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Application of "Dispersive Liquid-Liquid Micro Extraction Technique" for the Analysis of Piroxicam in Human Urine and Drug Formulation

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Abstract:- "Non-Steroidal Anti-inflammatory Drugs" (NSAIDs) are one of the most utilized sets of medicines in dentistry for the treatment of chronic and acute pain. Their therapeutic toxicity and effectiveness are well-documented. Demonstrating that NSAIDs give an appropriate therapeutic ratio of aching relief with fewer side effects than the opioid-mild pain-relieving combination medicines, they have essentially supplanted for dental uses. Many studies showed that by using the oral surgery model of acute pain, the single dosage of a NSAID is more efficacious than a combination of acetaminophen plus an opioid or aspirin with less side effects, making it the better choice for ambulatory patients. The combination of a "NSAID" with an opioid produces minimal analgesic efficacy but a higher rate of adverse effects, limiting its usage to individuals for whom the "NSAID" only provides insufficient analgesia.

"4–hydroxy–2–methyl–N–2–pyridinyl–2H–1,2–benzothiazine–3–carboxamide1,1–dioxide (PX)", is known as Piroxicam. It is non–steroidal anti–inflammatory and pain-relieving chemical that belongs to the oxicams, a novel family of pharmaceuticals. It's commonly utilised in dealing rheumatoid arthritis sufferers. Piroxicam is also called (NSAID), it relates to the oxicam family of drugs and is a Cyclooxygenase-1 (Cox-1) inhibitor. Piroxicam is a whitish colour crystalline substance found in nature. It is just marginally soluble in alcohols and is sparsely miscible in water, dilute acid, and organic solvents.

The advancement of accurate, sensitive, quick, secure, and automated analytical techniques of monitoring environmental has received a lot of attention in recent decades. Several analytes must be determined at trace levels in such applications. Despite extensive technical advancements, mainly analytical equipment does not allow direct assessment of the real ambient matrix composition. Furthermore, only few of sensitive testing methods enough to identify trace contaminants directly are present. In many situations, a preparatory step of analyte isolation, enrichment is necessary to analysis.

I. INTRODUCTION

Piroxicam

"4–hydroxy–2–methyl–N–2–pyridinyl–2H–1,2–benzothiazine–3–carboxamide1,1–dioxide (PX)", is known as Piroxicam. It is non–steroidal anti–inflammatory and pain-relieving chemical that belongs to the oxicams, a novel family of pharmaceuticals. It's commonly utilized in dealing rheumatoid arthritis sufferers. Piroxicam is also called (NSAID), it relates to the oxicam family of drugs and is a Cyclooxygenase-1 (Cox-1) inhibitor. Piroxicam is a whitish colour crystalline substance found in nature. It is just marginally soluble in alcohols and is sparsely miscible in water, dilute acid, and organic solvents [1].

In comparison to indomethacin, its efficacy (weight-for-weight) in non-specific animal models of inflammation was higher. Piroxicam is very powerful (weight-for-weight) than ibuprofen, naproxen, fenoprofen, phenylbutazone or aspirin as an analgesic. Piroxicam 20 milligram everyday led to less fecal blood loss in humans in early trials compare to the aspirin 3.8 gram daily. Piroxicam drug like many other non-steroidal anti-inflammatory medications, suppresses collagen-induced platelet and adenosine di-phosphate accumulation in the secondary phase. Piroxicam inhibits prostaglandin production in vitro and in vivo, and in the phase of arachidonic acid metabolism, cyclo-oxygenase behave as a selective reversible inhibitor [2].

Piroxicam has a longer plasma half-life than these agents, according to pharmacokinetic studies. When piroxicam is evaluated in the carrageenan rat paw oedema model, it showed potency similar to indomethacin. An intact adrenocorticoid system is not required for this action. Piroxicam's strong potency, long half-life, and lack of cardiovascular or central nervous system side effects have prompted clinical trials [3].

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In rheumatoid arthritis, spondylitis, ankylosing, osteoarthritis, acute gout and acute pain in musculoskeletal diseases, piroxicam is an efficient pain-relieving and anti-inflammatory drug. In fracture, dental, postoperative, and postpartum pain, it has been demonstrated to be an effective reliever. Piroxicam is a bitter-tasting odorless, white crystalline powder. Piroxicam comes in two crystal polymorphs that are interchangeable. Piroxicam crystallizes as needles when rapidly cooled from an ethanolic solution, but crystallizes as cubic form when slowly cooled from the same solution. Piroxicam is an NSAID (non-steroid anti-inflammatory) medication that is acidic. In humans, the chemical is effective in treating rheumatoid arthritis and other inflammatory diseases. The medication is extremely powerful and has a half-life of more than 30 hours, making it ideal for a single daily dose. It has no negative impact on the heart or the central nervous system [4].

NSAID (Non-Steroid Anti-Inflammatory Drugs)

The abbreviation NSAIDs stands for Non-Steroidal Anti-Inflammatory medicines. In common practice, drug names and classifications are often regarded at face value as natural categories that exist on their own. The word NSAID highlights the fact that medication names and classifications are complicated cultural and social artefacts developed by specific individuals for specific goals through specific historical processes, and that this is still applicable today. The term "non-steroidal" originally came in the early 1960s, when the iatrogenic tragedies associated with the use of corticosteroids became evident, and a clear distinction between these medicines and developing anti-inflammatory options were required [5]. NSAIDs (Non-Steroidal Anti-inflammatory medications) is one of the family of medicine that is frequently used with specific with a prescription [6]. NSAIDs are used to treat inflammation as well as the discomfort and fever that comes with it [7]. These medications are highly flexible and are typically considered safe for use in healthy and young people. Despite their different chemical structures, they have similar therapeutic activities and pharmacologically active drugs with negative effects. Aspirin is the oldest and first medicinal NSAID, having been launched in 1899. It is a salicylate and is still regarded as one of the most effective first-line agents.

NSAIDs have a long history, dating back to the dawn of civilization. Quinine, derived from cinchona bark is one of the oldest medicines for moderate fever and pain alleviation. Willow bark (Salik alba) has long been utilized in traditional medicine for a variety of ailments. Using a powdered version of willow bark used in the mid-eighteenth century, Reverend Edmund Stone recounted his effectiveness in treating ague marking the earliest entry in the records of NSAID literature. Leroux discovered the active component in willow bark, salicin and a bitter glycoside in 1829. Although acetylsalicylic acid (aspirin) was invented in 1853, it was not utilised until 1899. Synthetic chemicals quickly supplanted natural substances. Other NSAIDs were found at the end of the nineteenth century that shared the salicylates' effects. More than a hundred NSAIDs have been researched, and many of them are now available in a variety of formulations across the world.

Analgesics such as "Non-steroidal Anti-inflammatory medicines" (NSAIDs) are one of the most suggested pharmaceuticals in the world. Their usage, however, has been linked to significant dose-dependent GI problems such as upper gastro-intestinal (GI) hemorrhage. Because of the extensive use of NSAIDs, GI problems are one of the most prevalent medication adverse effects in the US. Even with short-term NSAID usage, the risk of upper GI problems exists, and the probability of events increases linearly with continuous use. Despite the availability of gastro-protective treatments, they are underutilized, physician and patient knowledge of few of the variables affecting the expansion of NSAID related upper gastrointestinal problems is low. Aspirin, corticosteroids, anticoagulants and selective serotonin reuptake inhibitors are among the risk factors, as are advanced age, a history of GI injury, and concurrent use of medicines including anticoagulants, aspirin, selective serotonin reuptake inhibitors and corticosteroids. Anti-secretory drugs, gastro-protective medicines, alternate NSAID formulations, and non-pharmacologic treatments are all used to avoid GI injuries. Greater understanding of the potential risk factor treatments for gastrointestinal problems caused by NSAID usage might assist patients who need NSAID therapy and can lead to better results [8].

"Non-Steroidal Anti-inflammatory Drugs" (NSAIDs) are one of the most utilized sets of medicines in dentistry for the treatment of chronic and acute pain. Their therapeutic toxicity and effectiveness are well-documented. Demonstrating that NSAIDs give an appropriate therapeutic ratio of aching relief with fewer side effects than the opioid-mild pain-relieving combination medicines, they have essentially supplanted for dental uses. Many studies showed that by using the oral surgery model of acute pain, the single dosage of a NSAID is more efficacious than a combination of acetaminophen plus an opioid or aspirin with less side effects, making it the better choice for ambulatory patients. The combination of a "NSAID" with an opioid produces minimal analgesic efficacy but a higher rate of adverse effects, limiting its usage to individuals for whom the "NSAID" only provides insufficient analgesia. The selective COX-2 enzyme inhibitors show promise in effectiveness from long-term use and clinical use, and may possibly useful in the treatment of chronic oro-facial pain. Because of the lack of proven effectiveness and the risk of severe gastrointestinal and renal damage with repeated dosage, the repeated use of "NSAIDs" doses for chronic oro-facial pain have to be reconsidered [9].

The following categories are used to classify and categorize the vast range of NSAIDs:

- The para-amino phenol derivatives (phenacetin, paracetamol).
- The salicylates and their eongenerts
- Phenyl acetic acid derivatives (diclofenac)
- The pyrazolone derivatives (phenyl butazone and related compounds)

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- Propionic acid derivatives (ibuprofen, fenoprofen, naproxen, ketoprofen and pirofen).
- Fenamates (flufenamic acid, mefenamic acid and enfenamic acid)
- Oxicams (piroxicam).
- Indole acetic acid derivatives (indomethacin, sulindac and tolmetin) [10].
- The compounds in groups (iv) to (vii) are called newer NSAIDs.

EARLY EXPLANATIONS FOR THE MECHANISM OF NSAIDs

Non-steroidal Anti-inflammatory medications (NSAIDs) are an alternative source of medicines used to treat the symptoms of rheumatic diseases. Since the early 1970s, it has been extensively recognized that the major mechanism of action of NSAIDs is suppression of cyclo-oxygenase (COX), a crucial enzyme in prostaglandin production, which is also responsible for the main adverse outcome of gastric mucosal injury. Prostaglandins are a class of hormone like lipid molecules that have a wide range of functional actions, including inflammatory control, pain sensitivity, and platelet aggregation, to name a few. NSAIDs, on the other hand, appear to offer additional anti-inflammatory effects, according to a growing body of data. NSAIDs' ability to penetrate biological membranes, which has been studied in vitro using membrane mimetic models, molecular dynamic and cell culture simulation systems where they disrupt normal signaling events and modify important cellular processes like cell adhesion, these are some effects which appear after consuming this drug.

NSAIDs have been shown to interfere with pathways of cell adhesion, which is essential for the inflammatory response, and also affecting L-selectin shedding on neutrophils or with leukocyte adhesion pathways and necessary for the inflammatory response, like cleavage of epithelial cell adhesion protein molecule on tumor cells. This anti-adhesive action of NSAIDs drug has been demonstrated to impact platelet adherence and it has been hypothesized that these chemicals might control coagulation, hemostasis, and thrombus formation independently of platelet release by pro-inflammatory mediators. In leukocytes, NSAIDs such as mefenamic, meclofenamic, and flufenamic acids, as well as aceclofenac and diclofenac have been shown to cause L-selectin down regulation, whereas oxicams and the phenylbutazone, meloxicam and piroxicam, have been shown to modulate the function of the integrin CD11b on neutrophils. Recent research has found that the action of anti-L-selectin in NSAIDs generates a substantial anti-inflammatory response in vivo, & this anti-inflammatory response is linked to the NADPH-oxidase-dependent production of super-oxide anion at the plasma membrane in human neutrophils in vitro [11].

Before 1971, few had known about the mechanism of action of "Non-Steroidal Anti-inflammatory drugs", that they had an Anti-inflammatory impact that was quantitatively and qualitatively distinct from that of the more potent anti-inflammatory glucocorticosteroids. Many of NSAIDs' biochemical effects had been established, but hypotheses based on these effects had been abandoned. The fact that salicylates may block numerous proteases was perhaps the most logical theory at the time. Several models of inflammation showed increased extracellular proteolytic activity, which was responsible for the tissue damage seen in chronic illnesses like rheumatoid arthritis. But it wasn't only biochemists who were curious about how these medications worked; pharmacologists were as well, and no one contributed more to the literature in this field throughout the 1960s than British pharmacologist Harry Collier. Because of its potential to inhibit the physiologic defence mechanisms of fever, pain and inflammation functioning normally, aspirin has been dubbed an "anti-defensive" medication by Collier. Collier hypothesised that the medicines worked by "inhibiting" various underlying cellular process that participates to varying degrees, in various reactions and is treated by various endogenous chemicals. The anodyne effect of "Non-steroidal Anti-inflammatory medicines" (NSAIDs) [12] has traditionally been attributed to their suppression of prostaglandin production enzymes. However, it is apparent that NSAIDs drug have numbing effects via several central and peripheral mechanism prostaglandin production addition by peripheral suppression. The enzyme cyclooxygenase is now recognised to have two structurally different variants, which is COX-1 and COX-2. In normal cells COX-1 is a naturally found enzyme, on the other hand in inflammatory cells enzyme COX-2 is increased. The most probable mechanism of action of NSAID-mediated analgesia is inhibition of COX-2 activity where-as the ratio of COX-1 & COX-2 inhibition by NSAIDs affects the probability of side effects. Furthermore, few NSAIDs chunk the pathway of lipoxygenase, which may lead to the formation of algogenic metabolites. NSAIDs' interference with G-protein-mediated signal transduction, might be the analgesic mechanism foundation independent to inhibition of prostaglandin production.

There's a rising body of proof that NSAIDs work in tandem with the peripheral mechanism because they have a central mechanism of action. In the CNS this impact might be caused by interfering with prostaglandin production. Inhibition of serotonin release or endogenous opioid peptides might also be involved in the central action (5-hydroxytryptamine). Stimulation of N-methyl-D-aspartate receptors or inhibition of excitatory amino acid has also been postulated as a mechanism [13].

Salicylates are included in all of these therapeutic treatments. Celsus defined the four basic symptoms of inflammation (calor, rubor, tumour and dolor or pain, redness, heat and swelling) around A.D. 30 and employed willow leaf extracts to treat them. The usage of plants containing-salicylate was further explored during the Roman era of Galen and Pliny the Elder Dioscorides, and the bark of Willow was suggested for mild to moderate discomfort. Salicylate-containing plants were also used medically in China and other regions of Asia. Early people South Africa and North America were aware of the medicinal properties of Spirea species and Salix. Salicylates were discovered to have other uses during the Middle Ages, including plasters to cure wounds and a variety of other internal and external

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applications, such as the treatment of monthly discomfort of dysentery and pain. On the other hand, willows were required for basket manufacturing, therefore women herbalists of the time resorted and related to other plants. For example, their herb gardens they planted meadowsweet (Spireaulmaria) and used the blossoms to make decoctions. Salicylic acid was firstly produced chemical 1859 in Germany and its widespread availability show to its use as an antibacterial, antipyretic, and rheumatism therapy. Felix Hoffman's father, a young Bayer chemist, pushed his son to develop a more palatable version of salicylate to cure his acute rheumatism. Felix prepared acetylsalicylate, also known as Aspirin, and gave it to his father to test. Dr. Heinrich Dreser, Bayer's research Director, recognized that he had a significant new medicine on his hands and presented it in 1899, while also authoring a paper stating that Aspirin was a simple means of providing the active ingredient Salicylate to the body. This is a subject of contention although most data currently indicate that aspirin is effective on its own. Antipyretic, anti-inflammatory, and analgesic properties were identified as the major therapeutic activities of Aspirin and sodium salicylate itself by the early 1900s. With the passage of time, numerous additional medicines that shared some or all of these effects were identified. Acetaminophen, Phenylbutazone, Antipyrine, Phenacetin (paracetamol) and more recently, the indomethacin, naproxen and Fenamates are among these medications. Because of their comparable therapeutic effects, these medicines were grouped together and dubbed aspirin-like medications. These medicines were dubbed "nonsteroidal anti-inflammatory drugs" because they were easily distinguished from glucocorticoids "the other major group of treatments used to treat inflammation". These medicines all have the same therapeutic effects, regardless of their chemical structures. They relieve inflammation's swelling, redness, and discomfort, as well as a general temperature and a headache. Furthermore, they have a number of comparable adverse effects, to a greater or lesser extent. They can induce stomach distress depending on the dose, delay the birth process in large doses, and harm the kidney in overdose. The antithrombotic effect is a very intriguing side effect, which is now recognized as a therapeutic activity. When a chemically varied collection of medicines not just has the same therapeutic features (which in and of themselves have nothing in common), but also the same adverse effects, it's safe to assume that their activities are based on single biochemical intervention. Biochemist and Pharmacologist had been looking for such a general mechanism of action for years but had yet to discover a scientific and valid explanation that was widely accepted [14].

DLLME (Dispersive liquid-liquid Micro-Extraction)

The advancement of accurate, sensitive, quick, secure, and automated analytical techniques of monitoring environmental has received a lot of attention in recent decades. Several analytes must be determined at trace levels in such applications. Despite extensive technical advancements, mainly analytical equipment does not allow direct assessment of the real ambient matrix composition. Furthermore, only few of sensitive testing methods enough to identify trace contaminants directly are present. In many situations, a preparatory step of analyte isolation, enrichment is necessary to analysis. As a result, sample preparation is still an important part of the total study. The primary objective is to purify the sample in sample preparation, raise the analyte concentration and perhaps alter the sample to meet the test equipment's criteria. In these conditions, the general rule holds true: the fewer the preliminary procedures, the more trustworthy and error-free the analysis' outcome. Furthermore, contemporary analytical chemistry has the problem of developing techniques that limit or even eliminate the usage of hazardous chemicals. As a result, there is a need to create extraction techniques that satisfy the standards of green chemistry. Dispersive Liquid Liquid Micro Extraction (DLLME) has recently been only of the approaches that has gotten a lot of attention. Rezaee et al. proposed this method from aqueous matrices in 2006 for the pre-concentration of organic analytes. The current aspect of DLLME consist modern analytical chemistry and is used in a variety of fields, along with biochemistry and chemistry, molecular biology and genetics, environmental science, chemical engineering, medicine, engineering, biological sciences and agricultural, toxicology, pharmaceutics and pharmacology, social sciences, and many others [15]. DLLME is easy to use, cheap, and ecologically safe and it can enrich a broad range of donor and acceptor phases.

Principle of DLLME

A solvent extraction is combined with a dispersive solvent in a standard DLLME technique, and into the aqueous sample the solvent combination is quickly injected, as well as the dispersive solvent mixture extraction is injected quickly, resulting the micro-droplets of extraction solvent made murky solution distributed throughout the aqueous sample. The development of an emulsion hazy solution enables the partitioning of analytes into the extraction phase in real time from the aqueous sample (a major benefit of this technique). This is due to the many micro-droplets creating a greater surface area compared to LLE. The centrifuged hazy solution breaking the emulsion into a two-phase system that allows the extraction solvent to be easily recovered for analysis. DLLME has a number of criteria that must be met in order for it to be useful. The extracted solvent must be aqueous with the dispersive solvent and insoluble in water, and these solvents also have a high affinity for the target analytes.

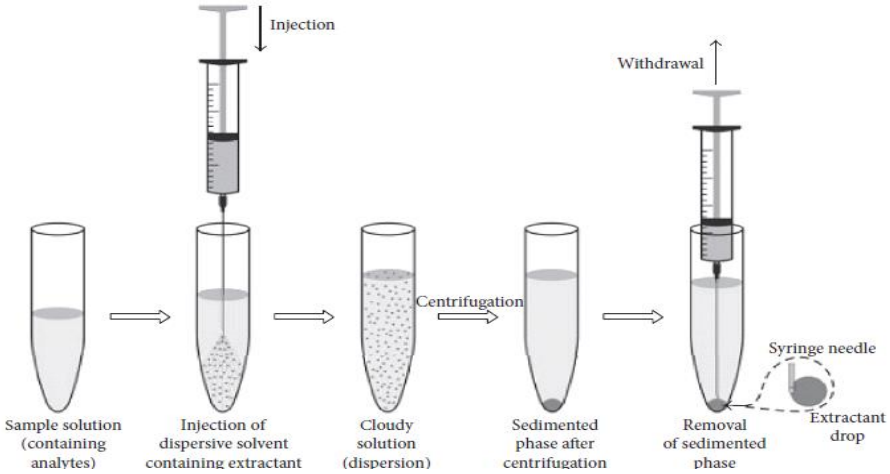

Figure 1: Schematic of DLLME technique [16]

The solvent extraction in what would be referred to as "conventional DLLME" is generally denser than water, forming a "sedimented phase" enabling simple collection with a small syringe needle and following centrifugation. The dispersive solvent, on the other hand, must be soluble with the extraction solvent as well as the aqueous sample. The solvent extraction should ideally be compatible with the analytical technique being employed; otherwise, the solvent extraction must be evaporated and reconstituted in a suitable solvent. Alternatively, the analytes might be extracted into a suitable solvent via in-syringe back extraction. To achieve quantitative extraction of analytes, dispersive solvent, the amount and type of extraction, ionic strength, aqueous phase pH, extraction duration and centrifugation time must all be optimized prior to analysis. A high enrichment factor and high Relative Recovery (RR) characterize an efficient DLLME technique. The enrichment factor is computed using C_0 as the analyte concentration in the original sample and C_{sed} as the analyte (con.) in the sedimented extraction solvent, as given below:

Enrichment factor = C_{sed}/C_0

The RR is computed, where C_{found} represents the total quantity of analyte found following standard addition, C_{add} represents the amount of standard spiked into the original sample and C_{real} represents the original analyte concentration in the sample [17, 18].

Relative recovery = $C_{found} - C_{real}/ C_{add}$ *100

Application of DLLME

DLLME Parabens found in beverages

Parabens are p-hydroxybenzoic acid esters that are employed as keepers in a wide range of cosmetic, food, and medicinal goods. Methylparaben, ethyl paraben, propylparaben, isopropyl paraben, butylparaben, isobutyl paraben, and benzyl paraben are all members of this category [19]. Since Routledge et al. initially revealed parabens' poor oestrogenicity [20], toxicological research and debate on parabens and their metabolic products' that may cause possible human health concerns to have gotten a lot of attention. Human breast, human serum and human urine all contained intact paraben esters. Several parabens have also been reported to bind to the human androgen receptor. Because it was discovered that parabens in the human body had androgen antagonist characteristics, anti-androgenic qualities and male reproductive problems have received increasing attention in recent years. Furthermore, recent studies have shown that propylparaben and butylparaben are carcinogenic, causing DNA damage and chromosomal abnormalities as **well** as sister-chromatid exchanges. For propylparaben from food the removal of the Acceptable Daily Intake (ADI) of 0 –10 mg per kg body weight has been advised due to reproductive and Endocerine harm. As a result, developing sensitive, easy, and quick techniques for detecting trace parabens in food items is important. Sample preparation is always crucial when analyzing parabens in other types of samples or food. Supercritical fluid extraction has been developed for the separation of parabens from cosmetic matrices [21]. SFE is a typical environment friendly approach with a high extraction efficiency, however it is not an easy way to use in the traditional sense. For the study of parabens in food items, "Solid Phase Extraction" is generally employed in sample preparation. To elute the adsorbed analytes, however, significant quantities of organic reagent were required. It was recently reported that Sonication-Assisted Extraction (SAE) was used to analyze parabens in solid environmental samples. Analyze parabens Solid Phase Micro-Extraction (SPME) has been also used because of its mobility and simplicity. However, the extraction fiber is very costly, brittle, and has a short lifespan. The technique of "Stir Bar Sorptive Extraction" (SBSE) was used to determine parabens in drinks; the process is simple to use, but the extraction takes time. The detection of parabens in cosmetic and water goods was reported using liquid phase micro-extraction, such as "Single-Drop Micro-Extraction". It has the advantages of simplicity, cost-effectiveness, and low solvent use. Assadi et al. proposed a new LPME technique called "Dispersive Liquid–Liquid Micro-Extraction" (DLLME) for the detection of "Polycyclic Aromatic Hydrocarbons" (PAHs) in water samples in 2006. To make an emulsified solution, disperser solvent and an organic extractant are rapidly injected into an aqueous sample. Extraction takes only a few seconds since the extractant is well distributed in the aqueous phase. Chromatography and

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spectrometry are used to identify the sedimented phase after centrifugation. This approach has been widely used to analyze numerous target chemicals in food samples [22]. This method uses a ternary component solvent solution and has the advantages of being simple, quick, and affordable. Parabens can be detected using a variety of methods, including "Gas Chromatography–Mass Spectrometry" (GC–MS) [23], liquid chromatography (LC), "LC–tandem mass spectrometry" (LC–MS–MS), "LC–time of-flight mass spectrometry" (LC–TOFMS), "Ion Mobility Spectrometry"(IMS), Micro Emulsion Electro Kinetic Chromatography (MEEKC), capillary electro chromatography (C For the measurement of four parabens (ethyl paraben, propylparaben, methylparaben, and butylparaben) in beverage samples, a technique based on (DLLME) combined with (GC–MS) was devised. The benefits of DLLME are that they are easy, quick, environmentally friendly, and cost-effective. DLLME combined with (GC–MS) is unquestionably an excellent option for paraben detection in beverage samples with simple matrix, like drinking water. However, DLLME also has disadvantages, such as a significant matrix effect, which necessitates sample dilution for samples with complex matrixes to minimize matrix-induced unfavorable effects. The analytical sensitivity of the technique is decreased in this scenario. As a result, it is strongly suggested that DLLME be combined with other successful separation methods, like solid phase extraction, to produce a very sensitive approach for the correct detection of detectable parabens in most complicated beverage samples [24].

Analysis of (PAHs) in fruit juice sample and water

The measurement and extraction of (PAHs) in water and fruit juice samples, a simple, fast, and effective technique known as DLLME coupled with liquid chromatography–fluorescence detection has been developed.

PAHs (Polycyclic Aromatic Hydrocarbons) are common environmental pollutants that can come from a number of sources, oil spills, including fossil fuel burning and various industrial operations [25]. Because of the teratogenic, mutagenic, and carcinogenic certain properties of these chemicals, environmental chemistry has given more attention to them [26]. As a result, they have been included to the priority pollution lists of the EU (European Union) and the Environmental Protection Agency (EPA). PAH determination and identification is a critical analytical challenge because of its hazardous properties. The primary challenge in monitoring PAHs is their low concentration and the environmental matrice complexity [27, 28]. To obtain the requisite selectivity and sensitivity, separation and pre-concentration are required. The most popular techniques for extracting PAHs from environmental samples are "Solid–Phase Extraction" (SPE) and "Liquid–Liquid Extraction" (LLE). LLE is a highly helpful method, laborious and involves a lot of hazardous and combustible solvents a with time consuming. Although SPE is a widely used method, it does have certain drawbacks, such as particle obstruction and a sluggish sample processing rate. Solid-Phase Micro-Extraction (SPME) has been developed to extract (PAHs) from aqueous samples. Solid phase micro-extraction is a solvent-free method that involves pre-concentration and simultaneous extraction of analytes from aqueous samples, as opposed to LLE. SPME, on the other hand, is costly, its fibre is brittle and has a short lifespan, and sample carry-over might be an issue. Headspace solvent micro-extraction, a kind of "liquid-phase micro-extraction" (LPME), was developed in recent years to detect "PAHs" in water samples. HSME is a sample-preparation process that is highly easy, low-cost, and nearly solvent-free when compared to traditional techniques. The drawbacks of this approach are: rapid stirring tends to create air bubbles; extraction technique is time-consuming and equilibrium in most situations cannot be achieved after a long time. Another approach for extracting PAHs from aqueous samples has been developed: cloud point extraction (CPE) [29]. Despite the many advantages of CPE, the primary drawback is that the extraction step is incompatible with various instrumental analyses such as GC and LC [30]. Assadi has invented a unique microextraction technique known as "DLLME". The DLLME technique has the following advantages: ease of use, speed, cheap cost, enrichment factor and high recovery. In liquid samples, DLLME has been used to extract triazine herbicides, chlorophenols, chlorobenzenes, antioxidants, volatile phenols, organo-phosphorus, lead, flame retardants and phthalate esters [31]. PAHs have previously been identified utilising chromatographic methods such as GC, LC, and supercritical fluid chromatography (SFC). Because of its sensitivity, LC-FLD is the most used technique. The goal of this investigation was to see if it was possible to extract and identify "PAHs" in fruit juice samples and water by using the LC-FLD and DLLME. The impacts of different experimental parameters including extraction solvent type and volume, dispersive solvent type and volume, effect of salt and extraction time were optimized. DLLME-LC-FLD was provided in the study for PAH determination and concentration in water and fruit juice samples. In comparison to previous approaches, the methodology produced greater repeatability, good recovery in a shorter period and a larger enrichment factor. Furthermore, the use of hazardous organic solvents was reduced while maintaining the method's sensitivity. This method's concept is simple, quick, and low-cost, and it may be effectively used to pre-concentrate and determine PAHs in fruit juice samples and water [32].

II. MATERIAL AND METHODS

Requirements:

- D–78532 Tuttlingen was utilized with 15 mL calibrated centrifuge tubes.
- The pH of the solution was checked using a Corning M-120 pH–meter “Halstead, Essex, England CO9 2DX“.
- Quartz cells, 1.5 mL to speed up the phase separation process, A Hettich centrifuge “EBA 20 model/ Andreas Hettich GmbH & Co. KG, Föhrenstr”.
- A UV–visible Recording Spectrophotometer “UV–160 model” was used to take spectral measurements with a 1–cm path length.

Reagents:

- A 500 g/ml Piroxicam stock solution was made by dissolving suitable quantities of pure PX in ethanol.
- It was stored in a refrigerator at around 4°C, away from light. Working standard solutions were made by diluting the stock standard solution appropriately.
- Sodium acetate trihydrate (Riedel–De Han) was used to make the Acetic-acid/acetate buffer 1 mol/L, with 3.0 pH.
- Merck (Darmstadt, Germany) provided all solvents, which included dichloromethane, carbon tetrachloride, chloroform, acetone, acetonitrile, methanol, and ethanol. The Glucuronidase, Type HP–2 from Helix pomatia (116,400 units/ml) provided by "Sigma-Aldrich".
- Acetic acid.
- Already prepared twenty percent (w/v) NaCl (Merck) solution.
- All of the other reagents were analytical grade or above.
- Throughout the work, "Milli–Q Advantage A 10 system, Millipore" Ultrapure water was used.

Pharmaceutical preparation procedure

- The comprising of 10 capsules containing 10 mg PX (Pursina Pharm. Co., Tehran, Iran) were properly measured and finely pulverized.
- This powdered sample containing 10mg of PX was weighed and put in a 15ml glass tube containing 10ml methanol.
- After that, it was rapidly agitated for 30 seconds in a vortex mixer.
- The solution was transferred to a volumetric flask with a 50ml capacity after filtration.
- The residue was washed with sufficient methanol and the solution was ultimately brought up to the required concentration with water. As a result, a 200g/ml PX solution was produced.
- This solution was quantitatively diluted to achieve concentrations within the working standard solution's range, and the PX content was determined using the technique outlined above.

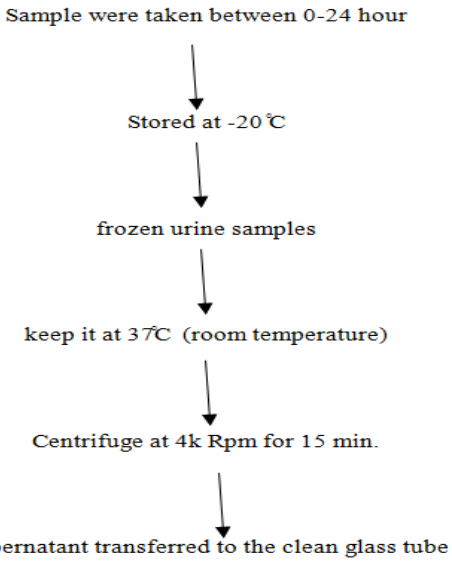
Urine sample

Collection of Urine sample

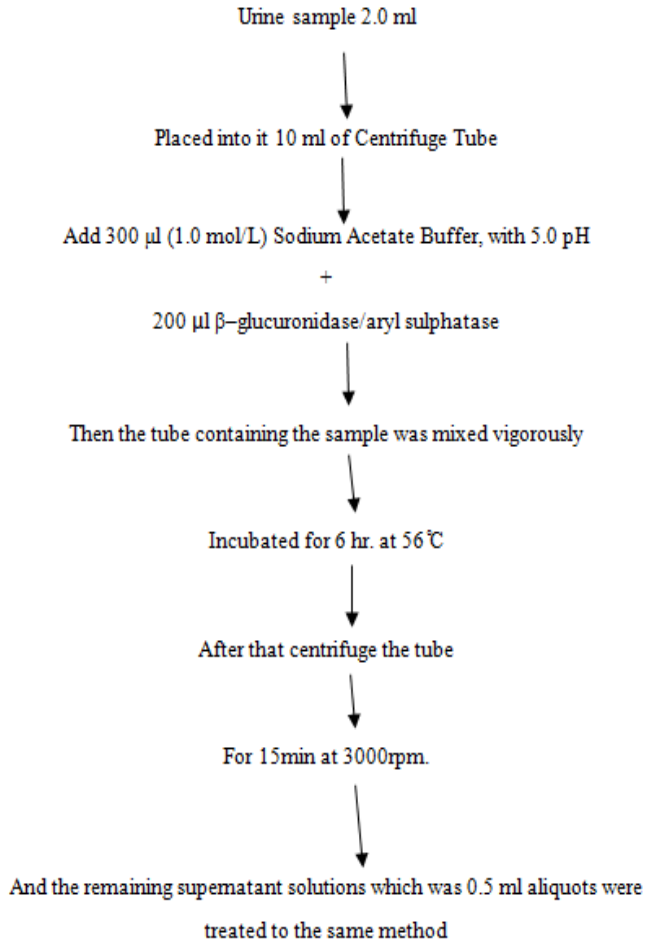
A healthy male volunteer provided urine sample after taking a single oral dosage of 10 mg PX pill.

Procedure

The samples were taken between 0–24 hours after delivery and stored at –20°C until analysis. The supernatants were transferred to clean glass tubes after the frozen urine samples were thawed at room temperature and centrifuged for 15 minutes at 4000 rpm. Enzymatic deconjugation was carried out with minor adjustments. 2.0 ml of urine was placed into a 10 ml centrifuge tube, along with 300 µl sodium acetate buffer (1.0 mol/L, pH 5.0) and 200 µl of "β–glucuronidase/aryl sulphatase (116400–1015 IU/ml). The tube was thoroughly stirred before being incubated at 56°C for 6 hours. The tube was then centrifuged for 15 minutes at 3000 rpm, and 0.5 ml aliquots of the supernatant solutions were treated to the method described above.



Enzymatic Deconjugation



Sample collection, preparation and storage

Twenty individuals were asked to provide urine samples (10 male). Anonymized samples were, frozen at 86 1C 210 F, and kept in our laboratory until analysis. To

participate in the study, all volunteers completed an informed consent form. A healthy participant in our lab gave simple pee. These samples were maintained frozen at -20°C before extraction according to *Shamsipur* and *Fattahi's* technique for deposition of undesired chemicals at the bottom of the conical test tube. The urine and plasma samples were thawed at room temperature before being centrifuged at 5000 rpm for 10 minutes. The supernatants were then filtered through a 0.45 mm pore sized filter and decanted into a clean glass tube. The extraction method used 500 L of filtration products diluted to 10.0 ml, as it was described in section 3.7.

Sample treatment

Treatment with enzymes Each sample was handled in two different ways to determine the free and total levels of EDCs (Endocerine Disrupting Chemicals) in urine. One sample was treated with glucuronidase/sulfatase whereas the other was handled without enzymes. An aliquot of urine (5.0 ml) was added to a centrifuge glass tube and mixed with 50 ml of ($EP^{-13}C_6$ and BPA-d16) standard solution for studies without enzymatic treatment (5 mg L^{-1} of $EP^{-13}C_6$, 2 mg L^{-1} of BPA-d_{16}). 5.0 ml of sample was mixed with 50 ml of ($EP^{-13}C_6$ and BPA-d_{16}) standard solution and 100 ml of enzyme solution (-glucuronidase/sulfatase) to determine the total (free-conjugated) concentration of the tested EDCs. To verify the extent of the deconjugation, 125 µl of 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate/13C4-4-methylumbelliferone standard combination (4 µg mL1) was added. The material was mixed and then incubated for 24 hours at 37°C. Free 4-methylumbelliferone was obtained by deconjugating 4-methylumbelliferyl sulphate and 4-methylumbelliferyl glucuronide. The 4-methylumbelliferone/$^{13}C_4$-4-methylumbelliferone peak area ratio was measured to ensure that the enzyme was working properly. In every case, deconjugation efficiencies were close to 100%.

DLLME procedure

- An aliquot of human urine (5 mL) was diluted to 10.0 ml with a 10% sodium chloride aqueous solution (w/v) prior to the DLLME process.
- With 0.1 M HCl, the pH was adjusted to 2.0. In a 15 ml screw-cap glass test tube, this solution was put.
- Next, a syringe was used to inject 0.5 ml of disperser solvent (acetone) and 750 ml of extraction solvent (TCM) into the aqueous sample.
- The mixture was gently mixed for 10 seconds before being centrifuged at 4000 rpm for 20 minutes (2600xg).
- Using a 1.0 ml micropipette, all the sedimented phase volume was transferred to a clean glass vial.
- A nitrogen stream was used to evaporate the organic phase. The residue was dissolved in 100 ml of an 80:20 (v/v) combination of ethyl acetate, BSTFA, and 1% TMCS. The solution was heated to 60 1C for 20 minutes after mixing. The extract was ready to be examined at this stage.
- For blank Urine In a 15 ml conical glass test tube equipped with a plastic cover, a 10.0 ml aliquot of water sample containing 100 ng/mL of methadone was inserted.
- Using a 5.0 ml syringe, a cloudy solution was created by rapidly injecting 2.5 ml methanol (as disperser solvent) and 250 mL chloroform (as extracting solvent) into a sample solution.
- The hazy solution was centrifuged at 3000 rpm for 3 minutes. The sedimented phase was entirely transferred into another test tube after centrifugation and evaporated to dryness under a moderate stream of nitrogen.
- The residue was diluted in 50 L of HPLC grade methanol and injected using a 20 L sample loop into the HPLC. All of the tests were repeated three times, with the average of the findings given.

Effect of disperser solvent type and extraction

For effective extraction, the kind of extraction solvent utilized in "DLLME" is critical. Comparison to the water the solvent must have a high density. It should also be better capable of extracting desired chemicals and have a low water solubility. As a result, the extraction solvents chloroform, dichloromethane, and carbon tetrachloride were investigated. The choice of a dispersive solvent, on the other hand, is restricted to solvents that are soluble with both extraction solvents and water, like ethanol, methanol, acetonitrile and acetone. All combinations of extraction solvents (50µl) carbon tetrachloride and ethanol, chloroform, dichloromethane and methanol, acetonitrile and acetone as (dispersive solvents) (500µl) were investigated in this study. When DCM (Dichloromethane) was employed as the extraction solvent in this technique, no hazy condition was noticed, and no extract of sediment droplet was discovered on the tube's bottom following centrifugation. With all four dispersive solvents, "A 2–phase system was produced with carbon-tetrachloride and chloroform, however with Carbon- Tetrachloride, low signals were detected, in this solution along with the analyte's limited extractability. In the case of methanol with chloroform, were found more stable 2–phase systems, as well as greater signals. In subsequent experiments, CH_3OH and $CHCL_3$ were chosen as disperser solvents and extraction, respectively.

Effect of disperser solvent volume and extraction

The impact of the solvent extraction volume on the analytical signals was studied. Experiments were carried out using varied amounts of chloroform (ranging from 30 to 90µl) as the solvent extraction, with the volume of methyl alcohol fixed at 500 µl. Observation shows that raising the volume of the chloroform to 70µl increased the absorbance, which then stayed rather constant when the volume was raised between 70 & 90 µl. In other tests, 70 µl of chloroform were employed. Solutions having varying quantities of methyl alcohol (in the range of 400 to 800 µl) including 70 µl of $CHCL_3$ were exposed to the identical DLLME technique to investigate the influence of the Disperser Solvent Volume.

Effect of salt addition

Various researches were carried out to examine the impact of ionic strength on DLLME extraction efficiency by adding varying amounts of sodium chloride (0–15%, w/v) while keeping other experimental conditions constant. The

absorbance was found to be enhanced by increasing the quantity of sodium chloride from 0-8%, and then progressively dropped by raising the salt concentration further. Based on these findings, the optimum salt content in the DLLME process was determined to be 8% (w/v) NaCl.

III. RESULTS AND DISCUSSION

According to a review of the literature, the most widely used methods for determining PX in pharmaceutical formulations are spectrophotometric and spectrofluorimetric approaches. After DLLME, spectrophotometric detection was used to monitor the extracted PX since it did not exhibit any substantial and sensitive fluorescence in the investigated circumstances.

The spectrophotometric methods used to determine PX are generally based on the oxidation of PX with different agents, like ceric ammonium sulfate, potassium iodate, ferric salts and solid–phase spectrophotometry, indirect spectrophotometric determination of the reaction products or chelating with ferric ion. In the present work, quantitative determination of piroxicam in human urine was investigated.

Figure 1 illustrates the target analyte's absorption spectra following dispersive liquid-liquid micro-extraction, which display an absorption band peaking at 355.3 nm. The impact of various variables such as pH and amount of extraction and dispersive solvents addition of salt and so on evaluated using the 1variable only one-time approach to achieve better extraction efficiency.

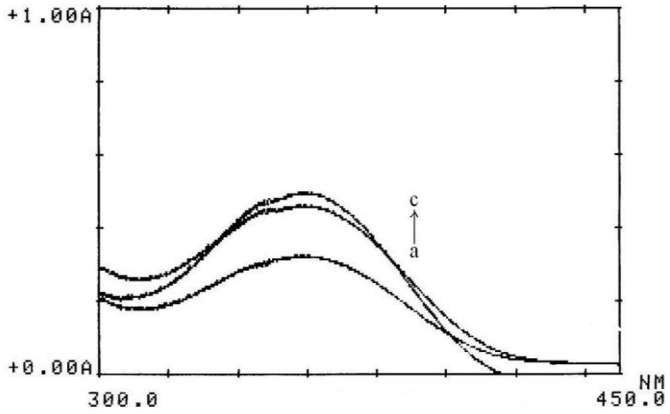


Figure 10 After DLLME absorption spectra of PX: a) 2.0 µg/mL Standard solution of PX (b) after addition of NaCl sample "a" (8%w/v), (c) 0.5 mL urine sample spiked with PX (2.0 µg/mL); other circumstances: acetate buffer 0.5 mL of 1.0 mol/L at pH 3.0; extraction with 500 µL of methanol containing 50 µL of chloroform.

Effect of pH

The pH of the sample solution was widely recognized to be one of the key variables influencing the states of analytes (as neutral forms or ions). The impact of pH on the absorption signal of the target analyte is shown in Figure 11. As can be observed, the signal strength of PX increased as pH increased from 2.0 to 0.3, then dropped as pH increased from 0.3 to 12.0. This may be described by the following factors: by extraction solvent neutral analytes are simpler to extract than ion analytes because of their high affinity. PX has pKa values of 1.81 and 5.12. When these values are considered, the enolic groups and pyridyl are mainly prorogued (LH2+, positive global charge) below pH 1.8, and deprotonated (L–, negative global charge) above 5.1pH. A tautomeric stability between the zwitterions (LH) and neutral molecule (LH0) and is established in the pH range 1.8–5.1 As a result, to analyte the neutral form pH solution in range of 1.8–5.1 in aqueous solution, which has a high tendency to be extracted into the extraction solvent. As a result, acetate buffer was used to keep the pH of the samples at 3.0 for further research.

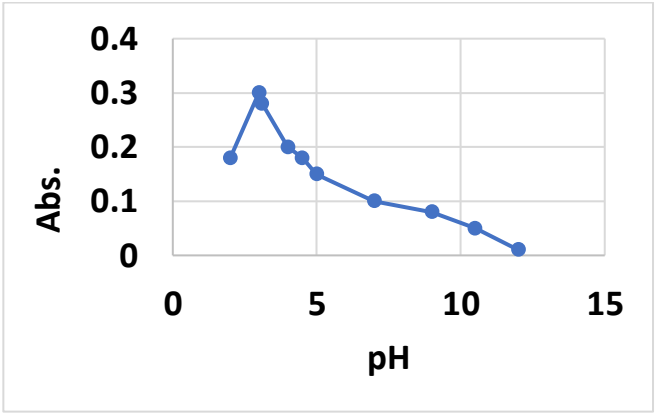


Figure 11. Effect of pH on the analytical responses 1.2 µg/mL PX; other situations: extraction with 500 µL of CH_3OH containing 50 µL of $CHCL_3$; 2.0 mL of 20% NaCl; acetate buffer 0.5 mL of 1.0 mol/L at pH 3.0.

Effect of extraction and other dispersive solvent

In the present study, we are using the all combinations of dispersive and extraction solvent such as chloroform ($CHCL_3$), dichloromethane CH_2CL_2 (DCM) and as extraction solvents (50 µL) of carbon tetra-chloride (CCL_4) and CH_3 OH (methanol), C_2H_5OH (ethanol) and as dispersive solvents (500 µL) of C_3H_6O(acetone) and acetonitrile (C_2H_3N) were tested.

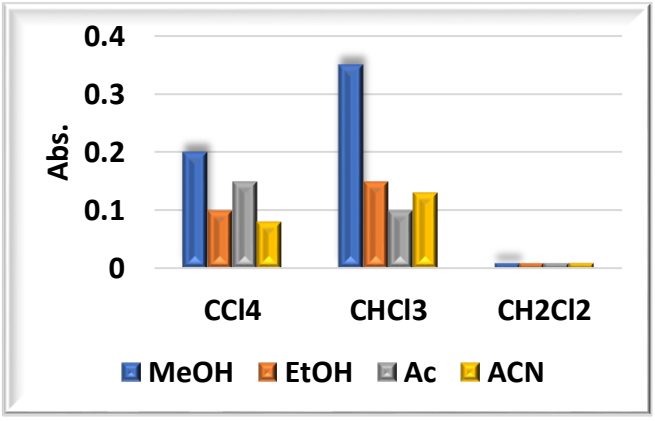


Figure 12 Effect of the form of dispersant solvents and extraction on the analytical responses, MeOH: methanol, EtOH: ACN: Acetonitrile, Ac: acetone, 1.8 µg/mL of PX; Figure 11 have been shown additional conditions.

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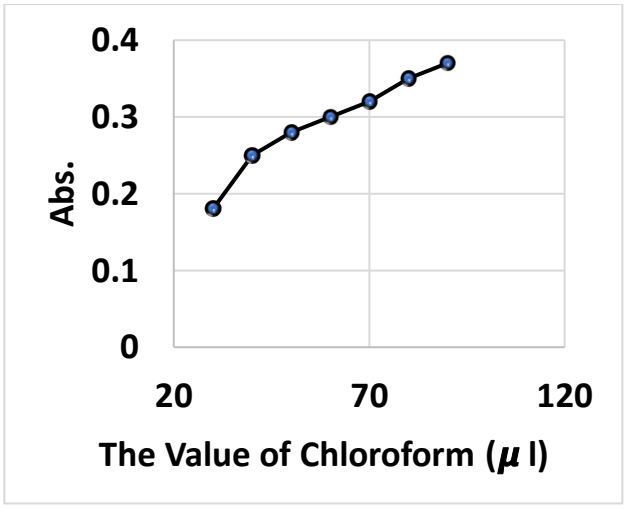

Figure 13. Effect of the (CHCl3) extraction solvent volume on the analytical signals, 1.8 µg/mL PX; additional conditions are represented in Figure 11.

As the amount of chloroform increased, the absorbance of piroxicam increased.

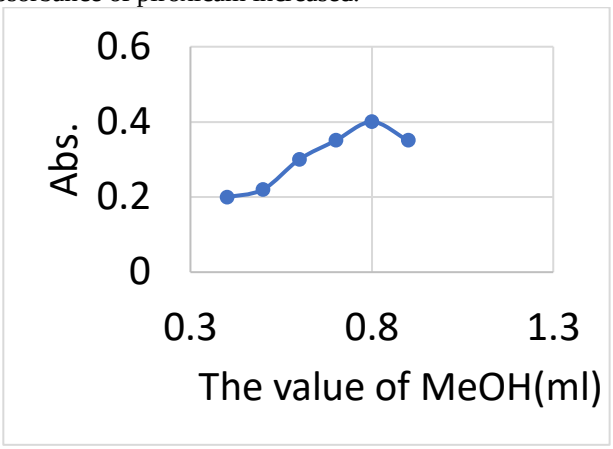

Figure 14. Effect of the (MeOH) dispersant solvent volume on the analytical signals, 1.8 µg/mL PX; additional conditions are represented in Figure 11.

The absorbance of piroxicam increased till 0.8 ml of methanol and then it decreased with further rise in the amount of solvent.

Method validation

Calibration graphs were produced by dispersive liquid-liquid microextraction containing known amounts of PX in the 5mL of standard solutions at the experimental situations described in the technique. The absorbance of the remaining phase (100 µl) was measured after it was diluted to 0.7 mL using water: ethanol (1:1 v/v) and diluted to 0.7mL. As a result, experimental and theoretical preconcentration factors of 7 & 50 were obtained, respectively. Over the range of concentration 0.2-4.8 µg/mL, the calibration curve for PX detection was linear.

The corresponding regression equation was:
Absorbance intensity = 0.1711C – 0.0154 (r = 0.9965)
Where, C is the PX concentration in micrograms per millilitre, and r is the correlation coefficient.

The LOD (limit of detection) was calculated as $3_{\sigma}s/R$, where $_{\sigma}s$ is the blank's "standard deviation" and R is the calibration curve's slope and was determined to be 0.058 µg/mL. This Limit of detection was small enough to be useful for determining PX levels in various bodily fluids. Furthermore, RSD, the linear range and LOD achieved were similar to those reported in other extractive techniques.

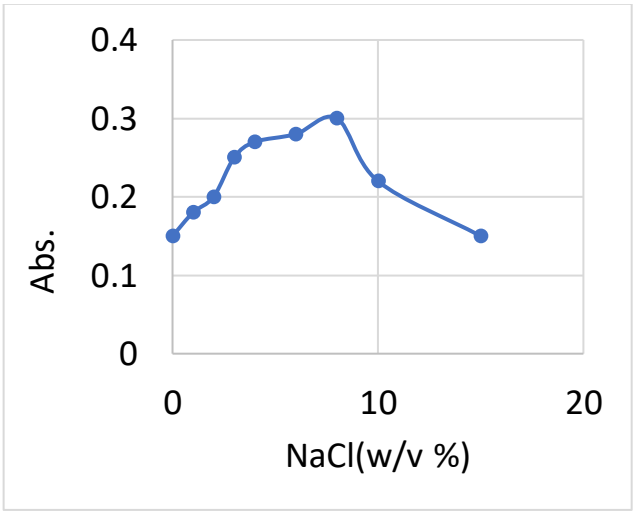

Figure 15. Salt amount effect on the analytical signals, 1.8 µg/mL of PX; additional conditions are represented in Figure 11.

Figure 6 shows that analytical signals obtained in the existence of urine are greater than those obtained in the absence of urine. Due to the salting out effect, this may be linked to the chemical makeup of the urine and the presence of NaCl, which can lead to greater PX extraction efficiency. The absorbance of piroxicam increased with rise in concentration of salt but up to a certain limit. Beyond that limit, the absorbance decreased. The addition of NaCl to normal PX solutions, approx. concentrations of 7.5% (w/v), was shown to improve extraction efficiency owing to the salting out effect, and therefore eliminate the interference effect.

Application to the human urine:

The recovery tests were conducted on urine sample aliquots of 0.5 mL of mixed with various doses of PX. Figure 7 shows typical spectra of a normal PX solution, a urine sample and blank urine obtained from the volunteer following α-glucuronidase therapy. At the analytical absorption wavelength, no extra picks owing to interferences were detected. As a result, the coincidence of absorption spectra and acceptable recoveries showed that the proposed technique did not meet any major matrix impact.

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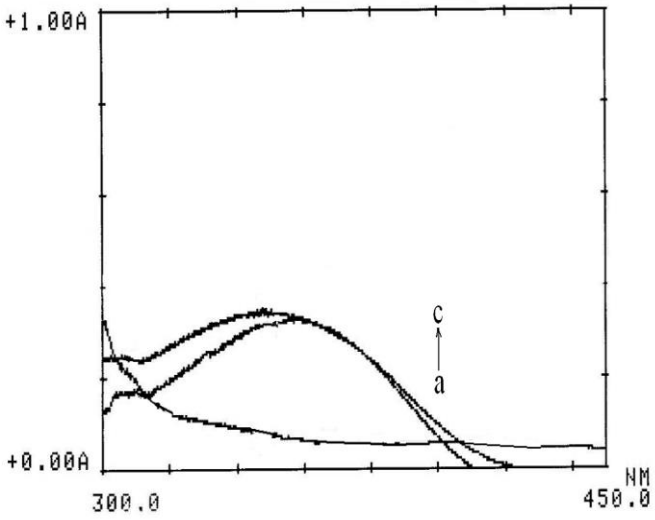

Figure 16: Absorption spectra of (a) urine blank, (b) Standard solution (1.8 µg/mL) of PX (c) Treatment of Oral administration of 10 mg of PX and β–glucuronidase than collect urine.

The suggested technique was used to determine piroxicam in human urine with great success. Urine was collected after twenty-four hours after a single oral dosage of piroxicam which is 10 mg was given to one participant. It's worth noting that, according to the literature2, the spectral bands of Piroxicam and 5–HP overlap quite a little. After –glucuronidase treatment and measurement in the analytical absorption wavelength of PX, the total expelled drug, i.e., unmodified piroxicam and its metabolites, may be measured as PX.

The average concentration of PX in a complete volume of 0.79 L of urine, was determined to be 2.99±0.09 µg/mL. Approximately 23.6% of the PX dosage was recovered in urine as the 5'–hydroxyl metabolite and its glucuronide conjugate in the current research, which was consistent with previous findings. In addition, unaltered PX excretion in the urine was minimal and below the assay's detection limit.

Optimize conditions of Dispersive liquid-liquid micro-extraction

The disperser-extractant solvent mix is critical in DLLME optimization. 5.0 mL of spiking human urine were treated with 10ng mL^{-1} concentration of all studied Endocerine disrupting chemical in the first step. 10 mL sample solution was injected with 1.0 mL certain disperser solvent (methanol, ethanol, acetone and acetonitrile), extractant solvent 0.1 mL (ClBz, TCM and TCC) (these are three experimental replicates). The acetone-TCM combo generated the most responses.

Effects of salt addition, extraction time and pH sample and the volumes of dispersant and extractant

The impact of sample pH percentage, sodium chloride, extraction time (After adding the binary extraction mixture before centrifugation, the sample is agitated for a length of time), volume of extractant and dispersant and on the performance of the method were investigated and the use of three replicates of the central axis simultaneously in 2 level 25^{-1} fractional factorial design. To decrease the quantity of Endocerine disrupting chemical naturally identified in human urine samples, a pool of these chemicals was spiked with 10ng mL^{-1} of all Endocerine disrupting chemical and used in optimization and diagnostic studies. The residue was dissolved in 100L of an 80:20 (v/v) mixture of $C_4H_8O_2$ (ethyl acetate), 10ng mL^{-1} $EP^{-13}C_6$ and BSTFA/1% TMCS and 10ng mL^{-1} BPA-d_{16}, as well as 10ng mL^{-1} $EP^{-13}C_6$ and 10ng mL^{-1} BPA-d_{16}. In these investigations, the relative area response variable was used. The experimental domain and standardized effects of variables are summarized. At least one analyte in the extraction process has a significant effect on all variables (95% confidence level). The effects sodium chloride content, extraction time and pH were the same for all compounds with the greatest results coming from lower pH values, higher NaCl percentages, and shorter extraction periods. Consequently, the optimum choices for easing the optimization method were a pH value of 2.5% (w/v) of NaCl and a shaking duration of 10 seconds. The samples were diluted 1:1 (v/v) in a 10% NaCl (sodium chloride) solution to a final concentration of 5% sodium chloride and the pH was adjusted to 2.0 using 0.1 M hydrogen chloride (HCl).

❖ **Discussion**

This improved DLLME technique was used to analyze piroxicam in urine samples from ten healthy men. Our earlier HPLC-MS/MS techniques for detecting phthalate metabolites in urine utilized APCI. APCI was sensitive enough for miBP and miMP. However, APCI did not ionize PA and mCPP, the only two metabolites with two carboxylic acid groups. So, we picked ESI. ESI is a gentler ionization method. ESI is used to quantify phthalate metabolites in urine. Study conducted by Koch M they show that these metabolites are secondary, chain oxidized mono esters of diethyl hexyl phthalate and 5-hydroxy-mono-2-ethyl-hexyl phthalate (50HMEHP) 5oxo-MEHP found in urine samples from the general population. They have devised a reliable, simple and quick method for detecting phthalates in human urine samples. For the first time secondary phthalate References metabolites have been utilized to estimate general population exposure. There is excellent reproducibility within and between days, even at low concentrations. The on-line clean-up process is extremely effective; thus, the matrix had no impact on the analytical findings. In this experiment using an established technique like dispersive liquid-liquid micro-extraction to analyze piroxicam in human urine sample is easy, inexpensive, and faster.

F. vela-soria studied “Endocerine disrupting chemical” in human urine sample. Due to the shortage human urine samples which is free from EDCs calibration urine was synthesized. This approach has been used before to determine EDCs.

In the absence of approved materials, a research using spiked synthetic urine samples was conducted on six consecutive days to assess the method's trueness and repeatability. The precision was represented as relative standard deviation (RSD) and the trueness was determined by a recovery test. The recovery of known quantities of the

tested chemicals in synthetic urine samples determined trueness. The concentration of each component was calculated by interpolation within the linear dynamic range and compared to the quantity of analytes previously added to the samples. A t-test for recovery was used. The null hypothesis seems to be true, since computed P-values were 0.05 (5%) or below in all instances. Inter-day precision (RSD) was less than 15%. Except for the LOQ (which should not vary by more than 20%), all substances were within acceptable ranges for bioanalytical technique validation. The technique used to identify target chemicals in human urine samples is extremely precise, and the existence of co–extracted matrix components, which usually reduce analyte signal in mass spectrometry, had no effect on the method's efficacy.

IV. CONCLUSION

It was examined if DLLME might be used as a simple and effective technique for extracting PX from a range of real-world samples. PX levels in human urine were determined using the method, which was evaluated on real samples. In contrast to HPLC, the proposed method allows for PX analysis with low operating costs, simple equipment, and no extra sample cleaning processes. Therefore, in addition to the other well–known advantages of the DLLME method, research time and cost may be significantly reduced. Combining DLLME technology with the proper HPLC method may enhance the approach for separating and determining each PX and its metabolites.

Using the DLLME technique, the detection and quantification of piroxicam in human urine samples was effectively accomplished. The method for isolating analytes from urine samples was precisely refined and verified. The suggested technique was utilised to determine the presence of these chemicals (both free and total content) in samples taken from ten people who were chosen at random (men). This is an analytical technique that may be used to determine human exposure to Endocerine disrupting chemicals in future research.

There are certain experimental parameters that effect the efficiency of the DLLME technique, with disperser solvents along with their volume & extraction, we are optimized pH of the solution and extraction time. In this work we are using the one variable at a time optimization method for obtaining precision, good sensitivity & selectivity for extraction and determination of piroxicam in human urine.

For the first time, a similar technique, DLLME–HPLC, was used to effectively pre-concentrate and quantify piroxicam in a biological sample of human urine. For example, the method consumes very little toxic organic solvent (2.75mL) (including sample preparation and centrifugation) the extraction operation time is < 5 minutes; the extraction technique is very useful, and the requirement of sample amount is depased reduced (urine500 L), making the method suitable for forensic investigations. The accuracy of the depicted technique was greater than conventional methods in all matrices in a comparison to other research and the linearity was in a broad appropriate variety. After using DLLME-HPLC in the case of urine samples, showed the lower LODs. The proposed analytical method can be used to determine piroxicam in the medical line for observing patients undergoing the maintenance treatment of piroxicam and studying bioavailability and pharmacokinetic of piroxicam studies with sufficient sensitivity, simplicity and specificity.

Using the DLLME technique and altering the size of the HPLC column and HPLC solvent gradient, we adapted our analytical method to analyse piroxicam in urine. The DLLME allows for the extraction of 10 urine samples without the need for supervision. Although our manual extraction method yielded a similar sample throughput, manual DLLME was time-consuming and needed continuous attention from the analyst conducting the extraction.

REFERENCES

[1]. Khalil S, Borham N, El-Ries MA. Piroxicam and tenoxicam selective membrane sensors. Analytica chimica acta. 2000 Jun 1;414(1-2):215-9.
[2]. Deore VA, Kumar RS, Gide PS. Development and statistical optimization of mucoadhesive buccal patches of indomethacin: In Vitro and ex vivo evaluation. Int. J. Adv. Pharm. Biol. Chem. 2013;2:405-22.
[3]. Brogden RN, Heel RC, Speight TM, Avery GS. Piroxicam. Drugs. 1984 Oct;28(4):292-323.
[4]. Mihalić M, Hofman H, Kuftinec J, Krile B, Čaplar V, Kajfež F, Blažević N. Piroxicam. InAnalytical profiles of drug substances 1986 Jan 1 (Vol. 15, pp. 509-531). Academic Press.
[5]. Buer JK. Origins and impact of the term 'NSAID'. Inflammopharmacology. 2014 Oct;22(5):263-7.
[6]. Giwa A, Jamiu MO. Utilization of Non-Steroidal Anti-Inflammatory Drugs among Physicians in a Nigerian Tertiary Health Facility.
[7]. Aronoff DM, Neilson EG. Antipyretics: mechanisms of action and clinical use in fever suppression. The American journal of medicine. 2001 Sep 1;111(4):304-15.
[8]. Goldstein JL, Cryer B. Gastrointestinal injury associated with NSAID use: a case study and review of risk factors and preventative strategies. Drug, healthcare and patient safety. 2015;7:31.
[9]. Dionne RA, Berthold CW. Therapeutic uses of non-steroidal anti-inflammatory drugs in dentistry. Critical Reviews in Oral Biology & Medicine. 2001 Jul;12(4):315-30.
[10]. Nayak VK, Desai NK, Kshirsagar NA, Bhandarkar SD, Satoskar RS. Antipyrine and doxycycline pharmacokinetics in patients with thyroid disorders. Journal of postgraduate medicine. 1991 Jan 1;37(1):5.
[11]. Díaz-González F, Sánchez-Madrid F. NSAIDs: learning new tricks from old drugs. European journal of immunology. 2015 Mar;45(3):679-86.

[12]. Vane JR, Botting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. The American journal of medicine. 1998 Mar 30;104(3S1):2S-8S.
[13]. Cashman JN. The mechanisms of action of NSAIDs in analgesia. Drugs. 1996 Nov;52(5):13-23.
[14]. Vane JR, Botting RM. Anti-inflammatory drugs and their mechanism of action. Inflammation Research. 1998 Oct;47(2):78-87.
[15]. Rykowska I, Ziemblińska J, Nowak I. Modern approaches in dispersive liquid-liquid microextraction (DLLME) based on ionic liquids: A review. Journal of molecular liquids. 2018 Jun 1;259:319-39.
[16]. Zgoła-Grześkowiak A, Grześkowiak T. Dispersive liquid-liquid microextraction. TrAC Trends in Analytical Chemistry. 2011 Oct 1;30(9):1382-99.
[17]. Quigley A, Cummins W, Connolly D. Dispersive liquid-liquid microextraction in the analysis of milk and dairy products: a review. Journal of Chemistry. 2016 Oct 31;2016.
[18]. Tabrizi AB, Tutunchi NS. Analysis of piroxicam in pharmaceutical formulation and human urine by dispersive liquid–liquid microextraction combined with spectrophotometry. Advanced pharmaceutical bulletin. 2013;3(1):37.
[19]. Darbre PD, Harvey PW. Paraben esters: review of recent studies of Endocerine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks. Journal of applied toxicology. 2008 Jul;28(5):561-78.
[20]. Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. Toxicology and applied pharmacology. 1998 Nov 1;153(1):12-9.
[21]. Scalia S, Games DE. Determination of parabens in cosmetic products by supercritical fluid extraction and high-performance liquid chromatography. Analyst. 1992;117(5):839-41.
[22]. Kozani RR, Assadi Y, Shemirani F, Hosseini MM, Jamali MR. Determination of trihalomethanes in drinking water by dispersive liquid–liquid microextraction then gas chromatography with electron-capture detection. Chromatographia. 2007 Jul;66(1):81-6.
[23]. Saraji M, Mirmahdieh S. Single-drop microextraction followed by in-syringe derivatization and GC-MS detection for the determination of parabens in water and cosmetic products. Journal of Separation Science. 2009 Apr;32(7):988-95.
[24]. Han Y, Jia X, Liu X, Duan T, Chen H. DLLME combined with GC–MS for the determination of methylparaben, ethylparaben, propylparaben and butylparaben in beverage samples. Chromatographia. 2010 Aug;72(3):351-5.
[25]. Woodhead RJ, Law RJ, Matthiessen P. Polycyclic aromatic hydrocarbons in surface sediments around England and Wales, and their possible biological significance. Marine Pollution Bulletin. 1999 Sep 1;38(9):773-90.
[26]. Fouillet B, Chambon P, Chambon R, Castegnaro M, Weill N. Effects of ozonation on mutagenic activity of polycyclic aromatic hydrocarbons. Bulletin of environmental contamination and toxicology. 1991 Jul 1;47(1):1-7.
[27]. García-Falcón MS, Cancho-Grande B, Simal-Gándara J. Minimal clean-up and rapid determination of polycyclic aromatic hydrocarbons in instant coffee. Food chemistry. 2005 May 1;90(4):643-7.
[28]. Charalabaki M, Psillakis E, Mantzavinos D, Kalogerakis N. Analysis of polycyclic aromatic hydrocarbons in wastewater treatment plant effluents using hollow fibre liquid-phase microextraction. Chemosphere. 2005 Jul 1;60(5):690-8.
[29]. Delgado B, Pino V, Ayala JH, González V, Afonso AM. Nonionic surfactant mixtures: a new cloud-point extraction approach for the determination of PAHs in seawater using HPLC with fluorimetric detection. Analytica Chimica Acta. 2004 Aug 2;518(1-2):165-72.
[30]. Berijani S, Assadi Y, Anbia M, Hosseini MR, Aghaee E. Dispersive liquid–liquid microextraction combined with gas chromatography-flame photometric detection: very simple, rapid and sensitive method for the determination of organophosphorus pesticides in water. Journal of Chromatography A. 2006 Aug 4;1123(1):1-9.
[31]. Naseri MT, Hosseini MR, Assadi Y, Kiani A. Rapid determination of lead in water samples by dispersive liquid–liquid microextraction coupled with electrothermal atomic absorption spectrometry. Talanta. 2008 Mar 15;75(1):56-62.
[32]. Zhao X, Fu L, Hu J, Li J, Wang H, Huang C, Wang X. Analysis of PAHs in water and fruit juice samples by DLLME combined with LC-fluorescence detection. Chromatographia. 2009 Jun;69(11):1385-9.
[33]. Vrečer F, Vrbinc M, Meden A. Characterization of piroxicam crystal modifications. International journal of pharmaceutics. 2003 Apr 30;256(1-2):3-15.
[34]. Vrecer F, Srcic S, Smid-Korbar J. Investigation of piroxicam polymorphism. International journal of pharmaceutics. 1991 Feb 1;68(1-3):35-41.
[35]. Nappi G, Micieli G, Tassorelli C, Viotti E, Altavilla T. Effectiveness of a piroxicam fast dissolving formulation sublingually administered in the symptomatic treatment of migraine without aura. Headache: The Journal of Head and Face Pain. 1993 Jun;33(6):296-300.
[36]. Consoli G, Covelli M, Di Matteo L, Marcolongo R, Tirri G, La Montagna G, Pipitone V. Fast-dissolving sublingual tablets of piroxicam versus naproxen in the treatment of recurrent acute osteoarthrosis. Multicenter clinical trial. Minerva medica. 1994 Mar 1;85(3):89-96.
[37]. Pan RN, Chen JH, Chen RR. Enhancement of dissolution and bioavailability of piroxicam in solid dispersion systems. Drug development and industrial pharmacy. 2000 Jan 1;26(9):989-94.
[38]. Sadi S, Jogiel J, inventors; Sadi Sonja J., Jogiel Jeanette M., assignee. Vitasolve dissolving film strips with supplements pharmaceutical drug (RX),+

vitamins. United States patent application US 10/894,486. 2005 Apr 28.
[39]. Upadhyay SK. Synthesis studies of non steroidal Piroxicam anti inflammatory drugs.
[40]. Cilurzo F, Cupone IE, Minghetti P, Selmin F, Montanari L. Fast dissolving films made of maltodextrins. European Journal of Pharmaceutics and Biopharmaceutics. 2008 Nov 1;70(3):895-900.
[41]. Mohammad S, Singh V, Wadhwani P, Tayade HP, Rathod OK. Sublingual piroxicam in the management of postoperative pain after surgical removal of impacted mandibular third molar. Indian Journal of Dental Research. 2012 Nov 1;23(6):839.
[42]. PATTEWAR SV. Fabrication and characterization of self microemulsifying drug delivery system based fast dissolving film for effective delivery of piroxicam.
[43]. Skiba M, Bouchal F, Boukhris T, Bounoure F, Fessi H, Fatmi S, Chaffai N, Lahiani-Skiba M. Pharmacokinetic study of an oral piroxicam formulation containing different molar ratios of β-cyclodextrins. Journal of Inclusion Phenomena and Macrocyclic Chemistry. 2013 Apr 1;75(3-4):311-4.
[44]. Osman HA. QSAR designing of new nonsteroidal antiinflammatory drugs nsaids considering ibuprofen diclofenac as lead compounds and selection of a good synthetic route through mathmetical modeling followed by their synthesis.
[45]. Patel P, Patel J, Patel K, Nihar S, Shah S. A Review on Fast Dissolving Sublingual Film. Journal of Pharmaceutical and Bioscientific Research. 2015;5(3):279-85.
[46]. Santosh P, Vaishali K. Interactive Mixture of Piroxicam and Polymers for Development of Mucoadhesive Fast Disintegrating Sublingual Tablet: In Vitro-In Vivo Evaluation.
[47]. Steinmeyer J. Pharmacological basis for the therapy of pain and inflammation with nonsteroidal anti-inflammatory drugs. Arthritis Research & Therapy. 2000 Aug;2(5):1-7.
[48]. Lesko SM, Mitchell AA. An assessment of the safety of pediatric ibuprofen: a practitioner-based randomized clinical trial. Jama. 1995 Mar 22;273(12):929-33.
[49]. MacDonald TM, Morant SV, Robinson GC, Shield MJ, McGilchrist MM, Murray FE, McDevitt DG. Association of upper gastrointestinal toxicity of non-steroidal anti-inflammatory drugs with continued exposure: cohort study. Bmj. 1997 Nov 22;315(7119):1333-7.
[50]. Strom BL, Schinnar R, Bilker WB, Feldman H, Farrar JT, Carson JL. Gastrointestinal tract bleeding associated with naproxen sodium vs ibuprofen. Archives of internal medicine. 1997 Dec 8;157(22):2626-31.
[51]. Moore N, Van Ganse E, Le Parc JM, Wall R, Schneid H, Farhan M, Verrière F, Pelen F. The PAIN study: paracetamol, aspirin and ibuprofen new tolerability study. Clinical drug investigation. 1999 Aug;18(2):89-98.
[52]. Galati G, Tafazoli S, Sabzevari O, Chan TS, O'Brien PJ. Idiosyncratic NSAID drug induced oxidative stress. Chemico-biological interactions. 2002 Nov 10;142(1-2):25-41.
[53]. Yang Z, Liang G, Xu B. Enzymatic hydrogelation of small molecules. Accounts of chemical research. 2008 Feb 19;41(2):315-26.
[54]. Toledano S, Williams RJ, Jayawarna V, Ulijn RV. Enzyme-triggered self-assembly of peptide hydrogels via reversed hydrolysis. Journal of the American Chemical Society. 2006 Feb 1;128(4):1070-1.
[55]. Adams DJ, Butler MF, Frith WJ, Kirkland M, Mullen L, Sanderson P. A new method for maintaining homogeneity during liquid–hydrogel transitions using low molecular weight hydrogelators. Soft Matter. 2009;5(9):1856-62.
[56]. E. K. Johnson, D. J. Adams and P. J. Cameron, J. Mater. Chem., 2011, 21, 2024–2027.
[57]. Suryawanshi SB, OSMAN HA, Shaikh YI, Nazeruddin GM. Synthesis of various esters of diclofenac (NSAIDs) as pro-drugs and their biological evaluation. Chemical Science Transactions. 2014;3(2):562-5.
[58]. Roy R, Deb J, Jana SS, Dastidar P. Peptide Conjugates of a Nonsteroidal Anti-Inflammatory Drug as Supramolecular Gelators: Synthesis, Characterization, and Biological Studies. Chemistry–An Asian Journal. 2014 Nov;9(11):3196-206.
[59]. Li X, Pu G, Yu X, Shi S, Yu J, Zhao W, Luo Z, He Z, Chen H. Supramolecular hydrogel of non-steroidal anti-inflammatory drugs: preparation, characterization and ocular biocompatibility. RSC advances. 2016;6(67):62434-8.
[60]. Cacciatore I, Marinelli L, Fornasari E, Cerasa LS, Eusepi P, Türkez H, Pomilio C, Reale M, D'Angelo C, Costantini E, Di Stefano A. Novel NSAID-derived drugs for the potential treatment of Alzheimer's disease. International journal of molecular sciences. 2016 Jul;17(7):1035.
[61]. Jervis PJ, Amorim C, Pereira T, Martins JA, Ferreira PM. Exploring the properties and potential biomedical applications of NSAID-capped peptide hydrogels. Soft Matter. 2020;16(44):10001-12.
[62]. Varga Z, rafay ali Sabzwari S, Vargova V. Cardiovascular risk of nonsteroidal anti-inflammatory drugs: an under-recognized public health issue. Cureus. 2017 Apr;9(4).
[63]. MacDonald TM, Beard K, Bruppacher R, Hasford J, Lewis M, Logan RF, McNaughton D, Tubert-Bitter P, Van Ganse E, Moore N. The safety of drugs for OTC use: what evidence is required for an NSAID switch?. pharmacoepidemiology and drug safety. 2002 Dec;11(7):577-84.
[64]. Bhagya N, Chandrashekar KR. Tetrandrine–A molecule of wide bioactivity. Phytochemistry. 2016 May 1;125:5-13.
[65]. Craig CR, Stitzel RE, editors. Modern pharmacology with clinical applications. Lippincott Williams & Wilkins; 2004.

[66]. Nagda CD. Design and characterization of mucoadhesive microspheres of some nsaids drugs.
[67]. Anastassiades M, Lehotay SJ, Štajnbaher D, Schenck FJ. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. Journal of AOAC international. 2003 Mar 1;86(2):412-31.
[68]. Lehotay SJ. Determination of pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate: collaborative study. Journal of AOAC International. 2007 Mar 1;90(2):485-520.
[69]. Lehotay SJ, Maštovská K, Lightfield AR. Use of buffering and other means to improve results of problematic pesticides in a fast and easy method for residue analysis of fruits and vegetables. Journal of AOAC International. 2005 Mar 1;88(2):615-29.
[70]. Rezaee M, Assadi Y, Hosseini MR, Aghaee E, Ahmadi F, Berijani S. Determination of organic compounds in water using dispersive liquid–liquid microextraction. Journal of Chromatography a. 2006 May 26;1116(1-2):1-9.
[71]. Abdulra'uf LB, Sirhan AY, Huat Tan G. Recent developments and applications of liquid phase microextraction in fruits and vegetables analysis. Journal of separation science. 2012 Dec;35(24):3540-53.
[72]. Fernández P, González C, Pena MT, Carro AM, Lorenzo RA. A rapid ultrasound-assisted dispersive liquid–liquid microextraction followed by ultra-performance liquid chromatography for the simultaneous determination of seven benzodiazepines in human plasma samples. Analytica chimica acta. 2013 Mar 12;767:88-96.
[73]. Fernández P, Regenjo M, Bermejo AM, Fernández AM, Lorenzo RA, Carro AM. Analysis of drugs of abuse in human plasma by dispersive liquid–liquid microextraction and high-performance liquid chromatography. Journal of Applied Toxicology. 2015 Apr;35(4):418-25.
[74]. Cabarcos P, Cocho JÁ, Moreda A, Míguez M, Tabernero MJ, Fernández P, Bermejo AM. Application of dispersive liquid–liquid microextraction for the determination of phosphatidylethanol in blood by liquid chromatography tandem mass spectrometry. Talanta. 2013 Jul 15;111:189-95.
[75]. M.A. Gardner, S. Sampsel, W.W. Jenkins, J.E. Owens, Analysis of Fentanyl in Urine by DLLME-GC-MS, J Anal Toxicol, (2014).
[76]. Berijani S, Assadi Y, Anbia M, Hosseini MR, Aghaee E. Dispersive liquid–liquid microextraction combined with gas chromatography-flame photometric detection: very simple, rapid and sensitive method for the determination of organophosphorus pesticides in water. Journal of Chromatography A. 2006 Aug 4;1123(1):1-9.
[77]. Satpathy G, Tyagi YK, Gupta RK. Development and validation of multi-residue analysis of 82 pesticides in grapes and pomegranate as per the requirements of the European Union (EU) and codex alimentarius using gcms/ms with compound based screening. American J. Food Sci. and Tech. 2014;2(2):53-61.
[78]. Yazdi AS, Razavi N, Yazdinejad SR. Separation and determination of amitriptyline and nortriptyline by dispersive liquid–liquid microextraction combined with gas chromatography flame ionization detection. Talanta. 2008 Jun 15;75(5):1293-9.
[79]. Xiong C, Ruan J, Cai Y, Tang Y. Extraction and determination of some psychotropic drugs in urine samples using dispersive liquid–liquid microextraction followed by high-performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis. 2009 Feb 20;49(2):572-8.
[80]. Lehotay SJ, Son KA, Kwon H, Koesukwiwat U, Fu W, Mastovska K, Hoh E, Leepipatpiboon N. Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. Journal of Chromatography a. 2010 Apr 16;1217(16):2548-60.
[81]. Wilkowska A, Biziuk M. Determination of pesticide residues in food matrices using the QuEChERS methodology. Food chemistry. 2011 Apr 1;125(3):803-12.
[82]. Maham M, Kiarostami V, Waqif-Husain S, Abroomand-Azar P, Tehrani MS, Sharifabadi MK, Afrouzi H, Shapouri M, Karami-Osboo R. Extraction and determination of cyproheptadine in human urine by DLLME-HPLC method. Iranian journal of pharmaceutical research: IJPR. 2013;12(2):311.
[83]. Andraščíková M, Hrouzková S, Cunha SC. Combination of QuEChERS and DLLME for GC-MS determination of pesticide residues in orange samples. Food Additives & Contaminants: Part A. 2013 Feb 1;30(2):286-97.
[84]. Campillo N, Viñas P, Férez-Melgarejo G, Hernández-Córdoba M. Liquid chromatography with diode array detection and tandem mass spectrometry for the determination of neonicotinoid insecticides in honey samples using dispersive liquid–liquid microextraction. Journal of agricultural and food chemistry. 2013 May 22;61(20):4799-805.
[85]. Cao X, Kong Q, Cai R, Zhu K, Ye X, Chen J, Mo W, Wang J. Solid-phase extraction based on chloromethylated polystyrene magnetic nanospheres followed by gas chromatography with mass spectrometry to determine phthalate esters in beverages. Journal of separation science. 2014 Dec;37(24):3677-83.
[86]. Fisichella M, Odoardi S, Strano-Rossi S. High-throughput dispersive liquid/liquid microextraction (DLLME) method for the rapid determination of drugs of abuse, benzodiazepines and other psychotropic medications in blood samples by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and application to forensic cases. Microchemical Journal. 2015 Nov 1;123:33-41.

[87]. Pallares N, Font G, Manes J, Ferrer E. Multimycotoxin LC–MS/MS analysis in tea beverages after dispersive liquid–liquid microextraction (DLLME). Journal of agricultural and food chemistry. 2017 Nov 29;65(47):10282-9.
[88]. Ferrone V, Genovese S, Carlucci M, Tiecco M, Germani R, Preziuso F, Epifano F, Carlucci G, Taddeo VA. A green deep eutectic solvent dispersive liquid-liquid micro-extraction (DES-DLLME) for the UHPLC-PDA determination of oxyprenylated phenylpropanoids in olive, soy, peanuts, corn, and sunflower oil. Food chemistry. 2018 Apr 15;245:578-85.
[89]. Galuch MB, Magon TF, Silveira R, Nicácio AE, Pizzo JS, Bonafe EG, Maldaner L, Santos OO, Visentainer JV. Determination of acrylamide in brewed coffee by dispersive liquid–liquid microextraction (DLLME) and ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Food chemistry. 2019 Jun 1;282:120-6.