compared to the aforementioned results from the several non-stressed sample runs. SB

cyst nematodes were selected as a biological insult because they represent a common source of infection within SB crops. Furthermore, it is thought that these types of infections invoke a sophisticated biochemical dialogue between host and parasite. Thus, an inbred strain of SB cyst nematodes was first extracted with methanol at ambient temperature according to procedure B. Populations containing about 5000 SB cyst nematode eggs/juveniles were shown to exhibit no significant peaks upon examination by HPLC using assay method D. The three SB cultivars were then inoculated with about 3000 SB cyst nematode eggs/juveniles at day 11 while being grown under conditions otherwise identical to all of the aforementioned, non-stress studies. Roots were harvested at 48 and 96 h after the inoculations, processed in the same manner as before, and submitted to extraction and HPLC assay according to procedure B and method D, respectively. This entire study was repeated once. Fig. 6 portrays the HPLC chromatographic fingerprints for the W, H and 14a stressed-root systems derived from the first run after 96 h. Table 3 provides a listing of all data from both runs.

Gross comparison of the respective chromatograms within Figs. 6 and 4 suggests that there is considerable similarity between the overall fingerprints of the stressed versus the non-stressed SB cultivars. Alternatively, closer comparison of all of the data contained in Table 3 to the corresponding values provided in Table 1 allows several distinctions to be drawn. Specifically, when peak area values for the two stressed runs consistently lie either above or below the peak area 95% confidence limits indicated for that same peak within Table 1, the preliminary stress studies suggest that an alteration in the amount of that particular chemical constituent may indeed have been prompted by the stress treatment. In this regard, it can be noted that all three of the stressed cultivars appear to produce a transient increase in the peak 11 constituent that can be observed 2 days after inoculation but then returns toward non-stressed levels by day 4. Likewise, all three cultivars show an apparent increase in the peak 5 constituent at day 4 after inoculation. Interestingly, the 14a hybrid appears to uniquely display a significantly increased constituent within the peak 12 area at day 4 after inoculation These same trends were also observed when more concentrated samples were examined at higher HPLC detection sensitivity (data not shown).

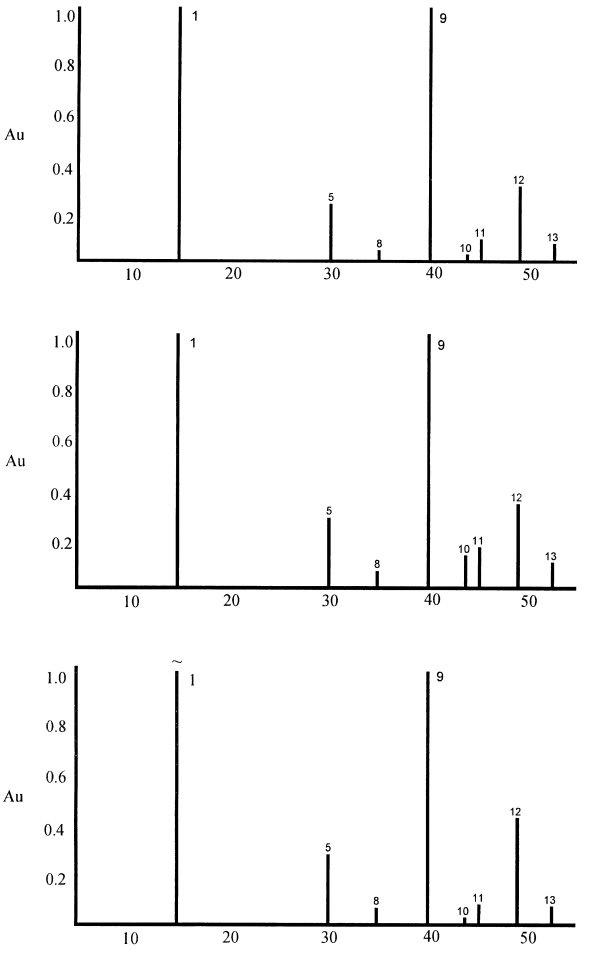

Fig. 6. Comparison of HPLC fingerprints for the Williams 82 (upper), Hartwig (middle) and their progeny 14a (lower) soybean cultivars after exposure of roots to a biotic stress for 96 h. Fingerprints were obtained by assay method D after performing methanolic extractions at ambient temperature (procedure B) and then using standard concentrations and detection sensitivity. Because of the considerable variance associated with the peak area at t_R 14.5, the responses observed at peak 1 should not be regarded as being different in any of the chromatograms. The gross similarity of these fingerprints to their respective non-stressed patterns can be ascertained by comparison to Fig. 4. A listing of the specific peak areas determined for all of the stressed studies is provided in Table 3 and allows for comparison on the subtle differences between these data and the results from the non-stressed runs (Table 1).

Table 3
Quantitative comparison of HPLC fingerprints for stressed W, H and 14a soybean cultivars at standard concentration and detection sensitivity

Peak	W		H		14a	
	2D	4D	2D	4D	2D	4D
1	85, 75	100, 93	110, 117	41, 70	151, 127	109, 139
5	21, 20	26, 23↑	19, 24	24, 25↑	14, 20	27, 30↑
8	12, 14	11, 10	12, 17	8, 9	10, 20	11, 10
9	100	100	100	100	100	100
10	6, 23	3, 8	7, 18	8, 14	7, 19	2, 16
11	30, 52↑	16, 12↓	39, 39↑	22, 32	29, 54↑	10, 21
12	26, 8	46, 31	23, 26	35, 17	19, 5	42, 35↑
13	13, 7↓	22, 6	13, 8↓	13, 9↓	18, 16↓	20, 13↓

Data represent all peak area determinations obtained from two studies. Both studies involved inoculation of W, H and 14a roots on day 11 using SB cyst nematodes that served as a biotic stress factor. Samples were taken at 2 days (2D) and at 4 days (4D) after inoculation. Peak numbering has been done so as to be consistent with the larger data set shown in Table 2. Corresponding retention times were in agreement with previous runs. Peak areas are in percents relative to that of peak 9 which was set as 100%. Cases where the values from both runs were either above or below the 95% confidence limit values for the corresponding peaks shown in Table 1 (non-stressed data) are noted as such by either an up-arrow or down-arrow, respectively, set immediately next to that pair of data. Comparisons of peak 1 areas were not undertaken because of its previously noted high degree of variation even in the non-stressed runs.

4. Summary

Methanolic extractions of SB root provide fingerprints that are representative of the chemical constituents that lend themselves to TLC and HPLC assay. Temperature-related differences in the fingerprints indicate that both the extraction procedures and the concentration of extracts for injection, need to be done within tightly specified protocols. We found it most practical to conduct extraction steps at ambient temperature (ca. 22°C) and rotary evaporator concentration steps at 40±2°C for 30±5 min while under water aspirator vacuum. Reproducible HPLC fingerprints can indeed be obtained for a given SB root cultivar when the latter is grown under well-controlled, reproducible greenhouse conditions. The fingerprints for HPLC runs with all peaks kept on-scale contain seven peaks. Statistical analyses indicate that when at least four experimental repetitions are performed, the 95% confidence limits for the relative area of a given peak varies by at most four units on a scale where all peaks are found within the range of 1 to 100 normalized units. Increasing the sample concentration and detection sensitivity allows for assessment of another six peaks along the baseline. In these cases, statistical analyses indicate that when at least six experimental runs are performed, the 95% confidence limits for the relative peak areas vary by at most 7 normalized units. Although comparisons across three different cultivars revealed only subtle differences in their overall fingerprints, statistically significant differences in the areas for certain of their more prominent peaks does allow them to be distinguishable (one-tailed *t*-test at $P<0.05$).

These studies demonstrate the reproducibility of an alcoholic extraction protocol for discerning the HPLC separable/UV detectable chemical constituents present within SB roots when the roots are grown in well-controlled environments. Furthermore, preliminary stress studies deploying this method indicate that detectable alterations of the fingerprints do occur upon these types of exposures. Thus, further optimization of the analytical protocol would appear to be warranted as a prelude to more thorough comparative investigations. Toward this end, the fingerprint variation associated with temperature differences appears to reflect alcoholysis-related events such that even the ambient extraction procedure should additionally utilize a bulky alcohol instead of methanol in order to further impede these types of conversions. Likewise, the HPLC assay should deploy an internal standard that is added just prior to analysis so that peak area normalizations and statistical comparisons can be more readily accomplished between different laboratories, as well as

within a single set of studies. Finally, a mobile phase buffering system that is more compatible with LC–MS–MS should probably be deployed at the onset in order to expedite any of such follow-up studies that may eventually be directed toward characterizing peaks that become of interest.

References

[1] R.A. Vierling, H.T. Nguyen, Crop Sci. 32 (1990) 370.
[2] R.A. Vierling, H.T. Nguyen, Crop Sci. 30 (1990) 1337.
[3] R.A. Vierling, H.T. Nguyen, Theor. Appl. Genet. 84 (1992) 835.
[4] S.W. King, R.A. Vierling, H.T. Nguyen, Crop Sci. 32 (1992) 822.
[5] F. Zhang, D.L. Smith, J. Exp. Botany 47 (1996) 785.
[6] G. Malmquist, R. Danielsson, J. Chromatogr. A 687 (1994) 71.
[7] R.V. Smith, P.W. Erhardt, Anal. Chem. 47 (1975) 2462.
[8] P.W. Erhardt, R.V. Smith, T.T. Sayther, J.E. Keiser, J. Chromatogr. 116 (1976) 218.
[9] J.G. Sarver, N. Peng, S. Lerdkanchanaporn, K.I. Oravecz-Wilson, K.A. Alexander, P.W. Erhardt, Int. J. Pharm. Compounding 3 (1999) 148.
[10] J. Liao, K. Golding, K. Dubischar, J. Sarver, P. Erhardt, H. Kuch, M. Aouthmany, Nat. Med. Chem. Symp. Abstr. B-32 (2000) 137.
[11] P. Kaufman, J. Duke, H. Brielmann, J. Boik, J. Hoyt, J. Altern. Complement. Med. 3 (1997) 7.
[12] U. Zahringer, J. Ebel, L.J. Mulheirn, R.L. Lyne, H. Grisebach, FEBS Lett. 101 (1979) 90.
[13] C. Kraus, G. Spiteller, A. Mithofer, J. Ebel, Phytochemistry 40 (1995) 739.
[14] J.L. Ingham, N.T. Keen, L.J. Mulheirn, R.L. Lyne, Phytochemistry 20 (1981) 795.
[15] T. Komives, J. Chromatogr. 261 (1983) 423.
[16] M.K. Bhattacharyya, E.W.B. Ward, Phys. Mol. Plant Pathol. 29 (1986) 227.
[17] J.-S. Huang, K.R. Barker, Plant Physiol. 96 (1991) 1302.
[18] R.M. Kosslak, J.R. Dieter, R.L. Ruff, M.A. Chamberlin, B.A. Bowen, R.G. Palmer, J. Hered. 87 (1996) 415.
[19] W.F. Osswald, J. Chromatogr. 333 (1985) 225.
[20] S. Kawai, Y. Sato, S.-I. Takagi, K. Nomoto, J. Chromatogr. 391 (1987) 325.
[21] T.W.-M. Fan, A.N. Lane, J. Pedler, D. Crowley, R.M. Higashi, Anal. Biochem. 251 (1997) 57.
[22] R.A. Vierling, J. Faghihi, V.R. Ferris, J.M. Feris, Theor. Appl. Genet. 92 (1996) 83.