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Analytical Methods

Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for detection of the furaltadone metabolite, AMOZ, in fortified shrimp samples ☆

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ABSTRACT

3-Amino-5-morpholinomethyl-2-oxazolidone (AMOZ) is a tissue bound metabolite of furaltadone, a synthetic nitrofuran antibiotic widely used in veterinary practices both to treat infectious diseases and as a growth promoter. Because AMOZ is carcinogenic and poses a potential health hazard for human consumers, its parent compounds, the nitrofurans, are banned from being used in animals destined for human food consumption in various countries. To enforce this, foods are routinely monitored for nitrofuran metabolite residues including AMOZ. Thus, reliable high throughput analytical methods to detect AMOZ have been developed but these are mostly based on chemical analysis. In contrast, sensitive and specific detection methods based on immunoassays for AMOZ using monoclonal antibodies have yet to be established. In this study, we report the generation of two monoclonal antibodies with high specificity for AMOZ and the development of a monoclonal antibody-based ELISA to detect AMOZ in shrimp samples using one of these clones (clone 2E5.1). Clone 2E5.1 yielded the highest sensitivity and specificity and cross reacted solely to furaltadone and AMOZ and not to other antibiotics. Competitive ELISA with 2E5.1 gave IC_{50} values for AMOZ, CPAMOZ and furaltadone of 5.33, 0.023 and 1.330 ng/ml, respectively. When applied in ELISA to detect AMOZ in fortified shrimp samples, the detection capability and limit of detection were 0.3 and 0.16 μg/kg, respectively. Taken together, a sensitive and specific monoclonal antibody-based ELISA for AMOZ detection has been developed that could facilitate the economic and reliable high throughput monitoring of AMOZ in shrimps and potentially other food.

1. Introduction

Nitrofurans are groups of synthetic antibiotics widely used as antibiotics in veterinary practices for treating infectious diseases such as salmonellosis, colibacilosis and coccidiosis. They exhibit broad spectrum antimicrobial activity against both Gram (+) and (−) bacteria, and some fungi. In addition to their efficacy as antibiotics, nitrofurans are also used as a growth promoter in feed additives for farm animals and in cultures of aquatic organisms (Auro, Sumano, Ocampo, & Barragan, 2004; McCracken & Kennedy, 2007).

Widely used parent drugs of nitrofurans are furaltadone (FTD), furazolidone (FZD), nitrofurazone (NZF) and nitrofurantoin (NFT) which all share 5-nitrofuraldehyde as a common chemical structure. All nitrofurans are light sensitive and metabolised immediately upon ingestion. While some metabolites are removed through excretion, some remain in the body covalently linked to proteins as tissue bound residues and can exhibit stability in tissues for more than 6 weeks after halting treatment. Cooper et al. (2005). FTD, FZD, NZF and NFT are metabolised to 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), 3-amino-2-oxazolidone (AOZ), semicarbazide (SEM) and 1-aminohydantonin (AHD), respectively (Cooper & Kennedy, 2005).

Animal studies have revealed the potential risks of nitrofurans as mutagens and carcinogens (Auro et al., 2004; Commision regulation, 1995; Van Koten, Wouters, & Van Leeuwen, 1993). In humans, tissue bound AMOZ were released upon ingestion as β-hydroxyethylhydrazine with both mutagenic and carcinogenic properties. Because of the potential health risks posed to consumers upon ingestion, many countries, including the European Union, USA, Canada, Australia and Japan, have banned the use of nitrofurans

Abbreviations: MRL, maximum residue limit; LOD, limit of detection.

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in feeds for farm animals destined for human consumptions. In restricting the use of nitrofurans, maximum residue limits (MRL) have been set for each parent drug of nitrofurans and its metabolites. MRL for AMOZ in the EU, for example, is set at 300 ng/kg in animal products. However, nitrofurans are still legally made and distributed for use in many countries of the world because they are cheap and effective antibiotics as well as growth promoters. Since these products with nitrofuran metabolites can make it to the market of those countries that have banned their use, the ability to reliably perform accurate high throughput screening in an economically feasible manner is required. For example, in 2002–2003 nitrofuran residues were found in poultry and aquaculture human food imports into Europe from Asia and South Americas, as well as from within the EU (Cooper, Caddell, Elliot, & Kennedy, 2004). Indeed, given the rapid rate of nitrofuran metabolism and clearance, it is the metabolites that are both the health risk and the required targets for detection in routine screening (McCracken, Blanchflower, Rowan, McCoy, & Kennedy, 1995; McCracken & Kennedy, 1997; Nouws & Laurensen, 1990). Therefore, the development of analytical methods for the reliable detection of both nitrofuran parent drugs in feeds and their metabolites in tissue-bound forms is increasingly and urgently in need to ensure food safety for consumers. Given the covalent attachment of nitrofuran metabolites to cellular proteins, their detection first requires their release through acidification. Currently, chemical methods for the detection of nitrofurans and their metabolites are widely used such as HPLC and LC–MS/MS (Cooper et al., 2005; Gottschall & Wang, 1995; McCracken & Kennedy, 2007; Horne, Cadogan, O'Keeffe, & Hoogenboom, 1996). The quantitative nature, precision and accuracy are the main advantages of these two analytical methods for detection of drug residues in food, whilst labour intensiveness, high cost, and the requirement of expensive equipments and trained professionals are their drawbacks. In addition to such technical drawbacks, the chemical based detection of nitrofurans, after being metabolised as tissue bound substance, requires an extra step of derivatisation. In the case of FTD metabolites, because of the small relative size of released tissue bound AMOZ, it needs to be first derivatised to NPAMOZ with addition of the 2-nitrobenzaldehyde group before subjecting to chemical analysis described above (Gottschall & Wang, 1995; Kumar, Toothill, & Ho, 1994).

In contrast to chemical detection by UV or MS detection, immunoassay-based methods for detecting drug residues in food are alternative methods and becoming increasingly important for screening large numbers of samples with equivalent or better sensitivity than chemical methods. The selectivity conferred by the antibody reduces the requirement for such stringent purification steps required by UV–HPLC (Hoogenboom, Berghmans, Polman, Parker, & Shaw, 1992), and theoretically, the requirement for 2-nitrobenzaldehyde conjugation to increase the size for LC–MS/MS analysis. However, it is not clear if the small sizes and conserved structures of these natural nitrofuran metabolites will allow specific antibody detection as opposed to aptamers, with the developed antibody for AOZ actually being to the derivative NPAOZ (Diblikova, Cooper, Kennedy, & Franek, 2005). In fact, several studies have reported on establishing immunoassay techniques for detection of nitrofurans and their derivatised metabolites such as NPAOZ, a metabolite of FZD, attaining sensitivity levels and, importantly, detection capabilities (no false negatives) as good as the more expensive and demanding LC–MS/MS methodologies in both shrimp and poultry tissues (Cooper, Elliott, & Kennedy, 2004; Diblikova et al., 2005; Franek et al., 2006). In addition, commercial test kits to detect nitrofurans, including AMOZ, are available. However, the more sensitive and greater threshold detection ability of monoclonal antibody-based ELISA for the detection of AMOZ has not been reported previously.

In this study, we report for the first time the generation of monoclonal antibodies specific for AMOZ and the development of a monoclonal antibody-based ELISA for AMOZ in shrimp samples.

2. Materials and methods

2.1. Reagents and animals

AMOZ, FTD and other nitrofurans and their metabolites (listed in Table 2) were purchased from Sigma–Aldrich (St. Louis, MO). Female BALB/c mice aged between 8 and 10 weeks were purchased from the National Laboratory Animal Centre (Mahidol University, Salaya, Thailand). All procedures involving laboratory animals were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the guidelines issued by Chulalongkorn University.

2.2. Preparation of immunogens

AMOZ was derivatised to CPAMOZ using 3-carboxybenzaldehyde and dry pyridine in a reflux reaction as described elsewhere (McCracken et al., 1995). CPAMOZ was detected by thin layer chromatography and kept at 4 °C until used. CPAMOZ was conjugated to carrier protein, cationised BSA (cBSA) or ovalbumin (OVA), using 1-ethy-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). After dialysis, CPAMOZ-cBSA and CPAMOZ-OVA conjugates were analysed by MALDI-TOF MS (National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand) to confirm protein-CPAMOZ conjugation. Protein conjugates were kept at −70 °C until used as immunising antigens and coating antigens for ELISA.

2.3. Immunisation and generation of hybridomas

BALB/c mice were immunised intraperitoneally with 50 μg of CPAMOZ-cBSA conjugates in complete Freund's adjuvants. Mice were later immunised a total of a further three or four times at 2-week intervals with CPAMOZ-cBSA in incomplete Freund's adjuvant. Tail bleeding was carried out to determine specific anti-NPAMOZ antibody serum titre before sacrificing the animals for spleens. Generation of hybridomas was carried out as described elsewhere (Harlow & Lane, 1988). In brief, isolated splenocytes were fused with the murine myeloma P3/NSI/1-Ag4-1 (TIB-18) obtained from ATCC, using polyethyleneglycol and selection for hybridomas were carried out in HAT media. Culture supernatants from each clone were subjected to screening by ELISA as described below. Positive clones were single cell cloned for multiple rounds by limiting dilution until monoclones were obtained.

2.4. ELISA

For screening of hybridomas, indirect ELISA was used. CPAMOZ-OVA dissolved in PBS (0.625 μg/ml) was used to coat Maxisorp ELISA plates (Corning) overnight at 4 °C. Plates were washed with washing buffer (5% Tween20 in 0.1 M Phosphate buffer saline pH 7.4; PBST) three times and blocked in blocking solution (5% skim milk in PBS) incubated at 37 °C for 1 h. After washing, culture supernatants or sera were added and plates were incubated at 37 °C for 2 h. HRP conjugated anti-mouse Fab rabbit Ab (Zymed, USA) was added and incubated at 37 °C for 1 h. Unbound antibodies were removed by washing solution as above and 150 μl of substrate *o*-phenylene diamine was added. The reactions were allowed to proceed for 10–15 min in dark and were terminated by the addition 100 μl of 2.5 M H_2SO_4 and the absorbance measured at 492 nm using a microplate reader (Titertrek). For

determining isotypes of monoclonal antibodies, antibody isotyping kits were used according to the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO).

2.5. Purification of monoclonal antibody

To obtain purified monoclonal antibody, hybridoma culture supernatants were collected, clarified of cells by centrifugation (1,000 rpm (168xg), 5 min) and antibody purified from the supernatant by Protein A affinity chromatography using a Step Elution System (Amersham Biosciences). A quantitative assay for the amount of protein was carried out using a BCA Protein Assay Kit (Pierce).

2.6. Cross reactivity and sensitivity

To determine the cross reactivity and specificity of each monoclonal antibody, antibody-captured indirect competitive ELISA was employed. CPAMOZ-OVA was coated onto Maxisorp ELISA plates as described above. Culture supernatants or purified antibodies were incubated with unconjugated free compounds at various concentrations for 2 h and the reactions were added to antigen pre-coated plates. After washing off unbound antibodies, the amounts of bound antibodies were determined using a similar procedure as that described above. The inhibition concentration 50 (IC_{50}) was determined using the following formula:IC_{50} = 50% B/B_0Where B_0 and B are the average absorbance obtained from the competitive indirect ELISA method without (B_0) and with (B) different concentrations of the competitors.Cross reactivity was determined by the following formula:Cross reactivity (%) = (CP-AMOZ $IC_{50} \times 100)/IC_{50}$ of compounds testedLimit of detection (LOD) was determined using the following formula:

$$\mathrm{LOD} = B_0 - 3\mathrm{SD}$$

where B_0 is the absorbance obtained in the competitive ELISA assay in reaction without any competitor, and SD is standard deviation obtained from experiments with 18 replicates.

2.7. Preparation of biotin-antibody conjugate

Biotin-antibody conjugate was prepared by adding biotin, dissolved in anhydrous DMSO, to 0.1 M carbonate buffer pH 8.4 containing antibody at a ratio of 100 μg biotin per mg of antibody. The solution mixture was stirred at room temperature for 4 h. Free biotin was then removed by dialysis against three changes of an excess volume of 0.1 M PBS (pH 7.4). The biotinylated antibodies were concentrated and stored at −20 °C.

2.8. Preparation of fortified shrimp samples

In this study, two methods of extraction were performed. Fresh shrimp samples were homogenised and spiked with AMOZ at different concentrations (0.125–80 μg/kg). For ethyl acetate extraction, determination of fortified AMOZ was based on the methods recommended by the United States Department of Agriculture Food Safety and Inspection Service (Office of Public Health Science) with some modifications. In brief, after homogenisation shrimp samples (1 g) were fortified with 100 μl of AMOZ at different concentrations and 4 ml of deionised water, 0.5 ml of 1 M HCl and 100 μl of 10 mM 2-nitrobenzaldehyde in DMSO were added. Samples were thoroughly mixed and incubated overnight in a water bath at 37 °C and then extracted by mixing with 5 ml of 0.1 M K_2HPO_4, 0.4 ml of 1 M NaOH and 5 ml of ethyl acetate, for 30 s, followed by centrifugation at 3000*g* for 10 min. The ethyl acetate fractions were collected and dried under nitrogen. Residues were dissolved in 2 ml of a 1:1 (v/v) mixture of hexane and 0.1 M PBS pH 7.4. The buffer phase, containing the AMOZ derivative, was separated by centrifugation at 3000*g*) for 10 min and collected for ELISA analysis.

For buffer extraction, the protocol was modified from a previous study (Cooper et al., 2004). In brief, shrimp samples were fortified with AMOZ diluted in Tris buffer (3.33 mM, pH 8.0) to the same final concentration as above. Protease Savinase 12T Type W (Novo Nordisk, Denmark) was added (final concentration of 0.625 mg/ml in Tris buffer), mixed thoroughly by vortexing for 1 min and incubated at 55 °C for 2 h in a shaking water bath. After allowing to cool down to room temperature the samples were mixed with 150 μl of 5 M HCl and 25 μl of 50 mM 2-nitrobenzaldehyde in DMSO, and incubated overnight at 37 °C. Samples were then mixed with 360 μl of 2 M NaOH in 10x concentrated PBS for 20 s, and centrifuged at 1640g at 4 °C for 15 min. The clear supernatants were collected and adjusted to pH 7.1–7.2 and used for ELISA analysis.

2.9. Detection of AMOZ in shrimp samples by competitive ELISA

AMOZ-OVA (0.625 μg/ml in 0.1 M PBS, pH 7.4) was coated on Maxisorp ELISA plates by incubation overnight at 4 °C. Plates were washed three times and blocked in blocking solution (1% BSA) at room temperature for 1 h. After washing as above, 50 μl of standard (AMOZ and NPAMOZ) or extracted samples and 50 μl of biotinylated Ab solution (1:4000 dilution) were added. Plates were incubated for 1 h at room temperature and unbound antigen and antibody were removed by washing three times in PBST. A 100 μl aliquot of HRP-labelled streptavidin solution (1:4000 dilution in 0.1 M PBS, pH 7.4) was added and plates were incubated for 10 min at room temperature. After removing unbound streptavidin by washing buffer, substrate developer solution (100 μl of 10% (w/v) TMB in DMSO dissolved into 10 ml of 0.1 M sodium acetate buffer, pH 6.0 and 3.4 μl of 30% (w/v) H_2O_2) was added. The enzymatic reaction was incubated for 30 min at room temperature and then stopped by the addition 100 μl of 1 M H_2SO_4. Absorbance values were measured at 450 nm and used to calculate the concentrations of the analyte in the samples.

For calculating the detection capability (CCβ) and the limit of detection (LOD) of ELISA, the methods described by Cooper et al. were followed (Cooper, Delahaut, Fodey, & Elliott, 2004).

Additionally, the samples were fortified with AMOZ at different concentrations (0.250–5 μg/kg) and comparatively analysed by both ELISA and LC–MS/MS.

3. Results and discussion

3.1. Generating monoclonal antibody to detect CPAMOZ

The desired hapten for generating monoclonal antibodies in this study was AMOZ, but in addition to its small conserved structure it has only one amino group available for conjugation to the protein carrier (ovalbumen or cBSA). Therefore, the 3-carboxybenzaldehyde derivative of AMOZ, CPAMOZ was used instead for the conjugation purpose and was derived using the condensation reaction, and the generation of CPAMOZ confirmed by MALDI-TOF MS. Conjugation of CPAMOZ yielded hapten-carrier proteins with a molar carrier protein:hapten ratio of 1:27 and 1:1.4 for cBSA and OVA (data not shown), respectively. Mice were repeatedly immunised by CPAMOZ-cBSA conjugate and the specific sera antibody titres were determined from tail bleeds by indirect ELISA using CPAMOZ-OVA conjugates (to exclude any antibodies against BSA) as coating antigens. Five mice were immunised and all showed varying sera titres ranging from 1:64 000 to 1:128 000. Sera from these mice showed a slight but insignificant cross reactivity with the

carrier protein cBSA, but not against OVA as expected (data not shown). When all sera were subjected to antibody-captured competitive ELISA using free CPAMOZ and AMOZ as competitors, they were found to be highly specific for CPAMOZ (data not shown). Multiple fusions of splenocytes with myeloma cell lines yielded a total of 20 hybridoma clones which produced monoclonal antibodies specific for CPAMOZ when screened by indirect ELISA using CPAMOZ-OVA as the coating antigen. Among these 20 hybridoma clones, five clones (2E5.1, 2H6, 4F1, 7H8 and 8B1.1) showed a high specificity for CPAMOZ and no cross reactivity with the carrier protein cBSA, and so were selected for further characterisation.

3.2. Characterisation of monoclonal antibodies

The data from the characterisation of the selected five clones are summarised in Table 1. All antibodies were of the IgG class, but showed variation in the subclass isotypes between IgG_1 and IgG_2. When assayed by antibody-captured competitive ELISA using CPAMOZ-OVA as the coating antigen, all antibodies showed cross reactivity to NPAMOZ and the parent drug FTD, in addition to reactivity to CPAMOZ. However, clones 4F1 and 7H8 revealed significantly higher IC_{50} values. Moreover, only 2E5.1 and 2H6 showed any detectable cross reactivity with AMOZ, albeit at only 7.9% and 16.9% relative reactivity, respectively (Table 1).

In order to use monoclonal antibodies generated in this study for the development of an immunoassay-based detection method, the sensitivity and cross reactivity of each monoclonal antibody were analysed by antibody-captured competitive ELISA using CPAMOZ-OVA as the coating antigen. As summarised in Fig. 1 and Table 1, the different antibodies showed different sensitivities against CPAMOZ, NPAMOZ, AMOZ and FTD. The inhibition concentrations 50 (IC_{50}) for CPAMOZ are in the range of 1.19–150 ng, whilst those for NPAMOZ are in the range of 1.59–72.4 ng/ml. The IC_{50} for AMOZ from monoclonal antibodies 2E5.1 and 2H6 were 15.0 and 90.6 ng/ml, respectively. A summary of the observed cross reactivity against other nitrofurans and unrelated compounds is shown in Table 2. β-Agonists and most antibiotic residues in foods pose a health hazard to consumers and are targeted for strict regulations in many countries. β-Agonists have been used in veterinary medicine as bronchodilators, but are also used illegally as a feed additative to promote rapid animal growth and to reduce the fat content in meat (Haughey et al., 2001). Similar to nitrofurans, these residues are routinely inspected for in food safety precautions. When reactivity to CPAMOZ is set as 100%, antibodies from clones 2E5.1 and 2H6 showed cross reactivity with AMOZ at 7.9% and 16.9%, respectively. On the other hand, all five clones and especially clones 2H6 and 4F1 produced antibodies that showed strong cross reactivity with the parent drug FTD. When cross reactivity with FZD, NFT and NFZ, their derivatives and metabolites were evaluated, no significant cross reactivity was detected at all (<0.01%), suggesting that all antibodies generated in this study are highly specific for FTD and its derivatives, but not other nitro-

Table 1
Summary of some characteristics of the five murine monoclonal antibodies derived from CPAMOZ-cBSA immunisation.

Clone#	Isotype	Competitor							
		CPAMOZ		NPAMOZ		AMOZ		FTD	
		IC_{50} (ng/ml)	LOD (ng/ml)	IC_{50} (ng/ml)	LOD (ng/ml)	IC_{50} (ng/ml)	LOD (ng/ml)	IC_{50} (ng/ml)	LOD (ng/ml)
2E5.1	IgG_{2b}	1.19 ± 0.03	0.17 ± 0.07	1.59 ± 0.02	0.14 ± 0.07	15.0 ± 0.02	1.42 ± 0.06	0.36 ± 0.02	0.12 ± 0.10
2H6	IgG_1	15.3 ± 0.04	1.35 ± 0.05	4.55 ± 0.01	0.38 ± 0.03	90.6 ± 0.03	4.71 ± 0.03	0.74 ± 0.01	0.19 ± 0.05
4F1	IgG_{2a}	43.4 ± 0.04	2.42 ± 0.05	57.6 ± 0.03	4.57 ± 0.03	NR*	NR*	2.19 ± 0.01	0.50 ± 0.10
7H8	IgG_{2b}	151 ± 0.05	22.5 ± 0.08	72.3 ± 0.05	3.76 ± 0.06	NR*	NR*	17.6 ± 0.03	2.55 ± 0.06
8B1.1	IgG_1	1.20 ± 0.03	0.20 ± 0.07	12.8 ± 0.04	0.14 ± 0.04	NR*	NR*	0.59 ± 0.02	0.12 ± 0.08

*NR = no reactivity.
IC_{50} and LOD were calculated using the formulas described in the Section 2.

Table 2
Cross reactivity of monoclonal antibodies with nitrofurans and other compounds.

Compounds		Cross Reactivity (%) for the indicated monoclonal antibody #				
		#2E5.1	#2H6	#4F1	#7H8	#8B1.1
Nitrofurans	CPAMOZ	100	100	100	100	100
	NPAMOZ	74.3	336	75.4	208	9.4
	AMOZ	7.91	16.9	NR	NR	NR
	FTD	338	2061	1981	859	202
	FZD	<0.01	<0.01	<0.01	<0.01	<0.01
	AOZ	<0.01	<0.01	<0.01	<0.01	<0.01
	NPAOZ	<0.01	<0.01	<0.01	<0.01	<0.01
	NFT	<0.01	<0.01	<0.01	<0.01	<0.01
	AHD	<0.01	<0.01	<0.01	<0.01	<0.01
	NPAHD	<0.01	<0.01	<0.01	<0.01	<0.01
	NFZ	<0.01	<0.01	<0.01	<0.01	<0.01
	SCA	<0.01	<0.01	<0.01	<0.01	<0.01
	NPSCA	<0.01	<0.01	<0.01	<0.01	<0.01
β-Agonists	Clenbuterol	<0.01	<0.01	<0.01	<0.01	<0.01
	Salbutamol	<0.01	<0.01	<0.01	<0.01	<0.01
Antibiotics	Norfloxacin	<0.01	<0.01	<0.01	<0.01	<0.01
	Penicillin G	<0.01	<0.01	<0.01	<0.01	<0.01
	Flumequine	<0.01	<0.01	<0.01	<0.01	<0.01
	Oxytetracycline	<0.01	<0.01	<0.01	<0.01	<0.01

*NR = no reactivity.
FZD = furazolidone, AOZ = 3-amino-2-oxazolidone, NPAOZ = 3-[(2-Nitro-benzylidene)-amino]-oxazolidin-2-one, NFT = N-[5-nitro-2-furfurylidene]-1-aminohydantoin, AHD = 1-aminohydantoin, NPAHD = 1-[(2-Nitro-benzylidene)-amino]-imidazolidin-2,4-dione, NFZ = 5-nitro-2-furaldehyde semicarbazone, SCA = semicarbazide, NPSCA = 2-nitro-benzaldehydesemicarbazone.

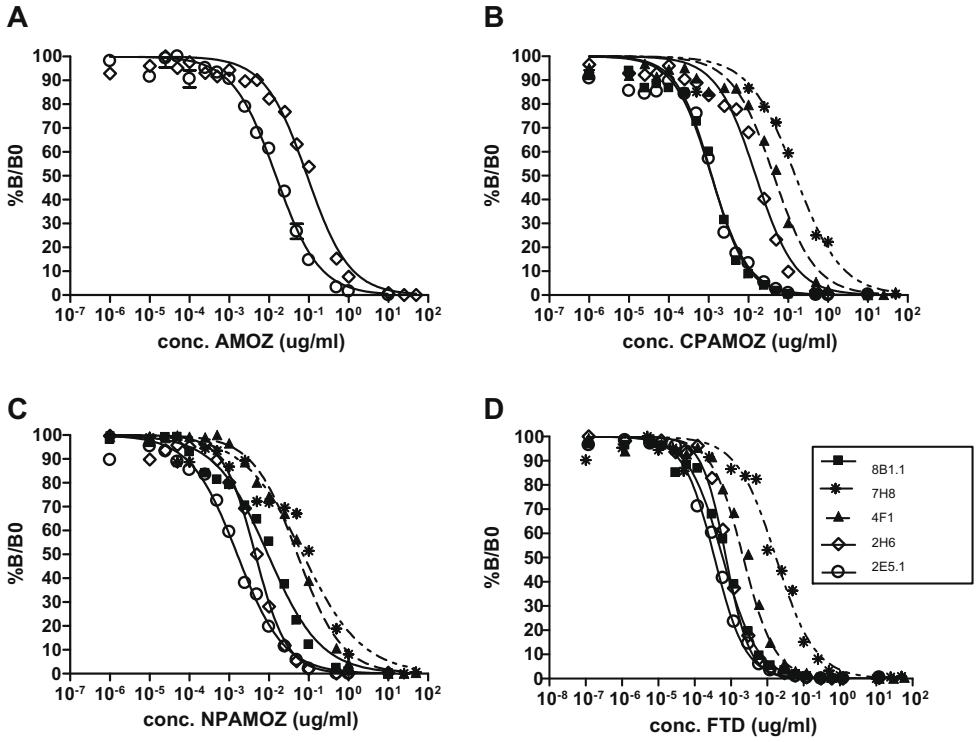

Fig. 1. Specificity of monoclonal antibodies to AMOZ, CPAMOZ, NPAMOZ and FTD. Monoclonal antibodies from five different clones were subjected to competitive ELISA using CPAMOZ-OVA as coating antigens and AMOZ (A), CPAMOZ (B), NPAMOZ (C) and FTD (D) as competitors. The% B/B_0 was calculated as described in Section 2. Out of five clones, only monoclonal antibodies 2E5.1 and 2H6 showed reactivity to AMOZ.

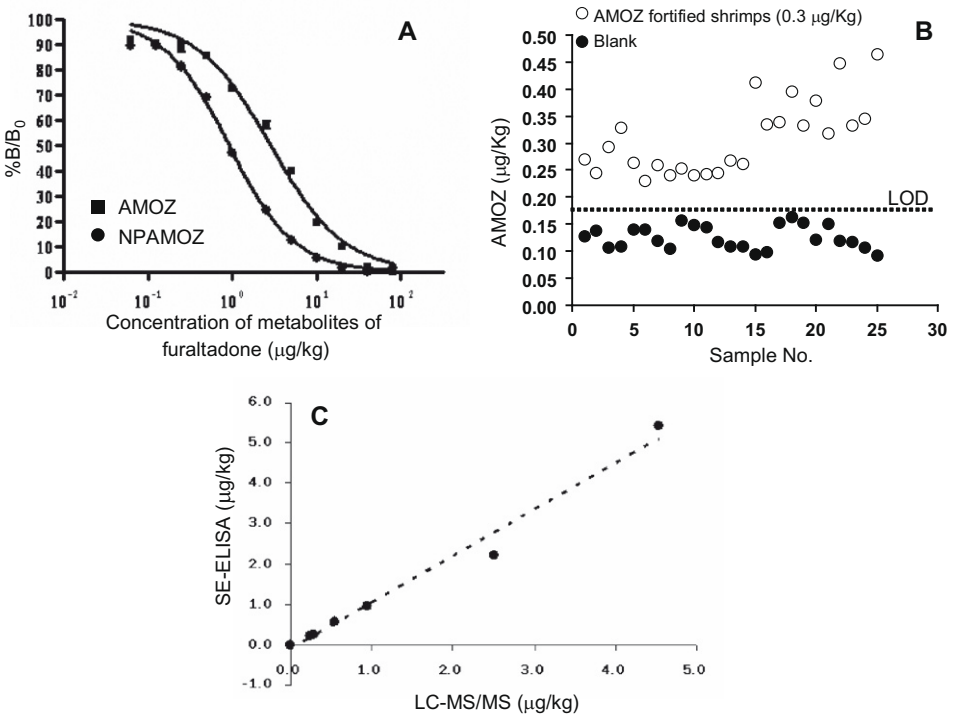

Fig. 2. Standard curve, CCβ and LOD for the detection of AMOZ in shrimp samples. (A) The standard curve was generated by using AMOZ-OVA as coating antigens (0.625 μg/ml) and AMOZ (closed rectangle) or NPAMOZ (closed circle) as competitors in an antibody-captured competitive ELISA. The% B/B_0 was calculated as described in Section 2. (B) Detection capability (CCβ) and limit of detection (LOD) of ELISA quantified by using 25 fortified shrimp samples with solvent extraction. Blank samples are represented by closed circles and fortified samples were represented by open circles. The LOD was determined as 0.17 ± 0.07 μg/kg. (C) Comparative detection of AMOZ by SE-ELISA and LC–MS/MS methods. The regression equation for the SE-ELISA was $y = 1.1403x - 0.075$.

furans. In addition, when unrelated antibiotics and β-agonists, two classes of prohibited food residues, were tested, no cross reactivity was detected (<0.01%), supporting the notion that the monoclonal antibodies generated in this study including 2E5.1 are capable of

specifically detecting FTD, its metabolites and metabolite derivatives.

After antibody was purified, the limit of detection (LOD) for competitive ELISA was also determined. Clone 2E5.1 showed the lowest LOD for AMOZ at 0.15 ng/ml, which is lower than the MRLs of 300 ng/kg which are currently enforced. Therefore, antibody clone 2E5.1 is highly specific and has sufficient sensitivity to NPAMOZ as to be able to detect AMOZ in food samples.

3.3. Development of ELISA to detect AMOZ in shrimp samples

The 2E5.1 monoclonal antibody was selected over 2H6 for ELISA development due to its fourfold lower LOD in assays using NPAMOZ, despite its twofold lower cross reactivity with AMOZ than 2H6. Thus to ascertain the applicability of the 2E5.1 monoclonal antibody generated in this study for the detection of derivatised AMOZ (NPAMOZ) in shrimp samples, direct competitive ELISA and different sample extraction methods were both investigated. Homogenised shrimps were spiked with known amounts of AMOZ (from 0.125 to 10 μg/kg) and the accuracy and precision of the developed ELISA was investigated. Samples were extracted by either the solvent (SE) or the buffer (BE) method and AMOZ was derivatised to NPAMOZ as described in Section 2. For generating a standard curve, the potential for matrix effects from the shrimp samples were investigated and no significant matrix effect was found, as in no effect from the shrimp samples themselves was detected in this system (data not shown). The amounts of NPAMOZ were analysed and calculated based on the standard curve using AMOZ and NPAMOZ as competitors (Fig. 2A). When samples were extracted with solvents, the intra-sample coefficients of variation (CV) and the percentages of recovery of the intra-assay for fortified AMOZ between 0.25 and 10 μg/kg were in the range of 0.03–25.6% and 80–124%, respectively, but at 0.125 μg/kg the CV rose to 51.2% potentially highlighting a possible limitation to the reliable detection threshold in this range (Table 3). However, the repeatability of the assay, as showed by analysis of the inter-sample assays over a three months period revealed a CV and yield recovery in the range of 0.01–7.43% and 72–157%, respectively, including for samples spiked at 0.125 μg/Kg (CV = 7.43%). Therefore, these results suggest that the developed ELISA can be used to screen AMOZ in shrimp samples with acceptable accuracy and precision following solvent extraction and derivatisation to NPAMOZ.

Although solvent extraction methods lead to antigen concentration, they are time consuming (especially in high throughout scenarios) and may in some cases adversely affect the precision of immunoassays due to differential extraction yields and solvent carry over. We, therefore, also investigated the reliability of the ELISA for detection of AMOZ in shrimps following buffer extraction (Table 3). Most of the% CV and% recovery values attained from buffer extracted samples were within an acceptable range. However, the% recovery showed a tendency to be under 100% (40% and 57% of intra- and inter-samples assayed were below the 80% recovery yield), which may potentially cause a false negative result. Therefore, perhaps in contrast to expectations (Diblikova et al., 2005), it is recommended that samples should be extracted by the solvent method.

Using this established ELISA, the detection capability (CCβ) and limit of detection (LOD) of ELISA were assessed. Analysis of 25

Table 3
Precision, recovery and repeatability in the determination of AMOZ levels in shrimp samples by ELISA methods.

Method		Fortified conc. (μg kg^{-1})	*N*	Mean ± SD (μg kg^{-1})	CV (%)	Recovery (%)
Intra-assay coefficient of variation						
SE	Sample 1	10	6	11.4 ± 0.00	0.03	114
	Sample 2	5	19	6.18 ± 0.02	0.25	124
	Sample 3	3	19	3.30 ± 0.07	1.32	110
	Sample 4	2.5	6	2.23 ± 0.01	0.26	89.1
	Sample 5	1	19	0.81 ± 0.09	8.14	81.3
	Sample 6	0.6	19	0.48 ± 0.13	15.3	80.4
	Sample 7	0.5	6	0.50 ± 0.02	4.05	100
	Sample 8	0.3	19	0.27 ± 0.07	16.1	90.7
	Sample 9	0.25	6	0.24 ± 0.06	25.6	97.2
	Sample 10	0.125	6	0.12 ± 0.06	51.2	98.4
BE	Sample 1	80	8	83.0 ± 0.00	0.00	104
	Sample 2	40	8	33.5 ± 0.02	0.06	83.6
	Sample 3	10	8	9.12 ± 0.02	0.17	91.2
	Sample 4	3	8	2.06 ± 0.06	2.92	68.6
	Sample 5	0.3	8	0.30 ± 0.10	33.6	99.6
Inter-assay coefficient of variation						
SE	Sample 1	10	8	10.2 ± 0.00	0.01	102
	Sample 2	5	8	6.47 ± 0.02	0.07	129
	Sample 3	3	8	4.71 ± 0.00	0.03	157
	Sample 4	2.5	8	3.00 ± 0.02	0.17	120
	Sample 5	1	8	1.11 ± 0.04	1.31	111
	Sample 6	0.6	8	0.66 ± 0.02	0.83	111
	Sample 7	0.5	8	0.63 ± 0.00	0.12	125
	Sample 8	0.3	8	0.36 ± 0.02	1.99	120
	Sample 9	0.25	8	0.18 ± 0.02	4.17	72.5
	Sample 10	0.125	8	0.15 ± 0.03	7.43	118
BE	Sample 1	80	8	91.8 ± 0.05	0.01	115
	Sample 2	40	8	39.7 ± 0.05	0.03	99.3
	Sample 3	20	8	16.5 ± 0.05	0.08	82.7
	Sample 4	10	8	6.74 ± 0.06	0.28	67.4
	Sample 5	2.5	8	1.79 ± 0.04	0.68	71.4
	Sample 6	1	8	1.48 ± 0.04	0.80	148
	Sample 7	0.5	8	0.45 ± 0.03	1.23	89.4
	Sample 8	0.25	8	0.16 ± 0.05	5.42	65.8

Notes: SE = solvent extraction.
BE = buffer extraction.
N = sample numbers.

Table 4
Comparison of quantitative analysis of AMOZ by ELISA and LC-MS/MS in fortified shrimp samples.

Fortified conc. (μg kg^{-1})	Measured conc. (μg kg^{-1})			
	ELISA		LC–MS/MS	
	Mean (μg kg^{-1})	Δ^2	Mean (μg kg^{-1})	Δ^2
5.00	5.41	0.1700	4.54	0.2116
2.50	2.21	0.0855	2.50	0.0000
1.00	0.97	0.0009	0.95	0.0025
0.60	0.59	0.0002	0.54	0.0036
0.30	0.26	0.0014	0.29	0.0001
0.25	0.22	0.0009	0.24	0.0001
Sum	–	0.26	–	0.22

Note: the results shown with ELISA represented mean of six replicates in an intra-assay.

samples of shrimps fortified with AMOZ at the current European MRL level of 300 ng AMOZ/kg tissue weight, and extracted by the solvent method yielded a CCβ of 0.3 μg/kg with a derived LOD of 0.17 ± 0.07 μg/kg, as shown in Fig. 2B.

The ability to detect AMOZ without the need for prior derivatisation with *o*-nitrobenzaldehyde would be a further advantage to both cost and time reduction in the detection of AMOZ in food samples, but the apparent low relative cross reactivity with AMOZ of these two antibodies (2E5.1 and 2H6) compared to that seen with NPAMOZ suggests that direct ELISA detection of AMOZ may not attain a reliable detection threshold within MRL levels, and indeed this may fall more into the domain of aptamer based detection, but this awaits experimental confirmation. In addition, AMOZ binds tightly to tissues, hampering the extraction step for direct detection.

Two other antibodies, #2H6 and #4F1, showed high relative binding activity to the parent nitrofuran FTD but not the metabolites and thus are under investigation as possible tools for detection of FTD in feedstocks.

In conclusion, we report the generation of monoclonal antibodies specific for the FTD metabolite, AMOZ and the derivatives CPAMOZ and NPAMOZ. Using one of these antibodies, 2E5.1, an ELISA-based detection method for NPAMOZ was developed and showed high specificity, sensitivity and precision sufficient for detecting AMOZ in shrimp samples within the range of the general MRL set for AMOZ when solvent extracted and derivatised with *o*-nitrobenzaldehyde (to NPAMOZ). This monoclonal antibody-based ELISA has, therefore, the potential to likely be useful for rapid screening of AMOZ in a high throughput economic manner. This is the first report to our knowledge of the development of a monoclonal antibody-based ELISA for the detection of AMOZ (via NPAMOZ) in shrimp samples.

3.4. Comparison of AMOZ detection between ELISA and LC–MS/MS method

The accuracy of the analysis was determined by the comparative detection of fortified AMOZ at different concentrations using ELISA and LC–MS/MS. The measurement correlation of the two methods was shown in Fig. 2C. The linear regression was obtained with an R^2 of 0.9809, indicating that both methods are in good agreement. In addition, the different values between the fortified and the analysed concentrations of both methods were also shown in Table 4. The results suggested that, like the LC–MS/MS method, the developed ELISA can be used to accurately detect AMOZ in shrimp samples.

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