Validation Data for the Analysis of 1-Napthylacetic acid and 1-Naphtylacetamide in tomato and zucchini Using Mini-Luke, Ethyl Acetate and QuEChERS methods Followed by LC-QqQ-MS/MS

UNIVERSITY OF ALMERIA

CITE I
Dpto. Hidrogeología y Química Analítica
Ctra. Sacramento s/n
La Cañada de San Urbano
04120 ALMERÍA
SPAIN

Tel (+34) 950 01 50 34/55 31

PESTICIDE RESIDUE LABORATORY
AGRO-FOOD ANALYSIS SERVICE

Dirección General de Innovación Agraria y Ganadería
Consellería de Agricultura, Pesca y Alimentación
GENERALITAT VALENCIANA
Pintor Goya, 8, 46100 Burjassot-VALENCIA
SPAIN

Tel (+34) 963 631 551

CONTENTS

1. Aim and Scope

This report describes the validation data for the analysis of plant growth regulators (PGR) 1-napthylacetic acid (NAA) and 1-naphtylacetamide (NAAm) in tomato and zucchini matrices by three different multiresidue methods.

2. Short Description

The analysis of NAA and NAAm was performed by using Mini-Luke, Ethyl Acetate and buffered acetate QuEChERS methods.

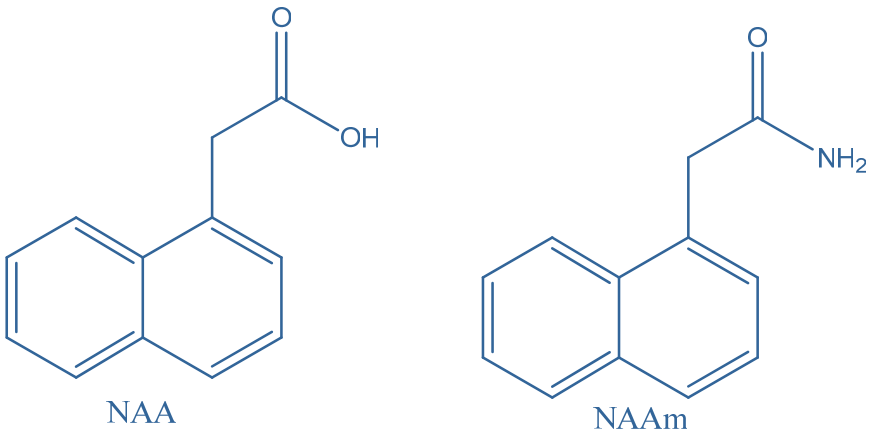


Fig. 1: Chemical structures of 1-napthylacetic acid (NAA)and 1-naphtylacetamide (NAAm).

In Mini-Luke method, the homogeneous sample is extracted with acetone followed by partition with dichlorometane / petroleum ether (1:1). The mixture is centrifuged and an aliquot of the extract is concentrated to dryness. The residue is redissolved with H_2O:MeCN (9:1), filtered in PTFE (0.45 µm) and injected in LC-MS/MS [1].

In Ethyl Acetate method, the homogeneus sample is extracted with EtAC using $MgSO_4$ and NaCl. The mixture is shaken, centrifuged and an aliquot of the extract is concentrated to dryness. The residue is redissolved with H_2O:MeCN (9:1), filtered in PTFE (0.45 µm) and injected in LC-MS/MS [2].

In QuEChERS acetate method [3], the homogeneus sample is extracted with MeCN (1% acetic acid) using $MgSO_4$ and NaAc. The mixture is shaken, centrifuged and an aliquot of the extract is cleaned-up with PSA and $MgSO_4$. An aliquot of the purified extract is concentrated to dryness, redissolved with H_2O:MeCN (9:1) and injected in LC-MS/MS.

3. Apparatus and Consumables

- Sample processing equipment, e.g. Sammic stainless-steel grinder.
- Homogeinizer, e.g. Polytron PT 10-35.
- Automatic axial extractor, e.g. AGYTAX®, Cirta Lab. S.L.
- Centrifuge suitable for Teflon flask of 50 and 15 mL with screw caps, e.g. Sarstedt, and capable of achieving at least 3500 rpm.
- Automatic pipettes, suitable for handing volumes of 10 to 100µl, 50 to 200 µl, 200 to 1000 µl, 1000 to 5000 µl.
- Pipettes of 5 and 10 mL.
- Test tubes 10 to 100 mL.
- Syringe, e.g. 2 mL, disposable syringes.
- PTFE syringes filters, 0.45 µm pore size.
- Concentration Workstation.
- Injection vials, 1.5 mL suitable for LC auto-sampler.

4. Chemicals

- TPP (triphenylphosphate), 1-napthylacetic acid (NAA) and 1-naphtylacetamide (NAAm) standards, e.g. Dr. Ehrenstorfer.
- Acetone, petroleum ether, dichloramethane and ethyl acetate of GC residue analysis grade.
- Acetonitrile, and methanol of LC residue analysis grade.
- Sodium acetate, sadium chloride pa, e.g. from Baker.
- Acetic acid, Formic acid pa, e.g. from Fluka.
- Milli-Q water e.g. Direct-Q™ 5 Ultrapure Water System from Millipore.
- TPP solution at 25mg/L was prepared in MeOH.
- Stock solutions of PGR were prepared in MeCN.
- PSA for dSPE, e.g. from Supelco.
- Magnesium sulphate anhydrous, for example from Merck. Phthalates and wet can be removed in a muffle furnace by heating to 550°C during 5 hours.

5. Procedure

5.1. Sample preparation

Sample was prepared according to the *"Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed" (Document No. SANCO/10684/2009)* [4].
Following this document, tomato and zucchini samples were perfectly homogenised by grinding finely at its arrival to the laboratory.
Sample was frozen for its storage immediately after grinding.

5.2. Spiking Procedure for Method Validation

Commodities employed for method validation should not contain any of the plant growth regultators analyzed. Organically grown samples are recommended. Of the analyzed samples no one was detected containing NAA naturally.

The validation method was performed at two fortification levels (0.02 mg/Kg and 0.1 mg/Kg for NAAm); (0.05 mg/Kg and 0.1 mg/Kg for NAA) in five fortified samples (n=5).
Blanks were spiked through the addition of appropriate standard solution and blending for 30 minutes.

5.3. Extraction procedures

5.3.1. Mini-Luke Method

1. Weigh 5 g ± 0.01 g of subsample in a wide-necked Teflon flask suitable for the centrifuge.
2. Add 10 mL of acetone and 3g of NaCl.
3. Add 100 µL TPP surrogate compound.
4. Blend the sample with Polytron homogeneizer for 30 sec.
5. Add 20 mL of petroleum ether – dichlorometane (1:1) mixture.
6. Blend again the sample with Polytron homogeneizer for 30 sec.
7. Centrifuge for 5 min at 3500 rpm.
8. Transfer 10 mL extract into a test tube. Evaporate to dryness under a nitrogen stream.
9. Add 2 mL H_2O:MeCN (9:1).
10. Vortex sample to mix it properly.
11. Filter slowly with 0.45µm PTFE into an injection vial suitable for LC-MS/MS.

5.3.2. Ethyl acetate method

1. Weigh 10 g ± 0.05 g of subsample in a wide-necked Teflon flask suitable for the centrifuge.
2. Add 10 mL of EtAc and 200 µL TPP surrogate compound.
3. Shake by hand during 3 sec.
4. Add 1.5g of NaCl and 8g of $MgSO_4$.
5. Shake with the automatic axial extractor during 15 min.
6. Centrifuge for 5 min at 3500 rpm.
7. Transfer 0.5 mL extract into vial and evaporate to dryness with mild N_2 stream.
8. Add 0.5 mL H_2O:MeCN (9:1).
9. Vortex vial during 30 sec.
10. Filter with 0.45µm PTFE into the injection vial for LC-MS/MS.

5.3.3. Acetate buffered QuEChERS

1. Weigh 15 g ± 0.1 g of subsample in a wide-necked Teflon flask suitable for the centrifuge.
2. Add 15 mL of MeCN (1% Hac) and 200 µL TPP surrogate compound
3. Shake by hand during 30 sec.
4. Add 2.5 g of NaAc and 6 g of $MgSO_4$.
5. Shake with the automatic axial extractor during 16 min.
6. Centrifuge for 5 min at 3500 rpm.
7. Transfer 5 mL of extract into dSPE tube containing 750 mg of $MgSO_4$ and 250 mg of PSA.
8. Vortex vigorously during 30 seconds.
9. Take up 0.5 mL of extract into a vial for LC-MS/MS and evoporate to dryness with mild N_2 stream.
10. Recompose with 0.5 mL H_2O:MeCN (9:1) and vortex the vial during 30 sec.
11. Filter with 0.45µm PTFE into the injection vial for LC-MS/MS.

5.4. Measurement

5.4.1. Instrumentation

- Agilent 6410 triple quad LC-MS system
- Agilent TOF MS
- Agilent 1200 HPLC

5.4.2. Analytical Conditions for the LC/QqQ

Settings for liquid chromatography:

- Mobile phase:
  - A: 0.05% formic acid MilliQ water
  - B: MeOH
- Injection volume: 10 µL
- Flow: 0.5 mL/min.
- Column: Zorbax C18 3 x 250 mm,di= 5 µm.
- Elution gradient:

Time (min)	B (%)
0	10
5	50
10	50
11	70
16	70
17	100
22	100
22.1	10
29	10

Settings for mass spectrometry:

The ESI source was operated in positive and negative ionization mode and its parameters were as follows:

Gas temperature	325°C
Gas flow	9 L/min
Nebulizer gas	40 psi
Capillary voltaje	4000 V

Tree time windows with a ±1min overlapping range around the borders were constructed, which have different polarities. The time windows are:

Time (min)	Polarity
0-15	Positive
15.1-19	Negative
19.1-22	Positive

Compound	Retention time (min)	Parent ion	Fragmentor (V)	Product ion 1	CE 1 (V)	Product ion 2	CE 2 (V)	Ratio
NAAm	14.07	186.2	120	141.1	15	115.1	20	3.7
NAA	16.35	185.1	60	140.9	5	116.7	-	-
TPP	21.07	322.0	120	77.2	35	152.2	30	77.7

5.4.3. Confirmation of NAA by LC-ESI(-)TOF

Settings for liquid chromatography:

- Mobile phase:
  - A: MeOH
  - B: 0.1% formic acid MilliQ water
- Injection volume: 20 µL
- Flow: 0.6 mL/min.
- Column: Zorbax Eclipse C8 4.6 x150 mm, id= 5 µm.
- Elution gradient:

Time (min)	B (%)
0	90
1	90
11	0
17	0
17.1	90
24	90

Settings for time of flight (TOF) mass spectrometry:

Compound	Retention time (min)	$[M+H]^+$ (m/z)	Fragment ion (m/z)
NAA	3.8	185.0608	141.0710

Polarity	Negative
Gas temperature	325ºC
Gas flow	9 L/min
Nebulizer pressure	40 psi
Capillary voltaje	4000 V
Fragmentor	120 V
Skimmer	60 V
OCT 1RF	250 V

TOF-MS internal mass calibration was performed using a calibration solution (ES-TOF reference mass solution, Agilent) that provided m/z 119.0363 and 966.0000 reference masses in negative mode.
Expected retention time for NAA: 3.8 min

6. Evaluation of results

In the table below are shown the results for the mean recovery (n=5) and RSD (%) for the quantified pesticide at both levels and by the three tested multiresidue methods.

Level / method	Analyte	TOMATO		ZUCCHINI	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
50 ppb **Mini-Luke**	NAA	107.0	10.1	86.7	1.8
20 ppb **Mini-Luke**	NAAm	73.8	15.1	93.1	2.4
50 ppb **EtAc**	NAA	98.2	10.7	95.9	4.4
20 ppb **EtAc**	NAAm	99.4	1.5	76.2	1.6
50 ppb **QuEChERS**	NAA	81.9	7.2	84.9	7.1
20 ppb **QuEChERS**	NAAm	88.4	4.6	76.8	3.7
100 ppb **Mini-Luke**	NAA	92.7	9.7	98.3	6.3
100 ppb **Mini-Luke**	NAAm	95.4	5.5	102.1	3.5
100 ppb **EtAc**	NAA	106.9	4.5	82.6	4.5
100 ppb **EtAc**	NAAm	97.8	4.0	91.6	2.9
100 ppb **QuEChERS**	NAA	81.5	5.8	76.2	7.3
100 ppb **QuEChERS**	NAAm	92.5	5.0	89.5	1.4

The validation results are acceptable for the recovery and RSD values by the Document No. SANCO/10684/2009 [4].
Linearity was evaluated in matrix-matched calibration in both matrices and by three tested method as shown in Appendix I. Appendix II shows a typical chromatogram for the analysis of the compounds and the required change in polarity of ESI source.

Method	Sample	LODs (ppb)	
		NAAm*	NAA**
Luke	Zucchini	5.0	3.2
	Tomato	6.0	6.7
EtAc	Zucchini	1.5	10.1
	Tomato	3.0	8.3
QuEChERS	Zucchini	4.8	6.0
	Tomato	5.0	6.5

* Refered to qualifier ion ** Refered to quantifier ion

Calculated LODs which are shown in the former table are in agreement to the MRLs permitted for these compounds [5].

However, NAA showed to have only one transition in LC-MS/MS (185.1→140.9) independently of the concentration of residues in the studied range. Accurate mass measurments by LC-TOF MS can be applied for confirmation purposes of NAA residues as seen in Appendix III. At the selected conditions positive findings were confirmed by LC-TOF at 20 ppb level.

7. Conclusion

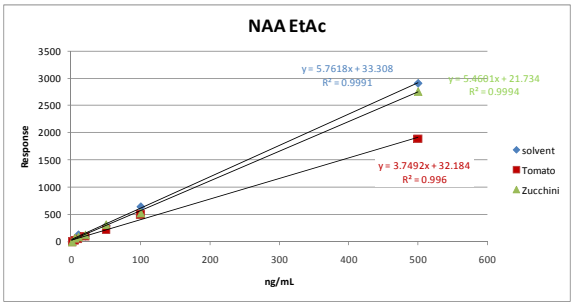
The results obtained are considered acceptable within the studied range. As these multiresidue methods are used at the present time by a large number of laboratories within the European Pesticide Residues Monitoring Programme, this survey can help to improve the analysis of PGR widely used in fruits and vegetables by inclusion of such analytes in these methods.

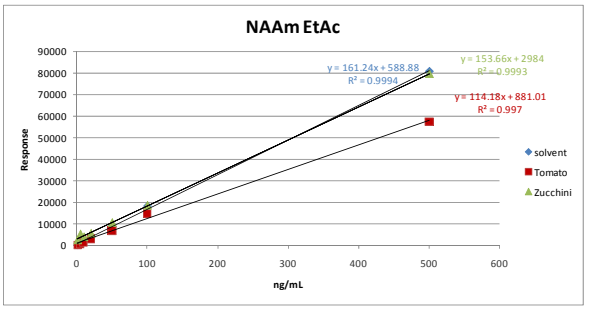
8. References

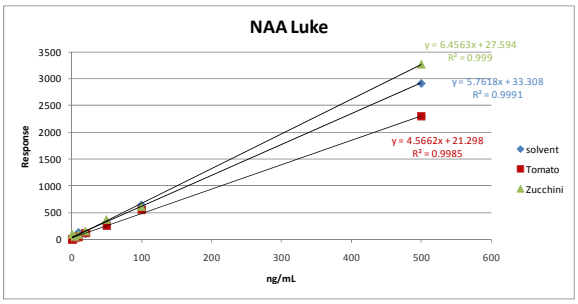
[1] http://www.crl-pesticides.eu, EURL-FV Method information and validation data.
[2] A. Andersson and H. Pålsheden (1991) Comparison of the efficiency of different GLC multi-residue methods on crops containing pesticide residues. Fresenius' Journal of Analytical Chemistry 339, 6, 365-367.
[3] S. Lehotay et al., (2007): Determination of Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate: Collaborative Study. Journal of AOAC International 90, 2, 485-520.
[4] Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed (Document No. SANCO/10684/2009).
[5] http://ec.europa.eu/sanco_pesticides/public/index.cfm

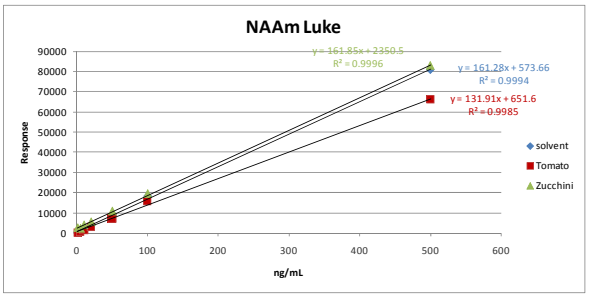
APPENDIX I

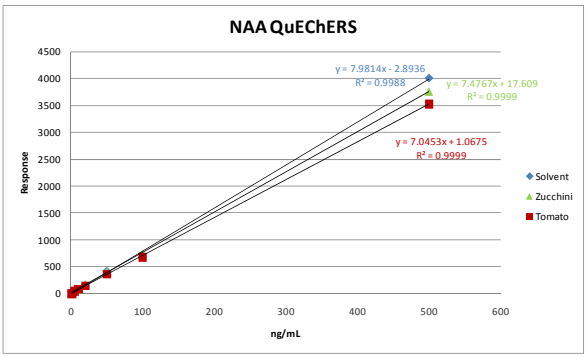
Linearity and matrix effect.

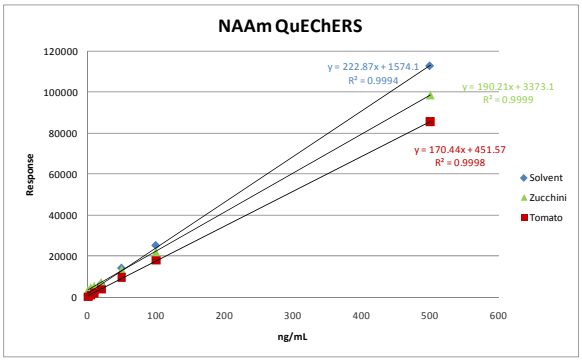






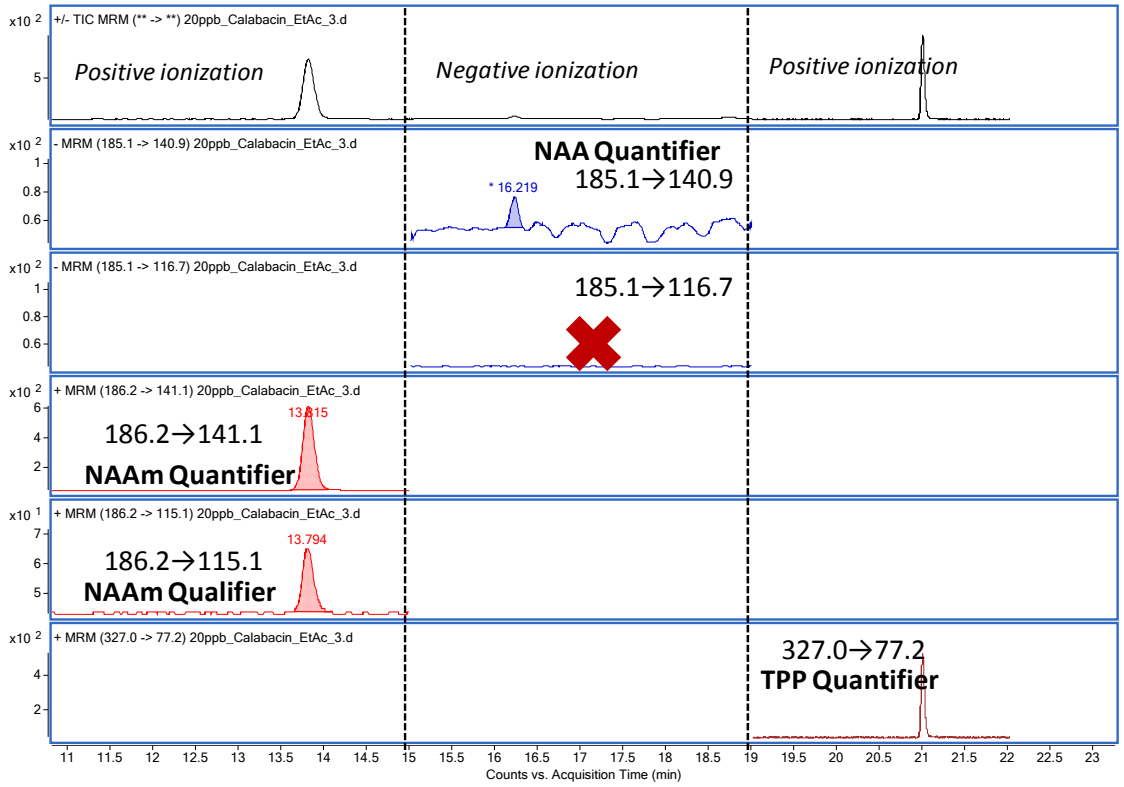






APPENDIX II

Typical LC-(QqQ)MS/MS chromatogram: Zucchini spiked at 20 ppb with NAA and NAAm using EtAc extraction, TPP surrogate compound.



APPENDIX III

LC-TOF: Extracted ion chromatogram for the confirmation of NAA residues at 20 ppb in incurred zucchini extract (buffered acetate QuEChERS extraction).