Assay Comparison for Measuring Shikimate in Glyphosate-Treated Plant Species

Keith Kretzmer, Mason Hughes, Laylonda Maines and Doug Sammons
Monsanto Company, 800 N. Lindbergh Blvd, St Louis, MO 63167

Abstract

Glyphosate inhibits 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase in the shikimate pathway of susceptible plant species, resulting in the accumulation of shikimate. Quantification of shikimate can be important in the determination of glyphosate resistance in putative resistant weed species. A limited survey has been underway to establish baseline levels of shikimate pathway intermediates, and specifically shikimate, in various crop and weed species treated with glyphosate.

Plants were established in the greenhouse and treated with Roundup WeatherMAX® (800 g/ha) and harvested 3 days after treatment. Plant tissue was ground to a frozen powder and extracted. Identification and quantification of shikimate and dehydroshikimate in extracts was done by HPLC-*uv* and confirmed by HPLC-MS, and quinic acid by HPLC-MS. Data for these metabolites are presented here.

The literature describes a spectrophotometric assay method for measuring shikimate levels in plants (1, 2, 3). A subset of samples was reextracted and assayed using this method in order to compare shikimate values between HPLC-*uv* and the spectrophotometric method.

As expected, shikimate accumulated in plants treated with glyphosate. The level of accumulation depended on the plant species, ranging from 230 ug/gFW in cotton to 7200 ug/gFW in soybean and alfalfa. In many species, quinic acid also accumulated in treated plants, and in certain species, to levels greater than shikimate. Further, in many species, there were significant levels of either shikimate or quinic acid, or both shikimate and quinic acid, in untreated plants.

It was also found that velvetleaf accumulated shikimate and dehydroshikimate at approximately equal levels after glyphosate treatment (808 and 883 ug/gFW, respectively). Of the other species tested, only cotton had measurable levels of dehydroshikimate.

The spectrophotometric plate assay results agreed reasonably well with those measured by HPLC-*uv* for the plant sample extracts tested. It is shown that the spectrophotometric method also detects quinic acid, especially over longer inbubation times such as 60 to 90 min. It is possible, then that quinic acid could interfere with the spectrophotometric assay method, especially if quinic acid levels in extracts are high.

Since plants treated with glyphosate may accumulate both shikimate and quinic acid, and in some cases more quinic acid than shikimate, a more sensitive analytical method such as HPLC-MS and HPLC-*uv* may be necessary for measuring a plant's response to glyphosate. In addition, there are likely several plant species which have significant levels of naturally occurring shikimate and quinic acid, so shikimate measurement alone may not necessarily be directly correlated to glyphosate treatment.

Method

Plants were established in the greenhouse and were treated between 18 and 24 days after planting. Glyphosate, formulated as Roundup WeatherMAX® (800 g ae/ha), was applied at 10 gpa using a track sprayer. Plants were harvested 3 days after treatment and stored at -80 C until extraction.

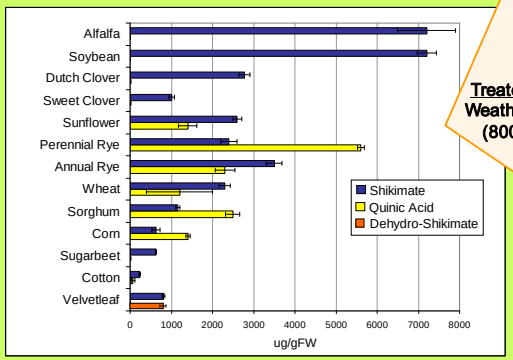
Plant tissue was ground to a frozen powder and 100 mg aliquots were extracted into 900 uL of 0.25N HCl. Extracts were diluted 1:10 in 0.1% formic acid and analyzed independently by HPLC-MS (shikimate anion mass 173, dehydroshikikate anion mass 171, quinic acid anion mass 191)and HPLC-*uv* (shikimate RT 3.4 min, 210nm and dehydroshikimate RT 4.0min, 236 nm), using a stepwise gradient of 0.1% formic acid:acetonitrile. N-methyl glyphosate was used as an internal standard in all samples. All analytes were expressed as ug/gFW.

For the spectrophotometric plate assay method (1, 2, 3), extracts were aliquoted into 100 uL of 0.5% periodate-0.5% *meta*-periodate (1:1) in a 96-well plate and incubated for 45 min at 37 C. After the addition of 100 uL of 0.6 N NaOH-0.22 MNa_2SO_3 (1:1) and mixing, the absorbance was immediately measured at 380 nm. For quinic acid, absorbance was read over successive 15 min intervals.

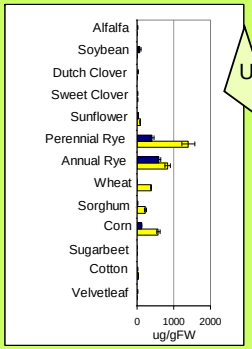
Specificity of HPLC-*uv*, HPLC-MS, and Spectrophotometric Plate Assays for Shikimate, Shikimate 3-Phosphate, Quinic Acid and Dehydroshikimate

Metabolite	Analytical Method		
	HPLC-uv	HPLC-MS	Spectrophotometric Plate Assay
Shikimate	+	+	+
Shikimate- 3-Phosphate	+	+	-
Quinic Acid	-	+	(+)
Dehydroshikimate	+	+	?

Shikimate, Quinic Acid and Dehydroshikimate (ug/gFW) Measured by HPLC-*uv* and HPLC-MS



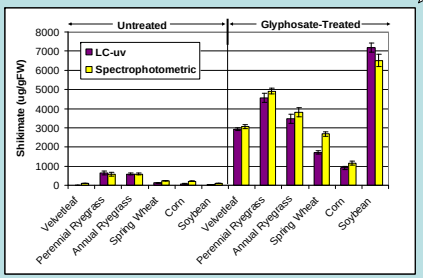
Treated with WeatherMAX® (800 g/ha)



Untreated

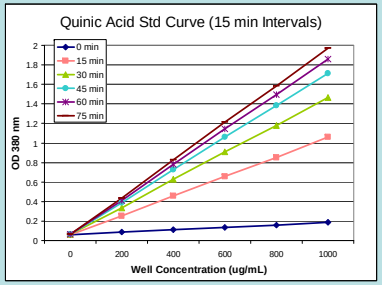
Shikimate and quinic acid, as well as dehydroshikimate, are found in plants by HPLC-uv and HPLC-MS, and increase in response to glyphosate treatment

Comparison between Two Assay Methods (HPLC-*uv* and Spectrophotometric Plate Assay) for Determination of Shikimate in Plant Extracts



For these extracts, quantification of shikimate by HPLC-*uv* method agrees with the Spectrophotometric method

Detection of Quinic Acid with Longer Incubation Times using Spectrophotometric Plate Assay

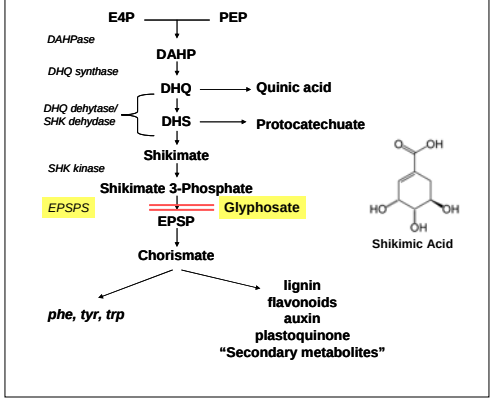


Quinic acid can be measured by the spectrophotometric assay, and may interfere with shikimate measurement if quinic acid levels in plants are high

Conclusions:

- Using HPLC-*uv* and HPLC-MS analysis, at least 3 metabolites were shown to accumulate in susceptible plants in response to glyphosate treatment: shikimate, quinic acid and dehydroshikimate
- The concentration of shikimate which accumulated in glyphosate-treated plants varied depending on the species, ranging from 230 ug/gFW in cotton to 7235 ug/gFW in alfalfa. Untreated plants of some species also contained significant levels of shikimate. For example, perennial ryegrass and annual ryegrass had 405 and 595 ug/gFW shikimate, respectively.
- As measured by HPLC-MS, in those species which accumulated quinic acid, the levels ranged from 1208 ug/gFW in wheat to 5621 ug/gFW in perennial ryegrass, after glyphosate treatment. In perennial ryegrass, sorghum and corn, more quinic acid than shikimate accumulated after glyphosate treatment.
- In velvetleaf, dehydroshikimate was identified by HPLC-MS and measured by HPLC-*uv*. It accumulated to levels approximately equal to shikimate in plants treated with glyphosate. Dehydroshikimate was also detected in treated cotton, but not in any other species in this study.
- The quantitative results for shikimate measured by the spectrophotometric plate assay agreed with those obtained by the more specific analytical methods of HPLC-*uv* and HPLC-MS in this study.
- It is possible that there could be interference by quinic acid in spectrophotometric plate determination of shikimate, especially if quinic acid concentrations in extracts are high.

Shikimate Pathway in Plants



Data Table

Plant Species	Shikimate (ug/gFW)		Quinic Acid (ug/gFW)	
	Untreated	WeatherMAX (800 g/ha)	Untreated	WeatherMAX (800 g/ha)
	Mean +/- Serr	Mean +/- Serr	Mean +/- Serr	Mean +/- Serr
Soybean	75 +/- 40	7188 +/- 233	ND	ND
Alfalfa	16 +/- 6	7235 +/- 701	ND	ND
Wheat	0 +/- 0	2306 +/- 80	381 +/- 9	1208 +/- 128
Annual Ryegrass	595 +/- 48	3465 +/- 238	836 +/- 75	2309 +/- 188
Perennial Rye	405 +/- 55	2382 +/- 75	1395 +/- 179	5621 +/- 200
Sunflower	33 +/- 4	2649 +/- 224	84 +/- 4	1449 +/- 111
Sugarbeet	0 +/- 0	615 +/- 89	3 +/- 2	7 +/- 1
Cotton	13 +/- 2	230 +/- 40	37 +/- 2	70 +/- 10
Corn	122 +/- 11	630 +/- 50	579 +/- 50	1400 +/- 100
Sorghum	19 +/- 1	1159 +/- 46	229 +/- 28	2488 +/- 167
Sweet Clover	12 +/- 2	1007 +/- 63	15 +/- 1	12 +/- 1
Dutch Clover	27 +/- 6	2776 +/- 132	0 +/- 0	15 +/- 4
Velvetleaf	13 +/- 1	808 +/- 29	ND	ND

Results

It had previously been determined that plants treated in the greenhouse with a lethal dose of glyphosate ceased growth at 3 days after treatment. In addition, it had been demonstrated that a glyphosate rate of 800 g/ha resulted in maximum herbicide effect.

The quantification of shikimate in all extracts was measured by HPLC-*uv* and and confirmed by HPLC-MS. Of the species tested, soybean and alfalfa accumulated the highest levels of shikimate, 7188 and 7235 ug/gFW, respectively, after glyphosate treatment. Other dicots tested such as cotton, sugarbeet, velvetleaf, sweet clover, sunflower and Dutch clover accumulated 230, 615, 808, 1007, 2649 and 2776 ug/gFW shikimate, respectively. Of the monocot species tested, corn, sorghum, spring wheat, perennial ryegrass and annual ryegrass accumulated 630, 1159, 2306, 2382 and 3465 ug/gFW shikimate, respectively.

At least three species, corn, perennial ryegrass and annual ryegrass had high levels of shikimate in untreated leaves, measuring 122, 405 and 595 ug/gFW, respectively. These levels of shikimate approach those measured in cotton, sugarbeet and velvetleaf which had been treated with glyphosate.

Quinic acid was found by HPLC-MS to accumulate in certain species after glyphosate treatment. In spring wheat, corn, sunflower, annual ryegrass, sorghum and perennial ryegrass, quinic acid was measured to be 1208, 1440, 1449, 2309, 2488 and 5621 ug/gFW, respectively. In perennial ryegrass, sorghum and corn, measured quinic acid levels were greater than measured shikimate levels. Also, there were high levels of quinic acid in untreated leaves of sunflower, sorghum, spring wheat, corn, annual ryegrass and perennial ryegrass, measured to be 84, 229, 381, 579, 836 and 1395 ug/gFW, respectively.

In velvetleaf, both shikimate and dehydroshikimate were measured by HPLC-*uv* in plant extracts treated with glyphosate, and at approximately equal levels (808 and 883 ug/gFW, respectively). Dehydroshikimate was also detected in cotton, but not in any other species tested.

The periodate/sulfite spectrophotometric plate assay developed by Cromartie and Polge has been used to measure shikimate in plant extracts (1, 3). Other substrates, including quinic acid and tryptophan, can also be detected by this assay (2). The results here show that absorbance due to quinic acid increases with time, probably because of the slow periodate oxidation of quinic acid.

References:

(1) Cromartie, T.H. and N.D. Polge. 2000. An improved assay for shikimicacid and its use as a monitor for the activity of sulfosate. Proc. Weed Sci. Am. 40:291.

(2) Cromartie, et al. 2002. Method of detecting shikimic acid. US Patent 6,482,654

(3) Singh, B.J. and D.L. Shaner. 1998. Rapid determination of glyphosate injury to plants and identification of glyphosate-resistant plants. Weed Technol. 12:527-530.