



Simultaneous detection of four nitrofurantol metabolites in honey using a multiplexing biochip screening assay

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ABSTRACT

A chemiluminescence-based biochip array sensing technique has been developed and applied to the screening of honey samples for residues of banned nitrofurantol antibiotics. Using a multiplex approach, metabolites of the four main nitrofurantol antibiotics could be simultaneously detected. Individual antibodies specific towards the metabolites were spotted onto biochips. A competitive assay format, with chemiluminescent response, was employed. The method was validated in accordance with EU legislation (2002/657/EC, 2002), and assessed by comparison with UHPLC–MS/MS testing of 134 honey samples of worldwide origin. A similar extraction method, based on extraction of the analytes on OasisTM SPE cartridges, followed by derivatisation with nitrobenzaldehyde and partition into ethyl acetate, was used for both screening and LC–MS/MS methods. The biochip array method was capable of detecting all four metabolites below the reference point for action of $1 \mu\text{g kg}^{-1}$. The detection capability was below $0.5 \mu\text{g kg}^{-1}$ for the metabolites AHD, AOZ and AMOZ; it was below $0.9 \mu\text{g kg}^{-1}$ for SEM. IC₅₀ values ranged from $0.14 \mu\text{g kg}^{-1}$ (AMOZ) to $2.19 \mu\text{g kg}^{-1}$ (SEM). This biosensor method possesses the potential to be a fit-for-purpose screening technique in the arena of food safety technology.

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1. Introduction

Nitrofurans are a class of broad-spectrum antibiotics that were widely used in food-producing animals (Vass et al., 2008) (see Figure S.1 (supplementary data) for structures). Due to concerns about their potency as carcinogens and mutagens (Van Koten-Vermeulen et al., 1993), they were banned outright from use (EC 1442/95, 1995). However, occurrences of nitrofurantol contaminants are the most frequent source of alerts in RASFF (the EU Rapid Alert System for Food and Feed Annual Reports, 2005 and 2006), although the number of transgressions is declining.

Nitrofurantol detection and quantification is typically performed using LC–MS/MS methods (Conneely et al., 2002; Conneely et al., 2003; O'Keefe et al., 2004), which provide unambiguous, confirmatory data in accordance with EU requirements (2002/657/EC, 2002). These methods involve detection of nitrofurans based on acid hydrolysis and chemical derivatisation of their side-chain metabolites (Horne et al., 1996). However, the analysis is time-consuming, requiring up to 20 min per sample. Immunochemical

assays have been proposed as a cheaper, rapid alternative for the screening of samples, reducing the need for lengthy LC–MS/MS analysis. An effective ELISA screening method for SEM has been described (Cooper et al., 2007), with a detection capability (CC_β) of $0.25 \mu\text{g kg}^{-1}$. Cheng et al. similarly developed an ELISA to detect AOZ (Cheng et al., 2009). Recent work (Li et al., 2009) presented simultaneous ELISA determination of the four nitrofurantol parent compounds in animal feed. However, given the inherently acidic nature of the honey matrix, and the possibility of honey being stored for months at room temperature prior to consumption, monitoring of side-chain metabolites is considered more appropriate in this instance. A drawback of analysis of all four metabolite using such an ELISA approach is that this would require four separate plates for testing, due to the limited cross-reactivity of the antibodies (Cooper et al., 2004).

In the current study of residues of nitrofurantol antibiotics in honey, a multiplex approach should be suitable to simultaneously detect the four main metabolites of nitrofurantol drugs. Examples of multiplexing platforms include those described by Lei et al. (2010) and Meimaridou et al. (2010). De Keizer et al. have also developed a multiplex technique for detection of sulfonamides in milk based on a flow cytometric immunoassay (De Keizer et al., 2008). Kloth et al. used a chemiluminescence-based microarray

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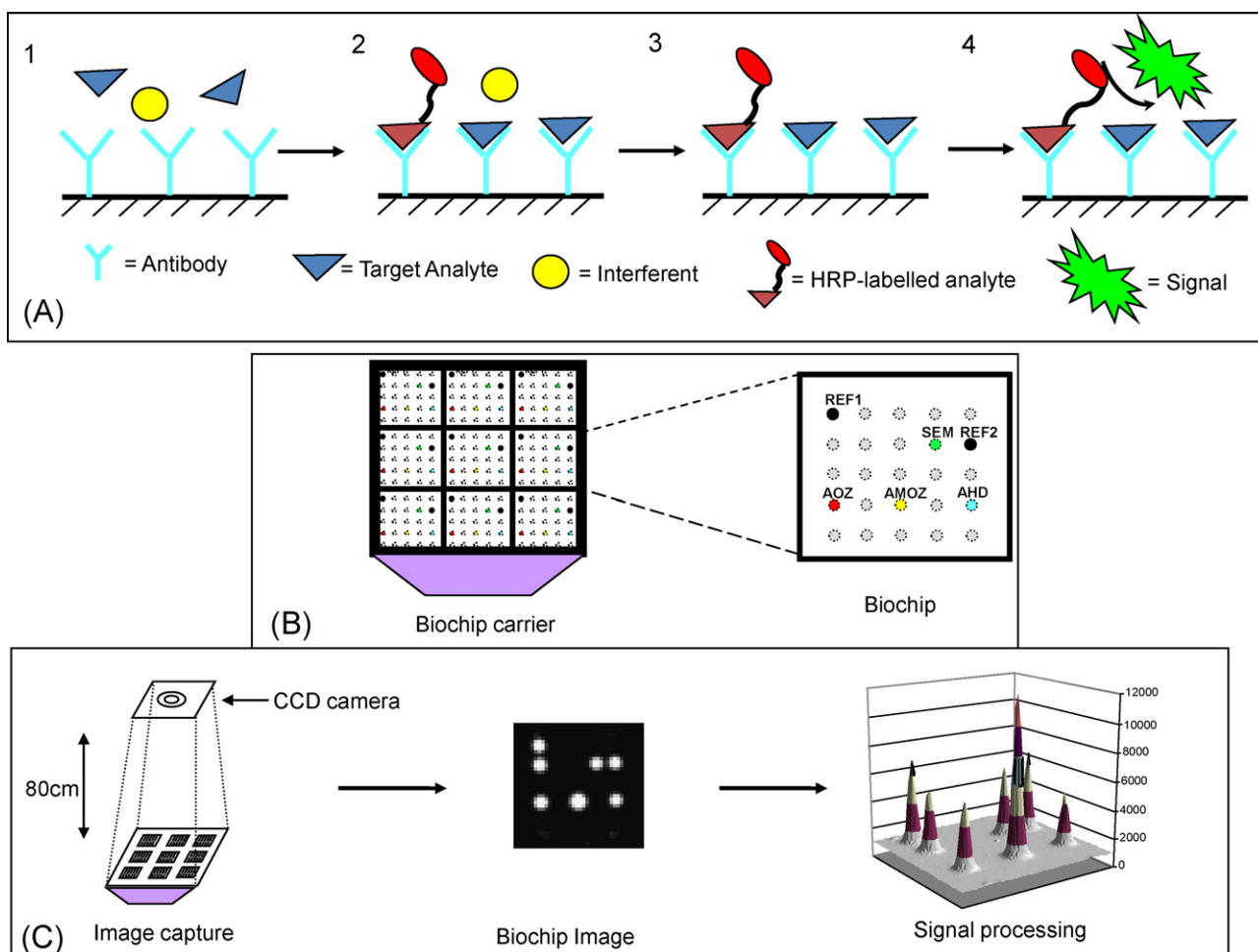


Fig. 1. (A) The assay employs a competitive format, which includes the following steps, (1) sample incubation, (2) conjugate incubation, (3) wash step, (4) addition of signal reagent leading to chemiluminescence response. (B) Biochip format, with 3 × 3 individual biochips per holder. Discrete test regions (DTRs) for SEM, AHD, AOZ, AMOZ are indicated. (C) Chemiluminescence is recorded by a CCD camera, signal intensity is interpreted by the software and plotted accordingly.

immunoassay for detecting up to 13 different antibiotics (Kloth et al., 2008). Here, a biochip array technique is exploited. Biochip array technology is an alternative immunochemical-based detection platform that allows the immobilisation of up to 25 different ligand molecules (i.e. antibodies, proteins, oligonucleotides) on the chip at specific locations, called Discrete Test Regions (DTRs) (see Fig. 1). The biochip array assay here employs a competitive format; antibodies selective for the analytes of interest are immobilised at the DTRs. Enzyme-labelled conjugate is applied; when this is captured by the relevant antibody, a complex is formed that outputs light upon addition of signal reagent. Any target analyte present in applied samples will compete with enzyme-labelled conjugate for complexation, resulting in a decrease in the quantity of recorded chemiluminescence.

The microarray format employed here exploits a piezoelectric nanodispense technique to deposit the relevant ligands at the DTRs (Fitzgerald et al., 2005). Detection is accomplished via imaging of a chemiluminescent signal with a CCD (charge-coupled device) camera (see Fig. 1). This technology has been used to screen for benzodiazepines, opiates, cocaine and cannabinoids in haemolysed whole blood (Grassin Delyle et al., 2008). Biochip array technology offers the advantage of multiplexing several specific antibodies on a single biochip to increase the number of analytes covered. The challenge explored in this work was to apply the microarray sensing technique to the simultaneous detection

of the four main nitrofurantol metabolites of interest in honey, at the concentration levels indicated by the appropriate EU legislation, thus exploiting this technique in a typical residue analysis application. As with LC–MS/MS analysis, nitrophenyl-labelled analogues of the compounds of interest are targeted. This is primarily due to the requirement for a distinctive fragmentation pattern in MS/MS analyses. Here, it is attributable to the inherent difficulty of raising antibodies against molecules of such relatively low molecular weight-derivatisation to increase molecular size has been found to improve antibody specificity in similar assays (Diblikova et al., 2005). Hydrolysis/derivatisation also ensures release of bound residues from the matrix, and prevents rebinding. The antibodies are raised against 4-nitrophenyl derivatives of the compounds, and the samples must thus also undergo the derivatisation process. Derivatisation of these compounds also inhibits their ability to rebind to proteins or other matrix components.

The assay was validated according to 2002/657/EC criteria. In addition, it was comprehensively evaluated through application to 134 honey samples of diverse geographical origin, which were also characterised by an accredited UHPLC–MS/MS assay. The combined approach allows an effective means of satisfying EU quality criteria specified for screening and confirmatory techniques, detecting below the reference point for action of $1 \mu\text{g kg}^{-1}$ identified (2003/181/EC and Regulation (EC) No.470/2009).

2. Experimental

2.1. Chemicals and reagents

1-Aminohydantoin (AHD), 3-amino-2-oxazolidinone (AOZ) and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), internal standards semicarbazide hydrochloride ($\text{SEM-}^{13}\text{C},^{15}\text{N}$), 3-amino-2-oxazolidinone- d_4 (AOZ- d_4), 3-amino-5-morpholinomethyl-2-oxazolidinone- d_5 (AMOZ- d_5) and 1-aminohydantoin- $^{13}\text{C}_3$ (AHD- $^{13}\text{C}_3$) were all purchased from Witega (Berlin, Germany). Nitrophenyl-labelled metabolites (NP-SEM, NP-AHD, NP-AOZ and NP-AMOZ) were also from Witega. Semicarbazide hydrochloride (VETANAL) was from Sigma–Aldrich (Steinheim, Germany). HPLC grade methanol, ethyl acetate and cyclohexane were from Fisher Scientific. Ammonium acetate, anhydrous potassium phosphate dibasic, sodium hydroxide, deuterated methanol, 2-nitrobenzaldehyde and 4-nitrobenzaldehyde were all from Sigma–Aldrich. Concentrated hydrochloric acid was from BDH. Pure water of $18.2\text{ M}\Omega\text{ cm}^{-1}$ was generated in-house from a Milli-Q water system. Oasis HLB SPE cartridges (60 mg, 3 mL) were obtained from Waters Corporation. Strata-X polymeric reverse-phase SPE cartridges were from Phenomenex (Macclesfield, UK). Millex-FG PTFE syringe filters (filter diameter 13 mm, pore size $0.2\text{ }\mu\text{m}$) were from Millipore (Cork, Ireland), and reagents for use with the Randox Investigator™ were supplied by Randox Laboratories Ltd. (Co. Antrim, Northern Ireland).

To conjugate the 4-nitrophenyl-labelled nitrofurans (AHD, AOZ, AMOZ, SEM) to horseradish peroxidase, 10 mg EDC hydrochloride was dissolved in 800 μL of water and immediately added to a solution of 2 mg of 4-CP-nitrofurans in 200 μL of DMF. After mixing, this solution was added to HRP (20 mg) in 1 mL of water. Sulfo-NHS (5 mg) was immediately added; the reaction mixture was incubated in the dark at room temperature overnight. Excess hapten was removed by desalting with 2 PD-10 columns (Pharmacia) in series, pre-equilibrated with PBS at pH 7.2. The hapten-HRP conjugate was then dialysed overnight against 10 L of PBS, pH 7.2, at 4°C .

Stock solutions of $50\text{ }\mu\text{g mL}^{-1}$ of each of the metabolites, the nitrophenyl-labelled metabolites, and the isotope-labelled analytes were prepared in methanol in 100 mL quantities. All concentrations were corrected for purity and salt forms. When stored at -20°C , standards were found to be stable for up to one year.

Working standard solutions were prepared by dilution of the stock standards with methanol, giving solutions containing the four side-chain metabolites at a concentration of $50\text{ }\mu\text{g L}^{-1}$, at $10\text{ }\mu\text{g L}^{-1}$ for the mixture of nitrophenyl-labelled derivatives and at $50\text{ }\mu\text{g L}^{-1}$ again for the isotope-labelled analytes for use as internal standard. All standard solutions were stored at -20°C .

2.2. Honey samples

All honey samples were sourced from retail outlets in Ireland. Honeys determined by UHPLC–MS/MS to be free of nitrofurans were used as negative controls. Several varieties of honey were examined in the survey, including types described simply as ‘clear’ (55), ‘set’ manuka (16), acacia (15), forest (9), wildflower (8). Samples of clover, eucalyptus, heather and lavender honey, as well as six samples of unprocessed honeycomb, were included.

2.3. Immunoassay on the biochip screening assay

2.3.1. Sample extraction and clean-up procedure

Honey samples (2 g) were weighed into 50 mL polypropylene centrifuge tubes. Samples were dissolved in 5 mL 0.1 M HCl, in a shaking bath at 37°C for 1 h. Samples were then applied to Oasis™ HLB SPE cartridges preconditioned with 2 mL methanol

and 2 mL water. The sample tubes were subsequently rinsed with 2 mL ultrapure water, and this too was applied to the SPE cartridges. The eluate was collected, and derivatised with 150 μL 10 mM 4-nitrobenzaldehyde in dimethylsulfoxide, for 16 h at 37°C in a shaking water bath.

Following derivatisation, samples washed with cyclohexane and neutralised as described in Section 2.4.1. Extraction was performed with one 15 mL ethyl acetate aliquot, before being vortexed, mechanically shaken for 20 min and then centrifuged at 2500 g for 10 min. Evaporation of the ethyl acetate was performed at 40°C under N_2 . As recommended by Lopez et al. (2007), once dried, 2 mL of methanol was added to each of the samples to rinse the walls of the tubes, which were vortex mixed for 30 s and then dried down again. Samples were reconstituted in 375 μL of the supplied sample diluent, before vortexing for 1 min.

2.3.2. Biochip assay

A Randox Evidence Investigator™ analyser was used for the biochip assay (Randox Laboratories Ltd., Crumlin, Co. Antrim). Individual biochip carriers contained nine wells for individual samples, capable of simultaneously testing for the four nitrofurans metabolites. Four individual antibodies were used for this assay, one for each of the individual nitrofurans metabolite analytes, with antibodies raised against the nitrobenzaldehyde-labelled analytes. The assay used four specific antibodies raised in sheep against SEM-bovine thyroglobulin (BTG), AHD-bovine serum albumin (BSA), AOZ-BTG and AMOZ-BTG, using the Ig fraction of the sheep polyclonal antiserum. These immunogens were prepared by conjugation of the 4-carboxybenzaldehyde derivatised nitrofurans haptens to the aforementioned carrier proteins and characterised by MALDI-TOF analysis. All antibodies (PAS9996, PAS9994, PAS9228, PAS9229) were sourced from Randox Life Sciences. The cross reactivity of each antibody was characterised against nitrofurans parent drugs and their side chain metabolites. For additional information on biochip preparation, please refer to Supplementary Data section (S.8).

Briefly, following preparation of the biochips, 10 nL volumes of diluted antibodies were deposited via a piezoelectric nanodispense technique onto the derivatised, silanised aluminium oxide surface of the biochips, at the relevant DTR sites. Immobilisation was achieved using the 3-glycidoxypolytrimethoxysilane (GOPTS) technique previously described (Fitzgerald et al., 2005). After antibody immobilisation, biochip surfaces were treated with 1% casein in 50 mM carbonate buffer at 25°C (1 h) to eliminate surface reactivity between the DTRs and to reduce non-specific binding. The biochips were then sealed in foil packets for storage. For analysis via Randox multiplexing biochip screening assay, 50 μL of sample/standard was applied to each well, along with 150 μL of assay diluent. The biochips were incubated with the sample/standard for 30 min at 25°C on a thermoshaker (shaking at 370 rpm). Subsequently, 100 μL of conjugate was added to each sample well, and samples were further incubated for 1 h at 25°C and 370 rpm. The conjugate solution comprised a mixture of the four nitrophenyl-labelled nitrofurans analytes, each labelled with horseradish peroxidase. Sample carriers were then subject to 2 quick wash cycles, followed by 4×2 -min wash cycles. 250 μL of signal (1:1 luminol in trizma buffer (pH 9.6) with 4-iodophenol as enhancer: peroxide, v/v) was then added to each well, and after 2 min the sample carrier was imaged in the biochip array analyser.

Each biochip incorporates a reference spot at DTR 5 and a correction spot at DTR 6. During image processing, the reference spot is located by the software at predefined x and y co-ordinates to validate the biochip. The correction spot is then used to define and locate each DTR.

2.3.3. Biochip array analyser: calibration

Calibration curves were prepared in matrix by fortifying negative honey samples at concentrations of 0.001, 0.01, 0.05, 0.1, 1, 4, 10 and 50 $\mu\text{g kg}^{-1}$ for AHD, AOZ and AMOZ, and for SEM at 0.001, 0.01, 0.05, 2, 20, 40, 100 and 500 $\mu\text{g kg}^{-1}$ prior to sample clean-up and derivatisation. Of the six carriers used in a run, the first carrier was employed as the calibration carrier (9 biochips). This leaves a further 45 spaces for samples/controls. One carrier (9 samples) is imaged at a time.

2.3.4. Biochip array analyser: data processing

For this particular biochip binding assay, calibrants are supplied with reagents, and the calibration curves are subsequently generated via the included software. However, given the complicated nature of honey as a sample matrix, all analyses were run alongside matrix-matched calibrants (a honey determined to be free of nitro-furan metabolites via UHPLC–MS/MS, spiked at the appropriate levels). Once the response of these calibrants (measured in Relative Light Units, RLUs) had been determined, a calibration curve was constructed, applying the 4-parameter logistic model (Findlay and Dillard, 2007).

$$Y = D + \frac{(A - D)}{(1 + (x/C)^B)}$$

where Y is the response generated, x is the concentration of the analyte, A is the response at zero analyte concentration, B is a slope factor, C represents the inflection point of the calibration curve, and D is the response at infinite analyte concentration. An initial estimate was made for each parameter, and this was then optimised by minimising the sum of square residuals via the Microsoft ExcelTM component, Solver. Correlation (R) values of >0.98 were obtained in all cases, though for SEM, lower R -values were obtained than for the other analytes.

2.3.5. Validation

A qualitative approach was used to determine the performance factor $CC\beta$ (detection capability) as described in 2002/657/EC and CRL Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines. A threshold value T was calculated by determining the mean response of 40 negative honey samples and adding 1.64 times the standard deviation. A cut-off factor Fm was determined by calculating the mean response of samples spiked at 0.5 $\mu\text{g kg}^{-1}$ (for AHD, AOZ, AMOZ) and 0.9 $\mu\text{g kg}^{-1}$ (for SEM), and subtracting 1.64 times the standard deviation. These values were chosen as they were below the reference point for action of 1 $\mu\text{g kg}^{-1}$, and the assay could reproducibly detect these quantities. If no more than two false-compliant results were observed at this level, it was taken that $CC\beta$ was below the assigned values of 0.5 $\mu\text{g kg}^{-1}$ (for AHD, AOZ, AMOZ) and 0.9 $\mu\text{g kg}^{-1}$ (for SEM).

Method applicability was demonstrated through application to various kinds of honey, including clear, acacia, manuka and clover.

The Commission Decision 2002/657/EC document does not require testing of repeatability as a part of validation of a qualitative screening method. However, repeatability was assessed for this method by spiking a single honey sample at 1 $\mu\text{g kg}^{-1}$ ($n=9$), and assaying in one run. This was repeated on three days, to also assess inter-assay variation (Table 1). Recovery was assessed by spiking 9 samples of negative control honey at 1 $\mu\text{g kg}^{-1}$ and quantifying the percentage found relative to the quantity spiked. For clarity, recoveries found via UHPLC–MS/MS and biochip array method are compared in Table S.7 (supplementary data).

Table 1

Inter and intra-assay variations in a honey sample, as determined via biochip assay ($n=9$, spiking at 1 $\mu\text{g kg}^{-1}$). Recovery ranges from 50% to 159% for intra-assay testing, and from 56% to 147% for inter-assay testing.

Analyte	Mean $\pm$ SD	CV(%)
Intra-assay variation		
AHD	0.54 $\pm$ 0.08	14.0
AOZ	0.50 $\pm$ 0.05	9.8
SEM	1.12 $\pm$ 0.20	17.9
AMOZ	1.16 $\pm$ 0.12	10.5
Inter-assay variation		
AHD	0.56 $\pm$ 0.07	13.3
AOZ	0.69 $\pm$ 0.14	20.5
SEM	0.80 $\pm$ 0.30	36.9
AMOZ	1.47 $\pm$ 0.16	10.9

2.4. UHPLC–MS/MS assay

2.4.1. Sample extraction and clean-up procedure

Honey samples (2 g) were weighed into 50 mL polypropylene centrifuge tubes and fortified with internal standard solution (80 μL). Cyclohexane (5 mL) was added to samples, followed by vortexing. Following centrifugation (5 min, 3000 g), the cyclohexane supernatant was removed and discarded. Samples were dissolved in 5 mL 0.1 M HCl, in a shaking bath at 37 °C for 1 h. Subsequently, samples were applied to OasisTM HLB SPE cartridges preconditioned with 2 mL methanol and 2 mL water. The sample tubes were rinsed with 2 mL ultrapure water, and this too was applied to the SPE cartridges. The eluate was collected, and derivatised with 250 μL of 50 mM 2-nitrobenzaldehyde in methanol for 16 h at 37 °C in a shaking water bath.

Following derivatisation, samples were allowed to cool, before again washing with 5 mL cyclohexane. The samples then underwent neutralisation with 0.1 M K_2HPO_4 and 1 M NaOH, adjusting to pH 7. Samples were next extracted with two 9 mL volumes of ethyl acetate. Samples were vortexed, shaken for 20 min on a mechanical shaker, and then centrifuged at 2500 g for 10 min. Evaporation of the ethyl acetate was performed at 40 °C under N_2 . Once dried, 2 mL of methanol was added to all samples to rinse the walls of the tubes, which were vortexed mixed for 30 s and then dried down again.

Samples were reconstituted in 500 μL of 0.5 mM ammonium acetate: methanol (80:20, v/v), before vortexing for 1 min and filtration through a 0.2 μm PTFE filter into a HPLC vial.

2.4.2. UHPLC–MS/MS detection conditions

The UHPLC–MS/MS system consisted of a Waters Acquity[®] separations module and a Quattro Premier triple quadrupole mass spectrometer equipped with electrospray interface (Waters, Milford, MA, USA). Separation was achieved on a Waters BEH C_{18} column, 2.1 mm $\times$ 5 mm, particle size 1.7 μm . A Waters VanguardTM guard column was installed between column and autosampler.

The nitrobenzaldehyde-derivatised nitrofurans side-chain metabolites were eluted via gradient elution, with mobile phase consisting of 100% 0.5 mM ammonium acetate and B being 100% methanol. Analysis was performed with a 9 min gradient at a constant flow rate of 0.5 mL min^{−1}. The gradient profile was as follows: (1) 0–0.6 min, 90% A; (2) 2 min, 50% A (3) 3–3.5 min 20% A (4) 5.5–6.0 min 90% A. An injection volume of 20 μL was used in all cases, and the column temperature was kept at 65 °C. The autosampler temperature was maintained at 5 °C.

Data was acquired in selective reaction monitoring (SRM) mode with two transitions selected for each target analyte to satisfy EU confirmatory criteria (see supplementary data, Table S.2).

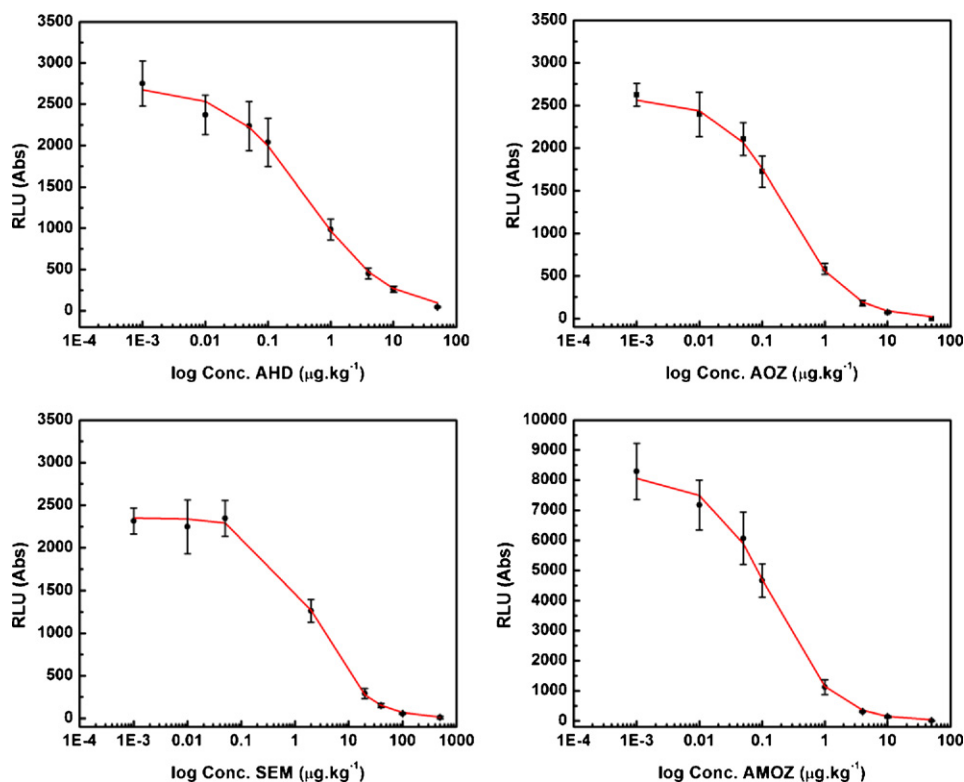


Fig. 2. Typical matrix-matched biochip assay calibration curves for AHD ($IC_{50} = 0.40 \mu\text{g kg}^{-1}$), AOZ ($IC_{50} = 0.24 \mu\text{g kg}^{-1}$), SEM ($IC_{50} = 2.19 \mu\text{g kg}^{-1}$) and AMOZ ($IC_{50} = 0.14 \mu\text{g kg}^{-1}$) in honey as obtained by biochip array analysis ($n = 5$ days). X-axis is in log scale.

Source temperature was at 140°C , while desolvation temperature was 400°C . The desolvation gas was set at 1100 L h^{-1} . *TargetLynx* software was used to process all ion chromatograms. Calibration curves were prepared by plotting the ratio of peak area of the analyte to peak area of the internal standard, against the concentration of the standards ($\mu\text{g kg}^{-1}$).

2.4.3. UHPLC–MS/MS calibration

An extracted five-point standard curve was prepared in matrix at concentrations of 0.2, 0.5, 1, 2 and $5 \mu\text{g kg}^{-1}$. A recovery control curve was also prepared, by spiking blank samples at the end of the sample preparation process, at 0.25, 0.5, 1 and $2 \mu\text{g kg}^{-1}$. Method recovery was assessed through comparison of results for the recovery control curve with the calibration curve.

3. Results and discussion

3.1. Method development

Initial experiments used simple dilution of the honey sample in acidic medium prior to derivatisation. Satisfactory calibration curves were obtained for AHD, AOZ and AMOZ. The yield for the nitrophenyl SEM derivative was improved by SPE clean-up, which is in agreement with previous findings in our laboratory with nitrofurans in honey by UHPLC–MS/MS, where a low yield of NPSEM was obtained without SPE clean-up. As a result, a SPE step was included in the sample preparation to improve assay response to SEM. As with the sample preparation steps for the UHPLC–MS/MS method, total sample preparation time was 1.5 days. Sample analysis time was significantly faster for the biochip assay method, with a set of 54 calibrants, controls and test samples being analysed in under 2 h. A higher throughput can be achieved (≥ 135 samples/day) by staggering the incubation times and thereby greatly increasing laboratory output per working day. An equivalent batch analysed

via the UHPLC–MS/MS method would require approximately 10 h (including retention time checks), given the sample run time of 9 min.

Confirmatory analysis here was performed via UHPLC–MS/MS. The extraction method was based on that of [Lopez et al. \(2007\)](#) with some modification. As little emulsion formation was observed during liquid-liquid extraction, we excluded NaCl from the samples. An additional cyclohexane wash step was included at the start of the sample preparation and following derivatisation to minimise matrix interference effects. The UHPLC assay could easily detect nitrofurans residues down to $0.2 \mu\text{g kg}^{-1}$ with signal to noise ratios of typically greater than 100.

3.2. Method validation

The method was validated according to Commission Decision 2002/657/EC criteria, wherein the criteria requisite for validation are identified as detection capability ($CC\beta$), selectivity/specificity, applicability and ruggedness/stability. The detection capability was determined within the lab to investigate how changes to sample preparation affect this.

Typical matrix-matched biochip assay calibration curves are displayed in [Fig. 2](#), for all analytes. Similar responses for all analytes were observed when multiplexed and individual analytes were tested. The assay β error was 0 for AHD, AOZ and AMOZ at a spike level of $0.5 \mu\text{g kg}^{-1}$, as no more than 2 false compliant results were obtained at this level, in accordance with the guidelines (CRL Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines). For SEM, the assay β error was 0, at a spike level of $0.9 \mu\text{g kg}^{-1}$ (see [Supplementary data Figure S.3](#)). This is compliant with the criterion for screening techniques identified in 2002/657/EC. Thus, the method may be applied for simultaneous screening of these analytes below the EU reference point for action of $1 \mu\text{g kg}^{-1}$. Specificity/selectivity had previously been

determined; the most significant observed cross reactivity was quoted for the 4-nitrophenyl-1-aminohydantoin antibody, which exhibited a cross-reactivity of 42% for nitrofurantoin, the parent drug (see Table S.4 (supplementary data)). Method applicability was demonstrated through application to various kinds of honey, including clear, set, manuka, acacia and clover. The method was also sufficiently rugged that the SPE cartridge (OasisTM HLB) could be replaced with an alternative (Phenomenex Strata-XTM reverse-phase polymeric) with no tangible decrease in assay response. Repeatability was assessed for this method by spiking a single honey sample at 1 $\mu\text{g kg}^{-1}$ ($n=9$), and assaying in one run. This was repeated on three days, to also assess inter-assay variation (Table 1). Intra-assay variation ranged from 9.8% (AOZ) up to 17.9% (SEM). For inter-assay variation, values from 10.9% (AMOZ) up to 36.9% (SEM) were observed. This indicates that the AHD, AOZ, AMOZ and SEM precision is acceptable. The biochip screening method precision is at least comparable to that achieved for the confirmatory UHPLC–MS/MS method. Recoveries were in the range 69–153% (see Table S.7 (supplementary data)), higher than those observed for UHPLC–MS/MS; however, %CV values were somewhat greater for the screening method.

The introduction of ultra high performance liquid chromatography, with tandem mass spectrometric detection, has proven to be an effective means of determining trace quantities of chemical residues in food matrices (Keegan et al., 2009). The UHPLC–MS/MS method for the analysis of the nitrofurans side-chain residues in honey was also validated. Decision limits of the order 0.09–0.14 $\mu\text{g kg}^{-1}$ were determined for the analytes, while detection capabilities ranged between 0.15 and 0.24 $\mu\text{g kg}^{-1}$ (see supplementary data, Table S.5).

These limits are of similar magnitude to those published for nitrofurans metabolites determined in, e.g., poultry muscle tissue (Verdon et al., 2007) ($\text{CC}\alpha=0.08\text{--}0.20\text{ }\mu\text{g kg}^{-1}$, $\text{CC}\beta=0.10\text{--}0.25\text{ }\mu\text{g kg}^{-1}$). The application of UHPLC in this particular assay results in an analysis time <50% that of, for example, the method reported by Lopez et al. (2007) (run time=20 min/injection), greatly improving the efficiency of the overall assay. Validation data both for within laboratory repeatability (same operator, three individual assay sets) and within laboratory reproducibility (three separate operators, each performing an individual assay set) was obtained (see Supplementary data, Table S.6). Within laboratory repeatability, determined as the %CV of the recoveries for individual analytes, ranged from 8.4% (AOZ) to 26.4% (AHD). For within laboratory reproducibility, accuracy ranged from 99.6–117.1%, while %CV of the recoveries of individual analytes ranged from 7.3 to 19.3%. Results from biochip array sensing technique for a survey of honey samples were assessed against UHPLC–MS/MS analysis of the same samples (see supplementary information for details).

4. Conclusion

Following extensive investigation and comparison with results obtained via the confirmatory technique (LC–MS/MS), the biochip array sensing technique has proven to be suitable for the rapid, simultaneous screening of the four main nitrofurans metabolites in honey. Validation of the method indicated that the detection capability was below 0.5 $\mu\text{g kg}^{-1}$ for AHD, AOZ and AMOZ, and below 0.9 $\mu\text{g kg}^{-1}$ for SEM. While sample preparation time remains equivalent to that for UHPLC–MS/MS (1.5 days), sample screening is significantly faster (45 samples in <2 h). This reduces the require-

ment for valuable LC–MS/MS time in the laboratory, as samples can be screened via this method, with suspected positives put forward to confirmatory testing. Use of microarray sensing techniques may thus hold much potential for the area of residue determination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.03.036.

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