DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR DETERMINATION OF NON-OPIOID ANALGESICS IN ADULTERATED HERBAL MEDICINES

The thesis is submitted in Fulfilment of the Requirements for Master`s Degree in Phytochemistry of Sokoine University of Agriculture, Morogoro

By

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EXTENDED ABSTRACT

Background: Herbal medicines are increasingly used worldwide despite the increased concern of their adulteration with conventional drugs. Non-opioid analgesics are among of the conventional drugs reported to be used in adulteration of anti-pain herbal medicines. This rise a need for developing reliable analytical method for determination of adulterated conventional drugs in herbal medicines for quality control. This study aimed at developing a high throughput Liquid Chromatography coupled with Tandem Mass Spectrometry for determination of non-opioid analgesics (acetaminophen, caffeine, acetylsalicylic acid, diclofenac, and ibuprofen) in herbal medicines in one run. The study also aimed at optimizing a sample preparation technique to enhance detection of the adulterants in herbal medicine obtained from the Tanzania market.

Methodology: Solid Phase Extraction (SPE) and Ultrasonic Assisted Extraction (UAE) methods were compared in respect of recoveries, extraction time, complexity, matrix effects, and expenses for sample preparation. Waters OASIS Hydrophilic-lipophilic balance (HLB) 200 mg 6 mL, a C_{18} sorbent was used in Solid Phase Extraction method optimization. The optimized parameters were effects of filters on sample filtration, sample loading pH, selection of suitable solvents for conditioning, washing, and elution. Ultrasonic bath and centrifuge were used for optimization of ultrasonic assisted extraction method parameters including effectives of organic solvent in extraction, extraction solvent content, and sample to extraction solvent volume ratio. Both methods were evaluated by calculating their matrix effects, absolute recoveries, and percentage recovery for sub-steps. The effective sample preparation method was used in development and validation of an advanced hyphenated method using an Agilent Technologies 1100 series Liquid Chromatography coupled with Waters Tandem Mass spectrometry (LC-MS/MS) with electrospray ionization. The LC-MS/MS method was validated for its selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effects, and stability. It was then used to analyse 132 samples collected from the markets of four regions in Tanzania.

Results and Discussion: The Ultrasonic Assisted Extraction method showed to be efficient to Solid Phase Extraction method for extraction of selected non-opioid analgesics. This was clearly evidenced by high recoveries, minimum extraction time, good peak shape, its simplicity in procedures, and use of less expensive consumables. The absolute recoveries for Ultrasonic Assisted Extraction were at acceptable range (>

60%) ranging from 60 % to 107 % while for Solid Phase Extraction method showed poor recovery except for

diclofenac and ibuprofen having 83% and 67%, respectively. The matrix effect expressed as signal suppression/enhancement ranged from 70% - 122% for Ultrasonic Assisted Extraction method and 3% to 124% for Solid Phase Extraction method. From the results the Ultrasonic Assisted Extraction method was chosen for sample extraction for analysis of Herbal Samples.

The developed LC-MS/MS method was linear with coefficient of determination of $R^2 \geq 0.9931$. The Limit of Detection (LOD) and Limit of Quantification (LOQ) for selected non-opioid analgesics were within the range of 0 – 3.7 µg/mL. The intra-precision of the LC-MS/MS method was expressed as Relative Standard deviation and was less than 9.5% with varying matrix effect among analytes. Twenty one percent (21%) of 132 collected herbal medicines were adulterated with caffeine and acetylsalicylic acid. 1,029.22 µg/mL was one of the highest concentration of caffeine detected in one of the analysed herbal medicines.

Conclusion and recommendation: This is the first study in Tanzania to report an analytical method for analysis of five (5) non opioid analgesics in herbal medicines in Tanzania. The developed LC-MS/MS method is suitable for the identification and quantification of 5 non opioid analgesics explored adulterants. The method can be adopted by the regulatory authorities for routine analysis of herbal medicines for monitoring its quality and safety. Further studies are recommended for Ultrasonic Assisted Extraction method using additional internal standards.

Key words: Herbal medicines, Adulteration, Analgesics, Solid Phase extraction, Ultrasonic assisted extraction, LC-MS/MS.

IKISIRI KUU

Usuli wa tatizo: Ongezeko la matumizi ya dawa zitokanazo na mimea-dawa linazidi kukua ulimwenguni licha ya wasiwasi uliopo wa kuathiriwa kwa dawa hizo kwa kuchanganywa na dawa za kisasa. Dawa za kutuliza maumivu za kisasa ni miongoni mwa dawa zinazoripotiwa kuongezwa katika dawa zitokanazo na mimea dawa za kupunguza maumivu na hivyo kushusha ubora wake. Hivyo, kuna uhitaji mkubwa wa kuunda njia ya kisayansi yenye kuaminika kwa ajili ya uchambuzi na utambuzi wa dawa zitokanazo na mimea dawa zilizochanganywa na dawa za kisasa ili kudhibiti ubora na kuongeza usalama kwa watumiaji. Utafiti huu ulilenga kutengeneza njia ya kisayansi ya uchambuzi inayotumia teknolojia ya LC-MS/MS ili kubaini aina na kiasi cha dawa za kisasa za kutuliza maumivu (asetaminopheni, kafeini, aspirini, diclofenaki, na ibuprofeni) zilizomo ndani ya dawa zitokanazo na mimea dawa. Utafiti huu pia ulilenga kuboresha mbinu ya utayarishaji wa sampuli ili kuboresha ugunduzi wa dawa za kisasa ndani ya dawa zitokanazo na mimea dawa zinazopatikana katika soko la Tanzania.

Methodolojia: Utafiti ulilinganisha njia mbili, *Solid Phase Extraction (SPE)* na *Ultrasonic Assisted Extraction (UAE)*, kwa kuzingatia uhuishaji, muda ambao ulitumika kuchukua kiziduo, athari za dutu za ziada, na gharama za uandaaji wa sampuli. Teknolojia ya *Waters OASIS Hydrophilic-lipophilic* balance (HLB) 200 mg 6 mL, sobanti C18 ilitumika katika mchakato wa kuchambua dutu lengwa. Vigezo stahiki vilipatikana kutokana na athari zilizojitokeza katika uandaaji wa sampuli. Pia teknolojia ya *ultrasonic assisted extraction* ilitumika kuweka sawa mchakato wa kikemikali wa kuchambua dutu lengwa kutoka kwenye sampuli. Njia zote mbili zilitathiminiwa kwa kukokotoa athari zilizojitokeza, kiasi cha dutu zilizopatikana katika mchakato, na kiasi cha dutu kilichopatikana katika hatua zilizofuata. Njia bora ya kuandaa sampuli stahiki ilitumika katika kutengeneza na kuthibitisha njia yenye ufanisi wa kuchambua dutu lengwa. Kukamilisha zoezi hili, teknolojia ya Agilent Technologies 1100 series Liquid Chromatography na ile ya Waters Tandem Mass spectrometry (LC-MS/MS) yenye electrospray ionization vilitumika. Njia ya LC-MS/MS ilihakikiwa uwezo wake katika kubaini dutu husika, usahihi, utoshelevu, na umadhubuti. Aidha, njia hii ilitumika kuchambua sampuli 132 zilizokusanywa kutoka kwenye masoko ya mikoa minne nchini Tanzania.

Matokeo na mjadala: Njia ya UAE ilionyesha kuwa na ufanisi katika kuandaa sampuli ya dawa za kupunguza maumivu zilizochaguliwa kwa ajili ya utafiti. Hii ilithibitishwa na uwezo mkubwa katika kuhuisha, kutumia muda

mchache, kutoa taswira nyoofu, urahisi katika kutumia pamoja na utumiaji wa malighafi

za gharama ndogo. Uhuishaji halisi wa UAE ulikuwa katika kiwango kinachokubalika, yani, kuanzia 60 % hadi 107 %, wakati njia ya SPE ilionesha uhuishaji dhaifu isipokuwa kwa diclofenac (83%) na ibuprofen (67%). Athari za ziada zilizooneshwa kama alama zilizodhibitiwa (fiche) zilianzia 70% - 122% kwa njia ya UAE na 3% hadi 124% kwa njia ya SPE. Kutokana na matokeo, UAE ilichaguliwa kwa ajili ya kuandaa sampuli zitokanazo na mimea dawa kwa ajili ya uchambuzi.

Njia ya LC-MS/MS ililandana na kizigeu-tambuzi, $R^2 \geq 0.9931$, upeo wa kubaini pamoja na upeo wa kiasi kwa dawa za kuzuia maumivu zilizotafitiwa zilikuwa kati ya 0 – 3.7 µg/mL. Utashelevu wa ndani wa njia ya LC-MS/MS ilijidhihirisha kama achano sanifu-wiano ambalo lilikuwa 9.5% iliyokuwa na athari za ziada mbalimbali katika dutu zilizochambuliwa. Asilimia ishirini na moja (21%) ya dawa 132 za asili zilikuwa zimechanganywa na kafeini na aspirini. Kiasi cha juu cha kafeini kilichogunduliwa katika dawa zilizochunguzwa ni 1,029.22 µg/mL.

Hitimisho na mapendekezo: Huu ni utafiti wa kwanza nchini Tanzania uliotumia njia changanuzi kuchunguza uwepo wa dawa tano za kisasa ndani ya dawa zitokanazo na mimea dawa za kutuliza maumivu. Njia iliyobuniwa ya LC-MS/MS ilifaa katika kutambua na kupima dawa tano za kupunguza maumivu zilizotumika kama viharibifu. Hivyo, njia hii inaweza kutumiwa na mamlaka za udhibiti wa ubora na usalama wa dawa za asili. Inapendekezwa kuwa tafiti zaidi zifanyike kutengeneza njia zingine za LC-MS/MS kwa dawa zingine ili kudhibiti ubora wake.

Maneno muhimu: Dawa za mimea-dawa, kuchanganywa, dawa za kutuliza maumivu, uziduzi, LC-MS/MS.

DECLARATION

I, Anna Mpanyakavili do hereby declare to the senate of Sokoine University of Agriculture that this thesis is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

Anna L. Mpanyakavili
(MSc. Candidate) **Date**

The above declaration is confirmed by;

_____ _____

Dr. F. P Mabiki (Supervisor) **Date**

_____

Prof. B. Styrishave (Supervisor) **Date**

LIST OF PUBLISHED MANUSCRIPT AND PAPER

I. Solid Phase Extraction and Ultrasonic Assisted Extraction methods for determination of non-opioid analgesics using LC-MS/MS in adulterated Herbal Medicines. Anna L. Mpanyakavili, Christopher J. Mwankuna, Faith P. Mabiki and Bjarne Styrishave. S*ubmitted: To the International Journal of Analytical Chemistry.*

II. LC-MS/MS Method for Determination of Non-opioid Analgesics Adulterants in Herbal Medicines. Anna L. Mpanyakavili, Christopher J. Mwankuna, Faith P. Mabiki and Bjarne Styrishave. *Journal of Chemistry Africa.*

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DEDICATION

This work is dedicated to my parents.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

ACM	Acetaminophen
ASA	Acetylsalicylic acid
CFN	Caffeine
DANIDA	Danish International Development Agency
DAD	Diode - array detector
DFC	Danida Fellowship Centre
DIC	Diclofenac sodium salt
EFSA	European Food Safety Authority
ESI	Electrospray ionization
FA	Formic acid
FDA	Food and Drug administration
Hcl	Hydrochloric acid
HLB	Hydrophilic lipophilic balance
HM	Herbal medicines
HPLC	High Performance Liquid Chromatograph
IBF	Ibuprofen
ICH	International Conference on Harmonization
LC	Liquid chromatograph
LC-MS/MS	Liquid chromatography with Tandem Mass Spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
ME	Matrix Effects
MeCN	Acetonitrile
MeOH	Methanol
MP	Mobile phase
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NOA	Non opioid analgesics
NSAIDs	Non-steroidal anti-inflammatory drugs
Rs	Peak Resolution
Rt	Retention time
SPE	Solid-phase extraction
SSE	Signal suppression/enhancement
STDs	Standard mixture
SUA	Sokoine University of Agriculture
TBS	Tanzania Bureau of Standards
TLC	Thin layer chromatography
TMDA	Tanzania Medicines and Medical Devices Authority

UAE	Ultrasonic-assisted extraction
UTI	Urinary tract infections
UV	Ultra-violet
WHO	World Health Organization

CHAPTER ONE

1. INTRODUCTION

1.1. Herbal medicines (HM)

Herbal medicines are one of the green resources products derived from plants or plant extracts containing therapeutic substances (Bost et al., 2010; Pavia, Lampman, Kriz, 1999). They are increasingly used worldwide as dietary supplements, cosmetics and medicines (Mir et al., 2017). Herbal medicines (HM) exclusively contain active ingredients made from plant parts such as leaves, roots or flowers which give pharmacological effect. They can be found in form of tablets, pills, granules, capsules, powder, or liquid. They naturally contain active ingredients for treatment of different health problems including pain relief (Bost et al., 2010). The consumption of HM is growing rapidly because of the perception that they are natural with no risks to human health and have high activity (Ekor, 2014). For that matter, the global market for HM is increasing. It has been projected by World Health Organization (WHO) that, the demand of HM will reach $5 trillion by 2050 (Mir et al., 2017).

1.2. Adulteration

Adulteration of HM is defined as deliberately fake practices in which a HM is substituted partially or fully with impure, extraneous, improper or inferior products into or onto a starting material, intermediate or finished herbal product during production, sampling, packaging or repackaging, storage or transport (Posadzki et al., 2013, Ichim, 2020). It as well refers to the practice of adding synthetic drugs in the formula of the products, which are marketed as “herbal medicine” (Al Lawati et al., 2017). Non-opioid analgesics such as acetaminophen, diclofenac, caffeine, ibuprofen, indomethacin, naproxen, and piroxicam have been reported to be among the adulterants in HM (Kim et al., 2014). The use of adulterated HM has resulted to a number of toxicity cases leading to gastrointestinal, cardiovascular diseases, nervous system, and renal effects (Calahan et al., 2016; Garg & Ferguson, 2002). In fact HM can be adulterated with synthetic chemical drugs to enhance the efficacy of respective products for the claimed indications (Kool, 2016). Adulterated HM have been reported to lack standardization, incorrect preparation, and inappropriate labelling (Sadgrove & Sadgrove, 2021).

Low capacity of monitoring of HM in many countries pave a way for adulteration. The state-of-the-art equipment such as Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) can be used to monitor the quality of HM in the market. Thus, the safety of HM becomes a major

concern to National health Control Authorities and general public monitoring Authorities (Traditional & Strategy, 2013). This calls for establishment of quality control of HM mechanisms globally and nationally through strengthening the analytical capacity.

1.3. Quality and safety of herbal medicinal

The demand for the herbal drugs has increased in recent times because contain bioactive compound(s) and so can be used as alternatives to synthetic drugs (Vadhana et al., 2015). Other than antimicrobial therapy, herbal drugs are used for treatment of age-related disorders, osteoporosis, and immune disorders (Ishtiyak & Hussain, 2018). They contain active ingredients that can be used as blood thinners and contain analgesic, antipyretic, insecticidal, antiseptic, and antimicrobial properties (Ishtiyak & Hussain, 2018). However, adulteration with undeclared conventional medicines and potent pharmaceutical substances such as corticosteroids and non-steroidal anti-inflammatory agents is a major safety concern (WHO, 2004).

1.4. Effect of uncontrolled use of non-opioid analgesics

Non-opioid analgesics (NOA) include acetaminophen (paracetamol) and non-steroidal anti-inflammatory drugs (NSAIDs). They have analgesic, antipyretic and anti-inflammatory activities that they inhibit prostaglandin synthesis (Zimmermann & Curtisa, 2017). Therefore, they are used to conditions such as mild to moderate inflammatory pain, rheumatoid arthritis, dysmenorrhea, migraine, headache, fever, and other ailments. Anti-inflammatory drugs provide greater comfort to the patient (Reis Nunes et al., 2020). Uncontrolled NOA usage is associated with gastrointestinal problems, kidney damages, asthma worsening in affected patients, increasing risk of post-surgery and post-partum bleeding, erectile dysfunction, and complications regarding conception and pregnancy (Amirimoghadam et al., 2017).

1.5. Choice of method of analyte extraction from herbal medicines

Sample preparation and clean-up are the key steps that must be involved in any analytical works. Depending on the sample type and analytes properties, different sample preparation methods have been reported to be used for sample preparation(Matuszewski & Constanzer, 1998) (Matuszewski & Constanzer, 1998)(Matuszewski & Constanzer, 1998). The best choice for the method of extraction will always provide the best chromatogram with good LC peak area, shape, width, resolution and high

MSMS signal. The popular reported sample preparation methods are liquid – liquid extraction (LLE), solid phase extraction (SPE), ultrasonic assisted extraction (UAE), protein precipitation, and quick, easy, cheap, effective, rugged and safe (QuEChERS) methods (Stone, 2017, Paíga et al., 2017, Chen et al., 2009;). For better results, methods need to be standardized according to the physical and chemical properties of analytes (Kang, 2012). In this study SPE and UAE methods were explored for higher recoveries of studied analytes.

1.5.1. Solid Phase Extraction method

Solid-phase extraction (SPE) has been a method of choice for extraction of non-opioid analgesics from HM. The ultimate goal of SPE is to concentrate and purify analytes from sample matrix by adsorption process and then elution with a solvent appropriate for instrumental analysis for longer column lifetime (Phenomenex, 2017). Different types of sorbents (stationery phase) can be applied for extraction of varieties of analytes from sample matrix. As the sample is slowly passed through the SPE cartridge, the analytes and some of the sample matrix compounds may be retained on the SPE material. Therefore, the choice of SPE sorbent depends on the properties of targeted analytes. The vacuum manifold can be applied to speed up the process (Corporation, 2008)

1.5.2. Ultrasonic Assisted Extraction method

Ultrasonic assisted extraction (UAE) method is the extraction method that use ultrasonic irradiation (20 to 1000 kHz) which allows simultaneous extraction of several samples (Albero et al., 2019). The main advantages of this method is that; it allows extraction of wide variety of compound with different polarity, have high extraction efficiency, operating time is shorter, and it is simple (Tabaraki et al., 2012). The method processes involved in ultrasonic extraction are centrifugation and evaporation.

1.6. Selection of the method for analysis of adulterants in herbal medicines

Different detection methods for adulterants have been reported including Thin Layer Chromatography (TLC) (Simaremare et al., 2018) and High Performance Liquid Chromatography (HPLC) with Diode array Detector (Patel et al., 2013). Mass Spectrometry is the most applied detection method for identification and structural elucidation of adulterants as it fulfils the requirement of selectivity and sensitivity needed for analysis (Pratiwi et al., 2021). Liquid Chromatography coupled with Tandem Mass Spectrometry is

a primary tool in the analysis of adulterated herbal products or food samples since it combines the separation capacity of LC with high sensitivity, resolution, and selectivity of MS detector for molecular identification (Vaclavik & Krynitsky, 2014; Zhu et al., 2014). It is therefore necessary to develop a rapid LC-MS/MS method for screening a large number of randomly selected samples.

Following the use HM by more than 50% of Tanzanians, assuring the quality through determining the extent of adulteration is inevitable (Liwa et al., 2017). The installation of LC-MS/MS machine at the department of Chemistry and physics opens an opportunity to contribute to the quality assurance of HM specifically non-opioid analgesics drugs in Tanzania. Therefore, the study aimed at developing a simple and advanced LC-MS/MS method for determination of five non-opioid analgesic adulterants in sampled liquid herbal medicines.

1.7. Problem Statement and Significance of the study

The adulteration of HM with synthetic drugs may cause health risks to consumers. Despite the increasing use of HM at primary health care levels in the country, there is still low regulation capacity in terms of screening technologies, limited analytical methods for analysis and identification of adulterants specifically NOA. This has limited the availability of information on the levels and extents of adulteration in the markets which exposes the users to both acute and chronic health effects. The presence of strengthened analytical capacity will produce scientific data that can be used by regulatory authorities. Those data can be used in setting strong standards and policy in ensuring the quality of HM. Therefore, the study focused at developing and validating a high throughput method for determination of five NOA in HM. This is contributing into strengthening regulatory capacity for quality control of HM for consumers' good health. The generated scientific data from this study could contribute in the government efforts in establishing benchmark standards for use by regulatory bodies such as Tanzania Bureau of Standards (TBS), Tanzania Medicines and Medical devices Authority (TMDA), and Traditional Medicine Practice Council. The LC-MS/MS method could be adopted by regulatory authorities for routine analysis of HM in Tanzania market and elsewhere.

1.8. Research Objectives

1.8.1. Overall objective

To develop and validate an LC-MS/MS method for determination of five (5) non-opioid analgesic adulterants in HM collected from Tanzanian market.

1.8.2. Specific objectives

i. To evaluate the extraction efficiency of SPE and UAE methods in enhancing recoveries of paracetamol, caffeine, aspirin, diclofenac, and ibuprofen from liquid HM Materials.
ii. To develop and validate the LC-MS/MS method for analysis of paracetamol, caffeine, aspirin, diclofenac, and ibuprofen adulterants in liquid HM.
iii. To identify and quantify the presence of paracetamol, caffeine, aspirin, diclofenac, and ibuprofen adulterants in liquid HM using the developed LC-MS/MS method.

1.9. Study design

The study was designed as an experimental study.

1.10. Sample collection

A total of 132 liquid HM declared for analgesic, antipyretic and anti-inflammatory activities were purposively collected from herbalists, shops, clinics, and open markets in Tanzania market. They were collected in four representative regions of Tanzania; including Dar es Salaam, (56 samples), Mwanza (20 samples), Morogoro (40 samples), and Njombe (16 samples).

1.11. Laboratory analysis

The general procedure for developing a method were followed that started by optimizing MS parameters, LC parameters and finally sample preparation method. All procedures are indicated in detail in chapter two and three. Generally, the developed LC-MS/MS method was of 20 minutes run time.

1.11.1. Optimization of MS parameters

MS parameters were optimized using a triple quadrupole Mass spectrometry (Waters, Micromass UK Limited) equipped with an electrospray ionization (ESI) interface and operated with Masslynx 4.1 software. Parameters were obtained by entering the molecular ion masses into the massLynx software. The default ranges for cone voltage and collision energy were used to enable MassLynx software to automatically determine other parameters,

such as capillary voltage, desolvation temperature, gas flows and multiple reaction monitoring (MRM) transitions. The nebulizing and collision gases were nitrogen and argon, respectively. Product ions and corresponding collision energies were stored in the method files. The most two intense MRM transitions were selected for quantification and qualification.

1.11.2. Chromatographic conditions and analysis

The use of LC-MS/MS was reported one of most effective method for reliable data compared to TLC or HPLC. Chromatographic separations were carried out on Agilent 1100 liquid chromatography system fitted with the Degasser, Autosampler, column and column oven, and Diode array detector. Agilent MassLynx was used for data analysis and handling. Acidified HPLC grade MeOH, MeCN, and water solvents at different compositions, flow rate, and column temperatures were tested for better resolution of analytes.

1.12. Limitation of the study

Use of a single internal standard which may limit the accuracy and precision of quantitation as well as the robustness of the developed method.

1.13. Thesis organization

This thesis has been developed in “Published paper formats” comprising of five chapters. Chapter one contains general introduction, problem statement and significance of the study, general and specific objectives, methodological, and study limitations. Chapter two presents a manuscript titled “Solid Phase Extraction and Ultrasonic Assisted Extraction methods in enhancing recoveries of non/opioid analgesics for LC-MS/MS analysis in Herbal Medicines”, submitted to International journal of analytical chemistry. Chapter three (3) presents a paper titled “LC-MS/MS method for determination of non-opioid analgesics adulterants in Tanzanian herbal market”, published in the journal of Chemistry Africa. Chapter four (4) contain general discussions and chapter five (5) have conclusion and recommendations.

CHAPTER TWO

Solid Phase Extraction and Ultrasonic Assisted Extraction methods for determination of non-opioid analgesics using LC-MS/MS in adulterated Herbal Medicines[1]

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[1] "The material contained in this chapter has been submitted to the International Journal of Analytical Chemistry"

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Manuscript submitted to International Journal of Analytical Chemistry

International Journal of Analytical Chemistry <lydia.kirubaidoss@hindawi.com> Fri, Sep 30, 2022 at 5:35 PM
To: anna.mpanyakavili@sua.ac.tz

Hindawi

Dear Dr. Mpanyakavili,

Congratulations, the manuscript titled "Solid Phase Extraction and Ultrasonic Assisted Extraction methods for determination of non-opioid analgesics using LC-MS/MS in adulterated Herbal Medicines" has been successfully submitted to International Journal of Analytical Chemistry.

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Abstract

This study aimed to compare the effectiveness of solid phase extraction (SPE) and Ultrasonic assisted extraction (UAE) methods for extraction of five (5) non-opioid analgesics (NOA) including acetaminophen, caffeine, acetylsalicylic acid, diclofenac, and, ibuprofen adulterants in herbal medicines. The two procedures compared in terms of extraction time, absolute recoveries, costs in terms of time and consumables used and complexity. The analysis was done using a Liquid Chromatography coupled by Triple Quadrupole Tandem mass spectrometer (LC-MS/MS). Waters OASIS Hydrophilic-lipophilic balance (HLB) 200 mg 6 mL, a C_{18} sorbent was used in SPE. It was observed that, the relative recovery for SPE ranged from 0% to 83% and UAE from 60% to 107%. The matrix effects for UAE were at accep range of $\geq 80\% \leq 120\%$ (70% – 122%), while that of SPE was poor (0% - 124%) with exceptional to ibuprofen (101%). It was found that UAE is economical in terms of time and consumables, simple method to operate, the LC chromatogram have good separation with good peak shape and have good recoveries at acceptable range. In SPE was shown that, the method is expensive in terms of consumables and time, complex method, and the LC chromatogram have poor peak shapes, tailing and interfering peaks observed. From the results it was shown that, the UAE is superior to SPE for extraction of five NOA from liquid HM. Further studies using other types of comparable methods are recommended to explore more extraction methods of NOA from herbal medicines.

1. Introduction

Herbal medicines (HM) are naturally occurring and plant derived medicines having active ingredients for medicinal purpose like anti-inflammatory agents for pain relief (Bost et al., 2010). Adulteration is among of the factors affecting the safety of HM resulting to health problems to consumers like antimicrobial resistance (Ichim, 2019; Pascale, 2019; Snyman et al., 2005). Non opioid analgesics (NOA) one of the conventional drug groups have been reported to be widely used for antipyretic and pain relief, (Ariffin et al., 2021; Zimmermann & Curtisa, 2017). They have been reported to be used in adulteration of anti-inflammatory, antipyretic and analgesic herbal medicines, (Kim et al., 2014). The quality of HM can be controlled by doing routine detection and quantification of adulterants present in HM using analytical methods. So, optimization of reliable, rapid, inexpensive, and simple methods is required.

Appropriate methods for determination of adulterants in HM depends on the properties of targeted analytes. Currently with the presence of advanced analytical tools like thin-layer chromatography (TLC), column liquid chromatography (CLC), and gas chromatography (GC) is possible to detect and identify several adulterants (Pratiwi et al., 2021). A combination of Liquid Chromatography (LC) with Tandem Mass Spectrometry (MS/MS) provides adequate sensitivity for the determination of adulterants in HM. This is because, it can be used for the identification and study the structure of unknown components of sample.

Development of sample preparation procedures for enrichment of the adulterants for LC-MS/MS analysis is important (Oral & Andaç, 2015). It is required for good performance of analytical method, adjust analyte concentration for detection limit of LC-MS/MS and serve half-life of LC column (Majors, 2013). It to save time and costs without losing sensitivity and reproducibility of the analytical method. Inappropriate sample preparation procedure may results to degradation of LC peak area, shape, width, resolution, retention time and less MS/MS signal due matrix interferences (Stone, 2017).

In traditional medicines analysis, the selection of optimal sample preparation procedures for extraction of pharmaceutical adulterants from herbal products and clean-up of crude extracts depends on several factors like complexity and nature of the sample matrix (Vaclavik & Krynitsky, 2014). Different sample preparation methods have been reported to be used (Danaceau & Ph, 2013; Finoulst et al., 2011). It includes simple – dilution (DIL), simple – protein precipitation (PPT), Liquid-liquid extraction (LLE), Ultrasonic assisted extraction (UAE) and Solid Phase extraction (SPE), (Zhou et al., 2017). LLE method is one of the sample preparation procedures which is inexpensive, but time consuming and polar compounds are poorly extracted. The SPE method overcome majority of the LLE method disadvantages, (Phenomenex, 2017a). SPE is a popular method which provide high recovery of polar compounds and have high capacity sample size (Madikizela & Chimuka, 2017). It is reported to be the commonly used in both environmental and clinical applications to clean-up and concentrates analytes. It purifies analytes from solution by adsorption onto a disposable solid phase cartridge, followed by elution of the analyte with a solvent appropriate for instrumental analysis (Murray K. J K., Boyd R. K., Eberlin M. N., Langley G. J., Li L., 2015). The SPE method with appropriate sorbent may reduce or eliminate interferences present in sample matrix producing a solution containing mostly analyte and concentrating analytes present at low (Phenomenex, 2017b). The vacuum manifold can be applied to speed up the process levels(Abdulkadir et al., 2019). SPE sorbents (stationery phase) are of different types depending on the properties of targeted analytes (Smith, 2015). In this study, the Oasis HLB cartridges which exhibit both hydrophilic and lipophilic retention characteristics was used (Rodríguez et al., 2003).

The extraction process in UAE can be assisted with the usage of ultrasonic waves. The mechanic effects of ultrasound increases the surface contact between the solvents and the sample components (Nn, 2015; Viana et al., 2018). The UAE method have been reported to be used for extraction of adulterants in HM (Chen et al., 2009). It was reported that, the UAE is superior than SPE in terms of reduction in extraction time and solvent consumption (Zahari et al., 2020).

Non-opioid analgesics are among of the most frequently used pharmaceutical drugs in human medicines. They can be used for reducing pains and are widely in demand and available (Kraemer & Maurer, 2000). They have a ceiling effect for analgesia (Mercadante et al., 1999). It has been reported that they are used to adulterate analgesic and antipyretic herbal medicines (Kim et al., 2014; Xu et al., 2019). The five (5) NOA including acetaminophen, acetylsalicylic acid, caffeine, diclofenac, and Ibuprofen were used as model compounds. The aim of this study was to compare the efficiency of the UAE versus SPE methods by finding the optimal conditions for extraction of non-opioid analgesics in liquid HM. It discusses on the experiences of the two methods in extraction of five analytes in HM and their salient advantages and disadvantages of the two methods. Both optimized UAE and SPE methods were used in sample preparation where the analysis were performed using LC-MS/MS.

2. Material and Methods

2.1. Materials

The chemical standards of NOA used were acetaminophen (99%), caffeine (98%), acetylsalicylic acid (99%), diclofenac sodium salt (98 %), ibuprofen (98%), and internal standard - acetaminophen-d4 (99%) from Sigma Aldrich. HPLC grade solvents; acetonitrile (MeCN) 99.9%, methanol (MeOH) 99.8%, and water (H_2O) from Finar ® Company were used. Hydrochloric acid (Hcl), 37%; sodium hydroxide (NaOH), 30%; formic acid (FA), 98% and tablets for basic and acidic pH. Also Oasis hydrophilic – lipophilic balance (HLB) cartridges (200 mg, 6 mL) from Waters Corporation Milford, Massachusetts, USA was used.

2.2. Methods

2.2.1. Instrumentation of SPE and UAE extraction methods

The Chromabond® SPE vacuum manifold with model No. 730150N was used in optimization of SPE method for extraction and pre-concentration of NOA from HM. The vacuum pump was connected to the SPE manifold. The ultrasonic bath (Bransonic Ultrasonic bath) from Bransonic Ultrasonic Corporation, USA and

centrifuge for separation of supernatant liquid and solid (matrixes) were used in optimization of UAE method. Vortex was used for mixing experimentations.

2.2.2. Preparation of stock and standard solutions

The stock solutions of each compound was prepared by dissolving 10 mg of individual solid powdered standard in 10 mL methanol with 0.1% formic acid. The stock solutions were stored at -20 °C prior to analysis. For LC-MS/MS experiments, the working standard solutions of 20 µg/mL for each compound was prepared in dilution solvent (95:5 (water: MeCN)) both with 0.1% FA.

2.2.3. Chromatographic conditions (MS and LC conditions) used

Detection and quantification of NOA were performed using an Agilent 1100 series LC coupled with Waters MS equipped with an electrospray ionization (ESI) interface. It was operated with Masslynx 4.1 software. An Agilent 1100 liquid chromatography system consisting of Degasser, G1379A; Binary pump, G1312A; Autosampler, G1313A; Column oven, G1316A; Diode array detector, G1315B; German was used. Separation were performed on a Kinetex® 2.6 µm, C18, 100 Å LC (75 mm x 2.1 mm) waters analytical column placed in column oven at 40 °C. The mobile phase (MP) was composed of A (0.1% FA in 95% water) and B (0.1% FA in 5% acetonitrile) with linear gradient elution: 0-11.5 minutes, 5 – 5% B; 11.5 – 13.5 minutes, 95% B; 13.5 – 13.6 minutes, 5% B; the initial conditions were held for 6.4 minutes as a requilibration step. The flow rate was set at 400 $\mu L min^{-1}$. The injection volume was 10µL.

Detection of acetaminophen and caffeine was done in positive mode while acetylsalicylic acid, ibuprofen and diclofenac in negative mode. The nebulizing and collision gases were nitrogen and argon, respectively. The ion source parameters were: capillary 3.2 Kv; exit potential 3 V; RF lens 0.2 V; source temperature 110 °C; and desolvation temperature 400 °C. The desolvation and cone gas flow were 650 L/hr and 130 L/hr, respectively. Product ions and corresponding collision energies were stored in the method files. The most two intense MRM transitions were selected for quantification and qualification with exception to ibuprofen where only one

precursor ion was observed because of the very low intensity of the second product ion.

2.2.4. Sampling

Spiked HPLC grade water and liquid HM were used in this study. Five (5) HM for method optimization were purchased from traditional medicine shops in Morogoro region, Tanzania. All liquid HM samples were stored in 4 °C until the day of preparation.

2.2.5. Sample preparation method optimization

2.2.5.1. Determination of absolute and relative recoveries for the evaporation step

Water bath temperature was optimized for solvent exchange from organic solvent to aqueous solution for LC-MS/MS analysis. The eluent and supernatant from SPE and UAE methods, respectively was evaporated in water bath to approximately 1mL and subsequently dissolved in aqueous solution. Different temperature of water bath including 80 °C, 83 °C, 85 °C, 90 °C, and 100 °C were tested. The expected final concentration was approximately. Its effectiveness were evaluated in context of calculating absolute recovery of each compound as described below:

i. 100 µL of 20 µg/mL NOA mixture was diluted up to 2 mL with acetonitrile. 1 mL of distilled water was added and the mixture was evaporated to 1 mL in a water bath. The residues was post spiked with 100 µl, 20 µg/mL of internal standard and diluted to 2 mL with a MP mixture before analysis.
ii. 100 µl of 20 µg/mL internal standard was diluted up to 2 mL of acetonitrile. 1 mL of distilled water was added and the mixture was evaporated to 1 mL in a water. The residues was post spiked with 100 µL, 20 µg/mL of NOA standard mixture and diluted with a MP to 2 mL before analysis.
iii. A mixture of 2 mL of acetonitrile and 1 mL of distilled water was evaporated to 1 mL in a water bath. The residues was post spiked with NOA standard mixture and internal standard each with 100 µL of 20 µg/mL, then diluted to 2 mL before analysis.

The formula used for recoveries experiments was as equation (1) and (2);

$$Absolute\ recovery\ (\%) = \frac{Area_{pre-spiked\ analyte}/\ Area_{post-spiked\ IS}}{Area_{post-spiked\ analyte}/\ Area_{post-spiked\ IS}} \times 100\ \% \quad (1)$$

$$Relative\ recovery\ (\%) = \frac{Absolute\ recovery\ of\ target\ analyte}{Absolute\ recovery\ of\ IS} \times 100\ \% \quad (2)$$

2.2.5.2. Optimization of Solid Phase Extraction method

The SPE was carried out using SPE column. Oasis HLB (6 mL, 200g) was used in the optimization of the SPE method. 100 mL of the liquid HM sample and pure water was spiked with mixture of standards. Both samples were filtered using whatman filters of 12 – 15 µm followed by 2 -3 µm to remove sample matrix interferences. The sample loading pH, conditioning, washing and elution solvent parameters were optimized. Three (3) mL of acetonitrile was used to activate the stationery phase followed by three (3) mL of HPLC grade water for equilibration of SPE bed. 100 mL of samples were forced to pass through the SPE cartridge for approximately 55 – 60 minutes for water and more than 2 hours for liquid HM. The cartridge were then dried in air for 30 minutes and eluted with 4 mL 3% of FA in acetonitrile. Each step in SPE was evaluate by calculating the percentage of analyte accordingly.

2.2.5.3. Effects of sample filtration

The efficient of filters was explored using a whatman filters. 100 mL of sample spiked with 100 µl (20 µg/mL of standard mixture was filtered using filters of two (2) size, i.e 12 – 15 µm followed by 2 – 3 µm, respectively. Filtration was performed to eliminate interferences that could clog SPE sorbent and affect column and instrument performance. The efficient was evaluated by calculating the percentage recovery as per equation 3.

$$\text{Percentage Recovery}\ (\%) = \frac{\boldsymbol{Peak\ area\ of\ analyte\ in\ filtered\ spiked\ sample}}{\boldsymbol{Peak\ area\ of\ analyte\ in\ unfiltered\ spiked\ MP}} \quad (3)$$

2.2.5.4. Optimization of sample loading pH

Sample loading pH was optimized in order to maximize the retention of analytes on stationary phase. It was optimized by using Hcl and NaOH. The effects of pH was investigated by loading 100 mL of the spiked sample with 100 µl (20 µg/mL) of

standard mixture onto Oasis HLB (6 mL, 200g) for approximately 55 minutes. 1 mL of the loaded eluate was taken for LC-MS/MS analysis to test the retention of analytes for pH of 2.5, 4.5, 6.0, 8.0, and 10.0 (n = 2). The conditioning solvent was kept constant. The selection of suitable pH was guided by calculating the percentage of analyte in loaded eluate for each pH using equation 4.

$$\text{Percentage of analyte } (\%) = \frac{\textit{Peak area of analyte in loaded eluate}}{\textit{Peak area of analyte spiked in MP}} \times 100\ \% \qquad (4)$$

2.2.5.5. Optimization of washing solvent

HPLC grade water with different percentage of acetonitrile of 0%, 5%, 10%, and 20% was used for optimization of washing step (n = 2). The wash solvents volume used was 3 mL. The loaded SPE columns with samples were washed to determine analyte retention. The conditioning solvent and sample pH of 4.0 were kept constant while varying the wash solution. 1mL of wash eluates were collected for analysis. The efficient of wash solvent was determined by calculating the percentage of analyte eluted during the washing step using equation 5. The one with small percentage was taken for further experiments.

$$\text{Percentage of analyte } (\%) = \frac{\textit{Peak area of analyte in wash eluate}}{\textit{Peak area of analyte spiked in MP}} \times 100\ \% \qquad (5)$$

2.2.5.6. Optimization of elution solvent

Four elution solvent of acetonitrile with 0.1%, 0.2%, 0.5% and 1.0% of formic acid were explored, (n = 2). The elution solvent was optimized to determine the elution patterns as a function of pH and percentage (%) of formic acid. The loaded and air dried SPE columns were eluted respectively each with 2.0 mL twice to make a total of 4 mL of the eluate. The column was eluted twice in order to increase the recovery of analytes. Other experimental parameters including conditioning, wash solvent, and loading pH were kept constant while varying the elution solvent. The eluate was diluted with 1 mL of water and evaporated at 85 °C to approximately 1mL. The residue was diluted with MeCN:water (5:95, v/v) with 0.1% FA for analysis. The

efficient of elution solvent was evaluated by calculating the percentage of analyte in eluent, % using equation 6.

$$\text{Percentage of analyte } (\%) = \frac{\textit{Peak area of analyte in eluate}}{\textit{Peak area of analyte spiked in MP}} \times 100\ \% \quad (6)$$

2.2.5.7. Determination of absolute and relative recoveries for SPE method using HPLC water and liquid herbal medicines

The pre and post spike approach was used for the determination of the absolute and relative recovery of the analytes. It was done using HPLC water and liquid HM independently. With exceptional to water sample, the liquid HM sample was filtered to remove interferences that could clog the cartridge or harm the LC column. The final concentration of analyte was expected to be 1.0 μgmL^{-1}. The steps were as described below, (n = 4):

i. A mixture of non-opioid analgesics standards of 100 µL, 20 µg/mL was spiked into four portions of sample. The pre-spiked samples were treated through the step of interest, and evaporated to approximately 1 mL in water bath of 85 °C temperature. Finally 100 µL of internal standard, 20 µg/mL was post spiked into the eluate and diluted to 2 mL with the dilution solvent.
ii. An internal standard of 100 µL, 20 µg/mL was spiked into four portions of a sample. The pre-spiked samples were treated, through the step of interest, and evaporated to approximately 1 mL in water bath of 85 °C temperature. Finally 100 µL of mixture of non-opioid analgesics standards, 20 µg/mL was post spiked into the eluate and diluted to 2 mL with the dilution solvent.
iii. Fur (4) portions of a sample was spiked with no pre-spiking instead both non-opioid analgesics (100 µL, 20 µg/mL) and internal standard (100 µL, 20 µg/mL) were post-spiked.

Absolute and relative recoveries were calculated as per equation 1 and 2. The peak area response of the target ion (Quantifier and qualifier ions) was used to identify and quantify analytes.

2.2.6. Optimization of Ultrasonic Assisted Extraction procedures

The method developed by (Chen et al., 2009) was used as starting point to optimize the extraction procedures. The sample preparation steps were sonication, centrifugation, and evaporation. The sonication was done for effectively extraction of analytes. The centrifugation was done for removal of particulate material that could harm or reduce the efficiency of the LC column and instrument performance. The evaporation was done for solvent exchange from organic to aqueous compatible with MP. The sonication process was done for effective extraction of analytes. Two (2) organic solvents, acetonitrile and methanol were explored to get the best solvent for extraction. The percentage recoveries were calculated in exploration of efficient of organic solvent in extraction, type of organic extraction solvent, percentage (%) of FA in extraction solvent, and volume ratio of sample to extraction solvent.

2.2.6.1. Determination of organic solvent for extraction

The efficient of organic solvent in extraction was determined by comparing the liquid HM sample extracted without organic solvent and liquid HM sample extracted with organic solvent (methanol) as follows:

i. For extraction without organic solvent; 5 mL of liquid HM was spiked with 50 µL of 20 µg/mL, standard mixture to make a final solution of 0.2 µg/mL. The mixture was sonicated for 20 minutes then centrifuged for 30 minutes.
ii. For extraction using organic solvent; 1.7 mL of methanol was added to 5.1 mL of liquid HM then spiked with 68 µL of 20 µg/mL, standard mixture to make a final solution of 0.2 µg/mL. The mixture was sonicated for 20 minutes then centrifuged for 30 minutes.

In each scenarios, 0.5 mL of it was diluted with MP to 1 mL for LC-MS/MS analysis. The percentage recovery was used to evaluate the two options using equation 7. The sample extracted with organic solvent (methanol) have better recoveries than the sample extracted without organic solvent, thus taken for further experiments.

$$\text{Percentage Recovery } (\%) = \frac{\textit{Peak area of pre-spiked analyte}}{\textit{Peak area of analyte spiked in dilution solvent}} \times 100\,\% \qquad (7)$$

2.2.6.2. Optimization of extraction organic solvent

Pure methanol, pure acetonitrile, acetonitrile with 0.1% FA and methanol 0.1% FA were explored to get appropriate solvent for effectively extraction of analytes. The solvent with high percentage recovery than others was taken for further experiments. Equation 7 was used for calculations. Acetonitrile with 0.1% FA were taken for further experiments because of its high recoveries than others.

2.2.6.3. Optimization of volume ratio of sample to extraction solvents

For highly recoveries, the volume ratio of sample to extraction solvent was determined. 5:0, 5:0.5, 5:2, 5:3, 5:5, and 5:7 and 5:10 rations were explored using equation 7. Sample to extraction solvent (1:2, v/v) had high recoveries, thus taken for further experiments.

2.2.6.4. Recoveries for the entire sample preparation method

The efficiency of analytes and internal standard extraction from liquid HM was performed by absolute and relative recoveries. The recoveries were calculated by the signal intensities of pre and post spiked samples. Relative recovery was calculated by comparing the mean peak area for samples spiked before extraction with that of samples spiked after extraction. The final expected concentration was expected to be 0.067 µg/mL. The formula used for calculation of recoveries was as per equation 1 and 2. The procedures are as described below:

i. Four (4) portions of 3.0 mL of sample were pre-spiked with 100 µL (20 ppm) of NOA standard mixture then vortex mixed for 1 minutes. 6 mL of extraction solvent was added in a mixture and ultrasonically extracted for 30 minutes followed by centrifugation process for 30 minutes at 4000 r/min. After centrifugation, 6 mL of a clear supernatant was transferred to a clean culture tube for evaporation to approximately 1 mL in water bath, 85°C. Finally the residue was post-spiked with 67.7 µL (20 µg/mL) of internal standard and diluted to 2 mL with the MP.

ii. Four (4) portions of 3.0 mL of sample were pre-spiked with 100 µL (20 µg/mL) of internal standard then vortex mixed for 1 minutes. 6 mL of extraction solvent was added in a mixture and ultrasonically extracted for 30 minutes followed by centrifugation process for 30 minutes at 4000 r/min. After centrifugation, 6 mL of a clear supernatant was transferred to a clean culture tube for evaporation to approximately 1 mL in water bath, 85°C. Finally the residue was post-spiked with 67.7 µL (20 µg/mL) of NOA standard mixture and diluted to 2 mL with the MP.

iii. Four (4) portions of 3 mL of sample with no pre-spiking instead both non-opioid analgesics and internal standard both with 67.7 µL (20 µg/mL) were post-spiked.

2.2.6.5. Overall Sample preparation procedure for combined all parameters

3 mL of liquid herbal medicinal product was spiked with 20 µL (25.165 µg/mL) of internal standard and vortex mixed for 1 minutes. 6 mL of extraction solvent was added in a mixture and ultrasonically extracted for 30 minutes followed by centrifugation process for 30 minutes at 4000 r/min. After centrifugation, a clear 6 mL of supernatant was collected and transferred to a clean culture tube for evaporation to approximately 1.0 mL in 85°C water bath. Finally the residue was diluted to 2 mL with the MP in acetonitrile: water (5/95, v/v) with 0.1% FA for LC-MS/MS analysis. Figure 1 shows the overlaid chromatogram for separation of all compounds.

2.3. Evaluation of the methods

The performances of SPE and UAE methods were evaluated in terms of visuals appearance of liquid chromatograms and calculating matrix effects. The matrix effect was expresses as signal suppression/enhancement (SSE), (Matuszewski et al., 2003). It was evaluated by comparing the mean area response of post-extraction spiked samples with the mean area of neat standard at 1 µg/mL. A SSE > 100%, signify ion enhancement, SSE < 100% signify ion suppression. The acceptable range of matrix effects is ≥ 80% ≤ 100%. In addition, the deuterated internal standard was used to correct the matrix effects as shown by equation 10.

$$SSE\ (\%) = \frac{Area\ (spiked\ sample)}{Area\ standard} \times 100\ \% \qquad (10)$$

3. Results and discussion

3.1. Optimized SPE Method procedures

3.1.1. The effects of filtering sample on the recoveries of analytes

From the results it has shown that, the filtration process can facilitate the adsorption properties of the analytes to the filter layer thus reducing recovery of analytes, (Pillai et al., 2016). Figure 2 show the experimental values on effects of filters in sample filtration. The analytes from filtered MP spiked with STDs and filtered HM spiked with STDs had low recoveries compared to unfiltered MP spiked with STDs.

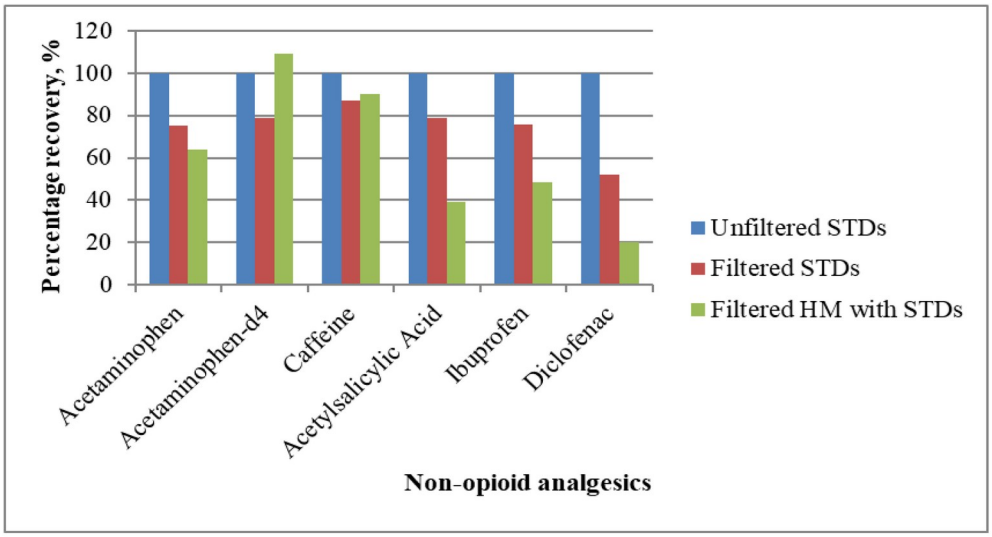

Figure 2: Percentage (%) effects of using filter 12-15 µm & 2-3 µm for sample filtration

3.1.2. Optimized sample loading pH

The pH of 4.5 was found better in helping the sorbent of SPE column to retain the analytes. This is may be because the pH of 4.5 made the SPE sorbent to strongly bind analytes. This was proven by having good recoveries as shown in figure 3.

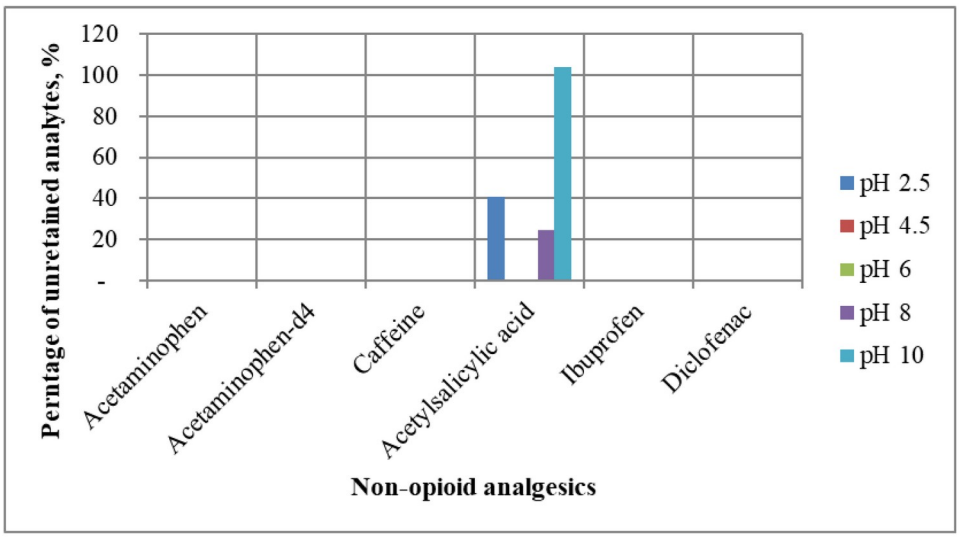

Figure 3: Percentage (%) effect of pH on sorbent retention efficiencies

3.1.3. Optimized washing solvent

Water with 0% of MeCN was used for washing step. This is because of the minimal concentration of analytes shown by its wash eluent after analysis. The other wash eluents with ≥ 5% of MeCN were shown to have analytes eluted with during washing step. This is may be because of the solubility properties of analytes in organic solvent but low in pure water. However, the solubility of analytes was increasingly as the percentage of organic solvent increase as shown in figure 4 below.

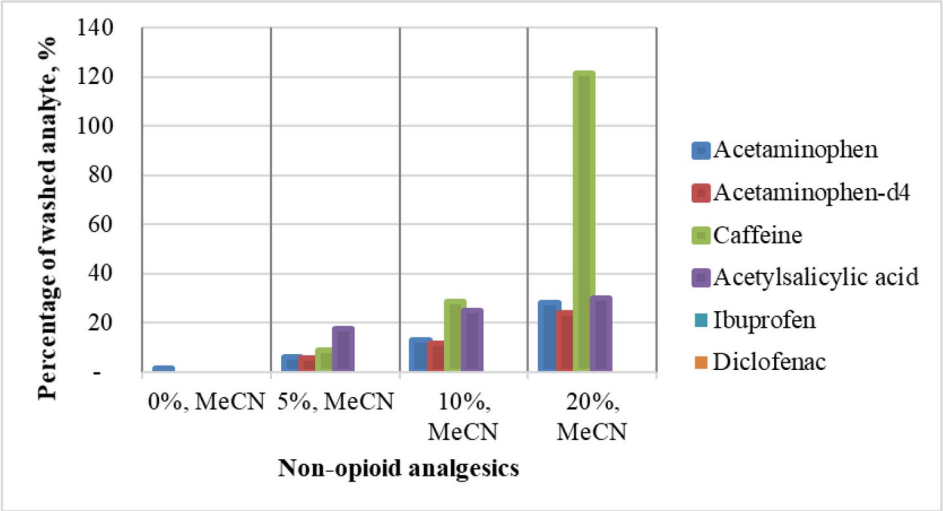

Figure 4: Percentage of washed analytes (%) in optimization of washing solvent

3.1.4. Optimized elution solvent

The use of acetonitrile with 0.1% FA resulted to high recovery for almost all analytes may be because acetonitrile has high elution capacity than methanol. The higher and lower pKa of basic and acidic compounds respectively may be caused the analytes to ionize and strongly eluted with 3% FA in acetonitrile. The pKa of acetaminophen, caffeine, diclofenac ibuprofen and acetylsalicylic acid were 8.9, 10.4, 4.15, 4.4, and 3.0 respectively. MeCN with 3% FA have high percentage of eluted analytes for almost all analytes and thus used for further experiments, see figure 5.

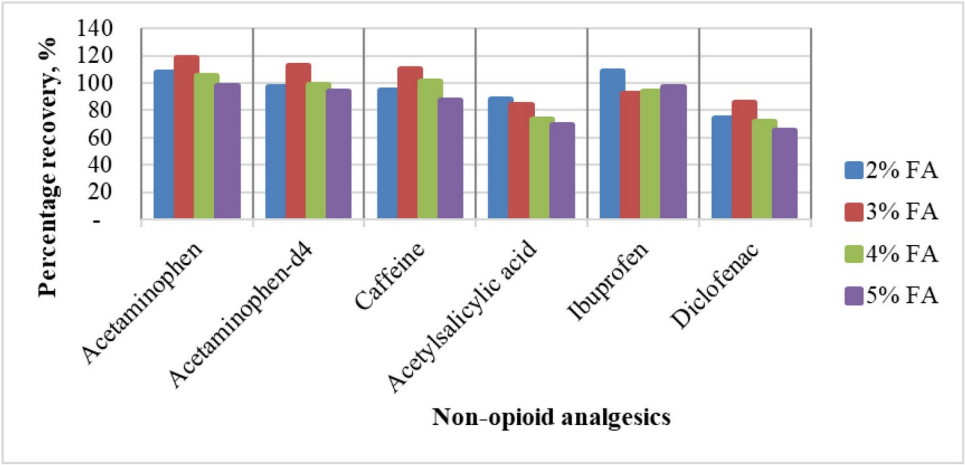

Figure 5: Percentage (%) recovery of analytes using acetonitrile with different % FA

3.1.5. The recovery of the entire SPE procedures in HPLC grade water sample

In this study, the SPE method failed to recover all analytes from liquid HM at acceptable range as shown in table 1 (0% - 83%), while in spiked water samples the recovery ranged from 74% to 135% and was at acceptable range >60%, table 2.

Table 1: Percentage (%) absolute and relative recoveries for overall SPE method for spiked HM matrix

Compound	Absolute recovery	Relative recovery
Acetaminophen: 151.90	2	922
Acetaminophen-d4: 155.92	0.2	11
Caffeine: 195.04	16	6,761
Acetylsalicylic Acid:294.07	-	-
Ibuprofen:205.08	67	27,908
Diclofenac:294.04	83	34,354

3.1.6. The recovery of the entire SPE procedures in real herbal medicines sample

The method showed poor absolute recovery for almost all analytes except for diclofenac and ibuprofen having 83% and 67% respectively but relative recovery was irrelevant due to poor absolute recovery of internal standard, see table 1. The poor recovery of acetaminophen, caffeine and Acetylsalicylic acid may be is due to the wrong sorbent bed which can cause stuck of analytes or analytes not full eluted. Leaching of analytes during washing ad elution steps can be also the cause of poor recovery, (Silicycle, 2021) . Also matrix interferences and reactions between the target analytes with other exogenous compounds, and the decomposition of analytes during the preparation procedures, (Buckner et al., 2016). Furthermore, the lower partition coefficient of -0.07, 0.48 and 1.14 for caffeine, acetaminophen and acetylsalicylic acid respectively can make them to be more polar with lower solubility in organic solvent. The two analytes, diclofenac and ibuprofen have high partition coefficient of 1.9 and 2.8 respectively which may be made them to be more soluble in organic solvent. Also, the good recovery of ibuprofen and diclofenac in SPE can be due to their resistance against the effects of sample matrixes. Also the adsorption properties of the analytes to the filter layer, may be contributed to low recoveries, (Pillai et al., 2016). Figure 6 shows the chromatogram of the separated compounds from sample extracted by SPE method.

Table 2: Percentage (%) absolute and relative recoveries for overall SPE method for spiked water sample

Compound	Absolute	Relative
Acetaminophen	97	95
Acetaminophen-d4	103	100
Caffeine	90	88
Acetylsalicylic Acid	74	72
Ibuprofen	135	131
Diclofenac	92	89

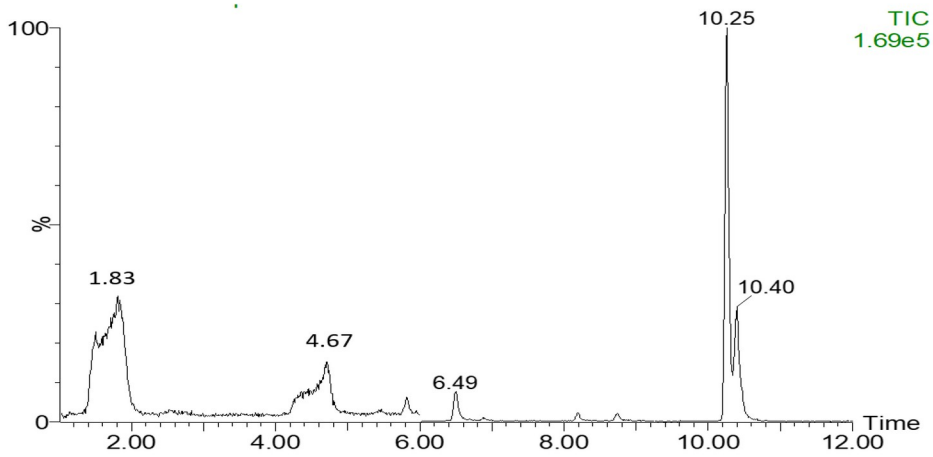

Figure 6: Representative chromatogram of analytes extracted by SPE method

3.2. Optimized UAE method procedures

3.2.1. Efficient of organic solvent in extraction

The use of organic solvent in UAE enhanced solubility of target analyte. Results in figure 7 shows that, organic extraction have advantageous than direct sample analysis. This is because Organic solvent increases solubility of organic compounds.

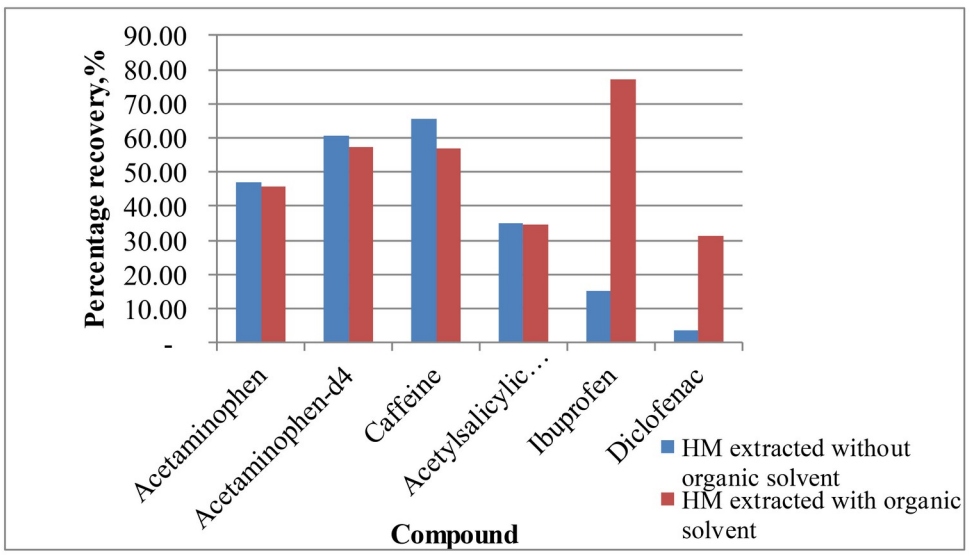

Figure 7: Percentage (%) efficiency of organic solvent in extraction of non-opioid analgesics

3.2.2. Optimization of extraction solvent

Figure 8 shows that, MeCN with 0.1% FA have maximum extraction efficiency for almost all analytes

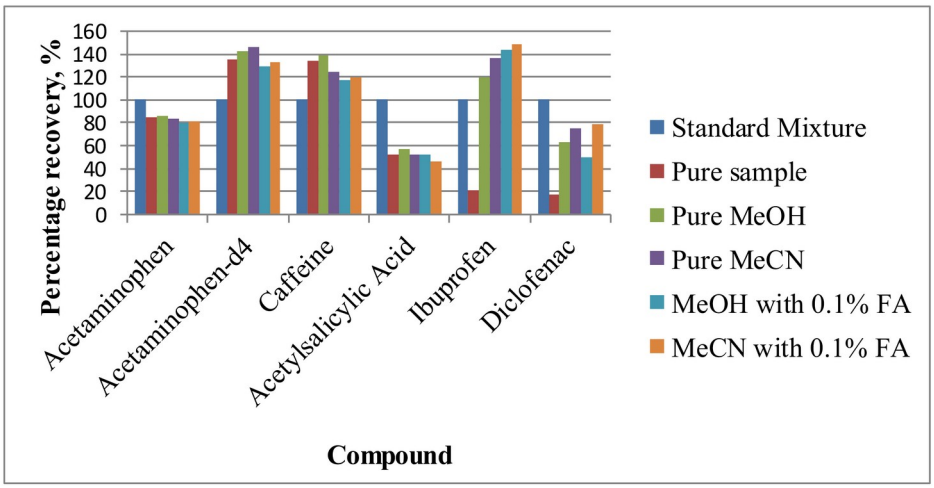

Figure 8: Percentage (%) efficiency of solvents in extraction of compound

3.2.3. Optimization of ratio of sample volume to extraction solvents

The sample volume ratio of 1:2 v/v had better recoveries as shown in figure 9. The fact that a ratio of 1:2 v/v sample:solvent extraction presented higher extraction capacity is explained by the increased contact area of organic solvent with sample (Buanasari, P. D. Palupi , Y. Serang, B. Pramudono, 2018).

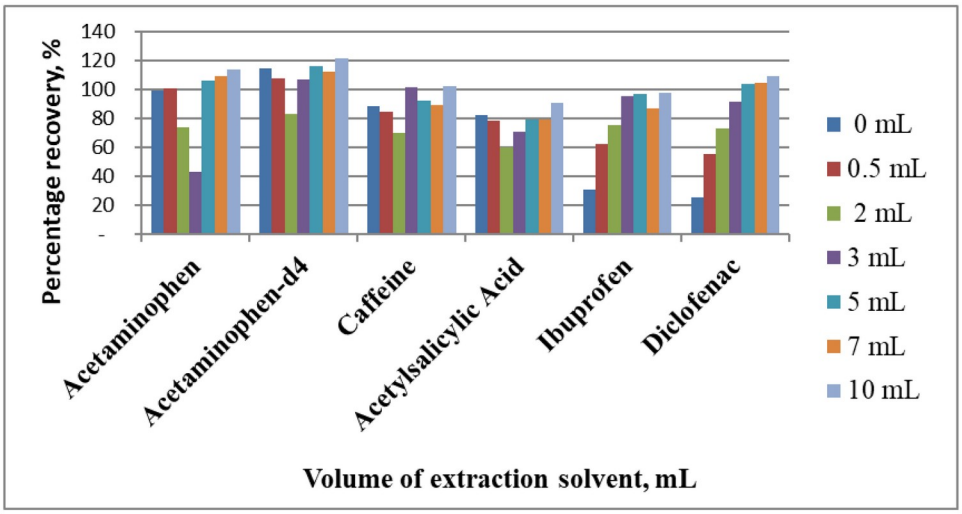

Figure 9: Percentage recovery (%) of analytes using different volume of extraction solvent

3.2.4. The recovery of the entire UAE procedures to HM samples

The absolute recoveries for the analytes ranged from 60 % to 107%. It was observed that almost all analytes had recoveries at acceptable range of ≥ 60% (Abarca, 2021) as shown in table 3. The relative recoveries were ranged from 56% to 105%. The high recoveries shown by NOA using UAE concur with the study done by (Kim et al., 2014) on determination of acetylsalicylic acid, ibuprofen, and acetaminophen in HM. Ultrasonic Assisted Extraction method have been found to be environmentally friendly in sample preparation and extraction of analytes from different matrices. For fast and sensitive detection of target analytes major efforts have been taken to develop robust extraction methods. The efficient sample/extractant contact area provided by sonication generally results in a good recovery of the analyte (Tadeo et al., 2019). Figure 10 shows the chromatogram of the separated compounds from the sample prepared by UAE method.

Table 3: Absolute and relative recoveries for the combined UAE procedures (%)

Compound	Absolute recovery	Relative recovery
Acetaminophen	102	95
Acetaminophen-d4	107	105
Caffeine	103	96
Acetylsalicylic Acid	60	56
Ibuprofen	78	73
Diclofenac	86	81

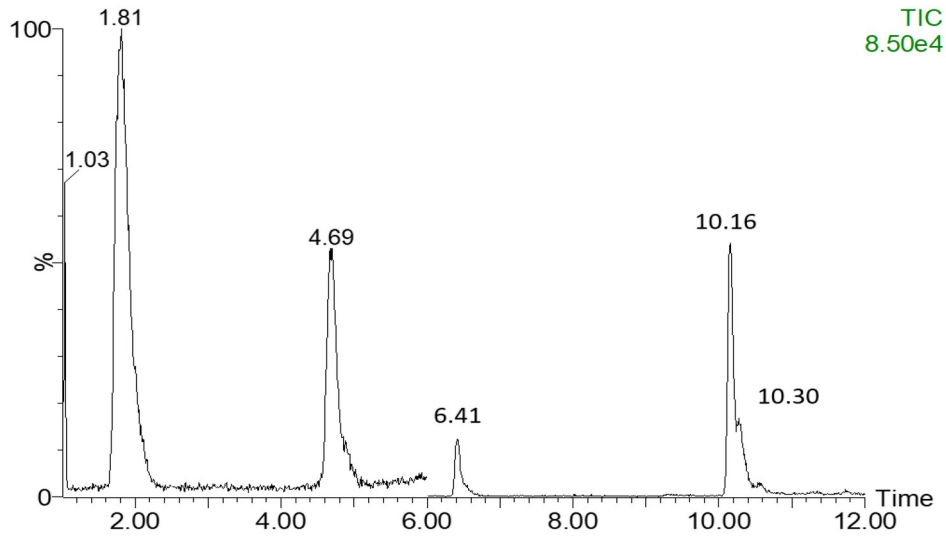

Figure 10: Representative chromatogram of analytes extracted by UAE method

3.3. Matrix effects

The reported acceptable range of matrix effects (ME) was ≥ 80% ≤ 120% (Zhou et al., 2017). The SPE was found to have poor matrix effect (ME) for almost all analytes except ibuprofen (101%). In UAE method, the matrix effects of four (4) analytes was found at acceptable range except that of acetylsalicylic acid < 80% and caffeine > 120%, see table 4. Comparing the two methods, UAE shown to be better than SPE.

Table 4: Matrix effects (ME) of SPE and UAE extraction methods

Compound	ME%, SPE	ME%, UAE
Acetaminophen	3	120
Acetaminophen-d4	0	106
Caffeine	24	122
Acetylsalicylic Acid	-	70
Ibuprofen	101	92
Diclofenac	124	102

Conclusion

This study showed that there is significant improvement on the recovery of all analytes with UAE when compared with SPE. The acetylsalicylic acid shown to have minimum recoveries than other analytes may be because its instability at room temperature. In this study the use of UAE method reduced time of extraction by 50% as compared to the SPE method. Other scientist has also reported that, UAE method have advantage of short time of extraction time, (Sricharoen et al., 2019). This could be attributed by the ability of the ultrasound waves penetrating the sample thus increases the rate of extraction which in turn enhance recoveries (Altemimi et al., 2016). Contrary to that SPE more processing steps increased the time of extraction thus contributes to the decomposition of analytes and hence lower recoveries.

Additionally, the UAE require inexpensive reagents as compared to SPE. The recorded high cost of SPE is due to costly consumables. These include SPE tubes, filters, HPLC vials, solvents and Eppendorf tubes. In addition, the presented chromatogram with poor peak shape, interfering peaks, wider peaks (decreased peak efficient), peak asymmetry and tailing disadvantage the SPE method as shown in figure 6. This in turn contribute to the method´s poor matrix effects as indicated in table 4. Therefore, the UAE method shown to be better than SPE method for extraction of 5 non-opioid analgesics from liquid HM and this was applied in preparation of liquid HM samples for LC-MS/MS analysis, (Mpanyakavili et al., 2022).

Recommendations

From this study it recommend that, the UAE is not the best method than SPE as seen in results since there are different types of SPE sorbent that could work better than Oasis HLB hydrophilic sorbent. However, this serve as a preliminary work which can be done at future. Also the use of selective method of extraction which target only one analyte could do better for higher recovery than extracting more than one analytes of different properties. Further studies should be done on optimization of sample preparation methods for extraction of non-opioid analgesics as adulterants in HM.

Conflict of Interest

The authors declare that there are no conflicts of interest which could potentially influence this work.

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Data Availability

The data used to support the findings of this research are available from the corresponding author upon request.

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CHAPTER THREE

LC-MS/MS Method for Determination of Non-opioid Analgesics Adulterants in Herbal Medicines[2]

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and practitioners [33]. The traditional Medicine Council of Tanzania regulates the HM safety and quality [26], however there is low capacity to monitor adulterants in HM. This needs analytical methods for screening and confirmatiom of adulterants in HM to be in place at the Tanzania Bureau Standards (TBS) and the Government of Chemistryr Laboratory Authority (GCLA) who are responsible to inform the traditional medicine Council. Different analytical techniques have been employed for screening of HM for quality check [13, 28, 43]. Analysis of adulterants have been achieved by chromatographic techniques such as such as Thin Layer Chromatography (TLC), High-performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) [5, 39].

These techniques are important at different levels to ensure the standards and quality of HM. A HPLC is considered to be simple, rapid, and inexpensive technique for preliminary screening of compounds but has low sensitivity and is applicable in a small number of samples or local health authorities [17, 29]. So far, some painkillers as adulterants have been determined in herbal products using an Ultra-performance liquid chromatography (UPLC) system. equipped with Xevo [16]. Liquid Chromatography with Tandem Mass Spectrometry (LC–MS/MS) is becoming the most popular analytical method of choice since it combines the separation capacity of LC with high sensitivity, resolution and selectivity of MS detector [36, 45].

Tanzania promotes high quality HM despite the existing limited information regarding the methods for detection of adulterants in HM and limited information on the levels and extent of adulteration. Due to these limitations, the country is striving towards attaining both high technology equipment and high skill human resources for setting benchmark methods for quality check of HM. The availability LC–MS/MS technology in Tanzania creates opportunity to set up specific analytical methods for detecting adulterants in HM. This study was conceived as one of the efforts in ensuring the safety of HM by developing a single high throughput LC–MS/MS method for the determination of acetaminophen, caffeine, acetylsalicylic acid, ibuprofen and diclofenac that are potentially used as adulterants in HM. Its availability will contribute in expanding the capability of doing routine detection of non-opioid analgesics in HM for quality check-up.

2 Materials and Methods

2.1 Solvents and Chemicals

Five (5) compounds of non-opioid analgesics including acetaminophen (99%), caffeine (98%), acetylsalicylic acid (99%), diclofenac ibuprofen (98%), sodium salt (98%), and internal standard (IS)—acetaminophen-d4 (99%) were bought from Sigma Aldrich (Fig. 1). For LC–MS/MS analysis, HPLC grade solvents including acetonitrile (MeCN, 99.9%), methanol (MeOH, 99.8%) and water (H_2O) from Finar® Company were used. Also analytical grade formic acid (FA, 98%) from Finar® Company was used.

2.2 Operating Conditions for Mass Spectrometry

A Triple Quadrupole Mass spectrometry (Waters, Micromass UK Limited) equipped with an electrospray ionization (ESI) interface operated with Masslynx 4.1 software was used. The MS detection was performed in both positive (+ ve) and negative (− ve) ion modes to capture the strongest ion. All compounds were first analysed individually in syringe infusion condition with a flow rate of 5µL/min. Optimization of parameters of the MS method was obtained by entering the molecular ion masses into the massLynx software. The default ranges for cone voltage and collision energy were used to enable MassLynx software to automatically determine all other parameters, such as capillary voltage, desolvation temperature, gas flows and Multiple Reaction Monitoring (MRM) transitions. Nitrogen and argon gases were used as nebulizing and collision gases respectively. The ion source parameters were capillary 3.2 kV; exit potential 3 V; radio frequency (RF) lens 0.2 V; source temperature 110 °C; and desolvation temperature 400 °C. The desolvation and cone gas flow were 650 and 130 L/hr. Product ions and corresponding collision energies were stored in the method files. The two most intense MRM transitions were selected for quantification and qualification except ibuprofen, of which one precursor ion was observed because of the very low intensity of the second product ion.

2.3 Instrumentation and Optimization of HPLC Operation Conditions

A chromatographic separation was carried out on Agilent 1100 liquid chromatography system consisting of Degasser, G1379A; Binary pump, G1312A; Autosampler, G1313A; Column oven, G1316A; Diode array detector (DAD), G1315B; German. Separation was performed on a Kinetex® 2.6 µm, C18, 100 Å LC (75 × 2.1 mm) waters analytical column placed in column oven at 40 °C. MassyLynx software was used for data collection and handling.

The chromatographic conditions like the composition of mobile phase, flow rate, and column temperature played a critical role in achieving good chromatographic separation and appropriate ionization. Different mobile phase composition of solvents mixture including HPLC grade methanol, acetonitrile and water using different percentages of formic acid as a buffer at different flow rates ranging from 0.25 to 0.4 mL/min in three different column temperatures (30, 35, and 40 °C) were tested for better resolution of all

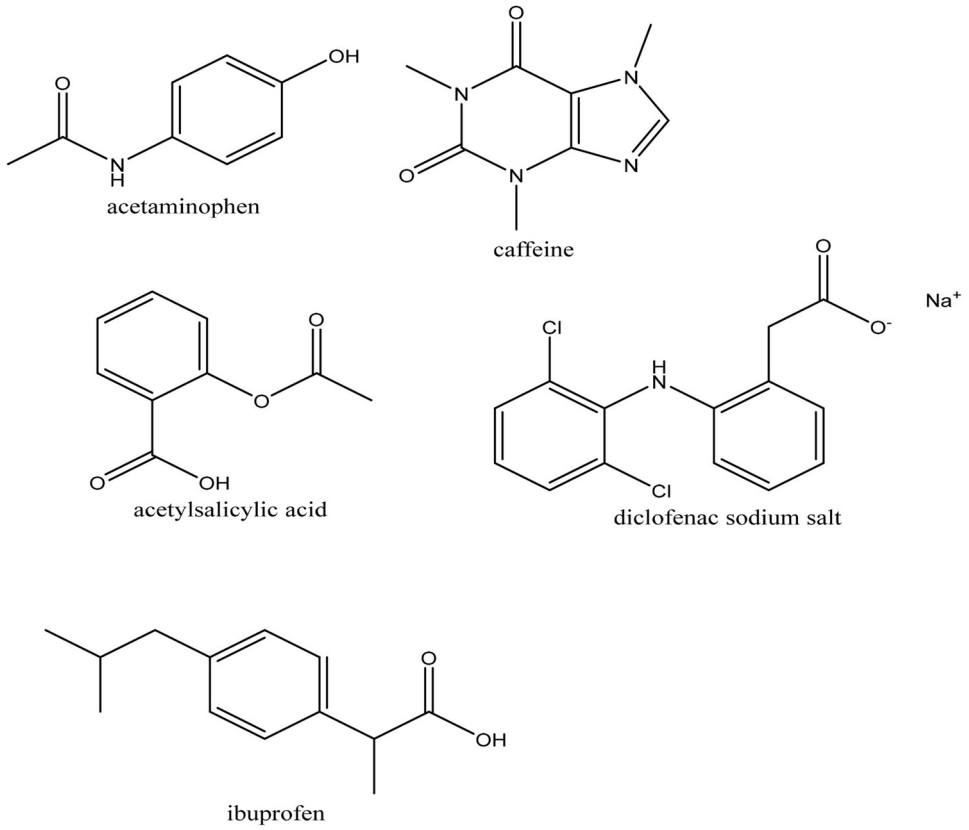

Fig. 1 Molecular structures of the studied non-opioid analgesics

compounds. The best conditions with good resolution were achieved for analysis, see Appendix 1. The mobile phase was composed of A (0.1% FA in 95% water) and B (0.1% FA in 5% acetonitrile) with linear gradient elution: 0–11.5 min, 5–5% B; 11.5–13.5 min, 95% B; 13.5–13.6 min, 5% B; the initial conditions were held for 6.4 min as a requilibration step. The injection volume was of sample 10 µL. Tthe effectiveness of the method was tested at higher, middle, and lower concentrations, i.e., 20.0, 2.0 and 0.2 µg/mL respectively.

2.4 Preparation of Standards

The stock solutions (1000 µg/mL) for acetaminophen, caffeine, acetylsalicylic acid, ibuprofen, and diclofenac were prepared in 10 mL MeOH with 0.1% FA. Besides, the stock concentration of acetaminophen-d4, internal standard was prepared by dissolving 5.033 mg in MeOH with 0.1% FA to make a solution of 2516.50 µg/mL. These solutions were stored at − 20 °C before analysis. During the LC–MS/MS experiments, the working standard solutions of 20 µg/mL for each compound was diluted in dilution solvent (95:5 (water: MeCN v/v) with 0.1% FA in both).

2.5 Optimization of Ultrasonic Assisted Extraction

The Ultrasonic Assisted Extraction (UAE) method was optimized for use in this study. Three parameters which are water bath temperature, type of organic solvent and sample to extraction solvent ratio were optimized sequentially. Each parameter was optimized at a time while the other parameters were kept constant. The effectiveness of the parameter

was determined by its recovery. In each parameter optimization procedure, a liquid sample was subjected to three processes of sonication, centrifugation and evaporation as per [7] with slight modification. Where by a minimum of 3 mL of a sample was spiked with 5 µL of 25.165 µg/mL IS. The sample was then vortexed for 1, 6 mL of extraction solvent was added and the mixture was sonicated for 30 min. After sonication the mixture was vortexed for 1 min followed by centrifugation for 30 min at 4000 rpm. Six (6 mL) of supernatant was transferred to a clean culture tube, evaporated in a water bath to 1 mL. The residues were diluted with mobile phase to 2 mL, then 10 µL was injected into the LC–MS/MS. Using the explained procedure, four types of organic solvents were explored including pure MeCN, MeCN with 0.1% FA, pure MeOH, and MeOH with 0.1% FA. The tested sample to extraction solvent ratio were 5:0, 5:0.5, 5:2, 5:3, 5:5, and 5:7 and 5:10 (v/v, mL) while the tested water bath temperatures were 80, 83, 85, 90 and 100 °C.

2.6 Sampling of Liquid Herbal Medicines

A total of 132 liquid herbal medicines (HM) indicated for having analgesic, antipyretic and anti-inflammatory activities were purposively collected from herbalists, shops, clinics, and open markets in Dar es Salaam, Mwanza, Morogoro and Njombe regions. The regions were selected representing specific hot spots for the Traditional medicines markets in Tanzania.

3 Method Validation

The method validation for detecting adulterants in HM was done referring to the United State (US) Food and Drug Administration (FDA) guidelines on biological method validation [14]. The validation parameters included specificity, selectivity, linearity, precision, accuracy, matrix effects and recovery.

3.1 Evaluation of Specificity and Selectivity of the Method

The specificity of the method was evaluated by comparing complete separated chromatograms of matrix blanks (HM without standard mixture (STDs)) with spiked blank (STDs in dilution solvent) and a spiked matrix (HM with STDs mixture). Selectivity was evaluated by comparing the retention time of compounds in dilution solvent with those in the sample matrix as per International Conference on Harmonization (ICH) guidelines, to ensure no matrix components co-elutes with analytes [12]. The selectivity of the method was confirmed by calculating peak resolution of all compounds using Eq. (1); where tRA is the retention time for peak 1 and tRB is the retention time for the peak 2, W1 and W2 are full width for peak 1 and 2 respectively.

$$Peak\ Resolution\ (Rs) = \frac{tRB - tRA}{0.5(W1 + W2)} \times 100\% \quad (1)$$

3.2 Determination of Recoveries from the Developed LC–MS/MS Method

The recoveries of five (5) analytes were calculated by comparing the mean area of prespiked samples (spiked before extraction) to that of post-spiked samples (spiked after extraction) in the same concentration. The recovery of IS was similarly estimated. Equations (2, 3) were used for recoveries experiments.

$$Absolute\ recovery\ (\%) = \frac{Area_{pre-spiked\ analyte} / Area_{post-spiked\ IS}}{Area_{post-spiked\ analyte} / Area_{post-spiked\ IS}} \times 100 \quad (2)$$

$$Relative\ recovery\ (\%) = \frac{Absolute\ recovery\ of\ target\ analyte}{Absolute\ recovery\ of\ IS} \times 100\% \quad (3)$$

3.3 Determination of Matrix Effect

The matrix effect was determined by comparing the retention time (Rt) and the MS response (peak areas) of analyte and IS into sample matrix spiked before extraction with the peak areas of the same analyte and IS spiked into the dilution solvent having the same concentration of 1 µg/mL. The competition between analyte ions and matrix components leads to an effective decrease (ion suppression < 100%) or increase (enhancement > 100%) in the ionization process. The matrix effects was expressed by signal suppression/enhancement (SSE) [20], Eq. (4).

$$SSE\ (\%) = \frac{Area\ (spiked\ sample)}{Area\ standard} \times 100\% \quad (4)$$

3.4 Determination of Precision and Accuracy

The intra-day precision of the method was determined by eight (8) replicates analysis at two concentrations (n = 8) of 0.1 and 1.0 µg/mL. It was expressed as relative standard deviation (%) of a series of eight (8) measurements [32]. Spike–and–recovery experiments were used in the determination of accuracy and recovery of the method. The accuracy of the method was determined by Eq. (5).

$$Accuracy\ (\%) = (Mean\ observed\ concentration - spiked\ concentration) \times 100\% \quad (5)$$

3.5 Preparation of Calibration Curve, LOD and LOQ of the Developed Method

The linearity of the method was investigated using dilution solvent spiked with different concentrations ranging from 0.001 to 10.0 µg/mL for acetylsalicylic acid, 0.003 to 10.0 µg/mL for caffeine and 0.01 to 10.0 µg/mL for acetaminophen, ibuprofen and diclofenac sodium salt. Linearity was determined by plotting the peak area ratio of the analytes to internal standard versus their relative concentrations' ratio of the analytes to IS. The calibration curves were best fitted using a least-square linear regression model $y = mx + b$, in which y is the peak area ratio of the analyte to IS, m is the slope of the calibration curve, b is the y-axis intercept of the calibration curve and x is the analyte concentration. The unknown sample concentrations were calculated from the weighted least-squares regression analysis of the standard curves. The LOD and LOQ were calculated using the formula recommended by The ICH Guidelines [8], using Eqs. (6, 7) respectively.

$$LOD = 3.3 * SD \tag{6}$$

$$LOQ = 10 * SD \tag{7}$$

3.6 Stability Test of Compounds

Stability of compounds was conducted to test their stability in working dilution solvent at different conditions. It was evaluated by preparing three (3) STDs in dilution solvent with an equal concentration of 20 µg/mL for each compound. The STDs was stored in different temperatures (at freezer, − 18 °C; fridge, 4 °C; and room temperature, 20 °C). The samples were analysed three (3) times within 4 weeks, after 3, 15 and 28 days. The stability was evaluated via peak areas using Eq. (8) [18]

$$ST\% = \frac{St}{S_0} \times 100\% \tag{8}$$

where, S_0 is the initial peak area, determined on the first day without introducing any extra pauses in the analysis process; St is the concentration obtained when analysis is carried out with making a pause with duration t in the analysis.

3.7 Analysis of HM

The presence of specific compounds was confirmed by comparing the retention times and fragments ion properties (quantifier and qualifier ions) of the sample from those of standards, Table 1.

4 Results

4.1 Optimized LC–MS/MS parameters

The results from ESI clearly illustrated that the compounds of interest were ionized in both positive and negative ion modes. ESI negative ion mode yielded the best sensitivity for ibuprofen, acetylsalicylic acid and diclofenac while acetaminophen and caffeine ionized in positive ion mode. The optimized MS/MS parameters were shown in Table 1. Reports were automatically generated specifying the optimized settings for the MRM method. The mass spectra of the compound of interest are shown in Appendix 2. The experimental information about the examined compounds with the fragment ions spectra along with cone voltages and collision energy values is shown in Table 1.

The LC analysis of the standard mixture shows that the positive ion mode compounds eluted first in the range of 0–6.0 min while the negative ion mode compounds eluted between 6 and 12 min. Figure 2 shows the overlaid chromatogram for separation of compounds. The method could analyse selectively studied compounds without interferences from any other compounds.

Table 1 Optimized MS parameters

Compound	Ionization mode	Parent ion (m/z)	Product ions	Cone voltage (V)	Collision energy (V)	Retention time (min)
Acetaminophen	+	151.87	64.40 109.70	20 20	30 19	1.84
Acetaminophen-d4	+	155.92	96.2 113.8	26 26	21 17	1.84
Caffeine	+	195.04	82.50 137.80	22 22	27 18	4.67
Acetylsalicylic acid	−	178.95	92.60 136.80	10 10	23 7	6.61
Ibuprofen	−	205.08	161.00	16	7	10.41
Diclofenac sodium salt	−	294.04	214.00 249.90	16 16	20 11	10.28

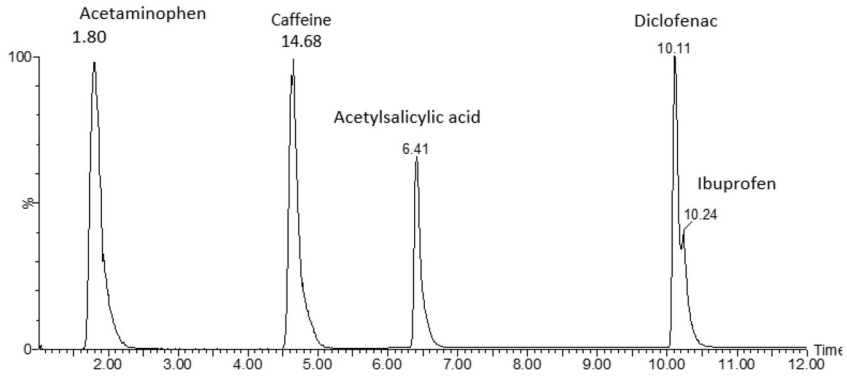

Fig. 2 Representative overlaid chromatogram showing the separation of acetaminophen, caffeine, acetylsalicylic acid, ibuprofen and diclofenac

Table 2 Absolute and relative recoveries (%) for water bath temperatures

Compound	Water bath temperature				
	80 °C	83 °C	85 °C	90 °C	100 °C
Acetaminophen	101.7	106.8	103.9	102.6	94.9
Acetaminophen-d4	99.9	98.8	98.2	92.1	101.3
Caffeine	105.8	97.3	91.4	100.3	91.9
Acetylsalicylic acid	11.1	22.8	55.1	30.7	21.6
Ibuprofen	110.5	96.9	90.9	99.3	65.9
Diclofenac	106.7	98.2	92.3	98.3	84.5

Table 3 Absolute and relative recoveries (%) for the overall extraction procedures

Compound	Entire sample preparation method	
	Absolute, %	Relative, %
Acetaminophen	102	95
Caffeine	103	96
Acetylsalicylic acid	60	56
Ibuprofen	78	73
Diclofenac sodium salt	86	81
Acetaminophen-d4	107	100

4.2 Optimized Sample Preparation Parameters

4.2.1 Optimized UAE Parameters for Extraction

The recoveries are at different temperatures as shown in Table 2. The high recovery of analytes were observed at the waterbath temperature of 85 °C and thus considered as the best water bath temperature. The Absolute recoveries of all compounds at this temperature were good except for ASA which had 55.1%. Recovery using MeCN with 0.1% FA was the highest (91.1–121.3%) and the best sample to extraction solvent ration was 2:1 (v/v) with recovery of 81.3–112.7% The overall absolute recoveries observed from sample preparation to analysis for the analytes ranged from 60 to 107%. These are acceptable recovery range as reported by other reseachres > 60% [1] as shown in Table 3.

4.3 Limit of Detection and Limit of Quantification

The Calibration curves were linear and the values of coefficient of determination (R^2) ranged from 0.9931 to 0.9982. The LOD and LOQ show that all analysed compounds are detectable and quantifiable at the level of microgram per millilitre as shown in Table 3.

4.4 Specificity and Selectivity

The method shows good chromatographic specificity and selectivity. No interfering peaks were detected at the RT of all analytes in the matrix, Fig. 3a, b. Also, good peak resolution (Rs) were observed to acetaminophen, caffeine and acetylsalicylic acid which was > 2 as required by ICH and FDA [18] guidelines. Poor resolution of < 2 was obserserved for diclofenac sodium salt and ibuprofen.

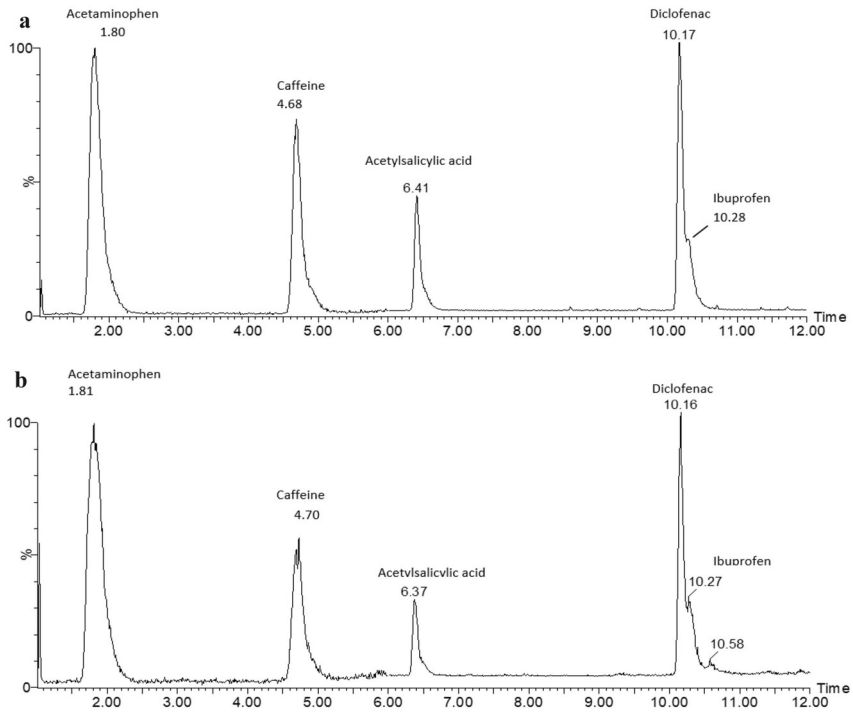

Fig. 3 **a** Overlaid chromatogram showing the separation of compounds in Dilution solvent, **b** representative overlaid chromatogram showing the separation of compounds in a sample matrix

4.5 Accuracy and Precision

The intra-day precision and accuracy of five (5) analytes ranged from 0.34 to 9.417% and 96.76 to 105.86% respectively. These accuracy and precision results were within acceptable criteria, showing that the developed method is reliable for quantification of five (5) analytes in HM, see Table 4.

4.6 Matrix Effects

The matrix effects ranged from 68.56 to 138.88%. Moderate signal enhancement was observed in acetylsalicylic acid with 118.96% while high signal enhancement was observed in acetaminophen, ibuprofen, and diclofenac with 138.88, 132.47, and 130.14% respectively. In contrast, higher signal suppression was observed in acetaminophen-d4 and caffeine

Table 4 The calibration equation, linearity (R^2), precision (%RSD), LOD and LOQ

Compound	Calibration equation	R^2	%RSD		Accuracy, %	LOD (µg/mL)	LOQ (µg/mL)
			1 µg/mL	0.1 µg/mL			
Acetaminophen	y = 1.0976x + 0.0141	0.998	7.30	3.896	100.27	0.1293	0.3919
Caffeine	y = 1.1494x + 0.0288	0.997	3.87	2.142	103.11	0.1513	0.4584
Acetylsalicylic acid	y = 0.294x + 0.026	0.997	0.54	6.239	105.86	0.1203	0.3645
Ibuprofen	y = 0.0793x + 0.0081	0.998	0.53	9.417	96.76	1.221	3.6998
Diclofenac sodium salt	y = 0.7216x + 0.2011	0.993	0.34	3.633	104.82	0.1938	0.5872

with 68.56 and 69.28% respectively. Thus, the majority of the compounds are affected by signal enhancement (≤ 39%).

4.7 Stability Test of Analytes in Dilution Solvent

Figure 4 shows that, the stability of analytes was decreasing as the time increase for four (4) weeks. The rate of stability decrease was higher in sample stored at room temperature compared to those stored in the fridge (4 °C) and freezer (− 18 °C) respectively.

4.8 Detected adulterants in HM samples

Caffeine was detected in 26 samples equal to 20% of all analysed samples, acetylsalicylic acid in 1 sample equal to 1%. Table 5 shows the adulterated HM and their concentrations, where the negative concentrations means adulterants were detected but not quantifiable. Appendix 3 shows chromatograms of some adulterated samples.

5 Discussion

This study report for the first-time combined methods for determination adulterants of acetaminophen, caffeine, acetylsalicylic acid, diclofenac and ibuprofen in liquid HM which allow for their quality check. The parameters optimised are well correlated with the ICH guideline [8]. The method run time was 20 min for analysis of five adulterants in a single run reducing the run time and solvents consumption. The good response, sharp peaks and short run time, and IS in chromatographic separation is important for efficient analysis [18]. The main fragments produced as a reference were comparable with other studies [4, 16]. The low recoveries of Acetylsalicylic acid have been reported by other scientists and are explained by its instability at room temperature [25]. The high sensitivity and selectivity demonstrated by this method qualify for screening of complex matrix [34].

The peak shape and resolution were the main criteria during the optimization of the LC method. The use of acetonitrile and water with 0.1% of FA, column temperature of 40 °C at 0.3 mL/min results to better separation and good peak shape compared to others, Appendix 1, Fig. 5a, b. In determining which compound is present in HM is accomplished by using precursor ions (both qualitative and quantitative ions) and retention time. The confidence level of identifying the targeted compounds in samples was configured as an error in retention time less than 0.5 min.

In the sample preparation procedure, acetonitrile with 0.1% FA in a ratio of 2:1 (extraction solvent to sample volume) was compatible with the mobile phase in the LC system used for analysis. A higher level of organic solvent was used for extraction because of high recoveries and low matrix effects. In the analysis of five compounds in HM, the standard mixture was used before, after, and in the middle of the batch analysis to check the consistency of the method. The shift of retention time ≤ 0.5 min was observed during a batch analysis that allow the use of replicates for the evaluation of results. Further, IS was added before sample extraction to compensate for matrix effects during measurements and random volume injections. The sensitivity of the instrument was stable throughout the analysis. Therefore, the specification of the developed LC–MS/MS method is suitable for qualitative and quantitative analysis of five compounds of interest in HM.

The reported quantifiable concentration range of up to 1029.22 µg/mL calls for the attention of the regulatory for quality and safety checks of these products. The presence of analytes as adulterants is reported in different parts of the world [5]. The Fact that HM analysed were adulterated with caffeine (20%) than other compounds can be attributed to the natural occurrence of caffeine on the other plants [38]. Caffeine can be found in leaves, seeds and fruits in many plants

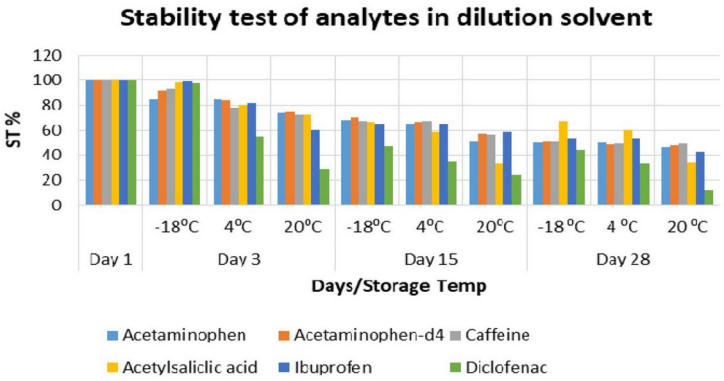

Fig. 4 Stability test of all compounds at – 18, 4 and 20 °C for 3, 15 and 28 days

Table 5 Summary of adulterated LHMs

S/No	Code	Adulterants	Conc. µg/mL	Demanded treatment	Source of sample
1	HM 01	Caffeine	− 0.1284	Malaria and typhoid	Shop
2	HM 02	Caffeine	30.9708	Coughing	Shop
3	HM 03	Caffeine	0.3679	Coughing	Shop
4	HM 04	Caffeine	0.2325	Coughing	Shop
5	HM 05	Caffeine	− 0.0339	Coughing	Shop
6	HM 06	Caffeine	0.5603	Skin diseases	Shop
7	HM 07	Caffeine	0.4866	UTI	Shop
8	HM 08	Caffeine	3.7453	Skin diseases	Shop
9	HM 09	Caffeine	3.3471	Malaria ^ typhoid	Street vendor
10	HM 10	Caffeine	0.2456	Women reproductive problems	Shop
11	HM 11	Caffeine	0.0519	Detoxification and anti-pain	Shop
12	HM 12	Caffeine	1.9256	Ant pain	Shop
13	HM 13	Caffeine	3.1309	Malaria ^ typhoid	Shop
14	HM 14	Caffeine	− 0.9649	Malaria ^ typhoid	Shop
15	HM 15	Caffeine	− 0.2640	Miscarriage	Shop
16	HM 16	Caffeine	1.3012	Malaria ^ typhoid	Shop
17	HM 17	Caffeine	2.5814	Malaria ^ typhoid	Shop
18	HM 18	Caffeine	− 0.0861	Anti-pains to organ	Street vendor
19	HM 19	Acetylsalicylic acid	− 0.8443	Coughing	Street vendor
20	HM 20	Caffeine	5.8062	Coughing	Street vendor
21	HM 21	Caffeine	1.6904	Skin diseases	Street vendor
22	HM 22	Caffeine	0.5993	Teeth problems	Street vendor
23	HM 23	Caffeine	13.9241	Anti-pains to organ	Street vendor
24	HM 24	Caffeine	3.7499	Coughing	Street vendor
25	HM 25	Caffeine	0.7056	Malaria ^ typhoid	Shop
26	HM 26	Caffeine	1029.22	UTI	Shop
27	HM 27	Caffeine	0.2897	UTI	Shop

such as tea leaves, cocoa beans, coffee beans, guarana and kola nuts. Also, caffeine can be added to foods and drinks to promote energy and mood. It is reported that 240 mL of coffee contain 100 mg, green tea 30–50 mg, energy drinks 80 mg of caffeine while 100 g of guarana leaves contain 4.5 g of caffeine [10]. It was reported that the normal intake of caffeine per day should not exceed 400 mg for adults, < 200 mg for pregnant/lactating women and < 2.5 mg/kg per body weight for adolescents and children [41]. Thus, the use of caffeinated products plus such kind of adulterated herbal medicine can exceed the recommended dose and put the user at high risks. The European Food Safety Authority (EFSA) reported that over-consumption of caffeine may lead to unwanted side effects including experiencing feelings of anxiety, hyperactivity, nervousness and sleep disturbance [6, 31]. Since consumers are unaware of adulterated HM, the risks of development of resistance, chronic diseases such as liver and other organ failures increase.

Caffeine can be added intentionally in skin care products because of its high biological activity and ability to penetrate the skin barriers characteristics [11] and its effectiveness in combination with other products [6]. The natural occurrence of caffeine in HM complicates its quantification, however, its presence at a higher level like the reported in this study may be caused by the intentional addition of synthetic caffeine [19].

6 Conclusion and Recommendation

The method developed met the ICH guidelines for detecton and quantification of acetaminophen, caffeine, acetylsalicylic acid, diclofenac and ibuprofen in HM. It is highly sensitive, accurate and time effective because it can analyse five compounds in one run. The information generated due to the method contribute to the country's first report on existence of adulteration of HM with non-opioid analgesics. It is recommended that, the method be used for routine quality check to expand monitoring of HM to ensure their quality and safety to consumers and consequently propagate the market and its use. Moreover, training of products producers and handlers is very important to reduce the extent of adulteration. Studies on the development of analytical methods for screening other types adulterants in HM are also recommended.

Appendix 1

See Fig. 5

Reprentative chromatograms for optimization oc column temperature and flor rate.

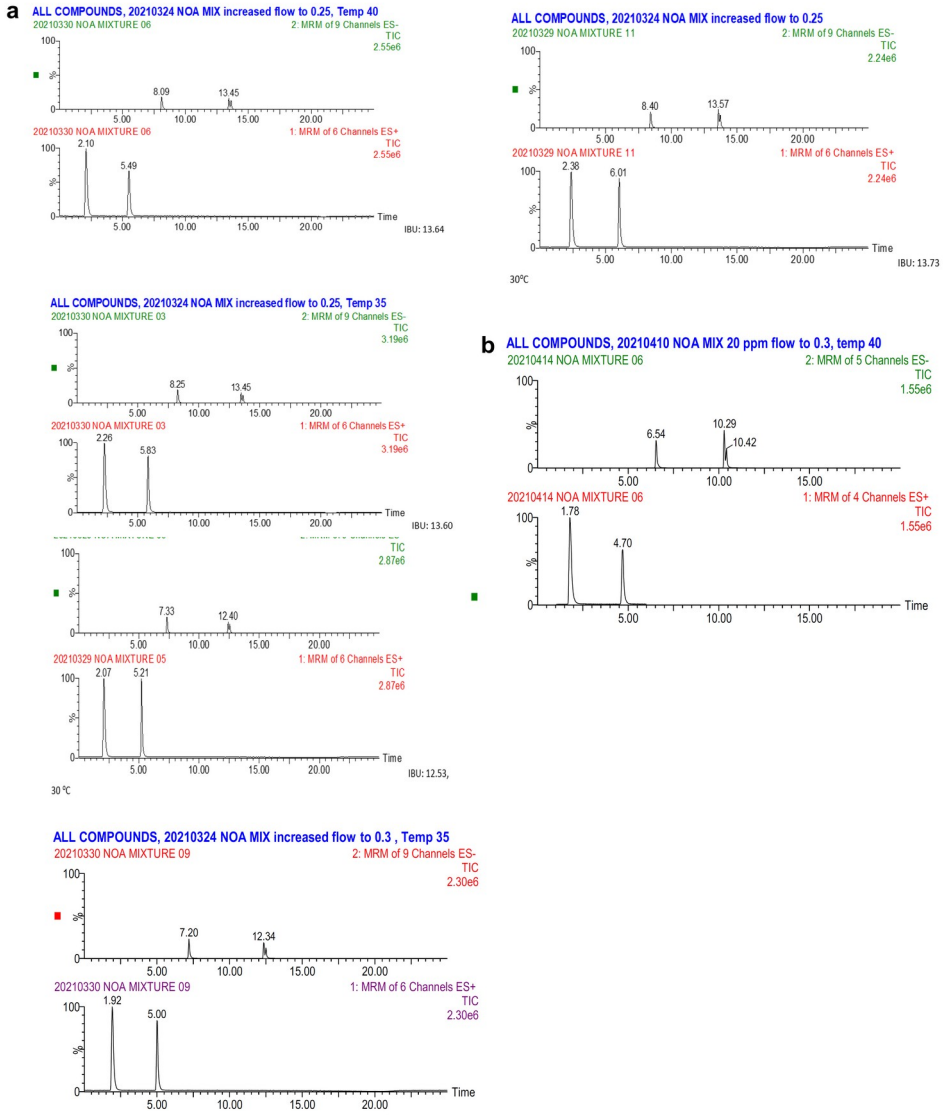

Fig. 5 **a** Chromatograms at 30, 35, 40 °C Column temperature at 2.5 mL/min flow rate, **b** Chromatograms for 30, 35, 40 °C Column temperature at 0.3 mL/min flow rate

Appendix 2

See Fig. 6

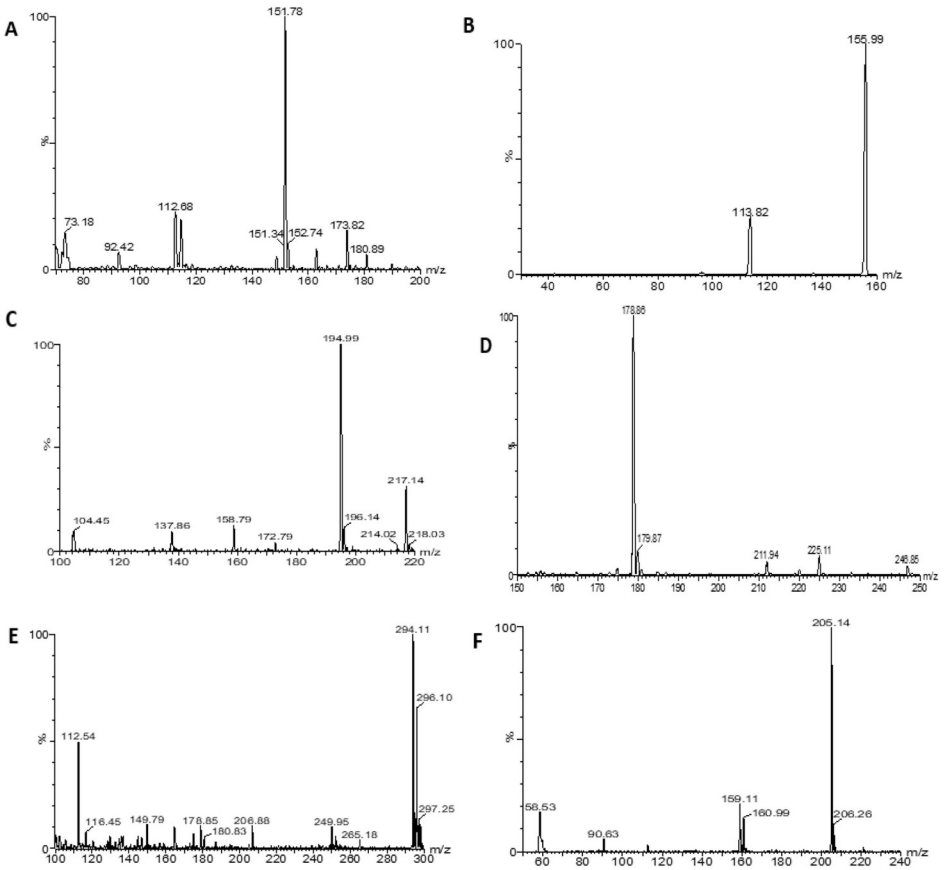

Fig. 6 Representative MS scan spectra for acetaminophen (**A**), acetaminophen-d4 (**B**), caffeine (**C**), acetylsalicylic acid (**D**), diclofenac (**E**) and, ibuprofen (**F**)

Appendix 3

See Fig. 7

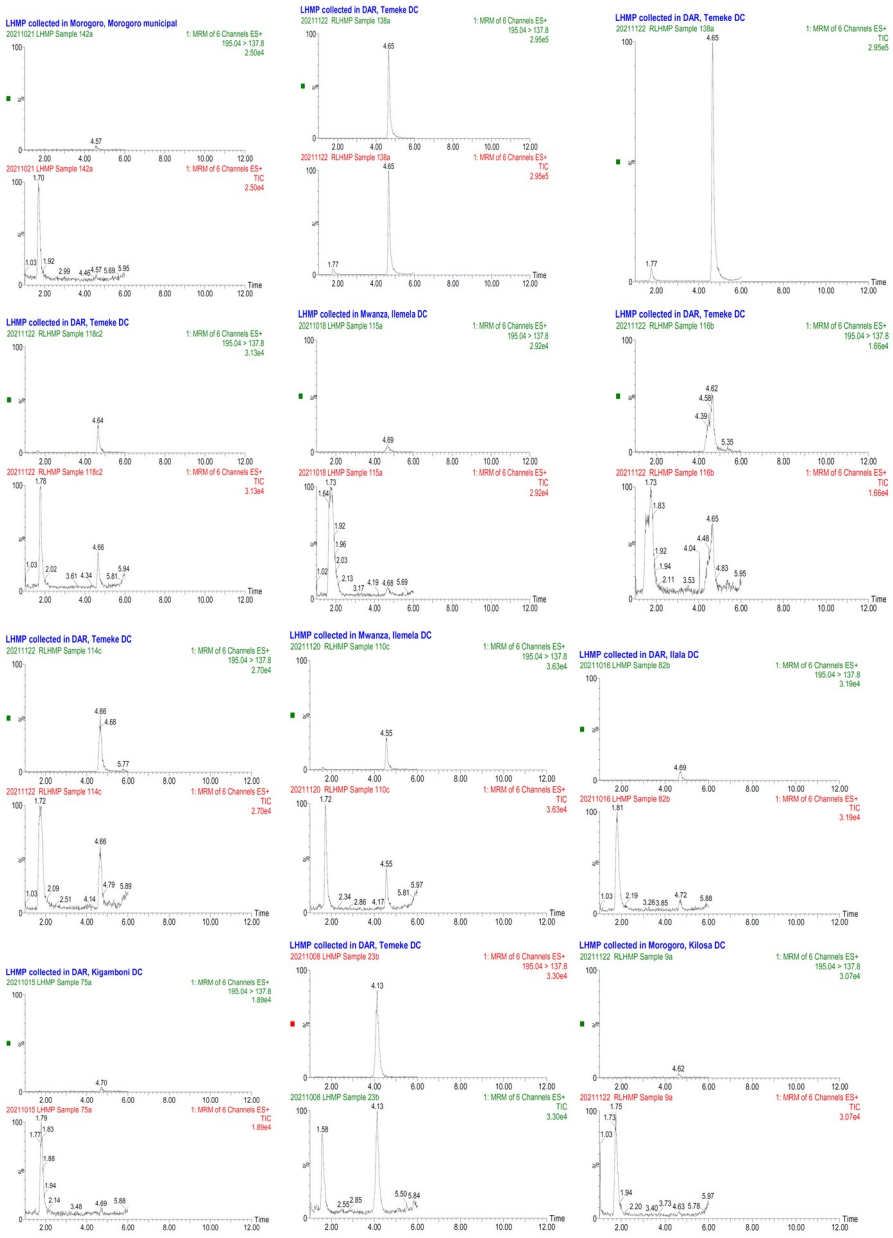

Fig. 7 Representative chromatograms showing the presence of Caffeine in HM

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Author contributions ALM Collected samples, collected data, performed the analysis and wrote the manuscript. CJM Technical assistance. FPM Conceived and designed the analysis. BS Conceived and designed the analysis.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest which could potentially influence this work.

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CHAPTER FOUR

GENERAL DISUSSION

General discussion

The amount of time spent on developing a sample preparation method and acceptable cost is a controversial topic between analytical chemists using LC-MS/MS. In order to maintain the good performance of LC-MS/MS and high robustness of the method intensive sample clean-up is important. Sample preparation methods can be simple and at low cost; more complex but low cost and complex but higher cost. The SPE and UAE methods were optimized for effective extraction of ACM, CFN, ASA, IBF and DFC in liquid HM.

The results showed that, the SPE method took long time; complex to perform (so laborious); more expensive as it uses the expensive consumables like SPE tubes. Its absolute recoveries were poor and is against the acceptable range except for diclofenac (83%) and ibuprofen (67%). The poor recovery of SPE method can be because of decomposition during the procedures, filtration process that reduce the recovery of analytes due to adsorption process (Pillai et al., 2016). The UAE method showed to be simple, less time consuming, low cost of analysis, and produced high recoveries. The absolute recoveries of UAE method was at acceptable range of ≥ 60% (Abarca, 2021) for almost all compounds except for acetylsalicylic acid (60%) and this may be due to its instability at room temperature (Paíga et al., 2015).

In LC-MS/MS, the parameters optimised are well correlated with the ICH guideline (European Medicines Agency, 2019). The main fragments produced during the optimization of MS parameter were comparable with other studies (Al Lawati et al., 2017; Kim et al., 2014). The LC method run time was 20 minutes with better separation and good peak shape for all compounds. During the analysis of HM, the standard mixture was used before, after, and in the middle of the batch analysis to check the

consistency of the method where. The shift of retention time between the analysis of liquid HM and the run of standard mixture before, in-between and after was ≤ 0.5 min. The sample was analysed in replicates for checking it's consistent. The internal standard (acetaminophen – d4) was used to compensate the matrix effects during measurements and random volume injections.

Out of 132 HM sample 20% was shown to contain caffeine and 1% have acetylsalicylic acid. The presence of caffeine in many HM sample can be ascribed due to the natural occurrence of caffeine on other plants (Verster & Koenig, 2018). It was reported that caffeine can be found in leaves, seeds and fruits in many plants such as tea leaves, cocoa beans, coffee beans, and guarana and kola nuts. Also, caffeine can be added to foods and drinks as preservative and for promotion of energy and mood. The acceptable intake of caffeine is reported not to exceed 400 mg for adults, < 200 mg for pregnant/lactating women and < 2.5 mg/kg per body weight for adolescents and children (WebMD, 2021). However, the use of caffeinated products such as 240 mL of coffee (100 mg), green tea (30 – 50 mg), energy drinks (80 mg)and 100 g of guarana leaves (4.5 g) plus adulterated HM with caffeine can exceed the recommended dose and put the user at high risks, (Healthline, 2021). The over-consumption of caffeine may lead to unwanted side effects like experiencing feelings of anxiety and sleep disturbance (Cappelletti et al., 2018; *Sources of Caffeine - Coffee and Health*, 2017). The unawares on the use of adulterated HM may results to development of resistance and chronic diseases.

This study opens an avenue to standardization of methods that are used for quality control of HM produced in Tanzania. The fact that the optimized extraction method and analytical methods developed met ICH standards allows the regulatory bodies in the country to set standards of quality of HM in the country. This will contribute in cubbing health risks associate with adulteration (Calahan et al., 2016; Rocha et al., 2016; Wang et al., 2018)**.**

CHAPTER FIVE

GENERAL CONCLUSIONS AND RECOMENDATIONS

Conclusions

It is concluded that in order to achieve better recoveries for the developed LC-MS/MS method, UAE method is best choice for HM. The developed method is highly sensitive, accurate and time effective because it can analyse the 5 compounds in one run. It can be used by regulatory authorities for routine analysis of 5 compounds of interest. These results contributes to the country database on extent of adulteration of liquid HM.

Recommendations

The study recommended other effective, rapid, and cost-effective analytical methods of adulterants detection studies to be done. More improvement of the method by using more than one Internal Standards to increase its sensitivity and instrumental set up is recommended. The fact that the samples collected were detected with caffeine and acetylsalicylic acid, we recommend that the method be adopted for the routine quality check to expand monitoring of HM to ensure their quality and safety to consumers. This will as contribute to marketability of the HM. Also education programs on good Production practices of HM to traditional healers are recommended.

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