# PREPARATION OF DUAL-DUMMY TEMPLATE MAGNETIC MOLECULARLY IMPRINTED POLYMER FOR MAGNETIC SOLID PHASE EXTRACTION OF FLUOROQUINOLONE ANTIBIOTICS IN FOOD

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**UNIVERSITI SAINS MALAYSIA**

**2025**

# PREPARATION OF DUAL-DUMMY TEMPLATE MAGNETIC MOLECULARLY IMPRINTED POLYMER FOR MAGNETIC SOLID PHASE EXTRACTION OF FLUOROQUINOLONE ANTIBIOTICS IN FOOD

**by**

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**Thesis submitted in fulfilment of the requirements**
**for the degree of**
**Master of Science**

**March 2025**

## ACKNOWLEDGEMENT

I would like to extend my gratitude to Allah for His blessings, which enabled me to wrap up my master's degree journey. I am incredibly appreciative to my supervisor, Assoc. Prof. Dr. Mazidatulakmam binti Miskam, for her support in structuring my research, particularly the drafting of my thesis, and for her stimulating recommendations and reassurance. I also owe many thanks to my co-supervisors Assoc. Prof. Dr. Noorfatimah Yahaya and Assoc. Prof. Dr. Atitaya Siripinyanond for their guidance, kindness and advice throughout my project. I would also like to express my greatest gratitude to the Ministry of Higher Education, Malaysia for financial assistance through Fundamental Research Grant Scheme (FRGS/1/2020/STG04/USM/02/5).

Furthermore, I am grateful for the chance to pursue a master's degree at Universiti Sains Malaysia (USM) and the Institute Postgraduate Studies (IPS). Along with my guardian, Noorhaliza binti Abdul Rahim, I would like to extend a special thanks to my grandparents, Abdul Rahim bin Jusoh and Fatimah binti Abu Bakar, for their financial and emotional support and for always being by my side during the highs and lows of this journey. I also want to thank my peers for their support and advice, as they have inspired me to finish my research and have offered me comments and critiques that have been useful and relevant to my thesis. Finally, I want to extend deep appreciation to everyone that helped me finish my thesis, both directly and indirectly.

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## LIST OF SYMBOLS

| | |
|---|---|
| $pK_a$ | Acid dissociation constant |
| Log *P* | Partition coefficient |
| $R^2$ | Coefficient of determination |
| °C | Degree celcius |
| $C_o$ | Initial concentration |
| $C_e$ | Equilibrium concentration |
| $V_i$ | Initial volume of the analytes |
| $V_f$ | Final volume of the analytes |
| $\sigma_1$ | Standard deviation of the peak from blank solution |
| $\sigma_2$ | Standard deviation of the replicates |
| $\bar{x}$ | Mean value of the replicates |
| v/v | Volume per volume |
| *Ms* | Saturation magnetization value |
| cm | Centimeter |
| g | Gram |
| mg | Milligram |
| L | Liter |
| µg | Microgram |
| mL | Mililiter |
| mm | Milimeter |
| mM | Millimolar |
| M | Molarity |
| N | Number of data point |
| nm | Nanometer |
| π | Pi |

| | |
|---|---|
| emu | Electromagnetic unit |
| θ | Theta |
| λmax | Wavelength of maximum absorbance |
| -COOH | Carboxyl group |
| -OH | Hydroxyl group |
| $H^+$ | Hydrogen ion |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Ammonium |
| AA | Acrylic acid |
| Ace | Acetic acid |
| AIBN | 2,2-azobisisobutyronitrile |
| BET | Brunauer-Emmett-Teller |
| CE | Capillary electrophoresis |
| CIP | Ciprofloxacin |
| COFs | Covalent organic frameworks |
| DAD | Diode array |
| DDMIP | Dual-dummy template molecularly imprinted polymer |
| DDMIP-MGO | Dual-dummy template molecularly imprinted polymer with magnetic graphene oxide |
| DDNIP | Dual-dummy template non-molecularly imprinted polymer |
| EDX | Energy dispersion X-ray |
| EFSA | European Food Safety Authority |
| EGDMA | Ethylene glycol dimethylacrylate |
| ENRO | Enrofloxacin |
| EU | European Union |
| FAs | Fluoroquinolones |
| FTIR | Fourier transform infrared spectroscopy |
| G | Graphene |
| GO | Graphene oxide |
| HPLC | High-performance liquid chromatography |
| IPS | Institute Postgraduate Studies |
| LC | Liquid chromatography |
| LLE | Liquid-liquid extraction |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| MAA | Methacrylic acid |
| MeOH | Methanol |
| MGO | Magnetic graphene oxide |
| MIP | Molecularly imprinted polymer |

| | |
|---|---|
| MNP | Magnetite |
| MOFs | Metal organic frameworks |
| MRL | Maximum residue limit |
| MS | Mass spectrometry |
| MSPE | Magnetic solid phase extraction |
| NA | Nalidixic acid |
| NPRA | National Pharmaceutical Regulatory Agency |
| OFL | Ofloxacin |
| PA | Pipemidic acid |
| SDG | Sustainable Development Goal |
| SEM | Scanning electron microscopy |
| SPAR | Sparfloxacin |
| SPE | Solid Phase Extraction |
| SULF | Sulfamethazole |
| TEM | Transmission electron microscopy |
| TGA | Thermogravimetric analysis |
| TRIM | Trimethylpropane trimethylacrylate |
| USM | Universiti Sains Malaysia |
| UV | Ultraviolet |
| VSM | Vibrating sample magnetometry |
| WHO | World Health Organization |
| XRD | X-ray Diffraction |

## LIST OF APPENDICES

**PENYEDIAAN POLIMER BERCETAKAN MOLEKUL BERTEMPLAT DUAL-DAMI UNTUK PENGEKSTRAKAN FASA PEPEJAL MAGNET ANTIBIOTIK FLOROKUINOLON DI DALAM MAKANAN**

## ABSTRAK

Antibiotik florokuinolon (FAs) digunakan secara meluas untuk merawat manusia dan sebagai perubatan veterinar. Hal ini menjadi kebimbangan utama kerana sisa FAs boleh menyerap ke dalam makanan yang berasal dari haiwan lalu menjejaskan keselamatan makanan dan menyebabkan ketahanan terhadap antimikrob kepada manusia. Oleh itu, penjerap inovatif dan serba boleh berdasarkan polimer tercetak molekul templat dual-dami – grafena oksida bermagnet (DDMIP-MGO) telah disediakan untuk pengekstrakan terpilih dan pembersihan FAs daripada sampel makanan. Dalam kajian ini, penjerap DDMIP-MGO berjaya disintesis dengan asid pipemidik dan asid nalidisik sebagai templat dual-dami polimer bercetak molekul (DDMIP) dengan nanopartikel grafena oksida bermagnet (MGO) yang disalut pada permukaan templat dual-dami yang disintesis. DDMIP-MGO memberikan kebolehlaksanaan penjerap dengan tapak pengecaman khusus untuk oflosaksin (OFL), enroflosaksin (ENRO) dan sparflosaksin (SPAR) dalam sampel daging ayam dan telur untuk memastikan pengekstrakan yang berkesan sebelum analisis kromatografi cecair berprestasi tinggi dengan pengesan ultraungu (HPLC-UV). Keputusan menunjukkan bahawa DDMIP-MGO menunjukkan saiz seragam, respon magnetik yang tinggi, kepekaan dan keupayaan pengecaman terpilih terhadap FAs. Kecekapan penjerapan setiap bahan penjerap sintetik dalam kajian awal menunjukkan bahawa penjerap DDMIP-MGO mempunyai peratusan penjerapan yang lebih tinggi berbanding dengan MGO dan DDMIP dengan 73.81%, 75.47%, dan 69.57% masing-masing untuk OFL,

ENRO dan SPAR. Keadaan optimum untuk parameter pengekstrakan fasa pepejal magnetic (MSPE) dinilai dan diperoleh dengan data berikut: jenis pelarut penyahjerap: MeOH: asid asetik nisbah (1:1, v/v), isipadu pelarut penyahjerap: 0.15 mL, masa penyahjerapan: 0.5 min untuk parameter penyahjerapan, manakala pH sampel: 7, kepekatan analit: 25 mg $L^{-1}$, masa penyerapan:60 mins dan dos penjerap: 20 mg untuk parameter penjerapan. Dalam kelinearan 1-100 µg $L^{-1}$, nilai $R^2$ dengan OFL, ENRO, dan SPAR adalah masing-masing 0.9916, 0.9950, dan 0.9904. Untuk OFL, ENRO, and SPAR, nilai had pengesanan (LOD) rendah dicapai dengan 0.0188, 0.0104 and 0.0108 µg $g^{-1}$ dengan ralat peratus 1.06%, 1.34% dan 1.67% manakala had kuantifikasi (LOQ) adalah 0.0627, 0.0346 and 0.0359 µg $g^{-1}$ dengan ralat peratus 1.12%, 0.97% dan 0.59%. Kebolehulangan %RSD dikira adalah antara 1.08-6.12% untuk OFL, 1.20-7.75% untuk ENRO dan 1.60-7.19% untuk SPAR. Peratusan perolehan semula yang cemerlang adalah antara 73.60% hingga 85.40% untuk daging ayam dan 75.64% hingga 84.24% untuk telur dalam kepekatan 5, 50, dan 100 µg $g^{-1}$. Data kinetik paling sesuai mengikut turutan pseudo-order kedua > Elovich > pseudo-order pertama manakala data isoterma paling sesuai mengikut turutan Freundlich > Temkin > Langmuir berdasarkan perbandingan nilai $R^2$ yang diperoleh. Nilai faktor pencetakan (IF) untuk OFL, ENRO dan SPAR pula masing-masing adalah 12.87, 13.22 dan 12.42 yang menunjukkan kapasiti penjerapan yang tinggi untuk kaedah yang ditetapkan. Oleh itu, penjerap DDMIP-MGO terbukti menjadi alternatif yang sangat baik sebagai penjerap kerana perolehan semula baik yang diperolehi, kestabilan dan pengecaman yang tinggi terhadap FAs dalam matriks makanan yang kompleks sambil pengunaan semula sebanyak empat kitaran.

**PREPARATION OF DUAL-DUMMY TEMPLATE MAGNETIC MOLECULARLY IMPRINTED POLYMER FOR MAGNETIC SOLID PHASE EXTRACTION OF FLUOROQUINOLONE ANTIBIOTICS IN FOOD**

## ABSTRACT

Fluoroquinolone antibiotics (FAs) are widely used to treat humans and as veterinary medicine. A major concern was raised as the residue of FAs may deposit in foods of animal's origin, jeopardizing the food safety and causing antimicrobial-resistant to humans. Thus, an innovative and versatile adsorbent based on dual-dummy-template molecularly imprinted polymer-magnetic graphene oxide (DDMIP-MGO) was fabricated for selective extraction and clean-up of FAs from food samples. In this study, DDMIP-MGO adsorbent was successfully synthesized with pipemidic acid and nalidixic acid as dual dummy templates molecular imprinted polymer (DDMIP) with magnetic graphene oxide (MGO) nanoparticles coated on the surface of the newly synthesized dual dummy template. The DDMIP-MGO provides feasibility of the adsorbent with specific recognition sites of ofloxacin (OFL), enrofloxacin (ENRO) and sparofloxacin (SPAR) in chicken meats and eggs samples to ensure efficient extraction prior to high performance liquid chromatography with ultraviolet detector (HPLC-UV) analysis. The results showed that the DDMIP-MGO exhibited uniform sizes, high magnetic responsivity, sensitivity and selectively recognition capability towards FAs. The adsorption efficiency of each of the synthetic adsorbent materials in preliminary adsorption studies showed that DDMIP-MGO adsorbent has a higher adsorption percentage compared to MGO and DDMIP with 73.81%, 75.47%, and 69.57% for OFL, ENRO and SPAR respectively. The optimal conditions for magnetic solid phase extraction (MSPE) parameters were assessed and

achieved with the following data: type of desorption solvent: MeOH: acetic acid in (1:1, v/v) ratio, desorption volume: 0.15 mL, desorption time: 0.5 min for desorption parameter; sample pH: 7, concentration analyte: 25 mg $L^{-1}$, adsorption time: 60 mins and adsorbent dosage: 20 mg. Within the linear range of 1-100 µg $L^{-1}$, the $R^2$ values for OFL, ENRO, and SPAR were 0.9916, 0.9950, and 0.9904, respectively. For OFL, ENRO, and SPAR, low limit of detection (LOD) were attained with 0.0188, 0.0104 and 0.0108 µg $g^{-1}$ with percent errors of 1.06%, 1.34% and 1.67% whilst limit of quantification (LOQ) were 0.0627, 0.0346 and 0.0359 µg $g^{-1}$ with percent errors of 1.12%, 0.97% and 0.59% respectively. The relative standard deviation % RSD calculated were ranged at 1.08-6.12% for OFL, 1.20-7.75% for ENRO and 1.60-7.19% for SPAR. Excellent recoveries ranged from 73.60% to 85.40% for chicken meat and 75.64% to 84.24% for egg in concentrations 5, 50, and 100 µg $g^{-1}$. The kinetic data best fitted in the order of pseudo-second order > Elovich > pseudo-first order while the isotherm data best fitted in the order of Freundlich > Temkin > Langmuir based on the comparison of $R^2$ values obtained. The IF values for OFL, ENRO and SPAR were 12.87, 13.22 and 12.42 respectively which indicated high adsorption capacity for the established method. Thus, DDMIP-MGO adsorbent proven to be an excellent alternative as adsorbent owing to its good recoveries obtained, high stability and selectivity towards the FAs in complex food matrices and reusable up to four cycles.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the study

The demand to breed greater numbers of livestock for consumption continues to rise in relation to the expanding population of the world, which in turn has surged the use of antibiotics in veterinary medicine (Pereira et al., 2018). Typically, with veterinary medicine, they are either used for therapeutic purposes, by giving high doses of antibiotics to the animals through feed or water supplies in a short period of time, or for prophylactic purposes, in which adequate doses of antibiotics are administered over a longer period of time. The animals are also given antibiotics for growth production in much lower doses than for therapeutic purposes (Mund et al., 2017). Fluoroquinolones (FAs) are veterinary antibiotics regulated by Malaysia's National Pharmaceutical Regulatory Agency (NPRA), which is also recognised by the World Health Organisation (WHO) as a source of highly essential drugs for human health (He & Blaney, 2015). Due to their potent antibacterial activity and low toxicity, FAs which are the new derivative of quinolones that include norfloxacin, ciprofloxacin, ofloxacin, gatifloxacin, and enrofloxacin, have grown to be heavily utilised in human and veterinary medical procedures for the preventive treatment of bacterial infections (Pham et al., 2019; Wan et al., 2021). It also ranked as the third-most widely applied class of antibiotics worldwide (Mathur et al., 2021). However, while certain nations persist permitting the use of antibiotics as growth promoters for livestock without strict regulation, there are several other regions and countries, such as the United States of America (USA) and Europe that have either constrained or outlawed the usage considering the accumulation of antibiotic residue in food can seriously risk human food security even at low dosages (Van et al., 2020). The Drug Control Authority

(DCA) in Malaysia and the US Food and Drug Administration both keep an eye on several types of FAs including ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin, and pefloxacin (Rawla et al., 2019). Antimicrobial resistance tends to be a subject of conversation and is frequently brought up when addressing the potential risks associated with administering FAs into poultry industry. Even at low levels of FAs (Figure 1.1) residue may cause permanent and constant exposure in humans, resulting in negative effects such as allergic reactions, intoxication, and antibiotic resistance (Mund et al., 2017). As a result, analytical measures ought to be taken to monitor the presence of FAs residues in food samples.

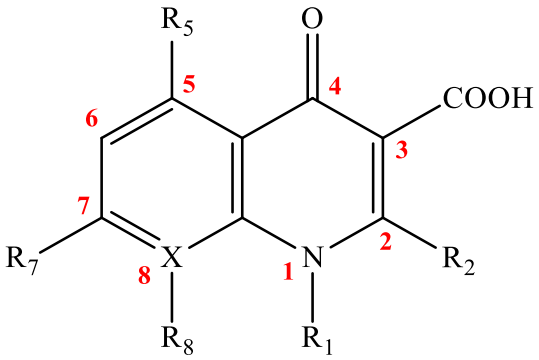


Figure 1.1 General chemical structure of FAs.

However, the determination of FAs at low concentration in food samples is a challenge considering their complex matrices. Traditionally, solid phase extraction (SPE) and liquid-liquid extraction (LLE) are common sample preparation techniques for food samples prior to the quantification analysis. Due to the substantial quantity of sorbent and volume of organic solvents consumption, the use of conventional methods has become less preferred, pushing researchers to branch out into more advanced methods such as magnetic solid phase extraction (MSPE) (Ye et al., 2019). MSPE, an enhanced form of SPE, is capable of promoting quick separation with high adsorption efficiency by using an external magnetic to replace the centrifugation step in traditional

SPE and help to hinder the sorbent packing, excessive back pressures, and packed bed clogging (Wen et al., 2020; Xiao et al., 2019). The incorporation of magnetic $Fe_3O_4$ nanoparticles into the synthesized product in this study allowed for superparamagnetic effects in MSPE while preserving its advantageous properties such as low toxicity, ease of preparation, and good biocompatibility with other compounds (Xiao et al., 2019).

Magnetic molecularly imprinted polymers are one of the cutting-edge technologies that have been introduced for the extraction of analytes from various matrices. Molecular imprinting materials (MIP) involved an approach where the template-shaped recognition sites designed specific pairs of forms, sizes, and functional groups of the analytes via polymerization. It typically involved covalent or non-covalent interactions between the target molecule and the functional monomer in the presence of a cross-linking agent, and the polymer with specific properties complementary to the desired analytes upon the removal of the template molecules via extensive washing (Hasanah et al., 2021). Yet there are some drawbacks when employing the typical MIP, such as the adsorption and desorption process kinetics being not ideal and mass transfer becoming delayed owing to the heterogeneous distribution of binding sites and the poor access of the target molecules to bind (Guo et al., 2019). Thus, dual dummy molecularly imprinted polymer (DDMIP) has been explored in this study for better selectivity towards FAs with the ability to extract multiple analytes from the same group using the same DDMIP adsorbent. For high binding capacity, surface imprinting into nanosized support materials with a large specific surface area is an excellent alternative. Graphene oxide is frequently preferred for incorporation with DDMIP adsorbents as it has superior chemical properties,

thermal stability, a larger specific surface area with small dimension, and a delocalized π-electron system (Ning et al., 2014; Xiao et al., 2019).

There have been multiple analytical methods that have been researched for tracing FAs in foods sampled from animals in order to monitor the concentration of FAs residues transmitted into food of originated from animals. Capillary electrophoresis (CE) (Martínez-Pérez-Cejuela et al., 2021), thin-layer chromatographic methods (Chen & Schwack, 2014), high performance liquid chromatographic methods (HPLC) (Lu et al., 2019; Wang et al., 2021), liquid chromatography equipped with a mass spectrometry detector (LC-MS) (Wang et al., 2021), and sensor assays (Wang et al., 2022) are among the most commonly applied for FAs in various sample matrices, and are now considered standard techniques for the analysis. The analytical technique for detection of FAs used in this study was HPLC method due to their fast separation in quicker time for complex matrices, versatility, and proven ability to determine multiple components in a single analysis (Abdu Hussen, 2022).

In the present research, a new adsorbent for MSPE based on DDMIP coated with magnetic graphene oxide (DDMIP-MGO) was synthesized by using pipemidic acid (PA) and nalidixic acid (NA) as dual dummy templates for the determination of FAs. The DDMIP adsorbent was prepared using acrylic acid (AA) as the functional monomer, ethylene glycol dimethylacrylate (EGDMA) as the crosslinker, and 2,2-azobisisobutyronitrile (AIBN) as the initiator for selective extraction and clean-up of FAs from food samples from animal derived. The synthesized DDMIP adsorbent was characterized using Fourier transform infrared spectrometer (FTIR), scanning electron microscopy-energy dispersion X-ray analysis (SEM-EDX), transmission electron

microscope (TEM), X-ray diffraction (XRD), thermogravimetric analysis (TGA), nitrogen adsorption-desorption analysis using Brunauer-Emmett-Teller (BET) isotherm model, and vibrating sample magnetometry (VSM). Several MSPE parameters were optimized including type of desorption solvent, ratio of desorption solvent, volume of desorption solvent, desorption time, pH level, analyte concentration, adsorption time, and adsorption dosage. The DDMIP-MGO adsorbent provided feasibility of the adsorbent with specific recognition sites of FAs to ensure selective extraction prior to HPLC-UV-Vis. The developed DDMIP-MGO MSPE technique showed excellent recovery percentage of ofloxacin (OFL), enrofloxacin (ENRO) and sparofloxacin (SPAR) in chicken meats and eggs samples.

## 1.2 Problem statement

When the utilisation of antibiotics in livestock is subject to debate, antimicrobial resistance has perpetually been an important point of focus to be concerned because it poses risks for toxicities, allergies, and, most importantly, the emergence of resistant bacterial strains (Pereira et al., 2018). The FAs residues transmissible to humans via consumption and react to a variety of microbes found in the human body, resulting in the development of FAs-resistant Campylobacter and Salmonella infections besides probable retinal detachment as well as the tenacity of major adverse effects involving the muscles, joints, and neurological system (Maris et al., 2021; Rawla et al., 2019). Antibiotics have been used in animal husbandry for economic gain for a long time without any limitations, enforcement, or oversight from any authority due to the occurrence of subpar and counterfeit antibiotics drugs that are easily accessible and attainable could additionally be contributing to an upsurge in antimicrobial resistance (Mathur et al., 2021). Despite the imposition of the maximum

residual limit (MRL) as one of the regulatory measures, the deposition and subsequent detection of residual levels of these antimicrobial substances above the regulated MRLs in food-derived products poses challenges to perform because of their complex matrices and low concentration. In accordance with recently published research, conventional SPE and LLE are less preferable for sample pre-treatment when it comes to trace analysis of FAs. The primary negative attributes of these approaches are their inherent complexity, high sample and solvent consumption, time requirements, high ecological impact, difficulty automating, high physical activity requirements, and high cost (Ghorbani et al., 2019). Some advancement on current methods that are often used for FA determination also posed some limitations. MIP materials on the other hand, due to their low binding capacity, difficult site accessibility, and slow binding kinetics caused by the deeply anchored recognition sites in highly cross-linked polymer networks, the majority of traditional MIPs produced using conventional imprinting techniques were insufficient. As a result, a significant amount of organic solvent was needed to completely remove the templates before the target molecules could bind which then could result in a high likelihood that template molecules will leak out during the extraction of FAs and that the template will not be completely removed despite extensive washing steps (Dai et al., 2015). MIP materials also required an appropriate surface imprinting materials for further stability. Proteins, lipids, carbohydrates, and other small molecules constitute some of the many substances found in food samples that might obstruct the detection and quantification of FAs (Wang et al., 2016). Inaccurate quantification can result from matrix effects that interfere with analytical measures by suppressing or enhancing signals, leading to inaccurate quantification (Lu et al., 2019). Determination of FAs in food sample can be difficult as non-specific binding can still occur as other compounds in the food

sample may bind to the polymer, leading to lower selectivity and potential contamination of the extracted FAs (Yu et al., 2019). Additionally, FAs levels in food samples are often relatively small. FAs need to be concentrated in order to achieve a good recovery rate (Lu et al., 2019). Thus, in this study a DDMIP coated with magnetic graphene oxide (DDMIP-MGO) was proposed. The dual-dummy-template were applied so that more than one analyte from the same group can be extracted using the same MIP adsorbent, allowing for specific detection of FAs. The chemical structure of both templates, pipemidic acid (PA) and nalidixic acid (NA) as dual-dummy templates in this synthesis complement the FAs analytes, which led to this selection. The strongly cross-linked three-dimensional network binding sites specific to the FAs were able to capture the corresponding analytes with the formation of the PA-imprinting cavities and the NA-imprinting cavities. Given that it possesses excellent physicochemical capabilities when paired with composite materials, the adhering of GO will promote the stability for the DDMIP-MGO adsorbent. When magnetic nanoparticles with superparamagnetic characteristics were coupled onto the GO, it has the potential to produce facile and quick separation of the solid phase extraction using external magnetic field thereby rendering the extraction process more convenient. Incorporation of MGO onto DDMIP adsorbent can also improve the carrier's specific surface area, leading to greater adsorption capability of the target molecule. The DDMIP-MGO application can be employed for reusability with the addition of MGO to the adsorbent, exhibiting its robustness. The use of MSPE in sample preparation can be beneficial for various reason for instance, MSPE can help to reduce matrix effects, separating the target fluoroquinolones from the interfering compounds. FAs also can be detected at trace levels by concentrating them using MSPE and achieving large

enrichment factors, while distinguishing FAs from other comparable substances with the specific recognition site in the food samples.

## 1.3 Objectives of the study

The following are the objectives of this study:

1. To synthesize and characterize DDMIP-MGO adsorbent using PA and NA as dual dummy templates.
2. To optimize DDMIP-MGO MSPE technique for the extraction of FAs prior to the analysis using HPLC-UV.
3. To investigate specific recognition ability (cross-selectivity) of DDMIP-MGO towards FAs.
4. To evaluate adsorption capacity and extraction performance of DDMIP-MGO for FAs in chicken and eggs food samples.

## 1.4 Outline of the thesis

There are five chapters covered in this thesis. First, **Chapter 1** provided a general overview of the problems that arise in food analysis and gave an overall view with objectives on the newly developed adsorbent and the selection of surface imprinting support materials. **Chapter 2** discussed the literature review that were carried out in relation to the study and evaluated on the compatible properties of the chemicals and reagents used to synthesize the adsorbent. The methodology of the study was described in **Chapter 3** and divided into three parts, which were the procedures for synthesis, characterization, and adsorption performances of DDMIP-MGO adsorbent along with determination of ofloxacin (OFL), enrofloxacin (ENRO), and sparfloxacin (SPAR) in chicken meats and eggs samples. Presented in three parts, **Chapter 4** addressed the results of the projects that were carried out. It discussed in-

depth on the synthesis, characterization, optimization, adsorption and extraction performances, method validation, analysis of real samples, selectivity, and reusability studies, with justification specified. Finally, **Chapter 5** gave a summary that concluded the overall results, limitations for future research, and suggestions.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Overview on the literation review

To develop an appropriate analytical technique for the extraction of FAs analytes in food samples, a number of research publications were examined for this work. The intricacy of the food matrix and the low level of analyte concentration necessitate pre-preparation treatment for samples, according to all the research publications examined. Therefore, one of the SPE modifications, magnetic solid phase extraction (MSPE), was selected for usage in order to prevent packed bed clogging, high back pressures, and sorbent packing. Because of its favourable characteristics, including low toxicity, ease of synthesis, and high biocompatibility with other substances, magnetic $Fe_3O_4$ nanoparticles with superparamagnetic effects can be used in MSPE to facilitate rapid and easy separation. A number of factors need to be assigned when creating MIP that can effectively target OFL, ENRO and SPAR analytes in food of animal origin, including template selection, functional monomer properties, cross linker choice, initiator and lastly porogen solvent selection. Pipemidic acid (PA) and nalidixic acid (NA) were employed as dual dummy templates, acrylic acid was used as the functional monomer, ethylene glycol dimethacrylate was used as the cross linker, azobisisobutyronitrile was utilized as the initiator, and methanol was used as the porogen solvent based on the literature review that were carried out. Dual dummy template molecularly imprinted polymer was selected in order to improve the selectivity of the FAs and due to their ability to generate multiple types of recognition sites in a single MIP format allowing them to be used for multiple analytes in a single sample, which reduced costs and shortened analysis times. Incorporation of MGO in DDMIP was selected to further improved adsorption efficiency and enhancing the

selectivity of the adsorbent due to the increased number of active sites and better interaction with the target molecules while facilitate easy separation.

## 2.2 Fluoroquinolones in food of animal origins

The initial function of antibiotics, a class of highly effective antimicrobial drugs, was to cure and avert various infectious in animals. However, their utilisation as prophylactics and growth promoters, as well as to control reproductivity and breeding performance, has greatly outstripped their current use as animal therapeutics (Ben et al., 2019). The majority of the veterinary drugs used in about 80% of animal husbandry is to treat and prevent diseases like mastitis, arthritis, respiratory issues, gastrointestinal tract infections, and other infections caused by germs, bacteria, and parasites (Bacanlı & Başaran, 2019). The United States Department of Agriculture (2022) reported that global meat production increased to 2% and 1% in 2023 for pork and chicken meat, respectively, resulting in demands for 102.7 and 110.0 million tonnes each. On a yearly basis, the global livestock implement an extensive amount of antibiotics, but when these drugs are misconstrued, long-term repercussions on human health and the environment befall (Majdinasab et al., 2020). The most common treatment for transmissible infections in humans and animals is usually administered by the synthetic antimicrobial drugs referred to as fluoroquinolone antibiotics (FAs) (Madikizela et al., 2022).

FAs are 6-fluorinated piperazinyl derivatives of nalidixic acid and first commercially marketed as quinolone antibacterial agents in 1963 (Zhanel et al., 2002). They are registered veterinary antibiotics under the National Pharmaceutical Regulatory Agency (NPRA) of the Ministry of Health Malaysia and recognised as the third-largest class of antibiotics that are extensively utilised worldwide (Mathur et al.,

2021). Given by the increasing population and per capita income, FAs have become prevalent in food animal husbandry to meet consumer's needs across the globe for livestock production, specifically pork, beef, and chicken meat (Kumar et al., 2019). In order to address the issue of the congestion and unhygienic living conditions in the cultivation, food animals typically added with subtherapeutic dosages of FAs antibiotics (Aguirre, 2017). Since animals can merely ingest a small portion of antibiotics, the remainders persist in the gastrointestinal tract and eventually prompt microorganisms to develop resistance to antibiotics, leading to the formation of resistant strains that are expelled in faeces (Wang et al., 2022). The significant portion of the residues are either deposited unaltered or after being metabolised (Sodhi & Singh, 2021). FAs residues are frequently present in animal-derived foods like meat, milk, and eggs due to the administration in poultry industry. The presence of counterfeit and subpar FAs antibiotics that are easily obtained could raise suspicions about the spread of antibiotic resistance (Mathur et al., 2021). By handling or consuming undercooked meat, people can introduce these bacteria into their bodies. Subsequently this microbe grows within human bodies, making the need for prescription antibiotics unnecessary and infections difficult to treat (Ghosh, 2014). The widespread implementation of antibiotics in the global scale has brought up a number of safety issues associated with their consume, toxicity, allergies, and, most importantly, the emergence of resistant bacterial strains (Pereira et al., 2018). Consequently, even though the exploitation of FAs may aid with the cultivation of livestock, their application in the veterinary sector ought to be constrained pursuant to appropriate regulations.

### 2.2.1 Antimicrobial resistance threats

The rationale behind the alarming administration of antibiotics among the food and agricultural industry, particularly in livestock, is the advent and stimulation of antimicrobial resistance brought by veterinary drug residues in animal-derived food and metabolites that enter the food chain (Tarannum et al., 2020). Because poultry are continuously given antibiotics, the residues of these drugs cause the bacteria within the livestock to become immune to the drugs. The World Health Organisation (WHO) has declared antibiotic resistance as being one of the foremost imperative public health concerns in the 21$^{st}$ century (Bacanlı & Başaran, 2019). Long-term and prolonged contact to relatively low concentrations of FAs can lead to adverse outcomes in humans, including allergic reactions, intoxication, and susceptibility to antibiotics (Mund et al., 2017). Extensive antibiotic residues have been discovered in animal blood and other tissues. These remnants may be consumed by humans and could come into contact with an array of microbes that are common in the human body to cause the spread of infections with Salmonella and Campylobacter that are resistant to FAs, in both cases being harmful to human health (Maris et al., 2021). Antibiotics were authorized to be used in animal husbandry for commercial purposes without restrictions, compliance, or surveillance from any entity for decades (Mund et al., 2017). Due to research conveying that both oral and injectable FAs are linked to incapacitating complications that affect tendons, muscles, joints, nerves, and the central nervous system and which might develop hours to weeks shortly after exposure and are potentially irreversible, the FDA announced on May 12, 2016, that FAs should only be given when no alternatives are available, noting that the risk of severe adverse effects outweighed the benefits (Meyer, 2016).

The NPRA has been issued 1,093 reports of adverse drug reactions (ADRs) in Malaysia to date. Of these, 2,066 potential complications have been linked to the continuous exposure to FAs, and six cases have involved hypoglycaemia resulting from the same cause (Rawla et al., 2019). These findings conclude that FA intake possesses a detrimental effect. What more pressing is, it is projected that by 2030, there will be a substantial spike in the amount of antibiotics consumed in food animals as a result of rising meat demand in middle-class and lower-class nations, highlighting the likelihood that these nations have higher chances in contributing to the global problem of antibiotic resistance which have been a serious issue that needed to be taken care of with the urgency (Van Boeckel et al., 2015). Thus, in alignment with Sustainable Development Goal 2 (SDG 2), which stresses the need to create strong ordinances that leverage the advantages of globalisation while minimizing the risks in order to combat hunger, enhance food security and nutrition, and advance sustainable agriculture, it is critical that antibiotic use be strictly monitored and prescribed in order to prevent the spread of antimicrobial resistance (Banik, 2019).

According to the European Medicines Agency, the indication "maximum residual limit" (MRL) refers to the highest concentration of residues in food derived from animals upon the usage of veterinary drug in animal husbandry and is potentially considered legally permissible for consumption in or on food. MRLs are standards that are imposed to ensure consumer safety by ensuring that food does not pose a risk to human health if consumed regularly over the course of a lifetime (Dinh et al., 2020). The European Food Safety Authority (EFSA) has set the MRLs for the FAs in foods of animal origin ranging 10-1900 µg/kg after gathering and analyzing information pertaining to antibiotic resistance from EU Member States. There are lack of laws controlling the administration of antimicrobials in animal feed for growth promotion

in middle-class and low-income countries, in contrast to Europe and the US, where guidelines have been placed (Van Boeckel et al., 2015).

## 2.3 Sample preparation

Since the presence of FAs antibiotic residues in food may severely undermine human food safety, it is of the utmost significance to develop straightforward, sensitive, and selective analytical techniques for monitoring their presence and limiting consumption. Regarding the efficiency of analytical methods for assessing the presence of organic residues and contaminants in living animals and animal products, the 657/2002/EC EU Commission Decision provides an explanation of the technical standards and fulfilment of criteria for residue control within the framework of the 96/23/EC Directive (Samanidou et al., 2008). For the detection of antibiotics, pre-preparation treatment and sample concentration are unavoidably essential due to the complexity of the food matrix and the low level of analyte concentration (Bergler et al., 2020). With regard to this, the development of quick and affordable sample preparation techniques has surged in popularity as a field of study in the past few years (Xu et al., 2023). In theory, sample preparation is the process of removing analytes from a sample matrix so that their distinctions in physical, chemical, and biological properties can be used to more effectively employ them for qualitative and quantitative evaluation (Xia et al., 2020). The extraction and purification of low levels of analytes in the food samples frequently necessitate intensive sample preparation due to the interference of complex matrices in foods of animal origin. While sample preparation remains a crucial step in the process, it also happens to be without a doubt the most labour- and time-intensive however, research into developing sensitive and quick

detection systems has focused on speeding up sample preparation, which is now the main obstacle to advance sample analysis.

The traditional approaches to sample preparation are generally associated with solid phase extraction (SPE) and liquid-liquid extraction (LLE), each of which has pros and cons based on the type of samples that need to be analyzed based on Figure 2.1. SPE has been recognized as a viable method for sample pre-treatment and matrix clean-up owing to its fast yet high recovery process.

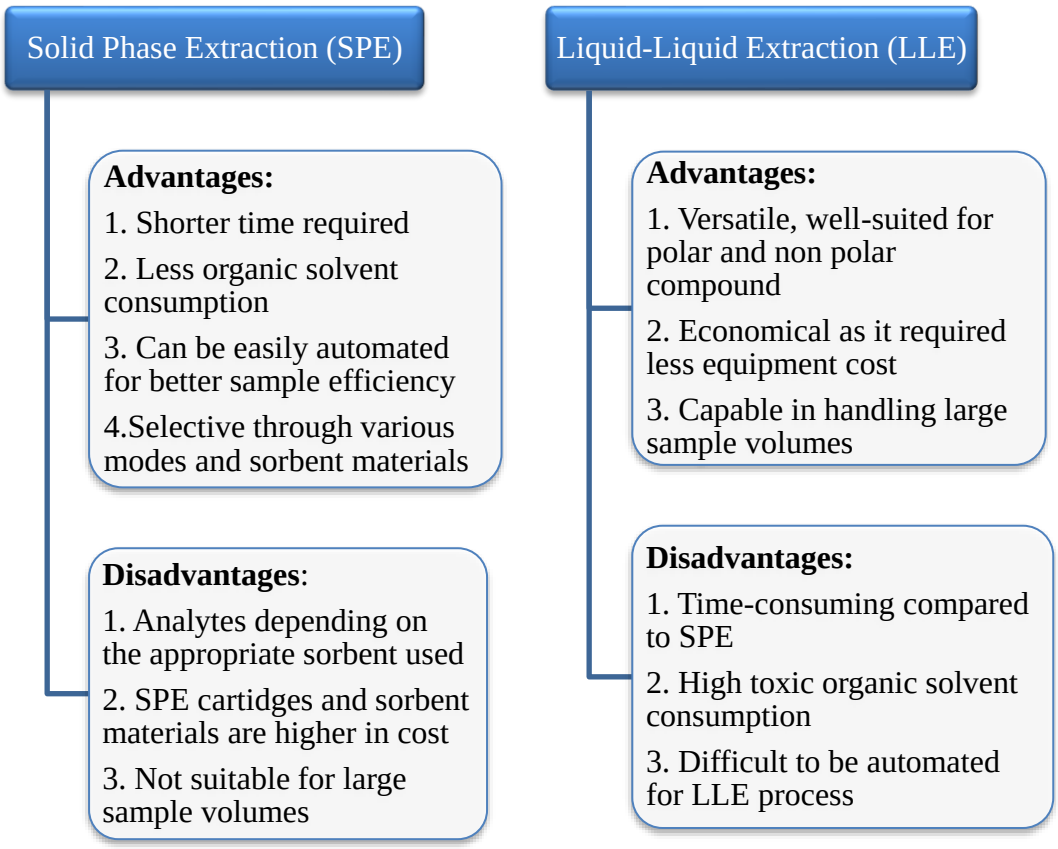


Figure 2.1 Solid phase extraction (SPE) vs liquid-liquid extraction (LLE)

Analytes, or solutes, are distributed in SPE by dispersing them between two liquid phases, the sample medium and the adsorbent, rather than by mixing the two liquid phases together. The pretreatment using SPE method are done by passing the sample through a cartridge or a packed column loaded with a solid sorbent where the

analytes are absorbed and subsequently eluted with an organic solvent (Sanagi et al., 2013). On the other hand, LLE takes place when immiscible or partially soluble liquid phases come into interaction with each other and specific components are transferred from one phase to another. According to various research papers in this field, SPE-based procedure sample preparation is a more prevalent and preferred option compared to others, as the vast range of sorbents with different polarity and functionality can be applied to obtain high potential for automation, less time consuming, high efficiency in pretreatment, ease of operation, and lower toxic solvent consumption, as well as, most pertinently, multiple modes of SPE are available for more selective target analytes (Arabi et al., 2020). However, it inherits drawbacks such as time consuming, required large amounts of toxic organic solvents, lack selectivity, sensitivity, and exhibit low capacity, leading to low sensitivity. Traditional SPE absorbents are readily contaminated by sample impurities and can only be used once.

### 2.3.1 Magnetic solid phase extraction

Magnetic solid phase extraction (MSPE) is one of the recent advances in SPE modifications that has been developed in order to overcome the limitations of the traditional SPE. Traditional SPE used cartilage which the solid phase is sorbent and serves as the stationary phase while the solvent phase that holds the analyte passes through the stationary phase to separate any mixture into the target analyte (Salve et al., 2023). MSPE is derived from conventional SPE by a modification in the dispersion mode based on the use of magnetic adsorbents and has received a lot of widespread attention due to its ability to facilitate separation (Xiao et al., 2019). Sample pretreatment using the MSPE method demonstrated immediate separation and great adsorption efficiency by the use of external magnetic field in the method to substitute

the centrifugation step in traditional dispersive SPE (Chen & Li, 2012). Through MSPE method, the sorbent packing, excessive back pressures, and packed bed clogging can be hindered (Wen et al., 2020). The properties, nature, and behaviour of the magnetic adsorbent play important factors in the development of MSPE technology as they govern the selectivity and efficiency of the method (Lian et al., 2018). The diverse types of magnetic absorbents, such as magnetic carbon materials, magnetic molecularly imprinted materials, $Fe_3O_4$@covalent organic frameworks (COFs), and $Fe_3O_4$@metal organic frameworks (MOFs) composites, have been introduced for the extraction of analytes from various matrices based on the extensive overview of published papers in this field. The use of magnetic $Fe_3O_4$ nano-particles with superparamagnetic effects in MSPE enables easy and quick separation, while its advantageous properties such as low toxicity, simple preparation, and good biocompatibility with other compounds make it a more preferable option (Xiao et al., 2019). The use of these magnetic properties as sorbent materials enables the desired analytes to be selectively extracted from complex samples such as food in addition to preconcentration which is necessary, especially when impurities may cause problems with quantification (Chen & Li, 2012). Numerous studies on MSPE have been conducted under the purview of food sample analysis for FAs. Table 2.1 summarizes the applications of different types of magnetic adsorbents for the extraction of antibiotics in food matrices.

It can be inferred from the compilation of modified MSPE procedures in Table 2.1 that the use of magnetic nanoparticles contributed to the improved stability of the adsorbent and the notable recovery rate observed in previous research. Table 2.1 indicates that the most common alteration of magnetic adsorbents involved bonds with various terminal groups of different compounds and surfactant, notably ligands and

nanocomposite materials (Khan et al., 2020). The surfactant magnetic adsorbents are a common substitute for classical adsorbents; however, their limited adsorption capacity due to their small surface area hinder their wide applicability (Gopal et al., 2019). Surfactant magnetic adsorbent synthesis is also a challenging procedure that calls for a high degree of skill (Khan et al., 2020). Moreover, the incorporation of nanocomposite materials in magnetic adsorbent has several disadvantages such as toxicity, prolonged synthesis process and difficult uniformity of nanocomposites (Mehmood et al., 2021). These drawbacks have prompted researchers to use the MIP approach to modify magnetic adsorbents in recent years.

Table 2.1 Application of different types of magnetic adsorbents for the extraction of antibiotics in food matrices.

| **Analytes** | **Magnetic adsorbent** | **Food Matrices** | **Instrument** | **Detection limit** | **Recovery (%)** | **Precision RSD (%)** | **Ref** |
|---|---|---|---|---|---|---|---|
| Marbofloxacin, norfloxacin, ciprofloxacin, lomefloxacin, enrofloxacin, sarafloxacin, sparfloxacin | Highly fluorinated and boron-rich | Milk | HPLC-DAD | 0.010–0.046 μg/kg | 78.9–119 | 4.4–9.1 | Ye et al., 2019 |
| Ciprofloxacin, enrofloxacin, lomefloxacin, norfloxacin | Ionic liquid modified $Fe_3O_4$/MWCNTs | Milk, pork | HPLC | 0.33–0.78 μg/L | 85.2–105.9 | 0.8–5.7 | Zhang et al., 2020 |
| Ciprofloxacin, enrofloxacin, lomefloxacin, gatifloxacin, levofloxacin, pefloxacin | Covalent organic frameworks with core-shell structure | Milk, pork | HPLC | 0.25-0.5 ng/g | 78.7-103.5 | 1.5–6.2 | Wang et al., 2019 |
| Enrofloxacin, danofloxacin, orbifloxacin, ofloxacin, enoxacin, ciprofloxacin | Sulphonate functionalized covalent organic framework-based | Pork, chicken and bovine | HPLC-MS/MS | 0.1–1.0 μg/kg | 82–110.2 | 1.7–7.7 | Wen et al., 2020 |
| Ofloxacin, norfloxacin, ciprofloxacin, enrofloxacin, difloxacin, sarafloxacin, pefloxacin, danofloxacin | Molecular sieve nanocomposite | Milk, pork, fish | HPLC-tandem mass spectrometry | 0.7–6.0 ng/L | 75.3–104.7 | 4.7–10.0 | Yu et al., 2019 |
| Enrofloxacin, norfloxacin, ofloxacin, sarafloxacin, difloxacin, nalidixic acid, oxolinic acid, flumequine, lomefloxacin, ciprofloxacin | Metal organic frameworks based | Fish | HPLC-tandem mass spectrometry | 0.02– 0.07 μg/kg | 80.8-112 | 5.95-14.5 | Wang et al., 2021 |

| Analytes | Magnetic adsorbent | Food Matrices | Instrument | Detection limit | Recovery (%) | Precision RSD (%) | Ref |
|---|---|---|---|---|---|---|---|
| Enrofloxacin, ofloxacin, ciprofloxacin | Polystyrene polymer composites | Milk | HPLC | 0.41–0.45 μg/kg | 96.5–118.6 | 1.2–4.2 | Yu et al., 2023 |
| Enoxacin, ofloxacin, prulifloxacin, gatifloxacin, difloxacin, tosufloxacin | g-$C_3N_4$/$Fe_3O_4$/$MoS_2$ | Chicken, eggs | HPLC | 0.5–2.0 μg/L | 89.3–99.6 | < 4.5 | Zhao et al., 2019 |
| Arafloxacin, ofloxacin, enrofloxacin, danofloxacin, lomefloxacin, pefloxacin, ciprofloxacin, enoxacin, norfloxacin, difloxacin | Accelerated solvent extraction (ASE) | Yellow croaker, grass carp, black fish, prawn, and macrobrachium | HPLC-tandem mass spectrometry | 0.02-0.29 μg/kg | 81.6–105.8 | 4.2–13.6 | Wei D et al., 2020 |
| Difloxacin, orbifloxacin, sparfloxacin, sarafloxacin, fleroxacin, marbofloxacin, ofloxacin, enrofloxacin, danofloxacin, lomefloxacin, pefloxacin, ciprofloxacin, enoxacin, norfloxacin, pipemidic acid, cinoxacin, oxolinic acid, nalidixic acid | Graphene oxide/nanoscale zerovalent iron | Milk | HPLC-tandem mass spectrometry | 3.1–13.3 ng/L | 82.4–103.9 | 2.9–15.1 | Wei & Guo. 2020 |

## 2.4 Molecular imprinting process

Molecular imprinting can be defined as a technique that create the artificial template-shaped of recognition sites which specifically pair the forms, sizes, and function-groups of the specific molecules in polymer matrix (Tarannum et al., 2020). The polymerization of functional monomer and crosslinker in the presence of a template molecule and agents that form pores is the underlying foundation of the imprinting process (Badawy et al., 2022). Molecularly imprinted polymer (MIP) is considered desirable to be an excellent alternative due to the unique recognition sites cross-linked on the surface of the substrate that selectively separate and concentrate target molecules from complex samples (Sanagi et al., 2013). The MIP also has a high potential to be an effective, simple, less pricey, stable, reusable, and template-specific option for determining antibiotic residues in food samples which outperform other analytical procedures since it can enrich trace targets molecules from complex matrix, making it suitable to extract targets with low limit of detection (LODs) while maintaining the target molecules and their structural analogues for higher specific and sensitivity of detection compounds (Sanagi et al., 2013; Tarannum et al., 2020). The MIPs were mostly synthesized in bulk polymerization. Usually, they are organic copolymers engulfed in a 3D network with recognition sites on a template in bulk polymerization. After that, the template is removed from the cavities by washing in order to match the molecular interaction, size, and shape. After sieving to obtain the desired particle sizes, the resulting monolithic polymer is packed into a SPE cartridge (Badawy et al., 2022). Polymerization generally involved the covalent or non-covalent interactions between the target molecule and the functional monomer with the presence of a cross-linking agent. Following the removal of the template molecules,

the polymer will be left with specific, recognizable three-dimensional (3D) voids which exhibit selective recognition for the specific target molecules (Cui et al., 2015). In addition, different type of polymerization for example, one pot reaction, precipitation, sol-gel multi-step swelling, and polymerization in the presence of surface-active agents can be utilised to obtain spherical particles with the desired size corresponding to what type of analytes of interest (Sajid & Płotka-Wasylka, 2018). The complementary cavities are likely expected to rebind to the template with high selectivity and high affinity owing to this molecular memory, and are capable of distinguishing between the molecule of interest and other molecules in the matrix through MIP approach.

In comparison to other identification systems, MIPs have contributed many adaptations in fields including purification and separation, artificial antibodies, drug delivery, and chemical sensors due to their low cost, simple preparation, high stability, and reusability (Xie et al., 2019). In spite of this, there are some drawbacks when using the traditional methods for preparation of MIPs. For example, the polymer generated by bulk polymerization must be crushed to a proper size, which reduces the polymer yield and might destruct the affinity sites. Subsequently, it is also rather tedious to extract the original templates located in the interior area of the bulk materials (template leakage), as it led to partial template removal, limited binding capacity, and slow mass transfer (Cui et al., 2015).

Without a doubt, selecting the materials for the component parts of MIP is a crucial step in the preparation of an efficient MIP since it will determine the type of analytes that can be extracted. In synthesizing MIP that can successfully targeted selective analytes, several components that have to be emphasized including template selection, functional monomer properties, cross-linker choice and lastly porogen

solvent selection in Figure 2.2. It is crucial to employ the right components with a specific binding affinity in preparing the ideal MIP with excellent adsorption performance and high selectivity to meet with different application requirements. The MIP strategy is beneficial for compounds that are tricky to purify or are costlier (Wang et al., 2010).

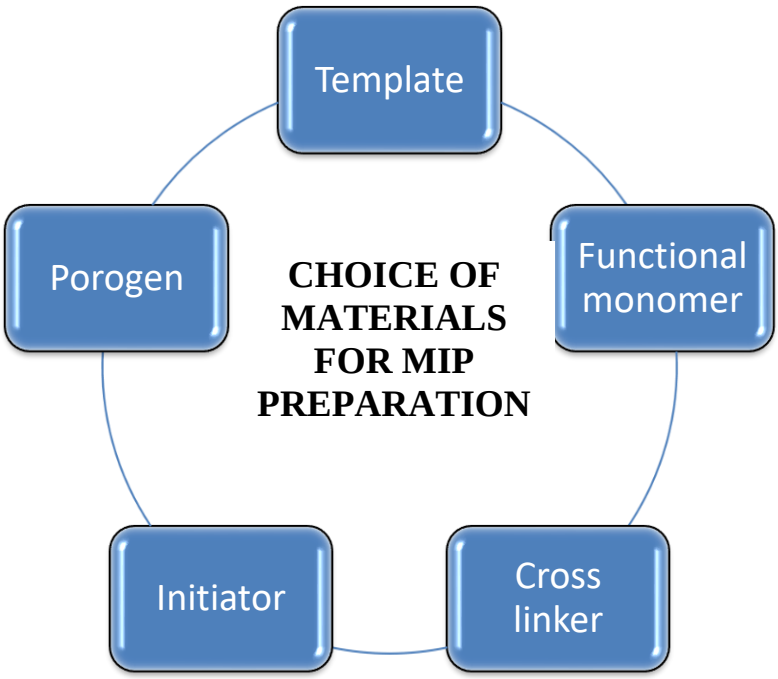


Figure 2.2 The materials for the component parts of MIP.

### 2.4.1 Template

The development of MIP is based on the incorporation of interactions between templates and functional compound monomers (Hasanah et al., 2021). It is significant to generate MIPs with affinity and specificity similar to target molecule so that they can easily replace such entities in real sample applications (Chen et al., 2016). Template is one of the components that has influenced the scientific interest and has been evolved for the advancement of separating, detecting, or loading for trace of small