

Study Title

**Analytical Method 00816/M002 for the Determination
of Residues of NNI-0001 and its des-iodo Metabolite A-1 in/on Plant Material
by HPLC-MS/MS Using Stable-Labelled Internal Standards**

Data Requirement

- EU Council Directive 91/414/EEC amended by Commission Directive 96/68/EC
- European Commission Guidance Document for Generating and Reporting Methods of Analysis in Support of Pre-Registration data Requirements for Annex II (part A, Section 4) and Annex III (part A, section 5) of directive 91/414, SANCO/3029/99
- US EPA Residue Chemistry Test Guideline OPPTS 860.1340: Residue Analytical Method

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Study Completion Date

2004-07-23

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Performing Laboratory

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Laboratory Project ID

P602030522

Data Confidentiality Statement

This page is intentionally left blank for the purpose of submitting administrative information that is required by regulations promulgated by various countries.

Certification of Good Laboratory Practice

Statement of Compliance

This study was conducted in compliance with the current OECD Principles of Good Laboratory Practice.

This study also meets the requirements of:

1. US EPA – FIFRA Good Laboratory Practice (40 CFR Part 160)
2. Principles of Good Laboratory Practice – German Chemical Law (Chemikaliengesetz), dated 2002-06-20, current version of Annex 1
3. JAPAN MAFF – Notification on the Good Laboratory Practice Standards for Agricultural Chemicals (JMAFF, 13 Nousan No. 6283, dated October 17, 2001)

with the exception that recognized differences exist between the GLP principles/standards of OECD and those of FIFRA and JMAFF (for instance, authority granted Agency inspectors).

The test facility has been inspected and certified as working in compliance with the Principles of Good Laboratory Practice by the competent authorities ("AktENZEICHEN VI-3-31.11.91.01, dated January 29, 2004", see Appendix 13).


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Certification of Authenticity

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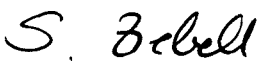
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BCS-D-AS-GLP/QAU	
Quality Assurance Statement	
Report No.: MR-121/03	Study No.: P 602 030522
Title of report: Analytical Method 00816/M002 for the Determination of Residues of NNI-0001 and its des-Iodo Metabolite A-1 in/on Plant Material by HPLC-MS/MS using Stable-Labelled Internal Standards	
The conduct of this study has been periodically inspected and this report has been audited by the Quality Assurance Unit. The dates of inspection and reporting to Principal Investigator(s) (if applicable), Study Director and Management are given below.	
Date of Study Plan Inspection:	Date of Report:
2003-08-04	no report
Date of Study Inspection:	Date of Report:
2003-08-08	2003-08-08
Date of Final Report Audit:	Date of Report:
2004-03-19	2004-03-19
The results reported in this study have been checked on the basis of our current SOPs and accurately reflect the raw data.	
S. Ziebell Quality Assurance Unit, BCS-D-AS-GLP/QAU	 Date: 2004-08-16 (yyyy-mm-dd)

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Acknowledgement

Thanks are expressed to Mr. G. Schuld and H. Wirkner, who carried out the practical work in the laboratory.

Schedule

Approval of the Study Protocol by the Study Director:	2003-08-04
Start of Experimental Phase:	2003-08-04
End of Experimental Phase:	2003-11-10

Archiving

All raw data pertaining to this study and a master copy of the final report are stored in the central GLP archive of Bayer CropScience AG, Alfred-Nobel-Str. 50, D-40789 Monheim for as long as required by GLP principles.

Reserve samples of test and reference items are stored in the archives of Bayer CropScience AG, Alfred-Nobel-Str. 50, D-40789 Monheim by the function which had certified the used substances. The test and reference items are stored as long as their quality still guarantees an evaluation.

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1 Summary

For the determination of residues of NNI-0001 and its metabolite NNI-0001-des-iodo (A-1) in/on plant material by HPLC-MS/MS, the corresponding internal stable-labelled standards were used for quantitation.

The active substance (a.s.) NNI-0001 and its des-iodo metabolite A-1 are extracted from the sample material using microwave with acidic acetonitrile and acidic acetonitrile/water mixture (2/1, v/v) in the second step. After evaporation the residues are cleaned-up using disposable columns filled with diatomaceous earth. The residues are eluted with cyclohexane/ethyl acetate (1/1, v/v).

For the determination of the analytes in/on plant material, the organic solution is evaporated to dryness. The residues are redissolved in acetonitrile/water mixture (1/1, v/v + 0.01% formic acid) and subjected to HPLC-MS/MS.

Vegetable (plant) oil samples are dissolved in n-hexane and extracted with acetonitrile (twice). The combined acetonitrile phases are reextracted with n-hexane. The acetonitrile phase is evaporated, the residues are redissolved in acetonitrile/water mixture (1/1, v/v + 0.01% formic acid) and subjected to HPLC-MS/MS.

Chromatography is performed by reversed-phase HPLC with MS/MS detection. For quantitation, the corresponding internal stable-labelled standards (d6-NNI-0001 and d6-NNI-0001-des-iodo) were used.

The limit of quantitation (LOQ) for both analytes is 0.01 mg/kg for all sample materials corresponding to the lowest fortification level of successfully conducted recovery experiments. The limit of detection (LOD) was estimated to be at least 10 times lower than the LOQ, as could be concluded from the linearity response data and matrix interference observed in control sample chromatograms.

The single recovery rates for NNI-0001 ranged from 72 to 101% at fortification levels of 0.01 and 0.1 mg/kg (overall means: 78 - 97%, overall RSDs = 2.8 – 8.8%).

The single recovery rates for NNI-0001-des-iodo (A-1) ranged from 72 to 103% at fortification levels of 0.01 and 0.1 mg/kg (overall means: 81 - 100%, overall RSDs = 1.7 – 5.8%).

As a measure for the precision of the method, the intra-laboratory repeatability (n = 5) is given for tomato (fruit), citrus (fruit), wheat (grain) and cotton (oil) at 0.01 and 0.1 mg/kg. The RSD of the repeatability tests at each recovery set ranged from 0.6 to 7.5%.

1 Summary (contd)

Blank values in control samples were well below 30% of the LOQ. The high selectivity (specificity) of the method results both from the clean-up steps and the HPLC separation in combination with the very selective MS/MS detection in the MRM mode.

An excellent linear correlation between the injected amount of both analytes and the detector response of HPLC-MS/MS was observed for standards in solvent for evaluation against the internal standards ranging from 0.5 to 200 µg/L. The correlation coefficient of the 1/x weighted linear regression was at 0.9999. The calculated peak areas from the linear regression function matched the experimentally obtained peak areas to a very high extent.

The occurrence of matrix effects was not monitored during method development and validation, as an internal standard procedure using stable-labelled standards was used which compensate matrix effects.

The calibration data obtained justify using the single point calibration method for calculation of the residues of NNI-0001 and NNI-0001-des-iodo. However, an appropriate bracketing standard concentration, corresponding to the order of magnitude of the residues should be used for quantification and it should be noted that the concentration of the stable-labelled internal standard in the analytical solution must be kept at a constant level.

The analytes and its deuterated derivatives are stable both in stock and standard solutions for at least three months of storage at $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$ in the darkness.

The stability of NNI-0001 and its des-iodo metabolite A-1 was determined at the LOQ level (0.01 mg/kg) for extracts of tomato (fruit), wheat (grain) and vegetable oil (cotton oil). Control samples were fortified with the analytes and after initial analysis, the analytical solutions were stored in a refrigerator and were reanalysed after periods of one and four weeks. Storage was conducted under the same conditions as used for analytical solutions (in a refrigerator at $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$). The results of this investigation show that NNI-0001 and its des-iodo metabolite A-1 are stable in representative matrix solutions for a period of at least four weeks.

The validation data obtained demonstrate the high sensitivity, selectivity and accuracy (= precision and trueness) of the method for determination of NNI-0001 and A-1 in/on material of plant origin. Since HPLC-MS/MS already offers maximum selectivity, i.e. specificity, a confirmatory procedure was considered to be unnecessary.

2 Introduction

2.1 Reason for the Modification

For the determination of residues of NNI-0001 and its metabolite NNI-0001-des-iodo (A-1) in/on plant material by HPLC-MS/MS, the corresponding internal stable-labelled standards were used for quantitation.

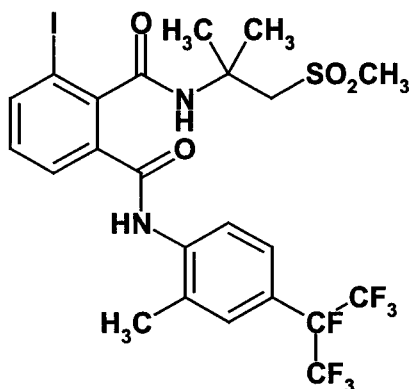
Further details were described in the analytical methods 00816 [6] and its modification 00816/M001 [7].

2.2 Properties of NNI-0001 and its des-iodo Metabolite A-1

NNI-0001

Empirical formula:
Molecular weight:

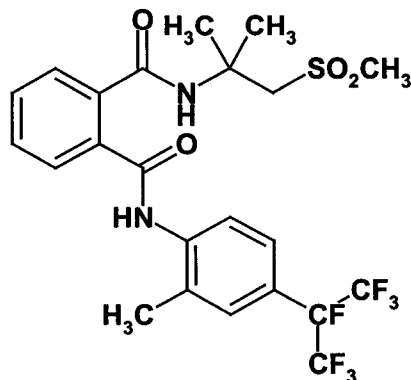
$C_{23}H_{22}N_2O_4F_7IS$
682.4 g/mol



NNI-0001-des-iodo (A-1)

Empirical formula:
Molecular weight:

$C_{23}H_{23}N_2O_4F_7S$
556.5 g/mol



2.3 Properties of d6-NNI-0001 and d6-NNI-0001-des-iodo

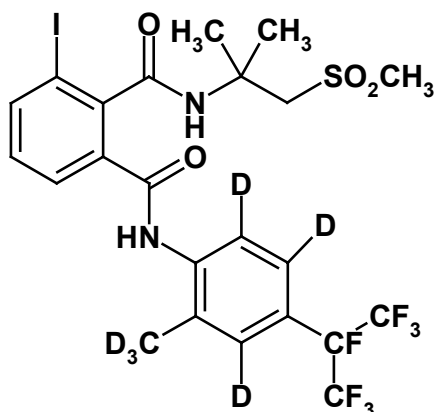
d6-NNI-0001

Empirical formula:

$C_{23}H_{16}N_2O_4F_7ISD_6$

Molecular weight:

688.4 g/mol



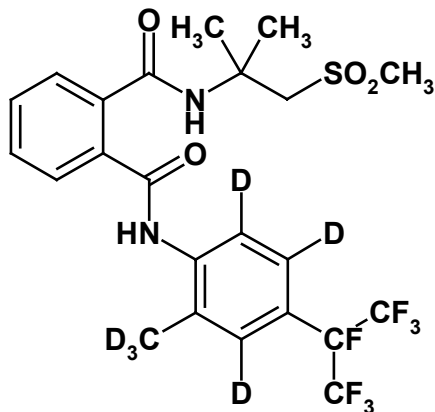
d6-NNI-0001-des-iodo

Empirical formula:

$C_{23}H_{17}N_2O_4F_7SD_6$

Molecular weight:

562.5 g/mol



3 Sampling and Preparation of Laboratory Samples

Analytical samples must be collected in a way that ensures the obtention of a representative sample. Guidance on adequate sampling may be obtained e.g. from the instructions of chapter VIII of the DFG method collection for the analysis of pesticide residues [1].

If the samples could not be analysed immediately, they were stored in a deep-freezer at -18°C or below. For the preparation of the laboratory samples, the deep-frozen samples were shredded with dry ice in a cutter. Parts of the frozen laboratory samples were transferred into polystyrene boxes and stored at -18°C or below until analysis. The homogeneity of a 5-g sample is sufficient if it has been prepared in a cutter with dry ice. In other cases it might be necessary to take larger sample amounts and to take adequate aliquots. Extraction volumes and glass hardware should then be adapted.

4 Experimental Section

When using this analytical method the German guidelines for laboratories of the Employees' Liability Insurance Association, e.g. Bulletin M006 [2] or comparable guidelines in other countries are to be observed.

The following chemicals were used, which are classified by the hazardous material regulations. The classification is based on the German guidelines [3] and has to be adapted to the respective national guidelines in case the method is used outside Germany.

- | | |
|-----------------|--|
| • Acetic acid | Corrosive |
| • Formic acid | Corrosive |
| • HCl conc. | Corrosive |
| • Acetonitrile | Toxic, highly flammable |
| • n-Hexane | highly flammable, harmful, dangerous for the environment |
| • Ethyl acetate | highly flammable, irritant |
| • Cyclohexane | highly flammable, harmful, dangerous for the environment |

The pertinent safety instructions must be observed when working with all compounds mentioned in this method (e.g. R- and S-phrases). It has to be made sure that the working place is properly ventilated when working with dry ice. Sample vessel and deep-freezing cabinet must guarantee pressure equalization.

4.1 Materials

4.1.1 Apparatus

- Liquid chromatograph (e.g. HP 1100 column compartment G1316A, HP 1100 binary pump G1312A, HP 1100 iso pump G1313A, HP 1100 degasser G1379A, Hewlett Packard, Ratingen, Germany); Autosampler (e.g. PAL CTC)
- Mass spectrometer (e.g. API 365 with turboionspray interface and mass spectrometric detector or higher systems, PE Applied Biosystems, Weiterstadt, Germany)
- Microwave oven (e.g. MLS-Ethos, MLS GmbH, Leutkirch, Germany)
- RP HPLC column (e.g. Luna 5 μ C18(2), 150 x 4.60 mm, 5 μ micron), Phenomenex, Aschaffenburg, Germany)
- Vacuum manifold (e.g. VacElut SPS 24, Varian, Darmstadt, Germany)
- Adapter (e.g. Baker, Groß-Gerau, Germany)
- Reservoir 60 mL (e.g. BondElut reservoir, Varian, Darmstadt, Germany)
- Rotary vacuum evaporator (e.g. Rotavapor R114, Büchi, CH-Flawil)
- Round-bottom flask (250-mL), Volumetric flask (e.g. 20-mL, 50-mL, 100-mL)
- Separating funnel (e.g. 50-ml, 100-ml, 250-ml)
- Variable dispenser (e.g. 10-mL, 50-mL)
- Graduated cylinder (e.g. 10-mL, 20-mL, 50-mL) and Glass beaker
- Calibrated pipettes (e.g. Brand Co., Wertheim, Germany)
- Small instruments (e.g. Pasteur pipettes, HPLC vials)

4.1.2 Reagents/Supplies

- Cyclohexane pesticide grade (e.g. Promochem, Wesel, Germany)
- Ethyl acetate pesticide grade (e.g. Promochem, Wesel, Germany)
- n-Hexane pesticide grade (e.g. Merck KGaA, Darmstadt, Germany)
- Acetonitrile HPLC grade (e.g. Merck KGaA., Darmstadt, Germany)
- Water, HPLC grade (e.g. purified with a milli-Q-water system, Millipore Co., Eschborn, Germany)
- Acetic acid, 100% (e.g. Suprapur, Merck KGaA, Darmstadt, Germany)
- Formic acid "extra pure" (e.g. Riedel-de-Haen, Seelze, Germany)
- HCl, Suprapur (e.g. Merck KGaA, Darmstadt, Germany)
- Filter aid, e.g. Celite 545 (e.g. Merck KGaA, Darmstadt, Germany)
- Disposable column filled with diatomaceous earth (e.g. ChemElute CE 1020, Varian, Darmstadt, Germany)
- Nitrogen 5.0, 99.9990% purity as bath, nebulizer, collision, curtain, and turbo gas (e.g. Linde AG, Höllriegelskreuth, Germany)

4.1.3 Reference Items

Generally, only sufficiently characterized and certified substances are used as reference items.

Table 1: Reference Items.

Reference Item	Certificate of Analysis	Date of Certification	Purity (%)	Expiry Date
NNI-0001	M 22919	2001-11-07	98.5	Oct 2003
NNI-0001	AZ 11114	2003-10-29	98.5	2006-10-21
NNI-0001-des-iodo	M 25064	2002-02-19	99	Feb 2004
NNI-0001-des-iodo	AD 03075	2003-06-20	99.3	2004-10-22
d6-NNI-0001	M 26477	2002-05-23	99.9*	2004-05-31
d6-NNI-0001-des-iodo	AD 03099	2003-07-15	97.9	---

*: 92.7% d6.

4.1.4 Stock and Standard Solutions of NNI-0001

The stock solution (primary standard) was prepared by weighing a defined amount of reference item into a 25-mL volumetric flask and filling up to the mark with acetonitrile. The final concentration was approx. 500 mg/L.

Table 2: Preparation Scheme of Stock Solution. Concentration is corrected for Purity.

Reference Item No.	Weight [mg]	Volume [mL]	Solvent	Final Concentration	
				required [mg/L]	actual [mg/L]
1 NNI-0001	12.62	25	acetonitrile	500	497
1 NNI-0001*	12.85	25	acetonitrile	500	506

*: ID No. AZ 11114

Standard solutions (secondary standards) were prepared from the stock solution by dilution with acetonitrile/water (1/1, v/v + 0.01% formic acid).

Table 3: Preparation Scheme for Reference Standards (Example).

No.	Target Concentration (mg/L)	Prepared by Removal of (mL)	No. of Solution	Dilution to (mL)	Solvent
10	2	0.4	1	100	acetonitrile/water*

*: (1/1, v/v + 0.01% formic acid)

4.1.5 Stock and Standard Solutions of NNI-0001-des-iodo

The stock solution (primary standard) was prepared by diluting a defined amount of reference item with about 10 mL of acetonitrile. The final concentration was approx. 500 mg/L.

Table 4: Preparation Scheme of Stock Solution. Concentration is corrected for Purity.

Reference Item No.		Weight [mg]	Volume [mL]	Solvent	Final Concentration	
					required [mg/L]	actual [mg/L]
2	NNI-0001-des-iodo	5.19	10.2	acetonitrile	500	504
2	NNI-0001-des-iodo	5.62	11	acetonitrile	500	507

** : ID No. AD 03075

Standard solutions (secondary standards) were prepared from the stock solution by dilution with acetonitrile/water (1/1, v/v + 0.01% formic acid).

Table 5: Preparation Scheme for Reference Standard Solution.

No.	Target Concentration (mg/L)	Prepared by Removal of (mL)	No. of Solution	Dilution to (mL)	Solvent
11	2	0.4	2	100	acetonitrile/water*

* : (1/1, v/v + 0.01% formic acid)

4.1.6 Stock Solutions of d6-NNI-0001 and d6-NNI-0001-des-iodo

The stock solutions (primary standards) were prepared by diluting a defined amount of reference item with about 10 mL of acetonitrile. The final concentration was approx. 500 mg/L.

Table 6: Preparation Scheme of Stock Solutions. Concentrations are corrected for Purity.

Reference Item No.		Weight [mg]	Volume [mL]	Solvent	Final Concentration*	
					required [mg/L]	actual [mg/L]
3	d6-NNI-0001	5.14	10.25	acetonitrile	~ 500	~ 500
4	d6-NNI-0001-des-iodo	5.16	10	acetonitrile	~ 500	~ 500

* : Due to the use of d6-NNI-0001 and d6-NNI-0001-des-iodo as internal standards only, the exact concentrations is not of importance.

4.1.7 Standard Mixture Solutions of NNI-0001 and NNI-0001-des-iodo

Standard mixture solutions (secondary standards) were prepared from the stock solutions by dilution with acetonitrile/water mixture (1/1, v/v + 0.01% formic acid). Standard solutions in the concentration range between 0.002 and 20 mg/L were used for data generation and preparation of recovery samples.

Table 7: Preparation Scheme for Reference Standards (Examples).

No.	Target Concentration (mg/L)	Prepared by Removal of (mL)	No. of Solution	Dilution to (mL)	Solvent
5	20	4 4	1 2	100	acetonitrile/water*
6	2	10	5	100	acetonitrile/water*

*: (1/1, v/v + 0.01% formic acid)

4.1.8 Standard Mixture Solutions of d6-NNI-0001 and d6-NNI-0001-des-iodo

Standard mixture solutions (secondary standards) were prepared from the stock solutions by dilution with acetonitrile/water mixture (1/1, v/v + 0.01% formic acid). Standard solutions in the concentration range between 0.05 and 5 mg/L were used for data generation.

Table 8: Preparation Scheme for Reference Standards (Examples).

No.	Target Concentration (mg/L)	Prepared by Removal of (mL)	No. of Solution	Dilution to (mL)	Solvent
12	5	1 1	3 4	100	acetonitrile/water*
13	0.5	10	12	100	acetonitrile/water*

*: (1/1, v/v + 0.01% formic acid)

4.1.9 Standard Mixture Solutions of NNI-0001, NNI-0001-des-iodo, d6-NNI-0001 and d6-NNI-0001-des-iodo

Standard mixture solutions (secondary standards) were prepared from the stock solutions by dilution with acetonitrile/water mixture (1/1, v/v + 0.01% formic acid). Standard solutions in the concentration range between 0.0005 and 0.5 mg/L (NNI-0001 and NNI-0001-des-iodo) were prepared and used for data generation.

Table 9: Preparation Scheme for Reference Standards (Examples).

No.	Target Concentrations (mg/L)	Prepared by Removal of (mL)	No. of Solution	Dilution to (mL)	Solvent
15	NNI-0001 (a): 0.5 NNI-0001-des-iodo (b): 0.5 d6-NNI-0001 (c): 0.05 d6-NNI-0001-des-iodo (d): 0.05	2.5 2.5 1 1	5 5 12 12	100	acetonitrile/water*
16	a: 0.2 b: 0.2 c: 0.05 d: 0.05	10 10 1 1	6 6 12 12	100	acetonitrile/water*
17	a: 0.1 b: 0.1 c: 0.05 d: 0.05	5 5 1 1	6 6 12 12	100	acetonitrile/water*
18	a: 0.05 b: 0.05 c: 0.05 d: 0.05	2.5 2.5 1 1	6 6 12 12	100	acetonitrile/water*
19	a: 0.02 b: 0.02 c: 0.05 d: 0.05	10 10 1 1	7 7 12 12	100	acetonitrile/water*

*: (1/1, v/v + 0.01% formic acid)

4.2 Analytical Method

Extraction Procedure

1. Weigh a representative aliquot of the sample material (5 g) into a 60-mL reservoir fitted with a frit and a plastic valve.
2. Add 20 mL of acetonitrile + 0.01% HCl and 1 g of filter aid to the sample and mix. For sample materials with higher contents of starch (e.g. wheat grain, corn kernel) add 0.25 mL of conc. HCl.
3. Place the reservoir into a microwave oven and heat 2 min at 200 W, corresponding to a temperature of approx. 40°C.
4. Place the reservoir on top of a 250-mL round-bottom flask and let the solvent percolate into the flask.
5. Add 20 mL of acetonitrile/water mixture (2/1, v/v + 0.01% HCl) to the remaining solids in the reservoir and mix with the pipette.
6. Repeat microwave extraction.
7. Place the reservoir again on top of a 250-mL round-bottom flask and let the solvent percolate into the flask, too.
8. Wash the remaining solids in the reservoir with 10 mL of acetonitrile/water mixture (2/1, v/v + 0.01% HCl). Let wash solution also percolate into the volumetric flask.
9. Evaporate the solution to an aqueous remainder (e.g. rotary evaporator).
10. Place remainder on top of a ChemElute 1020 cartridge and wait for about 15 min.
11. Elute residues with 80 mL of cyclohexane/ethyl acetate mixture (1/1, v/v).
12. Evaporate to dryness (e.g. rotary evaporator).
13. After addition of 0.1 mL of internal standard (d6-NNI-0001 and d6-NNI-0001-des-iodo, 5 mg/mL each) dissolve residues in acetonitrile/water mixture (1/1, v/v + formic acid 0.01%) and fill up to 10 mL (volumetric flask).
14. Subject to HPLC-MS/MS analysis.

Extraction Procedure for Vegetable Oil

1. Weigh 5 g oil of plant origin in a glass beaker (e.g. 100-mL) and dissolve with 10 mL of n-hexane.
2. Transfer into a separation funnel (e.g. 100-mL) and wash the beaker with 10 mL of n-hexane.
3. Extract n-hexane twice with 20 mL of acetonitrile.
4. Collect the acetonitrile phases in a further separation funnel and extract with 10 mL of n-hexane.
5. Transfer the acetonitrile phase into a round-bottom-flask (e.g. 100-mL) and evaporate to dryness (e.g. rotary evaporator).
6. After addition of 0.1 mL of internal standard (d6-NNI-0001 and d6-NNI-0001-des-iodo, 5 mg/mL each) dissolve residues in acetonitrile/water mixture (1/1, v/v + formic acid 0.01%) and fill up to 10 mL (volumetric flask).
7. Subject to HPLC-MS/MS analysis.

4.2 Analytical Method (contd)

Table 10: Extraction Scheme for NNI-0001 and NNI-0001-des-iodo (A-1).

Sample Material (5 g)	1 st Extraction	2 nd Extraction	Remarks
Citrus (fruit), Head cabbage (head), Bean (bean with pod), Tomato (fruit)	20 mL acetonitrile + 0.01% HCl. Microwave extraction.	20 mL acetonitrile/water (2/1, v/v + 0.01% HCl) Microwave extraction.	Clean-up with ChemElute.
Wheat (grain)	20 mL acetonitrile + 0.01% HCl + 0.25 mL HCl (conc.). Microwave extraction.	20 mL acetonitrile/water (2/1, v/v + 0.01% HCl) Microwave extraction.	Clean-up with ChemElute.
Vegetable Oil	LLE* of the n-hexane solution of the sample with acetonitrile (twice).	---	Clean-up of the acetonitrile phase by LLE* with n-hexane.

* LLE = Liquid/Liquid extraction.

4.3 Analysis by HPLC-MS/MS

4.3.1 Principle of Measurement

An aliquot of the prepared sample solution is injected into the high performance liquid chromatograph, chromatographed under reversed phase conditions and detected by tandem mass spectrometry with electrospray ionization. The protonated molecular ions of the analytes are accelerated by the adequate voltage regulation in the negative ion mode and filtered by the first quadrupole due to its mass-to-charge (m/z) ratio. These precursor ions (parent ions) are impulsed with nitrogen in the collision cell (second quadrupole) and each one fragment of these ions (product ions, daughter ions) is separated according to their m/z ratios in the third quadrupole and detected (multiple reaction monitoring, MRM).

4.3.2 HPLC Conditions

Instrument: e.g. Agilent 1100
Column: e.g. HPLC column, Luna 5 μ C18(2), 150 x 4.60 mm
5 μ micron
Solvent A Binary Pump: Water/acetonitrile (9/1, v/v) + 0.01% acetic acid
Solvent B Binary Pump: Acetonitrile + 0.01% acetic acid
Solvent Isocratic Pump: Water/acetonitrile (1/1, v/v) + 0.01% acetic acid
Oven Temperature: 40°C
Inject Volume: e.g. 20 μ L
Flow (Binary Pump): 1 mL/min
Flow (Isocratic Pump): 1 mL/min
Split: 150 μ L into MS from 1000 μ L

Time Table [min]	Module	Setting	Value
0	Binary Pump	% B	50
0.10	Binary Pump	External Contact 2	Closed
0.20	Binary Pump	External Contact 2	Open
1.00	Binary Pump	% B	50
5.50	Binary Pump	External Contact 1	Closed
5.60	Binary Pump	External Contact 1	Open
6.00	Binary Pump	% B	90
7.50	Binary Pump	External Contact 2	Closed
7.60	Binary Pump	External Contact 2	Open
10.20	Binary Pump	% B	90
10.30	Binary Pump	% B	50

External Contacts:

1 (Closed / Open) Solvent of Binary Pump into MS

2 (Closed / Open) Solvent of Binary Pump into waste, Solvent of Isocratic Pump into MS

4.3.3 MS/MS Conditions

The experiments were performed on a triple-quadrupole mass spectrometer system, fitted with an electrospray interface operated in the negative ion mode under MRM conditions.

Mass axis calibration was done by infusing a polypropylene glycol solution. Unit mass resolution was established and maintained in the mass resolving quadrupole by maintaining a full width at half-maximum of between 0.8 and 1.0 amu. After tuning and calibration, optimal collision-activated dissociation (CAD) conditions for fragmentation of the analytes were determined. These experiments were performed with nitrogen as collision gas.

4.3.3 MS/MS Conditions (contd)

Detector: e.g. Triple Quadrupole LC-MS/MS Mass Spectrometer
PE Applied Biosystems
API 365, Windows 2000, Analyst 1.3.1

Interface: Electrospray, Turbo Ion Spray™
Potential: - 4200 V
Temperature: 300°C (Source)

Gas: Nebulization gas: 1.48 L/min (liquid nitrogen 5.0)
Curtain gas: 1.44 L/min (liquid nitrogen 5.0)
Collision gas 0.87 L/min (liquid nitrogen 5.0)
Turbo gas: 7 L/min (liquid nitrogen 5.0)

Scan Type: MRM (Multiple Reaction Monitoring Mode)

Polarity: Negative

Table 11: Mass spectrometer operating parameters.

	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)
NNI-0001	681	254	250	-23
NNI-0001-des-iodo	555	254	250	-26
d6-NNI-0001	687	259	250	-32
d6-NNI-0001-des-iodo	561	259	250	-30

The detailed instrument settings for all analytes are presented in [Appendix 12](#).

4.3.4 Analytical Procedure

1. Transfer a prepared aliquot of the sample to a HPLC vial.
2. Inject e.g. 20 µL of standard solution using the prescribed HPLC, MS/MS and data acquisition conditions.
3. Inject e.g. 20 µL of the sample prepared in step 1. (The number of injections between the bracketing standard injections should not be higher than 10.)
4. Inject e.g. 20 µL of standard solution.
5. Compare the peak area ratios for the bracketing standards before and after each sample. If there is a variation of > 20% between the bracketing standards the analysis of that sample should be repeated. If there is a variation between 10 and 20% proceed calculation.

4.4 Calculation

4.4.1 Calculation of the Residues

Evaluation in this case is performed according to the external bracketing procedure against standards in solvent using a stable-labelled internal standard for the compensation of matrix effects.

1. Calculate the average peak area ratios for NNI-0001/d6-NNI-0001 and NNI-0001-des-iodo/d6-NNI-0001-des-iodo of the bracketing standards for each sample. This value will be used as F_{St} .
2. Determine the average peak area ratios for NNI-0001/d6-NNI-0001 and NNI-0001-des-iodo/d6-NNI-0001-des-iodo for the analysed sample. This value will be used as F_A .
3. Calculate the amount of residue R expressed in mg/kg as follows:

$$R = \frac{F_A * V_{End} * ST_A * V_E * D_F * C_F}{F_{St} * G * V_A}$$

- where:
- R : Determined amount of residue in **mg/kg**
 - F_A : Average peak area ratio of for NNI-0001/d6-NNI-0001 and NNI-0001-des-iodo/d6-NNI-0001-des-iodo for the analytical solution
 - V_{End} : Final volume of the sample solution in **mL**
(may be adjusted according to the expected residue level)
 - ST_A : Standard concentration in the external standard in **µg/mL**
 - V_E : Extract volume in **mL**
 - D_F : Dilution factor
 - F_{St} : Average peak area ratio of for NNI-0001/d6-NNI-0001 and NNI-0001-des-iodo/d6-NNI-0001-des-iodo for the bracketing standards
 - G : Sample weight of analytical sample in **g**
 - V_A : Aliquot (of extract) in **mL**
 - C_F : Conversion factor, here 1.000

4.4.2 Calculation of Recovery Rates

1. Calculate the average of the residues in the recovery sample according to 4.4.1.
2. Calculate the percent recovery rate as follows:

$$Rec = \frac{F_A * V_{End} * ST_A * V_E * D_F * C_F * 100}{F_{St} * V_A * A}$$

- where:
- Rec : Recovered amount found in fortified sample in %
 - F_A : Average peak area ratio of for NNI-0001/d6-NNI-0001 and NNI-0001-des-iodo/d6-NNI-0001-des-iodo for the analytical solution
 - V_{End} : Final volume of the sample solution in **mL**
 - ST_A : Standard concentration in the external standard in **µg/mL**
 - V_E : Extract volume in **mL**
 - D_F : Dilution factor
 - F_{St} : Average peak area of for NNI-0001/d6-NNI-0001 and NNI-0001-des-iodo/d6-NNI-0001-des-iodo for the bracketing standards
 - V_A : Aliquot (of extract) in **mL**
 - A : Fortified amount in **µg**
 - C_F : Conversion factor, here 1.000

5.3 Linearity of the detector (contd)

An excellent linear correlation between the injected amount of both analytes and the detector response of HPLC-MS/MS was observed for standards in solvent for evaluation against the internal standards ranging from 0.5 to 200 µg/L. The correlation coefficient of the 1/x weighted linear regression was at 0.9999. The calculated peak areas from the linear regression function matched the experimentally obtained peak areas to a very high extent.

The occurrence of matrix effects was not monitored during method development and validation, as an internal standard procedure using stable-labelled standards was used which compensate matrix effects.

The calibration data obtained justify using the single point calibration method for calculation of the residues of NNI-0001 and NNI-0001-des-iodo. However, an appropriate bracketing standard concentration, corresponding to the order of magnitude of the residues should be used for quantification and it should be noted that the concentration of the stable-labelled internal standard in the analytical solution must be kept at a constant level.

For details, cf. to [Appendix 3](#).

5.4 Limit of Quantitation (LOQ), Limit of Detection (LOD), Recovery Experiments and Repeatability

Determination of recoveries with fortification levels of 0.01 mg/kg (= LOQ) and 0.1 mg/kg has been performed for citrus (fruit), bean (bean with pod), head cabbage (head), vegetable oil (cotton oil), tomato (fruit), and wheat (grain).

The lowest fortification level experimentally providing a mean recovery of $\geq 70\%$ with a relative standard deviation of $\leq 20\%$ per definition corresponds to the LOQ, provided that blank values are below 30% of this level. The detection limit (LOD) was estimated to be at least 10 times lower than the LOQ, as could be concluded from the linearity response data of the lowest-concentration standards and matrix interference observed in control sample chromatograms.

Recovery experiments were conducted by fortification of untreated control samples with defined amounts of NNI-0001 and NNI-0001-des-iodo (A-1) prior to analysis, as shown in the following tables.

Table 14: Recoveries and Repeatability of NNI-0001, RSD: Relative Standard Deviation.

Sample Material	Fortifikation Level [mg/kg]	Recovery Rates [%]		
		Single Values	Mean	RSD [%]
Citrus (fruit)	0.01	85, 80, 90, 86, 84	85	4.2
	0.1	87, 87, 81, 85, 87	85	3.1
		Overall	85	3.5
Tomato (fruit)	0.01	78, 91, 88, 96, 89	88	7.5
	0.1	85, 90, 88, 89, 89	88	2.2
		Overall	88	5.2
Cotton (oil)	0.01	98, 97, 96, 95, 94	96	1.6
	0.1	101, 100, 98, 92, 97	98	3.6
		Overall	97	2.8
Wheat (grain)	0.01	80, 83, 82, 74, 85	81	5.2
	0.1	86, 81, 82, 81, 79	82	3.2
		Overall	81	4.1
Bean (bean with pod)	0.01	89, 72, 74	78	11.9
	0.1	77, 73, 84	78	7.1
		Overall	78	8.8
Head cabbage (head)	0.01	84, 83, 79	82	3.2
	0.1	87, 87, 85	86	1.3
		Overall	84	3.6

5.4 Limit of Quantitation (LOQ), Limit of Detection (LOD), Recovery Experiments and Repeatability (contd)

Table 15: Recoveries and Repeatability of NNI-0001-des-iodo (A-1), RSD: Relative Standard Deviation.

Sample Material	Fortifikation Level [mg/kg]	Recovery Rates [%]		
		Single Values	Mean	RSD [%]
Citrus (fruit)	0.01	88, 92, 91, 87, 89	89	2.3
	0.1	87, 89, 86, 89, 87	88	1.7
		Overall	89	2.6
Tomato (fruit)	0.01	79, 92, 92, 92, 91	89	6.4
	0.1	84, 89, 92, 88, 90	89	3.3
		Overall	89	4.8
Cotton (oil)	0.01	101, 103, 101, 102, 97	101	2.3
	0.1	99, 100, 99, 99, 100	99	0.6
		Overall	100	1.7
Wheat (grain)	0.01	84, 81, 85, 77, 86	83	4.4
	0.1	83, 76, 79, 85, 72	79	6.6
		Overall	81	5.8
Bean (bean with pod)	0.01	86, 84, 78	83	5.0
	0.1	89, 84, 90	88	3.7
		Overall	85	5.1
Head cabbage (head)	0.01	85, 87, 89	87	2.3
	0.1	93, 89, 91	91	2.2
		Overall	89	3.2

The single recovery rates for NNI-0001 ranged from 72 to 101% at fortification levels of 0.01 and 0.1 mg/kg (overall means: 78 - 97%, overall RSDs = 2.8 – 8.8%).

The single recovery rates for NNI-0001-des-iodo (A-1) ranged from 72 to 103% at fortification levels of 0.01 and 0.1 mg/kg (overall means: 81 - 100%, overall RSDs = 1.7 – 5.8%).

As a measure for the precision of the method, the intra-laboratory repeatability (n = 5) is given for tomato (fruit), citrus (fruit), wheat (grain) and cotton (oil) at 0.01 and 0.1 mg/kg. The RSD of the repeatability tests at each recovery set ranged from 0.6 to 7.5%.

All method validation data are in compliance with the guideline requirements for residue data generation and enforcement methods [4], [5].

5.5 Stability in Solutions

5.5.1 Stability in Standard and Stock Solutions

The stability in stock and standard solutions was tested over a period of about three months of storage. For this purpose aged standard and stock solutions were quantified against freshly prepared standard solutions. The aged solutions were stored in volumetric flasks in a refrigerator at $+4^{\circ}\text{C} \pm 3^{\circ}\text{C}$. Fresh solutions for quantitation were prepared at the date of analysis. The results indicate that both analytes are stable both in stock and standard solutions for at least three months of storage at $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$ in the darkness.

Table 16: Stability of NNI-0001 and A-1 in Stock Solutions. For determination, the original solutions of 500 µg/mL were diluted 1:10000 before analysis.

Sample Name	NNI-0001		NNI-0001-des-iodo (A-1)	
	Peak Area	Mean Area	Peak Area	Mean Area
Three Months				
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Aged Standard)	96057	95987 (= 100%)*	141952	145260 (= 90%)*
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Aged Standard)	95452		146483	
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Aged Standard)	96452		147346	
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Fresh Standard)	96761		161252	
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Fresh Standard)	95700		161045	
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Fresh Standard)	94335		95599	

Table 17: Stability of NNI-0001 and A-1 in Organic/Aqueous Solvents (Secondary Standard).

Sample Name	NNI-0001		NNI-0001-des-iodo (A-1)	
	Peak Area	Mean Area	Peak Area	Mean Area
Three Months				
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Aged Standard)	101355	99795 (= 103%)*	146421	145244 (= 89%)*
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Aged Standard)	101452		145619	
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Aged Standard)	96579		143691	
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Fresh Standard)	97769		166382	
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Fresh Standard)	96240		163637	
s_0.05 µg/mL each NNI-0001 and NNI-0001-des-iodo (Fresh Standard)	97071		97027	

*: AGED standard quantified using the freshly prepared standard solution as reference (= 100%)

5.5.1 Stability in Standard and Stock Solutions (contd)

Table 18: Stability of d6-NNI-0001 and d6-NNI-0001-des-iodo in Stock Solutions.

Sample Name	d6-NNI-0001		d6-NNI-0001-des-iodo	
	Peak Area	Mean Area	Peak Area	Mean Area
Three Months				
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	171570		145948	
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	179071		145623	
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	179368	176670 (= 95%)*	145799	145790 (= 99%)*
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	187918		147923	
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	186647		148977	
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	184370	186312	146153	147684

Table 19: Stability of d6-NNI-0001 and d6-NNI-0001-des-iodo in Organic/Aqueous Solvents (Secondary Standard).

Sample Name	d6-NNI-0001		d6-NNI-0001-des-iodo	
	Peak Area	Mean Area	Peak Area	Mean Area
Three Months				
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	182165		142629	
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	174291		141203	
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	178580	178345 (= 93%)*	139945	141259 (= 94%)*
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	192080		152351	
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	191415		148895	
s_0.05 µg/mL each d6-NNI-0001 and d6-NNI-0001-des-iodo (Aged Standard)	189853	191116	148384	149877

*: AGED standard quantified using the freshly prepared standard solution as reference (= 100%)

5.5.2 Stability in Plant Extracts

In addition, the stability of NNI-0001 and its metabolite A-1 was determined at the LOQ level of 0.01 mg/kg for extracts of wheat (grain), tomato (fruit) and cotton (oil). Control samples were fortified with the analytes and after initial analysis, the analytical solutions were stored in a refrigerator and were reanalysed after periods of one and four weeks. Storage was conducted under the same conditions as used for analytical solutions (in a refrigerator at 4°C ± 3°C). The results of this investigation show that both analytes are stable in representative plant extracts for a period of at least four weeks.

Table 20: Stability of NNI-0001 in Representative Plant Extracts; Fortification Level: 0.01 mg/kg.

Sample Material		Recovery Rates [%]		
		Single Values	Mean	RSD [%]
Cotton (oil)	initial analysis	98, 97, 96, 95, 94	96	1.6
	1 week reanalysis	81, 87, 85, 94, 93	88	6.2
	4 weeks reanalysis	94, 93, 97, 92, 90	93	2.8
Tomato (fruit)	initial analysis	78, 91, 88, 96, 89	88	7.5
	1 week reanalysis	102, 88, 92, 91, 93	93	5.7
	4 weeks reanalysis	75, 88, 87, 93, 91	87	8.1
Wheat (grain)	initial analysis	80, 83, 82, 74, 85	81	5.2
	1 week reanalysis	81, 81, 82, 79, 76	80	3.0
	4 weeks reanalysis	82, 82, 85, 76, 81	81	4.0

Table 21: Stability of A-1 in Representative Plant Extracts; Fortification Level: 0.01 mg/kg.

Sample Material		Recovery Rates [%]		
		Single Values	Mean	RSD [%]
Cotton (oil)	initial analysis	101, 103, 101, 102, 97	101	2.3
	1 week reanalysis	77, 83, 91, 95, 89	87	8.1
	4 weeks reanalysis	100, 100, 103, 102, 96	100	2.7
Tomato (fruit)	initial analysis	79, 92, 92, 92, 91	89	6.4
	1 week reanalysis	99, 97, 96, 98, 94	97	2.0
	4 weeks reanalysis	78, 90, 87, 93, 88	87	6.5
Wheat (grain)	initial analysis	84, 81, 85, 77, 86	83	4.4
	1 week reanalysis	79, 75, 81, 73, 72	76	4.8
	4 weeks reanalysis	81, 78, 79, 71, 78	77	4.9

6 References

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- [5] Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414 (working document), European Commission, Directorate General Health and Consumer Protection, SANCO/3029/99 rev. 4, 2000-07-11
- [6] Billian P., Analytical Method 00816 for the Determination of Residues of NNI-0001 in/on Material of Plant Origin by HPLC-MS/MS. Bayer CropScience AG Report No. MR-063/03, 2004-04-26
- [7] Billian P., Modification M001 to the Analytical Method 00816 for the Determination of Residues of NNI-0001 and its des-Iodo Metabolite A-1 in/on Plant Material by HPLC-MS/MS for Data Collection and Enforcement Purposes. Bayer CropScience AG Report No. MR-087/03, 2004-07-22

Appendix 1:
Representative Chromatograms
Cotton (Oil)

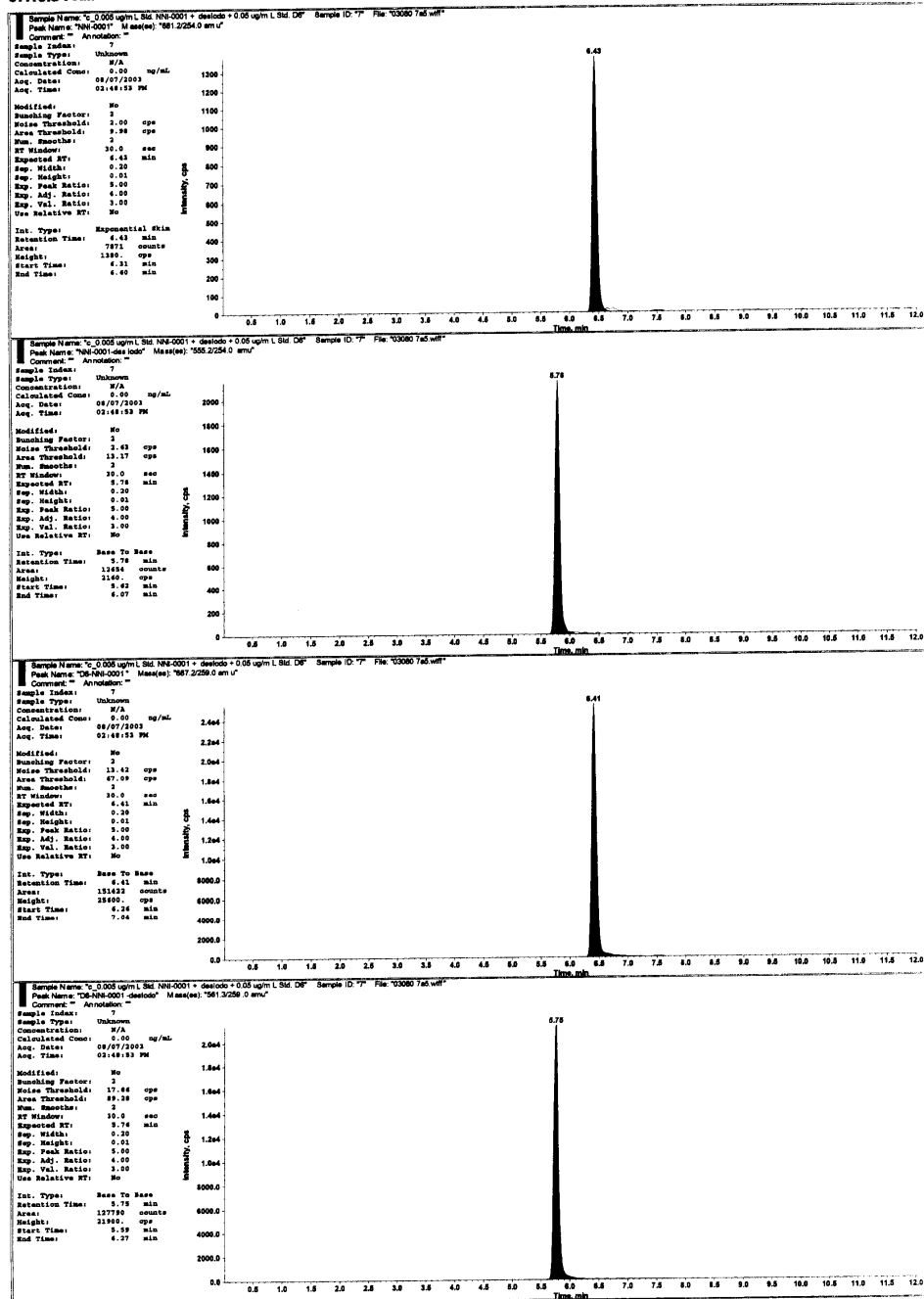
NNI-0001, NNI-0001-des-iodo (0.005 mg/L)
and d6-NNI-0001, d6-NNI-0001-des-iodo (0.05 mg/L) (from top to bottom)

2003-08-8

Study No.: P602030522

Operator: Gerhard Schuld (GS)

07:19:34 AM



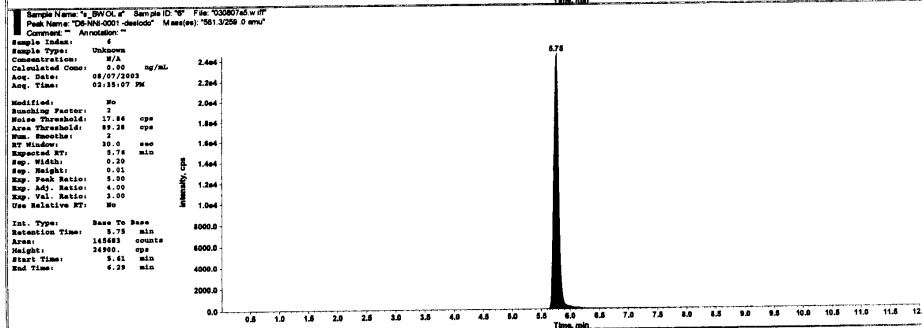
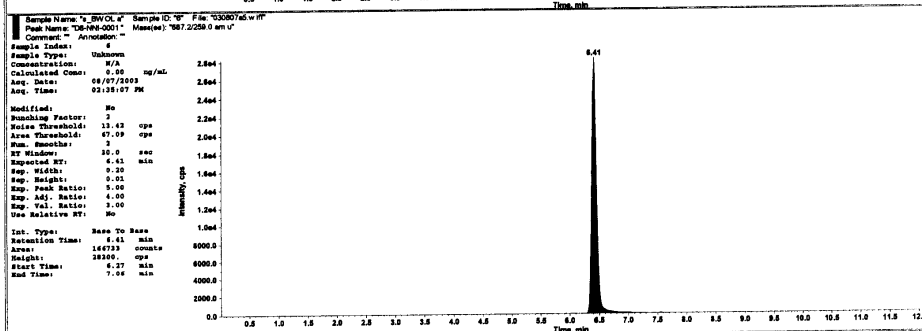
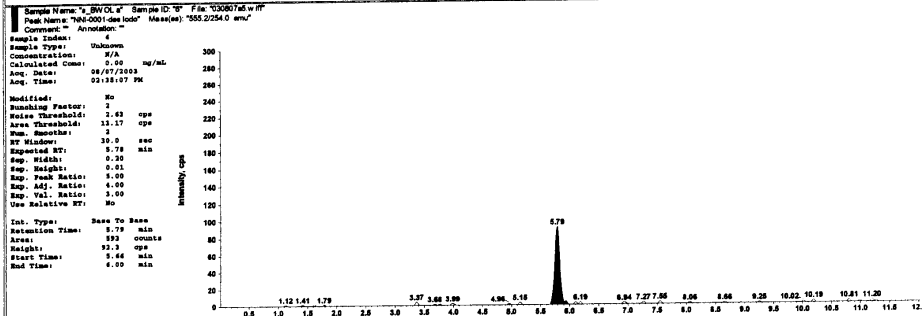
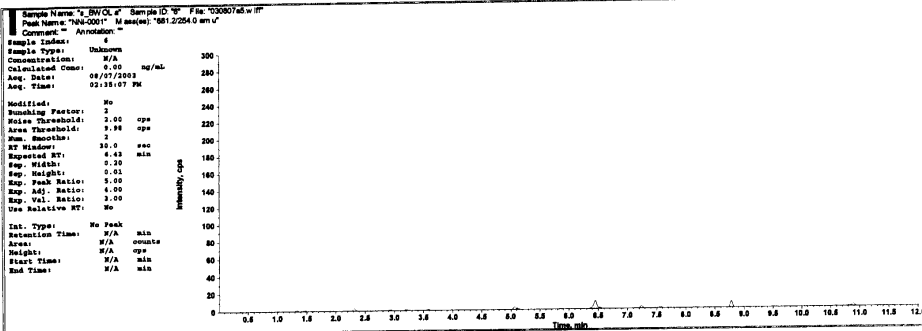
Appendix 1:
Representative Chromatograms (contd)
Cotton (Oil)
Control Sample

2003-08-8

Study No.: P602030522

Operator: Gerhard Schuld (GS)

07:19:34 AM



API 365 Serial: 2369802

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Appendix 1:
Representative Chromatograms (contd)
Cotton (Oil)

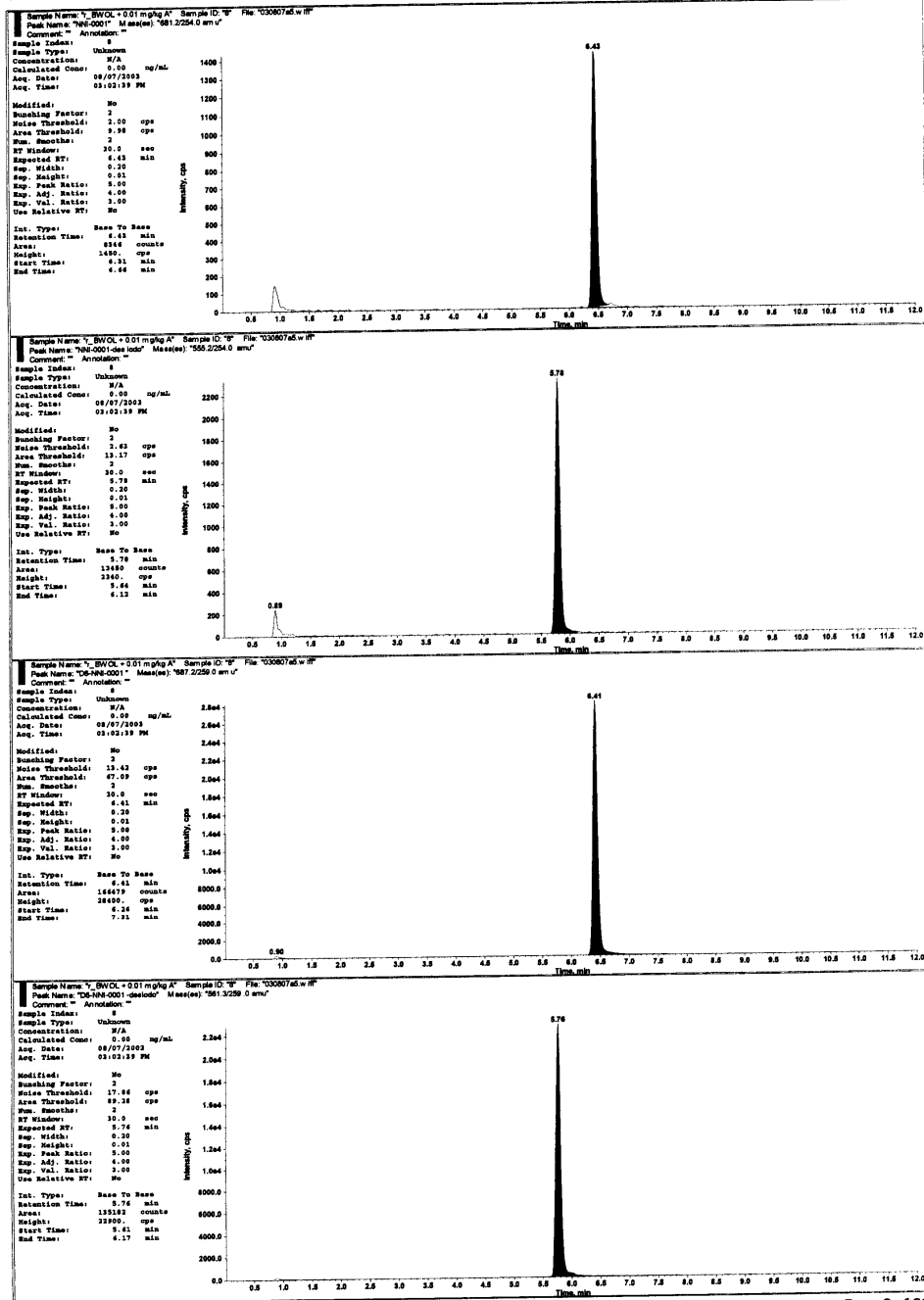
Recovery Sample (NNI-0001 and NNI-0001-des-iodo: 0.01 mg/kg, each)

2003-08-8

Study No.: P602030522

Operator: Gerhard Schulz (GS)

07:19:34 AM



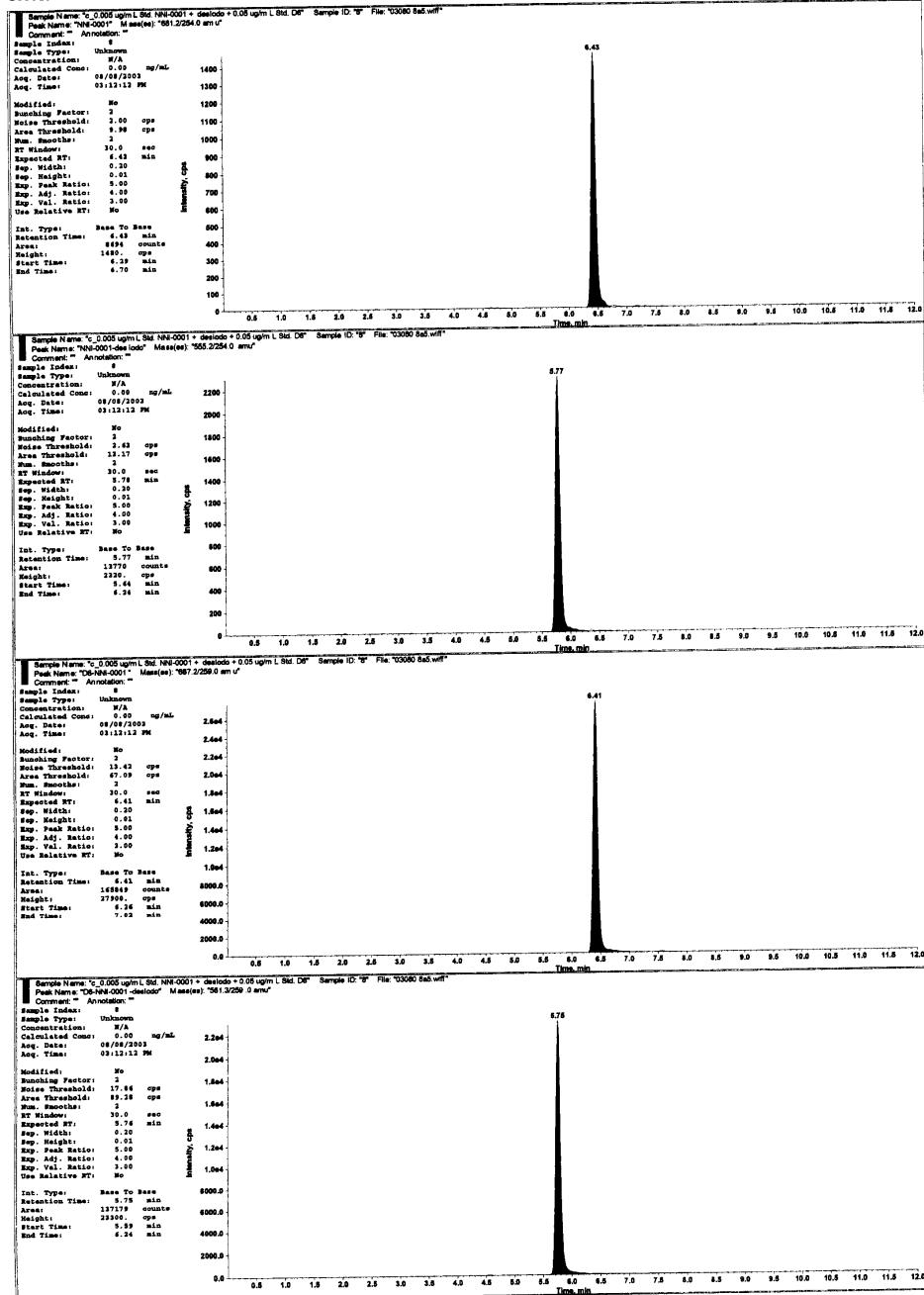
Appendix 1:
Representative Chromatograms (contd)
Tomato (Fruit)

NNI-0001, NNI-0001-des-iodo (0.005 mg/L)
and d6-NNI-0001, d6-NNI-0001-des-iodo (0.05 mg/L) (from top to bottom)

2003-08-11
07:13:10 AM

Study No.1 P602030522

Operator: Gerhard Schuld (GS)



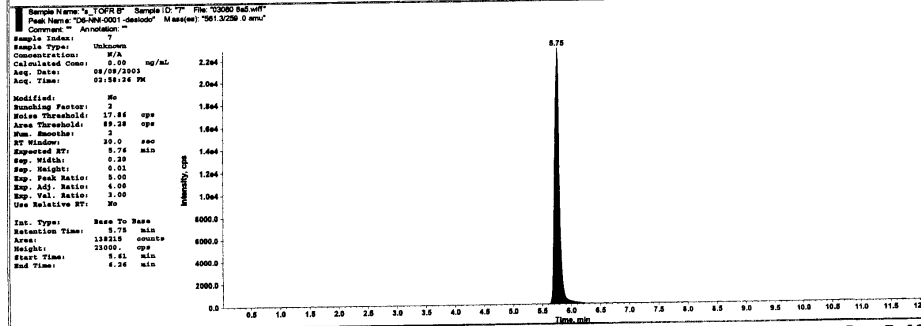
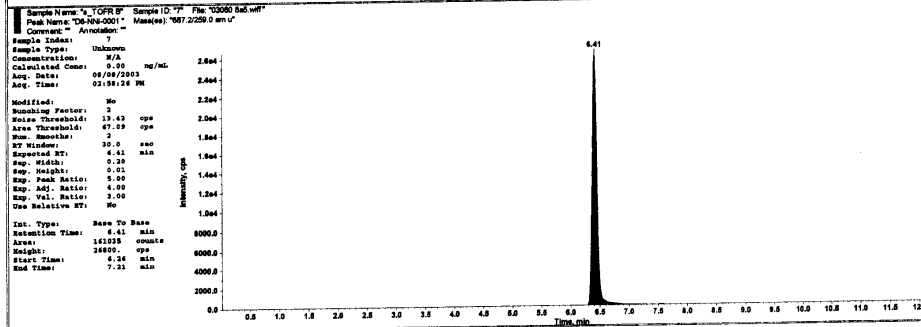
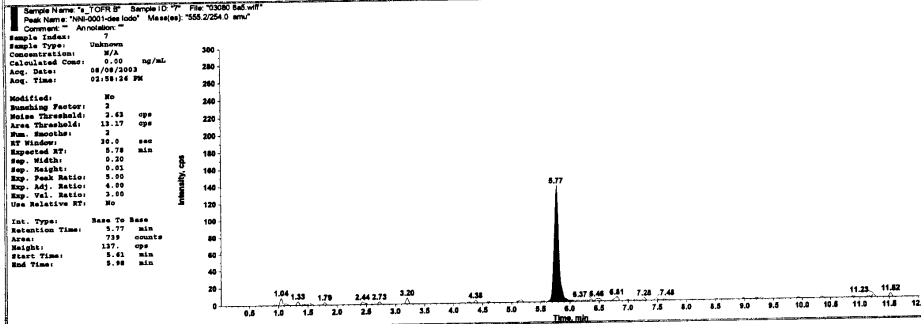
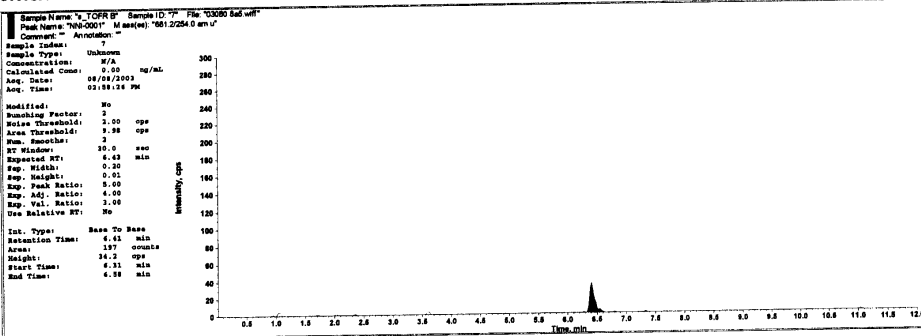
Appendix 1:
Representative Chromatograms (contd)
Tomato (Fruit)
Control Sample

2003-08-11

Study No.: P602030522

Operator: Gerhard Schulz (GS)

07:13:10 AM



API 365 Serial: 2369802

Page 7 of 21

Appendix 1:
Representative Chromatograms (contd)
Tomato (Fruit)

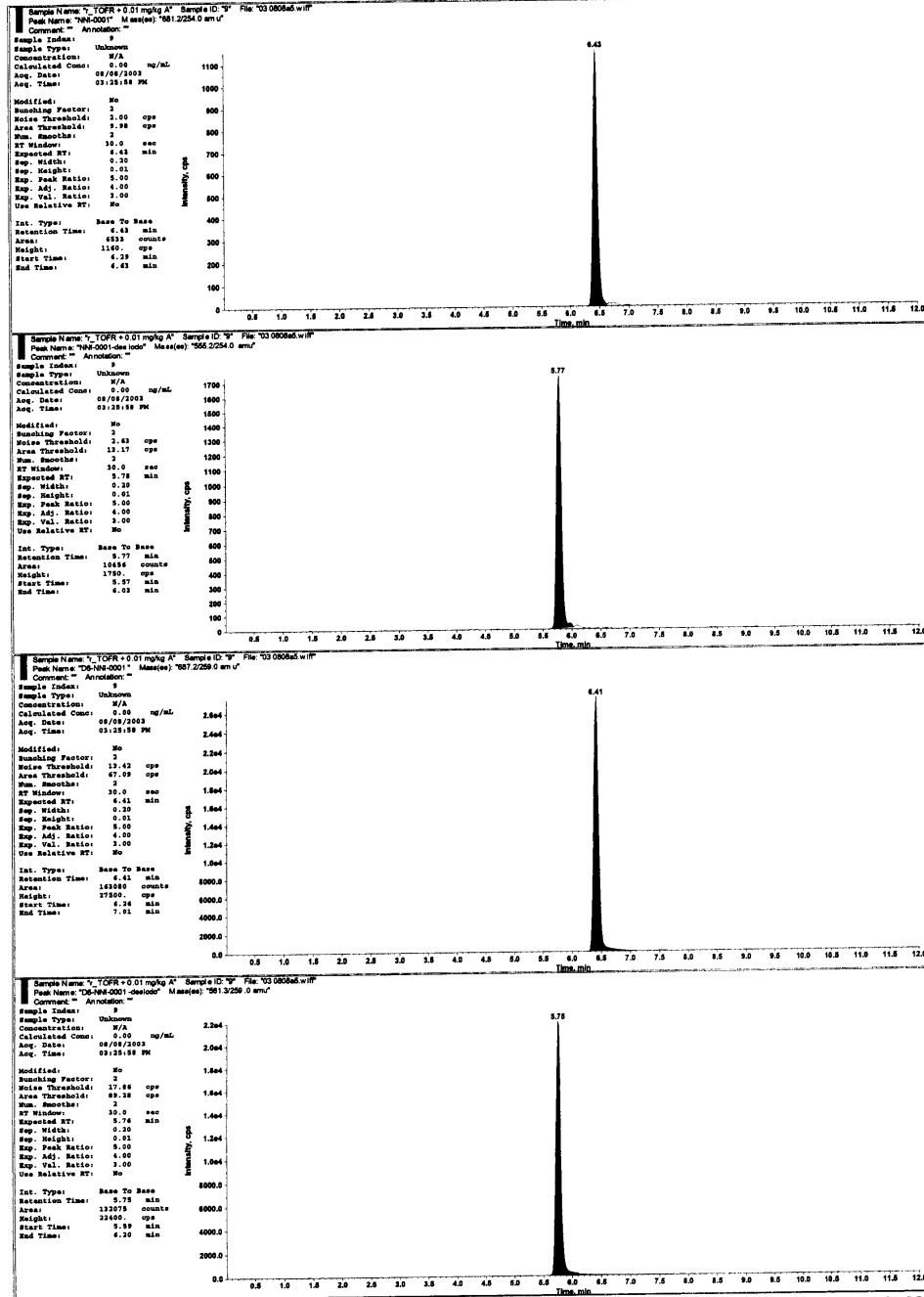
Recovery Sample (NNI-0001 and NNI-0001-des-iodo: 0.01 mg/kg, each)

2003-08-11

Study No.: P602030522

Operator: Gerhard Schulz (GS)

07:13:10 AM



Appendix 1:
Representative Chromatograms (contd)
Head Cabbage (Head)

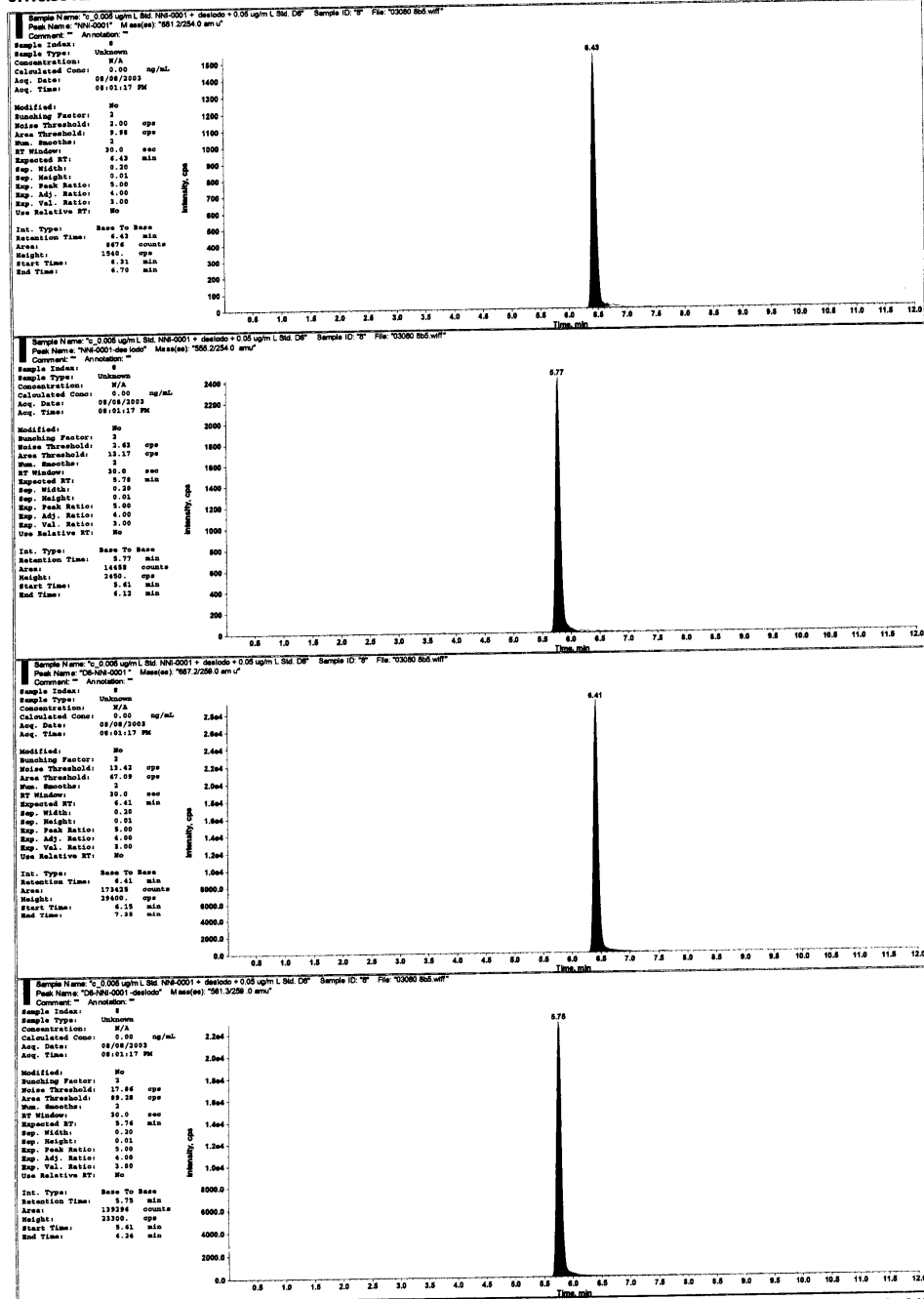
NNI-0001, NNI-0001-des-iodo (0.005 mg/L)
and d6-NNI-0001, d6-NNI-0001-des-iodo (0.05 mg/L) (from top to bottom)

2003-08-11

Study No.: P602030522

Operator: Gerhard Schuld (GS)

07:16:55 AM



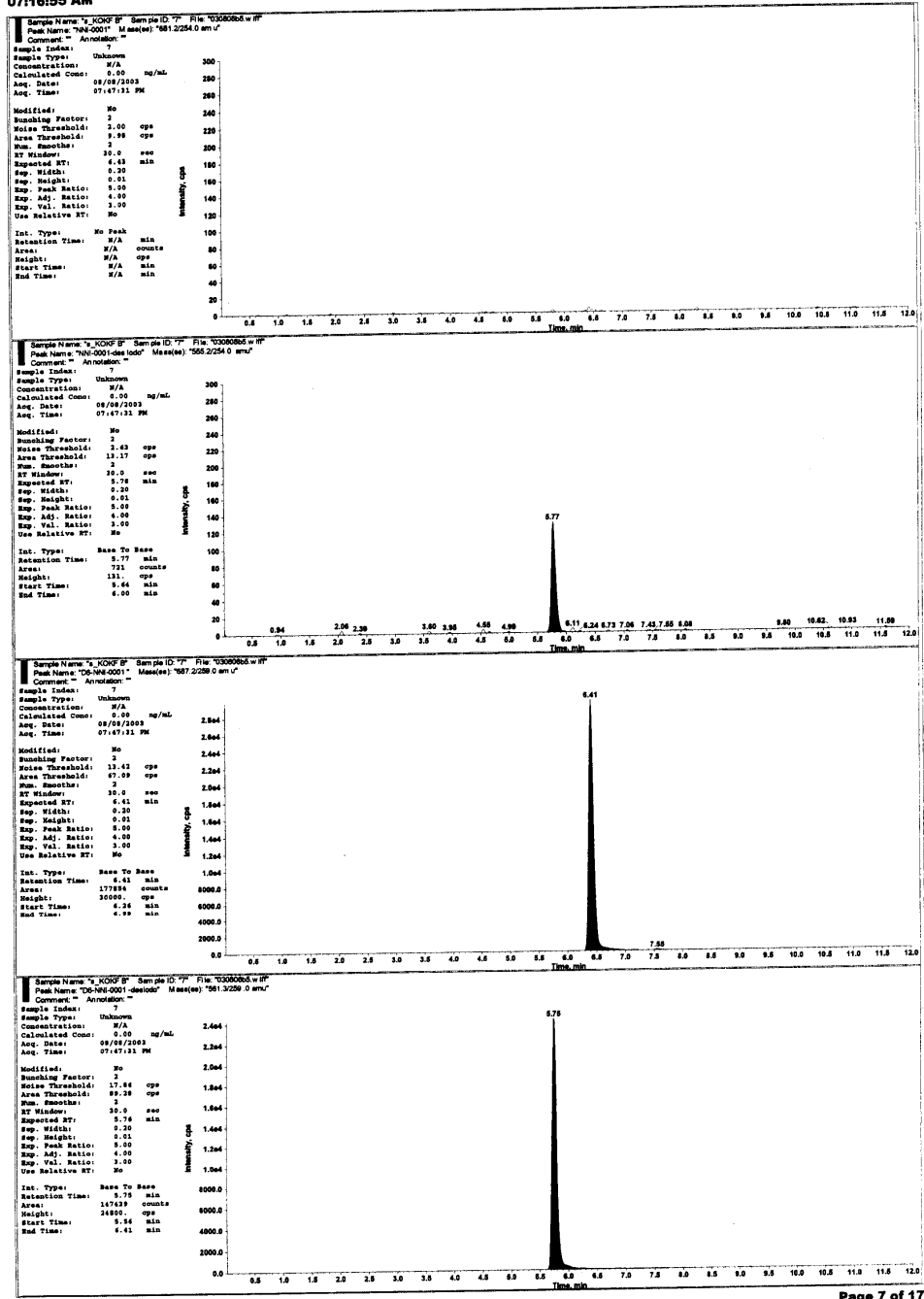
Appendix 1:
Representative Chromatograms (contd)
Head Cabbage (Head)
Control Sample

2003-08-11

Study No.: P602030522

Operator: Gerhard Schuld (GS)

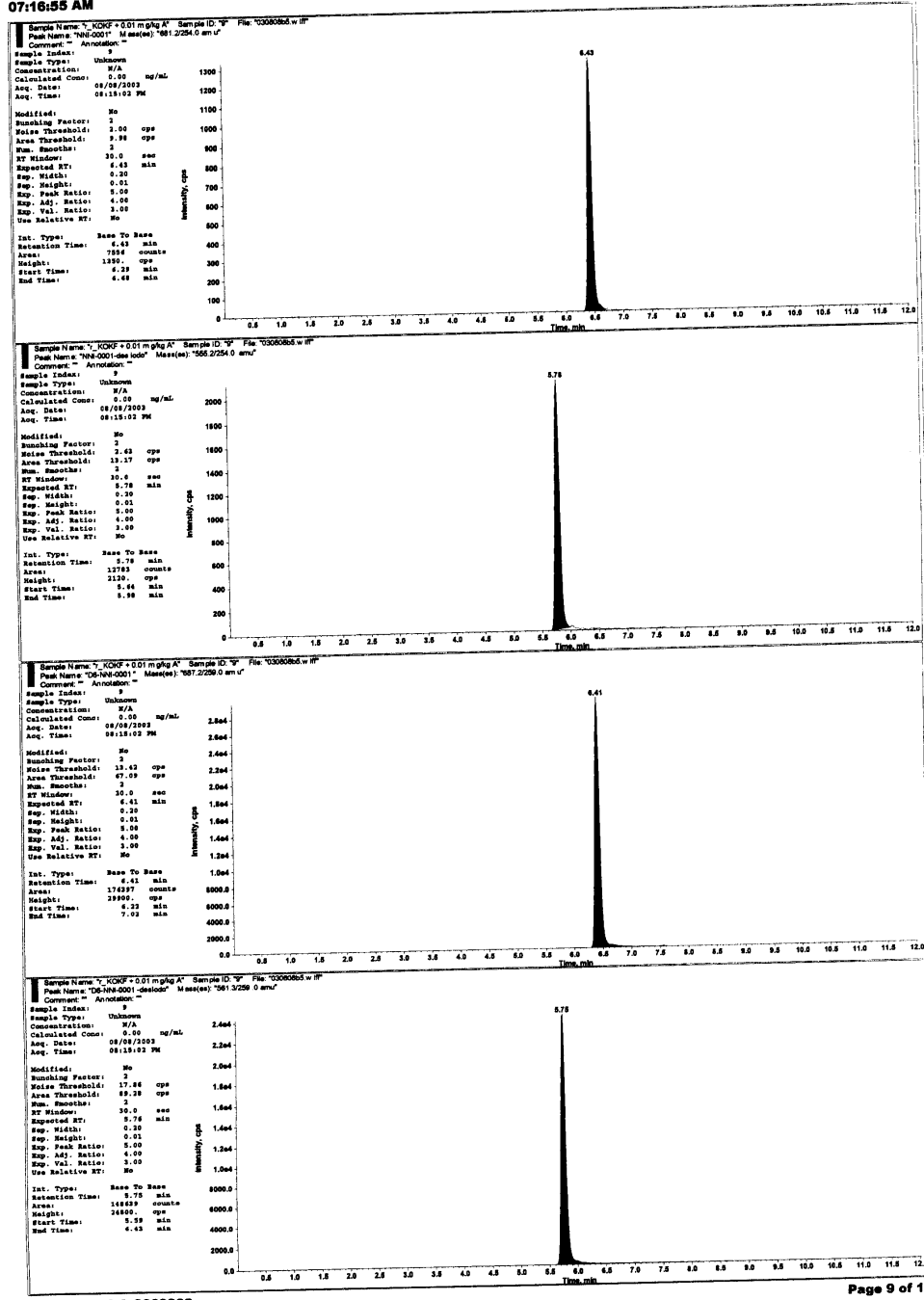
07:16:55 AM



Appendix 1:
 Representative Chromatograms (contd)
 Head Cabbage (Head)

Recovery Sample (NNI-0001 and NNI-0001-des-iodo: 0.01 mg/kg, each)

2003-08-11 Study No.: P602030522 Operator: Gerhard Schuld (G5)
 07:16:55 AM



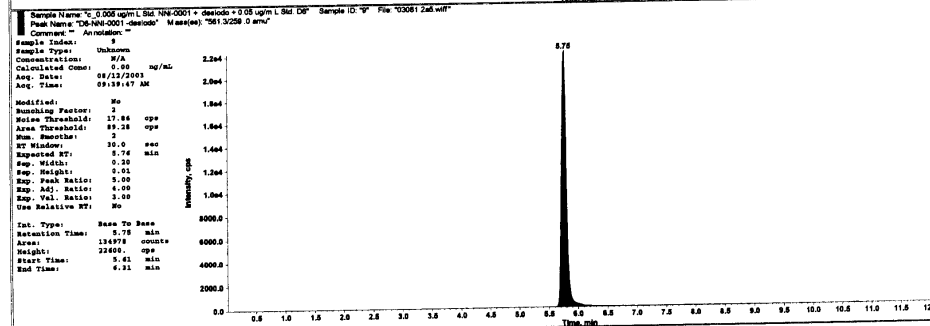
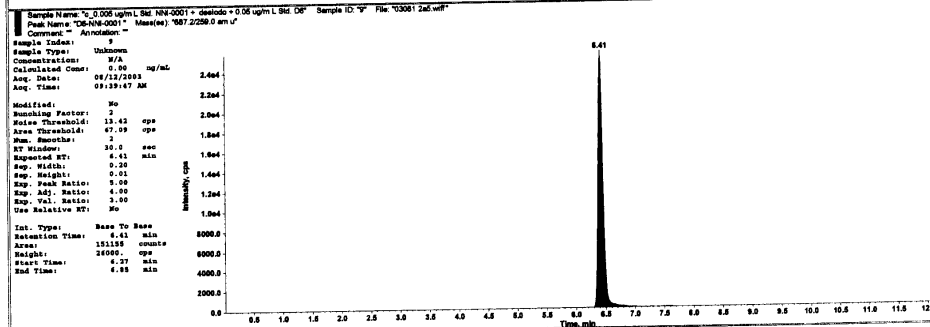
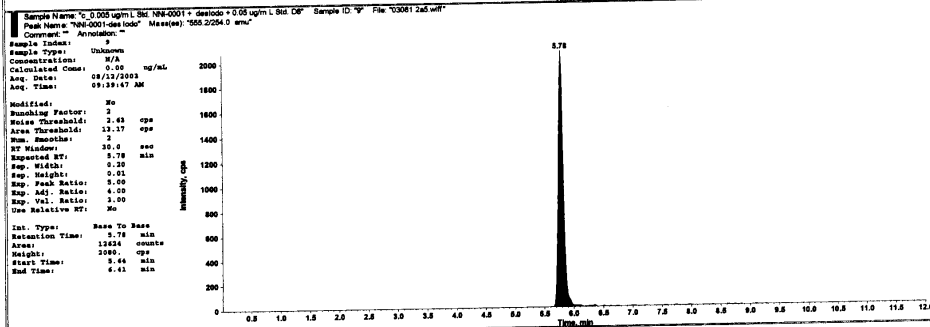
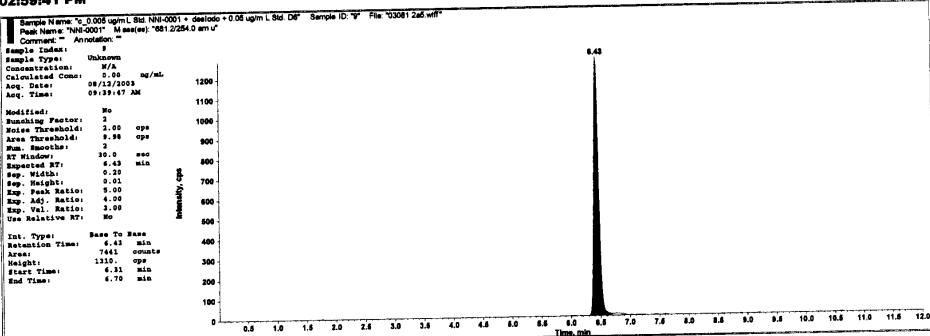
Appendix 1:
Representative Chromatograms (contd)
Wheat (Grain)

NNI-0001, NNI-0001-des-iodo (0.005 mg/L)
and d6-NNI-0001, d6-NNI-0001-des-iodo (0.05 mg/L) (from top to bottom)

2003-08-12
02:59:41 PM

Study No.: P602030522

Operator: Gerhard Schulz (GS)



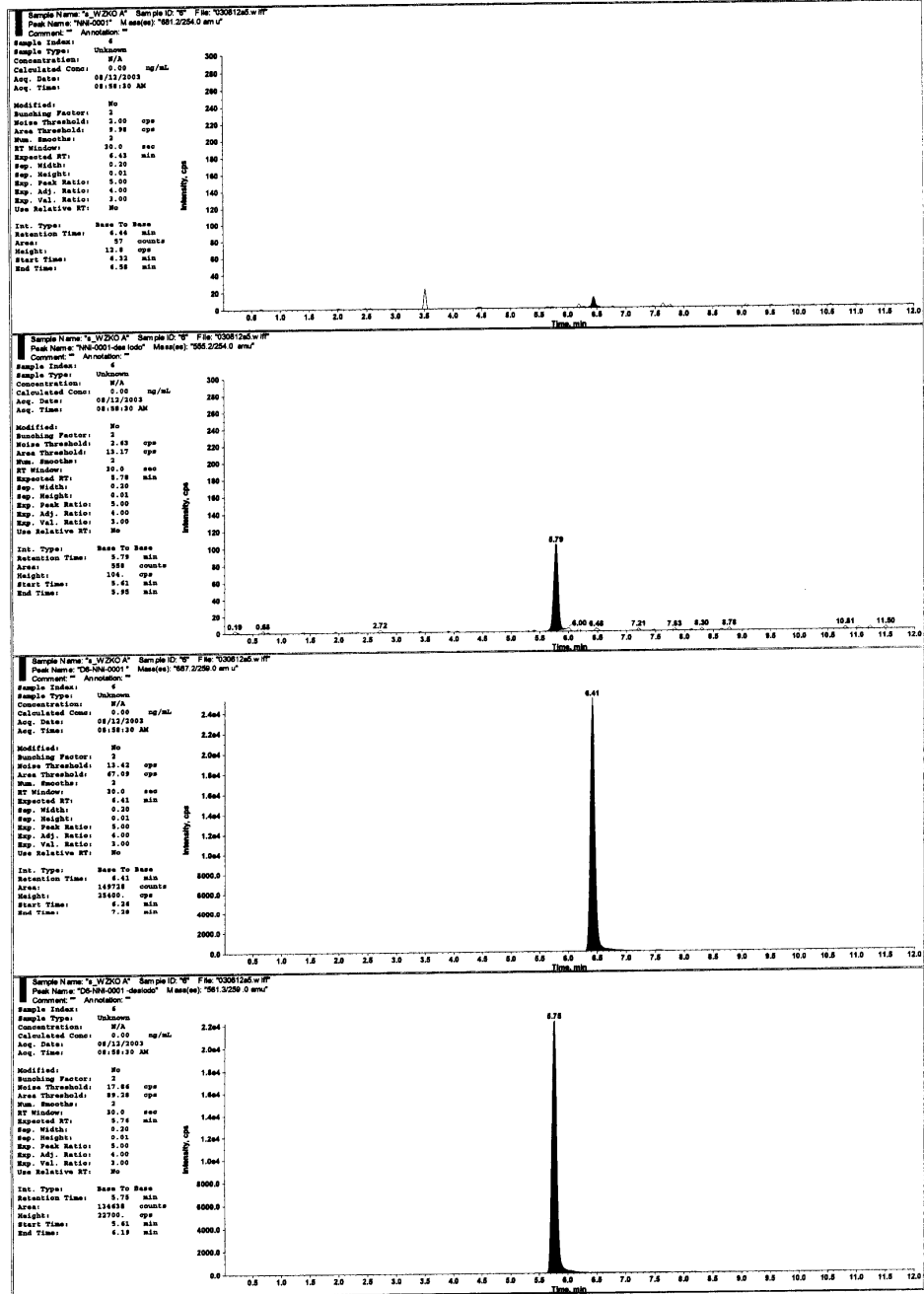
Appendix 1:
Representative Chromatograms (contd)
Wheat (Grain)
Control Sample

2003-08-12

Study No.: P602030522

Operator: Gerhard Schulz (GS)

02:59:41 PM



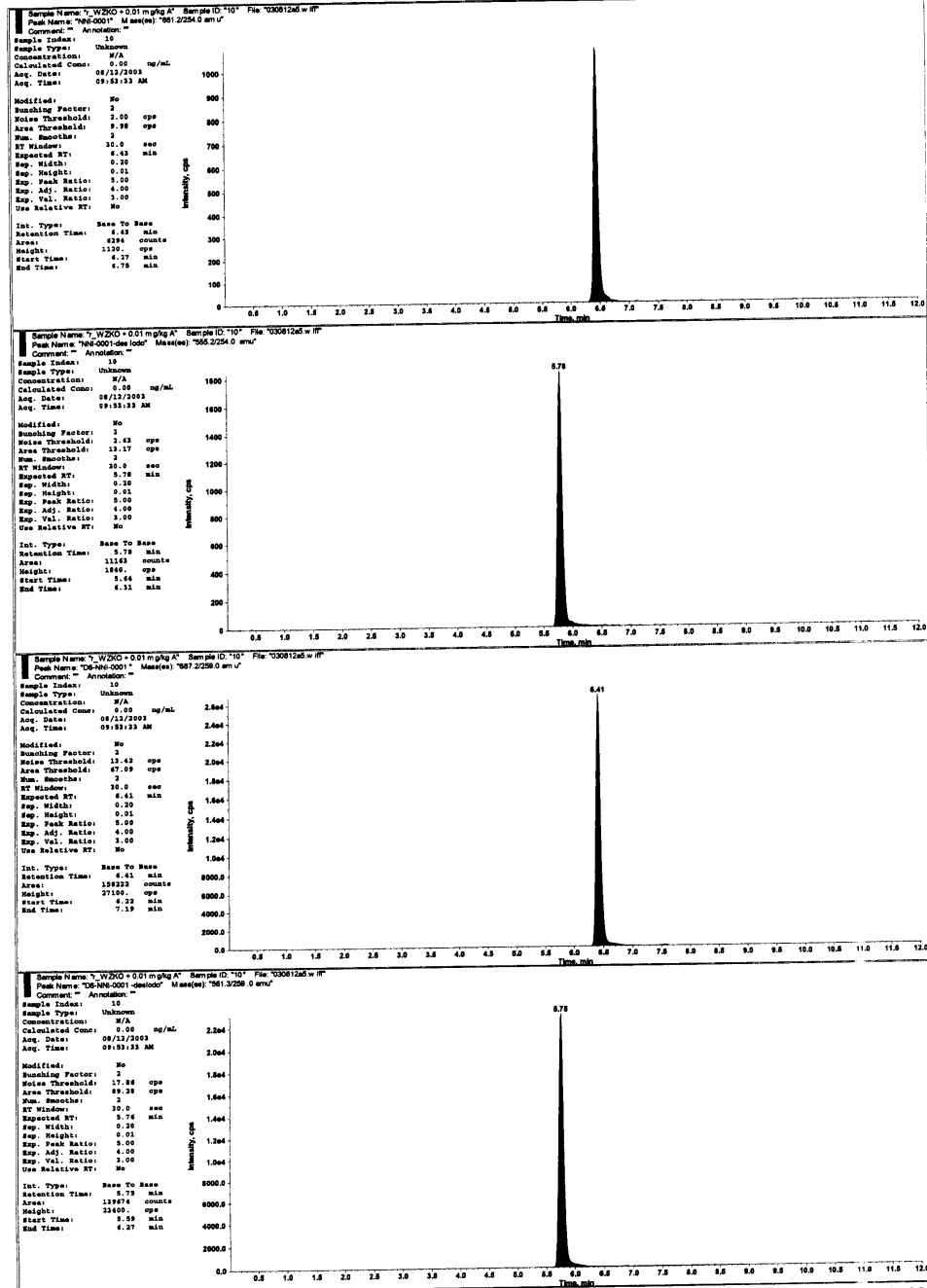
API 365 Serial: 2369802

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Appendix 1:
Representative Chromatograms (contd)
Wheat (Grain)

Recovery Sample (NNI-0001 and NNI-0001-des-iodo: 0.01 mg/kg, each)

2003-08-12 Study No.: P602030522 Operator: Gerhard Schuld (GS)
02:59:41 PM



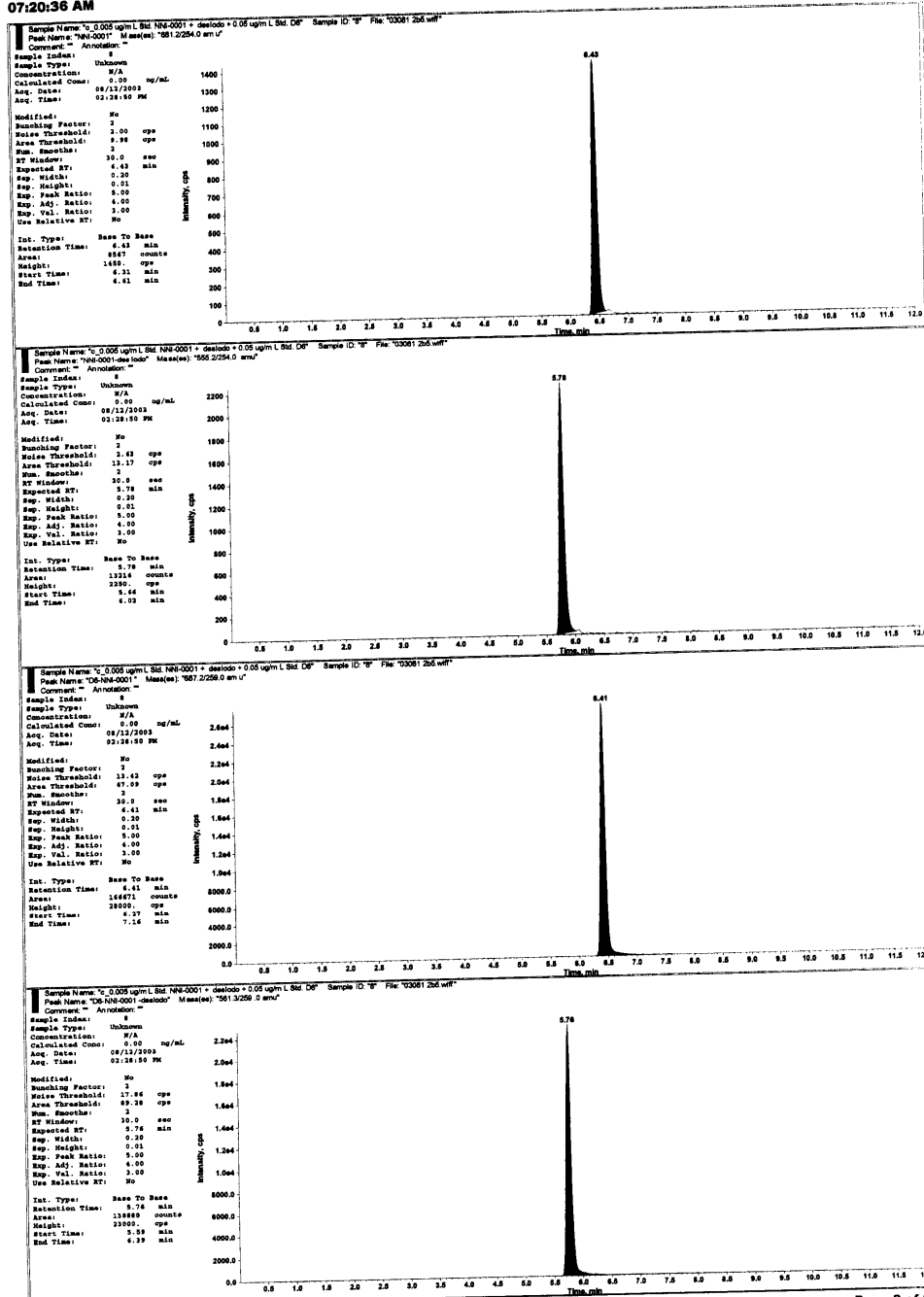
Appendix 1:
Representative Chromatograms (contd)
Citrus (Fruit)

NNI-0001, NNI-0001-des-iodo (0.005 mg/L)
and d6-NNI-0001, d6-NNI-0001-des-iodo (0.05 mg/L) (from top to bottom)

2003-08-13
07:20:36 AM

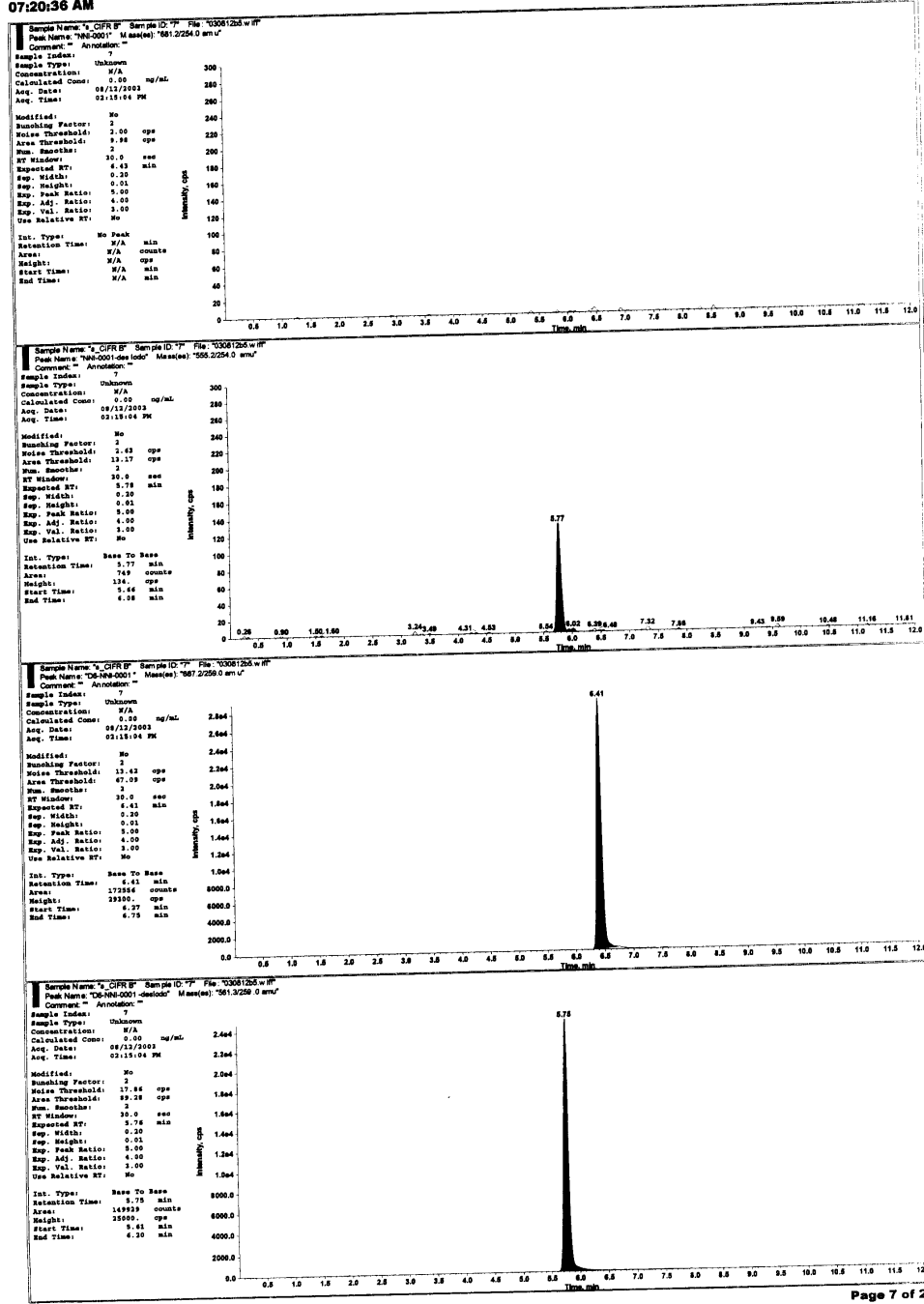
Study No.: P602030522

Operator: Gerhard Schulz (GS)



Appendix 1:
Representative Chromatograms (contd)
Citrus (Fruit)
Control Sample

2003-08-13 07:20:36 AM Study No.: P802030522 Operator: Gerhard Schuld (GS)



Appendix 1:
Representative Chromatograms (contd)

Bean (Bean with Pod)

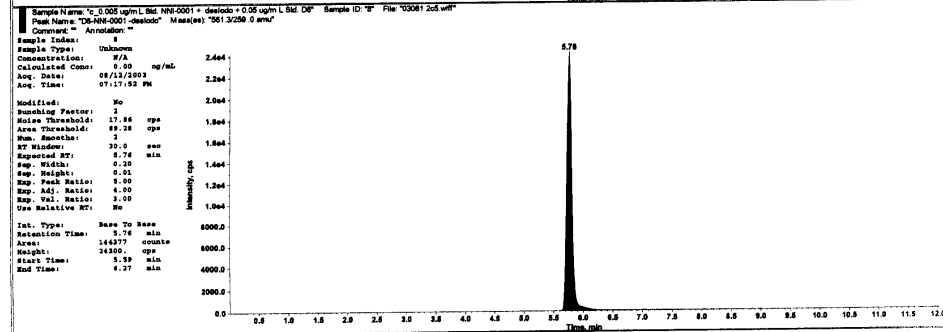
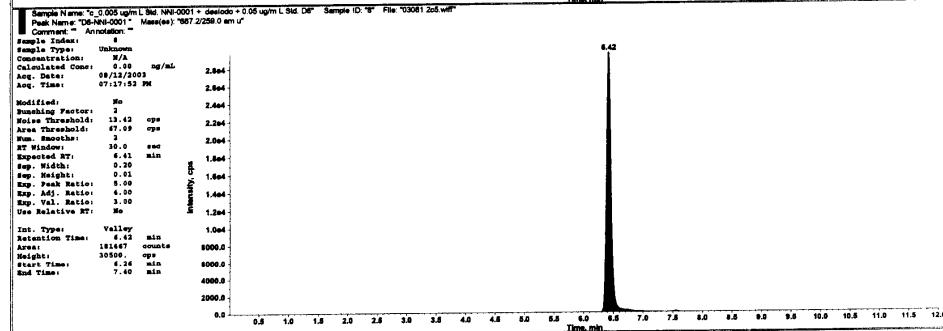
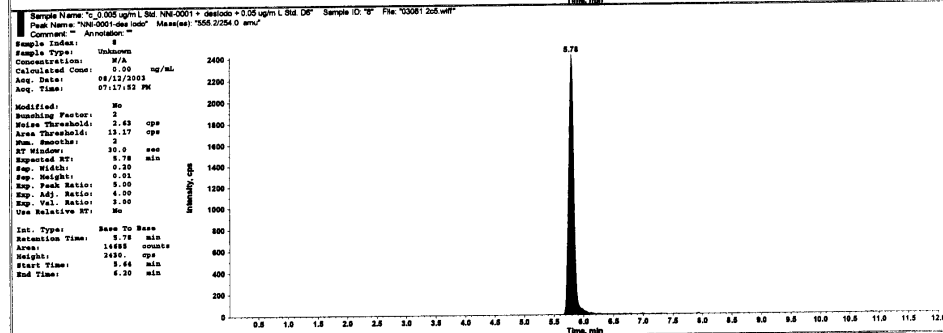
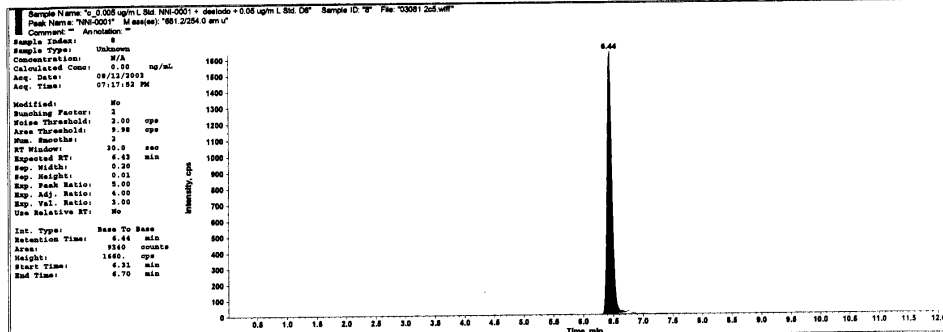
NNI-0001, NNI-0001-des-iodo (0.005 mg/L)
and d6-NNI-0001, d6-NNI-0001-des-iodo (0.05 mg/L) (from top to bottom)

2003-08-13

Study No.: P602030522

Operator: Gerhard Schuld (GS)

07:23:41 AM



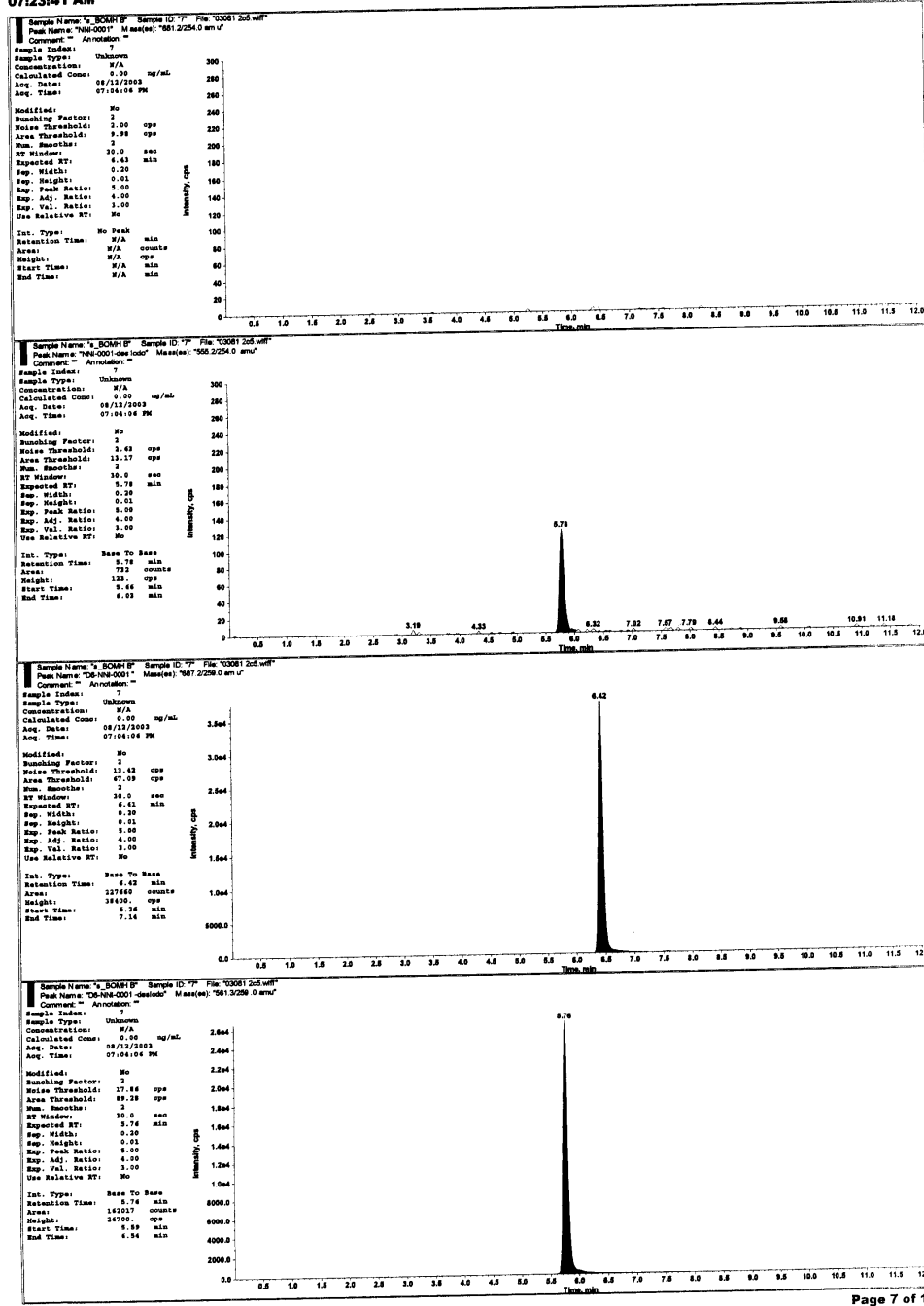
Appendix 1:
Representative Chromatograms (contd)
Bean (Bean with Pod)
Control Sample

2003-08-13

Study No.: P602030522

Operator: Gerhard Schuld (GS)

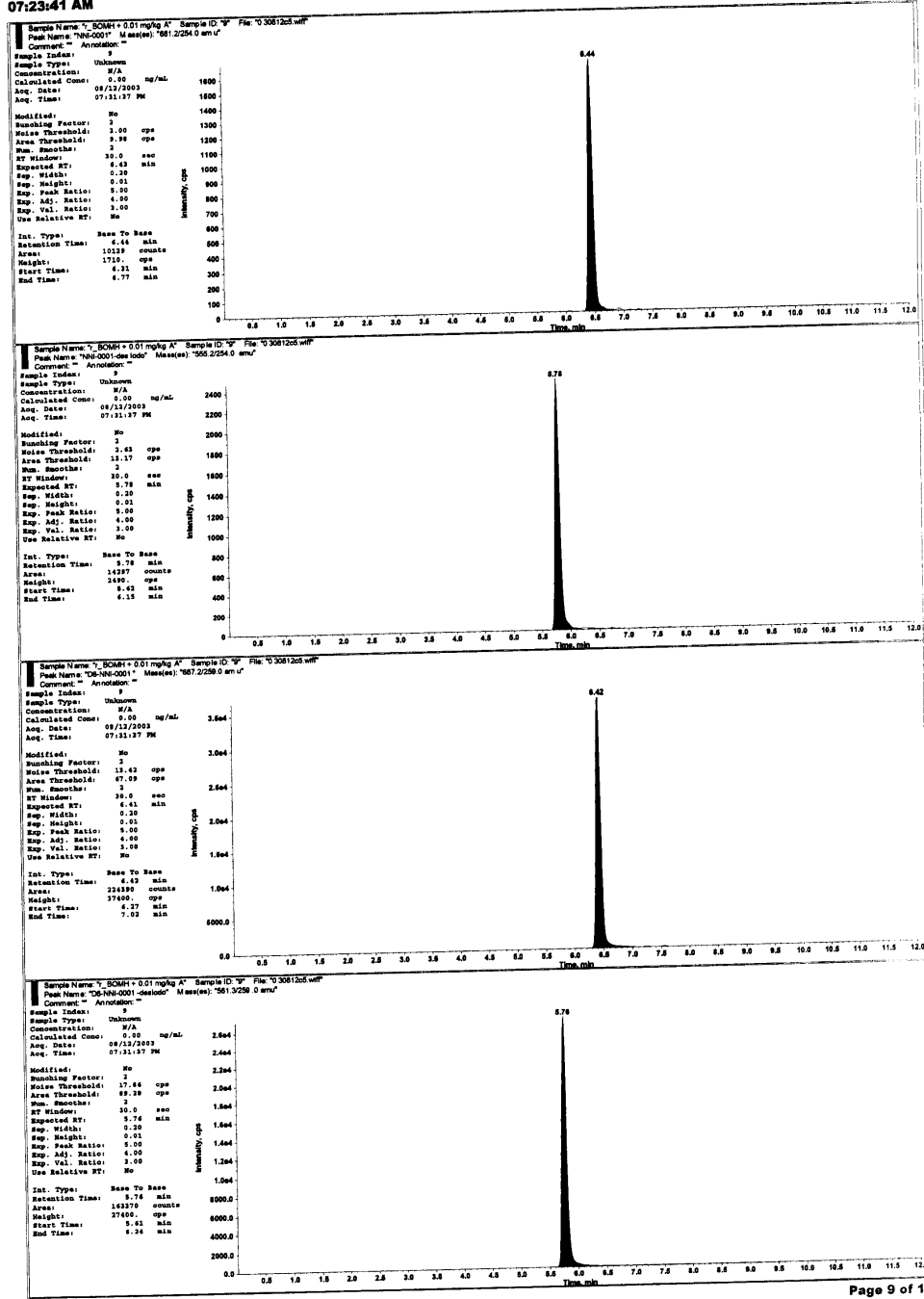
07:23:41 AM



Appendix 1:
Representative Chromatograms (contd)
Bean (Bean with Pod)

Recovery Sample (NNI-0001 and NNI-0001-des-iodo: 0.01 mg/kg, each)

2003-08-13 07:23:41 AM Study No.: P602030522 Operator: Gerhard Schuld (GS)



Appendix 2: Flow Diagrams of Residue Method 00816/M002

1. Weigh 5.0 g sample material for microwave extraction. Add 1 g of filter aid Celite 545 and 20 mL of acetonitrile incl. 0.01% HCL conc. Mix it.
2. Microwave extraction (2 min, 200 W)
3. Percolate the solvent onto a 250-mL round-bottom flask.
4. Add 20 mL of acetonitrile/water mixture (2/1, v/v + 0.01% HCl) and repeat microwave extraction under the same conditions.
5. Wash the remaining solids with 10 mL of acetonitrile/water mixture (2/1, v/v + 0.01% HCl) and combine the solvents in a 250-mL round-bottom flask.
6. Evaporate the solvent to an aqueous remainder.
7. Clean-up with Chem-Elut 1020 cartridges. Elute residues with 80 mL of cyclohexane/ethyl acetate (1/1, v/v).
8. Evaporate to dryness.
9. After addition of 0.1 mL of internal standard (d6-NNI-0001 and d6-NNI-0001-des-iodo, 5 mg/mL each) dissolve residues in acetonitrile/water mixture (1/1, v/v + formic acid 0.01%) and fill up to 10 mL.



HPLC-MSMS

Wheat (Grain)

1. Weigh 5.0 g sample material for microwave extraction. Add 1 g of filter aid Celite 545 and 40 mL of acetonitrile incl. 0.01% HCL conc. Add 0.25 mL HCl conc. Mix it.
2. Microwave extraction (2 min, 200 W)
3. Percolate the solvent onto a 250-mL round-bottom flask.
4. Add 30 mL of acetonitrile/water mixture (2/1, v/v + 0.01% HCl) and repeat microwave extraction under the same conditions.
5. Wash the remaining solids with 10 mL of acetonitrile/water mixture (2/1, v/v + 0.01% HCl) and combine the solvents in a 250-mL round-bottom flask.
6. Evaporate the solvent to an aqueous remainder.
7. Clean-up with Chem-Elut 1020 cartridges. Elute residues with 80 mL of cyclohexane/ethyl acetate (1/1, v/v).
8. Evaporate to dryness.
9. After addition of 0.1 mL of internal standard (d6-NNI-0001 and d6-NNI-0001-des-iodo, 5 mg/mL each) dissolve residues in acetonitrile/water mixture (1/1, v/v + formic acid 0.01%) and fill up to 10 mL.



HPLC-MSMS

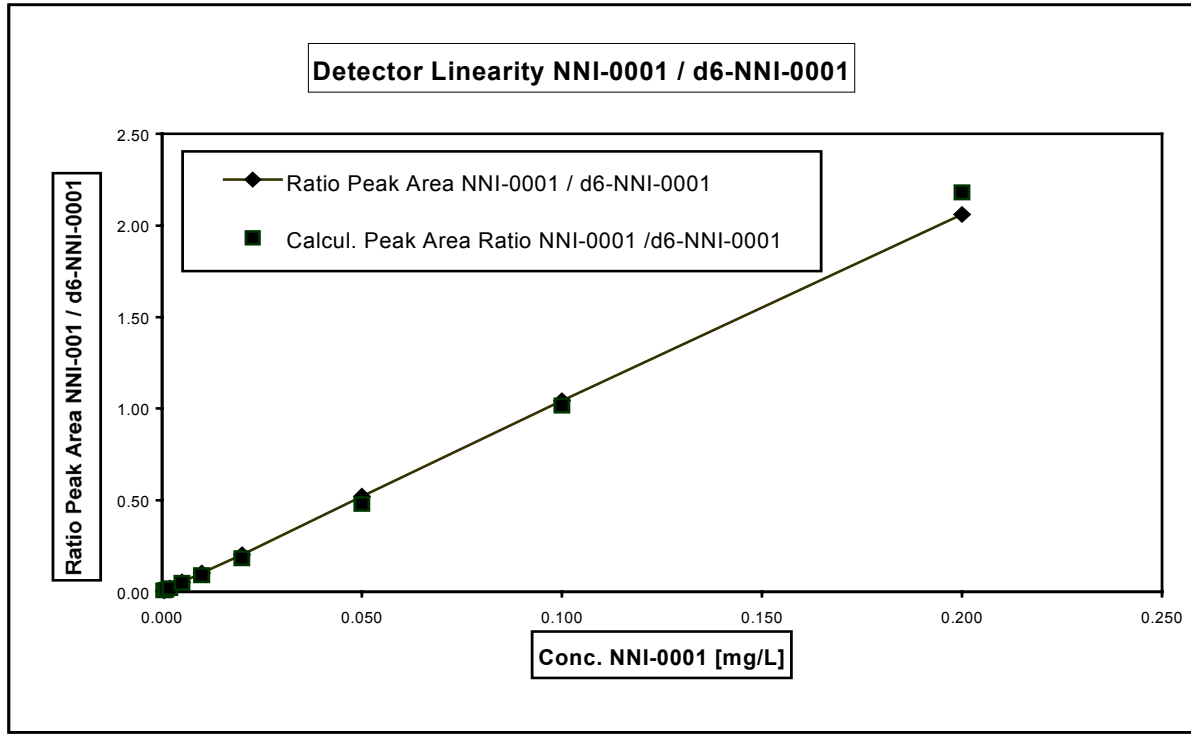
Vegetable Oil (e.g. Olive, Cotton)

1. Weigh 5.0 g sample material into a glass beaker and dissolve in 10 mL of n-hexane.
2. Transfer to a separation funnel. Wash the beaker with 10 mL of n-hexane. Extract the n-hexane twice with 20 mL acetonitrile.
3. Collect the acetonitrile in a separating funnel and extract with 10 mL of n-hexane.
4. After transfer to a round-bottom flask the acetonitrile phase is evaporated to dryness.
5. After addition of 0.1 mL of internal standard (d6-NNI-0001 and d6-NNI-0001-des-iodo, 5 mg/mL each) dissolve residues in acetonitrile/water mixture (1/1, v/v + formic acid 0.01%) and fill up to 10 mL.



HPLC-MSMS

Appendix 3:
 Representative Linearity Plots
 NNI-0001/d6-NNI-0001

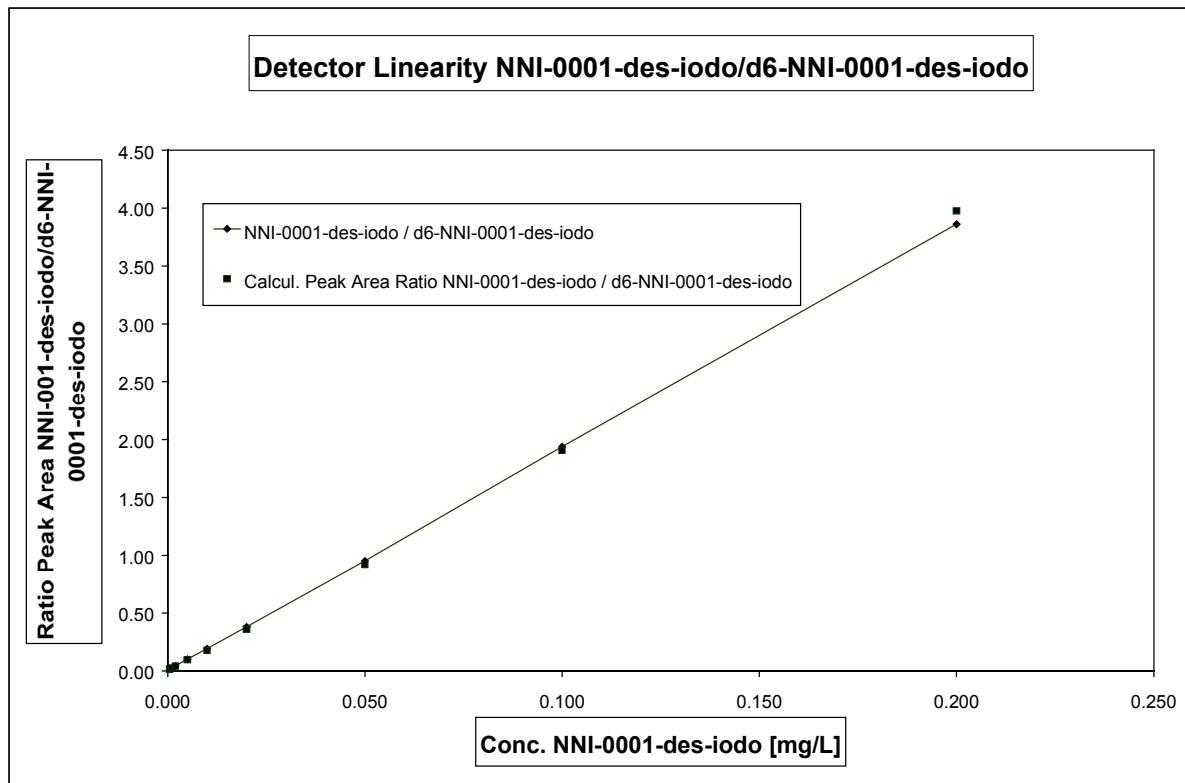


Regression Parameters (1/x weighted linear regression for the equation $y = mx + c$):

y-axis intercept (c) 0.0003
 slope (m) 10.3435
 correlation coefficient (r) 0.9999

Standard Amount		Peak Area			Peak Area			Mean of Peak Area Ratio	Calculated Ratio
[ng]	[mg/L]	NNI-0001			d6-NNI-0001			NNI-0001/ d6-NNI-0001	1/x weighted
0.01	0.0005	892	918	1031	169094	168152	170800	0.0056	0.0071
0.02	0.001	1828	1647	1807	165744	167004	168133	0.0105	0.0115
0.04	0.002	3561	3427	3451	166052	165644	166549	0.0210	0.0202
0.1	0.005	8802	8183	8727	163373	162735	161247	0.0528	0.0472
0.2	0.01	17014	16436	16551	164345	160404	162410	0.1026	0.0915
0.4	0.02	32598	32712	31757	160770	156341	159164	0.2038	0.1842
1	0.05	79704	78180	78606	150847	150510	152567	0.5210	0.4783
2	0.1	147897	149195	146944	144176	143945	137623	1.0430	1.0170
4	0.2	275209	273224	268498	133451	132671	130304	2.0607	2.1801

Appendix 3:
Representative Linearity Plots (contd)
NNI-0001-des-iodo/d6-NNI-0001-des-iodo

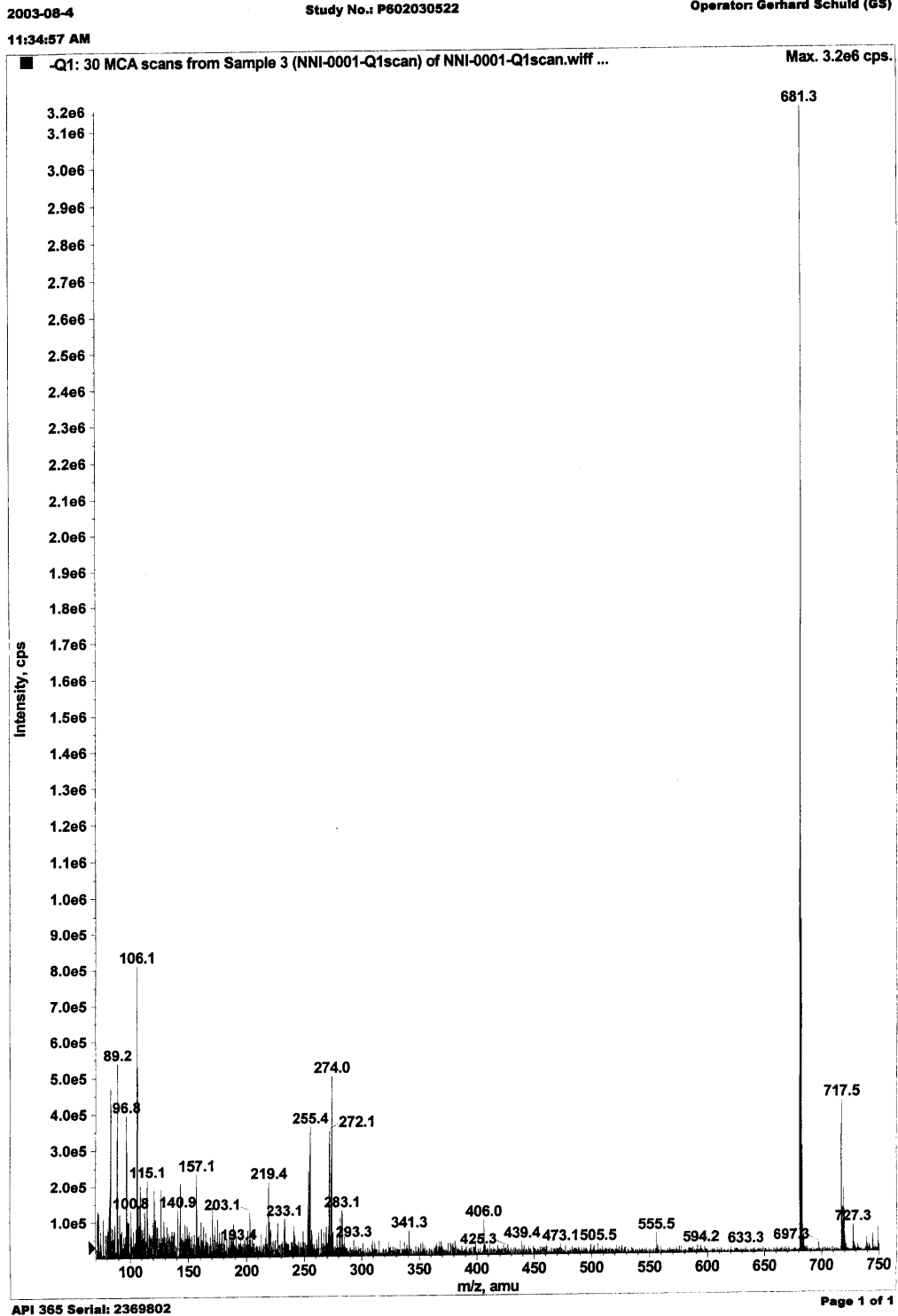


Regression Parameters (1/x weighted linear regression for the equation $y = mx + c$):

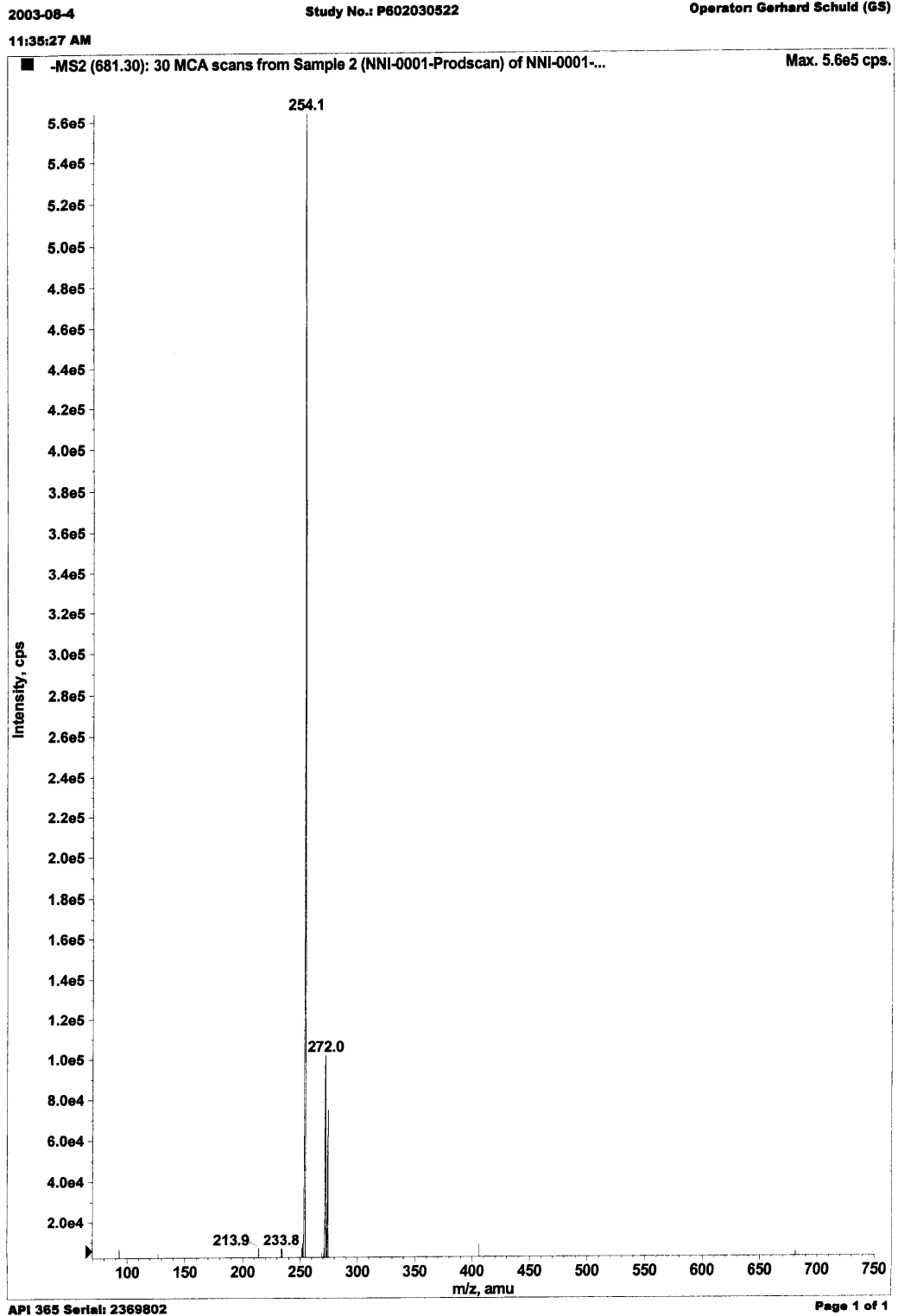
y-axis intercept (c) 0.0047
slope (m) 19.2193
correlation coefficient (r) 0.9999

Standard Amount		Peak Area			Peak Area			Mean of Peak Area Ratio	Calculated Ratio
[ng]	[mg/L]	NNI-0001-des-iodo			d6-NNI-0001-des-iodo			NNI-0001-des-iodo/ d6-NNI-0001-des-iodo	1/x weighted
0.01	0.0005	2007	1964	2085	133897	134937	138957	0.0149	0.0163
0.02	0.001	3296	3234	3039	136159	138172	133885	0.0234	0.0250
0.04	0.002	5864	6047	6033	136420	139711	136266	0.0435	0.0420
0.1	0.005	13788	13191	13199	132082	133599	130892	0.1013	0.0976
0.2	0.01	27072	27199	26629	139930	139951	138371	0.1934	0.1777
0.4	0.02	50841	49615	53061	133201	131827	137273	0.3816	0.3618
1	0.05	123976	125617	122363	130991	131321	128479	0.9518	0.9191
2	0.1	245777	242309	239747	126472	124280	124660	1.9388	1.9052
4	0.2	466867	465282	454206	121841	120258	117040	3.8602	3.9753

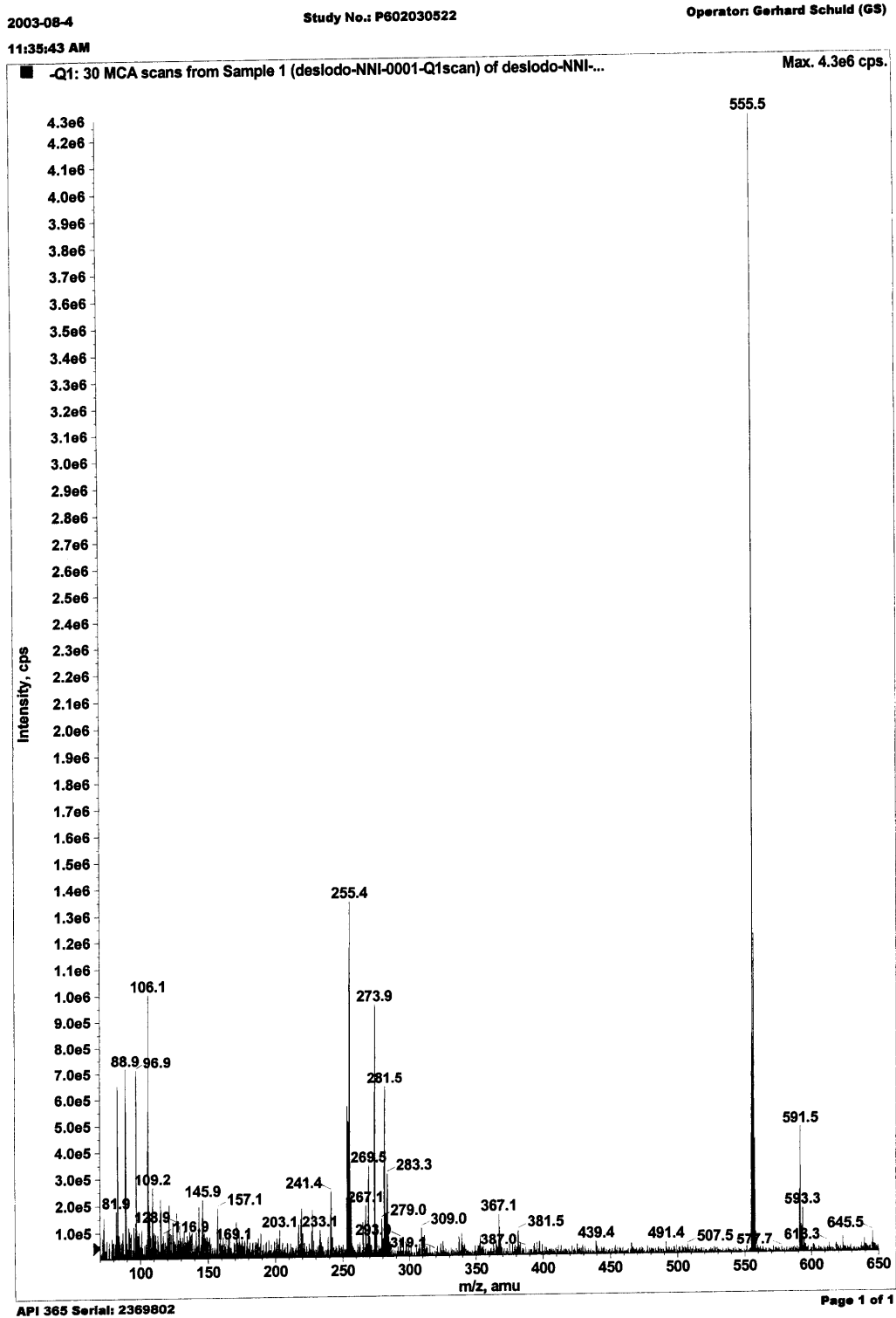
Appendix 4: Precursor Ion Mass Spectrum of NNI-0001



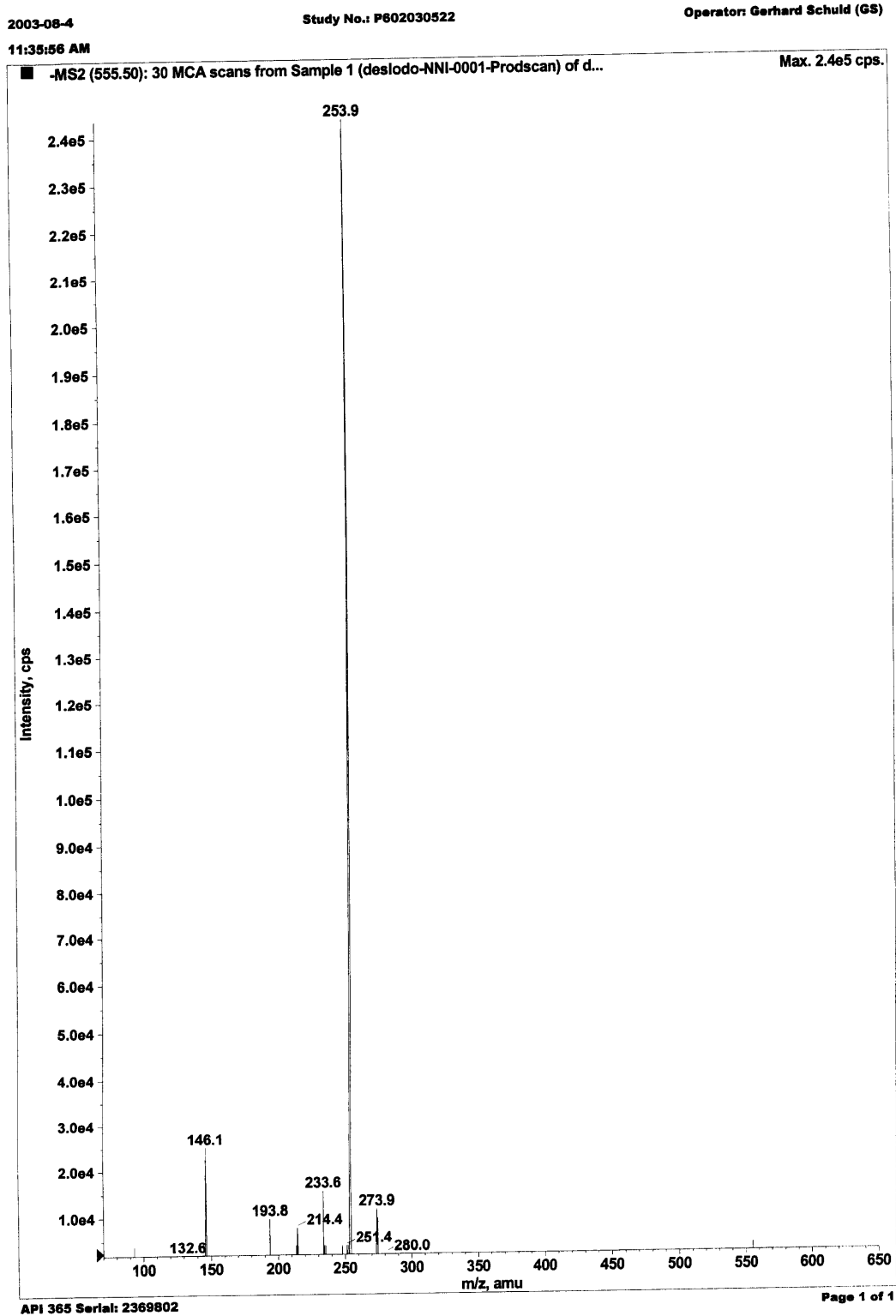
Appendix 5: Product Ion Mass Spectrum of NNI-0001 (Fragment $m/z = 681$)



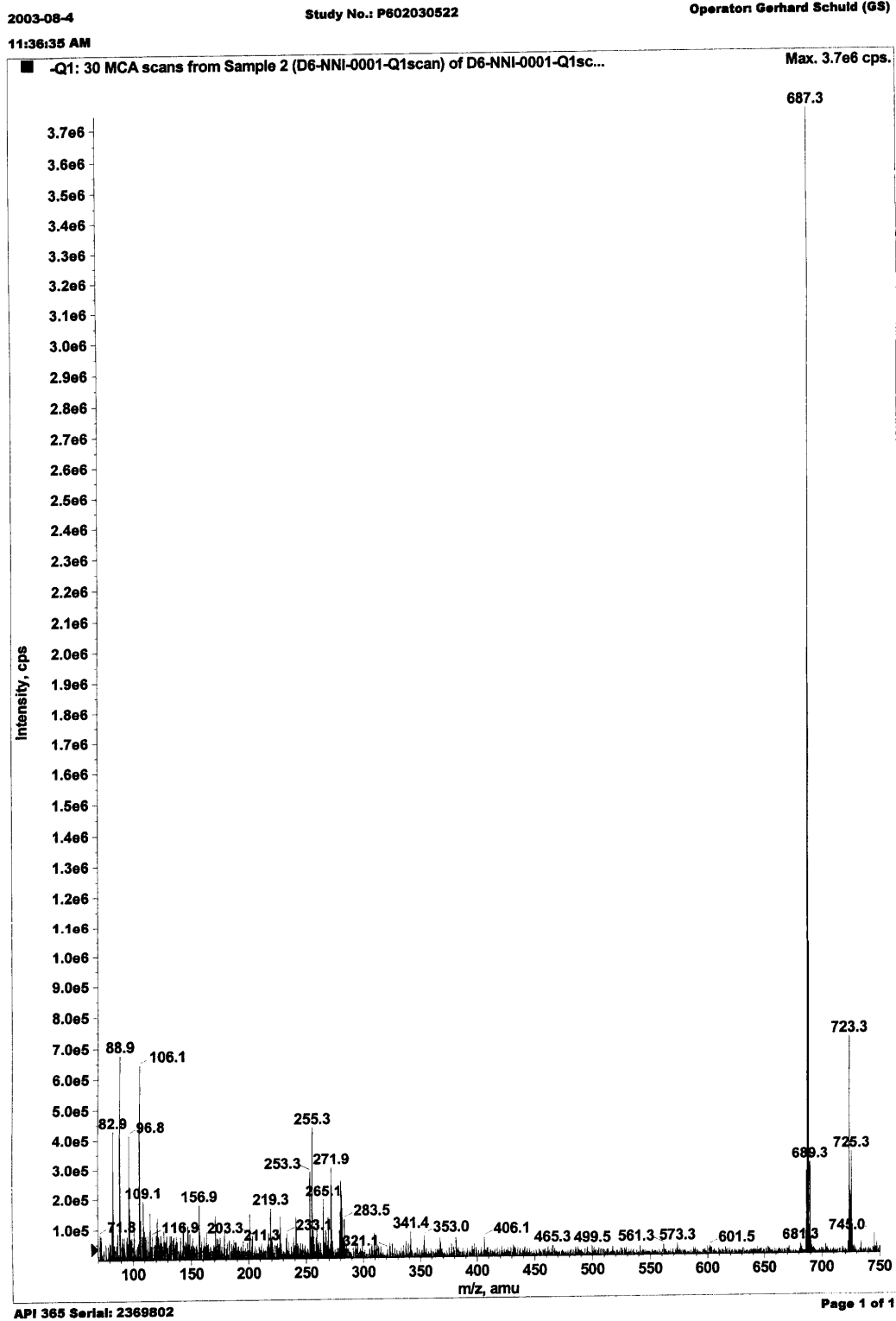
Appendix 6: Precursor Ion Mass Spectrum of A-1



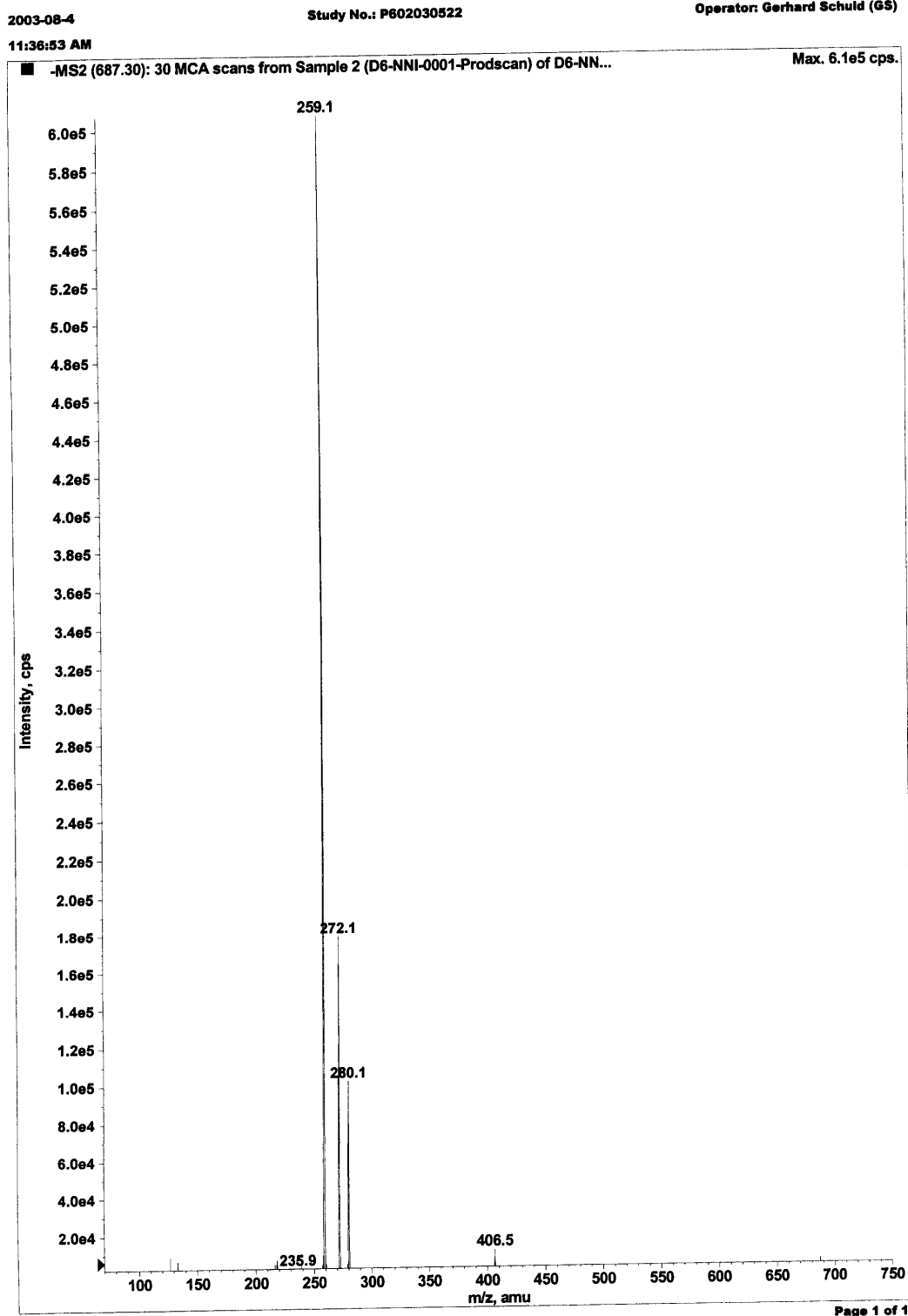
Appendix 7: Product Ion Mass Spectrum of A-1 (Fragment $m/z = 555.5$)



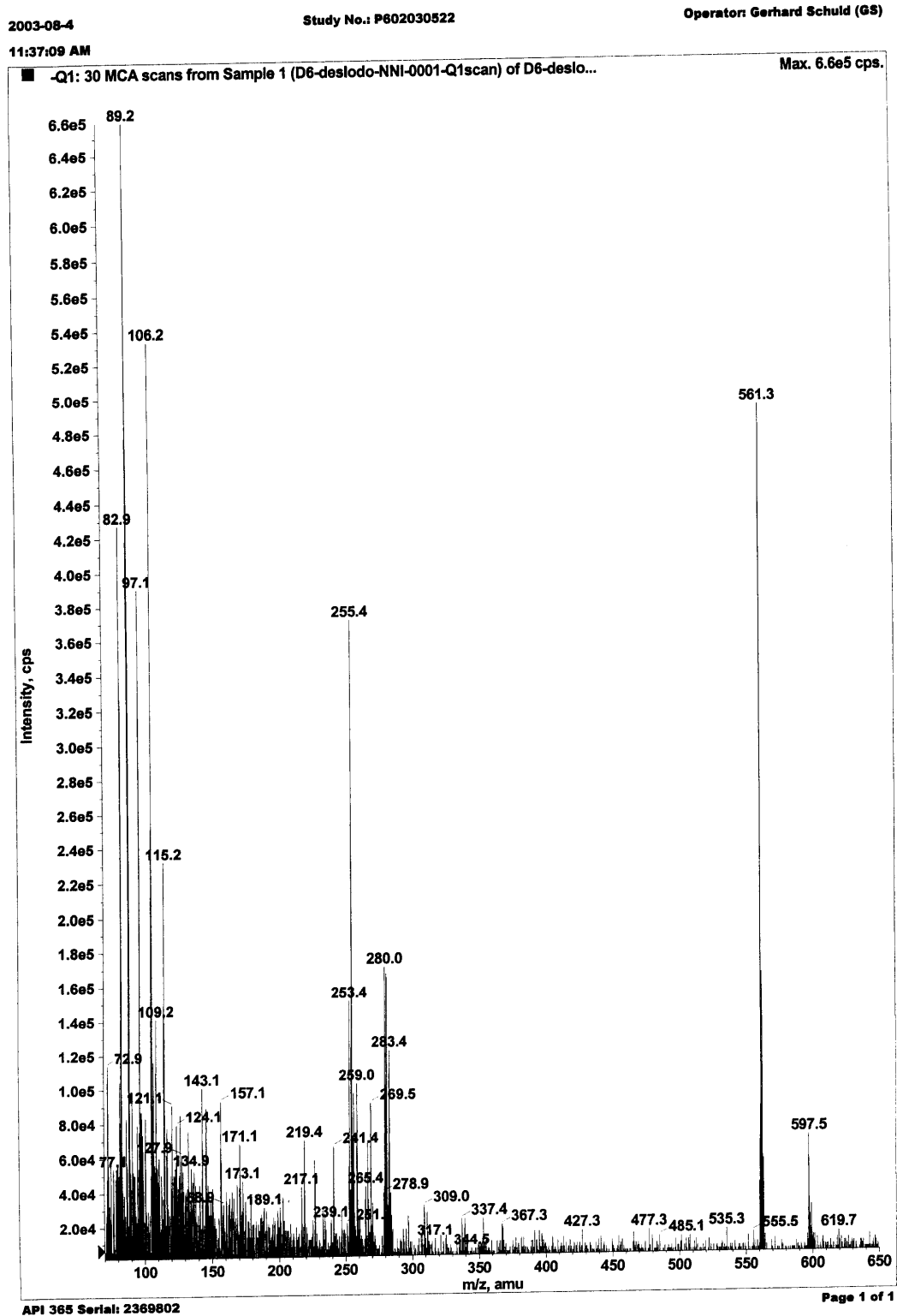
Appendix 8: Precursor Ion Mass Spectrum of d6-NNI-0001



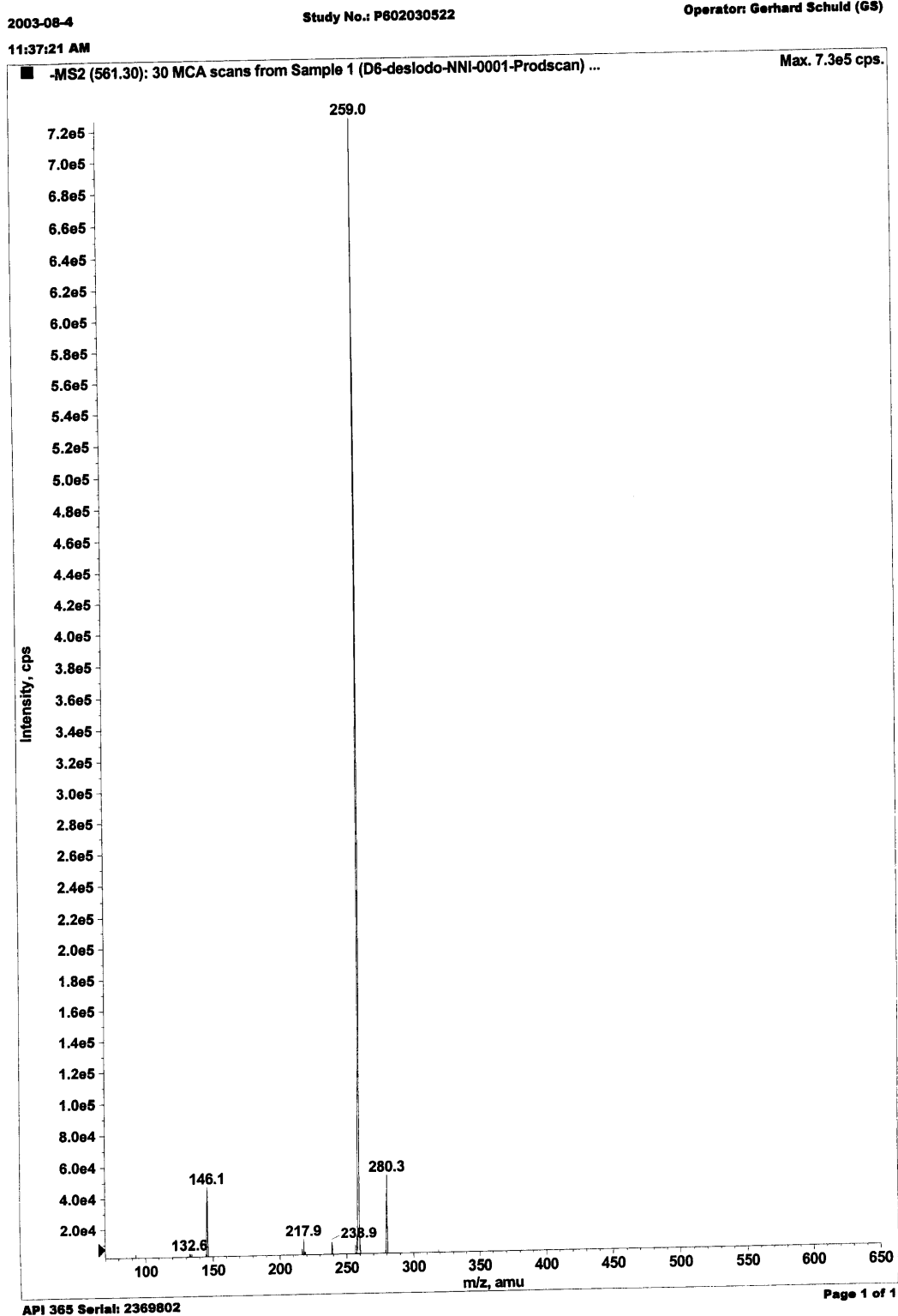
Appendix 9: Product Ion Mass Spectrum of d6-NNI-0001 (Fragment m/z = 687)



Appendix 10: Precursor Ion Mass Spectrum of d6-NNI-0001-des-iodo



Appendix 11: Product Ion Mass Spectrum of d6-NNI-0001-des-iodo
(Fragment m/z = 561)



Appendix 12: Detailed Instrument Settings

2003-08-4

Study No.: P602030522

Operator: Gerhard Schuid (GS)

12:15:31 PM

Acquisition Method

D6-NI0001

Mass Spec 12.009 min
Period 12.009 min
-MRM

Mass Spectrometer Method Properties

Period 1:

Scans in Period: 705
Relative Start Time: 0.00 msec
Experiments in Period: 1

Period 1 Experiment 1:

Scan Type: MRM (MRM)
Polarity: Negative
Scan Mode: N/A
Ion Source: Turbo Spray
Resolution Q1: UNIT
Resolution Q3: UNIT
Intensity Thres.: 0.00 cps
Smart Settling: On
Settling Time: 0.0000 msec
MR Pause: 5.0070 msec
MCA: No
Step Size: 0.00 amu

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
681.2C	254.0C	250.00	DE	-21.00	-21.00
			FE	-180.00	-180.00
			EE	-9.00	-9.00
			CEE	-22.00	-22.00
			CE	-23.00	-23.00
			CXF	-20.00	-20.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
555.2C	254.0C	250.00	DE	-21.00	-21.00
			FE	-180.00	-180.00
			EE	-8.50	-8.50
			CEE	-16.00	-16.00
			CE	-26.00	-26.00
			CXF	-10.00	-10.00

Appendix 12: Detailed Instrument Settings (contd)

2003-08-4

Study No.: P602030522

Operator: Gerhard Schuld (GS)

12:15:31 PM

Acquisition Method

D6-NNI0001

Mass Spec 12.009 min
Period 12.009 min
-MFM

Appendix 12: Detailed Instrument Settings (contd)

2003-08-4

Study No.: P602030522

Operator: Gerhard Schuld (GS)

12:15:31 PM

Acquisition Information:

Acquisition Method: D6-NNI0001.dam
 Created: Monday August 04 2003 12: 14: 46 PM
 Last Modified: Monday August 04 2003 12: 14: 46 PM
 Comment:
 Synchronization Mode: IC Sync
 Auto-Equilibration: Off
 Acquisition Duration: 12min1sec
 Number Of Scans: 705
 Periods In File: 1
 Acquisition Module: Acquisition Method
 Software version: Analyst 1.3.1

Period 1:

Scans in Period: 705
 Relative Start Time: 0.00 msec
 Experiments in Period: 1

Period 1 Experiment 1:

Scan Type: MRM (MRM)
 Polarity: Negative
 Scan Mode: N/A
 Ion Source: Turbo Spray
 Resolution Q1: UNIT
 Resolution Q3: UNIT
 Intensity Thres.: 0.00 cps
 Smart Settling: On
 Settling Time: 0.0000 msec
 MR Pause: 5.0070 msec
 MCA: No
 Step Size: 0.00 amu

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Param	Start	Stop
681.2C	254.00	250.0C	DE	-21.00	-21.00
			FE	-180.00	-180.00
			EE	-9.00	-9.00
			CEE	-22.00	-22.00
			CE	-23.00	-23.00
			CXE	-20.00	-20.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Param	Start	Stop
555.2C	254.00	250.0C	DE	-21.00	-21.00
			FE	-180.00	-180.00
			EE	-8.50	-8.50
			CEE	-16.00	-16.00
			CE	-26.00	-26.00
			CXE	-10.00	-10.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Param	Start	Stop
687.2C	259.00	250.0C	DE	-26.00	-26.00
			FE	-200.00	-200.00
			EE	-8.00	-8.00
			CEE	-26.00	-26.00
			CE	-32.00	-32.00
			CXE	-12.00	-12.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Param	Start	Stop
561.3C	259.00	250.0C	DE	-26.00	-26.00
			FE	-200.00	-200.00
			EE	-6.00	-6.00
			CEE	-18.00	-18.00
			CE	-30.00	-30.00
			CXE	-12.00	-12.00

Parameter Table (Period 1 Experiment 1):

NEB: 12.00
 CUR: 12.00
 TEM: 300.00
 CAD: 4.00
 IS: -4200.00

Appendix 13:
GLP-Certificate



Landesinstitut für Umwelt und Naturschutz, Landwirtschaft und
Verbraucherschutz
des Landes Nordrhein-Westfalen

Postanschrift: 40190 Düsseldorf

Aktenzeichen: VI-3- 31.11.91.01

GLP-Bescheinigung

Bescheinigung

Certificate

Hiermit wird bestätigt, dass die Prüfeinrichtung

It is hereby certified that the test facility

in D-40789 Monheim, Building 6610
(Ort, Anschrift)

in D-40789 Monheim, Building 6610
(location, address)

der Bayer CropScience Development
Residues, Operator and Consumer Safety (ROCS)
(Firma)

Of Bayer CropScience Development
Residues, Operator and Consumer Safety (ROCS)
(company name)

vom 30. Januar - 31. Januar 2003, 27. Juni 2003 und
20. August 2003
(Datum)

on 30 until 31 January 2003, 27 June and 20
August 2003
(date)

von der für die Überwachung zuständigen Behörde
über die Einhaltung der Grundsätze der Guten
Laborpraxis inspiziert worden ist.

was (were) inspected by the competent authority
regarding compliance with the Principles of Good
Laboratory Practice.

Es wird hiermit bestätigt, dass folgende Prüfungen in
dieser Prüfeinrichtung nach den Grundsätzen der
Guten Laborpraxis durchgeführt werden.

It is hereby certified that following studies in this
test facility are conducted in compliance with the
Principles of Good Laboratory Practice.

Appendix 13: GLP-Certificate (contd)

Kategorie 1	category 1
Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen	physical-chemical testing
Kategorie 4	category 4
Ökotoxikologische Prüfungen zur Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen	environmental toxicity studies on aquatic and terrestrial organisms
Kategorie 5	category 5
Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung	studies on behaviour in water, soil and air; bioaccumulation
Kategorie 6	category 6
Prüfungen zur Bestimmung von Rückständen	residue studies
Kategorie 8	category 8
Analytische Prüfungen an biologischen Materialien	analytical and clinical chemistry testing

Düsseldorf, 29. Januar 2004

Im Auftrag


(Prof. Dr. Heinrich David)

