JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Evaluation of Spectrophotometric and HPLC Methods for Shikimic Acid Determination in Plants: Models in Glyphosate-Resistant and -Susceptible Crops

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**ABSTRACT:** Endogenous shikimic acid determinations are routinely used to assess the efficacy of glyphosate in plants. Numerous analytical methods exist in the public domain for the detection of shikimic acid, yet the most commonly cited comprise spectrophotometric and high-pressure liquid chromatography (HPLC) methods. This paper compares an HPLC and two spectrophotometric methods (Spec 1 and Spec 2) and assesses the effectiveness in the detection of shikimic acid in the tissues of glyphosate-treated plants. Furthermore, the study evaluates the versatility of two acid-based shikimic acid extraction methods and assesses the longevity of plant extract samples under different storage conditions. Finally, Spec 1 and Spec 2 are further characterized with respect to (1) the capacity to discern between shikimic acid and chemically related alicyclic hydroxy acids, (2) the stability of the chromophore ($t_{1/2}$), (3) the detection limits, and (4) the cost and simplicity of undertaking the analytical procedure. Overall, spectrophotometric methods were more cost-effective and simpler to execute yet provided a narrower detection limit compared to HPLC. All three methods were specific to shikimic acid and detected the compound in the tissues of glyphosate-susceptible crops, increasing exponentially in concentration within 24 h of glyphosate application and plateauing at approximately 72 h. Spec 1 estimated more shikimic acid in identical plant extract samples compared to Spec 2 and, likewise, HPLC detection was more effective than spectrophotometric determinations. Given the unprecedented global adoption of glyphosate-resistant crops and concomitant use of glyphosate, an effective and accurate assessment of glyphosate efficacy is important. Endogenous shikimic acid determinations are instrumental in corroborating the efficacy of glyphosate and therefore have numerous applications in herbicide research and related areas of science as well as resolving many commercial issues as a consequence of glyphosate utilization.

**KEYWORDS:** alicyclic hydroxy acid, chromophore half-life ($t_{1/2}$), EPSPS EC 2.5.1.19, *Glycine max* (L.) Merr, glyphosate efficacy, herbicide resistance, molar absorptivity, periodate derivatization, shikimate, vicinal diol, *Zea mays* L.

## INTRODUCTION

*N*-[Phosphonomethyl]glycine (glyphosate) is a nonselective, postemergence, systemic herbicide that is used globally to control over 180 annual and perennial weed species.[1,2] The development of herbicide-tolerant crops has extended the use of glyphosate from conventional weed management strategies including noncrop and preplant burndown to allow for broadcast in-crop application. This has significantly increased the total area treated such that glyphosate is currently one of the most widely used herbicides in the world; glyphosate application rates have also increased in the past decade due to the evolution of tolerance and resistance in many monocotyledonous and dicotyledonous weed species.[3,4] Glyphosate kills plants by preventing the biosynthesis of aromatic amino acids through the inhibition of 3-phosphoshikimate 1-carboxyvinyl transferase (EPSPS; EC 2.5.1.19) in the shikimic acid biosynthetic pathway.[5,6]

The shikimic acid pathway, which exists in bacteria, fungi, and embryophyta, links the carbohydrate and aromatic biosynthetic pathways.[7,8] Products of the shikimic acid pathway include the essential aromatic amino acids L-phenylalanine (Phe), L-tyrosine (Tyr), and L-tryptophan (Trp), as well as many secondary metabolites, including cell antioxidant flavonoids, cell pigments (anthocyanins), photoinhibition deterrents (carotenoides), growth phytohormones (auxins), vital structural molecules (lignins), and plant defense molecules (phytoalexins and alkaloids).[9,10] The best studied enzyme in the shikimic acid pathway is EPSPS, which cleaves the $C_\beta$—O bond in phosphoenolpyruvate (PEP) and catalyzes a regiospecific transfer of the carboxyvinyl moiety to the $C_5$-hydroxyl in 3-phosphoshikimate (3PS). This reaction results in the production of 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate.[11] Glyphosate acts as a competitive inhibitor with respect to the carboxyvinyl moiety of PEP in the binary EPSPS·3PS complex, effectively blocking the shikimic acid pathway.[12,13] EPSPS inhibition by glyphosate therefore results in the rapid accumulation of endogenous 3PS, which in turn is stored in cell protoplasts and is subsequently dephosphorylated to shikimic acid (3*R*,4*S*,5*R*-trihydroxy-1-cyclohexene-1-carboxylic acid) by vacuolar phosphorylases.[14] Thus, endogenous shikimic acid measurements have been

**Received:** November 9, 2010
**Revised:** January 11, 2011
**Accepted:** January 12, 2011
**Published:** February 21, 2011





dx.doi.org/10.1021/jf1043426 | *J. Agric. Food Chem.* 2011, 59, 2202–2212

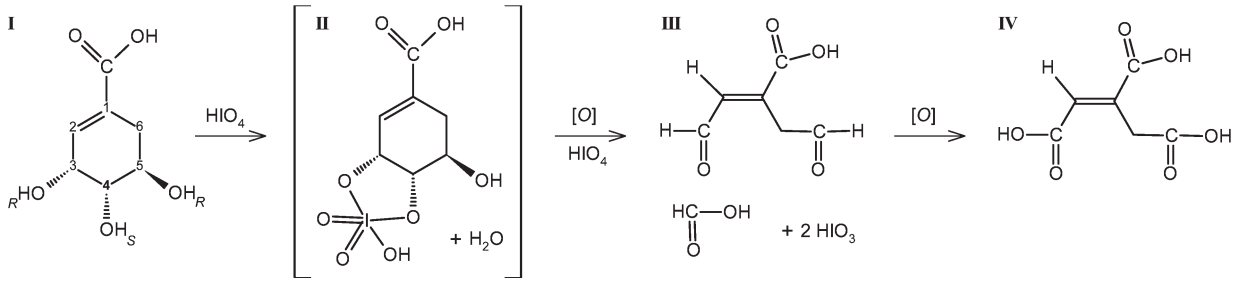


**Figure 1.** Reduced shikimic acid (**I**). Oxidation of shikimic acid by periodate ($IO_{-4}$) involves the formation of a transient cyclic periodate ester (**II**). Cleavage of the $C_3-C_4$ and $C_4-C_5$ bonds in shikimic acid yields *trans*-2-pentene-1,5-dialdehyde-3-carboxylic acid (PDC) (oxidized shikimic acid), two molecules of iodic acid, and one molecule of formic acid (**III**). Further oxidation of the carbonyl groups of PDC to carboxylic radicals yields *trans*-aconitic acid (**IV**). Enantiomers in stereogenic centers are designated according to the Cahn−Ingold−Prelog notation (**I**).

used in the past as an indirect estimate of the level of EPSPS inhibition by glyphosate. For example, following glyphosate application, shikimic acid and 3PS reach a maximum cytoplasmic concentration within 50 h at a ratio of 20:1,[15] indicating that EPSPS inhibition occurs rapidly following glyphosate exposure. Because endogenous shikimic acid concentrations correlate with the level of EPSPS inhibition by glyphosate, previous studies have measured changes in endogenous shikimic acid to (1) ascertain glyphosate drift to nontarget plants;[16−18] (2) discern between injuries caused by EPSPS and non-EPSPS inhibiting herbicides;[19,20] (3) evaluate the effects of glyphosate when applied preharvest to crops;[21] and (4) corroborate glyphosate resistance in weeds.[22−26]

Methods of shikimic acid determination include nuclear magnetic resonance (NMR),[15] thin layer chromatography (TLC),[27] spectrophotometry,[28] high-pressure liquid chromatography (HPLC),[29] mass spectroscopy (MS),[5] gas chromatography with MS (GC-MS),[30] micellar electrokinetic capillary chromatography (MECC),[31] and capillary zone electrophoresis (CZE) coupled with HPLC.[32] However, spectrophotometry- and HPLC-based methods are the most widely used, and many variations of these methodologies have been reported in the literature. HPLC methods include separation in a strongly basic anion-exchange solid phase, precolumn separation and differential refraction[33] and weak anion-exchange in organic solvents [14] or reversed-phase chromatography in a phosphate mobile phase.[34] Spectrophotometric methods are based on shikimic acid derivatization with periodate (Figure 1),[35] which generates a *trans*-2-pentene-1,5-dialdehyde-3-carboxylic acid (PDC) chromophore ($\lambda_{max}$ = 380−382 nm). A previous study compared spectrophotometric and HPLC assays and concluded that the latter was more quantitative.[36] In this investigation, we compared spectrophotometric and HPLC assays focusing on the sensitivity, specificity, and reproducibility of methods.

In this study, glyphosate-resistant and -susceptible maize (*Zea mays* L.) and soybean (*Glycine max* [L.] Merr) plants were treated with glyphosate, and the increment of endogenous shikimic acid levels and plant tissue necrosis was monitored over time. Plant tissues were then used to compare the versatility of shikimic acid extraction methods and the efficiency and reproducibility of determination methods. Specifically, two methods of shikimic acid extraction, extraction 1 [19,37] and extraction 2,[34] were adapted from previously reported protocols and were compared to determine method versatility and stability of shikimic acid in plant extract samples. Two spectrophotometry-based methods, Spec 1[37] and Spec 2,[19] were evaluated using plant extract samples and a shikimic acid standard to determine method (1) sensitivity, (2) specificity, (3) optimal absorbance ($\lambda_{max}$), and (4) stability of the PDC chromophore. Finally, an HPLC method[19] was compared to the two spectrophotometry-based methods to assess cost and simplicity of the analytical procedures and to optimize analysis of shikimic acid in glyphosate-treated plant extracts.

## MATERIALS AND METHODS

**Plant Growth Conditions.** Six glyphosate-resistant (Asgrow AG2901; AG2901RR) and -susceptible (Asgrow A2833; A2833) soybean and glyphosate-resistant (DeKalb 545RR; DK545RR) and -susceptible (Garst 8550Bt; G8550Bt) maize seeds were planted in 12 cm diameter pots containing a peat/perlite/loam (1:2:1) soil−mix medium. Following emergence, crops were thinned to four seedlings per pot and were grown under the following greenhouse conditions: a 16:8 h light/dark cycle of natural light supplemented with artificial illumination (600−1000 $\mu$mol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density), 28−35 °C diurnal and 20−25 °C nocturnal conditions, and a minimum of 50% relative humidity; plants were irrigated as needed and fertilized (Miracle Gro Excel, Scott-Sierra, Marysville, OH) 1 week after emergence.

**Glyphosate Application.** Twenty-one pots with 12−14 cm tall soybean (V2−V3) and 21 pots containing 12−14 cm tall maize (V3−V4) plants were used in this study. For both soybean and maize, 18 pots were treated with the isopropylamine salt of glyphosate (Roundup UltraMAX, Monsanto, St. Louis, MO), using the manufacturer's recommended field rate of 0.86 kg acid equivalents (AE) per hectare.[38] The application of glyphosate was performed inside a $CO_2$-powered spray chamber (model SB5-66, DeVries Manufacturing, Hollandale, MN), which delivered 187 L $ha^{-1}$ at a pressure of 2.8 kg $cm^{-2}$ through an 80015-E nozzle located 30 cm above the plant canopy. Care was taken to ensure uniform spray coverage and to minimize foliage overlap. Plants were sprayed in the morning and were allowed to grow in full sunlight during the initial 12 h after treatment (HAT). In addition to the pots sprayed with glyphosate, three soybean and maize pots were not sprayed, and these plants served as controls for the study. The experiment was repeated in time ($n$ = 42 maize and $n$ = 42 soybean plants).

**Plant Tissue Collection and Assessment of Phytotoxicity.** For both maize and soybean, three arbitrarily selected glyphosate-treated pots and the untreated control pots were assessed at each of the following time points: 0, 5, 12, 24, 72, and 120 HAT. Glyphosate efficacy was determined by comparing the degree of visual crop injury in the glyphosate-treated plants to the degree of crop injury in the untreated control plants (where 0% = asymptomatic and 100% = completely necrotic). Plant height was also estimated for both glyphosate-treated and untreated control plants by measuring the distance from the soil surface to the apex of soybean or the collar of the utmost expanded leaf in maize. Following glyphosate efficacy and plant height determinations, the glyphosate-treated maize and soybean plants were

sacrificed as follows: from each pot, the apex and the youngest fully expanded trifoliate from each of the four soybean plants and the basal 3 cm of the coleoptile of maize plants were excised and stored under dry or wet conditions. From each treated pot, the tissues of two sacrificed soybean or two sacrificed maize plants were placed in a paper bag and dried at 35 °C for 48 h (dry tissue), and the tissues of the remaining two plants were placed in a plastic bag and stored at −10 °C (wet tissue); these samples were used for shikimic acid extraction and analysis.

**Shikimic Acid Extraction.** Dry and wet tissue samples from both soybean and maize were ground in liquid nitrogen with a mortar and pestle, and shikimic acid was extracted following two methods: in the first method (extraction 1), 200 mg of plant tissue was ground in 2 mL of 0.25 N HCl for 5 min and then sonicated for 30 min at 25 °C (2210 Bransonic Ultrasonicator, Markham, ON, Canada). Plant cell components were then separated by centrifugation at 20000*g* for 15 min, and the supernatant was retained for shikimic acid determination. In the second method (extraction 2), 150 mg of plant tissue and 25 mL of 1 N HCl was refluxed at 100 °C for 1 h. Samples were removed from refluxing after 30 min, sonicated for 5 min, and then refluxed for an additional 30 min. The sample was then filtered through a no. 1 filter paper, the pH was adjusted to 2.5 with 1 M NaOH, and the volume was brought to 50 mL with deionized−distilled $H_2O$ ($_{dd}H_2O$). A 10 mL aliquot was passed through a 0.2 $\mu$m polytetrafluoroethylene (PTFE) membrane, and the filtrate was retained for shikimic acid determination. The stability of plant tissue extracts was investigated by storing samples at ambient temperature and −10 °C and determining the concentration of extracted shikimic acid in subsamples over a period of 6 months.

**HPLC Determination Method.** Soybean and maize plant extracts (10−25 $\mu$L) were filtered through a 0.22 $\mu$m nylon membrane and were analyzed by HPLC using Millennium software (Waters Corp., Milford, MA). Separation utilized a LiChrosorb $NH_2$ (250 × 4 mm, 5 $\mu$m internal diameter, Phenomenex, Torrance, CA) analytical column with a flow rate of 1 mL $min^{-1}$ mobile phase (95% acetonitrile and 5% 4:1 water/orthophosphoric acid).[19] The elution of shikimic acid was monitored at 210 nm, resulting in a retention time ($t_R$) of 7.1 min. Samples were assayed in duplicate, and standard curves were prepared (10 $\mu$L) with shikimic acid standard (>98% pure, Sigma-Aldrich) at a range of 1−900 $\mu$mol $mL^{-1}$.

**Spectrophotometric Determination Methods.** Two spectrophotometry-based methods for analysis of shikimic acid were evaluated using shikimic acid standard and samples of dry and wet tissues of maize and soybean extracted using extraction 1 and extraction 2 methods. For Spec 1, aliquots of sample extracts (5−15 $\mu$L for extraction 1; 50−250 $\mu$L for extraction 2) were diluted to a final volume of 250 $\mu$L with $_{dd}H_2O$ and oxidized with a 250 $\mu$L of aqueous solution of 0.5% periodate and 0.5% sodium *m*-periodate (w/v). The mixture was then vortexed, incubated at 37 C for 45 min, and quenched with 500 $\mu$L of a solution of 3:2 1 M NaOH/56 mM $Na_2SO_3$ (v/v). Absorbance (optical density; OD) was measured at 382 nm ($A_{382}$) in a Lambda 18 UV−vis spectrometer (Perkin-Elmer, Shelton, CT). For Spec 2, aliquot samples (5−15 $\mu$L for extraction 1; 50−250 $\mu$L for extraction 2) were diluted to a final volume of 250 $\mu$L in $_{dd}H_2O$ and oxidized with a 250 $\mu$L aqueous solution of 2% periodate (w/v). Samples were vortexed, incubated at ambient temperature for 3 h, and then quenched with a 500 $\mu$L solution of 1 M NaOH. Quenched samples were further diluted with a 300 $\mu$L solution of 0.1 M L-glycine and vortexed, and the absorbance was detected at 380 nm ($A_{380}$). The concentration of shikimic acid in the plant extract samples was quantified on the basis of standard curves created from a shikimic acid standard at a concentration range of 1−60 $\mu$mol $mL^{-1}$. In addition, standard curves were created by fortifying the extract samples of untreated control soybean and maize plants to assess shikimic acid recovery and potential background interference. All samples were analyzed in duplicate, and tests were repeated in time.

*Method Sensitivity Experiment.* The limits of detection for Spec 1 and Spec 2 were probed by assaying known concentrations of shikimic acid standard ranging from 0.001 to 1000 $\mu$mol $mL^{-1}$. Each concentration was assayed in six replicates, and experiments were repeated four times ($n$ = 24 per concentration per method).

*Chromophore Stability Experiment.* To assess the stability of the PDC chromophore, samples of shikimic acid standard with a concentration of 10, 30, and 60 $\mu$mol $mL^{-1}$ were assayed following the Spec 1 and Spec 2 protocols. After the initial OD determination at time zero ($y_0$), samples were placed in the dark at 4 °C or ambient temperature (22−24 °C). The OD was then determined every hour for the first 14 h and then every 12 h for 48 h. Ten replicates per shikimic acid concentration were assayed, and the experiment was repeated in time ($n$ = 20). The half-life ($t_{1/2}$) of the PDC chromophore at the different incubation temperatures and shikimic acid concentrations was determined on the basis of a nonlinear regression analysis (refer to Statistical Analysis).

*Optimal Absorbance (λ) Experiment.* Shikimic acid in its reduced form can be oxidized to PDC by periodate (oxidized shikimic acid) (Figure 1). To assess shifts in OD due to oxidation of the shikimic acid molecule by periodate, a range of wavelengths were tested to determine the absorption maximum ($\lambda_{max}$) of the reduced and oxidized samples. A 1 mL aliquot of shikimic acid standard or plant extract at 1 M concentration in $_{dd}H_2O$ (reduced) was analyzed at absorbances ranging from 190 to 900 nm, in 1 nm data interval and 120 nm $min^{-1}$ scan speed. All reduced samples were blanked against $_{dd}H_2O$. For the equivalent oxidized shikimic acid reference, a 30 $\mu$mol $mL^{-1}$ aliquot (10 $\mu$L) of shikimic acid standard or plant extract (15 $\mu$L for extraction 1; 250 $\mu$L for extraction 2) was assayed following the specification of Spec 1 and Spec 2, and the absorbance of each sample was analyzed from 190 to 900 nm, in 1 nm data interval and 120 nm $min^{-1}$ scan speed. All oxidized samples were blanked against a solution containing the constituents of Spec 1 or Spec 2 assays minus the shikimic acid sample. Reduced and oxidized samples were analyzed in five replicates, and the optimal absorbance experiment was repeated in time ($n$ = 10). Determinations were also conducted in reduced and oxidized extract samples of giant ragweed (*Ambrosia trifida* L.), woolly cupgrass (*Eriochloa villosa* [Thunb.] Kunth), common sunflower (*Helianthus annuus* L.), and shattercane (*Sorghum bicolor* [L.] Moench) treated with 0.86 kg of AE glyphosate per hectare.

*Specificity Experiment.* The following alicyclic hydroxy acids, gallic (3,4,5-trihydroxybenzoic acid), protocatechuic (3,4-dihydroxybenzoic acid), quinic (1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid), and syringic (3,5-dimethoxy-4-hydroxybenzoic acid) acid standards (Sigma-Aldrich), were prepared to 10, 20, 30, and 40 $\mu$mol $mL^{-1}$ concentrations, and the optimal absorbance spectrum was determined as described in the previous section. To assess whether Spec 1 and Spec 2 methods were specific to shikimic acid, the spectrophotometric methods were assayed with aliquots (10 $\mu$L) of each alicyclic hydroxy acid alone or in mixture with shikimic acid in a 1:1 concentration mixture. Five replicates of each sample were assayed, and assessments were repeated in time ($n$ = 10).

**Statistical Analysis.** Differences in plant shikimic acid concentrations between glyphosate-susceptible and -resistant plants were determined as follows: the experiment was set up as a 2 × 6 × $2^3$ factorial design. Crop variety (two levels) and sampling time (six levels) were the whole plot factors in a randomized complete block design (RCBD), and tissue moisture (two levels), extraction method (two levels), and determination method (two levels) were the split, split−split, and split−split−split plot factors, respectively. Shikimic acid concentrations ($\mu$mol per g of tissue) were analyzed by analysis of variance (ANOVA) with four variance components. For the whole plot portion of the experiment (two crop genetics at six sampling times), plant height and crop injury data were collected and analyzed independently by ANOVA as a RCBD. Finally, Spearman rank correlations ($\rho$) were

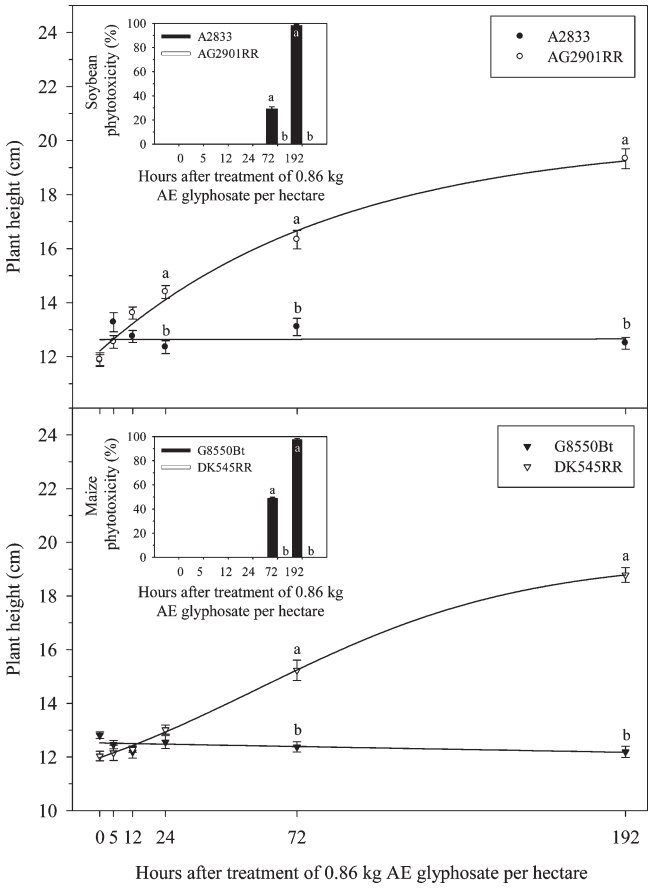


**Figure 2.** Plant height at 0, 5, 12, 24, 72, and 192 h after treatment (HAT) with 0.86 kg acid equivalents (AE) of glyphosate $ha^{-1}$. Glyphosate-susceptible (A2833; ●) and -resistant (AG2901RR; ○) soybean and glyphosate-susceptible (G8550Bt; ▼) and -resistant (DK545RR; ▽) maize plants were treated at the V2−V3 or V3−V4 phenological stages, respectively. Each data point represents the mean of three replications and two experiments ($n$ = 6); letters designate statistically different means within assessment timing according to Fisher's test ($LSD_{\alpha=0.05}$). (Insets) Crop phytotoxicity associated with glyphosate damage at 0, 5, 12, 24, 72, and 192 HAT.

calculated between shikimic acid concentration, crop phytotoxicity, and plant height.

PDC chromophore stability as a function of time was fitted to several nonlinear equations.[39] Of the models examined, the four-parameter sigmoidal and three-parameter exponential decay models had the best fit to the data of methods Spec 1 and Spec 2, respectively. Chromophore half-life ($t_{1/2}$), defined as the time at half that of time zero ($y_0$), was estimated from the following models:

four-parameter sigmoidal model

$$y = f(x) = y_0 + \left[\frac{c}{1 + e^{-\left(\frac{t - t_{1/2}}{b}\right)}}\right] \quad (1)$$

In eq 1 $y$ = OD as a function of $t$, $y_0$ = upper limit asymptote (OD at time zero), $c$ = lower limit asymptote (constant baseline), e = base of natural logarithms, $t$ = time, $t_{1/2}$ = half-life, and $b \neq 0$ = slope.

three-parameter exponential decay model

$$y = f(x) = a + b\, e^{-kt} \quad (2)$$

In eq 2 $y$ = OD as a function of $t$, $a$ and $b$ = arbitrary constants, e = base of natural logarithms, $k$ = >0 = decay constant, $t$ = time, and $t_{1/2}$ is calculated as

$$t_{1/2} = \left[\frac{\log n(2)}{k}\right] \Rightarrow k = \left[\frac{\log n(2)}{t_{1/2}}\right] \quad (3)$$

$\therefore$ $t_{1/2}$ was calculated by the parametrization

$$y = f(x) = a + b\, e^{-\left(\frac{\log n(2)}{t_{1/2}}\right)t} \quad (4)$$

For each sample, $t_{1/2}$ was estimated from the model corresponding to Spec 1 or Spec 2. These data were subjected to analysis of covariance (ANCOVA) with method, incubation temperature, and their interaction as factors and shikimic acid concentration entered as a covariate having a different coefficient for each method × incubation temperature combination. There was no evidence of lack of fit (LOF) for this model compared to the model treating shikimic acid concentration as a factor.

Differences in method specificity between Spec 1 and Spec 2 were determined as follows: the experiment was set up as a 5 × 4 factorial in a completely randomized design (CRD), with five alicyclic hydroxy acids alone at four concentrations or four mixtures at a 1:1 concentration ratio with shikimic acid. These data were analyzed in a two-way ANOVA, and Fisher's least significant difference test ($LSD_{\alpha=0.05}$) was used for post hoc comparison of means.[40]

## RESULTS AND DISCUSSION

**Crop Response to Glyphosate.** Prior to glyphosate application, the susceptible and resistant maize and soybean plants were not significantly different in terms of crop phytotoxicity or plant height (Figure 2). Following glyphosate application, susceptible soybean plants had significantly less growth compared to glyphosate-resistant plants at 24, 72, and 192 HAT ($P \leq 0.01$). Likewise, susceptible maize plants exhibited significantly decreased plant growth at 72 and 192 HAT with glyphosate ($P \leq 0.01$). Concurrently, a significant increase in crop phytotoxicity was observed for both glyphosate-susceptible maize and soybean plants at 72 and 192 HAT. Nearly 100% necrosis was observed at 192 HAT in susceptible soybean and maize plants, whereas the glyphosate-resistant plants were asymptomatic (Figure 2).

Glyphosate instigates a variety of direct and indirect physiological responses in susceptible plants. Within several hours of glyphosate application, reduced protein synthesis and stomatal conductance,[41] inhibition of photosynthesis and carbon allocation,[42] production of ethylene and $CO_2$,[43] and an alteration of indole-3-acetic acid[44] and phenolic compound metabolism[45] have been reported. These initial effects lead to larger responses, which include modifications of the rough endoplasmic reticulum,[46] dictyosomes, mitochondria,[47,48] and microtubules [49] and a reduction of sink water potentials due to dysfunctional root systems.[50] Indirectly, the accumulation of endogenous shikimic acid may further hinder plant cellular processes, specifically by inhibiting carbon and nitrogen fixation by PEP carboxylase[51,52] and nitrogenase,[53,54] respectively. These physiological alterations contributed to the significant growth differences between glyphosate-susceptible and -resistant maize and soybean observed in this study.

**Versatility of Extraction Methods.** When the times required to complete the extraction processes were compared, the reflux,

filtration, and pH-adjustment steps made the extraction 2 method a lengthier procedure compared to extraction 1. Furthermore, these procedures required additional steps that increased the opportunities for cross-contamination of samples. Moreover, the PTFE filtration step increased the cost of performing extraction 2 by at least 5 times compared to extraction 1. Overall, the extraction 1 method was more cost-effective, expeditious, and reproducible due to mitigation of potential sample cross-contamination compared to the extraction 2 method. Apart from the two extraction methods evaluated in this study, other methods have been reported in the literature, and these include shikimic acid extraction in $_{dd}H_2O$,[55] methanol,[56] or other organic solvents.[37] The advantages of shikimic acid extraction in $_{dd}H_2O$ include minimal cost and reagent requirements and the generation of less-hazardous waste. However, the efficiency of this extraction method is reportedly lower when compared to acid-based methods.[55] Additionally, $_{dd}H_2O$-based extraction methods require overnight incubation, double filtration, and larger aliquot volumes (5 mL) for the determination of shikimic acid.[57] A third category of extraction method relies on alkali conditions with low molecular weight $H_2O$-miscible organic solvents (NaOH/isopropanol).[37] These methods have the advantage of circumventing tissue grinding and prolonged refluxing as described in this study with HCl; however, these methods may not be suitable for extraction of shikimic acid from older lignified tissues, even with prolonged incubation of ground or intact plant samples. A fourth extraction method based on freeze−thawing of leaf disks is reported in the literature and requires no tissue grinding as described in extractions 1 and 2.[58] Although not evaluated in this investigation, freeze−thawing may provide extraction efficiencies comparable to tissue grinding methods, significantly simplifying the handling and processing of plant samples; however, the method may have limitations with regard to quantities of plant materials that can be processed.

**Stability of Plant Tissues and Extracts.** The storage period of dry or wet tissue samples kept at −10 °C was investigated by assaying subsamples over time with both Spec 1 and Spec 2. Results from these analyses suggested that the shikimic acid concentration in tissue samples remained stable for at least 6 months when extractions were performed on dry tissue stored at −10 °C; wet tissue samples tended to decompose after 1 month at −10 °C, and this obviously compromised the integrity of samples (data not shown). These results are consistent with previous reports purporting that shikimic acid in dry-ground tissue samples remains viable for several months at −10 °C.[55] The stability of shikimic acid plant extract samples was also assessed over time; overall, the stability of plant extract samples was inferior to that of intact tissue samples, and the consistency of shikimic acid determinations in plant extract samples decreased significantly over time. Samples generated by extraction 1 and extraction 2 methods could be stored for a maximum of 48 h at ambient temperature (22−24 °C), after which the detectable concentration of shikimic acid decreased by ≥20% relative to the concentration detected in the same samples shortly after extraction (data not shown). Microbial degradation is arguably the greatest factor limiting the stability of plant extract samples kept at ambient temperature. Nonetheless, the stability of shikimic acid plant extract samples increased significantly with storage at −10 °C, extending the viability of samples for at least 1 week (data not shown). A similar study reported no significant decrease in the concentration of detectable shikimic acid in plant samples extracted with extract 1 and stored at ≤ −4 °C for 1 month.[37] Overall, these results suggested that the best storage conditions for plant samples destined for shikimic acid extraction and determination comprised dry tissue sample storage at ≤ −10 °C.

**Comparison of Spectrophotometric Determination Methods.** *Basis for Spectrophotometric Determinations.* Periodate-mediated oxidations yield aldehydes by the selective cleavage of carbon bonds in vicinal (*vic*)-diol groups in a polymer;[59,60] this oxidation rate is dependent on the incubation temperature and periodate concentration in the reaction.[61] Periodate therefore oxidizes the $C_3-C_4$ and $C_4-C_5$ bonds in shikimic acid, thus yielding *trans*-2-pentene-1,5-dialdehyde-3-carboxylic acid (PDC), formic acid, water, and two molecules of iodic acid. Further oxidation of the carbonyl groups in PDC forms *trans*-aconitic acid (*trans*-propene-1,2,3-tricarboxylic acid) (Figure 1).[35] Neutralization of the excess periodate and iodic acids by alkalinization generates a yellow chromophore with maximal OD ($\lambda_{max}$) of 380 nm, which is the basis for spectrophotometric shikimic acid determination methods.[28] Importantly, the PDC chromophore generated by this reaction is reportedly photo-unstable.[61] Therefore, some spectrophotometric methods have explored whether conjugation of PDC with aniline[62] or 2-thiobarbituric acid[63,64] resulted in more photo-stable chromogens. However, these methods are less reproducible and therefore not popular at present.

*Evaluation of Method Sensitivity.* The sensitivity of Spec 1 and Spec 2 was assessed by determining the lowest shikimic acid concentration detectable in a reproducible manner. Previous papers suggested that the lowest detectable limits for spectrophotometric methods ranged from 0.1 to 1.0 $\mu$mol mL$^{-1}$ shikimic acid.[37] In this study, concentrations of 0.05 and 0.1 $\mu$mol mL$^{-1}$ shikimic acid in the reaction mixtures for Spec 1 and Spec 2 methods contributed to the molar absorptivity at 380−382 nm ($\varepsilon_{380-382}$) but often resulted in nonreproducible results. Consistent spectrophotometric readings at these absorption wavelengths were attained only at shikimic acid concentrations of ≥0.5 $\mu$mol mL$^{-1}$ for both methods, and we therefore recommend this as the minimum cutoff basal concentration for determinations in future studies (refer to Supporting Information). A different investigation reported a minimum shikimic acid detection limit of 1.7 $\mu$mol mL$^{-1}$ for periodate-based spectrophotometric methods.[28] Our data, however, suggested that a linear and reproducible response that obeyed the Beer−Lambert law ($I_1/I_0$ = 0.1−1.0) was obtained when periodate-based spectrophotometric methods were performed at concentrations ranging from 0.5 to 20 $\mu$mol shikimic acid mL$^{-1}$ (data not shown).

*Evaluation of Chromophore Stability.* Prior research determined that the stability of the chromophore formed from the periodate-based oxidation of shikimic acid was pH- and light-dependent.[35] Further studies demonstrated that alkali conditions favored the stability of PDC, yielding a maximum molar absorptivity of 4.76 × 10$^4$ L mol$^{-1}$ cm$^{-1}$ at 380 nm ($\varepsilon_{380}$) and a pH of 11−12; however, even with improved photostability from the alkali conditions the $t_{1/2}$ of the chromophore was only 30 min.[28,61] Consequently, two approaches have been developed to further increase the stability of the chromophore; these include incubation of PDC with sulfite or glycine, and these procedures are reflected in Spec 1 and Spec 2, respectively. Our investigation therefore focused on understanding which of these approaches resulted in a more stable chromophore and whether the time-dependent decay of PDC in Spec 1 and Spec 2

**Table 1. *trans*-2-Pentene-1,5-dialdehyde-3-carboxylic Acid (PDC) Chromophore Half-Life ($t_{1/2}$) Estimates for Spec 1 and Spec 2 Methods at Three Shikimic Acid Concentrations and Two Incubation Temperatures**

| method | shikimic acid ($\mu$mol mL$^{-1}$) | incubation temperature (°C) | $t_{1/2}$[a] (SE) (min) | ICS[b] |
|---|---|---|---|---|
| Spec 1 | 10 | 4 | 528 (23) | 1.96 |
| | | ambient | 269 (23) | |
| | 30 | 4 | 428 (23) | 1.61 |
| | | ambient | 266 (23) | |
| | 60 | 4 | 381 (23) | 1.52 |
| | | ambient | 251 (23) | |
| | av across concentrations | 4 | 442 (19) | 1.73 |
| | | ambient | 255 (19) | |
| Spec 2 | 10 | 4 | 301 (25) | 1.97 |
| | | ambient | 153 (24) | |
| | 30 | 4 | 375 (24) | 2.12 |
| | | ambient | 177 (24) | |
| | 60 | 4 | 438 (24) | 2.17 |
| | | ambient | 202 (24) | |
| | av across concentrations | 4 | 371 (20) | 2.10 |
| | | ambient | 177 (20) | |

[a] $t_{1/2}$ was estimated from the four-parameter sigmoidal and three-parameter exponential decay models for Spec 1 and Spec 2 methods, respectively. The standard error ($\sigma$; SE) associated with mean $t_{1/2}$ was estimated from an ANOVA of the individually estimated $t_{1/2}$ and had method, temperature, and amount of shikimic acid as factors. [b] Increase in chromophore $t_{1/2}$ stability (ICS); values were calculated by dividing the $t_{1/2}$ at 4° C by that obtained at ambient temperature.

was affected by incubation temperature. Because the PDC chromophore is purportedly photo-unstable, determinations of chromophore stability were conducted in the dark and samples were incubated at either 4 °C or ambient temperature (22−24 °C). Results from this investigation confirmed that the 4 °C incubation temperature nearly doubled the $t_{1/2}$ values and therefore the stability of the PDC chromophore compared to ambient temperature determinations, regardless of method or shikimic acid concentration (Table 1). Spec 1 had greater overall $t_{1/2}$ values compared to Spec 2. At 4 °C, the average $t_{1/2}$ value across shikimic acid concentrations was 442 min for Spec 1, and this contrasted to 371 min for Spec 2; these data suggested that Spec 1 had a more stable chromophore (Table 1). Similar results were attained at ambient temperature where the average $t_{1/2}$ value of 255 min for Spec 1 was approximately 1.5 higher compared to the average $t_{1/2}$ value of 177 min for Spec 2 ($P$ = 0.001). The difference in this comparison suggested that the addition of sulfite resulted in greater PDC chromophore stability compared to glycine, confirming a previous study.[37] There is evidence that increasing the sulfite concentration from 56 mM as used in this study to 220 mM further increases the stability of the PDC chromophore in Spec 1 (Nick Polge, personal communication). From a practical standpoint, however, most spectrophotometric readings are done within minutes of completion of assays, and thus both Spec 1 and Spec 2 methods provide reproducible results within this time frame. Improved chromophore stability may be important in high-throughput sample systems in which time to read is a limiting factor. In the event of protracted spectrophotometric readings, we recommend conducting assays with solutions chilled at 4 °C and completing reading within an hour of sample quenching; care is required to prevent condensation on cuvettes from interfering with spectrophotometric readings.

*Verification of Absorption Spectrum and Optimal Absorbance.* The oxidation of reduced shikimic acid by periodate forms PDC (oxidized shikimic acid) (Figure 1), which may change the absorption spectra of samples assayed with Spec 1 and Spec 2. To elucidate whether this absorbance shift occurred, reduced and oxidized shikimic acid samples were assayed and OD was monitored from 190 to 900 nm. Data reported in this section are restricted to maize samples as an identical result was obtained with soybean samples (data not reported). The absorption spectrum of reduced samples, in the form of either standard shikimic acid or glyphosate-treated maize samples, had an OD of $\leq$0.2 at wavelengths of $\geq$350 nm (Figure 3). From 190 to 260 nm the OD of reduced samples was $\leq$0.3, and at 270 nm the absorbance increased exponentially to a maximum OD of 0.8 at 283 nm; the molar absorptivity at this peak ($\varepsilon_{283}$) was 7.41 $\times$ $10^{-1}$ L mol$^{-1}$ cm$^{-1}$ ($\sigma_M$ = 0.0018). Importantly, the oxidation of shikimic acid by periodate caused a change in this observed absorbance spectrum (Figure 3). One peak with absorption maximum ($\lambda_{max}$) of 288−290 nm, analogous to that of reduced samples, was observed, and this had a molar absorptivity ($\varepsilon_{289}$) of 1.52 $\times$ $10^5$ L mol$^{-1}$ cm$^{-1}$ ($\sigma_M$ = 1.31 $\times$ $10^3$). A second, smaller, peak associated with the PDC chromophore appeared at an $\lambda_{max}$ of 382 nm, and the estimated molar absorptivity ($\varepsilon_{382}$) was 4.78 $\times$ $10^4$ L mol$^{-1}$ cm$^{-1}$ ($\sigma_M$ = 3.66 $\times$ $10^2$) (Figure 3). These results in spectrum shift were corroborated for Spec 1 and Spec 2 by assaying reduced and oxidized extract samples of giant ragweed, woolly cupgrass, common sunflower, and shattercane treated with glyphosate (data not shown). Results from these observations are in agreement with previous publications recommending detection at $A_{380-384}$ for quantification of the PDC chromophore in spectrophotometric methods.[19,37] To further assess the relevance of peak 2 ($\lambda_{max}$ = 380−384 nm) in PDC chromophore quantification, Spec 1 and Spec 2 assays were repeated with extract samples of untreated maize and soybean control plants, fortified with shikimic acid standard at a range of 1−60 $\mu$mol mL$^{-1}$. This resulted in a positive and linear increase in absorbance at $A_{380-384}$ with fortification of shikimic acid standard

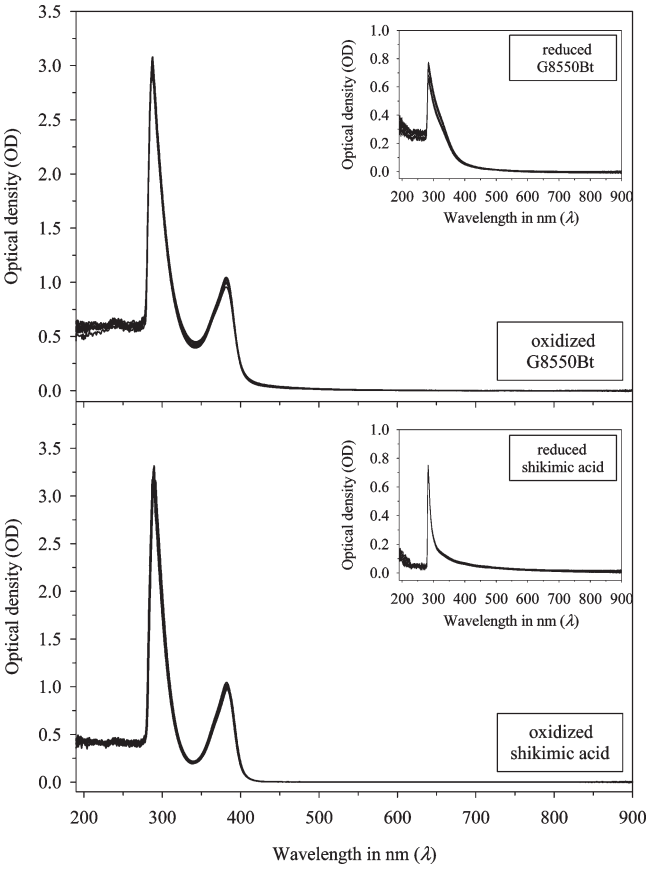


**Figure 3.** Absorption spectra of oxidized shikimic acid standard (bottom graph) and oxidized maize G8550Bt dry tissue sample extracted with extract 1 (top graph); the latter originated from plants treated with 0.86 kg acid equivalents (AE) of glyphosate $ha^{-1}$. A 10 $\mu$L sample of shikimic acid standard or a 15 $\mu$L sample of G8550Bt at a concentration of 30 $\mu$mol $mL^{-1}$ was assayed with Spec 1 and scanned from 190 to 900 nm in 1 mm data intervals (120 mm $min^{-1}$). The depicted lines represent the individual response of 10 independent samples runs. (Insets) Absorption spectra of reduced shikimic acid standard (bottom) and reduced maize G8550Bt (top) at a concentration of 1 M scanned in 1 mm data intervals at 120 mm $min^{-1}$.

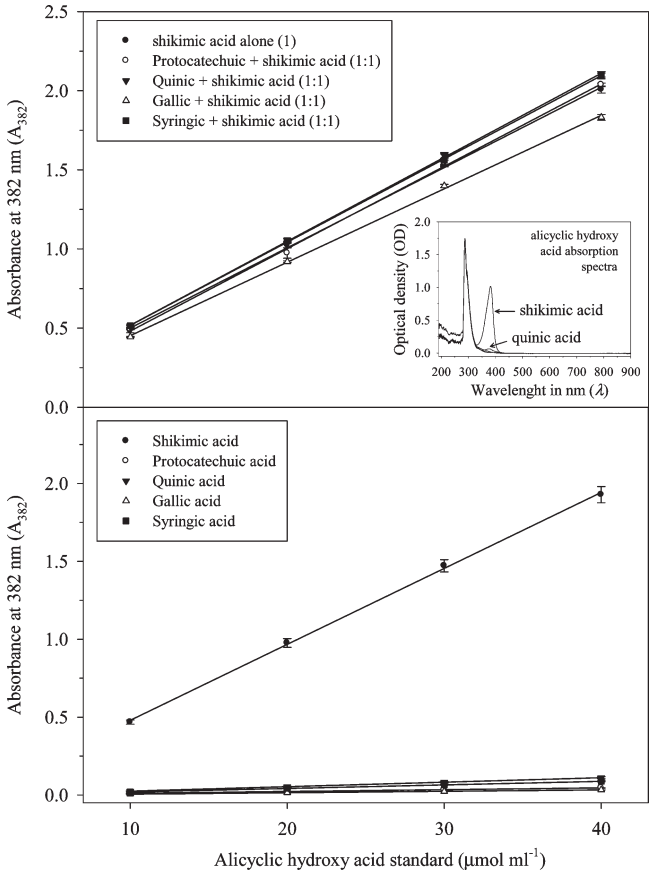


**Figure 4.** Specificity of Spec 1 to shikimic (●), protocatechuic (○), quinic (▼), gallic (△), and syringic (■) acids alone (bottom graph) or in a 1:1 concentration mixture of the alicyclic hydroxy acid and shikimic acid (top graph). Each data point represents the mean of three replications and two experiments ($n$ = 6); bars designate the standard error associated with each mean ($\sigma_M$). (Inset) Absorption spectra of oxidized shikimic and quinic acids; these data were determined at 1 mm data intervals and 120 nm $min^{-1}$ scan speed.

within extracts of the untreated plants (data not shown). Nevertheless, this increase in absorbance accounted for only 80−90% of that obtained when shikimic acid standard was assayed alone at equivalent concentrations. Similar data in untreated cotton (*Gossypium hirsutum* L.) tissue extracts spiked with shikimic acid reported only 73% recovery of shikimic acid compared to assays of shikimic acid alone.[36] These results suggested that the spectrophotometric methods were ≤90% effective in detecting fluctuations in shikimic acid concentrations within plant extract samples; presumably, compounds in these plant extract samples interfered with the oxidation of periodate, the formation of the PDC chromophore, or the determination of absorbance. High background absorbances at 382 nm were reported to occur in samples of glyphosate-untreated cotton extracted in acid; for this species, shikimic acid extraction with 0.05 M NaOH instead of 0.25 M HCl significantly improves sample quality and minimizes background absorbance at 382 nm.[65] The presence of molecules with primary amines may Schiff-base with the dialdehydes formed from the derivatization with periodate and hinder spectrophotometric determinations; imine bonding with glutathione may also reduce by 60% the $A_{380}$ of oxidized shikimic acid samples.[28] Other molecules without primary amines, importantly, epinephrine and ascorbic acid, may also hinder spectrophotometric shikimic acid determinations.[28]

*Evaluation of Method Specificity.* Analogous to the periodate-mediated oxidation of shikimic acid (Figure 1), the periodate anion can cleave *vic*-diols in other alicyclic hydroxy acids, thus yielding carbonyl-containing compounds. The inhibition of EPSPS by glyphosate causes the predominant accumulation of shikimic acid in tissues of treated plants. However, other alicyclic hydroxy acids common to the shikimic acid pathway are also reported to accumulate.[34,52] The origin of these alicyclic hydroxy acids is shikimic acid, which is converted to 3-dehydroshikimic and then to 3-dehydroquinic acids by the concomitant and reversible catalysis of the mono- or bifunctional shikimate 5-dehydrogenase (SKDH; EC 1.1.1.25) and 3-dehydroquinate dehydratase (DHQase; EC 4.2.1.10), respectively.[66] Protocatechuic and quinic acids also accumulate as the compounds are synthesized from 3-dehydroshikimic and 3-dehydroquinic acids, respectively, by the catalysis of DHQase and quinate 5-dehydrogenase (QDH; EC 1.1.1.24).[67] To investigate whether these alicyclic hydroxy acids interfered with spectrophotometric shikimic acid determinations, Spec 1 and Spec 2 were assayed with a

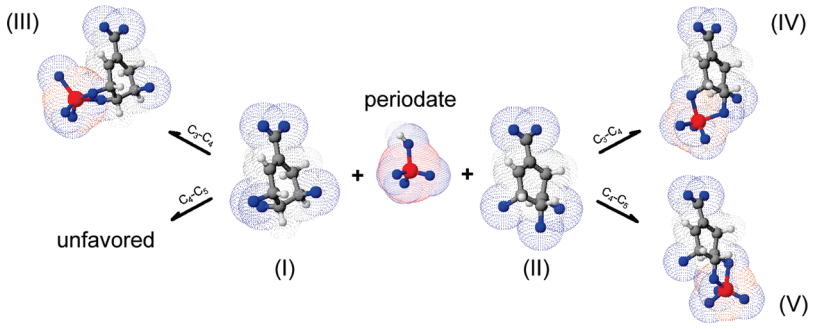


**Figure 5.** Electron density map defining the predicted conformation of shikimic acid and periodate. The $C_1-C_2$ double bond in shikimic acid restricts the cyclohexane to nonplanar (**I**) or near planar (**II**) conformation. Periodate attack of the $C_3-C_4$ *cis*-diol and formation of the transient−cyclic esters occurred in the nonplanar (**III**) and near planar (**IV**) conformations; however, attack of the $C_4-C_5$ *trans*-diol was favored only in the near planar (**V**) conformation. Hydrogen (white), carbon (gray), oxygen (blue), and iodine (red) atoms are depicted in nonbonded van der Waals interactions.

series of alicyclic hydroxy acids alone or in mixture with shikimic acid. Data herein reported are restricted to Spec 1 results as an identical outcome was obtained with Spec 2. The assay of gallic or protocatechuic acids alone at a range of 10−40 $\mu$mol mL$^{-1}$ resulted in limited contribution to the absorption spectra at $A_{382}$; similarly, negligible absorbance at $A_{382}$ was detected when syringic or quinic acid was assayed at these concentrations, and the maximum absorbance detected was ≤0.05 OD (Figure 4). The estimated $\varepsilon_{382}$values for syringic and quinic acids were 2.09 × 10$^3$ and 2.65 × 10$^3$ L mol$^{-1}$ cm$^{-1}$, respectively, and these values differed significantly ($P \leq 0.05$) from those attained for gallic and protocatechuic acids at 40 $\mu$mol mL$^{-1}$. Shikimic acid assayed alone yielded the highest $A_{382}$, which increased exponentially from 0.5 OD at 10 $\mu$mol mL$^{-1}$ to approximately 1.9 OD at 40 $\mu$mol mL$^{-1}$ (Figure 4). Shikimic acid therefore had the highest $\varepsilon_{382}$ (4.82 × 10$^4$ L mol$^{-1}$ cm$^{-1}$ at 40 $\mu$mol mL$^{-1}$) within the evaluated alicyclic hydroxy acids. When the alicyclic hydroxy acids were mixed with shikimic acid at a 1:1 ratio and assayed, only syringic and quinic acids marginally increased the $\varepsilon_{382}$, whereas protocatechuic acid had no effect on $\varepsilon_{382}$ values compared to those attained when shikimic acid was assayed alone (Figure 4). One treatment, gallic acid, hindered $\varepsilon_{382}$ determinations when mixed with shikimic acid, and this differed statistically ($P \leq 0.05$) from the shikimic acid alone treatment at concentrations of ≥30 $\mu$mol mL$^{-1}$ (Figure 4). To further evaluate the potential interference of gallic acid, mixtures of 0.01:1, 0.1:1, 1:1, 10:1, and 20:1 gallic acid/shikimic acid were assayed and compared to shikimic acid assayed alone at equal concentrations. These data confirmed that gallic acid at ratios of ≥10:1 significantly interfered with shikimic acid spectrophotometric determinations (data not shown). A more comprehensive specificity study of approximately 70 metabolic compounds is detailed elsewhere;[28] therein the authors purport that fewer than five compounds effectively interfere with $A_{382}$ determinations. Therefore, spectrophotometric determination based on derivatization with periodate is specific to shikimic acid and can discern between compounds of similar structure. Importantly, the specificity of periodate-based derivatization is used elsewhere in methods that facilitate the characterization of glycoproteins,[68] carbohydrates,[69] and specific peptide bonds[70] and methods that determine rates of antibody oxidation[71] and cell wall surface modification.[72]

To better understand the dynamics and specificity of the periodate-based derivatization, a biomolecular simulation analysis of the alicyclic hydroxy acids in this study was performed with CHARMM.[73] The double bond in $C_1-C_2$ restricts the configuration of the cyclohexene in shikimic acid (Figure 1). Concurrently, the molecular model predicted that shikimic acid adopted a half-boat configuration within an aqueous solution, with either the $C_3-C_4$ *cis*-diol away from the $C_5$ (nonplanar) or closer to $C_5$, in a more planar structure (Figure 5). Furthermore, the periodate ester formed at the $C_3-C_4$ *cis*-diol was predicted to occur in both the planar and nonplanar conformations, whereas esterification at the $C_4-C_5$ *trans*-diol occurred preferentially in planar conformation given the sizable distance between the $C_4-C_5$ *trans*-diol in the nonplanar arrangement (Figure 5). The fact that the nonplanar conformation had a lower energy geometry and that periodate esters in *cis*-diol formed in both the planar and nonplanar conformations suggested that periodate favored oxidation of the $C_3-C_4$ bond over the $C_4-C_5$ bond in shikimic acid. These results are consistent with previous reports that periodate oxidized *cis*-1,2-glycol more rapidly compared to the *trans* isomers;[60] kinetic estimates suggested that the periodate oxidation of *cis*-1,2-diol was 4−16-fold higher compared to glycosides with *trans*-1,2-diol.[74] Similar biomolecular modeling results were obtained with CHARMM for quinic acid, for which the lack of double bonds allowed the cyclohexane to adopt a chair or boat configuration and oxidation of the $C_3-C_4$ diol was favored over the $C_4-C_5$ diol. For gallic and protocatechuic acids, however, the planar configuration resulting from the alternating double bonds in the benzene ring favored a rigid conformation and thus rapid oxidation of both $C_3-C_4$ and $C_4-C_5$ *vic*-diol by periodate. The model predicted that the lack of *vic*-diol in syringic acid precluded the oxidation by periodate.

**Shikimic Acid Quantification in Plant Samples.** In the absence of glyphosate, basal quantities of detectable shikimic acid ranged from 0.0 to 0.4 $\mu$mol g$^{-1}$ of tissue for soybean and maize tissue samples regardless of tissue type and extraction or detection method. Similar quantities were estimated in the tissues of soybean and maize at 0 HAT. These estimates are consistent with determination in glyphosate-free tissues for weeds such as common buckwheat (*Fagopyrum esculentum* Moench)[6] and other crops species[16] for which detectable quantities ranged from 0.0 to 0.5 $\mu$mol shikimic acid g$^{-1}$ of tissue. No shikimic acid accumulated in the tissues of glyphosate-resistant crops treated with glyphosate at 0.86 kg AE ha$^{-1}$, and therefore these data were omitted from this paper. Conversely, large quantities of shikimic acid were detected in the tissue extract samples of glyphosate-susceptible crops. After glyphosate application, shikimic acid accumulation was detected at 5 h,

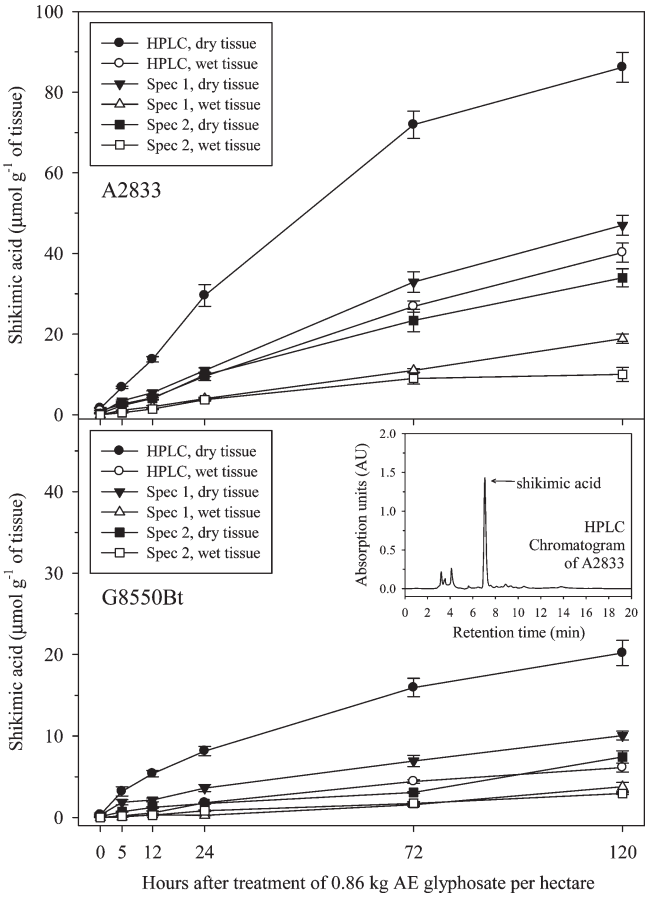


**Figure 6.** Comparison of high-pressure liquid chromatography (HPLC), spectrophotometric 1 (Spec 1) and spectrophotometric 2 (Spec 2) methods in detecting shikimic acid accumulation in time, in glyphosate-susceptible maize G8550Bt (bottom graph) and glyphosate-susceptible soybean A2833 (top graph) plants treated with 0.86 kg acid equivalents (AE) of glyphosate $ha^{-1}$. Determinations were conducted in identical samples of dry or wet tissue, and shikimic acid was isolated with extract 1. Each data point represents the mean of three replications and two experiments ($n$ = 6); bars designate the standard error associated with each mean ($\sigma_M$). (Inset) HPLC chromatogram of A2833 dry tissue sample (15 $\mu$L) extracted with extract 1; shikimic acid elution was monitored at 210 nm and resulted in a retention time ($t_R$) of 7.1 min.

increasing exponentially for the initial 24 h and plateauing at 72 h (Figure 6). Differential shikimic acid accumulation has been used to characterize glyphosate-resistant and -susceptible horseweed (*Conyza canadensis* [L.] Cronq)[22] and other glyphosate-resistant weed species.[23−26] Furthermore, variations of these methodologies based on an excised leaf disk tissue procedure exist, which permits characterization of in vivo shikimic acid accumulation in glyphosate-resistant plants.[58] When detection methods were compared, Spec 1 estimated more shikimic acid in identical plant sample extracts compared to Spec 2 and, likewise, HPLC determinations were more effective than spectrophotometric determinations, quantifying approximately 2 times more shikimic acid compared to Spec 1 and 4 times more compared to Spec 2 (Figure 6). Spectrophotometric methods have limitations in determining background levels of endogenous shikimic acid in glyphosate-untreated plant samples and are more susceptible to interferences caused by pigments absorbing at the same detection wavelength. Conversely, HPLC methods rely on an analytical column to concentrate and separate the compound(s) of interest, thus allowing quantification of lower levels of the compound(s) compared to spectrophotometric methods. Furthermore, spectrophotometric determinations require optimization of the chemical assay conditions (1) temperature, (2) incubation time, and (3) periodate/periodic acid concentrations to ensure maximal conversion of shikimic acid to the PDC chromophore; a 1:1 sensitivity between spectrophotometric and HPLC methods was reported in a different study.[65] More shikimic acid was detected in soybean apexes compared to maize coleoptile tissues regardless of the extraction method or tissue moisture content. As expected, almost twice as much shikimic acid was detected in extract samples of dry tissues compared to wet tissue samples for soybean and maize. Shikimic acid levels in these extract samples ranged from 0.3 to 86 $\mu$mol $g^{-1}$ of tissue, and these estimates were consistent with reports of 0.3−94 $\mu$mol $g^{-1}$ in the tissues of leguminous and nonleguminous species.[75]

Herein, we compared the most common spectrophotometric and HPLC shikimic acid determination methods cited in the literature and elucidated the strengths and weaknesses associated with these methodologies. Spectrophotometric methods were more cost-effective and simpler to execute, but had a narrower detection limit and, thus, quantification of shikimic acid compared to the HPLC method. Both spectrophotometric and HPLC methods were specific to shikimic acid and effectively discriminated between some compounds of analogous chemical structures. Furthermore, we provided guidance toward the appropriate storage conditions of plant samples to maximize integrity and thus consistency of shikimate detection. Endogenous shikimic acid determinations are instrumental in corroborating the efficacy of glyphosate in plants and therefore have countless practical applications in herbicide research and related areas of science as well as commercial agriculture.

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## ASSOCIATED CONTENT

**Supporting Information.** Additional tables concerning costs of shikimic acid extraction and other specifics relevant to this study. This material is available free of charge via the Internet at http://pubs.acs.org.

## ACKNOWLEDGMENT

Nick Polge critically reviewed an earlier version of the manuscript and provided practical advice and comments that supported the discussion and interpretation of this work.

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