Development and application of a LC-MS/MS method for analysing bound nitrofuran residues in meat, including new marker chemistries

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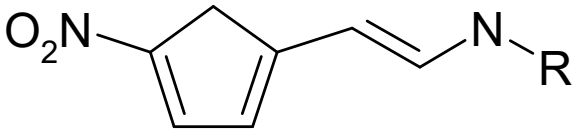
The Irish Agriculture and Food Development Authority

Nitrofuran Background

- Class of synthetic, broad spectrum antibiotic drugs
- Previously licensed uses:
  - Veterinary drugs for the prevention and control of disease
  - Feed additives for growth stimulation
- Characteristic 5-nitrofuran ring with various substituents in the 2-position



- Exact mode of antibacterial activity unknown but thought to inhibit several bacterial enzymatic systems
- Nitrofurans are prodrugs meaning that they are activated through metabolism

Nitrofuran Metabolism

- Nitrofurans are administered in their parent form
  - Short half-lives *in vivo*
  - Undetectable after a few hours
  - Rapidly metabolised to form highly stable **protein-bound** metabolites

- Metabolites persist for long periods of time and hence, are used as marker residues for nitrofuran analysis

- Pose a threat to consumer safety:
  - Carcinogenic
  - Genotoxic
  - Mutagenic

Carcinogenicity of 5-Nitrofurans and Related Compounds With Amino-Heterocyclic Substituents

Samuel M. Cohen, E. Ertürk, A. M. Von Esch, A. J. Crovetti, George T. Bryan

Mutagenicity studies of a carcinogenic nitrofuran and some analogues

R Jung, J Y Le, F Wengenmayer, E Wolf, M Kramer

Genotoxic action of nitrofuran derivative drugs

G. N. Zolotareva, L. P. Akin'shina & L. U. Radchenko

Current Legislation

- **Banned from use** in food producing animals due to concerns regarding their **undesirable toxicological properties**.

- To ensure food safety and consumer protection, strict legislation exists to monitor the levels of the marker residues in food.

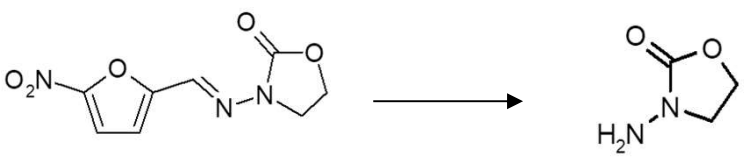
- Recently, the EU Reference Point for Action (RPA) has been reduced from 1.0 µg kg^{-1} to **0.5 µg kg^{-1}**.

Nitrofurans and their metabolites	0,5 (1)	0,5 µg/kg for each of the metabolites of furazolidone (AOZ or 3-amino-2-oxazolidinone), furaltadone (AMOZ or 3-amino-5-methylmorpholino-2-oxazolidinone), nitrofurantoin (AHD or 1-aminohydantoin), nitrofurazone (SEM or semicarbazide) and nifursol (DNSH or 3,5-dinitrosalicylic acid hydrazide)

Commission Regulation (EU) No. 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin

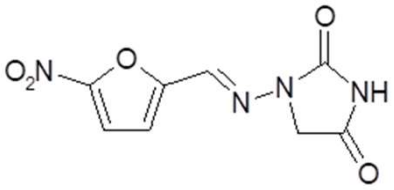
Chemistry: Nitrofuran Structures

- Majority of methodology focuses on **four main nitrofuran drugs** and their metabolites.

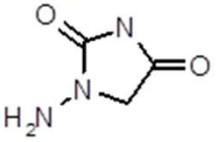


Furazolidone

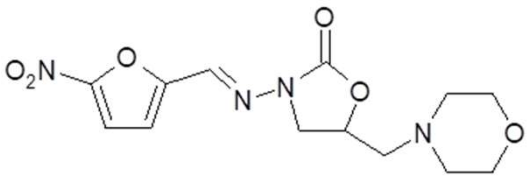
AOZ



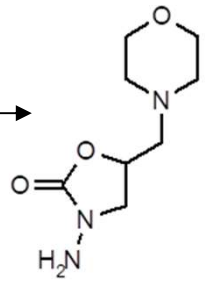
Nitrofurantoin



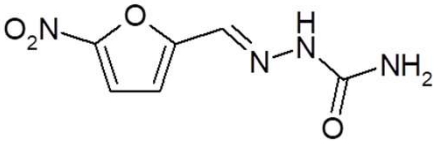
AHD



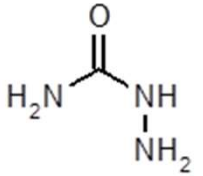
Furaltadone



AMOZ



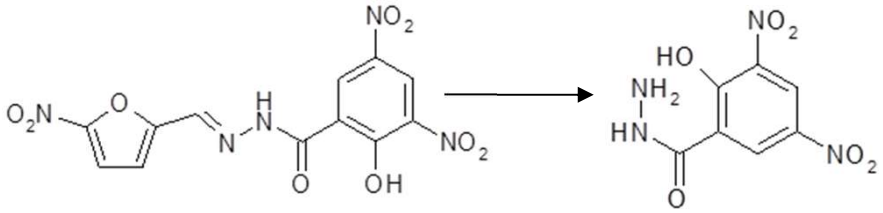
Nitrofurazone



SEM

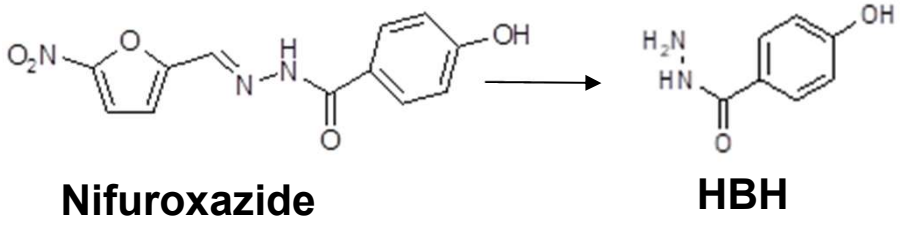
Additional 4 nitrofuran drugs

- Marker metabolites have been identified for **4 additional nitrofuran** drugs



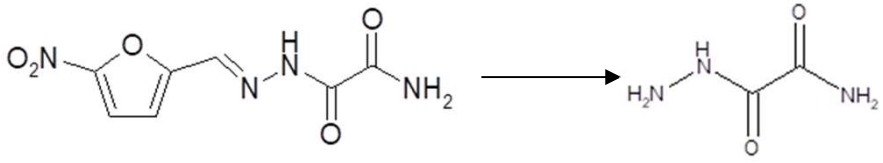
Nifursol → **DNSAH**

Added to the monitoring list in the EU

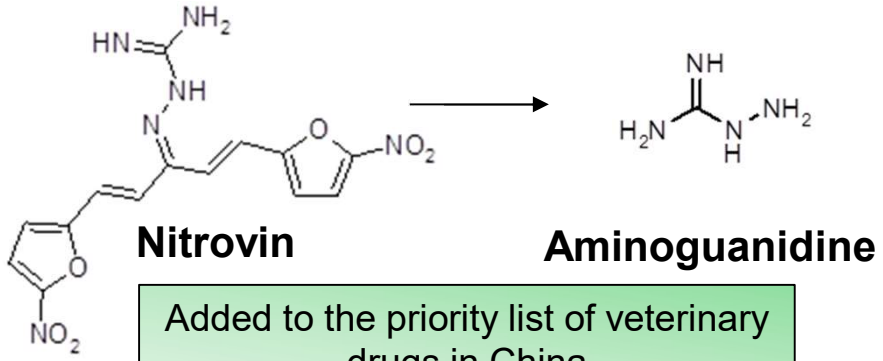


Nifuroxazide → **HBH**

Identified as a NF drug that can result in bound residues in animal tissues



Nifuraldezone → **Oxamic acid hydrazide**



Nitrovin → **Aminoguanidine**

Added to the priority list of veterinary drugs in China

Bound vs. Total Residues

- Nitrofuran residues can be monitored via “bound” analysis or “total” analysis

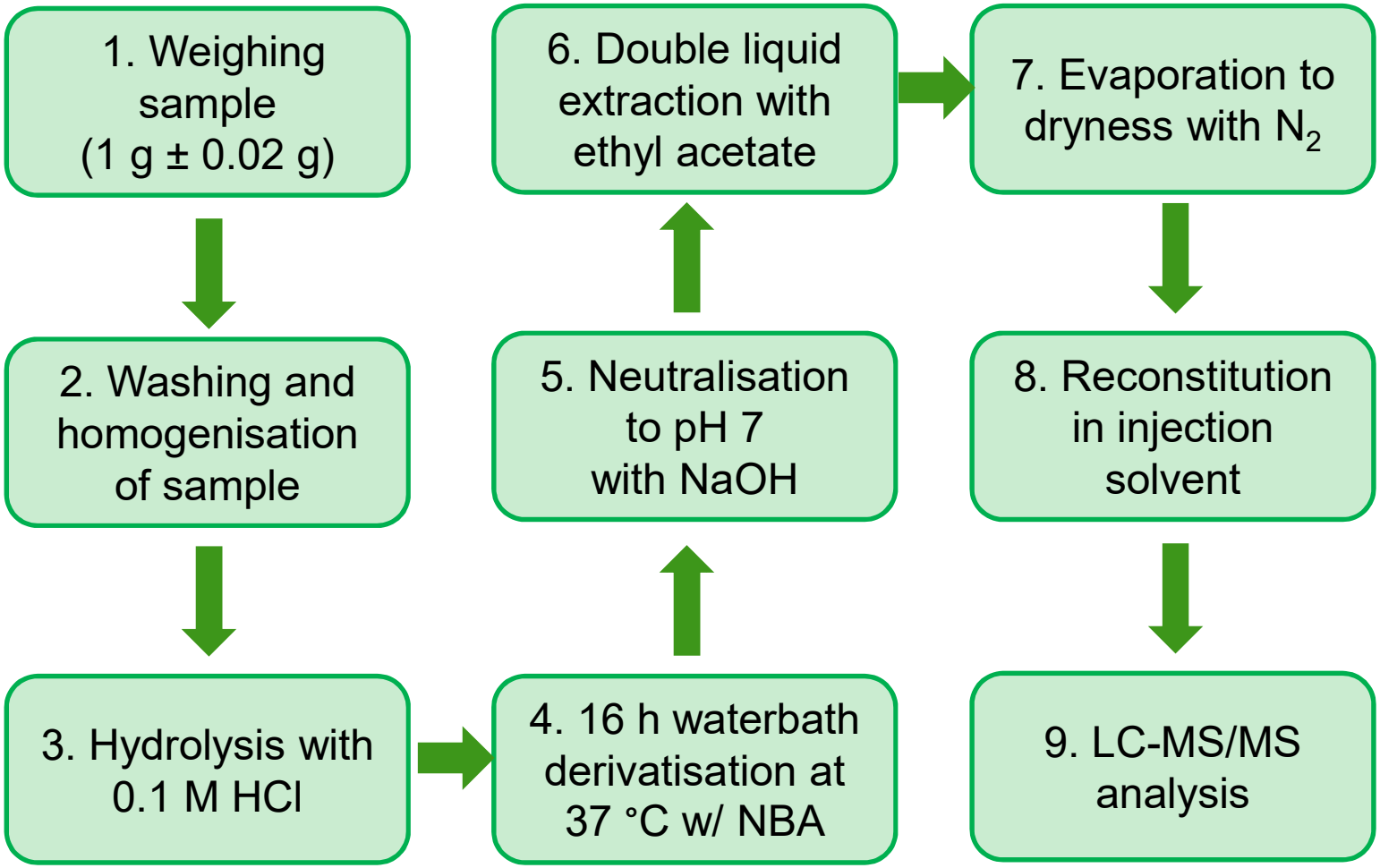
Bound

- Extensive washing with organic solvents
- Isolates the bound residues only
- Removes matrix interferences
- More sensitive analysis
- “Cleaner” analysis leads to less instrument downtime

Total

- No sample washing
- Bound and free residues (Total) brought through for analysis
- Quicker sample preparation
- Less sensitive analysis
- Shorter column lifetimes and more source contamination problems

Existing Bound Residue Method

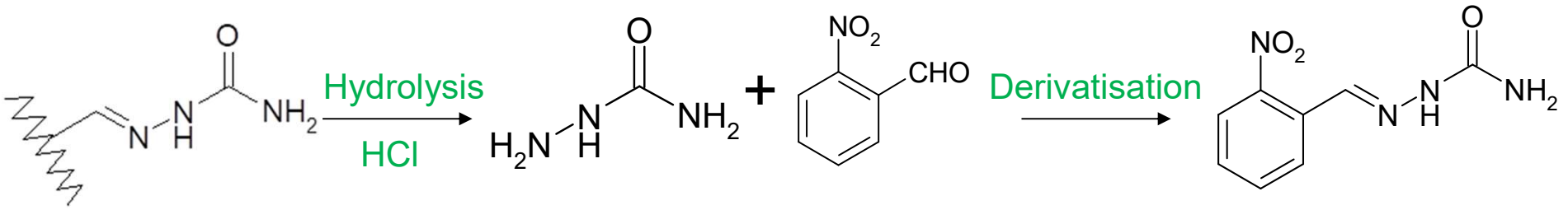


Standard sample preparation approach for the analysis of bound nitrofuran residues by LC-MS/MS

Derivatisation with NBA

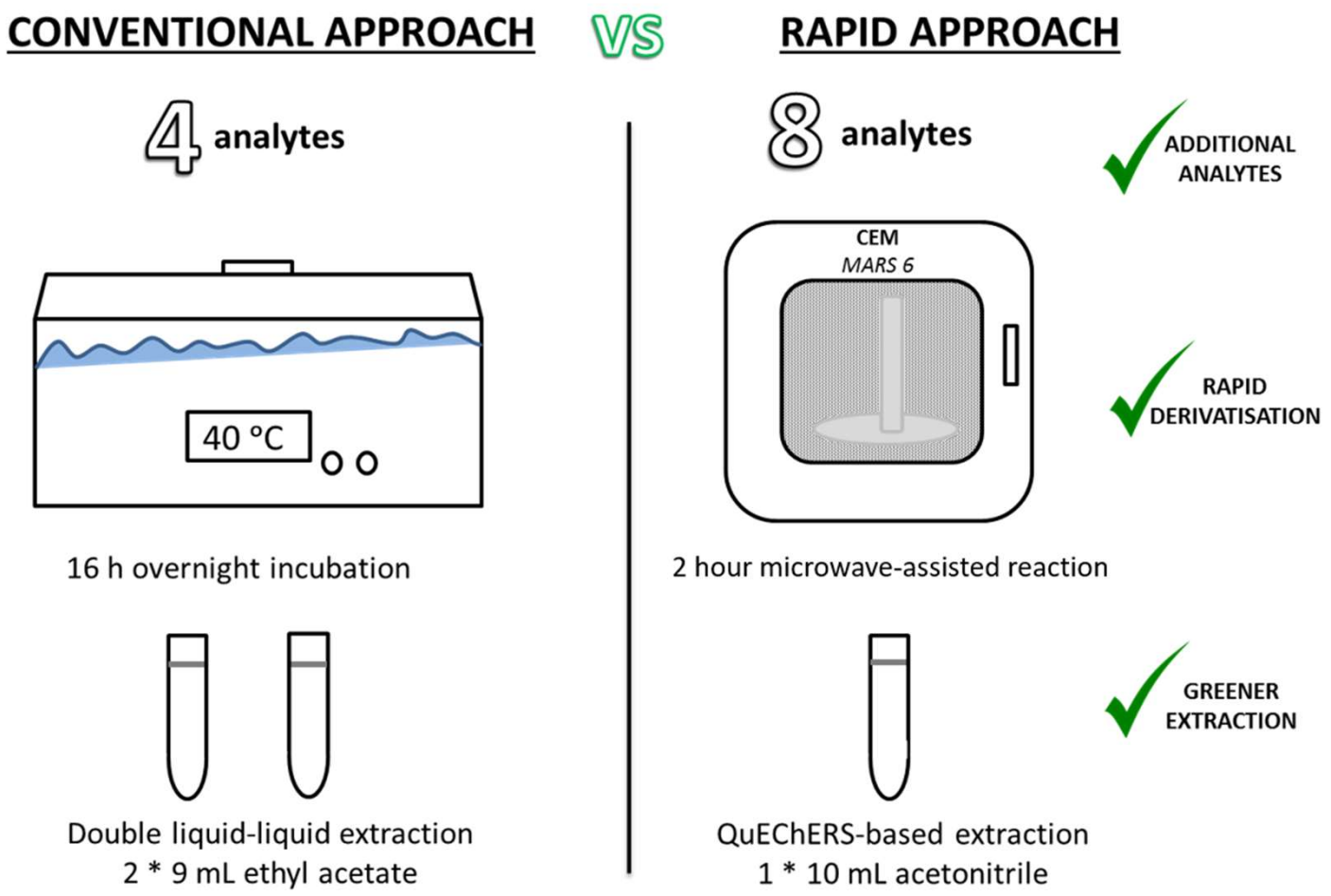
- To carry out nitrofuran analysis, the metabolites must undergo acid hydrolysis and subsequent derivatisation with nitrobenzaldehyde

- **Acid hydrolysis** → releases the bound metabolites from protein
- **Derivatisation** → produces nitrophenyl derivatives for detection, and prevents rebinding to the protein



Hydrolysis and derivatisation of tissue-bound SEM to form nitrophenyl derivative NPSEM

Method Development



LC Method Development
Why phenyl-hexyl?

- C_{18} column chemistry is very popular for the separation of four or fewer NF compounds, namely NPAHD, NPAOZ, AMOZ and NPSEM.
  - However, **C_{18} was unsuitable** for the eight compounds due to unsatisfactory peak shape and unresolved matrix interfering peaks.

- Phenyl-hexyl columns can provide **improved selectivity** for compounds containing aromatic functionalities.

- An **Agilent ZORBAX phenyl-hexyl** column was selected and **full chromatographic** separation was achieved for all eight compounds. Each analyte was successfully resolved from the other analytes and matrix interfering peaks.

Achieving selectivity

- Greater sensitivity introduced matrix interferences that were previously undetected

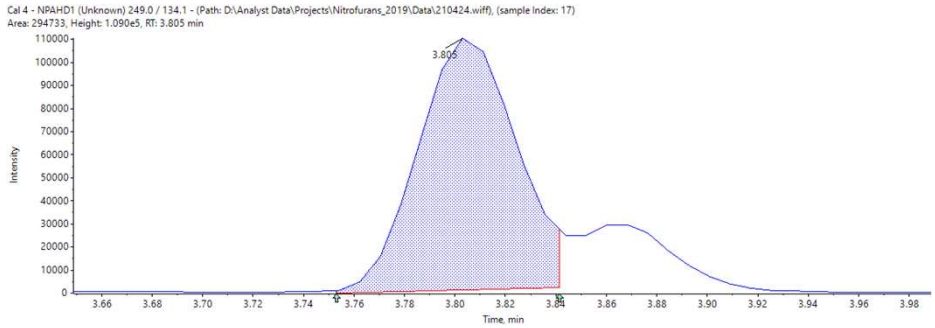


Fig A: Example of matrix interfering peak at the RT of NPAHD

- These interferences call for careful selection of column chemistries, mobile phase additives and LC gradients to achieve sufficient separation

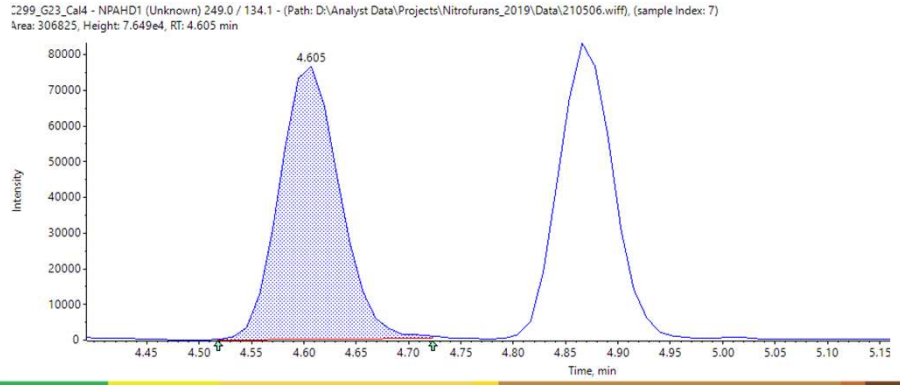
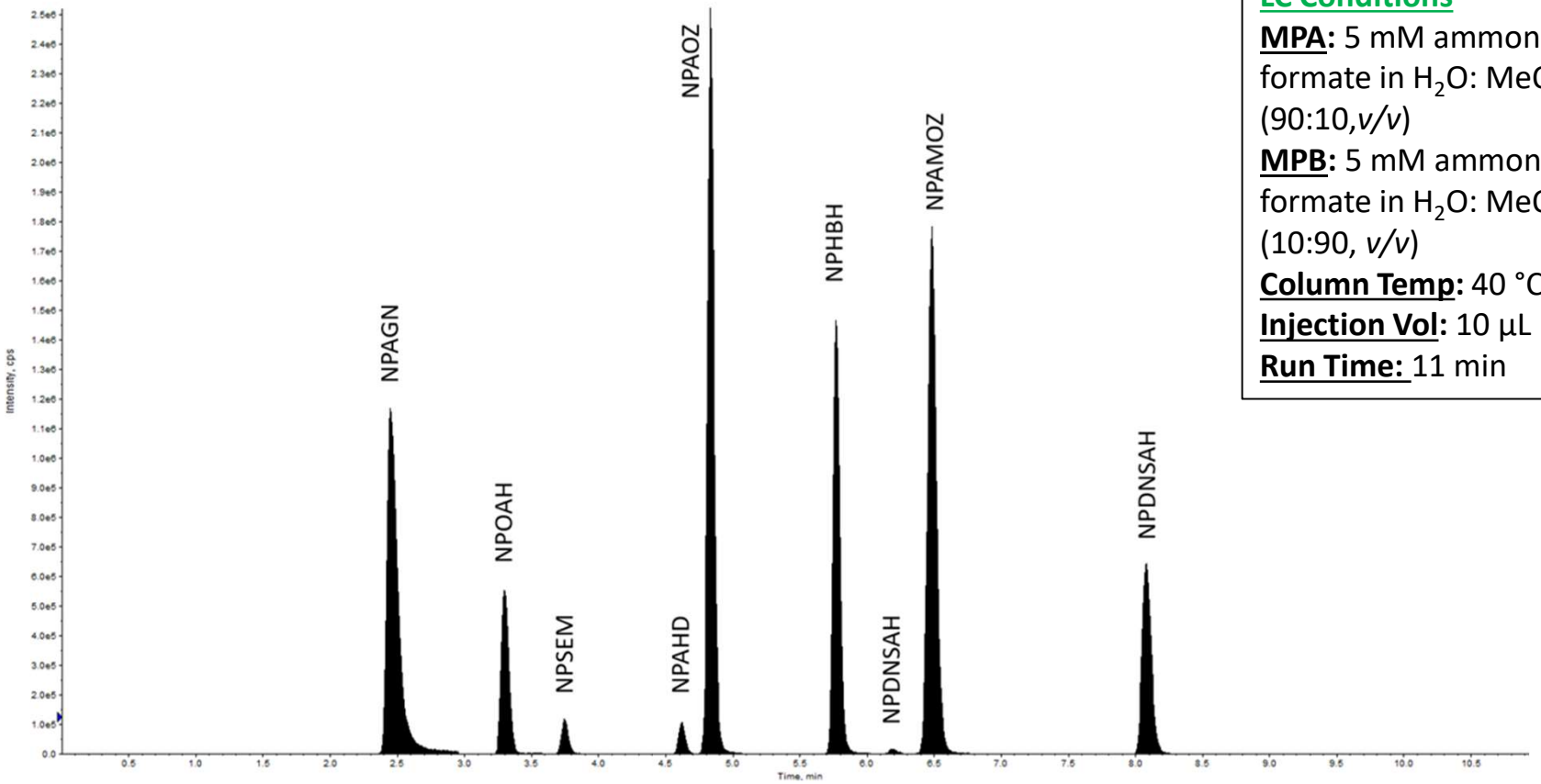


Fig B: Chromatographic separation achieved between NPAHD analyte and its interfering matrix peak

Chromatographic Separation



LC Conditions

MPA: 5 mM ammonium formate in H_2O: MeOH (90:10,*v/v*)

MPB: 5 mM ammonium formate in H_2O: MeOH (10:90, *v/v*)

Column Temp: 40 °C

Injection Vol: 10 µL

Run Time: 11 min

Chromatogram of a muscle sample spiked at 0.5 µg kg^{-1} for the quantifier transitions

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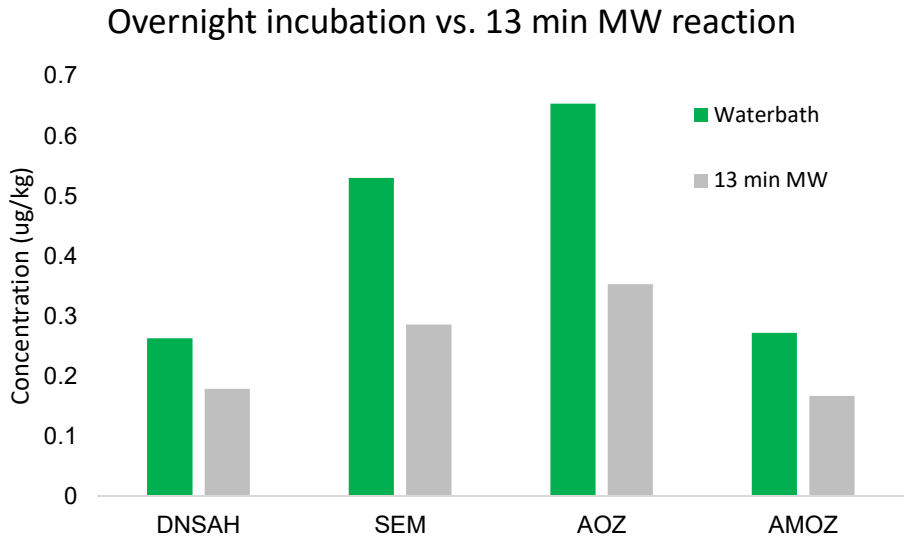
Microwave-assisted reaction

- Hydrolysis and derivatisation are key steps in nitrofuran analysis, and conventionally, the reaction is carried out as an overnight incubation in a waterbath for 16 h at 37 °C
  - Very time consuming
  - Limits sample throughput and longer sample turnaround times

- Developed an alternative approach using a microwave-assisted reaction, using **spiked material only**

- Proficiency test samples, with incurred material, highlighted a **major issue**
  - 13 min microwave derivatisation was **NOT** comparable to the overnight incubation when applied to real samples

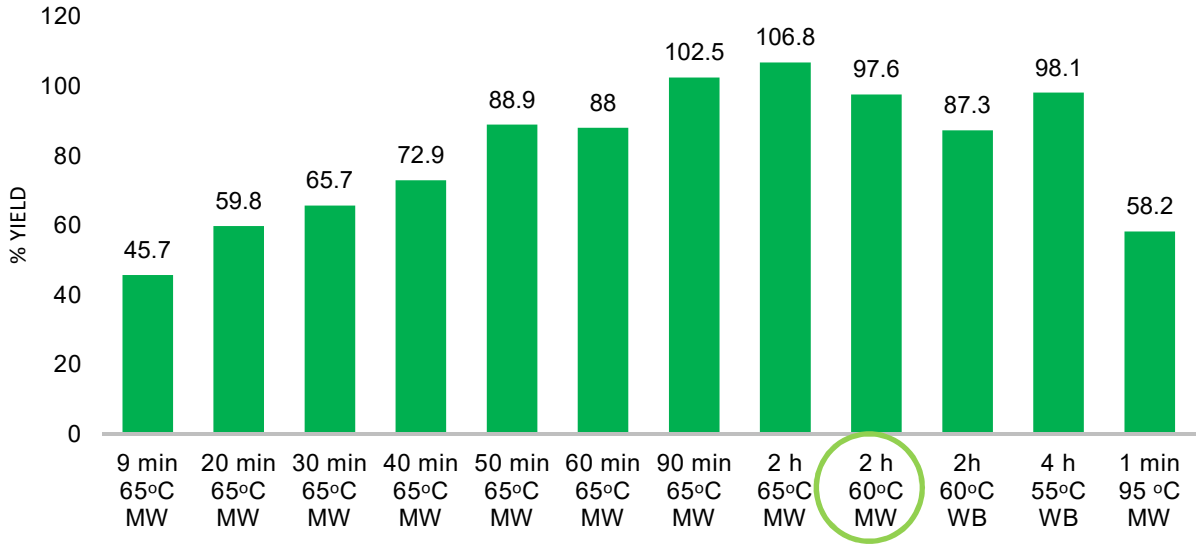


Further optimisation was needed

- Microwave parameters further optimised using **AOZ-incurred material**
- Various conditions were assessed, and their impact on analyte stability was evaluated.
- Final microwave conditions chosen:

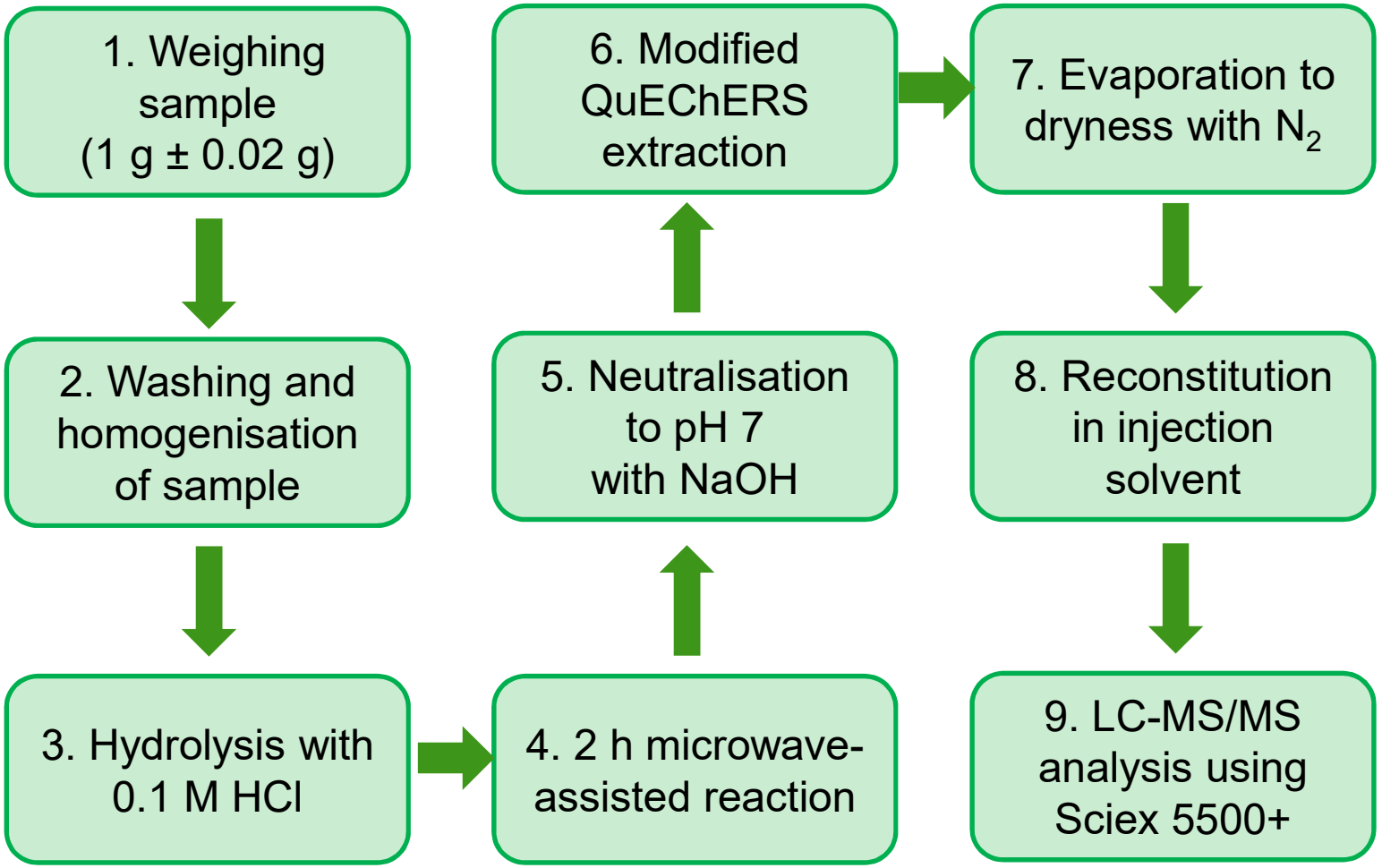
4 min ramp to 60 °C, with a 2 h hold time

Comparison of derivatisation conditions for AOZ incurred material



Comparison of the performance of various derivatisation conditions. % yield shown is determined by calculating the mean AOZ concentration (*n* = 3) measured with each set of conditions and expressing each value as a percentage of the AOZ concentration measured using the traditional overnight incubation at 37 °C. Time shown = hold time; MW = microwave reaction; WB = heated waterbath.

Final Method



Method Validation

- Method has been fully validated in accordance with the new legislative guidelines set out in **2021/808/EC**.
- The method met all the performance criteria for the following:
  - Identification
  - Selectivity
  - Linearity
  - Matrix effects
  - Trueness
  - Within-lab repeatability (WLr)
  - Within-lab reproducibility (WLR)
  - Decision limits (CCα)
- Multi-species validation for avian, bovine, ovine and porcine muscle samples.
- Awarded accreditation by the Irish National Accreditation Board (INAB) in conformity with the ISO/IEC 17025:2017 standards

Table: Validation results for the analysis of eight bound nitrofuran analytes in avian, bovine, ovine and porcine muscle samples.

Analyte	WLr Trueness (%) (RSDr) (%)			WLR Trueness (%) (RSDR) (%)				Verified CCα (µg kg-1)
	L1	L2	L3	L1	L2	L3	L4	
NPAHD	100 (2.8)	100 (1.7)	100 (1.9)	99 (2.4)	100 (2.0)	99 (3.9)	101 (4.0)	0.030
NPAOZ	101 (2.0)	100 (2.1)	100 (1.2)	100 (1.6)	100 (2.5)	99 (2.8)	99 (1.9)	0.019
NPAMOZ	100 (2.6)	100 (2.0)	100 (1.4)	101 (2.4)	100 (1.8)	100 (1.4)	101 (1.7)	0.013
NPSEM	100 (2.5)	101 (3.9)	99 (1.0)	101 (3.7)	100 (3.8)	100 (2.1)	100 (2.8)	0.200
NPHBH	101 (2.6)	101 (2.1)	100 (1.6)	100 (2.4)	99 (4.3)	100 (9.6)	98 (6.0)	0.070
NPAGN	100 (2.5)	101 (2.0)	100 (0.6)	101 (2.0)	101 (0.9)	101 (2.6)	101 (2.1)	0.017
NPOAH	100 (2.5)	100 (1.5)	100 (0.8)	101 (2.2)	100 (1.4)	100 (2.5)	100 (2.6)	0.200
NPDNSAH	101 (3.9)	102 (3.9)	101 (2.7)	99 (4.5)	101 (3.5)	105 (10.7)	100 (3.4)	0.058

L1: 0.5 times RPA = 0.2 µg kg^{-1} / **L2:** 1.0 times RPA = 0.5 µg kg^{-1} / **L3:** 1.5 times RPA = 0.75 µg kg^{-1} /**L4:** 2.0 times RPA = 1.00 µg kg^{-1}

Application to incurred tissues

- Method showed **satisfactory performance** when applied to incurred tissues.

- Participated in a FAPAS proficiency test in May 2021.
  - Tested chicken muscle incurred with SEM.
  - Assigned a z-score of 0.0.

- Additionally, incurred pig and muscle samples were analysed (supplied by ANSES Fougères).

Sample ID	Source	Analyte Detected	Species	Assigned Concentration (µg kg^{-1})	Measured Concentration (µg kg^{-1})	Proposed z-score
02429	FAPAS	NPSEM	Chicken	2.560	2.549	0.00
15JJ-9	EURL	NPAHD	Pig	1.701	1.435	-0.49
20QY-144	EURL	NPAOZ	Pig	0.456	0.563	+1.07
20QY-24	EURL	NPAMOZ	Turkey	0.294	0.313	+0.30
17NHD214	EURL	NPSEM	Pig	0.871	0.702	-0.88
20QY-89	EURL	NPSEM	Pig	0.558	0.470	-0.72
20QY-91	EURL	NPDNSAH	Turkey	0.239	0.234	-0.09

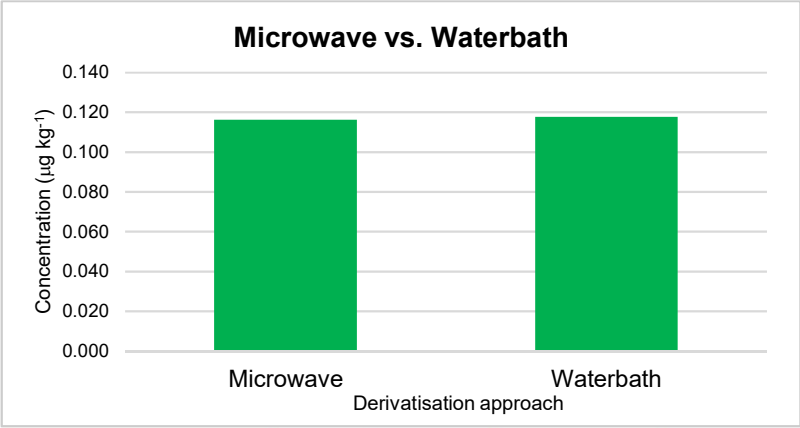
NTV-incurred tissues

- Method applied to NTV-incurred porcine tissue to confirm the formation of aminoguanidine (AGN) for use as a marker metabolite.

- Muscle, liver, kidney and plasma samples were collected from pigs fed with NTV-medicated feed (50 mg kg^{-1}).

- All incurred samples tested positive for AGN, whilst all control samples tested negative for AGN.
  - For the first time, this study has **confirmed the metabolism of nitrovin into its AGN metabolite**, and its subsequent accumulation in animal tissues

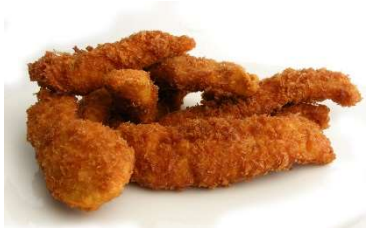
- These incurred tissues were also used to demonstrate the **equivalency of the microwave and waterbath derivatisation** when applied to NTV-incurred material.



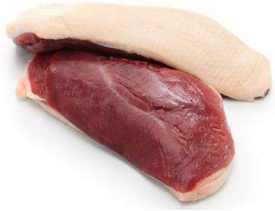
Poultry retail sampling survey

- Method was applied in the analysis of **118 poultry products**, comprised of chicken, turkey, duck and quail, with different countries of origin.

- All samples were found to be negative, given that no nitrofuran bound residues were detected.



- This selection of samples is only a snapshot representation of poultry at a certain point in time.
  - Further investigation, across a larger quantity of samples, would be required to gain an insight into potential nitrofuran misuse.



- The survey **highlights the suitability and robustness of the method**, given that it was fit to analyse a wide range of different processed and unprocessed sample types.

Conclusions

- A rapid and improved method, with greater sensitivity, for the detection of eight bound nitrofuran residues in meat has been developed:

  ✓Scope of analysis extended
  ✓Laboratory turnaround times shortened from 4 days to 2 days
  ✓Food safety and consumer confidence ensured

- Rigorous validation studies and proficiency tests have proven fitness for purpose, and is now an **accredited testing method for nitrofuran analysis** in the Irish National Residue Control Plan in Teagasc Food Research Centre.

- Method development highlighted the importance of applying newly developed methods to incurred materials, particularly when analysing bound residues, to ensure fitness for purpose.

Acknowledgements

- We wish to acknowledge staff in the National Reference Laboratory based at Teagasc in Dublin, Ireland for supporting this work through design of validation experiments, execution of experiments and data review.

- We also like to thank Ms Régine Fuselier and Dr Eric Verdon from the EU Reference laboratory at Fougères in France for supply of incurred material that was essential to evaluate the performance of the final methods.

- We wish to thank staff at the Agricultural and Bioscience Institute (AFBI) in Belfast for the production of NTV-incurred tissues and their assistance in inter-laboratory studies.

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