

Analytical Method for Glyphosate and AMPA in Raw Agricultural Commodities and their Processed Commodities

AMENDMENTS:

Overview

Purpose & Scope

This SOP describes the analytical method used by ESTC personnel to determine trace quantities of glyphosate (N-phosphonomethyl glycine) and AMPA in RACs and their processed commodities by HPLC.

Method Summary

Glyphosate and AMPA can be isolated from various matrices by extraction followed by application to a column containing the Fe(III) form of Chelex[®] 100 resin which complexes glyphosate and AMPA. The glyphosate and AMPA are eluted from the resin with HCl and the iron is removed using an anion exchange resin. After concentration to dryness to remove the HCl, samples are taken back up in either water or mobile phase, filtered, and analyzed by HPLC. Glyphosate and AMPA are separated and detected utilizing an HPLC equipped with a PCR system specific for primary amines. Glyphosate is oxidized to glycine in the presence of sodium hypochlorite, while AMPA remains unchanged. Glycine and AMPA are then each coupled with OPA in the presence of 2-mercaptoethanol. A fluorescence detector detects the resulting products of this reaction with excitation measured at 340 nm and emission at 455 nm. This method has been validated down to 0.05 ppm each of glyphosate and AMPA for sample sizes of 4.0 to 30.0 g. In some cases, it may be possible to validate down to the 0.05 ppm level in sample sizes of 2.0 to 4.0 g.

Abbreviations

The following abbreviations are used in this SOP:

Abbreviation	Definition
AMPA	aminomethylphosphonic acid
ARS	analytical reference standard
EDTA	ethylenediamine-tetraacetic acid
ESTC	Environmental Sciences Technology Center
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
OPA	o-phthalaldehyde
PCR	post-column reaction/reactor
RAC	raw agricultural commodity
SPE	solid phase extraction

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Materials

Equipment

The following equipment is used in this procedure. Specific brands are listed to aid the analyst in finding items. In most cases, equivalent equipment from other vendors can be used.

Sample Extraction/Preparation Equipment	Number/Specification
Balance (electronic, top-loading)	Mettler, Model PM-4800
Beaker (plastic, 500 mL)	suitable for procedure
Blender (motor)	Waring™, VWR Cat. No. 58977-227
Blender jar	Waring™ <ul style="list-style-type: none"> • VWR Cat. No. 58979-018 (glass) or • VWR Cat. No. 74800-002 (stainless steel)
Centrifuge (superspeed automatic refrigerated)	Sorvall Instruments (DuPont Co.), Model RC5B or RC5C
Centrifuge bottle (polypropylene, 250 mL)	<ul style="list-style-type: none"> • VWR Cat. No. 21010-614 or • Sorvall Instruments (DuPont Co.) Cat. No. 03937 or • Nalge Company (Nalgene product) Cat. No. 3141-0250
Centrifuge bottle sealing cap	<ul style="list-style-type: none"> • VWR Cat. No. 21010-035 or • Sorvall Instruments (DuPont Co.) Cat. No. 03278 or • Nalge Company (Nalgene product) Cat. No. 3132-0058

Columns (chromatography, glass): <ul style="list-style-type: none"> • 2.2 cm OD x 25 cm, with a 250 mL reservoir and a 1.5 cm delivery tip • 1.7 cm OD x 22 cm, with a 3 cm OD x 8 cm reservoir and a 1.5 cm delivery tip 	<ul style="list-style-type: none"> • Ace Cat. No. 5907-15 • Ace Cat. No. 5888-30
Dispensers (0-10 mL, 0-25 mL, 0-100 mL)	Brinkmann Dispensettes
Glass wool	suitable for procedure
Graduated cylinders (25 mL, 100 mL, 250 mL, 2 L)	suitable for procedure
Hot plate	Corning, Model PC-400, VWR Cat. No. 33920-224
pH meter	Corning, Model 445 and Corning 3-in-1 combination electrode
pH strips (range 0-6)	EM-Reagents colorpHast [®] , VWR Cat. No. EM-9586-3
Pipettes (adjustable: 40 µL-200 µL, 200 µL-1000 µL, 1000 µL-5000 µL)	suitable for procedure
Pipettes (transfer): <ul style="list-style-type: none"> • 5 ¾" length • 9" length 	<ul style="list-style-type: none"> • VWR Cat. No. 53283-910 • VWR Cat. No. 53283-914
Rotary evaporator power unit	Strong Innovations Inc.
Round bottom flask (250 mL)	suitable for procedure
Solid phase extraction cartridge (C ₁₈), 900 mg	Alltech Associates Inc., Prevail Maxi-Clean cartridges, Part No. 605942
Splash guard adapter	Aldrich Cat. No. Z14,779-6
Stainless steel utility bowl	Cole-Parmer Cat. No. SR-07274-00 (304 SS, heavy guage)
Syringe (disposable with Luer-Lok tip, 3 mL)	Becton-Dickinson, VWR Cat. No. BD309585
Syringe filter (13 mm disposable filter device, 0.45 µm pore size)	Whatman [®] , VWR Cat. No. 28137-564
Timer	GraLab, Model 171, VWR Cat. No. 62371-045
Transformer (variable, used to control blender speed)	VWR Cat. No. 62546-048
Vacuum pump	Sargent-Welch Duo Seal, Model 1400
Vacuum trap (Dewar-type, large volume)	Kimble Kontes Cat. No. 926910-1000
Water purification system	Millipore Compact Milli-Q Plus

HPLC Equipment	Number/Specification
Autosampler	Perkin-Elmer Series 200 (can be programmed to control valve switching)
Autosampler vials (12 x 35 mm, ½ dram, 2 mL)	Kimble Glass Inc. Cat. No. 60910-12
Autosampler vial caps (8mm screw top)	Varian Cat. No. 1600069800
Autosampler vial septa (red, Teflon-faced)	Varian Cat. No. 6900016900
Back-pressure (500 psi) regulator cartridge with PEEK holder	Upchurch Scientific
Back-pressure (100 psi) regulator cartridge with PEEK holder (put on end of waste line from detector)	Upchurch Scientific
Columns (glyphosate analysis; for use of another column, see “ Appendix ”): <ul style="list-style-type: none"> • Aminex, 30 cm x 4.6 mm ID • HRLC, 10 cm x 4.6 mm ID 	<ul style="list-style-type: none"> • Bio-Rad Labs Cat. No. 125-0104 • Bio-Rad Labs Cat. No. 125-0108
Columns (guard), 1.5 cm x 3.2 mm: <ul style="list-style-type: none"> • NewGuard cation, 7 µ or • NewGuard RP-18, 7 µ 	<ul style="list-style-type: none"> • Brownlee Columns Part No. 0711-0104 • Brownlee Columns Part No. 0711-0092
Columns (guard column holder): NewGuard Holder Complete	Brownlee Columns Part No. 0715-0001
Data collection system	suitable for procedure
Filter device (aqueous in-line filter/degasser)	Whatman® Cat. No. 6726-5002
Filter device (solvent filter/degasser, 0.2 µm pore size)	Gelman VacuCap® 90 filter unit, VWR Cat. No. 28143-315
Fluorescence detector	Jasco Model FP-920
Mixing tees (two required)	suitable for procedure
Pulse damper	SSI LO-Pulse®
Pumps (two)	Jasco Model PU-1580
Pumps (post column reagent, two required): require high backpressure to pump the post column reagents at low flow rates before they enter the detector. Pulse dampeners are utilized to reduce detector noise due to these pumps.	<ul style="list-style-type: none"> • Acuflow Series III Pumps or • SSI Model 222D liquid chromatography pumps with polymeric self-flushing heads
Reaction coil (1 mL, two required)	Kratos Cat. No. 1400-1324
Remote switch: a two position, six port, 60° rotation electrically actuated valve.	Valco Instruments Co., Inc., Model E60
Reservoir (solvent buffer)	Kontes 5L Ultra-ware HPLC reservoir
Temperature control module and column heaters (two) for the HPLC columns, the oxidation coil, and the OPA reaction coil	Waters

Tubing (stainless steel or PEEK): <ul style="list-style-type: none"> • 1/16" OD x 0.020" ID (before columns) • 1/16" OD x 0.010" ID (after columns) • 1/16" OD x 0.005" ID (may be used to deliver post column reagents to mixing tee, primarily used for additional back-pressure to post column pumps) 	suitable for procedure
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Chemicals, Reagents & Resins

The following chemicals, reagents, and resins are used in this procedure. Specific brands are listed to aid the analyst in finding items. In most cases, equivalent items from other vendors can be used.

Chemical/Reagent/Resin	Number/Specification
AMPA, analytical grade, >95% purity	Monsanto ARS Program
AG 1-X8 analytical grade anion exchange resin, chloride form, 200-400 mesh	Bio-Rad Laboratories Cat. No. 140-1451
Chelex [®] 100 analytical grade chelating resin, iron form, 100-200 mesh	Bio-Rad Laboratories Cat. No. 142-2825
Chloroform, HPLC grade	JT Baker, VWR Cat. No. JT9175-33
Deionized water	Milli-Q water
Glyphosate (N-phosphonomethyl glycine), analytical grade, >95% purity	Monsanto ARS Program
Hydrochloric acid (HCl), reagent grade: <ul style="list-style-type: none"> • 12 N (37%) • 0.2 N • 0.1 N 	<ul style="list-style-type: none"> • JT Baker, VWR Cat. No. JT9535-0 or EM Science HX0603-75 • JT Baker, VWR Cat. No. JT5612-07 • JT Baker, VWR Cat. No. JT5621-07
Methanol, HPLC grade	Burdick & Jackson Cat. No. 230-4
OPA (Fluoraldehyde [®]), (for stability information, see " Resin and Reagent Solution Preparation "/" OPA Solution ")	Pierce Chemical Company Cat. No. PI26025 (for alternative OPA solutions, see " Appendix ")
Phosphoric acid (85%), HPLC grade	EM Science, VWR Cat. No. EM-PX0996-6
Potassium dihydrogen phosphate (KH ₂ PO ₄), crystals, ultrapure bioreagent	JT Baker, VWR Cat. No. JT4008-01
Sodium chloride, crystals, ultrapure bioreagent	JT Baker, VWR Cat No. JT4058-01
Sodium hydroxide solution (50% w/w)	JT Baker, VWR Cat No. JT3727-01
Sodium hypochlorite solution (5%) Important: This solution deteriorates with time. Replace with a new unopened bottle, as needed.	JT Baker, VWR Cat No. JT9416-01

Resin and Reagent Solution Preparation

Overview The following describes the preparation and storage of the resin and reagent solutions used in this procedure. **Note:** Absolute amounts may be changed as long as proportions are maintained.

**Chelex®
100 Resin in
Fe(III) Form** Following are the steps to prepare Chelex® 100 resin in the Fe(III) form (for an alternative Chelex® resin preparation, see “**Appendix**”):

Step	Action
1	Transfer an appropriate amount of Chelex® 100 resin in the Fe(III) form into a clean bottle with lid.
2	Wash the Chelex® 100 resin by filling the bottle with deionized water and shaking until thoroughly mixed. Allow the resin to settle for at least 20 minutes before decanting the supernatant. Repeat two more times.
3	Store the resin under deionized water at room temperature until used.

AG 1-X8 Resin Following are the steps to prepare AG 1-X8 resin:

Step	Action
1	Transfer half of the contents of a bottle of AG 1-X8 resin to another clean plastic bottle with lid.
2	Wash each half by filling the bottle with deionized water and shaking until thoroughly mixed. Allow the resin to settle for at least 20 minutes before decanting the supernatant. Repeat two more times.
3	Store the resin under deionized water at room temperature until used.

6 N HCl Following are the steps to prepare 6 N HCl:

Step	Action
1	Add 1 L deionized water to a suitable container.
2	Add 1 L 12 N (37%) HCl to the same container.
3	Mix well. Store at room temperature.

**4% Methanol/
Deionized
Water** Following are the steps to prepare 4% methanol/deionized water:

Step	Action
1	Add 80 mL of methanol to a suitable container.
2	Add 1920 mL of deionized water to the same container.
3	Mix well. Store at room temperature.

**0.005 M
KH₂PO₄
HPLC Buffer**

Following are the steps to prepare 0.005 M KH₂PO₄ HPLC buffer:

Step	Action
1	Weigh 2.72 g of KH ₂ PO ₄ into a suitable container.
2	Add 4 L of 4% methanol/deionized water to the same container. Mix well to ensure complete dissolution.
3	Adjust the pH in the range of 1.5 to 2.4 with 85% phosphoric acid. Store at room temperature.

**Oxidative
Solution**

Following are the steps to prepare the oxidative solution (for an alternative oxidative solution preparation, see “**Appendix**”):

Step	Action
1	Add the following to a suitable container: <ul style="list-style-type: none"> • 2.72 g KH₂PO₄ • 23.2 g NaCl • 1.6 g 50% (w/w) NaOH solution • ~400-500 µL 5% sodium hypochlorite solution (the volume may need to be varied to provide the best peak response). • 2 L deionized water
2	Mix well to ensure complete dissolution.
3	Filter the solution through a 0.22 µm filter. Store at room temperature for up to ten days after preparation.

**OPA
Solution**

The OPA solution purchased from the Pierce Chemical Company is a specifically formulated solution. If unopened, it has an outstanding shelf life with no increase in background fluorescence over time for approximately one year.

After opening a new bottle of OPA solution and transferring it to a reservoir for use, the solution may be stored in a closed glass bottle under atmospheric conditions for up to two weeks without an appreciable increase in background fluorescence. When not in use (but previously opened), always put a head of nitrogen (or some other inert gas) over the solution, cap, and store in a refrigerator at <10°C to maximize shelf life.

For alternative OPA solutions, see “**Appendix**”.

Fortification and HPLC Standard Preparation

1000 µg/mL Mixed Standard Stock Solution

Glyphosate and AMPA are used to prepare mixed or unmixed standard solutions. Following are the steps to prepare a 1000 µg/mL **mixed** standard stock solution of glyphosate and AMPA:

Step	Action
1	Weigh 0.1000 ± 0.0005 g (weight adjusted for purity) each of glyphosate and AMPA and transfer into a single 100 mL volumetric flask.
2	Dissolve together and dilute to volume with deionized water.
3	Mix well (e.g., stir overnight) and/or sonicate to ensure complete dissolution. This stock solution contains 1000 µg/mL each of glyphosate and AMPA.

Working Standard Solutions

Following are the suggested serial dilutions to prepare the working standard solutions (i.e., fortification and HPLC) from the mixed standard stock solution. Other concentrations may be chosen, depending on the desired calibration range.

Aliquot the appropriate volume and concentration of the stock solution into a 100 mL volumetric flask. Dilute to volume with deionized and mix well. Store in appropriate bottles (e.g., polypropylene, amber glass) with airtight lids at <10°C.

Note: If the matrix requires that the final analysis samples be dissolved in a solvent other than deionized water (see “**Sample Extraction/ Preparation**” / “**Concentration and Preparation for Analysis**”), the HPLC standard solutions must be prepared in the same solvent.

Concentration of Stock Solution (µg/mL)	Volume of Stock Solution (mL)	Final Volume (mL)	Final Analyte Concentration (µg/mL)
1000	10.0	100	100.0
1000	3.00	100	30.0
1000	2.00	100	20.0
1000	1.50	100	15.0
1000	1.25	100	12.5
1000	1.00	100	10.0
1000	0.750	100	7.50
1000	0.500	100	5.00
100.0	3.00	100	3.00
100.0	2.00	100	2.00
100.0	1.00	100	1.00
100.0	0.500	100	0.500
100.0	0.250	100	0.250
100.0	0.100	100	0.100
5.00	1.00	100	0.050
5.00	0.500	100	0.025

Labeling

All standard solutions must be properly labeled with the following minimum information:

- description (e.g., “Glyphosate”, “AMPA”, “Mixed Standard Stock Solution”) or identity (e.g., unique lot or batch number(s))
- concentration of analyte(s) (e.g., “1000 µg/mL”)
- dilution solvent (e.g., “deionized water”)
- storage requirements (e.g., “<10°C”)
- preparation date
- expiration date (refer to the current version of “ES-PO-0897” for the procedures for setting expiration dates)
- notebook page or other unique identifier describing preparation
- signature/initials of preparer

Stability

Stock and working standard solutions are considered stable for up to six months when stored under refrigerated conditions (i.e., <10°C), unless further stability data is generated that supports a longer time period. Contact the Monsanto ESTC archivist for documentation. Working standard solutions cannot have an expiration date past that of the neat ARS or stock solution from which it was prepared.

Sample Extraction/Preparation**Preparation of
Chelex® Column**

Following are the steps to prepare a Chelex® column:

Step	Action
1	Add glass wool to the glass chromatography column (2.2 x 25 cm).
2	Measure 15 mL* of Chelex® 100 resin in the Fe(III) form (previously prepared in “ Resin and Reagent Solution Preparation ”/“ Chelex® 100 Resin in Fe(III) Form ”) into a 25 mL graduated cylinder. Document the lot number.
3	Allow the resin to settle and adjust the volume of resin to 15 mL.
4	Rinse the resin from the graduated cylinder into the column with deionized water.
5	If using the column within the next few hours, allow all the water to elute from the column. Otherwise, leave at least 5 mL of deionized water sitting on top of the resin bed and elute to waste before proceeding to “ Chelex Column Chromatography ”.

* More Chelex® resin (or less sample weight) may be needed when fortification recoveries are low (due to possible matrix competition for binding sites and Chelex® resin lot to lot variations of the binding sites). Volumes of 6 N HCl and concentrated HCl must be increased proportionately to compensate for the increase in Chelex® resin volume (see “**Chelex® Column Chromatography**”). The amount of Chelex® resin, 6 N HCl, and concentrated HCl used must be documented in the raw data.

**Sample
Extraction**

Following are the steps to extract the sample. **Note:** If the samples have not been prepared previously, they must be ground frozen in a Hobart chopper or a Waring blender with dry ice added. Allow the carbon dioxide to evaporate before weighing the samples. Samples that are liquid, powder, or fine granular form do not need sample preparation.

Step	Action
1	Weigh approximately 10 g of sample into a blender jar. Note: The sample weight may vary from 2 – 30 g depending on the type of sample. Bulky samples (e.g., dry hay and straw) and oily matrices (e.g., soybean grain, nutmeat, oil) may require smaller sample sizes to get higher extraction efficiencies and recoveries.
2	If required, fortifications must be made at this stage by adding the correct volume of the appropriate fortification standard solution to the designated control samples.
3	Add 50 mL of chloroform and 150 mL of 0.1 N HCl. Cover with a sheet of plastic and blend for 1 minute. Note: Blend cotton seed for at least 3 minutes.
4	Transfer the contents of the blender jar into a 250 mL centrifuge bottle. Do <u>not</u> rinse the blender jar. Balance pairs of centrifuge bottles by adding chloroform to the lighter bottle until its weight is within 1 g of the heavier bottle.
5	Centrifuge at 11,000 rpm for 20 minutes in a refrigerated centrifuge.
6	Decant off approximately 100 mL (document the actual volume) of the aqueous layer into a graduated cylinder. This volume may be adjusted depending on how much can be decanted off, or if sensitivity needs to be increased or decreased.
7	Transfer the sample extract to an appropriately sized beaker or bottle and dilute with 250 mL of deionized water. Check the pH of the diluted sample extract with a pH strip and ensure it is 2.0 ± 0.5 (if running multiple samples spot check at least three sample extracts). If the pH is too low or too high, adjust it with an acidic (HCl) or basic (NaOH) solution. The sample is now ready to be applied to the Chelex [®] column. Note: In the case of oily matrices (e.g., soybean grain, nutmeat, oil), the decanted sample extract may have to be diluted with 250 mL of 0.01 N HCl instead of deionized water to prevent flocculation of the cloudy sample extract which sometimes occurs with these samples during the Chelex [®] column chromatography. Extracting smaller weights may also eliminate flocculation. Use of this alternate procedure must be documented in the raw data.

Chelex[®] Column Chromatography Following are the steps to apply the sample extract to a Chelex[®] column:

Step	Action
1	Transfer the sample extract to the column previously prepared in " Preparation of Chelex[®] Column ". Important: The entire sample extract will <u>not</u> fit into the column all at once!! Allow the sample extract to elute at a rate of approximately 1 drop per second or less and allow the eluent to go to waste. Note: This is a convenient stopping point.
2	After the solution has eluted, rinse the walls of the column with approximately 50 mL of deionized water. Elute at approximately 1 drop per second or less and allow the eluent to go to waste. (If flocculation stops flow, stirring the top of the resin bed occasionally with a piece of copper wire (coiled at the end) or a glass rod will help the elution along. Rinse the wire or rod with deionized water before using on other columns.) Note: This is a convenient stopping point.
3	After the water rinse has eluted, rinse the column with 100 mL of 0.2 N HCl. Elute with the stopcock wide open and allow the eluent to go to waste.
4	Close the stopcock on the column and add 3 mL of 6 N HCl. The resin bed should be disturbed as little as possible. Adjust the elution rate to approximately 1 drop per 2-3 seconds and allow the eluent to go to waste.
5	After the 3 mL aliquot has eluted, add an additional 4 mL of 6 N HCl. Maintain the same elution rate as the previous step and allow the eluent to go to waste.
6	Place a graduated cylinder (or other suitable container) beneath the column. Add 5 mL of 6 N HCl and collect in the container. Repeat two more times. Allow each 5 mL aliquot to elute completely before adding the next.
7	Add 10 mL of 12 N (37%) HCl to the collected eluents.

Preparation of Anion Exchange Column Following are the steps to prepare an anion exchange column:

Step	Action
1	Add glass wool to the glass chromatography column (1.7 x 22 cm). Measure 5 cm above the top of the glass wool plug and mark the column.
2	Shake the container of AG 1-X8 resin (previously prepared in " Resin and Reagent Solution Preparation "/" AG 1-X8 Resin ") to mix well before adding the resin to the column. Add the resin (document the lot number) and allow it to settle to the mark. Remove any extra resin from the column and rinse down the sides with deionized water.
3	If using the column within the next few hours, allow all the water to elute from the column. Otherwise, leave at least 5 mL of deionized water sitting on top of the resin bed and elute to waste before proceeding to " Step 4 ".
4	Shortly before adding the sample extract, add a total of 15 mL of 6 N HCl to condition the columns. Allow the acid to flow through the column with the stopcock wide open and allow the eluent to go to waste. Important: Do <u>not</u> add the acid if the sample extract will not be applied that same day (see " Anion Exchange Column Chromatography ").

Anion Exchange Column Chromatography Following are the steps to apply the sample to an anion exchange column:

Step	Action
1	Place a 250 mL round bottom flask beneath the column (previously prepared in the " Preparation of Anion Exchange Column ") to collect the eluent.
2	Add the sample from the graduated cylinder (or other suitable container) from " Chelex[®] Column Chromatography "/" Step 7 " to the column with the stopcock wide open.
3	Rinse the sample container with 2 mL of 6 N HCl and apply to the column. Perform a final rinse of the column by adding 8 mL of 6 N HCl. Note: This is a convenient stopping point. The samples can sit in stoppered round-bottom flasks at room temperature over a night or weekend.

Concentration & Preparation for Analysis Following are the steps to concentrate and prepare the sample for analysis:

Step	Action
1	Concentrate the sample to dryness on a rotary evaporator by slowly increasing the temperature of the water bath from approximately 20°C to approximately 60°C. Note: As long as no bumping occurs, the water bath can be maintained at a higher temperature.
2	Dissolve the residue in 3.0 mL of deionized water*. A transfer pipette can be used to rinse the sides of the round-bottom flask with the aqueous mixture. Allow the sample to settle to the bottom of the flask.
3	Transfer the sample to a 3 mL syringe fitted with a C ₁₈ SPE cartridge (optional) and a 13 mm filter (0.45 µm pore size) and filter the sample into an autosampler vial (or scintillation vial prior to dilution, if necessary).
4	The sample is now ready for quantification of glyphosate and AMPA using the HPLC OPA PCR System. If the samples are not analyzed immediately, store the autosampler vials in a refrigerator at <10°C.

* Depending upon sample weights, extraction volumes, and the level of detection desired, the residue may be dissolved in more or less deionized water (measure volume to within 1%). If the residue is difficult to dissolve in water, HPLC buffer (with or without methanol) or 0.1 M EDTA may be used. If the residue still does not dissolve, concentrate to near dryness (approximately 0.5 mL), transfer to a graduated centrifuge tube (using part of the final volume desired to rinse the flask and bring up to volume), mix, and filter what is needed into an autosampler vial. Use of any of these alternate procedures must be documented in the raw data.

HPLC OPA PCR System

Overview

A general schematic of the components needed for the construction of the HPLC OPA PCR system are presented in Figure 1. The details are presented in Figure 2.

HPLC Conditions

The following HPLC parameters/conditions are instrument dependent and are considered nominal values. Different instruments may require modifications of these parameters in order to achieve satisfactory sensitivity and separation. Actual parameters/conditions must be documented in the raw data.

- Columns (glyphosate analysis; for use of another column, see “**Appendix**”):
 - ❖ Aminex, 30 cm x 4.6 mm ID
 - ❖ HRLC, 10 cm x 4.6 mm ID
- Columns (guard), 1.5 cm x 3.2 mm:
 - ❖ NewGuard cation, 7 μ or
 - ❖ NewGuard RP-18, 7 μ
- Column temperature: 50°C
- Reactor coil temperature: 38°C (may be increased to 40°C as needed for analysis).
Note: To prevent problems from temperature variation, the hypochlorite reaction coil must be placed in a temperature-controlled module. A temperature of 38-40°C gives the optimum response for both glyphosate and AMPA.
- Buffer solution flow rate: 1.0 mL/min total flow (0.50 mL/min per pump)
- Oxidative solution flow rate: 0.25-0.50 mL/min*
- OPA solution flow rate: 0.30-0.50 mL/min*
- Injection volume: 10-100 μ L
- Valve switch time: in the range of 10-20 min [The proper timing for the switching valve controller is determined by injecting a standard containing glyphosate and AMPA (at the highest standard level to be used in an analytical set) on the short column only.]
- Analysis time: Using the HPLC conditions described above, the analysis time between injections is typically 60 minutes, depending on the quality of the column. Retention times of glyphosate and AMPA increase with column use. Following the column manufacturers instructions on regenerating the column will usually restore retention times to their original values.

* The exact flow rates of the oxidative and OPA solutions will depend on the pH of the HPLC buffer solution. The flows should be balanced so that the glyphosate and AMPA peak heights are similar. A glyphosate peak in which the response is approximately <75% of the AMPA peak is an indication that not enough oxidative solution is being delivered. Too much hypochlorite in the oxidative solution will dilute the fluorescence and decrease sensitivity. A balance between oxidation and desired sensitivity must be achieved. The point at which this balance is acquired is left to the discretion of the analyst. For sample chromatograms, see Figure 5.

Air Bubbles

When starting the HPLC OPA PCR system, turn on all the pumps and the detector for thirty minutes prior to use. If an air bubble becomes trapped in the detector cell, it can be removed by disconnecting both lines to the detector and alternately drawing and forcing liquid through the cell with a syringe containing water or methanol. If an air bubble becomes trapped within the pumps, disconnect the pump outlet line from the remainder of the system and turn up the flow rate until the bubble has been forced out. A purge valve installed on the pump eliminates the need to disconnect the lines. An inline filter/degasser (buffer pumps) and bubbler (oxidative and OPA pumps) installed in the line will help prevent bubbles from entering the pump. The filter should be replaced approximately every three months. If an inline filter/degasser is not installed, the buffer solution must be filtered prior to use.

**Two-Column
Switching
System for
Late Eluting
Components**

Due to the late eluting components found in most samples, a two-column switching system consisting of an electronically actuated 6-port valve controlled by an autosampler (e.g., Perkin-Elmer Series 200, or other suitable controller) is connected to a 10 cm HPLC glyphosate analysis column and a 30 cm Aminex glyphosate analysis column. The switching system is used to divert the late eluting components to waste. A suggested configuration of the valve is shown in Figure 3, with the starting valve position shown on the bottom. The injected sample goes through both the 10 cm and 30 cm columns. When the last component of interest has entered the 30 cm analytical column, the valve is switched over to the position shown in the top of Figure 3. The second pump, also pumping the same analytical buffer, pumps through the 10 cm column either by back-flushing or through the column to flush late eluting components to waste (the figure shows through-column flushing). This prevents the late eluting components from entering the 30 cm analytical column.

If necessary, the 10 cm column may be back-flushed after switching the components of interest onto the longer column to flush highly retained material from the column. This is accomplished by a simple change of plumbing to the actuator controlled 6-port valve. Switch the input from HPLC Pump 2 with the waste output. See Figure 3.

**Alternate
Column
Switching
Systems**

Alternate column switching systems, such as switching early, middle, and late eluting components to waste and allowing only the glyphosate and AMPA components on the analytical column in order to enhance their separation from interfering components or speed-up chromatography time, may be used. For a suggested configuration using two 6-port switching valves, see Figure 4.

- Position A: The 10 cm column is connected directly to the detector; used to determine when the last component has eluted from the column.
- Position B: Both the 10 cm and 30 cm columns are connected in series; used at the beginning of the run.
- Position C: Pump 2 is used to back-flush the 10 cm column to waste, and only the 30 cm column is connected to the detector.

**Monitoring
Elution &
Setting Switch
Times**

The outlet line for the short column is channeled into the PCR system. Elution is monitored and the switch time is set at approximately the time the AMPA peak ends (i.e., when the detector response returns to baseline). There is a lag time from when the analyte exits the column until it enters the detector. The lag time is dependent on the flow rate, tubing, and reactor volume, and is typically from 1 to 2 minutes.

The time shown on the chromatograph is the time the peak end reaches the detector. Depending on how close the analyst wants to switch at the column peak end, the detector time (or that time minus a minute or two) would be used. A longer switch time ensures that all the AMPA has eluted, but a shorter switch time may be needed to optimize separation if there is a significant interferent eluting shortly after AMPA.

Injecting the sample again using the switch time selected and comparing the peak height (or area) to the previous non-switched chromatogram will indicate if the correct switch time has been selected. The switch time must be checked prior to each analysis, every time a new buffer is made, or when a different pH is used.

**Modifying
Chromatography**

Following are ways to modify the chromatography. Use of any of these modifications must be documented in the raw data.

- **Buffer Solution Flow Rate and Column Temperature:** Typically, a buffer solution of pH 1.9 or 2.0 and a column temperature of 50°C has been used for analyzing glyphosate and AMPA in most sample matrices. Sometimes, changing the pH of the buffer solution and/or the column temperature can further separate a nearby peak that interferes with the integration of the peak of interest. The buffer solution may be adjusted (typically, within the pH range of 1.8-2.4), as needed, to give the best separation. The column temperature limit is 60°C. The actual buffer solution pH and column temperature used for each analytical set must be documented in the raw data.
- **Oxidative (i.e., hypochlorite) and OPA Solution Flow Rates:** If it is necessary to change the pH of the buffer solution to achieve better separation, the oxidative (i.e., hypochlorite) and OPA solution flow rates also should be adjusted to optimize the greatest response for glyphosate and AMPA. The pH and the amount of excess oxidative (i.e., hypochlorite) and OPA solutions present affect the reactions that produce the fluorescence responses. When the pH of the buffer solution is changed, the buffer should be pumped through the system overnight for the column to come to equilibrium.
- **Anion Exchange Column:** For matrices in which there are glyphosate interferences, glyphosate has been successfully analyzed substituting an anion exchange column for the analytical column using the same mobile phase and HPLC system without the column switching (see “**Appendix**”). As with the other columns, the pH of the buffer solution may be adjusted to give the best separation. Analyzing AMPA on the same column is difficult because it comes out early with many interfering peaks.

**Improving
Sensitivity**

Following are some techniques to improve sensitivity. Use of any of these techniques must be documented in the raw data.

- **Sample Size:** Varying the sample weight, sample volume, and sample injection volume can help achieve the required sensitivity. For example, if the sample size is limited, a small sample weight can be extracted by loading as much of the aqueous extract as possible on the Chelex[®] resin column, using less final volume to dissolve the residue, and increasing injection volume to achieve the desired sensitivity needed to analyze the sample.
- **SPE:** Additional sample cleanup for chromatography may be achieved by filtering the final sample through a SPE cartridge. Reverse phase C₁₈ has had some success at eliminating interference peaks and other constituents (decolorizes the sample), which helps the column do a better job of separating sample constituents. Currently, the Alltech Associates Inc. Prevail Maxi-Clean cartridges (Part No. 605942) are considered the most effective means of decolorizing the sample.
- **Acid Hydrolysis:** Taking the eluent from the AG 1-X8 anion column, refluxing for four hours, evaporating to near dryness, dissolving in deionized water to the HPLC final volume, and pulling or pushing the sample through an SPE cartridge, has been shown to eliminate fluorescing background and fluorescing constituents in the chromatography without affecting the recovery of glyphosate and AMPA.

Troubleshooting

The chromatography of a mixed standard solution is the best indication of problems:

- If neither glyphosate nor AMPA peaks show up during chromatography, the most likely cause is a problem with the OPA solution or flow.
- If there is an AMPA peak but no glyphosate peak, the problem lies in the oxidative (i.e., hypochlorite) solution preparation or flow.
- If there is a glyphosate peak but no AMPA peak, the column switching time is too short and the AMPA does not make it off the short column before the flow is switched to the waste outlet.
- If the high standard causes a drop off in linearity, this is usually a sign that the oxidative solution is getting weak and a new source of sodium hypochlorite is needed.
- If the correlation coefficient of the curve fit (r^2 value) is <0.98, the instrument probably needs maintenance [e.g., changing of pump seals, auto-injector parts (seals, needle), or detector parts (lamp, flow cell)].

**Shutting Down
& Starting Up**

When shutting down the system for an extended period, the columns must be disconnected and stored in buffer. The pumps and detector must be flushed with deionized water followed by methanol. The post column reagent pumps must be flushed with deionized water thoroughly before being shut down for any length of time. When starting back up, the methanol must be flushed out with deionized water before introducing other solutions and installing the columns back in the system.

Methods of Calculation

Overview

The HPLC system is calibrated with the analytical standards within each analytical set. These standards are interspersed among the samples. This section contains the necessary steps for calculating the residue level of either glyphosate or AMPA in each sample. Each analyte is calculated independently of the other. The concentration of glyphosate and AMPA is determined based upon the detector response (peak height or area) of the elution peak of each analyte. The concentration is determined by interpolation of the external standard calibration curve. The calibration curve of the standards is generated by plotting the peak height or area of the detector response against the concentration of each standard. The residue levels are normally calculated using either a linear (least squares) or exponential calibration curve but a quadratic curve has also been used (other options may also be acceptable). Glyphosate and AMPA standard curve linearity has been achieved with standard amounts ranging from 0.05 through 30 µg/mL and 0.025 through 5 µg/mL. For example calibration curves for glyphosate and AMPA, see Figure 6.

All the samples from a study must be analyzed with the same type of calibration curve. When sample response exceeds the upper range of the standard response, appropriate sample dilutions must be made in order to keep the response within the standard range used.

ppm Added (i.e, Fortified) Calculation

The amount of analyte added (i.e., fortified) to a sample is determined using the following equation:

$$\text{ppm added} = \frac{\text{volume of standard added (mL)} \times \text{conc. of standard added (}\mu\text{g/mL)}}{\text{weight of sample (g)}}$$

Adjusted Final Volume Calculation

The adjusted final volume is determined using the following equation:

$$\text{adjusted final volume} = [\text{total extraction volume} / \text{aliquot volume}] * (\text{conc. volume})$$

- total extraction volume (150 mL, see “**Sample Extraction**”/“**Step 3**”)
- aliquot volume: Volume of the total aqueous phase taken prior to Chelex[®] column chromatography (100 mL is suggested, see “**Sample Extraction**”/“**Step 6**”). This becomes the fraction of the original extracted sample that is carried through the rest of the method.
- concentration volume: Volume used to dissolve the final residue (3.0 mL is suggested, see “**Concentration and Preparation for Analysis**”/“**Step 2**”).

If the method suggestions above are used, the aliquot factor is 1.5 (i.e., 150 ÷ 100) and the adjusted final volume is 1.5 x 3.0 = 4.5 mL.

**ppm Found
Calculation**

The concentration of analyte in a sample (ppm found) is determined by multiplying the concentration in the injected extract ($\mu\text{g/mL}$) by the adjusted final volume (mL). This gives the total amount found in μg for the sample. Division of this number by the weight of sample (g) results in the $\mu\text{g/g}$ or ppm found.

$$\text{ppm found} = \frac{(\mu\text{g/mL found}) \times \text{adjusted final volume (mL)}}{\text{weight of sample (g)}}$$

**Linear
Calibration
Curve**

The concentration of analyte in a sample may be determined using the following equation, which involves the use of a linear calibration curve:

$$\text{ppm found} = \frac{[(A) (\text{detector response}) + B] \times \text{adjusted volume (mL)}}{\text{weight of sample (g)}}$$

Where the concentration of analyte in the injected extract is:

$$\mu\text{g/mL found} = A (\text{detector response}) + B$$

The standards within each analytical set are used to determine the parameters A and B for the linear least squares regression curve from the following linear equation involving the slope (M) and the intercept (K):

$$\text{detector response} = K + (M) (\mu\text{g/mL found})$$

Solving for $\mu\text{g/mL}$ found gives:

$$\mu\text{g/mL found} = \frac{(\text{detector response} - K)}{M} = 1/M (\text{detector response}) - K/M$$

Where the A and B parameters for the linear least squares regression curve are:

$$A = 1/M \quad \text{and} \quad B = -K/M$$

**Exponential
Calibration
Curve**

The concentration of analyte in a sample may be determined using the following equation, which involves the use of an exponential calibration curve:

$$\text{ppm found} = \frac{[(A) (\text{detector response})^B] \times \text{adjusted volume (mL)}}{\text{weight of sample (g)}}$$

Where the concentration of analyte in the injected extract is:

$$\mu\text{g/mL found} = A (\text{detector response})^B$$

The standards within each analytical set are used to determine the parameters A and B for the exponential calibration curve from the following linear least squares regression curve, which is used to determine the slope (M) and the intercept (K):

$$\ln(\text{detector response}) = K + (M) \ln(\mu\text{g/mL found})$$

Rearranging to solve for $\ln(\mu\text{g/mL found})$ gives:

$$\ln(\mu\text{g/mL found}) = \frac{\ln(\text{detector response}) - K}{M}$$

Solving for $\mu\text{g/mL found}$ gives:

$$\begin{aligned} e^{\ln(\mu\text{g/mL found})} &= \mu\text{g/mL found} = e^{(\ln(\text{detector response})/M) + (-K/M)} = e^{(\ln(\text{detector response})^{1/M}) + (-K/M)} \\ &= e^{(-K/M)} \times (\text{detector response})^{1/M} \\ &= A(\text{detector response})^B \end{aligned}$$

Where the A and B parameters for the exponential calibration curve are:

$$A = e^{(-K/M)} \quad \text{and} \quad B = 1/M$$

**Quadratic
Calibration
Curve**

The concentration of analyte in a sample may be determined using a quadratic calibration curve. The parameters of the equation are generated from the response of the external standards included within each analytical set. The A, B, and C curve constants are obtained from a quadratic fit of the external standard responses to the equation:

$$\text{amount injected} = (A) (\text{detector response}^2) + (B) (\text{detector response}) + C$$

The amount found for each sample analyzed is calculated as follows:

$$\text{ppm found} = \frac{[(A) (\text{det. response}^2) + (B) (\text{det. response}) + C] (\text{adjusted final volume})}{\text{weight of sample (g)}}$$

Natural Log Transformation The concentration of analyte in a sample may be determined using a calibration curve involving natural log transformation generated from the response of the external standards included within each analytical set:

$$\text{amount injected} = e^{[(\ln(\text{response}) - A)/B]}$$

The amount found for each sample analyzed is calculated as follows:

$$\text{ppm found} = \frac{[e^{[(\ln(\text{response}) - A)/B}]] \times (\text{adjusted final volume})}{\text{weight of sample (g)}}$$

Percent Recovery Calculation Control (i.e., check) samples are fortified with a known amount of the analytes prior to extraction (i.e., ppm added). After the ppm is found in the fortified sample, that amount (after subtracting any ppm found in the unfortified control sample) is divided by the ppm value with which the samples were fortified. The percent recovery for each fortified sample is calculated as follows:

$$\% \text{ recovery} = \frac{(100) (\text{ppm found} - \text{ppm in control})}{\text{ppm added}}$$

Typical Results Recoveries for this method using RACs are typically in the 70 to 95% range for both glyphosate and AMPA. Recovery ranges and mean values generally vary from one matrix to the next. Coefficients of variation also vary, but are usually <30% at the lower fortification levels, and decrease with increasing level of fortification. Typically, the lower limit of method validation for glyphosate and AMPA is 0.05 ppm. This method has been used successfully for many crop matrices. Results of an interlaboratory validation study that included alfalfa forage, cabbage, grapes, and soybean grain as representative matrices can be found in Monsanto Final Report MSL-4268 (the results are also published in: J.E. Cowell, J. L. Kunstmann, P.J. Nord, J.R. Steinmetz, G.R. Wilson, J. Agric. Food Chem. **34**, 955, 1986.)

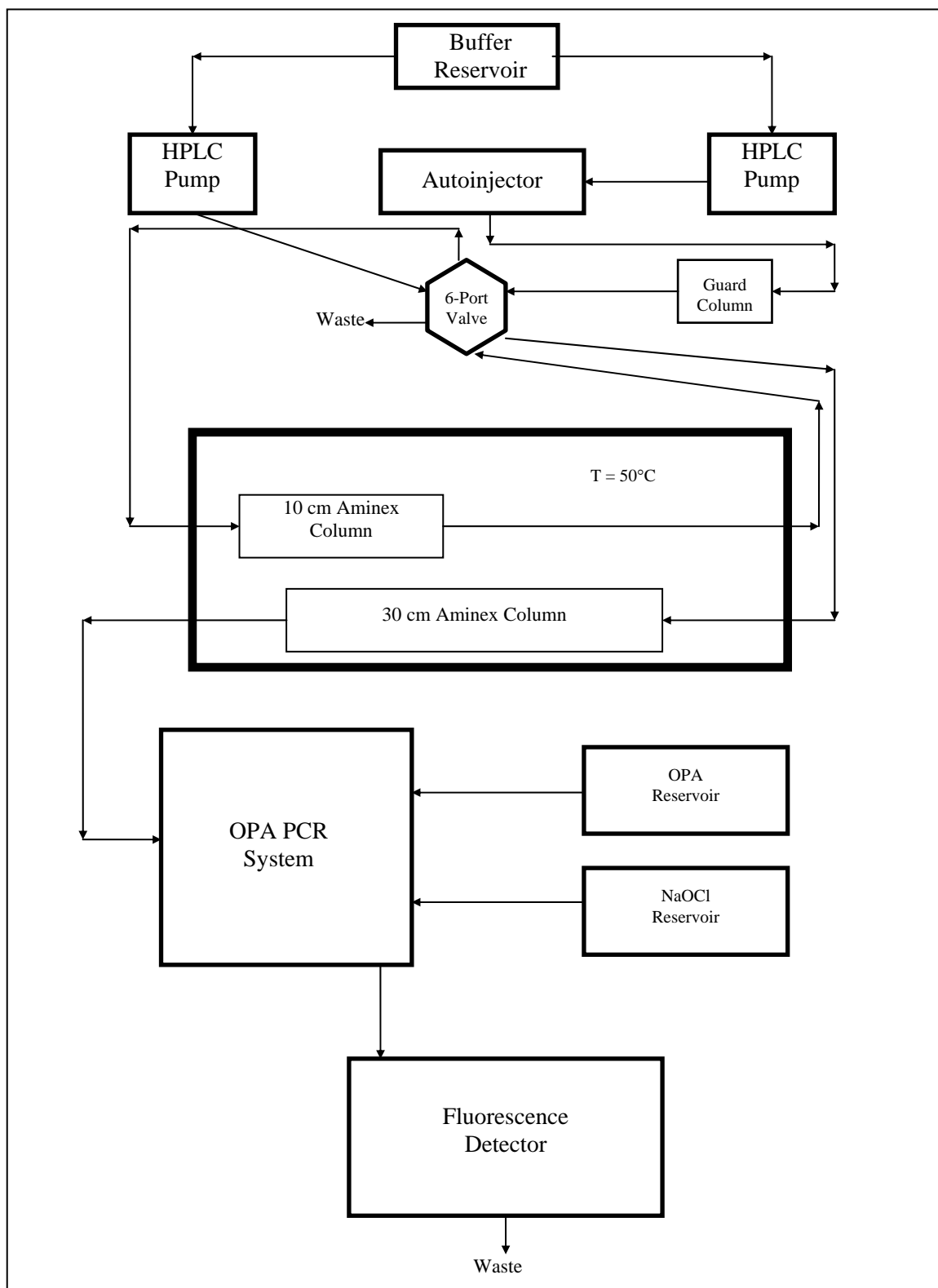
Figure 1: Two-Column Switching PCR System

Figure 2: OPA PCR System

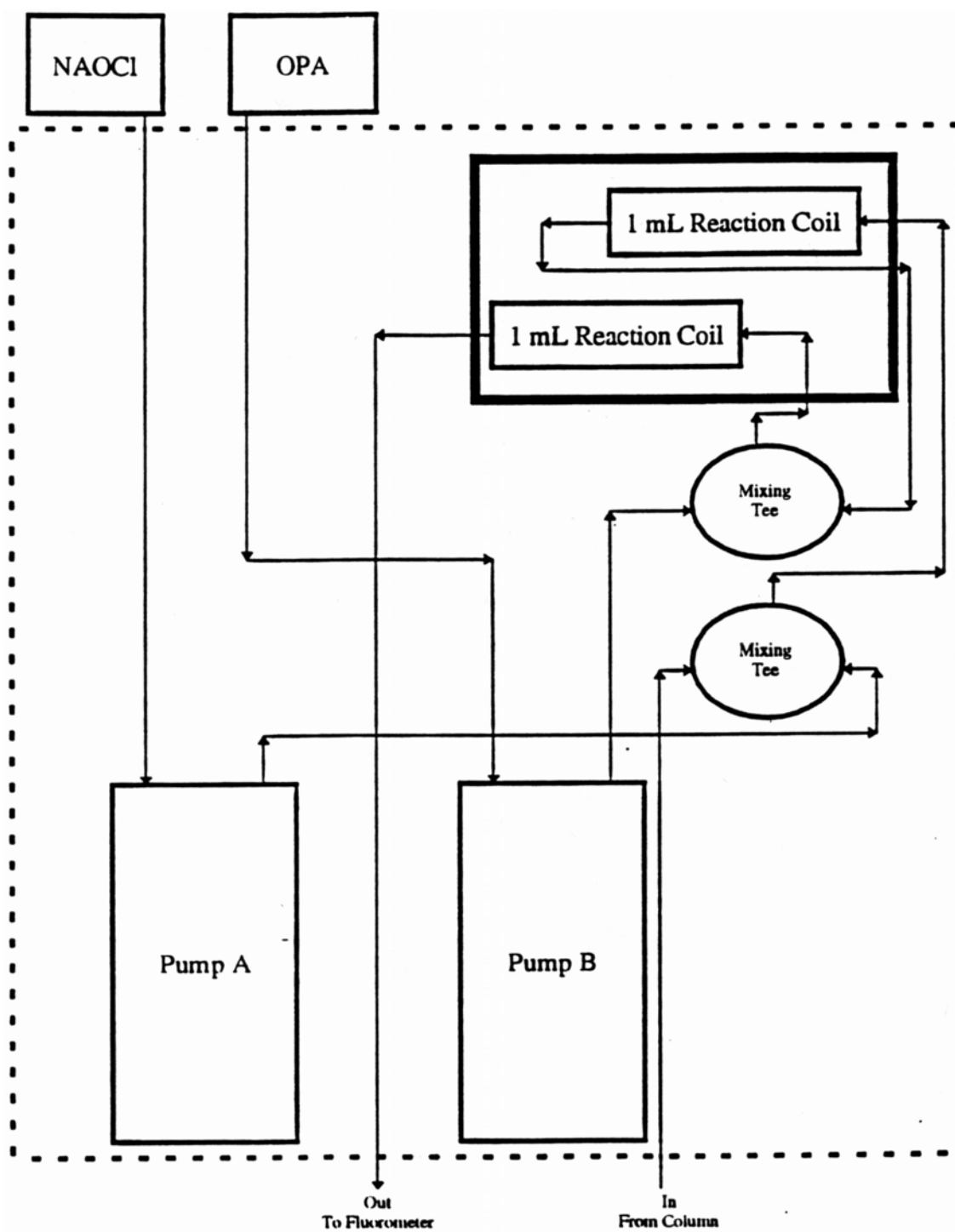


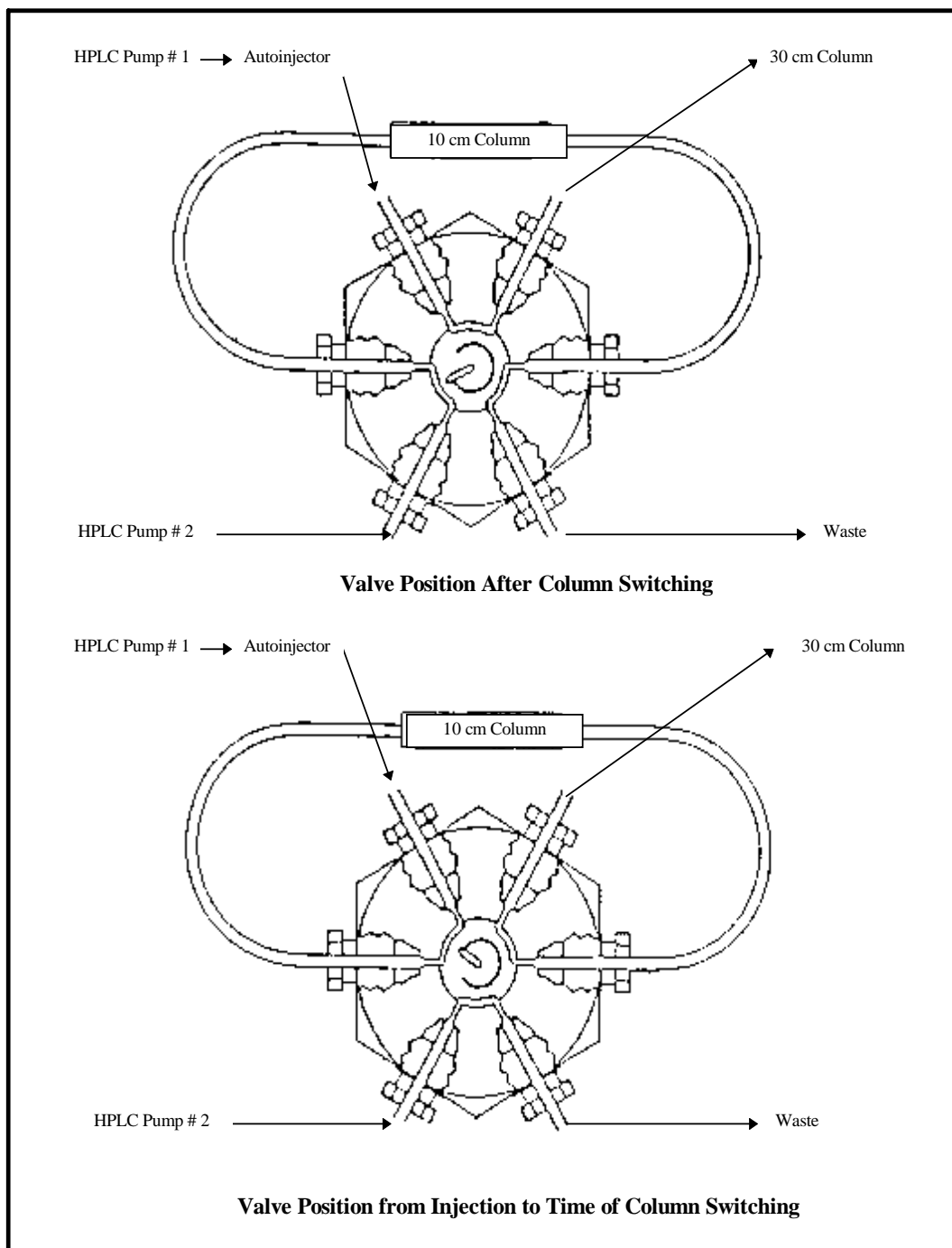
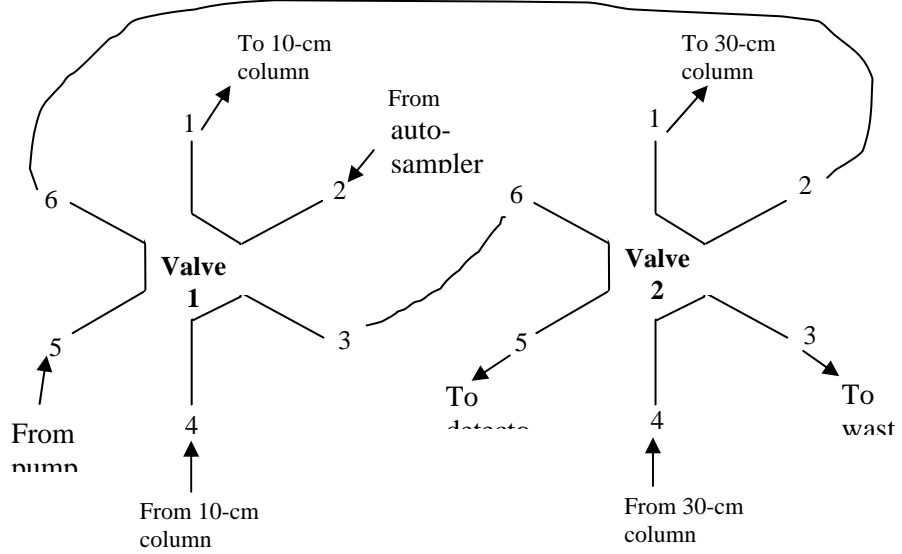
Figure 3: Typical Six Port Valve Configuration

Figure 4: Suggested Six Port Valve Configuration

Position A:

Pump 1 to autosampler to 10-cm column to detector

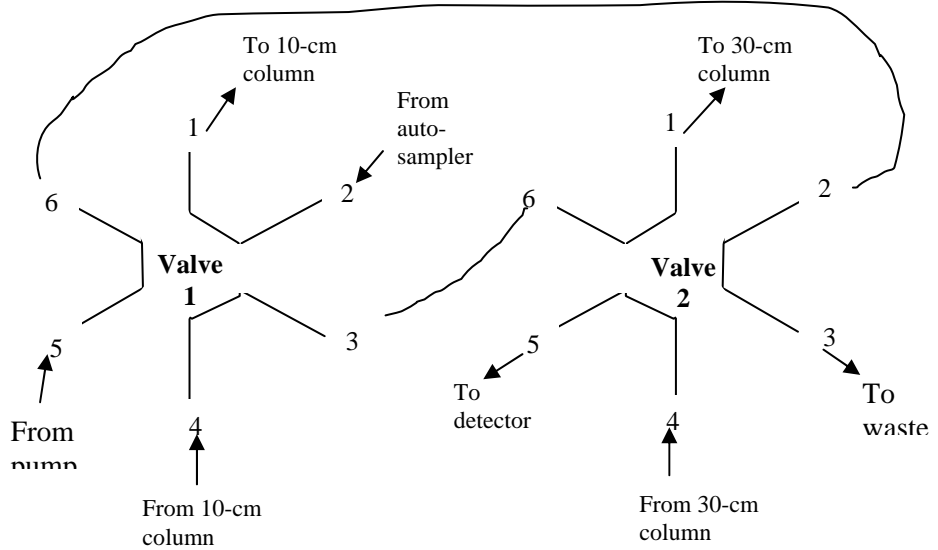
Pump 2 to 30-cm column to detector



Position B:

Pump 1 to autosampler to both columns to detector

Pump 2 to waste



Position C:

Pump 1 to autosampler to 30-cm column to detector

Pump 2 to back-flush 10-cm column to waste

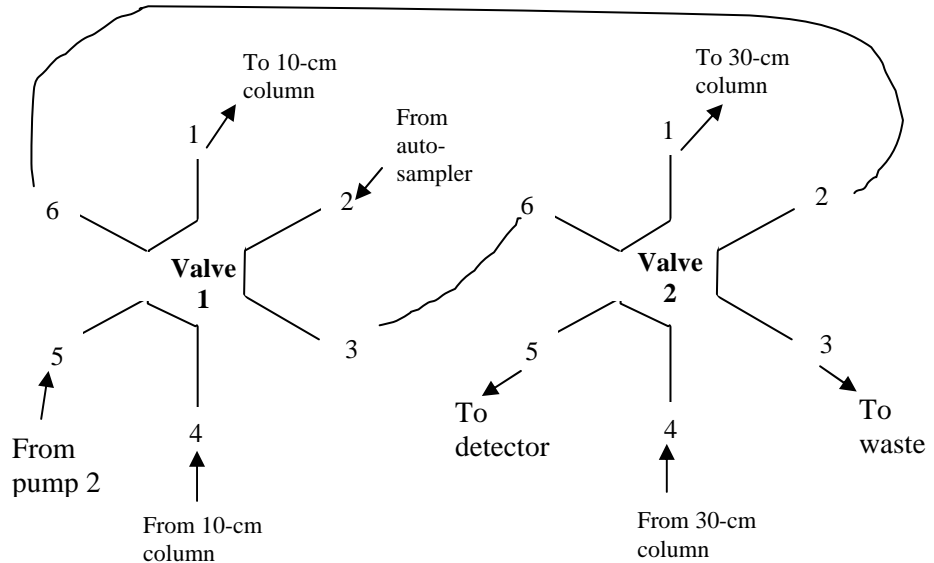
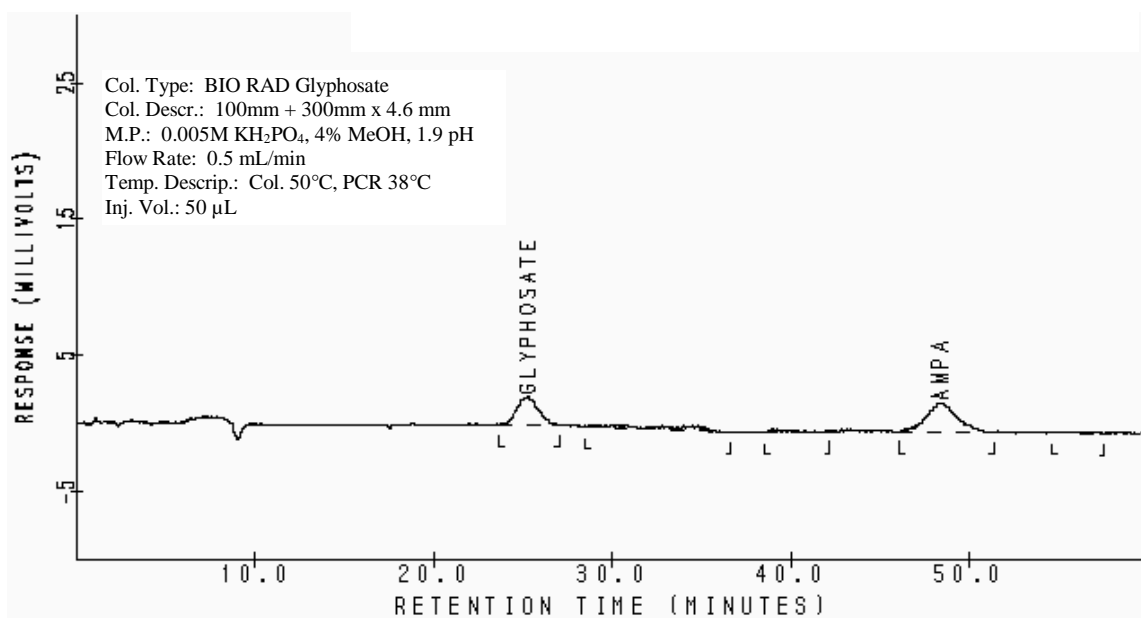


Figure 5: Sample ChromatogramsSTANDARD - 0.25 $\mu\text{g/mL}$
EACH OF GLYPHOSATE AND AMPA

CLOVER FORAGE CHECK

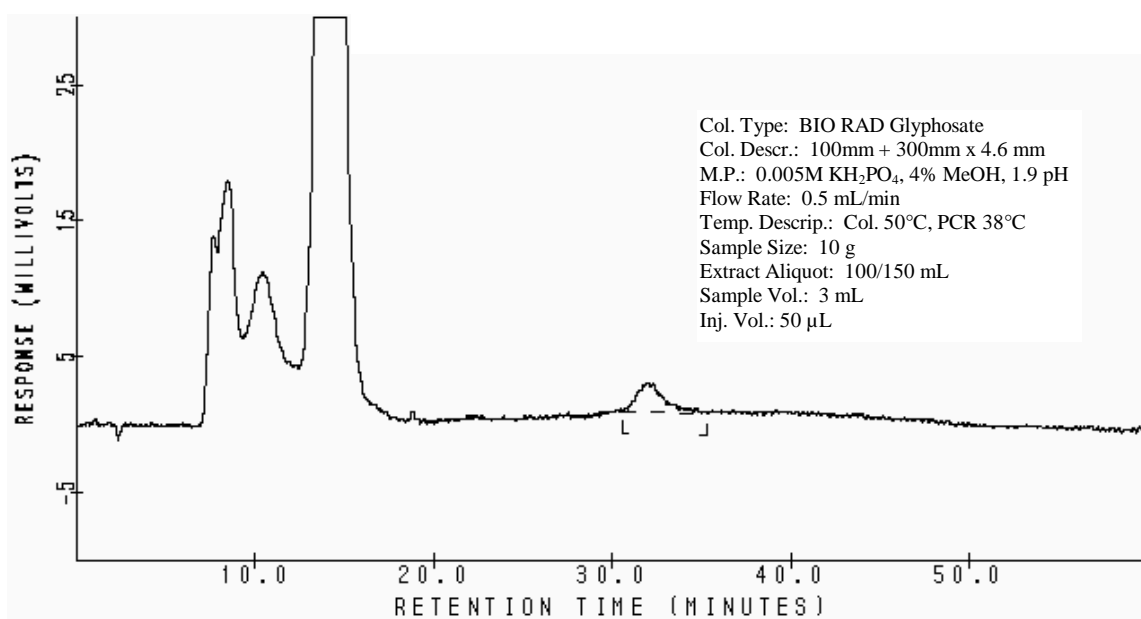
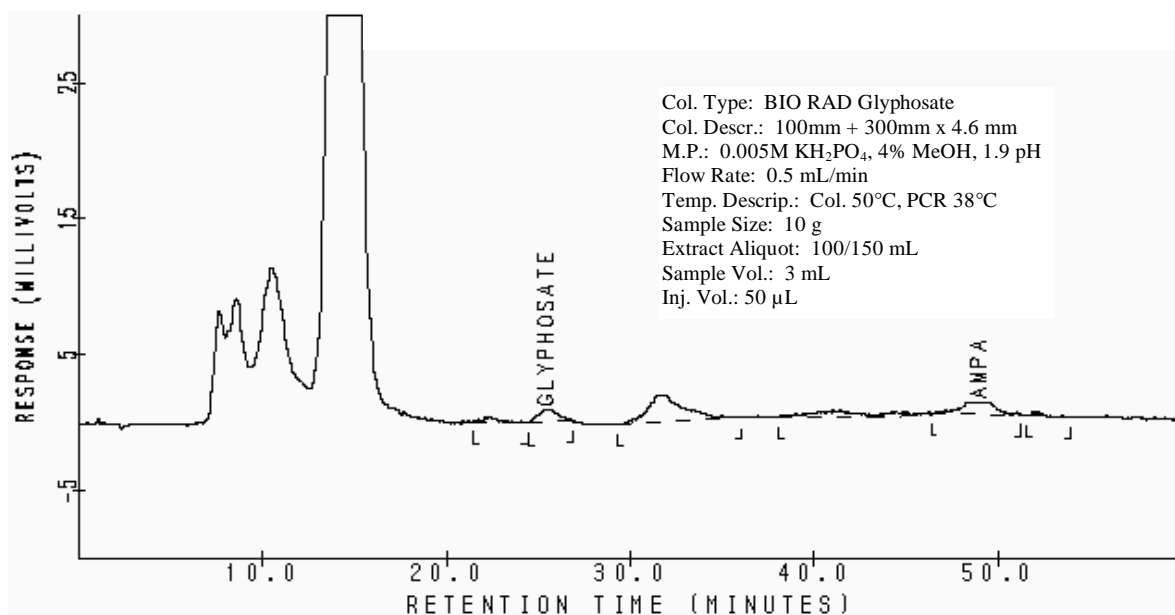


Figure 5: Sample Chromatograms (continued from previous page)

CLOVER FORAGE CHECK + 0.05 PPM
94.1% GLYPHOSATE RECOVERY AND 88.9% AMPA RECOVERY



CLOVER FORAGE CHECK + 30 PPM (1:20 DILUTION)
79.8% GLYPHOSATE RECOVERY AND 73.7% AMPA RECOVERY

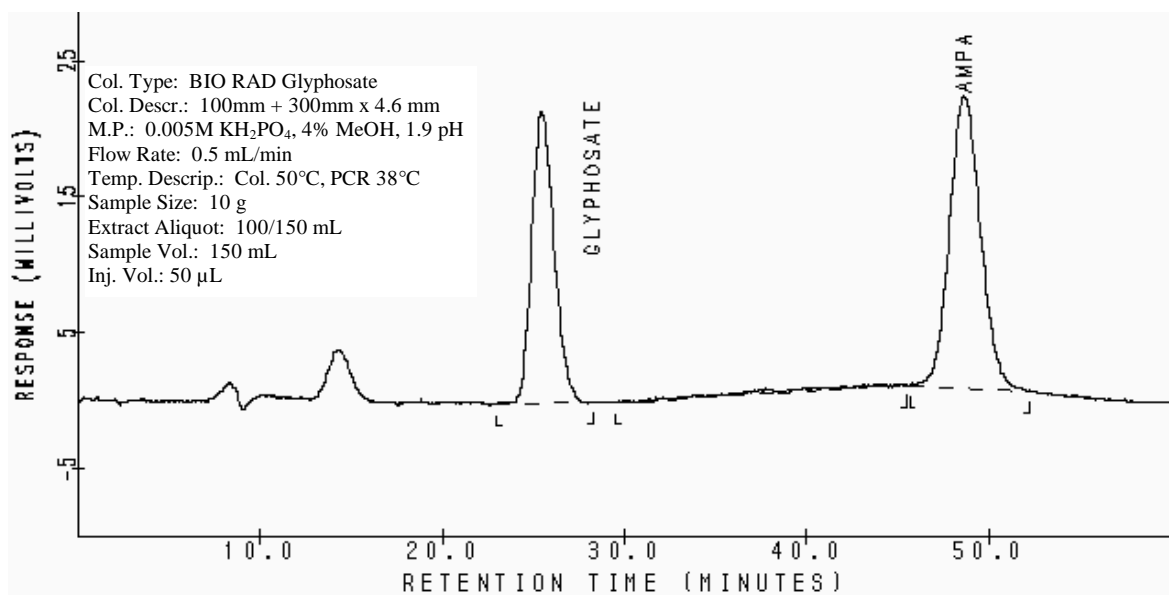


Figure 6: Calibration Curves for Glyphosate and AMPA

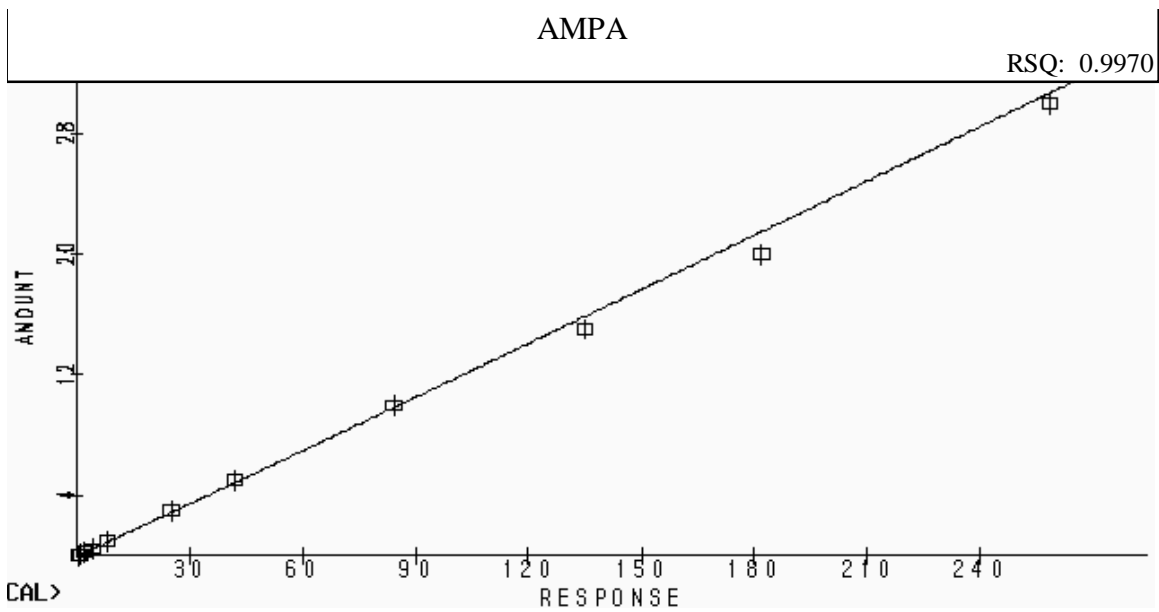
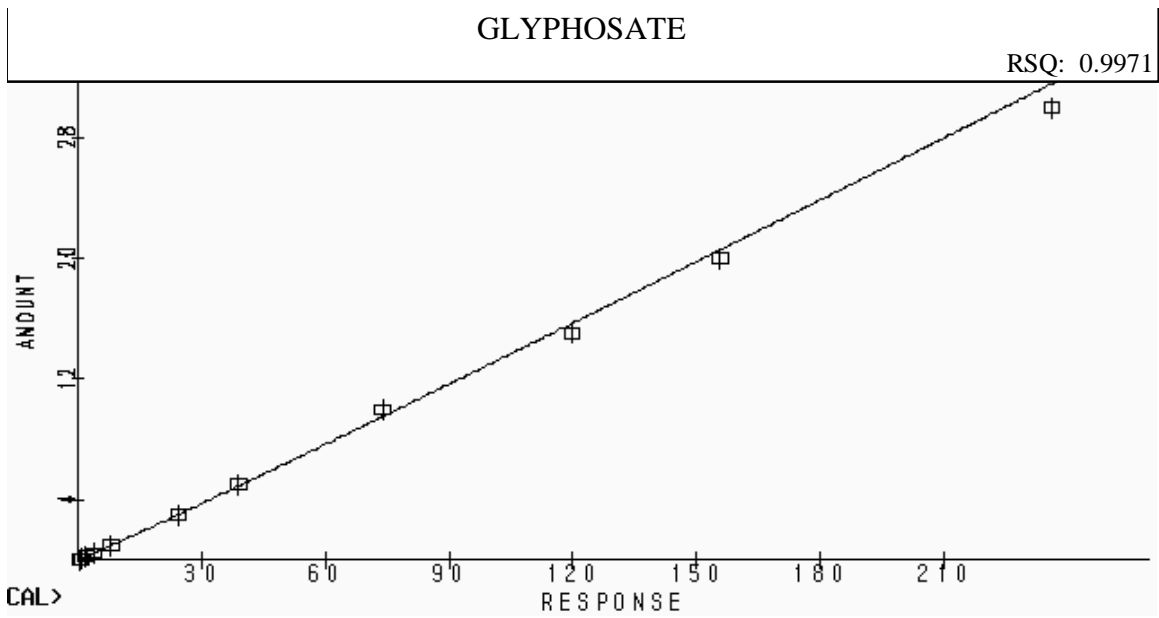
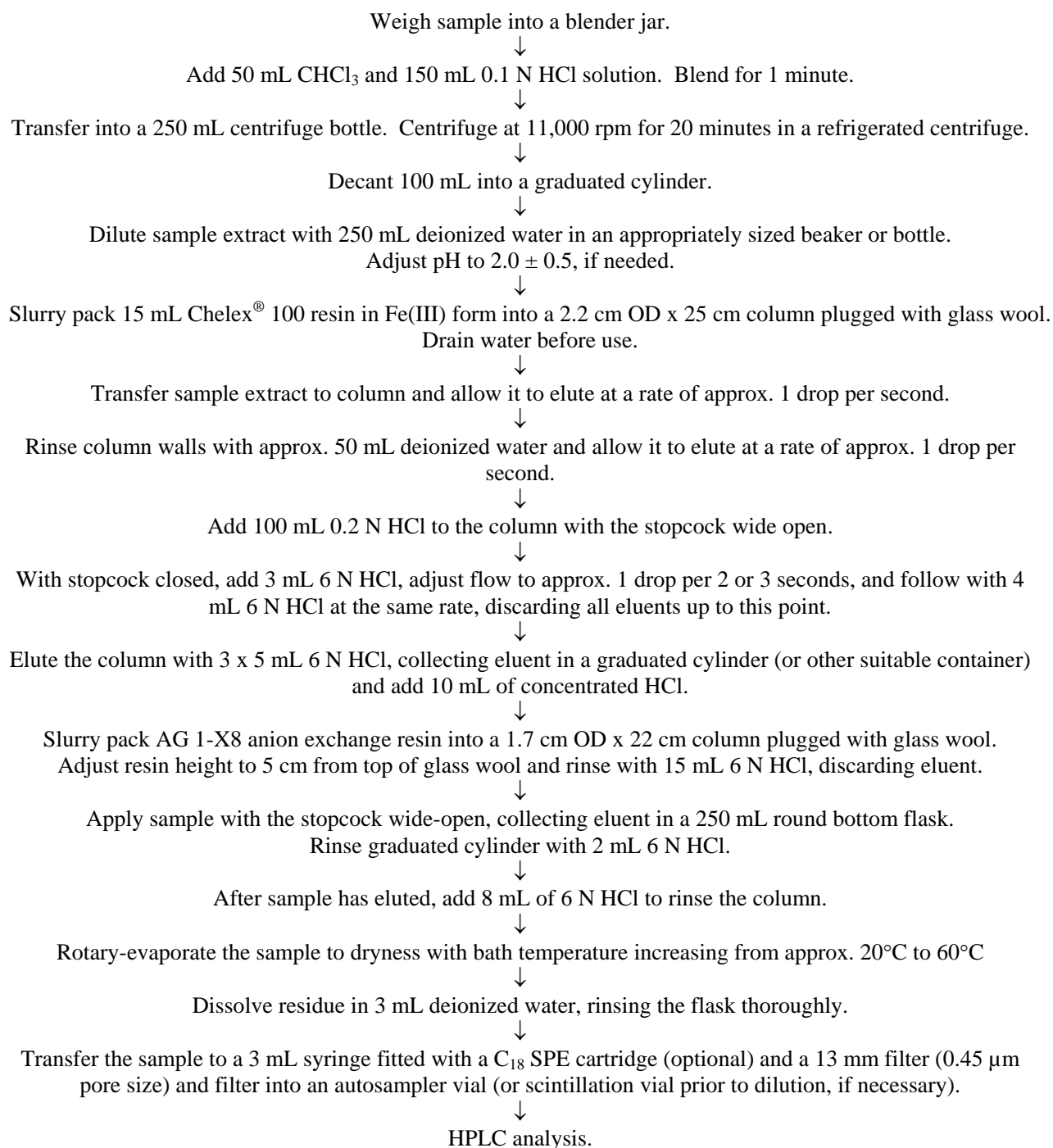


Figure 7: General Flowchart of the Analytical Method for the Determination of Glyphosate and AMPA in Raw Agricultural Commodities and their Processed Commodities

Appendix: Alternative Materials and Procedures

Overview The following materials and procedures are alternatives to those outlined in the preceding pages. Use of any of these alternative materials and procedures must be documented in the raw data.

Equipment The following is alternative equipment:

Equipment	Number/Specification
Column (glass), fitted with fritted-glass disk support	Ace Glass Cat No. 5889-40
Column (glyphosate analysis): Whatman [®] SAX, 25 cm x 4.6 mm ID*	Whatman [®] Cat. No. 4222-227

* For difficult matrices in which there are glyphosate interferences, glyphosate has been successfully analyzed substituting this anion exchange column for the 30 cm column using the same mobile phase and HPLC system without column switching.

**Chemicals,
Reagents, &
Resins**

The following chemicals, reagents, and resins are used in the alternative procedures described in this section.

Chemical/Reagent/Resin	Number/Specification
Boric acid	JT Baker, VWR Cat. No. JT0084-1
Brij [®] 35 solution (30%)	VWR Cat. No. VW3844-1
Calcium hypochlorite [Ca(OCl) ₂], powder, purified grade, available chlorine 68-72%. Note: Deteriorates with time, replace with a fresh bottle, as needed (e.g., 2 months).	JT Baker, VWR Cat. No. JT1378-1
Chelex [®] 100 analytical grade chelating resin, sodium form, 100-200 mesh	Bio-Rad Laboratories Cat. No. 142-2832
Ferric chloride (FeCl ₃), reagent grade: • 0.01 N • 0.1 N	• prepared appropriately • EM Science, VWR Cat No. EM-GC0094-4
Hydrochloric acid (HCl), reagent grade: 0.01 N 0.05 N	• JT Baker, VWR Cat. No. JT5611-2 • prepared appropriately
2-Mercaptoethanol	EM Science, VWR Cat. No. EM-6010
Methylene chloride, reagent grade	VWR Cat. No. VW4525-3
OPA	Pickering Laboratories Cat. No. O120
OPA diluent	Pickering Laboratories Cat. No. GA104
Potassium hydroxide (KOH) solution	VWR Cat. No. VW3622-2
Sodium hydroxide, pellets, reagent grade	VWR Cat. No. VW6720-1
Thiofluor	Pickering Laboratories Cat. No. 3700-2000

**Preparation of
Iron (III)
Loaded
Chelex®
100 Resin from
Resin in the
Sodium Form**

Following are the steps to prepare Iron (III) loaded Chelex® 100 resin from resin in the sodium form:

Step	Action
1	Divide a 1 lb bottle of Chelex® 100 resin (sodium form) in half.
2	Wash each half with 500 mL of 0.05 N HCl. Repeat 4 more times or until the rinse solution remains acidic (pH ~2) after mixing.
3	Rinse each half 2 times with 0.01 N FeCl ₃ , adjusting the solution to pH ~2 with 0.05 N HCl between the rinses.
4	Transfer both halves of resin to a large glass column fitted with a fritted-glass disk support.
5	Elute with 2 L of 0.01 N HCl, followed by 1 L of 0.1 N FeCl ₃ . Follow these treatments with 0.01 N HCl until the eluent is colorless.
6	Store the resin under deionized water at room temperature until used.

**AG 1-X8
Column
Regeneration**

Following are the steps to regenerate an AG 1-X8 column:

Step	Action
1	Rinse a used AG 1-X8 column with ca. 50 mL deionized water.
2	Rinse with 2 x 20 mL of 50:50 methanol/6 N HCl.
3	Rinse again with ca. 50 mL deionized water.
4	Store the column under deionized water at room temperature until used.

Note: The number of times the resin in a column can be regenerated has not been determined. Be alert to any adverse effects. If resin is still discolored after regeneration or becomes discolored after several uses, then discard and use new resin.

**Alternative
Oxidative
Solution**

Following are the steps to prepare an alternative oxidative solution:

Step	Action
1	Add the following to a suitable container and mix well: <ul style="list-style-type: none"> • 1.36 g KH₂PO₄ • 11.6 g NaCl • 0.4 g solid NaOH or 0.8 g 50% (w/w) NaOH solution • 500 mL deionized water
2	Dissolve 0.50 g Ca(OCl) ₂ in 500 mL of deionized water and store at room temperature for up to 1 week after preparation. Add 15 ml of this solution to the solution in “ Step 1 ” and dilute to 1 L with deionized water. Mix well.
3	Filter the solution through a 0.22 µm filter. Store at room temperature for up to ten days after preparation.

**Alternative
OPA Solutions**

Following are the steps to prepare two alternative OPA solutions:

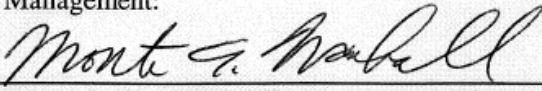
Step	Action
1	Acquire the following reagents from Pickering Laboratories: <ul style="list-style-type: none"> • o-Phthalaldehyde (OPA) • Thiofluor • OPA diluent (for glyphosate analysis)
2	Follow the directions for preparation that came with the reagents.
3	Mix well to ensure complete dissolution.

Step	Action
1	Dissolve 25 g boric acid in 950 mL deionized water using a magnetic stirrer.
2	While monitoring the pH with a pH meter, adjust to a final pH of 10.40 ± 0.2 with potassium hydroxide solution (approximately 30 mL will be required).
3	Add 3 mL 30% Brij [®] 35 solution and 2.0 mL 2-mercaptoethanol.
4	Dissolve 800 mg Fluoropa in 10 mL methanol at room temperature using gentle swirling. Add to the solution of Brij and 2-mercaptoethanol. Mix well.
5	Filter the solution through a 0.22 μm filter.

Storage: After transferring the OPA solution to a reservoir for use, the solution may be stored in a closed glass bottle under atmospheric conditions for up to two weeks without an appreciable increase in background fluorescence. When not in use, always put a head of nitrogen (or some other inert gas) over the solution, cap, and store in a refrigerator at $<10^{\circ}\text{C}$ to maximize shelf life. **Note:** Fluoropa and 2-mercaptoethanol are subject to atmospheric oxidation and these oxidation products can contribute to increased background fluorescence. If the reagent solution is not in use and not protected from atmospheric oxygen, it must be prepared fresh daily.

Superseded SOP(s): RES-008-90 version 6

Author(s) / Prepared by: Mitchell L. Kurtzweil, Diane L. Maher, and Cheryl A. Munie

Management:  Monte A. Marshall (TFM, Monsanto Regulatory)	Date: <u>6</u> / <u>24</u> / <u>04</u>
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