TRADE SECRET

49378205

Study Title

INDEPENDENT LABORATORY VALIDATION OF DUPONT-5856, "ANALYTICAL METHOD FOR THE DETERMINATION OF TRIBENURON METHYL AND METABOLITES IN-L5296, IN-A4098, IN-D5119, AND IN-00581 IN WATER USING LC/MS/MS"

Test Guidelines

OECD Guidance Document on Pesticide Residue Analytical Methods, ENV/JM/MONO (2007) 17

U.S. EPA Residue Chemistry Test Guidelines
OPPTS Ecological Effects Test Guidelines EPA Documents EPA 712-C-001 (OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation, January 2012

Pesticide Registration Notice 2011-3, January 6, 2012

EC Directorate General Health and Consumer Protection. "Guidance Document on Residue Analytical Methods", SANCO/825/00 rev.8.1, November 16, 2010

A single analyst completed sample sets consisting of 13 samples in the course of one eight-hour workday with LC/MS/MS analysis performed overnight.

2.0 INTRODUCTION

To satisfy EU and US regulatory ILV requirements, a residue analytical method must be validated at an independent laboratory prior to its submission to the appropriate regulatory authority. This study was conducted to fulfill those requirements.

The residue analytical method described in DuPont-5856, entitled "Analytical Method for the Determination of Tribenuron Methyl and Metabolites IN-L5296, IN-A4098, IN-D5119, and IN-00581 in Water Using LC/MS/MS", is applicable for the quantitation of tribenuron methyl and metabolites IN-L5296, IN-A4098, IN-D5119, and IN-00581 in water. In this study, the analytical method was validated on three representative matrices for which the method was designed: ground, surface, and drinking water.

Tribenuron methyl, IN-L5296, and IN-A4098 were extracted from the water samples by filtration through an Oasis HLB solid phase extraction (SPE) cartridge followed by a wash step, then eluted with base-adjusted acetonitrile. One (1) mL of water was added and the eluate was evaporated under a flow of nitrogen until the volume was less than 1 mL. The final volume was adjusted to 10 mL using water. Tribenuron methyl, IN-A4098, and IN-D5119 were separated from co-extracts by reversed phase liquid chromatrography (LC) and were detected by positive ion electrospray mass spectrometry/mass spectrometry (MS/MS). The Limit of Quantitation (LOQ) was 0.050 µg/L (ppb). The Limit of Detection (LOD) was estimated to be at 0.005 µg/L (ppb).

The analysis of IN-D5119 and IN-00581 required acidification of the sample prior to extraction followed by filtration through an Oasis HLB solid phase extraction (SPE) cartridge followed by three wash steps then eluted with 70:30 (v.v.) methanol:water. The extracts were evaporated until the volume was approximately 6 mL. The final volume was adjusted to 8 mL using water. IN-D5119 and IN-00581 were separated from co-extracts by reversed-phase liquid chromatography (LC) and were detected by negative ion electrospray mass spectrometry/mass spectrometry (MS/MS). The Limit of Quantitation (LOQ) was 0.10 µg/L (ppb). The Limit of Detection (LOD) was estimated to be 0.03 µg/L (ppb).

Except for the minor modifications discussed in Section 3.6 of this report, the method was performed as written. No communication, other than the approval of equivalent apparatus, reagents, and techniques; correction of typographical errors; extraction and chromatography issues; clarification of some technical aspects of the method; and recovery updates between the Sponsor Representative (Method Developer) and Study Director was required.

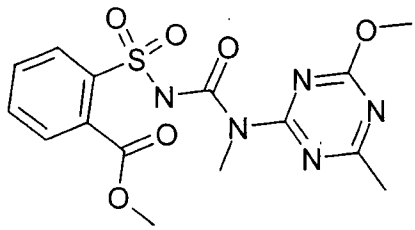
3.0 MATERIALS AND METHODS

3.1 Test Substances

The reference analytical standards (test substances) used for this study were:

Tribenuron Methyl:

DuPont Code: DPX-L5300
Chemical Name:
IUPAC: Methyl 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoyl sulfamoyl]benzoate
CAS: Methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)methylamino] carbonyl]amino] sulfonyl]benzoate
CAS No.: 101200-48-0
Chemical Structure:



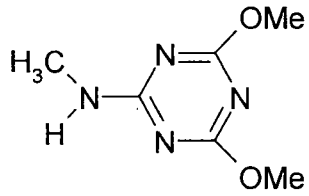
Tribenuron Methyl (DPX-L5300)

Molecular Weight: 395.39 g/mole
Source: E. I. du Pont de Nemours and Company
Purity: 98.7%
Lot No.: NOV00MA207
Receipt Date: July 5, 2013
Expiration Date: March 29, 2017
Storage: Freezer (-25 °C to -10 °C)

IN-L5296:

DuPont Code: IN-L5296
Chemical Name:
IUPAC: 4-methoxy-6-methyl-1,3,5-triazin-methylamine
CAS No.: Not available

Chemical Structure:

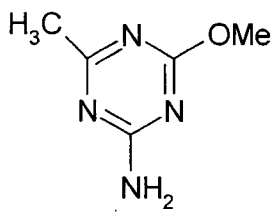


IN-L5296

Molecular Weight: 170.17 g/mole
Source: E. I. du Pont de Nemours and Company
Purity: 99.6%
Lot No.: E79048-55
Receipt Date: July 5, 2013
Expiration Date: January 25, 2021
Storage: Ambient (15 °C to 30 °C)

IN-A4098:

DuPont Code: IN-A4098
Chemical Name:
CAS: 2-amino-4-methoxy-6-methyl-1,3,5-triazine
CAS No.: 1668-54-8
Chemical Structure:



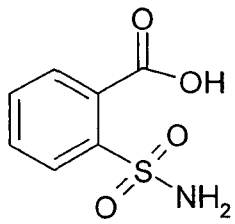
IN-A4098

Molecular Weight: 140.14 g/mole
Source: E. I. du Pont de Nemours and Company
Purity: 98.7%
Lot No.: 050942-015
Receipt Date: July 5, 2013
Expiration Date: September 2, 2019
Storage: Ambient (15 °C to 30 °C)

IN-D5119:

DuPont Code: IN-D5119
Chemical Name:
IUPAC: 2-(aminosulfonyl) benzoic acid
CAS No.: Not available

Chemical Structure:

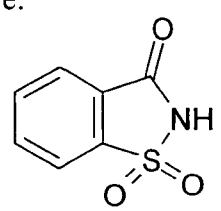


IN-D5119

Molecular Weight: 201.20 g/mole
Source: E. I. du Pont de Nemours and Company
Purity: 99.5%
Lot No.: E10035-086
Receipt Date: July 5, 2013
Expiration Date: March 29, 2021
Storage: Freezer (-25° C to -10 °C)

IN-00581:

DuPont Code: IN-00581 (Saccharin)
Chemical Name:
CAS: 1,2-benzisothiazol-3(*2H*)-one,1,1-dioxide
CAS No.: 81-07-2
Chemical Structure:



IN-00581

Molecular Weight: 183.19 g/mole
Source: E. I. du Pont de Nemours and Company (purchased from Sigma-Aldrich
Purity: 99.9%
Lot No.: 03111DD
Receipt Date: July 5, 2013
Expiration Date: April 30, 2015
Storage: Ambient (15 °C to 30 °C)

Tribenuron methyl (DPX-L5300) and metabolites IN-L5296, IN-A4098, IN-D5119, and IN-00581 standards were supplied by E. I. du Pont de Nemours and Company, DuPont Crop Protection, Newark, DE. Information pertaining to the characterization

and stability of the test substances is archived by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, Delaware with the exception of IN-00581 which was purchased from Sigma Aldrich and was not GLP characterized. The Certificates of Analysis are included in Appendix 1.

3.2 Test Systems

In this study, the analytical method was validated on the following matrices: ground, surface, and drinking water. These matrices were chosen because they are representative of the matrices for which the method was designed - water.

Control samples of ground, surface, and drinking water used in the study were provided by the laboratory. The ground water sample was collected from well water from Cool, California; the surface water sample was collected from the American River in Gold River, California; and the drinking water was collected as tap water from Morse Laboratories in Sacramento, California. The samples remained in refrigerated storage until removed for analysis. All water samples were GLP characterized, with the exception of the drinking water sample analyzed in Set 20. The characterization results are presented in Appendix 6. The Sponsor Monitor approved of the use of the drinking water sample analyzed without GLP characterization in an email dated August 27, 2013.

The samples were assigned unique identification by the laboratory. Additional designations such as "control" and "fortified control," as appropriate, were also assigned by the laboratory.

3.3 Equipment

Equipment used is the same as that specified in the analytical method, except as follows:

Balances:	Mettler Toledo, Model AB104, for weighing solid standards (Mettler Instrument Corp., Hightstown, NJ)
Centrifuge bottle:	250 mL polypropylene (PP), for extraction and storage (Fisher Scientific, Fairlawn, NJ)
Centrifuge tube:	50-mL glass conical with round glass stoppers (Pyrex, Fisher Scientific, Fairlawn, NJ)
	50-mL glass conical with PTFE-lined, plastic screw caps (Pyrex, Fisher Scienrific, Fairlawn, NJ)
Evaporator:	N-Evap® Model 115, attached to a nitrogen source (Organomation Associates, South Berlin, MA)
Graduated cylinder:	glass, various sizes
HPLC/MS/MS system:	API 4000 LC/MS/MS detector (Applied Biosystem/MDS Sciex) with an integrated Shimadzu chromatograph consisting of (2) LC-20AD Liquid Chromatograph units and a DGU-20AS Degasser, a Shimadzu SIL-20AC autosampler,

	a TC-50 controller, and a CH-30 column heater. The system is controlled and data processed by Applied BioSystems/MDS Sciex Analyst Software (Version 1.5).
HPLC vials:	Xpertek 12/32, clear glass, wide mouth, screw thread with graduation marks (P.J. Cobert Assoicates Inc., St. Louis, MO)
HPLC vial caps:	Xpertek 10-425, black with bonded PTFE/Sil Septa (P.J. Cobert Assoicates Inc., St. Louis, MO)
Mobile phase reservoir:	1000-mL, (Wheaton, Millville, NJ)
Pasteur pipets:	Glass, 9-inch and 5¾ inch, disposable
Pipets:	Glass, graduated, serological; various sizes
Pipets, digital:	Digital pipettors:
	5.0 - 40.0 µL: Fisher Scientific, Fairlawn, NJ 100 - 1000 µL: BrandTech Scientific, Essex, CT Eppendorf, Westbury, NY
	0.5 - 5.00 mL: BrandTech Scientific, Essex, CT 1.00 - 10.00 mL: BrandTech Scientific, Essex, CT Eppendorf, Westbury, NY
	Digital pipettor tips:
	1 - 200 µL: Fisher Scientific, Fairlawn, NJ 101 - 1000 µL: Fisher Scientific, Fairlawn, NJ 0.50 - 5.00 mL: PlastiBrand, Wertheim, Germany 1.00 - 10.00mL: Eppendorf, Westbury, NY
Vacuum manifold:	Visiprep 24-port SPE vacuum manifold (Supelco, Bellefonte, PA)
Volumetric flasks:	Glass, various sizes
Vortex mixer:	VWR Mini Vortexer (VWR Scientific Co., Bridgeport, NJ)
	Thermolyne Maxi Mix II type 37600 mixer (VWR Scientific Co., Bridgeport, NJ)

3.4 Reagents and Standards

Reagents and standards used were of equivalent grade as that specified in the analytical method.

3.5 *Principles of the Analytical Method*

The residue analytical method described in DuPont-5856, entitled "Analytical Method for the Determination of Tribenuron Methyl and Metabolites IN-L5296, IN-A4098, IN-D5119, and IN-00581 in Water Using LC/MS/MS," was used for the analyses in this study. The following is a summary of that method:

Tribenuron methyl, IN-L5296, and IN-A4098 were extracted from the water samples by filtration through an Oasis HLB solid phase extraction (SPE) cartridge followed by a wash step, then eluted with base-adjusted acetonitrile. One (1) mL of water was added and the eluate was evaporated under a flow of nitrogen until the volume was less than 1 mL. The final volume was adjusted to 10 mL using water. Tribenuron methyl, IN-A4098, and IN-D5119 were separated from co-extracts by reversed phase liquid chromatrography (LC) and were detected by positive ion electrospray mass spectrometry/mass spectrometry (MS/MS). The Limit of Quantitation (LOQ) was 0.050 µg/L (ppb). The Limit of Detection (LOD) was estimated to be at 0.005 µg/L (ppb).

The analysis of IN-D5119 and IN-00581 required acidification of the sample prior to extraction followed by filtration through an Oasis HLB solid phase extraction (SPE) cartridge followed by three wash steps then eluted with 70:30 (v.v.) methanol:water. The extracts were evaporated until the volume was approximately 6 mL. The final volume was adjusted to 8 mL using water. IN-D5119 and IN-00581 were separated from co-extracts by reversed-phase liquid chromatography (LC) and were detected by negative ion electrospray mass spectrometry/mass spectrometry (MS/MS). The Limit of Quantitation (LOQ) was 0.10 µg/L (ppb). The Limit of Detection (LOD) was estimated to be 0.03 µg/L (ppb).

3.6 *Modifications, Interpretations, and Critical Steps*

The analytical method was run exactly as written except as follows:

Step 1, Section 4.2.9. A 250-mL HDPE polypropylene centrifuge bottle was substituted for a 250-mL polycarbonate Erlenmeyer flask as the extraction vessel.

Step 2, Section 4.2.9. The adapter and 75-mL reservoir were not used.

3.7 *Instrumentation*

The quantitative analysis of tribenuron methyl and its metabolites was performed using Shimadzu LC-20AD Liquid Chromatograph units coupled to an Applied BioSystems/MDS Sciex API 4000 LC/MS/MS system. The system parameters are shown in the tables below. Peak area was used for quantitation.

HPLC Conditions: Tribenuron Methyl (DPX-L5300), IN-L5296, and IN-A4098

System:	API 4000 LC/MS/MS detector (Applied Biosystem/MDS Sciex) with an integrated Shimadzu chromatograph consisting of (2) LC-20AD Liquid Chromatograph units, a DGU-20AS Degasser, a Shimadzu SIL-20AC autosampler, a TC-50 controller, and a CH-30 column heater. The system is controlled and data processed by Applied BioSystems/MDS Sciex Analyst Software (Version 1.5).
Column:	4.6 mm i.d. × 150 mm, Phenomenex Aqua
Column Temperature:	35 °C
Injection Volume:	25 µL
Autosampler Temperature:	Ambient
Flow Rate:	1.0 mL/minute
Mobile Phase:	A: 0.005 M aqueous Formic acid B: Methanol
Mobile Phase Conditions:	Time %A %B Flow (mL/min) 0.0 90 10 1.0 1.0 90 10 1.0 3.5 70 30 1.0 12.0 20 80 1.0 12.5 5 95 1.0 14.5 5 95 1.0 15.0 90 10 1.0
Retention Times:	Tribenuron Methyl (DPX-L5300) ~13.1 minutes IN-L5296 ~7.2 minutes IN-A4098 ~5.7 minutes
Total Run Time:	~22.0 minutes

A six-port electronically activated switching valve was used to direct the flow to the MS or waste prior to and following the elution of the analytes. The valve switching times are given in the following table.

Time (minutes)	Column Eluate Flow
0.0 - 4.0	Waste
4.0 - 14.6	MS source
14.6 -22.0	Waste

Since electrospray LC/MS system performs optimally at low flow rates, the eluate was split following the switching valve. Approximately 100 µL/minute of eluate

(10:1 split) flowed into the ion source with remaining eluate flowing into a waste container.

The detection method utilized was LC/MS/MS employing electrospray (TIS) interface in the positive mode on a triple quadrupole instrument. The instrument was tuned by infusing the analytes into a TIS (turbo ion spray) source, then creating a tune file to maximize the response of each analyte using the TIS source. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for each analyte are shown in the table below:

MS Conditions:

System	Applied BioSystems/MDS Sciex API 4000 LC/MS/MS system						
Analytes Monitored	Ions Monitored (AMU)	Declustering Potential (volts)	Collision Energy (volts)	Dwell Time (seconds)	EP (volts)	CXP (volts)	Acquisition Timing (minutes)
Tribenuron Methyl (DPX-L5300)	396 → 155[a]	61	19	100	10	12	0 - 22
	396 → 181[b]		29	100	10	16	0 - 22
IN-L5296	155 → 71[a]	51	25	100	10	6	0 - 22
	155 → 57[b]		33	100	10	2	0 - 22
IN-A4098	141 → 57[a]	61	29	100	10	2	0 - 22
	141 → 85[b]		23	100	10	6	0 - 22

[a]Transistion ion used for quantitation
[b]Transistion ion used for confirmation.

Additional detector settings are shown in the table below:

Parameter	**Setting**
Acquisition Mode:	MRM
Ionization Mode:	Positive (+)
Source Temp.:	600 °C
Nebulizer (GS1):	50
Auxillary Gas (GS2):	50
Curtain Gas:	30
CAD Gas:	8
Ion Spray Voltage:	3000

The instrument was operated in the MS/MS (MRM) positive ion mode for quantitative analysis. Single transition chromatograms for each analyte were integrated and the peak areas used for quantitation. Quantitation was performed using a single transition for each analyte. Confirmation was determined using a secondary transition that was monitored throughout the validation.

HPLC Conditions: IN-D5119, and IN-00581

System:	API 4000 LC/MS/MS detector (Applied Biosystem/MDS Sciex) with an integrated Shimadzu chromatograph consisting of (2) LC-20AD Liquid Chromatograph units, a DGU-20AS Degasser, a Shimadzu SIL-20AC autosampler, a TC-50 controller, and a CH-30 column heater. The system is controlled and data processed by Applied BioSystems/MDS Sciex Analyst Software (Version 1.5).
Column:	4.6 mm i.d. × 150 mm, Phenomenex Aqua
Column Temperature:	35 °C
Injection Volume:	20 µL
Autosampler Temperature:	Ambient
Flow Rate:	1.0 mL/minute
Mobile Phase:	A: 0.005 M aqueous Formic acid B: Methanol
Mobile Phase Conditions:	Time %A %B Flow (mL/min) 0.0 90 10 1.0 1.0 90 10 1.0 3.5 70 30 1.0 12.0 20 80 1.0 12.5 10 90 1.0 15.5 10 90 1.0 16.5 90 10 1.0
Retention Times:	IN-D5119 ~6.6 minutes IN-00581 ~8.7 minutes
Total Run Time:	~25.0 minutes

A six-port electronically activated switching valve was used to direct the flow to the MS or waste prior to and following the elution of the analytes. The valve switching times are given in the following table.

Time (minutes)	Column Eluate Flow
0.0 – 3.6	Waste
3.6 – 11.0	MS source
11.0 -25.0	Waste

Since electrospray LC/MS system performs optimally at low flow rates, the eluate was split following the switching valve. Approximately 100 µL/minute of eluate (10:1 split) flowed into the ion source with remaining eluate flowing into a waste container.

The detection method utilized was LC/MS/MS employing electrospray (TIS) interface in the negative mode on a triple quadrupole instrument. The instrument was

tuned by infusing the analytes into a TIS (turbo ion spray) source, then creating a tune file to maximize the response of each analyte using the TIS source. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for each analyte are shown in the table below:

MS Conditions:

System	Applied BioSystems/MDS Sciex API 4000 LC/MS/MS system						
Analytes Monitored	Ions Monitored (AMU)	Declustering Potential (volts)	Collision Energy (volts)	Dwell Time (seconds)	EP (volts)	CXP (volts)	Acquisition Timing (minutes)
IN-D5119	200 → 156[a]	-40	-16	100	-10	-11	0 - 25
	200 → 92[b]	-40	-28	100	-10	-5	0 - 25
IN-00581	182 → 42[a]	-40	-58	100	-10	-1	0 - 25
	182 → 106[b]	-25	-26	100	-10	-7	0 - 25

[a]Transistion ion used for quantitation
[b]Transistion ion used for confirmation.

Additional detector settings are shown in the table below:

Parameter	**Setting**
Acquisition Mode:	MRM
Ionization Mode:	Negative (-)
Source Temp.:	600 °C
Nebulizer (GS1):	70
Auxillary Gas (GS2):	70
Curtain Gas:	40
CAD Gas:	8
Ion Spray Voltage:	-4500

The instrument was operated in the MS/MS (MRM) negative ion mode for quantitative analysis. Single transition chromatograms for each analyte were integrated and the peak areas used for quantitation. Quantitation was performed using a single transition for each analyte. Confirmation was determined using a secondary transition that was monitored throughout the validation.

3.8 Calculations

Calculations were performed as directed by the method. A validated software application was used to create a standard curve based on linear regression. Linear regression was monitored to support the response linearity of the mass spectrometer detector. The regression functions were used to calculate a best fit line (from a set of standard concentrations in ng/mL versus peak response) to demonstrate that a linear relationship exists between analyte concentration and peak response, and that a response factor approach to calculation was appropriate. All standards injected and their corresponding peak responses were entered into the program to construct the standard curve. Weighting (1/x) was used.

The equation used for the least squares fit is:

$$y = mx + b$$

where:

y	=	peak response
m	=	slope
x	=	ng/mL found for peak of interest
b	=	y-intercept

Equations

The calculations for ppb found and percent recovery (for fortified samples) were:

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

$$ppb = \frac{peak\ resp. \times Avg.\ Resp.\ Fact. \times mL\ FV \times HPLC\ dil.\ factor}{mL\ samp.\ vol.}$$

where:

peak resp.	=	peak area response of analyte in sample extract (corrected for control response, if applicable)
Avg. Resp. Fact.	=	average standard response factor of all the standards analyzed with the analytical set, where the standard response factor for each standard: $= \frac{standard\ concentration\ (ng/mL)}{Peak\ area\ response\ of\ standard}$
mL FV	=	mL volume of final extract submitted to HPLC (10.0 mL)
mL samp. vol.	=	milliliters of sample extracted (200 mL)
HPLC dil. factor	=	Magnitude of dilution required to bracket the response of the sample within the standard curve responses. No dilution = HPLC dilution factor of **1**

2. Percent recovery of fortified samples (procedural fortifications) was determined using the following equation:

$$\%\ Recovery = \frac{ppb\ found\ in\ fortified\ control\ sample}{ppb\ added} \times 100$$

Example Calculations

Tribenuron methyl (DPX-L5300) and metabolites IN-L5296, IN-A4098, IN-D5119 and IN-00581 were calculated in exactly the same manner for ground, surface, and drinking water. Only examples of tribenuron methyl (DPX-L5300) in ground water will be provided and thus serve to illustrate the calculations of all analytes in all three matrices.

1. LIMS ID# 139310, Tribenuron methyl (DPX-L5300), Ground Water, Set #13, Sample ID# 80140-1, **Control** 13:

$$sample\ peak\ response = 0$$

$$Avg.\ Resp.\ Fact. = 0.0000087840$$

$$ppb = \frac{0 \times 0.0000087840 \times 10.0 \times 1}{200\ mL}$$

$$ppb = 0$$

$$Reported\ ppb = ND$$

2. LIMS ID# 139310, Tribenuron methyl (DPX-L5300), Ground Water, Set #13, Sample ID# 80140-1, **Fortified Control** 3 @ 0.050 ppb:

$$sample\ peak\ response = 96299$$

$$Avg.\ Resp.\ Fact. = 0.0000087840$$

$$ppb = \frac{96299 \times 0.0000087840 \times 10.0 \times 1}{200\ mL}$$

$$ppb = 0.0422947 47$$

$$Reported\ ppb = 0.0423$$

5.0 CONCLUSIONS

Morse Laboratories, LLC successfully independently validated the residue analytical method described for tribenuron methyl (DPX-L5300) and its metabolites IN-L5296, IN-A4098, IN-D5119, IN-00581 in water as described in DuPont Study Number DuPont-36611.

The method was demonstrated to be applicable for the determination of tribenuron methyl (DPX-L5300) and its metabolites IN-L5296, IN-A4098, IN-D5119, and IN-00581 in water, representative of the matrices for which the method was intended. An LOQ of 0.050 µg/L (ppb) for tribenuron methyl (DPX-L5300), IN-L5296, and IN-A4098 was demonstrated for each matrix evaluated. An LOQ of 0.1 µg/L (ppb) for IN-D5119 and IN-00581 was demonstrated for each matrix evaluated.