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Utilization of Synthetic Antibody for Fumonisin Determination in Feed and Food

(Pemanfaatan Antibodi Sintetik untuk Determinasi Fumonisin dalam Pakan dan Pangan)

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ABSTRACT

Fumonisin contamination in food is limited around 2 – 4 ppm and in feed for different animals varies from 5 to 100 ppm. This regulation is to prevent animal and human from carcinogenic effect from fumonisins. Measurement of fumonisins frequently uses chromatography methods such as High-Performance Liquid Chromatography (HPLC) and Liquid chromatography tandem-mass spectrometry (LCMS/MS); however, the sample preparation and analysis process for these methods are costly and time consuming. Immunoassays have also been employed for detecting fumonisins in food or feed. Unfortunately, the instability of antibody to harsh condition such as high temperature and pH becomes the drawback for immunoassay method. Currently, the technology based on molecularly imprinting, which is called synthetic antibody, has been established for replacing antibody functions. Therefore, the aim of this review is to describe development of molecularly imprinted polymer (MIP) in fumonisin analysis in feed and food. Herein, the composition and production of MIP were described comprehensively. Bulk polymerization and solid phase synthesis were methods for production of MIP in micro and nano sizes. The application of MIP was reported for sample preparation as solid phase extraction measured continuously by HPLC showing the high recovery (> 60%). Then, MIP replaced antibody in direct competitive enzyme-linked immunosorbent assay (ELISA) for quantifying fumonisins in maize with high recovery (>90%) and limit detection (2 – 6 pM). Lastly, MIP was also employed in electrochemical sensor application as receptor for recognizing fumonisin in milk and maize. In conclusion, the performance of MIP has been applied successfully for fumonisin analysis comprehensively from sample preparation and quantification. The MIP would be developed for wider application for other toxins in feed or food such as veterinary drug, heavy metals, or pesticides.

Key words: Molecularly imprinted polymer, fumonisin, preparation, quantification

ABSTRAK

Kontaminasi fumonisin dalam pangan dibatasi sekitar 2 – 4 ppm dan batas maksimum dalam pakan untuk berbagai ternak/hewan bervariasi dari 5 hingga 100 ppm. Aturan ini dibuat untuk mencegah hewan dan manusia dari efek karsinogenitas dari fumonisin. Pengukuran fumonisin seringkali menggunakan metode kromatografi seperti Kromatografi Cair Kinerja Tinggi (KCKT) dan *Liquid chromatography tandem-mass spectrometry* (LCMS/MS). Tetapi, proses preparasi dan analisis sampel untuk metode kromatografi cukup mahal dan memerlukan waktu yang lama. Metode *immunoassay* juga sudah digunakan untuk mendeteksi fumonisin dalam pakan/pangan. Akan tetapi, ketidakstabilan antibodi terhadap kondisi suhu dan pH menjadi kendala dari metode *immunoassay*. Saat ini, teknologi berbasis *molecularly imprinting*, disebut juga antibodi sintetik, telah dikembangkan untuk mengganti fungsi dari antibodi. Oleh karena itu, tujuan dari penulisan artikel ini adalah untuk memaparkan pemanfaatan *molecularly imprinted polymer* (MIP) dalam analisis senyawa fumonisin dalam pakan dan pangan. Komposisi dan produksi MIP dijelaskan secara komprehensif. *Bulk polymerization* dan *solid phase synthesis* adalah metode untuk produksi MIP dengan ukuran mikro dan nano. Pemanfaatan MIP sudah digunakan untuk preparasi sampel sebagai *solid phase extraction* yang diukur lebih lanjut dengan KCKT yang menunjukkan hasil *recovery* yang tinggi (> 60%). Kemudian, MIP dapat menggantikan antibodi pada metode *direct competitive enzyme-linked immunosorbent assay* (ELISA) untuk mengkuantifikasi senyawa fumonisin dalam jagung dengan *recovery* yang tinggi (>90%) dan limit deteksi berkisar 2 – 6 pM. Selanjutnya, MIP juga digunakan pada aplikasi *electrochemical sensor* sebagai reseptor untuk mendeteksi fumonisin dalam susu dan jagung. Disimpulkan bahwa MIP dapat diaplikasikan dengan baik untuk mendeteksi fumonisin secara komprehensif dari preparasi sampel sampai kuantifikasi sampel sehingga MIP dapat diaplikasikan secara luas untuk senyawa racun lainnya dalam pakan dan pangan seperti obat hewan, logam berat atau pestisida.

Kata kunci: *Molecularly imprinted polymer*, fumonisin, preparasi, kuantifikasi

INTRODUCTION

Fumonisins are fungal secondary metabolites produced mostly by *Fusarium moniliforme* and *F. proliferatum* (Gaspardo et al. 2012). International Agency for Research on Cancer categorises fumonisins as group 2B carcinogenic (IARC 2002); therefore, these toxins have a potential risk not only for human but also for animal health. Moreover, the maximum level of fumonisins recommended in food is 2–4 ppm and in feed of different animals varies from 5 to100 ppm (FDA 2001; EC 2006; NSA 2009). Fumonisin B_1 (FB_1), which is the most common type of fumonisins, has been found in Indonesia since 1990s. It was reported that more than 50 % of analysed samples were contaminated with FB1 (Yamashita et al. 1995; Ali et al. 1998; Maryam 2000; Tangendjaja et al. 2008; Martindah & Bahri 2016). For these reasons, FB_1 contamination of maize could be considered a substantial issue especially in Indonesia.

The examination of FB_1 in maize mainly uses chromatography, such as High Performance Liquid Chromatography (HPLC) and Liquid chromatography tandem-mass spectrometry (LCMS/MS), and immunoassay (ELISA) techniques. Despite that the chromatography has good accuracy and sensitivity; unfortunately, the sample preparation for chromatography methods still uses long protocol and high volume of solvent; therefore, it would be costly and time consuming. Besides that, HPLC or LC-MS/MS are high-tech instruments demanding a skillful user to operate the machine and process the data comprehensively. Because of these reasons, determination of FB_1 by chromatograph methods is not simple. Similarly, the ELISA method has several drawbacks because of the use of monoclonal or polyclonal antibodies. These antibodies have a role for recognising FB_1 and their reactions result a colour change after enzymatic reaction, such as horseradish peroxidase (HRP). However, the antibodies are costly because their productions use animals such as mice and rabbits. Moreover, the procedure for obtaining pure antibodies is time consuming. Besides that, the characterisation of antibodies is unstable in harsh condition such as pH and temperature. Consequently, a better alternative approach will be required for determination of FB1.

Recently, molecularly imprinted polymer (MIP) has been developed. These polymers could be more efficient than monoclonal or polyclonal antibodies in terms of the time of preparation, cost and stability. To generate MIP, it is not necessary to use animals. Also, the MIP can be obtained in a short time and have a long shelf life and resistance to high temperature and extreme pH, where antibody could be easily denatured. MIP is potential to replace antibodies in assay and sensor applications for the determination of fumonisins. Hence, MIP is called also as plastic antibody (Piloto et al. 2018). Therefore, the aim of this review is to report the application of MIP as plastic antibody for determination of fumonisin in animal feed and food including introduction, production, and application of MIP, and MIP application for other toxins in supporting food/feed safety in the future. This review is the first information on the application of MIP reported in this article.

MOLECULARLY IMPRINTED POLYMER (MIP) AS "THE SYNTHETIC ANTIBODY"

Molecularly imprinted polymer (MIP) is a synthetic polymer owning recognition sites formed covalently or incovalently from mix monomers solution using a target compound as a template for recognition sites formation through polymerisation reaction. Schematically, the illustration of MIP formation can be seen in Figure 1. Alexander et al. (2006) described that the template could be atom, ion, molecule, or micro-organisms. It seems that the application of MIP is versatile for many targets including fumonisins. Because of those, MIP is potential for replacing the function of antibody as molecular recognition. Historically, the research about MIP has been initiated by a Polyakov's article in 1931.

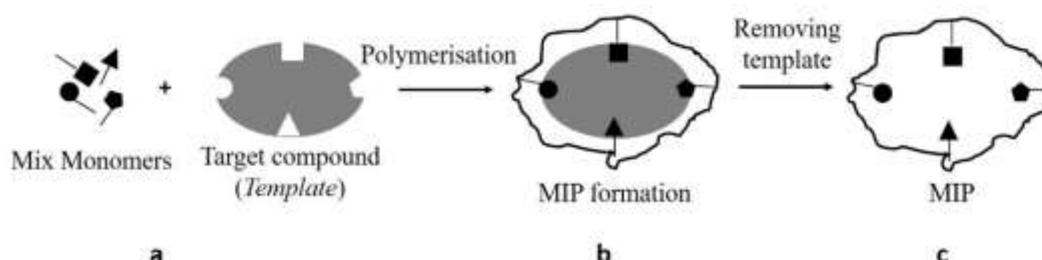


Figure 1. Illustration of molecularly imprinted polymer (MIP) formation: (a) Mixing all composition of MIP including functional monomers, crosslinkers, template, and solvent. (b) Generating MIP owning a specific cavity interacted covalently with template. (c) Removing a template to obtain MIP

This article mentioned the silica owning many pores because of the presence of the additive solvents such as benzene, toluene, and xylene (Alexander et al. 2006). This finding indicated that the silica might have the capability to uptake the additive agents and this phenomenon would become the first invention of molecular imprinting.

MIP COMPOSITION FOR FUMONISINS

The performance of MIP involves its composition role, including functional monomer, cross-linkers, initiator and solvent. All those materials are combined through polymerisation reaction surrounding the target compound in order to form the cavity of MIP. Also, the composition of MIP would affect the physical and chemical properties of MIP, such as particle size, solubility, selectivity, stability and rigidity. Therefore, in this part, the composition of MIP was mentioned in detail, including fumonisin as a target compound.

Fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3), have been used as a target for MIP production (De Smet et al. 2009; Smolinska-Kempisty et al. 2016; Munawar et al. 2018). This toxin has functional groups containing carboxyl ($-CO_2H$), hydroxyl (-OH), and primary amino ($-NH_2$) groups, as can be seen in Figure 2. These functional groups are the reactive sites of fumonisin and have the essential roles to cavity formation of MIP. Accordingly, the functional groups are one of the important factors considered for choosing the target compound.

Further, several functional monomers have been employed for initiating a cavity of MIP for fumonisin, such as 2-(diethylamino)ethyl methacrylate (DEAEM), 2-dimethylaminoethyl methacrylate (DMAEMA), methacrylic acid (MAA), Nisopropylacrylamide (NIPAm), ethylene glycol methacrylate phosphate (EGMP), and N-(3-Aminopropyl) methacrylamide hydrochloride (NAPMA) (De Smet et al. 2009; Appell et al. 2014; Smolinska-Kempisty et al. 2016; Munawar et al. 2018). The high selectivity of the MIP for fumonisin could be affected by the strength of the fumonisin-monomer complex interaction involving the functionalities of functional monomers, such as hydroxyl, carbonyl, carboxyl, and amide. Because of having different functional groups, the functional monomers are divided regarding to pH (acid, base, and neutral) and charge (positive and negative). Besides that, the ratio of functional monomers to the template can affect the selectivity of the imprinted polymer (Andersson et al. 1999; Yilmaz et al. 1999). Hence, the formulation of functional monomers should be calculated correctly to obtain the higher selectivity of the imprinted polymer for fumonisins.

The crosslinker is a monomer/compound used to obtain the rigid matrix of the polymer to maintain the template-functional monomers interaction. These materials are like a glue that has two or more double bonds to form the rigid crosslinked network. For fumonisin cases, trimethylolpropane trimethacrylate (TRIM), ethylene glycol dimethacrylate (EDMA), N-tert-butylacrylamide (TBAm), and N,N'- methylene-bis-acrylamide (BIS) have been used (De Smet et al. 2009; Smolinska-Kempisty et al. 2016; Munawar et al. 2018; Munawar et al. 2019). The crosslinkers have at least three functions, controlling the morphology of polymer (swelling and mesh size) (Wong et al. 2015), stabilising the binding sites of the polymer (Mayes & Mosbach 1996; Yu & Mosbach 2000) and conveying the mechanical stability of matrix polymer (Mane et al. 2015). Thus, the performance of MIP could be affected by crosslinkers. However, not all of them can be compatible for generating the imprinted polymer. Thus, using the different type of crosslinkers would obtain the different performance of molecular imprinting.

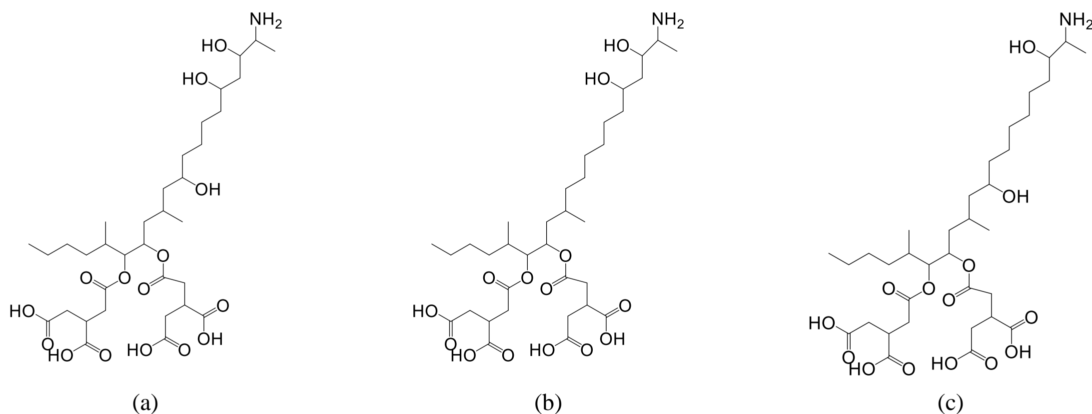


Figure 2. The structures of (a) fumonisin B1 (FB_1), (b) fumonisin B2 (FB_2), and (c) fumonisin B3 (FB_3)

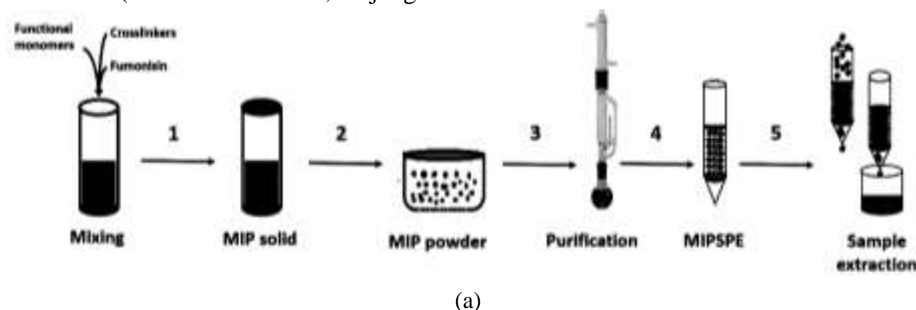
Source: Ren et al. (2011) with modification

The initiator is an important part for initiating polymerisation of MIP production for fumonisin. For examples, ammonium persulfate (APS) and tetramethyl ethylene diamine (TEMED) (Munawar et al. 2018), and 2,2'-Azobis (2-methylpropionitrile) (AIBN) (De Smet et al. 2009; Zhang et al. 2017). The initiator is employed in propagation and termination on polymerisation reaction. These parts are a crucial moment for generating MIP. Commonly, the initiator can be working optimally under light, thermal, or chemical condition, and become a free radical component (Mijangos et al. 2006).

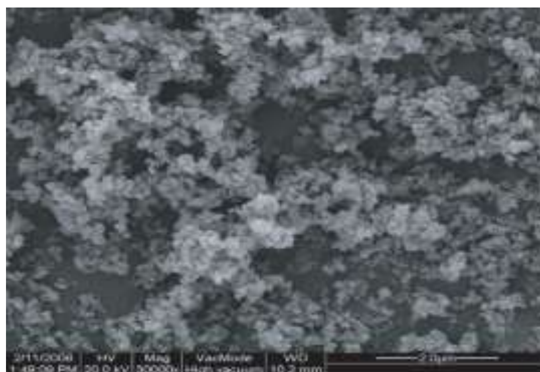
Finally, the solvent is the last component to generate the molecularly imprinting successfully. The solvent can facilitate a media for polymerisation reaction such as water, acetonitrile, and acetone (De Smet et al. 2009; Appell et al. 2014; Munawar et al. 2018). According to polarity, the type of solvent could be polar and nonpolar. Then, the polar solvent could be divided into protic and aprotic based on the abundance of O-H and N-H. Protic polar has more O-H and N-H than aprotic polar. This structure will impact to interaction with other components in polymerisation. The effect of those properties of solvent can influence binding capacity or interaction between the template and functional monomer (Turner et al. 2004; Mijangos et al. 2006). Despite that there is lack of certain data, the solvent will impact the performance of the imprinted polymer. As previously stated, the solvent contributes to a mesh size of the polymer (Turner et al. 2004) because of the conformation of polymer to solvent.

MIP SYNTHESIS

The molecular imprinting is produced by polymerisation including initiation, propagation and termination. Unlike polyclonal and monoclonal antibodies, the MIP production does not need animal experiment and long procedures including animal immunization, culture cell and antibody purification as conducted by Ling et al. (2014). Despite that the MIP production is shorter straightforward than antibody production, the finding appropriate composition of MIP is not straightforward. For instance, Munawar et al (2018) need molecular modelling, which needs skillful capacity for operating it, for screening method to select a functional monomer. Another way, the best functional monomer for generating MIP can be selected by many experiments. This approach is not as expensive as the antibody studies.



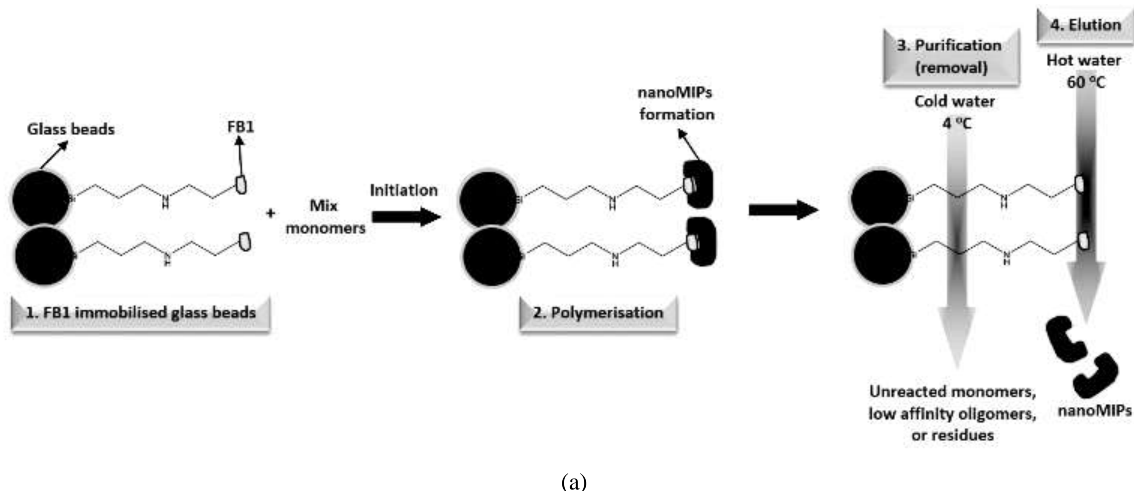
(a)



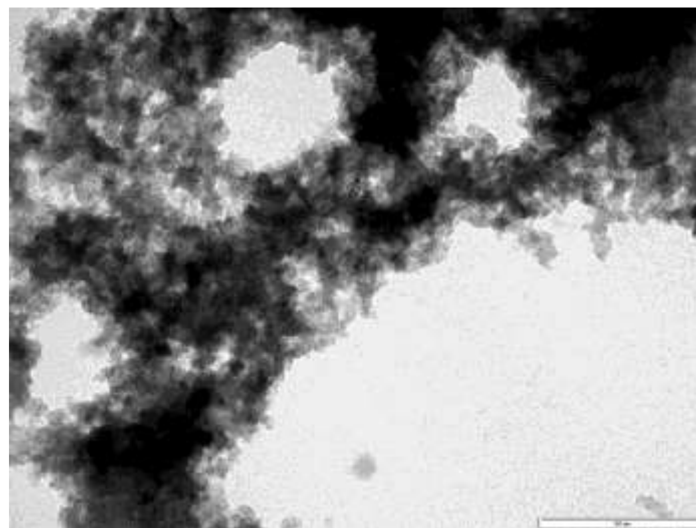
(b)

Figure 3. (a) Production of MIP in micro size; (1) the mix materials such as functional monomers, cross-linkers, and fumonisin, are polymerized for solid MIP formation, (2) The produced MIP is ground, (3) the ground MIP is purified by soxhlet extraction, (4) the purified MIP is packed as MIPSPE (MIP-based solid phase extraction), (5) The MIPSPE is applied for sample extraction. (b) SEM image of MIP from bulk poymerisation on 30k magnification

Source: Modified from Chen et al. (2014) and De Smet et al. (2009)



(a)



(b)

Figure 4. (a) production of MIP in nano size for FB_1: (1) FB_1 is bound on the glass beads, (2) the mix monomers are polymerized for nanoMIPs formation, (3) The residues, such as the low affinity MIP and unreacted monomers, are removed by cold water at 4°C and (4) The high-affinity nanoMIPs are collected by hot water at 60°C. (b) TEM image of nanoMIPs for FB_1 at 200k magnification

Source: Munawar et al. (2018)

De Smet et al. (2009) stated that the MIP for fumonisin was produced by bulk polymerisation. This simple procedure combines all materials in a vial glass. Afterward, the solution will be polymerized continuously being a solid polymer. This polymer will be ground to become powder. Finally, the MIP for fumonisin is used for binding fumonisin through solid phase extraction process (Figure 3(a)). The MIP produced by bulk polymerisation is in micro size. It seems that the MIP is visible clearly captured by scanning electron microscope (SEM). The image of the MIP for fumonisin B analogue is hard and random granular (Figure 3(b)). De Smet at al. (2009) showed that MIP is small anomalous polymer particles without circular particles.

Production of MIP in nano size can not be made by bulk polymerisation. Canfarotta et al. (2016) showed that solid phase synthesis method can be used for generating MIP in nano size, which is called nanoMIPs, using fumonisin-immobilised glass beads. These glass beads were employed for producing nanoMIPs through initiating the mix monomers to those glass beads. The thermal polymerisation was reached and its results had high affinity nanoMIPs, including monomer or compound residues. Consequently, cold water at 4°C was needed to remove the unknown materials and finally, hot water at 60°C was eluted to collect the nanoMIPs. Figure 4(a) illustrated the brief on how the nanoMIPs was produced by solid phase synthesis method. In addition, the image of MIP is different when MIP produced in

nano size. The image of nanoMIPs is usually seized by Transmission Electron Microscopy (TEM). Figure 4(b) is an example of the nanoMIPs for FB_2 appeared spherical and regular particles. Therefore, MIP can be more flexible size than antibody because the particle could be produced in either micro size or nano size. From this flexibility, MIP could be used for chemical or biological purposes.

MIP FOR FUMONISIN ANALYSIS IN ANIMAL FEED AND FOOD

MIP for sample preparation

The synthetic antibody or MIP has been used for extracting mycotoxin in rice, bell pepper, and maize by De Smet et al. (2009) and this material were called MISPE (molecularly imprinted solid-phase extraction). The performance of MISPE could be evaluated from the percentage of recovery signing the total of target compound extracted by MIP. Table 1 showed that the recovery of FB_1, FB_2, and FB_3 in rice and corn flakes is more than 60%; however, the high percentage of recovery >80% of those fumonisins is from bell pepper. Moreover, Bryla et al. (2013) reported that the percentage of recovery of fumonisin is higher than 90% in maize. These results are similar to previous experiment using clean-up column based on antibody and obtanin the recovery percentage higher than 80% (De Girolamo et al. 2010). Accordingly, MIP based solid phase extraction enable to bind fumonisins with high selectivity and sensitivity.

Table 1. The percentage of recovery of FB_1, FB_2 and FB_3 from extraction sample of feed and food by molecularly imprinted based solid phase extraction (MISPE)

Samples	Recovery (%)	RSD (%)	References
Rice			De Smet et al. 2009
FB1	66 – 89	1 – 9	
FB2	62 – 75	1 – 8	
FB3	67	2	
Bell pepper			De Smet et al. 2009
FB1	81 – 86	1 – 5	
FB2	80 – 83	2 – 10	
FB3	80 - 81	4 – 6	
Corn flakes			De Smet et al. 2009
FB1	62 – 72	2 – 9	
FB2	71 – 75	2 – 8	
FB3	65 – 70	3 – 7	
Maize			Bryła et al. 2013
FB1	102 – 111	6 – 15	
FB2	91 – 101	13 – 20	
FB3	98 – 109	8 – 14	

De Smet et al. (2009) provided the powerful data for cross reactivity of produced MIP for fumonisins with other mycotoxins such as deoxynivalenol (DON), aflatoxin B_2 (AFB_2), aflatoxin G_2 (AFG_2), citrinin (CIT), T-2 toxin (T-2), ochratoxin A (OTA), zearalenone (ZEN) and HT-2 toxin (HT-2). Figure 5 showed that the comparison among MIP and commercial clean up columns such as C-18 and SAX catridges are very competitive.

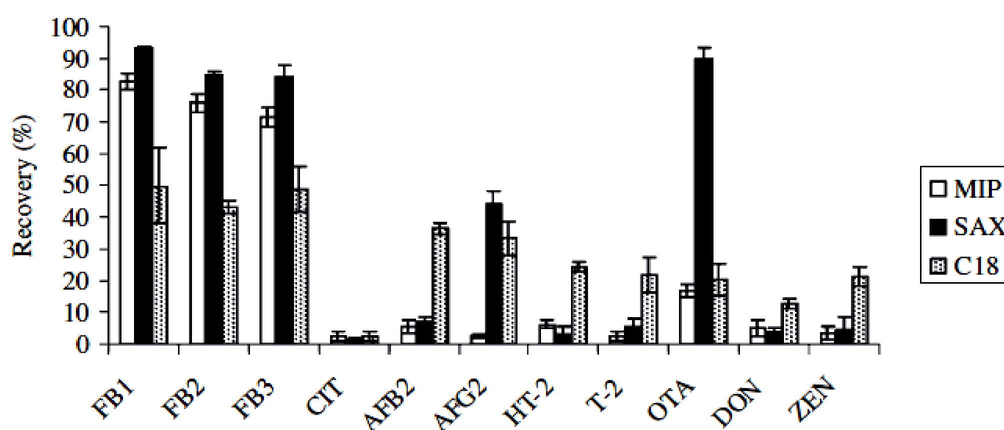


Figure 5. The percentage of recovery and cross reactivity of mycotoxins-spiked rice samples using MIP, C18 and SAX cartridges

Source: De Smet et al. (2009)

It means that MIP is reliable for sample extraction. Also, the interaction between MIP and other mycotoxins are negligible because the recovery percentage is lower than 20% in average for all mycotoxins. MIP could be employed alternatively for sample preparation and replacing the function of commercial materials.

MIP for fumonisin quantification

NanoMIP has been used for quantifying fumonisins. In the first application, nanoMIPs were replacing the function of antibody as molecular recognition for fumonsins in immunoassay system. Figure 6 illustrated how nanoMIPs was employed for replacing antibody in direct competitive assay. The MIP method for quantifying fumonisins is the same as the conventional ELISA including immobilisation, blocking reaction, enzyme-substrate addition, stopover process, and microplate reader measurement. Obviously, the MIP could be applied in immunoassays for quantifying fumonisins.

Munawar et al. (2018) reported that FB_1 can be detected in concentration range from 10 pM to 10 nM (Table 2). This interval was smaller than conventional ELISA based on antibody. Clearly, the sensitivity and selectivity of MIP in assay application were high, with recovery >90% (in maize samples) and detection limit was around 1.9 pM. Additionally, Smolinska-Kempisty et al. (2016) showed the same MIP application for fumonisin B2 (FB2) (Table 2). Unfortunately, there was no data explaining the recovery percentage in the sample.

In addition, Munawar et al. (2019) mentioned that the correlation between Molecularly imprinted polymer nanoparticle-based assay (MINA) and other conventional methods (HPLC and ELISA) is relatively high even though the total of samples were limited (< 10 maize samples). MINA was positively correlated (0.5-0.9) with ELISA and HPLC (Figure 7). These results indicate that the performance of MINA is realible for measuring FB1 quantitatively. Unfortunately, this test needs more improvement because the data obtained from their study was few. Thus, it will be a challenge for other researchers for finding more information about the correlation between MINA and other conventional methods.

Next application, MIP could be used as receptor in electrochemical sensor platform. It was called MINES (molecularly imprited polymer nanoparticles based electrochemical sensor) (Munawar 2018). Previously, Zhang et al. (2017) have used MIP for detecting fumonisin in milk and maize. The outstanding results were reported that the percentage recovery was higher than 90% and limit detection was 0.35 pg/mL in concentration interval from 0.001 to 100 ng/mL. Munawar (2018) stated that electrochemical sensor application has more benefits than immunoassays. For instance, the sensor system could be shorter in analysis time, easier in production, simpler in using chemical reagents, such as blocking solution, washing solution, enzymatic conjugate (Horseradish peroxidase-fumonisins complex), the enzymatic substrate (3,3',5,5'-Tetramethylbenzidine (TMB)) and stop solution.

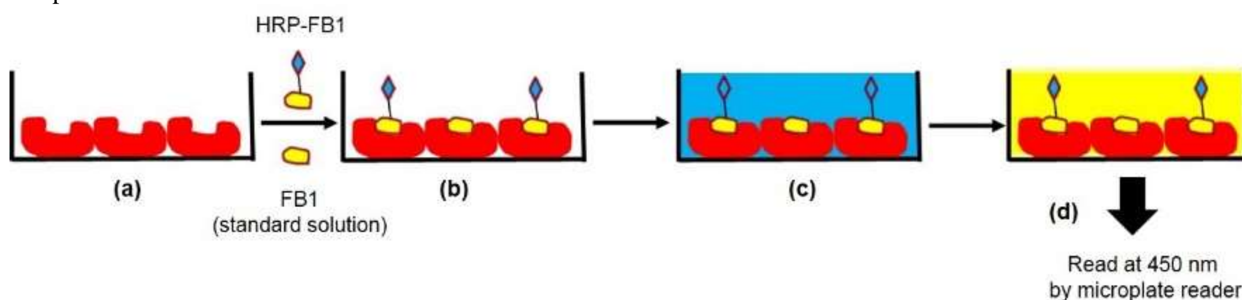


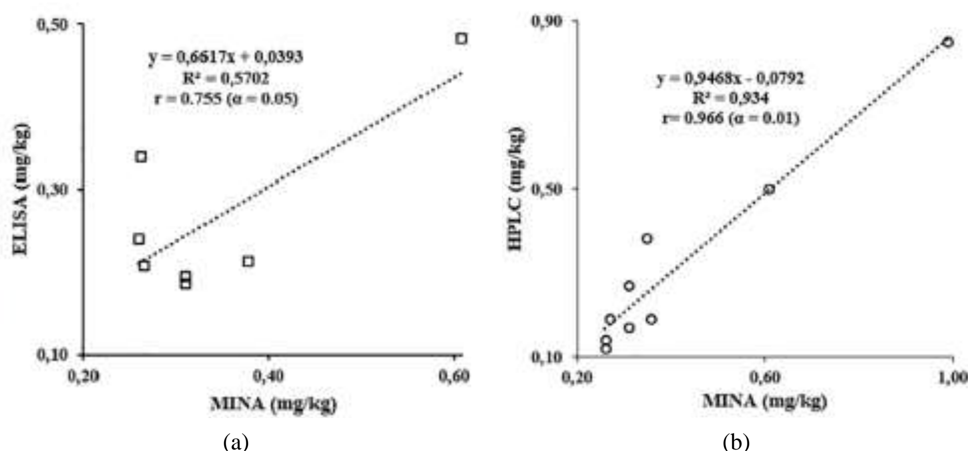
Figure 6. Illustration of MINA protocol; (a) NanoMIPs immobilisation, (b) HRP-FB_1 conjugate and FB_1 standard reaction competition, (c) Substrate addition. (d) stop solution addition, then measured the absorbance at 450 nm by microplate reader

Source: Munawar et al. (2018)

Table 2. The recovery of molecularly imprinted polymer based assays

Fumonisins	Concentration range	Recovery (%)	Limit detection	Samples	References
FB_1	10 pM – 10 nM	108 – 113	1.9 pM	Maize	Munawar et al. (2018)
FB_2	1 pM – 10 nM	NA*	6.1 pM	NA	Smolinska-Kempisty et al. (2016)

NA = not available



(a) (b)

Figure 7. The correlation (a) between ELISA and MINA, and (b) between HPLC and MINA

Source: Munawar et al. (2019)

THE USE OF MIP FOR SUPPORTING FOOD/FEED SAFETY PROGRAMS

The MIP application is not only useful for fumonisin but also for other toxins in animal feed or food. Currently, the MIP technology could be used for supporting food/feed safety to prevent both human and animal health from contaminated food. By developing MIP, the innovation of detection techniques is more flexible, effective, and efficient than those based on antibody; therefore, developing the innovative detection techniques woud be faster and these techniques would give a result immediately. Accordingly, the main purposes of food/feed safety program for maintaining animal and humal healths would be achieved.

For the near future, Indonesian Research Centre for Veterinary Science will produce MIP to detect other toxins such as antibiotics, pesticides, and heavy metals for preventing toxicity in animal feed and products. Furthermore, MIP will also be applied in microbiology agents such as bacteria, fungal, virus, or other pathogen agents. Unfortunately, few MIP researchs have been reported in Indonesia and the obtained MIP is already made in micro size (Royani et al. 2012; Nurhamidah et al. 2017). Consequently, this issue will be a challenge for Indonesia, especially in Ministry of Agriculture to provide a facility and funding for producing MIP such as the software for finding the suitable composition of MIP and focus research on MIP development in nano scale for wider application both chemical and biological purposes for supporting feed/food safety program in Indonesia.

CONCLUSION

This review has demonstrated the application of MIP for fumonisin in feed and food comprehensively. This article also illustrated how the MIP was produced in micro or nano sizes. The MIP benefits could be applied for sample preparation/extraction, such as molecularly imprinted solid phase synthesis (MISPE) for extracting rice, bell pepper, maize, and corn flakes with high recovery percentage. Furthermore, the MIP could be used for sample quantification such as molecularly imprinted polymer based assay (MINA) and sensing technologies with high recovery percentage and low detection limit compared to conventional methods (HPLC and Immunoassay). In future, MIP application for biological purposes will be useful to detect microorganism-based contamination in food.

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