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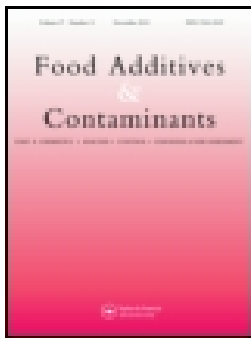
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Feasibility of a novel multispot nanoarray for antibiotic screening in honey

**McNamee, Sara E.¹, Rosar, Giulia², Persic, Lidija², Elliott, Christopher T.¹,
Campbell, Katrina^{1*}**

¹Institute for Global Food Security, School of Biological Sciences, Queen's University,
David Keir Building, Stranmillis Road, Belfast, United Kingdom, BT9 5AG

²Tecna s.r.l., c/o Area Science Park, Località Padriciano, 99 - 34149 Trieste, Italy.

*** Corresponding author**

Dr Katrina Campbell

Institute for Global Food Security, School of Biological Sciences, Queen's University, David
Keir Building, Stranmillis Road, Belfast, UK BT9 5AG

Telephone: +44 (0) 2890976535

Fax: +44 (0) 2890976513

Email: katrina.campbell@qub.ac.uk

Abstract

Practical solutions for multiple antibiotic determination in food is required by the food industry and regulators for cost effective screening purposes. This study describes the feasibility in development and preliminary performance of a novel multispot nanoarray for antibiotic screening in honey. Using a multiplex approach the metabolites of the four main nitrofurans including morpholinomethyl-2-oxazolidone (AMOZ), 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), 1-aminohydantoin (AHD) and chloramphenicol (CAP) were simultaneously detected. Antibodies specific to the five antibiotics were nano-spotted onto microtitre plate wells and a direct competitive assay format was employed. The assay characteristics and performance were evaluated for feasibility as a screening tool for antibiotic determination in honey to replace traditional ELISAs. Optimisation of the spotting and assay parameters were undertaken with both individual and multiplex calibration curves

generated in PBS buffer and a honey matrix. The limits of detection as determined by the 20% inhibitory concentrations (IC_{20}) were determined as 0.19, 0.83, 0.09, 15.2 and 35.9 ng/mL in PBS buffer, 0.34, 0.87, 0.17, 42.1 and 90.7 ng/mL in honey (fortified at the start of the extraction) and 0.23, 0.98, 0.24, 24.8 and 58.9 ng/mL in honey (fortified at the end of the extraction) for AMOZ, AOZ, CAP, SEM and AHD respectively. This work has demonstrated the potential of multiplex analysis for antibiotics with results available for 40 samples within a 90 min period for antibiotics sharing a common sample preparation. Although both the SEM and AHD assay do not show the required sensitivity with the antibodies available for use to meet regulatory limits, with further improvements in these particular antibodies this multiplex format has the potential to show a reduction in cost with reduced labour time in combination with the high throughput screening of samples. This is the first 96 well spotted microtitre plate nanoarray for the semi-quantitative and simultaneous analysis of antibiotics.

Keywords: nanoarray, screening method, immunoassay, antibiotic, nitrofurans, AMOZ, AOZ, SEM, AHD, chloramphenicol, honey.

Introduction

Antibiotics are a vital component for the treatment and elimination of disease in human, plants and animals. There are many concerns regarding the human consumption of food products contaminated with drug residues such as antibiotics. Antibiotic residues from agricultural use must be carefully monitored as they can adversely impact public health due to allergenic and carcinogenic factors and may also contribute to bacterial resistance. Nitrofurans belong to a class of synthetic broad spectrum antibiotics which all contain the characteristic five-membered nitrofuran ring. They were commonly used as feed additives for growth promotion and used mainly for livestock, aquaculture and apiculture for the prophylactic and therapeutic treatment of bacterial infections. Parent nitrofuran compounds are quickly metabolised after ingestion and have very short *in vivo* half-lives. Monitoring of nitrofurans is therefore based on the monitoring of the tissue-bound metabolites of nitrofurans as they remain in the body for many weeks after treatment (McCracken and Kennedy, 1997; Cooper et al., 2005). The four main nitrofuran antibiotics are furaltadone, furazolidone, nitrofurazone and nitrofurantoin with the resulting tissue bound metabolite residues AMOZ, AOZ, SEM and AHD produced respectively. There is however increasing concern in the use of SEM as a marker for nitrofurazone due to its reported natural occurrence in seafood, eggs, whey and heather honey (Crews, 2012) but recommendations in the measurement of total and

comparison of between free and protein bound SEM may offer a solution until an alternative marker is identified (Points et al., 2015). The nitrofurans metabolites have also been shown to be stable after cooking with 67-100 % of the residues remaining after conventional cooking techniques and additionally there was no significant change in residue concentration after storage at -20 °C (Cooper and Kennedy, 2007). In 1995, the use of nitrofurans for livestock production was completely prohibited in the European Union (EU) due to concerns about the carcinogenicity and their potential harmful effects on human health. Nitrofurans antibiotics have been included in Commission Regulation (EC) 1442/95 as compounds that are not permitted for use in the livestock industry. The EU has established a minimum required performance limit (MRPL) of 1 µg/kg for tissue of animal origin. There is however increasing concern in the use of SEM as a marker for nitrofurazone due to its natural occurrence in seafood, eggs, whey and heather honey (Crews et al., 2012). CAP is a broad spectrum antibiotic with excellent antibacterial and pharmacokinetic properties. This antibiotic is proven to be valuable for the treatment of bacterial infections in human and veterinary medicine as well as being administered to animals for disease prevention. It is often associated with serious side effects such as the development of aplastic anaemia and bone marrow suppression (Jimenez et al., 1990). In 1994, CAP was included in the Annex IV of the Council Regulation (EEC) No. 2377/90 and is therefore banned in the EU for the treatment of food producing animals. Consequently, CAP may not be present in animal products for human consumption and a MRPL of 0.3 µg/kg in meat, eggs, milk, urine, aquaculture products and honey has been set.

As both the nitrofurans and CAP are prohibited substances, zero tolerance applies. Methods of detection therefore require very low detection limits and high sample throughput. The development of rapid, inexpensive, accurate, multi residue and easy to use screening methods are therefore of primary importance in the food industry. Sensitive methods for nitrofurans metabolites for both screening (ELISA) and confirmatory methods (LC-MS/MS) were first developed by the EU FoodBRAND project (Cooper et al., 2004a; 2004b; Cooper et al., 2005). Analytical procedures for nitrofurans analysis in various matrices by screening and confirmation methods with respect to EU regulations are reviewed in Vass et al. (2008). Although analytical methods, HPLC-UV and LC-MS/MS, are available for both nitrofurans and CAP (Conneely et al., 2003; O'Keeffe et al., 2004; Cooper et al., 2005; Vivekanandan et al., 2005; Han et al., 2011; Douny et al., 2013), it is acknowledged that these methods are expensive, require skilled personnel and are time consuming. The need therefore for simple, rapid and efficient analytical methods for antibiotics that can be handled by relatively

unskilled operators has been recognised. Additionally, affordable monitoring of antibiotics to ensure food safety requires high throughput and economical methods of detection. To date a range of methods for the determination of nitrofurans metabolites and CAP residues in foodstuff have been developed. Independent enzyme linked immunosorbent assays (ELISAs) are widely used for screening purposes for each nitrofurans metabolite (Cooper et al., 2004; Diblikova et al., 2005; Cooper et al., 2007; Cheng et al., 2009; Li et al., 2009) and CAP (Fodey et al., 2007; Liu et al., 2014; Guo et al., 2015) thereby requiring five ELISA assays to detect all targets. Commercial screening tests are available for honey from Abraxis, Europroxima, Randox Food Diagnostics, R-Biopharm, Romer Labs and Tecna for nitrofurans detection and from Europroxima, Randox Food Diagnostics, R-Biopharm, Tecna and Bio Scientific for CAP detection. Biosensors have also been developed for honey for the independent detection of individual nitrofurans metabolites (Jin et al., 2011; Yang et al., 2011; Jin et al., 2014) and CAP (Ferguson et al., 2005; Yan et al., 2012; Kara et al., 2013; Gao et al., 2014). There is, however, an increasing necessity for the development and implementation of multiplexed tests that can detect a range of antibiotics simultaneously thus serving to reduce operator time and costs. Multiplex assays for nitrofurans include both antibody based screening methods (Thompson et al., 2011; O'Mahony et al., 2011; Liu et al., 2015) and confirmatory physicochemical LC-MS/MS methods (Tribalat et al., 2006; Lopez et al., 2007) for the detection of all four nitrofurans simultaneously. LC-MS/MS multiple analyte tests are also described for the simultaneous detection of the nitrofurans metabolites and CAP (An et al., 2015, El-Demerdash et al., 2015, Veach et al., 2015; Kaufmann et al., 2015). The only commercial test available for multiple antibiotic detection is from Randox Food Diagnostics based on a customised biochip technology with chemiluminescent detection for the four main nitrofurans metabolites..

In recent years innovative nano science and technology with state of the art sensing equipment have allowed the emergence of novel detection platforms. Nanoarrays have become unique and important tools for high-throughput analysis providing promising methods in which several targets are separately detected in spatially defined zones simultaneously. This enables miniaturization, higher sensitivity and simplified sample preparation in combining multiple extraction procedures into a single procedure. Nanoarrays may be employed in a number of applications such as medical diagnosis, genetic testing, environmental monitoring and food safety. There are very few studies that effectively employ this promising technology for the detection of antibiotics. An immunoassay using the microarray chip reader 3 platform has been described for the determination of antibiotic

residues in milk (Kloth et al., 2009a; 2009b) and honey (Wutz et al. 2011). A novel multiplex nanoarray based on planar waveguide with fluorescence detection was developed for the detection of a number of antibiotics including CAP (McGrath et al., 2015) in milk. Nanoarray formats in 96 well microtitre plates provide very promising and powerful detection methods in which several targets can be detected simultaneously. The benefits of ELISA and nanoarrays can thus be combined to produce a multiplex test capable of conducting large surveys and high throughput but also the simultaneous semi-quantitative detection of many antibiotics by users already familiar with ELISA methods.

Apiculture relies on antibiotics to prevent disease spreading through bee colonies, however, the overuse of these antibiotics can cause residues in honey products. Honey is one of the many foods that are monitored for antibiotic residues worldwide. Honey producers, importers, exporters and regulators, therefore, need simple, fast and effective ways to test honey for antibiotics. The development of sensitive, multi residue, rapid and high throughput screening methods are therefore important in the area of food safety and residue determination. The aim of this research was to demonstrate feasibility of a direct competitive multiplex nanoarray in a 96 well microtitre plate for the simultaneous detection of five key banned antibiotics (AMOX, AOZ, SEM, AHD and CAP) in honey as a rapid and easy to use detection system.

Materials and methods

Instrumentation

A sciFLEXARRAYER S5 (Sciencion, Germany) was used for spotting microtitre plates and a sciReader CL colorimetric nanoarray reader (Sciencion, Germany) was used for scanning and analysing spot intensities.

Reagents

Antibodies and enzyme labelled HRP conjugates for AMOX, AOZ, SEM, AHD and CAP were provided by Tecna (Trieste, Italy). Nunc 96 well microtitre plates (442404 flat bottom; 473768 breakable) were purchased from VWR (Leicestershire, UK). SciColor T2, enzyme substrate solution, was purchased from Sciencion (Berlin, Germany). Methanol, hexane, dimethyl sulfoxide, ethyl acetate (all HPLC grade), 2-nitrobenzaldehyde, dipotassium phosphate, bovine serum albumin, CAP, 2-NP-AMOX, 2-NP-AOZ, 2-NP-SEM and 2-NP-AHD were all purchased from Sigma-Aldrich (Dorset, UK). CAP, 2-NP-AMOX, 2-NP-AOZ,

2-NP-SEM and 2-NP-AHD were all prepared as 1mg/mL stock standards in methanol and stored at -20°C until required for use.

Spotting nanoarrays

Purified antibodies were diluted in filtered printing buffer (100 mM sodium phosphate, 50 mM sodium chloride, 100 µg/mL BSA, 0.005 % Tween-20, pH 8.0) to the required concentration (depending on the antibody). A spotting volume of 1 nL for each antibody was spotted onto a 96 well microtitre plate using a sciFLEXARRAYER S5. For part one of the study (optimisation) a nine spot matrix format (3x3 array) was arrayed with a 1000 µm spot to spot pitch composing of nine replicates of each target in separate wells. For part two of the study (multiplex spotting analysis) a 25 spot matrix format (5x5 array) was arrayed with a 500 µm spot to spot pitch composed of five replicates of each target (x 5 targets) in the same well. All spotting was carried out at room temperature and 65 % humidity. Microtitre plates were left at 65 % humidity for 1 hr on the nanospotter before being stored at 25 °C and 30 % humidity overnight in a humidity chamber (Deny, China).

Assay protocol

Enzyme labelled HRP conjugate (50 µl) diluted in bovine serum albumin (0.2 %) and sample/standard (50 µl) were applied to each well. The microtitre plate was incubated for 60 min at room temperature. The microtitre plate was washed 4 times with ELISA wash solution (0.15 M NaCl, 0.0125 % Tween) and dried with lint-free paper. SciColor T2 (50 µl) was added to each well and incubated for 30 min at room temperature. Finally, the microtitre plate was washed 2 times with ELISA wash solution and dried with lint-free paper. The microtitre plate was scanned using the sciReader CL colorimetric nanoarray reader.

Data processing

Microtitre plates were scanned using the sciReader colorimetric nanoarray reader at an exposure of 100 ms. An image of each well of the microtitre plate was taken and saved as a TIFF file. One microtitre plates takes approximately 1 min to scan. The images are opened and processed using sciANA software from Scienion. The spotting matrix of each well is defined by the number of blocks (1x1) and number of spots (5x5) so that the software knows the spotting configuration. Next the image is evaluated and the software attempts to find the spotting configuration that has been specified. If the software successfully detects the spots it will draw grids around each spot automatically. If the intensity difference between the spots

and the background is too low the software will not be able to find the spots and instead the grids must be manually aligned by the user. Once grids are aligned then the data can be exported into Excel. The Excel sheet will contain information on each spot of the well including X and Y coordinates, diameter, median intensity of spot and intensity of background. The median intensity (with background removed) measured in pixels was used for further data analysis.

Optimisation of assay set-up

Key materials and reagents were assessed to determine the optimum assay set-up. These included the microtitre plate supplier for spotting (Scienion (Clear Scienion Type 1 (CPH-5511); Clear Scienion Type 2 (CPH-5521)), Nunc (White plates 463201; Flat bottom 442404; Breakable 473768) and Millipore (MSFBN6B50), and the TMB/E supplier for the assay (Millipore and Scienion).

Optimisation of spotting volume

Both 1 nL and 5 nL spotting volumes were initially assessed for AMOZ, AOZ and CAP assays to determine any effects on assay performance. By applying the optimised antibody dilutions of AMOZ (1/2000), AOZ (1/1000) and CAP (1/1000) and enzyme labelled HRP conjugates at AMOZ (1/4000), AOZ (1/100) and CAP (1/1000), eight-point calibration curves, from 0 to 50 ng/mL were prepared in PBS buffer for each antibiotic and assessed as an individual system (only one antibiotic spotted) with no interaction from any other antibiotic.

Optimisation of antibodies and enzyme labelled HRP conjugates

A chequerboard design was employed to optimise the assay parameters. Microtitre plates were spotted in an individual system using antibodies to determine optimum parameters for each antibiotic. Microtitre plates were spotted using purified antibodies at a spotting volume of 1 nL at dilutions 1/50 – 1/500 for AHD, 1/250 – 1/2000 for AOZ and CAP and 1/500 – 1/4000 for AMOZ and SEM. Spotted antibodies were assessed as a chequerboard with individual enzyme labelled HRP conjugates at different dilutions (1/50 – 1/8000). A negative (0 ng/mL) and positive (10 ng/mL) standard were assessed for each parameter to determine optimum assay parameters for each antibiotic. The optimised antibody dilutions were for AMOZ (1/2000), AOZ (1/1000), SEM (1/4000), AHD (1/250) and CAP (1/1000) and the

optimised enzyme labelled HRP were for AMOZ (1/4000), AOZ (1/100), SEM (1/1000), AHD (1/100) and CAP (1/1000).

Individual and multiplex calibration curves

Microtitre plates were spotted in a multiplex system (AMOZ, AOZ, SEM, AHD and CAP) and both individual and multiplex calibration curves were assessed. Spotting and assay parameters were determined during the optimisation stage. The microtitre plates were spotted using purified antibodies at a spotting volume of 1 nL using different antibody dilutions depending on the antibody. Antibody dilutions for each antibody were as follows; AMOZ (1/2000), AOZ (1/1000), SEM (1/4000), AHD (1/250) and CAP (1/1000). Enzyme labelled HRP conjugates were used at the following dilutions for each antibody; AMOZ (1/4000), AOZ (1/100), SEM (1/1000), AHD (1/100) and CAP (1/1000) during the assay. Eight-point individual calibration curves for AMOZ, AOZ, SEM, AHD or CAP were prepared in PBS buffer (10 mM, pH 7.4) at concentrations 0, 0.05, 0.1, 0.5, 2, 5, 10 and 50 ng/mL (AMOZ, AOZ and CAP) and at concentrations 0, 1, 5, 10, 25, 50, 100 and 200 ng/mL (SEM and AHD). Eight-point multiplex calibration curves for AMOZ, AOZ, SEM, AHD and CAP were also prepared in PBS buffer. Where necessary further standards were added as additional calibration points to improve the curve shape for the multiplex calibration curves especially for SEM and AHD. Calibration curves were assessed and examined (n=2 analysis, 5 spots per analysis) and sensitivity as either the midpoint (IC_{50}) for full curves or the 50% inhibition concentration from the zero response determined from a 4 parameter fit curve using BIAevaluation version 4.1 software (Biacore, GE Healthcare).

Sample preparation

Hexane (5 mL), 1 M HCl (0.5 mL) and distilled water (4 mL) were added to honey (1 g) and vortex mixed for 1 min. The sample was centrifuged at 3000 g for 10 min and frozen at -80 °C for 2 hr in order to separate the phases. The upper phase was discarded and the lower aqueous phase was allowed to defrost. 2-Nitrobenzaldehyde (10 mM) in dimethyl sulfoxide (200 µl) was added to the aqueous phase and the sample was incubated overnight at 37 °C. Dipotassium phosphate (0.1 M, 5 mL), NaOH (1 M, 0.4 mL) and ethyl acetate (5 mL) were added to the sample and vortexed for 1 min. The sample was centrifuged at 3000 g for 10 min. The upper organic phase (2.5 mL) was transferred to a glass test tube and evaporated at 50°C under a slow nitrogen stream. Finally, the residue was dissolved in 1 mL PBS (10 mM, pH 7.4).

Matrix effects

Microtitre plates were spotted for the multiplex format and assay parameters examined were as described previously. Blank material for honey was sourced by Queen's University Belfast and was confirmed as blank for the analytes of interest. Calibration curves for AMOZ, AOZ, SEM, AHD and CAP covering the concentration range of 0 – 1000ng/mL were prepared in PBS buffer to achieve full calibration curves. Calibration curves were also prepared, equivalent in final concentration to the buffer curve for honey samples fortified at the start of extraction, and honey fortified at the end of extraction to determine matrix and recovery effects in the assay compared to the buffer curve.

Results and Discussion

Optimisation of assay set up

Based on the selection of the microtitre plates assessed the Nunc flat-bottomed plates were chosen for the assay development based on the assay performance achieved and price compared to the competitor plates. Millipore TMB/E (E-S001) was deemed not suitable for this multiplex assay as this reagent re-mobilised the spots whereby the development reaction was observed throughout the well, merging spots instead of as individual spots. Alternatively, when using the SciColor T2 special enzyme substrate solution the reaction was observed on the individual immobilised spots within the well and the liquid could be removed prior to scanning.

Optimisation of Spotting Volumes

For the two spotting volumes of 1 nL and 5 nL individual eight-point calibration curves for each antibiotic (AMOZ, AOZ or CAP) were successfully achieved (Figure 1). Similar curve shapes were observed between the two spotting volumes and there was minimal difference in sensitivity when applying the 1 nL or 5 nL spotting volume. Nonetheless, the smaller spots appeared sharper and were more clearly spatially defined. Initially reagents were only available for the AMOZ, AOZ and CAP targets and therefore it was decided that the final nanoarray would be spotted at 1 nL; the smaller spotting volume enabled the addition of the further two targets AHD and SEM when these reagents became available.

Optimisation of antibodies and enzyme labelled HRP conjugates

For the optimisation of the pairing of antibody and enzyme labelled HRP conjugate for each target the dilutions were optimised to give a spot intensity for the 0 ng/mL at approximately 20,000 pixels. At this level of signal there was significant improvements in the ease of grid alignments and the processing of data as the software was able to find the spotted nanoarrays and carry out grid alignments with the images automatically. Final assay parameters for the spotted antibodies were 1/2000, 1/1000, 1/4000, 1/250 and 1/1000 for AMOZ, AOZ, SEM, AHD and CAP respectively. Final assay parameters for the enzyme labelled HRP conjugates were 1/4000, 1/100, 1/1000, 1/100 and 1/1000 for AMOZ, AOZ, SEM, AHD and CAP respectively. These optimum parameters selected provided suitable results in terms of spot intensity and inhibition with the 10 ng/mL positive standard to develop further each assay. A relatively high concentration of 10 ng/mL was applied compared to the MRPLs for the positive control to observe a working dynamic range for AMOZ, AOZ and CAP between negative and positive controls and to observe inhibition for the AHD and SEM assays with detectable spot intensities.

Individual and multiplex calibration curves

Eight-point individual calibration curves determined for each antibiotic (AMOZ, AOZ, SEM, AHD or CAP) as individual curves in the multiplex format with all targets spotted in the assay were successfully achieved (Figure 2). Additionally, eight-point multiplex calibration curves for all five antibiotics (AMOZ, AOZ, SEM, AHD and CAP) analysed simultaneously in the multiplex assay were completed (Figure 3). Table 1 presents the limit of detection (LOD) of the assays, as the IC_{20} , and midpoint, IC_{50} , based on the midpoint of a full sigmoidal curve or the 50% inhibition concentration for partial curves for each target both in the individual assay and multiplex format. For AMOZ, AOZ and CAP the LODs achieved at 0.09, 0.26 and 0.04 ng/mL as individual assays is suitable to meet the MRPL values of 1 ng/mL and 0.3 ng/mL for the nitrofurans and CAP respectively. The LODs of these three individual assays are lower compared to when they are multiplexed at 0.19, 0.34 and 0.09 ng/mL for AMOZ, AOZ and CAP respectively. For multiplexing the detection of these targets at the MRPLs is realistic. The IC_{50} was similar for CAP at approximately 0.8 ng/mL for both the individual and multiplex calibration curves. The IC_{50} increased from 1.2 ng/mL to 3.2 ng/mL for AMOZ and similarly for AOZ from 1.9 ng/mL to 3.1 ng/mL between the individual and multiplex calibration curves showing a decrease in sensitivity for AMOZ and AOZ. Multiplexing assays with different antibodies may lead to some degree of cross talk

between targets which accounts for the increase in the IC_{20} and IC_{50} for the multiplex curves. The benefits of multiplexing can be a suitable compromise to a minimal loss of sensitivity in most applications. However, for SEM and AHD the antibodies utilised do not allow the assays to reach the desired LODs for these targets as individual assays with IC_{20} values established of 35.9 and 15.2 ng/mL respectively. Nonetheless, the proof of principle in multiplexing has still been established. With better-quality antibodies the LODs for these targets could be improved. The IC_{50} was greater than 200 ng/mL for both SEM and AHD for the individual calibration curves. For the multiplex calibration curves for the matrix study the calibration range was increased to 1000 ng/mL for both SEM and AHD to determine sensitivity (Figure 4). As expected the dynamic range for both SEM and AHD was improved and 50 % inhibition of 166.6 g/mL and 553.8 ng/ml respectively were obtained for the multiplex curves. Additionally, when assessed as individual calibration curves on multiplex spotted microtitre plates both SEM and AHD conjugates showed a degree of cross reactivity with other spotted antibodies. SEM was detected by the AMOZ, AOZ and AHD spotted antibodies and AHD was detected by the AOZ spotted antibody. Reduced sensitivities between the individual and multiplex calibration curves is possibly due to minor interference and non-specific binding of reagents between the assays. There was extremely good repeatability shown with the IC_{50} values shown for the first multiplex study in PBS buffer (Figure 3) compared to the PBS buffer data from the matrix study (Figure 4) as outlined in Table 1 though the LODs were more variable.

Matrix effects

For the examination of matrix and recovery effects for the multiplex assay, over the concentration range for the sample preparation method applied, calibration curves of equivalent concentration prepared in PBS buffer, honey fortified before extraction and honey fortified after extraction were generated (Figure 4). It should be noted that for nitrofurans analysis there is the added complexity in sample preparation in the derivatisation of the marker metabolites for their detection. Therefore the extraction and derivatisation remains an overnight process for all methods though the advantage of the nanoarray is that it offers the simultaneous analysis of the five targets from the one sample extract. Due to the sensitivity required to achieve the desired MRPL detection levels the matrix and recovery effects appear more pronounced at the LODs (IC_{20} values) compared to the midpoints (IC_{50} values) of the calibration curves (Table 1). The comparison of matrix effects is determined by differences observed in the calibration curves prepared in PBS buffer and honey matrix fortified at the

end of the extraction based on 100% recovery of the targets. Low matrix effects over the concentration range were evident for each antibiotic with differences observed in both LODs and IC_{50} s in calibration curves prepared in honey matrix (fortified at the end) compared to PBS buffer. Generally, the LODs were higher in fortified honey matrix curves though for AMOZ, AOZ and CAP still achieving the target MRPLs. An indication of the recovery of each assay can be observed on comparison of the difference between the calibration curves fortified before and after extraction. Based on the extraction method applied full recovery of the targets when fortified at the beginning of the extraction may not have been achieved based on the assumptions to attain 100% recovery. If the curves are overlaying there is excellent recovery in the assay but if they vary substantially then there are issues in recovery of the extraction method being applied. As can be observed from Figure 4 for each of the targets there are marginal differences in the overlay of the calibration curves highlighting minimal losses in recovery from the extraction method applied. When blank honey extract was fortified at the end of the extraction the IC_{50} improved for the AMOZ target to 1.4 ng/mL compared to 3.0 ng/mL in honey fortified at the beginning of the extraction. For other targets the sensitivity was marginally improved to that previously determined in honey fortified at the start of the extraction. For SEM the IC_{50} was 166.6 ng/mL in PBS buffer decreasing to 272.7 ng/mL in honey (fortified at the start) and 138 ng/mL (fortified at the end). For AHD the IC_{50} was quite high in PBS buffer at 553.8 ng/mL increasing to > 1000 ng/mL in honey matrix. Neither SEM nor AHD show the required sensitivity that would meet regulatory limits to be used successfully in a testing laboratory. Based on the comparison of the calibration curves determined it may be possible to apply a PBS buffer calibration curve to determine the concentration of each target in real samples as opposed to having a supply available of known blank honey for all targets to prepare extracted curves. The suitability of buffer curves for calibrations would need to be determined in a full validation study. The incorporation of a calibration curve for each target allows for the semi-quantitative analysis for the target nitrofurans and CAP as opposed to a qualitative yes / no response reflective of the LOD and associated MRPL.

Conclusions

The feasibility of a novel multiplexed nanoarray for the semi-quantitative and simultaneous screening of AMOZ, AOZ, CAP, SEM and AHD in honey has been illustrated whereby these antibiotics were successfully multiplexed into one assay. It was realised that antibodies for SEM and AHD were not as sensitive as for the other antibiotics and further improvements to

the sensitivity to make this multiplex nanoarray more applicable for regulatory testing would be necessary. Similarly, with the considerations of the natural occurrence of SEM it may be appropriate for rapid methods to source an antibody that differentiates between protein bound SEM compared to free SEM as protein bound is believed to be more indicative of nitrofurazone usage compared to natural occurrence. The identification of an alternative marker would also be important.

This study has demonstrated the proof of concept of a multiplex nanoarray for multiple nitrofurans and chloramphenicol detection with results available for 40 samples within 90 min after extraction. The benefit of this assay system is that it follows established ELISA protocols, whereby laboratories with immunological screening methods already in place have end users familiar with the steps of the analysis. The simplicity and sensitivity of the antibiotic nanoarray means it could be used as a multiple target screening technique for many antibiotics within the area of residue determination and food safety. This study has shown some very promising data that is worthy of further research to determine if this approach is suitable for a commercial diagnostic test in the food industry. As the multiplex reader technology is relatively new, uptake and implementation would require replacement of existing ELISA plate readers. In the food safety diagnostics sector this investment should be comparable to existing readers to have potential interest for future users, though consideration should also be given to the simultaneous high throughput detection of five targets on an established ELISA platform compared to five individual assays which has the potential to minimise labour time improving throughput reducing the overall cost of analysis in the long term. As traditional antibody based screening methods are now competing in a number of laboratories with LC-MS/MS methods as the new screening tools the cost of kits for multiplex analysis should be relatively low to be competitive with these LC-MS/MS methods. Improvements to the sensitivity for SEM and AHD, the addition of other antibiotics to the nanoarray and the detection in different food matrices are also all worthy of further evaluations. Additionally, prior to implementation a full validation and inter-laboratory trial of the nanoarray should be conducted following accreditation guidelines. To meet with the criteria of the EU this would be according to the guidelines for the validation of screening methods as outlined in the European decision EC/2002/657. For the collaborative trial the availability of multiplex readers would also need consideration.

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Table

Table 1. Limit of detection (LOD), as the 20 % inhibition concentration (IC₂₀), and mid-point for the calibration curve (IC₅₀) for each antibiotic (AMOZ, AOZ, SEM, AHD and CAP) expressed as ng/mL for both individual and multiplex analysis using PBS buffer and a honey matrix (honey samples fortified at the start and end of the extraction).

Antibiotic	Individual Analysis		Multiplex Analysis		Multiplex Analysis					
	PBS Buffer		PBS Buffer		PBS Buffer		Fortified at start of extraction		Fortified at end of extraction	
	IC ₂₀ (ng/mL)	IC ₅₀ (ng/mL)	IC ₂₀ (ng/mL)	IC ₅₀ (ng/mL)	IC ₂₀ (ng/mL)	IC ₅₀ (ng/mL)	IC ₂₀ (ng/mL)	IC ₅₀ (ng/mL)	IC ₂₀ (ng/mL)	IC ₅₀ (ng/mL)
AMOZ	0.09	1.2	0.65	3.2	0.19	3.0	0.34	4.1	0.23	1.4
AOZ	0.26	1.9	1.0	3.1	0.83	3.5	0.87	4.0	0.98	3.5
CAP	0.04	0.7	0.16	1.0	0.09	0.8	0.17	1.1	0.24	0.9
SEM	45.8	> 200*	34.8	161.4*	15.2	166.6*	42.1	272.7*	24.8	138.0*
AHD	6.9	> 200*	25.4	>200*	35.9	553.8*	90.7	> 1000*	58.9	> 1000*

*A full calibration curve was not obtained therefore the 50 % inhibition concentration was calculated from the 0 ng/mL standard.

Figures

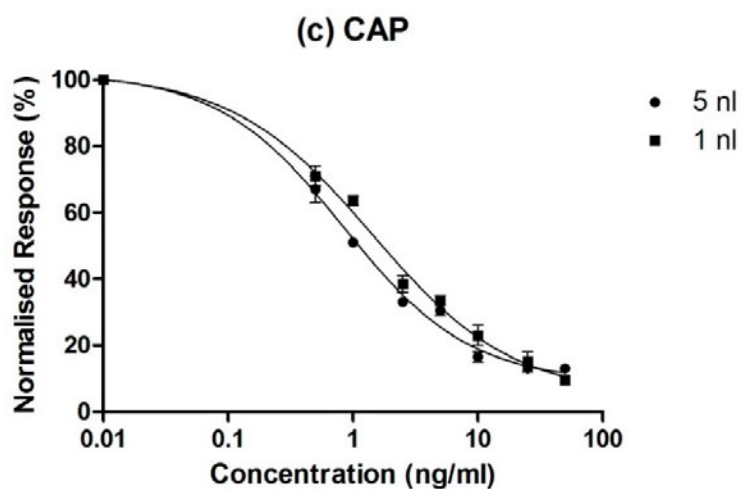
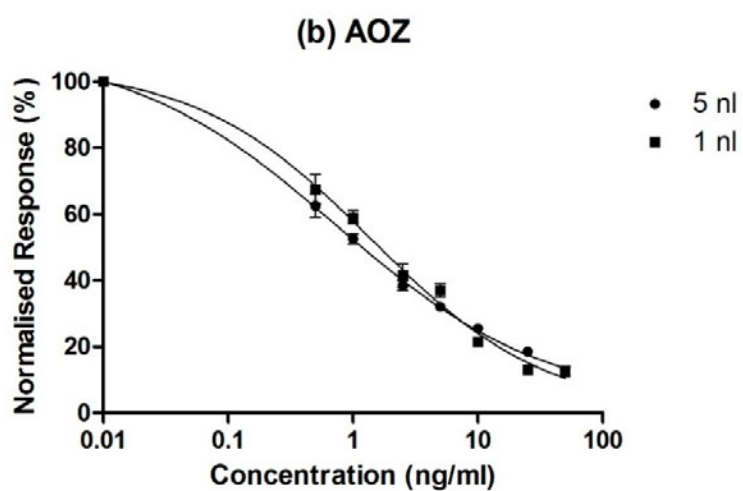
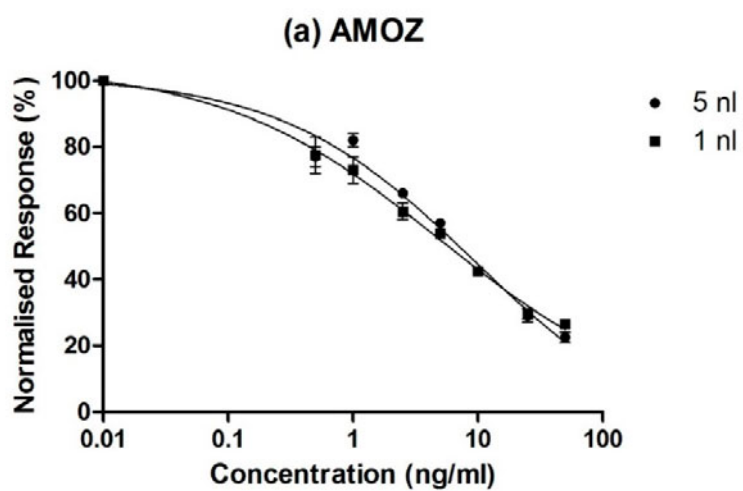


Figure 1: Individual calibration curves for AMOZ, AOZ and CAP in PBS buffer using individual spotted microtitre plates at 1 and 5 nL spotting volume (n=2 analysis, 9 spots per analysis).

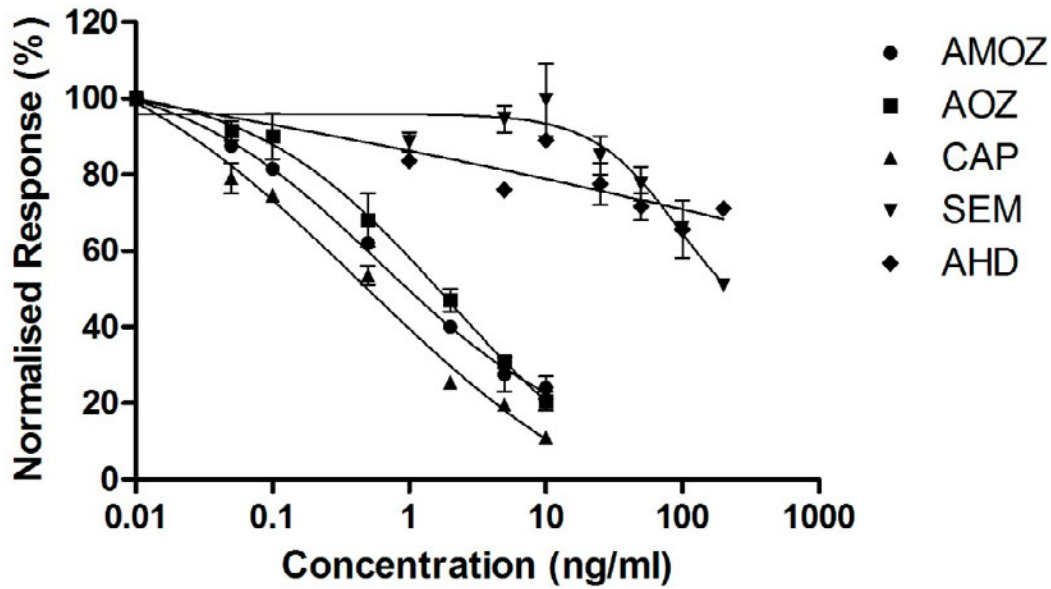


Figure 2: Individual calibration curves for the independent detection of AMOZ, AOZ, CAP, SEM and AHD in PBS buffer using a multiplex spotted microtitre plate (n=2 analysis, 5 spots per analysis).

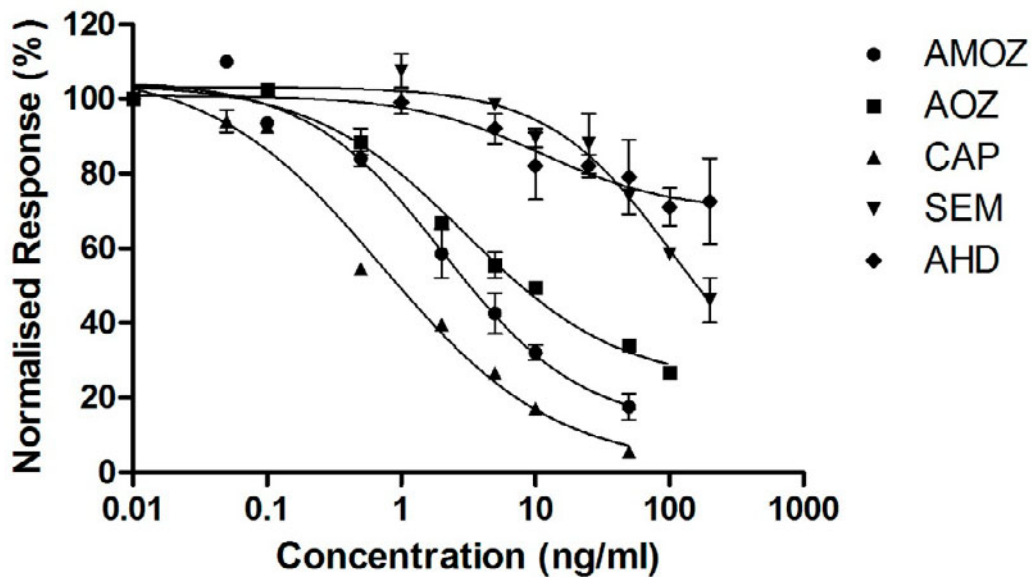


Figure 3: Multiplex calibration curves based on the simultaneous detection of AMOZ, AOZ, CAP, SEM and AHD in PBS buffer using a multiplex spotted microtitre plate (n=2 analysis, 5 spots per analysis).

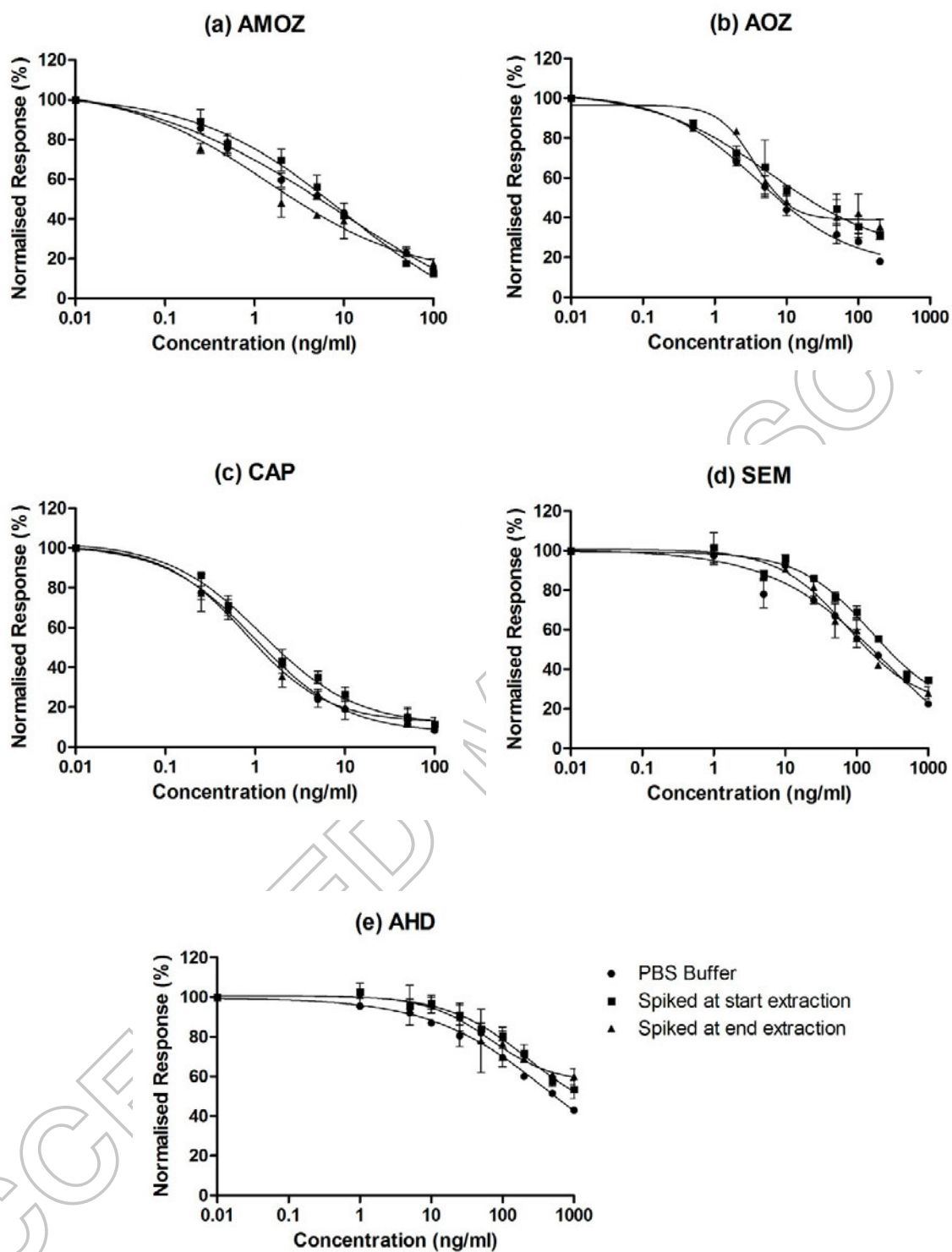
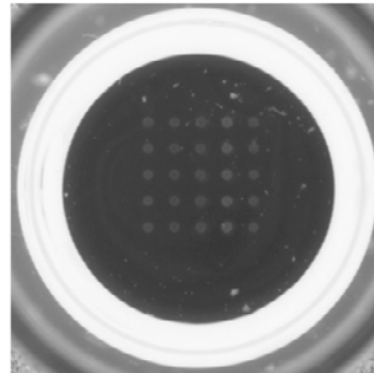
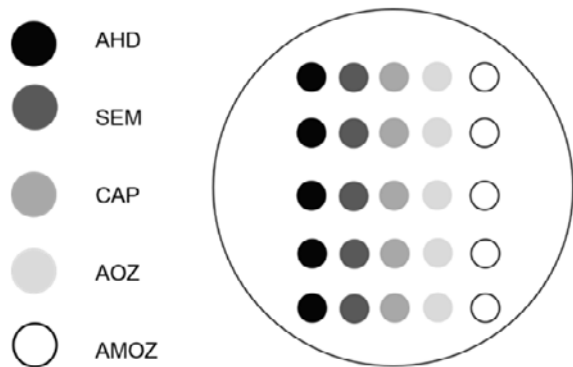


Figure 4: Matrix and recovery effects for AMOZ, AOX, CAP, SEM and AHD in PBS buffer, honey fortified at the start of the extraction and blank honey extracts fortified after extraction using multiplex spotted microtitre plates (n=2 analysis, 5 spots per analysis).

Graphical abstract



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