Chemosphere xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Commercially available chemicals as immunizing haptens for the development of a polyclonal antibody recognizing carbendazim and other benzimidazole-type fungicides

Christos Zikos [a], Alexandra Evangelou [a], Chrysoula-Evangelia Karachaliou [a], Georgia Gourma [a], Petros Blouchos [b], Georgia Moschopoulou [b], Constantinos Yialouris [c], John Griffiths [d], Graham Johnson [d], Panagiota Petrou [a], Sotirios Kakabakos [a], Spyridon Kintzios [b], Evangelia Livaniou [a,*]

[a] Institute of Nuclear and Radiological Sciences & Technology, Energy and Safety (INRaSTES), NCSR "Demokritos", 153 10 Athens, Greece
[b] Agricultural University of Athens, Faculty of Biotechnology, Iera Odos 75, 118 55 Athens, Greece
[c] Agricultural University of Athens, Faculty of Agricultural Economics and Development, Laboratory of Informatics, Iera Odos 75, 118 55 Athens, Greece
[d] Uniscan Instruments LTD, 1 Burlow Road, Buxton SK17 9JB, United Kingdom

ARTICLE INFO

Article history:
Received 3 January 2014
Received in revised form 7 March 2014
Accepted 10 March 2014
Available online xxxx

Handling Editor: J. de Boer

Keywords:
Benzimidazole and benzimidazole derivatives
Benzimidazole-type fungicides (carbendazim, benomyl, thiabendazole)
Immunizing haptens
Polyclonal antibody
ELISA
Biosensors

ABSTRACT

Carbendazim is a fungicide widely used for controlling fungi affecting fruits, vegetables, field crops etc. Determination of carbendazim in water, soil and various crops is frequently required to assure compliance with national/European regulations. A polyclonal antibody recognizing carbendazim was developed by using commercially available 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid and 2-mercaptobenzimidazole as immunizing haptens; each of the above derivatives was directly conjugated to the carrier protein keyhole limpet hemocyanin and a mixture of the conjugates was administered to New Zealand white rabbits. Immunochemical functionality of the antisera and the corresponding isolated antibody (whole IgG fraction) was evaluated through titer and displacement curves in an in-house developed ELISA, which employed a 2-mercaptobenzimidazole – functionalized lysine-dendrimer as the immobilized hapten. As shown with ELISA-displacement curves, the above antibody could recognize carbendazim as well as other benzimidazole-type fungicides, i.e. benomyl and thiabendazole, and also intact benzimidazole, while it did not cross-react with the structurally different pesticides carbaryl and imazalil. Considering the rather simple approach which has led to its development and its highly promising immunochemical profile, the new antibody may be exploited in immunoanalytical systems for detecting benzimidazole-type pesticides e.g. in samples of environmental interest. The above antibody is being currently tested as a biorecognition element in the novel FOODSCAN cell biosensor platform for pesticide residue detection based on the Bioelectric Recognition Assay technology.

1. Introduction

Carbendazim [methyl 2-benzimidazole carbamate] is applied to a wide range of cereals, fruits, vegetables, field crops etc. as a broad spectrum benzimidazole fungicide (Davidse, 1986), being one of the most commonly used pesticides in modern agriculture.

Despite their great contribution to the improvement of crop production yields, pesticides – including benzimidazole fungicides – have been recognized as being possibly hazardous to human health (McCarroll et al., 2002). Maximum residue limits (MRLs) have been established in the EU for benzimidazole residues in food commodities, in order to protect public health according to Commission Regulation 2010/37/EC, while annual surveillance

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BCA, bicinchoninic acid; BERA, bioelectric recognition assay; BSA, bovine serum albumin; DCM, dichloromethane; DIC, N,N′-diisopropyl carbodiimide; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; eq, equivalent; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IgG, γ-immunoglobulin; KLH, keyhole limpet hemocyanin; LC–MS, liquid chromatography–mass spectrometry; MRL, maximum residue limit; OD, optical density; oxyma, ethyl 2-cyano-2-(hydroxyimino)acetate; PB, phosphate buffer; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sSMCC, sulfo-succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate; TFA, trifluoroacetic acid.

* Corresponding author. Tel.: +30 210 650 3839; fax: +30 210 654 3526.
E-mail address: livanlts@rrp.demokritos.gr (E. Livaniou).

http://dx.doi.org/10.1016/j.chemosphere.2014.03.049
0045-6535/

programs are carried out in member states under Council Directive 96/23/EC (Keegan et al., 2011). Analytical methodologies that enable fast and accurate determination of pesticide levels in water, soil and various food commodities are therefore needed and frequently required to assure compliance with national/European legislation. Instrumental analysis including LC–MS has been used for the determination of carbendazim in various samples (Hiemstra and deKok, 2007). As an alternative, assays based on the immunoanalytical methodology have also been developed and used (Gough et al., 2010), due to rather low cost, simple assay protocols and a high sample throughput capacity (Chan et al., 2008).

A challenging step in pesticide immunoanalysis is the development of specific antibodies, since most pesticides are small molecules (haptens) that should be conjugated through a suitable, active chemical group to a carrier protein in order to elicit an immune response (Erlanger, 1980; Singh et al., 2004). If the parental pesticide does not contain any suitable active groups in its molecule, then special derivatives should be synthesized, following often laborious and skill-demanding organic chemistry protocols; these special synthetic derivatives should be then coupled to the carrier protein.

In the present work we describe a rather simple experimental approach to develop a polyclonal antibody for carbendazim; more specifically, we employed a combination of commercially available benzimidazole derivatives as immunizing haptens, thus avoiding the need for extended synthesis of special carbendazim-hapten derivatives. Each of the above derivatives was directly conjugated to the carrier protein KLH and a mixture of the protein-conjugates was administered to New Zealand white rabbits for antibody development. As shown experimentally, the new antibody could recognize other benzimidazole-type fungicides as well.

2. Materials and methods

2.1. Immunizing haptens

Commercially available benzimidazole derivatives, namely 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid and 2-mercaptobenzimidazole, all products of Sigma–Aldrich, were used as immunizing haptens. The aforementioned reagents (stock solutions in H_2O, DMSO, and 0.1 M $HCl_{(aq)}$/ethanol, respectively) were conjugated to the carrier protein KLH (product of Thermo Scientific), through glutaraldehyde (product of Sigma–Aldrich), EDC (product of Sigma–Aldrich) and sSMCC (product of Thermo Scientific), respectively, following well-established conjugation chemistry protocols (Avrameas and Ternynck, 1969; Mattson et al., 1993; Singh et al., 2004).

2.2. Immunization of rabbits

New Zealand white rabbits (two rabbits, 2-month old) were immunized with a mixture of the KLH-conjugates of the aforementioned benzimidazole derivatives. The mixture of the KLH-conjugates had been emulsified with an equal volume of Complete Freund's Adjuvant (Papasarantos et al., 2010) and then administered subcutaneously to the host-animals, according to the method of Vaitukaitis (1981). The animals were boosted initially six weeks after first exposure and subsequently every four weeks. Incomplete Freund's Adjuvant was used in boosting immunizations (Harlow and Lane, 1988). Blood was collected two weeks after each booster injection. Antisera were obtained with low speed centrifugation of whole blood.

Care of animals was provided in accordance to the corresponding European legislation.

2.3. Isolation of antibody (whole IgG-antibodies fraction) from the antisera

Isolation of the antibody (whole IgG-antibodies fraction) from the antisera was accomplished through sequential precipitation with caprylic acid and ammonium sulfate, as previously described (Perosa et al., 1990) with slight modifications. Briefly, 1 mL of antiserum was added to 3 mL of 60 mM acetate buffer and the pH adjusted to 4.5 with 1 N NaOH. One hundred μL of caprylic acid was then added drop-wise while stirring at room temperature. After 30 min of stirring at room temperature, the mixture was centrifuged for 45 min at 10,000×*g* at room temperature. The supernatant was then harvested, filtered through a 0.45 μm filter to remove fine precipitates and the pH was adjusted to 7.4 with 1 N NaOH. The sample was then cooled on ice and, while stirring vigorously, 1.1 g of ammonium sulfate was added to it very slowly. After stirring at 0 °C for 30 min, the mixture was centrifuged (10,000×*g*, 30 min, 4 °C) and the precipitated IgG-antibodies were resuspended in 0.01 M PBS, pH 7.4. The IgG-antibodies were finally dialysed for 72 h against 0.01 M PBS, pH 7.4. IgG purity was tested with SDS–PAGE (Chevalier, 2010). Protein concentration was measured using the BCA method (Sorensen and Brodbeck, 1986).

2.4. ELISA evaluation

2.4.1. ELISA coating

2-Mercaptobenzimidazole coupled to a suitable lysine-dendrimer, which had been pre-functionalized with 3-maleimidopropionic acid (Ogawa and Taneda, 1979; Bayer et al., 1985), was employed as the immobilized hapten in the in-house developed ELISA system. The lysine-dendrimer was prepared with a solid phase peptide synthesis protocol (Amblard et al., 2006), appropriately modified as previously described by our team (Papasarantos et al., 2010).

2.4.2. ELISA buffers

Coating buffer: 0.01 M PB, pH 7.4; *washing buffer* (PBS-T): 0.01 M PBS, pH 7.4, containing 0.05% (v/v) Tween-20; *diluting buffer 1*: PBS-T containing 0.2% (w/v) BSA and 5% (v/v) ethanol; *diluting buffer 2*: PBS-T containing 0.2% (w/v) BSA; *diluting buffer 3*: PBS-T containing 0.2% (w/v) BSA and 10% (v/v) ethanol.

2.4.3. ELISA titration experiments

ELISA microtiter plates were coated with the above described benzimidazole-bearing lysine-dendrimer (1 μg mL^{-1} in coating buffer, 100 μL per well, overnight, 37 °C). The following day, the liquid was discarded and the wells were washed once with 0.01 M PB, pH 7.4 (250 μL per well). Blocking was performed with a 2% BSA solution in PBS-T (200 μL per well, 1 h, room temperature). After blocking, the liquid was discarded, the wells were washed three times with PBS-T and then incubated (100 μL per well, 2 h, 37 °C) with serial dilutions (1:1000–1:50,000) of the antiserum under evaluation or with increasing concentrations of the corresponding antibody solution (0.01–20 μg mL^{-1}) in diluting buffer 1. After incubation, the liquid was discarded, the wells were washed three times with PBS-T and then incubated (100 μL per well, 2 h, 37 °C) with a commercially available anti-rabbit IgG coupled to horseradish peroxidase (anti-rabbit IgG/HRP, product of Sigma–Aldrich), at 1:3000 dilution in diluting buffer 2. Afterwards, the liquid was discarded, the wells were washed three times with PBS-T and finally incubated (100 μL per well, 30 min, 37 °C) with an ABTS (product of Sigma–Aldrich, 1 mg mL^{-1})/H_2O_2 (0.003%) solution in 0.1 M citrate/phosphate buffer, pH 4.5. After color development, the OD was measured (405 nm) in a microtiter plate reader (Sirio S, SEAK) and the corresponding titer curves were plotted.

Please cite this article in press as: Zikos, C., et al. Commercially available chemicals as immunizing haptens for the development of a polyclonal antibody recognizing carbendazim and other benzimidazole-type fungicides. Chemosphere (2014), http://dx.doi.org/10.1016/j.chemosphere.2014.03.049

2.4.4. ELISA displacement experiments

ELISA microtiter plates were coated, washed, blocked and washed again as described above. Then, the wells were incubated (2 h, 37 °C) with 50 μL of the antibody solution, diluted in accordance with its titer value in diluting buffer 2, and 50 μL of a series of standard solutions (1–10 μg mL^{-1}) of the substances under evaluation (benzimidazole, carbendazim, benomyl, thiabendazole, carbaryl, imazalil), which had been preincubated (18 h, 4 °C) before being added to the wells. All standard solutions were prepared by diluting a 10 mg mL^{-1} stock solution, in 0.1 N $HCl_{(aq)}$/ethanol (carbendazim, benomyl), H_2O [2-(2-aminoethyl) benzimidazole], or ethanol (carbaryl, imazalil, thiabendazole), with diluting buffer 3. Afterward, the procedure described in the previous paragraph was followed. Finally, the OD (405 nm) was measured and the displacement curves were plotted.

3. Results and discussion

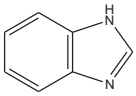
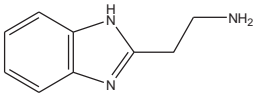
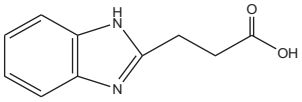
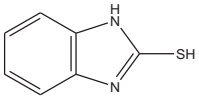
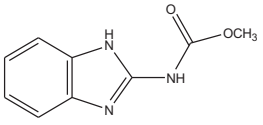
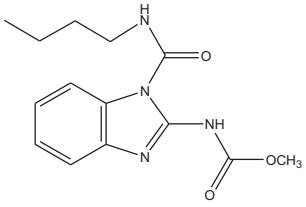
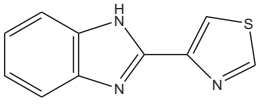
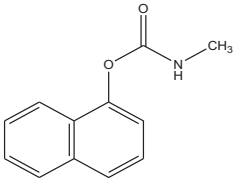
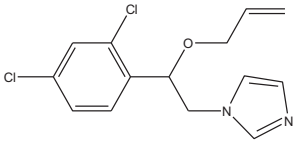
In this work we present a polyclonal antibody developed against a combination of commercially available -for a low price- benzimidazole derivatives as immunizing haptens. Each of the above derivatives was directly conjugated to the carrier protein KLH following well-established conjugation chemistry protocols, suitable to each derivative, and the mixture of the protein-conjugates was administered to New Zealand rabbits. This approach considerably accelerated the antibody development procedure and reduced cost. On the other hand, by employing different benzimidazole derivatives as immunizing haptens (each owing a different solubility profile and bearing a different chemically active group, i.e. $–NH_2$, –COOH, or –SH, linked either through a $–CH_2CH_2–$ chain or directly to the benzimidazole moiety, as shown in Table 1) we tried to increase the possibilities of obtaining an overall functional mixture of hapten-protein conjugates, for successful immunization.

The immunochemical characteristics of the antibody were evaluated in an in-house developed ELISA through titer and displacement curves. Commercially available 2-mercaptobenzimidazole coupled to a lysine-dendrimer (Papasarantos et al., 2010), which had been pre-functionalized with 3-maleimidopropionic acid (Ogawa and Taneda, 1979; Bayer et al., 1985), was used as the immobilized hapten. All the steps leading to the synthesis of the final dendrimer – bearing benzimidazole groups at the end of its "branches" – were performed on a solid phase resin, as described in Section 2, which highly facilitated the synthetic procedure.

As revealed by the ELISA experiments, the titer values of the antisera collected – corresponding to four consecutive bleedings – were higher than 1:10,000. The titer-curves for the corresponding IgG-antibodies, isolated as described in Section 2, are shown in Fig. 1.

As shown by the results of the ELISA-displacement experiments, the antibody developed against the commercially available benzimidazole derivatives was able to recognize the intact benzimidazole molecule (Fig. 2). Moreover, this antibody was able to recognize various benzimidazole-type fungicides (Table 1), such as carbendazim, benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate], and thiabendazole [4-(1*H*-1,3-benzodiazol)-2-yl)-1,3-thiazole] in standard solutions (1–10 μg mL^{-1}, Fig. 2). Benomyl is a highly toxic fungicide currently banned from use (since 2002) that is known to rapidly decompose to carbendazim in the environment (Römbke et al., 2007), while thiabendazole is a widely used fungicide found in high concentrations in fruits and vegetables (Santovito et al., 2011). Due to the variety of the benzimidazole derivatives used as immunizing haptens for its development (Table 1), the above antibody is expected to recognize not only carbendazim, benomyl and thiabendazole, but other benzimidazole-type fungicides, too. On the other hand, the

Table 1
Chemical structures of benzimidazole, 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid, 2-mercaptobenzimidazole and the pesticides carbendazim, benomyl, thiabendazole, carbaryl and imazalil.

Benzimidazole	
2-(2-Aminoethyl) benzimidazole	
2-Benzimidazole propionic acid	
2-Mercaptobenzimidazole	
Carbendazim	
Benomyl	
Thiabendazole	
Carbaryl	
Imazalil	

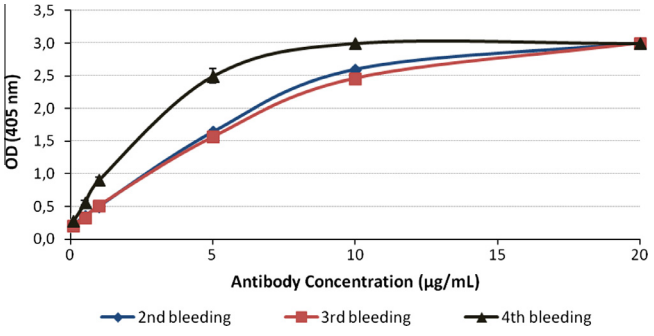


Fig. 1. Indicative ELISA-titer curves of the isolated antibodies (whole IgG-antibodies fraction); antisera collected after the last three consecutive bleedings were used as source for antibody isolation.

Please cite this article in press as: Zikos, C., et al. Commercially available chemicals as immunizing haptens for the development of a polyclonal antibody recognizing carbendazim and other benzimidazole-type fungicides. Chemosphere (2014), http://dx.doi.org/10.1016/j.chemosphere.2014.03.049

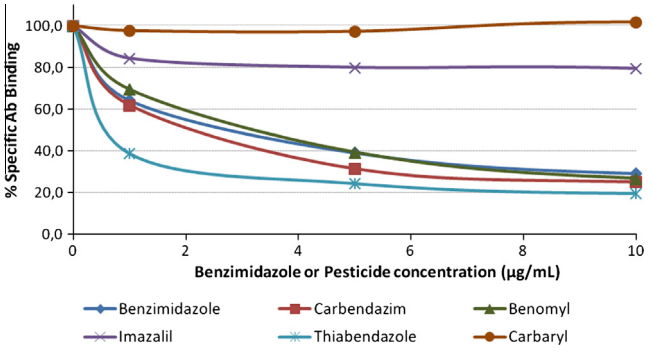


Fig. 2. Indicative ELISA-displacement curves obtained in the presence of increasing concentrations of intact benzimidazole as well as the pesticides carbendazim, benomyl, imazalil, thiabendazole and carbaryl.

new antibody did not cross-react with the fungicides carbaryl (1-naphthalenyl methylcarbamate) and imazalil [(*RS*)-1-(β-allyloxy-2,4-dichlorophenyl)-2-imidazol-1-ylethyl ether], which did not bear the benzimidazole moiety in their structure (Table 1). Although cross-reactivity studies with additional fungicides are currently underway, it should be noticed that carbendazim and thiabendazole (both benzimidazole-type) and imazalil (conazole-type) are among the most widely used fungicide molecules (Gough et al., 2010).

Considering its highly promising immunochemical profile, and especially its potential ability to recognize several bioactive substances bearing the benzimidazole group, the antibody described here may be a useful tool in the development of immunoassay systems for detecting toxic benzimidazole residues in food or samples of environmental interest. Detection limits of such immunochemical analytical systems may be considerably improved by optimizing the assay format, detection system, etc. A perspective example in this direction is given by preliminary experiments using the new antibody in order to construct target-specific cellular biorecognition elements, based on a technology known as Bioelectric Recognition Assay (BERA) technology or Molecular Recognition through Membrane Engineering technology (Moschopoulou and Kintzios, 2006). A bioelectric assay biosensor (BERA-type) measuring changes of the membrane potential of cells engineered with the new antibody is currently being developed by our group: preliminary results show that it is possible to quantitatively determine carbendazim in standard solutions in just 3 min using a sample volume of less than 10 μL; in such preliminary experiments, at least 5 ppb of carbendazim could be detected, while an improved limit of detection (LOD) may be achieved by carefully optimizing certain system parameters, such as the electric field intensity

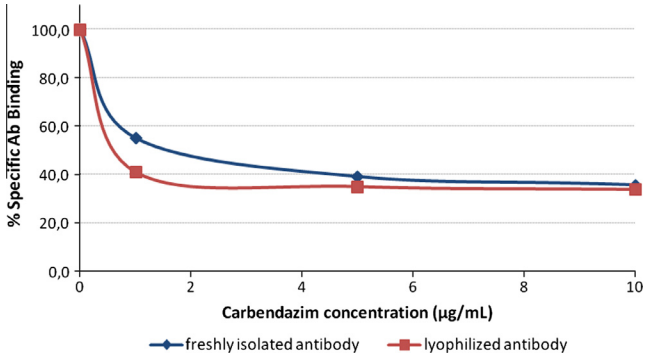


Fig. 3. Indicative ELISA-displacement curves obtained in the presence of increasing concentrations of carbendazim using freshly isolated or lyophilized antibodies. The latter had been reconstituted with distilled water immediately before use.

during antibody electroinsertion (Kokla et al., 2013). By further increasing either the density of the molecular biorecognition elements, i.e. antibodies, or the number of the membrane-engineered cells (Moschopoulou et al., 2012), a LOD in the sub-picomolar range could be achieved. Therefore, the feasibility of highly versatile, at the same selective and ultra-high throughput screening for benzimidazole residues has been demonstrated. It should be also noticed that the above antibody, probably due to its polyclonal nature, seems to fully retain its immunochemical functionality after lyophilisation (Fig. 3), which renders it especially suitable for commercial applications.

4. Conclusions

In conclusion, the development of a polyclonal antibody recognizing carbendazim as well as benomyl and thiabendazole is presented in this work. The antibody was developed by using 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid and 2-mercaptobenzimidazole, in combination, as immunizing haptens. By employing the above, low cost and easily accessible chemicals for direct coupling to the carrier protein KLH, synthesis of new, special immunizing haptens for each and every of the aforementioned benzimidazole-type fungicides was avoided; this approach considerably accelerated the antibody development procedure and reduced the overall cost.

The above antibody is being currently tested as a specific biorecognition element in a cell biosensor platform for pesticide residue detection based on the BERA technology, which has been developed in the framework of the EU Capacities Project FOODSCAN; recent data have demonstrated the feasibility of highly versatile, group-selective and ultra-high throughput screening for benzimidazole residues.

Acknowledgements

This work was supported in part by the EU FOODSCAN project (FP7-SME-2011, No. 286442).

The authors thank the personnel of the Animal House of NCSR "Demokritos" for excellent technical assistance during animal immunization.

References

Amblard, M., Fehrentz, J.-A., Martinez, J., Subra, G., 2006. Methods and protocols of modern solid phase peptide synthesis. Mol. Biotechnol. 33, 239–254.
Avrameas, S., Ternynck, T., 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 6, 53–66.
Bayer, E.A., Zalis, M.G., Wilchek, M., 1985. 3-(*N*-Maleimido-propionyl) biocytin: a versatile thiol-specific biotinylating reagent. Anal. Biochem. 149, 529–536.
Chan, C.P.-Y., Cheung, Y.-C., Renneberg, R., Seydack, M., 2008. New trends in immunoassays. Adv. Biochem. Eng./Biotechnol. 109, 123–154.
Chevalier, F., 2010. Highlights on the capacities of "Gel-based" proteomics. Proteome Sci. 8, 23. <http://www.proteomesci.com/content/8/1/23>.
Davidse, L.C., 1986. Benzimidazole fungicides: mechanism of action and biological impact. Ann. Rev. Phytopathol. 24, 43–65.
Erlanger, B.F., 1980. The preparation of hapten-carrier conjugates: a survey. Methods Enzymol. 80, 85–104.
Gough, K.C., Jarvis, S., Maddison, B.C., 2010. Development of competitive immunoassays to hydroxyl containing fungicide metabolites. J. Environ. Sci. Health B 46, 581–589.
Harlow, E., Lane, D. (Eds.), 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, USA.
Hiemstra, M., deKok, A., 2007. Comprehensive multi-residue method for the target analysis of pesticides in crops using liquid chromatography – tandem mass spectrometry. J. Chromatogr. A 22, 3–25.
Keegan, J., O'Kennedy, R., Crooks, S., Elliott, C., Brandon, D., Danaher, M., 2011. Detection of benzimidazole carbamates and amino metabolites in liver by surface plasmon resonance-biosensor. Anal. Chim. Acta 700, 41–48.
Kokla, A., Blouchos, P., Livaniou, E., Zikos, C., Kakabakos, S.E., Petrou, P.S., Kintzios, S., 2013. Visualization of the membrane engineering concept: evidence for the specific orientation of electroinserted antibodies and selective binding of target analytes. J. Mol. Recognit. 26, 627–632.

Mattson, G., Conklin, E., Desai, S., Nielander, G., Savage, M.D., Morgensen, S., 1993. A practical approach to crosslinking. Mol. Biology Rep. 17, 167–183.
McCarroll, N.E., Protzel, A., Ioannou, Y., Stack, H.F., Jackson, M.A., Waters, M.D., Dearfield, K.L., 2002. A survey on EPA/OPP and open literature on selected pesticide chemicals III. Mutagenicity and carcinogenicity of benomyl and carbendazim. Mutation Res. 512, 1–35.
Moschopoulou, G., Kintzios, S., 2006. Application of "membrane-engineering" to bioelectric recognition cell sensors for the detection of picomole concentrations of superoxide radical: a novel biosensor principle. Anal. Chim. Acta 573–574, 90–96.
Moschopoulou, G., Valero, T., Kintzios, S., 2012. Superoxide determination using membrane-engineered cells: an example of a novel concept for the construction of cell sensors with customized target recognition properties. Sens. Actuat. 175, 88–94.
Ogawa, H., Taneda, A., 1979. Characteristic distribution of sulfhydryl groups in human epidermal cancer by the new histochemical staining method. Arch. Dermatol. Res. 264, 77–81.
Papasarantos, I., Klimentzou, P., Koutrafouri, V., Anagnostouli, M., Zikos, C., Paravatou-Petsotas, M., Livaniou, E., 2010. Solid-phase synthesis of a biotin derivative and its application to the development of anti-biotin antibodies. Appl. Biochem. Biotechnol. 162, 221–232.
Perosa, F., Carbone, R., Ferrone, S., Dammacco, F., 1990. Purification of human immunoglobulins by sequential precipitation with caprylic acid and ammonium sulphate. J. Immunol. Methods 128, 9–16.
Römbke, J., Garcia, M.V., Scheffczyk, A., 2007. Effects of the fungicide benomyl on earthworms in laboratory tests under tropical and temperate conditions. Arch. Environ. Contam. Toxicol. 53, 590–598.
Santovito, A., Cervella, P., Delpero, M., 2011. In vitro aneugenic effects of the fungicide thiabendazole evaluated in human lymphocytes by the micronucleus assay. Arch. Toxicol. 85, 689–693.
Singh, K.V., Kaur, J., Varshney, G.C., Raje, M., Raman Suri, C., 2004. Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. Bioconjugate Chem. 15, 168–173.
Sorensen, K., Brodbeck, U., 1986. A sensitive protein assay method using micro-titer plates. Experientia 42, 161–162.
Vaitukaitis, J.L., 1981. Production of antisera with small doses of immunogen: multiple intra-dermal injections. Methods Enzymol. 73, 46–52.

Please cite this article in press as: Zikos, C., et al. Commercially available chemicals as immunizing haptens for the development of a polyclonal antibody recognizing carbendazim and other benzimidazole-type fungicides. Chemosphere (2014), http://dx.doi.org/10.1016/j.chemosphere.2014.03.049