KU Leuven

Biomedical Sciences Group

Faculty of pharmaceutical sciences

Department of pharmaceutical and pharmacological sciences

QUALITY CONTROL OF SELECTED TRADITIONAL ETHIOPIAN PHARMACEUTICAL FORMULATIONS

Gereziher Sibhat

Promotor:

Prof. Dr. Erwin Adams

Dessertation presented in partial fulfilment of the requirements for the degree of Doctor in pharmaceutical sciences

September 2022

KU Leuven

Biomedical Sciences Group

Faculty of pharmaceutical sciences

Department of pharmaceutical and pharmacological sciences

QUALITY CONTROL OF SELECTED TRADITIONAL ETHIOPIAN PHARMACEUTICAL FORMULATIONS

Gereziher Sibhat

Jury:

Promotor:	Prof. Dr. Erwin Adams
Co-promotors:	Prof. Ann Van Schepdael
	Prof. Dr. Getu Kahsay
Chair:	Prof. Dr. Isabelle Huys
Jury members:	Prof. Dr. Peter de Witte
	Prof. Dr. Mathy Froeyen
	Prof. Dr. Joelle Quetin-Leclercq
	Prof. Dr. Kris De Braekeleer

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in pharmaceutical sciences

September 2022

Curriculum vitae

Gereziher Sibhat was born on the 28th of December 1984 in Enticho, Tigray, Ethiopia. He completed his primary school in Enticho primary school in Enticho and secondary school in Atse Yohannes high school in Mekelle, Ethiopia. He has obtained his bachelor's degree in pharmacy from Mekelle University and MSc degree in pharmacognosy from Addis Ababa University, Ethiopia in 2009 and 2013, respectively. Upon completion of his master program, he then started working in Mekelle University, Ethiopia as an academic staff. From March 2015 to September 2018, he was head of the department of pharmacognosy. He has been involved in teaching both undergraduate and post graduate courses in Mekelle University and in guest lectures in Adigrat University. Besides, he has been conducting basic and applied research activities and giving community services through different ways including regional medias. As a result, he has been recognized as best performing teacher in 2015 and accelerated promoted into Assistant Professor in 2016. In 2018, he started his Ph.D. research at the Pharmaceutical Analysis laboratory of KU Leuven in Belgium under the supervision of Prof. Dr. Erwin Adams and co-promoters Prof. Dr. Ann Schepdael and Prof. Dr. Getu Kahsay. The focus of the research was on quality control of selected traditional Ethiopian pharmaceutical formulations. Besides, he was also a member of the KU Leuven student ambassadors.

Acknowledgements

My trajectory towards a PhD degree would have been impossible without the support and motivation from various people.

First of all, my special thank goes to my supervisor Prof. Dr. Erwin Adams for giving me the opportunity to pursue my PhD, for the continuous advice, effective supervision and support. You were always happy in sharing your hands, and helpful during the difficult times I had. You are a wonderful promoter! My heartfelt thanks also go to my co-supervisor Prof. Ann Van Schepdael for her continuous supervision and collegial approach during my PhD trajectory. Besides, I am grateful to co-supervisor Prof. Getu Kahsay, my undergrade teacher and later my colleague at Mekelle University, for the continuous support starting from the inception of my PhD study, mentoring during the hard times and for being an inspiring teacher and supervisor. I would like also to thank Prof. Dr. Dierdre Cabooter for sharing her hands when needed and for being a nice colleague. Further, I am very much grateful to my jury members (Prof. Dr. Isabelle Huys, Prof. Dr. Peter de Witte, Prof. Dr. Mathy Froeyen, Prof. Dr. Joelle Quetin-Leclercq, and Prof. Dr. Kris De Braekeleer) for the constructive comments during the evaluation moments, for reading my PhD thesis, and for the best wishes.

My special thanks also go to Mr. Kris Wolfs, for the technical help throughout my PhD work. Besides, I have always been fascinated by his general talks during the breaks, and touched by his kindness. I am also indebted to Dr. Adissu Alemayehu for the warm reception and familiarizing me with KU Leuven and the city of Leuven during my first trip to Belgium.

I am so grateful to the Interfaculty Council for Development Co-operation (IRO, KU Leuven) for granting me the scholarship to pursue my PhD study and Mekelle University for the study leave.

During my study, I was lucky to meet different lab collaborators in different times and I am very much thankful for being part of the wonderful crew and memorable path: Maxim, Asmin, Juan, Yaxin, Lynch, Jasper, Huiying, Ayele, Marwa, Shengyun, Derick, Luna, Alex, Donatela, Surbhi, Ihtsham, Wenping, Suraya, Marie, Anke, Tam, Gilles, Zhiqi, Allison, Emery, Karyna, Xiongwei and Shrooq. Thank you everyone! Bedankt iedereen!

My family and my stay in Leuven and KU Leuven were mesmerizing. Leuven (Belgium) is the city where my kids, my wife and I start going to school (again). Besides, during my stay, I have

got an opportunity to experience several events in Leuven and KU Leuven. I was part of the Tegaru football team in Leuven. Special thanks to all members of the team. Besides, I have participated in the KU Leuven trails and become member of KU Leuven student ambassadors. It helped me to network with students from different corners of the globe in different circumstances and improve my visibility. Besides, KU Leuven has always been with me and my family during the good and hard times. “at KU Leuven at home”. KU Leuven and the beautiful city of Leuven will always stay printed on my mind wherever I am.

Last but not least, my special thanks go to my wife Fikaden, a resilient and hardworking paediatrician, for being a wonderful wife, and to my kids (Yohana, Lilia and Caleb), my magic medicines, for being potent antioxidants at all times. Besides, I am very much grateful to my father and my mother (ሓለፋይ). Your dream became real and your investment in me resulted in success. Thank you ሓለፋይ! Congratulations ሓለፋይ! Further, I am thankful to my siblings (esp. Kibrom and Rahel) who have always been my backbone during my whole trajectory.

Publications

Publications in scientific journals

[1] G. Sibhat, G. Kahsay, A. Van Schepdael, E. Adams. Fast and easily applicable LC-UV method for analysis of bioactive anthrones from Aloe leaf latex. J. Pharm. Biomed. Anal. 195 (2021) 113834.

[2] G. Sibhat, G. Kahsay, A. Van Schepdael, E. Adams. Evaluation of aloins, pH and moisture in aloe leaf gel based personal care products. Int J Cosmet Sci. 44 (2022) 74-81.

[3] G. Sibhat, L.D. Montalvo, G. Kahsay, A. Van Schepdael, E. Adams. Quality of African moringa (*Moringa stenopetala*) leaf samples by liquid chromatography of phenolics, loss on drying and ash content. J. Food Process. Preserv. (*In print*).

[4] G. Sibhat, G. Kahsay, A. Van Schepdael, E. Adams. Fast and easily applicable LC-UV analysis of glucosinolates and phenolics in *Moringa stenopetala* leaf powder (*submitted to J. Herb. Med.)*.

Conferences (presentations and posters)

[1] G. Sibhat, G. Kahsay, A. Van Schepdael, E. Adams. Fast and easily applicable LC-UV method for analysis of bioactive anthrones from Aloe leaf latex. ULLA summer school, Uppsala, Sweden, 06-08 July 2021. *Poster presentation*. *1 min pitch* was also conducted.

[2] G. Sibhat, G. Kahsay, A. Van Schepdael, E. Adams. Fast and easily applicable LC-UV method for analysis of bioactive anthrones from Aloe leaf latex. RDPA, 6^{th}-8^{th} Sep. 2021, Modena, Italy. *Poster presentation.*

[3] G. Sibhat, G. Kahsay, A. Van Schepdael, E. Adams. Quality evaluation of aloe leaf gel based personal care products by liquid chromatography. RDPA, 6^{th}-8^{th} Sep. 2021, Modena, Italy. *Poster presentation.*

Contents

List of abbreviations and symbols

%B	percent of organic solvent/mobile phase B
%v/v	volume percent
µg	microgram
µg/mL	micro gram per milliliter
µL	microliter
µm	micrometer
ACN	acetonitrile
CI	confidence interval
CIR	Cosmetic Ingredient Review
EDQM	The European Directorate for the Quality of Medicines & Healthcare
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ESI	electrospray ionization
ESM	external standard method
FDA	Food and Drug Administration
g	gram
GACP	Good Agricultural and Collection Practice
GC	gas chromatography
h	hour
HM	herbal medicines
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IASC	International Aloe Science Council

ICH	International Council for Harmonisation
ID	internal diameter
IUPAC	International Union of Pure and Applied Chemistry
kV	kilovolt
L	liter
L/min	liter per minute
LC-UV	liquid chromatography with ultraviolet detection
LOD	limit of detection
LOQ	limit of quantification
M. stenopetala	*Moringa stenopetala*
m/*z*	mass-to-charge ratio
mg/mL	milligram per milliliter
min	minute
mL	milliliter
mL/h	milliliter per hour
mL/min	milliliter per minute
MS	mass spectrometry
MS^n	multistage mass spectrometry
nm	nanometer
Ph. Eur.	European Pharmacopoeia
ppm	parts per million
psi	pounds per square inch
PTFE	polytetrafluoroethylene
QAMS	quantitative analysis of multi-components by single marker

QC	quality control
r^2	coefficient of determination
RCF	relative correction factor
Rs	resolution
RSD	relative standard deviation
S/N	signal-to-noise ratio
SDMC	simultaneous determination of multi-components
T (°C)	temperature in degree Celcius
t (min)	time in minutes
TLC	thin layer chromatography
TM	traditional medicine
USP	United States Pharmacopeia
UV-VIS	ultraviolet visible
V	volume
WHO	World Health Organization

Chapter 1: General introduction

An overview of quality control of traditional medicine and herbal medicines used in Ethiopia

1.1. Traditional medicines

Traditional medicines (TM) have been used since antiquity and the interest among the community is ever-increasing, not only in developing, but also in developed countries. Traditional and complementary medicine is an important and often underestimated health care resource having diverse applications, especially in prevention and management of chronic diseases. The World Health Organization (WHO) defines traditional medicine as the sum of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, even if not all effects are explicable. They are used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses (1). Although, the terms complementary/alternative/non-conventional medicine are used interchangeably with TMs in some countries, WHO defines "complementary medicine" or "alternative medicine" as a broad set of health care practices that are not part of that country's own tradition or conventional medicine and are not fully integrated into the dominant health-care system (2). Hence, traditional medicine is a broad and diverse domain with different forms of indigenous healing methods. For instance, traditional African medicine (TAM), one of the oldest and most used TM, is a holistic approach comprising diverse TM disciplines, mainly divination, spiritualism, and herbalism. Herbalism or herbal medicine is the oldest and still the most widely used kind of medicine (3).

1.2. Quality control of herbal medicines

1.2.1. General aspects

Traditional medicine, particularly herbal medicines (HM), have been increasingly used worldwide in recent years. At the same time, the demand for safe and qualitative HM has become a major concern of health authorities, pharmaceutical industries and the public at large (4). According to WHO, HM include herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, other plant materials or combinations (1). Although HM are being used for the treatment of a number of diseases with promising efficacy, many of them remain untested and their use is either poorly monitored or not even monitored at all (5).

Among consumers, there is a widespread misconception that "natural" always means "safe", and a common belief that remedies from natural origin are harmless and carry no risk. However, some medicinal plants are inherently toxic. Further, as with all medicines, HM are expected to

have side effects, which may be of an adverse nature. Some adverse events reported in association with herbal products are attributable to problems of quality (6). The overall quality of a HM may be affected by many factors, including seasonal changes, harvesting time, cultivation sites, post-harvesting processing, adulterants or substitutes of raw materials, and procedures in extraction and preparation (7). Moreover, other major causes of such events are adulteration of herbal products with undeclared ingredients like potent pharmaceutical substances. Adverse events may also arise from the mistaken use of the wrong species of medicinal plants, incorrect dosing, errors in the use of HM both by health-care providers and consumers, interactions with other medicines, and use of products contaminated with potentially hazardous substances, such as toxic metals, pathogenic microorganisms and agrochemical residues such as pesticides, fumigants and residual solvents possibly sourced from polluted air, soil and water, during cultivation/growth and/or manufacturing process (1, 4). As a result, WHO promotes the safe and effective use of TM by regulating, researching and integrating these products, practitioners and practice into health systems and conducting an extensive phytovigilance to ensure the surveillance of safe use of HM and to minimize harm from their misuse (1).

According to the European Medicines Agency (EMA) guideline on quality of herbal medicinal products, for herbal substances and herbal preparations consisting of comminuted or powdered herbal substances, the grade of comminution has to be given. Furthermore, the following has to be indicated:

(i) in the case of standardisation: the quantity of the herbal substance/preparation shall be given as a range corresponding to a defined quantity of constituents with known therapeutic activity;

(iia) in the case of quantification: the quantity of the herbal substance/preparation shall be stated as a distinct content and the content of the quantified substance(s) shall be specified in a range and

(iib) for all other cases: the quantity of the herbal substance or the quantity of the genuine herbal preparation shall be stated as a distinct content (8).

1.2.2. Quality control methods for HM

Quality control (QC) of articles of botanical origin is an important issue (9-14) as plant materials have been among the top consumed products throughout developed and developing countries as home remedies, over-the-counter drug products and raw materials for the pharmaceutical industry. They represent a substantial proportion of the global drug market and

so the demand for quality is also rising. To ensure the quality of medicinal plant products by using modern QC techniques and applying suitable standards, WHO developed a manual that describes a series of tests such as determination of foreign matter, macroscopic and microscopic examination, thin-layer chromatography, determination of ash, determination of water etc. for assessing the quality of medicinal plant materials (12). Besides, WHO (1999) also adopted a monograph for medicinal plants with focus on identity tests, purity tests and chemical assay (9). Furthermore, EMA details universal tests and acceptance criteria for herbal substances (including leaves, herbs, roots, flowers, seeds, bark, etc), herbal preparations (such as simple, comminuted plant material as well as extracts, tinctures, oils and resins) and herbal medicinal products. This is completed by the definition (a qualitative statement of the medicinal plant), characters (a qualitative statement about the organoleptic character(s)), identification (such as macroscopical and microscopical characters), tests for contaminants (such as inorganic impurities and microbial limits) and assay of active markers (8).

For QC of HM, selecting a relevant analytical method, from the many available, is a crucial point that mainly depends on the set analytical goals (10). Moreover, according to EMA, universal tests such as foreign matter, total ash and water content should always be applied to herbal substances, while tests such as extractable matter, residual solvents, microbiological testing and swelling index may be skipped (8).

Moisture content, total ash, and acid-insoluble ash contents in HM are important indicators for any contamination by external sources that must be considered during QC of HM. High moisture content is a sign for a poorly dried and/or stored product and the possibility of stability issues and microbial contamination. Besides, total ash content indicates whether a given herbal medicine is free of contamination by foreign materials while acid insoluble ash content signals any contamination of heavy metals from soil and sand during the course of production (11, 12). Total ash and loss on drying methods are among the commonly used QC tests mentioned in herbal monographs of the European Pharmacopoeia (13). Percentage loss on drying and ash content of the herbal samples are calculated using equations (1.1) and (1.2), respectively:

$$\%\, loss = \frac{weight\ \ before\ drying\ - weight\ after\ drying}{weight\ before\ drying} \times 100 \qquad (1.1)$$

$$\%\, ash = \frac{weight\ of\ ash}{weight\ of\ sample} \times 100 \qquad (1.2)$$

1.2.3. Herbal marker compounds in QC of HM

Traditionally, qualitative (e.g., identification and chromatographic profile) and quantitative (e.g., content analyses) markers are applied for QC purposes. As reviewed by Klein-Junior et al., quantification of one or a few markers (sometimes randomly selected) remains the main instrument for quality assurance of HM although multivariate techniques have also been regularly applied in the study of natural products, especially for qualitative purposes (14).
EMA defines herbal markers as chemically defined constituents or groups of constituents of a herbal substance, a herbal preparation or a herbal medicinal product, which are of interest for control purposes regardless whether they have any therapeutic activity. Besides, according to the EMA herbal quality guidelines, when a herbal medicinal product contains herbal substances and/or herbal preparations with 'constituents with known therapeutic activity', then these constituents should be used for identification and quantification (15). From harvesting to manufacturing, chemical markers play a vital role in evaluating the quality of herbal medicines. Moreover, chemical markers are useful for authentication of genuine species and purity determination (7).

According to the WHO, the criteria for the selection of reference substances and QC of HM should consider that various ingredients may have different levels of influence on the final quality, safety and efficacy of the medicine. As a result, the order of selection of the substances for identification and quantification should be: first, therapeutically active constituents should be used as markers. If this does not work and if constituent(s) with recognized pharmacological activity (activities) is (are) known, they should be used as markers. If the above two scenarios are not applicable, the identity and quantity of herbal materials, preparations and medicines may be established by the production process and by analysing marker substance(s) containing other characteristic constituent(s) (16).

During quality evaluation, often liquid chromatography (LC) is used. After proper sample treatment, the content of bioactive compounds in the portion of sample taken should be determined using equation (1.3):

$$\text{Content} = \left(\frac{A_u}{A_s}\right) \times C_s \times \left(\frac{V}{W}\right) \qquad (1.3)$$

Where the content is expressed in mg of analyte per g of sample, A_u is the peak area for the respective bioactive marker compound from the sample solution, A_s is the peak area for the

respective bioactive marker from the standard solution, and C_s is the concentration of the respective bioactive marker in the standard solution (mg/mL) taking also into account the purity of the standard, while *V* represents the final volume (mL) of the sample solution and *W* the weight of sample taken to prepare the sample solution (g). Unknown peaks should be quantified by using one of the known compounds as standard.

1.2.4. Chromatographic fingerprinting of HM

A chemical fingerprint is a unique pattern that indicates the presence of multiple chemical markers within a sample (7). A chromatographic fingerprint of a HM is a chromatogram of an extract containing chemical components (whether pharmacologically active or not), which create a characteristic profile of the sample including raw materials, slices, semi-finished and finished products after appropriate processing (17).

Compared to the use of one or two markers or pharmacologically active components in herbs or herbal mixtures, chemical fingerprinting gives a more complete picture of a herbal product as multiple constituents are usually responsible for its therapeutic effects. Nevertheless, the approach using only a few markers is currently often employed for evaluating the quality and authenticity of herbal medicines, in the identification of the single herb or HM preparations, and in assessing the quantitative herbal composition of a herbal product (18).

1.2.5. Quantitative analysis of multi-components by a single marker for QC of HM

Quantitative analysis of multi-components by a single marker (QAMS) is a method also known as the simultaneous determination of multiple components or as the use of a single marker to determine multi-components. It is becoming a popular method in QC of HM such as in Chinese herbal medicine (CHM). The reason for the development of QAMS is the scarcity and high cost of high-purity reference substances. So, quantification of multiple components based on individual standards in routine QC is not convenient. QAMS is a low-cost method using a single standard which is inexpensive and easy to purchase. QAMS allows to calculate the contents of multiple components versus the single marker component on condition that the response factor of each component versus the marker has been determined. QAMS has been adopted by the United States Pharmacopeia (USP), European Pharmacopoeia (Ph. Eur.) and Chinese Pharmacopoeia (ChP) to build QC specifications of HM (19). Besides, QAMS has been accepted as a strategy for the quality assessment of HM and preparations by the US Food and Drug Administration, State Food and Drug Administration of China, and EMA (20). Although

the use of multiple reference standards for the simultaneous determination of multiple components in HM is a more scientific and reasonable approach than a single component method, its application is restricted due to the availability of affordable standards (21). By using a single marker, QAMS reduces the operating costs (19, 20). To properly validate the method, the content of the components measured by QAMS should be compared with results using multiple standards (21, 22).

1.2.6. Chromatographic methods for QC of HMs

In general, one could use chromatographic techniques to obtain a relatively complete picture of an HM to illustrate the so-called phytoequivalence. Several chromatographic techniques, such as high performance liquid chromatography (HPLC), gas chromatography (GC) and thin layer chromatography (TLC) are recommended for the purpose of QC of HM. They indicate appropriately the "chemical integrities" of the HM and can therefore be used for their authentication and identification (18).

HPLC (Figure 1.1) is one of the most widely used techniques for QC of HM. Among its advantages are: the ability to provide high resolution of a wide variety of analytes present in HM, the versatility due to the range of stationary phases available, as well as the possibility to couple to different detector types. This way, it may provide different levels of selectivity and sensitivity (14, 18).

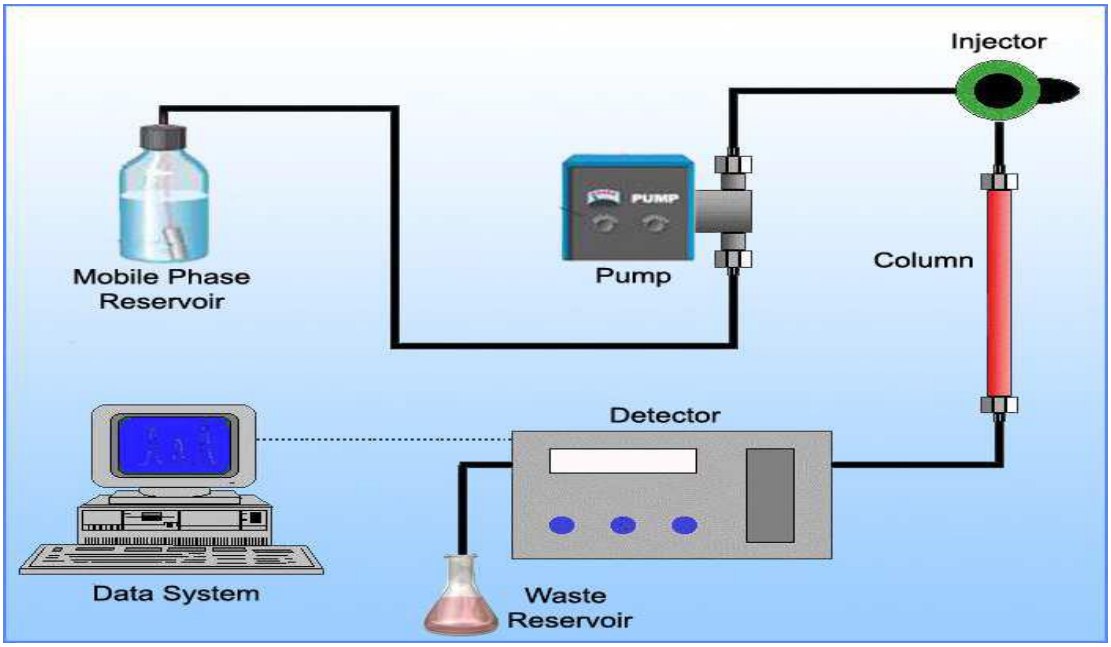


Figure 1.1: Schematic overview of HPLC system (23).

The HPLC system comprises a solvent reservoir, a high-pressure pump, a sample injector and a stationary phase or column – an important component, sometimes called “the heart of the HPLC system”. As per the review by Žuvela et al., based on the mode of separation, there are several types of chromatography such as normal-phase (NP), reversed phase (RP), size-exclusion (SEC), ion-exchange (IC) and hydrophilic interaction liquid chromatography (HILIC). RP columns are the most widely used stationary phases, covering for more than 90% of all separations conducted in several sectors, mainly pharmaceutical, environmental, food industry, clinical, biomedical and life sciences, including natural products analysis. This is attributed to their versatility to (simultaneously) separate nonpolar and polar compounds. Moreover, the mobile phase contains relatively safe and easily accessible solvents, like (buffered) water and a water-miscible mobile phase organic modifier, such as methanol or acetonitrile (24). Hence, column selection is a crucial step of RP-HPLC method development as the chromatographic performance considerably depends on the chosen column. Monolithic columns have proven to be a good alternative to particle packed columns. The high porosity of monolithic columns (Figure 1.2) leads to high permeability and low flow resistance, which will enable faster separation with shorter analysis times compared to particle packed columns. Physically, monolithic columns have better organic solvent resistances and are more robust. Owing to the high flow rate possible, monoliths have also great potential for the clean-up and preparation of complex mixtures. Online extraction possibilities are another advantage of monoliths. When only a limited amount of sample is available, miniaturized monolithic columns can be used (25-28).

Hence, due to the relatively short analysis time, monolithic columns are used in several areas, such as in the analysis of prohibited drugs and their metabolites, complex mixtures such as food, food additives, environmental pollutants, biological samples, enantiomeric analytes, proteomics, as well as in the purification and analysis of bioactive natural products of plant origin (25, 28-30).

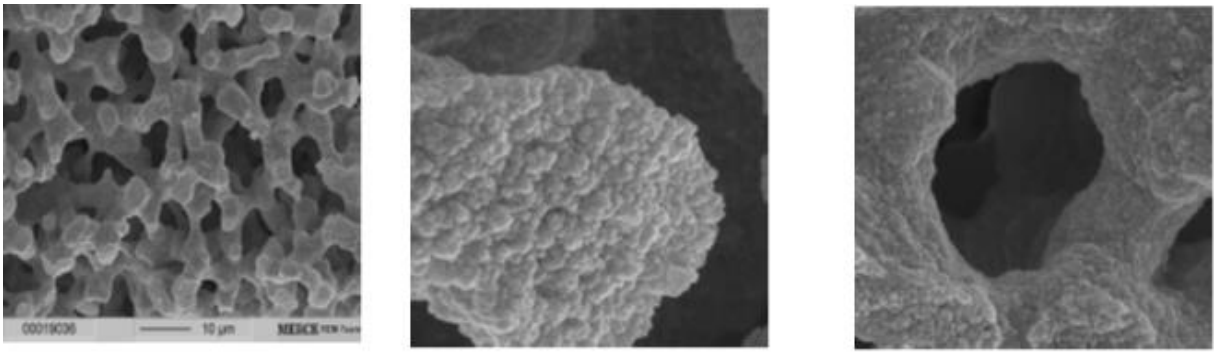


Figure 1.2: Pictures of the porous structure of monolithic silica columns (A); the mesoporous structure of the silica skeleton (B); and the macropores or throughpores (C) (29).

According to the size and function of their pores, monolithic columns are biporous. The macropores are responsible for the permeability of the monolith and allow LC separations at low pressures while the mesopores are filled with the "stagnant" mobile phase, in which the solute molecules migrate to access the active adsorption sites (28). According to the International Union of Pure and Applied Chemistry (IUPAC), mesopores range from 2 to 50 nm, with sub-2 nm pores being classified as micropores, and pores larger than 50 nm to be macropores (31). The inner pores of the particles in the packed columns correspond to the mesopores in the monolithic columns (28). The presence of mesopores increases the total pore surface area and sample capacity of monolithic beds. The structure of monolithic media can be represented as a network of small mesopores, which are responsible for the retention and separation selectivity, interconnected by large flow-through pores, and this should lead to higher permeability (28, 32).

1.3. QC of HM in Ethiopia

In Ethiopia and in Africa in general, TM and in particular HM are still widely used besides modern medicines. According to a WHO report, over 80% of the Ethiopian population still rely on TM for a whole gamma of diseases (33-35). However, QC on this kind of products is rather limited or even absent. Herbal products are often promoted to the public as being "natural" and completely "safe" alternatives to conventional medicines. However, many herbs are potentially toxic and the arguments are often commercially misused to increase the trade. Safety, efficacy, and quality of herbal medicines can be compromised by physicochemical and biological factors during any of the production stages. Some are even adulterated with modern drugs (36).

Though the policy on Ethiopian TM gives enough recognition and research in this domain is encouraged, it lacks strong regulation and HM are being sold with medical claims. Regulatory requirements for manufacturing and safety assessments are only in their draft phase. Even to date, the Ethiopian government does not effectively regulate TM. They are not included in an essential medicines list nor is there a post-market surveillance system. There is no restriction on the sale and no monograph or national pharmacopoeia is available for HM in Ethiopia (1, 37). Many medicinal plants are also used as food plants in Ethiopia. For instance, *Moringa stenopetala* is an important food plant and usually named as "cabbage tree" as its leaves are nutritionally rich and it appears leafy towards the end of the dry season when other green vegetables are scarce. As a result, it is often promoted as potential tree for food supply in case of shortage, cultivated as food crop and consumed in different types of food formulations (38). In general, HM are categorized as non-prescription medicines, self-medication or over-the-counter medicines, which are mostly sold in outlets other than pharmacies (1). Aloes and *Moringa stenopetala* are among those extensively used medicinal plants and will be discussed more in detail in this thesis (39-41).

1.4. *Moringa* and *Aloe* based Ethiopian HM

1.4.1. Aloe based Ethiopian herbal medicines

Aloe, from the Asphodelaceae family, is a perennial succulent or xerophyte plant known for a wide spectrum of treatment in folk medicine, as ingredient of food and food supplements and cosmetics. *Aloe vera* (*Aloe barbadensis* Miller) and *Aloe ferox* (*Aloe capensis*) are the most studied and commercialized *aloe* species with established monographs (9, 13, 42). *Aloe* leaves (Figure 1.3) consist of two liquid materials, a bitter yellow latex composed of mainly anthraquinone compounds and a clear mucilaginous gel consisting of polysaccharides as principal components (43, 44). Mechanical extraction of the inner aloe leaf pulp yields a 70% mucilaginous gel mainly (99–99.5%) composed of water (44).

Aloe is a commonly used plant in traditional Ethiopian medicine for an array of diseases. There are 46 *Aloe* species identified in Ethiopia, out of which 67% are endemics (45). The yellowish sap, aloe leaf latex, has been traditionally used to treat different diseases like malaria, inflammation, burn, wounds, and diabetes mellitus (39). *Aloe* gel is commonly used in cosmetics, as food (the fresh gel is eaten as source of water in extremely hot areas) and as

medicine to treat an array of diseases such as wound, skin inflammation, skin and hair infections, burn, pain, gall stone, gastric problem, infertility and dehydration (45).

According to Yeshak et al., different *in vivo* and *in vitro* biological tests showed that the Ethiopian aloes possess antimicrobial, antioxidant, antileishmanial and antimalarial activities that justify the traditional claims. Upon bioassay guided fractionation and characterization studies, glycosylated anthrones were found to be the principal components of the species (46).

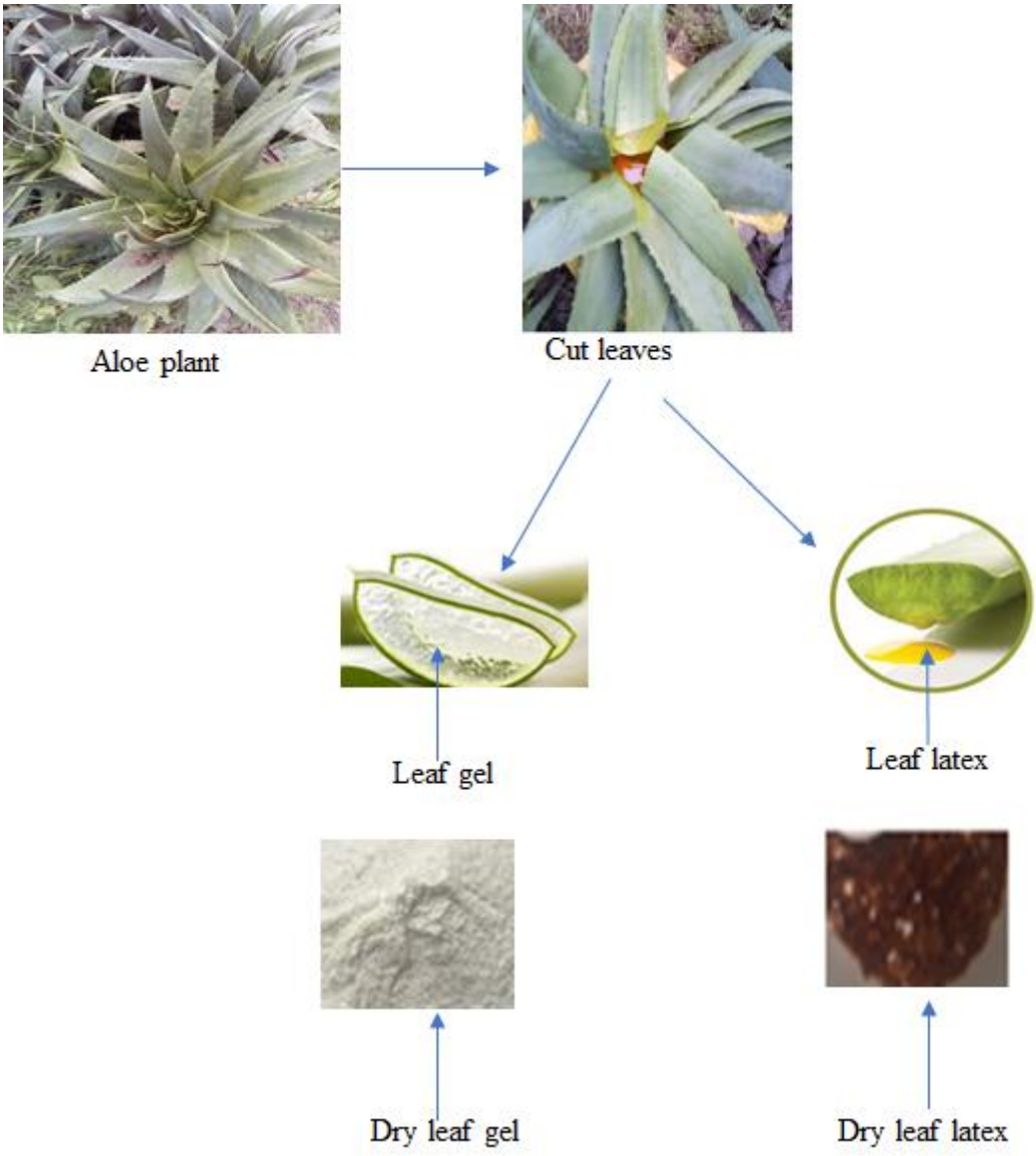


Figure 1.3: Illustration of aloe plant and its leaf composition

Aloe latex also called *Aloe* juice is a dry opaque mass ranging from reddish black to brownish black to dark brown in colour, and yellowish brown to dark reddish brown when powdered. Moreover, the dried latex possesses a characteristic and disagreeable odour and somewhat sour, nauseating and very bitter taste. The principal bioactive components of aloe leaf latex are hydroxyanthracene derivatives, where aloin (barbaloin), 1,8-dihydroxyanthracene-C glycoside, is the main bioactive which constitutes up to 40% of the aloe leaf latex by dry weight bases. The powder or dried juice and preparations thereof are traditionally used for the treatment of constipation, seborrheic dermatitis, peptic ulcers, tuberculosis, and fungal infections, and for reduction of blood sugar (glucose) levels (9, 44). Aloe leaves juice is sometimes also used to refer to the whole leaf extract (44).

However, an overdose or chronic exposure of *Aloe* juice has been reported to cause different toxicities and side effects including griping and severe diarrhea with consequent losses of fluid and electrolytes (mainly potassium) (9), acute eczema, contact urticaria, and dermatitis in individuals who applied aloe-derived ingredients topically (47). Besides, the International Agency for Research on Cancer (IARC) put the whole leaf extract of *Aloe vera* under Group 2B as possibly carcinogenic to humans (44). Hence, for the treatment of short-term occasional constipation, the standard oral dose for adults and children over 10 years old is limited to 40–110 mg (Curacao or Barbados Aloe) or 60–170 mg (Cape Aloe) of the dried juice, corresponding to 10–30 mg hydroxy anthracenes per day, or 0.1 g as a single dose in the evening (9) or as per EMA, the maximum daily dose recommended is 1 to 3 coated tablets containing 27.5-35.0 mg dry extract (corresponding to 10-30 mg hydroxy-anthracene derivatives calculated as aloin) for adults and children > 12 years of age (48). Besides, in Aloe-derived ingredients used in cosmetics, regardless of species, anthraquinone levels should not exceed 50 ppm (49).

Freshly prepared *Aloe vera* gel has been traditionally used as a natural remedy for burns, the treatment of acne, haemorrhoids, psoriasis, anaemia, glaucoma, peptic ulcer, tuberculosis, blindness, seborrhoeic dermatitis, and fungal infections. *Aloe vera* gel is reported to possess different pharmacological activities such as wound healing, anti-inflammatory, and anti-burn. Medicinal *Aloe* gel is defined as fresh gel or preparations containing 10–70% fresh gel (9). Moreover, *Aloe vera* gel is used as ingredient in health foods and beverages, and hydrating ingredient in cosmetics. For medicinal use of *Aloe vera* gel, 25 to 100 mL per day of a 4.5:1 gel concentrate is suggested as typical oral dose range in adults (44) or a total daily consumption

of *Aloe vera* of 2–8 fluid ounces (59–237 mL) of single strength leaf gel (50). For topical use, pure *Aloe vera gel* is often used liberally on the skin. Besides, *Aloe vera* may be safely used as a flavouring substance in food according to FDA regulations and classified as a List 3 substance (inerts of unknown toxicity) and as an inert ingredient of pesticide products as per The Environmental Protection Agency (EPA) (44). However, *Aloe gel* could be contaminated by the latex containing aloin during collection and production stages leading to different side effects. As a result, different organizations such as the International Aloe Science Council (IASC) set a maximum limit for leaf latex expressed as aloins. According to IASC, aloin A and B are limited to 10 ppm in aloe leaf preparations for oral consumption (50).

Despite the wide medicinal values of aloes, different studies revealed an array of side effects and toxicities upon excessive use or chronic exposure to aloe preparations (47). Moreover, the synthesis and accumulation of phytochemicals in plants depend on several factors such as genetics, environmental conditions, harvest and processing operations, and storage factors (51) which result in variation in biological activities.

1.4.2. Moringa based Ethiopian HM

Moringa stenopetala (*M. stenopetala*) from the family of Moringaceae, is one of the 13 species that belong to the genus *moringa*. A genus that has been widely cultivated throughout Asia and Africa for its multiple uses (52). *M. stenopetala,* commonly called 'African moringa', is originating from the southern regions of Ethiopia and the northern part of Kenya (53).

Moringa leaf preparations are widely used nowadays in tropical Africa as source of diet and medicine. In the Ethiopian and Kenyan traditional medicine, *M. stenopetala* leaves, both fresh and as dry powder, are extensively employed for the treatment of a range of illnesses such as diabetes, hypertension, stomach pain, malaria, leishmaniasis, leprosy, epilepsy, diarrhoea, asthma, colds (53) and flu (54) upon oral consumption of a boiled soup prepared from fresh leaves. Moreover, *M. stenopetala* leaves were investigated and found to have various pharmacological activities such as antibacterial, antidiabetic, antioxidant, antimalarial, antihypertensive and anti-inflammatory (55).

Phytochemical screening tests revealed that *M. stenopetala* leaves are reported to contain various bioactive phytoconstituents such as alkaloids, saponins, terpenoids, anthraquinones, flavonoids, polyphenols and phytosterols (56, 57), glucosinolates (58-60) and cyanogenic glucosides (61). Different bioactive compounds such as rutin, quercetin, chlorogenic acid (3-

O-caffeoylquinic acid), neochlorogenic acid (5-O-caffeoylquinic acid), caffeic acid (58, 62, 63), and glucomoringin (GMG) (58), are reported from the leaves of *M. stenopetala*.

Although moringa leaf products are often promoted for their nutritional and medicinal values, different surveys (61, 64, 65) indicated an association between moringa leaf consumption and a negative effect on the thyroid function. Moreover, *in vivo* (mice and rat models) and *in vitro* studies (66-68) reported dose and time dependent liver and cellular toxicity of moringa leaf extract. Furthermore, as mentioned above, a cyanogenic glucoside was also encountered in *M. stenopetala* leaves although the amount was less than that expected to cause goitre. However, it may have a health risk in areas with high incidence of endemic goitre as an exacerbating factor if consumed for a long period of time (61).

1.5. Aim of the study

Several people with chronic illness commonly use HM as an alternative to modern drugs. Due to increased consumer usage and media promotion, expectations by the public have been enlarged, leading to a more stringent demand for quality. It is not easy to have an overview of the total size of problems since those are not always reported, but it is known among users that safety and quality issues arise rather frequently. For this reason, QC needs to be carried out according to established principles. Moreover, it is also important that the government imposes certain quality demands, based on scientific results.

Hence, this project was aimed to evaluate the quality of *aloe* and *moringa* leaves based herbal medicinal preparations sourced from different species and areas following general QC tests for HM such as loss on drying, ash content, and pH. Moreover, fast and easily applicable HPLC-UV methods were developed and validated to determine bioactive marker compounds therein. The use of monolithic columns has been explored. It is also important that these methods can be easily executed in Ethiopian QC laboratories. This way, the study will contribute to the general health and well-being of the people in Ethiopia and at large in Africa. Moreover, since well trained personnel is scarce and resources are limited, tests should be rather simple and relatively cheap. So, techniques like for example ultra HPLC combined with mass spectrometry for routine analysis should be avoided.

1.6. References

1. World Health Organization (WHO). WHO global report on traditional and complementary medicine. Geneva: World Health Organization; 2019.
2. World Health Organization (WHO). Traditional and complementary medicine in primary health care. Geneva: World Health Organization; 2018.
3. Ozioma, E-OJ, Chinwe, OAN. Herbal Medicines in African Traditional Medicine. In: Builders PF., editor. Herbal Medicine. London: Intechopen; 2019. https://www.intechopen.com/chapters/64851.DOI: 10.5772/intechopen.80348.
4. World Health Organization (WHO). WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues. Geneva: World Health Organization; 2007.
5. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front. Pharmacol. 2014, 4: 1-10. doi:10.3389/fphar.2013.00177.
6. World Health Organization (WHO). WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems. Geneva: World Health Organization; 2004.
7. Li S, Han Q, Qiao C, Song J, Cheng CL, Xu H. Chemical markers for the quality control of herbal medicines: an overview. Chinese Med. 2008, 3(7): 1-16. doi:10.1186/1749-8546-3-7.
8. European Medicines Agency (EMA). Guideline on specifications: test procedures and acceptance criteria for herbal substances, herbal preparations and herbal medicinal products /traditional herbal medicinal products. London: EMA; 2011.
9. World Health Organization (WHO). WHO monographs on selected medicinal plants, volume1. Geneva: World Health Organization; 1999.
10. Muyumba NW, Mutombo SC, Sheridan H, Nachtergael A, Duez P. Quality control of herbal drugs and preparations: The methods of analysis, their relevance and applications. Talanta Open 2021, 4:100070. Doi:10.1016/j.talo.2021.100070.
11. Kim DG, Kim BS, Kim, YC, Hwang YO, Chae YZ, Park SK. Selection of Herbal Medicines Requiring Quality Control for Loss on Drying, Total Ash, and Acid-insoluble Ash in Korea. Nat. Prod. Sci. 2011, 17(1): 38-44.

12. World Health Organization (WHO). Quality control methods for herbal materials. Geneva: World Health Organization; 2011.
13. European Pharmacopoeia. 10th ed. Strasbourg: EDQM Council of Europe; 2020.
14. Klein-Junior LC, de Souza MR, Viaene J, Bresolin TMB, de Gasper AL, Henriques AT, Vander Heyden Y. Quality Control of Herbal Medicines: From Traditional Techniques to State-of-the-art Approaches. Planta Med. 2021, 87: 964–988. DOI: 10.1055/a-1529-8339.
15. European Medicines Agency (EMA). Reflection paper on markers used for quantitative and qualitative analysis of herbal medicinal products and traditional herbal medicinal products. London: EMA; 2008.
16. World Health Organization (WHO). Technical Report Series, No. 1003. Geneva: World Health Organization; 2017.
17. Yongyu Z, Shujun S, Jianye D, Wenyu W, Huijuan C, Jianbing W, Xiaojun G. Quality Control of Herbal Medicines and Related Areas- Chemical Fingerprint Analysis. In: Shoyama Y., editor. Quality Control of Herbal Medicines and Related Areas. London: Intechopen; 2011. https://www.intechopen.com/chapters/23472. (Accessed on 06 January 2022).
18. Liang Y-Z, Xie P, Chan, K. Quality control of herbal medicines. J. Chromatogr. B 2004, 812: 53–70. doi: 10.1016/j.jchromb.2004.08.041.
19. Zhu C, Li X, Zhang B, Lin Z. Quantitative analysis of multi-components by single marker—a rational method for the internal quality of Chinese herbal medicine. Integr Med Res. 2017, 6 (1): 1-11. https://doi.org/10.1016/j.imr.2017.01.008.
20. Li T, Zhou H, Ma J, Dong L, Xu F, Fu X. Quality Assessment of Licorice Based on Quantitative Analysis of Multicomponents by Single Marker Combined with HPLC Fingerprint. Evid. Based Complementary Altern. Med. 2021, 2021: 1-12. https://doi.org/10.1155/2021/8834826.
21. Xu R, Mao F, Zhao Y, Wang W, Fan L, Gao X, Zhao J, Tian H. UPLC Quantitative Analysis of Multi-Components by Single Marker and Quality Evaluation of *Polygala tenuifolia* Wild. Extracts. Molecules 2017, 22: 2276. doi:10.3390/molecules22122276.
22. Li Y, Zhang Y, Zhang Z, Hu Y, Cui X, Xiong Y. Quality Evaluation of Gastrodia Elata Tubers Based on HPLC Fingerprint Analyses and Quantitative Analysis of Multi-

Components by Single Marker. Molecules 2019, 24: 1521. doi:10.3390/molecules24081521.

23. Salvato F, de Carvalho MCCG, Leite AL. Strategies for Protein Separation. In: Leung H-CE, Man T-K, Flores RJ., editors. Integrative Proteomics. Intechopen; 2012. https://www.researchgate.net/publication/279957463. (Accessed on 03 April 2022).
24. Žuvela P, Skoczylas M, Liu JJ, Baczek T, Kaliszan R, Wong MW, Buszewski B. Column Characterization and Selection Systems in Reversed-Phase High-Performance Liquid Chromatography. Chem. Rev. 2019, 119: 3674−3729. https://doi.org/10.1021/acs.chemrev.8b00246.
25. Khoo HT, Leow CH. Advancements in the preparation and application of monolithic silica columns for efficient separation in liquid chromatography. Talanta 2021, 224: 121777. https://doi.org/10.1016/j.talanta.2020.121777.
26. Sharma G, Tara A, Sharma VD. Advances in monolithic silica columns for high-performance liquid chromatography, J. Anal. Sci. Technol. 2017, 8(16): 1-11. DOI 10.1186/s40543-017-0125-x.
27. Yu B, Zhang H, Cong H, Chen G, Xu T, Liu Y. Recent development and application of monolithic columns, Rev. Adv. Mater. Sci. 2017, 48: 58–67.
28. Díaz-Bao M, Barreiro R, Miranda JM, Cepeda A, Regal P. Recent Advances and Uses of Monolithic Columns for the Analysis of Residues and Contaminants in Food. Chromatography 2015, 2:79-95. doi:10.3390/chromatography2010079.
29. Cabrera K. Applications of silica-based monolithic HPLC columns. J. Sep. Sci. 2004, 27: 843–852.
30. Zhu T, Row KH. Monolithic materials and their applications in hplc for purification and analysis of bioactive compounds from natural plants: a review. Instrum. Sci. Technol 2012, 40:78–89. DOI: 10.1080/10739149.2011.651667.
31. Wouters S, Hauffman T, Mittelmeijer- Hazeleger MC, Rothenberg G, Desmet G, Baron GV, Eeltink S. Comprehensive study of the macropore and mesopore size distributions in polymer monoliths using complementary physical characterization techniques and liquid chromatography. J. Sep. Sci. 2016, 39: 4492–4501.
32. Ali I, Gaitonde VD, Aboul-Enein HY. Monolithic Silica Stationary Phases in Liquid Chromatography. J. Chromatogr. Sci. 2009, 47: 432-442.

33. Gemeda N, Tadele A, Girma B, Teka F. Ethiopian traditional medicine: Past, current, and future: Proceeding; 2015. https://www.ephi.gov.et. (Accessed on 17 January 2022).
34. d'Avigdor E, Wohlmuth H, Asfaw Z, Awas T. The current status of knowledge of herbal medicinal plants in Fiche, Ethiopia. J. Ethnobiol. Ethnomedicine 2014, 10(38): 1-32.
35. Wassie SM, Aragie L., Taye BW, Mekonnen LB. Knowledge, Attitude and utilization of traditional medicine among the communities of Meraw town, Northwest Ethiopia: Cross-sectional study: Evidence-based CAM 2015, 2015: 1–7. https://doi.org/10.1155/2015/138073.
36. Adewunmi CO, Ojewole JAO. Safety of traditional medicines, complementary and alternative medicines in Africa. Afr J Tradit Complem 2004, 1:1-3. DOI: 10.4314/ajtcam.v1i1.31090.
37. Kassaye KD, Amberbir A, Getachew B, Mussema Y. A historical overview of traditional medicine practices and policy in Ethiopia. Ethiop. J. Health Dev. 2006, 20: 127–134. DOI: 10.4314/ejhd.v20i2.10023.
38. Seifu E. Actual and Potential Applications of *Moringa stenopetala*, Underutilized Indigenous Vegetable of Southern Ethiopia: A Review. J. Agric. Food Res. 2014, 3 (4): 8-19.
39. Oda BK, Erena BA. *Aloes* of Ethiopia: A Review on Uses and Importance of Aloes in Ethiopia. Int. J. Plant Biol. Res. 2017, 5: 1059-1066.
40. Moges A, Moges Y. Ethiopian Common Medicinal Plants: Their Parts and Uses in Traditional Medicine - Ecology and Quality Control. In: Gonzalez A, Rodriguez M, Sağlam NG., editors. Plant Science. London, UK, Intechopen; 2019. https://www.intechopen.com/chapters/66996. DOI: 10.5772/intechopen.86202.
41. Meshesha SG, Yeshak MY, Gebretekle GB, Tilahun Z, Fenta TG. Concomitant Use of Herbal and Conventional Medicines among Patients with Diabetes Mellitus in Public Hospitals of AddisAbaba, Ethiopia: A Cross-Sectional Study. Evid. Based Complementary Altern. Med. 2020, 2020: 1-9. https://doi.org/10.1155/2020/4871459.
42. Salehi B, Albayrak S, Antolak H, Kregiel D, Pawlikowska E, Sharifi-Rad M, Uprety Y, Fokou PVT, Yousef Z, Zakaria Z A, Varoni EM, Sharopov F, Martins N, Iriti M, Sharifi-Rad J. *Aloe* Genus Plants: From Farm to Food Applications and Phytopharmacotherapy. Int. J. Mol. Sci. 2018, 19: 2843. doi:10.3390/ijms19092843.

43. Christaki EV, Florou-Paneri PC. Aloe vera: A plant for many uses. J Food Agric Environ 2010, 8 (2): 245-249.
44. International Agency for Research on Cancer (IARC) monograph-108. Evaluation of Carcinogenic Risks to Humans: some drugs and herbal products. Lyon: IARC; 2016.
45. Belayneh A, Demissew S, Bussa NF, Bisrat D. Ethno-medicinal and bio-cultural importance of aloes from south and east of the Great Rift Valley floristic regions of Ethiopia. Heliyon 2020, 6: e04344. https://doi.org/10.1016/j.heliyon.2020.e04344.
46. Yeshak MY, Bisrat D, Asres K. Pharmacognostic studies on *Aloes* of Ethiopia. 6th Nordic Natural Products Conference, Visby, Gotland, Sweden; 2015. (Abstract book). https://findresearcher.sdu.dk:8443/ws/files/118237329/Abstract_book_NNPC2015.pdf
47. Guo X, Mei N. Aloe vera: a review of toxicity and adverse clinical effects, J. Environ. Sci. Health Pt C – Environ. Carcinog. Ecotoxicol. Rev. 2016, 34: 77–96. doi: 10.1080/10590501.2016.1166826.
48. European Medicines Agency (EMA). Assessment report on *Aloe barbadensis* Mill. and on *Aloe* (various species, mainly *Aloe ferox* Mill. and its hybrids), *folii succus siccatus*. London: EMA; 2016.
49. Cosmetic Ingredient Review (CIR). Final report on the safety assessment of *Aloe andongensis* extract, *Aloe andongensis* leaf juice, *Aloe arborescens* leaf extract, *Aloe arborescens* leaf juice, *Aloe arborescens* leaf protoplasts, *Aloe barbadensis* flower extract, *Aloe barbadensis* leaf, *Aloe barbadensis* leaf extract, *Aloe barbadensis* leaf juice, *Aloe barbadensis* leaf polysaccharides, *Aloe barbadensis* leaf water, *Aloe ferox* leaf extract, *Aloe ferox* leaf juice, and *Aloe ferox* leaf juice extract. Int. J. Toxicol. 2007, 26(2): 1–50.
50. The international Aloe science council (IASC). https://www.iasc.org/Portals/19/Documents/Certification/AloeVeraQualityStandard.pdf?ver=2015-09-01-113340-987. Accessed on 02 April 2022.
51. Tiwari U, Cummins E. Factors influencing levels of phytochemicals in selected fruit and vegetables during pre-and post-harvest food processing operations. Food Research International 2013, 50: 497-506.
52. Abd Rani NZ, Husain K, Kumolosasi E. *Moringa* Genus: A Review of Phytochemistry and Pharmacology. Front. Pharmacol. 2018, 9(108): 1-26. doi: 10.3389/fphar.2018.00108.

53. Habtemariam S, Varghese GK. Extractability of Rutin in Herbal Tea Preparations of *Moringa stenopetala* Leaves. Beverages 2015, 1: 169-182. doi: 10.3390/beverages1030169.
54. Teklehaymanot T, Giday M. Quantitative ethnobotany of medicinal plants used by Kara and Kwego semi-pastoralist people in lower Omo River Valley, Debub Omo Zone, Southern Nations, Nationalities and Peoples Regional State, Ethiopia. J. Ethnopharmacol. 2010, 130: 76–84. doi: 10.1016/j.jep.2010.04.013.
55. Hadis M, Gebreyohannes Y, Gemeda N. Potential therapeutic uses of *Moringa stenopetala*: a scoping review. J Glob Health Sci. 2020, 2(2): e26. https://doi.org/10.35500/jghs.2020.2.e26.
56. Teshome M, Adane L, Tariku Y. Phytochemical Screening and Evaluation of Antibacterial Activities of Root Bark Extracts of *Moringa stenopetala*. Res. J. Med. Plants 2021, 15 (1), 1-6. DOI: 10.3923/rjmp.2021.1.6.
57. Udofia NE, Misonge OJ, Mworia M, William N, Apiri MG. Chemical Composition of *Moringa oleifera* Lam. and *Moringa stenopetala* Bac. Leaves from Kenya. Int. J. Plant Res. 2020, 10(1):1-10.
58. Bennett RN, Mellon FA, Foidl N, Pratt JH, Dupont MS, Perkins L, Kroon PA. Profiling Glucosinolates and Phenolics in Vegetative and Reproductive Tissues of the Multi-Purpose Trees *Moringa oleifera* L. (Horseradish Tree) and *Moringa stenopetala* L. J. Agric. Food Chem. 2003, 51: 3546-3553.
59. Mekonnen Y, Drager B. Glucosinolates in *Moringa stenopetala*. *Planta Med.* 2003, 69: 380–382. DOI: 10.1055/s-2003-38881.
60. Mekonen A, Gebreyesus T. Chemical investigation of the leaves of *Moringa stenopetala*. Bull. Chem. Soc. Ethiop. 2000, 14: 51–55. DOI: 10.4314/bcse.v14i1.72018.
61. Abuye C, Urga K, Knapp H, Selmar D, Omwega AM, Imungi JK, Winterhalter P. A compositional study of *moringa stenopetala* leaves. East Afr Med J. 2003, 80 (5): 247-252.
62. Habtemariam S. Investigation into the Antioxidant and Antidiabetic Potential of *Moringa stenopetala*: Identification of the Active Principles. Nat. Prod. Commun. 2015, 10 (3): 475 – 478.

63. Abo El-Fadl SR, Osman A, Al-Zohairy A M, Dahab AA, Abo El Kheir ZA. Assessment of total phenolic, flavonoid content, antioxidant potential and hplc profile of three *moringa* species leaf extracts. Scientific J. Flowers & Ornamental Plants 2020, 7(1):53-70. doi: 10.21608/sjfop.2020.91397.
64. Golla AZ. Thyroid function profile, and its association to consumption of cassava and *Moringa stenopetala* in pregnant women. Adv. biol. chem. 2013, 3: 448-454. DOI: 10.4236/abc.2013.35048.
65. Abuye C, Omwega AM, Imungi JK. Familial tendency and dietary association of goitre in Gamo-Gofa, Ethiopia. East Afr Med J. 1999, 76 (8): 447-51.
66. Geleta B, Makonnen E, Debella A. Toxicological Evaluations of the Crude Extracts and Fractions of *Moringa stenopetala* Leaves in Liver and Kidney of Rats. J Cytol. Histol. 2016, 7(1): 1-6. DOI:10.4172/2157-7099.1000383.
67. Sileshi T, Makonnen E, Debella A, Tesfaye B. Antihyperglycemic and subchronic toxicity study of *Moringa stenopetala* leaves in mice. J. coast. life med. 2014, 2(3): 214-221. DOI:10.12980/JCLM.2.2014C300.
68. Mekonnen N, Houghton P, Timbrell J. The Toxicity of Extracts of Plant Parts of *Moringa stenopetala* in HEPG2 Cells *In Vitro*. Phytother. Res. 2005, 19: 870–875. DOI: 10.1002/ptr.1720.

Chapter 2: Fast and easy applicable LC-UV method for analysis of bioactive anthrones from aloe leaf latex

Gereziher Sibhat, Getu Kahsay, Ann Van Schepdael, Erwin Adams

KU Leuven, Department of Pharmaceutical and Pharmacological Sciences, Pharmaceutical Analysis, Herestraat 49, O&N2, PB 923, 3000 Leuven, Belgium

J. Pharm. Biomed. Anal. 195 (2021) 113834

Abstract

Aloe leaf latex is a commonly used plant preparation in traditional medicine. However, quality control on the content of medicinally important constituents is often limited. Hence, establishing a reliable quality control method to identify and quantify bioactive markers is important to ensure safety and efficacy.

In the present study, a novel liquid chromatographic (LC) method was developed and validated for efficient analysis of bioactive markers to evaluate the quality of aloe leaf latex. Quantification of marker compounds was possible in only 7 min on a monolithic column using gradient elution with 0.1% formic acid in acetonitrile and water as mobile phases. The major compounds (aloins A and B) could be baseline separated together with related compounds within 10 min. The method showed excellent linearity with determination coefficients (r^2) of 0.9999. Detection limits were 0.017 and 0.013 μg/mL, while quantification limits were 0.057 and 0.043 μg/mL for aloin A and aloin B, respectively. Relative standard deviation (RSD) values for intra- and inter-day precision were less than 2% and recoveries for both aloins were close to 100%. The robustness was evaluated using an experimental design. The method was applied to some aloe leaf latex samples from Ethiopia. Aloin contents varied from 14 to 35% and two unknown peaks were tentatively identified as aloinoside and microdontin.

Keywords: Liquid chromatography, Method validation, Aloe, Leaf latex, Aloin

2.1 Introduction

The genus *Aloe (A.)*, which comprises more than 500 species (1), is used as a traditional medicine for treatment of different diseases and as ingredient of food products and cosmetics (2). Aloin, the main anthraquinone-C glycoside found in a variety of aloe species, has been reported to constitute up to 40% of the aloe leaf exudates (3). Aloin occurs naturally as a mixture of two diastereoisomers termed as aloin A (barbaloin) (10S) and aloin B (isobarbaloin) (10R) (**Figure 2.1**) (3, 4). Due to its large amount in aloe species and multiple pharmacological properties, aloin is used as a marker compound to evaluate the occurrence of aloe in food, drinks and drugs (5, 6).

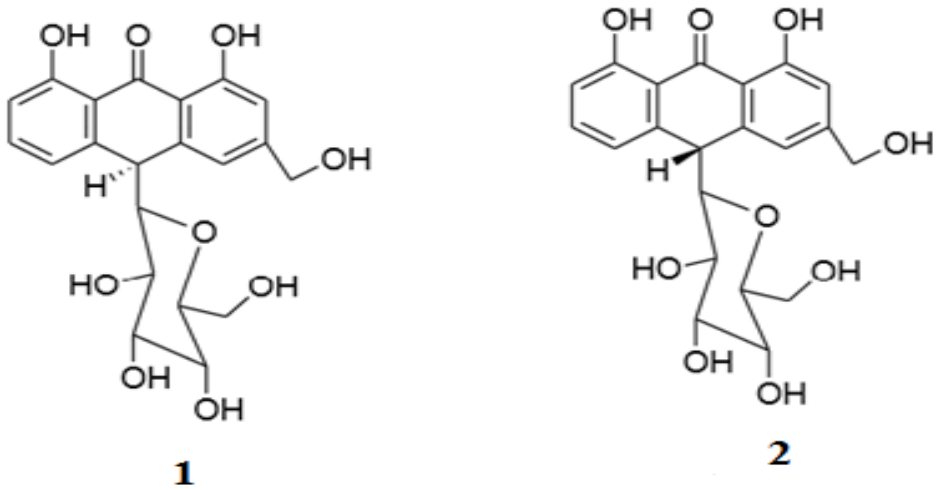


Figure 2.1. Chemical structure of aloin A (**1**) and aloin B (**2**)

Aloe is a commonly used plant in traditional medicine for a range of diseases. The inner sap, aloe leaf latex, has been traditionally used to treat different diseases like malaria, inflammation, burn, wounds and diabetes mellitus (7). However, different studies revealed an array of side effects and toxicities of leaf latex upon excessive use or chronic exposure (8). Due to this reason, different pharmacopoeias stipulated analytical methods for the detection and quantification of aloins and set limits in aloe based preparations. According to the United States Pharmacopeia (USP) (9), medicinal *A. vera* contains not less than 16% of aloin, while the European Pharmacopoeia (Ph. Eur.) (10) describes not less than 28% and 18% of hydroxyanthracene derivatives (expressed as aloin) in the dried juice of the leaves of *A. barbadensis* and *A. capensis*, respectively.

Only a few LC–UV methods and spectrophotometric methods have been described for the analysis of aloin and related compounds in raw and finished aloe-based products. The USP (9) uses an LC method for the assay of aloin in aloe leaf latex while the Ph. Eur. (10) prescribes spectrophotometry. However, the LC method of the USP with isocratic elution was not able to separate well aloin A from its epimer aloin B and from other related compounds. On the other hand, the spectrophotometric method described in the Ph. Eur. was found to be time consuming as it requires several sample preparation steps. Moreover, the method is not specific to aloins A and B as it quantifies total hydroxyanthracene derivatives.

Reducing the overall analysis time is good practice nowadays, but this is not evident in phytochemical studies seen the complex nature of plant extracts (11). In this framework, Azaroual et al. (5) compared three reversed phase chromatographic methods using a conventional particulate column (150 mm × 3 mm, 5 µm), a monolithic type (100 mm × 4.6 mm) and an ultra HPLC column (100 mm × 2.1 mm, 1.7 µm). The analysis times on the latter two columns were 5 and 10 times shorter respectively, compared to the first one with a total run time of over 40 min. In the present study, preference has been given to monolithic columns since they can be operated at high flow rates while maintaining high efficiency and low backpressure. As a result, they are compatible with classical LC systems (12, 13). Unfortunately, poor separation of aloins, low sensitivity (limit of detection (LOD) = 10 mg/L and limit of quantification (LOQ) = 33.2 mg/L), as well as considerable baseline drift were observed when we applied the method described by Azaroual et al. (5).

Besides, most papers about the analysis of aloins did not report which aloin diastereoisomer they considered, took the sum or only one (mostly aloin A) into account. Possible differences in bio-activities and kinetics between aloins A and B were neglected. This is partially due to the lack of proper separation methods (14).

In this work, it was the intention to develop and validate an LC-UV method that is able to separate well aloins A and B and related anthrones in a fast way without the need of ultra HPLC equipment or complicated sample pretreatment. So, the method should be easily applicable in low income countries like Ethiopia, where aloe leaf latex is commonly used, but barely controlled for the moment due to the absence of a suitable analytical method.

2.2 Materials and methods

2.2.1 Materials

Aloe (locally known as "ere") leaf latex was collected from six aloe species, *A. elegans* Todaro, *A. adigratana* Reynolds, *A. percrassa* Todaro, *A. megalacantha* Baker, *A. macrocarpa* Todaro, and *A. monticola* Reynolds in 2019 from Tigray region, Ethiopia. Aloin reference standard (97%) was obtained from Alfa Aesar (Thermo Fisher Scientific, Karlsruhe, Germany). HPLC grade acetonitrile (ACN), methanol (MeOH), and formic acid (99.9%) were procured from Acros Organics (Geel, Belgium). A Milli-Q water purification system obtained from Millipore (Bedford, MA, USA) was used to further purify demineralized water.

2.2.2 Methods

2.2.2.1 Plant material extraction

The yellowish sap was collected in situ by cutting cleaned leaves using a knife transversely near the bottom part and allowing to drain the exudates into plastic containers. The latex was then allowed to dry at room temperature for three days. Dried samples were powdered using mortar and pestle and stored in sealed amber colored bottles at 4 to 8 ˚C.

2.2.2.2 Preparation of sample and reference solutions

Accurately weighed powder (0.1 g) of each tested sample was transferred into a 100 mL volumetric flask, mixed with 50 mL methanol – water (50:50, v/v) and then sonicated (Branson ultrasonic, Danbury, CT, USA) for 10 min. After cooling to room temperature, the extraction solution was adjusted to volume using the same solvent mixture. Next, an aliquot of the sample solution was filtered through a 0.45 μm Chromafil® Xtra membrane filter (Düren, Germany) before analysis to remove particulates that can clog the chromatographic system, and discarding the first few mL of the filtrate to avoid any loss of the analyte due to adsorption to the filter membrane. A standard stock solution (1 mg/mL) was prepared by dissolving aloin standard in methanol – water (50:50, v/v). Moreover, the effect of filtration as sample pretreatment was investigated by comparing the peak areas of filtered with those of unfiltered standard solution. Working solutions were prepared by diluting the standard solution with the same solvent mixture immediately before chromatographic analyses. All test and standard solutions were stored in amber glass containers at -20 ˚C.

2.2.2.3 Instrumentation and chromatographic conditions

LC analyses were performed on a Merck-Hitachi apparatus (Darmstadt, Germany) equipped with an intelligent pump (L-6200), autosampler (L-2200) and UV/VIS detector (L-2400). For data processing and acquisition, Chromeleon software version 6.70 from Dionex (Sunnyvale, CA, USA) was used. Chromatographic separations were achieved on a Chromolith performance RP-18e (100 mm × 3 mm, i.d.) column from Merck (Darmstadt, Germany). A Julabo EM immersion thermostat (Seelbach, Germany) was used to keep the water bath of the column at 35 °C. The mobile phase was a gradient mixture of mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) pumped at a flow rate of 0.8 mL min^{-1}. The gradient program (time (min), % B) was set as (0, 20), (2, 20), (5, 60), (7, 60), followed by a few minutes equilibration time to return to the initial conditions. The injection volume was 5 µL. Quantification was performed at a detection wavelength of 295 nm using the external standard method.

2.2.2.4 Method validation

The developed method was validated for its selectivity, sensitivity, linearity, precision, accuracy and robustness based on the ICH guidelines (15).

2.2.2.4.1 Selectivity

Selectivity of the developed method was examined for the separation of aloin A and aloin B from each other by comparing chromatograms of standard solution of aloin, aloe samples and the blank solvent consisting of methanol – water (50:50, v/v).

2.2.2.4.2 Calibration curve, limits of detection and quantification

The calibration curve for the quantification of aloin A, aloin B and the sum of aloins A and B in the studied aloe samples was established by diluting aloin standard solution. Twelve concentrations of standard solution ranging from the LOQ to 125% (1 mg/mL = 100%) were analyzed in triplicate. The calibration curve was represented by the equation $y = ax + b$, where y represents the peak area (mAU*min) and x the corresponding concentration (mg/mL) of the compound. The LOD and LOQ were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

2.2.2.4.3 Precision

Precision of the developed method was evaluated by the intra- and inter-day variations expressed as %RSD of peak areas for six repetitive injections of a sample solution with a concentration of 1 mg/mL (100%). For the intra-day precision, injections were made on the

same day. The inter-day precision of the method was evaluated from 18 injections of the same sample solution for three consecutive days, where results of the third day were obtained by a different analyst.

2.2.2.4.4 Accuracy

Accuracy, expressed as recovery of the developed method, was determined by adding four different concentration levels (80, 160, 240 and 320 µg/mL) of standard solution to a test sample. Samples were analyzed in triplicate at each level. The percent recoveries were calculated using equation (2.1):

$$\text{Recovery (\%)} = \frac{\text{total amount after spiking} - \text{amount original}}{\text{amount spiked}} \times 100 \quad (2.1)$$

2.2.2.4.5 Robustness

A robustness study was performed by means of an experimental design and multivariate analysis. In this study, three chromatographic factors (column temperature, concentration of formic acid in mobile phases A and B, and percentage of acetonitrile in the initial gradient step) were investigated.

A two-level full factorial design was applied so that the number of runs is equal to $2^k + n$, where k is the number of factors and n is the number of times that the center point is repeated. Here, 11 experiments, including 3 at the center point (nominal value), were performed. The lower and higher values for each factor in the design are given in Table 2.1. The mathematical relationship between a response y and the experimental variables x_i, x_j, ... can be represented as first order equation (2.2):

$$y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \ldots + E \quad (2.2)$$

Where the letters β represent the regression coefficients and E is the overall experimental error. The linear coefficients β_i and β_j describe the quantitative effect of the experimental variables in the model while the cross coefficient, β_{ij}, measures the interaction effect between the variables x_i and x_j. As responses, the peak areas of aloins A and B as well as the resolution (Rs) between them were selected.

Table 2.1: Chromatographic parameter settings applied in the experimental design of the robustness study.

Parameter	Low value (−)	Central value (0)	High value (+)
Column temperature (°C)	33	35	37
% Formic acid in mobile phase	0.09	0.1	0.11
% Acetonitrile in initial gradient step	19	20	21

2.2.2.5 Quantification of aloin in aloe samples

The percentage of aloin in the portion of aloe taken was determined using equation (2.3):

$$Percentage\ of\ aloin\ content = (\frac{A_u}{A_s}) \times C_s \times (\frac{V}{W}) \times 100 \qquad (2.3)$$

Where A_u is the peak area for aloin from the sample solution, A_s is the peak area for aloin from the standard solution, and C_s is the concentration of aloin in the standard solution (mg/mL) taking also into account the purity of the standard, while V and W represent the final volume (mL) of the sample solution, and the weight of aloe taken to prepare the sample solution (mg), respectively.

2.2.2.6 MS characterization

Identification of aloins in all species was achieved by comparison of retention times of aloin A and aloin B with the reference standard using LC. Additionally, MS analyses were performed on an Esquire 3000^{plus} ion trap (Bruker Daltonics GmbH, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Esquire control and Bruker compass version 1.3 software were used to control the equipment and for data analysis, respectively. MS experiments were performed with the ESI source operated in negative ion mode, using a capillary voltage of 4.0 kV, end plate voltage of −500 V and nebulizing gas at 8.0 psi. Nitrogen gas was pumped into the ion source at a flow rate of 6 L/min and the dry gas temperature was set at 300 °C. The effluent from LC-UV corresponding to unknown peaks was collected and injected into the MS using a Hamilton 0.5 mL syringe (Reno, NV, USA). The syringe flow rate was set at 0.5 mL/h.

2.3 Results and discussion

2.3.1 Aloe leaf collection and extraction

To minimize contamination, sample leaves were cleaned, and the dried latexes collected from the leaves were stored in well-closed amber colored bottles. To assess the effect of extraction time on the extraction efficiency of the marker compounds, the leaf latex was treated with methanol – water (50:50, v/v) for 10, 20, 30 and 60 min. Results have shown that the marker compounds (aloins) were extracted within 10 min. A sonication time longer than 10 min did not significantly increase the quantity of aloins in the extracts. This was shorter than reported by Wu et al. (16) and Li et al. (17), where the sonication time was 30 and 45 min respectively, but in agreement with Azaroual et al. (5). As a result, test solutions were prepared by ultrasonic extraction with 50% methanol for 10 min.

2.3.2 Development and optimization of the LC method

To establish optimal and reliable chromatographic conditions for the separation of the targeted compounds, the effects caused by different mobile phase compositions, column dimensions, column temperature and detection wavelength were examined. In order to obtain fast separation with limited back pressure, Chromolith performance RP18e columns (100 mm × 4.6 mm i.d. and 100 × 3 mm i.d.) were evaluated. Both columns yielded comparable retention times. The 3 mm column was selected for further study because of its higher peak response and lower mobile phase consumption which is interesting from an economic and ecological point of view. Next, different mobile phase compositions, flow rates and elution modes (i.e., isocratic and gradient) were investigated. It was found that the peak shape and separation efficiency of aloins and related compounds could be improved considerably using gradient elution. Good separation of aloins and related compounds was obtained using a gradient mixture of mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) pumped at a flow rate of 0.8 mL/min. The gradient program (time, % B) was set as (0, 20), (2, 20), (5, 60), (7, 60), followed by a return to 80% of mobile phase A. Formic acid at a concentration of 0.1% (v/v) was added to improve the peak shape of the analytes. The effect of column temperatures (25, 30, 35 and 40 °C) on the separation process was assessed too. It was found that at 35 °C, aloins and other peaks in the chromatogram were better separated. Samples monitored at 295 nm revealed a higher and more stable UV absorbance for aloins compared to 270 and 350 nm. Therefore, a wavelength of 295 nm was selected for detection of the studied anthrones. Stability of standard and sample solutions in the autosampler (kept at 5 °C) was checked at different time intervals:

0, 3, 6, 9, 12, 15, 18, 24 and 48 h after preparation. Results revealed that both solutions were stable over 48 h. Moreover, standard and sample solutions were found to be stable for at least a month in the freezer (-20 °C) and for 12 h at room temperature. Mean peak areas of filtered and unfiltered solutions were compared using a t-test and the difference was found to be statistically not significant.

2.3.3 Chromatographic fingerprints of the leaf latex of aloe species

Using the chromatogram of aloin standard, the peaks corresponding to aloins A and B in the test solution were identified. Moreover, MS based identification of the selected peaks was performed. The optimized LC method with UV detection was used for the separation, identification and simultaneous quantification of aloins and related compounds contained in the studied aloe leaf latex (Figure 2.2).

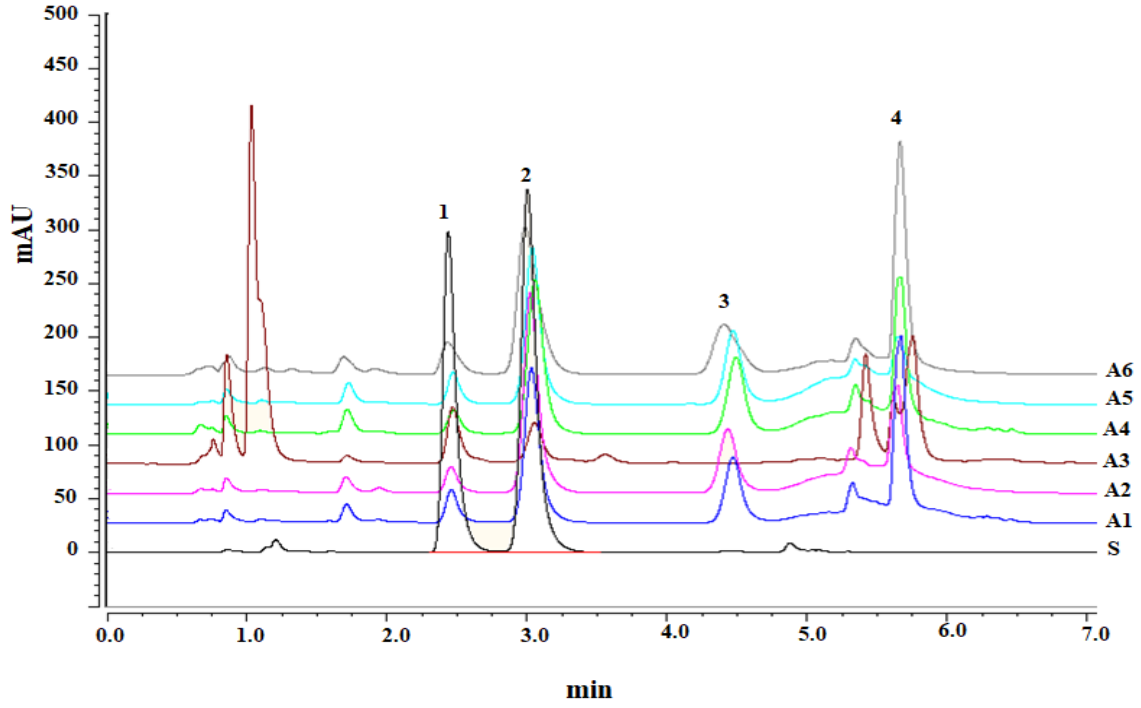


Figure 2.2: Chromatographic fingerprints of aloin standard and plant samples; S: Aloin standard, A1: *A. elegans*, A2: *A. macrocarpa*, A3: *A. monticola*, A4: *A. percrassa*, A5: *A. adigratana*, A6: *A. megalacantha*, 1: aloin B, 2: aloin A, 3: unknown 1, 4: unknown 2. Peaks 3 and 4 could later be characterized by MS as aloinoside and microdontin, respectively.

2.3.4 Method validation

2.3.4.1 Selectivity

An overlay of chromatograms of blank, standard solution of aloin and test sample showed no interfering peaks at the retention times of aloin. The peaks corresponding to aloins A and B were baseline separated.

2.3.4.2 Calibration curve, LOD and LOQ

Linearity of the detector response was examined using linear regression of the peak areas versus analyte concentrations. The residuals were randomly distributed around the horizontal zero axis suggesting that the linear model gives a good fit of the data (Table 2.2). Moreover, the 95% confidence interval of the intercepts included zero so that the intercepts are statistically not significant (18). Sensitivity of the method was evaluated based on the LOD and LOQ. The LOD values were 0.017 and 0.013 µg/mL, while the LOQ values were 0.057 and 0.043 µg/mL for aloin A and aloin B, respectively. The values indicate that the method is sensitive and enables to quantify small amounts of aloins in aloe samples.

Table 2.2: Regression analysis for aloin A, aloin B and the sum of aloins A and B.

Analyte	**Range (mg/mL)**	**Regression equation**	r^2	$S_{y,x}$
Aloin A	$0.057\ 10^{-3} – 0.71$	$y = 107.19\ x + 0.0034$	0.9999	0.29
Aloin B	$0.043\ 10^{-3} – 0.54$	$y = 105.88\ x - 0.0291$	0.9999	0.25
Aloins A+B	$0.10\ 10^{-3} – 1.25$	$y = 106.63\ x - 0.0258$	0.9999	0.54

$S_{y,x}$: standard error of estimate; y: peak area (mAU*min); x: concentration (mg/mL); r^2: determination coefficient

2.3.4.3 Precision

Precision of the developed method was evaluated for intra- and inter-day variations as %RSD on the peak areas (18). Results obtained are summarized in Table 2.3. The %RSD values for the intra-day (n = 6) and inter-day (n = 18) precision were found to be lower than 2.0%. The low RSD values of the peak areas indicate the good precision of the method.

Table 2.3: Results of intermediate precision determinations for peak areas of aloin A, aloin B and their sum, expressed as %RSD (n = 6).

Day	Aloin A	Aloin B	Aloins A+B
Day 1	1.4	1.8	1.4
Day 2	1.5	1.7	1.5
Day 3	0.9	1.8	0.8
Day 1–3	1.3	1.9	1.3

2.3.4.4 Accuracy

Accuracy was determined as percent recovery of the known added amount of standard to the sample. It was checked for aloin A, aloin B, as well as the sum of aloins A and B, at four different concentration levels (80, 160, 240 and 320 µg/mL). The percent recovery values ranged from 98.8% to 101.6% with a mean recovery of 100.2%. This indicates that the marker compounds could be recovered completely from the plant leaf latex.

2.3.4.5 Robustness study

Variations in LC operating conditions were made deliberately to demonstrate the robustness of the method. The effect of small changes in the chromatographic parameters on the results was investigated by means of an experimental design using R software (version 3.6.3) from R Studio (Boston, MA, USA). The different chromatographic parameter settings in the design and the factors investigated were described in Table 2.1. The results are shown in Figure 2.3 where each bar indicates the 95% confidence interval of the regression coefficient corresponding to the respective variable. It can be drawn from Fig. 2.3 that the change of individual variables within the investigated ranges has no significant effect on the responses as most intervals include zero, except for the percentage of acetonitrile in the initial gradient step. The latter has a significant negative effect on Rs (Figure 2.3(a)), which means that the resolution between the peaks of aloins A and B will decrease by increasing the amount of acetonitrile, and vice versa. Concerning interactions between two variables, no significant effect was observed as all intervals included zero except for the interaction between acetonitrile and formic acid which resulted in a significant effect on Rs (Figure 2.3(a)). However, it can be derived from the response surface plot illustrating the response as a function of the amount of acetonitrile and

formic acid (Figure 2.4), that under the examined conditions, the resolution between aloins A and B is always above 2.4. This means that small variations in the chosen chromatographic conditions will not have a deleterious effect on the resolution between aloins A and B.

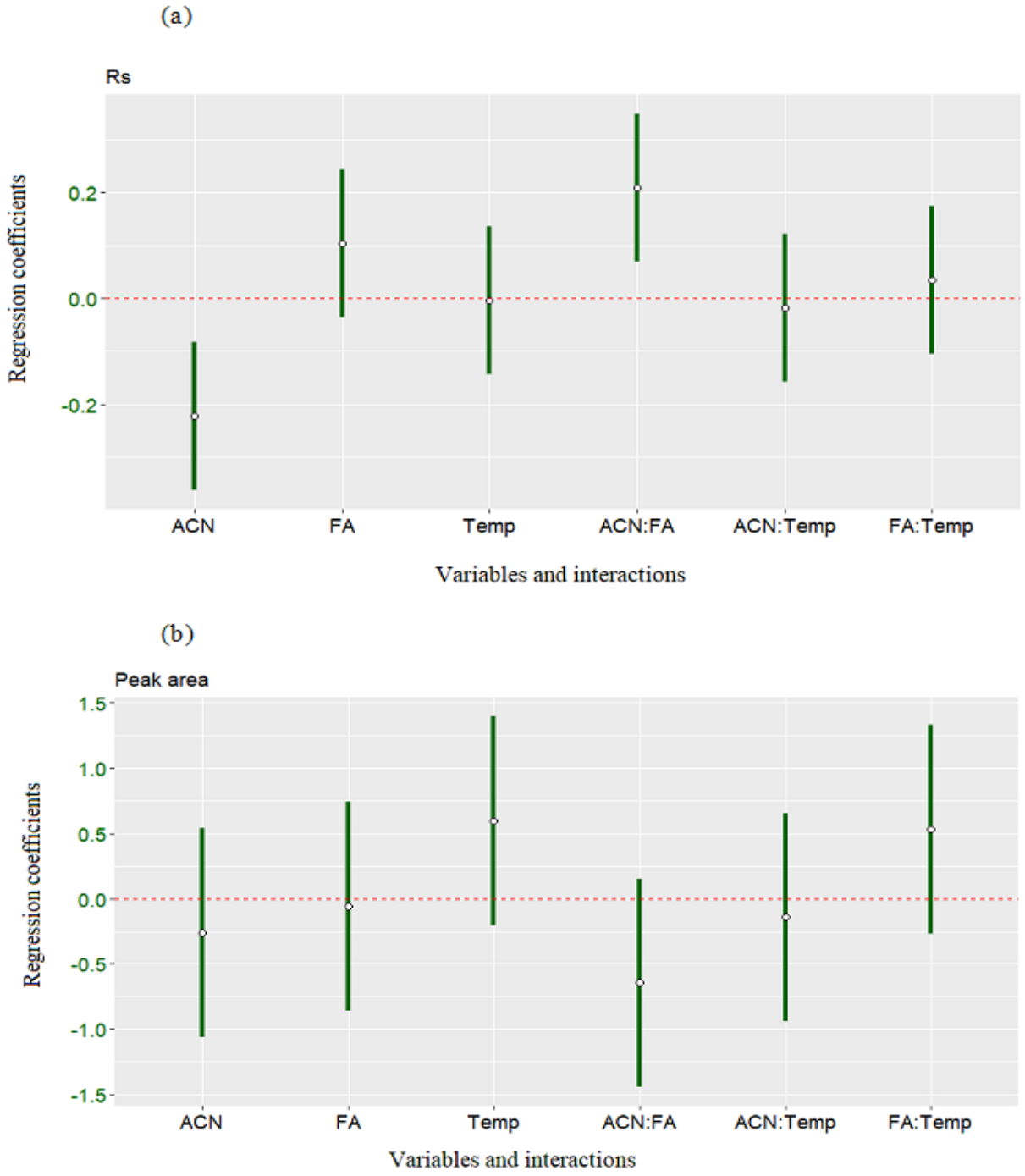


Figure 2.3: Regression coefficient plots obtained from the robustness study for (a) Rs: resolution between aloins A and B; (b) peak area as sum of peak areas of aloins A and B. Temp stands for column temperature, ACN for acetonitrile and FA for formic acid.

Since quantification is based on the peak areas, it is good that none of the factors examined was found to have an influence on the peak areas (Figure 2.3(b)). Therefore, the developed method is considered as robust in the examined domain of these three variables.

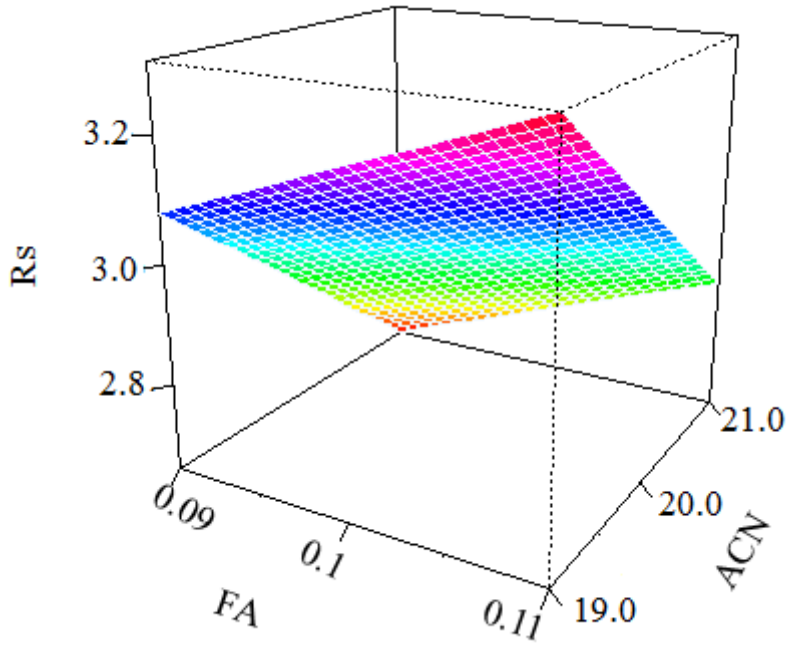


Figure 2.4: Response surface plot showing the influence of percentage of acetonitrile (ACN) and percentage of formic acid (FA) on Rs. Other parameters were kept constant at their central value.

2.3.5 Quantification of aloin in aloe samples

The developed LC method with UV detection was successfully applied for the quantification of aloins A and B in crude aloe leaf latex samples from the Tigray region in Ethiopia. From the results summarized in Table 2.4, it can be concluded that most samples contain around 30% (300 mg/g) of aloins, except *A. monticola* with only 14%. The minimum acceptance limit of aloins in aloe samples for medicinal purposes is generally accepted to be 16%, as also specified in the USP (9) for *A. vera*. Regarding the various encountered amounts of aloin in samples and the absence of an upper acceptance limit, it is advisable to calculate the dose of leaf latex to be processed in pharmaceutical formulations based on the aloin content. Unfortunately, this is not always common practice nowadays while high amounts of aloins can lead to unwanted side effects.

Table 2.4: Quantification results (n = 3) of aloins in the aloe samples.

Sample name	Aloin A mg/g (%RSD)	Aloin B mg/g (%RSD)	Aloins A+B mg/g (%RSD)	Aloins A+B %	ratio of aloin A/B
A. elegans	284 (1.0)	51 (1.2)	335 (1.0)	33.5	5.6
A. macrocarpa	315 (0.7)	34 (0.9)	349 (0.7)	34.9	9.3
A. monticola	63 (1.4)	77 (1.6)	140 (1.3)	14.0	0.8
A. percrassa	244 (1.5)	30 (0.7)	274 (1.3)	27.4	8.1
A. adigratana	241 (1.2)	42 (0.9)	283 (1.1)	28.3	5.7
A. megalacantha	279 (0.4)	49 (0.3)	328 (0.4)	32.8	5.7

Viljoen et al. (19) reported that the two aloin isomers are usually found in a quantitative imbalance with less aloin B than aloin A. A similar finding was reported by Wu et al. (16) who wrote that aloin A was found to represent the largest portion of anthrones in *A. barbadensis*. The same trend was observed in the present study, except for the leaf latex of *A. monticola*, which showed a different profile.

The quantity of a chemical marker is an indicator of the quality of a given herbal medicine (17). However, the types and levels of the chemical components present in the medicinal plants can vary depending on the geographical source or origin of the plants (20, 21). The Tigray region covers an area of over 50 000 km² with altitudes between 600 and 2500 m, with *A. monticola* collected from the higher areas. It has also been described that extracts of different species of the same genus can have a different composition (22), and plants of the same species occurring in different environmental conditions may differ significantly in their content of secondary metabolites (21). The variation on the content of aloins in the studied aloe species may be attributed to one or more of the factors mentioned above.

2.3.6 MS characterization of unknown compounds

MS analysis was performed in order to obtain structural information of unknown compounds corresponding to peaks 3 and 4 in Figure 2.2. The MS spectra of aloins A and B (corresponding to peaks 2 and 1 respectively in Figure 2.2) were used as interpretative templates.

The ESI-MS spectrum of Peak 1 (t_r = 2.4 min) and peak 2 (t_r = 3.0 min) showed a deprotonated molecule $[M - H]^-$ with a mass-to-charge ratio (*m/z*) of 417. Their MS/MS spectra gave rise to fragment ions at *m/z* 297 (= $[M - H - 120]^-$) indicating the loss of $C_4H_8O_4$ and *m/z* 268 (= $[M - H - 120 - 29]^-$) as the result from an additional loss of CHO through the sugar moiety. These findings were in agreement with previously reported data for aloins A and B by Wu et al. (16) and Zhong et al. (23), who reported cleavages between C-1'/O and C-2'/C-3' as a characteristic fragmentation for C-glycosides (Figure 2.5A).

The mass spectra of peak 3 (unknown 1, t_r = 4.5 min) obtained in negative ion mode revealed a deprotonated molecule $[M - H]^-$ at *m/z* 563, indicating a relative molecular mass of 564. A fragment thereof was observed at *m/z* 443 (= $[M - H - 120]^-$) indicating the loss of $C_4H_8O_4$. Further fragmentation of the $[M - H - 120]^-$ ion resulted in the formation of ions with *m/z* 425 (= $[M - H - 120 - 18]^-$), *m/z* 251 (= $[M - H - 120 - 164 - 28]^-$) and *m/z* 278 (= $[M - H - 120 - 165]^-$) (Figure 2.5B). The first could be formed by loss of an additional water molecule (18 u). The second could be produced by loss of the rhamnosyl moiety ($C_6H_{12}O_5$ = 164 u) and a carbonyl group (28 u). The loss of 164 Da for rhamnose was attributed to bond breaking at the O-glycosidic linkage. The third ion is probably also related to the combined loss of the rhamnosyl moiety (147 u), and water molecule (18 u) although there is a difference of 1 u. Similar MS data (including the ion with *m/z* 278) were reported by Wu et al. (16), so that this compound was analogously characterized as aloinoside. Peak 3 was present in all of the analyzed samples except in the leaf latex of *A. monticola* (Table 2.5).

Table 2.5: Content (n = 3) of unknown 1 and unknown 2 (corresponding to peaks 3 and 4 respectively in the chromatogram of Figure 2.2) in the aloe samples expressed as aloin.

Sample name	unknown 1 mg/g (%RSD)	unknown 2 mg/g (%RSD)	unknown 1 (%)	unknown 2 (%)
A. elegans	132.0 (0.9)	242.0 (1.7)	13.2	24.2
A. macrocarpa	120.0 (0.8)	141.0 (1.1)	12.0	14.1
A. monticola	ND	114.0 (1.0)	ND	11.4
A. percrassa	132.0 (1.2)	234.0 (0.3)	13.2	23.4
A. adigratana	124.0 (0.8)	58.0 (1.9)	12.4	5.8
A. megalacantha	116.0 (0.3)	203.0 (1.0)	11.6	20.3

ND: Not detected

Peak 4 (unknown 2, t_r = 5.7 min), which was detected in all species investigated (Table 2.5), revealed a deprotonated molecule $[M - H]^-$ at *m/z* 563. MS/MS analysis showed fragment ions at *m/z* 399 (= $[M - H - 164]^-$) produced by loss of a *p*–coumaroyl unit. In addition, a fragment ion at *m/z* 297 (= $[M - H - 266]^-$) was observed. This could be due to the loss of a $C_{13}H_{14}O_6$ unit containing the *p*–coumaroyl group produced by a cross-ring cleavage in the hexosidic part between C-1'/O and C-2'/C-3' (Figure 2.5C**)**. These mass data were comparable with those reported by Zhong et al. (23) for microdontin. So, peak 4 was tentatively identified as microdontin.

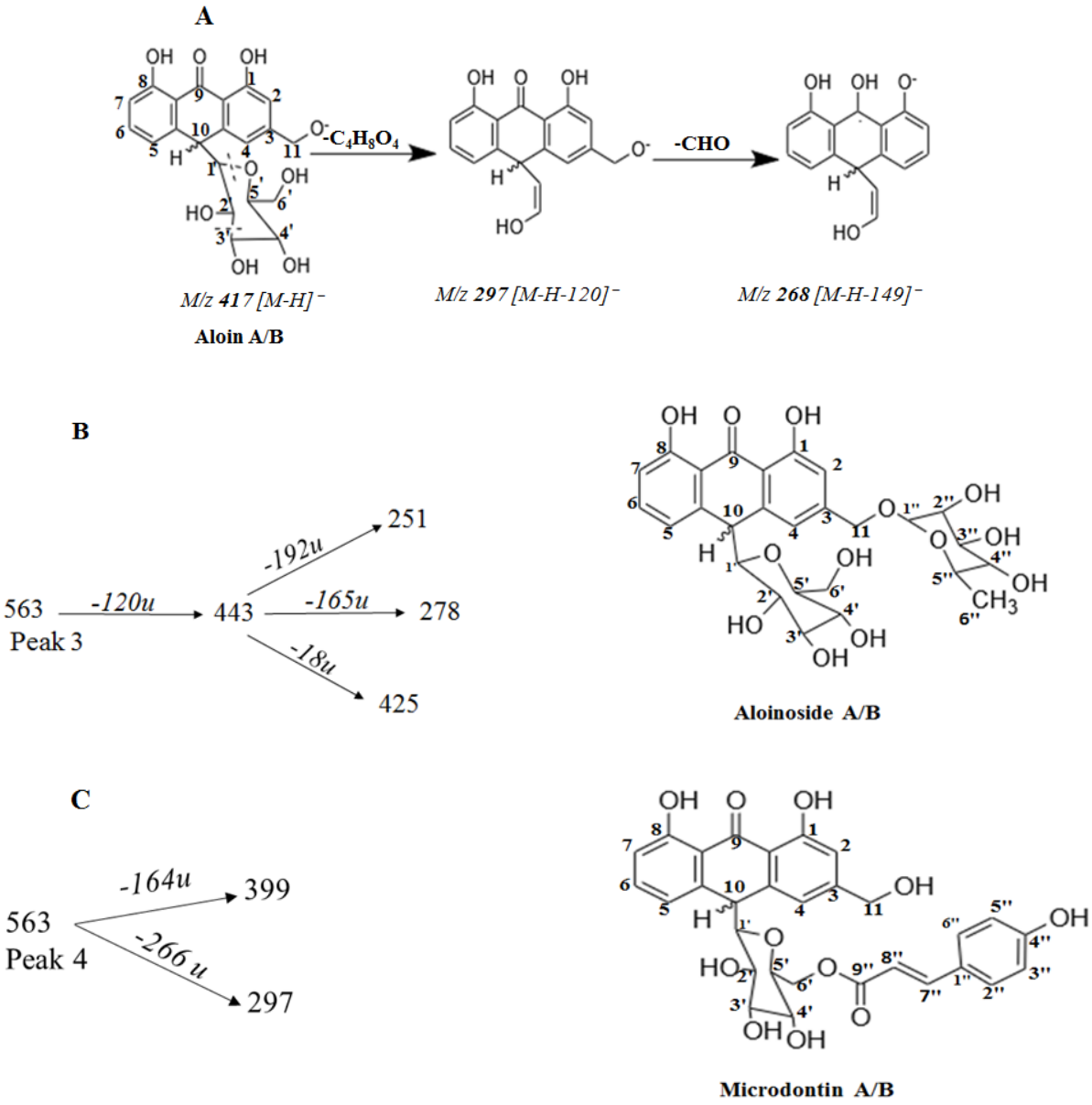


Figure 2.5: Schematic representation of the fragmentation of (A) aloin A/B, (B) unknown 1 eluted as peak 3 and (C) unknown 2 eluted as peak 4 in Figure 2.2 and their proposed structures.

These findings are in accordance with a paper of Viljoen et al. (19) on the taxonomic distribution of anthrones in *Aloe*, where different aloe species (including *A. elegans* and *A. megalacantha*) collected from Ethiopia were found to contain aloin, aloinoside and microdontin. Those compounds are considered among the major bioactive ingredients which were found to possess multiple pharmacological activities such as *in vivo* anti-inflammatory (24), antimalarial (25, 26), anti-constipation (27, 28) and *in vitro* antimicrobial activities (29).

Hence, it makes sense to consider these anthrones as characteristic components during quality control of aloe preparations.

2.4 Conclusion

The newly developed LC–UV method was able to quickly separate and quantify the pharmacologically active constituents (aloin, aloinoside and microdontin) in the leaf latex of aloe species. Hence, it can be applied to monitor quality consistency of different aloe species that are potentially used for commercialisation and for incorporation in aloe based medicinal, nutraceutical and cosmetic preparations. Moreover, the method does not require complicated sample pretreatment and it can be executed using a conventional LC apparatus, making it affordable for low income countries so that they can easily control their harvested aloe leaf latex.

2.5 References

1. Frodin DG. History and concepts of big plant genera, Taxon. 2004,53:753–776.
2. Eshun K, He Q. Aloe vera: a valuable ingredient for the food, pharmaceutical and cosmetic industries – a review, Crit Rev Food Sci. Nutr. 2004, 44: 91–96.
3. Brown PN, Yu R, Kuan CH, Finley J, Mudge EM. Determination of Aloin A and Aloin B in Aloe vera Raw Materials and Finished Products by High-Performance Liquid Chromatography: Single-Laboratory Validation, J. AOAC Int. 2014, 5: 1323-1328.
4. Ding WJ, Wu XF, Zhong JS, Wan JZ. Effects of temperature, pH and light on the stability of aloin A and characterization of its major degradation products, Int. J. Food Sci. Technol. 2014, 49: 1773–1779.
5. Azaroual L, Liazid A, Barbero GF, Brigui J, Palma M, Barroso CG. Improved chromatographic methods for determination of bioactive compounds from aloe vera leaves, ISRN. 2012, 2012: 1–7.
6. Zhao Y, Kim YH, Lee W, Lee YK, Kim KT, Kang JS. A simple and simultaneous identification method for aloe, catechu and gambir by high performance liquid chromatography, J. Pharm. Biomed. Anal. 2016 ,117: 73–78.
7. Oda BK, Erena BA. Aloes of Ethiopia: A review on uses and importance of aloes in Ethiopia, Int. J. Plant Biol. Res. 2017, 5: 1059.
8. Guo, X., Mei, N. Aloe vera: A review of toxicity and adverse clinical effects, J. Environ. Sci. Health Pt C – Environ. Carcinog. Ecotoxicol. Rev. 2016, 34: 77–96.

9. United States Pharmacopeia. USP 37 - National Formulary 32. Rockville (MD): United States Pharmacopeial Convention; 2014.
10. European Pharmacopoeia. 10th ed. Strasbourg: EDQM Council of Europe; 2020.
11. Kaufmann B, Souverain S, Christen P, Veuthey JL, Cherkaou S. Rapid liquid chromatographic-mass spectrometric analysis of withanolides in crude plant extracts by use of a monolithic column, Chromatographia 2002, 56: 137–141.
12. Yu B, Zhang H, Cong H, Chen G, Xu T, Liu Y. Recent development and application of monolithic columns, Rev. Adv. Mater. Sci. 2017, 48: 58–67.
13. Sharma G, Tara A, Sharma VD. Advances in monolithic silica columns for high-performance liquid chromatography, J. Anal. Sci. Technol. 2017, 8: 16.
14. Xiao M-W, Bai X-L, Liu Y-M, Yang L, Hu Y-D, Liao X. Rapid quantification of aloin A and B in aloe plants and aloe-containing beverages, and pharmaceutical preparations by microchip capillary electrophoresis with laser induced fluorescence detection, J. Sep. Sci. 2018, 41: 3772–3781.
15. ICH. Validation of analytical procedures: text and methodology Q2 (R1). London: EMA; 2006.
16. Wu X, Ding W, Zhong J, Wan J, Xie Z. Simultaneous qualitative and quantitative determination of phenolic compounds in *Aloe barbadensis* Mill by liquid chromatography-mass spectrometry-ion trap-time-of-flight and high performance liquid chromatography-diode array detector, J. Pharm. Biomed. Anal. 2013, 80: 94–106.
17. Li S, Han Q, Qiao C, Song J, Cheng LC, Xu H. Chemical markers for the quality control of herbal medicines: an Overview, Chin. Med. 2008, 3: 7.
18. Ermer J, Ploss HJ. Validation in pharmaceutical analysis. Part II: central importance of precision to establish acceptance criteria and for verifying and improving the quality of analytical data, J. Pharm. Biomed. Anal. 2005, 37: 859–870.
19. Viljoen AM, van Wyk BE, Newton LE. The occurrence and taxonomic distribution of the anthrones aloin, aloinoside and microdontin in Aloe, Biochem. Syst. Ecol. 2001, 29: 53–67.
20. Kumar S, Yadav M, Yadav A, Rohilla P, Yadav JP. Antiplasmodial potential and quantification of aloin and aloe-emodin in Aloe vera collected from different climatic regions of India, BMC Complement. Altern. Med. 2017, 17: 369.

21. Quispe C, Villalobos M, Borquez J, Simirgiotis M. Chemical Composition and Antioxidant Activity of Aloe vera from the *Pica Oasis* (Tarapaca, Chile) by UHPLC-Q/Orbitrap/MS/MS, J. Chem. 2018, 2018: 1–12.
22. Hoai NN, Dejaegher B, Tistaert C, Thi Hong, VN, Rivière C, Chataigné G, Phan Van K, Chau Van M, Quetin-Leclercq J, Vander Heyden Y. Development of HPLC fingerprints for Mallotus species extracts and evaluation of the peaks responsible for their antioxidant activity, J. Pharm. Biomed. Anal. 2009, 50: 753–763.
23. Zhong JS, Wan JZ, Ding WJ, Wu XF, Xie ZY. Multi-responses extraction optimization combined with high-performance liquid chromatography–diode array detection–electrospray ionization-tandem mass spectrometry and chemometrics techniques for the fingerprint analysis of Aloe *barbadensis* Miller, J. Pharm. Biomed. Anal. 2015, 107: 131–140.
24. Tsegay M, Tewabe Y, Bisrat D, Asres K. *In vivo* antiinflammatory activity of two anthrones from the leaf latexes of *Aloe adigratana* Reynolds and *Aloe elegans* Todaro, Ethiop. Pharm. J. 2018, 34: 1–8.
25. Geremedhin G, Bisrat D, Asres K. Isolation, Characterization and *In Vivo* Antimalarial Evaluation of Anthrones from the Leaf Latex of *Aloe percrassa Todaro*. J. Nat. Remedies 2014, 14: 120-125.
26. Minale G, Bisrat D, Asres K. Antimalarial anthrones from the leaf latex of *Aloe sinana*. J. Pharma. Care Health Syst. 2015, 4: 58.
27. Oda BK, Erena BA. *Aloes* of Ethiopia: A Review on Uses and Importance of Aloes in Ethiopia. Int. J. Plant Biol. Res. 2017, 5: 1059-1066.
28. International Agency for Research on Cancer (IARC) monograph-108. Evaluation of Carcinogenic Risks to Humans: some drugs and herbal products. Lyon: IARC; 2016.
29. Minale G, Bisrat D, Asres K, Mazumder A. *In vitro* antimicrobial activities of anthrones from the leaf latex of *Aloe sinana* Reynolds, Int. J. Green Pharm. 2014, 8: 7– 12.

Chapter 3: Evaluation of aloins, pH and moisture in aloe leaf gel based personal care products

Gereziher Sibhat, Getu Kahsay, Ann Van Schepdael, Erwin Adams

KU Leuven, Department of Pharmaceutical and Pharmacological Sciences, Pharmaceutical Analysis, Herestraat 49, O&N2, PB 923, 3000 Leuven, Belgium

Int. J. Cosmet. Sci. 2022; 44:74–81.

Abstract

Some easily applicable analytical methods were explored to evaluate the quality of personal care products containing aloe leaf gel. Aloins should be absent in these products in view of their side effects. To check this, liquid chromatography (LC) was applied. The LC method used a C18 monolithic column combined with gradient elution and ultraviolet (UV) detection. The mobile phase consisted of a mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The method was validated with respect to specificity, linearity, precision, and accuracy. Next, it was practically applied for the analysis of commercial samples. The results indicated that aloins were detected in 25% of the analysed commercial samples. In addition, the pH and moisture content were determined. It turned out that 42% of the test samples were found to be in the basic pH range and 33% of them contained excessive moisture.

Keywords: control of aloins; quality evaluation; liquid chromatography

3.1. Introduction

Aloe vera gel may be used as emollient and moisturizer in cosmetics and personal care products in concentrations which vary widely from less than 0.1% up to 20%. High quality gel is opaque, slightly off white in color, and viscous (1). However, during its production stage, the gel could be contaminated by the latex, which contains aloin, an anthraquinone-C glycoside. It can be prevented from entering the production process if the outer layers of the Aloe leaves, containing the highest quantities of aloin, are discarded before extracting the gel (2). Aloin occurs naturally as a mixture of two diastereoisomers termed as aloin A (barbaloin) and aloin B (isobarbaloin) (3, 4). Aloin has been reported to cause an array of side effects and toxicities upon excess use or chronic exposure (5) including acute eczema, contact urticaria, and dermatitis in individuals who applied aloe-derived ingredients topically (6).

Anthraquinone rich *Aloe vera* extracts may appear yellow to yellowish green in color. Although they may function as absorbers of ultraviolet radiation in sunscreens, most of the manufacturers of *Aloe vera* gel for cosmetic use take care to supply a product containing not more than 50 ppm of anthraquinones. This maximum level is also demanded in a safety assessment of the cosmetic industry (1). So, in Aloe-derived ingredients used in cosmetics, regardless of species, anthraquinone levels should not exceed 50 ppm (6). As part of the quality control of commercial *Aloe vera* gel products for cosmetic use, it should be checked whether the aloin content is not exceeded (1). To realise this, a validated and reliable analytical method is essential.

Cosmetics containing *Aloe vera* gel are very common in several countries, among others the Ethiopian population. As these products have no medical indication, they are used without passing through strict safety evaluations or laboratory assessments. In view of the possible presence of aloin, this may possibly lead to unsafe cosmetic products which could pose public health risks to the consumers (7). In a cross-sectional study on utilization of cosmetics among university students and residents in Ethiopia, allergic reactions, acne, brittleness, breakage/loss of hair, sore on skin and face etc. have been reported (8, 9, 10). These adverse effects were found to be highly associated with traditional herbal cosmetics, often bought from local shops and supermarkets.

Similarly, traditional medicine practitioners, pharmacists and druggists mentioned similar cases of adverse effects from their clients upon using cosmetics made from *Aloe vera* which are

among the top herbal preparations for topical use. These products from natural origin are often promoted as safe alternatives to treat a number of dermal problems.

To carry out quality control of commercial *Aloe vera* gel products for cosmetic use, determination of the aloin content is recommended as well as control of the labelling. The pH of a topical formulation is also among the main parameters to be investigated. Indeed, acidic and alkaline solutions may cause hair problems, dehydration, irritability, etc. (11, 12). Excessive dilution with water is also a common problem as the moisture content is an important parameter determining the shelf life of a product (13).

In this study, a fast, selective and sensitive LC method was applied to check for the absence of aloins in cosmetics containing *Aloe vera* gel. Focus was on samples produced and sold in Ethiopia. Besides LC, attention was also paid to visual inspection, labelling, pH and moisture content to assess the quality of the cosmetic products.

3.2. Materials and Methods

3.2.1. Materials

3.2.1.1. Chemicals and reagents

Aloin reference standard (97%) was purchased from Alfa Aesar (Thermo Fisher Scientific, Karlsruhe, Germany). HPLC grade acetonitrile (ACN), methanol (MeOH), and formic acid (99.9%) were procured from Acros Organics (Geel, Belgium). Glacial acetic acid (100%) was obtained from VWR Chemicals (Fontenay-sous-Bois, France). A Milli-Q water purification system obtained from Millipore (Bedford, MA, USA) was used to further purify demineralized water. pH-Fix 0-14 test strips were from Macherey–Nagel (Düren, Germany)

3.2.1.2. Samples

Twelve commercial cosmetic (skin and hair care) samples consisting of five different soaps (S1-S5), a hair conditioner (HC), two liquid shampoos (SH1, SH2), three *Aloe vera* powders (P1-P3) for multipurpose use and a treatment oil (TO) were purchased from local markets and retail drug outlets in Ethiopia. For this study, traditional healers and drug sellers were surveyed to identify the most utilized and locally made herbal cosmetics. Issues on traditional medicinal preparations reported by users were an additional motivation to select the products for investigation. The samples were from different brands. Their names are not shown for reasons of confidentiality. Samples were kept in their original containers at 4 to 8 $^{\circ}$C until analysis. The labels on the packages did not indicate the presence of aloin. Before analysis, liquid samples were vortexed while soap samples were cut into pieces using a stainless steel spatula, and

levigated using mortar and pestle to obtain homogeneity. Finally, all samples were kept in amber coloured glass bottles to minimize degradation by light.

3.2.2. Methods

3.2.2.1. Preparation of standards and sample solutions

Five of the previously mentioned samples (SH1, S1, TO, P2 and HC) were used for method optimization and validation. Except in P2, no aloins were detected. A stock standard solution containing aloin A (570 µg/mL) and aloin B (430 µg/mL) was prepared using methanol – water (50:50, v/v) as solvent. From this solution, working calibration solutions for aloin A (0.057–570 µg/mL) and aloin B (0.043–430 µg/mL) were prepared by proper dilution with methanol – water (50:50, v/v).

Sample solutions were prepared by reverse pipetting 1 mL into a 10 mL volumetric flask for liquid samples or weighing 0.1 g into a 15 mL Falcon tube for solid samples, followed by adding 6 mL of methanol – water (50:50, v/v). After that, the mixture was placed in an ultrasonic bath (100 W, 42 kHz) from Branson (Danbury, CT, USA) and sonicated for 10 min in order to achieve lixiviation of the analyte from the sample matrix. Next, the mixture was allowed to cool. The supernatant of the Falcon tubes was transferred into a 10 mL volumetric flask and made up to volume with methanol – water (50:50, v/v). Finally, all solutions were filtered through a 0.45 µm PTFE filter from Chromafil® (Düren, Germany) to remove non-soluble compounds, prior to injection in the LC-UV system. Samples P2 and P3 were ten times further diluted so that their amount of aloins fell within the validated linear range.

3.2.2.2. Liquid chromatographic analysis

LC analyses were performed on a Merck-Hitachi apparatus (Darmstadt, Germany) equipped with a pump (L-6200), autosampler (L-2200) and UV/VIS detector (L-2400). For data processing and acquisition, Chromeleon software version 6.70 from Dionex (Sunnyvale, CA, USA) was used. Chromatographic separations were achieved on a Chromolith performance RP-18e (100 mm × 3 mm, i.d.) column from Merck (Darmstadt, Germany). A Julabo EM immersion thermostat (Seelbach, Germany) was used to keep the water bath of the column at 35 °C. The mobile phase was a gradient mixture of mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) pumped at a flow rate of 0.8 mL min^{-1}. The gradient program (time (min), % B) was set as (0, 20), (2, 20), (5, 60), (13, 60), followed by a few minutes equilibration time to return to the initial conditions. The injection volume was 10 µL.

The detection wavelength was 295 nm. Calibration curves were constructed by plotting the peak area of the target analytes versus concentration.

3.2.2.3. Development and validation of the liquid chromatographic method

The method is based on that described previously for the determination of aloins A and B in Aloe leaf latex (14). However, the extraction procedure had to be revised and the LC method had to be revalidated for the new matrices.

Extraction of aloins from the complex matrix of the test samples was performed using methanol – water (50:50, v/v).

The LC method was validated in terms of selectivity, sensitivity, linearity, precision and accuracy. The selectivity of the method was established by injecting aloin reference solution, sample solutions and blank to check for possible interferences and the resolution between aloins A and B. Samples wherein no aloins were detected were used as blanks for spiking. So, for a first screening of the suitability of the LC method, 1 mL of a 1 µg/mL solution of aloins A and B (0.57: 0.43) was added to 0.1 g of sample before dilution to 10 mL. This corresponded to spiking at a level of 10 ppm.

The sensitivity is expressed as limit of detection (LOD) and limit of quantification (LOQ), determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Linearity of the proposed method was determined using a ten-point calibration covering a range from LOQ to 1000 µg/mL of aloins A and B. Regression equations were obtained through linear regression analysis, using peak area as a function of concentration. The calibration curve was represented by the equation $y = ax + b$, where y represents the peak area (mAU*min) and x the corresponding concentration (µg/mL) of the compound.

The accuracy of the method, expressed as percent recovery, was examined by spiking four different concentration levels of aloin standard solution to the validation samples (10, 20, 40 and 60 µg of aloin per 100 mg of sample). Each concentration was injected three times.

Precision of the method was evaluated by analyzing SH1, S1, TO and HC (each spiked with 10 µg of aloin per 100 mg of sample) and P2 (which contained already aloins A and B). Each sample solution was prepared in duplicate. Both preparations were injected in triplicate ($n = 6$) and analysed on 2 consecutive days ($n = 12$). The within-day and between-day variations for aloin A and aloin B were calculated for each of the samples.

3.2.2.4. Determination of pH

The pH of the products was determined as per the procedure described by Tarun *et al.* (12). Accordingly, a 1% soap solution and a 10% shampoo solution in water, producing as less as possible lather, were used for this test. The shampoo solutions were kept for 30 min and the soap solutions were left undisturbed for 24 h. Then the pH of each sample was measured using pH test strips with 4 colour panels. These were preferred because they are quick and easy to use and they are easily available in labs of low resource countries like Ethiopia, compared to a pH meter which is more expensive and has to be calibrated daily. The pH value of the powder samples was determined following the procedure for soaps while for the hair conditioner and treatment oil, the procedure of the shampoos was followed.

3.2.2.5.Determination of moisture content

The moisture content of the samples was determined by loss on drying as described in the European Pharmacopoeia (15) by taking 1.000 g of test sample and drying in an oven at 105 °C for 2 h.

3.3. Results and discussion

3.3.1. Liquid chromatographic method

3.3.1.1. Extraction procedure

Cosmetic formulae are too complex for complete extraction of all components by a single solvent. So, pre-treatment before quantitation is a key process for analysis (16). In this study, water, methanol, and methanol – water (50:50, v/v) were investigated as extraction solvents. Among them, a mixture of methanol – water (50:50, v/v) was found to be an effective solvent to extract aloins from the samples followed by sonication. A single extraction was sufficient for almost complete recovery (at least 98.0% of both aloins A and B). Water was the least efficient and in addition, samples dissolved in water were foamy and yielded a high back pressure during filtration.

3.3.1.2. Method validation

The selectivity was evaluated based on chromatograms from the injection of standard, solvent blank and samples with no detectable aloins, representing a shampoo (SH1), soap (S1), treatment oil (TO) and hair conditioner (HC). They showed no significant interference at the retention times (RTs) of the peaks of aloins A and B. When those samples were spiked with 10 ppm of aloins A and B, peaks were clearly observed (matching the RTs of the aloin peaks

following injection of the standard solution) indicating that the method is specific (Figure 3.1). The gradient elution was continued up to 13 min in order to elute other constituents from the preparations.

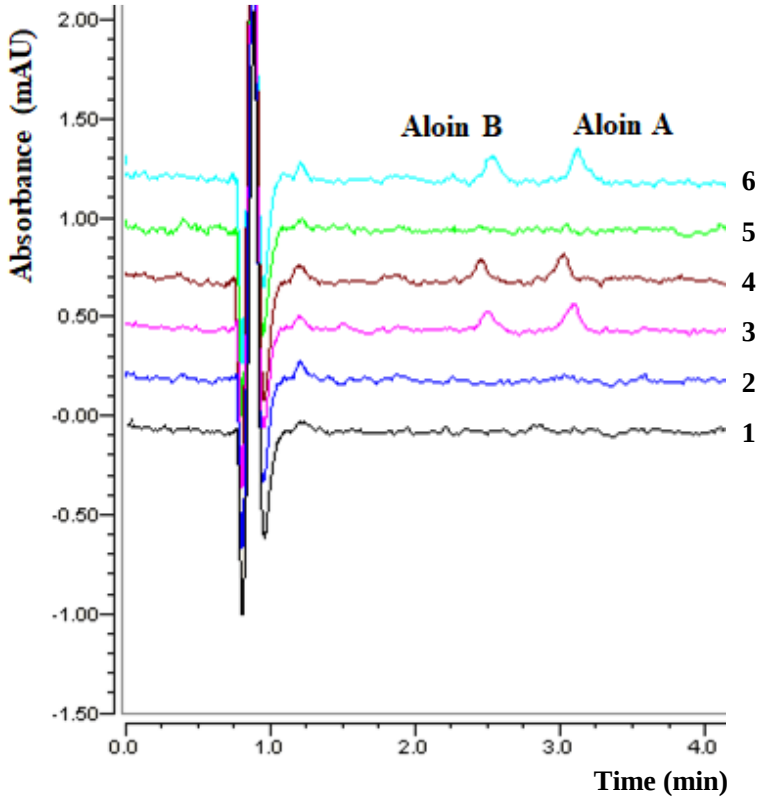


Figure 3.1: Representative overlay of chromatograms from 0 to 4 min, focussing on the region where aloins A and B are eluted. 1: solvent blank, 2: Aloin free shampoo, 3: 0.1 µg/mL standard mixture of aloins A and B (in a ratio 57:43), 4: Aloin free shampoo spiked with 10 ppm aloin (A + B), 5: Aloin free hair treatment oil, 6: Aloin free hair treatment oil spiked with 10 ppm aloin (A + B).

The LOD and LOQ of the method were determined by measuring the S/N of aloin from standard solutions and from spiked placebo samples. The LOD values were found to be 0.017 µg/mL for aloin A and 0.013 µg/mL for aloin B, respectively, while the LOQ values were 0.057 µg/mL for aloin A and 0.043 µg/mL for aloin B, respectively. The low values indicate good sensitivity of the method (Table 3.1). Compared to a sample concentration of 10 mg/mL, the LOQ for aloin A corresponds to 5.7 ppm and this of aloin B to 4.3 ppm. Thus, determinations below the safety level of 50 ppm for anthraquinones are well feasible.

Table 3.1: Regression analysis, LOD and LOQ of the proposed LC method for the determination of aloins A and B

Parameters	Aloin A	Aloin B
Range (µg/mL)	0.057 – 570	0.043 – 430
Regression equation	$y = 0.2038\ x + 0.0299$	$y = 0.1989\ x + 0.0238$
R^2	0.999	0.999
Standard error	0.154	0.109
LOD (µg/mL)	0.017	0.013
LOQ (µg/mL)	0.057	0.043

Quantification of aloin A and aloin B was carried out following linear regression at ten concentration levels (from LOQ to 570 µg/mL for aloin A and from LOQ to 430 µg/mL for aloin B, respectively). The residual plots indicated a random distribution of residuals around the horizontal axis suggesting that the linear model gave a good fit of the data. Moreover, the 95 % confidence interval of the intercepts included zero so that the intercepts were statistically not significant. The coefficients of determination (r^2) were all ≥ 0.999. Therefore, the method is considered linear and acceptable for quantifying eventually present aloins in the different test materials.

SH1, S1, HC, TO and P2 were spiked with aloins A and B at 4 levels. Next, those samples were analysed in triplicate and the percentage recoveries for the aloins were calculated (Table 3.2). The average recoveries were 98.5–101.6% for aloin A and 98.0–101.3% for aloin B. These were within the acceptable limit range of 98–102%.

Table 3.2: Recovery results (%) for aloin A and aloin B after spiking of a shampoo (SH1), soap (S1), hair conditioner (HC), treatment oil (TO) and powder (P2)

Spike level (µg/100 mg)	SH1		S1		HC		TO		P2	
	Aloin A	**Aloin B**	**Aloin A**	**Aloin B**	**Aloin A**	**Aloin B**	**Aloin A**	**Aloin B**	**Aloin A**	**Aloin B**
10	100.7	99.7	99.7	98.1	97.0	100.7	100.5	99.7	99.1	100.6
20	97.0	96.5	99.8	98.7	98.8	101.3	101.6	102.3	99.6	101.5
40	97.3	97.1	100.1	98.3	99.3	100.7	102.5	101.8	99.4	100.4
60	100.2	99.6	99.5	96.8	98.7	100.2	103.7	102.3	100.8	102.8
Mean	98.8	98.2	99.8	98.0	98.5	100.7	101.6	101.3	99.7	101.3
RSD (%)	1.9	1.7	0.3	0.9	1.0	0.4	1.3	1.2	0.8	1.1
95% CI	95.7-101.9	95.6-100.8	99.4-100.2	96.7-99.3	96.9-100.1	100.0-101.4	99.4-103.8	99.3-103.3	98.5-100.9	99.6-103.0

CI: confidence interval

Precision of the method was studied with respect to both within-day and between-day variations on the determination of aloins A and B in liquid and solid matrices. Test solutions were analysed using 2 preparations with 3 injections per preparation for two consecutive days with a total of 12 determinations per sample. The relative standard deviations (RSD) for the intra and inter-day precisions are shown in Table 3.3. RSD values lower than 2% indicate an acceptable precision of the developed method.

Table 3.3: Precision results for aloin A and aloin B after spiking

Sample	Formulation	Precision					
		Day 1 %RSD (*n* = 6)		Day 2 %RSD (*n* = 6)		Day 1-2 %RSD (*n* = 12)	
		Aloin A	Aloin B	Aloin A	Aloin B	Aloin A	Aloin B
SH1	Shampoo	0.4	0.1	1.3	0.4	1.4	1.8
S1	Soap	0.8	0.8	1.4	1.4	1.1	1.8
HC	Conditioner	1.2	0.9	1.3	1.7	1.3	1.7
TO	Oil	0.5	1.6	1.3	0.8	1.2	1.3
P2	Powder	0.8	0.9	1.2	1.1	1.0	1.0

3.3.1.3. Stability of sample solutions

The stability of spiked sample solutions was determined by storing them at room temperature for 24 h. After 24 h, the peak areas of aloins A and B in SH1, HC, TO and P2 and in the reference solution indicated an average difference of less than 1%. However, the peak areas of aloin A and aloin B in soap (S1) decreased by about 25% and 44%, respectively. A similar observation was made for soap S2. It has been described that the aloin stability significantly decreased at alkaline pH (4). This could be the reason for the decrease in aloin peak areas in soaps which were found to be alkaline.

Based on the above test results, solutions from the analysed skin care products were stable for 24 h except for the soap solutions. Hence, it is better to prepare the soap solutions immediately before analysis.

3.3.2. Analysis of commercial samples

Samples were prepared in duplicate and analysed in triplicate under the chromatographic conditions described in the method section. Sample chromatograms are shown in Figure 3.2.

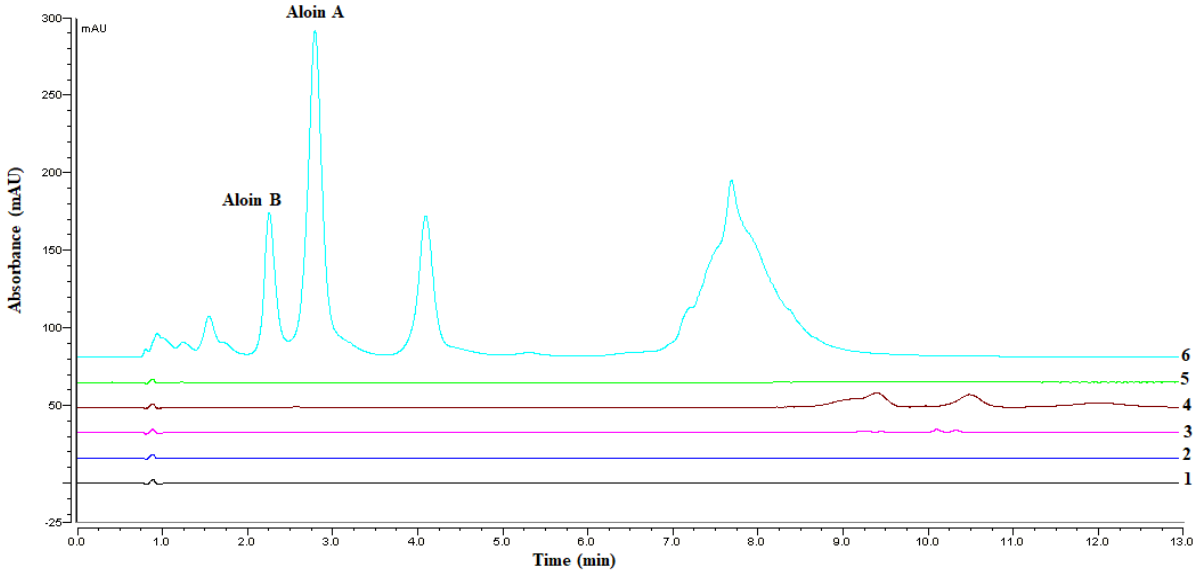


Figure 3.2: Overlay of sample chromatograms. 1: solvent blank, 2: shampoo (SH1), 3: soap (S1), 4: hair conditioner, 5: treatment oil, 6: powder (P2).

The results of this study revealed that aloins were detected in five of the twelve samples analysed (Table 3.4). All powder samples were found to contain aloin contents above the tolerable limit for cosmetic products. According to the cosmetic ingredient review expert panel, in Aloe-derived ingredients used in cosmetics, regardless of species, anthraquinone levels should not exceed 50 ppm or 0.05 mg/g (6). However, in this study, 25% of the products (i.e. the powders), were found to contain more than the maximum limit for aloins in cosmetic formulations. Visual inspection of the powder samples learned that latex parts could be noticed in P2 and P3. Also, the characteristic smell of aloe latex was observed. Finally, SH1 and S1 were found to contain traces of aloins. Hence, sufficient information should be included on the label describing the aloin content to safeguard users.

The pH and moisture content are also important parameters to check the quality of cosmetic products (13). Acid balanced shampoos and soaps are generally recommended by experts to maintain physiological skin and hair pH values (12). The appropriate pH of shampoo (pH 5.5 – 6.0) also helps in minimizing eye irritation, enhancing hair wellness and maintaining the physiology of the scalp (20). Results of this study revealed that 7 out of the 12 samples tested (58.3%) were found to have a pH within the range of the skin (pH 5 – 6) while 5 samples

(41.7%) had a basic pH deviating from the normal skin pH (Table 3.5). An alkaline pH is favorable for Propionibacterium, a bacterium involved in the pathogenesis of acne (12). Surprisingly, 3 soaps (S1, S3 and S4) promoted as anti-acne soaps, were found to show a basic pH.

Table 3.4: Aloin content (n = 6) of cosmetic products

Test sample	Formulation	Content	
		Aloin A, mean* (%RSD)	**Aloin B, mean* (%RSD)**
SH1	Shampoo	<LOQ	<LOQ
SH2	Shampoo	ND	ND
S1	Soap	<LOQ	<LOQ
S2	Soap	ND	ND
S3	Soap	ND	ND
S4	Soap	ND	ND
S5	Soap	ND	ND
P1	Powder	0.61 (1.4)	0.56 (1.3)
P2	Powder	195.8 (1.7)	64.3 (1.4)
P3	Powder	170.5 (1.7)	56.9 (1.1)
HC	Conditioner	ND	ND
TO	Oil	ND	ND

*****mg/g for solid samples and mg/mL for liquid samples; ND: not detected; <LOQ: below limit of quantification

The moisture content in soaps should be limited to avoid hydrolysis during storage leading to free fatty acids and glycerol. Moisture contents lower than 15% are in general considered as acceptable (13). The primary ingredient in all shampoos is water, which amounts to about 70-80% of the entire formula (21). From the loss on drying results, all soap samples fall within

these limits, while the shampoos, the hair conditioner and one powder (P1) were found to contain too much water (Table 3.5).

Table 3.5: Moisture content and pH values of aloe gel-based skin and hair care formulations

Sample	Type	pH	Moisture content (%) (% RSD, *n* = 3)
SH1	Shampoo	5	87.4 (0.1)
SH2	Shampoo	6	91.0 (0.1)
S1	Soap	8	12.6 (0.4)
S2	Soap	10	4.8 (4.3)
S3	Soap	9	7.1 (1.2)
S4	Soap	9	4.5 (6.1)
S5	Soap	10	4.9 (1.3)
P1	Powder	5	33.4 (0.1)
P2	Powder	5	8.6 (3.2)
P3	Powder	5	8.2 (2.5)
HC	Hair conditioner	6	94.3 (1.4)
TO	Oil	6	0.4 (0.1)

Reports from healthcare professionals and reports from consumers on adverse reactions are accepted as a serious source of information during pharmaco-vigilances of herbal medicines (17). This way, druggists, pharmacists, traditional medicine practitioners and consumers can help to avoid cosmetics with unwanted effects. So, cosmetic related adverse events were revealed by Jigjiga town residents in Eastern Ethiopia with allergic reactions, the appearance of acne, and hirsutism as the most common ones (10). For the samples used in this study, pharmacists and traditional healers mention regularly cases of adverse effects from their clients upon using Aloe powder.

According to the Cosmetics Labeling Guide, information on the outer container should be shared on a principal display panel (PDP) and information panels (18). Cosmetics that bear false or misleading label statements or that are not labelled in accordance with the requirements,

are considered misbranded and may be subject to regulatory actions (19). Moreover, therapeutic claims such as antifungal, antiperspirant, and/or disinfectant, removing lice, relieving itches, removing earwax and treating acne, haemorrhoids, or swimmers ear, are drug claims and prohibited. In this study, nine out of 12 products were found to exhibit misleading information on the label.

On the other hand, required information like species and part of plant used, origin, manufacturer, storing temperature and expiry date were often missing. Among the 12 cosmetic products selected, the powder formulations contain no expiry date, which could pose public health risks as consumers could use them at any time. The other products indicated a shelf life of 2-3 years with no clue on storing temperature. Such a case was reported on a correctional study where body care cosmetic products without ingredient label or expiry dates were sold in Jimma town, Ethiopia (7).

3.4. Conclusions

Most of the cosmetics studied here were found to contain aloin contents below the acceptance limit. However, adequate labeling should be considered taking into account the side effects and toxicities of aloin for those exceeding the limit (50 ppm), which was the case for 3 out of 12 samples. In terms of pH content and loss on drying, 42% of test samples were found to be in the basic pH range and 33% of them contained excessive moisture.

3.5. References

1. International Agency for Research on Cancer (IARC) monograph-108. Evaluation of Carcinogenic Risks to Humans. Lyon, France; 2016.
2. Lachenmeier K, Kuepper U, Musshoff F, Madea B, Reusch H, Lachenmeier DW. Quality control of *Aloe vera* beverages. Electron J Environ Agric Food Chem. 2005, 4(4): 1033–1042.
3. Brown PN, Yu R, Kuan CH, Finley J, Mudge EM. Determination of aloin a and aloin b in aloe vera raw materials and finished products by high-performance liquid chromatography: single-laboratory validation. *J AOAC Int.* 2014, 97(5): 1323–1328.
4. Ding WJ, Wu XF, Zhong JS, Wan JZ. Effects of temperature, pH and light on the stability of aloin A and characterization of its major degradation products. Int J Food Sci Technol. 2014, 49: 1773–1779.
5. Guo X, Mei N. Aloe vera: a review of toxicity and adverse clinical effects. *J Environ* Sci Health Pt C – Environ Carcinog Ecotoxicol Rev. 2016, 34: 77–96.

6. Cosmetic Ingredient Review (CIR). Final report on the safety assessment of *Aloe andongensis* extract, *Aloe andongensis* leaf juice, *Aloe arborescens* leaf extract, *Aloe arborescens* leaf juice, *Aloe arborescens* leaf protoplasts, *Aloe barbadensis* flower extract, *Aloe barbadensis* leaf, *Aloe barbadensis* leaf extract, *Aloe barbadensis* leaf juice, *Aloe barbadensis* leaf polysaccharides, *Aloe barbadensis* leaf water, *Aloe ferox* leaf extract, *Aloe ferox* leaf juice, and *Aloe ferox* leaf juice extract. Int J Toxicol. 2007, 26(2): 1–50.
7. Amasa W, Santiago D, Mekonen S, Ambelu A. are cosmetics used in developing countries safe? Use and dermal irritation of body care products in Jimma town, Southwestern Ethiopia. J Toxicol. 2012, 2012: 1-8.
8. Dibaba H, Yadesa D, Legesse B, Shewamene Z, W/Gerima B. Cosmetics utilization pattern and related adverse reactions among female university students. IJPSR. 2013, 4(3): 997-1004.
9. Meharie BG, Ambaye AS, T/haimanot YM, Atnafe SA. A cross sectional study on assessment of cosmetics utilization and self-reported adverse reactions among Wollo University Dessie campus female students, Dessie, North East Ethiopia. EJPMR. 2014, 2(2): 49-63.
10. Bilal AI, Tilahun Z, Osman ED, Mulugeta A, Shekabdulahi M, Derbew Fikadu Berhe DF. Cosmetics use-related adverse events and determinants among Jigjiga town residents, Eastern Ethiopia. Dermatol Ther (Heidelb). 2017,7: 143–153.
11. Krunali T, Dhara P, Meshram DB, Mitesh P. Evaluation of standards of some selected shampoo preparation. World J Pharm Pharm Sci. 2013, 2(5): 3622-3630.
12. Tarun J, Susan J, Suria J, Susan VJ, Criton S. Evaluation of pH of bathing soaps and shampoos for skin and hair care. Indian J Dermatol. 2014, 59(5): 442-444.
13. Mahesar SA, Chohan R, Sherazi STH. Evaluation of physico-chemical properties in selected branded soaps. Pak J Anal Environ Chem. 2019, 20(2): 177-183.
14. Sibhat GG, Kahsay G, Van Schepdael A, Adams E. Fast and easily applicable LC-UV method for analysis of bioactive anthrones from Aloe leaf latex. J Pharm Biomed Anal. 2021, 95:113834.
15. European Pharmacopoeia. 10th ed. Strasbourg, France: EDQM Council of Europe; 2021.
16. Wen KC, Chan SL, Liu IL, Lai PY, Lin YT, Hsiu SL, Chiag HM. An HPLC method for the simultaneous determination of marker compounds of *Aloe* and *Scutellariae* radix in cosmetics. J Food Drug Anal. 2010, 18(5): 328-338.

17. WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems. Geneva, Switzerland; 2004.
18. FDA; https://www.fda.gov/cosmetics/cosmetics-labeling-regulations/cosmetics-labeling-guide. (Accessed on 16 April 2021).
19. FMHACA; http://www.fmhaca.gov.et/wp-content/uploads/2019/03/cosmetics. (Accessed on 16 April 2021).
20. Gubitosa J, Rizzi V, Fini P, Cosma P. Hair care cosmetics: from traditional shampoo to solid clay and herbal shampoo, a review. Cosmetics 2019,6(13):1-16.
21. https://www.encyclopedia.com/science-and-technology/technology/technology-terms-and-concepts/shampoo. (Accessed on 16 April 2021).

Chapter 4: Quality of African moringa (*Moringa stenopetala*) leaf samples by liquid chromatography of phenolics, loss on drying and ash content

Gereziher Sibhat, Laura Díaz Montalvo, Getu Kahsay, Ann Van Schepdael, Erwin Adams

KU Leuven, Department of Pharmaceutical and Pharmacological Sciences, Pharmaceutical Analysis, Herestraat 49, O&N2, PB 923, 3000 Leuven, Belgium

J. Food Process. Preserv. (In print)

Abstract

The aim of this study was to evaluate the quality of African moringa (*Moringa stenopetala*) leaf preparations using easily applicable analytical methods. For this purpose, a simple and fast LC-UV method was developed and validated to determine the most common phenolic compounds. The method showed good linearity with determination coefficients (r^2) ≥ 0.999. Detection limits were 0.2, 0.06, 0.3 and 0.4 µg/mL, while quantification limits were 0.6, 0.2, 1.0 and 1.2 µg/mL for rutin, quercetin, caffeic acid and chlorogenic acid, respectively. Relative standard deviation (RSD) values for intra- and inter-day precision were less than 2% and recoveries were close to 100%. Robustness of the method was evaluated using an experimental design.

The developed method was applied to analyse some commercial moringa leaf samples obtained from Ethiopia. Unknown compounds were tentatively identified as neochlorogenic acid and kaempferol 3-O-rhamnosylglucoside (KRG) by means of mass spectrometry (MS). Rutin was found to be the most important compound. Its content in samples varied from 2 to 18 mg/g. The amounts of (neo)chlorogenic acid and KRG were less than 2 mg/g, while quercetin and caffeic acid were not detected. The method allowed to observe differences in phenolic composition between regions. Results for loss on drying and ash content revealed that 40% and 50%, respectively, of the samples were not compliant.

Keywords: Liquid chromatography; method validation; quality control; *Moringa stenopetala* leaves; phenolic compounds; MS characterization; loss on drying; ash content

4.1. Introduction

Moringa stenopetala (*M. stenopetala*), commonly called 'African moringa', is originating from several regions in Ethiopia and the northern part of Kenya. *Moringa* leaf preparations are widely used nowadays in tropical African countries as source of diet and as medicine. In the Ethiopian and Kenyan traditional medicine, *M. stenopetala* leaves, both fresh and as dried powder, are extensively employed for treating a range of illnesses such as diabetes, hypertension, stomach pain, malaria, leishmaniasis, leprosy, epilepsy, diarrhoea, asthma and colds (1). Although moringa leaf products are often promoted for their nutritional and medicinal values (2), different survey studies (3,4,5) reported an association between moringa leaf consumption and a negative effect on the thyroid function. Moreover, *in vivo* (mice and rat models) and *in vitro* studies (6, 7,8) reported dose and time dependent liver and cellular toxicity of moringa leaf extract.
M. stenopetala leaves are reported to contain bioactive phenolic compounds such as rutin, quercetin, quercitrin (quercetin 3-O-rhamnoside), isoquercitrin (quercetin 3-O-glucoside), chlorogenic acid or 3-O-caffeoylquinic acid (3-CQA), neochlorogenic acid or 5-O-caffeoylquinic acid (5-CQA) and caffeic acid (2,9,10). Variations in contents could be due to several reasons like environmental and agronomic conditions, harvest and processing operations, and storage factors (11).

Pharmacologically active phytoconstituents should serve as marker substances for standardization and quality control of herbal medicines (12,13). Rutin, a common dietary natural flavonoid, is the principal phenolic constituent in *M. stenopetala* leaves. Respectable levels of 2.3% in *M. stenopetala* dry leaves have been reported, making it commercially interesting and distinctively different from Indian Moringa (1). Moreover, Habtemariam correlated antidiabetic and antioxidant effects of *M. stenopetala* with rutin (major active compound) and neochlorogenic acid (minor antioxidant compound) (2). Hence, rutin was used as major bioactive marker compound to differentiate commercial moringa leaf samples.

Although plant flavonoids are described as common health-promoting and disease-preventing components with many potent biological properties ascribed to them, several *in vivo, in vitro*, and survey studies indicated possible toxicities and side effects of flavonoids. These could be individual and/or by interacting with several drugs and receptors upon (excessive) consumption (14-18). Rutin and its metabolic aglycone quercetin were reported to have mutagenic effects

(19). Moreover, an *in vivo* study with rats indicated the inhibitory effect of quercetin on the thyroid function (14).

Hence, analysis of the bioactive phenolic compounds in *M. stenopetala* is important to control the quality of different moringa leaf preparations to assure their safety and efficacy. Dessalegn and Rupasinghe studied the phenolic compounds and antioxidant activity of *M. stenopetala* using LC-MS (20). However, this equipment is not very convenient for routine analysis in developing countries regarding its complexity and costs. LC with ultraviolet detection (LC-UV) is the most preferred technique for quality control of herbal medicines. Bennett et al. published an ion-pair reversed phase LC method using gradient elution for tissue profiling of *M. oleifera* and *M. stenopetala* (10). The mobile phases consisted of 0.1% v/v trifluoroacetic acid in both water and methanol. The analysis time lasted 40 min. Another reversed phase LC-UV method was developed by Habtemariam and Varghese for the analysis of rutin (eluted at 26 min) in *M. stenopetala* leaves (1). The mobile phase composition was a mixture of water and methanol with the latter rising from 10% to 90% over a period of 50 min. To the best of our knowledge, there were no other studies describing an LC method for quality control of phenolic compounds in *M. stenopetala* leaves and no monograph for the analysis of *M. stenopetala* could be found in official compendia.

Loss on drying and ash content are among the mandatory tests recommended during quality check of herbal medicines. Excessive moisture is a sign of poorly dried and/or stored plant material. It has a negative impact on the stability and is conducive to microbial contamination. The total ash content points towards possible contamination by residues of extraneous matter (e.g. sand and soil) adhering to the plant surface while acid insoluble ash indicates the presence of silica, especially as sand and siliceous earth, which has a correlation with dangerous heavy metals (21,22).

In this study, a fast and sensitive LC-UV method using a monolithic column was developed and validated for the analysis of phenolic compounds in *M. stenopetala* leaves. This was supplemented with loss on drying, total ash and acid insoluble ash. Hence, the methods can be easily used in low-income countries like Ethiopia, where moringa leaf is commonly consumed, but rarely controlled due to the absence of a suitable analytical procedure.

4.2. Methods and materials

4.2.1. Materials and reagents

Moringa leaf samples (MLS) were purchased from local markets in Ethiopia. MLS 1 to 4 were obtained from the central and southern part of Ethiopia while MLS 5 to 10 were from the north. Analytical standards used during this study were: rutin trihydrate (95%) and chlorogenic acid (98%) from Thermo Fisher (Kandel, Germany) and quercetin (99%) and caffeic acid (98%) from Sigma-Aldrich (Steinheim, Germany). HPLC grade acetonitrile (ACN), methanol (MeOH), formic acid (99.9%) and hydrochloric acid (37%) were procured from Acros Organics (Geel, Belgium). Ultrapure water was obtained in-house using a Milli-Q system from Millipore (Bedford, MA, USA).

4.2.2. Methods

4.2.2.1. Preparation of solutions for chromatography

Coarse moringa leaf samples were powdered using an electric herb grinder (850 W, 50-300 mesh) and passed through a 180-mesh sieve to obtain homogeneity. In order to obtain uniform mixtures, different blends were prepared, each consisting of three moringa samples from the same geographic origin and mixed in the same proportion. The mixed samples were stored in sealed amber colored bottles at 4 to 8 $^{\circ}$C during the study. Before analysis, 100 mg of powdered sample was weighed (executed in triplicate and processed in parallel) and mixed with 3 mL of 50% aqueous methanol and sonicated for 20 min (Branson ultrasonic (100 W, 42 kHz), Danbury, USA). Extraction of each sample was repeated three times to ensure maximum recovery. The three supernatants were collected in a 10 mL volumetric flask and made up to volume with methanol-water (50:50, v/v). After that, an aliquot of each sample solution was filtered through a 0.45 μm Chromafil® Xtra membrane filter (Düren, Germany) into a 1.5 mL LC vial before analysis.

Standard stock solutions of rutin (0.5 mg/mL), caffeic acid (0.5 mg/mL) and chlorogenic acid (0.5 mg/mL) were prepared by adding methanol-water (50:50, v/v) and vortexed for 30 s. A quercetin solution (0.3 mg/mL) was prepared in methanol as it did not dissolve well in methanol-water (50:50, v/v). All solutions were further diluted with the same solvent to obtain a series of standard solutions for the establishment of calibration curves.

4.2.2.2. Instrumentation and chromatographic conditions

LC analyses were performed on a Merck-Hitachi apparatus (Darmstadt, Germany) equipped with an intelligent pump (L-6200), autosampler (L-2200) and UV/VIS detector (L-2400). For data processing and acquisition, Chromeleon software version 6.70 from Dionex (Sunnyvale, CA, USA) was used. Chromatographic separations were achieved on a Chromolith performance RP-18e (100 mm × 4.6 mm, i.d.) column from Merck (Darmstadt, Germany). During method development, a Zorbax Eclipse XDB C18 (250 mm × 4.6 mm, 5 µm) from Agilent (Santa Clara, CA, USA) was used too. A Julabo EM immersion thermostat (Seelbach, Germany) was used to keep the water bath of the column at 30 °C. The mobile phase was a gradient mixture of mobile phase A (0.1% formic acid in water) and B (acetonitrile) pumped at a flow rate of 1 mL/min. The gradient program (time (min), % B) was set as (0, 10), (1, 10), (12, 70), (15, 70), followed by some minutes to return to the initial mobile phase composition. The injection volume was 10 µL. Quantification was performed at a detection wavelength of 254 nm.

4.2.2.3. Chromatographic method validation

Following the ICH guidelines (23), the developed method was validated with respect to selectivity, sensitivity, linearity, precision, accuracy and robustness.

Selectivity of the developed method was examined for possible interferences by comparing chromatograms of standard solutions, moringa samples and the blank solvent consisting of methanol – water (50:50, v/v).

The limit of detection (LOD) and limit of quantification (LOQ) for rutin, quercetin, caffeic acid and chlorogenic acid were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The calibration curves for the quantification of these four compounds in the studied moringa samples were established by diluting the stock solutions of these reference standards. Six concentrations of standard solutions, namely, rutin (LOQ to 0.5 mg/mL), quercetin (LOQ to 0.3 mg/mL), caffeic acid (LOQ to 0.5 mg/mL) and chlorogenic acid (LOQ to 0.5 mg/mL), were analyzed in triplicate. The calibration curve was represented by the equation $y = ax + b$, where y represents the peak area and x the corresponding concentration (mg/mL) of the compound.

Precision of the method, expressed as RSD of peak areas, was evaluated by six repetitive injections of chlorogenic acid, caffeic acid, rutin and quercetin solutions. For the intra-day precision, injections were made on the same day. The inter-day precision of the method was

evaluated from 18 injections of the same solution over three consecutive days, where results of the third day were obtained by a different analyst.

Accuracy, expressed as percent recovery (equation 4.1) of the developed method, was determined at three levels by spiking 50, 100 and 150 µg/mL of standard solutions to a test sample. Solutions were analyzed in triplicate at each level and the percent recovery of the amount added to the sample was calculated.

$$\text{Recovery (\%)} = \frac{\text{total amount after spiking} - \text{amount original}}{\text{amount spiked}} \times 100 \qquad (4.1)$$

To evaluate the influence of LC operating conditions on the responses, a robustness study was performed using a two-level full factorial design and multivariate analysis using R software (version 3.6.3) from R Studio (Boston, MA, USA). In this study, three chromatographic factors were investigated at two levels (-1 and +1) around their central level (0). The number of runs was equal to $2^k + n$, where k is the number of factors and n is the number of times that the center point is repeated. Hence, 11 experiments, including 3 at the center point (nominal value), were performed in a random order (Table 4.1). As responses, the peak area of rutin as well as the resolution (Rs) between rutin and quercetin were selected.

Table 4.1: Chromatographic parameter settings applied in the experimental design of the robustness study.

Parameter	**Low value (−)**	**Central value (0)**	**High value (+)**
Column temperature (°C)	25	30	35
% Formic acid in mobile phase	0.09	0.1	0.11
% Acetonitrile in initial gradient step	9	10	11

The mathematical relationship between a response y and the experimental variables x_i, x_j, ... can be represented as first order equation (4.2):

$$y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \ldots + E \qquad (4.2)$$

where the letters β represent the regression coefficients and E is the overall experimental error. The linear coefficients β_i and β_j describe the quantitative effect of the experimental variables in the model while the cross coefficient, β_{ij}, measures the interaction effect between the variables x_i and x_j.

4.2.2.4. MS characterization of peaks

MS investigations were performed on a Bruker Esquire 3000 Plus ion trap mass spectrometer (GmbH, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Esquire control (version 5.2) and Bruker compass version 1.3 software were used to control the equipment and for data analysis, respectively. MS experiments were performed with the ESI source operated in negative ion mode, using a capillary voltage of 4.0 kV, end plate voltage of 200 V and nitrogen as nebulizing gas at 10.0 psi. Nitrogen gas was pumped into the ion source at a flow rate of 5 L/min and the dry gas temperature was set at 300 °C. The column effluent from the LC-UV corresponding to the most important peaks (i.e. ≥ 0.2 mg/g) was collected and infused into the MS using a Hamilton 0.5 mL syringe (Reno, NV, USA) and a KD Scientific syringe pump (Holliston, MA, USA). The syringe flow rate was set at 0.2 mL/h. Mass spectra were recorded in full scan mode in the range of 50 –1500 *m/z* and manual MS^n mode to obtain fragment ions.

4.2.2.5. Quantification of phenolics in moringa samples

The content of rutin, quercetin, chlorogenic acid and caffeic acid in the portion of moringa taken was determined using equation (4.3):

$$\text{Content} = \left(\frac{A_u}{A_s}\right) \times C_s \times \left(\frac{V}{W}\right) \qquad (4.3)$$

Where the content is expressed in mg of phenolic per g of MLS, A_u is the peak area for the respective bioactive marker compound from the sample solution, A_s is the peak area for the respective bioactive marker from the standard solution, and C_s is the concentration of the respective bioactive marker in the standard solution (mg/mL) taking also into account the purity of the standard, while V represents the final volume (mL) of the sample solution and W the weight of sample taken to prepare the sample solution (g). Unknown peaks were quantified using rutin as standard.

4.2.2.6. Loss on drying

Loss on drying of samples was determined following the European Pharmacopoeia (24) by taking 1.000 g of test sample and drying in an oven (Memmert ULE400, Schwabach, Germany) at 105 °C for 2 h. The percentage loss on drying of the samples was calculated using equation (4.4):

$$\%\ loss\ = \frac{weight\ \ before\ drying\ - weight\ after\ drying}{weight\ before\ drying} \times 100 \qquad (4.4)$$

4.2.2.7. Total ash and ash insoluble in hydrochloric acid

Total ash and acid insoluble ash of samples were determined following the procedure described in the European Pharmacopoeia (24). To determine the total ash, 1.00 g of powdered moringa leaf samples were examined by igniting to constant mass in a muffle furnace at 600 °C. Acid insoluble ash content of the samples was determined by adding 25 mL of hydrochloric acid (15%) to the crucible containing the residue from the determination of total ash. After the mixture was gently boiled for 10 min, it was cooled and filtered through a hardened ashless filter paper grade 540 (Whatman, England). The residue was washed with hot water until the filtrate was neutral. Then, the filter paper containing the insoluble matter was transferred to the original crucible, dried in an oven at 105 °C for about 1 h and ignited to constant mass until the difference between two consecutive weighings was not more than 1 mg. Finally, the percentage of acid insoluble ash was determined with reference to the sample weighed using equation (4.5):

$$\%\ ash\ = \frac{weight\ of\ ash}{weight\ of\ sample} \times 100 \qquad (4.5)$$

4.3. Results and discussion

4.3.1. Optimization of the chromatographic determination

The LC–UV method was initially developed for the simultaneous quantification of 4 phenolic compounds (chlorogenic acid, caffeic acid, rutin and quercetin) in *M. stenopetala* leaf samples. Those compounds were selected according to previous studies on moringa leaf samples and their availability as reference substances.

4.3.1.1. Extraction procedure

Factors such as solvent composition, extraction time, extraction temperature, solvent to solid ratio and extraction cycle may significantly influence the extraction efficiency (25, 26). In this study, to find optimum extraction conditions for phenolics, moringa leaf samples were extracted for 10, 20, 30 and 60 min using ethanol, water or 30, 50, 70 and 100% methanol. Each extraction was repeated 4 times. To enhance extraction, sonication was preferred over other techniques to reduce any loss of flavonoids due to oxidation, ionization, and hydrolysis during extraction that could be caused by conventional heating, boiling, or refluxing (27). An extraction by sonication using 50% methanol for 20 min was found to be effective for the selected compounds. The peak area of the major compound (rutin) in the extraction cycle was compared each time with the previous step. At the third extraction step, the peak area was found < 1% of the peak area in the second step. Hence, at that point the extraction was considered as complete so that the 4^{th} step was omitted in further experiments.

4.3.1.2. Chromatographic method

To select the initial chromatographic conditions, two methods for the analysis of phenolics in *M. stenopetala* leaves described in literature (1,10) were considered. Both papers used gradient elution with methanol as organic modifier and showed an analysis time of at least 40 min. Bennett et al. added trifluoroacetic acid as ion-pairing agent to the mobile phase. At the start of our study, effects due to mobile phase composition were evaluated using a Zorbax Eclipse XDB C18 column. It was found that more baseline drift and less well separated peaks were observed with methanol than with acetonitrile. Further, formic acid caused a more stable baseline than trifluoroacetic acid. So, further experiments were performed with acetonitrile as organic modifier and formic acid as volatile additive to install an acidic pH.

Next, the different samples were screened and sample 1 (MLS 1) was included for further method development since it yielded most peaks.

To reduce the analysis time, a monolithic column (Chromolith performance RP18e (100 mm × 4.6 mm)) was investigated. Compared to particle packed columns, monolithic columns show a lower backpressure so that they can be used with a higher flow rate, resulting in a shorter analysis time (28,29).

Next, various flow rates (i.e., 0.8, 1.0, 1.2 mL/min) and elution modes (i.e., isocratic and gradient with different mobile phase compositions) were investigated. A mixture of mobile phases A (0.1 % formic acid in water) and B (acetonitrile) using gradient elution at a flow rate

of 1.0 mL/min gave the best results in terms of separation efficiency. The low concentration of formic acid was added to mobile phase A to improve the peak shape of the phenolic compounds by restraining ionization. It was also found that at a column temperature of 30 °C, peaks were better separated compared to 25 and 35 °C. The detection wavelength was finally set at 254 nm as it was found to give a higher and more stable UV absorbance than 215, 270 and 350 nm, all selected after scanning the sample solution using a UV/VIS spectrophotometer in the range of 200 to 800 nm. An example chromatogram of sample 1 obtained under the final conditions is illustrated in Figure 4.1.

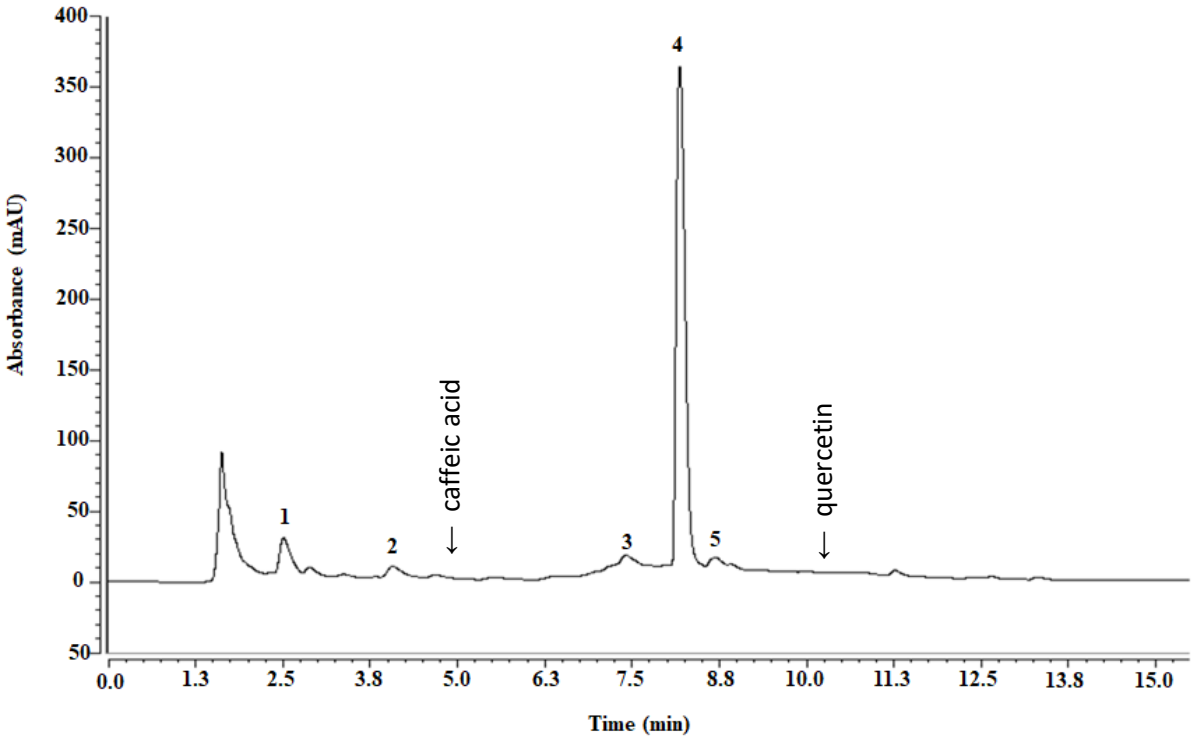


Figure 4.1: Chromatogram of moringa leaf extract (sample 1) obtained under the optimized chromatographic conditions. The elution times of caffeic acid and quercetin are indicated with an arrow, but were not encountered in any of the samples.
Peak 1: unknown 1, peak 2: chlorogenic acid, peak 3: unknown 2, peak 4: rutin, peak 5: unknown 3. Peaks 1 and 5 were later characterized by MS as neochlorogenic acid and kaempferol 3-O-rhamnosylglucoside, respectively.

Identification of chlorogenic acid, caffeic acid, rutin, and quercetin in the chromatogram was achieved by comparison of retention times with the respective reference.

Stability of the sample solution with respect to rutin (major bioactive marker) was verified at 0, 24, 48 and 72 h at room temperature. After 72 h, peak areas of rutin decreased by less than 2.0%, indicating that the sample solution was stable for 3 days.

4.3.2. Chromatographic method validation

Selectivity of the method was assessed by overlaying chromatograms of blank, standard solutions of rutin, quercetin, caffeic acid, chlorogenic acid and test sample. No interfering peaks were noticed at the retention times of rutin, quercetin, caffeic acid and chlorogenic acid, demonstrating the good selectivity of the method.

Table 4.2: Method validation results for chlorogenic acid, caffeic acid, rutin and quercetin

Compound	**Linear equation***	**Range (µg/mL)**	**r^2**	**recovery, % (% RSD, *n*=3)**	**LOD (µg/mL)**	**LOQ (µg/mL)**
Chlorogenic acid	$y = 0.165x - 0.192$	1.2 - 500	0.9997	99.3 (1.9)	0.4	1.2
Caffeic acid	$y = 0.262x - 0.731$	1.0 - 500	0.9998	100.0 (1.3)	0.3	1.0
Rutin	$y = 0.235x + 0.490$	0.6 - 500	0.9998	99.6 (0.9)	0.2	0.6
Quercetin	$y = 0.575x - 0.546$	0.2 - 300	0.9989	99.7 (2.3)	0.06	0.2

* with *y*: peak area and *x*: concentration (µg/mL)

Sensitivity of the method was evaluated based on the LOD and LOQ. The values indicated that the method was sensitive and enabled to quantify small amounts of phenolic compounds in moringa leaf samples (Table 4.2).

Linearity of the method was examined using linear regression of the peak areas versus analyte concentrations for rutin, quercetin, caffeic acid and chlorogenic acid. Results are given in Table 4.2. In addition, analysis of the residuals of the regression line indicated that they were randomly distributed around the horizontal zero axis, suggesting that the linear model gave a good fit of the data. Moreover, the 95% confidence interval of the intercepts included zero so that the intercepts were statistically not significant.

Precision of the method was checked by six repetitive injections of the standard solutions. Intra- and inter-day variations expressed as RSD of the peak areas of rutin, quercetin, caffeic acid and chlorogenic acid were assessed (Table 4.3). In all cases the RSD was less than 2.0%, indicating good precision of the method.

Accuracy of the method, determined as percent recovery, was close to 100% (Table 4.2) indicating that the marker compounds could be recovered completely from the leaf powder samples.

Table 4.3: Results of precision studies for peak areas of chlorogenic acid, caffeic acid, rutin and quercetin, expressed as %RSD.

Day	Chlorogenic acid	Caffeic acid	Rutin	Quercetin
Day 1 (n = 6)	1.2	1.2	1.3	1.8
Day 2 (n = 6)	1.7	1.5	0.8	1.4
Day 3 (n = 6)	1.4	1.0	1.8	1.3
Day 1–3 (n = 18)	1.8	1.9	1.6	1.6

In the robustness study, variations in LC operating conditions were made deliberately. The effect of small changes in the chromatographic parameters on the results was investigated by means of an experimental design. The results are shown in Figure 4.2 where each bar indicates the 95% confidence interval of the regression coefficient corresponding to the respective variable. It can be drawn from Figure 4.2 that the change of individual variables within the investigated ranges has no significant effect on the peak area of rutin as all intervals include zero (Figure 4.2(a)). Concerning the resolution between rutin and quercetin, the percentage of acetonitrile in the initial gradient step as well as the column temperature had a significant effect (Figure 4.2(b)). The column temperature showed a negative effect, which means that the resolution between the peaks of rutin and quercetin will decrease by increasing the temperature, and *vice versa*. The positive effect of the amount of acetonitrile implies that the resolution will increase by increasing the parameter, and *vice versa*. Concerning interactions between two variables, no significant effect was observed as all intervals included zero. Since quantification

is based on the peak areas, it is good that none of the factors examined was found to have an influence on the peak areas. In conclusion, it is important to consider the effect of temperature and acetonitrile when eventually transferring the developed method. The influence of temperature and acetonitrile on the peak area of rutin and the resolution between rutin and quercetin is illustrated in the response surface plots of Figure 4.3.

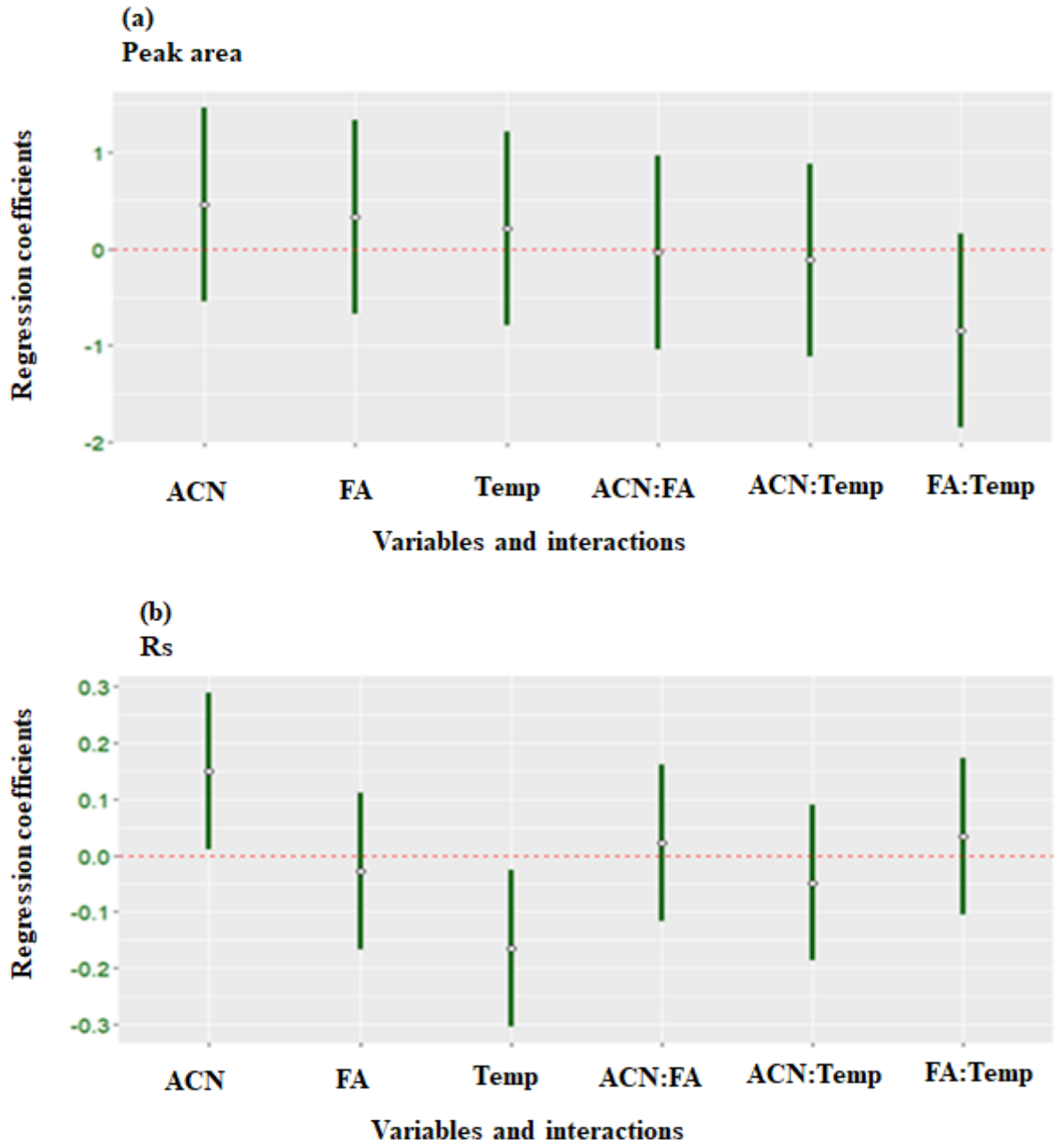


Figure 4.2: Regression coefficient plots obtained from the robustness study for (a) peak area of rutin; (b) Rs: resolution between rutin and quercetin. Temp stands for column temperature, ACN for acetonitrile and FA for formic acid.

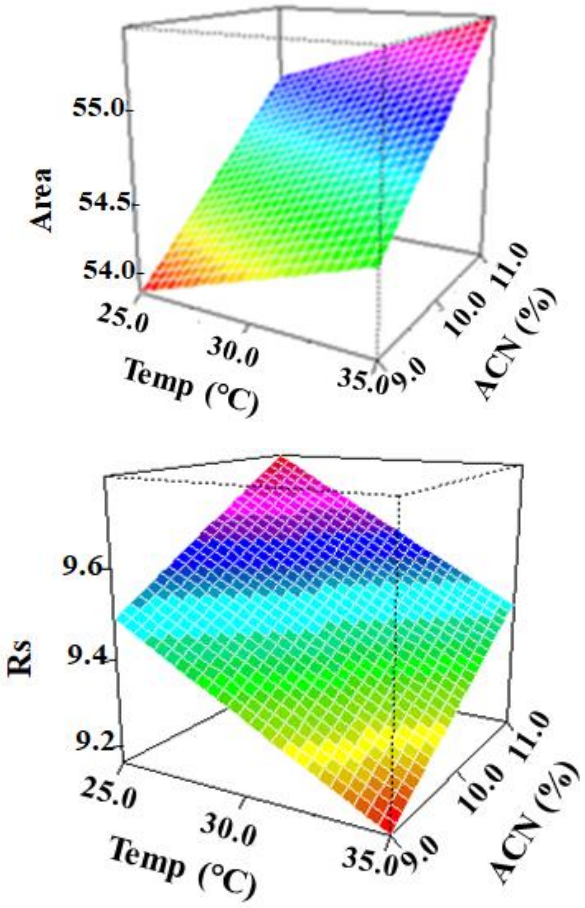


Figure 4.3: Response surface plots showing the influence of percentage of acetonitrile (ACN) and column temperature (Temp) on the peak area of rutin (upper) and the resolution (Rs) between rutin and quercetin (lower). Other parameters were kept constant at their central value.

4.3.3. MS characterization of chromatographic peaks

MS has the advantage of providing structural information about the eluted compounds based on their mass-to-charge ratio (*m/z*) and fragmentation behaviour. Negative ionization resulted in a higher sensitivity for phenolics than the positive mode (30). So, the former was used in this study. Mass spectra of chlorogenic acid and rutin, corresponding to peak 2 and peak 4 (Figure 4.1) respectively, were used as interpretative templates in an attempt to characterize unknown compounds (peaks 1, 3 and 5 in Figure 4.1).

Peak 2 (Fig. 4.1), which was detected in all the samples, produced a $[M–H]^-$ at *m/z* 353. Its MS/MS fragments at *m/z* 191 (= $[M - H – 162]^-$) and *m/z* 179 (= $[M - H – 174]^-$) indicated the presence of quinic acid and caffeic acid moieties. Additional fragment ions at *m/z* 135 (= [M −

H – 179 – 44]$^-$) could be due to neutral loss of CO_2 (Figure 4.4B). By comparing with the retention time and MS data of an authentic standard and the mass spectral data reported by (31) and (32), peak 2 was identified as 3-CQA or chlorogenic acid.

Peak 4 (Figure 4.1) was the major peak in all samples and produced a deprotonated molecule $[M – H]^-$ at *m/z* 609, indicating a relative molecular mass of 610. The MS/MS spectrum produced a predominant ion at *m/z* 300 indicating the combined loss of rhamnose (Rha) and glucose (Glu) due to homolytic cleavage of the 3-O-glycosidic bond. The radical aglycone ion at *m/z* 300 (quercetin), further fragmented to yield product ions at *m/z* 271, *m/z* 255, *m/z* 179 and *m/z* 151 (Figure 4.4C). These MS data were comparable with the papers of (15), (33) and (34). The latter proposed a fragmentation pathway for rutin explaining the different fragments that were formed. Hence, based on the retention time compared to the reference and mass spectral data, peak 4 was characterized as rutin.

The ESI-MS spectrum of Peak 1 (unknown 1, Figure 4.1) showed a precursor ion $[M - H]^-$ at *m/z* 353 (Figure 4.4A). Its MS/MS fragment at *m/z* 191 (= $[M - H – C_9H_6O_3]^-$) indicated the presence of a quinic acid residue and was formed by loss of caffeic acid. The spectrum also showed a fragment with very low abundance at *m/z* 179 (= $[M - H – C_7H_{10}O_5]^-$) corresponding to caffeic acid and formed by loss of quinic acid. These mass data were comparable with those reported by (31) and (32) for 5-CQA (or neochlorogenic acid). The small signal at *m/z* 179 is characteristic for 5-CQA and a diagnostic ion to differentiate from its 3-CQA isomer (31,32). So, peak 1 was tentatively identified as 5-CQA.

Peak 3 could not be characterized because the peak intensity was too low.

Peak 5 (unknown 3, Figure 4.1) showed a $[M – H]^-$ at *m/z* 593, which was 16 *u* less than peak 4 (rutin). The deprotonated molecule fragmented into the product ions at *m/z* 447 following the loss of a rhamnosyl moiety and a predominant ion at *m/z* 285 (= $[M - H – 308]^-$) by cleavage of the 3-O glycosidic bond indicating the loss of the disaccharide rutinose (combined loss of glucosyl and rhamnosyl moiety). This is characteristic for the flavonol called kaempferol (35, 34). MS^3 of the ion at *m/z* 285 lost 29 *u* (H + CO) to *m/z* 256, with further loss of CO (28 *u*) yielding the ion at *m/z* 228 (Figure 4.4D).

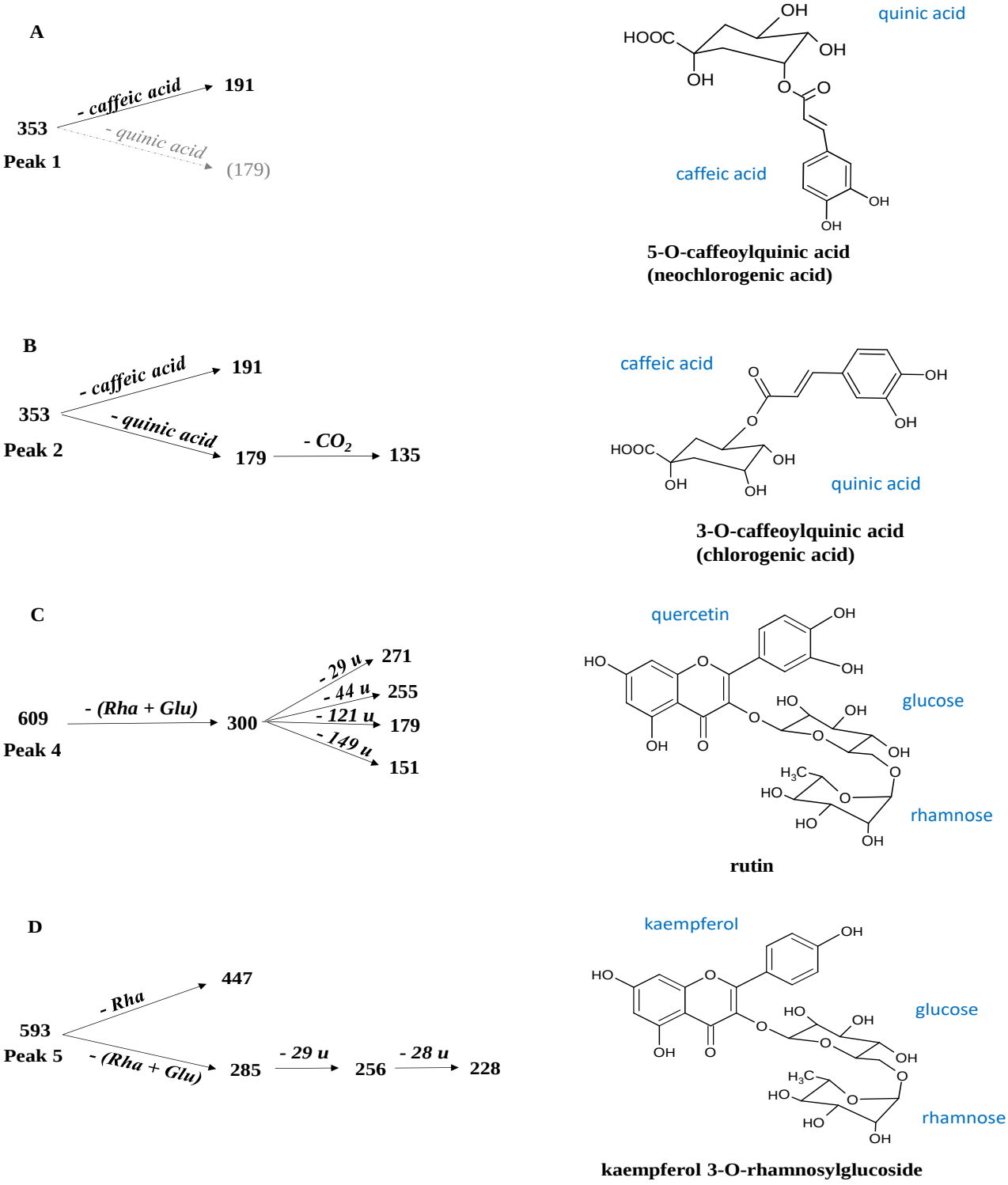


Figure 4.4: Schematic representation of the MS fragmentation of (A) unknown 1, (B) chlorogenic acid, (C) rutin and (D) unknown 3, corresponding to peaks 1, 2, 4 and 5 respectively in Figure 4.1, as well as their (proposed) structures.

The mass data were comparable with the report of (15) and (33) reporting fragments at *m/z* 255 and *m/*z 227. The difference of 1 *u* can be explained by the formation of a radical, as described for rutin by (34). So, unknown 3 was characterized as kaempferol 3-O-rhamnosylglucoside (KRG).

4.3.4. Evaluation of commercial samples

The developed LC method was applied for the quantification of neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, quercetin and KRG, as well as an unknown compound in moringa leaf samples collected from Ethiopia. Chromatograms of the samples are shown in Figure 4.5.

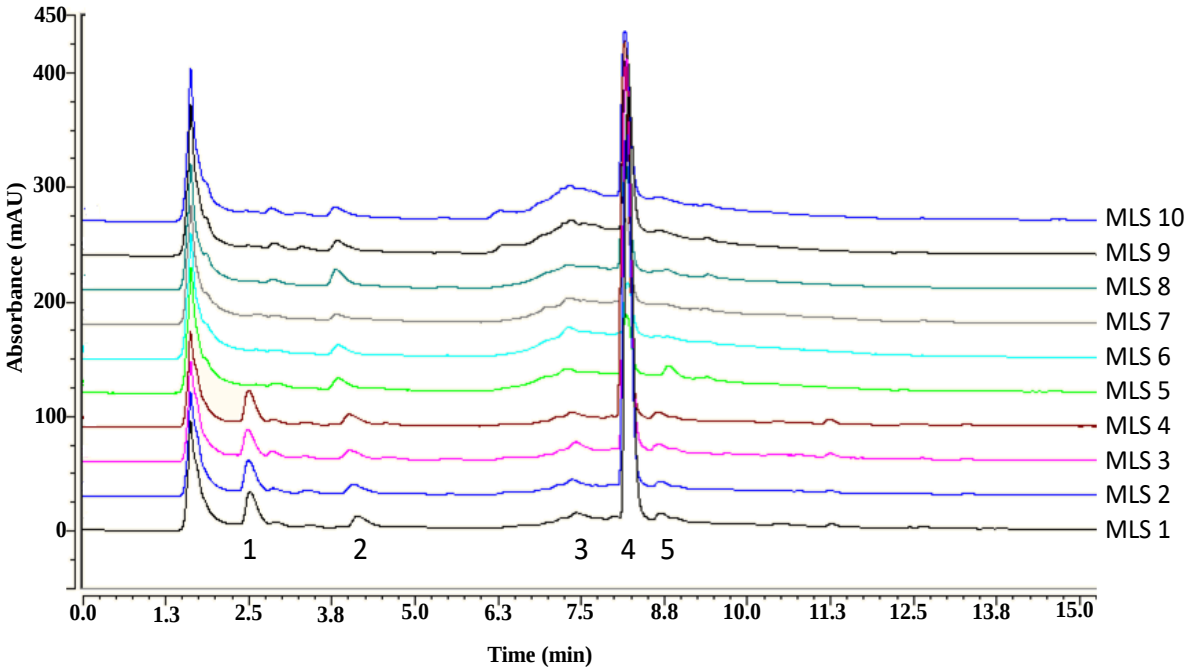


Figure 4.5: Chromatographic fingerprints of the 10 moringa leaf samples (MLS) that were investigated (peak numbering according to Figure 4.1).

Applying a reporting threshold of 0.2 mg/g, the results are summarized in Table 4.4. The content of rutin varied from 2.4 mg/g to 17.9 mg/g. This corresponds to the results of (1) who reported 2.3% (or 23 mg/g) of rutin in *M. stenopetala* leaves. Assuming a consumption of 150 g of moringa leaves per day, this results in maximum 2700 mg of rutin, which is clearly below the safety level of 2000 mg/kg body weight (36). Nevertheless, it would be good to indicate the

amount of rutin on the label of *M. stenopetala* leaf preparations so that people suffering from goiter can avoid the ones with higher amounts. Neochlorogenic acid, KRG and unknown 2 were present for not more than 2 mg/g (expressed as rutin), while quercetin and caffeic acid were not detected.

Table 4.4: Content of phenolic compounds in Ethiopian moringa leaf samples. Quercetin and caffeic acid were not detected. RSD was calculated on 3 values.

Sample	Neochlorogenic acid mg/g (% RSD)	Chlorogenic acid mg/g (% RSD)	Unknown 2 mg/g (% RSD)	Rutin mg/g (% RSD)	KRG mg/g (% RSD)
MLS 1	1.8 (1.1)	1.5 (0.2)	0.4 (1.6)	17.9 (1.5)	0.3 (0.7)
MLS 2	1.7 (1.1)	1.2 (0.5)	0.4 (1.1)	15.2 (0.6)	0.3 (0.5)
MLS 3	1.4 (1.8)	1.0 (1.1)	0.4 (0.4)	16.2 (1.9)	0.3 (1.0)
MLS 4	1.7 (1.5)	1.2 (0.8)	0.3 (1.2)	16.1 (1.5)	0.3 (0.9)
MLS 5	BT	1.3 (1.8)	0.2 (0.8)	2.7 (1.1)	0.5 (0.6)
MLS 6	BT	1.2 (0.9)	0.4 (0.3)	2.4 (1.4)	BT
MLS 7	BT	0.6 (0.8)	0.3 (1.7)	3.8 (0.5)	BT
MLS 8	BT	2.0 (0.9)	BT	5.1 (0.7)	BT
MLS 9	BT	1.3 (0.6)	BT	7.4 (1.5)	BT
MLS 10	BT	1.3 (1.0)	BT	7.8 (0.7)	BT

BT: below threshold (< 0.2 mg/g).

Concerning loss on drying, total ash and ash insoluble in hydrochloric acid, 10%, 14.0% and 2.0%, respectively, are specified as maximum tolerable limit for a given herbal drug, according to the technical guide for the elaboration of monographs on herbal drugs and herbal drug preparations of the EDQM (37). In this study, 4 out of 10 samples exceeded the limit for

moisture content, and 5 out of 10 resulted in excessive ash, while all samples complied for acid insoluble ash (Table 4.5).

Table 4.5: Loss on drying and ash content results of moringa leaf samples

Sample	Loss on drying (%)	Total ash (%)	Acid insoluble ash (%)
MLS 1	6.9	12.1	1.2
MLS 2	6.4	13.0	1.9
MLS 3	5.9	12.6	1.5
MLS 4	3.3	12.3	1.0
MLS 5	7.5	13.2	1.0
MLS 6	13.4	16.4	0.8
MLS 7	9.3	17.2	0.2
MLS 8	11.8	17.9	1.0
MLS 9	11.0	14.8	1.2
MLS 10	13.9	15.3	0.7

MLS 1 to 4 contain considerably higher amounts of rutin and neochlorogenic acid compared to MLS 5 to 10 (Table 4.4). In addition, their moisture content and total ash (Table 4.5) are lower. This can be explained by the fact that samples 1 to 4 originate from another geographical region than samples 5 to 10, as indicated in section 4.2.1.

4.4. Conclusions

In this work, a simple and sensitive LC-UV method was developed and validated for the potential determination of 6 bioactive phenolic compounds in *M. stenopetala* leaf samples. The analysis time was only 15 min. The method was applied to evaluate the quality of ten samples collected in Ethiopia. The content of rutin varied between 2 and 18 mg/g, while the contents of

four more compounds (including chlorogenic acid, neochlorogenic acid and KRG) were below 2 mg/g. Quercetin and caffeic acid were not detected. Further, 4 and 5 out of 10 samples did not comply for moisture and ash content, respectively. The proposed tests gave a good indication of the quality and allowed to distinguish between samples from different regions.

4.5. References

1. Habtemariam S, Varghese, GK. Extractability of Rutin in Herbal Tea Preparations of *Moringa stenopetala* Leaves. Beverages 2015, 1: 169–182.
2. Habtemariam S. Investigation into the Antioxidant and Antidiabetic Potential of *Moringa stenopetala*: Identification of the Active Principles. Nat. Prod. Commun. 2015, 10(3): 475 – 478.
3. Abuye C, Omwega AM, Imungi J K. Familial tendency and dietary association of goitre in Gamo-Gofa, Ethiopia. East Afr Med J. 1999, 76(8): 447–51.
4. Abuye C, Urga K, Knapp H, Selmar D, Omwega AM, Imungi JK, Winterhalter P. A compositional study of *moringa stenopetala* leaves. East Afr Med J. 2003, 80(5):51–56.
5. Golla AZ. Thyroid function profile, and its association to consumption of cassava and *Moringa stenopetala* in pregnant women. Adv. biol. chem. 2013, 3: 448–454.
6. Geleta B, Makonnen E, Debella A. Toxicological Evaluations of the Crude Extracts and Fractions of *Moringa stenopetala* Leaves in Liver and Kidney of Rats. J Cytol Histol 2016, 7(1): 1–6.
7. Mekonnen N, Houghton P, Timbrell J. The Toxicity of Extracts of Plant Parts of *Moringa stenopetala* in HEPG2 Cells *In Vitro*. Phytother. Res., 2005,19: 870–875.
8. Sileshi T, Makonnen E, Debella A, Tesfaye B. Antihyperglycemic and subchronic toxicity study of *Moringa stenopetala* leaves in mice. J Coast Life Med 2014, 2(3): 214–221.
9. Abo El-Fadl SR, Osman A, Al-Zohairy AM, Dahab AA, Abo El Kheir ZA. Assessment of total phenolic, flavonoid content, antioxidant potential and hplc profile of three moringa species leaf extracts. Scientific J. Flowers & Ornamental Plants 2020, 7(1): 53–70.
10. Bennett RN, Mellon FA, Foidl N, Pratt JH, Dupont MS, Perkins L, Kroon PA. Profiling Glucosinolates and Phenolics in Vegetative and Reproductive Tissues of the Multi-

Purpose Trees *Moringa oleifera* L. (Horseradish Tree) and *Moringa stenopetala* L. J. Agric. Food Chem. 2003, 51:3546–3553.

11. Tiwari U, Cummins E. Factors influencing levels of phytochemicals in selected fruit and vegetables during pre-and post-harvest food processing operations. Food Res. Int. 2013, 50: 497-506.
12. Li S, Han Q, Qiao C, Song J, Cheng CL, Xu H. Chemical markers for the quality control of herbal medicines: an overview. Chinese Med 2008, 3(7): 1-16.
13. World Health Organization (WHO). Fifty-first report of the WHO Expert Committee on Specifications for Pharmaceutical Preparations. WHO Technical Report Series, No. 1003. Geneva: World Health Organization; 2017.
14. Giuliani C, Bucci I, Santo SD, Rossi C, Grassadonia A, Piantelli M, Monaco F, Napolitano G. The flavonoid quercetin inhibits thyroid-restricted genes expression and thyroid function. Food Chem Toxicol. 2014, 66: 23–29.
15. Li Z-H, Guo H, Xu W-B, Ge J, Li X, Alimu M, He D-J. Rapid Identification of Flavonoid Constituents Directly from PTP1B Inhibitive Extract of Raspberry (*Rubus idaeus* L.) Leaves by HPLC–ESI–QTOF–MS-MS. J Chromatogr Sci. 2016, 54(5): 805–810.
16. Li Y, Ning J, Wang Y, Wang C, Sun C, Huo X, Yu Z, Feng L, Zhang B, Tian X, Ma X. Drug interaction study of flavonoids toward CYP3A4 and their quantitative structure activity relationship (QSAR) analysis for predicting potential effects. Toxicol Lett. 2018, 294: 27–36.
17. Ma Q, Kim E-Y, Lindsay EA, Han O. Bioactive Dietary Polyphenols Inhibit Heme Iron Absorption in a Dose-Dependent Manner in Human Intestinal Caco-2 Cells. J Food Sci. 2011, 76(5): H143–H150.
18. Morris ME, Zhang S. Flavonoid–drug interactions: Effects of flavonoids on ABC transporters. Life Sci. 2006, 78: 2116–2130.
19. Yang C-H, Huang Y-C, Chen C-Y. Degradation of rutin by *Thermoactinomyces vulgaris* and other thermophilic compost isolates. J Agric Food Chem. 2009, 57: 5095–5099.
20. Dessalegn E, Rupasinghe HPV. Phenolic compounds and *in vitro* antioxidant activity of *Moringa stenopetala* grown in South Ethiopia. Int. J. Food Prop. 2021, 24(1): 1681–1692.

21. Kim DG, Kim BS, Kim YC, Hwang YO, Chae YZ, Park SK. Selection of Herbal Medicines Requiring Quality Control for Loss on Drying, Total Ash, and Acid-insoluble Ash in Korea. Nat. Prod. Sci. 2011, 17(1): 38–44.
22. World Health Organization (WHO). Quality control methods for herbal materials. Geneva: World Health Organization; 2011.
23. ICH. Validation of Analytical Procedures: Text and Methodology Q2 (R1). London: EMA, 2006.
24. European Pharmacopoeia. 10th ed. Strasbourg, France: EDQM Council of Europe; 2020.
25. Bilek SE. The effects of time, temperature, solvent: solid ratio and solvent composition on extraction of total phenolic compound from dried olive (*Olea europaea* L.) leaves. The Journal of Food 2010, 35(6): 411– 416.
26. Tchabo W, Ma Y, Kwaw E, Xiao L, Wu M, Apaliya MT. Impact of extraction parameters and their optimization on the nutraceuticals and antioxidant properties of aqueous extract mulberry leaf. Int. J. Food Prop. 2018, 21(1): 717–732.
27. Xie Z, Sun Y, Lam S, Zhao M, Liang Z, Yu X, Yang D, Xu X. Extraction and isolation of flavonoid glycosides from Flos *Sophorae Immaturus* using ultrasonic-assisted extraction followed by high-speed counter current chromatography. J Sep Sci. 2014, 37: 957–965.
28. Khoo HT, Leow CH. Advancements in the preparation and application of monolithic silica columns for efficient separation in liquid chromatography. *Talanta* 2021, 224: 121777.
29. Sharma G, Tara A, Sharma VD. Advances in monolithic silica columns for high-performance liquid chromatography. J Anal Sci Technol 2017, 8(16): 1-11.
30. Willems JL, Khamis MM, Saeid WM, Purves RW, Katselis G, Low NH, El-Aneed A. Analysis of a series of chlorogenic acid isomers using differential ion mobility and tandem mass spectrometry. Analytica Chimica Acta 2016, 933: 164–174.
31. Rainha N, Koci K, Coelho AV, Lima E, Baptista J, Fernandes-Ferreira M. HPLC–UV–ESI-MS analysis of phenolic compounds and antioxidant properties of *Hypericum undulatum* shoot cultures and wild-growing plants. Phytochemistry 2013, 86:83–91.
32. Ncube EN, Mhlongo MI, Piater LA, Steenkamp PA, Dubery IA, Madala NE. Analyses of chlorogenic acids and related cinnamic acid derivatives from *Nicotiana tabacum* tissues with the aid of UPLC-QTOF-MS/MS based on the in-source collision-induced dissociation method. Chem. Cent. J. 2014, 8(66): 1–10.

33. Chen Y, Yu H, Wu H, Pan Y, Wang K, Jin Y, Zhang C. Characterization and Quantification by LC-MS/MS of the Chemical Components of the Heating Products of the Flavonoids Extract in Pollen Typhae for Transformation Rule Exploration. Molecules 2015, 20: 18352–18366.
34. Wang J, Ren X, Wen C, Xu Y, Chen Y. Separation and characterization of unknown impurities in rutin tablets using trap-free two-dimensional liquid chromatography coupled with ion trap/time-of-flight mass spectrometry. Rapid Commun Mass Spectrom. 2020, 34: e8739.
35. Farias LS, Mendez ASL. LC/ESI-MS method applied to characterization of flavonoids glycosides in *B. forficata* subsp. Pruinose. Quimica Nova 2014, 37(3): 483–486.
36. Tiwari R, Siddiqui MH, Mahmood T, Farooqui A, Bagga P, Ahsan F, Shamim A. An exploratory analysis on the toxicity & safety profile of polyherbal combination of curcumin, quercetin and rutin. Clin. Phytoscience 2020, 6(82): 1-18.
37. Technical guide for the elaboration of monographs on herbal drugs and herbal drug preparations. Strasbourg, France: EDQM Council of Europe; 2007.

Chapter 5: Fast and easily applicable LC-UV analysis of glucosinolates and phenolics in *Moringa stenopetala* leaf powder

Gereziher Sibhat, Getu Kahsay, Ann Van Schepdael, Erwin Adams

KU Leuven, Department of Pharmaceutical and Pharmacological Sciences, Pharmaceutical Analysis, Herestraat 49, O&N2, PB 923, 3000 Leuven, Belgium

Submitted to J. Herb. Med.

Abstract

In Ethiopia, *Moringa stenopetala* leaf powder is extensively used as food and medicine. These days, its use is still growing and being promoted as a potential plant to ensure food supply in case of shortage, as well as for preparation of nutraceuticals and herbal medicines. As a result, the demand for safety and quality product is an important issue. In this study, a fast and easily applicable LC-UV method was developed for simultaneous determination of glucosinolates and phenolics in *Moringa stenopetala* leaf powders. The method was linear with determination coefficients (r^2) ≥ 0.998. Detection limits were below 1.5 µg/mL and quantification limits below 5 µg/mL. Relative standard deviation (RSD) values for intra- and inter-day precision were less than 1.5% and recoveries were close to 100%. Besides the use of external standards, quantitative analysis of multicomponents by a single marker (QAMS), an emerging method for herbal materials analysis, was developed and applied to commercial moringa samples collected from Ethiopia. The correlation coefficient (r ≥ 0.9993) between the QAMS and the external standard method proved the consistency of the two methods. Rutin was used as single marker for the simultaneous determination of 5 bioactive components in *Moringa stenopetala* leaf samples. Furthermore, a radar plot was used to differentiate samples collected from different geographical origin. Analysis results of the commercial samples revealed that rutin was found to be the highest constituent (varying from 6.6 mg/g to 18.8 mg/g) among the phenolics while glucomoringin (varying from 0.2 mg/g to 4.2 mg/g) was the highest glucosinolate.

Keywords: Liquid chromatography; QAMS method; quality control; *Moringa stenopetala* leaves; phenolic compounds; glucosinolates, MS characterization

5.1. Introduction

Moringa stenopetala (*M. stenopetala*) belongs to the family of Moringaceae and is one of the 13 species of the genus *moringa* (1). *M. stenopetala*, commonly known as cabbage-tree or locally as *Aleko* or *Shiferaw* in Ethiopia, is a perennial, drought resilient, multipurpose food plant with nutritious leaves. It is often promoted as a potential plant for hidden hunger and consumed in different ways, including boiled fresh leaves and leaf powder used in tea or mixed with other dishes (2, 3). Nowadays, *M. stenopetala* is one of the greatest socioeconomic plants for nutritional and medicinal use in Ethiopia and Northern Kenya due to its ability to grow in poor soil and its short harvest time (4).

M. stenopetala leaves are reported to contain glucosinolates (GLSs) and phenolic compounds (5, 6, 7) which are widely reported for their diverse beneficial health effects. GLSs are sulfur-containing secondary plant metabolites considered as botanical biomarkers and used as bioindicators of herbal freshness (8) and food flavor (9). GLSs and their break down products called isothiocyanates (ITCs) (Figure 5.1) are known for the prevention and management of oxidative stress-related diseases due to their capacity to activate detoxification enzymes. ITCs derived from moringa GLSs are stable compared to ITCs found in other crops as they contain an additional sugar in their structure (10).

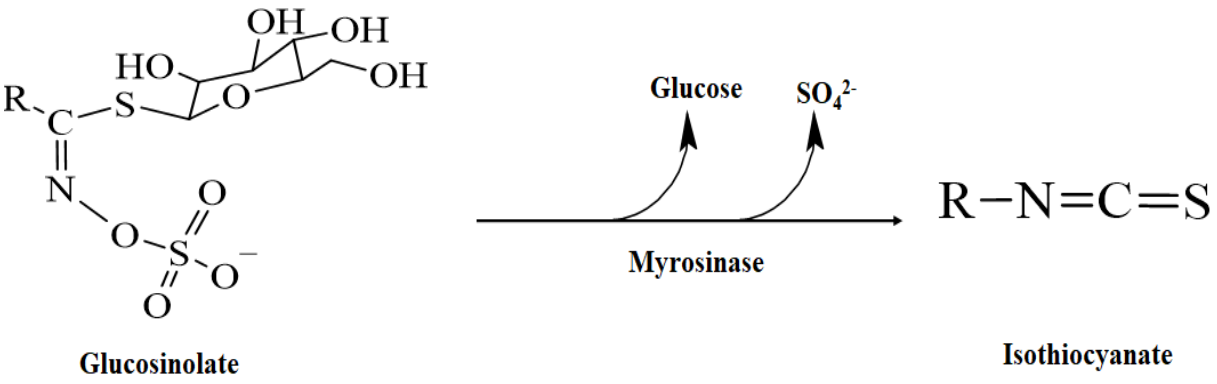


Figure 5.1. General structure of glucosinolates and their conversion to isothiocyanates.

In the intact plant cell, GLSs are stable as they are separately found from the major degrading enzymes (myrosinase isoenzymes). In cases of plant damage or stress during harvest, storage and processing, as well as during chewing by animals, enzymatic and non-enzymatic reactions

yield various transformation products, including ITCs, thiocyanates, nitriles, epithionitriles and oxazolidinethiones (11, 8).

In literature, some detrimental effects of GLSs and their ITCs have been described since they can interfere with the iodine uptake and the synthesis of thyroid hormones (triiodothyronine (T3) and plasma thyroxine (T4)), leading eventually to hypothyroidism and enlargement of the thyroid gland (goitre) (11). In addition to their potential to cause "cabbage" goiter, GLSs are also known for their antinutritive properties, especially indole glucosinolates (12). An *in vitro* study by Habza-Kowalska et al. (13) revealed that polyphenols such as chlorogenic acid, quercetin, and rutin are found to be thyroid peroxidase (TPO) inhibitors and are considered as etiological factors for hypothyroidism. Moreover, Giuliani et al., (14) reported an inhibitory effect of quercetin on the thyroid function in rats at 50 mg/kg/day i.p, for 14 days. These chemicals are also reported to be present in *M. stenopetala* leaves (5, 6, 15, 16).

The European Food Safety Authority (EFSA) recommends an extensive control on plants that contain GLSs, as type and content of GLSs are dependent on many factors such as species, geographic origin, plant preparation methods etc. (11). It also states that the GLSs content in vegetables expressed in terms of daily needs and activity should be limited to 20 mg (17).

Despite the high consumption of moringa leaves as food, medicine and nutraceutical, quality control to substantiate the claims is limited due to lack of validated analytical method for the determination of its bioactive chemicals such as GLSs and flavonoids. Hence, in this study, the most important GLSs and phenolic compounds were determined to ensure safety and efficacy of different moringa leaf preparations.

Bennett et al. (6) used an ion-pair LC/UV-Vis method to analyze GLSs in *M. stenopetala* leaf samples with a total analysis time of 60 min. However, focus was on profiling and no validation data were reported. Habtemariam (18) developed an LC-UV method for the analysis of moringin, an ITC of glucomoringin, in *M. stenopetala* seed samples with a total analysis time of 50 min. Furthermore, Mekonnen and Dräger (7) used an LC-ESI-MS method for the quantification of desulpho-glucosinolates in *M. stenopetala*. To the best of our knowledge, no validated LC method has been reported for the analysis of intact GLSs in *M. stenopetala* leaf powders. To avoid long analysis times, ion-pairing agents, complex sample preparation procedures and costly MS detectors, it would be interesting to develop a fast LC-UV method

that could be easily applied in low resource analytical laboratories like in Ethiopia. Hence, in this study, a fast and sensitive LC-UV method was developed and applied to determine simultaneously GLSs and phenolic compounds in moringa leaf powders from Ethiopia using external standards. Moreover, a quantitative analysis of multicomponents by QAMS has been developed as alternative method for quality control of moringa leaf preparations seen the scarcity and high cost of high-purity reference substances.

5.2. Methods and materials

5.2.1. *Materials and reagents*

Moringa leaf preparations (MLP) were purchased from local markets in Ethiopia. MLP1 to 4 were obtained from the central and southern part of Ethiopia while MLP 5 to 9 were from the North. Analytical standards used during this study were: rutin trihydrate (95%) and chlorogenic acid (98%) from Thermo Fisher (Kandel, Germany) and caffeic acid (98%) from Sigma-Aldrich (Steinheim, Germany), glucomoringin (99%) and progoitrin (98%) from Phytolab (Vestenbergsgreuth, Germany). To perform the LC analysis, HPLC grade acetonitrile (ACN), methanol (MeOH), and formic acid (99.9%) were procured from Acros Organics (Geel, Belgium) and ultrapure water was obtained in-house using a Milli-Q system obtained from Millipore (Bedford, MA, USA).

5.2.2. Preparation of sample and reference solutions

An amount of 50 mg of powdered *Moringa* samples, previously sieved (Fackelmann sieve 18/10) and stored in the freezer, were transferred into a 1.5 mL Eppendorf tube. Extraction was performed in duplicate by adding 1 mL of 80% MeOH at 70°C (sample to solvent ratio 1:20 w/v), vortexing for 10 s and using an Eppendorf thermo mixer (Hamburg, Germany) maintained at 1400 rpm for 30 min. After being cooled, the supernatants were combined and filtered using a PTFE filter (0.45 µm) into an HPLC vial for analysis. To optimize the extraction procedure, progoitrin (200 µL of 0.1 mg/mL) and quercetin (200 µL of 0.4 mg/mL) dissolved in 80% MeOH were used as glucosinolate and phenolic extraction standards, respectively. MLP1 (Arba Minch organic moringa) was used for method development as it was found to contain a higher number of peaks. Moreover, the product was properly labeled and most commercialized compared to the other samples collected.

Stock solutions of progoitrin, glucomoringin, chlorogenic acid, caffeic acid and rutin references were prepared by dissolving each reference in 80% methanol and the working solutions were

prepared from the standard stock solutions by mixing an appropriate volume with water to the desired concentrations for the construction of calibration curves. All solutions were stored at -20 °C before use.

5.2.3. LC operating conditions

LC analyses were performed on a Merck-Hitachi apparatus (Darmstadt, Germany) equipped with an intelligent pump (L-6200), autosampler (L-2200) and UV/VIS detector (L-2400). For data processing and acquisition, Chromeleon software version 6.70 from Dionex (Sunnyvale, CA, USA) was used.

All samples were analyzed in triplicate using a Luna C18 column (150×4.6 mm, 5 µm) from Phenomenex (Torrance, CA, USA). The mobile phase solutions and extraction solvent were degassed using helium (Air Liquide, Brussels, Belgium). A Julabo EM immersion thermostat (Seelbach, Germany) was used to keep the water bath of the column at 30 °C. The mobile phase was a gradient mixture of mobile phase A (0.1% formic acid in water) and B (acetonitrile) pumped at a flow rate of 1 mL/min. The gradient program (time (min), % B) was set as (0, 7), (2, 7), (10, 20), (17, 70), (18, 7) and (20, 7). The injection volume was 10 µL and the detection wavelength was set at 230 nm.

5.2.4. Method validation

Following the ICH guidelines (19), the developed LC-UV method was validated with respect to selectivity, sensitivity, linearity, precision, accuracy, and robustness. Linear calibration curves were constructed by six different concentrations of mixed standard solutions. Limits of detection (LODs) and limits of quantification (LOQs) of progoitrin, glucomoringin, chlorogenic acid, caffeic acid and rutin were determined at signal-to-noise ratios of 3 and 10, respectively. Intra- and inter-day precision of peak areas expressed as relative standard deviations (RSDs) were evaluated by injecting six sample solutions on 3 days. Accuracy of the method expressed as percentage recovery (equation (5.1)) was evaluated by adding a known amount of standard solutions at different concentration levels.

$$\text{Recovery (\%)} = \frac{\text{total amount after spiking - amount original}}{\text{amount spiked}} \times 100 \qquad (5.1)$$

To evaluate the effect of slight changes in LC operating conditions on the outcome variables, a robustness study was performed using an experimental design with three chromatographic factors at two levels (-1 and +1) around their central level (0). The number of runs was equal to $2^k + n$, where k is the number of factors and n is the number of times that the center point was repeated. Hence, 11 experiments, including 3 at the center point (nominal value), were performed in a random order (Table 5.1). The areas of glucomoringin and rutin were selected as response factors. Besides, the resolution (Rs1) between UNK1 and glucomoringin as well as the resolution (Rs2) between glucomoringin and neochlorogenic acid were also evaluated.

The mathematical relationship between a response y and the experimental variables x_i, x_j, ... can be represented as first order equation (5.2):

$$y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \ldots + E \quad (5.2)$$

where the letters β represent the regression coefficients and E is the overall experimental error. The linear coefficients β_i and β_j describe the quantitative effect of the experimental variables x_i and x_j in the model while the cross coefficient, β_{ij}, measures the interaction effect between the variables x_i and x_j.

The stability of the sample and standard solutions at room temperature were examined at 0, 2, 4, 6, 8, 12, and 24 h after preparation of the solution.

Table 5.1: Chromatographic parameter settings applied in the experimental design of the robustness study.

Parameter	**Low value (−)**	**Central value (0)**	**High value (+)**
Column temperature (°C)	28	30	32
% Formic acid in mobile phase	0.09	0.1	0.11
% Acetonitrile in mid gradient step	19	20	21

5.2.5. MS characterization of peaks

Full scan MS spectra were obtained using a Bruker Esquire 3000 Plus ion trap mass spectrometer (Bremen, Germany) equipped with an electrospray ionization (ESI) source

controlled by Esquire control (version 5.2). For data analysis Bruker compass version 1.3 software was used. The ESI source was operated in negative ion mode and MS operating conditions were optimized by infusing glucomoringin standard (10 µg/mL) for glucosinolates and rutin (10 µg/mL) standard for phenolics at a flow rate of 180 µL/h. MS conditions were as follows: capillary voltage; 4.0 kV, nebulizing gas pressure; 15.0 psi, dry gas flow rate; 5 L/min, dry gas temperature; 300 °C, capillary exit voltage; 208 V, trap drive; 66 V, Oct 2 dc; 1.1 V. Accumulation time was set at 25 ms. For characterization of the peaks, the column effluent from the LC-UV corresponding to the most important peaks (i.e. ≥ 0.2 mg/g) was collected and infused into the MS using a Hamilton 0.5 mL syringe (Reno, NV, USA) and a KD Scientific syringe pump (Holliston, MA, USA). The syringe flow rate was set at 180 µL/h. Mass spectra were recorded in full scan mode in the range of 50–1500 *m/z* and manual MS^n mode to obtain fragment ions.

5.2.6. Quality evaluation of moringa leaf samples

5.2.6.1. External standard method

The content of GLSs and phenolics in the portion of moringa leaf taken was first determined by applying the external standard method (ESM) using equation (5.3):

$$\text{Content} = \left(\frac{A_u}{A_s}\right) \times C_s \times \left(\frac{V}{W}\right) \qquad (5.3)$$

Where the content is expressed in mg of bioactive marker per g of MLP, A_u is the peak area for the respective bioactive marker compound from the sample solution, A_s is the peak area for the respective bioactive marker from the standard solution, and C_s is the concentration of the respective bioactive marker in the standard solution (mg/mL) taking also into account the purity of the standard, while V represents the final volume (mL) of the sample solution and W the weight of sample taken to prepare the sample solution (g). Unknown peaks were quantified using rutin for phenolics and glucomoringin for GLSs as standards.

5.2.6.2. QAMS

In a second stage, rutin was selected as single marker since it is an easily available and affordable component of *M. stenopetala* leaves. It was used to calculate the relative correction factor (RCF) (equation (5.4)) and content (equation (5.5)) of progoitrin, glucomoringin, chlorogenic acid and caffeic acid.

$$f_{si} = \frac{A_s/C_s}{A_i/C_i} = \frac{a_s}{a_i} \qquad (5.4)$$

$$C_i = \frac{A_i \times C_s}{A_s} \times f_{si} \qquad (5.5)$$

where f_{si} is the correction factor for component "*i*" versus the single marker "*s*", A_s is the peak area of the single marker, C_s is the concentration of the single marker, A_i is the peak area of the tested component and C_i is the concentration of component *i*. The ratio of a_s and a_i is also a measure of f_{si} where a_s is the slope of the calibration curve of the single marker and a_i is the slope of the calibration curve of the measured component.

5.3. Results and discussion

5.3.1. Optimization of the extraction procedure

Until now, few studies reported on the analysis of GLSs and phenolics in *M. stenopetala* leaves. However, all of them used different extraction methods (6, 7, 15, 20). To prevent myrosinase activity and subsequent loss of glucosinolates, extraction using 70-80% cold or hot MeOH or hot water (60–100°C) are reported as effective extraction methods to keep the GLSs intact (8, 21, 22, 23). In this study, to compare extraction efficiencies, 50 mg of sample was extracted using 1 mL of 80% methanol, at room temperature or at 70°C. All samples were extracted for 30 min. This was repeated three times to maximize yield. Although methanol extracts at room temperature were found to contain the targeted GLSs and phenolics, the yield was less compared to warm methanol extracts. Hence, 80% methanol at 70°C was preferred as extraction solvent for the samples.

Measurement of desulfoglucosinolates, an alternative method for analyzing GLSs, was considered as time consuming due to its several sample processing steps such as enzymatic desulfation (24). Moreover, according to Förster et al. (25) desulfo glucosinolate extraction resulted in the production of artifacts and loss of acetylated GLSs. So, extraction of intact GLSs was recommended.

5.3.2. LC operating conditions

Optimization of chromatographic parameters was done to reduce the overall analysis time while considering good separation among the GLSs and phenolics. Two solvent systems consisting of water – methanol and water – acetonitrile as mobile phases were tested during the preliminary studies. The combination of water and acetonitrile was found to give better separation of GLSs

and phenolics. To minimize ionization of GLSs and phenolics and enhance separation, 0.1% v/v of formic acid or 0.05% v/v of trifluoroacetic acid (TFA) were added. However, formic acid produced a more stable baseline than trifluoroacetic acid. As a result, formic acid was preferred as volatile additive to install an acidic pH. After screening different samples, MLP1 was employed for further method development as it yielded more peaks. A Luna C18 (150 mm × 4.6 mm, 5 µm) column was used for the analysis as it was found to retain better early eluting analytes, especially the targeted glucosinolates, compared to a monolithic column (Chromolith performance RP18e (100 mm × 4.6 mm)).

Better LC performance was achieved using a gradient mixture of mobile phase A (0.1% formic acid in water) and B (acetonitrile). The fine tuned gradient program is described in section 5.2.3. The injection volume was set at 10 µL as it was found to give better separation between analytes compared to 5 µL and 20 µL. Simultaneous determination of GLSs and phenolics was performed at a detection wavelength of 230 nm as it was found to give better and more stable absorbance for the GLS than at 215, 227 or 254 nm. Phenolics are typically detected at 254 nm, but since their absorbance was comparable with this at 230 nm, the latter was selected here. A better separation of peaks was achieved at a column temperature of 30 °C compared to 25 and 35 °C. Using the optimum chromatographic conditions, it was possible to separate the five bioactive compounds within 20 min as shown in the chromatogram of Figure 5.2.

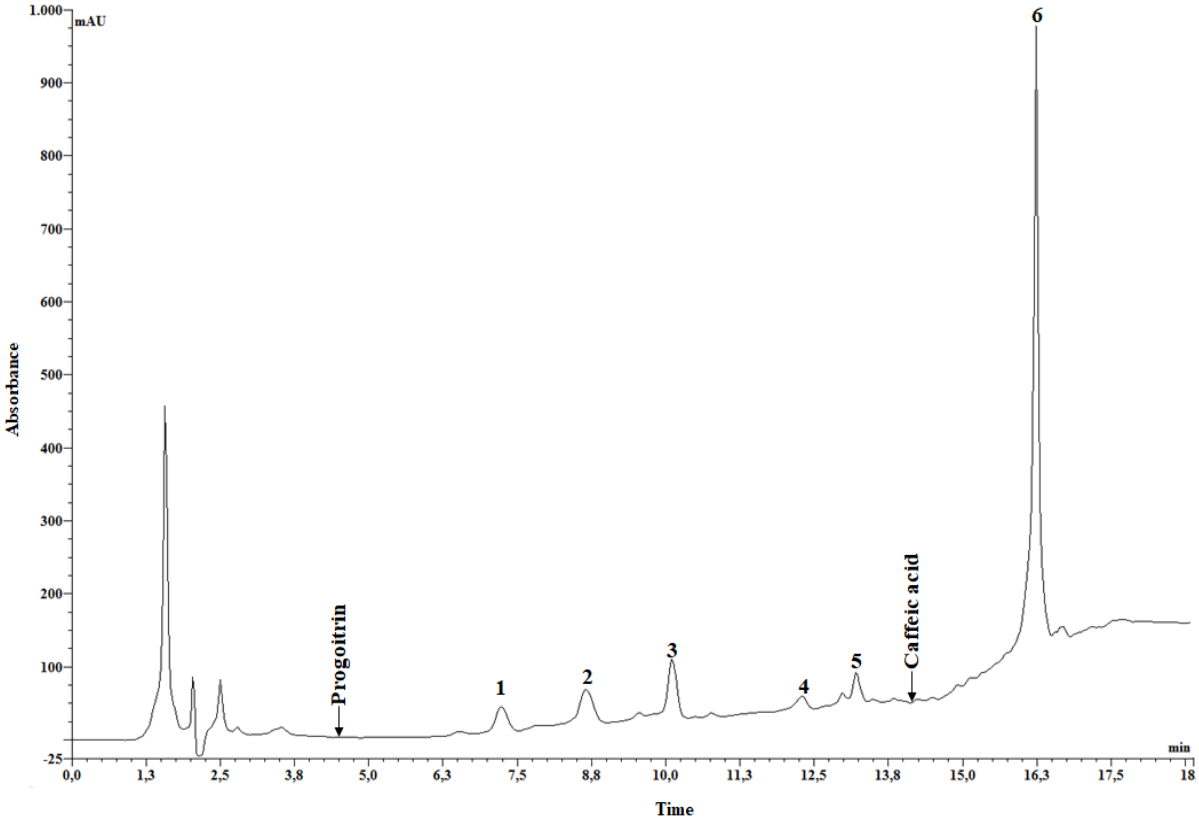


Figure 5.2: Chromatogram of *M. stenopetala* leaf extract (MLP1) under optimized chromatographic conditions (see section 5.2.3.). 1: UNK1, 2: glucomoringin, 3: neochlorogenic acid, 4: acetylated glucomoringin (see also section 5.3.4.), 5: chlorogenic acid, 6: rutin. Progoitrin and caffeic acid were not detected in any of the samples, but the position of their peaks in the chromatogram are indicated with an arrow.

5.3.3. HPLC method validation

5.3.3.1. Linearity, LOD and LOQ

The calibration curves of the five reference compounds were constructed using a series of dilutions of mixed standard solutions. Then the peak areas (y) vs. concentrations (x) were plotted to obtain a calibration curve ($y = ax + b$). Results are illustrated in Table 5.2. The high determination coefficient values ($r^2 > 0.998$) showed a good linear relationship over the concentration range examined. The limits of detection (LOD) and limits of quantitation (LOQ) were determined at signal-to-noise ratios of 3:1 and 10:1, respectively. The low values of LOQ and LOD indicated that the method is sensitive and capable of determining small quantities of the compounds in the samples.

Table 5.2: Linearity, limit of quantification (LOQ), limit of detection (LOD) and relative correction factors (RCFs) *vs.* rutin for progoitrin, glucomoringin, chlorogenic acid and caffeic acid (n = 3)

Analyte	**Linear equation***	**Range (µg/mL)**	**r^2**	**LOD (µg/mL)**	**LOQ (µg/mL)**	**RCFs**
Progoitrin	$y = 0.150\,x - 0.184$	1.3 - 200	0.9995	0.45	1.3	1.437
Glucomoringin	$y = 0.273\,x + 0.585$	5.0 - 200	0.9986	1.5	5.0	0.792
Chlorogenic acid	$y = 0.214\,x - 0.509$	2.5 - 200	0.9988	1.0	2.5	1.022
Caffeic acid	$y = 0.434\,x - 0.925$	2.5 - 200	0.9986	0.8	2.5	0.484
Rutin	$y = 0.216\,x - 1.189$	2.5 - 600	0.9996	1.0	2.5	1.000

* with y: peak area and x: concentration (µg/mL)

5.3.3.2. Stability, precision and accuracy

Stability of the samples (MLP1) and standard solutions were investigated by storing them at room temperature for 0, 2, 4, 6, 12 and 24 h. The results indicated that all solutions were stable for at least 24 h. Intra-day precision of the method was checked by six repetitive injections of standard solutions containing progoitrin, glucomoringin, chlorogenic acid, caffeic acid and rutin while the inter-day precision was evaluated over 3 days. RSD values were not exceeding 1.5%, indicating acceptable precision of the method. Accuracy of the method, determined as percent recovery, was close to 100% indicating that the compounds could be recovered completely from the leaf powder samples (Table 5.3).

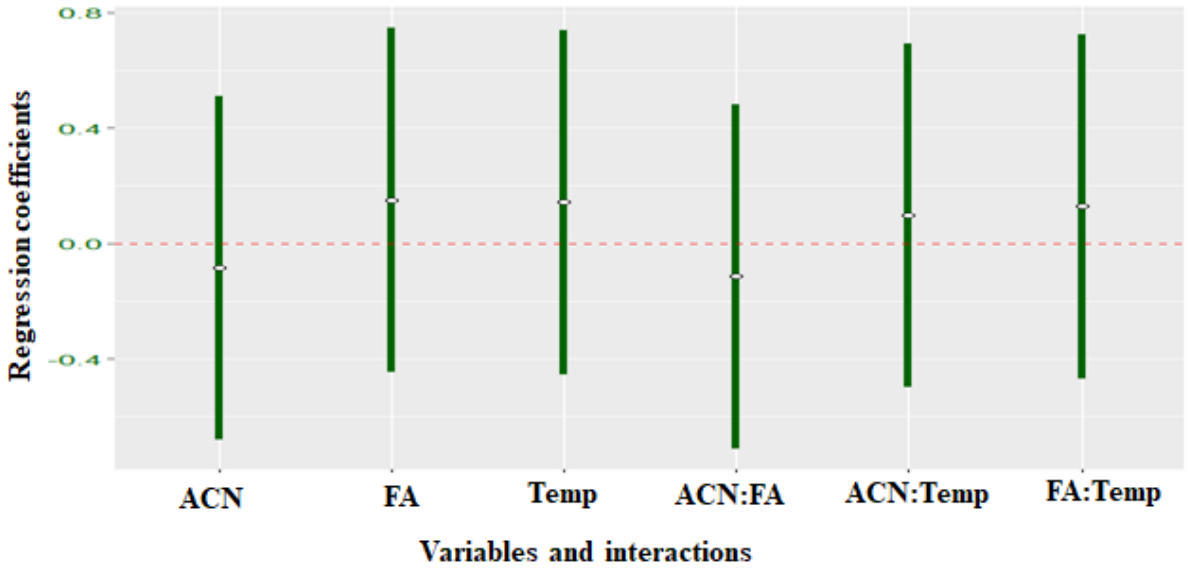
Table 5.3: Precision, accuracy and stability test results for progoitrin, glucomoringin, chlorogenic acid, caffeic acid and rutin

Analyte	Precision (%RSD)		Stability (%RSD, *n* = 3) 24 h RT	%Recovery (%RSD, *n* = 3)
	Intra-day (*n* = 6)	Inter-day (*n* = 18)		
Progoitrin	1.3	0.8	0.1	100.6 (2.8)
Glucomoringin	1.4	1.0	1.2	101.4 (2.4)
Chlorogenic acid	0.7	1.2	1.0	98.6 (3.8)
Caffeic acid	1.5	1.2	0.2	99.9 (0.2)
Rutin	1.1	1.2	1.3	98.5 (0.6)

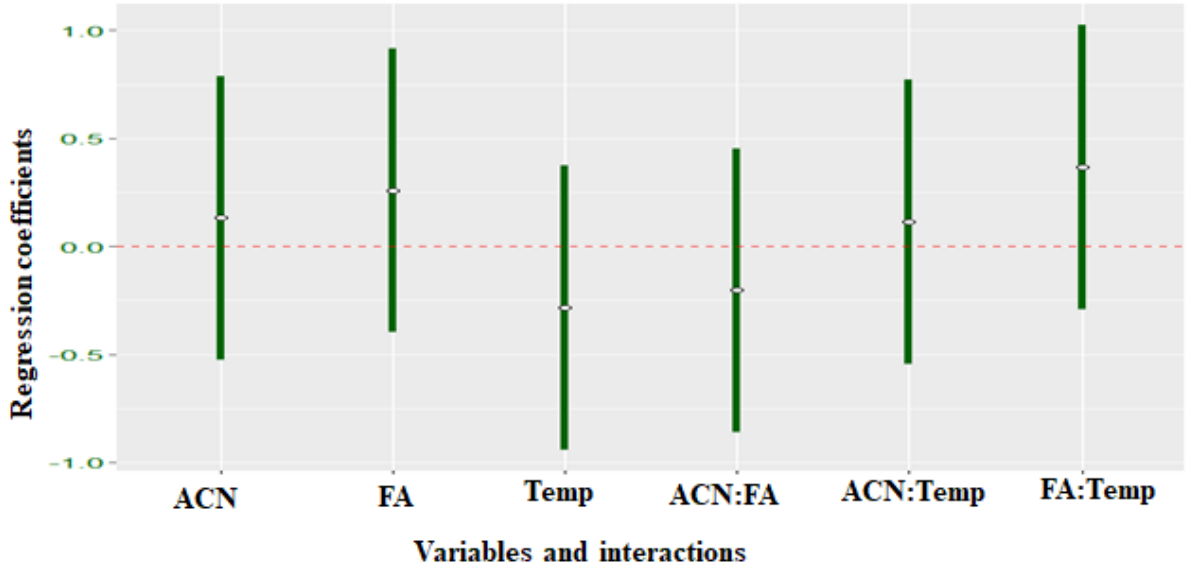
5.3.3.3. Robustness

The robustness of the developed method towards small changes that could be caused when changing between laboratories, instruments or analysts was investigated by intentionally varying the LC operating conditions. As can be seen in Figure 5.3 (a) and (b), the change of individual variables within the investigated ranges, has no significant effect on the peak area of glucomoringin as well as on the peak area of rutin since all intervals include zero. However, it was found that the resolution between the peaks due to UNK1 and glucomoringin (Rs1) (Figure 5.3(c)) was found to be negatively affected by the percentage of formic acid in the mobile phase. On the other hand, the resolution between the peaks due to glucomoringin and neochlorogenic acid (Rs2) was impacted positively by the percentage of formic acid in the mobile phase and negatively by a change in column temperature (Figure 5.3(d)). Hence, by increasing the percentage of formic acid, the resolution between the peaks of UNK1 and glucomoringin will decrease while the separation between glucomoringin and neochlorogenic acid will increase and *vice versa*.

(a) Peak area glucomoringin



(b) Peak area rutin



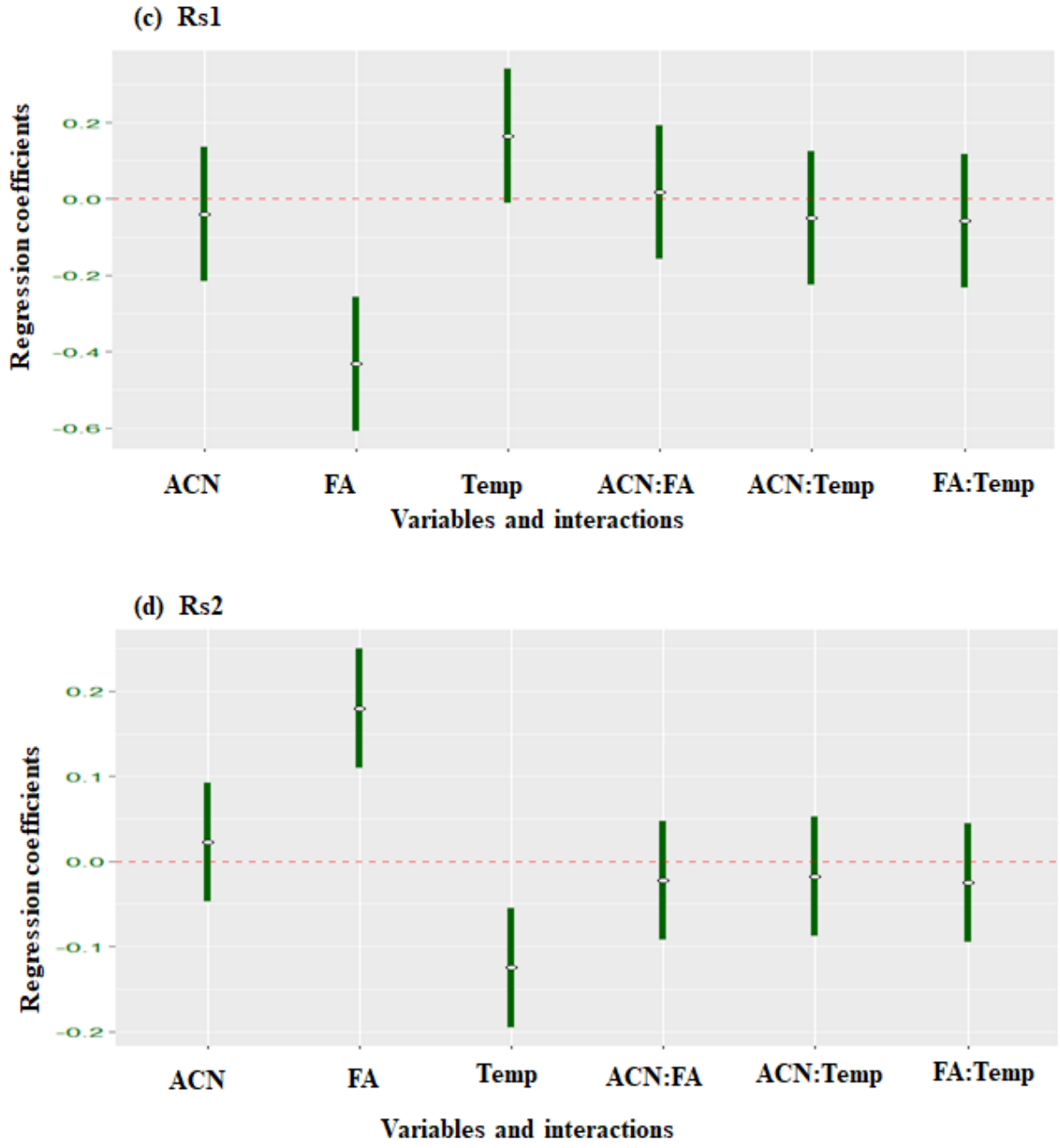
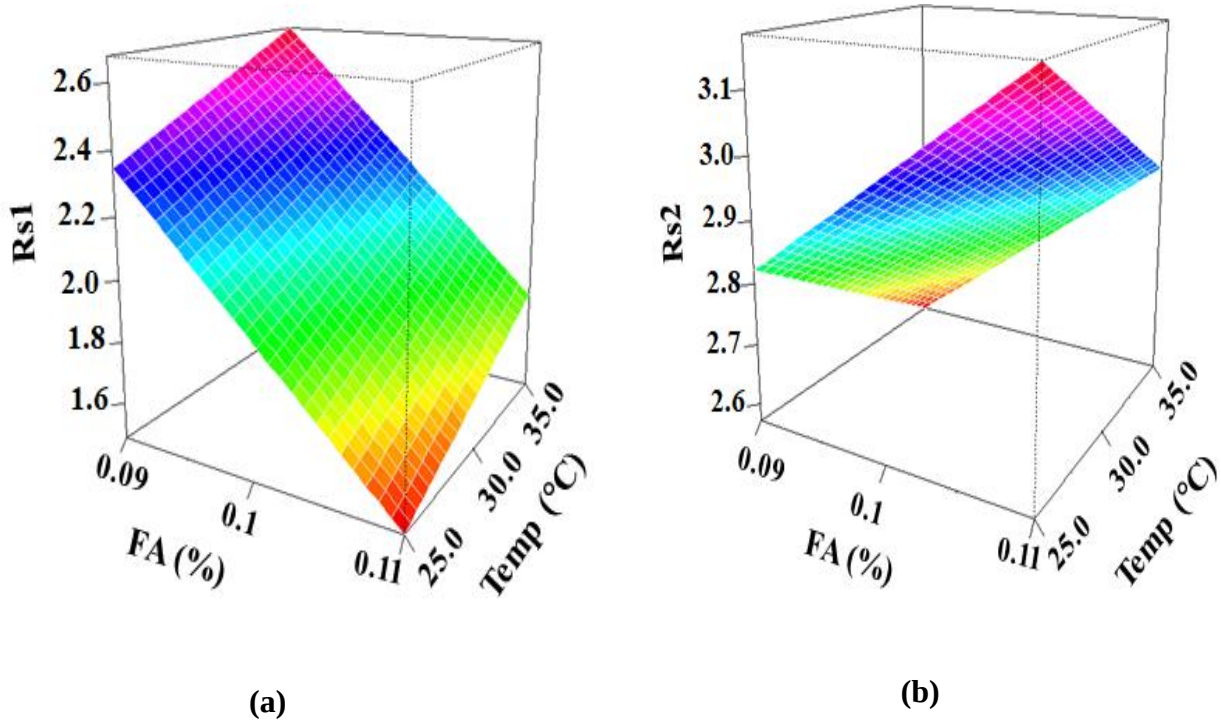


Figure 5.3: Regression coefficient plots obtained from the robustness study for (a) peak area of glucomoringin; (b) peak area of rutin; (c) Rs1: resolution between UNK1 and glucomoringin; (d) Rs2: resolution between glucomoringin and neochlorogenic acid. Temp stands for column temperature, ACN for percentage acetonitrile in mid gradient step and FA for percentage of formic acid in the mobile phase.

Concerning interactions between two variables, no significant effect on the peak areas and resolutions was observed as all intervals included zero. Since quantification is based on the

peak areas, it is good that none of the factors examined was found to have an influence on the peak areas. So, it is important to consider the effect of formic acid and column temperature when eventually transferring the developed method.

The response surface plots shown in Figure 5.4 visualize the influence of temperature and formic acid on the resolution between UNK1 and glucomoringin (Rs1), as well as on the resolution between glucomoringin and neochlorogenic acid (Rs2). In the examined range, Rs1 was always above 1.5 and Rs2 above 2.5.



(a) **(b)**

Figure 5.4: Response surface plots showing the influence of percentage of acetonitrile (ACN), percentage of formic acid (FA) and column temperature (Temp) on Rs1 and Rs2; (a) resolution (Rs1) between UNK1 and glucomoringin, (b) resolution (Rs2) between glucomoringin and neochlorogenic acid. Other parameters were kept constant at their central value.

5.3.4. MS characterization of chromatographic peaks

ESI-MS in negative ion mode was used to obtain structural information of the eluted compounds. Mass spectra of glucomoringin, chlorogenic acid and rutin, corresponding to peak

2, peak 5 and peak 6 (Figure 5.2) respectively, were used as interpretative templates to characterize unknown compounds (peaks 1, 3 and 4 in Figure 5.2).

Peak 2, which was detected in all the samples, resulted in an intense deprotonated molecule ($[M–H]^-$) at *m/z* 570. Its MS/MS resulted in fragments at *m/z* 424 due to loss of a rhamnose sugar and at *m/z* 311 due to a combined loss of a glucose and hydrogen sulphate moiety. Further, a fragment ion at *m/z* 259 was produced by loss of 311 *u*. The ion at *m/z* 259 corresponds to glucose sulphate, a typical fragment ion and one of the diagnostic ions for GLSs as described by Fabre et al. (26). The MS data obtained corresponded to the report by Bennett et al. (6), Förster et al. (25) and Maldini et al. (27) for 4-(α-l-rhamnopyranosyloxy)-benzylglucosinolate or glucomoringin (Figure 5.5A). The identity of this compound was also confirmed by comparing with the MS data of the available standard.

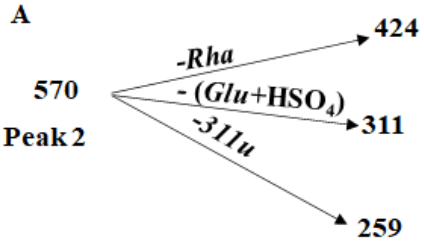
Peak 5, which was also present in all the samples, produced a $[M – H]^-$ at *m/z* 353. Its MS/MS fragments at *m/z* 191 (= $[M - H – 162]^-$) and *m/z* 179 (= $[M - H – 174]^-$) indicated the presence of quinic acid and caffeic acid moieties. Besides, a fragment ion at *m/z* 135 (= $[M - H – 179 – 44]^-$) was observed that could be due to neutral loss of CO_2. By comparing with the retention time and MS data of an authentic standard and the MS data reported by Bennett et al. (6), Rainha et al. (28) and Ncube et al. (29), peak 5 was identified as chlorogenic acid (Figure 5.5D).

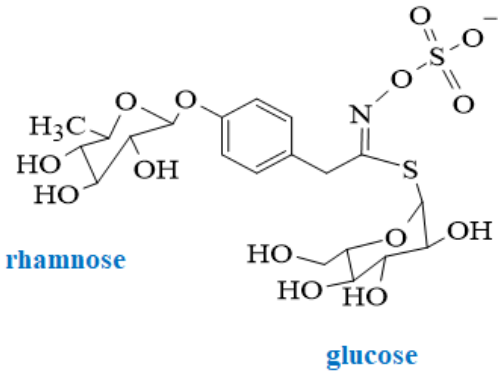
Peak 6 was the major peak in all samples and yielded a $[M – H]^-$ at *m/z* 609. The MS/MS spectrum showed a predominant ion at *m/z* 300 indicating the combined loss of glucose and rhamnose as a result of 3-O-glycosidic bond cleavage. Further fragmentation of the product ion at *m/z* 300 (quercetin) resulted in fragment ions at *m/z* 271, *m/z* 255, *m/z* 179 and *m/z* 151. These MS data were comparable with the MS data reported by Li et al. (30), Chen et al. (31) and Wang et al. (32) for rutin. Further, the retention time and MS data were comparable with the reference. Hence, peak 6 was characterized as rutin (Figure 5.5E).

Peak 1 could not be characterized because the peak intensity was too low.

Peak 3 showed a precursor ion $[M - H]^-$ at *m/z* 353 (Figure 5.5B). Its MS/MS fragment at *m/z* 191 (= $[M - H – C_9H_6O_3]^-$) indicated the presence of a quinic acid residue and was formed by loss of caffeic acid. The spectrum also showed a fragment with very low abundance at *m/z* 179 (= $[M - H – C_7H_{10}O_5]^-$) corresponding to caffeic acid and formed by loss of quinic acid. These mass data were comparable with those reported by Bennett et al. (6), Rainha et al. (28) and Ncube et al. (29) for neochlorogenic acid.

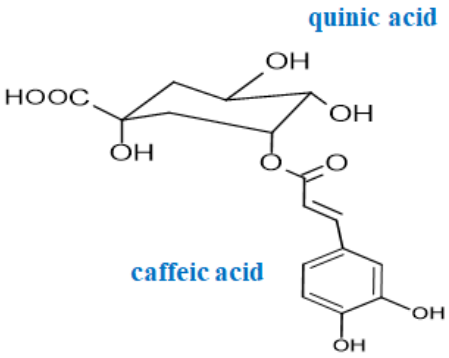
A





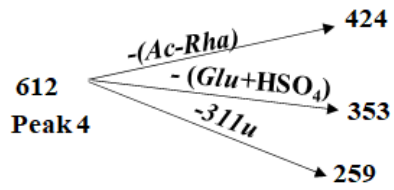
Glucomoringin

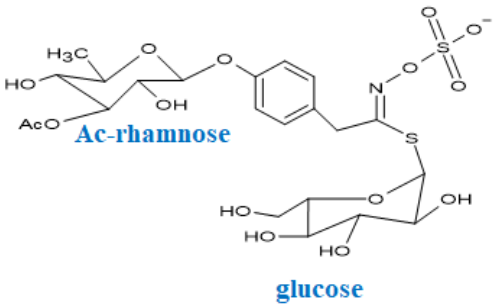
B



Neochlorogenic acid
(5-O-caffeoylquinic acid)

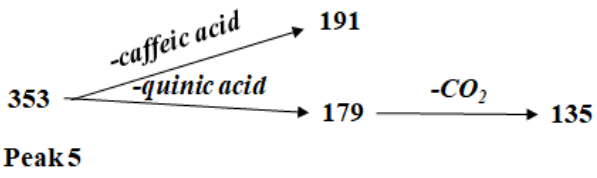
C

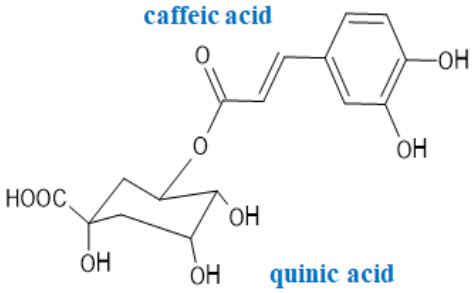




acetyl-glucomoringin isomer III

D





Chlorogenic acid
(3-O-caffeoylquinic acid)

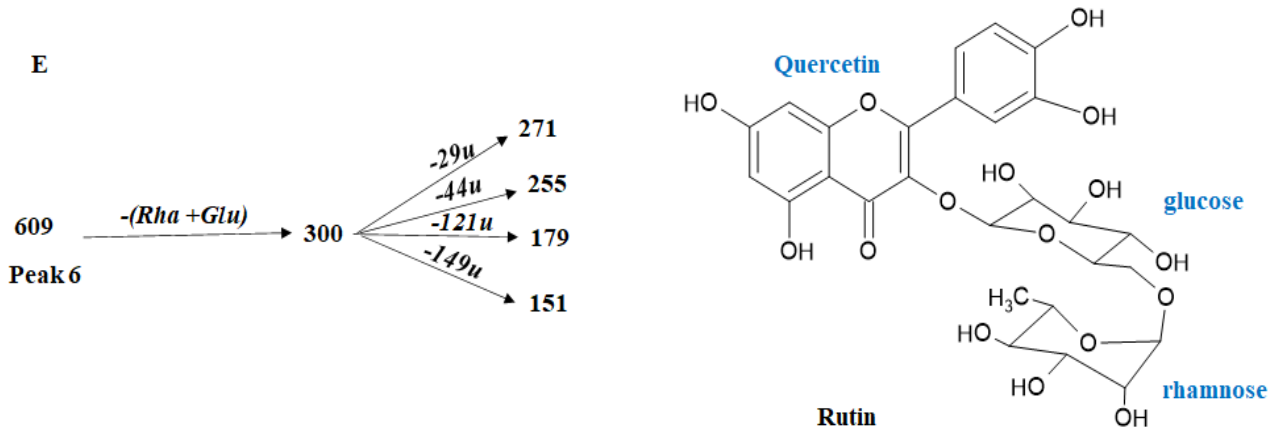


Figure 5.5: Schematic representation of the MS fragmentation of (A) glucomoringin, (B) neochlorogenic acid, (C) Acetyl glucomoringin, (D) chlorogenic acid and (E) rutin, as well as their (proposed) structures.

Peak 4 showed a deprotonated molecule $[M - H]^-$ at *m/z* 612 indicating a relative molecular mass of 613 (Figure 5.5C). Its MS/MS resulted in fragments at *m/z* 424, due to loss of an acetyl rhamnose moiety, and at *m/z* 353, due to a combined loss of a glucose and hydrogen sulphate moiety. Besides, a fragment ion at *m/z* 259 was produced. This could be due to the glucose sulphate ion which is characterstic for GLSs (26). The MS data agreed with those reported by Bennett et al. (6), Förster et al. (25) and Maldini et al. (27) for acetyl-4-(R-L-rhamnopyranosyloxy)-benzylglucosinolate isomer III. Hence, peak 4 was tentatively identified as acetyl-glucomoringin Isomer III (Ac-GMG). Identification as isomer III was done based on the relative elution order and relative amount compared to isomer I and II, as reported by Bennett et al. (6) and Förster et al. (25).

5.3.5. Quality evaluation of moringa samples by ESM and QAMS

The developed LC method was applied for the quantification of GLSs and phenolics in moringa leaf samples collected from Ethiopia. In this study, applying a reporting threshold of 0.2 mg/g, the assay results of the five components in all moringa leaf samples are presented in Table 5.4. Among the five components, rutin was the analyte with the highest mean concentration. Progoitrin, a goitrogenic and antinutritional indole GLS, was not detected although it is reported in the members of the cabbage family (Brassicaceae or Cruciferae). The results of this study complement this of Bennett et al. (6) who reported the absence of aliphatic and indole GLSs in the leaves of *M. stenopetala*. Caffeic acid was also not detected in any of the analysed samples.

Glucomoringin, an aromatic GLS, was present in all samples (Table 5.4). The content of glucomoringin ranged from 0.2 mg/g to 4.2 mg/g, while the content of acetyl-glucomoringin-isomer III was maximum 1.2 mg/g. In this study, the contents of GLSs were found to be a bit higher than those published by Bellostas et al. (20), who reported 2.65 µmol/g in old leaves and < 5 µmol/g in young leaves of *M. stenopetala*. This corresponds to 1.6 mg/g and 3.0 mg/g, respectively when converted using MW (609 g/mol) for the potassium salt of glucomoringin. However, it was somewhat less compared to the paper of Bennett et al. (6) who mentioned 5.3 mg/g in old leaves and 8.2 mg/g of glucomoringin in young leaves and total GLSs to be 11.5 mg/g in old leaves and 21.4 mg/g in young leaves. Different extraction and analysis methods could be one of the reasons why results of this study varied compared to the previous studies. So, it is important to define well the extraction and analytical conditions before specifications can be set.

Table 5.4: Content of glucosinolates and phenolic compounds in Ethiopian moringa leaf powders determined using the ESM and QAMS method (mg/g (% RSD), *n* = 3).

Sample	UNK1 ESM	GMG ESM	GMG QAMS	Neochlorogenic acid ESM	Ac-GMG ESM	Chlorogenic acid ESM	Chlorogenic acid QAMS	Rutin ESM
MLP 1	1.7 (0.3)	2.3 (0.3)	1.8 (0.3)	3.3 (2.2)	0.9 (1.9)	2.6 (1.3)	2.6 (1.3)	18.8 (1.2)
MLP 2	0.9 (0.8)	2.9 (1.5)	2.3 (1.5)	3.6 (1.3)	1.2 (1.9)	2.6 (0.6)	2.6 (0.6)	17.0 (1.7)
MLP 3	0.9 (0.6)	3.5 (1.2)	2.8 (1.2)	3.4 (1.7)	0.9 (2.1)	2.6 (1.6)	2.6 (1.6)	18.4 (1.6)
MLP 4	1.0 (2.3)	4.2 (0.5)	3.4 (0.5)	3.5 (0.5)	0.8 (2.0)	2.7 (0.9)	2.9 (0.9)	16.9 (1.7)
MLP 5	2.2 (1.7)	0.4 (2.0)	0.3 (2.0)	0.5 (0.6)	0.4 (0.9)	0.4 (0.9)	0.4 (0.9)	11.0 (1.4)
MLP 6	1.3 (0.7)	0.2 (1.9)	0.1 (1.9)	0.2 (0.2)	0.3 (1.1)	BT	BT	6.6 (0.5)
MLP 7	2.2 (0.3)	0.2 (1.3)	0.2 (1.3)	0.8 (0.7)	0.3 (1.7)	0.4 (0.9)	0.4 (0.9)	7.0 (0.9)
MLP 8	1.5 (2.0)	0.2 (1.7)	0.2 (1.7)	0.3 (3.2)	0.4 (0.2)	0.2 (2.1)	0.2 (2.1)	7.9 (0.7)
MLP 9	2.3 (0.9)	0.2 (1.1)	0.2 (1.1)	0.5 (3.3)	0.5 (2.3)	0.5 (2.6)	0.5 (2.6)	9.3 (1.8)

BT: below threshold (< 0.2 mg/g), GMG: glucomoringin, Ac-GMG: acetylated glucomoringin isomer III

Bennett et al. (6) and Bellostas et al. (20) reported a higher content of GLSs and phenolics in young leaves compared to old leaves of *M. stenopetala*. The concentration of GLSs can further be influenced by growing conditions, the plant's growth stage, harvesting, drying and storage conditions (6, 20, 25). Hence, the content variation observed in this study, could also be due to the reasons mentioned above, as the information related to agricultural and collection practices such as growth conditions, collection, production, post-harvest processing and storage was not indicated on the labels of the samples.

According to EFSA, the GLS content in vegetables expressed in terms of daily needs and activity is limited to 20 mg (17). Hence, although there is a lack of adequate exposure assessment studies on moringa consumption in Ethiopia, it would be good to indicate the GLSs content on the label of moringa leaf preparations and precautionary information to avoid undesirable effects upon frequent and/or excessive consumption of moringa samples with higher contents of GLSs.

The average content of rutin, the principal component, ranged from 6.6 mg/g to 18.8 mg/g. This finding was comparable with the findings by Habtemariam & Varghese (15) who reported 2.3% (or 23 mg/g) of rutin in *M. stenopetala* leaves and significantly higher than those published by Bennett et al. (6) and Dessalegn and Rupasinghe (16), which were 3.0 mg/g (highest in young leaves) and 1.155 mg/g (in methanol extract), respectively. Moreover, the content of neochlorogenic acid varied from 0.2 to 3.6 mg/g while the content of chlorogenic acid ranged from 0.2 mg/g to 2.7 mg/g. The content of neochlorogenic acid was found to be less compared to the results (4.2 mg/g and 6.2 mg/g in old and young leaves, respectively) obtained by Bennett et al. (6). On the other hand, chlorogenic acid was found to be higher than the results of Dessalegn and Rupasinghe (16) which were 0.165 mg/g in an aqueous extract and 0.159 mg/g in a methanolic extract. Varations among the findings could be explained due to differences in extraction methods, extraction solvents, analysis method and growth stages among others.

A radar plot (also known as spider plot or web plot) is commonly used for quick discrimination of samples based on multiple parameters. It was used in this study to further evaluate the moringa samples collected from different regions based on the GLSs and phenolics contents. As can be seen from the radar plot in Figure 5.6, samples 1-4 collected from southern Ethiopia were found to contain a higher content of glucomoringin, neochlorogenic acid, acetyl-glucomoringin, chlorogenic acid and rutin, compared to samples 5 to 9 collected from the

northern part of Ethiopia. Hence, radar plot analysis could be used to discriminate *M. stenopetala* leaf samples collected from different geographical origin. However, information about collection time and further processing were not available.

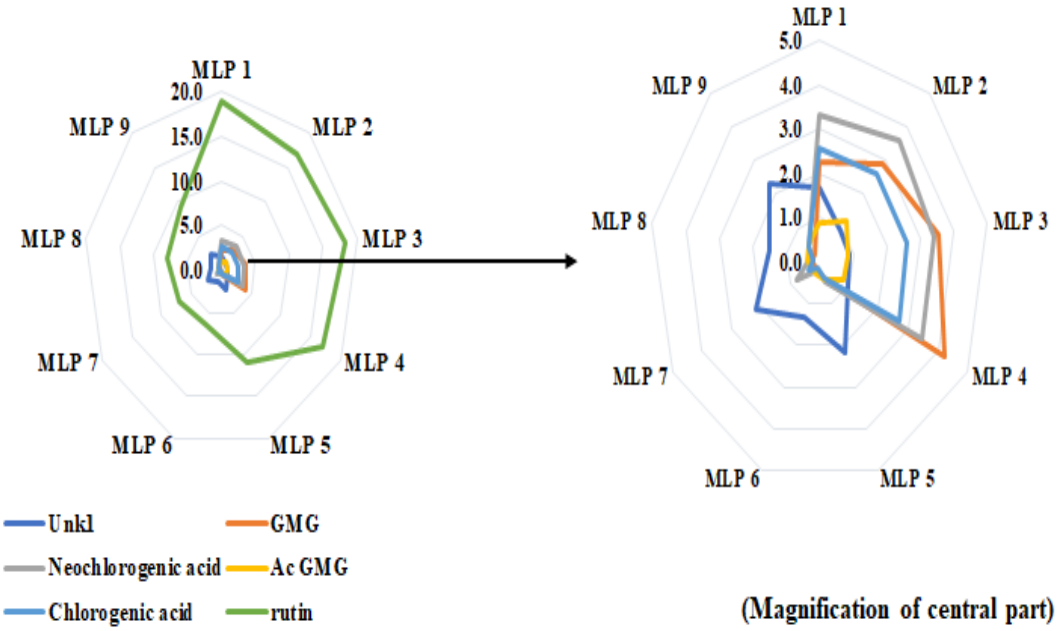


Figure 5.6: Radar plots showing the distribution of glucosinolates and phenolics among moringa leaf samples collected from different regions in Ethiopia

A component of a given herbal medicine that is stable, inexpensive, easily available, pharmacologically active and easy to separate under conventional chromatographic conditions is an ideal candidate for a marker compound (33). In this study, rutin, the most abundant and pharmacologically active component of *M. stenopetala*, was selected as single marker for the quantification of GLSs and phenolics in moringa leaves. Correction factors for each component in *Moringa* leaf samples were determined with respect to rutin (Table 5.2).

To check the validity of QAMS, the contents of the components measured by QAMS were compared with those from the ESM method. Besides, similarity between the two methods was compared using a correlation test (r). The results obtained using QAMS for glucomoringin and chlorogenic acid were similar ($r \geq 0.9993$) with the ESM contents (Table 5.4) demonstrating that QAMS was effective and reliable for quantitative analysis of glucomoringin and neochlorogenic acid in moringa leaf samples. Hence, in view of the cost and scarcity of

reference standards, this method could be applied as alternative method to ESM during quality control of moringa samples. However, it has to be remarked that the compounds should be available as standards during the QAMS method development to determine the RCF. Here we focused on the most important compounds.

5.4. Conclusions

In this study, a fast and easily applicable LC-UV method was developed and validated for the simultaneous determination of GLSs and phenolics in *M. stenopetala* leaf samples. Besides the ESM method, the QAMS method was developed as alternative for the analysis of GLSs and phenolics in *M. stenopetala* leaf samples. The two methods were found to give similar results. Hence, both can be applied for quality control and standardization of herbal medicines from *M. stenopetala* leaves. When comparing results or setting specifications, it is also impotant to define well the extraction conditions. Finally, radar plot analysis was applied to further discriminate the samples based on their geographical origin.

5.5. References

1. Abd Rani, NZ, Husain K, Kumolosasi E. *Moringa* Genus: A Review of Phytochemistry and Pharmacology. Front. Pharmacol. 2018, 9(108): 1-26. doi: 10.3389/fphar.2018.00108.
2. Seifu E. Actual and Potential Applications of *Moringa stenopetala*, Underutilized Indigenous Vegetable of Southern Ethiopia: A Review. International Journal of Agricultural and Food Research 2014, 3(4): 8-19.
3. Kumssa DB, Joy EJM, Young SD, Odee DW, Ander EL, Magare C, Gitu J, Broadley MR. Challenges and opportunities for Moringa growers in southern Ethiopia and Kenya. PLoS ONE 2017, 12 (11): e0187651. https://doi.org/10.1371/journal.pone.0187651.
4. Habtemariam S, Varghese GK. Extractability of Rutin in Herbal Tea Preparations of *Moringa stenopetala* Leaves. Beverages 2015, 1: 169-182. doi:10.3390/beverages1030169.
5. Mekonen A, Gebreyesus T. Chemical investigation of leaves of *Moringa stenopetala*. Bull. Chem. Soc. Ethiop. 2000, 14: 51-55.
6. Bennett RN, Mellon FA, Foidl N, Pratt JH, Dupont MS, Perkins L, Kroon PA. Profiling Glucosinolates and Phenolics in Vegetative and Reproductive Tissues of the Multi-

Purpose Trees *Moringa oleifera* L. (Horseradish Tree) and *Moringa stenopetala* L. J. Agric. Food Chem. 2003, 51: 3546-3553. https://doi.org/10.1021/jf0211480.

7. Mekonnen Y, Dräger B. Glucosinolates in *Moringa stenopetala.* Planta Med. 2003, 69(4): 380-382. DOI: 10.1055/s-2003-38881.
8. Ares AM, Valverde S, Nozal MJ, Bernal JL, Bernal J. Development and validation of a specific method to quantify intact glucosinolates in honey by LC–MS/MS. J. Food Compos. Anal 2016, 46: 114–122. http://dx.doi.org/10.1016/j.jfca.2015.12.002.
9. Hanschen FS, Schreiner M. Isothiocyanates, Nitriles, and Epithionitriles from Glucosinolates Are Affected by Genotype and Developmental Stage in *Brassica oleracea* Varieties. Front. Plant Sci. 2017,8: 1095. doi:10.3389/fpls.2017.01095.
10. Lopez-Rodriguez NA, Gaytán-Martínez M, Reyes-Vega ML, Loarca-Piña G. Glucosinolates and Isothiocyanates from *Moringa oleifera*: Chemical and Biological Approaches. Plant Foods Hum Nutr. 2020, 75: 447–457. https://doi.org/10.1007/s11130-020-00851-x.
11. European Food Safety Authority (EFSA). Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the European Commission on glucosinolates as undesirable substances in animal feed. The EFSA Journal 2008, 590: 1-76. DOI: https://doi.org/10.2903/j.efsa.2008.590.
12. Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 2001,56: 5–51. doi: 10.1016/s0031-9422(00)00316-2.
13. Habza-Kowalska E, Kaczor AA, Zuk J, Matosiuk D, Gawlik-Dziki U. Thyroid Peroxidase Activity is Inhibited by Phenolic Compounds—Impact of Interaction. Molecules 2019, 24: 2766. doi:10.3390/molecules24152766.
14. Giuliani C, Bucci I, Santo SD, Rossi C, Grassadonia A, Piantelli M, Monaco F, Napolitano G. The flavonoid quercetin inhibits thyroid-restricted genes expression and thyroid function. Food Chem Toxicol. 2014, 66: 23–29. https://doi.org/10.1016/j.fct.2014.01.016.
15. Habtemariam S. Investigation into the Antioxidant and Antidiabetic Potential of *Moringa stenopetala*: Identification of the Active Principles. Nat. Prod. Commun. 2015, 10 (3): 476-478.

16. Dessalegn E, Rupasinghe HPV. Phenolic compounds and *in vitro* antioxidant activity of *Moringa stenopetala* grown in South Ethiopia. Int. J. Food Prop. 2021 24(1):1681–1692.
17. European Food Safety Authority. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on the substantiation of health claims related to various foods/food constituents and ―immune function/immune system‖ (ID573, 586, 1374, 1566, 1628, 1778, 1793, 1817, 1829, 1939, 2155, 2485, 2486, 2859, 3521, 3774, 3896), ―contribution to body defences against external agents‖ (ID 3635), stimulation of immunological responses (ID1479, 2064, 2075, 3139), reduction of inflammation (ID 546, 547, 641, 2505, 2862), increase in renal water elimination (ID 2505), treatment of diseases (ID500),and increasing numbers of gastro-intestinal microorganisms (ID762, 764, 884)pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal 2011, 9(4): 2061. doi: 10.2903/j.efsa.2011.2061.
18. Habtemariam S. Methodology for Rapid Isolation of Moringin: Potential Anticancer Compound from the Seeds of *Moringa stenopetala.* Pharm Anal Acta 2017, 8:8. DOI: 10.4172/2153-2435.1000558.
19. ICH. Validation of Analytical Procedures: Text and Methodology Q2 (R1). London: EMA; 2006. http://www.emea.eu.int.
20. Bellostas N, Sørensen JC, Nikiema A, Sørensen H, Pasternak D, Kumar S. Glucosinolates in leaves of Moringa species grown and disseminated in Niger. Afr. J. Agric. Res. 2010, 5(11): 1338-1340.
21. Tian Q, Rosselot RA, Schwartz SJ. Quantitative determination of intact glucosinolates in broccoli, broccoli sprouts, Brussels sprouts, and cauliflower by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry. Anal. Biochem. 2005, 343: 93–99. doi:10.1016/j.ab.2005.04.045.
22. Ishida M, Kakizaki T, Ohara T, Morimitsu Y. Development of a simple and rapid extraction method of glucosinolates from radish roots. Breed. Sci. 2011, 61: 208–211. doi:10.1270/jsbbs.61.208.
23. Doheny-Adams T, Redeker K, Kittipol V, Bancroft I, Hartley SE. Development of an efficient glucosinolate extraction method. Plant Methods 2017, 13:17. DOI 10.1186/s13007-017-0164-8.
24. Mellon FA, Bennett RN, Holst B, Williamson G. Intact Glucosinolate Analysis in Plant Extracts by Programmed Cone Voltage Electrospray LC/MS: Performance and

Comparison with LC/MS/MS Methods. Anal. Biochem. 2002, 306: 83–91. doi:10.1006/abio.2002.5677.

25. Förster N, Ulrichs C, Schreiner M, Müller CT, Mewis I. Development of a reliable extraction and quantification method for glucosinolates in *Moringa oleifera*. Food Chem. 2015, 166: 456–464. http://dx.doi.org/10.1016/j.foodchem.2014.06.043.
26. Fabre N, Poinsot V, Debrauwer L, Vigor C, Tulliez J, Fourasté I, Moulis C. Characterisation of Glucosinolates using Electrospray Ion Trap and Electrospray Quadrupole Time-of-flight Mass Spectrometry. Phytochem. Anal. 2007, 18: 306–319. DOI: 10.1002/pca.983.
27. Maldini M, Maksoud SA, Natella F, Montoro P, Petretto GL, Foddai M, De Nicola GR, Chessaa M, Pintore G. '*Moringa oleifera*: study of phenolics and glucosinolates by mass spectrometry'. J. Mass Spectrom. 2014, 49: 900–910.
28. Rainha N, Koci K, Coelho AV, Lima E, Baptista J, Fernandes-Ferreira M. HPLC–UV–ESI-MS analysis of phenolic compounds and antioxidant properties of Hypericum undulatum shoot cultures and wild-growing plants. Phytochemistry 2013, 86: 83–91.
29. Ncube EN, Mhlongo MI, Piater LA, Steenkamp PA, Dubery IA, Madala NE. Analyses of chlorogenic acids and related cinnamic acid derivatives from Nicotiana tabacum tissues with the aid of UPLC-QTOF-MS/MS based on the in-source collision-induced dissociation method. Chemistry Central Journal 2014, 8: 1–10.
30. Li Z-H, Guo H, Xu W-B, Ge J, Li X, Alimu M, He D-J. Rapid Identification of Flavonoid Constituents Directly from PTP1B Inhibitive Extract of Raspberry (Rubus idaeus L.) Leaves by HPLC–ESI–QTOF–MS-MS. J. Chromatogr. Sci. 2016, 54: 805–810.
31. Chen Y, Yu H, Wu H, Pan Y, Wang K, Jin Y, Zhang C. Characterization and Quantification by LC-MS/MS of the Chemical Components of the Heating Products of the Flavonoids Extract in Pollen Typhae for Transformation Rule Exploration. Molecules 2015, 20: 18352–18366.
32. Wang J, Ren X, Wen C, Xu Y, Chen Y. Separation and characterization of unknown impurities in rutin tablets using trap-free two-dimensional liquid chromatography coupled with ion trap/time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 2020, 34: e8739.

33. Cui K, CaoY, Shao J, Lu Z, Wang L. Quantitative analysis of multicomponents by a single marker and quality evaluation of *Venenum Bufonis* from different geographical origins. Biomed. Chromatogr. 2019, 33: e4555. https://doi.org/10.1002/bmc.4555.

Chapter 6: General discussion

6.1. General aspects

Herbal medicine is the oldest, and still a predominant part of African traditional medicine. Herbal medicines (HM) are also called botanical medicines, vegetable medicines, or phytomedicines and the traditional healer specialized in herbal medicine is called a herbalist (1). According to the WHO, HM include herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or combinations (2).

Herbs include crude materials which could be derived from lichen, algae, fungi or higher plants, such as leaves, flowers, fruit, fruiting bodies, seeds, stems, wood, bark, roots, rhizomes or other parts, which may be entire, fragmented or powdered. Herbal materials include, in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting or stir baking with honey, alcoholic beverages or other materials. Herbal preparations are the basis for finished herbal products and may include comminuted or cut herbal materials, or extracts, tinctures and fatty oils of herbal materials produced by extraction, fractionation, purification, concentration, or other physical or biological processes (3).

Good agricultural and collection practices for medicinal plants is the first step in quality assurance of herbal medicinal products where the safety and efficacy are directly linked (4). The overall quality of a HM may be affected by several factors, including seasonal changes, cultivar variation, environmental factors (such as sunshine radiation, temperature variation and climatic circumstances) and agronomic conditions (such as sowing date, cultivation sites, fertilisation, irrigation and harvesting time), post-harvest processing operations, and storage conditions, adulterants or substitutes of raw materials, and procedures in extraction and preparation (5,6), leading to variations in secondary metabolites, a variety of bio active phytochemicals naturally occurring in different parts of plants (6).

QC of articles of botanical origin, including plant materials, plant extracts, and HM, remains a challenge (7). As plant materials are still widely consumed products in developed and developing countries and as they represent a substantial proportion of the global drug market, the demand for quality is also rising. To ensure the quality of medicinal plant products by using modern QC techniques and applying suitable standards, WHO developed a manual that describes a series of tests such as determination of foreign matter, macroscopic and microscopic

examination, thin-layer chromatography, liquid chromatography, determination of ash, determination of water etc. for assessing the quality of medicinal plant materials (8).
According to the EMA guideline on quality of herbal medicinal products, for herbal substances and herbal preparations consisting of comminuted or powdered herbal substances, the grade of comminution has to be given. Furthermore, the following has to be indicated:
(i) in the case of standardisation: the quantity of the herbal substance/preparation shall be given as a range corresponding to a defined quantity of constituents with known therapeutic activity.
(iia) in the case of quantification: the quantity of the herbal substance/preparation shall be stated as a distinct content and the content of the quantified substance(s) shall be specified in a range.
(iib) for all other cases: the quantity of the herbal substance or the quantity of the genuine herbal preparation shall be stated as a distinct content (9).

Markers are chemically defined constituents of a herbal material utilized for control purposes. They may or may not contribute to the clinical efficacy. When they contribute to the clinical efficacy, however, evidence that they are solely responsible for the clinical efficacy may or may not be available. Markers are generally employed when constituents of known therapeutic activity are not known or are not clearly identified, and may be used to identify the herbal material or preparation or calculate their quantity in the finished product (3).

Selecting a relevant analytical method for QC of HM, from the many available, is a crucial step that mainly depends on the set analytical goals. Herbal identity confirmation is an essential step during QC of HM. This can be done visually and/or through microscopic observation on the key morphological features of the species. Microscopic evaluation is a preferred method for the identification of similar species or the verification of adulteration as it helps to identify herbal materials at the cellular level. Besides, it is the only morphological alternative for the analysis of HM presented in the form of powders. However, microscopic authentication is not always useful for formulations and should be supported by chromatographic data for completeness. Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC), in combination with macroscopic and microscopic evaluations, are other herbal chemical profiling methods based on the affinity of chemicals in the mixture towards a stationary and mobile phase. HPTLC has become a reference method in modern pharmacopoeias for the identification of herbal drugs, in conjunction with macroscopic and microscopic examinations (10). For quantitative tests, LC is superior. According to EMA, universal tests such as foreign matter, total ash and water content should be applied to herbal substances while tests such as

extractable matter, swelling index and tests for contaminants (e.g. heavy metals, pesticides, mycotoxins, fumigants, microbial contamination and residual solvents) should be performed when found relevant (9). Besides, Ph. Eur. herbal monographs describe tests such as foreign matter determination, loss on drying, total ash, hydrochloric acid insoluble ash and assay of bio-active marker compounds for dry cut or powdered herbal samples (11). Loss on drying, total ash, and acid-insoluble ash are basic tests for evaluating the quality state of commercial HM (12). However, regulatory limits are not established for HM used in Ethiopia including *Aloe* and *Moringa* based products, making it difficult for QC of commercialized HM.

According to the technical report by EFSA (2019), as there was lack of information on the quality and quantity of undesirable substances and the absence of an exposure assessment, EFSA didn't conclude whether or not *M. stenopetala* leaf powder could be used as traditional food and raises safety objections to the placing on the market (13).

Likewise, in this project, due to lack of local monographs and specifications and limited information on the label present on commercial *Aloe* and *Moringa* leaf based herbal products, it was a challenge to fully apply all quality evaluation parameters. As it was not feasible and convenient to do all QC tests, some tests such as residual solvent determination were skipped. Hence, in this project, relevant general QC tests for HM mentioned in WHO guidelines, EMA guidelines and Ph. Eur. were used as references to evaluate the quality of *Aloe* and *Moringa* leaf based medicinal products. As a result, macroscopic identification, the fastest and simplest method of authentication during quality evaluation of HM (14,15), like appearance or colour change, smell, and foreign matters were performed. Moreover, to further evaluate internal quality of the samples, selected tests like loss on drying, ash content, acid insoluble ash, and pH were performed depending on the nature of samples (solids/powders). Macroscopic identification was performed first by naked eye and using a hand lens (10× magnification). Besides, to further validate the genuinity of samples, botanical identification was also considered. Excessive moisture in a given HM indicates the likelihood that the sample may be degraded and/or contaminated by pathogenic microbes. On the other hand, excessive ash content indicates that the product is contaminated by sand or soil during the course of its production. Furthermore, LC methods were developed and applied to determine the content of bioactive constituents in the samples. To finally decide whether a sample is compliant or not, all test results should comply with the specifications.

6.2. Specific aspects

As the consumption of botanicals as medicine, functional foods and cosmetics is continuously growing, users are demanding for safe and qualitative herbal products and different stakeholders are promoting standardization and QC to ensure safety and efficacy of herbal materials.

Despite the availability of a large number of traditional HMs in Africa, the legal basis for establishing them as part of the drug legislation is very limited. As a result, in most parts of Africa, HMs are being sold everywhere without any scientific proof of safety and efficacy. In the sub-Saharan African countries like Nigeria, South Africa, Ghana, and Uganda, many gaps have been identified in the policy design and practice. In Kenya, HMs are not registered and sold without any restriction. In Ethiopia, however, although policies, legislations and regulations about traditional medicines are recently established, many HMs are still sold without any restrictions in the open market without proven safety, efficacy and quality. Besides, there is no traditional healer licensed by the Ethiopian Food and Drug Administration (EFDA), a regulating agency, indicating a clear gap in controlling HMs (16).

Moringa and *Aloe* are among the most used and scientifically studied medicinal plants in Ethiopia. Their leaves are used as food, medicine and in cosmetics in different ways. As a result, they are often promoted as potential plants for the development of medicines, nutraceuticals or incorporated into functional foods.

In this project, quality of *Moringa* and *Aloe* based herbal products, which are widely used botanicals in Africa in general and in Ethiopia in particular, were evaluated following the general QC methods for identity, purity, and content of bioactive chemicals. General identity evaluation tests (such as botanical identification, colour, shape, odour), purity tests (such as foreign matter determination, loss on drying, ash content), stability tests (such as odour, pH) and assay of bioactive phytochemicals by LC were performed.

LC-UV methods using monolithic columns were developed, validated, and applied to *Moringa* and *Aloe* leaves based herbal preparations to determine bioactive marker compounds such as glycosylated anthrones in *Aloe* and phenolic compounds in *Moringa*. For the determination of glucosinolates in *Moringa stenopetala* leaf powder, a Luna C18 column was found to be more suitable. A Luna column is compatible with highly aqueous mobile phases and capable of

separating highly polar analytes. So, it gave a better selectivity for the targeted compounds compared to a monolithic column.

LC-UV was selected as it is known for its versatility, selectivity, and feasibility to determine a wide range of secondary phytochemicals. Moreover, in this project, monolithic columns were selected as it is becoming a popular type of stationary phase in different fields including herbal product analysis for their better performance (high selectivity and sensitivity), online cleaning and causing low back pressure compared to particle packed columns. Besides, monoliths are compatible with conventional LC systems making them feasible in low-income countries.

In this study, a fast and easily applicable LC-UV method has been developed and validated for the determination of aloins in *Aloe* leaf latex and *Aloe* leaf gel based personal care products. According to the analysis results, the content of aloins in the leaf latex ranged from 14 to 35 %. Besides, aloins were detected in a quarter of the aloe gel based personal care commercial samples.

Further, quality of commercial *Moringa* leaf preparations was evaluated using a validated LC-UV method to determine GLS and phenolics, loss on drying, total ash and acid insoluble ash contents. Nearly half of the samples was found to be compliant.

The developed LC-UV methods were found to differentiate samples from different sources indicating their feasibility for the analysis of commercial samples.

Furthermore, quantitative analysis of multi components by a single marker (QAMS) method, adopted by different pharmacopoeias such as the European, United States and Chinse Pharmacopoeia for standardization and quality evaluation of HMs and becoming a preferable method over external standardization methods for its feasibility reason in herbal analysis, was applied in this project.

The developed QAMS method for simultaneous determination of glucosinolates and phenolics in *Moringa* leaves was found to give comparable results with the external standard method.

The results of this study will be disseminated to relevant stakeholders such as regional and national regulatory agencies like for example EFDA which is situated in the 9 administrative states. This will be done through capacity building trainings (hosted by Mekelle University, School of Pharmacy). They can then impose a more stringent regulation on *Aloe* and *Moringa* based preparations. This way, the project will contribute to the general health and well-being

of the people in Ethiopia and at large in Africa. Besides, the developed LC methods are state-of-the-art, fast and easy applicable. Hence, they can be easily executed in Ethiopian QC laboratories to ensure efficacy and safety of users. Furthermore, the findings could be used as good input for standardization of raw materials and finished products and/or for developing monographs in local languages for *Aloe* and *Moringa* leaf based herbal products. Hence, they may play a big role in supporting the national direction towards commercialization and internationalization of quality HM.

In addition, awareness creation to traditional medicine practitioners, herb growers, herbal sellers and consumers (the public in general) and establishing a QC lab for phytomedicines at Mekelle University will also be realized. This will be done in consultation with university level offices such as the center for research and development, and the office for knowledge and technology transfer. Hence, the results of this study will be used to prepare a protocol useful for standardization and QC of *Aloe* and *Moringa* formulations in the QC lab for phytomedicines.

Finally, this study was somewhat negatively impacted by the corona crisis and ongoing civil war in Ethiopia. Otherwise, more samples could have been collected to perform a more extensive market surveillance study. This would allow to further finetune the specifications to be set.

6.3. References

1. Ozioma EJ, Chinwe OAN. Herbal Medicines in African Traditional Medicine. https://www.intechopen.com/chapters/64851. (Accessed on 26 April 2022).
2. World Health Organization (WHO). WHO global report on traditional and complementary medicine. Geneva: World Health Organization; 2019.
3. World Health Organization (WHO). WHO guidelines on good manufacturing practices (GMP) for herbal medicines. Geneva: World Health Organization; 2007.
4. World Health Organization (WHO). WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants. Geneva: World Health Organization; 2003.

5. Li S, Han Q, Qiao C, Song J, Cheng CL, Xu H. Chemical markers for the quality control of herbal medicines: an overview. Chinese Med. 2008; 3(7): 1-16. doi:10.1186/1749-8546-3-7.
6. Tiwari U, Cummins E. Factors influencing levels of phytochemicals in selected fruit and vegetables during pre-and post-harvest food processing operations. Food Res. Int. 2013; 50: 497-506.
7. Klein-Junior LC, de Souza MR, Viaene J, Bresolin, TMB, de Gasper AL, Henriques AT, Vander Heyden Y. Quality Control of Herbal Medicines: From Traditional Techniques to State-of-the-art Approaches. Planta Med. 2021; 87: 964–988. DOI: 10.1055/a-1529-8339.
8. World Health Organization (WHO). Quality control methods for herbal materials. Geneva: World Health Organization; 2011.
9. European Medicines Agency (EMA). Guideline on quality of herbal medicinal products1 /traditional herbal medicinal products. London, UK; 2011. www.ema.europa.eu. (Accessed on 25 March 2022).
10. Muyumba NW, Mutombo SC, Sheridan H, Nachtergael A, Duez P. Quality control of herbal drugs and preparations: The methods of analysis, their relevance and applications. Talanta Open 2021; 4:100070. https://doi.org/10.1016/j.talo.2021.100070.
11. European Pharmacopoeia. 10th ed. Strasbourg, France: EDQM Council of Europe; 2020.
12. Kim DG, Kim BS, Kim YC, Hwang YO, Chae YZ, Park SK. Selection of Herbal Medicines Requiring Quality Control for Loss on Drying, Total Ash, and Acid-insoluble Ash in Korea. Nat. Prod. Sci. 2011, 17(1): 38-44.
13. EFSA. Technical Report on the notification of leaf powder of *Moringa stenopetala* as a traditional food from a third country pursuant to Article 14 of Regulation (EU) 2015/2283; 2019. DOI: https://doi.org/10.2903/sp.efsa.2019.EN-1672.
14. van der Valk JMA, Leon, CJ, Nesbitt, M. Macroscopic authentication of Chinese materia medica (CMM): A UK market study of seeds and fruits. J. Herbal Med. 2017; 8: 40–51.
15. Zhao Z, Liang Z, Guo P. Macroscopic identification of Chinese medicinal materials: Traditional experiences and modern understanding. J Ethnopharmacol. 2011; 134: 556–564.

16. Demeke H, Hasen G, Sosengo T, Siraj J, Tatiparthi R, Suleman S. Evaluation of policy governing herbal medicines regulation and its implementation in Ethiopia. Multidiscip. Healthc. 2022;15: 1383–1394.

Summary

As the consumption of herbal materials is dramatically increasing worldwide, the demand for quality control is becoming a global issue. The quality could be compromised by genetic, physicochemical, and biological factors. More control on herbal medicines is especially evident in developing countries where this is limited or even absent due to a number of reasons like for example lack of state-of-the-art analytical instruments, relevant monographs, easily applicable methods and trained man power. *Aloe* and *Moringa* leaf based herbal products are commonly used and commercially important herbal products in Africa in general and in Ethiopia in particular. Despite their wide use for medicinal, food and cosmetic purposes, their quality is barely controlled. Hence, in this study, quality control of *Aloe* and *Moringa* leaf based herbal preparations collected from Ethiopia was performed.

In chapter 1, a brief introduction on traditional medicine and herbal medicines as well as their quality control methods is presented. Besides, literatures on the traditional medicinal use, phytochemistry and pharmacological activities of *Aloe* and *Moringa* from Ethiopia were reviewed.

In chapter 2, a fast and easily applicable LC-UV method for the determination of anthrone glycosides, the principal bioactive marker components in *Aloe* leaf latex, has been developed. The method used a monolithic column, a stationary phase known for advantages such as low back pressure, stability, fast equilibration and compatibility with conventional LC systems. The developed method was validated with respect to specificity, linearity, precision, accuracy, and robustness and practically applied to determine aloins and their derivatives in *Aloe* leaf latex (*Aloe* juice products) collected from Ethiopia. In this study, the aloin contents of the samples were found to vary from 14 to 35 %. Besides, two unknown peaks in the chromatogram were tentatively identified as aloinoside and microdontin.

In chapter 3, the above developed LC-UV method has been applied to determine aloins and related anthrones in *Aloe* leaf gel based personal care products with a modification on the extraction procedure considering the new matrix. *Aloe* leaf gel-based products are expected to be free from any contamination by the latex seen the diverse dermal toxicities and side effects. Moreover, additional quality parameters such as pH, loss on drying and ash content were also applied. According to the results of this study, aloins were detected in 25% of the analysed

commercial samples. Besides, 42% of the tested samples were found to be in the basic pH range and 33% of them contained excessive moisture, indicating that their quality is compromized.

In chapter 4, a sensitive LC-UV method with short analysis time has been developed to determine phenolics in *Moringa* leaf preparations. After validation, it was practically applied to products commercialized in Ethiopia. Moreover, loss on drying, total ash and acid insoluble ash contents of samples were determined too as part of the quality evaluation. Further, MS characterization of unknown peaks in the chromatogram was performed. In this study, rutin was found to be the principal component and its content in samples varied from 2 to 18 mg/g. The method allowed to observe differences in phenolic composition between regions in Ethiopia. Results for loss on drying and ash content revealed that 40% and 50%, respectively, of the samples were not compliant.

In chapter 5, an LC-UV method with quantitative analysis of multicomponents by a single marker (QAMS) has been developed for simultaneous determination of glucosinolates and phenolics in *Moringa* leaves. Rutin, the principal bioactive component, was used as a single marker for QAMS – an emerging method for quality control of herbal medicines. The results obtained with the developed QAMS method were comparable with the external standard method, proving its validity and feasibility for use. From the analysis of commercial samples, rutin was found to show the highest concentration (6.6 mg/g to 18.8 mg/g) among the phenolics while glucomoringin (0.2 mg/g to 4.2 mg/g) was the most present glucosinolate. The two methods were capable of differentiating samples by geographical origin. Hence, in view of the cost and scarcity of reference standards, QAMS could be applied as alternative to the external standard method for quality control of *Moringa* leaf preparations.

Samenvatting

Aangezien de consumptie van plantaardige preparaten wereldwijd aanzienlijk toeneemt, is de vraag naar kwaliteitscontrole een globale aangelegenheid. De kwaliteit kan beïnvloed worden door genetische, fysicochemische en biologische factoren. Meer controle van plantaardige geneesmiddelen is in het bijzonder van belang in ontwikkelingslanden waar deze beperkt tot nagenoeg onbestaande is omwille van een aantal redenen zoals een gebrek aan hedendaagse analytische instrumenten, relevante monografieën, eenvoudig toepasbare methoden en getraind personeel. Kruidenpreparaten op basis van de bladeren van *Aloë* en *Moringa* worden vaak gebruikt en zijn commercieel belangrijke plantaardige producten in Afrika in het algemeen en Ethiopië in het bijzonder. Ondanks hun wijd gebruik in geneesmiddelen, voedsel en cosmetica, wordt hun kwaliteit nauwelijks gecontroleerd. Vandaar dat in deze studie de kwaliteitscontrole van kruidenpreparaten of basis van de bladeren van *Aloë* en *Moringa* nader aan bod kwamen.

In hoofdstuk 1 werd een korte introductie gegeven over traditionele geneeskunde en plantaardige preparaten, evenals over kwaliteitscontrolemethoden. Daarnaast werd ook literatuur over het traditionele medische gebruik, fytochemie en farmacologische activiteit van *Aloë* en *Moringa* uit Ethiopië weergegeven.

In hoofdstuk 2 werd een snelle en gemakkelijk toepasbare LC-UV methode ontwikkeld voor de bepaling van antronglycosiden, de voornaamste bioactieve merkercomponenten in de latex van *Aloë* bladeren. De methode maakte gebruik van een monolithische kolom, een stationaire fase gekend omwille van voordelen zoals lage tegendruk, stabiliteit, snelle evenwichtsinstelling en verenigbaarheid met conventionele LC systemen. De ontwikkelde methode werd gevalideerd met betrekking tot specificiteit, lineariteit, precisie, accuraatheid en robuustheid. Ze werd praktisch toegepast voor de bepaling van aloïnes en hun derivaten in latex van *Aloë* bladeren (product met sap van *Aloë*) verzameld in Ethiopië. In deze studie werden aloïne gehaltes gevonden die varieerden van 14 tot 35%. Daarnaast werden 2 onbekende pieken in het chromatogram met grote waarschijnlijkheid geïdentificeerd als aloïnoside en microdontine.

De hierboven ontwikkelde LC-UV methode werd in hoofdstuk 3 toegepast voor de bepaling van aloïnes en verwante antrones in persoonlijke verzorgingsproducten gebaseerd op gel van *Aloë* bladeren. Aangezien het een andere matrix betreft, werd een aanpassing van de extractieprocedure doorgevoerd. Producten gebaseerd op gel van *Aloë* bladeren worden verondersteld vrij te zijn van elke contaminatie door latex gezien de diverse huidproblemen en

neveneffecten die kunnen optreden. Vervolgens werden ook kwaliteitsparameters zoals pH, massaverlies na drogen en hoeveelheid as bepaald. Volgens de resultaten van deze studie werden in 25% van de geanalyseerde commerciële monsters aloïnes teruggevonden. Daarnaast waren 42% van de onderzochte monsters te alkalisch en 33% van hen bevatten te veel vocht, wat een negatieve invloed kan hebben op de kwaliteit.

In hoofdstuk 4 werd een gevoelige LC-UV methode ontwikkeld om fenolen te bepalen in preparaten van *Moringa* bladeren. Na validatie werd de methode praktisch toegepast op producten gecommercialiseerd in Ethiopië. Bovendien werden ook massaverlies na drogen, totale verassing en in zuur onoplosbare hoeveelheden as in de monsters bepaald als onderdeel van de kwaliteitsevaluatie. Verder werden onbekende pieken in het chromatogram gekarakteriseerd met massaspectrometrie. Uit deze studie bleek dat rutine de meest voorkomende component was in hoeveelheden variërend van 2 tot 18 mg/g. De methode liet toe om verschillen waar te nemen in fenolische samenstelling tussen regio's in Ethiopië. Resultaten voor massaverlies na drogen en hoeveelheid as brachten aan het licht dat respectievelijk 40% en 50% van de monsters niet voldeden.

In hoofdstuk 5 werd een LC-UV methode met kwantitatieve analyse van meerdere componenten door een enkele merker (KAME) ontwikkeld voor de gelijktijdige bepaling van glucosinolaten en fenolen in bladeren van *Moringa.* Rutine, de belangrijkste bioactieve component, werd gebruikt als enkele merker voor KAME, een methode die opgang maakt voor de kwaliteitscontrole van plantaardige geneesmiddelen. De resultaten bekomen met de ontwikkelde KAME methode waren vergelijkbaar met de externe standaardmethode, wat de geldigheid en geschiktheid voor gebruik bewijst. Uit de analyse van commerciële monsters bleek dat rutine de hoogste concentratie (6,6 mg/g tot 18,8 mg/g) vertoonde voor de fenolen terwijl glucomoringine (0,2 mg/g tot 4,2 mg/g) het meest voorkomende glucosinolaat was. De twee methoden waren in staat om monsters te onderscheiden op basis van hun geografische oorsprong. Wanneer de kost en zeldzaamheid van referentiestandaarden beschouwd worden, is KAME een waardig alternatief in vergelijking met de externe standaardmethode voor de kwaliteitscontrole van bereidingen op basis van *Moringa* bladeren.

Scientific acknowledgements and conflict of interest statement

All authors gratefully acknowledge the Interfaculty Council for Development Co-operation (IRO, KU Leuven) for the grant.

All authors involved declare that they have no conflicts of interest.