Development and validation of a HPLC-MS/HRMS method for the screening of NSAIDs in oral fluid and dried blood spots : application on the study of the prevalence of consumption by ultratrail runners

Mohammad Shafiq Mashal

THESE de DOCTORAT DE L'UNIVERSITE CLAUDE BERNARD LYON 1

Ecole Doctorale N° 205
(Ecole Doctorale Interdisciplinaire Sciences Santé)

Discipline : Pharmacologie-Toxicologie

Soutenue publiquement le 29/09/2022, par :
Mohammad Shafiq MASHAL

Développement et validation d'une méthode HPLC-MS/HRMS pour le criblage d'AINS dans la salive et le sang capillaire. Application à l'étude de la prévalence des AINS dans l'ultratrail.

Devant le jury composé de :

Pr Pelissier Alicot Anne-Laure, Faculté de Médecine, Aix Marseille Université, Président.e, Rapporteure
Pr Coudoré François, Faculté de Pharmacie, Université Paris Saclay, Rapporteur
Dr Gaulier Jean-Michel, CHU de Lille, Rapporteur
Dr Marcel Chatelain Dominique, Faculté de Pharmacie, UCB Lyon1, Examinatrice

Pr Guitton Jérôme, Faculté de Pharmacie, UCB Lyon1, Directeur de thèse
Dr Machon Christelle, Faculté de Pharmacie, UCB Lyon1, Co-directrice de thèse

Résumé

Les anti-inflammatoires non stéroidiens (AINS) sont l'un des médicaments les plus utilisés dans le monde, accessibles sur ordonnances ou en vente libre. Plusieurs articles ont fait état d'une forte consommation d'AINS par des coureurs pendant les ultratrails. Plusieurs études montrent qu'environ 50% des coureurs utillisent des AINS sans ordonnance médicale, sans forcément connaître leurs effets indésirables et leur toxicité.

A ce jour, seulement des données basées sur des questionnaires ont été rapportées sur la prévalence des AINS chez les ultratrailers. Or, en raison de la méconnaissance des coureurs concernant les AINS, l'utilisation du questionnaire peut entraîner des résultats faux positifs ou faux négatifs.

Dans cette étude, nous avons étudié la prévalence des AINS chez les utratrailers via des prélèvements de salive et sang capillaire. Des protocoles de préparation d'échantillon et une méthode LC-MS/HRMS ont été développés puis validés pour 19 AINS dans la salive et pour 18 AINS dans le sang capillaire. La méthode a été appliquée avec succès sur des échantillons collectés auprès de participants de l'Ultratrail du Mont Blanc® (UTMB®) 2021. Les résultats ont montré une prévalence des AINS supérieure dans les prélèvements de sang capillaire (46,6%) que dans les prélèvements salivaires (18,5%) ainsi qu'à partir des données du questionnaire (13,8%).

En conclusion, ce travail est le premier rapportant l'identification et la quantification d'autant d'AINS dans la salive et le sang capillaire. De plus, l'étude menée lors de l'UTMB® 2021 a montré que la recherche d'AINS dans le sang capillaire est le moyen le plus pertinent pour étudier la prévalence dans l'ultratrail.

Mots clés :
Anti-inflammatoires non stéroidiens, LC-HRMS, salive, sang capillaire, ultratrail

Intitulé et adresse du laboratoire de recherche : Laboratoire de Pharmaco-toxicologie, Hospices Civils de Lyon, Centre de Biologie Sud, 69495 Pierre-Bénite Cedex

Development and validation of a HPLC-MS/HRMS method for the screening of NSAIDs in oral fluid and dried blood spots. Application on the study of the prevalence of consumption by ultratrail runners.

Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most used drugs worldwide accessible through medical prescription or over-the-counter (OTC). Several published articles have reported a high consumption of NSAIDs by runners during ultratrail. Pain relief and prevention are the main reasons for these drugs consumption by runners. Several studies reported that around 50% runners used NSAIDs without medical prescription, these data show that half of runner may have low information or even lack of information about NSAIDs adverse effects. Up to date, only questionnaire-based data reported about NSAIDs prevalence by the runners. Therefore, using questionnaire may not provide the right information and leads to false positive or false negative results.

In this study, we used oral fluid (OF) and dried blood spots (DBS), as biological samples for the study of NSAIDs consumption prevalence by the runners. Sample preparation workflows and a LC-MS/HRMS analytical method were developed and validated according to European Medicines Agency guideline for 19 and 18 different NSAIDs in OF and DBS, respectively. The method was successfully applied on OF and DBS samples collected from Ultramarathon du Mont Blanc® 2021. Results showed a higher prevalence of NSAIDs using DBS (46.6%) than OF (18.5%) and questionnaire (13.8%).

To conclude, this work is the first one that reported a LC-MS/HRMS method for identification and quantification of as many NSAIDs in oral fluid and dried blood spots. Moreover, the prospective study conducted on UTMB® 2021 showed that the analysis of drugs directly in DBS is the most relevant tool to determine the prevalence in ultratrail events.

Keywords:
Non-steroidal anti-inflammatory drugs, LC-HRMS, oral fluid, dried blood spots, ultratrail

Résumé substanciel

La pratique d'efforts physiques intenses et longs en continu (pouvant aller bien au-délà de 24 h) dans le cadre d'ultratrails peut conduire chez les coureurs à differents troubles physiques. En effet divers biomarqueurs indiquent que l'organisme subit des aggressions tels que réduction de la fonction rénale (élévation de la créatinine plasmatique), dommages musculaires (élévation de la créakine kinase), souffrance cardiaque (élévation de la troponine), dommages hépatiques (élévation des enzymes hépatiques) et perturbation des troubles hydroélectrolytiques tels que la déshydratation ou l'hyponatrémie (si l'apport en sel est insuffisant par rapport à l'apport en eau). Dans la grande majorité des cas, ces troubles disparaissent après quelques jours de repos. Les anti-inflammatoires non stéroïdiens (AINS) sont une famille de médicaments qui peuvent être utilisés pour la prévention des douleurs musculaires, le soulagement de la douleur, des courbatures, la réduction des phénomènes inflammatoires qui sont des situations fréquement rencontrées dans la pratique de l'ultratrails. Il n'est donc pas étonnant que de nombreuses études rapportent une prévalence élevée de la consommation d'AINS pendant les ultratrails. Dans ces études, la prévalence est mesurée à l'aide de questionnaires réalisés auprès des ultratraileurs.

L'objectif de ce travail de thèse était de mettre au point des méthodes analytiques permettant de déterminer la présence d'AINS dans des fluides biologiques de manière à disposer d'une mesure directe de la prévalence de la consommation d'AINS en ultratrails. La salive et le sang capillaire ont été utilisés pour faciliter le receuil des matrices biologiques.

La partie bibliographique rapporte les données concernant les AINS (pharmacocinétique, toxicité, prévalence de leur consommation en ultratrail et ses conséquences) ainsi que les méthodes de dosages dans les fluides biologiques.

La partie expérimentale se décompose en 2 principales parties : le développement de 2 méthodes analytiques de dosage de près de 20 AINS à partir de prélèvements salivaires et de sang capillaire, puis une partie application proprement dite dans laquelle les résultats obtenus à partir de prélèvements effectués au cours de l'UTMB® 2021 sont présentés et discutés. Les applications de ce travail de thèse se sont faites dans le cadre du programme QUARTZ Elite qui a pour objectif de promouvoir la philosophie d'un Sport sans médicament et de permettre ainsi de contribuer à un Sport sans dopage. Ce programme consiste en un suivi biologique et toxicologique unique destiné en premier lieu à protéger la santé du Sportif. Il a été développé par l'Association « Athletes For Transparency » (AFT). Des prélèvements salivaires et de sang capillaire ont été réalisés à l'arrivée de différentes courses de l'UTMB®. Une autorisation auprès d'un Comité de Protection des Personnes a été obtenu préalablement pour l'utilisation des résultats.

Les dosages des AINS ont été réalisés avec un appareil de chromatographie liquide couplé à un spectromètre de masse haute résolution : Ultimate 3000 system et un Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific™,) equipé d'une source d'ionisation « electrospray ». Pour le développement de la technique de chromatographie liquide, nous avons testé trois colonnes différentes (Accucore RP-MS, Accucore AQ et Accucore Phenyl-hexyl), ainsi que plusieurs compositions de phases mobiles. En ce qui concerne la partie spectrométrie de masse, les AINS ont été analysés avec une ionisation

positive ou négative, en mode « full scan» avec un rapport masse/charge (m/z) de 100 à 650 et une résolution de 35,000, associé au mode « ddMS2 » avec une résolution de 17,500. L'énergie de collision a été optimisée pour chaque AINS. En ce qui concerne les prélèvements salivaires, le dispositif Quantisal a été sélectionné pour sa capacité à collecter un volume adéquat de salive (1 mL ± 10 %), un temps de collecte de salive court (moins de 2 min) et un tampon stabilisateur pour le transport et le stockage des échantillons. Le tampon Quantisal a cependant été remplacé par un mélange contenant de l'acétonitrile et de l'eau (50/50) et 2% d'acide acétique. Ceci a permis d'obtenir de meilleurs rendements d'extraction et un effet matrice globalement plus faible. La préparation d'échantillon est basée sur une extraction liquide/liquide réalisée avec deux fois 500 µL de chloroforme. Plusieurs conditions d'extraction ont été préalablement testées. La validation la méthode a suivi les recommandations de l'Agence Européenne du Médicament (EMA). Les critères suivants ont été validées pour 19 AINS : sélectivité, précision, justesse, stabilité, limite de détection, limite de quantification, contamination et effet matrice. En ce qui concerne le sang capillaire, celui-ci a été prélevé à l'extrémité du doigt par le dispositif HemaXis DB 10. Ce dispositif permet de prélever exactement 10 µL de sang qui seront déposés sur un papier filtre conduisant à un « spot » de sang qui sera ensuite découpé pour réaliser l'analyse des AINS. La préparation des échantillons fait appel d'abord à la désorption des composés en plongeant le spot de sang dans un mélange d'eau et d'acetonitrile qui est soniqué pendant 15 mn puis une extraction liquide/liquide avec du chloroforme est réalisée. Les conditions chromatographiques et de spectrométrie de masse sont identiques à celles mises au point pour les prélèvements salivaires. Pour quatre AINS, les critères de l'EMA n'ont pu être atteint (acide salicylique, celecoxib, flurbiprofene, acide mefenamic) et une validation qualitative a été retenue. Le paracétamol a également été validé sur des critères qualitatifs.

Concernant la partie application, 81 ultratraileurs (61 hommes et 20 femmes) ayant un âge moyen de 38.8 années (+/- 8.8) ont participé à cette étude. Tous les participants ont accepté le prélèvement salivaire, 80 participants ont répondu au questionnaire leur demandant s'ils avaient pris des AINS durant la course et 73 participants ont accepté le prélèvement capillaire. Dans 15 prélèvements salivaires (18,5 %) et dans 34 prélèvements capillaires (46,6 %), un ou plusieurs AINS ont été identifiés. D'après le questionnaire, 11 coureurs (13,8 %) ont déclaré avoir pris un AINS 24 h avant et/ou pendant la course. Au totalt, des AINS ont été identifiés dans 36 échantillons provenant de 81 coureurs (44,4%). Dans 21 cas, les AINS ont été retrouvés dans le sang, et pas dans la salive. Pour 4 d'entre eux, l'hydroxyibuprofène a été retrouvé dans la salive mais pas l'ibuprofène. Le nombre de cas positifs étaient beaucoup plus élevés dans le sang que dans la salive. Cela peut s'expliquer par le fait que les concentrations sanguines d'AINS sont supérieures aux concentrations salivaires. Comme les AINS présentent un pourcentage de liaison aux protéines plasmatiques d'environ 95-99%, seulement une très petite fraction non liée aux protéines plasmatiques peut passer du sang à la salive. Six AINS différents ont été identifiés dans le sang : l'ibuprofène (n=22), le diclofénac (n=6), le kétoprofène (n=3), l'acide salicylique (n=3), le naproxène (n=2) et l'acide niflumique (n=2). Dans la plupart des cas (80,6 % des cas positifs), un AINS a été observé. Cependant, 2 AINS ont été trouvés pour six coureurs et 3 AINS pour un coureur. Dans la majorité des échantillons, les concentrations retrouvées étaient inférieures aux concentrations maximales après la prise d'une dose thérapeutique. Pour cinq coureurs, les concentrations d'ibuprofène dans le sang étaient inférieures à la limite de quantification (LdQ) mais supérieures à limite de détection (LdD). Il

en était de même pour quatre coureurs pour le diclofénac, pour un coureur pour le kétoprofène et le naproxène et pour deux coureurs pour l'acide niflumique. Le paracétamol a été détecté dans 25 cas (30,9%) dans la salive, dans 22 cas (30,1%) dans le sang et 18 coureurs (22,5 %) déclarent l'avoir pris. Basé sur l'analyse dans le sang, 12 coureurs (16,4%) ont consommé à la fois de l'ibuprofène et du paracétamol. On le voit la prévalence était plus élevée pour les AINS que pour le paracétamol. Ceci est en accord avec les données rapportées précédemment pour des distances similaires d'ultratrails. La prévalence observée pour les AINS (46,6 %) dans notre étude est assez élevée mais reste inférieure à celles rapportées dans la littérature pour des ultratrails de plus de 100 km. La prévalence plus faible des AINS dans la présente étude peut s'expliquer par la mise en place du programme Quartz dans le règlement de l'UTMB®. Les campagnes d'information sur les risques des AINS peuvent également avoir modifié les mentalités. Une autre hypothèse est que d'autres analgésiques puissent être consommés à la place des AINS. Le paracétamol peut être exclu mais le tramadol ou la codéine ne sont pas analysés dans notre travail. Une estimation de l'intervalle de temps maximum (ITM) pendant lequel les AINS pourraient être détectable dans le sang a été proposé en fonction de la LOD obtenue avec le présent travail et en tenant compte de la concentration maximale, de la demi-vie et de la limite de détection analytique. Les ITM étaient : 26 h pour l'ibuprofène, 22 h pour le diclofénac, 28 h pour le kétoprofène, 60 h pour l'acide niflumique et 150 h pour le naproxène. Ainsi, pour les AINS présentant une demi-vie autour de 2 h (ibuprofène, diclofénac, kétoprofène), seule la consommation pendant la course peut être détectée car les coureurs mettent entre 24 et 48 heures pour terminer le parcours de 171 km. L'urine pourrait également être une matrice pertinente puisqu'elle est non invasive et que les médicaments pourraient être détectés dans un intervalle de temps plus long après l'ingestion que dans le sang et la salive. Cependant, certaines limites peuvent également être identifiées, telles que des difficultés de collecte : déshydratation des coureurs, organisation pratique du prélèvement, caractère intrusif de l'échantillonnage.

La prévalence des AINS déterminée à partir des résultats de sang capillaire est proche de 50%, ce qui est élevé au regard des critères du programme Quartz (pas de consommation dans les 24 h avant le départ et pendant la course). Il est donc nécessaire de continuer à informer les coureurs concernant la consommation d'AINS en trail, tant du point de vue de l'éthique que sur les aspects de santé et de risques.

Le travail présenté fait l'objet de 2 articles publiés :

- Simultaneous quantification of 19 nonsteroidal anti-inflammatory drugs in oral fluid by liquid chromatography-high resolution mass spectrometry: Application on ultratrail runner's oral fluid. Mashal MS, Nalin M, Bevalot F, Sallet P, Guitton J, Machon C. Drug Test Anal. 2022 Apr;14(4):701-712. doi: 10.1002/dta.3216.

- Comparative study between direct analysis in whole blood, oral fluid, and declaration of consumption for the prevalence of nonsteroidal anti-inflammatory drugs and acetaminophen in ultratrail runners Mohammad Shafiq Mashal, Fabien Bevalot, Antony Citterio-Quentin, Pierre Sallet, Qand Agha Nazari, Jérôme Guitton, Christelle Machon. Drug Test Anal. 2022 Sep 27. doi: 10.1002/dta.3374. Online ahead of print.

.

Acknowledgments

For sure, words can't express my true feeling and gratitude to all individuals who directly or indirectly contributed to this work.

I would like to thank cordially and express my immeasurable appreciation to my thesis director Pr. Jerome GUITTON, for accepting me to work on this topic, for his kindness, constructive advices and time. My biggest thanks and deepest gratitude to my thesis co-director Dr.Christelle MACHON, for her supports, cooperation, effective and professional advices. In addition of Dr. MACHON great hand on my professional work, she fully supported me in administrative affairs related to my professional and personal life.

I would like to express my deepest gratitude to jury members Pr. François COUDORE, Pr. Anne Laure PELISSIER ALICOT, Dr. Jean-Michel GAULIER and Dr. Dominique MARCEL-CHATELAIN, for their time and effort they spend for evaluating my thesis and giving me the honour of judging my work.

I thank Dr. Fabien BEVALOT from LatLumTox laboratory and Dr. Pierre SALLET from Quartz program for their cooperation and support during this work.

I would like to thank my thesis follow up committee members Pr. Sylvain GOUTELLE and Dr. Lars Petter JORDHEIM for their cooperation and technical advices during this work.

A great thanks to the Cultural center of the French embassy in Kabul, for sure without financial (bourses du gouvernement français (BGF)) and administrative support of French embassy, this work was not possible for me.

I thank LYKAPHA association, especially Dr. Dominique MARCEL-CHATELAIN for her full support, continuous and fruitful work on academic relations between Lyon and Kabul universities.

I would like to thank Campus France authorities for their administrative support and for providing accommodation.

I thank Marie NALIN, for her cooperation in the analytical part of my work and effective role in my lab life.

I would like to thank Biochemistry and Pharmaco-toxicology laboratory, Lyon Sud Hospital, senior staff Dr. Anne MIALON, Pr. Léa PAYEN, Dr. Charlotte CUERQ, Dr. Antony CITTERIO-QUENTIN and Dr. Aurélien MILLET.

I would like to thank all staff of the Biochemistry and Pharmaco-toxicology laboratory, Lyon Sud Hospital.

I would like to thank Paola RABENDA, Sylvie COULOMB FLASHER and Mona Dosh ASLANOGLOU lab secretary for their kindness and cooperation.
I would like to thank my colleagues at the pharmacy faculty of Kabul University, especially Mr. Aga Mohammad Zhakfar and Mr. Qand Agha Nazari, Farid Habibyar and Masood Noori for their support and cooperation.

I would like to thank my family members especially doctor Mohammad Tawfiq MASHAL and commissar Mohammad Tariq MASHAL for their support and advices.

I would like to thank Jean Pierre GUITTON, Julie GUITTON and Christine DUMAS for their kindness, support and advices.

I would like to thank my Afghan colleagues Dr. Haji Mohammad NAIMI, Dr. Waisudin BADRI and Dr. Khalil ELAJ for being around and giving me morale.

Great thanks to my wife assistant professor Hamida MASHAL for accepting all problems and responsibilities in very hard conditions and providing me the opportunity to complete my thesis. Thanks to my kids Moqadas MASHAL and Moqset MASHAL and so sorry for being far from you.

Table of contents

List of tables

List of figures

List of annexes

Annexe 1. Quartz event program rules
Annexe 2. Summary of the study send to Comité de Protection des Personnes

Abbreviations

ACE	Angiotensin-Converting Enzyme
ACN	Acetonitrile
ADRs	Adverse Drug Reactions
AKI	Acute Kidney Injury
ATP	Adenosine Triphosphate
BCE	Before Common Era
Caspase	Cysteine-Aspartic Proteases
CE	Capillary Electrophoresis
CK	Creatine kinase
CNS	Central Nervous System
COX-I	Cyclooxygenase I
COX-II	Cyclooxygenase II
CV	Cardiovascular
CYP	Cytochrome P450
DBS	Dried Blood Spots
DMPK	Drug Metabolism and Pharmacokinetics
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
FDA	Food and Drug Administration
FTA	Flinders Technology Associates
GC	Gas Chromatography
GI	Gastrointestinal
HPLC	High-Performance Liquid Chromatography
IL	Interleukine
IS	Internal Standard
ITRA	International Trail Running Association
LC	Liquid Chromatography
LC/ESI	Liquid Chromatography-Electrospray Ionisation
LC-HRMS	Liquid Chromatography High Resolution Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LLME	Liquid-Liquid Microextraction
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
MeOH	Methanol
MI	Myocardial Infraction
MP	Mobile phase
MS	Mass Spectrometry
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OF	Oral Fluid
OTC	Over The Counter

PEG	Polyethylene Glycol
PGs	Prostaglandins
QueChers	Quick, Easy, Cheap, Effective, Rugged and Safe
ROS	Reactive Oxygen Species
RP	Reversed Phase
SOD	Superoxide Dismutase
SPE	Solid-Phase Extraction
TDM	Therapeutic Drug Monitoring
TNF-α	Tumor Necrosis Factor Alpha
TOF	Time of Flight
TVA	ToxiVials Type A
TVB	ToxiVials Type B
TXA2	Thromboxane A2
TXB2	Thromboxane B2
UGTs	Uridine-Glucuronosyltransferases
UTMB®	Ultratrail du Mont Blanc®
UV	Ultra-Violet
VAMS	Volumetric Absorptive Microsampling

INTRODUCTION OF THESIS WORK

The practice of trail and ultratrail has been increased since last two decades. The range of running distance proposed by several events are 60 up to more than 100 km. By increase in running range, ultratrail runners need to ameliorate and increase their mental and physical abilities. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are frequently used by runners for pain prevention or reduction. A French study in 2014 reported that among 279 endurance runners, NSAIDs were the most used therapeutic class consumed by participants before or during the race (Didier et al., 2017). According to a Spanish study which reported the results of questionnaire answered by participants of 112 km, 67 km and 44 km events, 48.3 % of runners consumed NSAIDs just before and/or during and/or immediately after the event. Among NSAIDs consumers, 38.5 % of participants reported taking these drugs following medical prescription. If event distance increases, the percentage of NSAIDs consumption also increases (Martínez, Aguiló, Moreno, Lozano, & Tauler, 2017). Indeed, the prevalence of these drugs consumption at 160 km events reported by Hoffman et al and Nieman et al, were 60.5 % and 72%, respectively (Hoffman & Fogard, 2011; Nieman et al., 2005). NSAIDs intake is associated with adverse effects on renal, gastrointestinal and cardiovascular systems. Their consumption during extensive practice by ultratrail runners increase the risk of gastrointestinal disorder, hyponatremia and renal injuries such as renal insufficiency and renal failure (Lambert, Boylan, Laventure, Bull, & Lanspa, 2007; Lipman et al., 2017; S. McAnulty et al., 2007; Wharam et al., 2006). Up to date, all studies concerning the prevalence of NSAIDs consumption in ultratrail events are based on a questionnaire. No study reported prevalence based on the analysis of these drugs in ultratrail runner's biological samples.
The ITRA (International Trail Running Association) is an association whose goal is to promote ultratrail worldwide and defend ethical values. One of ITRA goals is runner's health protection through clean sport by supporting the Quartz Event program. According to the Quartz Event program, participants do not have to consume NSAIDs the day before and during the race. In order to know that the participants are respecting the rule, biological samples are taken for analysis.

For this purpose, my thesis objectives were:

- The development of a liquid chromatography coupled with high resolution mass spectrometer (LC-HRMS/MS) method for the simultaneous identification and quantification of 20 NSAIDs
- The development and the validation of amethod for NSAIDs quantification in oral fluid (OF) and dried blood spots (DBS).
- The application of this method on samples collected during 2021 Ulratrail du mont-Blanc® (UTMB®)

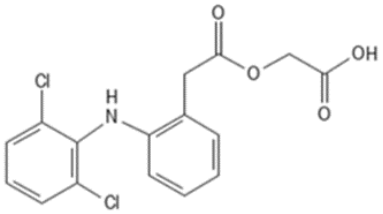
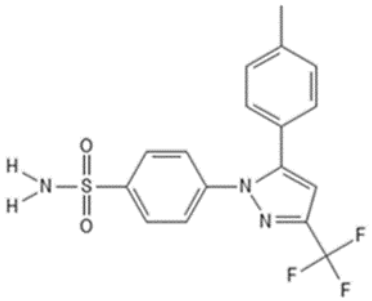
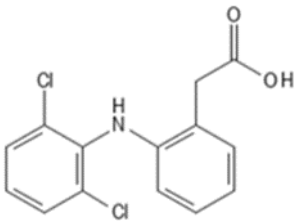
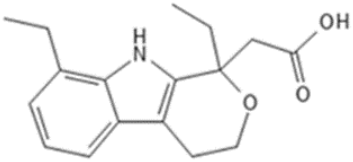
1.BIBLIOGRAPHIC PART

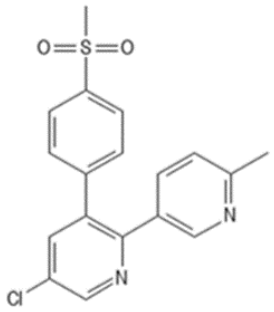
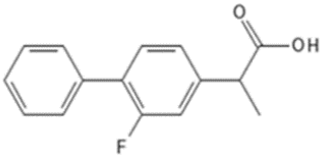
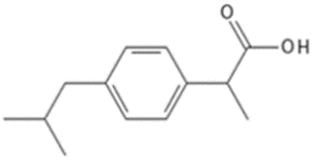
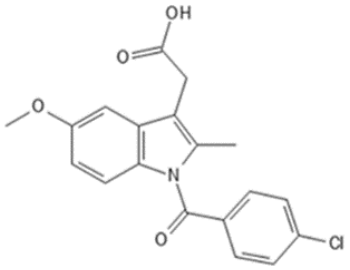
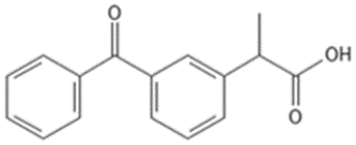
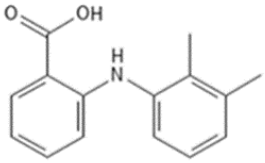
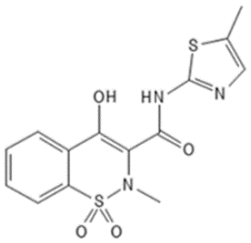
1.1. Non-steroidal anti-Inflammatory drugs

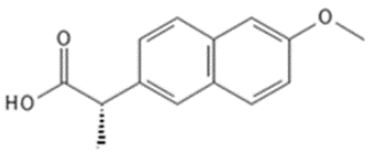
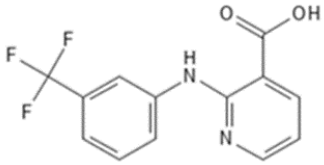
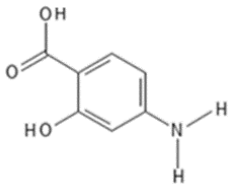
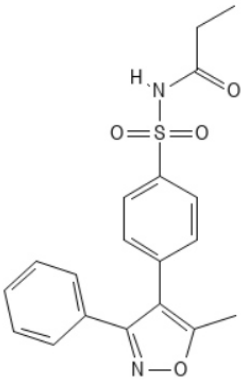
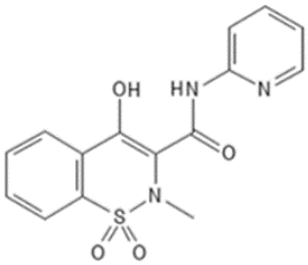
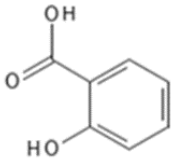
1.1.1. Definition and brief history

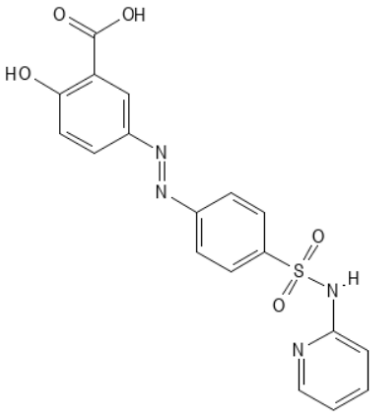
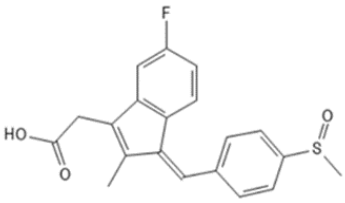
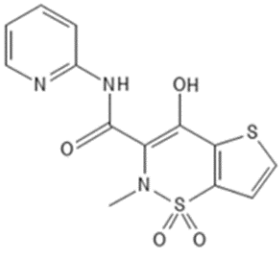
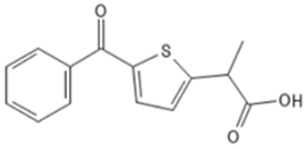
The term Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) refers to the non-steroidal and non-narcotic group of drugs used for pain, fever and inflammation management (Mehanna, 2003). However they are one of the oldest medicines, they remain classified among the top level of drugs in the market worldwide for their diverse pharmacological indications (Rao & Knaus, 2008). According to the classic classification, NSAIDs are divided into Aspirin and non-Aspirin NSAIDs, but an acceptable classification is based on their chemical structure. They correspond to a therapeutic class composed of chemically heterogeneous organic acids (Table 1).

Table 1. Chemical specifications of studied NSAIDs
(PubChem and Drug Bank Online, 16/04/2022)

Compound	Molecular Weight Formula	Chemical Structure	Log P	pKa
Aceclofenac	354.183 $C_{16}H_{13}Cl_2NO_4$		4.88	3.44
Celecoxib	381.373 $C_{17}H_{14}F_3N_3O_2S$		3.99	10.6
Diclofenac	296.147 $C_{14}H_{11}Cl_2NO_2$		4.98	4.00
Etodolac	287.359 $C_{17}H_{21}NO_3$		3.39	4.73

Etoricoxib	358.84 $C_{18}H_{15}ClN_2O_2S$		3.7	16.19
Flurbiprofen	244.265 $C_{15}H_{13}FO_2$		3.57	4.42
Ibuprofen	206.285 $C_{13}H_{18}O_2$		3.5	4.85
Indomethacin	357.79 $C_{19}H_{16}ClNO_4$		4.25	3.79
Ketoprofen	254.285 $C_{16}H_{14}O_3$		3.29	3.88
Mefenamic Acid	241.29 $C_{15}H_{15}NO_2$		5.58	3.89
Meloxicam	351.395 $C_{14}H_{13}N_3O_4S_2$		2.28	4.47

Naproxen	230.263 $C_{14}H_{14}O_3$		3.29	4.19
Niflumic Acid	282.222 $C_{13}H_9F_3N_2O_2$		4.33	1. 88
Para-aminosalicylic Acid	153.137 $C_7H_7NO_3$		0.62	3.68
Parecoxib	370.423 $C_{19}H_{18}N_2O_4S$		3.42	4.24
Piroxicam	331.346 $C_{15}H_{13}N_3O_4S$		2.2	4.76
Salicylic acid	138.122 $C_7H_6O_3$		1.69	2.79

Sulfasalazine	398.393 $C_{18}H_{14}N_4O_5S$		2.92	3.23
Sulindac	356.411 $C_{20}H_{17}FO_3S$		2.96	4.09
Tenoxicam	337.368 $C_{13}H_{11}N_3O_4S_2$		2.42	2.21
Tiaprofenic Acid	260.307 $C_{14}H_{12}O_3S$		3.22	4.03

The history of NSAIDs corresponds mostly to the history of salicylic compounds. It began with the use of some trees barks and leaves belonging to Salicaceae family such as *Salix alba* and *Salix fragilis* in ancient time (Desborough & Keelin, 2017). Hippocrate (460–377 BCE), prescribed willow barks for the treatment of inflammatory pain, gout and ear pain and to relieve childbirth pain. Reverend Edward Stone from England (1758) who looked for an alternative to cinchona barks in malaria symptom treatment, applied *Salix alba* barks aqueous extract every 4 hours to 50 patients and discovered that *Salix alba* bark is cheaper and more effective than cinchona (Marson & Pasero, 2006).

For the first time, the Italian pharmacists Francesco Fontana and Bartolommeo Rigatell extracted the active ingredient of willow barks in 1824. It was latterly named Salicin. Josef Buchner (1783–1852) a German pharmacologist, extracted yellow crystals from willow barks and he also labelled it Salicin. In 1829, the French pharmacist Henri Leroux extracted an important quantity of pure salicin as yellow crystals from willow barks. Raffaele Piria (1814–1865) an Italian chemist, separated salicylic acid from salicin and defined its molecular formula. From 1818 up to1876, sodium salicylate became a popular drug due to its both antipyretic and anti-rheumatic effects (Desborough & Keelin, 2017). The main problem with sodium salicylate

was its side effects like tinnitus, nausea and gastric irritation. In the 19th century, Bayer, a German drug manufacturing company, decided to produce a more effective and safer derivative of salicylate. Felix Hoffmann (1868–1946), who was assigned by Bayer company, found a stable molecule named Acetylsalicylic acid by acetylation of the salicylic acid phenolic ring. Due to Aspirin selectivity on Cyclooxygenase I and some side effects (gastritis and Reye syndrome), scientists discovered different other NSAIDs with less noxious effects during the 20th century (Desborough & Keelin, 2017; Douthwaite, 1938).

NSAIDs are one of the most used medicine worldwide. Data collected during 2 years (2009-2010) from the French National Healthcare Insurances System, showed that 229 477 of 526 108 patients (43.6%) received at least one NSAID. Among them, 44 484 patients (19%) received these drugs without prescription (ibuprofen in 93 % of cases) and 121 208 (53%) after a medical prescription (Duong et al., 2014).

1.1.2. Mechanism of action

NSAIDs are potent blockers of COX-I and COX-II enzymes which lead to inhibition of prostaglandins production, and thus anti-inflammatory effects (Rao, Kabir, & Mohamed, 2010). Cells damaged by mechanical, chemical or biologic agents, release inflammatory mediators called prostaglandins (PGs). PGs are cell membrane phospholipid derivatives produced by specific enzymes called cyclooxygenase (COX). Cell membrane phospholipids are converted into arachidonic acid by phospholipase and then to prostaglandins by two types of COX enzymes called COX-I and COX-II (Figure 1) (Brunton, Parker, MD, PhD, Blumenthal, PhD, & Buxton, PharmD, FAHA, 2012). A third COX, named COX-III is described but its function is not fully understood (Gunaydin & Bilge, 2018). COX-I is a constitutive enzyme existing in all tissues and responsible for some physiologic activities (cytoprotective), whereas the COX-II isoform is upregulated in inflammatory conditions.

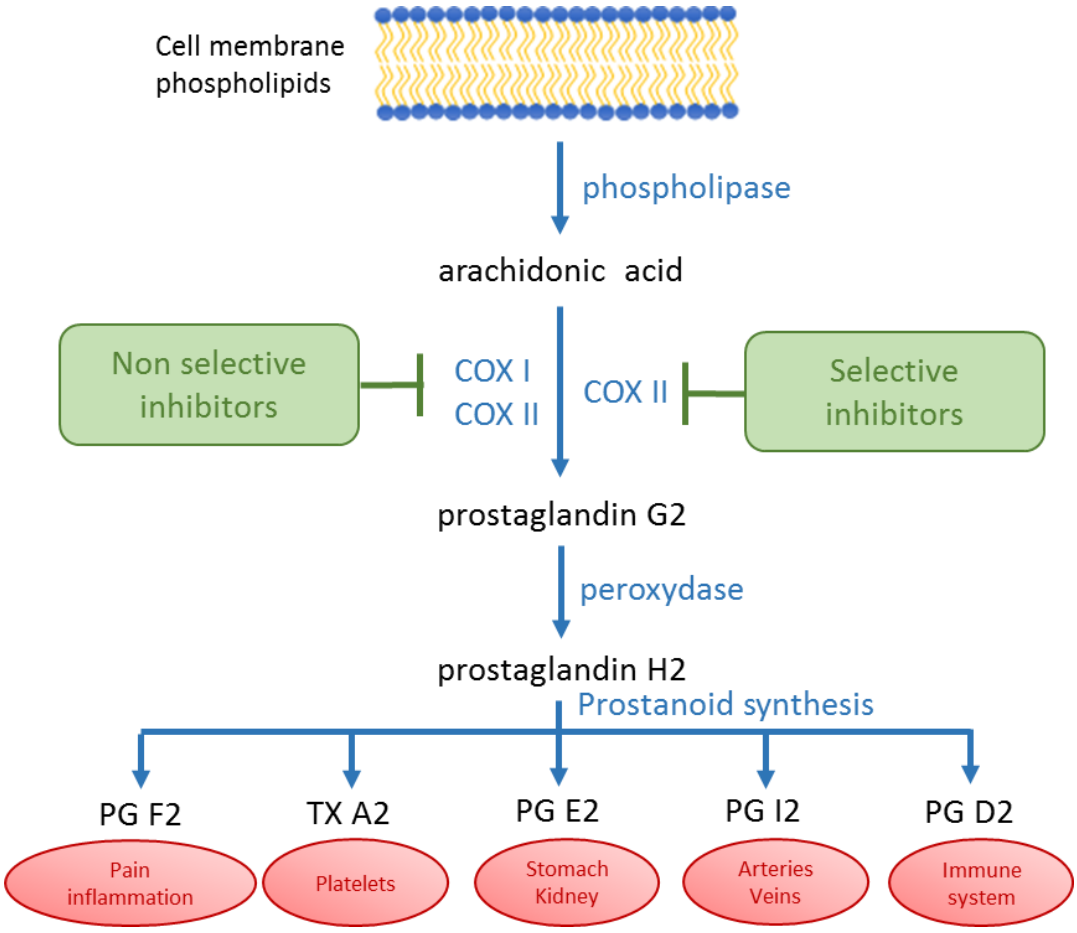


Figure 1. Synthesis of prostaglandins and mechanism of action of NSAIDs on cyclooxygenase

NSAIDs are generally divided into two groups. In one hand, classic NSAIDs (discovered before 1995) which have inhibitory effects on both COX-I and II with more affinity to COX-I, are also called non-selective NSAIDs. And in the second hand, new NSAIDs which present high selectivity to COX-II (Figure 2). Selectivity of NSAIDs against COX could be calculated by the formula [-log (concentration inhibiting TX B2 by 50% in human platelets)) (Figure 2) (Bonnesen & Schmidt, 2021; Driver, Marks, & van der Wal, 2019; Grossman, Wiseman, Lucas, Trevethick, & Birch, 1995; Haag et al., 2008).

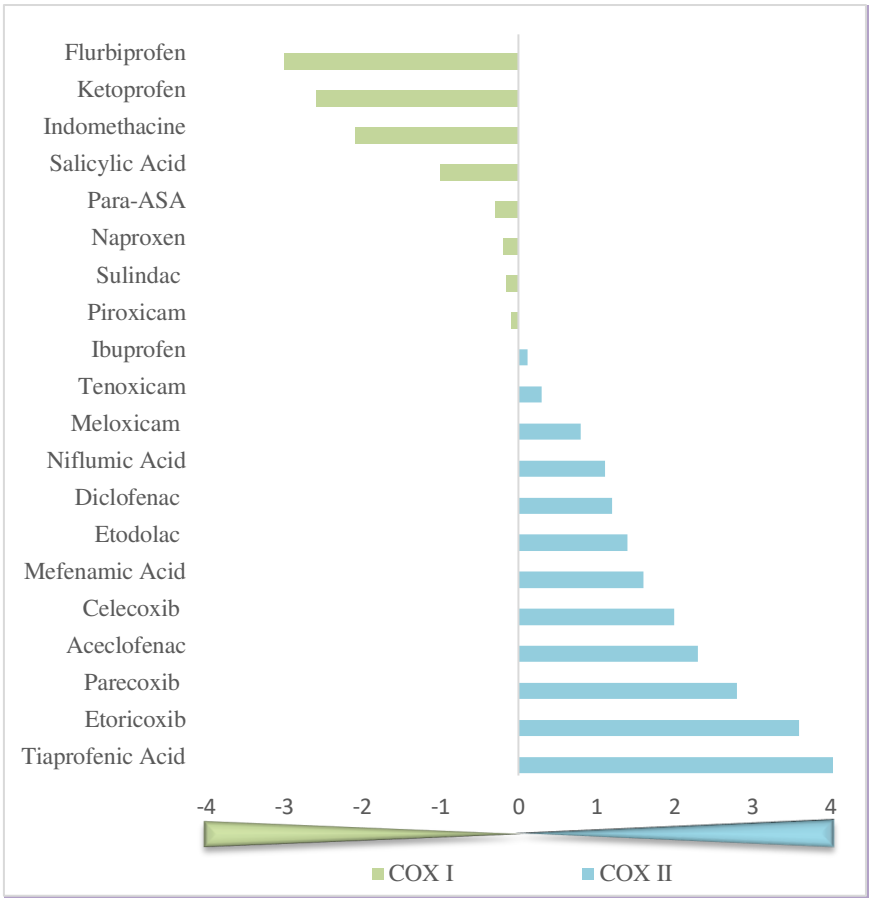


Figure 2. Selectivity of NSAIDs against COX-I and COX-II enzymes.
Results are expressed as log (concentration inhibiting TX B2 by 50% in human platelets). Left site dashed lines indicate NSAIDs affinity for COX-I and the right site dashed lines for COX-II enzyme (Antman, DeMets, & Loscalzo, 2005; Bonnesen & Schmidt, 2021; Brune & Patrignani, 2015; Grossman et al., 1995).

NSAIDs drugs exert other effects: anti-platelet, analgesic, antipyretic, and anti-cancer effects, and against Alzheimer disease (Gunaydin & Bilge, 2018).

1.1.3. Indications

NSAIDs are indicated for the treatment of acute or chronic pain and inflammation due to various diseases and some of them are available as over the counter drugs (OTC) in most countries (Day & Graham, 2013). In addition to the indications mentioned above, some NSAIDs are used for other clinical purposes such as inhibition of platelets aggregation as anti-thrombotic agents (Undas, Brummel-Ziedins, & Mann, 2007), Alzheimer disease (Gasparini, Ongini, & Wenk, 2004) and in colorectal cancer treatment (Stolfi, De Simone, Pallone, & Monteleone, 2013). NSAIDs are one of the most prescribed drugs globally in different health care settings. These drugs consist in 5-10% of all medicines prescribed each year (Wongrakpanich, Wongrakpanich, Melhado, & Rangaswami, 2018).

1.1.4. Pharmacokinetics

1.1.4.1. Absorption

Different routes of administration such as oral, rectal, local (spray, eye drop, ointment…) and injection are used for NSAIDs administration. All NSAIDs are lipophilic compounds, therefore their absorption through the gastrointestinal tract is rapid and complete (80-100%) (Calatayud & Esplugues, 2016; Mehanna, 2003). High GI absorption rates and low hepatic clearances due to week first-pass effects by liver increase drugs bioavailability (except diclofenac and aspirin with high first-pass metabolism of 54% and 70%, respectively) (Day, Graham, Williams, & Brooks, 1988). NSAIDs are acidic compounds, therefore they are ionized more than 99% at physiologic pH. Thus, both lipophilic and ionization characters of NSAIDs facilitate their local absorption (Haroutiunian, Drennan, & Lipman, 2010).

Peak plasma concentrations after oral administration of these drugs are often about 2-3 hours, except for etoricoxib and naproxen (20-26 hours) and for piroxicam and meloxicam (50-75 hours). Most of NSAIDs are poor water-soluble compounds and different factors like beverages and anti-acids delay or reduce their absorption. (Brunton et al., 2012). Local application of some NSAIDs, showed very low plasma concentration, for example the percutaneous absorption of ibuprofen cream (400mg) was 3% with a Tmax of 11 hours (Davies, 1998).

1.1.4.2. Distribution

Majority of NSAIDs are highly bound to plasma proteins (usually albumin): the percentage of binding to plasma proteins is 95-99%, with except for para-aminosalicylic acid (50-60%) (Calatayud & Esplugues, 2016). Basic and lipophilic residues present in albumin protein interacts with acidic function and lipophlic groups of NSAIDs, resulting in both ionic and hydrophobic interactions of albumin with NSAIDs. Binding of these drugs with albumin leads to potent drug-drug interaction especially with drugs that bind highly on the same site of albumin such as oral anticoagulants, anticancer agents and thyroid hormones. Simultaneous administration of drugs mentioned above with NSAIDs may increase their toxicity due to their displacement from albumin (Mehanna, 2003).

Drugs lipophilic character allows them to diffuse passively through cell membranes (Day et al., 1988)**.** Most of NSAIDs are distributed through the body and easily penetrate in the affected area. NSAIDs are able to reach at a sufficient concentration in the central nervous system (CNS). NSAIDs with pKa values around 4 or 5 such as ibuprofen, diclofenac and ketoprofen, easily penetrate and accumulate in inflamed tissues and synovial fluid. Inflammation leads to an acidic microenvironment, increases local blood flow and high level of albumin in damaged tissue and synovial fluid facilitate drug accumulation in the affected area. Slightly acidic extracellular pH, lead to these drugs dissociation from plasma proteins (Calatayud & Esplugues, 2016).

Only two studies reported the distribution and the pharmacokinetic of NSAIDs in saliva. The first one has determined and compared the pharmacokinetic parameters of piroxicam and its major metabolite hydroxypiroxicam in plasma and saliva (Calvo et al., 2016). The two compunds are found in saliva. However, the maximum concentrations in saliva are largely lower than those in plasma, with a plasma/saliva concentration ratio calculated to 0.003 for piroxicam (Figure 3).

Piroxicam	Plasma		Saliva
	Mean ± SD		Mean ± SD
AUC_{0-72} (h ng/mL)	64819 ± 9722		214 ± 59
AUC_{∞} (h ng/mL)	78653 ± 42454		356 ± 128
Clt/F (L/h)	0.2 ± 0.1		64.9 ± 25.3
Vd/F (L)	14.8 ± 3.7		4167.6 ± 1318.2
$t_{1/2}$ (h)	50.7 ± 8.8		50.9 ± 24.8
C_{max} (ng/mL)	2275.9 ± 367.7		7.4 ± 2.4
T_{max} (h)	4.0 ± 1.2		4.7 ± 2.4
SL/PL		0.003	
5′-Hydroxypiroxicam	Plasma		Saliva
	Mean ± SD		Mean ± SD
AUC_{0-72} (h ng/mL)	6213 ± 1856		247.6 ± 92.7
Clt/F (L/h)	110.3 ± 39.9		5.2 ± 2.9
C_{max} (ng/mL)	133 ± 47.2		5.9 ± 2.8
T_{max} (h)	53.6 ± 14.7		54.9 ± 14.2
SL/PL		0.04	

AUC $_{0-72and0-\infty}$: area under the concentration versus time curve from the first to the last concentration observed; Vd/F: estimated distribution volume in the total AUC; Clt/F: total clearance; $t_{1/2}$: terminal half-life time of the drug; T_{max} and C_{max}: time and the maximum concentration observed, respectively. SL/PL: saliva/plasma concentration ratio. SD: standard deviation.

Figure 3. Pharmacokinetics parameters from plasma (n=10) and saliva (n=12) of piroxicam and 5'-hydroxypiroxicam in health volunteers **(Calvo et al., 2016)**

The second study reported plasmatic and salivary concentrations of salicylic acid (Cheng et al., 2022). They showed a correlation between plasmatic and salivary concentration, with concentrations in saliva lower than in plasma (saliva/plasma concentration ratio estimated to 0,03).

1.1.4.3. Metabolism

NSAIDs undergo both phases I and II reactions. Hydroxylation is the most important phase I reaction which leads to the production of hydrophilic metabolites. By hydroxylation, metabolites lose their pharmacologic effects because hydrophilic metabolites are not able to compete with lipophilic arachidonic acid for COX enzyme. For some NSAIDs, metabolism leads to activation of these agents. For example, sulindac, a hydrophilic prodrug, changes to highly lipophilic metabolite by reduction its sulfoxide group to sulfide. Aspirin rapidly change to salicylate which has anti-inflammatory effect and then salicylate is eliminated slowly. Some NSAIDs such as ketoprofen, ibuprofen, naproxen, tiaprofenic acid, and flurbiprofen has chiral isomers activated by metabolism. For example, 60% of ibuprofen inactive R-enantiomers are metabolically converted to its active S-enantiomers (Day et al., 1988).

Glucuronic acid conjugation and sulfate conjugation are the main phase II reactions (Mehanna, 2003). First-pass effects for some NSAIDs such as diclofenac and aspirin, significantly decrease their bioavailability, while it increases for some prodrugs such as sulindac and parecoxib (Calatayud & Esplugues, 2016; Wongrakpanich et al., 2018).

Different cytochrome enzymes are involved in NSAIDs metabolism: CYP2C9 and CYP3A4 (at ibuprofen high concentration) play major role, while CYP2C8 and CYP2C19 play minor

role in ibuprofen metabolism (Mazaleuskaya et al., 2015). For prodrugs NSAIDs metabolism and anti-inflammatory activities are decreased by cytochrome enzyme inhibitors such as cimetidine and valproic acid and increase by enzyme inducers such as carbamazepine and phenobarbital (Mehanna, 2003). Even NSAIDs metabolites are generally considered as non-toxic, some NSAIDs' metabolism leads to toxic metabolite production. For exemple, the acylglucuronid metabolite of ibuprofen is highly reactive and able to covalently bind to cellular macromolecules, which contributes to ibuprofen toxicity (Figure 4).

Genetic polymorphism of CYP2C8 and CYP2C9 could be a reason for variation in NSAIDs metabolism. Genetic variation in mentioned enzymes causes a decrease of enzyme activity against NSAIDs (Agúndez, García-Martín, & Martínez, 2009). Individuals with genetic variation in CYP2C9*2 and CYP2C9*3 are poor metabolizers for NSAIDs. Therefore, these drugs metabolism will be longer which leads to high plasma concentration and may increase their adverse effects (Katar, Usman, & Aliska, 2022). For example, NDAIDs increase the risk of gastrointestinal bleeding in poor metabolizer with variant gene (single nucleotide polymorphism, mutation, copy number variation) for CYP2C8 and CYP2C9 (Agúndez et al., 2009).

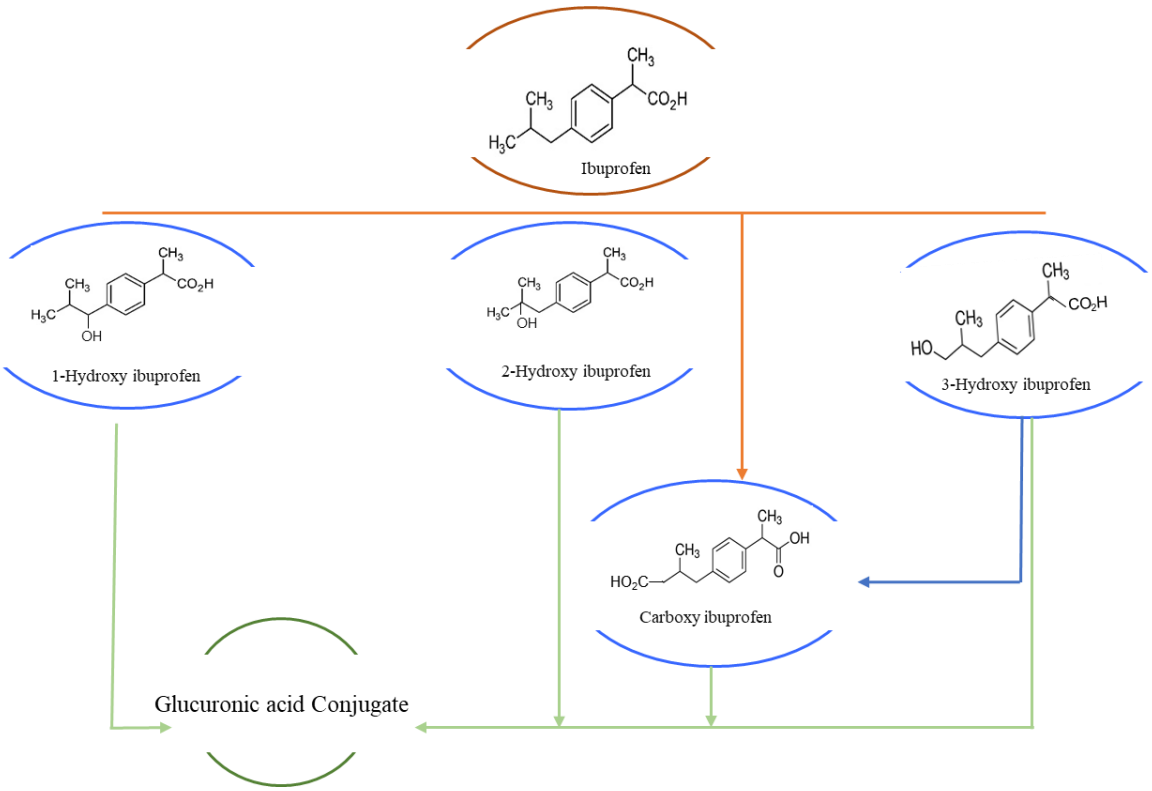


Figure 4. Metabolism of ibuprofen in humans.

Broun flash depicts ibuprofen metabolism in phase I, the blue flash shows hydroxylated metabolite transformation to carboxy metabolite and the green flash indicates phase II conjugation.

Carboxylated and hydroxylated metabolites are able to conjugate with glucuronic acid and eliminate from body. Significant amount (about 30% of administrated dose) of Hydroxy and Carboxy ibuprofen eliminate through urine without conjugation. CYP 2C9 and CYP 2C8, play

important role in ibuprofen biotransformation. Uridine-glucuronosyltransferases (UGTs) enzymes such as UGT1A3, UGT1A9, UGT2B4, UGT2B7, and UGT2B17, are able to conjugate glucuronic acid with ibuprofen (Mazaleuskaya et al., 2015).

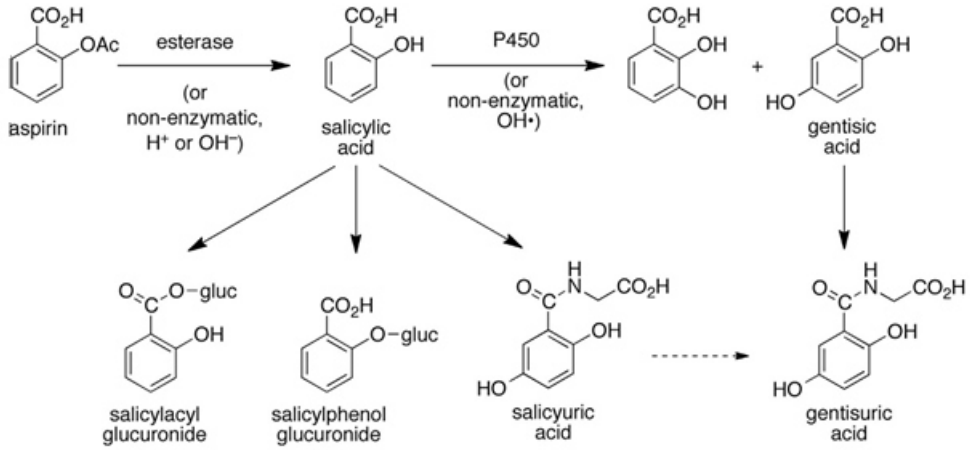


Figure 5. Metabolism of acetylsalicylic acid in humans (Bojić, Sedgeman, Nagy, & Guengerich, 2015)

1.1.4.4. Elimination

Hepatic biotransformation and renal excretion are the main routes of NSAIDs elimination. Most NSAIDs are excreted as glucuronic acid or sulfate conjugates and in a small amount as unchanged drugs in urine (Mehanna, 2003). Hepatic metabolism alteration in elders or individuals with liver problem, may reduce the clearance for most of these drugs (Calatayud & Esplugues, 2016). The risk of decrease urinary output or renal failure by NSAIDs in older subjects (aged >75), increase with pre-existing renal impairment (Monteiro, Silvestre, Duarte, & Alves, 2022).

Pharmacokinetic parameters of NSAIDs studied are summarized in Table 2.

Table 2. Pharmacokinetic parameters of NSAIDs

NSAIDs	Dosage Form	Serum Level	Phase I Metabolites	Phase II Metabolites	Excretion	References
Aceclofenac	100 mg	Cmax=1.25 µg/ml Tmax= 3 h	4'-hydroxy aceclofenac 5-hydroxy aceclofenac 5-hydroxy diclofenac 4'-hydroxy diclofenac diclofenac	Diclofenac sulfate Diclofenac acyl glucuronide	In Urine as hydroxyl metabolites and 1% unchanged	RCP ANSM - Mis à jour le : 10/10/2013
	100 mg	Cmax=13.39 µg/ml Tmax= 1.5 h				(Naz et al., 2011)
	100 mg	Cmax= 10.3 µg/ml Tmax of 1.5-3 h Half-life = 2.1-5 h				(Bae et al., 2012)
	200 mg	Cmax= 705 ng/ml Tmax= 3 h Half-life = 8-12 h				(Gong et al., 2012)
Celecoxib	25 mg	Cmax= 0.88 mg/ L Tmax= 1.75 h Half-life = 3-6 h	Hydroxy celecoxib Celecoxib carboxylic acid	Acyl-glucuronide conjugates	57% in feces and 27% in urine	(Sarkar et al., 2017)
Diclofenac	50 mg	Cmax= 1.5 mg/L Tmax= 1.56 h	4-hydroxydiclofenac 5-hydroxydiclofenac 3-hydroxydiclofenac 4,5-dihydroxydiclofenac	Glucuronic acid conjugation and taurine amino acid conjugation Sulfate Conjugation	Urine	RCP ANSM - Mis à jour le : 02/05/2017
	140 mg	Cmax= 3 µg/ml				
	50 mg	Cmax= 1.7 mg/L Tmax = 1.5, 2.4 h half-life= 0.75-01.05 h				(Kirchheiner et al., 2003)
	200 mg	Cmax= 17.4-19.7 µg/ml Tmax= 1.2-1.3 h Half-life = 6-7.5 h				(Brocks & Jamali, 1994)
Etodolac	400 mg	Cmax= 10.4-49.6 µg/ml Tmax= 1-1.5 h Half-life = 6.4-9.7h	6-Hydroxy etodolac 7-hydroxy etodolac 8- hydroxyethyl etodolac	Acyl-glucuronide 4-ureidoetodolac	Urine	(Boni et al., 1999)
	200 mg	Cmax= 15.2-31.4 µg/ml Tmax= 1-1.5 h Half-life = 4.9-7.3 h				(Boni et al., 1999) (Tjandrawinata, Setiawati, Nofiarny, Susanto, & Setiawati, 2018)
	120 mg	Cmax= 3.155 µg/ml Tmax = 0.40-2 h Half-life = 20.95 h				
Etoricoxib	120 mg	Cmax= 1.36 µg/ml Tmax = 1 h Half-life = 27 h	6-Hydroxymethyl etoricoxib 6-Carboxy etoricoxib	6′-hydroxymethyl glucuronidated	Urine (70%) and Feces (20%)	(Escudero-Contreras, Cervantes, & Collantes-Estevez, 2007)

	120 mg	Cmax= 1.56 µg/ml Tmax = 1 h				(Takemoto, Reynolds, Remsberg, Vega-Villa, & Davies, 2008)
	120 mg Fasting	Cmax= 2.43 µg/ml Tmax = 2 h				(Takemoto et al., 2008)
	100 mg 200 mg	Cmax= 14.2 µg/ml Tmax = 1.9 h Half-life = 3.3-4 h Cmax= 25.8-36.1µg/ml Tmax = 0.94-1.73 h				(Davies, 1995)
Flurbiprofen	100 mg	Cmax= 15 µg/ml Tmax = 1.35 h Half-life = 5.37 h	4'-hydroxy-flurbiprofen 3',4'–dihydroxy-flurbiprofen 3'–hydroxy 4'methoxy-flurbiprofen	Acyl-glucuronide conjugates	Urine	Product Monograph February 7, 2019
	200 mg	Cmax= 19-22 µg/ml Tmax = 1-1.5 h Half-life = 1.8-3.5 h				(Davies, 1998)
Ibuprofen	400 mg	Cmax= 28-30 µg/ml Tmax = 1.1-1.25 h	1-Hydroxy ibuprofen 2-Hydroxy ibuprofen 3-Hydroxy ibuprofen 2,4carboxy ibuprofen	Acyl-glucuronide conjugates	Urine	(Davies, 1998) (Boyer et al., 2009; Vree, Biggelaar-Martea, Verwey-van Wissen, & Ewijk-Beneken Kolmer, 1994)
	100 mg	Cmax= 2.95 µg/ml Tmax = 1.64 h Half-life = 2.6-11.2 h				
Indomethacin	100 mg	Cmax= 4.71 µg/ml Tmax = 1.4 h	Desmethyl indomethacin Desbenzoyl indomethacin Desmethyl-desbenzoyl indomethacin Deschlorobenzoylindomethacin	Glucuronic acid conjugate indomethacin acyl glucuronide, DMI acyl glucuronide,	Urine and Feces	(Yeh, 1985)
	100 mg	Cmax= 8.84 µg/ml Tmax = 3.5 h in men Half-life = 3 h				(Dennis et al., 1985; Skordi, Wilson, Lindon, & Nicholson, 2004)
Ketoprofen	200 mg	Cmax= 8.4 µg/ml Tmax = 8 h in women Cmax= 7.3 µg/ml Tmax = 5 h in men	2-[3-(3-hydroxy benzoyl)phenyl]-propanoicacid 2-[3-(4-hydroxy	Glucuronic acid Conjugation (acyl-glucuronide conjugates)	Urine	(Magallanes et al., 2016)

	1000 mg	Cmax= 10-20 µg/ml Tmax = 2-4 h Half-life = 2 h	benzoyl)phenyl]-propanoicacid 2[3(hydroxy(phenyl)methyl) phenyl]-propanoicacid			(Ullah, Baloch, Ullah, & Mustaqeem, 2014)
Mefenamic Acid	2 mg/kg in infants	Cmax= 1.2-6.1 µg/ml Tmax = 2-18 h Half-life = 3.8- 43.6 h	3- hydroxymethyl MEF 3-carboxy MEF	Acyl-glucuronide conjugates	Urine and Feces	(Ito et al., 1994)
	30 mg	Cmax= 1.4 µg/ml Tmax = 9.7 h Half-life = 26 h				(Davies & Skjodt, 1999)
Meloxicam	500 mg	Cmax= 77.3 µg/ml Tmax = 2 h Half-life = 18.84 h	5-hydroxymethyl 5-carboxy	Not mentioned in this article	Urine and Feces	(Choi et al., 2015)
Naproxen	500 mg	Cmax= 62.2 µg/ml Tmax = 1.5 h Half-life = 24.7 h	O-desmethylnaproxen	O-desmethylnaproxen acyl glucuronide	Urine	(Vree, Van den Biggelaar-Martea, Verwey-Van Wissen, Vree, & Guelen, 1993)
	250 mg	Tmax = 2 h Half-life = 4 - 6 h				RCP du produit (26/05/22)
Niflumic Acid	700 mg Morniflumate (pro-drug)	Cmax= 20.47 µg/ml Tmax = 2.6 h Half-life = 4 h	5-hydroxyniflumic acid 4-hydroxyniflumic acid	Glucuro and sulfoconjugates	Urine and feces	(Cho, Park, & Lee, 2013)
	370 mg Talniflumate (pro-drug)	Cmax= 158 ng/ml Tmax = 2.8 h Half-life =3.2 h	Not mentioned in this article	Not mentioned in this article	Urine	(Park, Na, Shin, & Lee, 2008)
	6 g In fasting	Cmax= 21.4 µg/ml Tmax = 4.43 h Half-life = 1.21-3.91 h	hydroxyniflumic acid	Not mentioned in this article	Urine	(Bilecen et al., 2003)
Para-aminosalicylic acid	6 g With food	Cmax= 32.5 µg/ml Tmax = 6.56 h Half-life = 1.1-6 h	*N*-acetyl-PAS	Glycyl-PAS (glycine-conjugation of aminosalicylic acid)	Urine	(Peloquin, Zhu, Adam, Singleton, & Nix, 2001) (Momekov, Momekova, Stavrakov, Voynikov, & Peikov, 2015)
	4 g	Cmax= 49.98 µg/ml Tmax = 3.54 h Half-life = 26 min				
Parecoxib	40 mg (i.v and i.m)	Cmax= 1.681 µg/ml Tmax = 0.5-1.5 h Half life= 0.87 h	Valdecoxib (as an active metabolite)	Acyl-glucuronide conjugates	Urine	(Stichtenoth, 2004; Tacconelli, Capone, & Patrignani, 2004)
Piroxicam	In plasma after 20 mg	Cmax= 2.28 µg /ml Tmax = 4 h Half-life = 50.7 h	5-hydroxypiroxicam	Acyl-glucuronide conjugates	Urine and feces	(Calvo et al., 2016) (Nagelschmitz et al., 2014)

	In whole saliva after 20 mg 500 mg	Cmax= 7.4 ng /ml Tmax = 4.7 h Half-life = 50.9 h Cmax= 22.85 µg/ml Tmax = 1.5 h Half-life = 2.54 h				
Salicylic acid*	500 mg 14.3 mg/kg in man	Cmax= 22.85 µg/ml Tmax = 1.5 h Half-life = 2.54 h Cmax= 68 µg/ml Tmax = 1.57 h Half-life = 0.46 h	2,3-dihydroxybenzoic Acid 2,5-dihydroxybenzoic acid (Gentisic acid)	Salicylacyl glucuronide Salicylphenol glucuronide salicyluric acid	Urine	(Bojić et al., 2015; Trnavska & Trnavska, 1983) (Bae et al., 2008)
	14.3 mg/kg in women	Cmax= 83.5 µg/ml Tmax = 1.23 h Half-life = 0.31 h				
	100 mg	Cmax= 3.78 µg/ml Tmax = 4.75 h Half-life = 2 h				
	100 mg	Cmax= no data Tmax = 2-4 h Half-life = ~ 8 h				(Sung et al., 2020)
Sulindac	200 mg	Cmax= 6.6 µg/ml Tmax = 1.2 h	Not mentioned in this article	Sulf-conjugation (Sulindac sulfone and Sulindac sulfide) Glucuronide-conjugate	Urine	(Davies & Watson, 1997)
	20 mg	Cmax= 2.7 µg/ml Tmax = 67 h Half-life = ~70 h	Not mentioned in this article	Not mentioned in this article		RCP du produit, 07/04/2022
Tenoxicam	40 mg	Cmax= 5.91 µg/ml Tmax = 1.56 h Half-life = 42 h	5-hydroxypyridyl	5-hydroxypyridyl glucuronide-conjugate	Urine and feces Metabolites mostly eliminated in urine	(Bird, Allen, Dixon, & Wright, 1985)
	300 mg	Cmax= 29.6 µg/ml Tmax= 1.5 min Half-life = 2.9 h	Not mentioned in this article	Not mentioned in this article	Not mentioned in this article	(Malátková, Skarka, Musilová, & Wsól, 2017)
Tiaprofenic acid			4-hydroxybenzyl 5-benzylalcohol-2-thienyl	Acyl-glucuronide conjugates	Urine	

*After Acetyl Salicylic acid administratio

1.1.5. Toxicity of NSAIDs

Both acute overdose and chronic usage of these drugs can cause several adverse health effects and toxicity (Bjarnason et al., 2018). According to a France pharmacovigilance report (data collected from 2002 to 2006), defined daily doses of eight oral NSAIDs used in France led to different adverse drug reactions (ADRs) ranging from 0.3 up to 0.78 cases per million. The incidence of ADRs was higher for ketoprofen (0.78 case per million) followed by diclofenac (0.58), naproxen (0.50), piroxicam (0.47), tenoxicam (0.42), meloxicam (0.41) and aceclofenac (0.30) (Lapeyre-Mestre, Grolleau, Montastruc, & Association Française des Centres Régionaux de Pharmacovigilance (CRPV), 2013). In Poland, 80 (30.5%) out of 232 ADR collected by the Regional Centre for the Monitoring of Adverse Drug Reactions during 2007-2009 were about NSAIDs. Among these 80 reports, 53 (66.3%) were observed in women (Jaźwińska-Tarnawska et al., 2012). In the United States of America and the United Kingdom, NSAIDs are reported to be responsible for 21% and 25% of ADR, respectively (Zhang, Donnan, Bell, & Guthrie, 2017). NSAIDs adverse effects are an important reason for hospitalization and mortality due to their short or long-term usage in most countries. In the United States of America, NSAIDs are responsible for around 107,000 hospitalizations and 16,500 deaths annually. This number is about 10,000 hospital admissions and 2,000 deaths annually in United Kingdom (Kuritzky & Samraj, 2012).

Toxic effects of NSAIDs are highly variable, depending on factors such as the type of NSAID, dose, individual susceptibilities (metabolizing enzymes, age…). Both COX-I and II inhibitors are able to induce toxicities, but the risk of GI, CV, and renal ADR is lower with COX-II selective NSAIDs than with nonselective (Monteiro et al., 2022). A meta-analysis on cardiovascular toxicity of NSAIDs including 116,429 patients from 31 trials *versus* placebo showed that estimated rate ratios of cardiac strokes were higher for ibuprofen, diclofenac and etoricoxib than naproxen and celecoxib (3.36, 2.86, 2.67, 1.76 and 1.12, respectively). CV death rate was higher for etoricoxib and diclofenac (4.07, 3.98, 2.39, 2.07 and 0.89 for etoricoxib, diclofenac, ibuprofen, celecoxib and naproxen, respectively) (Trelle et al., 2011).
Cardiac stroke were higher for ibuprofen and diclofenac (3.36, 2.86) than etoricoxib, and celecoxib (2.67, 1.12), however cardiovascular death rate were higher for etoricoxib and diclofenac (4.07, 3.98, 2.39, 2.07 and 0.89 for etoricoxib, diclofenac, ibuprofen, celecoxib and naproxen respectively) (EMA, 2018; Trelle et al., 2011).
They are commonly observed among chronic users (Modi et al., 2012; Moore, Pollack, & Butkerait, 2015).

1.1.5.1. Renal toxicity

After aminoglycosides, NSAIDs are the second cause of nephrotoxicity, accounting for 15.5% of all drug induced renal failure. Nephrotoxicity represents a serious challenge of NSAIDs uses worldwide. For example, in the United States of America, among 50 million NSAIDs users, around 0.5 up to 2.5 million developed nephrotoxicity (Nolin & Himmelfarb, 2010).
PG are essential for renal blood volume control, electrolytes balance, controlling renin release, and renal vasodilation. Prostaglandins produced by COX-I (PGI2) regulates renal perfusion and glomerular filtration and by COX-II (PGE2) regulates salt and water excretion. In the human

kidney, COX-I expresses in collecting duct, interstitial cells, vasa recta and Bowman's capsule, and COX-II in renal vessels, loop of Henle, podocytes, macula densa, inner medulla and papilla. Inhibition of thromboxane and PG synthesis by these drugs leads to vasoconstriction and reduction of renal perfusion. PGE2 by binding to its EPI receptor inhibits sodium and chloride transport in the collecting duct and ascending loop of Henle. Furthermore, inhibition of the antidiuretic hormone receptor by PGE2 increases diuresis. Therefore, chronic usage of these drugs is associated with edema due to sodium and water retention (Lucas et al., 2019).
The kidney is a small organ (makes about 0.4% of body weight) but receives a huge amount of blood (about 25% of cardiac output). Decrease in renal blood supply is the main mechanism of acute renal failure (Lucas et al., 2019). Nearly all NSAIDs may induce acute renal toxicity, and decrease in renal blood flow is the key mechanism of acute renal failure. Decrease in renal blood supply can cause acute renal failure in 3-7 days (Nørregaard, Kwon, & Frøkiær, 2015). Patients with cardiovascular, hepatic and renal diseases are more susceptible to acute renal failure induced by NSAIDs (Drożdżal et al., 2021).

Other mechanisms are implicated in renal toxicity of NSAIDs:

- *Renal damage by free radicals***:** Free radicals formation is a crucial feature of NSAIDs in renal failure. For example, after indomethacin administration, activated neutrophils infiltration occurring through gastrointestinal tract produces reactive oxygen species (ROS) which could destroy nephrons by lipids peroxidation (Basivireddy, Jacob, Pulimood, & Balasubramanian, 2004). In addition to ROS generation, indomethacin inactivates antioxidant system of renal cells. Histological study of rat's kidney treated with indomethacin (20mg/kg by gavage and sacrificed after 24h) indicated a significant decrease in renal cells antioxidant system such as superoxide dismutase (SOD), catalase, glutathione reductase and glutathione peroxidase (Basivireddy et al., 2004).
Kidneys are very susceptible against all chemicals that affect energy supply or energy production. NSAIDs affect some mitochondrial functions in renal cells and disturb energy processes by different mechanisms. Some NSAIDs are uncoupler of oxidative phosphorylation and block ATP synthesis in mitochondria. Respiratory enzymes gene mutation, direct inactivation of these enzymes by ROS produced, or inhibition of electron transport by NSAIDs (Salicylate and indomethacin) are other mechanisms of impairing ATP synthesis. ROS have a big hand on mitochondrial damage and renal cell death. (Fosslien 2001; Nolin and Himmelfarb 2010).
- *Renal cell death by apoptosis*: Renal cell death by apoptosis due to ROS production or lowering its anti-oxidant system is another renal toxic effect of NSAIDs. For example, diclofenac is associated with nuclear DNA fragmentation and renal cells death by apoptosis. Female Wistar albino rats (160–190 grams body weight) treated with diclofenac (50 mg/kg b.w./day, i.p.) for two days have shown a reduction in antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione-S-transferase (GST), glutathione peroxidase (Gpx) and elevation of ROS. Another apoptotic mechanism of NSAIDs is the cellular accumulation of arachidonic acid, which activates sphingomyelinase enzyme leading to cell death by apoptosis. COX-II plays a vital role in the inhibition of cell death by apoptosis through death receptor and p53 genes down-regulation. Thus, selective COX-II inhibitors may be implicated in cell death by apoptosis through elevation of death

receptor and p53 level (Johar D et al., 2004). Activation of caspase pathway is another mechanism of renal cell death by apoptosis. ROS produced by diclofenac lead to caspase-3 activation and cytokines release (TNF-α, IL-6, IL-1β, and NF-κB) in renal cells. Caspase-3 and NF-κB elevation by diclofenac are associated with renal cell death by apoptosis (Basivireddy et al., 2004). NSAIDs effects on plasma membrane permeability is another mechanism of renal cell death via necrosis. The study of 10 different NSAIDs (Nimesulide, Celecoxib, Mefenamic Acid, Flufenamic Acid, Flurbiprofen, Indomethacin, Diclofenac, Etodolac, Ibuprofen and Ketoprofen) on calcein-loaded liposomes have shown that all of these drugs affect plasma membrane permeability. Alteration of plasma membrane permeability increases Ca^{2+} influx and elevates intracellular level of calcium. Increase in intracellular Ca^{++} leads to activation of apoptotic proteins and cell death by apoptosis (Mizushima, 2010).

- *Electrolytes Disorders*: Electrolytes imbalance and acidosis are important toxic effects of NSAIDs. In a context of a renal perfusion diminution, renin released from juxtaglomerular cells by PGI2 leads to aldosterone production through the renin-angiotensin process. Aldosterone increases sodium absorption and potassium excretion through the kidney. Therefore, inhibition of PGI2 synthesis by COX-II inhibitors is associated with hyperkalemia. (Lucas et al., 2019).

1.1.5.2. Gastrointestinal toxicity

About 30-50% of NDAIDs chronic users (for at least 6 weeks) present endoscopic lesions located in gastric antrum, which generally reduce or disappear after some time due to mucosa adaptation. More than 50% patients with serious peptic ulcer had no preliminary warning symptoms (Sostres, Gargallo, & Lanas, 2013).

Mucus production by PG is necessary for stomach inner wall protection against acid. Inhibition of PG synthesis are common GI adverse effects associated with NSAIDs chronic use (Wongrakpanich et al., 2018). As COX-I constitutively expressed in all tissue include GI tract and COX II induced mostly during inflammation, therefore COX II selective NSAIDs do not affecting GI protective effects of COX I (inhibition gastric acid secretion and mucosa production), this is why COX II selective inhibitors are less GI toxic than COX I. In a study including patients treated with celecoxib (400 mg 2 times per day), diclofenac (75 mg 2 times per day) and ibuprofen (800 mg 3 times daily), it has been shown that ulcer was significantly less among celecoxib and diclofenac users than ibuprofen users (Day & Graham, 2013; Sostres, Gargallo, Arroyo, & Lanas, 2010). However, it does not mean that COX II selective inhibitors are not GI toxic. Prostaglandins produced by COX II play important role in GI ulcer healing, therefore COX II selective inhibitors delay gastric ulcer healing (Sostres et al., 2010). Moreover, Bindu et al reported that COX II selective inhibitors has sufficient COX I inhibitory capacity to induce GI toxicity through inhibition of PGE2 (Bindu, Mazumder, & Bandyopadhyay, 2020). Among 15,396 patients who received COX II selective and 41,512 patients nonselective NSAIDs from 1999 up to 2004, both COX selective and non selective NSAIDs induced upper GI toxicity, but the incidences were lower in COX II selective users than nonselective (Davies, Smith, Windmeijer, & Martin, 2013).

Long term usage of non-selective NSAIDs can cause a small intestine obstruction and ulceration.The incidences of small intestine inflammation and perforation in NSAIDs long-term

users was reported to be about 50-70% (Ghosh, 2021).
NSAIDs may also damage intestinal epithelial cells. The exact mechanism of NSAIDs toxicity on intestinal epithelial cells remains unknown. However, glucuronic acid conjugated metabolites may play a role in these drugs intestinal toxicity (A. Boelsterli & Ramirez-Alcantara, 2011).
In addition to PG inhibition, blocking thromboxane production in platelets increases bleeding induced by damaged gastric epithelial layer, gastric mucosal inflammation and increase in its permeability frequently occur in NSAIDs users (Sostres et al., 2013).
Older age (> 70 years) and H. pylori are risk factors in NSAIDs GI toxicity (Sostres et al., 2010).

1.1.5.3. Cardiovascular toxicity

Both COX-I and II inhibitors are associated with CV toxicity. Oedema, myocardial infarction, thrombotic events, stroke and hypertension are cardiovascular adverse effects associated to NSAIDs (Wong, Chowienczyk, & Kirkham, 2005).
The type of NSAIDs, dose, treatment duration and frequency of administration are factors contributing to cardiovascular toxicity. Cardiotoxicity is frequently observed with naproxen, ibuprofen, diclofenac, celecoxib and etoricoxib (Singh, Haque, & Pillai, 2014). No significant differences of CV incidences were observed by application of COX-II selective such as celecoxib (400mg, twice daily) and non-selective COX-II such as ibuprofen (800 mg, three times a day) and diclofenac (75mg, twice daily) in 8,059 patients (Harirforoosh, Asghar, & Jamali, 2014).
According to a case-control study reported by Page et al, NSAIDs increase the risk of congestive heart failure in older subjects (Page & Henry, 2000). Inhibition of PG synthesis and sodium retention due to serum aldosterone elevation especially by COX-I inhibitors increase blood pressure and reduce some antihypertensive drugs effects such as angiotensin-converting enzyme inhibitor (ACE) (Wongrakpanich et al., 2018). Inhibition of platelets aggregation and vasodilatation by PGI_2 play an important role in CV protection system. PGI_2 inhibition by COX-II inhibitors, especially coxibs, increases CV events like myocardial infarction (Schellack, Schellack, Schellack, Fourie, & Fourie, 2015; Vostinaru, 2017).

1.1.5.4. Hepatotoxicity

Hepatotoxicity by NSAIDs is rare, but can lead to a serious health problem. According to a cohort study conducted in the United Kingdom, NSAID-related hepatotoxicity was found in 1–8 out of 100,000 individuals each year. Hepatoxicity occurs within 12 weeks after starting NSAID but it is more common with diclofenac and sulindac (5-10 fold higher than other NSAIDs) (O'connor, Dargan, & Jones, 2003).
Nausea, vomiting, upper abdominal pain, fatigue, and jaundice are the documented symptoms associated with treatment by both COX-I and II inhibitors (Vostinaru, 2017). Cholestasis, hepatic necrosis, and liver failure are also observed in NSAIDs users.
Mitochondrial damages and protein adduct produced by reactive metabolites are the key mechanisms of NSAIDs hepatotoxicity. Indeed, NSAIDs are week acids and they are able to easily penetrate the mitochondrial outer membrane. These drugs interfere with proton transport from intermembranous space back to the mitochondrial matrix and block ATP synthesis

(uncoupling of oxidative phosphorylation) which lead to hepatocytes death. Acidic NSAIDs are also able to form protein adducts by binding to different cellular proteins with their carboxyl group. For example, protein adduct of these drugs with bile salts transporter eventuated to toxic bile salts accumulation in hepatocytes and hepatocytes death due to oxidative stress and apoptosis (Boelsterli, 2002). Inhibition of β-oxidation due to aspirin chronic administration, lead to fat accumulation and hepatocytes death (liver steatosis and fibrosis) (Fosslien n.d. 2001).

1.2. NSAIDs consumption in ultratrail

1.2.1. Prevalence

Many athletes of different sports like mountain or ultra-mountain bike, endurance (Didier et al., 2017), ultra-endurance (Martínez et al., 2017), marathon and ultra-marathon running (André, Girard, Gautier, Derambure, & Rochoy, 2020; Joslin, Lloyd, Kotlyar, & Wojcik, 2013), triathlon (Gorski et al., 2011) use NSAIDs during sport. NSAIDs are the most drugs consumed by ultratrailers, before acetaminophen (Didier et al., 2017; Hoffman & Fogard, 2011). In ultratrail, the prevalence of the use of NDAIDs ranged usually from 35 to 70 % (Table 3).
As shown by Martinez et al. in a study conducted in Spain including runners who participated to races with different distances (44 km, 67 km and 112 km), the prevalence of NSAIDs increases with distance (Martínez et al., 2017).

Table 3. Prevalence of NSAIDs in ultratrail. Prevalence was estimated by questionnaire filled by runners.

Events	Distance	Number of participants	Prevalence of NSAIDs	References
6000D trail 2016	65 km	212	26 %	(André et al., 2020)
2011 Desert Race Across the Sand (RATS)	238 km on 6 days	73	53.4%	(Joslin et al., 2013)
Infernal Trail des Vosges 2014	72 km 160 km	297	9.8 %	(Didier et al., 2017)
2005 Western States 100-Mile Endurance Run	160-km	60	72 %	(S. McAnulty et al., 2007)
Ultra Mallorca Serra de Tramuntana	112 km (Ultra) 67 km (Trail) 44 km (Matathon)	238	60.3 % for Ultra 49.2 % for Trail 35.5 % for Marathon	(Martínez et al., 2017).
2016 London Marathon	42 km	109	45.9 %	(Whatmough, Mears, & Kipps, 2017)
Western States Endurance Rune	160 km	60	72%	(Nieman et al., 2005)

Ibuprofen is the most consumed NSAID (60-80 % of cases), followed by diclofenac, ketoprofen salicylic acid, naproxen, piroxicam (Martínez et al., 2017; S. McAnulty et al., 2007; Nieman et al., 2005; Whatmough et al., 2017).
Athletes use NSAIDs before, during, and/or after the race or training, but mainly during the race, as shown by Martinez et al. (Figure 6) (Martínez et al., 2017).

	All Consumers (n = 115)	Ultra (n = 35)	Trail (n = 58)	Marathon (n = 22)	p Value
Only before competition (n, %)	18 (15.6%)	2 (5.7%)	7 (12.1%)	9 (40.9%) $^{\&,*}$	0.046
Only during competition (n, %)	62 (53.9%)	23 (65.7%)	31(53.4%)	8 (36.4%) $^{\&}$	0.004
Only after competition (n, %)	8 (7.0%)	3 (8.6%)	3 (5.2%)	2 (9.1%)	0.659
Before and during competition (n, %)	18 (15.6%)	4 (11.4%)	13 (22.4%)	1 (4.5%)	0.075
Before and after competition (n, %)	1 (0.9%)	0	1 (1.7%)	0	0.600
During and after competition (n, %)	7 (6.1%)	3 (8.6%)	3 (5.2%)	1 (4.5%)	0.482
Before, during and after competition (n, %)	1 (0.9%)	0	0	1 (4.5%)	0.240

Notes: Values are expressed as n (% with respect to number of consumers). $p < 0.05$ was considered statistically significant (Chi square test). $^{\&}$ indicates significant differences between Ultra and Marathon; * indicates significant differences between Trail and Marathon (Z-test).

Figure 6. Distribution of non-steroidal anti-inflammatory drugs consumers depending on the time-point. Ultra, trail and marathon refer to running distances of 112, 67 and 44 km **(Martínez et al., 2017)**.

Pain prevention (56.4%), pain relief (30.9%), reducing muscle injury or muscle injury treatment (9.1%) and cold or headache treatment (3.6%) were the main reasons for these drugs usage by athletes (Martínez et al., 2017). The dose consumed increases also with distance. For example, a Spanish study conducted on 3 distances reported that the dose ingested by runners was significantly higher in the 112 km race than in the 67 km and 44 km races (Figure 7).

	All Consumers (n = 62)	Ultra (n = 23)	Trail (n = 30)	Marathon (n = 9)	p Value
mg	835.5 ± 400.9	1052 ± 456	713 ± 206 $^{\#}$	689 ± 163 $^{\&}$	0.03
mg/hour competition	70.3 ± 31.2	61.3 ± 32.9	68.9 ± 27.0	96.7 ± 27.4 $^{\&,*}$	0.013

Notes: The values are the mean ± S.D. $p < 0.05$ was considered statistically significant (one-way ANOVA). $^{\#}$ indicates significant differences between Ultra and Trail; $^{\&}$ indicates significant differences between Ultra and Marathon; * indicates significant differences between Trail and Marathon.

Figure 7. Ibuprofen consumption during the Ultra Mallorca Serra de Tramuntana (Mallorca, Spain). Ultra, trail and marathon refer to running distances of 112, 67 and 44 km **(Martínez et al., 2017)**.

A considerable number of athletes use different types of NSAIDs without medical prescription (Gorski et al., 2011; Martínez et al., 2017). According to a study in Brazil 2008, 48.5% of Ironman Triathlon athletes used NSAIDs without medical prescription and most of athletes are not aware about NSAID effects (Gorski et al., 2011). However, NSAIDs are not free from adverse effects and only 30-40% of athletes have sufficient knowledge about them (André et al., 2020).

1.2.2. Consequences of NSAIDs use by trailers

The practice of a long and intense effort such as ultratrail leads to perturbations in biomarkers indicating organ damages: elevation of plasmatic creatinine (= reduction of renal function), elevation of creakine kinase (= muscular damages), elevation of troponin (= cardiac suffering), elevation of liver enzymes (= liver damages) and hydroelectrolytic disorders such dehydration or hyponatremia (if salt intake is insufficient compared to water intake). Abnormalities are also observed on imagery techniques like echography. Additionnaly, runners suffer from digestive problems. All of these troubles are usually temporary, and normalize one or few days after the race (Knechtle & Nikolaidis, 2018). Most of these troubles are also reported as adverse effects of NSAIDs. Thus, it appears difficult to distinguish adverse effects link to ultratrail and those link to NSAIDs use.

Some studies have been performed based on double-blind placebocontrolled trials.

- Lipman et al. have conducted a study on the effect of ibuprofen on acute kidney injury during a 80 km ultratrail (Lipman et al., 2017). Two groups of runners were constituted: ibuprofen group (= intake of 400 mg ibuprofen every 4 hours) and placebo group (= intake of placebo every 4 hours). The average dose of ibuprofen ingested was 1200 mg (75% of runners taking between 1,200 and 1,800 mg). Results showed a higher prevalence of acute kidney injury in the ibuprofen group (52 %) than in placebo group (34 %). The severity of acute kidney injury was also greater in the ibuprofen group. The association between NSAIDs use and the alteration of renal function was also reported when NSAIDs were consumed 24h prior the race (Reid et al., 2004).
- Nieman et al. have compared levels of plasmatic and urinary F_2-isoprostanes between user and nonusers of ibuprofen during an ultratrail. They reported higher levels on F_2-isoprostanes in users of ibuprofen, indicating an increased oxidative stress (S. R. McAnulty et al., 2007).
- Lambert et al. have shown that aspirin intake (1,300 mg) increases gastrotintestinal permeability during prolonged running (Lambert et al., 2007)

Moreover, Davies et al. and Wharam et al. have identified NSAIDs use as an additional risk factor for hyponatremia during long efforts (Davis et al., 2001; Wharam et al., 2006).

All of these studies have concluded that NSAIDs intake worsens adverse effects of ultratrail.

1.3. Analysis of NSAIDs

Many analytical methods are published concerning the analysis of NSAIDs in different matrix: blood, urine for pharmacokinetic study, meat and milk for food controls. Liquid chromatography (LC), gas chromatography (GC) and electrophoresis coupled with different detectors, especially mass spectrometer (MS), are the most used methods for NSAIDs analysis in different samples (Gentili, 2007).

1.3.1. Analytical methods

1.3.1.1. Separative methods

Liquid Chromatography

Liquid chromatography (LC) is a fundamental technique and the most used analytical device for chemicals analysis in different matrix. From 1999 up to 2010 the usage of LC was mentioned in about 70% of research papers related to NSAIDs analysis (I. Olives, Gonzalez-Ruiz, & Antonia Martin, 2012).

The reversed phase mode is the most used one for NSAIDs analysis (Table 4). As NSAIDs are weak acids with pKa values mostly around 4-5, these drugs are in non-ionized form at pH around 2-3 and interact well with lipophilic stationary phase (Farré, Petrovic, & Barceló, 2007). Thus, mobile phases used for NSAIDs analysis in reversed phase mode are composed of an acidic solvent. There is also a publication which reported anion exchange mode (Ayano et al., 2006).

Many methods reported the simultaneous analysis of more than 10 NSAIDs in human samples (plasma and urine) and other biologic and non biological samples such bovine milk and tissues (Gentili et al., 2012; Jedziniak, Szprengier-Juszkiewicz, Pietruk, Śledzińska, & Żmudzki, 2012; Nemoto et al., 2014; van Pamel & Daeseleire, 2015) or waste-water (Márta et al., 2018; Paíga et al., 2015). Whole saliva and capillary whole blood appear only in few works (Table 4).

Table 4. Characteristics of LC methods used for analysis of NSAIDs in whole saliva and capillary whole blood (DBS).

MP: mobile phase, LOD: limit of detection, LLOQ: limit of quantification, ACN: acetonitrile, PDA: PhotoDiode Array Detector, DAD: Diode Array Detector, TFA: trifluoroacetic acid, APy-PPZ: (S)-1-methyl-4-(5-(3-aminopyrrolidin-1-yl)-2,4-dinitrophenyl) piperazine, DAPAP: (S)-1-(4-dimethylaminophenylcarbonyl)-3-aminopyrrolidine,

NSAIDs studied	Matrix	Analytical Method	LOD & LLOQ	Reference
Furprofen, Indoprofen, Ketoprofen, Fenbufen, Flurbiprofen, and Ibuprofen	Saliva	HPLC-PDA Column: Symmetry C18 (75 × 4.6 mm, 3.5 μm) MP: phosphate buffer (pH= 2.5) and Acetonitrile	LOD: 30 ng/ml LLOQ: 80 ng/ml	(Tartaglia et al., 2020)

NSAIDs studied	Matrix	Analytical Method	LOD & LLOQ	Reference
Ketoprofen, Etodolac, Flurbiprofen and Ibuprofen	Saliva	HPLC-DAD column: Grom-Sil 80 Octyl-4 FE (250 × 4.6 mm, 3 µm) MP: ACN /1.0% TFA (40:60 v/v)	LOD: 0.07-0.18 µg/ml LLOQ: 0.22-0.61 µg/ml	(Hassan & Alshana, 2019)
Flurbiprofen	Saliva	HPLC-UV Column: Agilent ZORBAX SB-C18 column (250 × 4.6 mm, 5 µm,) MP: ACN / 0.001 M citric acid (90:10, v/v)	LOD: 0.01 µg/ml LLOQ: 0.03 µg/ml	(Aminu, Chan, Khan, & Toh, 2018)
Ketoprofen (*R*)- and (*S*)-isomers	Saliva	LC/ESI-MS/MS Column: Mightysil RP-18 GP (150 × 4.6 mm, 3 µm) chiral derivatization with APy-PPZ MP: water / ACN (58/42, v/v) containing 0.1% formic acid	LOD: 0.5 fmol injected	(Jin, Zhang, Jin, & Lee, 2018)
Ibuprofen (*R*)- and (*S*)-isomers	Saliva	LC/ESI-MS/MS Column: A YMC-Pack Pro C18 RS (150 × 2.0 mm, 5 µm) chiral derivatization with DAPAP MP: ACN /10 mM ammonium formate (11:9, v/v)	LOD: 0.15 fmol injected	(Ogawa, Tadokoro, Sato, & Higashi, 2014)
Salicylic acid, Ketoprofen, Naproxen, Diclofenac and Ibuprofen.	Saliva	HPLC-UV Column: STAR RP-18 (75 × 4.0 mm, 3 µm) MP: 0.1% formic acid (pH 2.6) and methanol	LOD: 0.09-0.3 µg/ml LLOQ: 0.1-0.5 µg/ml	(Ramos-Payan, Maspoch, & Llobera, 2016)
Piroxicam and 5-Hydroxypiroxicam	Saliva	LC-MS/MS Column: LiChroCART 125-4 RP Select-B Sorbent C18 (205 × 4.6 mm, 5 µm) MP: methanol and 2% phosphoric acid (70:30 v/v) pH 2.7	Piroxicam LLOQ: 0.15 ng/ml Hydroxypiroxicam LLOQ: 0.15 ng/ml	(Calvo et al., 2016)
Ibuprofen, Acetylsalicylic acid and Salicylic acid	Saliva	LC-MS/MS Column: Imtakt Cadenza CD-C18 HT (150 × 2 mm, 3 µm) MP A: 5 mM ammonium acetate containing 0.5% formic acid MP B: ACN	LOD: 100 ng/ml LOD for acetylsalicylic acid was not determined	(Kuwayama et al., 2016)
Ibuprofen	DBS	HPLC-MS/MS Column: Varian Polaris C18 (50 × 2.0 mm, 5 µm) MP : ACN /10 mM ammonium (62:38 v/v) pH4.2	Not mentioned	(Abu-Rabie & Spooner, 2009)

NSAIDs studied	Matrix	Analytical Method	LOD & LLOQ	Reference
Ketoprofen, Fenoprofen, Flurbiprofen, and Ibuprofen	DBS	UHPLC-MS/MS Column: Acquity UPLC® HSS T3 (100 × 2.1 mm, 1.8 µm) MP A: 20mM formic acid and methanol (95:5, v/v) MP B: 20mM formic acid and methanol (5:95, v/v)	Not mentioned	(Ask, Øiestad, Pedersen-Bjergaard, & Gjelstad, 2018)
Ibuprofen, Salicylic acid	DBS	LC-MS/MS Column: Acquity BEH C18 (100 × 2.1 mm, 1.7 µm) MP A : H2O and 0.1% formic acid MP B: ACN	LOD: 1.6 µg/ml for Ibuprofen and 0.07 µg/ml for Salicylic acid	(Gaissmaier, Siebenhaar, Todorova, Hüllen, & Hopf, 2016)
Naproxen	DBS	LC-MS/MS Column:Waters XBridge™ C18 (30 × 2.1 mm, 3 µm) MP: 0.1% propionic acid and methanol	LLOQ: 0.5 µg/ml	(Youhnovski, Bergeron, Furtado, & Garofolo, 2011)
Flurbiprofen and 4-OH-flurbiprofen	DBS	LC/MS/MS Column: RP-18 (25 × 4.6 mm) MP: H2O / methanol (90:10, v/v)	LLOQ: 1 ng/ml	(Déglon et al., 2011)

Gas Chromatography

For NSAIDs analysis by GC coupled with mass spectrometry (GC-MS), these drugs must change to less polar and more volatile molecules by a process called derivatization. For this purpose, the carboxyl group of NSAIDs is a good target group. Tri- or dimethylsilyl and amide are the most widely derivates produced (Migowska, Caban, Stepnowski, & Kumirska, 2012; Noche, Laespada, Pavón, Cordero, & Lorenzo, 2011; Sebők, Vasanits-Zsigrai, Palkó, Záray, & Molnár-Perl, 2008). As for LC, GC methods are mostly applied to milk or waste-waters. Concerning huma matrices, plasma is the most reported one and ibuprofen is the most analysed NSAID (Jack, Rumble, Davies, & Francis, 1992; Yilmaz, Sahin, & Erdem, 2014). To the best of my knowledge, no work reports the analysis of NSAIDs in saliva and capillary whole blood by GC.

Capillary Electrophoresis

Various modes of capillary electrophoresis are used for the analysis of NSAIDs in pharmaceutical preparations, biological samples and water samples. UV is the most detection system used. Since recently, MS is more and more popular (Macià, Borrull, Calull, & Aguilar, 2007).

Among all publications, only few methods used capillary electrophoresis for the analysis of NSAIDs in whole saliva (Table 5). To the best of my knowledge, no work reports the analysis of NSAIDs in capillary whole blood by capillary electrophoresis.

Table 5. Summary of some methods used for the analysis of NSAIDs in whole saliva by capillary electrophoresis

CE: capillary electrophoresis

NSAIDs	Matrix	Analytical Method	LOD & LOQ	Reference
Ketoprofen, Fenbufen and Indomethacin	Saliva	CE-DAD narrow-bore silica with id of 75 µm, an od of 375 µm detection wavelength of 214 nm separation voltage of 20 kV capillary conditioned by rinsing with 1 M HCl for 5 min, water for 1 min, 0.1 M NaOH for 10 min, water for 1 min and separation buffer for 10 min.	LOD*:* 1-50 ng/L	(Almeda, Arce, & Valcárcel, 2008)
Ketoprofen, Fenbufen and Indomethacin	Saliva	Micellar Electrokinetic Capillary Chromatography (MEKC). DAD with wavelength of 214nm capillary conditioned by rinsing with 1 M HCl for 5 min, water for 1 min, 0.1 M NaOH for 10 min, water for 1 min and separation buffer for 10 min	LOD: 0.1 µg/L	(Almeda et al., 2008)

1.3.1.2. Detection

Mass spectrometry (MS) is largely the most detection method used for NSAIDs analysis. MS is coupled with LC. Different types of MS analyzers are used: simple quadrupole, triple quadrupole, ion trap, Orbitrap® and time of flight (TOF). Orbitrap® and time of flight (TOF) could be coupled with a quadrupole to performed ms^2 analysis. Table 6 summarizes the type of analyzer, ionization mode and MS mode reported in the literature for the analysis of NSAIDs in whole saliva and DBS.

Table 6. Data on MS methods used for the analysis of NSAIDs in whole saliva and capillary whole blood

NSAIDs	Matrix	Type of analyzer	Ionization mode	MS mode	References
Ibuprofen	DBS	Triple Quadrupole	$[M-H]^-$	MS/MS	(Abu-Rabie & Spooner, 2009)
Flurbiprofen and 4-OH-flurbiprofen	DBS	Linear ion trap	$[M-H]^-$	MS/MS	(Déglon et al., 2011)
Ibuprofen	DBS	Triple Quadrupole			(Manicke, Abu-Rabie, Spooner, Ouyang, & Cooks, 2011)
Naproxen	DBS	Triple Quadrupole	$[M-H]^-$	MS/MS	(Youhnovski et al., 2011)
Ibuprofen, Salicylic acid,	DBS	Orbitrap®	$[M-H]^-$	MS/MS	(Gaissmaier et al., 2016)
ketoprofen, fenoprofen, flurbiprofen and ibuprofen	DBS	Triple quadrupole and ion trap	$[M+H]^+$	MS/MS	(Ask et al., 2018)
Ibuprofen	Saliva	Triple Quadrupole	$[M+H]^+$	MS/MS	(Ogawa et al., 2014)
Ibuprofen, Acetylsalicylic	Saliva and plasma	Triple Quadrupole	$[M-H]^-$	MS/MS	(Kuwayama et al., 2016)

acid and Salicylic acid					
Piroxicam and 5_ Hydroxypiroxicam	Saliva and Plasma	Triple Quadrupole	$[M+H]^+$	MS/MS	(Calvo et al., 2016)
Ketoprofen	Saliva	Triple Quadrupole	$[M+H]^+$	MS/MS	(Jin et al., 2018)

As shown in Table 6, the ionization mode is negative or positive depending on the NSAID. All methods used a ms² mode.

1.3.2. Sample preparation

1.3.2.1. Whole saliva

Whole saliva also called oral fluid (OF) is a mixture of different major and minor salivary and non-salivary glands secretion in the oral cavity (Crouch, 2005).

1.3.2.1.1. Composition of whole saliva

OF consists of water (98.4-99 %) and organic and inorganic substances. Inorganic compounds of OF are mainly composed of gas (CO_2, N_2, O_2) and minerals (ion hydrogenium, sodium, potassium, calcium, magnesium, chlorides, carbonates, phosphates, thiocyanates, iron, copper). Organic compunds, representing about 0.3 % of OF, are mostly proteins. In addition to organic and inorganic substances, OF also consists of food residue, endothelial and immune cells and microorganisms. Its pH is about 5.8 – 7.1, and its density about 1.004 – 1.012, OF is hypotonic compared to plasma.

Daily amount of the whole saliva produced by a healthy person reachs to about 500-1500 mL. In a normal adult, the flow rate of stimulated and unstimulated OF is 0.7-3 ml/min and 0.1-0.35 ml/min, respectively (Azevedo et al., 2008; White & Moore, 2018).

The components and the flow rate of OF produced in a specific time is very variable, and depend on physiologic (age, hydratation state, circadian rhythm) and pathologic parameters such as diabete. Exercise (hard exercise for 30 minutes) increases saliva viscosity through increase of salivary proteins (1,518 µg/mL before exercise and 2,244 µg/mL after high exercise), pH (6.9 before exercise and 7.2 after high exercise) and flow rate (0.62 mL/min before exercise and 0.94 mL/min after high exercise) (Ligtenberg, Liem, Brand, & Veerman, 2016).

Substances transport from blood into OF is the main reason of its usage as an analytical specimen. Passive diffusion, active transport and ultrafiltration through intracellular tight junctions are the mechanisms of substances transport form blood into whole saliva. Molecules size, pKa, lipophilicity and the degree of binding with plasma proteins are important factors which affect substance transport into the whole saliva (Crouch, 2005). Only non-ionized and free drugs can pass from blood to whole saliva through cell membrane. As NSAIDs present percentages of binding to plasmatic proteins about 95-99%, only a very small free fraction (unbound to plasmatic proteins) can cross from blood into OF. This can be illustrated by results published by Calvo et al.. They reported the analysis of piroxicam and its major metabolite (5-

hydroxypiroxicam), in saliva and blood samples. Piroxicam mean concentration (after 20 mg oral dose) from 10 volunteers after around 4 h was 308 times higher in plasma than saliva (2275.9 and 7.4 ng/ml in plasma and saliva, respectively). This ratio for 5-hydroxypiroxicam after about 53h, where 22.5 (133 and 5.9 ng/ml in plasma and saliva, respectively). The study also showed that after about 50 h, there is very big difference of piroxicam and its metabolite in plasma (piroxicam concentration is 27 time higher than its metabolite in plasma) but a very small difference (piroxicam concentration is 1.25 times higher than its metabolite in saliva) in OF. (Calvo et al., 2016)

1.3.2.1.2. Advantages and challenges linked to whole saliva

OF is a good biological matrix for analysis of different drugs and their metabolites.
Since the past 30 years, the saliva became a more popular matrix in pharmacology and toxicology (Figure 8).

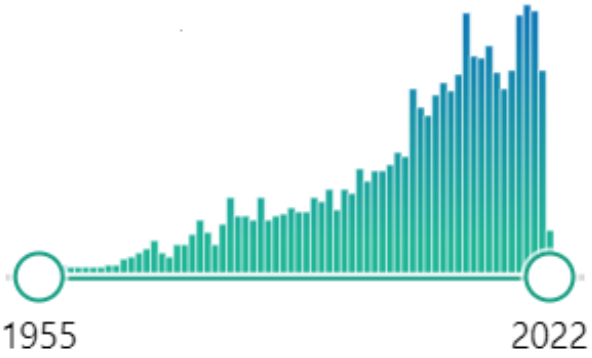


Figure 8. Number of results obtained per year in Pubmed using the terms "drugs and analysis and saliva"

OF samples have several advantages over blood for testing of different analytes. Some advantages of whole saliva as matrix are mentioned bellow (Hofman, 2001; Nagler, Hershkovich, Lischinsky, Diamond, & Reznick, 2002; Slowey, 2015):

- noninvasive and easy sampling
- possibility of repeated sampling from the same person
- sampling not necessarily made by a health professional
- no risk of infection
- good accessibility because sample collection could be performed during normal daily activity (in the work area, on the street, in the shop, during a trail…) (Gröschl, 2017).
- OF proteins level is considerably low than blood. This issue facilitates extraction process and decreases the risk of the interfering molecule during instrumental analysis (Liu & Duan, 2012)
- availability of different devices for whole saliva sampling

However, whole saliva presents also challenges for analysis:

- qualitative and quantitative intra-individual and inter-individual variability (pH, viscosity, flow rate, composition) (White & Moore, 2018)
- different factors like food, beverages, exercises, diseases, age, sex, poor oral hygiene, smoking, and drugs can change saliva components or flow rate (Bhattarai, Kim, & Chae, 2018; Crouch, 2005)
- difficulty in pipetting linked to the viscosity of whole saliva
- matrix effect due to preservative present in the collection devive

1.3.2.1.3. Collection of whole saliva

Generally, two types of saliva are used for analytical purpose: OF and specific gland saliva. OF collection is the mostly used method because it is the easier one.
Passive drooling, active spitting, suction or using saliva collection devices are the methods used for OF sample collection. (Bellagambi et al., 2020; Bhattarai et al., 2018; Crouch, 2005).
Different devices with various collecting methods exist for whole saliva sampling (Gröschl, 2017). Most of these devices are equipped with cotton as an absorbent material (Table 7).

Table 7. Different types of whole saliva collecting devices

Device Name	Indication	Collection mechanism	Volume adequacy indicator	Collected volume	References
Quantisal™	Drugs testing	Pad with buffer	Yes	1 ± 0.1 ml	Manufacturer
Salivette	Drugs testing	Collecting pad	No	1.1 ± 0.1 ml	Manufacturer
UltraSal-2™	Drugs testing	Whole saliva drooling	No	24 ml	Manufacturer
Versi•SAL®	Hormones and substances of abuse	Collecting pad	Yes	0.5 -1.4 ml	Manufacturer
Super•SAL™	Drugs of abuse	Collecting pad	Yes	1.0 ml	Manufacturer
Accu•SAL™	Hormones and Drugs testing	Collecting pad	Yes	0.75-0.90 ml	Manufacturer
Super•SAL™	Hormones and Drugs testing	Collecting pad	Yes	> 1.0 ml	Manufacturer
Intercept®	Drug of abuse	Collecting pad	Yes	1 ml	(Gröschl, 2017)
Versi SAL®	Proteomics	Collecting pad	Yes	1.2-1.4 ml	Manufacturer
Pedia•SAL™	For sampling from neonatal	Collecting pad	No	Not mentioned	Manufacturer

Cozart RapiScan®	Drugs of abuse	Collecting pad with buffer	No	1 ml	(Moore, Wicks, Spiehler, & Holgate, 2001)
Oral-Diffusion-Sink	Hormones testing	Plastic cylinder with many tiny ports and cellulose membrane	No	2 ml every 10 min	(Wade & Haegele, 1991)
OmniSal®	DNA detection	Pad with stabilizing buffer	Yes	1 ml	(Chohan et al., 2001)
Muddler	Drugs and biomarkers	Plastic swab with many tiny ports	No	Around 40 µl	(Takagi, Ishikura, Hiramatsu, Nakamura, & Degawa, 2013)

Despite the advantages of the devices such as easy sampling, collection of an accurate volume, analyte absorption on the cotton and rate of analyte recovery are the potential challenging points for some of these devices. The volume of saliva collected by the different device may be the same but there is a considerable difference between saliva volumes recovery. For example, the saliva volume collected by Salivette® and Hooded collector® was 1.86 ml and 1.69ml, but recovered volume was 1.48ml and 0.3 ml respectively (Crouch, 2005). Extraction recovery from saliva collected by devices depends on analyte and devices. For example, by comparing anticonvulsants and Busulfan extraction recovery in saliva collected with Quantisal®, extraction recovery for anticonvulsants were < 90 %, while for Busulfan, it was 106 % (Gröschl, Köhler, Topf, Rupprecht, & Rauh, 2008).
Some chemicals are stable in the OF for a long time, while others are not (Chiappin, Antonelli, Gatti, & De Palo, 2007). Therefore, some collecting devices contain a preservative.

Advantages and disadvantages of saliva collection devices are presented in Table 8.

Table 8. Advantages and disadvantages of saliva collection devices

Advantages	**Disadvantages**
Fast sampling	Quantitative errore due to saliva dilution
Hygienic sample collection	Increased matrix effect by device buffer (White & Moore, 2018)
Adequate volume sampling by most devices	Some devices without volume adequacy indicator
Storage and transport	Cost

Dry saliva spot (DSS) is another saliva collection method which is carried out by drying a low volume of saliva (50 µL) at room temperature on a saliva collection card's filter paper. Saliva

sampling by this method do not need pre-treatment procedure. Fast sampling, low sample volume, high stability of analyte and long storage are the advantages of saliva sampling by this method (Bellagambi et al., 2020; White & Moore, 2018).

The following points are important for saliva sample collection:

- Collect saliva sample at less 1 hour after taking food, beverage, oral hygiene or tooth brush.
- Saliva donor have to rinse their mouth with distilled water for 1 min, about 5 min before sampling.
- Analyst have to emphasis on saliva donor to avoid nasal and pharynx fluids mixing with saliva.
- In the case of saliva collection with cotton or polystyrene swab, inform the donor to avoid swab chewing.

For the storage of samples, in the case of saliva collection with direct spitting or drooling in a collecting tube, it is better to store it in a refrigerator (4°C) as soon as possible. If saliva sample immediate analysis is not intended, it is recommended to freeze saliva sample at -20 °C (or at -80 °C for keeping more than 6 month). To avoid repeated freezing and defreezing cycle, it is better to prepare aliquot of collected sample (Bhattarai et al., 2018; Enomoto, Nambu, Kashiwagi, Okinaga, & Baba, 2020).

1.3.2.1.4. Preparation of whole saliva before analysis

The pre-treatment phase is an important step for substances analysis in saliva. Saliva viscosity, matrix impurities and interferences are the main aims of this stage. Even saliva sample pre-treatment is advantageous; this step often requires time and most of the analytical errors could be made in this phase. Some interventions in this step such as protein precipitation, centrifugation, sonication and extraction may lead to analyte loss and affect quantitative results (Tartaglia et al., 2020).

If saliva is not collected wih a device, in order to decrease saliva viscosity, freshly collected saliva could be to diluted 1:1 with 150 mM NaCl, homogenized for 1 min with a vortex and centrifugation for 5 min at 10,000 g at room temperature (Ligtenberg et al., 2016).

If saliva is collected by a device, the first step is the desorption of studied analytes from the pad. Inappropriate squeezing eventuates sample contamination or affects quantitative results. Using disposable needle-less plastic, direct sequeezing in collecting tube or manual squeezing with pipette tip are saliva recovery methods from collecting swab (Sobczak & Goryński, 2020).

After desorption from the pad, an extraction step is performed in order to decrease interfering substances. Both liquid/liquid extraction (LLE) and solid phase extraction (SPE) are used for NSAIDs extraction from saliva. LLE is the most used one (Table 9). Among the different extraction methods indicated in table 9, 1 out of 9 study used SPE and 8 out of 9 used LLE methods. Nowadays due to large solvent volume consumption, long operation time and environmental toxicity of traditional LLE solvents, different microextraction methods (LLME)

which needs microliter volume of solvent are used for these drugs extraction (Seidi, Rezazadeh, & Alizadeh, 2019). Ethyl acetate is the most used solvent for NSAIDs LLE from saliva. Extraction recoveries of NSAIDs ranged from 76 to 100 % depending on NSAID and extraction method (Table 9).

Table 9. Methods used for extraction of NSAIDs in saliva.
FPSE: fabric phase sorptive extraction, LLME : liquid/liquid microextraction

NSAIDs	Collecting device	Sample pretreatment	Extraction Method	Extraction recovery (%)	Reference
Furprofen, Indoprofen, Ketoprofen, Fenbufen, Flurbiprofen, and Ibuprofen	Drooling	No pretreatment has been applied.	**FPSE extraction technique (C18)** 2 ml acetonitrile:methanol (50:50), remove organic solvent with 2ml deionized water 2-3 times.	85.6 – 105.6 %	(Tartaglia et al., 2020)
Ketoprofen, Etodolac, Flurbiprofen and Ibuprofen	Not mentioned	No pretreatment has been applied.	**LLME** 4 ml ACN and 1ml NaCl saturated solution 500 µl dimethylcyclohexylamine (DMCA) 500 µl of 20 M sodium hydroxide.	96.8-104.5 %	(Hassan & Alshana, 2019)
Flurbiprofen	Simulated saliva	No pretreatment has been applied.	1ml spiked simulated saliva, diluted with 15 ml MeOH.	97.98 %	(Aminu et al., 2018)
Ketoprofen (*R*)- and (*S*)-isomers	Drooling/ spitting	No pretreatment has been applied.	**LLE** saliva samples diluted with 200 µL 0.02 M acetic acid and extracted with 200 µL ethyl acetate.	100.5-105.9 %	(Jin et al., 2018)
Ibuprofen (*R*)- and (*S*)-isomers	Drooling/ spitting	No pretreatment has been applied.	**LLE** saliva samples diluted with 100 µL 0.02 M acetic acid and extracted with 100 µL ethyl acetate.	101.3– 106.1%	(Ogawa et al., 2014)
Salicylic acid, Ketoprofen, Naproxen, Diclofenac and Ibuprofen.	Not mentioned	Saliva pH adjusted to 1.5 with HCl	**Liquid phase microextraction (LPME)** Analytes pass from a donor phase of HCl to acceptor phase of NaOH	76.1-98.8 %	(Ramos-Payan et al., 2016)
Piroxicam and 5-Hydroxypiroxicam	Not mentioned	Acidification with 400 µL of 0.5 M HCl	**LLE** Extraction with 2 mL ethyl acetate	91.7 and 99.2 %	(Calvo et al., 2016)
Ibuprofen, Acetylsalicylic acid and Salicylic acid	Drooling/ spitting	Dilution and deproteinization with ACN	**LLE** Exraction with 200 µL ACN	94.6-117.5 % ER for Acetyl salicylic acid was not determined	(Kuwayama et al., 2016)
Ketoprofen, fenbufen and indomethacin	Spitting	Saliva samples are centrifugal ultra-filtered at 8050 × 6g for 30 min.	**SPE** RP C-18 sorbent conditioned with 1 mL of methanol and 1 mL of ultrapure water. 1 mL ultrafiltered saliva sample was transferred SPE washed with 1 ml water and analytes are eluted with 1 ml MeOH.	92 to 98 %	(Almeda et al., 2008)

The entire workflow for preparation of OF samples from sampling to analysis is summarized in figure 9.

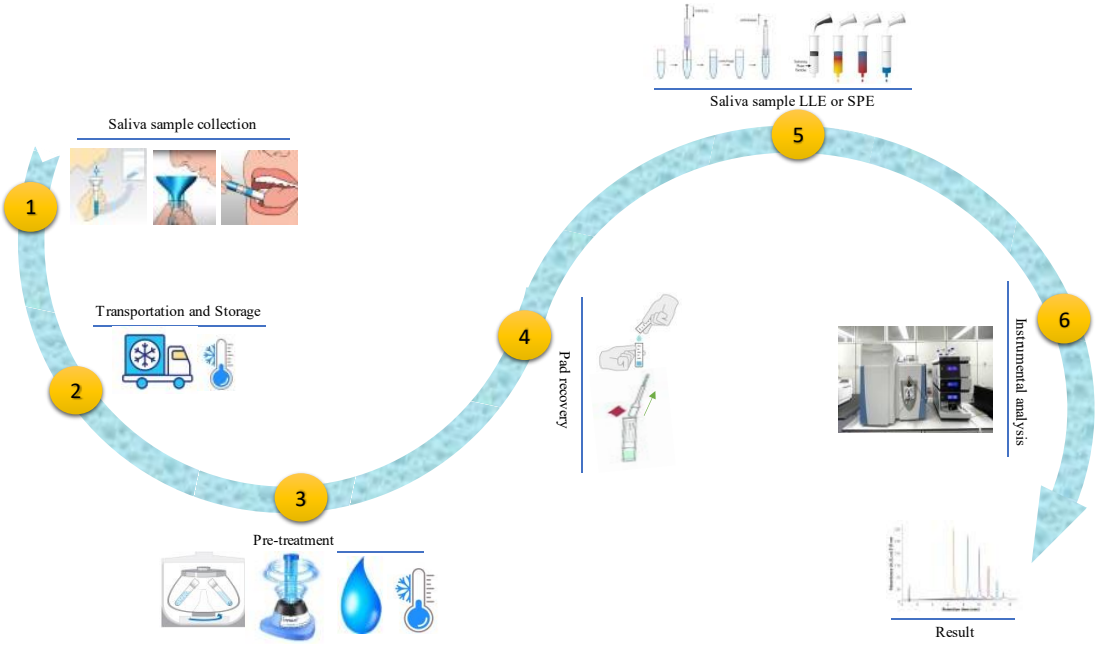


Figure 9. Steps of the workflow for analysis of NSAIDs in oral fluid, from sample collection to analysis.

1.3.2.2. Dried blood spots

For the first time a new blood sampling method called Dried Blood Spot (DBS) was used by Robert Guthrie in 1963 (Lehmann, Delaby, Vialaret, Ducos, & Hirtz, 2013). DBS is a micro-sampling method require only10-50 µl blood (Henion, Oliveira, Li, Foley, & Pomponio, 2013). Transportation and storage facility and analytes stability are the important features of DBS (Sharma, Jaiswal, Shukla, & Lal, 2014).

As depicted in table X, DBS is a technique used in therapeutic drugs monitoring, pharmacokinetics, toxicokinetics, doping control, forensic sciences, immunology, clinical trials (Sharma et al., 2014) and some infectious diseases diagnosis such as HIV, hepatitis B and C (Tuaillon et al., 2020). In addition of blood sampling, DBS is also used for plasma, urine, breast milk and saliva (Nakadi, Garde, da Veiga, Cruces, & Resano, 2020; Tey & See, 2021; Wilhelm, den Burger, & Swart, 2014).

New blood collecting devices which are able to generate volumetric blood spot from nonvolumetric blood drop, are the most using. These devices are used to overcome common DBS challenges such hematocrit, spot inhomogeneity and volume related bias. HemaXis, hemaPEN, Capitainer-B, and volumetric absorptive microsampling (VAMS) are different devices used for this purpose (Denniff & Spooner, 2014; Nakadi et al., 2020; Velghe & Stove, 2018). In the present presentation we focused on DBS card. For our experimental work was used HemaXis DB 10, a microsampling device presentingMme Florence ignon cadre serviceMm four microfluidic channels with filling capacity of 10µl finger pricked blood.

DBS cards/filter paper

DBS cards are made of cellulose or non cellulose matrix with specific pore sizes and thickness (Locatelli et al., 2020). Type 903 Cards (GE Healthcare Life Sciences, formerly Whatman) and the grade 226 (PerkinElmer Health Sciences, formerly Ahlstrom Filtration LLC) are the most used papers cards and registered by the Food and Drug Administration (FDA) (Wilhelm et al., 2014). Different Whatman filter papers such as Whatman 903, Flinders Technology Association or FTA DMPK type-A, B, C cards and FTA Elute cards are commercially available. DMPK A and B are chemically treated Whatman 903 DBS cards and DMPK C is non-treated. Chemically treated cards are used for cells lyses, proteins denaturation, enzymes inactivation and bacteria growth prevention in the matrix. Untreated cards are mostly used for proteins based molecules analysis (Gaissmaier et al., 2016).

Cellulose path thickness, pore size and particles retention are DBS card related factors which play important role in blood spot thickness, density and spreadability (Wagner, Tonoli, Varesio, & Hopfgartner, 2016). Moisture in DBS due to improper drying, chemicals leaking from DBS package (plasticizers for example) in to the sample due to improper packaging or transportation, may increase the risk of interference or matrix effects (Edelbroek, Heijden, & Stolk, 2009).

Drying Effects on DBS Card

Spot drying after blood application on DBS card, is crucial for analytical test results. Blood spot drying highly depend on the type of paper card, spot volume and ambient temperature. It is very important to keep away blood spot from sun light, dust and other factors during drying (Sharma et al., 2014). At room temperature, appropriate blood sample drying require 2-3 h. Inappropriate blood spot drying highly affect analytical test results due to enzymatic or bacterial activity.

According to Van Baar et al, 2013, drying time for spot have to be at least 2h and then DBS card have to stored in a plastic bag at room temperature in a desiccator prior analysis (Keevil, 2011; van Baar et al., 2013).

DBS Storage and Transportation

In contrast to conventional blood sample, DBS sample transportation and storage is very simple and don't require special transportation mean or equipments such as freezer or refrigerator. Even analyte stability is an important advantage of DBS, but storage condition humidity significantly affects substances stability and provide the chance for bacterial growth (Sharma et al., 2014). In order to avoid enzymatic reaction and bacterial effects, it is generally recommended to dry DBS samples completely before storage and transportation (Malátková et al., 2017).

Sample collection

Blood from finger, toe, earlobes or heel (mostly in neonates) pricking with sterile disposal lancet is used for DBS preparation (Keevil, 2011; Sharma et al., 2014; Tey & See, 2021). For digital sampling, 3rd 4th or 5th finger are generally preferred due to their innervation by ulnar nerve which is less sensitive than fingers innervated by median nerve (Tuaillon et al., 2020). Warming or massaging individuals sampling area (finger in most case), cleaning the sampling site with 70% isopropyl alcohol, finger pricking with sterile lancet (lancet is better than needle),

wiping out the first blood drop (due to high level of intera cellular fluid in first drop) with sterile tissue (Edelbroek et al., 2009; Milosheska, Grabnar, & Vovk, 2015), hand gently rubbing and worming before puncture and applying intermediate pressure after puncturing (Tuaillon et al., 2020) are important before blood sampling (Antunes, Charão, & Linden, 2016).
Blood sampling in this method fulfilled by two different ways, direct collection of whole blood drop on sampling paper and blood pipetting directly onto DBS paper. DBS sample preparation by direct contact of blood drop with filter paper, is not an effective method. Poor repeatability, blood equal dispersion and spot homogeneity are the main challenges of this method (Edelbroek et al., 2009; Wilhelm et al., 2014). Even using capillary pipette for DBS generation, relatively solved the mentioned problems, however, spots overlapping, variation in collected blood volume and uniform samples collection, are still key challenges of DBS (Milosheska et al., 2015). Furthermore, capillary tube or using of micro-pipette is not practical for on-field blood sample collection.

Hematocrit Effect

Hematocrit is the volume and percentage of red blood cells in blood, which has important effect on blood viscosity (Wilhelm et al., 2014). Hematocrit range is 0.40-0.50 and 0.35-0.45 for adult men and women, respectively (Antunes et al., 2016). Hematocrit affect blood spot formation, homogeneity, drying time, analyte recovery and analytical test reproducibility. By increase in hematocrit (blood viscosity), the size of DBS decreases proportionally.

1.3.2.2.1. Sample preparation

Different blood spot punching methods such as manual, semi-automated and automated punching are used for blood dried spot preparation at various diameter (Sharma et al., 2014).

DBS Desorption/Extraction

Generally, DBS extraction procedure involves spot punching, spot immersion in desorptive solvent, sonication or vortex mixing, centrifugation, solvent evaporation and then subjecting to instrumental analysis. Desorption solvent selection is a crucial point for analyte separation from DBS. Methanol, acetonitrile, or mixture of both at different proportions are the most effective solvents for analyte extraction. Using water as a solvent will increase matrix effects due to different agents co-extraction (Edelbroek et al., 2009; Keevil, 2011).
Both off-line (all separation procedure conducted by analyst) and online extraction (solvent desorption of DBS by analytical instruments) methods are used for analytes separation from DBS card (Henion et al., 2013). Automation of DBS has the advantage of simultaneous extraction and analysis of dried blood spots into one integral process (Déglon et al., 2011; Tey & See, 2021).

Internal standard introduction

In the case of analytical method validation by using DBS samples, improper usage of internal standard (IS) can affect extraction recovery and quantitative analytical results. There are different ways for adding IS on DBS sample such as adding IS small volume in extraction solvent, application of IS on DBS card paper before sampling, and IS applying on blood before

its absorption by DBS card. According Abu-Rabie et al. 2011, there were no significant difference in accuracy and precision by using above mentioned techniques for IS application on DBS samples, but application of IS into extraction solvent is the most used method (Antunes et al., 2016).

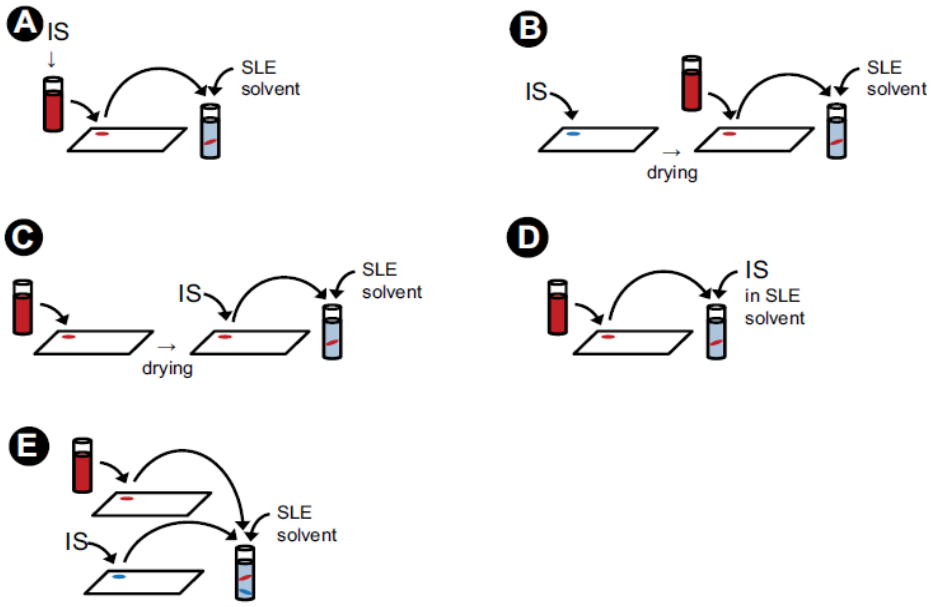


Figure 10. Different methods of IS introduction **(Wagner et al., 2016)**

Table 10. Summary of methods used for the analysis of drugs in DBS

Drugs	DBS Paper	Spotting volume and method	Extraction Method	Extraction recovery LOD & LLOQ	References
Ibuprofen, acetaminophen, and other drugs	Ahlstrom grade 237 paper	15 µL	Direct desorption with the TLC-MS interface	Not mentioned	(Abu-Rabie & Spooner, 2009)
ketoprofen, ibuprofen, flurbiprofen, fenoprofen,	Whatman® FTA® DMPK-C cards	5, 10 or 20 µL Entire spot for 5 an 10 µL samples and 3 mm discs for 20 µL samples were punched out	Desorption with 20mM formic acid and extraction with dihexyll ether centrifugation 15 min at 14 000 rpm.	ER= 58-74.7% LOD and LLOQ not mentioned	(Ask et al., 2018)
Acetaminophen and Ibuprofen	Whatman DBS card	10 µL	No sample preparation	LOD = >100 ng/mL for acetaminophen and ibuprofen	(Manicke et al., 2011)
Naproxen	Whatman® FTA Classic Card And DBS DMPK-B card	20 µL 3-mm disk was punched out	Desorption with 1 mL MeOH containing IS	ER=DBS classic = 73.5% DBS DMPK-B= 113.2% LLOQ= 0.5 µg/ml	(Youhnovski et al., 2011)
Acetaminophen, ibuprofen, salicylic acid and other drugs	Whatman™ 589 DBS filter paper	2 µL 8 mm diameter was punched	On-line desorption electrospray ionization	ER= 85% to 104% LOD = 0.07 to, 5 µg/ml	(Gaissmaier et al., 2016)

Drugs	DBS Paper	Spotting volume and method	Extraction Method	Extraction recovery LOD & LLOQ	References
Flurbiprofen and 4-OH-flurbiprofen	Whatman filtre paper card	5 µL	On-line desorption with MeOH	LLOQ = 10 and 100 ng/mL for OH-FLB and FLB, respectively LOD = 1 ng/mL for each compound ER= 50.2- 56.6 for both FLB and OH-FLB	(Déglon et al., 2011)

Volumetric Absorptive Microsampling (VAMS), Therapeutic drug monitoring (TDM), Pentafluoropropionic anhydride (PFPA), Pharmacokinetic (PK)

1.3.2.2.2. Advantages and challenges linked to DBS

DBS Advantages

- Sampling with minimum pain
- Self-sampling (Wilhelm et al., 2014)
- Long term storage of sample at ambient conditions (Gaissmaier et al., 2016)
- Cost effective (especially from transportation and storage point of view) (Locatelli et al., 2020)
- Analyte stability in sample (Wilhelm et al., 2014)
- No risk of sample lose during transport or storage (Niemiec, 2021)

DBS Disadvantages

- Blood viscosity (hematocrit) effects on analyte concentration
- Strong affinity between some analyte and DBS card (Locatelli et al., 2020)
- Analyte trapping by blood different components (proteins, fats, blood cells) (Locatelli et al., 2020)
- Need of sensitive analytical methods (LC-MS/MS) (Locatelli et al., 2020)
- Variability in the nature of sampling paper of different brands (Edelbroek et al., 2009)
- No extra sample if required for additional tests (Wilhelm et al., 2014)
- Long drying time (at least 2 h) (Niemiec, 2021)

2.EXPERIMENTAL PART

The experimental part of my thesis is presented in 3 parts:

- Firstly, the development of the LC method and the MS method for the screening of about 20 NSAIDs. The majority of this work was performed on pure solutions of NSAIDs.
- Secondly, the development of a method for the preparation of OF and the validation of all the workflow, including the sample preparation and the analysis
- Finally, the development of a method for the preparation of DBS and the validation of all the workflow, including the sample preparation and the analysis

2.1. Development of a LC-MS/HRMS method for the analysis of NSAIDs

2.1.1. Aims

The aim of this part of my thesis was to develop a LC-MS/HRMS method for the identification and quantification of NSAIDs.

2.1.2. Material

The liquid chromatography apparatus is a Ultimate 3000® system (Thermo Fisher Scientific). It is composed of 2 ternary pumps, a sampler and 2 ovens (a multi-column oven and an oven for on-line extraction coupling).

The mass spectrometer is a Q Exactive Plus® (Thermo Fisher Scientific). It is composed of a heated electrospray ionization source, a quadrupole, a collision cell and an Orbitrap®. Analysis on a Q Exactive Plus® could be performed in full scan mode and in ms² mode.

2.1.3. Results

LC method development

Column selection

Chromatographic conditions were studied on individual NSAIDs and internal standards pure standard solution at 100 ng/ml MeOH. ACN and H_2O, both with 0.1% acetic acid was used as mobile phase for the first tests.

Based on the structure of tested molecules and previously published articles, we selected three chromatographic columns with different characteristics. NSAIDs have high carbon to heteroatom ratio, therefore reversed phase C18 is mostly used columns for their separation. Hydrophobic and π-π interactions are the main separation mechanism with C18 stationary phase. Even NSAIDs contain different chemical functions such as halogens, amines and carboxylic acid groups, they all share a six-carbon aromatic ring. Therefore, reversed stationary phase with polar mobile phase is an effective strategy for their separation by hydrophobic, electrostatic π-π interactions mechanism. Phenyl stationary phases provide π-π interactions between the phenyl groups of the stationary phase and any unsaturated bonds in the analyte (Yang, Fazio, Munch, & Drumm, 2005). The acid in mobile phase keeps NSAIDs in non-ionized form and ameliorates their hydrophobic interaction with stationary phase.

The three columns were tested: Accucore Phenyl Hexyl C6 RP (100 × 2.1mm, 2.6 µm with phenyl groups that are bound to silica surface using a 6-carbon chain) (Thermo Scientific, USA), Accucore RP-MS (100 × 2.1mm, 2.6 µm with C18 silica surface) (Thermo Scientific, USA) and Accucore aQ (150 × 2.1mm, 2.6 µm with C18 on silica surface and endcapping) (Thermo Scientific, USA). Chromatographic results for salicylic acid (good peak shape, peak area and fit on baseline) and para-aminosalicylic acid (good peak separation from mesalazine with same exact mass (145.04987 for both) and very close retention time) in pure solution were better by using Accucore aQ column than the two others. Therefore, Accucore aQ column was selected.

After the choice of the column, 4 gradients were tested (Table 11).

Table 11. Gradient programs tested during LC method development
The flow rate was set to 200 µL/min. MP A: acetonitrile with 0.1% acetic acid, MP B: H_2O with 0.1% acetic acid

Gradient 1			**Gradient 2**			**Gradient 3**			**Gradient 4**		
Time	MP A	MP B	Time	MP A	MP B	Time	MP A	MP B	Time	MP A	MP B
0.000	40	60	0.000	20	80	0.000	30	70	0.000	25	75
0.000	40	60	0.000	20	80	0.000	30	70	0.000	25	75
1.000	40	60	1.000	20	80	1.000	30	70	1.000	25	75
2.500	95	5	3.000	95	5	2.800	95	5	2.900	95	5
6.000	95	5	6.500	95	5	6.300	95	5	6.400	95	5
6.100	40	60	6.600	20	80	6.400	30	70	6.500	25	75
10.000	40	60	10.500	20	80	10.300	30	70	10.400	25	75

Chromatographic results achieved with gradient 4 was better than those obtained with other gradient programs. Therefore, gradient 4 was selected.

In our method, some NSAIDs (salicylic acid, para-aminosalicylic acid and tenoxicam) have shorter retention time than others. As mentioned by Hassan et al, the carboxyl and amin group in para-aminosalicylic acid and carboxyl group in salicylic acid increase their polarity and decrease their retention time. Other NSAIDs such as aceclofenac, diclofenac, etodolac, flurbiprofen, indomethacin, ketoprofen, naproxen, niflumic acid, sulindac and tiaprofenic acid, also have carboxyl group. Unlike all these molecules with two aromatic rings, salicylic acid and para-aminosalicylic acid has only one aromatic ring, therefore there is a weaker hydrophobic interaction (phenyl π-π interactions) for these molecules with stationary phase.
According to De Ruiter et al, in the case of salicylic acid and para-aminosalicylic acid, the carboxyl group attached directly on aromatic ring is more acidic. However, arylpropanoic acid in ibuprofen makes this molecule more lipophilic with a higher log P value (Log P for salicylic acid, para-aminosalicylic acid and ibuprofen is 1.69, 0.62 and 3.5, respectively). Therefore, even ibuprofen has only one aromatic ring, like salicylic acid and para-aminosalicylic acid, it has a relatively long retention time.
Log P play an important role in analytes retention by reversed phase chromatography. As demonstrated in figure 11, NSAIDs with low Log P value are eluted earlier than NSAIDs with greater Log P value. The relationship between Log and retention time, is demonstrated in figure 11.

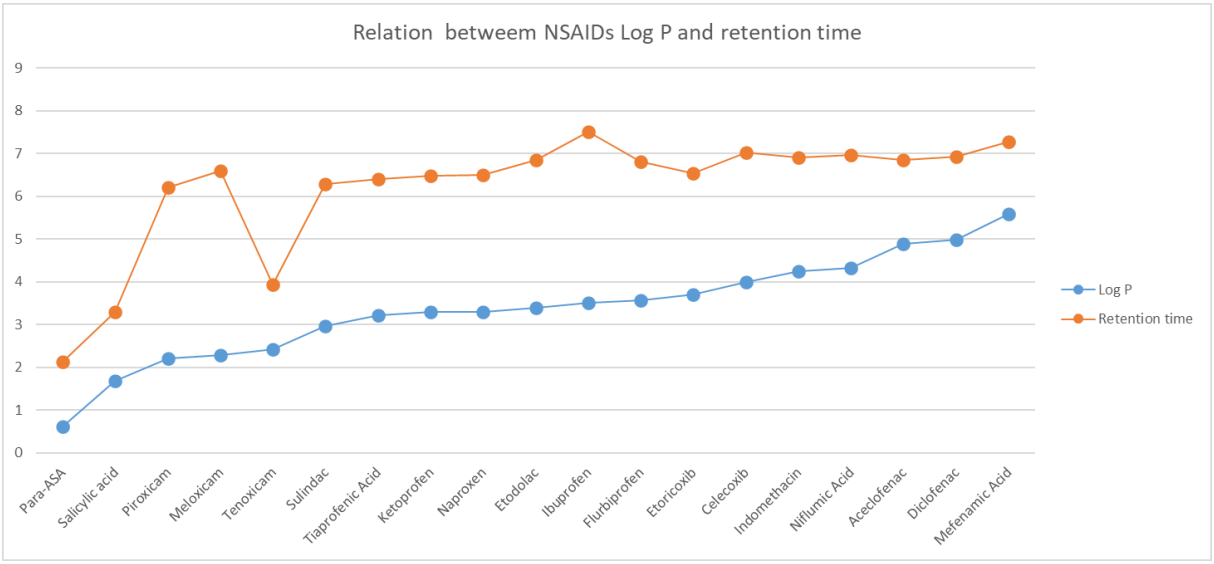


Figure 11. Relation between log P and retention time for NSAIDs using Accucore aQ column

As depicted in figure 11, if Log P value increases, NSAIDs retention time increases with except of piroxicam and meloxicam. Tenoxicam, piroxicam and meloxicam are all belong to oxicam family with chemical structure closed to each other. But, both piroxicam and meloxicam have high retention time with low Log P value. The same results were reported by Ji et al, for tenoxicam with shorter retention time (2.1 min) than piroxicam and meloxicam (3.3 min and 4.7 min respectively) (Ji, Lee, Kim, Jeong, & Lee, 2005). Piroxicam and meloxicam higher retention time than tenoxicam maybe related to their chemical structure. As demonstrated in table 1, piroxicam and tenoxicam has a couple of phenyl ring on the one site of their molecular structure, which may lead to increased π-π interactions between these molecules and stationary phase and increased their retention time. Lack of double phenyl rings in tenoxicam may eventuated to its low retention time compared to other molecules in the same chemical group.

Mobile phase optimization

We compared acetic acid and formic acid (0.1%) in the mobile phase composed of ACN/H_20 to evaluate effects of the nature of acid on chromatofraphic results. No significant changes in analytes retention time and peak resolution using both acids. But, with mobile phase acidification with formic acid, ibuprofen and ibuprofen $^{13}C_6$ were not observable on the chromatogram. Furthermore, peak areas for most of analytes were better using acetic acid.

A chromatogram presenting retention and chromatographic peak of all NSAIDs is presented in the 1st article related to the validation of the method in OF.

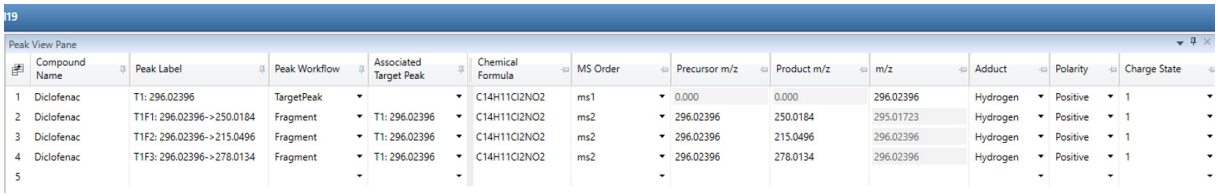
MS method development

The Q Exactive Plus mass spectrometer operated in full scan mode (from m/z 100 to 650 at a resolution of 35,000) and in ddMS² mode (at a resolution of 17,500) after positive and negative

electrospray ionization. MS conditions such as spray voltage (2.5 kV), nitrogen gas pressure (25 arbitrary units), auxiliary gas pressure (10 arbitrary units) and capillary temperature (320 °C) were optimized for individual NSAIDs standard solution at 100 ng/ml in MeOH.
Both positive and negative ionization modes were tested and the optimal ionization mode was selected by comparing the signal to noise ratio for each NSAIDs and ISs after injection/infusion at 100 ng/ml MeOH. Peak area values for all analytes were greater in positive ionization mode ($M+H^+$), excepted for flurbiprofen, ibuprofen, ibuprofen $^{13}C_6$, naproxen and salicylic acid.
Mostly positively charged NSAIDs contain basic nitrogen atom with an unbounded electron pair which can attached to H^+ to produce positive ion. The lack of nitrogen atom in flurbiprofen, ibuprofen, naproxen and salicylic acid eventuated to lose of H^+ by their carboxyl group and negative charge during ionization (Márta et al., 2018).

Tracefinder software

Data processing including identification and quantification was carried out using Trace Finder (Version 4.0) software.
As depicted in figure 12, a database was created for each NSAIDs. It contains name, exact mass, ionization mode, m/z of mother molecule and fragments, acquisition mode (full scan or $ddMS^2$).


Peak View Pane

	Compound Name	Peak Label	Peak Workflow	Associated Target Peak	Chemical Formula	MS Order	Precursor m/z	Product m/z	m/z	Adduct	Polarity	Charge State
1	Diclofenac	T1: 296.02396	TargetPeak		C14H11Cl2NO2	ms1	0.000	0.000	296.02396	Hydrogen	Positive	1
2	Diclofenac	T1F1: 296.02396->250.0184	Fragment	T1: 296.02396	C14H11Cl2NO2	ms2	296.02396	250.0184	295.01723	Hydrogen	Positive	1
3	Diclofenac	T1F2: 296.02396->215.0496	Fragment	T1: 296.02396	C14H11Cl2NO2	ms2	296.02396	215.0496	296.02396	Hydrogen	Positive	1
4	Diclofenac	T1F3: 296.02396->278.0134	Fragment	T1: 296.02396	C14H11Cl2NO2	ms2	296.02396	278.0134	296.02396	Hydrogen	Positive	1
5												

Figure 12. TraceFinder database. Example of diclofenac

mzVault software

A database containing the spectrum of each NSAIDs was created using mzVault 1.0sp1 (Thermo Fischer, USA) software (Figure 13). Collision energy and NSAIDs fragmentation was optimized with infusion of standard solution at 100 ng/ml MeOH. Different collision energies were tested for each NSAID and internal standards. Fragments intensity was the criteria for collision energy selection.

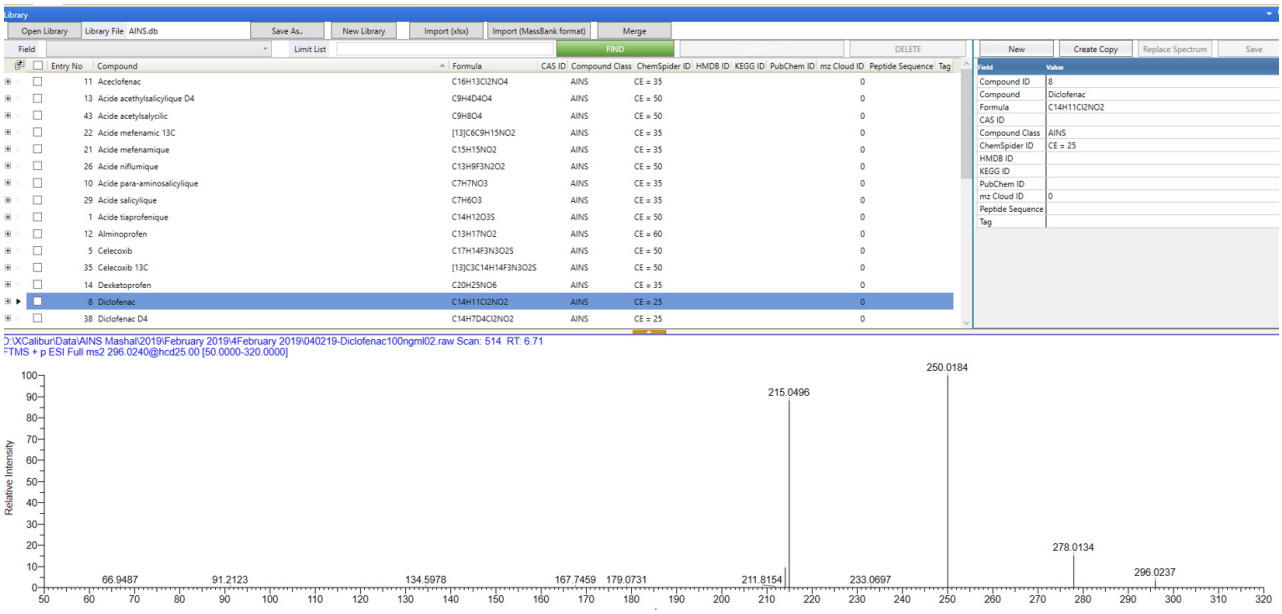


Figure 13. mzVault database. Example of diclofenac

Identification of NSAIDs

Identification of NSAIDs on chromatogram was performed using TraceFinder software. Criteria for identification were retention time, m/z of molecular ion (full scan mode), m/z of fragments (ddm² mode) based on TraceFinder database (Figure 14). TraceFinder was linked to mzVault database in order to use the fragmentation spectra for identification.

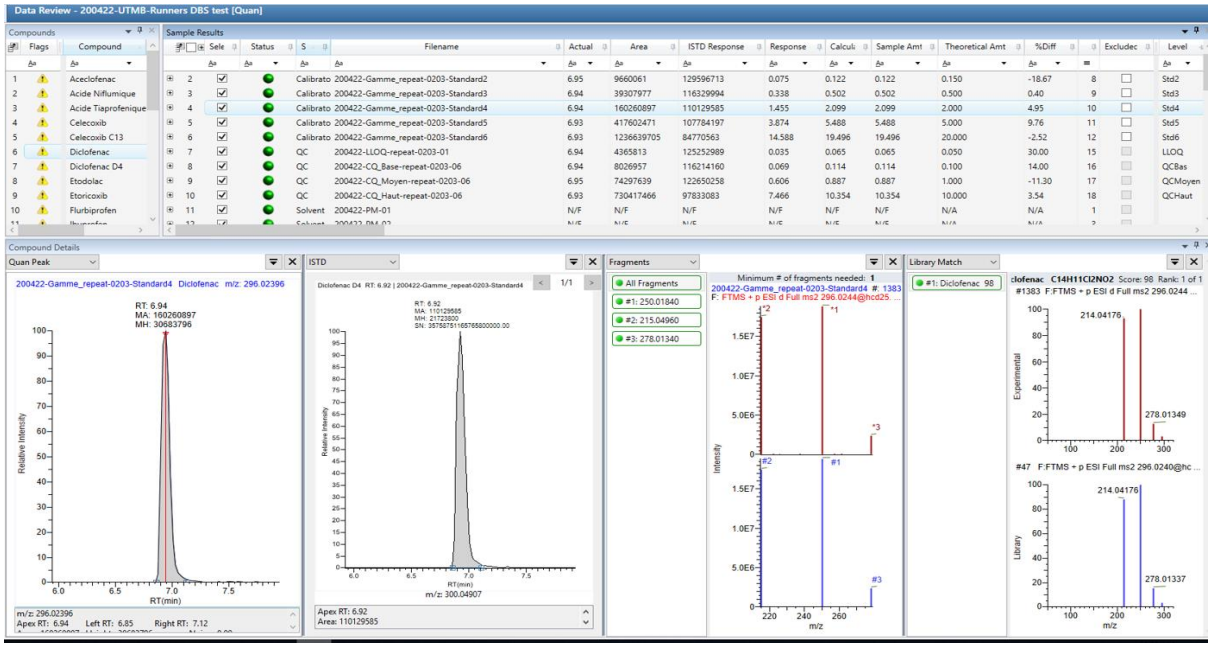


Figure 14. An example of identification of diclofenac using TraceFinder software.

2.2. Validation of the LC-MS/HRMS for the quantification in OF

2.2.1. Aims

The aims of this part of my thesis were:

- the development of a sample preparation method for analysis of NSAIDs in OF
- the validation of the method in OF
- the application of the method in OF collected from trailers

2.2.2. Results and discussion

2.2.2.1. Sample preparation

Sample collection

Among all devices commercially available, we have chosen the Quantisal® device. Quantisal is a whole saliva collection device containing saliva collecting swab and a plastic tube with a preservative buffer. Collecting swab made of cellulose pad is fixed to a polypropylene stem with a volume adequacy indicator at the top. The cotton pad absorbs 1 mL (± 10 %) of OF. The volume saturation is indicated by a blue color appearing at the flag windows. After OF collection, the cotton pad is plunged in a stabilizing buffer for analyte storage, stability and transport (Figure 15).

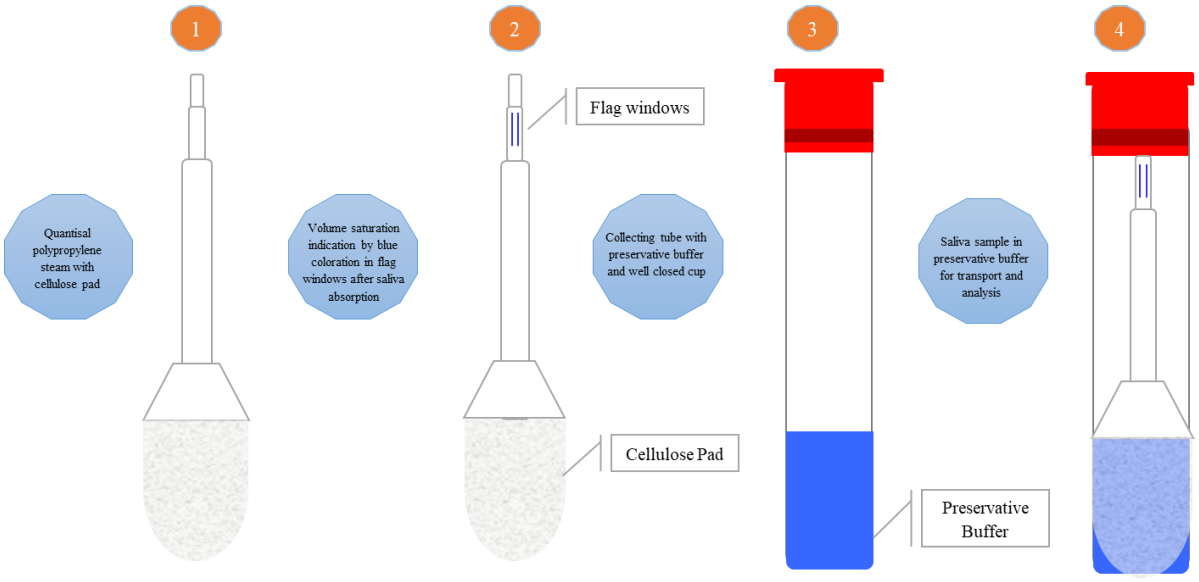


Figure 15. Schematic presentation of Quantisal device different parts.

Quantisal device was selected for its ability to collect an adequate volume of OF (1 mL ± 10 %), short saliva collecting time (less than 2min) and stabilizing buffer for OF sample transport and storage. Quantisal device appears to be the best choice for saliva sample collection from ultramarathon runner and drugs analysis. Sample volume repeatability plays important role in quantitative analytical results. The mean of saliva volume collected from 50 subjects, was 0.993 ml with a standard division of 0.029 ml (Coulter, Taruc, Tuyay, & Moore, 2010).

Saliva desorption from pad

Desorption of NSAIDs from the pad is the first step of sample preparation.

Extraction recovery of NSAIDs were significantly low in OF samples collected with Quantisal cotton pad and desorbed using the Quantisal buffer (up to 50from 12 to 55% of extraction recovery for all NSAIDs, except salicylic acid with 95%). Thus, we replaced Quantisal buffer by 2.8 ml ACN:H2O (50:50 v/v) + 2% acetic acid. Values of extraction recovery ranged from 41 to 100 % with this homemade mix. That's why we have choosen to replace the Quantisal buffer by the following homemade mix: ACN:H2O (50:50 v/v) + 2% acetic acid.

The low recovery of desoprtion of drugs from collector pad is already reported. Coulter et al., found that extraction recovery of 11 commonly used antipsychotics and their 5 metabolites spiked on collector pad and then plunged in stabilizing buffer were lesser (ranged between 51.4% and 87.4%) than spiked OF directly added on stabilizing buffer (range between 89.2%–97.0%) without using collector pad (Coulter et al., 2010). Quantisal stabilizing buffer contain salts, including bicarbonate, mono/dibasic sodium phosphate or citrate, antimicrobial agents, usually ProClin 300 or 950, and an excipient or emulsifying like Tween 20 or long chain polyethylene glycol (PEG). All of these compounds may increase matrix effect in mass spectrometry.

The final protocol was : 1 mL blank or NSAIDs spiked (100ng of each) OF → Quantisal Swab → immersion in homemade solvent (2.8 ml ACN:H2O (50:50 v/v) + 2% acetic acid) for 10 min → sonication for 1 min → vortex for few seconds → cotton pad squeezing → Centrifugation at 3,900 g for 5 min at 20°C → transfer supernatant in a borosilicate tube → apply extraction method (LLE, TVA, TVB and QueChers)

Extraction

Based on bibliographic studies, LLE and SPE methods could be tested.

Nevertheless, as we have replaced Quantisal buffer by a homemade buffer containing 50% ACN, SPE methods based on reversed phase interactions were not relevant. Thus, SPE methods were excluded.

Based on previously published article, four different LLE methods such as liquid-liquid extraction (LLE), QueChers, ToxiVials® Type A (TVA) and ToxiVials® Type B (TVB) were tested. As developed in the first article, LLE method was selected.

In order to achieve good extraction recovery for all NSAIDs, especially for acetylsalicylic acid and salicylic acid, we planned to ameliorate extraction results by some modifications in the method:

- Firstly, extracting solvent effects were evaluated on 300µl Chloroform, Cyclohexane and Ethyl Acetate, with or without 2% acetic acid. Cyclohexane was removed from the study because of very low extraction result (extraction recovery 0.1 up to 3.92% for all NSAIDs). Ethyl acetate was also excluded from the study because we observed just only one solvent phase of about 2.5 ml (not extraction, just a precipitation). Extraction recoveries using chloroform ranged from 30 up to 99% (with the except 20% for sulfasalazine and 27% for acetyl salicylic acid).

- Secondly, extraction solvent volume effects were evaluated using 100µl, 300µl and 500µl and 800µl of chloroform. As demonstrated in figure 16, better extraction recovery values were achieved using 500µL. Then a double step extraction with 500 µL each step was also tested. As depicted in figure 16, the result of double extraction with 500µl chloroform is better than all used volumes.

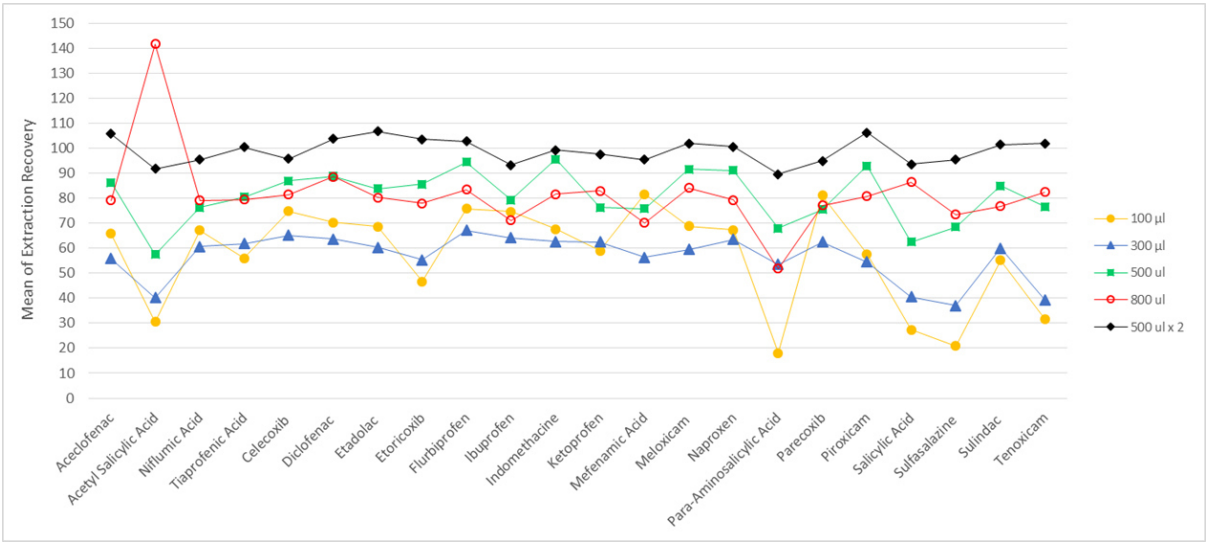


Figure 16. Line plot depicting percentage of extraction recoveries with different volume of chloroform (n=4).

Extraction recovery reported by previous studies (Hassan et al, 96.8-104.5% for 4 NSAIDs, Aminu et al, 97.98% for 1 NSAIDs, Jin et al, 100.5- 105.9 % for 1 NSAIDs, Ogawa et al, 101.3-106.1% for 1 NSAIDs, Calvo et al, 91.7-99.2% for 1 NSAIDs and its metabolite, Kuwayama et al, 94.6-117.5% for 3 NSAIDs) are closed to the results obtained by our method (Calvo et al., 2016; Hassan & Alshana, 2019; Kuwayama et al., 2016; Ogawa et al., 2014). While extraction yield obtained by our method is better than the results reported by Payan et al (76.1-97.8% for 5 NSAIDs) and Tartaglia et al (85.6-105.6% for 6 NSAIDs) (Ramos-Payan et al., 2016; Tartaglia et al., 2020).

The entire workflow for preparation of OF us summarized in figure 17.

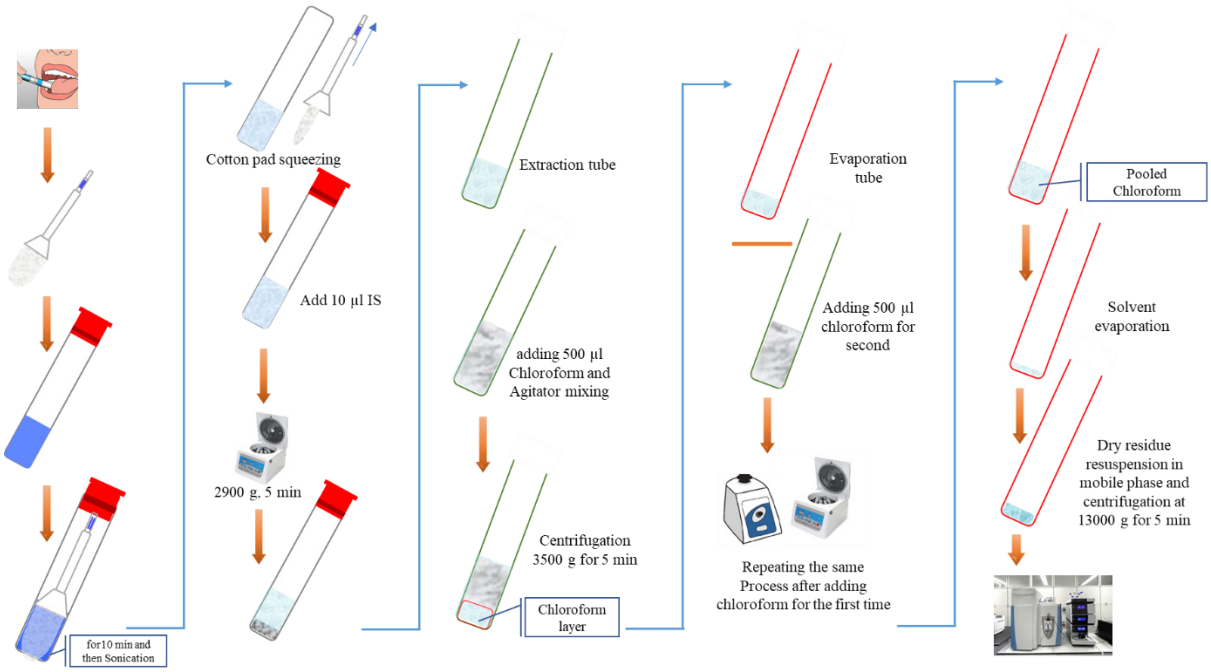


Figure 17. Schematic presentation of NSAIDs extraction workflow from OF collected with Quantisal device and double extraction with 500 µl chloroform

2.2.2.2. Method validation

The development of the LC-MS/HRMS method and the validation of this method in OF was published in the article:
Simultaneous quantification of 19 non-steroidal anti-inflammatory drugs in oral fluid by liquid chromatography-high resolution mass spectrometry: Application on ultratrail runner's oral fluid. Mashal MS, Nalin M, Bevalot F, Sallet P, Guitton J, Machon C. Drug Test Anal. 2022 Apr;14(4):701-712. doi: 10.1002/dta.3216. Epub **2022** Jan 19 (Mashal et al., 2022).

2.2.2.3. Conclusion

A rapid and sensitive LC-MS/HRMS method was developed and validated for simultaneous analysis of 19 NSAIDs in OF. Several previously studies have used saliva as a biological matrix for different NSAIDs analysis. Among 8 different studies mentioned in table 4, the maximum number of these drugs is studied by Tartaglia et al, 2020 (6 different NSAIDs) (Tartaglia et al., 2020). This present study is the first one reporting as much NSAIDs simultaneously analysed in OF. Quantisal device was adapted for NSAIDs analysis in OF and for the first time, runner4s samples were collected with this device.

Moreover, as indicated in the article published in Drug Testing and Analysis, the LC-MS/HRMS method allows also the analysis of phase I and phase II metabolites of NSAIDs.

2.3. Validation of the LC-MS/HRMS for the quantification in DBS

2.3.1. Aims

The aims of this part of my thesis were:

- the development of a sample preparation method for analysis of NSAIDs in DBS
- the validation of the method in DBS

2.3.2. Material and method

The LC-MS/HRMS method published in Drug Testing and Analysis was used for the screening of NSAIDs in DBS.

For the development of the sample preparation protocol, NSAIDs free real blood from lab volunteers were collected in 4 mL K_2 EDTA coated tubes and congelated at -20 °C. 1 mL of decongelated blood was spiked with individual NSAIDs and IS at 100 ng/mL and 10 ng/mL, respectively. DBS were prepared by depositing 10 µL of spiked blood on the HemaXis paper, dried and stored at room temperature overnight. The entire spot was cut out and desorption method was applied.

For method validation, analytical parameters were validated according to European Medicine Agency 2011 guideline.

- Stock standard solution of individual NSAIDs and internal standards were prepared at 1 mg/ml MeOH and stored during method validation at -20 °C.
- Calibration curves were created according the peak area ratio (area of the peak of NSAID / area of the peak of internal standard) versus the known concentrations of the calibration standards. The linearity of all analytes was determined in spiked NSAIDs free blood at six concentration levels. Calibration standard was tested 3 timesonce a day in 3 different days. Ranges of calibration curves were established based on NSAIDs pharmacokinetics (Cmax).
    - For tiaprofenic acid, ibuprofen, naproxen, para-aminosalicylic acid and flurbiprofen gamme range was 0.1, 0.4, 1.6, 3.5, 10 and 50 µg/ml blood.
    - For aceclofenac, diclofenac, etodolac, indomethacin, ketoprofen, mefenamic acid and sulindac, gamme range was 0.05, 0.15, 0.5, 2, 5 and 20 µg/ml blood.
    - For niflumic acid, celecoxib, etoricoxib, meloxicam, parecoxib, piroxicam and tenoxicam, gamme range was 0.05, 0.05, 0.2, 0.7, 2 and 5 µg/ml blood.
- The LLOQ corresponded to the lowest calibation standard
- The limit of detection (LOD) was estimated for all 18 NSAIDs. LOD for individual analyte were calculated according to peak area in 6 blank DBS. The lowest concentration of NSAIDs spiked in real blood with a peak area three time greater than peak area in blank DBS samples was accepted as LOD. For NSAIDs with no noise in blank DBS, the concentration with three scans in full scan mode was accepted as LOD.
- Selectivity was evaluated on six blank DBS by comparing the response (peak area) of the blank samples to response of spiked samples at the LLOQ. According to EMA guideline, acceptance interval of co-eluting interferences for individual NSAIDs and internal standards was 20 % and 5% of the LLOQ, respectively. Selectivity calculated according to the equation 1.

$$Selectivity = \frac{Peak\ area\ in\ blank\ DBS}{Peak\ area\ of\ LLOQ} \times 100 \qquad \text{Equation 1}$$

- Matrix effects was tested on six blank DBS sample spiked or not with QC low and QC high concentration of individual NSAIDs. Ion suppresion or enhancement was calculated according to the equation 2.

$$ME\ \% = \left(\frac{Peak\ area\ in\ the\ presence\ of\ matrix}{Peak\ area\ in\ pure\ solution} \times 100\right) - 100 \qquad \text{Equation 2}$$

- Accuracy and precision: Quality control samples calculated at four different concentrations (LLOQ, QC low, QC medium and QC high), 3 times on the same day (within-day accuracy and precision) and once a day in 3 different days (between-day accuracy) were used for accuracy and precision.

2.3.3. Results and discussion

2.3.3.1. Sample preparation

Sample collection

HemaXis DB 10 dried blood spot kit was selected for blood sample collection due to its ability to change non-volumetric blood sample to a volumetric sample, on-field sampling and the capacity of 4 blood spot collection in one DBS card.

Desorption of NSAIDs from DBS

Based on previously published articles, different solvents were selected for NSAIDs desorption from DBS. Different volumes (500 µl and 1000 µl) and proportions of MeOH:H2O (60:40 v/v), ACN:H2O (60:40 v/v), with or without acetic acid, MeOH pure and ACN pure, were tested. High desorption recovery and low matrix effects were the criteria for the desorption method selection.

The entire spot (blank or spiked) was cut out and placed in an eppendorf then desorption solvent was added. 10 µl of internal standard mix containing 10 ng of each IS was added and vortex for few seconds. After sonication for 15 min, centrifugation for 5 min at 13,000 g, the supernatant was collected, dried under nitrogen and resuspended in 100 µL of mobile phase.

As demonstrated in figure 18, desorption results for most NSAIDs using MeOH pure, MeOH:H2O (60:40 v/v) and ACN:H2O (60:40 v/v) are close to each others. Firstly, ACN pure was excluded due to its very low desorption recovery.

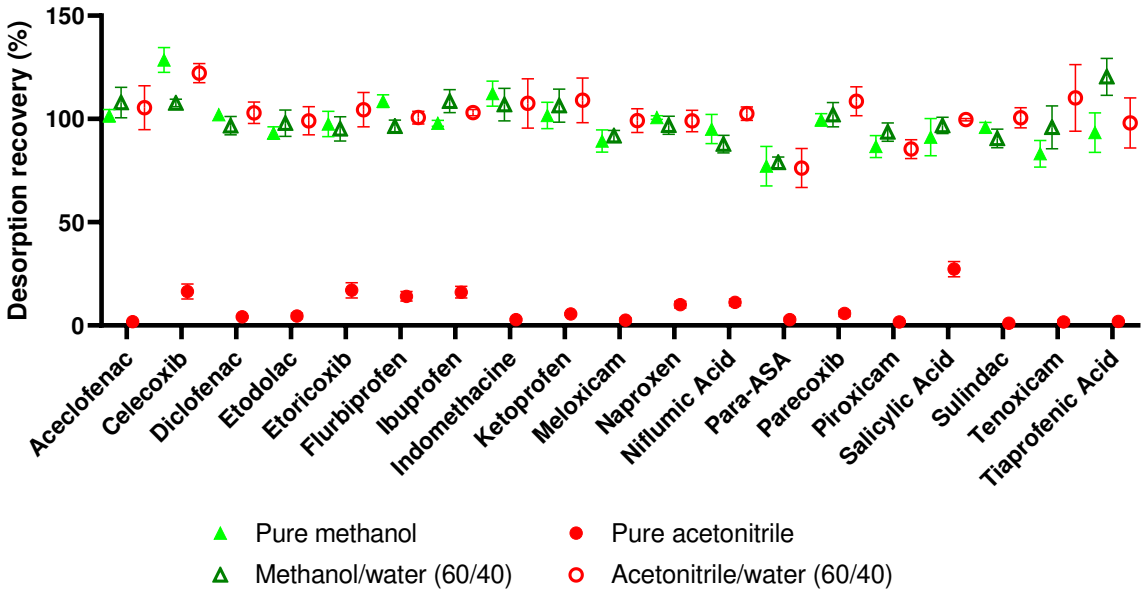


Figure 18. Desorption recoveries of NSAIDs from DBS using 1 mL of four different solvents

Secondly, MeOH:H2O (60:40 v/v) and ACN:H2O (60:40 v/v) are excluded from study due to their dirty extracts (Figure 19) and their long solvent evaporation time.

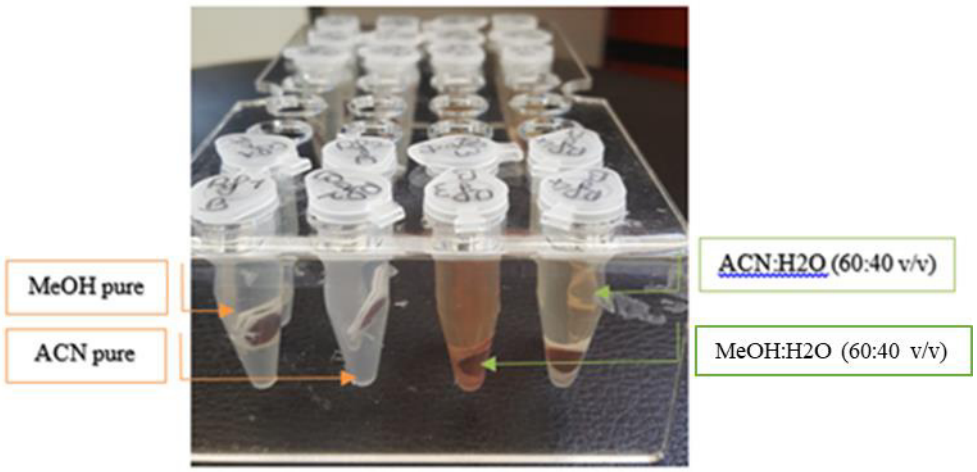


Figure 19. Images of DBS extracts after desoprtion with 1 mL of solvent

After the choice of 1 ml MeOH as desorptive solvent, we have compared results between analysis of 1 DBS (100 ng of each NSAIDs) and 2 DBS (50 ng of each NSAIDs in each. As a result, there is no significant difference in desorption yield for most of NSAIDs, but the matrix effect was higher using 2 DBS than 1 DBS. Therefore, we have chosen 1 ml MeOH for NSAIDs desorption from 1 DBS for analytical method development and validation.

Liquid-Liquid Extraction

As depicted in figure 18, even desorption with 1 ml MeOH appears clean, extracts were dirty after resuspension in mobile phase and the signal to noise ratio was not acceptable for lower

calibration curves of some NSAIDs . In order to decrease the signal to noise ratio, we decided to apply LLE after desorption from DBS. The solvents below were tested for LLE:

- MeOH + Chloroform + 50µl Acetic acid
- MeOH+ 500µl H2O + 500µl Cyclohexane + 50µl Acetic acid
- ACN:H2O (60:40) + 500µl Cyclohexane + 50µl Acetic acid
- ACN:H2O (60:40) + 500µl Chloroform + 50µl Acetic acid

Solvents were compared using peak area of NSAIDs (Figure 20). Among all 5 different extraction methods, better result was obtained using ACN:H2O (60:40) + 500µl Chloroform + 50µl Acetic acid.

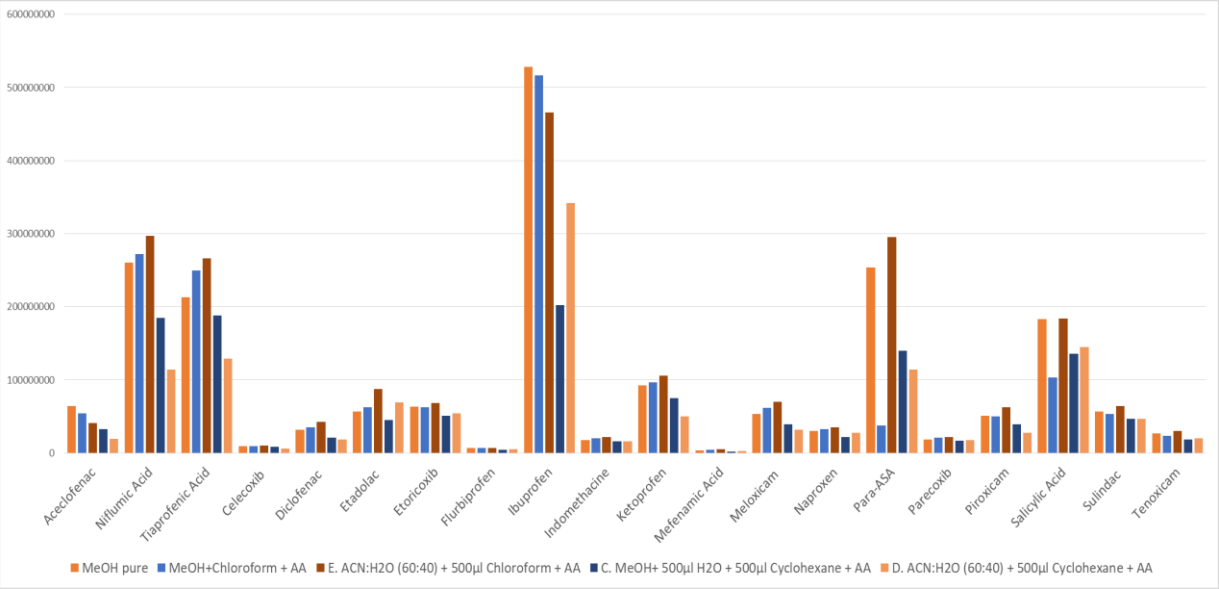


Figure 20. Results of LLE with different solvent. Data are presented as a peak area of individual NSAIDs.

LLE extraction recoveries using chloroform were calculated and are presented in figure 21. In addition to getting high extraction recovery, extracts were clean before injection.

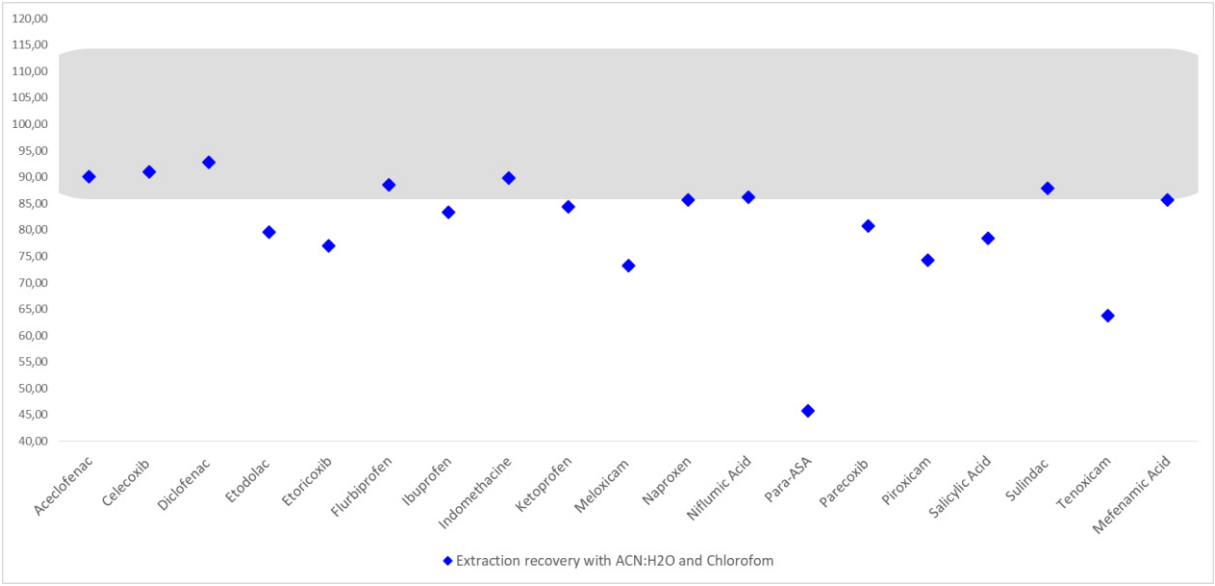


Figure 21. Extraction recovery of 18 NSAIDs after LLE with 500 µl chloroform.

2.3.3.2. Method validation

Values obtained for selectivity were according to criteria proposed by EMA guideline for all NSAIDs except for salicylic acid with value equal to 50 %. Therefore, salicylic acid was only qualitatively validated with LOD of 0.10 µg/mL.

Quadratic regression was selected for all NSAIDs by using 1/x or $1/x^2$ weighting factor (Table 12). Linearity was validated for all NSAIDs, except mefenamic acid, according to EMA guideline. Data related to LOD for individual NSAIDs also depicted in table 12.

Table 12. LOD, calibration curves and levels of QC of NSAIDs in DBS

NSAIDs	LOD (µg/mL)	Calibration range (µg/mL)	Internal standard	Regression	Weighting	QC low (µg/mL)	QC medium (µg/mL)	QC high (µg/mL)
Aceclofenac	0.001	0.05 - 20	Diclofenac D_4	Quadratic	1/x	0.09	0.95	9.7
Celecoxib	0.006	0.05 - 20	Ketoprofen D_3	Quadratic	$1/x^2$	0.12	0.41	3.7
Diclofenac	0.001	0.05 - 20	Diclofenac D_4	Quadratic	$1/x^2$	0.09	0.95	9.7
Etodolac	5.10^{-4}	0.05 - 20	Sulindac D_6	Quadratic	$1/x^2$	0.09	0.95	9.7
Etoricoxib	2.10^{-4}	0.05 - 20	Ketoprofen D_3	Quadratic	$1/x^2$	0.12	0.41	3.7
Flurbiprofen	0.1	0.1 - 50	Ibuprofen $^{13}C_6$	Quadratic	1/x	0.28	3.00	31.5
Ibuprofen	0.004	0.1 - 50	Ibuprofen $^{13}C_6$	Quadratic	1/x	0.28	3.00	31.5
Indomethacin	0.004	0.05 - 20	Diclofenac D_4	Quadratic	$1/x^2$	0.09	0.95	9.7
Ketoprofen	5.10^{-4}	0.05 - 20	Ketoprofen D_3	Quadratic	$1/x^2$	0.09	0.95	9.7

Meloxicam	1.10^{-4}	0.05 - 20	Sulindac D_6	Quadratic	1/x	0.12	0.41	3.7
Naproxen	0.006	0.1 - 50	Ketoprofen D_3	Quadratic	1/x	0.28	3.00	31.5
Niflumic acid	4.10^{-5}	0.05 - 20	Diclofenac D_4	Quadratic	$1/x^2$	0.12	0.41	3.7
Para-ASA	4.10^{-4}	0.1 - 50	Sulfasalazin D_4	Quadratic	1/x	0.28	3.00	31.5
Parecoxib	9.10^{-4}	0.05 - 20	Ketoprofen D_3	Quadratic	1/x	0.12	0.41	3.7
Piroxicam	2.10^{-4}	0.05 - 20	Piroxicam D_3	Quadratic	$1/x^2$	0.12	0.41	3.7
Sulindac	9.10^{-4}	0.05 - 20	Sulindac D_6	Quadratic	1/x	0.09	0.95	9.7
Tenoxicam	3.10^{-4}	0.05 - 20	Sulfasalazin D_4	Quadratic	1/x	0.12	0.41	3.7
Tiaprofenic acid	7.10^{-4}	0.1 - 50	Diclofenac D_4	Quadratic	1/x	0.28	3.00	31.5

Within-day and between-day accuracy and precision were in acceptable range for all NSAIDs (Figure 22), with the except for the within day accuracy (A) for celecoxib at LLOQ, low QC and medium QC, between-day accuracy (B) for celecoxib, flurbiprofen and ibuprofen at LLOQ and low QC levels. Within-day precision (C) for meloxicam at LLOQ and between-day precision (D) for diclofenac and flurbiprofen at low QC levels were also out of acceptable range.

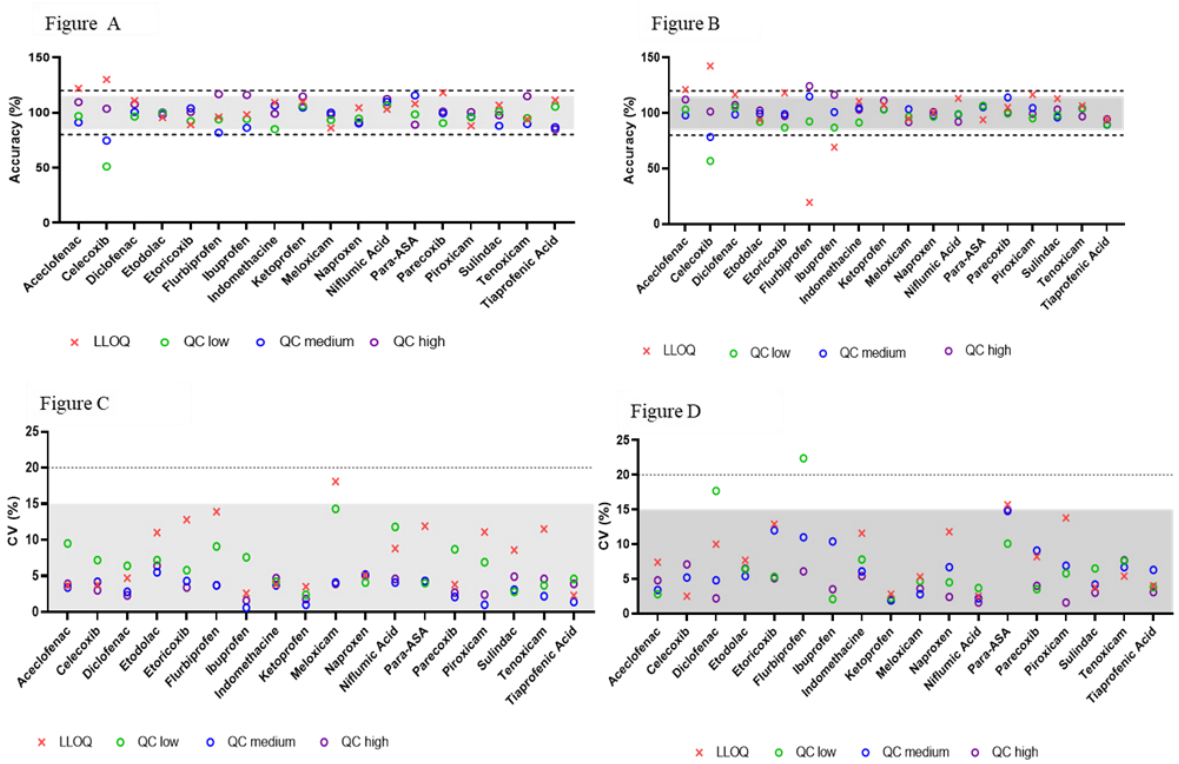


Figure 22. Within-day and between-day accuracy and precision. Within day accuracy (A), between-day accuracy (B), within-day precision (C) and between-day precision (D).
The highlighted area (gray area) shows the zone where data are in the acceptable range for QC low, QC medium and QC high (± 15) and dotted line shows acceptable range for LLOQ (± 20).

Matrix effects results are presented in Table 13. As quantification is made using internal standards, ion supression or enhancement did not affect quantitative results.

Table 13. Matrix effects at QC low and QC high level (n = 6)
nd: not determined

NSAIDs	QC Low mean	CV (%)	QC High mean	CV (%)
Aceclofenac	-16,88	7,29	-3,13	4,99
Niflumic Acid	-18,10	13,15	-3,79	4,90
Tiaprofenic Acid	-10,94	6,73	-1,91	4,27
Celecoxib	-9,12	15,71	22,06	6,78
Diclofenac	-27,89	11,01	-6,07	2,80
Etodolac	14,05	21,88	3,42	4,19
Etoricoxib	-5,22	7,08	0,63	2,79
Flurbiprofen	nd	nd	-1,25	2,26
Ibuprofen	-1,32	7,67	71,68	5,95
Indomethacine	-13,52	12,48	-0,35	4,61
Ketoprofen	-15,91	13,57	-2,79	2,59
Meloxicam	35,29	18,72	4,26	1,64
Naproxen	-23,81	31,16	-1,78	4,40
Para-ASA	-26,22	15,52	-5,75	4,96
Parecoxib	-16,20	10,56	-5,79	3,68
Piroxicam	38,95	21,92	-5,95	7,31
Sulindac	-8,07	12,34	-5,50	7,60
Tenoxicam	15,54	16,49	8,85	6,63

2.3.3.3. Discussion

ISs introduction into the sample is a serious question when using DBS. In this study, adding IS in extraction solvent was a reasonable choice for DBS sample collection from ultramarathon runners. Hematocrit (Hct) is another challenge of using DBS, which affects analytical method accuracy and reproducibility. Whole DBS analysis is a strategy to nullify Hct impacts on analytical method accuracy and precision (Henion et al., 2013). Therefore, in this study, the whole DBS was cut for sample preparation

Among 6 previous studies on NSAIDs analysis in DBS, only in one study 4 different NSAIDs have been tested (ketoprofen, fenoprofen, flurbiprofen and ibuprofen) (Ask et al., 2018), in other works, the analysis of 1 or 2 NSAIDs have been reported. Unlike previous studies, in this work, 18 different NSAIDs were analysed in a single DBS.

According to EMA guideline 2011, the method is validated quantitatively for 15 out of 19 analytes and only qualitatively for 4 NSAIDs (Salicylic acid, celecoxib, flurbiprofen, mefenamic acid).

3.APPLICATIONS

In this last part of my thesis, i present the application of the method presented above on OF and DBS samples from ultratrailers. For this purpose, a prospective study was conducted during the UTMB® 2021. UTMB® participates to the Quartz Event program. The aim of this program is to contribute to a cleaner sport using rules that athletes must respect (Annexe 1).
The fisrt step of the study was to obtain ethical authorization to conduct it. Documents (Annexe 2) were written and sent to Comité de Protection des Personnes (CPP) on July 2021. The authorization from the CPP was received on August 2021.
The second step was the collection of samples at UTMB® 2021. 81 runners were included in the study: all of them accepted OF collection, 74 of them filled the questionnaire (Table 14) and 73 of them accepted DBS collection. Samples were collected just after the arrival of runners. Finally, OF and DBS samples were analysed.

Table 14. Questionnaire filled by runners

N° inclusion :	
Date de prélèvement :	
Prélèvement : □ salive □ blood spot	
Age :	Sexe : □ Homme □ Femme
Nombre d'ultratrails déjà effectués (> 60 km) :	
Avez-vous pris des AINS ces 3 derniers jours ? □ Oui □ Non Si oui : □ par automédication □ suite à une ordonnance médicale	

As acetamoniphen is the second drug consumed by ultratrailers (Didier et al., 2017; Hoffman & Fogard, 2011), this drug was added in the study.
All results are presented in a research article published: **Comparative study between direct analysis in whole blood, oral fluid, and declaration of consumption for the prevalence of nonsteroidal anti-inflammatory drugs and acetaminophen in ultratrail runners Mohammad Shafiq Mashal, Fabien Bevalot, Antony Citterio-Quentin, Pierre Sallet, Qand Agha Nazari, Jérôme Guitton, Christelle Machon. Drug Test Anal. 2022 Sep 27. doi: 10.1002/dta.3374. Online ahead of print.**

CONCLUSION - PERSPECTIVES

NSAIDs are one of the most used drugs in different sports. Indeed, high prevalence of these drugs consumption by ultratrail runners was reported in previous studies, based on data collected by questionnaire. ITRA (International Trail Running Association) works for runner's health protection through the Quartz Event program. According to the Quartz Event program, NSAIDs consumption is prohibited and ultratrail participants are not allowed to use these drugs the day before or during the race.

Due to runners low information or lack of information about NSAIDs, questionnaire based data appear to not be a reasonable tool to reveal accurate information about the prevalence of these drugs consumption by ultratrail runners. Furthermore, classic biological samples such as blood collection by venipuncture and urine have limitations and are even not possible for runners on-field sampling.

For the first time, a LC-MS/HRMS analytical method was developed and validated for simultaneous analysis of a huge number of NSAIDs in OF and DBS. The non-invasive Quantisal® device for whole saliva collection and the low invasive HemaXis DB 10 for capillary blood were the best choice and compatible for on-field runners sample collection. Development and validation of an analytical method for a broad range of NSAIDs in runner's biological samples enable this work to detect and quantify all NSAIDs which may possibly be used by runners.

Data obtained by questionnaire, OF and DBS samples analysis demonstrated that questionnaire based data could eventuate false negative or false positive information about NSAIDs consumption prevalence. Even OF samples are non-invasive and easy to collect, however due to analytes very low concentrations in OF and some NSAIDs short half-life, OF samples may lead to false negative results. Reliable information were obtained using DBS samples. Detection of NSAIDs in the runner's samples with negative results in the questionnaire and OF, looking for NSAIDs metabolites and detection of more than one NSAIDs, are the main advantages of DBS samples over the questionnaire. Therefore, DBS is the more reliable and best choice for the study of NSAIDs prevalence in runners.

Fear of finger pricking in some runners (in this study, 9.87% of runners rejected DBS samples), incomplete sampling, coast and some NSAIDs short half-life are the limitations of DBS samples. DBS may not be an effective method if runners consumed NSAIDs two or three days before the race.

Unchanged NSAIDs or their metabolites are mainly execrated in urine. Therefore, the use of dried urine spots (DUS) may provide effective information even time interval between NSAIDs intake and sample collection is relatively long.

In addition to Quartz program implementation by ITRA, increasing runners awareness about NSAIDs adverse effects and the association of these effects with heavy exercise may decrease these drugs consumption by runners.

ANNEXES

Annexe 1. Quartz event program rules

Quartz Event Program

In order to protect the health of the participants and to contribute to a clean sport, the organization of the **UTMB® Mont-Blanc** has set up since 2008 the QUARTZ Event Program which implies specific medical rules; in particular, runners agree not to take part in any of the races when using:

Within 60 days before the start of the competition and during the competition:

- Intravenous iron infusion

Within 7 days before the start of the competition and during the competition:

- Intravenous infusion
- Gas inhalation
- Substance subject to a Therapeutic Use Exemption (TUE) according to the WADA Prohibited List
- All glucocorticoids regardless of the mode of administration
- Thyroid synthesis hormones except in case of partial or total removal of the thyroid or hypothyroidism of medical origin.

Within 24 hours before the start of the competition and during the competition:

- All beta-2-agonists regardless of the mode of administration
- All painkillers including Tramadol and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) regardless of the mode of administration
- All substances included in the WADA Monitoring Program

In order to verify that the above rules are respected, different types of samples (blood, saliva, hair, urine) are collected before and after the main races of the UTMB® Mont-Blanc: UTMB®, CCC®, OCC, TDS®.

The official rules of the UTMB® Mont-Blanc state that any unreasonable failure, presence of an unauthorized substance in a sample, refusal or transmission of fake information related to the QUARTZ Event Program may result in the participant being **disqualified before or after competition**.

Annexe 2. Summary of the study send to Comité de Protection des Personnes

Titre du projet : PAINSTRAIL

Détermination de la prévalence de la prise d'anti-inflammatoires non stéroïdiens dans les compétitions de trail-running par dosage salivaire et de sang capillaire

°

Version 1 du 07/06/2021

Promoteur : Association "Athletes For Transparency" (AFT) / Programme QUARTZ
03 cours d'Herbouville 69004 Lyon
Tél : 04 82 33 55 90
Fax. : 04 50 53 47 44
Courriel : contact@athletesfortransparency.com

Investigateur : Dr Patrick BASSET
ULTRA SPORTS SCIENCE
109 Boulevard de l'Europe
69310 Pierre-Benite
Tél : 07 52 60 82 84
Courriel : p.basset@ultrasportsscience.org

Collaborateur scientifique: Docteur Pierre SALLET, Ph.D.
Tél : 04 82 33 55 90
Fax. : 04 50 53 47 44
Courriel : p.sallet@quartzprogram.org

Collaborateur scientifique: Docteur Jérôme GUITTON, Pharm D, Ph.D
Laboratoire de Biochimie et Pharmaco-toxicologie. Bâtiment 3D. 165 chemin du Grand Revoyet 69495 PIERRE BENITE Cedex
Tél : 04-72-66-64-00
Fax : 04-78-86-66-54
Courriel : jerome.guitton@chu-lyon.fr

Collaborateur scientifique: Docteur Christelle MACHON, Pharm D, Ph.D
Laboratoire de Biochimie et Pharmaco-toxicologie. Bâtiment 3D. 165 chemin du Grand Revoyet 69495 PIERRE BENITE Cedex
Tél : 04-72-66-64-00
Fax : 04-78-86-66-54
Courriel: christelle.machon@chu-lyon.fr

RESUME

TITRE	Etude PAINSTRAIL Détermination de la prévalence de la prise d'anti-inflammatoires non stéroïdiens dans les compétitions de trail-running par dosage salivaire et de sang capillaire
PROMOTEUR	**Association "Athletes For Transparency" (AFT) / Programme QUARTZ** **03 cours d'Herbouville** **69004 Lyon**
INVESTIGATEUR PRINCIPAL	Dr Patrick BASSET
VERSION DU PROTOCOLE	V1 du 07/06/2021
JUSTIFICATION / CONTEXTE	Les Anti-Inflammatoires Non Stéroïdiens (AINS), subsance ne figurant pas sur la liste des interdictions de l'Agence Mondiale Antidopage, sont largement utilisés dans les sports d'ultra-endurance comme le trail-running leur potentiel effet ergogénique. Les études rapportent donc une prévalence élevée (de 50 à 70% selon les épreuves). Les AINS sont connus pour provoquer des effets indésirables. Ils peuvent également aggraver les dommages liés à la pratique du trail-running : troubles gastro-intestinaux, hyponatrémie, insuffisance rénale. Actuellement, les études présentant la prévalence de la consommation des AINS chez les participants à des épreuves de trail longue distance et ultra-longue distance se sont basées sur des questionnaires. A notre connaissance, aucune étude n'a objectivé la prise d'AINS par une recherche d'AINS chez les participants.
OBJECTIFS	<u>Objectif principal :</u> L'objectif principal de notre projet est d'évaluer la prévalence de la consommation d'AINS chez des participants de trail longue distance, en nous basant sur la réponse à un questionnaire et une recherche salivaire et sanguine des AINS. <u>Objectif secondaire :</u> L'objectif secondaire est d'identifier, grâce aux dosages et aux données du questionnaire, une population à risque, c'est-à-dire une catégorie de trailers (selon âge, sexe, expérience en trail...) ayant plus recours aux AINS que le reste des trailers, afin d'envisager des actions de prévention ciblées.
METHODOLOGIE / SCHEMA DE LA RECHERCHE	Etude monocentrique, prospective, non-randomisée, non interventionnelle selon le 3° de la Loi Jardé.
CRITERES DE JUGEMENT	<u>Critère d'évaluation principal :</u>

	Recherche et identification des AINS pré-compétition et post-compétition dans la salive et le sang capillaire à l'aide d'une méthode de chromatographie liquide couplée à la spectrométrie de masse haute résolution (LC-HRMS). <u>Critère(s) d'évaluation secondaire(s) :</u> Questionnaire déclaratif relatif à la prise d'AINS en compétition.
POPULATION CIBLE	Ultratrailers participant à des compétitions de trail-running adhérant au programme QUARTZ Event.
CRITERES D'INCLUSION	- Homme ou Femme inscrits à une compétition de trail-running adhérant au programme QUARTZ Event. L'inscription implique l'acceptation du règlement de la compétition indiquant que des prélèvements biologiques peuvent être réalisés dans le cadre du programme QUARTZ Event. - Age supérieur ou égal à 18 ans
CRITERES DE NON INCLUSION	Refus du participant de répondre au questionnaire
CRITERES DE SORTIE D'ETUDE	Souhait du participant de ne plus participer à l'étude.
NOMBRE DE SUJETS	250
DUREE DE L'ETUDE	Durée totale de la recherche **:** 18 mois. Période d'inclusion **: 15** mois. Durée de participation pour un sujet **:** 1 compétition
LIEU DE LA RECHERCHE	Les dosages seront réalisés au Centre Hospitalier Lyon Sud, Hospices Civils de Lyon (laboratoire de Biochimie et pharmaco-toxicologie)
RETOMBEES ATTENDUES	Identifier une population cible afin de mettre en place des action de prévention ayant pour but de diminuer la consommation d'AINS chez les trailers.

BIBLIOGRAPHIC REFERENCES

A. Boelsterli, U., & Ramirez-Alcantara, V. 2011. NSAID Acyl Glucuronides and Enteropathy. ***Current Drug Metabolism***, 12(3): 245–252.

Abu-Rabie, P., & Spooner, N. 2009. Direct Quantitative Bioanalysis of Drugs in Dried Blood Spot Samples Using a Thin-Layer Chromatography Mass Spectrometer Interface. ***Analytical Chemistry***, 81(24): 10275–10284.

Agúndez, J. A., García-Martín, E., & Martínez, C. 2009. Genetically based impairment in CYP2C8- and CYP2C9-dependent NSAID metabolism as a risk factor for gastrointestinal bleeding: Is a combination of pharmacogenomics and metabolomics required to improve personalized medicine? ***Expert Opinion on Drug Metabolism & Toxicology***, 5(6): 607–620.

Almeda, S., Arce, L., & Valcárcel, M. 2008. Combination of solid-phase extraction and large-volume stacking with polarity switching in micellar electrokinetic capillary chromatography for the determination of traces of nonsteroidal anti-inflammatory drugs in saliva. ***ELECTROPHORESIS***, 29(14): 3074–3080.

Aminu, N., Chan, S.-Y., Khan, N. H., & Toh, S.-M. 2018. Concurrent determination of triclosan and flurbiprofen by high-performance liquid chromatography in simulated saliva and its application in dental nanogel formulation. ***Acta Chromatographica***, 30(4): 219–224.

André, C., Girard, J., Gautier, S., Derambure, P., & Rochoy, M. 2020. Training modalities and self-medication behaviors in a sample of runners during an ultratrail. ***Science & Sports***, 35(1): 48.e1-48.e7.

Antman, E. M., DeMets, D., & Loscalzo, J. 2005. Cyclooxygenase inhibition and cardiovascular risk. ***Circulation***, 112(5): 759–770.

Antunes, M. V., Charão, M. F., & Linden, R. 2016. Dried blood spots analysis with mass spectrometry: Potentials and pitfalls in therapeutic drug monitoring. ***Clinical Biochemistry***, 49(13–14): 1035–1046.

Ask, K. S., Øiestad, E. L., Pedersen-Bjergaard, S., & Gjelstad, A. 2018. Dried blood spots and parallel artificial liquid membrane extraction-A simple combination of microsampling and microextraction. ***Analytica Chimica Acta***, 1009: 56–64.

Ayano, E., Nambu, K., Sakamoto, C., Kanazawa, H., Kikuchi, A., et al. 2006. Aqueous chromatography system using pH- and temperature-responsive stationary phase with ion-exchange groups. ***Journal of Chromatography A***, 1119(1): 58–65.

Azevedo, L. R., De Lima, A. A. S., Machado, M. Â. N., Grégio, A. M. T., & de Almeida, P. D. V. 2008. Saliva Composition and Functions: A Comprehensive Review. ***The Journal of Contemporary Dental Practice***, 9(3): 72–80.

Bae, S. K., Kim, S.-H., Lee, H. W., Seong, S. J., Shin, S.-Y., et al. 2012. Pharmacokinetics of a New Once-Daily Controlled-Release Formulation of Aceclofenac in Korean Healthy Subjects Compared with Immediate-Release Aceclofenac and the Effect of Food: A Randomized, Open-Label, Three-Period, Crossover, Single-Centre Study. ***Clinical Drug Investigation***, 32(2): 111–119.

Bae, S. K., Seo, K. A., Jung, E. J., Kim, H.-S., Yeo, C.-W., et al. 2008. Determination of acetylsalicylic acid and its major metabolite, salicylic acid, in human plasma using liquid chromatography–tandem mass spectrometry: Application to pharmacokinetic study of Astrix® in Korean healthy volunteers. ***Biomedical Chromatography***, 22(6): 590–595.

Basivireddy, J., Jacob, M., Pulimood, A. B., & Balasubramanian, K. A. 2004. Indomethacin-induced renal damage: Role of oxygen free radicals. ***Biochemical Pharmacology***, 67(3): 587–599.

Bellagambi, F. G., Lomonaco, T., Salvo, P., Vivaldi, F., Hangouët, M., et al. 2020. Saliva sampling: Methods and devices. An overview. ***TrAC Trends in Analytical Chemistry***, 124: 115781.

Bhattarai, K. R., Kim, H.-R., & Chae, H.-J. 2018. Compliance with Saliva Collection Protocol in Healthy Volunteers: Strategies for Managing Risk and Errors. ***International Journal of Medical Sciences***, 15(8): 823–831.

Bilecen, D., Schulte, A.-C., Kaspar, A., Kustermann, E., Seelig, J., et al. 2003. Detection of the non-steroidal anti-inflammatory drug niflumic acid in humans: A combined 19F-MRS in vivo and in vitro study. ***NMR in Biomedicine***, 16(3): 144–151.

Bindu, S., Mazumder, S., & Bandyopadhyay, U. 2020. Non-steroidal anti-inflammatory drugs (NSAIDs) and organ damage: A current perspective. ***Biochemical Pharmacology***, 180: 114147.

Bird, H. A., Allen, J. G., Dixon, J. S., & Wright, V. 1985. A pharmacokinetic comparison of tenoxicam in plasma and synovial fluid. ***Rheumatology***, 24(4): 351–356.

Bjarnason, I., Scarpignato, C., Holmgren, E., Olszewski, M., Rainsford, K. D., et al. 2018. Mechanisms of Damage to the Gastrointestinal Tract From Nonsteroidal Anti-Inflammatory Drugs. ***Gastroenterology***, 154(3): 500–514.

Boelsterli, U. A. 2002. Mechanisms of NSAID-Induced Hepatotoxicity: Focus on Nimesulide. ***Drug Safety***, 25(9): 633–648.

Bojić, M., Sedgeman, C. A., Nagy, L. D., & Guengerich, F. P. 2015. Aromatic hydroxylation of salicylic acid and aspirin by human cytochromes P450. ***European Journal of Pharmaceutical Sciences***, 73: 49–56.

Boni, J. P., Korth-Bradley, J. M., Martin, P., Simcoe, D. K., Richards, L. S., et al. 1999. Pharmacokinetics of etodolac in patients with stable juvenile rheumatoid arthritis. ***Clinical Therapeutics***, 21(10): 1715–1724.

Bonnesen, K., & Schmidt, M. 2021. Recategorization of Non-Aspirin Nonsteroidal Anti-inflammatory Drugs According to Clinical Relevance: Abandoning the Traditional NSAID Terminology. ***Canadian Journal of Cardiology***, 37(11): 1705–1707.

Boyer, D., Bauman, J. N., Walker, D. P., Kapinos, B., Karki, K., et al. 2009. Utility of MetaSite in Improving Metabolic Stability of the Neutral Indomethacin Amide Derivative and Selective Cyclooxygenase-2 Inhibitor 2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-phenethyl-acetamide. ***Drug Metabolism and Disposition***, 37(5): 999–1008.

Brocks, D. R., & Jamali, F. 1994. Etodolac clinical pharmacokinetics. ***Clinical Pharmacokinetics***, 26(4): 259–274.

Brune, K., & Patrignani, P. 2015. New insights into the use of currently available non-steroidal anti-inflammatory drugs. ***Journal of Pain Research***, 8: 105–118.

Brunton, L. L. P., Parker, MD, PhD, K. L., Blumenthal, PhD, D. K., & Buxton, PharmD, FAHA, I. L. O. 2012. ***Goodman and Gilman's Manual of Pharmacology and Therapeutics***. McGraw-Hill.

Calatayud, S., & Esplugues, J. V. 2016. Chemistry, Pharmacodynamics, and Pharmacokinetics of NSAIDs. In A. Lanas (Ed.), ***NSAIDs and Aspirin***: 3–16. Cham: Springer International Publishing.

Calvo, A. M., Santos, G. M., Dionísio, T. J., Marques, M. P., Brozoski, D. T., et al. 2016. Quantification of piroxicam and 5'-hydroxypiroxicam in human plasma and saliva using liquid chromatography–tandem mass spectrometry following oral administration. ***Journal of Pharmaceutical and Biomedical Analysis***, 120: 212–220.

Cheng, S., Xu, X., Kong, X., Jiang, Y., Mo, L., et al. 2022. Monitoring of salicylic acid content in human saliva and its relationship with plasma concentrations. ***Journal of Pharmaceutical and Biomedical Analysis***, 219: 114961.

Chiappin, S., Antonelli, G., Gatti, R., & De Palo, E. F. 2007. Saliva specimen: A new laboratory tool for diagnostic and basic investigation. ***Clinica Chimica Acta***, 383(1–2): 30–40.

Cho, H.-Y., Park, G.-K., & Lee, Y.-B. 2013. Simultaneous determination of morniflumate and its major active metabolite, niflumic acid, in human plasma by high-performance liquid chromatography in stability and pharmacokinetic studies: Simultaneous assay of morniflumate and niflumic acid using HPLC. ***Biomedical Chromatography***, 27(11): 1438–1443.

Chohan, B. H., Lavreys, L., Mandaliya, K. N., Kreiss, J. K., Bwayo, J. J., et al. 2001. Validation of a Modified Commercial Enzyme-Linked Immunoassay for Detection of Human Immunodeficiency Virus Type 1 Immunoglobulin G Antibodies in Saliva. ***Clinical and Diagnostic Laboratory Immunology***, 8(2): 346–348.

Choi, H.-G., Jeon, J.-Y., Kwak, S.-S., Kim, H., Jin, C., et al. 2015. Pharmacokinetic Comparison Study of a Combination Containing 500 mg of Naproxen and 20 mg of Esomeprazole: A Randomized, Single-dose, 2-way Crossover, Open-label Study in Healthy Korean Men. ***Clinical Therapeutics***, 37(1): 83–93.

Coulter, C., Taruc, M., Tuyay, J., & Moore, C. 2010. Antidepressant Drugs in Oral Fluid Using Liquid Chromatography-Tandem Mass Spectrometry. ***Journal of Analytical Toxicology***, 34(2): 64–72.

Crouch, D. J. 2005. Oral fluid collection: The neglected variable in oral fluid testing. ***Forensic Science International***, 150(2–3): 165–173.

Davies, N. M. 1995. Clinical Pharmacokinetics of Flurbiprofen and its Enantiomers. ***Clinical Pharmacokinetics***, 28(2): 100–114.

Davies, N. M. 1998. Clinical pharmacokinetics of ibuprofen. The first 30 years. ***Clinical Pharmacokinetics***, 34(2): 101–154.

Davies, N. M., & Skjodt, N. M. 1999. Clinical Pharmacokinetics of Meloxicam: A Cyclo-Oxygenase-2 Preferential Nonsteroidal Anti-Inflammatory Drug. ***Clinical Pharmacokinetics***, 36(2): 115–126.

Davies, N. M., Smith, G. D., Windmeijer, F., & Martin, R. M. 2013. COX-2 Selective Nonsteroidal Anti-inflammatory Drugs and Risk of Gastrointestinal Tract Complications and Myocardial Infarction: An Instrumental Variable Analysis. ***Epidemiology***, 24(3): 352–362.

Davies, N. M., & Watson, M. S. 1997. Clinical Pharmacokinetics of Sulindac: A Dynamic Old Drug. ***Clinical Pharmacokinetics***, 32(6): 437–459.

Davis, D. P., Videen, J. S., Marino, A., Vilke, G. M., Dunford, J. V., et al. 2001. Exercise-associated hyponatremia in marathon runners: A two-year experience. ***The Journal of Emergency Medicine***, 21(1): 47–57.

Day, R. O., & Graham, G. G. 2013. Non-steroidal anti-inflammatory drugs (NSAIDs). ***BMJ (Clinical Research Ed.)***, 346: f3195.

Day, R. O., Graham, G. G., Williams, K. M., & Brooks, P. M. 1988. Variability in Response to NSAIDs: Fact or Fiction? ***Drugs***, 36(6): 643–651.

Déglon, J., Thomas, A., Daali, Y., Lauer, E., Samer, C., et al. 2011. Automated system for on-line desorption of dried blood spots applied to LC/MS/MS pharmacokinetic study of flurbiprofen and its metabolite. ***Journal of Pharmaceutical and Biomedical Analysis***, 54(2): 359–367.

Denniff, P., & Spooner, N. 2014. Volumetric Absorptive Microsampling: A Dried Sample Collection Technique for Quantitative Bioanalysis. ***Analytical Chemistry***, 86(16): 8489–8495.

Dennis, M., French, P., Crome, P., Babiker, M., Shillingford, J., et al. 1985. Pharmacokinetic profile of controlled release ketoprofen in elderly patients. ***British Journal of Clinical Pharmacology***, 20(6): 567–573.

Desborough, M. J. R., & Keelin, D. M. 2017. The aspirin story – from willow to wonder drug—Desborough—2017—***British Journal of Haematology—Wiley Online Library***. https://onlinelibrary.wiley.com/doi/epdf/10.1111/bjh.14520.

Didier, S., Vauthier, J.-C., Gambier, N., Renaud, P., Chenuel, B., et al. 2017. Substance use and misuse in a mountain ultramarathon: New insight into ultrarunners population? ***Research in Sports Medicine (Print)***, 25(2): 244–251.

Douthwaite, A. H. 1938. Recent Advances in Medical Diagnosis and Treatment. ***British Medical Journal***, 1(4038): 1143–1146.

Driver, B., Marks, D. C., & van der Wal, D. E. 2019. Not all (N)SAID and done: Effects of nonsteroidal anti-inflammatory drugs and paracetamol intake on platelets. ***Research and Practice in Thrombosis and Haemostasis***, 4(1): 36–45.

Drożdżal, S., Lechowicz, K., Szostak, B., Rosik, J., Kotfis, K., et al. 2021. Kidney damage from nonsteroidal anti-inflammatory drugs—Myth or truth? Review of selected literature. ***Pharmacology Research & Perspectives***, 9(4): e00817.

Duong, M., Salvo, F., Pariente, A., Abouelfath, A., Lassalle, R., et al. 2014. Usage patterns of 'over-the-counter' vs. Prescription-strength nonsteroidal anti-inflammatory drugs in France. ***British Journal of Clinical Pharmacology***, 77(5): 887–895.

Edelbroek, P. M., Heijden, J. van der, & Stolk, L. M. L. 2009. Dried Blood Spot Methods in Therapeutic Drug Monitoring: Methods, Assays, and Pitfalls. ***Therapeutic Drug Monitoring***, 31(3): 327–336.

EMA. 2018, September 17. European Medicines Agency finalises review of recent published data on cardiovascular safety NSAIDs. ***European Medicines Agency***. https://www.ema.europa.eu/en/news/european-medicines-agency-finalises-review-recent-published-data-cardiovascular-safety-nsaids.

Enomoto, A., Nambu, T., Kashiwagi, K., Okinaga, T., & Baba, S. 2020. Impact of short-term saliva storage at room temperature on the microbial composition. ***Journal of Osaka Dental University***, 54(1): 9.

Escudero-Contreras, A., Cervantes, J. V.-M., & Collantes-Estevez, E. 2007. Update on the clinical pharmacology of etoricoxib, a potent cyclooxygenase-2 inhibitor. ***Future Rheumatology***, 2(6): 545–565.

Farré, M., Petrovic, M., & Barceló, D. 2007. Recently developed GC/MS and LC/MS methods for determining NSAIDs in water samples. ***Analytical and Bioanalytical Chemistry***, 387(4): 1203–1214.

Fosslien, E. 2001. Review: Mitochondrial Medicine – Molecular Pathology of Defective Oxidative Phosphorylation. ***Annals of Clinical Laboratory Science***, 31: 43.

Gaissmaier, T., Siebenhaar, M., Todorova, V., Hüllen, V., & Hopf, C. 2016. Therapeutic drug monitoring in dried blood spots using liquid microjunction surface sampling and high resolution mass spectrometry. ***The Analyst***, 141(3): 892–901.

Gasparini, L., Ongini, E., & Wenk, G. 2004. Non-steroidal anti-inflammatory drugs (NSAIDs) in Alzheimer's disease: Old and new mechanisms of action. ***Journal of Neurochemistry***, 91(3): 521–536.

Gentili, A. 2007. Determination of non-steroidal anti-inflammatory drugs in environmental samples by chromatographic and electrophoretic techniques. ***Analytical and Bioanalytical Chemistry***, 387(4): 1185–1202.

Gentili, A., Caretti, F., Bellante, S., Mainero Rocca, L., Curini, R., et al. 2012. Development and validation of two multiresidue liquid chromatography tandem mass spectrometry methods based on a versatile extraction procedure for isolating non-steroidal anti-inflammatory drugs from bovine milk and muscle tissue. ***Analytical and Bioanalytical Chemistry***, 404(5): 1375–1388.

Ghosh, S. 2021. Metabolomic Studies for Metabolic Alterations Induced by Non-Steroidal Anti-Inflammatory Drugs: Mini Review. ***Biomolecules***, 11(10): 1456.

Gong, L., Thorn, C. F., Bertagnolli, M. M., Grosser, T., Altman, R. B., et al. 2012. Celecoxib pathways: Pharmacokinetics and pharmacodynamics. ***Pharmacogenetics and Genomics***, 22(4): 310–318.

Gorski, T., Cadore, E. L., Pinto, S. S., da Silva, E. M., Correa, C. S., et al. 2011. Use of NSAIDs in triathletes: Prevalence, level of awareness and reasons for use. ***British Journal of Sports Medicine***, 45(2): 85–90.

Gröschl, M. 2017. Saliva: A reliable sample matrix in bioanalytics. ***Bioanalysis***, 9(8): 655–668.

Gröschl, M., Köhler, H., Topf, H.-G., Rupprecht, T., & Rauh, M. 2008. Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs. ***Journal of Pharmaceutical and Biomedical Analysis***, 47(3): 478–486.

Grossman, C. J., Wiseman, J., Lucas, F. S., Trevethick, M. A., & Birch, P. J. 1995. Inhibition of constitutive and inducible cyclooxygenase activity in human platelets and mononuclear cells by NSAIDS and Cox 2 inhibitors. ***Inflammation Research***, 44(6): 253–257.

Gunaydin, C., & Bilge, S. S. 2018. Effects of Nonsteroidal Anti-Inflammatory Drugs at the Molecular Level. ***The Eurasian Journal of Medicine***, 50(2): 116–121.

Haag, M. D. M., Bos, M. J., Hofman, A., Koudstaal, P. J., Breteler, M. M. B., et al. 2008. Cyclooxygenase Selectivity of Nonsteroidal Anti-inflammatory Drugs and Risk of Stroke. ***Archives of Internal Medicine***, 168(11): 1219–1224.

Harirforoosh, S., Asghar, W., & Jamali, F. 2014. Adverse Effects of Nonsteroidal Antiinflammatory Drugs: An Update of Gastrointestinal, Cardiovascular and Renal Complications. ***Journal of Pharmacy & Pharmaceutical Sciences***, 16(5): 821.

Haroutiunian, S., Drennan, D. A., & Lipman, A. G. 2010. Topical NSAID Therapy for Musculoskeletal Pain. ***Pain Medicine***, 11(4): 535–549.

Hassan, M., & Alshana, U. 2019. Switchable-hydrophilicity solvent liquid–liquid microextraction of non-steroidal anti-inflammatory drugs from biological fluids prior to HPLC-DAD determination. ***Journal of Pharmaceutical and Biomedical Analysis***, 174: 509–517.

Henion, J., Oliveira, R. V., Li, F., Foley, T. P., & Pomponio, R. J. 2013. Dried blood spots: The future. ***Microsampling in Pharmaceutical Bioanalysis***: 48–66. Unitec House, 2 Albert Place, London N3 1QB, UK: Future Science Ltd.

Hoffman, M. D., & Fogard, K. 2011. Factors related to successful completion of a 161-km ultramarathon. ***International Journal of Sports Physiology and Performance***, 6(1): 25–37.

Hofman, L. F. 2001. Human Saliva as a Diagnostic Specimen. ***The Journal of Nutrition***, 131(5): 1621S-1625S.

I. Olives, A., Gonzalez-Ruiz, V., & Antonia Martin, M. 2012. Isolation and Quantitative Methods for Analysis of Non-Steroidal Anti-Inflammatory Drugs. ***Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry***, 11(1): 65–95.

Ito, Kaiichi, Niida, Y., Sato, J., Owada, E., Ito, Keiji, et al. 1994. Pharmacokinetics of mefenamic acid in preterm infants with patent ductus arteriosus. ***Pediatrics International***, 36(4): 387–391.

Jack, D. S., Rumble, R. H., Davies, N. W., & Francis, H. W. 1992. Enantiospecific gas chromatographic—mass spectrometric procedure for the determination of ketoprofen and ibuprofen in synovial fluid and plasma: Application to protein binding studies. ***Journal of Chromatography B: Biomedical Sciences and Applications***, 584(2): 189–197.

Jaźwińska-Tarnawska, E., Wiela-Hojeńska, A., Wolańczyk-Medrala, A., Patkowski, J., Nittner-Marszalska, M., et al. 2012. Monitoring of NSAIDs adverse effects based on spontaneous reports from Wrocław regional adverse drug reactions monitoring center. ***Acta Poloniae Pharmaceutica***, 69(5): 979–984.

Jedziniak, P., Szprengier-Juszkiewicz, T., Pietruk, K., Śledzińska, E., & Żmudzki, J. 2012. Determination of non-steroidal anti-inflammatory drugs and their metabolites in milk by liquid chromatography–tandem mass spectrometry. ***Analytical and Bioanalytical Chemistry***, 403(10): 2955–2963.

Ji, H. Y., Lee, H. W., Kim, Y. H., Jeong, D. W., & Lee, H. S. 2005. Simultaneous determination of piroxicam, meloxicam and tenoxicam in human plasma by liquid chromatography with tandem mass spectrometry. ***Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences***, 826(1–2): 214–219.

Jin, X., Zhang, C., Jin, D., & Lee, Y.-I. 2018. Enantioselective analysis of ketoprofen in human saliva by liquid chromatography/tandem mass spectrometry with chiral derivatization. ***Microchemical Journal***, 143: 280–285.

Johar D, Roth JC, Bay GH, Walker JN, Kroczak TJ, et al. 2004. Inflammatory response, reactive oxygen species, programmed (necrotic-like and apoptotic) cell death and cancer. ***Annales Academiae Medicae Bialostocensis***, 49: 9.

Joslin, J., Lloyd, J. B., Kotlyar, T., & Wojcik, S. M. 2013. NSAID and other analgesic use by endurance runners during training, competition and recovery. ***South African Journal of Sports Medicine***, 25(4): 101-104–104.

Katar, Y., Usman, E., & Aliska, G. 2022. Genetic Variation of the CYP2C9 Genetic of Minangkabau as a Predictor of Side Effect Providing Indications of Non-steroidal Anti-inflammatory Drugs. ***Open Access Macedonian Journal of Medical Sciences***, 10(A): 548–551.

Keevil, B. G. 2011. The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. ***Clinical Biochemistry***, 44(1): 110–118.

Kirchheiner, J., Meineke, I., Steinbach, N., Meisel, C., Roots, I., et al. 2003. Pharmacokinetics of diclofenac and inhibition of cyclooxygenases 1 and 2: No relationship to the CYP2C9 genetic polymorphism in humans. ***British Journal of Clinical Pharmacology***, 55(1): 51–61.

Knechtle, B., & Nikolaidis, P. T. 2018. Physiology and Pathophysiology in Ultra-Marathon Running. ***Frontiers in Physiology***, 9: 634.

Kuritzky, L., & Samraj, G. P. 2012. Nonsteroidal anti-inflammatory drugs in the treatment of low back pain. ***Journal of Pain Research***, 5: 579–590.

Kuwayama, K., Miyaguchi, H., Yamamuro, T., Tsujikawa, K., Kanamori, T., et al. 2016. Effectiveness of saliva and fingerprints as alternative specimens to urine and blood in forensic drug testing: Saliva and fingerprints as alternative specimens in forensic drug testing. ***Drug Testing and Analysis***, 8(7): 644–651.

Lambert, G., Boylan, M., Laventure, J.-P., Bull, A., & Lanspa, S. 2007. Effect of Aspirin and Ibuprofen on GI Permeability during Exercise. ***International Journal of Sports Medicine***, 28(9): 722–726.

Lapeyre-Mestre, M., Grolleau, S., Montastruc, J.-L., & Association Française des Centres Régionaux de Pharmacovigilance (CRPV). 2013. Adverse drug reactions associated with the use of NSAIDs: A case/noncase analysis of spontaneous reports from the French pharmacovigilance database 2002-2006: Adverse drug reactions related to oral NSAIDs' use. ***Fundamental & Clinical Pharmacology***, 27(2): 223–230.

Lehmann, S., Delaby, C., Vialaret, J., Ducos, J., & Hirtz, C. 2013. Current and future use of "dried blood spot" analyses in clinical chemistry. ***Clinical Chemistry and Laboratory Medicine***, 51(10). https://doi.org/10.1515/cclm-2013-0228.

Ligtenberg, A. J. M., Liem, E. H. S., Brand, H. S., & Veerman, E. C. I. 2016. The Effect of Exercise on Salivary Viscosity. ***Diagnostics***, 6,40.

Lipman, G. S., Shea, K., Christensen, M., Phillips, C., Burns, P., et al. 2017. Ibuprofen versus placebo effect on acute kidney injury in ultramarathons: A randomised controlled trial. ***Emergency Medicine Journal***, 34(10): 637–642.

Liu, J., & Duan, Y. 2012. Saliva: A potential media for disease diagnostics and monitoring. ***Oral Oncology***, 48(7): 569–577.

Locatelli, M., Tartaglia, A., D'Ambrosio, F., Ramundo, P., Ulusoy, H. I., et al. 2020. Biofluid sampler: A new gateway for mail-in-analysis of whole blood samples. ***Journal of Chromatography B***, 1143: 122055.

Lucas, G. N. C., Leitão, A. C. C., Alencar, R. L., Xavier, R. M. F., Daher, E. D. F., et al. 2019. Pathophysiological aspects of nephropathy caused by non-steroidal anti-inflammatory drugs. ***Brazilian Journal of Nephrology***, 41(1): 124–130.

Macià, A., Borrull, F., Calull, M., & Aguilar, C. 2007. Capillary electrophoresis for the analysis of non-steroidal anti-inflammatory drugs. ***TrAC Trends in Analytical Chemistry***, 26(2): 133–153.

Magallanes, L., Lorier, M., Ibarra, M., Guevara, N., Vázquez, M., et al. 2016. Sex and Food Influence on Intestinal Absorption of Ketoprofen Gastroresistant Formulation: Sex and Food Influence on Intestinal Absorption. ***Clinical Pharmacology in Drug Development***, 5(3): 196–200.

Malátková, P., Skarka, A., Musilová, K., & Wsól, V. 2017. Reductive metabolism of tiaprofenic acid by the human liver and recombinant carbonyl reducing enzymes. ***Chemico-Biological Interactions***, 276: 121–126.

Manicke, N. E., Abu-Rabie, P., Spooner, N., Ouyang, Z., & Cooks, R. G. 2011. Quantitative Analysis of Therapeutic Drugs in Dried Blood Spot Samples by Paper Spray Mass Spectrometry: An Avenue to Therapeutic Drug Monitoring. ***Journal of The American Society for Mass Spectrometry***, 22(9): 1501–1507.

Marson, P., & Pasero, G. 2006. The Italian contributions to the history of salicylates. ***Reumatismo***, 66–75.

Márta, Z., Bobály, B., Fekete, J., Magda, B., Imre, T., et al. 2018. Simultaneous determination of ten nonsteroidal anti-inflammatory drugs from drinking water, surface water and wastewater using micro UHPLC-MS/MS with on-line SPE system. ***Journal of Pharmaceutical and Biomedical Analysis***, 160: 99–108.

Martínez, S., Aguiló, A., Moreno, C., Lozano, L., & Tauler, P. 2017. Use of Non-Steroidal Anti-Inflammatory Drugs among Participants in a Mountain Ultramarathon Event. ***Sports***, 5(1): 11.

Mashal, M. S., Nalin, M., Bevalot, F., Sallet, P., Guitton, J., et al. 2022. Simultaneous quantification of 19 nonsteroidal anti-inflammatory drugs in oral fluid by liquid chromatography-high resolution mass spectrometry: Application on ultratrail runner's oral fluid. ***Drug Testing and Analysis***, 14(4): 701–712.

Mazaleuskaya, L. L., Theken, K. N., Gong, L., Thorn, C. F., FitzGerald, G. A., et al. 2015. PharmGKB summary: Ibuprofen pathways. ***Pharmacogenetics and Genomics***, 25(2): 96–106.

McAnulty, S., McAnulty, L., Nieman, D., Morrow, J., Dumke, C., et al. 2007. Effect of NSAID on Muscle Injury and Oxidative Stress. ***International Journal of Sports Medicine***, 28(11): 909–915.

McAnulty, S. R., Owens, J. T., McAnulty, L. S., Nieman, D. C., Morrow, J. D., et al. 2007. Ibuprofen use during extreme exercise: Effects on oxidative stress and PGE2. ***Medicine and Science in Sports and Exercise***, 39(7): 1075–1079.

Mehanna, A. S. 2003. NSAIDs: Chemistry and Pharmacological Actions. ***American Journal of Pharmaceutical Education***, 67(2): 63.

Migowska, N., Caban, M., Stepnowski, P., & Kumirska, J. 2012. Simultaneous analysis of non-steroidal anti-inflammatory drugs and estrogenic hormones in water and wastewater samples using gas chromatography–mass spectrometry and gas chromatography with electron capture detection. ***Science of The Total Environment***, 441: 77–88.

Milosheska, D., Grabnar, I., & Vovk, T. 2015. Dried blood spots for monitoring and individualization of antiepileptic drug treatment. ***European Journal of Pharmaceutical Sciences***, 75: 25–39.

Mizushima, T. 2010. Molecular Mechanism for Various Pharmacological Activities of NSAIDS. ***Pharmaceuticals***, 3(5): 1614–1636.

Modi, C. M., Mody, S. K., Patel, H. B., Dudhatra, G. B., Kumar, A., et al. 2012. Toxicopathological overview of analgesic and anti-inflammatory drugs in animals. ***Journal of Applied Pharmaceutical Science***, 9.

Momekov, G., Momekova, D., Stavrakov, G., Voynikov, Y., & Peikov, P. 2015. ***Para-aminosalicylic acid—Biopharmaceutical, pharmacological, and clinical features and resurgence as an anti-tuberculous agent***, 62(1): 27.

Monteiro, C., Silvestre, S., Duarte, A. P., & Alves, G. 2022. Safety of Non-Steroidal Anti-Inflammatory Drugs in the Elderly: An Analysis of Published Literature and Reports Sent to the Portuguese Pharmacovigilance System. ***International Journal of Environmental Research and Public Health***, 19(6): 3541.

Moore, L., Wicks, J., Spiehler, V., & Holgate, R. 2001. Gas Chromatography-Mass Spectrometry Confirmation of Cozart RapiScan Saliva Methadone and Opiates Tests. ***Journal of Analytical Toxicology***, 25(7): 520–524.

Moore, N., Pollack, C., & Butkerait, P. 2015. Adverse drug reactions and drug-drug interactions with over-the-counter NSAIDs. ***Therapeutics and Clinical Risk Management***, 11: 1061–1075.

Nagelschmitz, J., Blunck, M., Kraetzschmar, J., Ludwig, M., Wensing, G., et al. 2014. Pharmacokinetics and pharmacodynamics of acetylsalicylic acid after intravenous and oral administration to healthy volunteers. ***Clinical Pharmacology: Advances and Applications***, 51.

Nagler, R. M., Hershkovich, O., Lischinsky, S., Diamond, E., & Reznick, A. Z. 2002. Saliva Analysis in the Clinical Setting: Revisiting an Underused Diagnostic Tool: ***Journal of Investigative Medicine***, 50(3): 214–225.

Nakadi, F. V., Garde, R., da Veiga, M. A. M. S., Cruces, J., & Resano, M. 2020. A simple and direct atomic absorption spectrometry method for the direct determination of Hg in dried blood spots and dried urine spots prepared using various microsampling devices. ***Journal of Analytical Atomic Spectrometry***, 35(1): 136–144.

Naz, A., Beg, A. E., Ahmed, K. Z., Ali, H., Naz, S., et al. 2011. Pharmacokinetics Study of Aceclofenac in Pakistani Population and Effects of Sucralfate Co-administration on Bioavailability of Aceclofenac. ***The Journal of Applied Research***, 11(1): 55.

Nemoto, T., Lee, X.-P., Kumazawa, T., Hasegawa, C., Fujishiro, M., et al. 2014. High-throughput determination of nonsteroidal anti-inflammatory drugs in human plasma by HILIC-MS/MS. ***Journal of Pharmaceutical and Biomedical Analysis***, 88: 71–80.

Nieman, D. C., Dumke, C. L., Henson, D. A., McAnulty, S. R., Gross, S. J., et al. 2005. Muscle damage is linked to cytokine changes following a 160-km race. ***Brain, Behavior, and Immunity***, 19(5): 398–403.

Niemiec, A. 2021. Dried Blood Spot in Toxicology: Current Knowledge. ***Separations***, 8(9): 145.

Noche, G. G., Laespada, M. E. F., Pavón, J. L. P., Cordero, B. M., & Lorenzo, S. M. 2011. In situ aqueous derivatization and determination of non-steroidal anti-inflammatory drugs by salting-out-assisted liquid–liquid extraction and gas chromatography–mass spectrometry. ***Journal of Chromatography A***, 1218(37): 6240–6247.

Nolin, T. D., & Himmelfarb, J. 2010. Mechanisms of Drug-Induced Nephrotoxicity. In J. Uetrecht (Ed.), ***Adverse Drug Reactions***, vol. 196: 111–130. Berlin, Heidelberg: Springer Berlin Heidelberg.

Nørregaard, R., Kwon, T.-H., & Frøkiær, J. 2015. Physiology and pathophysiology of cyclooxygenase-2 and prostaglandin E2 in the kidney. ***Kidney Research and Clinical Practice***, 34(4): 194–200.

O'connor, N., Dargan, P. I., & Jones, A. L. 2003. Hepatocellular damage from non-steroidal anti-inflammatory drugs. ***QJM: An International Journal of Medicine***, 96(11): 787–791.

Ogawa, S., Tadokoro, H., Sato, M., & Higashi, T. 2014. Enantioselective determination of ibuprofen in saliva by liquid chromatography/tandem mass spectrometry with chiral electrospray

ionization-enhancing and stable isotope-coded derivatization. ***Journal of Pharmaceutical and Biomedical Analysis***, 98: 387–392.

Page, J., & Henry, D. 2000. Consumption of NSAIDs and the development of congestive heart failure in elderly patients: An underrecognized public health problem. ***Archives of Internal Medicine***, 160(6): 777–784.

Paíga, P., Lolić, A., Hellebuyck, F., Santos, L. H. M. L. M., Correia, M., et al. 2015. Development of a SPE–UHPLC–MS/MS methodology for the determination of non-steroidal anti-inflammatory and analgesic pharmaceuticals in seawater. ***Journal of Pharmaceutical and Biomedical Analysis***, 106: 61–70.

Park, E. J., Na, D. H., Shin, Y.-H., & Lee, K. C. 2008. Liquid chromatography–mass spectrometric method for the sensitive determination of niflumic acid in human plasma and its application to pharmacokinetic study of talniflumate tablet. ***Journal of Chromatography B***, 876(2): 159–162.

Peloquin, C. A., Zhu, M., Adam, R. D., Singleton, M. D., & Nix, D. E. 2001. Pharmacokinetics of para-Aminosalicylic Acid Granules under Four Dosing Conditions. ***Annals of Pharmacotherapy***, 35(11): 1332–1338.

Ramos-Payan, M., Maspoch, S., & Llobera, A. 2016. An effective microfluidic based liquid-phase microextraction device (μLPME) for extraction of non-steroidal anti-inflammatory drugs from biological and environmental samples. ***Analytica Chimica Acta***, 946: 56–63.

Rao, P., & Knaus, E. E. 2008. Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): Cyclooxygenase (COX) inhibition and beyond. ***Journal of Pharmacy & Pharmaceutical Sciences***, 11(2): 81s–110s.

Rao, P. P. N., Kabir, S. N., & Mohamed, T. 2010. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs): Progress in Small Molecule Drug Development. ***Pharmaceuticals***, 3(5): 1530–1549.

Reid, S. A., Speedy, D. B., Thompson, J. M. D., Noakes, T. D., Mulligan, G., et al. 2004. Study of hematological and biochemical parameters in runners completing a standard marathon. ***Clinical Journal of Sport Medicine***, 14(6): 344–353.

Sarkar, U., Ravindra, K. C., Large, E., Young, C. L., Rivera-Burgos, D., et al. 2017. Integrated Assessment of Diclofenac Biotransformation, Pharmacokinetics, and Omics-Based Toxicity in a Three-Dimensional Human Liver-Immunocompetent Coculture System. ***Drug Metabolism and Disposition***, 45(7): 855–866.

Schellack, N., Schellack, G., Schellack, G., Fourie, J., & Fourie, J. 2015. A review of nonsteroidal anti-inflammatory drugs. ***South African Pharmaceutical Journal***, 82(3): 8–18.

Sebők, Á., Vasanits-Zsigrai, A., Palkó, Gy., Záray, Gy., & Molnár-Perl, I. 2008. Identification and quantification of ibuprofen, naproxen, ketoprofen and diclofenac present in waste-waters, as their trimethylsilyl derivatives, by gas chromatography mass spectrometry. ***Talanta***, 76(3): 642–650.

Seidi, S., Rezazadeh, M., & Alizadeh, R. 2019. Miniaturized sample preparation methods for saliva analysis. ***Bioanalysis***, 11(2): 119–148.

Sharma, A., Jaiswal, S., Shukla, M., & Lal, J. 2014. Dried blood spots: Concepts, present status, and future perspectives in bioanalysis: An overview on dried blood spots. ***Drug Testing and Analysis***, n/a-n/a.

Singh, B. K., Haque, S. E., & Pillai, K. K. 2014. Assessment of nonsteroidal anti-inflammatory drug-induced cardiotoxicity. ***Expert Opinion on Drug Metabolism & Toxicology***, 10(2): 143–156.

Skordi, E., Wilson, I. D., Lindon, J. C., & Nicholson, J. K. 2004. Characterization and quantification of metabolites of racemic ketoprofen excreted in urine following oral administration to man by 1H-NMR spectroscopy, directly coupled HPLC-MS and HPLC-NMR, and circular dichroism. ***Xenobiotica***, 34(11–12): 1075–1089.

Slowey, P. D. 2015. Saliva Collection Devices and Diagnostic Platforms. In C. F. Streckfus (Ed.), ***Advances in Salivary Diagnostics***: 33–61. Berlin, Heidelberg: Springer Berlin Heidelberg.

Sobczak, Ł., & Goryński, K. 2020. Evaluation of swabs from 15 commercially available oral fluid sample collection devices for the analysis of commonly abused substances: Doping agents and drugs of abuse. ***The Analyst***, 145(22): 7279–7288.

Sostres, C., Gargallo, C. J., Arroyo, M. T., & Lanas, A. 2010. Adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and coxibs) on upper gastrointestinal tract. ***Best Practice & Research Clinical Gastroenterology***, 24(2): 121–132.

Sostres, C., Gargallo, C. J., & Lanas, A. 2013. Nonsteroidal anti-inflammatory drugs and upper and lower gastrointestinal mucosal damage. ***Arthritis Research & Therapy***, 15(Suppl 3): S3.

Stichtenoth, D. 2004. The Second Generation of COX-2 Inhibitors: Clinical Pharmacological Point of View. ***Mini-Reviews in Medicinal Chemistry***, 4(6): 617–624.

Stolfi, C., De Simone, V., Pallone, F., & Monteleone, G. 2013. Mechanisms of Action of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and Mesalazine in the Chemoprevention of Colorectal Cancer. ***International Journal of Molecular Sciences***, 14(9): 17972–17985.

Sung, J. W., Yun, H., Park, S., Kim, Y. J., Yee, J., et al. 2020. Population Pharmacokinetics of Sulindac and Genetic Polymorphisms of FMO3 and AOX1 in Women with Preterm Labor. ***Pharmaceutical Research***, 37(3): 44.

Tacconelli, S., Capone, M. L., & Patrignani, P. 2004. Clinical pharmacology of novel selective COX-2 inhibitors. ***Current Pharmaceutical Design***, 10(6): 589–601.

Takagi, K., Ishikura, Y., Hiramatsu, M., Nakamura, K., & Degawa, M. 2013. Development of a saliva collection device for use in the field. ***Clinica Chimica Acta***, 425: 181–185.

Takemoto, J. K., Reynolds, J. K., Remsberg, C. M., Vega-Villa, K. R., & Davies, N. M. 2008. Clinical Pharmacokinetic and Pharmacodynamic Profile of Etoricoxib: ***Clinical Pharmacokinetics***, 47(11): 703–720.

Tartaglia, A., Kabir, A., D'Ambrosio, F., Ramundo, P., Ulusoy, S., et al. 2020. Fast off-line FPSE-HPLC-PDA determination of six NSAIDs in saliva samples. ***Journal of Chromatography B***, 1144: 122082.

Tey, H. Y., & See, H. H. 2021. A review of recent advances in microsampling techniques of biological fluids for therapeutic drug monitoring. ***Journal of Chromatography A***, 1635: 461731.

Tjandrawinata, R. R., Setiawati, A., Nofiarny, D., Susanto, L. W., & Setiawati, E. 2018. Pharmacokinetic equivalence study of nonsteroidal anti-inflammatory drug etoricoxib. ***Clinical Pharmacology : Advances and Applications***, 10: 43–51.

Trelle, S., Reichenbach, S., Wandel, S., Hildebrand, P., Tschannen, B., et al. 2011. Cardiovascular safety of non-steroidal anti-inflammatory drugs: Network meta-analysis. ***BMJ***, 342(jan11 1): c7086–c7086.

Trnavska, Z., & Trnavska, K. 1983. Sex differences in the pharmacokinetics of salicylates. ***European Journal of Clinical Pharmacology***, 25(5): 679–682.

Tuaillon, E., Kania, D., Pisoni, A., Bollore, K., Taieb, F., et al. 2020. Dried Blood Spot Tests for the Diagnosis and Therapeutic Monitoring of HIV and Viral Hepatitis B and C. ***Frontiers in Microbiology***, 11: 373.

Ullah, Irfan, Baloch, M. K., Ullah, Imran, & Mustaqeem, M. 2014. Enhancement in Aqueous Solubility of Mefenamic Acid using Micellar Solutions of Various Surfactants. ***Journal of Solution Chemistry***, 43(8): 1360–1373.

Undas, A., Brummel-Ziedins, K. E., & Mann, K. G. 2007. Antithrombotic properties of aspirin and resistance to aspirin: Beyond strictly antiplatelet actions. ***Blood***, 109(6): 2285–2292.

van Baar, B. L., Verhaeghe, T., Heudi, O., Rohde, M., Wood, S., et al. 2013. IS addition in bioanalysis of DBS: Results from the EBF DBS-microsampling consortium. ***Bioanalysis***, 5(17): 2137–2145.

van Pamel, E., & Daeseleire, E. 2015. A multiresidue liquid chromatographic/tandem mass spectrometric method for the detection and quantitation of 15 nonsteroidal anti-inflammatory drugs (NSAIDs) in bovine meat and milk. ***Analytical and Bioanalytical Chemistry***, 407(15): 4485–4494.

Velghe, S., & Stove, C. P. 2018. Evaluation of the Capitainer-B Microfluidic Device as a New Hematocrit-Independent Alternative for Dried Blood Spot Collection. ***Analytical Chemistry***, 90(21): 12893–12899.

Vostinaru, O. 2017. Adverse Effects and Drug Interactions of the Non-Steroidal Anti-Inflammatory Drugs. In A. G. A. Al-kaf (Ed.), ***Nonsteroidal Anti-Inflammatory Drugs***. InTech. https://doi.org/10.5772/intechopen.68198.

Vree, T. B., Biggelaar-Martea, M., Verwey-van Wissen, C. P. W. G. M., & Ewijk-Beneken Kolmer, E. W. J. 1994. Probenecid inhibits the glucuronidation of indomethacin andO-desmethylindomethacin in humans: A pilot experiment. ***Pharmacy World & Science***, 16(1): 22–26.

Vree, T., Van den Biggelaar-Martea, M., Verwey-Van Wissen, C., Vree, M., & Guelen, P. 1993. The pharmacokinetics of naproxen, its metabolite O-desmethylnaproxen, and their acyl glucuronides in humans. Effect of cimetidine. ***British Journal of Clinical Pharmacology***, 35(5): 467–472.

Wade, S. E., & Haegele, A. D. 1991. Time-integrated measurement of corticosteroids in saliva by oral diffusion sink technology. ***Clinical Chemistry***, 37(7): 1166–1172.

Wagner, M., Tonoli, D., Varesio, E., & Hopfgartner, G. 2016. The use of mass spectrometry to analyze dried blood spots. ***Mass Spectrometry Reviews***, 35(3): 361–438.

Wharam, P. C., Speedy, D. B., Noakes, T. D., Thompson, J. M. D., Reid, S. A., et al. 2006. NSAID use increases the risk of developing hyponatremia during an Ironman triathlon. ***Medicine and Science in Sports and Exercise***, 38(4): 618–622.

Whatmough, S., Mears, S., & Kipps, C. 2017. The Use of Non-Steroidal Anti-Inflammatories (nsaids) at the 2016 London Marathon. ***British Journal of Sports Medicine***, 51(4): 409–409.

White, R. M., & Moore, C. M. 2018. Introduction. Detection of Drugs and Their Metabolites in Oral Fluid: 1–9. ***Elsevier***.

Wilhelm, A. J., den Burger, J. C. G., & Swart, E. L. 2014. Therapeutic drug monitoring by dried blood spot: Progress to date and future directions. ***Clinical Pharmacokinetics***, 53(11): 961–973.

Wong, M., Chowienczyk, P., & Kirkham, B. 2005. Cardiovascular issues of COX-2 inhibitors and NSAIDs. ***Australian Family Physician***, 34(11): 945–948.

Wongrakpanich, S., Wongrakpanich, A., Melhado, K., & Rangaswami, J. 2018. A Comprehensive Review of Non-Steroidal Anti-Inflammatory Drug Use in The Elderly. ***Aging and Disease***, 9(1): 143.

Yang, M., Fazio, S., Munch, D., & Drumm, P. 2005. Impact of methanol and acetonitrile on separations based on pi-pi interactions with a reversed-phase phenyl column. ***Journal of Chromatography. A***, 1097(1–2): 124–129.

Yeh, K. C. 1985. Pharmacokinetic overview of indomethacin and sustained-release indomethacin. ***The American Journal of Medicine***, 79(4): 3–12.

Yilmaz, B., Sahin, H., & Erdem, A. F. 2014. Determination of naproxen in human plasma by GC-MS: Gas Chromatography. ***Journal of Separation Science***, 37(8): 997–1003.

Youhnovski, N., Bergeron, A., Furtado, M., & Garofolo, F. 2011. Pre-cut dried blood spot (PCDBS): An alternative to dried blood spot (DBS) technique to overcome hematocrit impact: Pre-cut dried blood spot: an alternative to dried blood spot. ***Rapid Communications in Mass Spectrometry***, 25(19): 2951–2958.

Zhang, X., Donnan, P. T., Bell, S., & Guthrie, B. 2017. Non-steroidal anti-inflammatory drug induced acute kidney injury in the community dwelling general population and people with chronic kidney disease: Systematic review and meta-analysis. ***BMC Nephrology***, 18(1): 256.