

CYPROCONAZOLE: METHOD

TITLE

Determination of 1,2,4-Triazole, Triazole Alanine and Triazole Acetic Acid Residues in
Plant and Animal Matrices

DATA REQUIREMENTS

OPPTS 860.1340

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COMPLETION DATE

April 13, 2005

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LABORATORY STUDY IDENTIFICATION

Morse Laboratories Meth-160, Revision 2
Syngenta Number T002388-06

SUBMITTER/SPONSOR

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VOLUME 1 OF 1 OF STUDY

PAGE 1 OF 51

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA section 10 (d) (1) (A), (B), or (C).

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Date: 5-May-2006

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Since this volume contains a method and is not a study per se, a separate Good Laboratory Practice Compliance Statement as defined by 40 CFR Part 160 is not applicable.

Study Director: There is no GLP Study Director for this volume.

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Date

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MORSE LABORATORIES, INC.

**DETERMINATION OF 1,2,4-TRIAZOLE, TRIAZOLE ALANINE
AND TRIAZOLE ACETIC ACID RESIDUES IN
PLANT AND ANIMAL MATRICES**

Analytical Method# Meth-160, Revision #2

April 13, 2005

APPROVED BY: Sam Westing

Date: April 13, 2005

TABLE OF CONTENTS

	<u>Page</u>
TITLE PAGE	1
TABLE OF CONTENTS	2
1 PRINCIPLE	3
2 EQUIVALENCE STATEMENT	3
3 APPARATUS AND EQUIPMENT	4
4 REAGENTS AND MATERIALS	6
5 TEST ITEMS/REFERENCE STANDARDS/INTERNAL STANDARDS	8
6 STANDARD PREPARATION	12
7 SAMPLE FORTIFICATION	18
8 SAMPLE EXTRACTION	18
9 SOLID PHASE EXTRACTION (SPE) CARTRIDGE CLEANUPS	20
10 DERIVATIZATIONS	21
11 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS	25
12 CALCULATIONS	27
13 REFERENCE	29
APPENDIX I Analysis Flowcharts	30
APPENDIX II Quality Control for SPE Cartridges	33
APPENDIX III Mass Spectra for Parent and Product-ions	36

DETERMINATION OF 1,2,4-TRIAZOLE, TRIAZOLE ALANINE AND TRIAZOLE ACETIC ACID RESIDUES IN PLANT AND ANIMAL MATRICES

Reason for Revision:

- 1) To change the primary column and update the chromatographic conditions.

1 PRINCIPLE

The method described herein is capable of determining 1,2,4-triazole, triazole alanine and triazole acetic acid in a variety of plant and animal matrices. It is derived from a Bayer CropScience draft method, dated May 15, 2003 (Reference 1).

Residues of 1,2,4-triazole (T), triazole alanine (TA) and triazole acetic acid (TAA) are extracted from 5.0 grams of sample with 60 mL of methanol:water (80:20, v/v). Celite is added to the mixture and the crude extract is vacuum filtered. Following several rinsings of the filter cake, the extract is brought to a final volume of 100 mL with methanol:water (80:20, v/v). (For milk samples, 5.0 g of milk are diluted to 100 mL with methanol:water (80:20, v/v) and handled as solid sample extracts.)

One (1.0) mL aliquots of the sample extract are processed separately through solid phase extraction (SPE) cleanup and/or derivatization steps, which are specific for each analyte. The TAA aliquot is purified through a C-18 SPE cartridge, then derivatized using HCl/butanol esterification. The TA aliquot is purified through a Bond Elut Certify II SPE cartridge, then undergoes two derivatizations; the first an esterification using HCl/butanol and the second an acylation using HFBA (heptafluorobutyric anhydride). The T aliquot does not undergo further cleanup and is directly derivatized with dansyl chloride to produce the dansyl derivative of 1,2,4-triazole. The derivative is partitioned into ethyl acetate which is evaporated to dryness, then redissolved in 2.5 mL of acetonitrile:water (30:70, v/v). The TA and TAA derivatized extracts are evaporated to dryness as well, then brought to a final volume of 2.5 mL in acetonitrile:water (30:70, v/v). All three derivatized extracts are submitted to HPLC for individual analysis.

During routine analysis, determination and quantitation for derivatives of 1,2,4-triazole, triazole alanine and triazole acetic acid are conducted using HPLC employing mass spectrometric (MS/MS) detection. The limit of quantitation (LOQ) for all analytes (as respective parent equivalents) in plant and animal matrices is 0.01 ppm, with the exception of 1,2,4-triazole in milk. The LOQ for 1,2,4-triazole in milk is 0.005 ppm.

2 EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques (such as sample extract evaporation) may be substituted for those described in this method, except where specifically noted otherwise. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

3 APPARATUS AND EQUIPMENT

Assorted laboratory glassware

Balances:	Analytical balance capable of weighing to ± 0.1 mg Top-loading balance capable of weighing to ± 0.01 g
Dry block heater:	VWR Scientific, Bridgeport, NJ
Dry block thermometer:	50-110°C (VWR Scientific, Bridgeport, NJ)
Dry heating block:	12/13 mm (VWR Scientific, Bridgeport, NJ)
Evaporator:	N-Evap Laboratory Sample Evaporator, Model 115, attached to a nitrogen source (Organomation Associates, South Berlin, MA)
Extraction jars:	Wide-mouth glass jars with PTFE-lined lids: 4 oz. (Qorpak, Pittsburgh, PA)
Filter flasks:	Glass, 125 mL
Funnels:	Büchner type, 56 mm diameter Powder, glass, 75 mm diameter
Graduated cylinders:	Glass; 1000, 500, 250 and 100 mL
Graduated mixing cylinders:	Glass: 250 and 100 mL
Homogenizer:	Omni Mixer Model 17105 with Generator Probe (Omni International, Waterbury, CT)

HPLC/MS system:	PE Sciex API 2000 LC/MS/MS system with a Perkin Elmer series 200 autosampler, an integrated Shimadzu chromatograph consisting of (2) LC-10ADvp Liquid Chromatograph units, a DGU-14A Degasser, and a SCL-10Avp System Controller. The system is controlled and data processed by PE Sciex Analyst Software.
Microliter syringes:	Various sizes, (Hamilton Co., Reno, NV)
Oven:	Blue M, Model MO1426A (Blue M, Asheville, NC)
Pasteur pipets:	Glass, 5.75 inch, disposable
Pipets:	Glass, graduated, serological; various sizes
Pipets, adjustable:	Finnpipette [®] digital pipettors (VWR Scientific Products, Bridgeport NJ)
	Pipettors: 5-40 μ L volume range Catalog No. 53515-038
	40-200 μ L volume range Catalog No. 53515-052
	200-1000 μ L volume range Catalog No. 53515-044
	Tips: 1-200 μ L, Catalog No. 53508-810
	100-1000 μ L, Catalog No. 53516-164
Syringe:	Glass, 2.5 mL, Hamilton Teflon [®] Luer-Lok (Hamilton Co., Reno, NV)
Solid phase extraction (SPE) apparatus:	Vac Elut SPS 24 (Varian Sample Preparation Products, Harbor City, CA) with associated support apparatus.
SPE cartridges (empty):	20 mL, polyethylene (Varian Sample Preparation Products, Harbor City, CA)
Test (culture) tubes:	Glass; 13 \times 100 mm, 16 \times 100 mm and 16 \times 150 mm

Ultrasonic bath:	Branson Model 2210 ultrasonic bath (VWR Scientific, Bridgeport, NJ)
Volumetric flasks:	Glass; 100, 50 and 25 mL
Vortex mixer:	Vortexer 2 (VWR Scientific, Bridgeport, NJ)
Vials, autosampler:	Target [®] DP [™] Amber vials, Part# C4000-188W (National Scientific Company)

4 REAGENTS AND MATERIALS

Acetone:	OmniSolv [®] (EM Science, Gibbstown, NJ)
Acetonitrile:	OmniSolv [®] (EM Science, Gibbstown, NJ)
Ammonium hydroxide:	28-30% concentrate GR ACS (EM Science, Gibbstown, NJ)
Celite 545:	"Baker Analyzed" (J.T. Baker Chemical Company, Phillipsburg, NJ)
Dansyl-chloride:	98%, Part #: AC115850050 (Acros Organics, Fairlawn, NJ)
Ethyl acetate:	OmniSolv [®] (EM Science, Gibbstown, NJ)
Filter paper:	Whatman GF/A, 55 mm (VWR Scientific, Bridgeport, NJ)
Formic acid:	GR ACS (EM Science, Gibbstown, NJ)
Glasswool:	VWR Scientific
3N HCl in n-butanol:	Part #: 201009 (Regis Technologies, Inc. Morton Grove, IL)
Heptafluorobutyric anhydride:	98+%, Part #: AC169260250 (Acros Organics, Fairlawn, NJ)
Hexane:	OmniSolv [®] (EM Science, Gibbstown, NJ)
HPLC column:	150 mm × 2.0 mm i.d. Phenomenex Luna C18(2), 3.0μ particle size (Phenomenex, Torrance, CA)

HPLC sample filter:	Nylon, 13 mm, 0.45µm (Thompson Instrument Co., San Diego, CA)
Methanol:	OmniSolv [®] (EM Science, Gibbstown, NJ)
Solid phase extraction cartridges:	Bond Elut- C18 SPE cartridges, 500 mg/6 mL (Varian Sample Preparation Products, Harbor City, CA) Bond Elut-Certify II SPE cartridges, 1.0 g/6mL (Varian Sample Preparation Products, Harbor City, CA)
Sodium bicarbonate:	GR ACS (EM Science, Gibbstown, NJ)
Sodium sulfate:	Analytical grade, anhydrous, granular (Mallinckrodt, St. Louis, MO)
Water:	Deionized (DI) water (Polymetrics System, Morse Laboratories, Inc.) HPLC Grade water (Fisher Scientific, Fairlawn, NJ)

4.1 Reagents and Materials to be Prepared (including typical preparation instructions)

- 4.1.1 Acetonitrile:DI water (80:20, v/v): To a 1000-mL graduated cylinder, add 200 mL of DI water. Bring to a final volume of 1000 mL with acetonitrile. Transfer to a properly labelled secondary container. Mix well. Sufficient for approximately 100 samples.
- 4.1.2 Acetonitrile:HPLC water (50:50, v/v): To a 1000-mL graduated cylinder, add 500 mL of DI water. Bring to a final volume of 1000 mL with acetonitrile. Transfer to a properly labelled secondary container. Mix well.
- 4.1.3 Acetonitrile:HPLC water (30:70, v/v): To a 1000-mL graduated cylinder, add 300 mL of acetonitrile. Bring to a final volume of 1000 mL with HPLC water. Transfer to a properly labelled secondary container. Mix well.
- 4.1.4 0.5 M Ammonium hydroxide: To a 100-mL graduated cylinder, add 5.9 mL of concentrated ammonium hydroxide. Bring to a final volume of 100 mL with DI water. Transfer to a properly labelled secondary container. Mix well. Sufficient for approximately 100 samples.

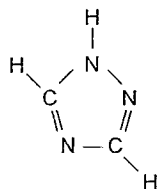
- 4.1.5 2.0 mM Dansyl chloride: Weigh 135 mg of dansyl chloride and transfer to a 250-mL graduated mixing cylinder. Bring to a final volume of 250 mL with acetone. Mix well. Transfer to a properly labelled secondary container. Sufficient for approximately 60 samples. Prepare weekly.
- 4.1.6 0.1 M Sodium bicarbonate: Weigh 2.1 g of sodium bicarbonate and transfer to a 250-mL graduated mixing cylinder. Bring to a final volume of 250 mL with DI water. Mix well. Transfer to a properly labelled secondary container. Sufficient for approximately 60 samples. Prepare weekly.
- 4.1.7 HPLC mobile phase:
- 0.1% formic acid in water*: To a 1 liter graduated cylinder, add HPLC grade water to the 1000 mL mark. Add 1.0 mL of formic acid using a 2.0 mL graduated pipet. Transfer entire solution to the HPLC solvent reservoir and once transferred, mix thoroughly.
- 4.1.8 Methanol:DI water (80:20, v/v): To a 1000-mL graduated cylinder, add 200 mL of DI water. Bring to a final volume of 1000 mL with methanol. Transfer to a properly labelled secondary container. Mix well. Sufficient for approximately 10 samples.

5 TEST ITEMS/REFERENCE STANDARDS/INTERNAL STANDARDS

5.1 Native targeted compounds

5.1.1 *1,2,4-Triazole, (T)*

Common Name: 1,2,4-Triazole
Chemical Name: 1*H*-1,2,4-Triazole
Structural Formula:

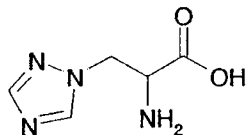


CAS No.: 288-88-0
Source: Bayer CropScience
Molecular weight: 69.07

5.1.2 *Triazole Alanine, (TA)*

Common Name: Triazolylalanine

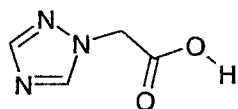
Chemical Name: α -Amino-1*H*-1,2,4-triazole-1-propanoic acid
 Structural Formula:



CAS No.: 114419-45-3
 Source: Bayer CropScience
 Molecular weight: 156.10

5.1.3 Triazole Acetic Acid, (TAA)

Common Name: Triazolylacetic Acid
 Chemical Name: 1*H*-1,2,4-Triazole-1-acetic acid
 Structural Formula:

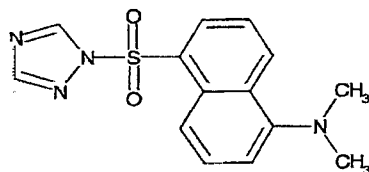


CAS No.: 28711-29-7
 Source: Bayer CropScience
 Molecular weight: 127.10

5.2 Derivatized targeted compounds

5.2.1 Dansyl Triazole, (TD)

Common Name: Dansyl 1,2,4-Triazole
 Chemical Name: 4-[[5-(Dimethylamino)-1-naphthalenyl]sulfonyl]-1*H*-1,2,4-triazole
 Structural Formula:



CAS No.: Not available
 Source: Bayer CropScience

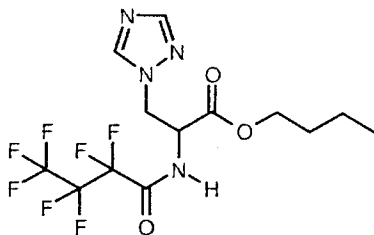
Molecular weight: 302.36

5.2.2 Triazole Alanine BEHFBA, (TAD)

Common Name: Triazolylalanine BEHFBA

Chemical Name: Butyl α -[(2,2,3,3,4,4,4-heptafluoro-1-oxobutyl)amino]-1H-1,2,4-triazole-1-propanoate

Structural Formula:



CAS No.: Not available

Source: Bayer CropScience

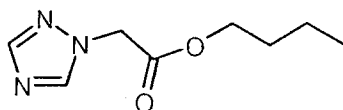
Molecular weight: 408.28

5.2.3 Triazole Acetic Acid Butyl Ester (TAAD)

Common Name: Triazolylacetic Acid Butyl Ester

Chemical Name: Butyl 1H-1,2,4-triazole-1-acetate

Structural Formula:



CAS No.: Not available

Source: Bayer CropScience

Molecular weight: 183.20

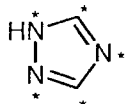
5.3 Native internal standard compounds

5.3.1 1,2,4-Triazole-15N3 (TIS)

Common Name: 1,2,4-Triazole-15N3, 13C2

Chemical Name: 1H-1,2,4-Triazole-3,5-¹³C₂-1,2,4-¹⁵N₃

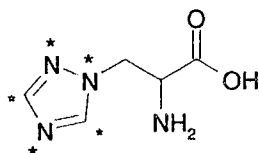
Structural Formula:



CAS No.: Not available
 Source: Bayer CropScience
 Molecular weight: 74.00

5.3.2 *Triazole-15N3-13C2-Alanine (TAIS)*

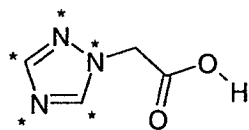
Common Name: Triazolylalanine-triazole-3,5-13C2-1,2,4-15N3
 Chemical Name: α -Amino-1H-1,2,4-triazole-3,5-¹³C₂-1,2,4-¹⁵N₃-1-propanoic acid
 Structural Formula:



CAS No.: Not available
 Source: Bayer CropScience
 Molecular weight: 161.00

5.3.3 *Triazole-15N3-13C2-Acetic Acid (TAAIS)*

Common Name: Triazolylacetic Acid-triazole-3,5-13C2-1,2,4-15N3
 Chemical Name: 1H-1,2,4-Triazole-3,5-¹³C₂-1,2,4-¹⁵N₃-1-acetic acid
 Structural Formula:



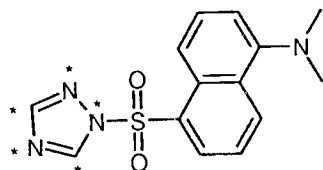
CAS No.: Not available
 Source: Bayer CropScience
 Molecular weight: 132.00

5.4 Derivatized internal standard compounds

5.4.1 *Dansyl-1,2,4-triazole-15N3 (TDIS)*

Common Name: Dansyl-1,2,4-triazole-triazole-3,5-13C2-1,2,4-15N3
 Chemical Name: 4-[[5-(Dimethylamino)-1-naphthalenyl]sulfonyl]-1H-1,2,4-triazole-3,5-¹³C₂-1,2,4-¹⁵N₃

Structural Formula:

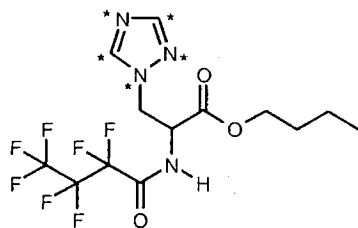


CAS No.: Not available
 Source: Bayer CropScience
 Molecular weight: 307.40

5.4.2 *Triazole-15N3-13C2-Alanine BEHFBA (TADIS)*

Common Name: Triazolylalanine BEHFBA-triazole-3,5-13C2-1,2,4-15N3
 Chemical Name: Butyl α-[(2,2,3,3,4,4,4-heptafluoro-1-oxobutyl)amino]-1H-(1,2,4-triazole-3,5-¹³C₂-1,2,4-¹⁵N₃-1-propanoate

Structural Formula:

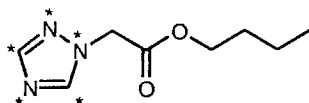


CAS No.: Not available
 Source: Bayer CropScience
 Molecular weight: 413.00

5.4.3 *Triazole Acetic Acid Butyl Ester IS (TAADIS)*

Common Name: Triazolylacetic Acid Butyl Ester-triazole-3,5-13C2-1,2,4-15N3
 Chemical Name: Butyl 1H-1,2,4-triazole-3,5-¹³C₂-1,2,4-¹⁵N₃-1-acetate

Structural Formula:



CAS No.: Not available
Source: Bayer CropScience
Molecular weight: 188.00

6 STANDARD PREPARATION

6.1 Stock Standard Solutions

Because it is difficult to accurately weigh small amounts (generally <20 mg) of both liquid and solid analytical standards to specific predetermined values, they may be weighed to $\pm 10\%$ of the target value. When calculated, the actual concentration of the stock solution produced is expressed to three significant figures. Appropriate adjustments in the preparation of subsequent dilutions can be made in order to produce concentrations that are more manageable to work with. All solutions are to be stored in the dark at approximately 1 to 8°C when not in use.

6.1.1 *Native compounds*

Weigh approximately 10 mg (corrected for purity) of analytical standard directly into a 100-mL volumetric flask. Record actual weight of standard added and bring to volume with HPLC grade water. The resulting concentration, corrected for purity and actual amount weighed should be approximately 100 $\mu\text{g/mL}$.

6.1.2 *Derivatized compounds*

Weigh approximately 10 mg (corrected for purity) of analytical standard directly into a 100-mL volumetric flask. Record actual weight of standard added and bring to volume with acetonitrile:HPLC grade water (50:50, v/v). The resulting concentration, corrected for purity and actual amount weighed should be approximately 100 $\mu\text{g/mL}$.

For TAADIS, the analytical standard is supplied as an $\sim 2\%$ (w/w) solution in acetonitrile. Transfer a suitable volume of the solution, corrected for purity and density, to a 100-mL volumetric flask to make an approximate 100 $\mu\text{g/mL}$ solution. Bring to volume with acetonitrile: HPLC grade water (50:50, v/v).

6.2 Intermediate Solutions

6.2.1 *Native targeted compounds (T, TA and TAA)*

10 µg/mL: Transfer a volume of the ~100 µg/mL stock standard solution, equivalent to 500 µg of the applicable compound, to a 50-mL volumetric flask. Bring to volume with HPLC grade water. Mix well. The appropriate volume (x) is determined as follows:

$$x = 500 \mu\text{g} \div \text{concentration of stock standard } (\mu\text{g/mL})$$

For example: concentration of stock standard = 102 µg/mL

$$\begin{aligned} x &= 500 \mu\text{g} \div 102 \mu\text{g/mL} \\ x &= 4.90 \text{ mL} \end{aligned}$$

6.2.2 *Derivatized targeted compounds (TD, TAD and TAAD)*

Prepare as respective parent equivalents (PE).

10 µg/mL PE: Transfer a volume of the ~100 µg/mL stock standard solution, equivalent to 500 µg of the respective parent equivalent, to a 50-mL volumetric flask. Bring to volume with acetonitrile:HPLC grade water (50:50, v/v). Mix well. The appropriate volume (x) is determined as follows:

$$x = 500 \mu\text{g PE} \times \text{MWCF} \div \text{concentration of stock standard } (\mu\text{g/mL})$$

MWCF = molecular weight conversion factor

$$\text{TD} = \text{T} \times \mathbf{4.38}$$

$$\text{TAD} = \text{TA} \times \mathbf{2.62}$$

$$\text{TAAD} = \text{TAA} \times \mathbf{1.44}$$

Example volume calculations:

TD: $x = 500 \mu\text{g} \times 4.38 \div 109 \mu\text{g/mL}$
 $x = 20.09 \text{ mL}$
 20.09 mL (109 µg/mL TD) → 50 mL FV = 10 µg/mL TD expressed as T equivalents

TAD: $x = 500 \mu\text{g} \times 2.62 \div 119 \mu\text{g/mL}$
 $x = 11.01 \text{ mL}$
 11.01 mL (119 µg/mL TAD) → 50 mL FV = 10 µg/mL TAD expressed as TA equivalents

TAAD: $x = 500 \mu\text{g} \times 1.44 \div 107 \mu\text{g/mL}$
 $x = 6.73 \text{ mL}$
 6.73 mL (107 $\mu\text{g/mL}$ TAAD) \rightarrow 50 mL FV = 10 $\mu\text{g/mL}$ TAAD expressed
 as TAA equivalents

6.2.3 Native internal standard compounds (TIS, TAIS and TAAIS)

Same procedure as described in Section 6.2.1.

6.2.4 Derivatized internal standard compounds

Prepare as respective parent equivalents (PE).

10 $\mu\text{g/mL}$ PE: Transfer a volume of the \sim 100 $\mu\text{g/mL}$ stock standard solution, equivalent to 500 μg of the respective parent equivalent, to a 50-mL volumetric flask. Bring to volume with acetonitrile:HPLC grade water (50:50, v/v). Mix well. The appropriate volume (x) is determined as follows:

$$x = 500 \mu\text{g PE} \times \text{MWCF} \div \text{concentration of stock standard } (\mu\text{g/mL})$$

MWCF = molecular weight conversion factor

$$\text{TDIS} = \text{TIS} \times \mathbf{4.15}$$

$$\text{TADIS} = \text{TAIS} \times \mathbf{2.57}$$

$$\text{TAADIS} = \text{TAAIS} \times \mathbf{1.42}$$

Example volume calculations:

TDIS: $x = 500 \mu\text{g} \times 4.15 \div 103 \mu\text{g/mL}$
 $x = 20.15 \text{ mL}$
 20.15 mL (103 $\mu\text{g/mL}$ TDIS) \rightarrow 50 mL FV = 10 $\mu\text{g/mL}$ TDIS expressed
 as TIS equivalents

TADIS: $x = 500 \mu\text{g} \times 2.57 \div 99.6 \mu\text{g/mL}$
 $x = 12.90 \text{ mL}$
 12.90 mL (99.6 $\mu\text{g/mL}$ TADIS) \rightarrow 50 mL FV = 10 $\mu\text{g/mL}$ TADIS expressed as
 TAIS equivalents

TAADIS: $x = 500 \mu\text{g} \times 1.42 \div 98.8 \mu\text{g/mL}$
 $x = 7.19 \text{ mL}$
 7.19 mL (98.8 $\mu\text{g/mL}$ TAADIS) \rightarrow 50 mL FV = 10 $\mu\text{g/mL}$ TAADIS expressed
 as TAAIS equivalents

6.3 Fortification Solutions

These solutions can be prepared to contain either individual or mixed native compound (T, TA and/or TAA) components. Internal standards (TIS, TAIS and/or TAAIS) must be prepared individually.

Typically the following concentrations of native targeted compound and internal standard compound solutions are prepared. All solutions are stored in the dark at approximately 1 to 8°C when not in use.

Native targeted compounds:

1.0 µg/mL: Transfer 5.0 mL of a 10 µg/mL standard solution to a 50-mL volumetric flask. Bring to volume in HPLC grade water. Mix well.

0.1 µg/mL: Transfer 5.0 mL of a 1.0 µg/mL standard solution to a 50-mL volumetric flask. Bring to volume in HPLC grade water. Mix well.

Internal standard compounds:

0.1 µg/mL: Transfer 5.0 mL of a 1.0 µg/mL standard solution to a 50-mL volumetric flask. Bring to volume in HPLC grade water. Mix well.

6.4 HPLC (Calibration) Standard Solutions

All calibration standards are derivatized. They are prepared as their respective parent equivalents (i.e., the concentrations are expressed in terms of their underivatized precursors). They may be prepared as individual solutions or as mixtures. All are prepared to contain specified amounts of applicable derivatized internal standards, as their respective parent equivalents. All standard solutions prepared in this section are to be stored in the dark at 1 to 8°C when not in use.

Prepare the following intermediate concentrations of all applicable derivatized targeted analyte standards and internal standards:

Derivatized targeted analytes:

1.0 µg/mL: Transfer 5.0 mL of a 10 µg/mL (as PE) derivatized standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (50:50, v/v). Mix well.

0.1 µg/mL: Transfer 5.0 mL of a 1.0 µg/mL (as PE) derivatized standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (50:50, v/v). Mix well.

Internal standards:

1.0 µg/mL: Transfer 5.0 mL of a 10 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (50:50, v/v). Mix well.

Typically the following concentrations of derivatized targeted analytes (TD, TAD and/or TAAD), as HPLC standard solutions, are prepared:

6.4.1 Milk

0.00003 µg/mL:
(TD and TAD only) Transfer 30 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 100 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 100-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.00005 µg/mL:
(TAAD only) Transfer 25 µL of a 0.1 µg/mL TAAD (as PE) standard solution and 50 µL of a 1.0 µg/mL TAADIS (as PE) internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.0002 µg/mL: Transfer 100 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.0005 µg/mL: Transfer 250 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.001 µg/mL: Transfer 500 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.002 µg/mL: Transfer 1000 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.01 µg/mL: Transfer 500 µL of each 1.0 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

6.4.2 Crops and processed commodities

0.00003 µg/mL:
(TD and TAD only) Transfer 30 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 100 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 100-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.00005 µg/mL:
(TAAD only) Transfer 25 µL of a 0.1 µg/mL TAAD (as PE) standard solution and 50 µL of a 1.0 µg/mL TAADIS (as PE) internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.0002 µg/mL: Transfer 100 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.0005 µg/mL: Transfer 250 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.001 µg/mL: Transfer 500 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.002 µg/mL: Transfer 1000 µL of each 0.1 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

0.02 µg/mL: Transfer 1000 µL of each 1.0 µg/mL (as PE) derivatized target compound standard solution to be included and 50 µL of each respective 1.0 µg/mL (as PE) derivatized internal standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:HPLC grade water (30:70, v/v). Mix well.

7 SAMPLE FORTIFICATION

1. Weigh 5.00 g (± 0.02 g) of macerated (ground) sample or well-mixed milk sample into a 4 oz. Qorpak jar.
2. Fortify the sample with the appropriate amount of standard solution(s) containing either individual or mixed components. Use a volume of fortification solution ≤ 1.0 mL. Allow 5 minutes for standard(s) to soak into sample.
3. Proceed with Step 8.1.2 for crops and processed commodities or Step 8.2.2 for milk.

8 SAMPLE EXTRACTION

8.1 Crops and processed commodities

1. Weigh 5.00 g (± 0.02 g) of macerated (ground) sample into a 4 oz. Qorpak jar.
2. Add 60 mL of methanol:DI water (80:20, v/v) and allow it to soak into the sample for 30 minutes.
3. Blend mixture using a high speed homogenizer at high speed for approximately 2 minutes. Rinse blender probe with 5-10 mL of methanol: DI water (80:20, v/v) and combine with the extract.
4. Add 1.0 g of Celite[®] to the sample. Mix thoroughly.
5. Vacuum filter the entire mixture through an Whatman GF/A filter in a 56 mm diameter Büchner funnel into a 125-mL filter flask.

6. Rinse the extraction jar and filter cake with approximately 5 mL of methanol:DI water (80:20, v/v), collecting the rinse in the filter flask. Repeat this step two additional times.
7. Rinse the filter cake one last time with 5 mL of methanol: DI water (80:20, v/v).
8. Transfer the extract to a 100-mL graduated mixing cylinder and bring to a final volume of 100 mL with methanol: DI water (80:20, v/v).
9. Transfer ~50 mL of extract to the original extraction jar and seal with PTFE-lined lid. Proceed with SPE (solid phase extraction) clean-ups for triazole alanine and triazole acetic acid (Sections 9.1 or 9.2) or derivatization for 1,2,4-triazole (Section 10.1) or store extract in the freezer until analysis can be performed.

8.2 Milk

1. Weigh 5.00 g (± 0.02 g) of well-mixed milk sample into a 4 oz. Qorpak jar.
2. Add 60 mL of methanol: DI water (80:20, v/v). Swirl to mix.
3. Transfer the mixture to a 100-mL graduated mixing cylinder.
4. Rinse the extraction jar with approximately 5 mL of methanol: DI water (80:20, v/v), collecting the rinse in the graduated cylinder. Repeat this step two additional times.
5. Bring the mixture to a final volume of 100 mL with methanol: DI water (80:20, v/v).
6. Transfer ~50 mL of extract to the original extraction jar and seal with PTFE-lined lid. Proceed with SPE (solid phase extraction) clean-ups for triazole alanine and triazole acetic acid (Sections 9.1 or 9.2) or derivatization for 1,2,4-triazole (Section 10.1) or store extract in the freezer until analysis can be performed.

9 **SOLID PHASE EXTRACTION (SPE) CARTRIDGE CLEANUPS**

Note: Check or calibrate the SPE cartridges prior to use in order to ensure optimum method performance. In general, check one column per lot number. This assessment should be conducted well in advance of needing the cartridges for sample analysis. Recovery of >90% is desired to ensure that a box of columns is suitable for use. The analyses are conducted on an "analyte with no matrix present" basis. See Appendix II for detailed instructions on assessment of the SPE cartridges.

9.1 Triazole alanine (TA). Bond Elut-Certify II SPE cartridge cleanup

Procedure:

1. Set up Vac Elut system and support apparatus and proceed with Bond Elut-Certify II SPE cleanup. In general, set vacuum to produce a flow rate of approximately 3-4 distinct drops/second (not continuous flow) for all elutions.
2. Condition a 1.0 g Bond Elut-Certify II SPE cartridge by passing 5 mL of acetonitrile followed by 5 mL methanol: DI water (80:20, v/v) through the cartridge. Do not let the cartridge go to dryness after conditioning. (Stop elution when conditioning solvent reaches top of frit, by closing stopcock.) Discard eluate.
3. Place a 13 × 100 mm test tube under the SPE cartridge.
4. Transfer a 1.0 mL aliquot of sample extract from either Step 8.1.9 or Step 8.2.6 to a 13 × 100 mm test tube. Add 25 µL of a 0.10 µg/mL solution of TAIS internal standard (equivalent to 0.0025 µg). Vortex mix the solution for ~5 seconds.
5. Pour the solution onto the SPE cartridge (with stopcock closed). Add 1 mL acetonitrile: DI water (80:20, v/v) to the test tube as a wash. Open stopcock and start elution. When loading solvent reaches top of frit, pour the 1 mL wash of the test tube onto the SPE. Stop elution when wash reaches top of frit.
6. Wash the test tube with an additional 2 mL of acetonitrile: DI water (80:20, v/v) and transfer the wash to the SPE. Start and continue elution until the cartridge goes dry.
7. Evaporate the eluate (load + combined washes) to dryness on an N-Evap evaporator set at 45-50°C.
8. Proceed with TA derivatization (Section 10.2).

9.2 Triazole acetic acid (TAA). Bond Elut-C18 SPE cartridge cleanup

Procedure:

1. Set up Vac Elut system and support apparatus and proceed with Bond Elut-C18 SPE cleanup. In general, set vacuum to produce a flow rate of approximately 3-4 distinct drops/second (not continuous flow) for all elutions.

2. Condition a 0.5 g Bond Elut-C18 SPE cartridge by passing 5 mL of acetonitrile followed by 5 mL methanol: DI water (80:20, v/v) through the cartridge. Do not let the cartridge go to dryness after conditioning. (Stop elution when conditioning solvent reaches top of frit, by closing stopcock.) Discard eluate.
3. Place a 13 × 100 mm test tube under the SPE cartridge.
4. Transfer a 1.0-mL aliquot of sample extract from either Step 8.1.9 or Step 8.2.6 to a 13 × 100 mm test tube. Add 25 µL of a 0.10 µg/mL solution of TAAIS internal standard (equivalent to 0.0025 µg). Vortex mix the solution for ~5 seconds.
5. Pour the solution onto the SPE cartridge (with stopcock closed). Add 1 mL acetonitrile: DI water (80:20, v/v) to the test tube as a wash. Open stopcock and start elution. When loading solvent reaches top of frit, pour the 1 mL wash of the test tube onto the SPE. Stop elution when wash reaches top of frit.
6. Wash the test tube with an additional 2 mL of acetonitrile: DI water (80:20, v/v) and transfer the wash to the SPE. Start and continue elution until the cartridge goes dry.
7. Evaporate the eluate (load + combined washes) to dryness on an N-Evap evaporator set at 45-50°C.
8. Proceed with TAA derivatization (Section 10.3).

10 DERIVATIZATIONS

10.1 1,2,4-triazole (T). Dansyl chloride derivatization.

Procedure:

1. Transfer a 1.0-mL aliquot of sample extract from either Step 8.1.9 or Step 8.2.6 to a 16 × 150 mm test tube. Add 25 µL of a 0.10 µg/mL solution of TIS internal standard (equivalent to 0.0025 µg). Vortex mix the solution for ~5 seconds.

Note: For soybeans and other oily matrices, transfer a 0.5-mL aliquot of sample extract from Step 8.1.9 a 16 × 150 mm test tube. Add 0.5 mL methanol: DI water (80:20, v/v). Add 12.5 µL of a 0.10 µg/mL solution of TIS internal standard (equivalent to 0.00125 µg). Vortex mix the solution for ~5 seconds.

2. Add 4 mL of 2.0 mM dansyl chloride and 4 mL of 0.1 M sodium bicarbonate.
3. Vortex the tube for ~10 seconds and sonicate for ~30 minutes.

Note: Do not expose sample to direct sunlight during this procedure since the dansyl (Dns) derivatives are light sensitive.

4. Following sonication, add 1 mL of 0.5 M NH₄OH to the sample and vortex mix for 10-20 seconds. Let sample sit for 4 minutes.

Note: Do not allow samples to sit for more than 4 minutes at this step, as significant hydrolysis of the dansyl triazole derivative may occur.

5. After 4 minutes, add 6 mL of ethyl acetate to the sample. Shake vigorously for ~20 seconds. Allow the two phases to separate.
6. Place a 20-mL SPE cartridge, filled with ~10 g of anhydrous sodium sulfate retained with a glasswool plug, in a support rack so it can drain into a 16 × 100 mm test tube by gravity.
7. Using a pipet, draw off the ethyl acetate (top) layer from the sample and transfer to the SPE drying cartridge. Drain the ethyl acetate into a 16 × 100 mm test tube. Rinse the sodium sulfate with 4 mL ethyl acetate and collect the rinse as well.
8. Concentrate the dried ethyl acetate extract to ~0.2 mL on an N-Evap evaporator set at 45-50°C. Continue evaporating to dryness with manual nitrogen blowdown.
9. Add 0.75 mL of acetonitrile, rinsing the walls of the tube in the process. Vortex mix for ~10 seconds and sonicate for ~30 seconds.

Note: If a 0.5-mL aliquot of the original sample extract (Step 1) was taken through the procedure, add 375 µL of acetonitrile, rinsing the walls of the tube in the process. Vortex mix for ~10 seconds and sonicate for ~30 seconds.

10. Add 1.75 mL of HPLC grade water, rinsing the walls of the tube in the process. Vortex mix for ~10 seconds and sonicate for ~10 seconds.

Note: If a 0.5-mL aliquot of the original sample extract (Step 1) was taken through the procedure, add 875 µL of HPLC grade water, rinsing the walls of the tube in the process. Vortex mix for ~10 seconds and sonicate for ~10 seconds.

11. Mix well and submit to HPLC analysis. Final concentration of HPLC-ready extract is 1.0 mL = 0.02 g sample.

Note 1: Samples require a 0.45µm filtration just prior to HPLC analysis. Instrumentation personnel will filter the solution through a 0.45 µm Nylon filter using a Hamilton Teflon[®] Luer-lock syringe just prior to HPLC analysis.

Note 2: Some HPLC autosampler vials permit/promote degradation of the dansyl derivative of 1,2,4-triazole. The following HPLC vial has been found suitable for use: Target[®]DP[™] Amber vials, Part# C4000-188W (National Scientific Company).

10.2 Triazole alanine (TA). HCl/butanol esterification and HFBA acylation.

Procedure:

1. Add 0.5 mL of 3N HCl in n-butanol to the test tube containing the dried residue from Step 9.1.7. Rinse the walls of the tube with this addition. Sonicate in a beaker of water for ~10 minutes.
2. Heat the tube in a heat block at ~110°C for 30 minutes, mixing the tubes at ~10-minute intervals. Remove from the heat and cool.
3. Evaporate the sample to dryness on an N-Evap evaporator set at 45-50°C.
4. Add 0.2 mL of heptafluorobutyric anhydride to the dried residue in the test tube. Rinse the walls of the tube with this addition. Cap tightly. Sonicate in a beaker of water for ~10 minutes. Heat the tube in an oven at ~140°C for 10 minutes. Remove from the heat and cool.
5. Evaporate the sample to dryness on an N-Evap evaporator set at 45-50°C. Add 0.5 mL of hexane to redissolve/resuspend the residue, then re-evaporate to dryness at on the N-Evap evaporator at 45-50°C.
6. Add 0.75 mL of acetonitrile, rinsing the walls of the tube in the process. Vortex mix for ~10 seconds and sonicate for ~10 seconds.
7. Add 1.75 mL of HPLC grade water, rinsing the walls of the tube in the process. Vortex mix for ~10 seconds and sonicate for ~10 seconds.
8. Mix well and submit to HPLC analysis. Final concentration of HPLC-ready extract is 1.0 mL = 0.02 g sample.

Note: Samples require a 0.45µm filtration just prior to HPLC analysis. Instrumentation personnel will filter the solution through a 0.45 µm Nylon

filter using a Hamilton Teflon[®] Luer-lock syringe just prior to HPLC analysis.

10.3 Triazole acetic acid (TAA). HCl/butanol esterification.

Procedure:

1. Add 0.5 mL of 3N HCl in n-butanol to the test tube containing the dried residue from Step 9.2.7. Rinse the walls of the tube with this addition. Sonicate in a beaker of water for ~10 minutes.
2. Heat the tube in a heat block at ~110°C for 30 minutes, mixing the tubes at ~10-minute intervals. Remove from the heat and cool.
3. Evaporate the sample to dryness on an N-Evap evaporator set at 45-50°C.
4. Add 0.75 mL of acetonitrile, rinsing the walls of the tube in the process. Vortex mix for ~10 seconds and sonicate for ~10 seconds.
5. Add 1.75 mL of HPLC grade water, rinsing the walls of the tube in the process. Vortex mix for ~10 seconds and sonicate for ~10 seconds.
6. Mix well and submit to HPLC analysis. Final concentration of HPLC-ready extract is 1.0 mL = 0.02 g sample.

Note: Samples require a 0.45µm filtration just prior to HPLC analysis. Instrumentation personnel will filter the solution through a 0.45 µm Nylon filter using a Hamilton Teflon[®] Luer-lock syringe just prior to HPLC analysis.

11 **HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS**

Note: The column and conditions stated in the method have been satisfactory for the matrices being analyzed. The specific column packing, mobile phase, column temperature and flow rate listed are typical conditions for this analysis. Alternate columns may be used depending on the need to resolve analyte and/or interfering responses. Specific conditions used will be noted on each chromatographic run and will not otherwise be documented.

11.1 Operating Conditions

Instrument: PE Sciex API 2000 LC/MS/MS system with a Perkin Elmer series 200 autosampler, an integrated Shimadzu chromatograph

consisting of (2) LC-10ADvp Liquid Chromatograph units, a DGU-14A Degasser, and a SCL-10Avp System Controller. The system is controlled and data processed by PE Sciex Analyst Software.

HPLC Column: Phenomenex Luna C18(2) 150 mm × 2.0 mm, 3 micron particle size

Mobile Phase: (Fisher Water, Burdick and Jackson Acetonitrile, EM Science Formic Acid)

Gradient:

1,2,4Triazole (T):

<u>Time (min)</u>	<u>Percent of 0.1% formic acid in water</u>	<u>Percent of 100% acetonitrile</u>
0.0	50	50
5.0-7.5	30	70
7.6-10.1	10	90
10.2-13.0	50	50

Triazole Alanine (TA):

<u>Time (min)</u>	<u>Percent of 0.1% formic acid in water</u>	<u>Percent of 100% acetonitrile</u>
0.0	50	50
5.0-6.7	30	70
7.0-9.4	10	90
9.5-12.5	50	50

Triazole Acetic Acid (TAA):

<u>Time (min)</u>	<u>Percent of 0.1% formic acid in water</u>	<u>Percent of 100% acetonitrile</u>
0-0.3	80	20
2.5	60	40
7.0-9.8	10	90
9.9-12.5	80	20

Divert Valve:

T: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 5.8 minutes and again from 7.5 to 13 minutes. LC flow is directed to detector during the 5.8 to 7.5 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

TA: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 5.3 minutes and again from 7.1 to 12.5 minutes. LC flow is directed to detector during the 5.3 to 7.1 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

TAA: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 5.1 minutes and again from 6.7 to 12.5 minutes. LC flow is directed to detector during the 5.1 to 6.7 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

Flow Rate: 200 μ L/min

Interface: TIS (turbo ion spray)

Ionization Mode: positive (+)

Acquisition Mode: MRM

Resolution: Q1 – unit, Q3 – low (Note: Unit equivalent to medium)

Source Temperature: T and TAA: 350°C
TA: 300°C

Curtain Gas: Nitrogen: T: @ 15 psi
TA: @ 50 psi
TAA: @ 40psi

Collision Gas: Nitrogen @ setting of "8"

Transitions Monitored:		Ion, <i>m/z</i>		Time, <i>ms</i>	CE, <i>v</i>
		Q1	Q3		
TD:		303.1	169.8	150	27 (confirmation)
		303.1	180.9	150	41 (quantitation)
		303.1	195.5	150	41 (confirmation)
TDIS:		307.9	170.0	150	29 (confirmation)
		307.9	184.0	150	43 (quantitation)
TAD:		409.1	70.0	150	47 (quantitation)
		409.1	284.2	150	29 (confirmation)
TADIS:		414.0	75.0	150	47
TAAD:		184.0	70.0	150	39 (quantitation)
		184.0	128.1	150	23 (confirmation)
TAADIS:		189.0	133.0	150	23

Injection Volume: 50 μ L

Column

Temperature: 35°C

Retention Times: TD: ~6.6 minutes
 TDIS: ~6.6 minutes
 TAD: ~6.1 minutes
 TADIS: ~6.1 minutes
 TAAD: ~5.9 minutes
 TAADIS: ~5.9 minutes

11.2 Sample Analysis

Prepare, at minimum, a six-point standard curve for each analyte by injecting constant volumes of individual or mixed standard solutions. Use constant volume injections for sample extracts as well. Inject a curve check standard every 4-5 sample injections.

12 CALCULATIONS

Calculations for instrumental analysis are conducted using a validated software application to create a standard curve based on linear regression. The regression functions are used to calculate a best fit line and to demonstrate linearity of the HPLC

detector system. The best fit line is calculated from a set of analyte/internal standard concentration ratios versus their respective quantitation ratios (analyte response/internal standard response). Because all calibration standards are based on the respective parent equivalent of each analyte, no molecular weight conversion from derivative to native compound is required. **Use 1/x weighting.**

The equation used for the least squares fit is:

$$y = mx + b$$

where:

m = slope of the line

b = the y-intercept

y = Analyte Response (AR)/Internal Standard Response (ISR) ratio

x = Analyte Concentration (AC)/Internal Standard Concentration (ISC) ratio

In order for the specific HPLC software being employed to calculate "µg/mL of analyte found", this equation is transformed as follows:

$$AR/ISR = m \times AC/ISC + b$$

$$AC = \left(\frac{AR/ISR - b}{m} \right) \times ISC$$

where:

AC = µg/mL found for analyte

The calculations for ppm found and percent recovery (for fortified samples) are:

1. The amount of analyte (in ppm) found in the sample is calculated according to the following equation:

$$ppm = \mu\text{g/mL found} \times \frac{FV(\text{mL})}{\text{sample wt. (g)}} \times \frac{\text{ext. solv. (mL)}}{\text{aliq. (mL)}}$$

where:

$\mu\text{g/mL}$ found = $\mu\text{g/mL}$ analyte found from standard curve

FV (mL) = mL volume of final extract submitted to HPLC analysis (2.5 mL)

sample wt. (g) = gram weight of sample extracted (5.00 g)

ext. solv. (mL) = total volume of extraction solvent (100 mL)

aliq. (mL) = aliquot of extract taken through procedure (1.0 mL)

2. The percent recovery for fortified control samples is calculated as follows:

$$\% \text{ Rec.} = \frac{\text{ppm found in fortified control (spike)} - \text{ppm found in control}}{\text{fortification level (ppm) added}} \times 100$$

13 REFERENCE

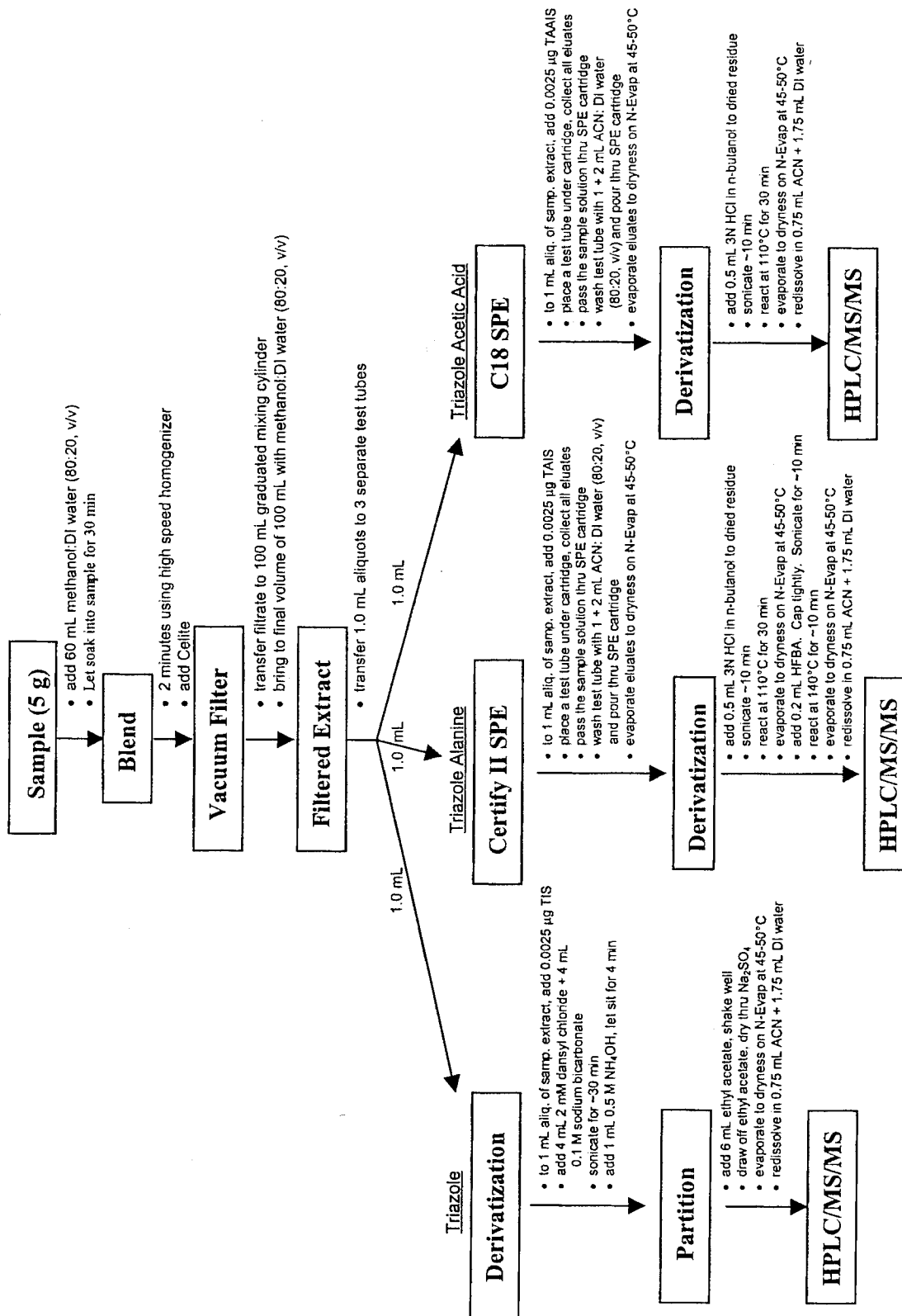
1. *Working Residue Analytical Method for the Determination of Triazole, Triazole Alanine, and Triazole Acetic Acid Residues in Plant and Animal Matrices*, Draft dated May 15, 2003, Bayer CropScience, Agriculture Division, Kansas City, MO.

Method author: Gary L. Westberg

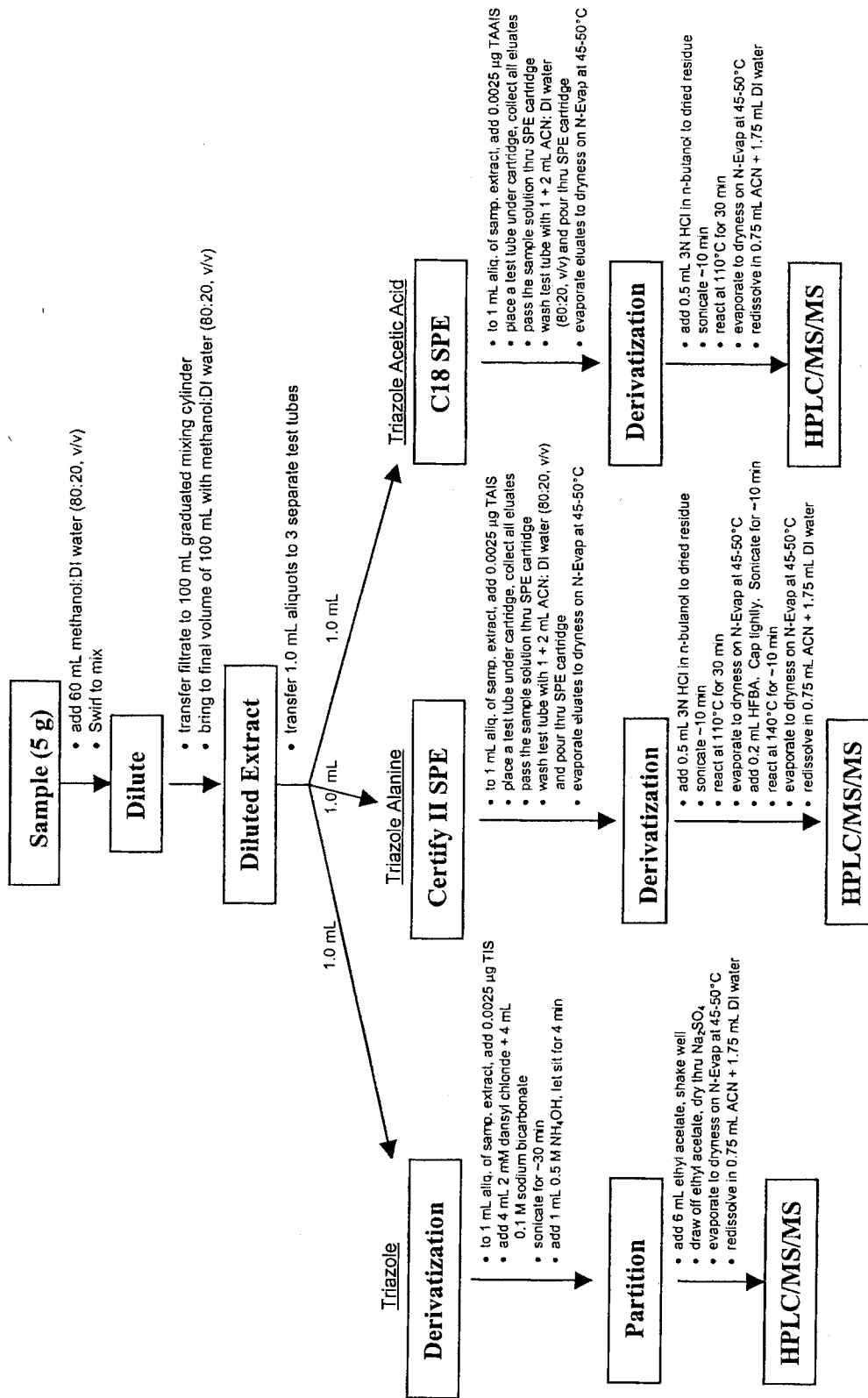
APPENDIX I

Analysis Flowcharts

ANALYSIS FLOWCHART- Crops and Processed Commodities



ANALYSIS FLOWCHART- MIK



APPENDIX II

Quality Control for SPE Cartridges

Bond Elut-Certify II SPE Cartridges

Add 200 μL of a TA standard solution @ 10 $\mu\text{g}/\text{mL}$ (in HPLC grade water) to a 13 \times 100 mm test tube. Add 800 μL of methanol. Vortex mix. Follow Steps 9.1.1. through 9.1.7. of the procedure. Redissolve the residue in 1.0 mL of HPLC grade water. Vortex mix and sonicate. Submit to HPLC/UV analysis (See conditions below). Final concentration is 2.0 μg TA/mL.

HPLC Analysis:

1. Operating Conditions

Instrument: Thermo Separation Products P4000 quaternary gradient elution high pressure liquid chromatograph equipped with a Thermo Separation Products UV 1000 UV/vis detector.

HPLC Column: 25 cm \times 4.6 mm Agilent Zorbax Rx-C8, 5 micron particle size

UV Detector
Wavelength: 210 nm

Column
Temperature: 35 $^{\circ}\text{C}$

Flow Rate: 0.5 mL/min.

Mobile Phase: Isocratic: 100% HPLC water

Injection Volume: 50 μL

Retention Time: ~6.0 minutes

2. Sample Analysis

Inject sample against a standard having the same concentration, i.e., 2.0 μg TA/mL.

3. Calculation

Use ratio proportion to determine concentration of TA found in sample:

$$\text{conc. TA found in sample} = \frac{\text{sample peak response}}{\text{standard peak response}} \times 2.0 \mu\text{g/mL}$$

$$\% \text{ Recovery} = \frac{\text{concentration of TA found in sample}}{\text{concentration of standard}} \times 100$$

Bond Elut-C18 SPE Cartridges

Add 200 µL of a TAA standard solution @ 10 µg/mL (in HPLC grade water) to a 13 × 100 mm test tube. Add 800 µL of methanol. Vortex mix. Follow Steps 9.1.1. through 9.1.7. of the procedure. Redissolve the residue in 0.5 mL of HPLC grade water. Vortex mix and sonicate. Submit to HPLC/UV analysis (See conditions below). Final concentration is 4.0 µg TAA/mL.

HPLC Analysis:

1. Operating Conditions

Instrument: Thermo Separation Products P4000 quaternary gradient elution high pressure liquid chromatograph equipped with a Thermo Separation Products UV 1000 UV/vis detector.

HPLC Column: 25 cm × 4.6 mm Agilent Zorbax Rx-C8, 5 micron particle size

UV Detector
Wavelength: 215 nm

Column
Temperature: 35 °C

Flow Rate: 0.8 mL/min.

Mobile Phase: Isocratic: 0.1% formic acid in HPLC water

Injection Volume: 50 µL

Retention Time: ~5.8 minutes

2. Sample Analysis

Inject sample against a standard having the same concentration, i.e., 4.0 µg TAA/mL.

3. Calculation

Use ratio proportion to determine concentration of TA found in sample:

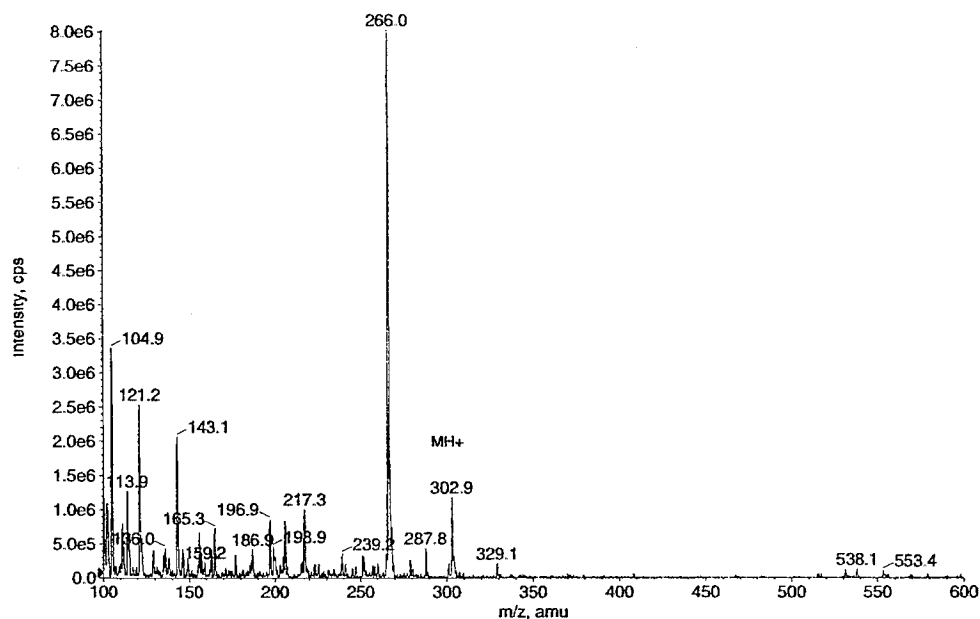
$$\text{conc. TAA found in sample} = \frac{\text{sample peak response}}{\text{standard peak response}} \times 4.0 \mu\text{g/mL}$$

$$\% \text{ Recovery} = \frac{\text{concentration of TAA found in sample}}{\text{concentration of standard}} \times 100$$

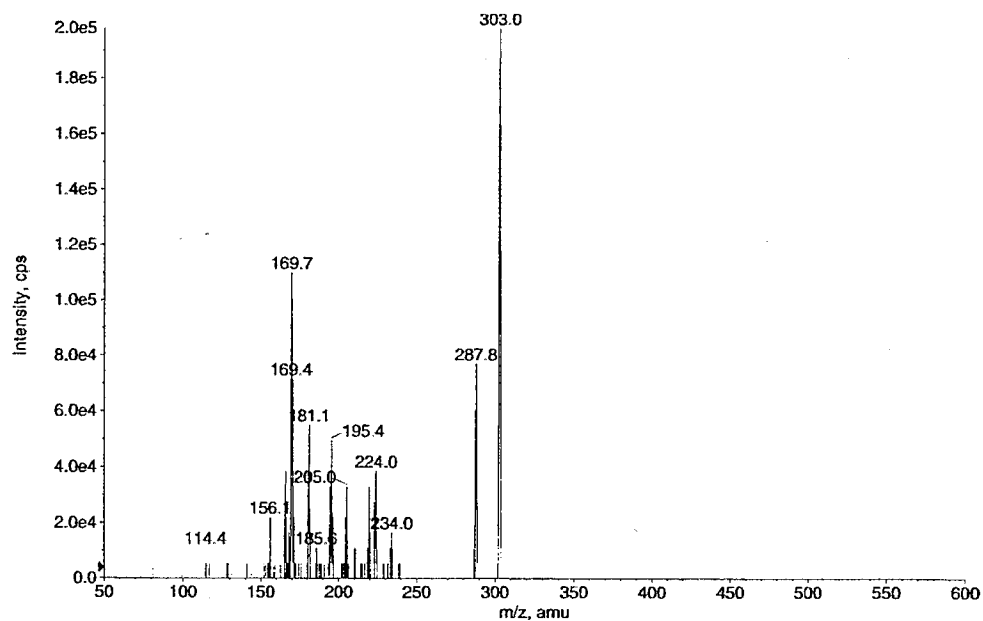
APPENDIX III

Mass Spectra for Parent and Product-ions

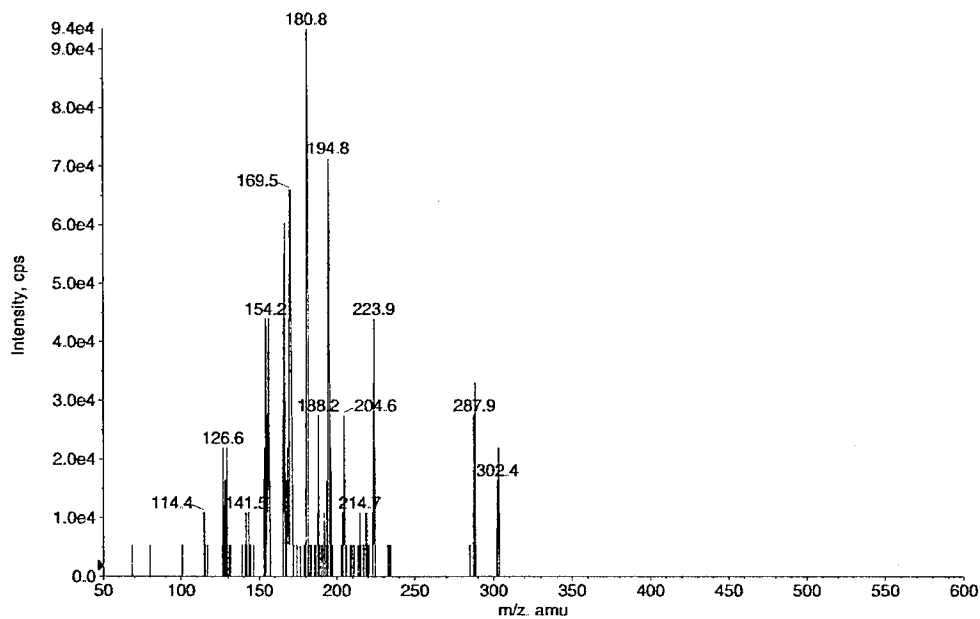
MS Full Scan (Q1 Scan) Spectrum of a 5 µg/mL TD Standard



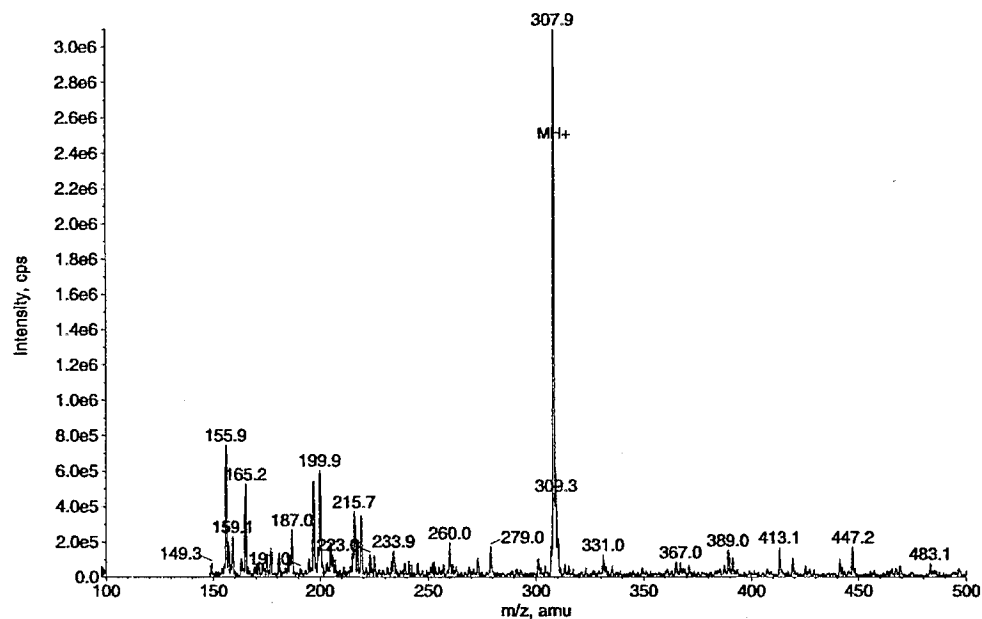
MS/MS Full Scan (Q3 Scan) Spectrum of a 5 µg/mL TD Standard (Collision Energy Set to 27V)



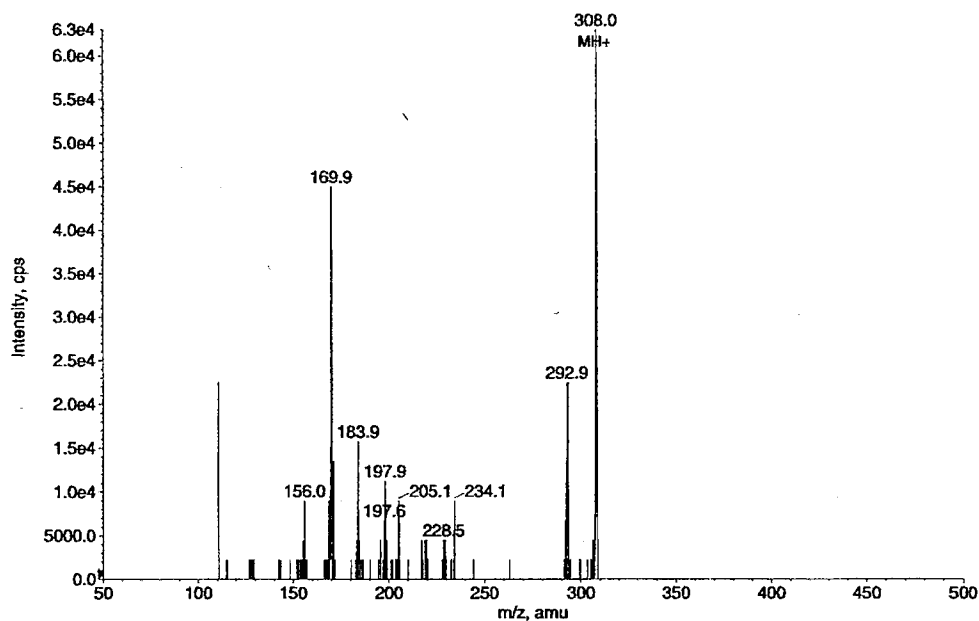
MS/MS Full Scan (Q3 Scan) Spectrum of a 5 µg/mL TD Standard (Collision Energy Set to 41V)



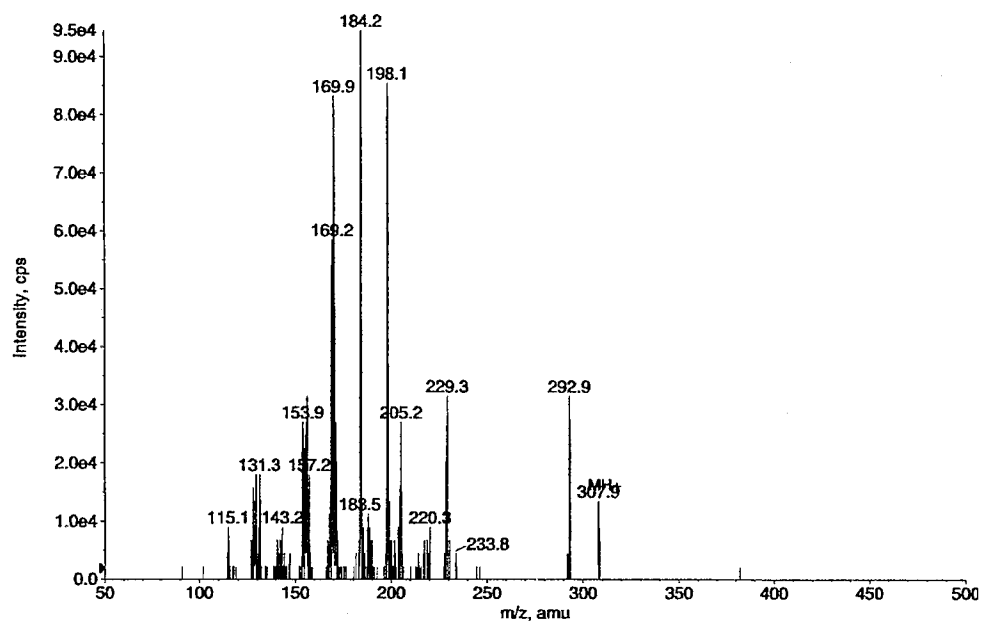
MS Full Scan (Q1 Scan) Spectrum of a 1 µg/mL TDIS Standard



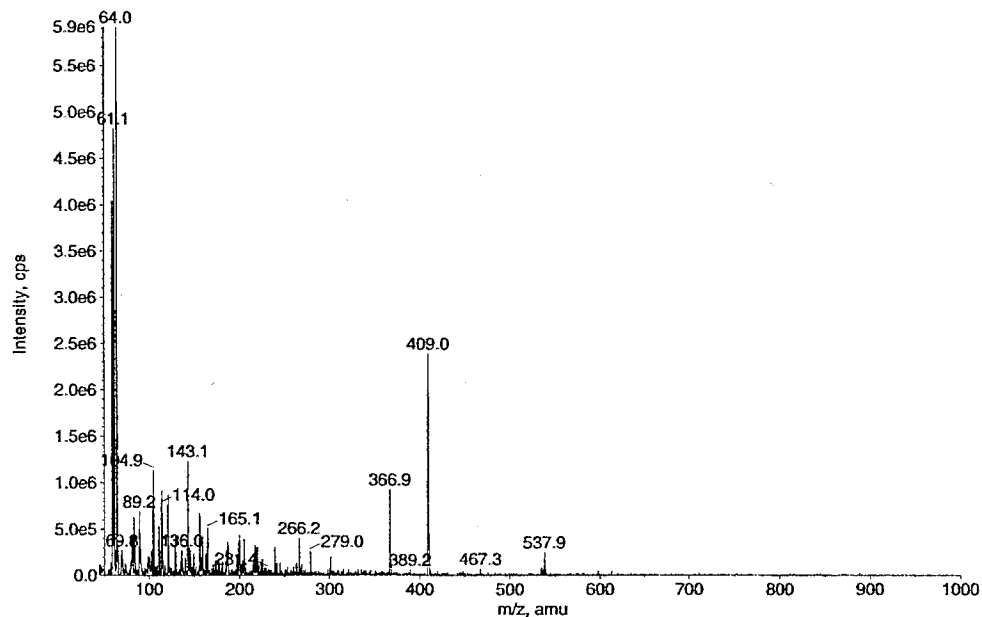
MS/MS Full Scan (Q3 Scan) Spectrum of a 0.5 µg/mL TDIS Standard (Collision Energy Set to 29V)



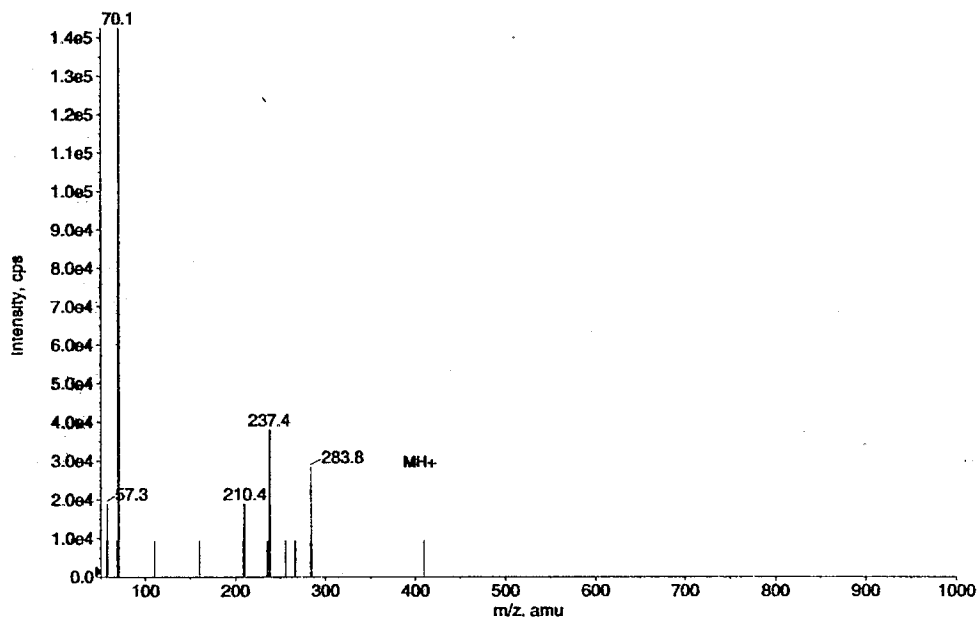
MS/MS Full Scan (Q3 Scan) Spectrum of a 0.5 µg/mL TDIS Standard (Collision Energy Set to 43V)



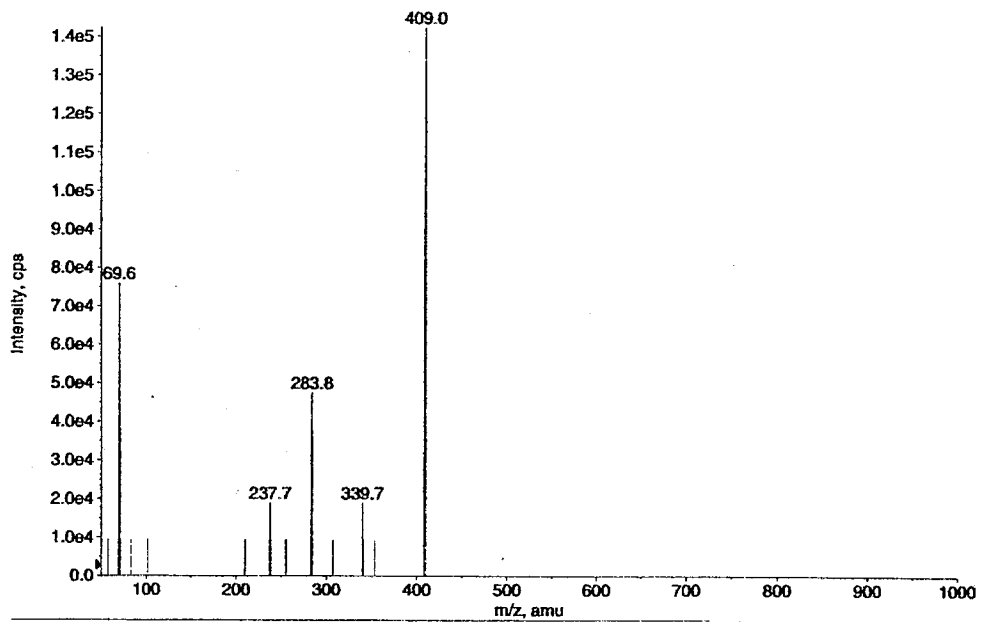
MS Full Scan (Q1 Scan) Spectrum of a 0.5 µg/mL TAD Standard



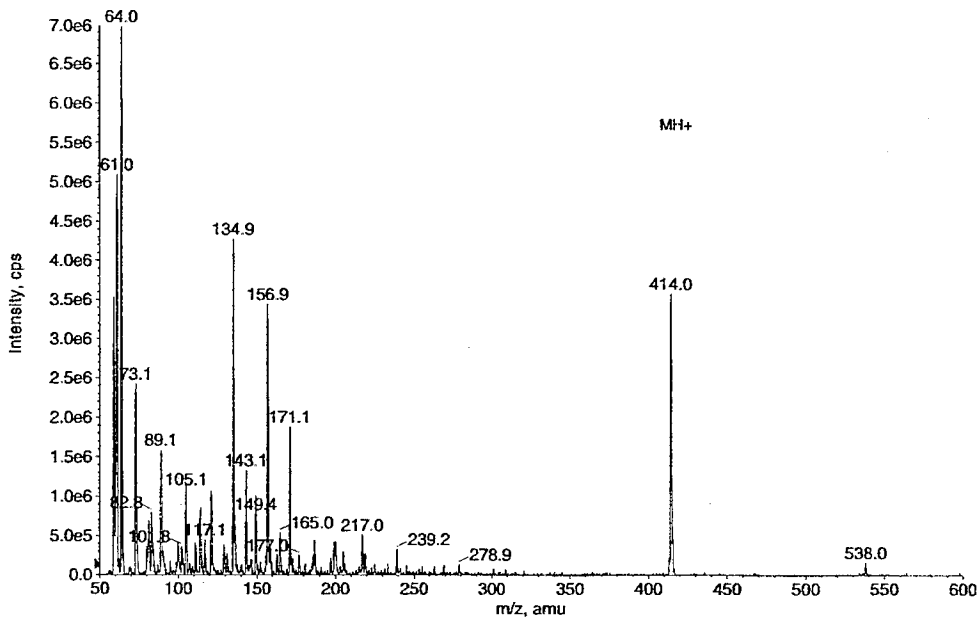
MS/MS Full Scan (Q3 Scan) Spectrum of a 0.5 µg/mL TAD Standard (Collision Energy Set to 47V)



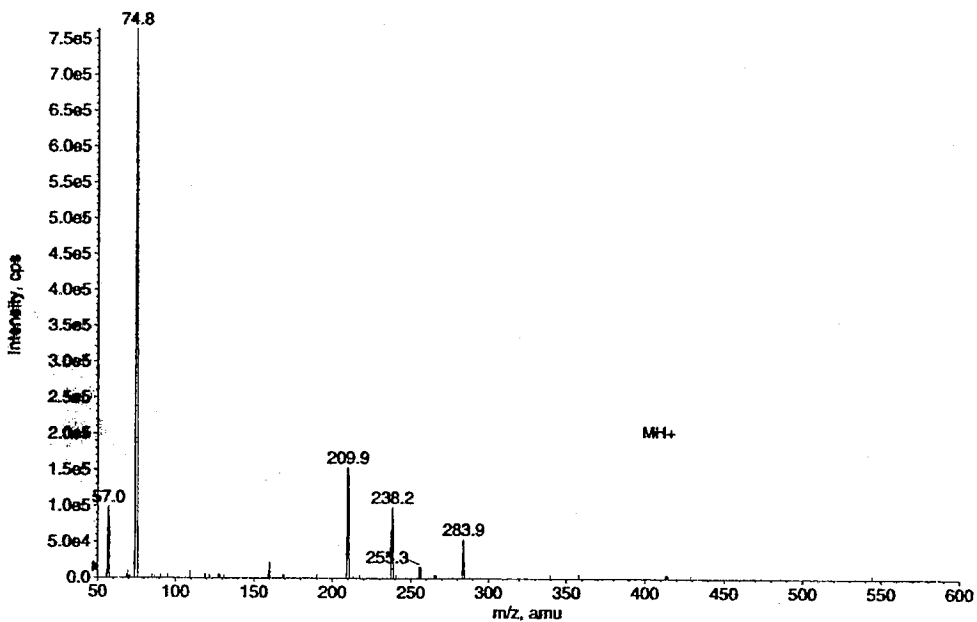
MS/MS Full Scan (Q3 Scan) Spectrum of a 0.5 µg/mL TAD Standard (Collision Energy Set to 29V)



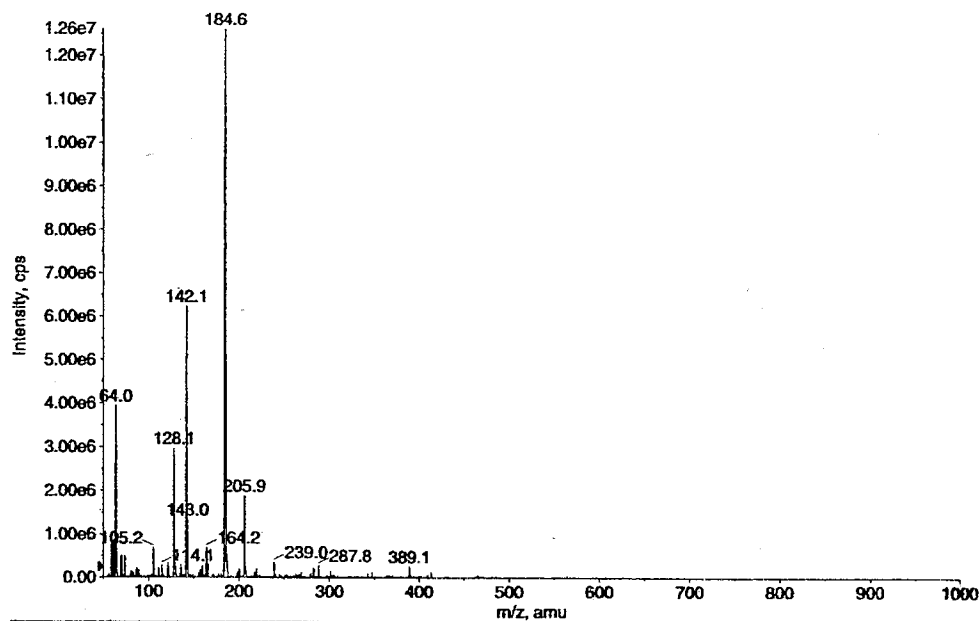
MS Full Scan (Q1 Scan) Spectrum of a 1 µg/mL TADIS Standard



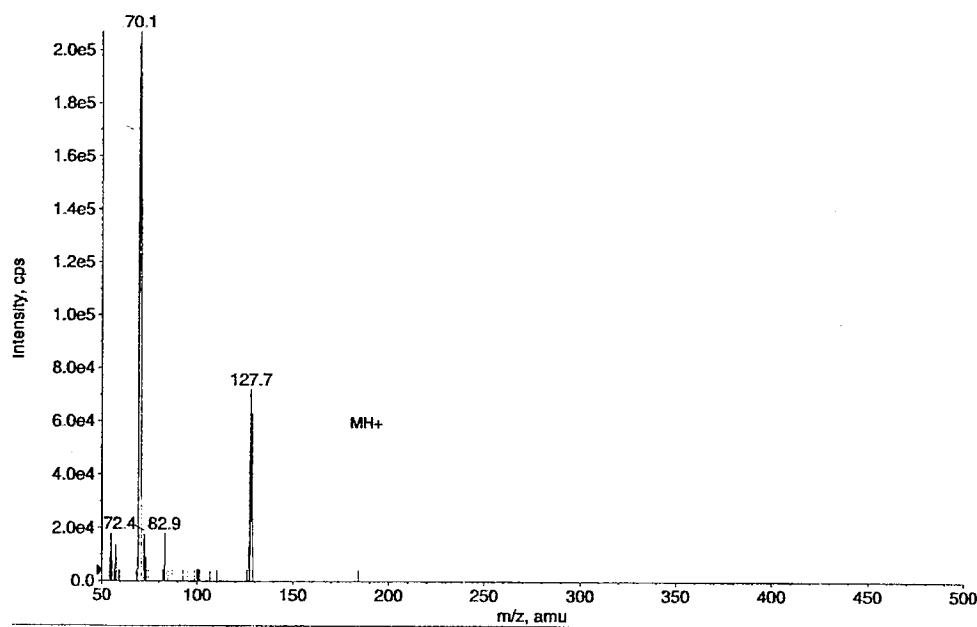
MS/MS Full Scan (Q3 Scan) Spectrum of a 1 µg/mL TADIS Standard (Collision Energy Set to 47V)



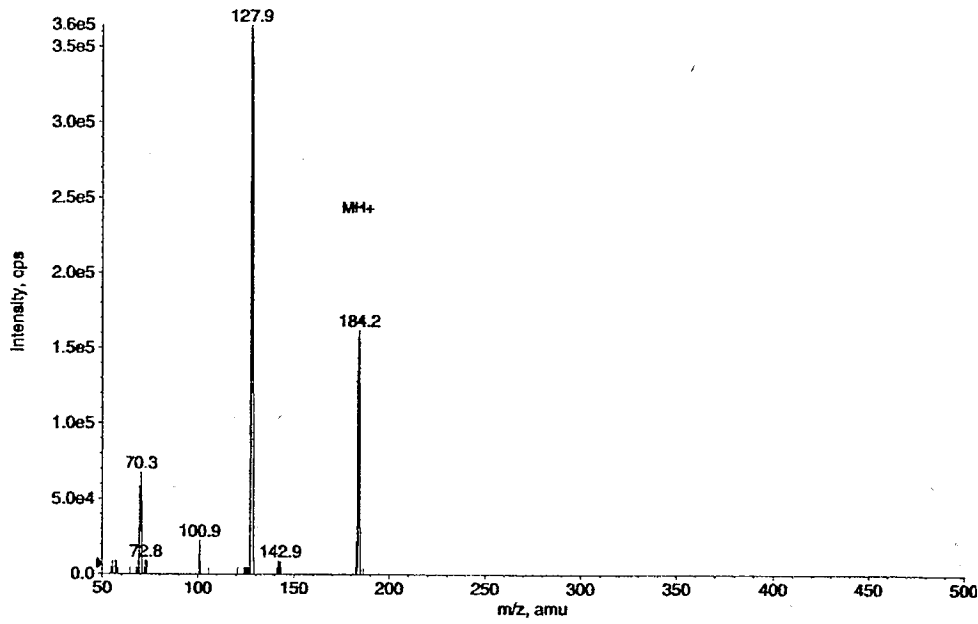
MS Full Scan (Q1 Scan) Spectrum of a 50 µg/mL TAAD Standard



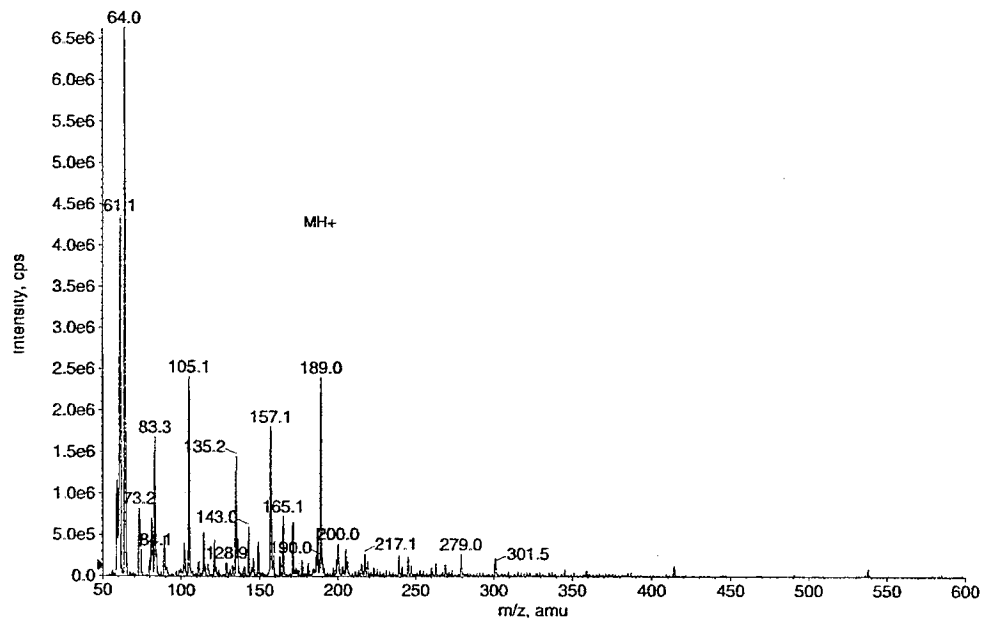
MS/MS Full Scan (Q3 Scan) Spectrum of a 0.5 µg/mL TAAD Standard (Collision Energy Set to 39V)



MS/MS Full Scan (Q3 Scan) Spectrum of 0.5 µg/mL TAAD Standard (Collision Energy Set to 23V)



MS Full Scan (Q1 Scan) Spectrum of a 1 µg/mL TAADIS Standard



MS/MS Full Scan (Q3 Scan) Spectrum of a 1 µg/mL TAADIS Standard (Collision Energy Set to 23V)

