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Simultaneous detection of four nitrofuran 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 nitrofuran antibiotics. Using a multiplex approach, metabolites of the four main nitrofuran 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 Oasis™ 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 μg kg^{-1}. The detection capability was below 0.5 μg kg^{-1} for the metabolites AHD, AOZ and AMOZ; it was below 0.9 μg kg^{-1} for SEM. IC_{50} values ranged from 0.14 μg kg^{-1} (AMOZ) to 2.19 μg kg^{-1} (SEM). This biosensor method possesses the potential to be a fit-for-purpose screening technique in the arena of food safety technology.

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 nitrofuran 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.

Nitrofuran detection and quantification is typically performed using LC–MS/MS methods (Conneely et al., 2002; Conneely et al., 2003; O'Keeffe 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 ($CC\beta$) of 0.25 μg 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 nitrofuran 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 nitrofuran antibiotics in honey, a multiplex approach should be suitable to simultaneously detect the four main metabolites of nitrofuran 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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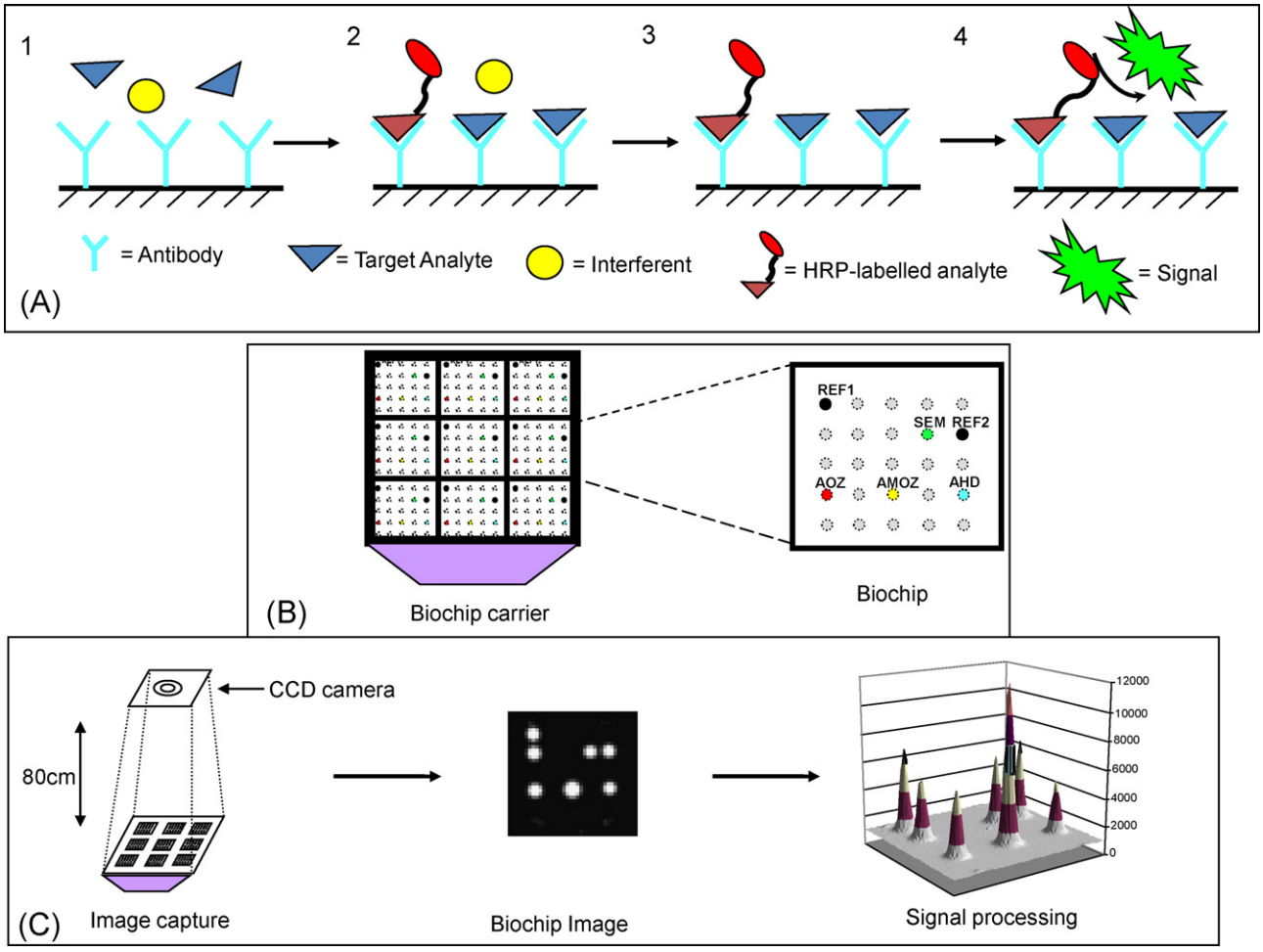

Fig. 1. (A) The assay employs a competitive format, which includes the following steps, (1) sample incubation, (2) conjugate incubation, (3) wash step, (4) addition of signal reagent leading to chemiluminescence response. (B) Biochip format, with 3×3 individual biochips per holder. Discrete test regions (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 nitrofuran 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 $\mu g\,kg^{-1}$ identified (2003/181/EC and Regulation (EC) No.470/2009).