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PHARMACEUTICAL ACTIVE COMPOUNDS (PhACs) IN FISH: A REVIEW OF ANALYTICAL STRATEGIES FOR THEIR DETERMINATION

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Abstract: Pharmaceutical contaminants bioaccumulate in fish tissue after being introduced into the food chain, posing a health risk to consumers due to its potential toxicity. Suitable extraction techniques remain a key issue due to the matrix interference from fish tissues. This research paper reviews and evaluates analytical approaches for extracting and analysing amlodipine, simvastatin, hydrochlorothiazide, diclofenac, acetylsalicylic acid, and paracetamol in fish tissue. Articles were screened according to preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines to ensure rigorous selection criteria. Extraction methods that use polar solvents were found to be effective in extracting these compounds due to the solvents polarity and capacity for hydrogen bonding, though acidified solvents exhibited high recovery rates for basic compounds. Ultrasonic-Assisted Extraction (UAE) with polar solvents emerged as a dominant technique owing to its simplicity, ability to minimise handling time and solvent use, high extraction yield, and preservation of thermolabile compounds. Furthermore, compound separation and detection were predominantly conducted using High-Performance Liquid Chromatography (HPLC) with C18 columns as the stationary phase, offering advantages over Gas Chromatography (GC) methods by eliminating the derivatisation steps. Future research endeavours should optimise and develop new extraction techniques to minimise matrix interference in biota sample analysis, thereby enhancing the accuracy of pharmaceutical contaminant detection in fish tissues.

Keywords: Analytical methods, pharmaceuticals, fish, emerging contaminants.

Introduction

Pharmaceutical active compounds (PhACs) belong to the group of Emerging Contaminants (ECs) aimed at combating diseases in humans and animals (Dey *et al*., 2019). They are more likely than other ECs to harm the environment and water quality because they are bioactive and make harmful toxic byproducts when they are released into the environment at different stages of their lifecycle (Adeleye *et al*., 2022). Pharmaceutical active compounds primarily originate from wastewater treatment plants (WWTPs), agricultural runoff, pharmaceutical manufacturing sites, aquaculture, and human consumption (Ebele *et al*., 2017; Świacka *et al*., 2022).

The release and accumulation of pharmaceutical pollutants in the environment are viewed as an inevitable by-product of a modern lifestyle, with most medicines eventually finding their way into aquatic systems after entering wastewater systems because of the relatively low effectiveness of pharmaceutical contaminant removal processes from wastewater systems (0%-40%) (Zhou *et al*., 2023). All pharmaceuticals come from manufacturing facilities and many of these toxins are released into the environment through wastewater that has not been properly treated or has not been treated at all (Patel *et al*., 2019). A significant number of medicines and their metabolites are expelled with urine and faeces from our homes, hospitals, livestock farms, and poultry farms. This is because neither humans nor animals completely metabolise and eliminate these substances, which eventually

find their way to wastewater treatment facilities where pharmaceuticals and their metabolites are present (Zhou *et al*., 2021; 2023).

Pharmaceutical active compounds can be defined as active substances with complete chemical structures, metabolites, and degradation products that result from enzyme activities in people and the environment (Sadutto & Picó, 2020). Previous studies have detected PhACs in various environmental compartments, including aquaculture (Thiang *et al*., 2021), drinking water (Wee *et al*., 2020), rivers and lakes (Yin *et al*., 2019; da Costa Filho *et al*., 2022), and various fish organs (Cravo *et al*., 2022; Manjarrés *et al*., 2022; Nozaki *et al*., 2023). Aquatic organisms are continuously exposed to contaminants, leading to harmful effects on their health, as supported by scientific evidence (Peivasteh-Roudsari *et al*., 2020; Hashempour-Baltork *et al*., 2023). Effective extraction, clean-up, and detection methods are essential for accurate results, particularly in recovery, precision, and sensitivity (Cravo *et al*., 2022).

However, the greatest challenge lies in efficiently extracting target analytes due to the high lipid, protein, and organic content in biota samples and the low concentration of analytes. Thus, the objective is to evaluate the analytical techniques used to analyse amlodipine, simvastatin, paracetamol, diclofenac, acetylsalicylic acid, and hydrochlorothiazide in wild fish samples by various scholars across the globe between 2019 and 2023 (five years) to produce a simple database for future researchers.

Relevant data searches were conducted in Scopus, Web of Science, Google Scholar, and ScienceDirect, using different keyword combinations. The researchers identified 153 articles; after removing duplicate copies, following the articles screening based on their relevance to the research goal, 135 articles were excluded, and only 18 research articles were included as they described techniques for analysing pharmaceuticals from the target analyte in the fish. For the specific literature search period covering (2019-2023), the reviewers chose to focus on "wild fish", excluding any fish raised in aquaculture farms, cages, or shellfish, as well as those purchased from the market. Figure 1 shows the literature identification and screening process using the PRISMA flow chart.

The review process focuses on amlodipine, simvastatin, diclofenac, acetylsalicylic acid, paracetamol, and hydrochlorothiazide. These pharmaceuticals were selected based on the prescription level in private and public hospitals in Malaysia according to Malaysian statistics of medicine and ranked at the top of the list, which may result in potential detections due to their continued release into the aquatic environment through various pathways.

Sample Collection

The sampling method is a crucial component of monitoring studies, as it provides a detailed explanation of the selection or collection of samples for the intended study. The fish sampling process employed various approaches based on the nature and availability of the target samples. Omar *et al.* (2021) used a fishing net during the sampling process while Yin *et al.* (2019) decided to collect from local markets and, in some cases, from fishermen in coastal areas, as reported by Miossec *et al*. (2020). Fish samples are typically collected by researchers using fishing gear, from local markets, or from local fishermen in coastal areas.

Sample Storage

Sample management must focus on storage and preservation to ensure accuracy and minimise biological, chemical, and physical changes. The best way to ensure proper sample storage and preservation are generally choosing the appropriate sample container, keeping the sample in the refrigerator at the proper temperature, and adding preservatives. While researchers can minimise contamination, it cannot be avoided entirely (Omar *et al*., 2016). The reviewed articles did not report the use of preservatives for fish sample storage; instead, they recommended storing fish tissue samples

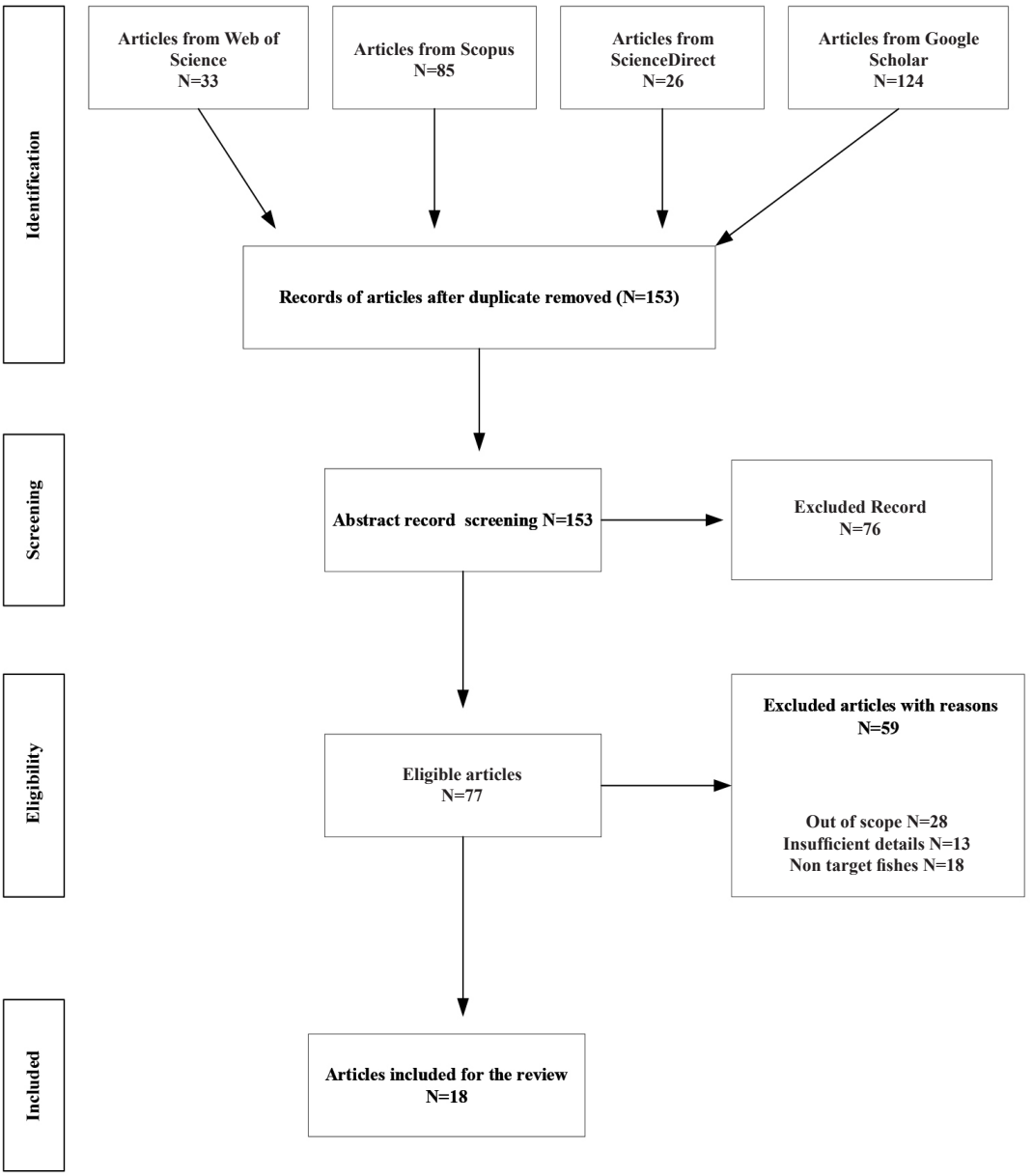

Figure 1: Literature identification and screening

at a very low temperature, of between -4°C and -80°C, without adding any preservatives.

Sample Dissection

Fish dissection is an important preliminary step in sample pretreatment (Di Fiore *et al*., 2024). By carrying out the dissection process under hygienic conditions with sterilised instruments, it aids in obtaining the actual target fish tissue (muscles, liver, kidney, intestine, gills) separated from other organs without contamination. The dissection process requires knowledge of fish internal and external morphology, which helps to obtain the exact target tissue. It is crucial to use sterilised instruments like knives and

scissors, as well as measuring tools like rulers and weighing balances to collect morphometric data.

Sample Freeze Drying

Freeze drying, also referred to as lyophilisation is a dehydration technique that uses low temperatures. The process involves freezing the product and then reducing the pressure, allowing the ice to convert directly into vapour through sublimation (Pardeshi *et al*., 2023). The main purpose is to retain the stability of heat-sensitive compounds, which can easily decompose via the use of alternative drying processes. Before mounting the sample in a freeze dyer with a vacuum pump, it must solidify in the fridge. The pump soaks away tissue moisture during the sublimation process, leaving behind the dried sample ready for analysis.

Analytical Techniques for the Evaluation of Pharmaceuticals in the Fish Tissue

The environment has been extensively exposed to pharmaceutical contaminants from numerous sources and through diverse pathways. Subsequently, these contaminants have a potential implication on biota such as aquatic animals. Therefore, analytical evaluation using appropriate sample preparation and instrumentation is required to access these contaminants at trace levels.

Sample Preparation

Sample preparation is crucial to justify perfect results during the analysis. It is important to employ methods that can eliminate lipids and other interfering contaminants while preserving the target analytes (Huerta *et al*., 2012; Omar *et al*., 2016). Sample preparation is a crucial step in analytical procedures as it makes the sample matrix more suitable for analysis, releases the analytes from the matrix, removes interfering compounds, concentrates or dilutes the matrix to meet the detection limits of the analytical method, and improves accuracy while minimising contamination during analysis. Some sample preparation methods include extraction and clean-up techniques such as Ultrasonic-assisted Extraction (UAE), Mechanical Extraction (ME), Pressurised Liquid Extraction (PLE), Gel Permeation Chromatography (GPC), Solid Phase Extraction (SPE), and sample concentration methods like the use of rotary evaporators and solvent evaporation techniques such as the use of a nitrogen evaporator to reduce the solvent volume for analysis as shown in Figure 2.

Extraction Methods

Numerous studies have reported the analytical evaluation of the pharmaceuticals from these therapeutic classes in different fish tissues (Rojo *et al*., 2019; Yin *et al*., 2019; Miossec *et al*., 2020; Omar *et al*., 2021). Several sample preparation methods have been tested for their extractions, including UAE, PLE, ME, Cell Disruption Extraction (CDE), Tissue Laser Extraction (TLE), Soxhlet Extraction (SE), Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction while instrumentation was mainly dominated by Liquid Chromatography (LC) using different detectors such as Mass Spectrometry (MS), tandem mass spectrometry (MS-MS), and photodiode array detector (PDA). Numerous extraction solvents were tested such as methanol (MeOH), acetonitrile (ACN), acetone, isopropyl alcohol, dichloromethane, and N-hexane. Depending on the chemical properties of the compound, polar analytes can best be extracted by polar solvents and vice versa.

The polarity of MeOH, ACN, and their corresponding acidified solutions gives them a high potential to extract a wider range of polar compounds from a sample (Yin *et al*., 2019). This property makes MeOH and ACN popular choices as solvents in various analytical techniques. Additionally, their ability to form hydrogen bonds with polar analytes enhances their extraction efficiency even further. When the target compounds are basic, the acidified solvents have high extraction recoveries. This is because the acidification makes it easier for the compounds to break apart, which makes it easier to separate them from the matrix. As revealed earlier, the major challenge in the analytical

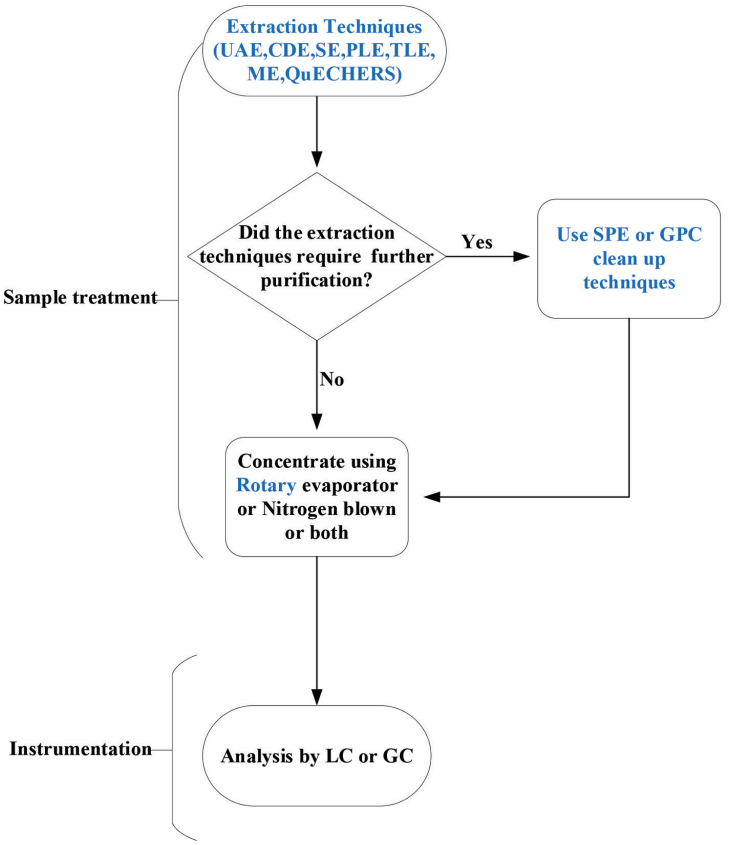

Figure 2: Sample preparation and instrumentation

evaluation of pharmaceutical compounds in fish tissue is the complex nature of these matrices owing to lipids, proteins, and organic compounds. These issues make analytical assessment much more demanding and require good sample preparation before monitoring the study. Several sample extraction techniques were developed successfully such as PLE, QuEChERS, Microwave-Assisted Extraction (MAE), and UAE for numerous biota samples (Ojemaye & Petrik, 2019; Arguello-Pérez *et al*., 2020; Pemberthy *et al*., 2020; Baesu *et al*., 2021).

Ultrasound Assisted Extraction

Ultrasound has gained popularity over the last decade and has been successfully used as step-up extraction technology owing to the cavitational effect that led to improved heat as well as mass transfer.

Ultrasound is a sound wave that vibrates in an alternating pattern of rarefaction and compression, leading to cavitation. The UAE process speeds up due to the interaction of ultrasound with supply material and the extraction solvent because of cavitational bubble collapse, which results in the extraction of the target analytes (Gómez Regalado *et al*., 2021; Jadhav *et al*., 2023). Sonication can be done directly by using a probe or indirectly using a sonication water bath. Compared to traditional extraction methods, UAE has a high efficiency in extracting target compounds, and it requires less solvent volume during sample preparation and requires less time for extraction, making it an effective and environmentally friendly technique.

Although there are several benefits to using UAE over conventional approaches, it is important to regulate the experimental conditions to prevent analyte deterioration throughout the extraction process (Albero *et al*., 2019). Amlodipine, diclofenac, and other compounds of different therapeutic classes in the whole fish body tissue samples were extracted using UAE techniques with an acidified mixture of MeOH/CAN as an extraction solvent (Tanoue *et al*., 2020).

The method has acceptable accuracy with extraction recovery of between 70% and 120% and sensitivity with low detection limits of between 0.075 ng/g and 0.024 ng/g for amlodipine and diclofenac, respectively. Peña Herrera *et al*. (2020) used a mixture of acidified ACN and isopropanol to evaluate the level of paracetamol and other pharmaceuticals in the fish muscles. The method achieved the recovery of > 60% of most of the target analytes with paracetamol limit of detection (LOD) 1.7 ng/g. Moreover, Zheng *et al*. (2022) employed UAE using a solution of ammonium acetate/acetonitrile (1:1 v/v) to examine diclofenac and other PhACs in eight different fish species. Research findings revealed an extraction recovery of between 54.0% and 120.50% with a diclofenac LOD of between 0.16 ng/g and 0.22 ng/g.

Similarly, Yin *et al*. (2019) used UAE using a mixture of ACN and ultra-pure water, UPW (8:2 v/v) to check diclofenac, paracetamol, and other ECs in the fish. The method is sensitive with a low LOD of 0.01 ng/g for both diclofenac and paracetamol, but with a recovery of 43% to 127%, the low recovery of some compounds may be attributed to the use of non-acidified extraction solvents. Equally, Fabunmi *et al*. (2020) used UAE with acidified MeOH to evaluate paracetamol in different fish tissue (muscles, gills, and eyes). The optimised method yields recoveries between 46% and 113%, with a detection limit of between 0.22 ng/g and 25 ng/g for most of the compounds. Similarly, Arguello Pérez *et al*. (2020) utilised UAE with MeOH and dichloromethane as solvents to evaluate diclofenac in the fish muscles. The method has a recovery of between 92% and 95% with a LOD of 1.15 ng/g w.w.

Pressurised Liquid Extraction

Also known as Accelerated Solvent Extraction (ASE), it combines the use of various solvents with high temperatures and pressures to extract analytes from solid or semi-solid matrices, which reduces the extraction times and increases recoveries (Qin *et al*., 2023). Methanol (MeOH), acetonitrile (ACN), and water, or a combination of them, have all been employed as extractant solvents regularly, with around 50ºC as the working temperature (Andreu & Picó, 2019; Gómez-Regalado *et al*., 2022). This technique has some advantages, including minimum extraction time, low solvent and energy consumption, and the ability to perform multiple extracts concurrently (Soriano *et al*., 2024). The main limitation of this extraction method is the high temperature used, which may degrade the target compound and subsequently decrease the recovery of the target compound (Višnjevec *et al*., 2024).

Solvent polarity should be close to that of target analytes. Thus, a nonpolar compound can best be extracted by nonpolar solvents while polar compounds by polar solvents. Generally, a mixture of low and high-polar solvents provides efficient extraction compared to single solvents for analytes with a wider range of polarities (Carabias Martínez *et al*., 2005). Hydrochlorothiazide and diclofenac in the muscles of three fish species were extracted by PLE using mixed extraction solvents MeOH, ACN, and water (Rojo *et al*., 2019). The method is sensitive in terms of the LOD of between 0.062 µg/kg and 0.072 µg/kg and between 2.1 µg/kg and 2.7 µg/kg for hydrochlorothiazide and diclofenac, respectively with extraction recovery of between 50% and 118%.

Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS)

The QuEChERS was introduced in 2003 by Anastassiades, Lehotay, Stajnbaher, and Schenck (Santana-Mayor *et al*., 2019). The method

gained popularity owing to its simplicity, less time, and solvent consumption. It was originally developed for pesticide extraction on fruits and vegetables, despite some method limitations such as selective recovery and limited clean-up, but the application was extended to many environmental matrices and organic pollutants.

The extraction approach is based on ACN followed by liquid-liquid partition and employing dispersive solid-phase extraction for the clean-up method (Omar *et al*., 2021). Baesu *et al*. (2021) used an acidified mixture of ACN and water as extraction solvents for the analysis of paracetamol in the fish liver tissue. The developed method performed well in terms of precision (< 22), with paracetamol LOD of 1.9 ng/g w.w. and recovery of between 102% and 107%. The use of an acidified mixture of ACN results in optimum analyte recovery. Similarly, Omar *et al*. (2021) uses the same extraction approach using ACN as solvent to extract diclofenac in the fish and Mollusc samples collected from different sampling sites in the Klang River Estuaries Malaysia. The optimised method is sensitive with a low LOD of 0.35 ng/g and satisfactory extraction recovery of between 64% and 114%. Pemberthy *et al.* (2020) utilised ACN as a solvent to quantify diclofenac in fish muscles and achieved a recovery of between 63% and 108% with an LOD of 20 µg/kg.

Solid Phase Extraction

The SPE is based on the distribution of the analyte between a solid phase, which is typically a sorbent contained in a column, and a liquid phase, which is a solvent with analytes (Zhang *et al*., 2022). The liquid sample is passed through adsorbent particles, to which the analytes have a stronger affinity than the bulk liquid; the analytes are then extracted by elution with the appropriate solvent (Badawy *et al*., 2022). The method was invented as an alternative to Liquid-Liquid Extraction (LLE) to eliminate several disadvantages of LLE such as the use of a large amount of solvent, time consumption, operational steps, and cost. The method is excellent in the selective extraction of target compounds but has limited capacity for high throughput.

The technique is also a useful tool for clean-up, isolation, purification, and isolation in different sample matrices (Ötles & Kartal, 2016). Solid phase extraction has been used as a clean-up method in several studies. Different adsorbents are used depending on the physicochemical properties of the analytes. Some of these studies employed SPE for fish plasma samples as an extraction technique. One such study used a hybrid SPE phospholipid cartridge, acetate buffer (pH 4), and a mixture of ACN and MeOH (1:1 v/v) as extraction solvents to analyse diclofenac in the plasma of three fish species. The method demonstrated a LOD for diclofenac 0.0048 ng/ml - 0.8 ng/ml, with recovery rates of between 70% and 120% and good precision of less than 15% (Nozaki *et al*., 2023).

Soxhlet Extraction

Soxhlet extraction is the extraction method that involves the extraction of the compound of low solubility from the solid mixture by using a distillation flask. The solvent condenses together with dissolved analytes. In this approach, solvents continuously wash the solid samples, which results in extracting the desired compound. Despite the advantage of thorough extraction of lipids and compounds, the main limitations of the method are that it is time-consuming and that the process requires a higher temperature, which may affect the stability of target analytes (Luque de Castro & Priego-Capote, 2010; Gómez-Regalado *et al*., 2021). Ojemaye and Petrik (2019) employed Soxhlet extraction using MeOH:ACN (3:1 v/v) as an extracting solvent to evaluate paracetamol and diclofenac in the fish tissue (muscles, gills, liver, and intestine). The method achieved a satisfactory recovery of between 68.8% and 107.5% and a low LOD of between 0.010 ng/g and 0.036 ng/g. Also, Martins *et al*. (2020) used a similar approach but used n-hexane and dichloromethane (1:1 v/v) for 12 hours as an extraction solvent to evaluate diclofenac in fish

tissue. The method has a recovery of between 75% and 91%.

Tissuelyser Extraction

The method involves agitation of the samples with the solvent mixture at high speed to lyse biological tissue samples using bead mill equipment to extract the target compound. The approach can be applied in animal and plant tissue with good extraction efficiency, this method has the advantage of effective disruption of touch samples, but the process requires specialised equipment and protocols (Gómez Regalado *et al*., 2022). Manjarrés *et al*. (2022) used bead beating tissue homogenisation using acidified acetonitrile: isopropanol (3:1 v/v) to evaluate hydrochlorothiazide, paracetamol, and diclofenac in different parts of the fish tissue (muscles, brain, kidney, pancreas, liver, skin, and heart). The optimised method is sensitive with a very low detection limit of between 0.0004 ng/g and 14.91 ng/g but a moderate extraction recovery of between 40% and 70%.

Cell Disruption Extraction

The approach is normally carried out in high-speed shaking equipment, which operates for a very short time. This method is very effective in breaking cell walls, which results in higher extraction efficiency of the target compound, but this high-speed shaking at a short time may lead to sample degradation. The approach was employed by Boulard *et al*. (2020) using heptane and ACN as solvents to extract hydrochlorothiazide and diclofenac in the fish tissues (muscles and liver). The method shows good extraction recoveries ranging between 70% and 130% with an LOD of between 0.1 ng/g and 48 ng/g d.w.

Mechanical Extraction

The mechanical method involves stirring the sample with solvent for a certain time to complete the migration of the sample analytes to the extraction solvent. The technique is usually accompanied by centrifugation to achieve better separation of layers, which leaves analytes in the solvent (Gil-Solsona *et al*., 2021; Gómez-Regalado *et al*., 2022). The extraction approach is simple and cost-effective but may result in low yield compared to other methods. Miossec *et al*. (2020) used a vortex mixer with 0.1% acetic acid in MeOH as an extraction solvent to determine paracetamol, hydrochlorothiazide, diclofenac, and acetylsalicylic acid in fish muscles. The method is sensitive, with a LOD of between 0.1 and 40.2 ng/g and extraction recovery of between 45% and 131%. Similarly, Lan *et al*. (2019) employed a similar approach using dichloromethane and potassium hydroxide (1:1 v/v) as extraction solvents to assess the level of ibuprofen in Tilapia fish muscle. The developed method has an LOD of between 1.29 ng g−1 and 79.45 ng g−1.

Clean Up Method

Good and efficient sample preparation should remove a significant amount of the lipids from biota extracts devoid of removing target analytes (Omar *et al*., 2016). The clean-up technique is one of the most crucial segments in sample preparation, especially in biota matrices, owing to the complexity of the samples (Omar *et al*., 2016). The clean up technique involves various methods such as SPE, Gel Permeation Chromatography (GPC), HPLC fractionation, and QuE Z-Sep/C18 sorbent for clean-up in the QuEChERS method, which helps with selectively removing these interfering substances. These techniques not only enhance the sensitivity of the analysis but also ensures the preservation of analytes of interest. Solid phase extraction is a well-established clean up method that removes lipids from biological samples (Farahin *et al*., 2023).

This method involves the use of a solid sorbent material such as silica or polymer that selectively adsorbs the lipids while allowing other analytes to pass through (Hajeb *et al*., 2022). It is widely used in various fields, including environmental analysis, pharmaceutical research, and forensic toxicology, due to its efficiency and versatility (Kanu, 2021). The application of the SPE

method in the analysis of pharmaceuticals from various therapeutic classes in fish tissue has been widely reported in numerous studies (Lan *et al.*, 2019; Yin *et al.*, 2019; Tanoue *et al.*, 2020; Manjarrés *et al.*, 2022). Arguello-Pérez *et al.* (2020) utilised a C18 SPE cartridge for the clean-up process to detect diclofenac in fish muscle, achieving a recovery rate of 92% for the target compound. The C18 cartridge is one of the most popular reversed-phased SPE sorbents due to the high retention of a non-polar compound such as lipid.

Similarly, Omar *et al*. (2021) also used a polymeric reversed phase C18 SPE cartridge (strata x) for the clean-up process in the analysis of diclofenac and other emerging contaminants in the fish muscles. The method achieved a recovery rate of between 64% and 112% of this type of polymeric SPE sorbent, which strongly retains basic, acidic, and neutral compounds under extreme organic wash conditions. Another important SPE cartridge used for SPE clean up is the HybridSPE phospholipid cartridge, which contains dual sorbents, zirconium-coated silica particle sorbents. Tanoue *et al.* (2020) used a hybrid SPE phospholipid cartridge as an SPE clean up cartridge to remove the interfering compounds during the extraction of amlodipine, diclofenac, and other pharmaceuticals from different therapeutic classes in different fish tissues. The clean up approach yields a satisfactory extraction recovery in the range of 70%-120%. Zirconium bonded to a hybrid silica surface act as Lewis's acid due to vacant d-orbitals that interact with phospholipid components by Lewis's acid-base interactions; hence, phospholipid will be retained chemically by the zirconia site while protein will be precipitated and retained physically by sorbent material.

A little amount of acid, especially formic acid, is normally added as a modifier for most basic, acidic, and neutral compounds. The formate ion produced is a strong lewis base, and for this reason, it prevents the interaction of most acids with zirconia sites, and proton H^+ produces neutralised silonol effect and hence, improves the extraction recovery. The Oasis HLB cartridge is a widely accepted, polymeric reversed-phase cartridge used for purifying and cleaning compounds from various matrices. It is made from two monomers: Hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene (Wleklinski *et al.*, 2024).

This versatile polymeric sorbent is suitable for extracting a broad range of basic, acidic, and neutral compounds. It offers excellent retention of polar compounds and is water-wettable (Tomai *et al.*, 2020; Murtada & Pawliszyn, 2023). Additionally, it delivers high recovery rates, even with dry adsorbents, eliminating the need for extra steps to keep the sorbents wet. Various research papers have reported using these cartridges in their clean-up processes and achieved a satisfactory recovery of their target pharmaceutical compounds (Ojemaye & Petrik, 2019; Yin *et al*., 2019; Fabunmi *et al*., 2020).

Optimisation of LC Parameters

The LC is the category of chromatography that can be applied to any sample analytes soluble in a liquid phase like MeOH, ACN, water, and their mixture at different proportions and is among the most extended approaches in the analysis of emerging contaminants (Álvarez-Ruiz & Picó, 2020). Over the last decades, LC-MS and LC-MS/MS have been the most popular chromatography techniques due to compatibility with both polar and nonpolar volatile compounds like PhACs (Huerta *et al*., 2012). LC has an advantage over Gas Chromatography (GC) and has become the choice for researchers, as it does not require derivatisation steps (Zeki *et al*., 2020), which are difficult and time-consuming and must be carried out by trained personnel to obtain the best results (Omar *et al*., 2016). Several researchers used LC in their analysis (Yin *et al*., 2019; Fabunmi *et al*., 2020; Pemberthy *et al*., 2020; Zheng *et al*., 2022).

Column dimensions (length, internal diameter, and particle size) are important factors to consider during HPLC method optimisation (Lawlor *et al*., 2024). The above factors play a significant role in compound separation during method development. Column length

has similar effects as column internal diameter; a shorter column results in a shorter run time and low solvent consumption; a longer column retains the target compound longer and requires more solvent but results in better compound separation. Column particle size proved to be an important determining factor for proper compound resolution (Jiang *et al*., 2024); smaller adsorbent particle size in the column delayed the interaction of the mobile phase and target compounds in the column, hence, the better peak separation. Several researchers reported the use of shorter columns with small internal diameters (50 mm x 2.1 mm) in the analysis of their target compounds in the fish tissue and achieved good separation of their target pharmaceutical compounds (Rojo *et al*., 2019; Yin *et al*., 2019; Miossec *et al*., 2020; Peña Herrera *et al*., 2020).

Similarly, the use of longer columns was also reported (Lan *et al*., 2019; Boulard *et al*., 2020; Fabunmi *et al*., 2020) and they also achieved good compound resolution. Generally, in the method optimisation, selecting a shorter column for compound separation within the shortest possible time, but for many compounds of different polarities, a longer column is the right choice for the best compound separation, though the analysis time will be longer. Another important parameter to consider during method optimisation is the mobile phase. The choice of mobile phase is crucial in HPLC analysis, as it plays a key role in eluting target compounds from the stationary phase (column) and achieving effective separation of the analytes (De Luca *et al.*, 2024).

The physicochemical properties of the target compound must be taken into consideration before making the right decision on the mobile phase choice. For the reverse phase chromatography, the mobile phases are polar solvents such as UPW, MeOH, ACN, and tetra hydro furan, THF in their single phase or combined in different proportions, and sometimes are added to chemical additives such as formic acid, acetic acid, and ammonium acetate. To improve the ionisation especially for the MS detector, the mobile phase flow rate is also an important parameter to consider during HPLC method optimisation, the higher mobile phase flow rate reduces the analysis time and vice versa. Also, another parameter in the optimisation process is the sample injection volume; a higher sample injection results in higher sensitivity of the HPLC and vice versa while at the same time, a higher injection volume results in a broader peak, especially at high concentrations, so, these factors are also crucial in the HPLC optimisation process.

Optimisation of GC Parameters

The GC is a powerful analytical technique utilised for separating and analysing chemicals that can be vaporised without undergoing breakdown (Cardoso Rial, 2024). Due to its high sensitivity, selectivity, and precision in complex mixtures, it finds extensive applications in various industries such as pharmaceuticals, forensics, environmental analysis, and food and beverage sectors (Kabir & Furton, 2021). Particularly advantageous for volatile organic compounds and low molecular weight molecules (Xie *et al*., 2023). Optimising GC parameters is crucial for achieving optimal separation and analysis of chemicals within a sample. Multiple factors can be fine-tuned to improve the efficiency of a gas chromatography system. Choosing the appropriate GC column is essential for achieving optimal separation (Garcia *et al*., 2024).

Several factors can be fine-tuned to enhance the efficiency of a GC system. Key considerations include selecting the appropriate GC column, which involves parameters like column length, diameter, stationary phase, film thickness, and polarity. These parameters are chosen based on the properties of the compounds of interest such as volatility, polarity, and molecular weight (Duarte *et al*., 2023). The temperature of the gas chromatography column plays a significant role in affecting the volatility of chemicals and their interactions with the stationary phase (Yusuf *et al*., 2023).

Adjusting the column temperature can optimise compound resolution (Sudol *et al*., 2020). Moreover, the flow rate of the carrier gas

influences the speed at which compounds move along the column. Higher flow rates can decrease analytical time but may compromise resolution (Nassini *et al*., 2023). It is worth noting that among the reviewed articles, only one used GC, potentially due to the challenges associated with the derivatisation process involved in using this method.

Detection System

A detection system identifies and quantifies separated compounds using different detection techniques such as UV-visible detection (measuring light absorbance), fluorescence detection (detecting emitted light), Mass Spectrometry (MS) (measuring mass-to-charge ratios for high sensitivity), and refractive index detection (measuring changes in refractive index), each suited to different analytical needs. Among these detection systems, the MS detector is the most widely used due to the sensitivity and selectivity; nearly all reviewed articles used the MS detector, except for one study. Additionally, electrospray ionisation (ESI) is the predominant interface based on the reviewed literature. The ESI interface generates ions by applying a higher voltage to liquid, which generates an aerosol, mostly used in large molecules that fragment after ionisation (Gómez-Regalado *et al.*, 2022).

Mass spectrometry is classified into Low-Resolution Mass Spectrometry (LRMS) and High-Resolution Mass Spectrometry (HRMS), each serving distinct purposes. Low resolution mass spectrometry is utilised for targeted analysis, whereas HRMS is employed for non-target analysis, benefiting from its superior resolution capabilities. Notably, tandem mass spectrometry (MS/MS) using a triple quadrupole mass analyser is the most frequently referenced technique in the reviewed literature. Contrary to the function of HRMS, which is mainly for non-target analysis, Manjarrés *et al.* (2022) used the same instrument for target analysis of paracetamol, diclofenac, and hydrochlorothiazide and achieved a remarkably low LOD of between 0.0004 ng/g and 14.91 ng/g.

Similarly, Nozaki *et al.* (2023) used HRMS (UFLC-QTRAP-MS) to evaluate diclofenac in the fish plasma and attained a low LOD in the range of between 0.0048 ng/ml and 0.8 ng/ml; also, in the same manner, Tanoue *et al.* (2020) applied high-resolution UFLC-QTRAP-MS to evaluate diclofenac and amlodipine in the whole-body fish sample and attained a remarkably low LOD of 0.024 ng/g and 0.075 ng/g for diclofenac and amlodipine, respectively. Table 1 provides a summary of the analytical methods reported in the reviewed articles for the analysis of amlodipine, simvastatin, diclofenac, paracetamol, hydrochlorothiazide, and acetylsalicylic acid, along with some members of the same therapeutic classes. Furthermore, Tables 2 and 3 depicted the merits and demerits of the extraction and instrumental analysis, respectively.

Conclusions

The review critically evaluates various analytical techniques used to detect pharmaceuticals in fish. Several extraction methods have been explored with polar solvents, particularly in combination with other solvents, showing higher recovery rates. Satisfactory results have also been obtained for some non-polar organic compounds. Among the extraction techniques reviewed, UAE emerged as the preferred method during the study period. This preference is attributed to UAE's simplicity, requiring less time and solvent use during sample preparation while effectively extracting high yields of the target compounds. Additionally, UAE is noted for being environmentally friendly and preserving thermolabile compounds due to its operation at relatively low temperatures, which prevents compound decomposition. Most separations and detections were conducted via LC coupled with different detection systems, which is preferred over GC because it eliminates the need for derivatisation, a cumbersome, and time-consuming process that requires trained personnel. Despite the efforts by various researchers, the challenge of matrix interference persists as the extraction recoveries obtained from various results are not satisfactory.

Table 1: Summary of analytical methods

Matrices	Analytes	Sample Storage	Extraction and Clean Up	Instrumental Analysis	Accuracy	LOD	Reference
Fish muscle	DIC	Wrapped in aluminium foil and stored at -20°C until analysis	QUECHERS with ACN Followed by SPE clean up using polymeric reversed phase cartridge	LCMS/MS with Reverse phase C18 column (50 mm × 2.0 mm, 3 µm). Mobile phases include. (A = UPW, B-ACN: MeOH (60:40) for (ESI-) and (ESI+) using 0.2% NH_4OH and 0.2% CH_2O_2 as chemical additives	64-114%	0.02-3.5 ng/g	Omar *et al.* (2021)
Fish muscle	DIC PCM NPX IBF	Stored at -20°C until analysis	Ultrasonic using ACN: UPW (8:2 v/v) followed by SPE clean up. Oasis prime HLB cartridge	UHPLC MS/MS using ZORBAX Eclipse Plus C18 column (RR HD 2.1 × 50 mm × 1.8 µm). Mobile phases include. (ESI-) A = 100%UPW, B = 100% ACN While (ESI+) A-UPW + 0.1% formic acid B = 100% CAN	43-127%	0.01-1.9 ng/g	Yin *et al.* (2019
Fish muscle	AMP DIC DIL LOV	-	Mechanical shaking with ACN acidified with 0.1% formic acid as solvents and clean up with C18 sorbent	LC-QTRAP-MS with RP-18 column (100 mm × 4.6 mm, 2.6 µm). The mobile phase includes. A-UPW with 0.2% formic acid B-Acetonitrile with 0.2% formic acid	46-97%	0.01-0.10 ng/g	Bobrowska *et al.* (2021)
Fish muscles	PCM HCTZ ASA DIC	Freeze-dried, grind, and stored at -80°C until analysis	Mechanical shaking with MeOH + 1% Acetic Acid	LC-MS/MS using C18 UPLC HSST3 (size, 50 mm × 2.1 mm i.d. 1.8 µm) Mobile phases include. (ESI+) mode A-UPW with 0.1% formic acid B-ACN (ESI-) mode A-UPW with 0.01% formic acid B-ACN	45-131%	0.1-40.2 ng/g	Miossec *et al.* (2020)

Fish muscle, heart, skin, liver, kidney, pancreas, brain	HCTZ PCM DIC DIL	Stored at -40°C until analysis	Tissuelyser extraction with ACN: iPrOH (3:1) Acidified with 1% formic acid Followed by a zirconia-based clean up step (Z-Sep/C18)	LC-HRMS (Q-Exactive Orbitrap) with EVOC18 KINETEX column (100 × 2.1 mm, 2.6 µm) ESI (+) A = 5mM of Ammonium acetate an 0.1% acetic acid B- = ACN ESI (-)	40-70%	0.0004-14.91 ng/g	Manjarrés *et al.* (2022)
Plasma		Stored at -80°C until analysis	Mechanical extraction with cold ACN	A = 2 mM ammonium fluoride B- = CAN		0.01-1.56 ng/ml	
Biles		Stored at -80°C until analysis	Mechanical extraction with cold ACN			0.02-28.71 ng/ml	Manjarrés *et al.* (2022)
Fish liver	PCM	Stored at -80°C until analysis	QUECHERS using. ACN: UPW (84:16 v/v) Acidified with 1% acetic acid as solvent. dSPE clean up	UHPLC-QTOP-MS using Poroshell 120 (PhenylHexyl,3.0 × 100 mm, 2.7 µm, With a Poroshell (4.6 mm) column. Mobile phases include. (ESI+) A = UPW with 1% formic acid B = ACN (ESI-) A = 0.05M Ammonium acetate B- = CAN	102-107 %	1.9 ng/g w.w.	Baesu *et al.* (2021)
Fish whole body tissue	DIC AMP DIL IDM	Stored at -20°C until analysis	Ultrasonic extraction using acetic acid-ammonium acetate buffer and ACN:MeOH (1:1 v/v) followed by SPE clean up using Hybrid SPE phospholipid cartridge (containing zirconia coated silica particles)	UFLC-QTRAP-MS operating in (ESI) with Multiple Reaction Monitoring (MRM) using express C18 analytical column (2.7 µm,100 × 2.1 mm). The mobile phase includes. A = 0.1%(v/v) acetic acid in UPW/ methanol (95:5, v/v) B = 0.1%(v/v) acetic acid in methanol/ acetonitrile (1:1, v/v)	70-120%	0.024 ng/g w.w. 0.075 ng/g 0.014 ng/g 0.0096 ng/g	Tanoue *et al.* (2020)

Fish plasma		Stored at -20°C until analysis	SPE on Hybrid SPE Phospholipid Ultra cartridge using acetic acid-ammonium acetate buffer and acetonitrile/ methanol (1:1 v/v) as solvents			(0.0077 to 0.93 ngmL−1)	
Fish muscle, gills, liver, intestine, brain, kidney	DIC IBF	Stored at -80°C until analysis	Ultrasonic extraction with ammonium acetate: ACN (1:1 v/v) followed by SPE clean up	UPLC-MS/MS with AQUITY BEH C18 column (2.1 mm x 100 mm, 1.7 m). The mobile phase includes. (ESI +) A = (UPW: MeOH 98:2 + 0.05% methicillin) B = (methanol + 0.05% formic acid) (ESI-A = (UPW: MeOH 98:2+5 mol/L ammonium acetate) B = (100% acetylene)	54.0-120.50%	0.16-0.22 ng/g 0.16-0.36 ng/g	Zheng *et al.* (2022)
Fish plasma	DIC IBF IDM MEF	Stored at -20°C until analysis	SPE using Hybrid SPE phospholipid cartridge. With acetate buffer (pH 4) and ACN: MeOH (1:1 v/v) as solvents	UFLC-QTRAP -MS operating in (ESI) modes with (MRM) using AscentisExpress C18 analytical column (2.7 µm,100 × 2.1 mm). The mobile phase includes. A = 0.1% acetic acid in UPW/ methanol (95:5, v/v) B = 0.1% acetic acid in methanol/ acetonitrile (1:1, v/v)	70-120%	0.0048-0.8 ng/ ml	Ruan *et al.* (2020)

Fish muscles	HCTZ DIC	Wrapped with aluminium foil, placed in a plastic bag, and stored at -80°C until analysis	PLE with MeOH, ACN, and water as solvent followed GPC clean up with chromatography (HPLC) system.	UPLC-QTRAP-MS with BEH C18 column (50 mm, 2.1 mm i.d., 1.8 mm) in negative ionisation mode while HSS T3 Column (50 mm2.1 mm i.d., 1.7 mm particle size) positive ionisation mode. Mobile phase (ESI+) A = Methanol B = 10 Mm formic acid/ammonium formate (pH 3.2) (ESI-) A = Acetonitrile B = 5 mM ammonium acetate/ ammonia (pH 8)	50-118%	0.062-0.072 µg/kg 2.1-2.7 µg/kg	Rojo *et al.* (2019)
Fish muscles and liver	HCTZ DIC	Homogenised and stored at -20°C until analysis	Cell disruption at 4 m/s for 40 s using n-Heptane and ACN as solvents, followed by clean up with RAM chromatography	LC-MS/MS with Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 3.5 µm) with a mobile phase A = 0.1% acetic acid B = Acetonitrile	70-130%	0.1-48 ng/g	Boulard *et al.* (2020)
Fish muscles	IBF	-	Mechanical shaking with CH_2CL_2: KOH (1:1 v/v) followed by alumina gel column clean up	HPLC-PDA with Xterra C18 (25 mm × 4.6 mm, 5 µm) with mobile phase include A = 0.1% v/v trifluoroacetic acid (TFA) in water (55% v/v) and B: Acetonitrile	56-105%	0.02-1.3 ng/g	Lan *et al.* (2019)
Fish muscles Gills eyes	PCM DIL	Wrapped with aluminium foil, insert into the plastic bag, and stored at -20°C until analysis	Ultrasonic extraction with 0.1 M acetic acid: MeOH(1:1 v/v) followed by SPE clean up with Oasis HLB SPE cartridge	UPLC-MS/MS and (ESI source) using reversed phase BEH C18 column (150 x 1.0 mm, 1.7 mm particle size). The mobile phase includes. A = UPW: Methanol (80:20 v/v) containing 5 mM NH_4OAc and 3 Mm CH3COOH B = 100% Methanol	113.2% 89.3%	2.33 ng/g dw 0.27 ng/g	Fabunmi *et al.* (2020)

Fish muscles	DIC IBF	Stored at -20°C until extraction	QuEChERS using ACN as solvent dSPE clean up	UPLC-MS/MS with ESI source in (MRM) mode using C18 Column (2.1 x 100 mm, 2.7 µm). Mobile phase A-UPW with 0.5 M acetic acid, B-MeOH: ACN (1:1 v/v)	63-108%	0.32 ng/g 0.35 ng/g	Pemberthy *et al.* (2020)
Fish muscles gills liver intestine	DIC PCM	Freeze-dried ground and store at -20°C until analysis	Soxhlet extraction using MeOH: acetone (3:1 v/v) followed by SPE clean up with Oasis HLB cartridges.	UPLC-MS/MS with ESI source using Acquity BEH C18 1.7 mm column (2.1 mm x 100 mm). The mobile phase includes. A = 0.02 M formic acid in water B = Acetonitrile	68.8 to 107.5%	0.010-0.036 ng/g	Ojemaye *et al.* (2019)
Fish muscles	PCM DIL KET MEF	Stored at -25°C until analysis	Ultrasonic extraction with ACN/isopropanol 0.1% Formic acid as solvent followed by dSPE clean up using Z-Sep/C18 sorbents	UPLC-QTOP-MS using EVOC18 KINETEX packed column (50 mm × 2.1 mm, 2.6 µm). The mobile phase includes. A = 5 mmolL−1 ammonium acetate + 0.05% V/V formic in water B = 0.05% V/V formic acid in acetonitrile	> 60%	1.7 ng/g 0.6 ng/g 1.7 ng/g 1.3 ng/g	Peña-Herrera *et al.* (2020)
Fish muscles	DIC IBF KET NAP	Stored at -16°C to 18°C until analysis	Ultrasonic extraction using MeOH: dichloromethane (60:40) followed by clean up with C18 SPE column	UHPLC-MS/MS	92-95%	1.15 ng/g 3.21 1.32 0.97	Arguello-Pérez *et al.* (2020)
Gills Liver Muscles Ovaries Blood	DIC	Stored at -80°C until analysis	Soxhlet extraction with dichloromethane and n-hexane (1:1 v/v) for 12 hour SPE	GC-MS	75-91%	0.3 ng/g	Martins *et al.* (2020)

DIC=diclofenac, PCM=paracetamol, AMP=Amlodipine, HCTZ=Hydrochlorothiazide, ASA=Acetylsalicylic Acid, SIM=Simvastatin, IBF=ibuprofen, KET=Ketoprofen, NAP=Naproxen, MEF=Mefenamic acid, DIL=Diltiazem, IDM=Indomethacin, LOV=lovastatin.

Table 2: Extraction Methods' Merit and Demerit

S/N	Extraction Methods	Merit	Demerit	References
1	UAE	- High efficiency to extract target compounds - Requires less solvent volume - Requires less time	- Experimental conditions (frequency of the sound wave, temperature, and time) must be regulated to prevent analyte deterioration throughout process	Albero *et al.* (2019)
2	PLE	- Minimum extraction time - Ability to perform multiple extracts concurrently	- Required high temperature, which may degrade the target compound and decrease the extraction recovery	Soriano *et al.* (2024) Višnjevec *et al.* (2024)
3	QuEChERS	- Simplicity - Less time and solvent consumption	- Selective recovery and limited clean up	Santana-Mayor *et al*. (2019)
4	SPE	- Excellent in selective extraction of target compounds - Useful tool for clean-up, isolate, purification, and isolation in different sample matrices	- Limited capacity for high throughput	Ötles & Kartal (2016)
5	SE	- In depth extraction of lipids and compounds	- It is time consuming method - Required higher temperature, which may affect the stability of compound	Luque de Castro & Priego-Capote. (2010) Gómez-Regalado *et al*. (2021)
6	TLE	- High extraction efficiency - Effective disruption of touch samples	- Requires specialised equipment and protocols	Manjarrés *et al*. (2022) Gómez Regalado *et al.* (2022)
7	CDE	- Effective in breaking cell walls, results in higher extraction recovery - Required short extraction time	- The high-speed shaking at a short time may result in sample degradation	Gómez Regalado *et al.* (2022)
8	ME	- Simple and cost-effective	- Low yield compared to other methods	Gil-Solsona *et al.* (2021)

Table 3: Instrumental analysis methods' merit and demerit (UHPLC)

S/N	Instrumentation Methods	Merit	Demerit	Reference
1	LC	- Sensitive and selective - Compatibility with both polar and non-polar volatile compounds - Does not require derivatisation steps	- Periodic maintenance cost - Mobile phases are toxic and non eco friendly - Need trained personnel to operate	Zeki *et al.* (2020)
2	GC	- Highly sensitivity - Very selective and precise - Compatible with volatile organic compounds and low molecular weight molecules	- High operational and maintenance costs - Required derivatisation steps in case of non-volatile compounds - Required trained personnel to operate	Xie *et al.* (2023)

Current Trends and Future Perspectives

In recent years, chromatographic methods have seen significant advancements. Ultra-High-Performance Liquid Chromatography UHPLC has largely replaced traditional HPLC due to its faster speed, greater efficiency, and higher resolution. With the ability to use smaller sample volumes and less solvent, UHPLC is particularly well suited for high-throughput analysis of pharmaceuticals in fish. In the realm of MS, LC-MS/MS has emerged as the gold standard for detecting trace pharmaceuticals in fish, thanks to its high sensitivity, selectivity, and ability to quantify multiple compounds simultaneously. More recently, HRMS techniques such as quadrupole-time of flight (Q-TOF) and orbitrap have gained prominence. These methods offer ultra-high resolution and accurate mass measurements, allowing for the identification of unknown pharmaceuticals and metabolites through non-target screening.

Additionally, MS/MS with triple quadrupole instruments has further enhanced the precision of pharmaceutical quantification, making it indispensable for environmental monitoring. However, the review identified several research gaps. Notably, no data is available from the Australian continent, and only limited data exists from Africa. Furthermore, there is very limited research data on the concentration of pharmaceutical compounds in fish skin and bones, areas often neglected despite their importance. Future research should focus on these two continents to develop a more comprehensive global dataset, as well as investigate the levels of pharmaceutical contaminants in fish skin and bones to fully understand fish exposure across all body parts. Finally, we recommend developing novel and optimising existing analytical methods to drastically minimise the challenges of matrix inference in aquatic biota analysis.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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