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APPROVAL

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name **:** SOAD ALSOUL

Signature **:**

Date **:** 28 June 2018

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To my mother for her tender care, love and a great example of motherhood

To my father for being a pillar of support throughout my life

To my husband for his unconditional love and support

To my son for bringing overflowing and meaning to our lives

ABSTRACT

Alsoul S. Determination of nonsteroidal anti-inflammatory drugs in human milk by DLLME-HPLC.

Near East University, Institute of Health Sciences, Analytical Chemistry Program, Master of Science Thesis, Nicosia, 2018.

Dispersive liquid-liquid microextraction (DLLME) with back-extraction was used prior to high-performance liquid chromatography (HPLC) for the extraction of four nonsteroidal anti-inflammatory drugs (NSAIDs) [i.e., ketoprofen (KET), etodolac (ET), flurbiprofen (FBP) and ibuprofen (IBU)]. Optimum extraction conditions were achieved using 200 μL chloroform (as an extraction solvent), 2.5 mL acetonitrile (as a disperser solvent) and 3% w/v NaCl completed to 11 mL with DI and extracted for 30 s. Back-extraction of NSAIDs into 100 μL of an aqueous solution (30% acetonitrile: 70% 1.0 M NaOH) within 1 min facilitated direct injection of the final extract into HPLC. The analytes were separated at 40 °C using a reversed-phase column, [i.e., Grom-Sil 80 Octyl-4 FE, 4.6 mm ID x 250 mm (3 μm)], a mobile phase of acetonitrile: 1.0 %TFA in H_2O, 40:60 (%, v/v), a flow rate 0.8 mL min^{-1} and an injection volume of 20 μL. The analytes were monitored using a diode-array detector at 256 nm for KET, 224 nm for ET, 246 nm for FBP and 230 nm for IBU. Enrichment factors ranged between 7.2 and 10.0 resulting in limits of detection (LOD) as low as 0.2 mg L^{-1}. Calibration graphs showed good linearity with coefficient of determination (R^2) in the range of 0.9951 and 0.9999. Three different mother milk samples with different age of milk (2, 6 and 12 months) from 3 healthy volunteers were studied to understand the effect of different matrices on the extraction efficiency and resulted in very low to no matrix effect. Finally, the proposed method was applied to determine FBP in genuine samples of different time intervals (between 0.5 and 3.45 hours); the highest concentration was found to be at 2.30 hours. DLLME-HPLC was demonstrated to be a simple and rapid method for the determination of NSAIDs in mother milk with percentage relative recoveries in the range of 93.1-104.6%.

Keywords: Dispersive liquid-liquid microextraction, HPLC, Human milk, Nonsteroidal anti-inflammatory drugs

ÖZET

Alsoul S. Anne sütünde nonsteroid antiinflamatuar ilaçların DLLME-HPLC ile belirlenmesi.

Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Analitik Kimya Programı, Yüksek Lisans Tezi, Lefkoşa, 2018.

Dört farklı nonsteroidal antiinflamatuar ilaçların (NSAID) (ketoprofen (KET), etodolak (ET), flurbiprofen (FBP) ve ibuprofen (IBU)) ekstraksiyonu için yüksek performanslı sıvı kromatografisi (HPLC) öncesinde, geri ekstraksiyonlu dispersif sıvı-sıvı mikro ekstraksiyon (DLLME) yöntemi uygulanmıştır. Optimum ekstraksiyon koşulları; 200 uL kloroform (ekstraksiyon çözücüsü olarak) ve 2.5 mL asetonitril (dağıtıcı çözücü olarak) kullanılarak % 3 w/v NaCl eklenmiş ve deiyonize su ile 11 mL'ye tamamlanarak 30 s boyunca ekstraksiyon sonucunda elde edilmiştir. NSAID'lerin geri ekstraksiyonu için 100 µL çözelti karışımı (%30 asetonitril: %70 1.0 M NaOH), son eksrenin doğrudan HPLC ile enjeksiyonunu kolaylaştırmıştır. Analitler, 40 °C'de Grom-Sil 80 Octyl-4 FE, 4.6 mm ID x 250 mm (3 µm) kolonu kullanılarak ayrıştırılmış ve 0.8 mL min^{-1} akış hızında, ACN: %1 TFA (sulu) 40:60 (% h/h) hareketli fazla ve 20 µL enjeksiyon hacminde enjekte edilmiştir. Örneklerin görüntülenmesi diode-array dedektör ile KET için 256 nm'de, ET için 224 nm'de, FBP için 246 nm'de ve IBU için 230 nm'de gerçekleşmiştir. Zenginleştirme fakörü 7,2 ile 10 arasında edilerek en düşük tayin limiti (LOD) 0,2 mg L^{-1} olarak bulunmuştur. Doğrusal kalibrasyon grafikleri gözlenmiş ve tamamlayıcılık katsayısı (R^2) 0,9951 ile 0,9999 aralığında hesaplanmıştır. Üç farklı sağlıklı anneden farklı yaşlardaki (2, 6 ve 12 ay) anne sütü örneği, farklı matrislerin ekstraksiyon verimi üzerindeki etkisini incelemek üzere analiz edilmiş ve sonuç olarak matris etkisi oldukça düşük bulunmuştur. Son olarak örnekler üzerinde FBP'nin belirlenmesi için farklı zaman aralıklarında (0.5 ile 3.45 saat arasında) önerilen yöntem kullanılmış ve en yüksek konsantrasyon 2.30 saat olarak belirlenmiştir. Yapılan çalışma sonucunda, uygulanan yöntemin (DLLME-HPLC) anne sütündeki NSAID'lerin %93.1-104.6 aralığında nispi geri kazanımlarla belirlenmesi için basit ve hızlı bir yöntem olduğu kanıtlanmıştır.

Anahtar sözcükler: Anne sütü, Dispersif sıvı-sıvı mikroekstraksiyonu, HPLC, Nonsteroidal anti-inflamatuar ilaçlar

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LIST OF ABBREVIATIONS

Abbreviation	Definition
1-DO	1-Dodecanol
1-UN	1-Undecanol
ACN	Acetonitrile
ADME	absorption, distribution, metabolism and elimination
AS	Asymmetry
BES	Back-extraction solvent
C	Concentration
CE	Capillary electrophoresis
CF	Chloroform
COX	Cyclooxygenase
COXIBS	COX-2 inhibitors
DI	Deionized water
DLLME	Dispersive liquid-liquid microextraction
DPE	Diphenylether
EF	Enrichment factor
ET	Etodolac
FASS	Field-amplified sample stacking
FBP	Flurbiprofen
GC	Gas chromatography
GC-FID	Gas chromatography with flame ionization detection
HF-LPME	Hollow-fiber-liquid phase microextraction technique
HPLC-DAD	High performance liquid chromatography with diode array detection
IBU	Ibuprofen
k´	Capacity (retention) factor
KET	Ketoprofen
LDS-DLLME	Low-density solvent-based dispersive liquid-liquid microextraction

LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LPME	Liquid phase microextraction
MP	Mobile phase
MRLs	Maximum Residue Limits
MS	Mass spectrometry
MSPD	Matrix solid phase dispersion
N	Efficiency
NSAIDs	Non-steroidal anti-inflammatory drugs
OHF	4-Hydroxyflurbiprofen
PUFAs	Polyunsaturated fatty acids
R^2	Coefficient of determination
Rs	Resolution
RSD	Relative standard deviation
SOE	Salting-out extraction
SPE	Solid-phase extraction
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
UPLC	Ultra-performance liquid chromatography
USE-AALLME	Ultrasound-enhanced air-assisted liquid-liquid microextraction
UV-VIS	Ultraviolet- visible
α	Selectivity

CHAPTER 1
INTRODUCTION

1.1 Non-steroidal anti-inflammatory drugs (NSAIDS)

Recently, the use of pharmaceutical drugs by humans has increased significantly. Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used drugs with several kilotons of NSAIDs are produced annually in the world. NSAIDs are used to remedy conditions that involve inflammation, mild to moderate pain, and pyrexia [1]. Additionally, epidemiological studies suggest that long-term usage of NSAIDs depreciates the risk of developing Alzheimer's disease and delays its onset [2]. NSAIDs can be divided into nine sub-classes taking into account their inhibitory selectivity and structures, which include; Cyclooxygenase (COX) - non-selective inhibitors - (aniline derivatives, salicylic acid derivatives, pyrazole derivatives, nicotinic acid derivatives, anthranilic acid derivatives, propionic acid derivatives, acetic acid derivatives, and enolic acid derivatives), and COX-2 selective inhibitors (COXIBS) [3]. It has been reported that NSAIDs can inhibit prostaglandin synthesis and reduce kinin cascades and could, therefore, be used to decrease propofol injection pain [4].

Due to the widespread use of these drugs, there is a potential risk for the consumers if food-containing residues enter the food chain considering the fact that NSAIDs are also authorized for food-producing animals. This presents a need to control the residues and develop analytical methods to monitor their compliance with legislations [5]. Physiological development in neonates, infants and children has substantial impact on the absorption, distribution, metabolism and elimination (ADME) of drugs [6]. The European Union (EU) has set Maximum Residue Limits (MRLs) for some NSAIDs to minimize the risk to human health associated with their consumption [7]. Although NSAIDs are perceived to be safe drugs, they may lead to severe toxic effects in cases of acute over-dosage or chronic abuse [8].

1.1.1 NSAIDs determined in this study

Four NSAIDs were determined in this study namely; etodolac (ET), ketoprofen (KET), ibuprofen (IBU) and flurbiprofen (FBP).

1.1.1.1 ET

ET, also known as Lodine, is a non-steroidal anti-inflammatory compound that first became available in 1986 which was prepared by chemical synthesis. The compound has a white crystalline appearance and is stable under normal conditions. ET is an inhibitor of prostaglandin cyclooxygenase but not of lipoxygenase. It is used for symptomatic control of inflammatory rheumatoid and osteoarthritis [9]. It also exhibits activity against several cancer cells [10]. Et is also effective in the treatment of ankylosing spondylitis, and in the alleviation of postoperative pain [11]. The use of ET is sometimes accompanied with gastrointestinal side effects [10].

1.1.1.2 KET

KET is a derivative of propionic acid, (R, S)-2-(3-Benzylphenyl) propionic acid. It has analgesic and antipyretic action. It inhibits cyclooxygenase active with a reduction in the tissue production of prostaglandins [9]. It has been demonstrated to be effective in the treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, gout, pain associated with inflammation, dental pain, trauma, and postoperative pain. The analgesic effect of (S)-KET has been demonstrated in animal models and in humans [12]. KET has limited use due to its significant adverse effects, which include gastrointestinal side effects, such as dyspepsia, gastrointestinal bleeding, and even perforation, and renal side effects [13].

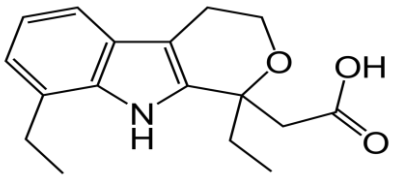
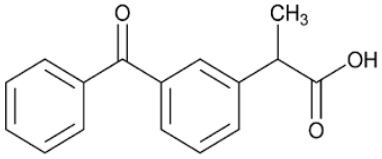
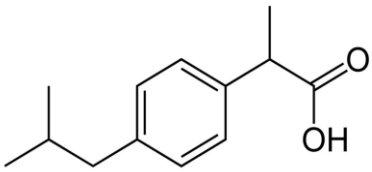
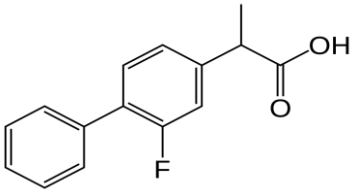
1.1.1.3 IBU

IBU, $(\pm)2-(4-\text{Isobutylphenyl})$ propionic acid, was the first of the propionic acid derivative NSAIDs [9]. It is among the most widely used NSAIDs for treatments for pain and fever [14]. IBU is probably the least toxic [6]. It has white powdery or crystalline appearance with a slight odor and taste; it is non-hygroscopic [9]. Racemic IBU has half the potency of S-(+)-IBU in inhibiting platelet aggregation and thromboxane formation, while R-(–)-IBU was about two orders of magnitude less active [15].

1.1.1.4 FBP

The role of FBP is to inhibit the synthesis of prostaglandin [16]. FBP is used in inflammation, rheumatic diseases and mild to moderate pain of migraine, sore throat and also in primary dysmenorrheal [17]. It is also used in surgery and cancer pain management [16]. FBP is one of the possible probe drugs for CYP2C9 activity, due to the rate of hydroxylation of FBP to 4'-hydroxyflurbiprofen (OHF) [18]. It causes gastrointestinal side effects after prolonged use [19]. The structure of the analytes and some of their physicochemical properties are given in **Table 1.1**.

Table 1.1: Chemical structure, $logP$ and molecular weight (M_r) of the studied analytes.

Analyte	Chemical Structure	$logP$	M_r (g mol^{-1})
ET		3.44	287.350
KET		3.61	254.285
IBU		3.84	206.285
FBP		3.94	244.265

1.2 Mother Milk

Breast milk is the universally preferred form of nutrition for newborn human infants [20]. This is due to the presence of antibodies that protect the newborn against diseases [21]. It contains a variety of indigenous enzymes, many complex proteins, lipids and carbohydrates, the concentrations of which alter dramatically over a single feed, as well as over lactation, to reflect the needs of the infant. Additionally, breast milk contains a multitude of biologically active components [22,23]. Human milk of healthy mothers is also characterized by the optimal ratio of n-3 and n-6 long chain polyunsaturated fatty acids (PUFAs) [24]. Human milk enhances the development of the immune system [25]. It is associated to lower manifestation of type 2 diabetes and obesity in adulthood [26]. With

these myriad of importance of breast milk, it is important to ensure that it is not contaminated with impurities such as the case where lactating mothers are treated for certain diseases. The drugs taken by the mother can contaminate the milk which in turn will affect the baby.

1.3 Liquid Chromatography

A Russian botanist, named Michael Tswett, was the first to use column liquid chromatography in 1903, where the column was packed with a solid adsorbent representing the stationary phase, while the mobile phase was a liquid. The word 'chromatography' is derived from the Greek word Khroma, which means color and graphos meaning writing.

In 1913 an American, named L.S. Palmer, was able to successfully separate dyes in plant and dairy products by column liquid chromatography, also the pigments in egg yolk were identified in Germany in 1930 using chromatography by Edgar Lederer. During 1938, planar chromatography was carried out by Eastern European workers whereby a powder was spread on a glass plate. This could be identified as the early origin of thin-layer chromatograph (TLC).

In 1941, there were developments in chromatography when Martin and Synge carried out partition chromatography of amino acids using silica and most importantly, the mathematical treatment of chromatographic theory that predicted future developments in chromatography. In addition, Martin and James were able to apply gas-liquid chromatography using a gaseous mobile phase and liquid stationary phase in 1952. During the 1950s, chromatography entered the oil field. Stahl was used TLC in 1956.

In the mid-1960s, chromatography became important in the pharmaceutical, agricultural and chemical industries, while in late-1960s, it was necessary to develop

chromatography in relation to volatile compounds through the development of gas chromatography (GC).

High-performance liquid chromatography (HPLC) was then developed for the purpose of analyzing non-volatile compounds. The development of the column necessitated the use of small particles for the stationary phase as well as the use of a pump to generate pressure to produce a rapid flow rate. Hence, the technique was called HPLC [27]. Also packings with particles as small 3 to 10 µm were used instead of larger particle sizes used earlier in columns [28].

1.4 HPLC

HPLC is among the most commonly used separation techniques nowadays. It is employed for the separation of analytes in a sample by distributing them in a column between a stationary phase (SP) and a mobile phase (MP). The separation depends on adsorption to the SP and/or solubility in the MP [28].

The SP in HPLC is supported by a bed of either spherical or irregular particles packed into a stainless steel tube (column) or coated on the internal surface of a quartz capillary tube. The MP flows across the SP as a result of pressure difference (P) across the column.

Separation of analytes injected into the column is due to differential retention of the analytes by the SP. The types of analyte-SP interactions involved in chromatographic retention include hydrogen bonding, van der Waal's forces, electrostatic forces or hydrophobic forces [27]. The major components of the instrument include; a solvent delivery system, solvent degassing, sample injection port, column oven, and detector. These components will be discussed in the following sections [29].

1.4.1 Solvent delivery systems

The role of the solvent delivery system is to deliver the MP (eluent) through the chromatographic column, in an accurate and reproducible manner. This comprises of the pump and solvent reservoir. A pulse-free delivery of the MP is desired to ensure that baseline noise is minimal from the pump, check valves, flow controllers, pulse dampeners, and pressure transducers, each needs to be maintained to ensure that flow rates are constant for better reproducible results.

1.4.2 Solvent degassing

A critical parameter especially in gradient elusion is the quality of the solvents used. Dissolved gases appear in solution when the eluents are pumped through the system, increasing the probability of gases bubbling out of solution when two or more liquids are mixed. Offline degassing can be done by sonication but modern instruments come with degassing modules to ensure efficient removal of gases in the solvent.

1.4.3 Sample injection port

The sample is introduced into the chromatograph via a sample injector. The injection devices can be manual, where a single injection can be made at a time using an HPLC syringe, or automatic injectors via an autosampler, where injection vials are used and the injection can be set automatically and unattended. Up to 100 injections in some instruments can be performed which is especially useful for routine analysis.

1.4.4 Column oven

Even though for many applications close control of temperature is not necessary, a more reproducible result is obtained by keeping column temperature constant. Modern HPLC instruments are equipped with temperature modules that can control temperature from a

few tenths to 150 ˚C. A water bath can be attached to water jackets with constant temperature for temperature control.

1.4.5 Detector

Detectors used in HPLC are analytical instruments adapted with flow cells with the capability of measuring low concentrations of solutes in liquid streams. The ideal detector should have high sensitivity to be able to detect very low concentration in a solution, good stability and high reproducibility, a short response time that is independent of flow rate, reliable and easy to used, have similar response to all solutes, and it should be non-destructive. The most common detectors used in HPLC are ultraviolet-visible (UV-VIS), mass spectrometry and fluorescence detectors [29].

1.5 Parameters Describing the Peak

1.5.1 Peak shape

Theoretically, a single chromatographic band will assume a Gaussian distribution in which the concentration (C) of the solute is related to the time elapsed from the point of injection of the solute (t_o), the retention time (t_R) volumetric flow rate (F_v), standard deviation of distribution (σ), and mass injected (m). Then, C can be described by **Equation 1.1**:

$$C = \frac{1}{F_v \sigma} \frac{m}{\sqrt{2\pi}} e^{-\frac{1}{2}\left(\frac{t_R - t_0}{\sigma}\right)^2} \qquad \textbf{Equation 1.1}$$

1.5.2 Peak area and peak height

Most analytical determinations employed using liquid chromatography are based on the measurement of peak area (A) or peak height (h). Peak area is measured by electronic integration of the signal from an on-line detector, while peak height can be measured electronically or manually from the chromatogram. The choice of peak area or peak height for quantitation is based on peak broadening. Generally, peak height gives more reproducible results for narrow peaks, while peak area is more appropriate for relatively broad peaks. A relationship between the peak height and peak area is given in **Equation 1.2**.

$$h = \frac{A}{\sqrt{2\pi}} \qquad \textbf{Equation 1.2}$$

1.5.3 Peak asymmetry

In theory, chromatographic peaks are predicted to be symmetrical, but peak asymmetry is common, even in the most carefully controlled analytical and preparative separations. Therefore, a more practical measure of peak asymmetry (AS) involves the comparison of the width of the tail, b_f, of the peak to its front, a_f. Then, AS can be described by **Equation 1.3**. An example illustrating how AS can be calculated is shown in **Figure 1.1**.

$$AS = \frac{b_f}{a_f} \qquad \textbf{Equation 1.3}$$

In analytical applications of HPLC, common causes of peak asymmetry include; mixed mechanisms of retention, incompatibility of the sample with the chromatographic MP, or

development of excessive void volume at the head of the column. The causes in analytical application should be identified and corrected because they are frequently accompanied by concentration-dependent retention, non-linear calibration curves and poor precision. Additionally, peak asymmetry can significantly compromise column efficiency leading to reduced resolution and lower peak capacity.

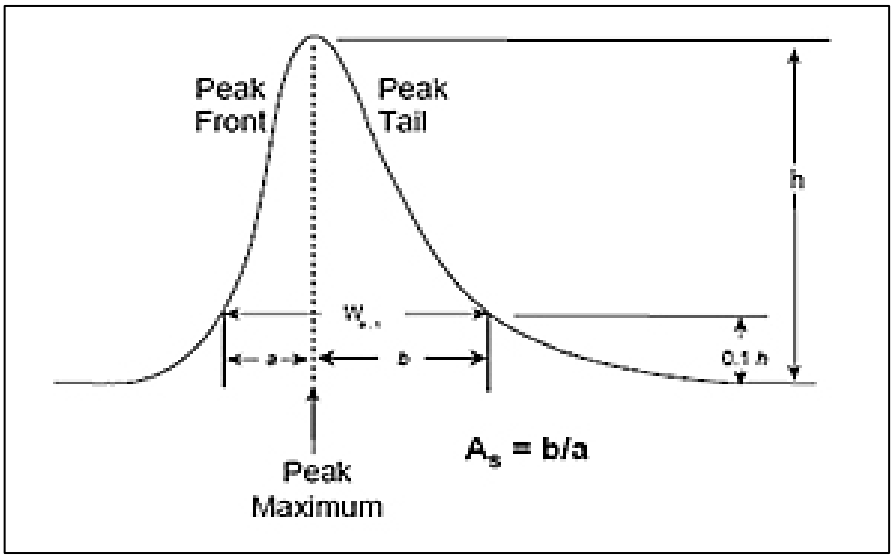


Figure 1.1: Calculating peak asymmetry.

1.6 Retention Relationships

1.6.1 Retention time (t_R)

The retention time (t_R) of a chromatographic peak is defined as the first moment of the Gaussian distribution and is measured from the point of injection to the peak maximum. This is used for qualitative determination of the analyte. Generally, the position of the peak on the time axis in a chromatogram under optimized HPLC conditions is used to identify an analyte even from different samples.

1.6.2 Retention volume (V_R)

The retention volume (V_R) of a peak is the volume occupied by the liquid from the point of injection to the point at which the peak maximum exits the column.

1.7 Relative Retention Parameters

Although the retention volume (V_R) is independent of flow rate, relative retention parameters are preferred because they utilize dimensionless parameters as well as provide additional information about the chromatographic process. These parameters include:

1.7.1 Retention (Capacity) factor (k')

The retention factor (k') of an analyte to its retention time (t_R) and the elution time, t_M, of an unretained compound is given in **Equation 1.4**.

$$k' = \frac{t_R - t_M}{t_M} \qquad \textbf{Equation 1.4}$$

1.7.2 Relative retention time (R_{t_R})

The relative retention time of a solute *j* (R_{t_R}) may be defined by **Equation 1.5**.

$$R_{t_R} = \frac{t_{R\,J}}{t_{R\,i}} \qquad \textbf{Equation 1.5}$$

Where, t_{R_J} and t_{R_i} are the retention time of the solute j and reference compound i, respectively.

1.7.3 Theoretical plates number (N)

The column efficiency or Theoretical plate number (N) can be used as a measure of band broadening. N is an index that also indicates column efficiency. It describes the number of plates as defined according to plate theory, and can be used to determine column efficiency based on calculation in which the larger the theoretical plate number, the sharper the peaks. N is represented by **Equation 1.6**.

$$N = \frac{L}{H} \qquad \textbf{Equation 1.6}$$

where, L is the column length (usually in centimeters) and H is the plate height. Efficiency can be practically calculated from a chromatogram using **Equation 1.7**:

$$N = 16\left(\frac{t_R}{W}\right)^2 \qquad \textbf{Equation 1.7}$$

1.7.4 Selectivity factor (α)

The selectivity factor ($\propto$) of a column is defined as the degree of separation between successive peaks (generally called as critical pair). For the two species A and B, α can be defined by **Equation 1.8:**

$$\propto = \frac{k'_B}{k'_A} = \frac{(t_R)_B - t_M}{(t_R)_A - t_M} \qquad \textbf{Equation 1.8}$$

where, k'_A and k'_B are the retention factors of A and B, and $(t_R)_A$ and $(t_R)_B$ are the retention times of A and B, respectively. This factor is used to indicate how much two adjacent peaks are resolved from each other.

1.7.5 Resolution (R_s)

Resolution (R_s) between a critical pair can be described in terms of retention factor ($\frac{k'av}{(k'av+1)}$), selectivity factor ($\frac{(\alpha-1)}{\alpha}$) and a column efficiency factor ($\sqrt{N}$). If the assumption that the two peaks widths are unequal is not made, then R_s can be obtained using **Equation 1.9**.

$$R_S = \frac{1}{4}\sqrt{N}\ \frac{k'av}{(k'av+1)}\ \frac{(\alpha-1)}{\alpha} \qquad \textbf{Equation 1.9}$$

Fortunately, each element in **Equation 1.9** can be optimized individually until an optimum value for resolution between two adjacent peaks is achieved which is usually from 1.5 and above.

Figure 1.2 indicates that the optimum range for k' is between 2 and10, meaning that in an ideal situation, the solvent strength should be adjusted so that the retention of the peak of interest lies within this range. Clearly, decreasing the solvent strength to increase k' to value of greater than 10 has no significant effect on the resolution. k' can be adjusted experimentally by changing the mobile phase composition, pH, buffer concentration or column temperature. These parameters need to be adjusted first until an optimum k' value is reached.

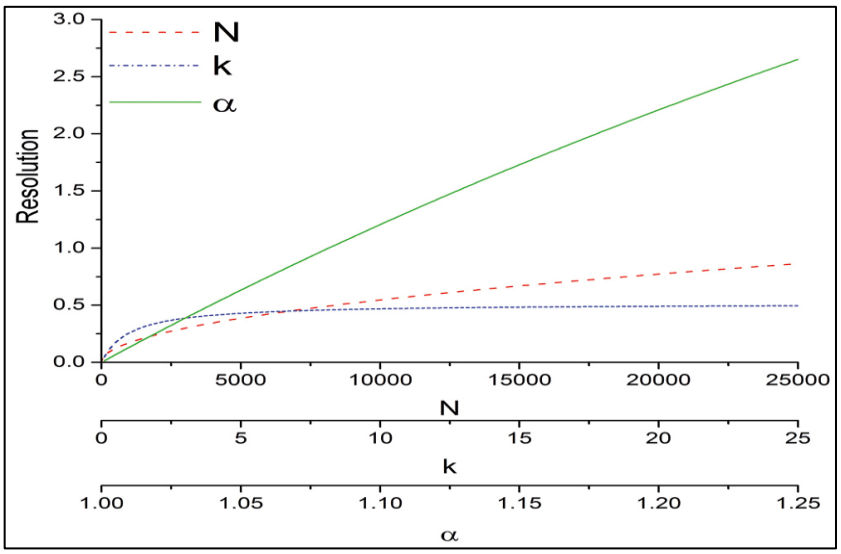


Figure 1.2: Relationship between resolution ($\boldsymbol{R_s}$), retention factor ($\boldsymbol{k'}$), selectivity ($\boldsymbol{\alpha}$) and efficiency ($\boldsymbol{N}$).

Increasing column efficiency (N) is the least attractive method of increasing resolution because it can only be achieved with packed columns at the expense of increased pressure and longer analysis time. The easiest way to increase N experimentally is by adjusting the flow rate. **Figure 1.2** indicates that increasing N will have a positive effect on R_s indefinitely.

Increasing α has a rapid effect on resolution and should be treated with care. Experimentally, α can be adjusted by changing the MP identity or the column identity, making it difficult to predict [27].

1.8 Modes of Elution in HPLC

There are two modes of elution in HPLC known as isocratic and gradient elusion. The isocratic elution is achieved by keeping a constant composition of the MP during experiment. This makes it easily predictable to adjust k'. Most instruments available in

analytical laboratories only have isocratic elution making this mode the method of choice for most analytical chemists.

The gradient elution, which is available in modern instruments, is achieved by varying the composition of the mobile phase during analysis. This is necessary to solve the general elution problem which is long analysis time or poor resolution. The solution of such problem is not possible in isocratic elution [29].

1.9 Dispersive liquid-liquid microextraction (DLLME)

Large volumes of costly and harmful organic solvents are used for conventional extraction methods. This brought the necessity for the recent research trends to miniaturize traditional extraction methods such as liquid-liquid extraction (LLE), aiming at decreasing the volume ratio between the acceptor-to-donor phase [30]. Solid-phase extraction (SPE) might seem better in this context, due to smaller amounts of organic solvents used. However, SPE cartridges are replaced frequently in ultra-trace analysis which generate enormous waste during the process [31].

Recently, liquid-phase microextraction (LPME) techniques have been promoted for both removal of interferences, analyte enrichment in samples, and for simplifying the matrix composition [30]. LPME has evolved prompting the development of different extraction modes. Notable among these procedures is DLLME proposed in 2006 by Assadi and his team [32]. It offers various advantages such as simple operation, rapidity, low capital cost, high efficient extraction, and no need of instrumentation or special equipment [33].

DLLME involves two simple steps: First, a mixture containing the extraction solvent (in μL) and disperser solvent (in μL to few mL) are rapidly injected to an aqueous sample solution (in mL). Dispersion of the extraction solvent is formed in the form of a cloudy colloidal solution (emulsion) containing micro droplets of the extractant to facilitate rapid extraction of analytes due to the infinitely large surface area between the donor

and acceptor phase. The second step involves breaking the colloidal solution by centrifugation or other mechanical means and collecting the extractant containing the analytes usually by means of a micro syringe for analysis [31].

The extraction solvent is chosen on the basis of its ability to extract target analyte(s), higher-density than water (chlorinated solvents such as chloroform, dichloromethane, tetrachloromethane, etc) in conventional DLLME and also low miscibility in water. There are situations where lower-density solvents are used, a case that results in difficulty in collecting the extractant at the top of the centrifuge tube but can be overcome if solvents that solidify at room temperature such as 1-undecanol, 1-dodecanol etc. are used by solidification of a floating organic drop in a freezer for few minutes or by using ice bath around the centrifuge tube. However, the disperser solvent should be miscible in the extraction solvent and water, thus enabling the formation of fine droplets of extraction solvent in the aqueous phase. Common disperser solvents used include acetonitrile, methanol, acetone, and ethanol, etc. [34].

The extracting solvent volume has a significant effect on the enrichment factor (EF). Increasing the volume of extraction solvent results in increase of the volume of the sedimented phase recovered after centrifugation. Hence, preconcentration will also decrease because the analytes are diluted, once the maximum extraction efficiency has been achieved. Therefore, optimum volume of the extraction solvent should ensure both high EFs and enough volume of the sedimented phase for effective extraction and subsequent analysis after centrifugation [33].

1.10 Literature Review

A look at the literature reveals that NSAIDs have been extensively studied due to their various importance in pharmaceutical industry and their inherent dangers as mentioned in the Introduction part. The main focus here will be on development of analytical methods used for the analysis of NSAIDs, specifically in the context of their extraction

from various matrices. It will be challenging to exhaust all the literature, so the focus of instrumentation will be chromatography with various detectors while for the extraction, we will begin by taking a look at the general extraction methods before focusing on DLLME-based techniques in the context of NSAIDs analysis.

HPLC is the instrument of choice for the analysis of NSAIDs in literature for obvious reasons as far as separation is concerned. Beginning with the analysis of NSAIDs in dairy products using HPLC, Pasquale Gallo et al. in 2010 [35] carried out a confirmatory analysis of NSAIDs in bovine milk by RP-HPLC with fluorescence detector. A total of nine drugs related to different sub-classes of NSAIDs namely; FBP, carprofen, naproxen, vedaprofen, 5- hydroxy-flunixin, niflumic acid, mefenamic acid, meclofenamic acid and tolfenamic acid were analyzed using an extraction method involving 10 mL ACN/MeOH (90/10, v/v), followed by acid hydrolysis for 10 min using ascorbic acid and HCl. C_{18} SPE was then carried out. The eluent was evaporated to dryness under a stream of nitrogen at room temperature before dissolving the residue in 500 μL HPLC-grade MeOH. The sensitivity of the method was relatively high with limit of quantitation (LOQ) as low as 0.25 μg kg^{-1}, which can be attributed to the higher sensitivity of fluorescence detector as compared to UV.

In another study carried out by Estelle Dubreil-Chéneau et al. in 2011 [7] for the analysis of NSAIDs in bovine milk by liquid chromatography-tandem mass spectrometry (LC-MS/MS) where 12 NSAIDs (phenylbutazone, oxyphenylbutazone, naproxen, mefenamic acid, vedaprofen, flunixin, 5-hydroxyflunixin, tolfenamic acid, meloxicam, diclofenac, carprofen and KET where resolved and validated according to the European commission decision 2002/657/EC. The extraction method used 8 mL of MeOH as extraction solvent before evaporation of the solvent under a stream of nitrogen at 40 ˚C for 1.5 h. The method showed high sensitivity also due to the high sensitivity of the MS detector used with LOQ as low as 0.69 μg kg^{-1}.

Ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used by Tao Peng et al. in 2013 [3] to resolve sub-classes of NSAIDs in milk (milk beverage and milk powder) and dairy products (yogurt and processed cheese). The extraction method involved an initial step of extraction and deproteinization with ascorbic acid (pH 3, 0.01 M) and ACN-ethyl acetate mixture before centrifugation and evaporation to dryness. High sensitivity was achieved with an LOQ as low as 0.10 μg kg^{-1}.

Another sample matrix that has been analyzed for NSAIDs using HPLC is urine that can be used for pharmacokinetic studies, clinical and therapeutic studies, doping analysis and forensic toxicology. A method proposed by Yong Byoung Cha and Seung-Woon Myung in 2013 [36] demonstrated a three-phase hollow-fiber-liquid phase microextraction technique (HF-LPME), a relatively green sample preconcentration and clean-up technique as compared to conventional LLE or SPE. The extraction method involved using 4 mL of aqueous solution at pH 3 as the donor phase, into dihexyl ether stagnated in the porous hollow fiber wall, followed by extraction into the acceptor phase at pH 13 positioned at the lumen of the hollow fiber. After successful extraction, an aliquot of the acceptor phase was injected directly into HPLC with UV detection. LOQ was as low as 15 ng mL^{-1}, a relatively high sensitivity for UV detector, but less for MS or fluorescent detector as shown in the previous studies which can be attributed to the HF-LPME method with enrichment factor (EF) between 59 and 260.

Wastewater is another important matrix for the analysis of NSAIDs due to danger to human health and aquatic environment. A study by Aguilar-Arteaga et al. in 2010 [37] demonstrated the use of magnetic matrix solid phase dispersion (MSPD) extraction method for the analysis of four NSAIDs (acetaminophen, naproxen, diclofenac and IBU) in wastewater by HPLC-UV. The extraction method involved using SiO_2–Fe_3O_4 of different polarities to enable rapid separation by a simple magnetic extraction. The method showed high extraction efficiency and sensitivity with LOQ as low as 3 μg L^{-1}.

The focus was then shifted to DLLME in the analysis of NSAIDs. Notable among the studies is the one carried out by Alshana et al. in 2013 [38] for the analysis of NSAIDs in bovine milk and dairy products (yogurt and cheese) by combining DLLME with field-amplified sample stacking (FASS) for capillary electrophoresis (CE) to achieve EFs ranging from 46-229. Conventional DLLME procedure was carried out using 2.0 mL of ACN as the disperser solvent and 150 μL of chloroform as the extraction solvent to achieve very rapid extraction in just one min. The solution was back-extracted into 70 μL of a basic aqueous solution before injection into CE for FASS. LOQ was as low as 9.0 μg kg^{-1}, a very high sensitivity for UV detector due to the synergistic preconcentration effects of DLLME and FASS.

Recent trends in DLLME are geared towards the elimination of toxic organic solvent despite the relatively low volume used in the microliter range for extraction solvent to a few mL for disperser solvent. Alternative solvents are being adopted away for heavy toxic organic solvent and mechanical means are adopted for dispersion such as vortex-assisted extraction or ultrasound-assisted extraction. A study conducted by Behruz Barfi et al. in 2015 [8], compared ultrasound-enhanced air-assisted liquid-liquid microextraction (USE-AALLME) with low-density solvent-based dispersive liquid-liquid microextraction (LDS-DLLME) methods for determination of NSAIDs in human urine samples. Both methods were rapid, convenient, and simple with minimum consumption of organic solvent. However, USE-AALLME gave better results with an optimum volume of 30 μL of 1-octanol as extraction solvent and five extraction cycles by 20 s ultrasonication, each making a total extraction time of 100 s. Analysis was carried out using gas chromatography with flame ionization detection (GC-FID). LOQ for this method was as low as 0.3 μg L^{-1} with EFs ranging from 115 to 135.

In line with the use of greener solvents, ionic liquids are becoming popular for use as extraction solvent. This is due to their tunable physiochemical properties, thermal stability, low viscosity, and low vapor pressure. A method proposed by Carla Toledo-Neira and Alejandro Álvarez-Lueje in 2015 [39] used ionic liquids as extraction solvents in

DLLME prior to high performance liquid chromatography-diode array-fluorescence (HPLC-DAD-Fl) detection for the analysis of three NSAIDs (KET, IBU and diclofenac) in tap water. MeOH (210 μL) was added to the extraction protocol as disperser solvent. The lowest LOQ achieved was 51 ng mL^{-1}. EF was in the range of 49-57.

A major limitation of conventional DLLME is inability to automate the process and also the centrifugation step which is considered the most time consuming step. To overcome this limitation and open up a possible new horizon for DLLME automation, Marta Cruz-Vera et al. in 2009 [32] proposed an in-syringe ionic liquid-based DLLME for the analysis of NSAIDs in urine sample prior to analysis by HPLC-UV in a single step. The method involved using a 10 mL plastic syringe as the extraction unit. The sample solution is loaded into the extraction unit placed upside down. The extraction mixture containing 720 μL of MeOH as disperser solvent and 280 μL of 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim]PF_6) as the extractant were rapidly injected into the extraction unit using a 1000 μL glass syringe to form a cloudy solution. Ionic liquid containing the analyte could be collected at the tip of the syringe. Different adapters could be coupled to the tip of the syringe depending on the amount of the extractant expected to be collected. The duration of the process was below 5 min. LOQ of this method was as low as 24.9 ng mL^{-1}.

This brief review of the literature reveals that the different variations of DLLME have advantages above other methods discussed, such as, least consumption of organic solvent, least extraction time, cheapest, highest sample pre-concentration and clean up efficiency, and simple execution. It is due to these facts that DLLME is the method of choice for this study.

1.11 Aim of this Study

The aim of this study is to develop a rapid, efficient and economical method based on DLLME combined with HPLC for the determination and quantitation of NSAIDs in human milk.

CHAPTER 2
EXPERIMENTAL

2.1 Instrument

An Agilent technologies 1200 series HPLC System (USA) was used for chromatographic separation. The instrument is equipped with quaternary pump, a degasser, autosampler, column oven, diode array detector and controlled by Agilent ChemStation for LC 3D systems (Rev. B.03.01) software. The column used was a reversed phase [Grom-Sil 80 Octyl-4 FE, 4.6 mm ID x 250 mm (3 µm)].

2.2 Reagents

An HPLC gradient-grade ACN and MeOH with purity equal or above 99.9% were used for chromatographic separation from Sigma-Aldrich (Germany). Chloroform, diphenylether, 1-undecanol and 1-dodecanol, sodium hydroxide, sodium chloride, and phosphoric acid were purchased from Sigma-Aldrich (Germany), while n-hexane was purchased from Fluka (USA).

2.3 Apparatus

Ultrasonication was performed with Iso-lab Laborgerate GmbH digital ultrasonic bath (Germany), centrifugation with Hettich Eba 20 centrifuge (Germany), and vortex with Heidolph Reax top Vortex. Sample collection and transfer were performed with Eppendorf micropipettes (Sigma-Aldrich, USA) and tips from Iso-lab (Germany). Centrifuge tubes were from Iso-Lab (Germany). For filtration of the MP, GE infrastructure (0.2 µm) nylon filters (China) were used. A Blomberg refrigerator was used for preservation of the sample.

2.4 Sampling

Mother milk was collect from three volunteers. The first is a 30 years old breastfeeding for twelve months; the second is 24 years old breastfeeding for six months, while the third is a 31 years old breastfeeding for two months. About 10 mL of the milk were collected and discarded before collecting for sampling and stored in the freezer at -15 ˚C.

2.5 Sample Pretreatment

The milk sample was removed from the freezer and kept to thaw at room temperature. Two 14 mL portion of the sample were collected in two centrifuge tubes and centrifuged at 6000 rpm for 15 min. A precipitate formed at the bottom of the tube, while lipids floated on the top of the solution. A clear solution formed in the middle and was collected as the sample solution. Representative pictures of the milk samples after centrifugation are shown in **Figure Figure** 2.1**.**

Figure 2.1: Mother milk samples after centrifugation.

2.6 Salting-Out Extraction (SOE)

2.0 mL of the sample solution were collected into a centrifuge tube, 100 µL of phosphoric acid were added, the solution was vortexed for 1 min, 1.0 mL of saturated NaCl solution and 4 mL of ACN were added. The solution was vortexed for 1 min and

centrifuged for 3 min. The ACN salted-out at the top and 3 mL were collected. 1.0 mL of n-hexane was added to defatten the ACN, the solution was vortexed and centrifuged for 1 min each. *N*-hexane in the upper phase was discarded and the ACN at the bottom was collected for DLLME.

2.7 DLLME

2.5 mL of the ACN milk extract were transferred into a centrifuge tube, with the ACN acting as a disperser solvent. 200 µL of chloroform were added as the extraction solvent, 3.33 mL of 10.0% (w/v) NaCl solution making the final percentage of NaCl 3% (w/v) after the solution was completed to 11.0 mL with deionized water and 100 µL phosphoric acid were added. The solution was vortexed for 30 s and centrifuged for 3 min at 6000 rpm. The chloroform layer settled at the bottom of the flask and was collected with an HPLC syringe for back-extraction.

2.8 Back-Extraction

The chloroform extract was subjected to back-extraction by adding 100 µL of 30/70, (%v/v) ACN/1.0 M NaOH in an Eppendorf micro tube. The solution was vortexed and centrifuged for 1 min each. 20 µL of the upper aqueous phase was injected into HPLC for analysis.

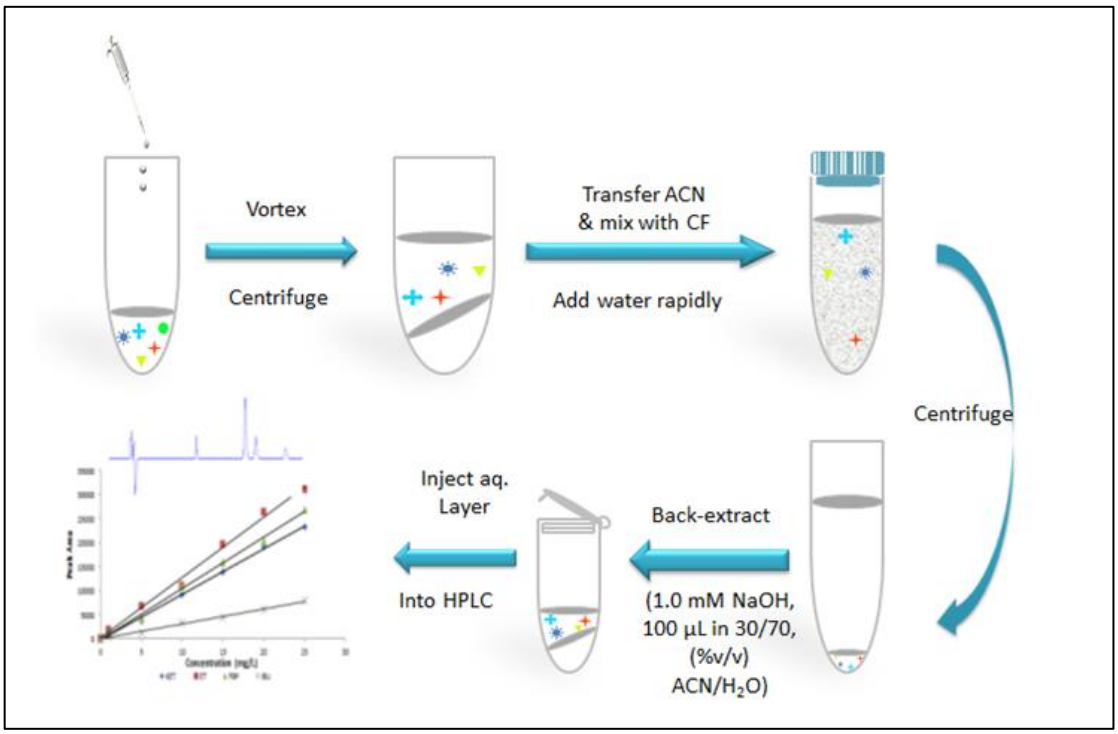


Figure 2.2: Schematic diagram of the DLLME procedure with back-extraction.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Selection of Wavelength of Maximum Absorption

The wavelength of maximum absorbance (λ_{max}) is very important in spectroscopy because it ensures maximum sensitivity of the method and robustness because the absorbance remains relatively constant when there is a little variation in wavelength.

To determine the wavelength of maximum absorption, 15.0 ppm of the standards were prepared in the MP and their UV spectra were obtained after they were injected into the HPLC-DAD instrument. The four analytes have absorption maxima with KET at 256 nm, ET at 224 nm, FBP at 246 nm and IBU at 230 nm as shown in **Figure 3.1**. λ_{max} was used in quantitation studies.

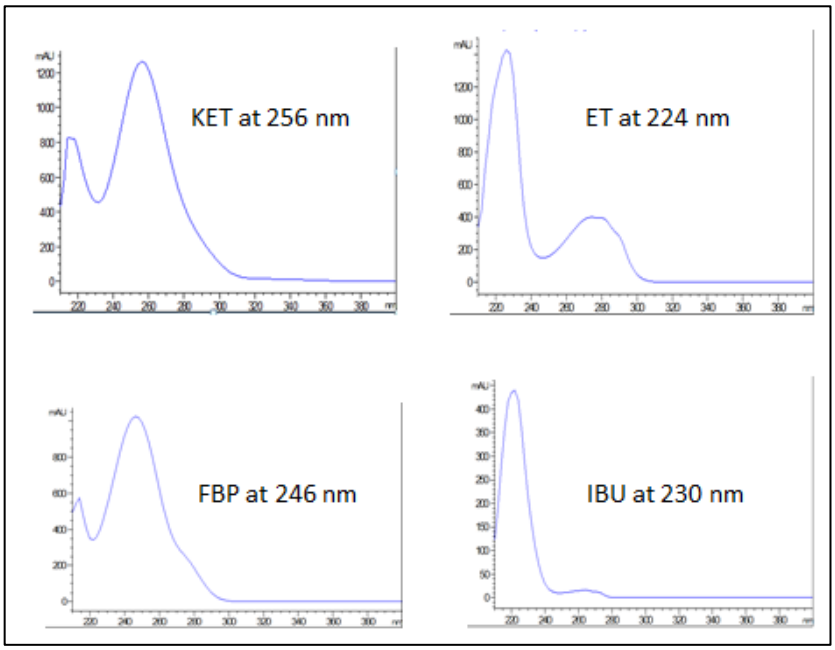


Figure 3.1: UV Absorption profile of analytes showing absorption maxima.

To optimize the extraction and HPLC parameters of the four analytes, it is more convenient and practical to use single chromatogram for the four analytes at the same wavelength. The 3D plot of the four analytes (**Figure 3.2**) gave an optimum absorption at 230 nm, though it was a compromise of sensitivity of some analytes but it was sufficient for optimization of the different parameters. Hence, 230 nm was used for all optimization purposes.

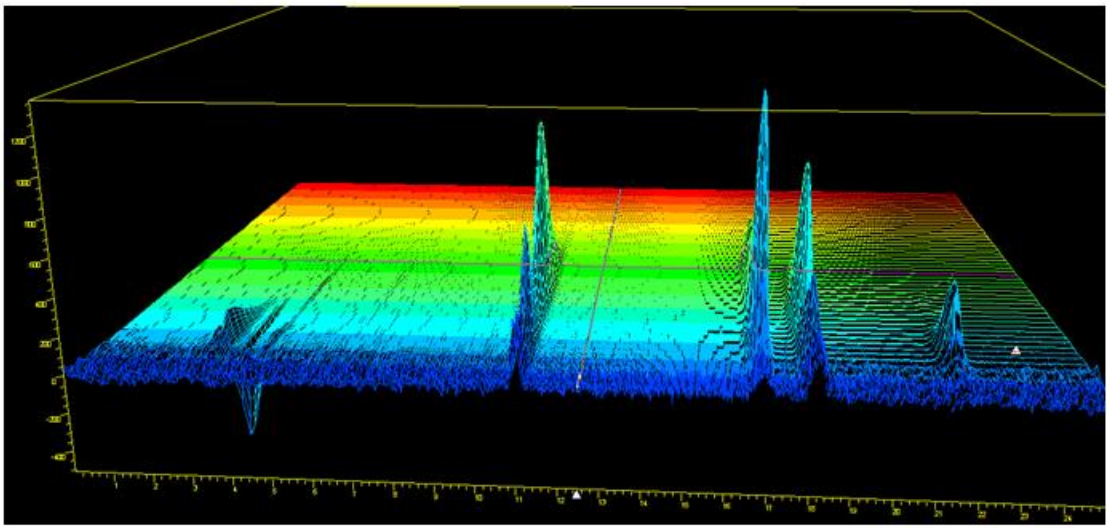


Figure 3.2: 3D plot of four NSAIDs.

3.2 Determination of Initial Extraction Parameters

To determine the extraction parameters of the analytes, it was necessary to examine the polarity of each specie. The $logP$ value of the NSAIDs of interest ranged from 3.44 to 3.94 as shown in **Table 1.1.** The values suggested that the analytes are relatively non-polar. This implies that a relatively non-polar solvent is required for their extraction. However, we are faced with a dilemma of not being able to inject a non-polar solvent directly into RP-HPLC, this is because the MP of RP-HPLC is relatively polar, requiring also a relatively polar solvent for injection. We were left with two options to either evaporate the solvent to dryness and reconstitute into the MP, or to back-extract the

analytes into an aqueous solution that is compatible with the MP. Evaporation to dryness takes a relatively longer time; it is relatively more expensive, and tedious as compared to back-extraction. Evaporation to dryness would be used as a last resort in the case that back-extraction could not be used (e.g., for non-ionizable analytes).

To determine if the analytes can be back-extracted, the Henderson-Hasselbalch plot from MavinSketch, shown in **Figure 3.3**, indicates that the NSAIDs can exist in either neutral or charged form in acidic and basic medium, respectively. Acidifying the solution when extracting the analyte into a non-polar organic solvent will ensure that the analytes are in their neutral form, thereby increasing the extraction efficiency into a non-polar solvent. Back-extracting the analytes in a basic solution will ensure that the analytes are charged to be able to leave the non-polar solvent into the polar one. This influenced the decision to acidify the sample in the extraction step and treat it with a basic solution in the back-extraction step.

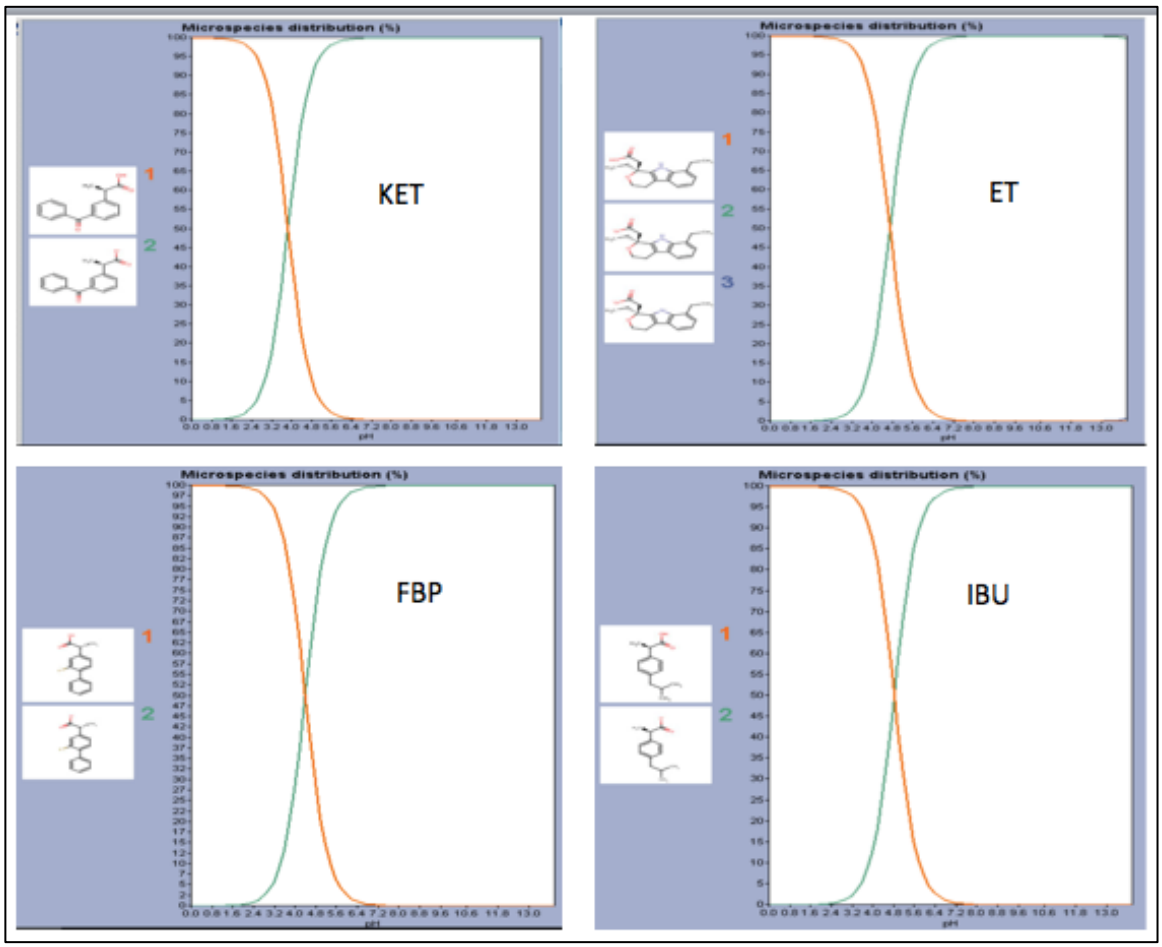


Figure 3.3: Henderson-Hasselbalch plots of NSAIDs.

3.3 DLLME Optimizations

In order to be able to perform analysis in the shortest possible time, using the minimum amount of solvents and chemical without sacrificing extraction efficiency, it is necessary to optimize the parameters that are involved in the extraction method. Some of the most important factors that affect extraction efficiency in DLLME that have been examined here include: Type and volume of extraction solvent, volume of disperser solvent, extraction time, volume of deionized water added to the solution, and ionic strength.

3.3.1 Type of extraction solvent

For the type of extraction solvent, two high-density solvents, chloroform (CF) and diphenyl ether (DPE) were considered as the extraction solvent, while for low-density solvents, 1-undecanol and 1-dodecanol were considered by applying solidification of floating organic drop by freezing the solvent in a freezer and collecting the solvent as a solid drop for analysis. Peak areas of obtained with the different types of extraction solvents were compared as shown in **Figure 3.4.** Chloroform (CF) showed the highest extraction efficiency and was used for further analysis.

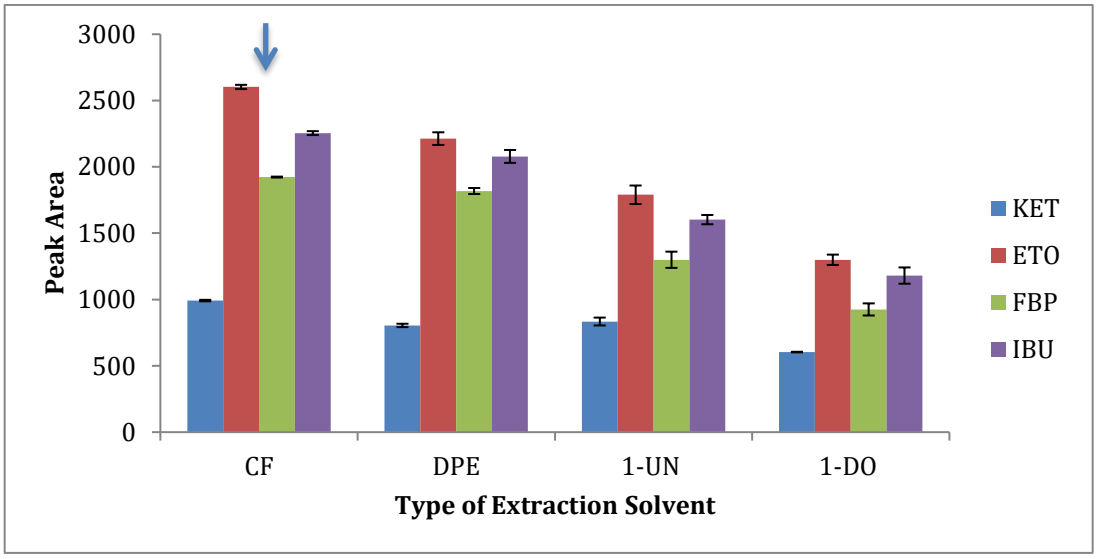


Figure 3.4: Optimization of the type of extraction solvent.

3.3.2 Volume of extraction solvent

The volume of extraction solvent (chloroform) was examined from 100-300 µL. The extraction efficiency started to increase with increase in the volume of the solvent because, increase in the volume of CF extract more of the analytes, until equilibrium was reached at 200 µL of the CF. This is the point where all possible analytes have been extracted. Further increase in the solvent volume does not increase extraction of the

analytes but rather diluted the analytes in the solution, which explains the subsequent decrease in peak area as shown in **Figure 3.5.** 200 µL CF was taken as an optimum volume of the extraction solvent and was used for further analysis.

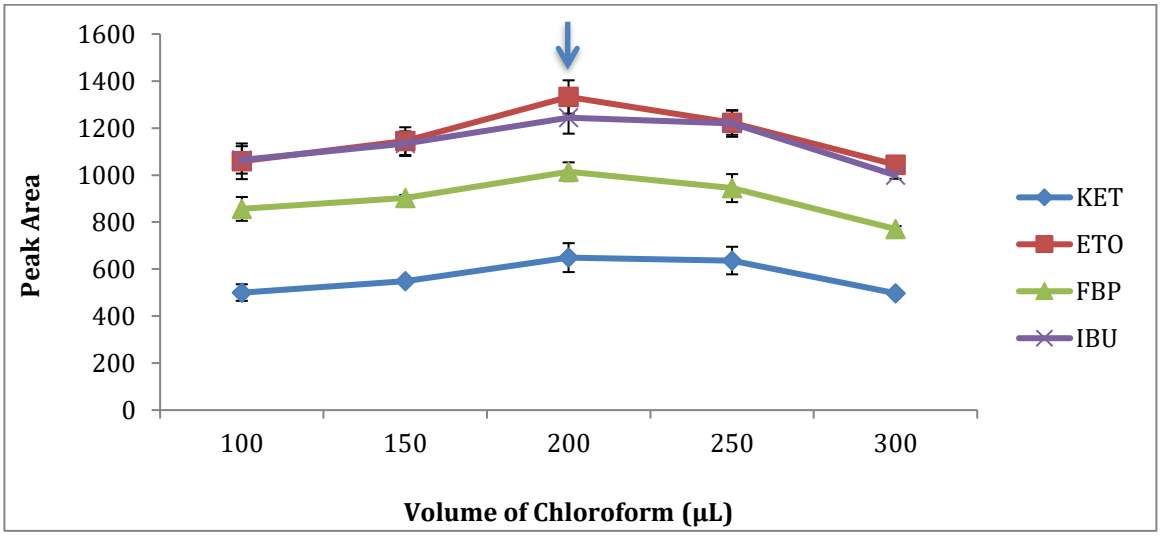


Figure 3.5: Optimization of the volume of Extraction solvent.

3.3.3 Volume of disperser solvent

An experiment was not performed to determine the type of disperser solvent because prior to DLLME, SOE was performed to clean-up the mother milk sample due to the extremely complex nature of the sample matrix. The type of disperser solvent experiment was not performed because ACN is the most suitable solvent for SOE (ref). The effect of ACN volume on extraction efficiency was monitored. Increasing the volume of ACN increased the degree of dispersion of the CF into the aqueous solution which resulted into higher extraction efficiency. Maximum extraction efficiency was observed with 2.5 mL of ACN added, above which the peak area began to decrease as shown in **Figure 3.6**. This is because with increase in the volume of ACN, solubility of CF in the donor solution increases causing less volume of CF to be recovered after centrifugation. It was observed that at 3.5 mL of ACN, the CF floated at the top of the

centrifuge tube, probably due to the changes in the density of CF due to the presence of high concentration of ACN. 2.5 mL of ACN was taken as the optimum disperser solvent in subsequent experiments.

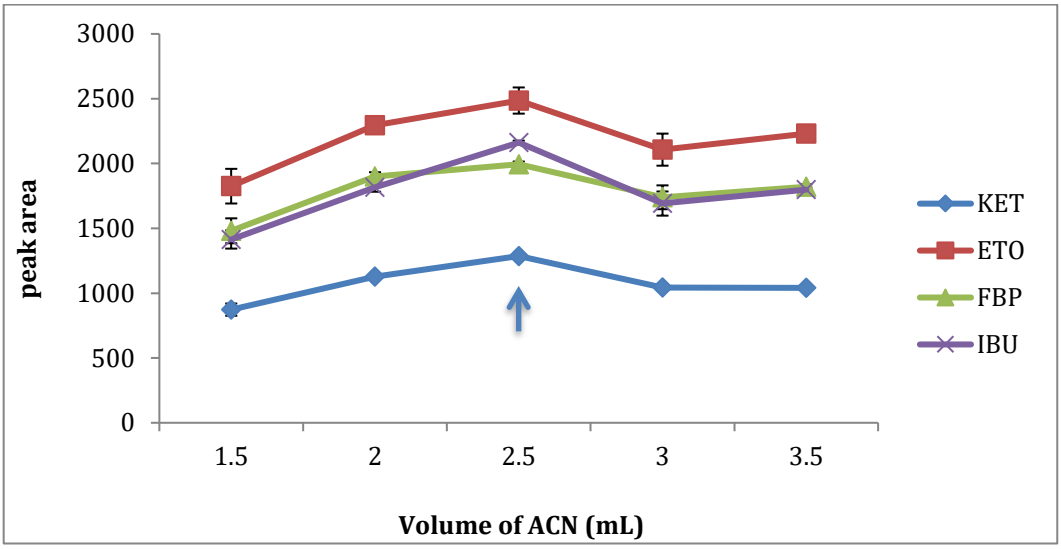


Figure 3.6: Optimization of the volume of disperser solvent.

3.3.4 Volume of deionized water (DI)

The volume of DI water is another important parameter in DLLME. An appropriate volume is required for adequate dispersion of the extraction solvent into the aqueous phase to ensure maximum interaction of the analyte with the extraction solvent. The volume of DI was varied from 8.0 to 12.0 mL. At 11.0 mL of DI water, maximum extraction was achieved as shown in **Figure 3.7**, above which the solution was relatively too polar for proper dispersion of the extraction solvent in the aqueous phase. Above the optimum volume of the disperser solvent, increase in the overall volume of the sample solution decreases the probability of dispersion of the analytes into the extraction solvent. Hence, 11.0 mL of DI water was taken as optimum.

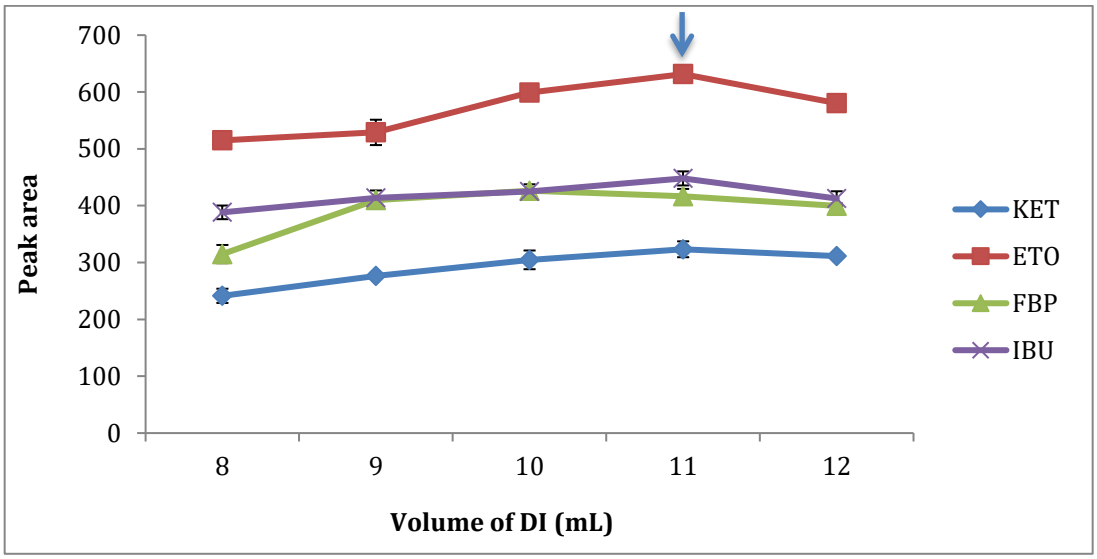


Figure 3.7: Optimization of the volume of DI water added.

3.3.5 Ionic strength

The ionic strength of the solution helps in the form of repelling the organic phase from the aqueous phase through the salting-out effect. An interesting paradox occurs here. This is because it is desired that the organic and aqueous phase be adequately in contact with the help of the disperser solvent to ensure maximum extraction of the analyte. However, after the extraction, it is desired that the two phases are adequately separated after centrifuging to ensure maximum recovery of the analyte-rich organic phase where the ionic strength comes into play. An appropriate volume of the salt solution that will result in high recovery of the organic phase after centrifugation without inhibiting the dispersion of the organic phase in the aqueous phase is desired. In this experiment, 3.0% (w/v) of the salt solution was shown to be optimum, above which the extraction efficiency drops due to the salt preventing adequate dispersion of the two phases. In addition, the Nernst diffusion film around the organic droplet will be thicker due to surface tension, thereby making it harder for the analyte to diffuse through. The effect of ionic strength (i.e., concentration of NaCl in the sample solution) is shown in **Figure 3.8**.

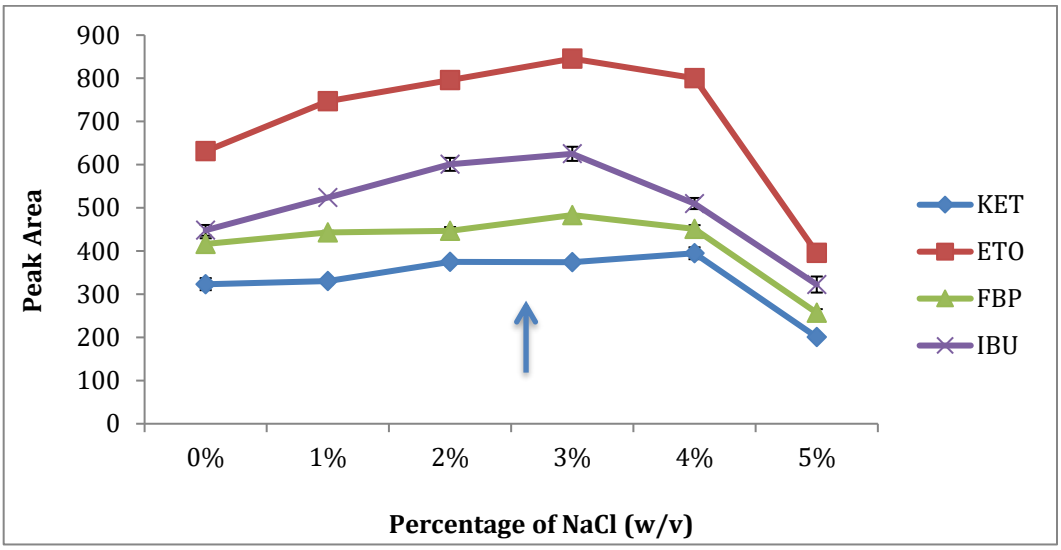


Figure 3.8: Optimization of ionic strength.

3.3.6 Extraction time

Extraction time in DLLME is defined as the time taken after addition of the extraction and disperser solvents to the time before centrifugation, in our case being the vortex time. The vortex time was monitored by first of all manually shaking of the solution which is the zero time and next within 30 s intervals. After 30 s, the trend was relatively constant as shown in **Figure 3.9**, this is due to the high surface area of contact between the extraction and aqueous phases that significantly reduced the extraction time. Increasing the extraction time after equilibrium was reached did not result in any increase in the extraction efficiency. Therefore, 30 s vortex time was taken as the optimum extraction time.

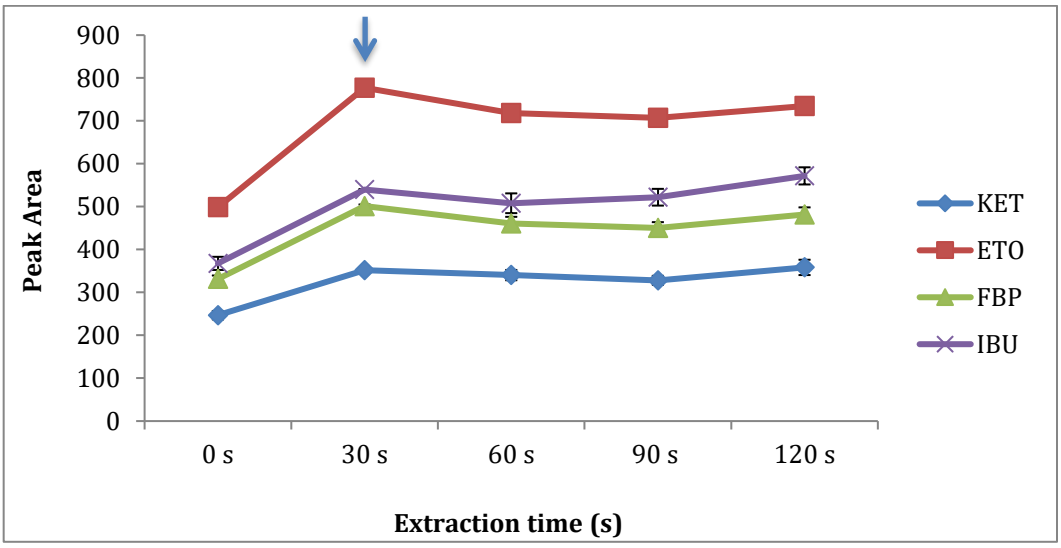


Figure 3.9: Optimization of DLLME time.

3.4 Back-Extraction

3.4.1 Addition of organic disperser

Even though 1.0 M of NaOH can extract the analytes from the CF, the efficiency is greatly affected by the presence of micro volume of a disperser solvent in the BES due to immiscibility of CF with water. A disperser solvent would aid in ensuring a higher degree of contact is achieved between the two phases. ACN was selected as the disperser because it is the solvent used as the MP and thus it would reduce background noise and/or the appearance of extraneous peaks in the chromatogram. The percentage of ACN was varied in the BES from 0 to 40% (v/v). Maximum extraction efficiency was achieved at 30% (v/v) of ACN as shown in **Figure 3.10**, above which its dispersion did not lead to further improvement of extraction efficiency. There is also a risk that high dispersion of the CF in the aqueous phase would prevent phase separation after centrifugation resulting into the injection of CF into the instrument. CF would then stick to the stationary phase of the RP column irreversibly and would result into

irreproducible results and in worst cases permanently damage the column. Therefore, 30% (v/v) of ACN was taken as optimum.

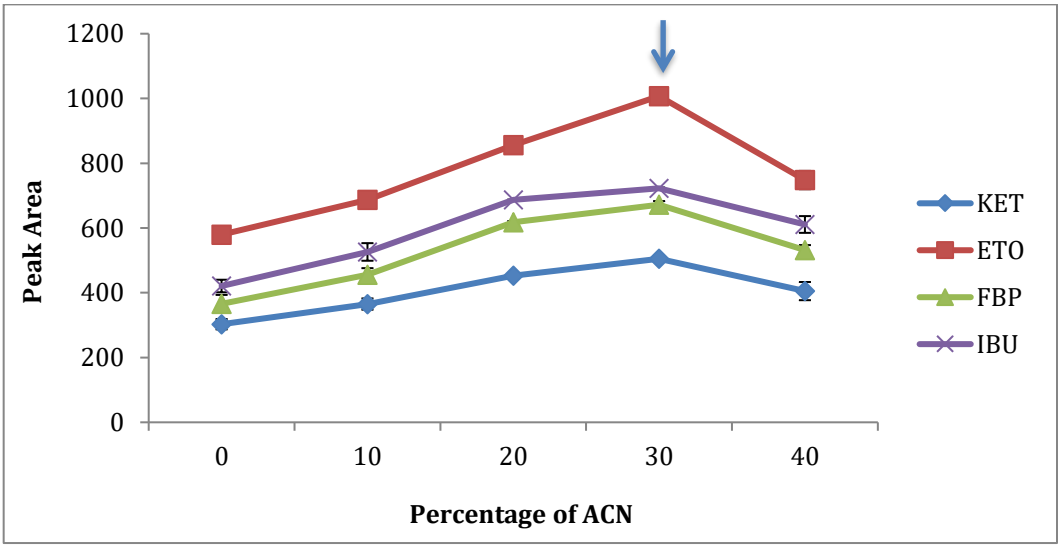


Figure 3.10: Optimization of the percentage of disperser solvent in BES.

3.4.2 Volume of BES

An appropriate volume of BES is required to ensure maximum back-extraction of the analyte. The volume of BES was varied from 50 to 250 µL with maximum extraction efficiency being observed at 100 µL as shown in **Figure 3.11**. Above this volume, further increase in BES did not increase the extraction of the analytes, but rather resulted in dilution of the analytes, which explained the reduction in the extraction efficiency beyond the optimum volume. Therefore, 100 µL of BES was taken as the optimum volume.

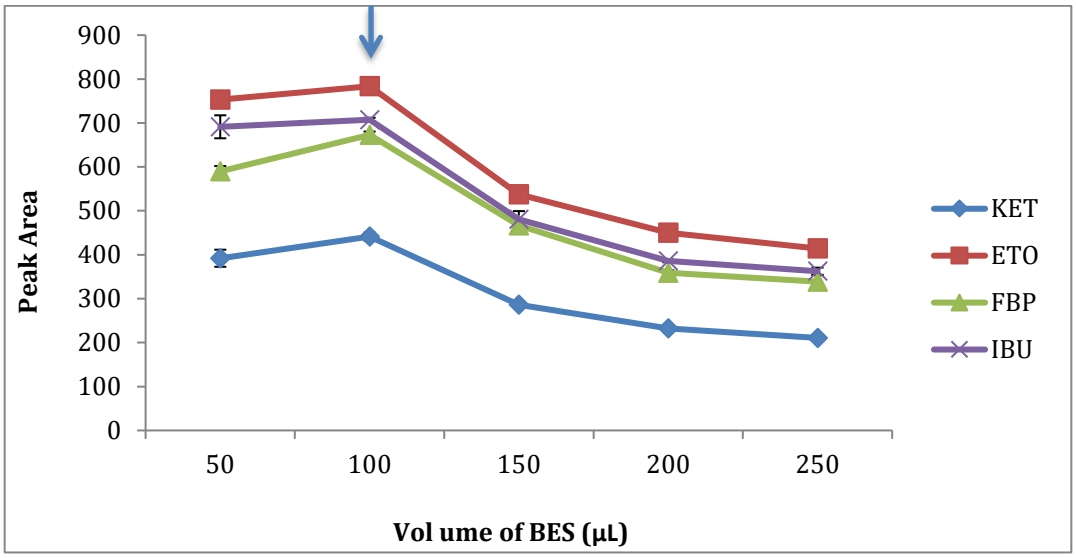


Figure 3.11: Optimization of the volume of BES.

3.4.3 Back-extraction time

Just as in the case of DLLME, the vortex time is equivalent to the extraction time. 60 s were enough to ensure maximum back-extraction efficiency of the analyte, above which no significant improvement was made in extraction efficiency as shown in **Figure 3.12**. Hence, 60 s was taken as the optimum back-extraction time.

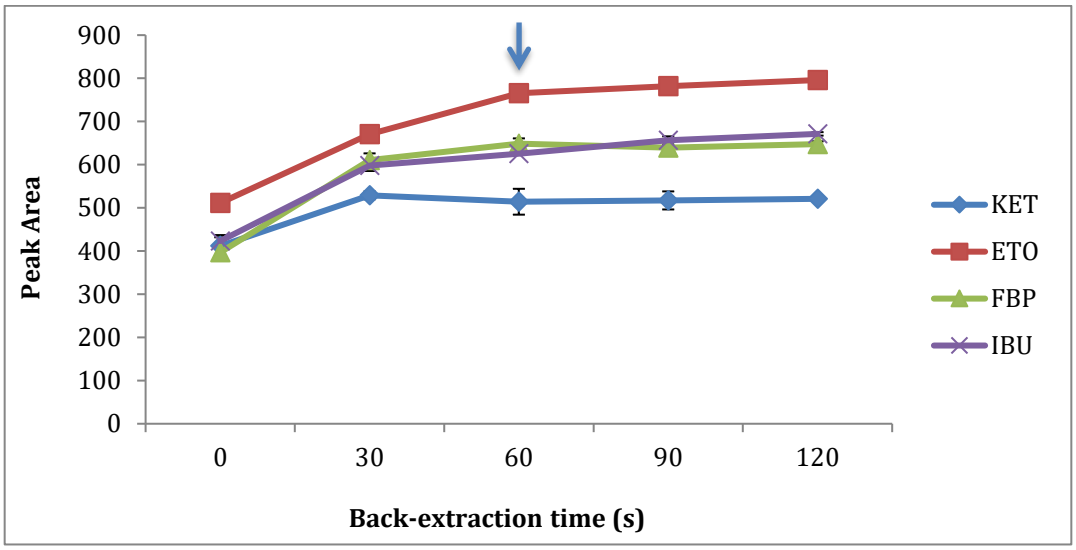


Figure 3.12: Optimization of Back extraction time.

3.5 Optimum DLLME-BE

The optimum DLLME-BE conditions are summarized in **Table 3.1.**

Table 3.1: Optimum DLLME-BE conditions.

DLLME	Extraction solvent	Chloroform
	Volume of extraction solvent	200 µL
	Disperser solvent	ACN, 250 µL
	Salt addition	NaCl, 3% (w/v)
	Acidification	with phosphoric acid, 100 µL
	Extraction time	30 s
BE	Back-extraction solution	30/70 (%, v/v) ACN/ 1.0 M NaOH
	Volume of back-extraction solution	100 µL
	Back-extraction time	60 s

3.6 Optimization of HPLC Parameters

3.6.1 Retention factor (k')

The retention factor (k') is an important parameter that is used to describe the migration rate or the degree to which the analyte is retained in the column. A short k' implies that the analyte elutes very fast. A high k' value, on the other hand, indicates that the analyte is strongly retained in the column. Generally, a value within the rage of 2-10 for k' is acceptable and ideally between 5 and 7. Two among other HPLC parameters that affect k' were monitored to find its optimum. These parameters were the MP composition and column temperature.

3.6.1.1 Effect of MP composition on k'

The effect of the percentage of ACN in the MP was monitored. k' of each analyte was calculated separately at different compositions of ACN/H_2O. The average k' (k'_{av}) of the four analytes at each composition was also calculated to be used for the final decision. The MP composition was not made below 40% organic to avoid damaging the column. At 40% ACN, k'_{av} was calculated as 5.82, which was an ideal condition despite the fact that analysis time (i.e., the time it took for the last analyte to be eluted from the column) was 28 min. Increasing the composition of ACN decreased k' and analysis time but priority was given to k' because other parameters were thought to reduce the analysis time. A MP composition of 40% ACN was taken as optimum. The effect of percentage of ACN in the MP is shown in **Figure 3.13**.

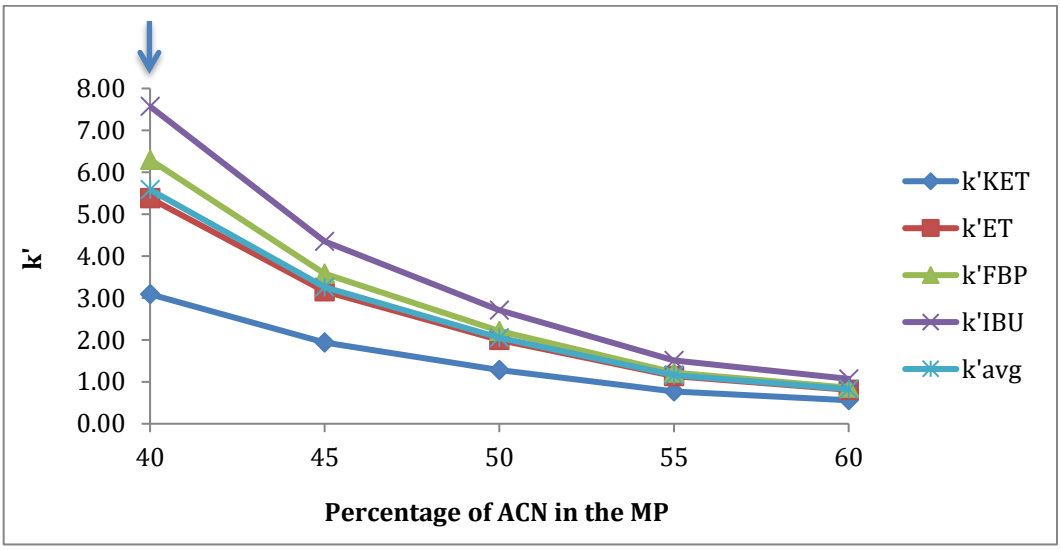


Figure 3.13: Concentration of ACN in the MP.

3.6.1.2 Effect of temperature on k'

Temperature helps to reduce diffusion in the column by decreasing the viscosity of the MP and also reducing back-pressure. As a rule of thumb, it is advised to perform experiments at as high temperature as the column permits and if the analytes are thermally stable. k'_{av} decreased upon increasing the temperature as shown in **Figure 3.14** and the analysis time greatly decreased from 28 min at 20 ^{0}C to 22 min at 40 ^{0}C. Moreover, there was reduction in back-pressure. 40 ^{0}C was taken as the optimum column temperature as k'_{av} was still within the ideal range and above 40 ^{0}C, the lifetime of the column can be reduced.

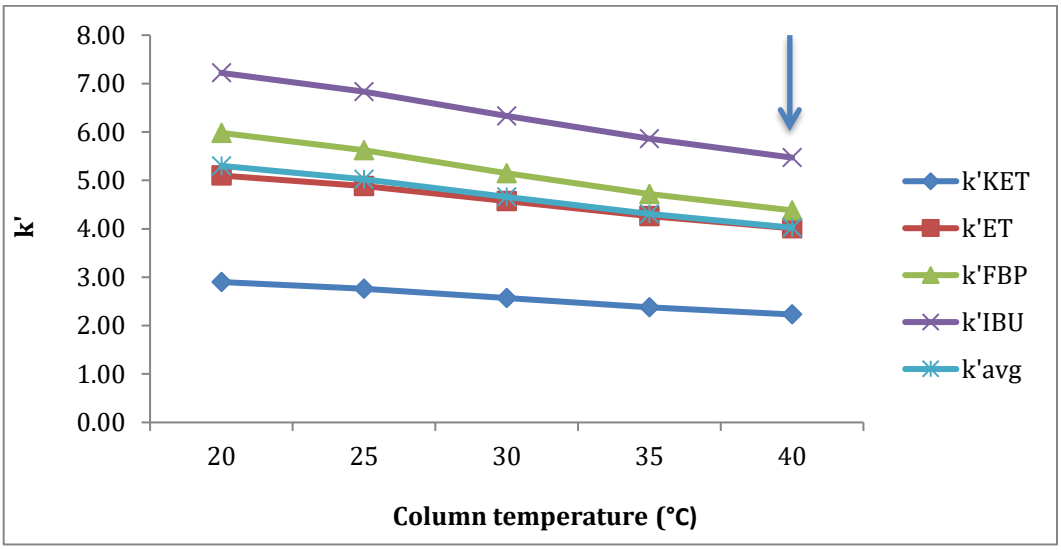


Figure 3.14: Optimization of Column temperature.

3.6.2 Flow rate

The flow rate has a direct effect on efficiency (N). The flow rate can significantly reduce the analysis time but also affect resolution. Corrected peak area is a useful tool obtained by dividing the peak area by t_R for each analyte and plotting this ratio against the flow rate. This factor accounts for the effect of flow rate on both t_R and resolution. The optimum flow rate is then taken as the point where a constant trend is obtained even with increase in flow rate in this case at 0.8 mL min^{-1} as shown in **Figure 3.15**.

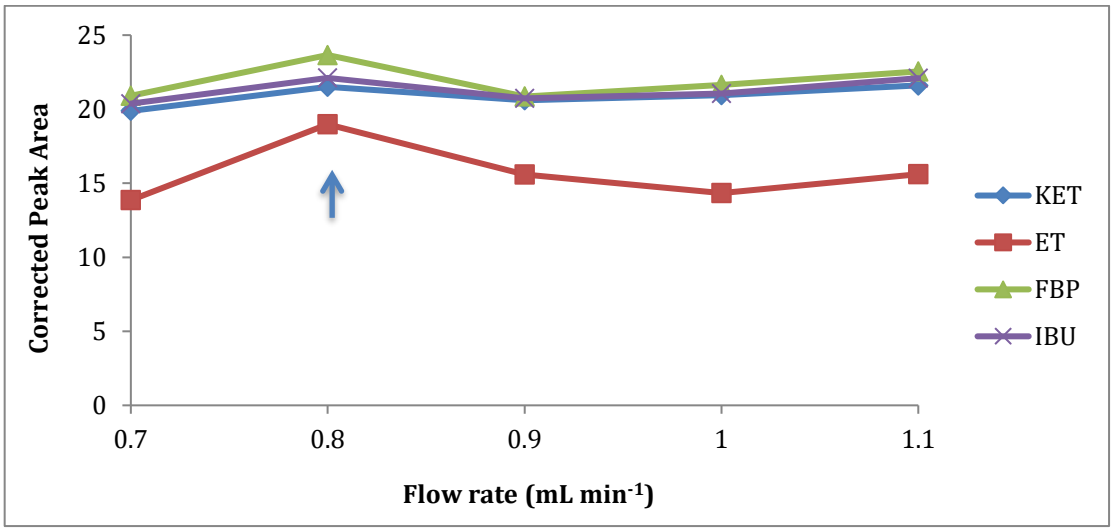


Figure 3.15: Optimization of flow rate.

3.7 Optimum HPLC Conditions

The optimum HPLC conditions are summarized in **Table 3.2.**

Table 3.2: Optimum HPLC Conditions.

Physical parameters	Column	Grom-Sil 80 Octyl-4 FE, 4.6 mm ID x 250 mm (3 µm)
	Flow Rate	0.8 $mL\ min^{-1}$
	Temperature	40 $^{\circ}$C
	Detector/wavelength	DAD, 256 for KET, 224 for ET, 246 for FBP and 230 nm for IBU
	Injection volume	20 µL
Chemical parameters	Mobile phase	40:60 (%, v/v), ACN: 1.0 % TFA in H_2O

3.8 Peak Characterization

An individual standard of each analyte was prepared in the MP at a concentration of 5 mg L^{-1} and was injected under the optimized HPLC conditions to compare its retention time with the retention times in the chromatogram obtained for the mixed standard solution as a means of identifying the peaks. The resulting chromatograms are shown in **Figure 3.16**. The order of elution was KET, ET, FBP, and IBU.

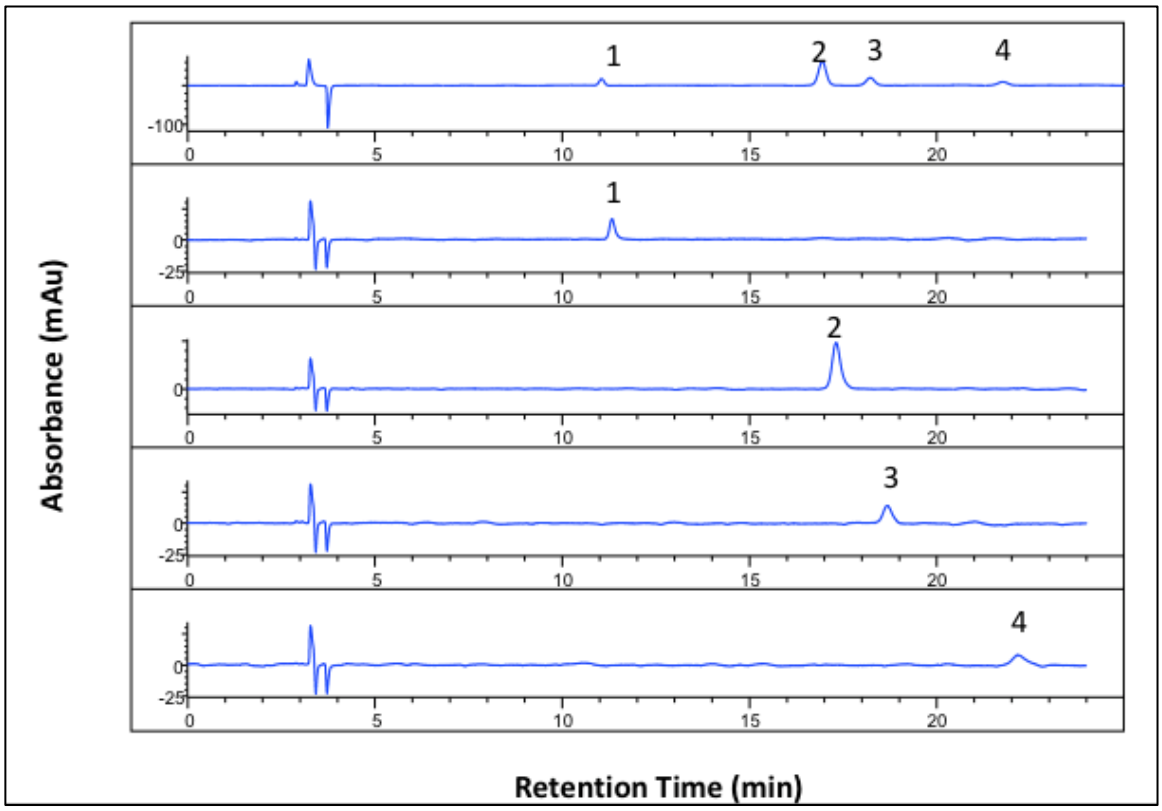


Figure 3.16: Peak Characterization; Peaks: 1, KET; 2, ET; 3, FBP; 4, IBU.

3.9 Calibration, Quantitation and figures of merit

The optimized RP-HPLC conditions were used to plot aqueous external calibration graphs using standard solutions of target NSAIDs to evaluate the performance of the

method. Individual stock solutions of the standards was prepared in 10 mL ACN, with a concentration of 1000 mg L^{-1}. An aqueous calibration curve was plotted by preparing various concentrations of analytes in BES as shown in **Figure 3.17**. From the calibration graph, the sensitivity of each analyte can be deducted from the slope. ET had the highest sensitivity while IBU had the lowest. The graph showed good linearity with R^2 equal to or greater than 0.99773 with a linearity range from 2.07 to 150 mg L^{-1} for each analyte.

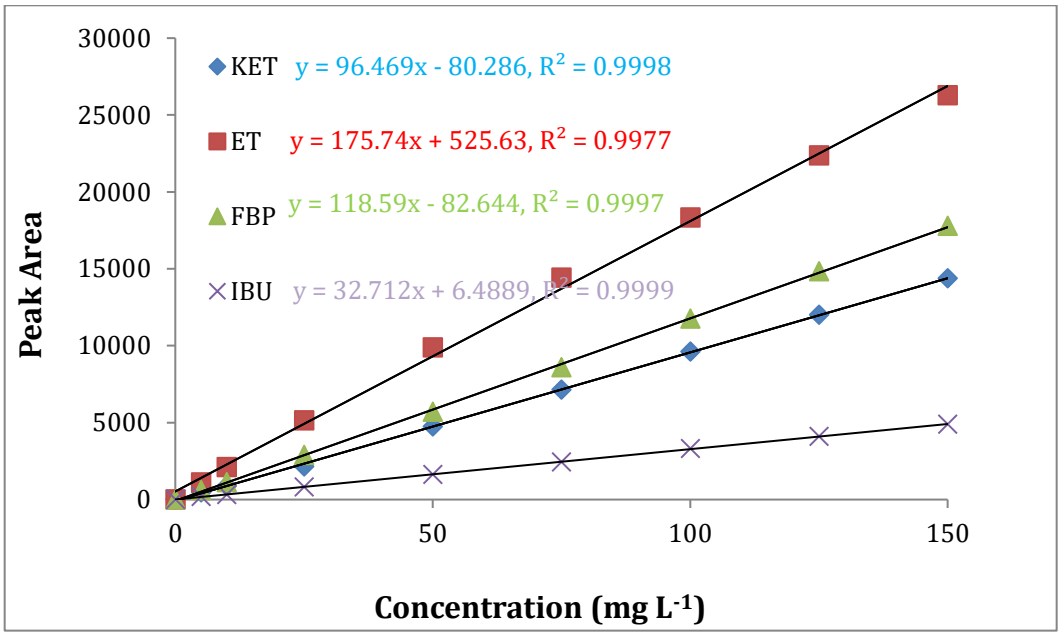


Figure 3.17: Aqueous external calibration graph.

A matrix-matched calibration graphs were also prepared for each sample by spiking known concentrations of the standard in the mother milk sample void of the analyte and carrying out the optimized DLLME-BE-HPLC procedure. This helped to evaluate the applicability, recovery and possible matrix effect on the proposed method. By comparing the aqueous calibration with the matrix-matched calibration graph, it was observed that the slope of the matrix-matched calibration curve was higher than that of aqueous calibration indicating higher sensitivity. This was attributed to the pre-concentration effect of DLLME when applied to real samples, being one of the strengths of this method. The three matrix-matched calibration graphs for the three different

samples indicated no matrix effect, which implies that this method can be applied to different samples without any variation of the procedure, another great strength of the proposed method. Matrix-matched calibration graphs are shown in **Figure 3.18** for 12-month milk sample, **Figure 3.19** for 2-month milk sample, and **Figure 3.20** for 6-month milk sample.

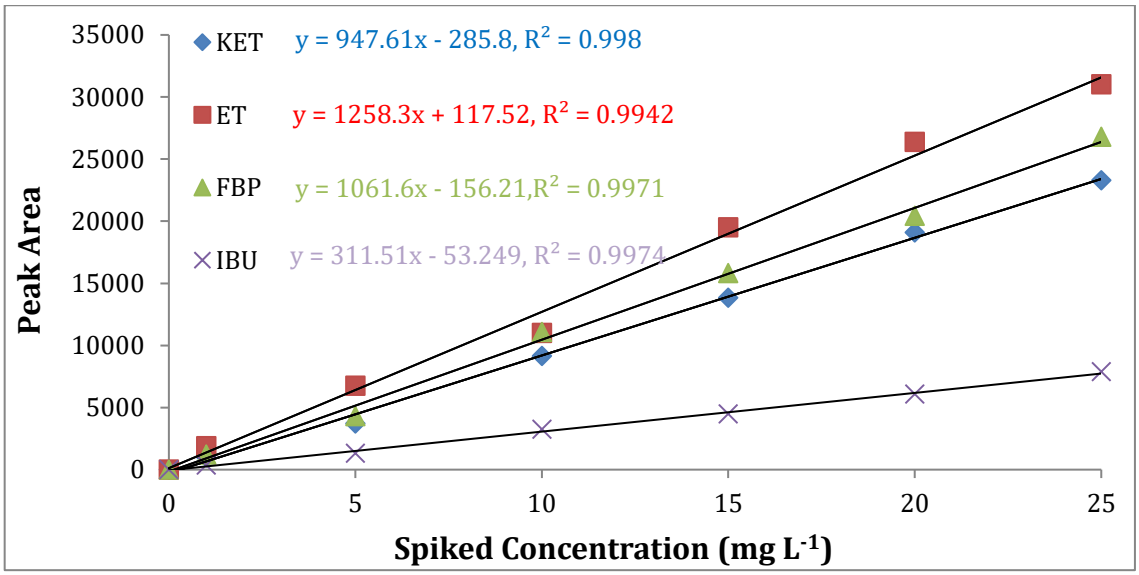


Figure 3.18: Matrix-matched calibration graph for 12-month milk sample.

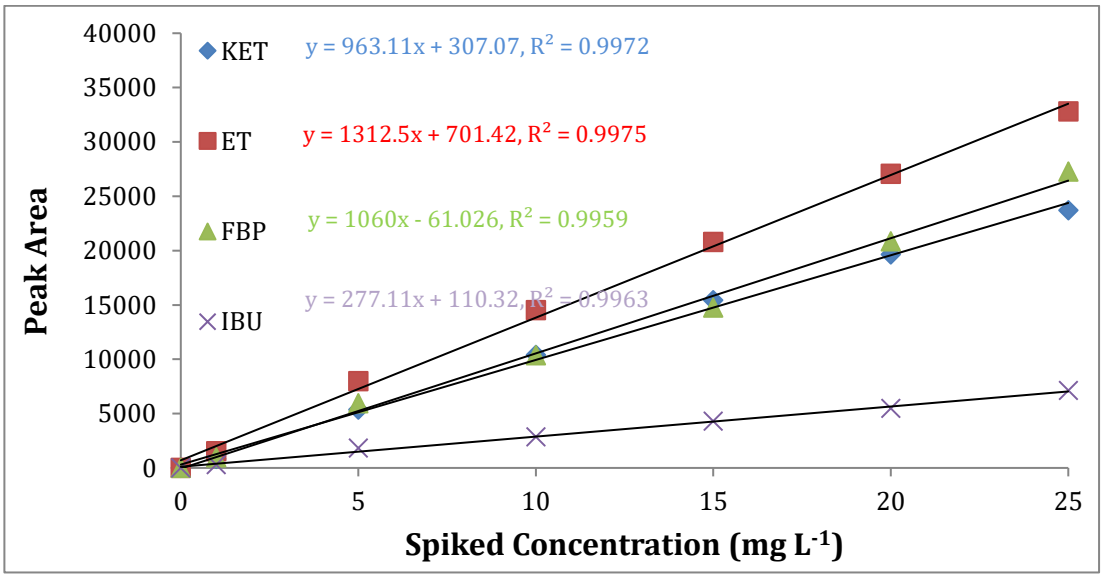


Figure 3.19: Matrix-matched calibration graph for 2-month milk sample.

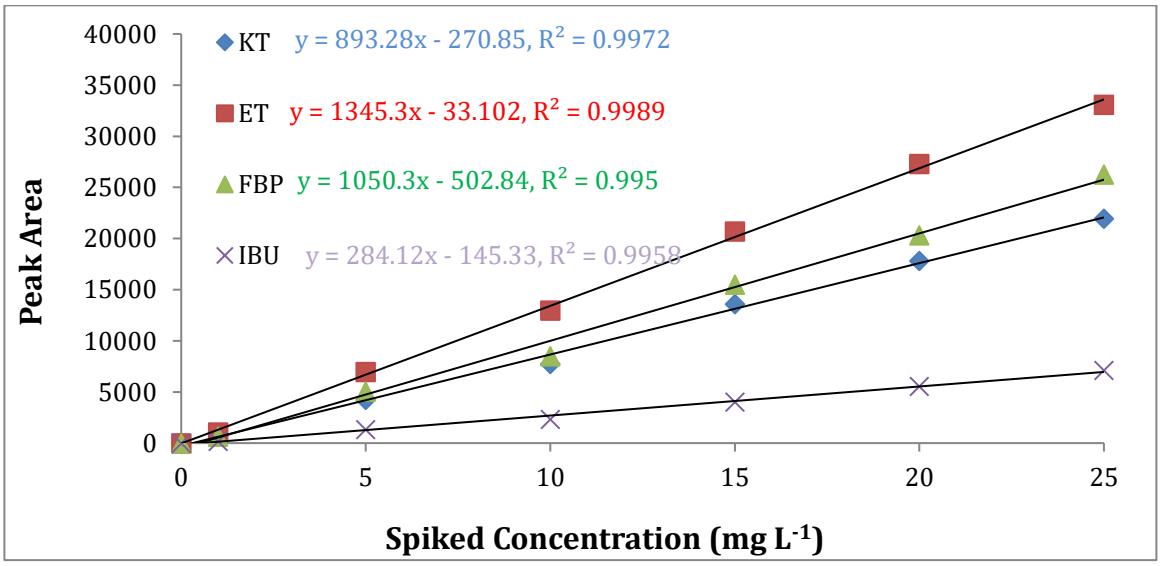


Figure 3.20: Matrix-matched calibration graph for 6-month milk sample.

From the regression equations, the limit of detection (LOD) was calculated for each analyte and aqueous calibration curve based on $3s_b/m$, where, s_b is the standard deviation of the intercept and m is the slope of the regression equation. The LOD was lower for the matrix-matched calibration graphs for the analytes than those obtained

with external aqueous calibration graphs. LODs ranged from 0.62 to 1.95 mg L^{-1} for the aqueous calibration curve and from 0.20 to 0.85 mg L^{-1} for the matrix-matched calibration graphs. Limit of quantitation (LOQ), calculated based on $10s_b/m$, ranged from 2.07 to 6.50 mg L^{-1} for external aqueous calibrations and 0.67 to 2.83 mg L^{-1} for matrix matched-calibrations. The coefficient of determination (R^2) was greater than 0.9977 and 0.9950 for external aqueous and matrix-matched calibrations, respectively. Figures of merit are summarized in **Table 3.3**. The method showed good reproducibility and repeatability as indicated by the intraday precision with percentage relative standard deviation (%RSD) ranging from 1.1 to 5.5 and interday precision ranging from 2.0 to 10.9. Enrichment factors (EF) were in the range of 7.2 to 10.0 with a %RR ranging from 93.1 to 104.6 % as shown in **Table 3.4**.

Table 3.3: Figures of merit of external aqueous and matrix-matched calibration methods.

Sample	Analyte	Regression equation[(a)]	R^2	LOD[(b)] (mg L^{-1})	LOQ[(c)] (mg L^{-1})	LDR[(d)] (mg L^{-1})
Aqueous	KET	$y = 96.5(\pm 0.29)x - 80.3(\pm 23.25)$	0.9998	0.62	2.07	2.07 – 150
	ET	$y = 175.7(\pm 1.68)x + 525.64(\pm 19.8)$	0.9978	1.95	6.50	6.50 – 150
	FBP	$y = 118.6(\pm 0.49)x - 82.6(\pm 38.91)$	0.9998	1.19	3.97	3.97 – 150
	IBU	$y = 32.7(\pm 0.09)x + 6.5(\pm 7.00)$	0.9999	0.78	2.60	2.60 – 150
12 months	KET	$y = 947.6(\pm 9.62)x - 285.8(\pm 134.8)$	0.9980	0.32	1.07	1.07 – 25
	ET	$y = 1265.1(\pm 11.47)x + 246.89(\pm 144.8)$	0.9981	0.29	0.97	0.97 – 25
	FBP	$y = 1058.0(\pm 13.84)x - 91.2(\pm 171.12)$	0.9971	0.58	1.93	1.93 – 25
	IBU	$y = 311.5(\pm 4.63)x - 53.2(\pm 64.87)$	0.9974	0.67	2.23	2.23 – 25
6 months	KET	$y = 893.3(\pm 10.80)x - 270.85(\pm 151.36)$	0.9972	0.38	1.27	1.27 – 25
	ET	$y = 1344.5(\pm 8.72)x - 19.3(\pm 107.83)$	0.9989	0.20	0.67	0.67 – 25
	FBP	$y = 1038.7(\pm 18.24)x - 293.4(\pm 225.51)$	0.9951	0.78	2.60	2.60 – 25
	IBU	$y = 284.12(\pm 5.33)x - 145.39(\pm 74.73)$	0.9958	0.85	2.83	2.83 – 25
2 months	KET	$y = 963.1(\pm 11.79)x + 307.1(\pm 165.24)$	0.9972	0.39	1.30	1.30 – 25
	ET	$y = 1328.6(\pm 13.34)x + 409.3(\pm 164.95)$	0.9975	0.31	1.03	1.03 – 25
	FBP	$y = 1058.6(\pm 16.35)x - 35.6(\pm 202.13)$	0.9959	0.69	2.30	2.30 – 25
	IBU	$y = 277.1(\pm 4.86)x + 110.3(\pm 68.09)$	0.9963	0.79	2.63	2.63 – 25

[a] $Peak\ area = slope(\pm SD) * concentration(mg\ L^{-1}) + intercept(\pm SD)$
[b] Limit of detection
[c] Limit of quantitation
[d] Linear dynamic range

Table 3.4: Figures of merit showing %RSD, EF and %RR.

Sample	Analyte	%RSD[(a)]		EF[(b)]	%RR
		Intraday	Interday		
Aqueous	KET	1.6	4.8	-	-
	ETO	4.4	7.6	-	-
	FBP	1.1	2.0	-	-
	IBU	1.1	2.4	-	-
12 months	KET	4.0	10.7	9.8	99.2
	ETO	5.0	10.7	7.2	101.4
	FBP	5.1	10.9	8.9	100.3
	IBU	5.2	10.0	9.5	96.7
6 months	KET	3.0	6.8	9.3	103.5
	ETO	2.6	3.6	7.7	102.7
	FBP	3.7	7.8	8.8	101.5
	IBU	4.4	9.2	8.7	97.9
2 months	KET	3.5	5.4	10.0	104.6
	ETO	5.0	10.5	7.6	102.3
	FBP	5.3	9.7	8.9	93.1
	IBU	5.1	13.5	8.5	100.5

[a]Percentage relative standard deviation $(n = 3)$
[b]Enrichment factor, calculated as the ratio of slope of matrix-matched calibration to that of external aqueous calibration.

Representative chromatograms obtained with real samples for 12-month, 6-month and 2-month milk samples are given in **Figure 3.21**. The spiked chromatogram is given at the top, while the unspiked chromatogram is given at the bottom. Because the mothers were not taking any treatment with NSAIDs, the analytes were not detected in the unspiked samples. But, spiked concentration below 15.0 mg L^{-1} could be detected by the method indicating the high sensitivity of the method as further highlighted by the low LODs. The clean baseline for all chromatograms highlights the selectivity and high efficiency of DLLME-BE for sample clean-up.

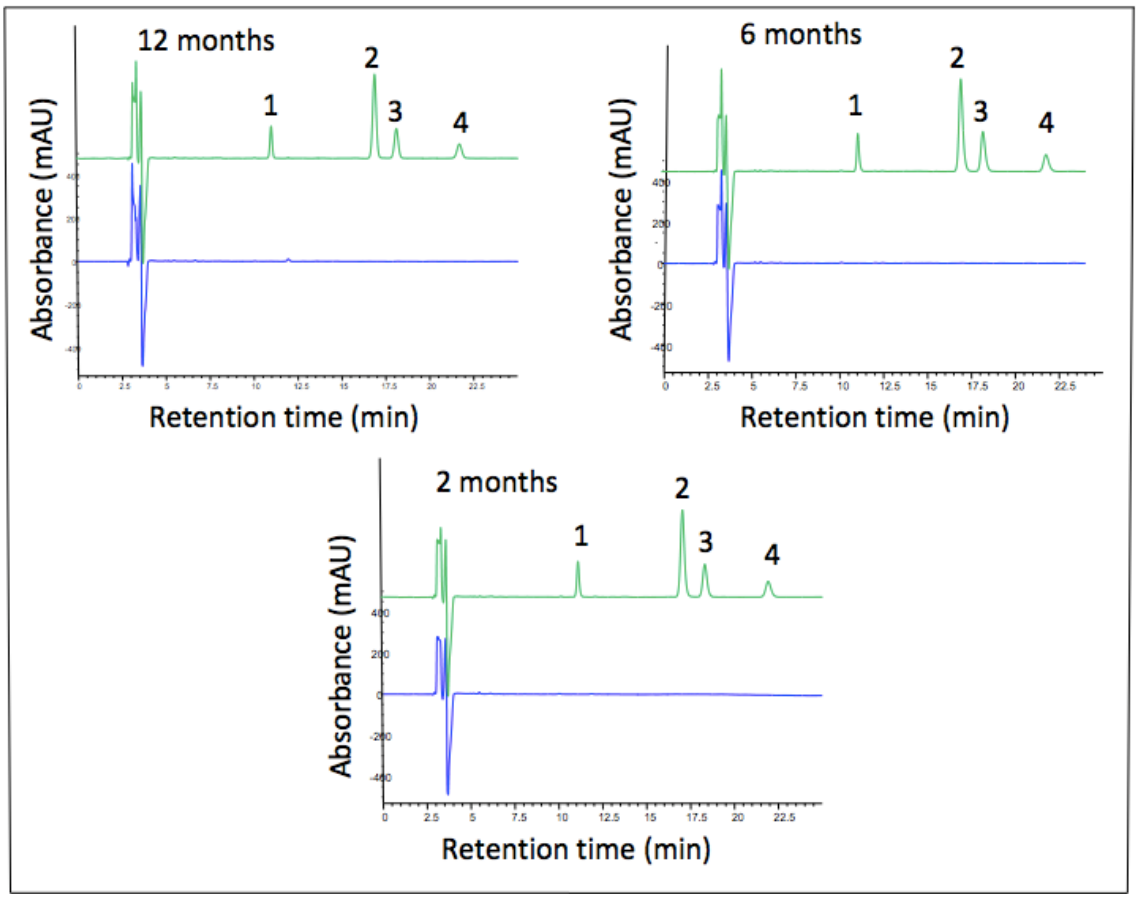


Figure 3.21: Representative chromatograms of extracted samples under optimized DLLME-BE-HPLC conditions.

Top; spiked. Bottom; unspiked. Peaks: 1, KET; 2, ET; 3, FBP; 4, IBU. HPLC conditions; Grom-Sil 80 Octyl-4 FE Reversed phase column; mobile phase 40:60 (%, v/v), ACN: 1.0 % TFA in H_2O; 0.8 $mL\ min^{-1}$ flow rate; 40 ˚C column temperature; 20 µL injection volume; DAD detector at 230 nm wavelength.

3.10 Pharmacokinetic Study

Pharmacokinetic study was also carried out through a 30-year-old volunteer (12-month breastfeeding) by taking a tablet containing 200 mg of FBP and sampling the milk at different time intervals to be analyzed for checking the variation in the concentration of

the analyte in the mother milk against time. The analyte was detected in the milk sample at 30 min with a concentration of 0.16 mg L^{-1}. The concentration continued to increase with time until it reached a maximum concentration of 0.39 mg L^{-1} after 150 min of administration, then started to decrease until it was not detected at 225 min as shown in **Figure 3.22.** This study is significant as it shows the duration of the analyte in the mother milk after administration of the drug. It is valuable information to nursing mothers to know the time interval they can breastfeed their babies after administrating the drug, in this case 225 min.

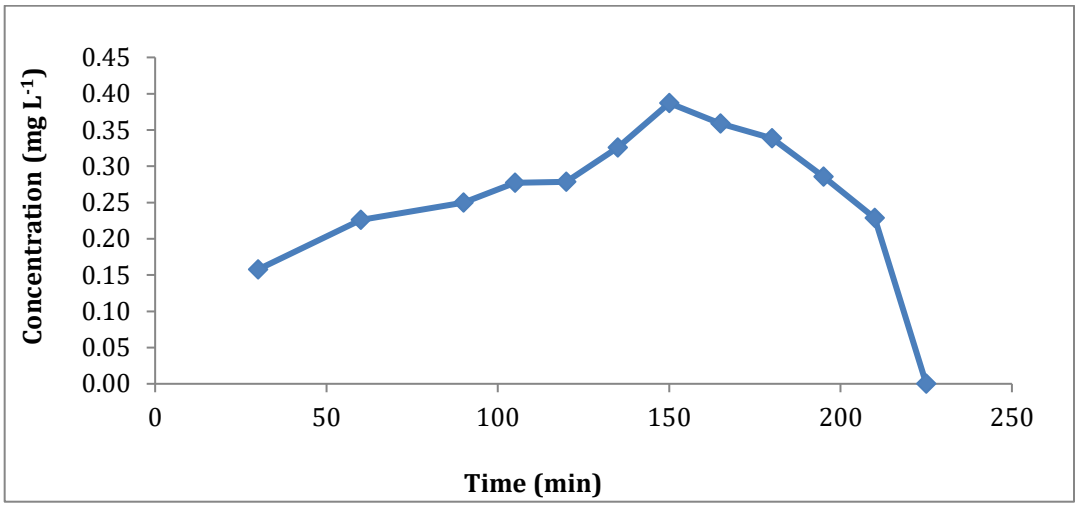


Figure 3.22: Pharmacokinetic study of FBP in a milk sample of 12-month breastfeeding.

3.11 Comparison with other studies

This study was compared with other similar studies from the literature to appreciate the strength of this method. The extraction time is least for this study at 90 s with the closest time being 2 min in a study conducted by Alshana and coworkers [38] employing DLLME-FASS-CE, further proving the rapidity of DLLME. In terms of volume of organic solvent, this study employed an amount of organic solvent of 4.20 mL, the least being 50 µL in a study by Yong Byoung Chan and coworkers [36] using hollow fiber-liquid phase microextraction (HF-LPME). However, HF-LPME generally requires much longer time

(i.e., 45 min in this case) and special instrumentation unlike DLLME, the implication being higher capital cost. In terms of sensitivity, a fair comparison will take into consideration the type of detector used, and of the studies considered; two studies used a UV detector [36,38]. The LOD of our proposed method is lower at 0.2-1.95 mg L^{-1} compared with 5-15 ng mL^{-1} of that study, indicating the superior preconcentration capability of DLLME over HF-LPME. The other studies used MS detectors and it is expected that they provide lower LODs. The study by Alshana and coworkers [38] combined the pre-concentration capability of DLLME with the online preconcentration of FASS-CE with the result being lower LOD than this study despite the fact that a DAD detector was used.

The combination of speed, sensitivity, low volume of organic solvent used for this DLLME-HPLC-DAD method has placed our proposed method shoulder to shoulder with similar studies if not better. All data of comparison is given in Table 3.5.

Table 3.5: Comparison between related studies with this study.

Sample	Analyte	Extraction/ Instrumentation	Extraction time	Volume of org. solvent	LOD	Ref.
Urine	FBP, NAX, KET, DLF, IBU. TLF, MFN	HF-LPME-HPLC-UV	45 min	50 µL	5-15 ng mL^{-1}	[36]
Bovine milk	PBZ, OPB, NP, MF, VDP, FLU, TLF, MLX, DC, CPF, KTP, FLU-OH	LLE-LC-MS/MS	10 min and 1.5 hours evaporation	8 mL	0.1-3.43 µg L^{-1}	[7]
Bovine milk and dairy products	ET, NAX, KTP, DIC, FBP	DLLME-FASS-CE	2 min	4.15 mL	3-13.1 µg kg^{-1}	[38]
Mother milk	KET, ET, FBP, IBU	DLLME-HPLC-DAD	90 s	4.20 mL	0.2-1.95 mg L^{-1}	This study

CHAPTER 4
CONCLUSION AND RECOMMENDATION

The result of this research is a testament of how much dispersive liquid-liquid microextraction (DLLME) followed by a back-extraction step as evolved as a robust, efficient, cheap, simple and relatively green sample pre-concentration and clean-up technique in over a decade of existence.

The total volume of organic solvent used in the extraction method was 4.2 mL with an extraction time of 30 s for DLLME and 60 s for back extraction making a total of 90 s extraction time to obtain a clean baseline in the chromatogram, demonstrating an exception power of this method as a sample clean-up technique. Up to ten times enrichment was obtained that can be attributed to the extraction technique when matrix-matched graphs with mother milk were compared to external aqueous calibration graphs. The %RSD below 13.5 % demonstrated a good repeatability of the method. The method is sensitive with an LOD ranging from 0.2 to 1.95 mg L^{-1} which was low enough to detect the analytes in milk through a pharmacokinetic study, showing that this method can be used to monitor patients under treatment.

The proposed DLLME-BE-HPLC method offered numerous advantages, such as simplicity, low cost, ease of operation, use of small amounts of organic solvents and high enrichment factors within a very short analysis time. Despite the complexity of the matrices studied, good recoveries, high reproducibility and interference-free chromatograms were achieved. The results indicates that this method could be of great interest, especially for determination of NSAIDs in human milk in routine analytical laboratories and health centers.

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