Application of modern extraction methods for determining toxic phytochemical compounds contained in *Solanum* plants

by

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Thesis for Doctor of Philosophy Degree in

Analytical Chemistry

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August 2021

Declaration

I, **Tebogo Mphatlalala Mokgehle (18009725)** declare that this thesis entitled 'Application of modern extraction methods for determining toxic phytochemical compounds contained in *Solanum* plants' is my own original work. This work is being submitted for the Doctor of Philosophy degree in Chemistry at the University of Venda and has not been submitted for any degree at any other university or institution. The thesis does not contain other persons' writing unless specifically acknowledged and referenced accordingly.

Candidate's signature: 

List of publications

This thesis is based on the following papers:

I. The effect of kosmotrope and chaotrope salts during aqueous two phase extraction (atpe) of polyphenolic compounds and glycoalkaloids from the leaves of a nutraceutical plant, *Solanum retroflexum*, with the Aid of UPLC-qTOF-MS

Tebogo Mphatlalala Mokgehle, Ntakadzeni Madala, Wilson Mugera Gitari, Nikita Tawanda Tavengwa (Applied Biological Chemistry, 2021, 64 (1), 1-15)

II. Hyphenation of aqueous two phase and microwave extraction of solasonine and solamargine from *Solanum* mauritianum via UHPLC-qTOF-MS

Tebogo Mphatlalala Mokgehle, Ntakadzeni Madala, Wilson Mugera Gitari, Nikita Tawanda Tavengwa (Under review)

III. The effect of microwave assisted aqueous two phase extraction of α-solanine from *S. retroflexum* and analysis on UHPLC-qTOF-MS

Tebogo Mphatlalala Mokgehle, Ntakadzeni Madala, Wilson Mugera Gitari, Nikita Tawanda Tavengwa (submitted to journal)

IV. Optimization in the aqueous two phase extraction of solasodine, ATPE of solasodine from *Solanum mauritianum* and analysis via UHPLC-qTOF- Tebogo Mphatlalala Mokgehle, Ntakadzeni Madala, Wilson Mugera Gitari, Nikita Tawanda Tavengwa (submitted to journal)

V. Evaluation of the effect of a chaotrope and kosmotrope in the multivariate optimization of PHWE-ATPE of solasodine from leaves of *Solanum mauritianum*, a UHPLC-qTOF-MS study

Tebogo Mphatlalala Mokgehle, Ntakadzeni Madala, Wilson Mugera Gitari, Nikita Tawanda Tavengwa (submitted to journal)

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VI. Advances in the development of biopolymeric adsorbents for extraction of metabolites from nutraceuticals with emphasis on Solanaceae, and subsequent pharmacological applications

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Tebogo Mphatlalala Mokgehle, Ntakadzeni Madala, Wilson Mugera Gitari, Nikita Tawanda Tavengwa (Under review)

Contribution of the authors

Paper I: Principal author, designed performed the ATPE experiments, evaluation, and analysis of extraction of polyphenols and glycoalkaloids from *Solanum retroflexum*, was also involved in writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement.

Paper II: Principal author, formulated and performed MAE, ATPE + MAE, MA-ATPE coupled experiments. Co-authors checked the draft manuscript and added their inputs for improvement.

Paper III: Main author in the central composite design and execution of the MA-ATPE experiments on the extraction of solanine from *S. retroflexum* and subsequent analysis on UHPLC-qTOF-MS

Paper IV: Main author in the central composite design and execution of the ATPE experiments on the extraction of solasodine from *S. retroflexum* and subsequent analysis on UHPLC-qTOF-MS

Paper V: Principal author, initiated and conducted PHWE-ATPE adsorption studies. Co-authors edited the draft manuscript and added their inputs for improvement.

Paper VI: Principal author participated in planning and writing of the manuscript. Co-authors revised the draft manuscript and made suggestions for improvement (Appendix).

Paper VII: Principal author, involved in planning, performed ATPE and HP-TLC experiments, evaluation of the results and writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement (Appendix)

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Preface

In this work, modern extraction techniques, such as aqueous two phase extraction (ATPE), pressurized hot water extraction (PHWE), microwave assisted extraction (MAE) were used for the extraction of nutraceutical compounds from *Solanum retroflexum* and *Solanum mauritianum*. Analysis of the metabolites obtained was performed using ultra-performance liquid chromatography quadrupole time of flight mass spectroscopy (UPLC-qTOF-MS) and included amongst other flavonoids, polyphenols, and toxic compounds such as glycoalkaloids. The thesis contains seven papers, five are within the scope of the project, while two were performed during the PhD study period and are included in the appendix section.

The effect of kosmotrope and chaotrope salts during ATPE of polyphenolic compounds and glycoalkaloids from the leaves of a nutraceutical plant, *Solanum retroflexum* was evaluated in **Paper I**. This work demonstrated that a comprehensive metabolome of *S. retroflexum,* more than what was previously reported on the same plant, can be achieved by application of kosmotropes (Na_2CO_3) and chaotropes (NaCl) as precipitating agents with the aid of the ATPE approach. The best-performing salts, for glycoalkaloids were the kosmotrope and chaotrope. The ATPE technique was found to be efficient in simultaneous extraction of multiple metabolites.

In **Paper II,** optimization of microwave and aqueous two phase-based extraction techniques which involved MAE, ATPE + MAE and MA-ATPE for the extraction of solasonine and solamargine from leaves of *S. mauritianum* was evaluated. Application of NaCl with CaO-dried ethanol in MA-ATPE yielded a two-fold increase in intensity observed for both solamargine and solasonine in comparison to MAE + ATPE. The synergy of microwaves and salting-out principle in the 'one-pot' MA-ATPE technique was shown to be a contributing factor for enhanced extraction of solamargine and solasodine from leaves of *S. mauritianum,* with the Na_2CO_3 being a better extractor then NaCl. The MA-ATPE technique was a promising extraction method that could be applied on a large scale.

In **Paper III,** a hyphenated microwave assisted aqueous two phase extraction (MA-ATPE) was applied in the extraction of a solanine from *Solanum retroflexum*. Fitting the central composite design response surface model to the data generated a quadratic model with a good fit ($R^2 = 0.920$). The response surface methodology model indicated that the maximal extraction of α-solanine was 82.21 mg kg^{-1} and 77.81 mg kg^{-1} for Na_2CO_3 and NaCl, respectively. The shorter optimal

extraction times of MA-ATPE makes it a potential technique that could meet market demand as a quick and green approach where no organic solvents are applied.

In **Paper IV,** aqueous two phase extraction (ATPE) was applied in the extraction of a toxic metabolite, solasodine, from *Solanum mauritianum*. Central composite design (CCD) was performed which included numerical parameters such as time and mass of plant. The categorical factors included the type of salt used, chaotrope (NaCl) or kosmotrope (Na_2CO_3). Fitting the central composite design response surface model to the experimental data generated a quadratic model with a good fit (R^2 = 0.925). Maximal extraction of solasodine was 233.65 mg kg^{-1} and 413.50 mg kg^{-1} for NaCl and Na_2CO_3, respectively. The application of ATPE, under these conditions, in conjunction with the use of a kosmotrope was shown to enhance the extraction of pharmacologically relevant solasodine.

Paper V focused on the effect of a pressurized hot water extraction (PHWE) and ATPE for enrichment of solasodine from *Solanum mauritianum*. Fitting the central composite design response surface model for PHWE-ATPE to the data generated a model with a good quadratic fit (R^2 = 0.901). The statistically significant ($p < 0.05$) parameters such as the linear and quadratic effect of the concentration of salt (%) had a significant impact on the extraction of solasodine, in the presence of the kosmotrope. Furthermore, the kosmotrope was almost twice a more efficient extractor of solasodine than NaCl with maximal extractions of 300.79 mg kg^{-1} and 162.34 mg kg^{-1} for Na_2CO_3 and NaCl, respectively.

Paper VI highlighted new trends in the development of biopolymers such as polysaccharides and proteins as adsorbents of nutraceutical compounds, with emphasis on metabolites derived from Solanaceae, were discussed. The application of polysaccharides/protein containing the adsorbed *Solanum* derived nutraceutical compound for drug delivery was discussed. Additionally, the nature of molecular interactions between the biopolymer and the drug being adsorbed, were also reviewed (Appendix).

Paper VII was directed at the use of UPLC-qTOF-MS for simultaneous extraction of HP-TLC fluorescent compounds obtained from a *Solanum retroflexum*. This work attempted to evaluate correlation between two independent chromatographic techniques such as HPTLC and ultra high performance liquid chromatography quadrupole time of flight mass spectroscopy (UHPLC-qTOF-

MS). Paper VII also demonstrated that tomatidine galactoside and tomatine commonly associated with *Solanum lycopersicum*, could also be found in *Solanum retroflexum* (Appendix).

Abstract

The *Solanum* genus is among the most diverse and valuable in terms of agricultural utility and vegetable crops. This study was directed at the characterization of toxic metabolites contained in *Solanum retroflexum* and *Solanum mauritianum* following extraction by aqueous two phase extraction (ATPE), microwave assisted extraction (MAE) and pressurized hot water extraction (PHWE) with the aid of UHPLC-qTOF-MS. The application of qTOF-MS offered unprecedented sensitivity for thorough identification of similar metabolites such as solanelagnin, solanine, solamargine, solasonine and solasodine following ATPE. Furthermore, the application of ATPE in the presence of precipitating agents in a form of kosmotropes and chaotropes enabled simultaneous extraction of multiple glycoalkaloids in a single step. The ATPE technique was also observed to be a versatile technique which saw it being compatible with PHWE and MAE. In particular, the application of microwave assisted aqueous two-phase extraction (MA-ATPE) was quantitatively shown to be a better extractant of solasonine and solamargine compared to MAE and MAE+ATPE. Additionally, the synergy of microwaves and salting-out in the 'one-pot' MA-ATPE technique was a contributing factor for enhanced extraction of glycoalkaloids at shorter extraction periods. Multivariate chemometric studies were designed using Design Expert 11 for optimizing the extraction of solasodine (*m/z* 414 → 396) and solanine (*m/z* 868 → 722) based on MRM quantification in MA-ATPE, ATPE and PHWE-ATPE. Comparison of ATPE and PHWE-ATPE for the extraction of solasodine from *Solanum mauritianum* indicated that ATPE was a better extractor of solasodine by a factor of approximately 1.5. The effect of temperature in PHWE-ATPE was shown to be insignificant ($p > 0.05$) and could account for the lower extraction of solasodine compared to ATPE. Furthermore, the effect of mass of plant powder during ATPE was a statistically significant ($P < 0.05$) parameter behind the enhanced extraction of solasodine. Quantification studies based on MRM transition showed that the kosmotrope-Na_2CO_3 was a better extractant than the chaotrope-NaCl for solanine in MA-ATPE and solasodine in ATPE and PHWE-ATPE. This observation, herein, was due to the greater negative charge density of the divalent carbonate ion from Na_2CO_3, which was pivotal in salting-out of the analyte (solanine or solasodine) through the formation of strong hydrogen bonds among water molecules surrounding the solute. As a prototype, ATPE and MA-ATPE could be quick, green purification and enrichment methods for phytochemicals with strong pharmaceutical relevance, which could meet the insatiable appetite for affordable medicines in the market.

Dedications

This work is dedicated to my family, Mom (Winkie) and siblings Elijah and Dineo, there are no words to describe the love and encouragement you provided me. Thank you very much for your continuous prayer and support throughout this project. I am truly inspired by this united spirit and may God continue to strengthen it.

A special thank you goes to my father William for unwavering support and invaluable encouragement during my PhD studies.

Acknowledgements

Above all, I would like to thank my Lord, God Almighty for this awesome opportunity to further my studies. I would like to express my gratitude for the strength, wisdom, determination, and health He gave me to conduct my work.

To my supervisor, Dr N.T Tavengwa, I am extremely grateful for your guidance throughout the project. It has been such a great privilege to work under your supervision. I thank you for stepping out of your comfort zone to make sure I had what I needed to get through as a student when faced with finanacial difficulties. I also thank you for your consistent motivation to ensure that I was mentally and emotionally sound to thoroughly complete the project. May God richly bless you and your family.

My sincere gratitude goes to my other co-supervisor Dr Ntakadzeni Madala. There is so much I have learnt from you particularly as to how one conducts himself as a researcher, the approach one should take when planning a set of experiments, giving insights into the chemistry of metabolites in *Solanum* plants as well as recent developments in instrumental techniques for distinguishing isomeric compounds, the list is simply endless. Thanks indeed for motivating me to discover my true potential as a researcher; I guess one does not really know himself until you take the time to discover what you are truly capable of.

I would like to thank my co-supervisor, Prof Wilson Mugera Gitari for your expert advice and priceless contribution to this project. Your help is sincerely appreciated!

I would like to extend my appreciation to the Department of Biochemistry Research Group at the University of the Johannesburg and Dr Tugizimana for opening their doors for me to conduct metabolic profiling of my samples using the UPLC-qTOF-MS. It is because of your open arms that this project was able to be completed. Thank you so much!

To the Chemistry Department at the University of Venda, thank you for your assistance throughout this project.

Conference presentations

Tebogo Mphatlalala Mokgehle, Ntakadzeni Madala, Wilson Mugera Gitari, Nikita Tawanda Tavengwa. The effect of chaotrope and kosmotrope salts on the extraction of phytochemicals from three *Solanum* species during aqueous two phase extraction (ATPE), SACI North region 2019 Conference, 30 October, Thohoyandou, South Africa, *Oral presentation*

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List of abbreviations

ATPE	Aqueous two phase extraction
LC	Liquid chromatography
MS	Mass spectroscopy
MAE	Microwave assisted extraction
PHWE	Pressurized hot water extraction
qTOF	Quadrupole time of flight
UPLC	Ultra high performance liquid chromatography
DLLME	Dispersive liquid-liquid microextraction
ERK	Extracellular signal-regulated kinases
PVX	Potato virus X
ERK	Extracellular regulated kinases
MASE	Membrane assisted solvent extraction
MIPs	Molecular imprinted polymers
CCD	Central composite design

List of tables

Chapter 1 - Introduction and background

This chapter introduces the reader and gives a background to the study as well as the problem statement. This section concludes by providing an outline on the work conducted in this thesis.

1.1 Background of study

The lifestyles of human beings have drastically changed since the industrial revolution. Increased working hours and various socio-economic pressures have led to people opting for fast foods with depreciable amounts of essential nutrient necessary for healthy living. Additionally, the emergence of the industrial age has resulted in soil and food contamination due to the emission of toxic chemicals into the air and harmful electromagnetic radiation such as UV, gamma, or X rays. These man-made problems have resulted in increased cases of diseases such as diabetes, high blood pressure, neurodegenerative illnesses, and various types of cancers. These sicknesses are always associated with increased health care costs due to the demand for synthetic medicines. This has prompted researchers to turn to a much cheaper source of nutrition, plants. The use of plants as a source of nutrients has been an ancient practice, with Egyptians, Jews, Arabs, Chinese, Greeks, and Romans being at the forefront (Hussain, 2019; Sharma et al., 2019). Currently, there is an increase of skin care products, supplements and oral or injected drugs which are primarily derived from plants (Apone et al., 2018; Pérez-Sánchez et al., 2018; Rezaeiamiri, et al., 2020). Additionally, Rauf and Jehan (2015) reported that more than 80% of the world's population is reliant on medicinal plants to maintain their health.

The efficacy of medicinal plants in human health is derived from the bioactive phytochemicals produced from the plant's primary or secondary metabolism which generally play a role in growth or defense against pathogens or predators (Latif et al., 2017; Hammerbacher et al., 2019). One such family with a wide range of bioactive compounds is Solanaceae (Sinani et al., 2017); these phytocompounds have been discussed to a greater extent in **paper I**. Solanaceae have been studied, to be one of the largest groups of angiosperms (Ramírez et al., 2018). The Solanaceae family is composed of approximately 100 genera and over 3500 species according to Samuels (2015) and Rasul et al. (2019). The diversity of *Solanum* genus has broadened its significance as it includes edible, medicinal and ornamental plant species. Economically, Solanaceae is the third most valuable taxon in essential nutrients found in food supplements and medicines (Rasul et al., 2019). Furthermore, it is regarded as the most diverse and valuable in terms of agricultural utility and vegetable crops (Samuels, 2015; Rasul et al., 2019). The *Solanum* genus is known for its toxic metabolites which varies in concentration. amongst different species; resulting in it still being edible by some communities or inedible by others (Ramírez et al., 2018). The Solanaceae family

is a rich source of nutrients derived from common vegetables such as *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato) and *Solanum melongena* (eggplant) as shown in Figure 1.1.

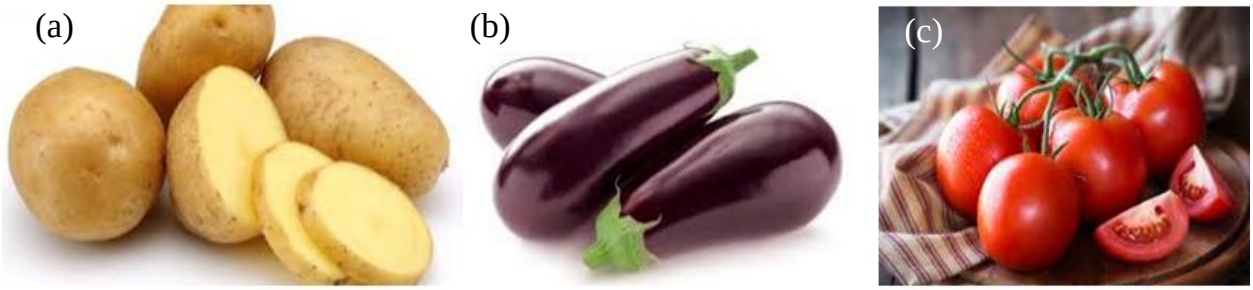


Figure 1.1: Some common *Solanum* plants (a) *Solanum tuberosum* (b) *Solanum melongena* (c) *Solanum lycopersicum*

Solanum species have been studied to synthesize secondary metabolites to protect themselves against phytopathogens (Sanchez-Maldonado et al., 2016; Al-Ashaala et al. 2018; Sinani et al., 2019). Secondary metabolites have been renowned for their activity against herbivores, bacteria, viruses, insects, and fungi (Chen et al., 2020; Sucha et al., 2016). For example, phenolics metabolites commonly found in Solanaceae have gained interest from researchers due to their protective role through antioxidant potential (Verma et al., 2016). Additionally, phenolic compounds are essential for the growth and reproduction of plants and are produced as a response for defending injured plants against pathogens (Verma et al., 2016). Plant phenolics are characterized by hydroxylated aromatic rings such as flavanols (Dzailo et al., 2016). Some *Solanum* species have been studied for quantification and identification of phenolic compounds in *Solanum scabrum* and *Solanum burbankii* berries for antioxidant effects (Oszmainski, 2014).

A variety of factors such as pH, light, temperature, water deficit, soil texture and moisture content influence the secretion of secondary metabolites from *Solanum* plants (Morillo et al., 2020). Another class of secondary metabolites primarily found in Solanaceae, are glycoalkaloids widely studied for their desirable effects. For instance, glycoalkaloids have been reported to show significant antidiabetic (Al-Ashaala et al. 2018), antifungal (Sanchez-Maldonado et al., 2016), antiparasitic (Anwar et al., 2020), antimicrobial (Kalimuthu et al., 2018), hepatoprotective (Chester et al., 2019) and anticancer (Arslan et al., 2018). Hence, glycoalkaloids have extensively been applied in the manufacture of contraceptives and steroidal anti-inflammatory drugs (Tiossi et

al., 2012; Morias et al., 2020). Further applications of glycoalkaloids are detailed in **papers I** and **VI**.

Chemically, glycoalkaloids are naturally occurring N-containing secondary metabolites found in plants of Solanaceae. Glycoalkaloids consist of two structural components, an aglycone unit and a carbohydrate sugar side chain which are polar and non-polar, respectively. The aglycone unit consists of hydrophobic C27 steroid skeleton of cholestane with nitrogen incorporated into the F ring. The second unit is a hydrophilic carbohydrate side chain attached at the 3-OH position (Sinani et al., 2017; Patel et al., 2021). The aglycones are divided into five different categories depending on their structure: solanidanes (with fused indolizidine rings), spirosolanes (with an oxa-azaspirodecane alkaloid portion) epiminocholestanes, epiminocyclohemiketals and 3-aminospirostanes (Väänänen, 2007; Sinani et al., 2019; Hassan et al., 2020). For instance, structurally, similar glycoalkaloids, solasonine and solamargine, have the same aglycone, solasodine which are derived from spirosolanes, but differ in the type of the carbohydrate side chain as shown in Figure 1.2. Furthermore, solasodine due to its water-insoluble characteristic, is considered as a promising compound for synthesis of steroidal drugs, having exhibited impressive anticancer activity, insecticide property and an important anti-accelerator cardiac action (Fekry et al., 2019, Carvalho et al., 2019; Hassan et al., 2020). The carbohydrate side chain is attached to the 3-hydroxyl position of aglycones as shown in Figure 1.2 and consists of diverse arrangements of D-glucose, D-galactose, D-xylose and L-rhamnose generally reaching a maximum of tri or tetrasaccharides (Sinani et al., 2017).

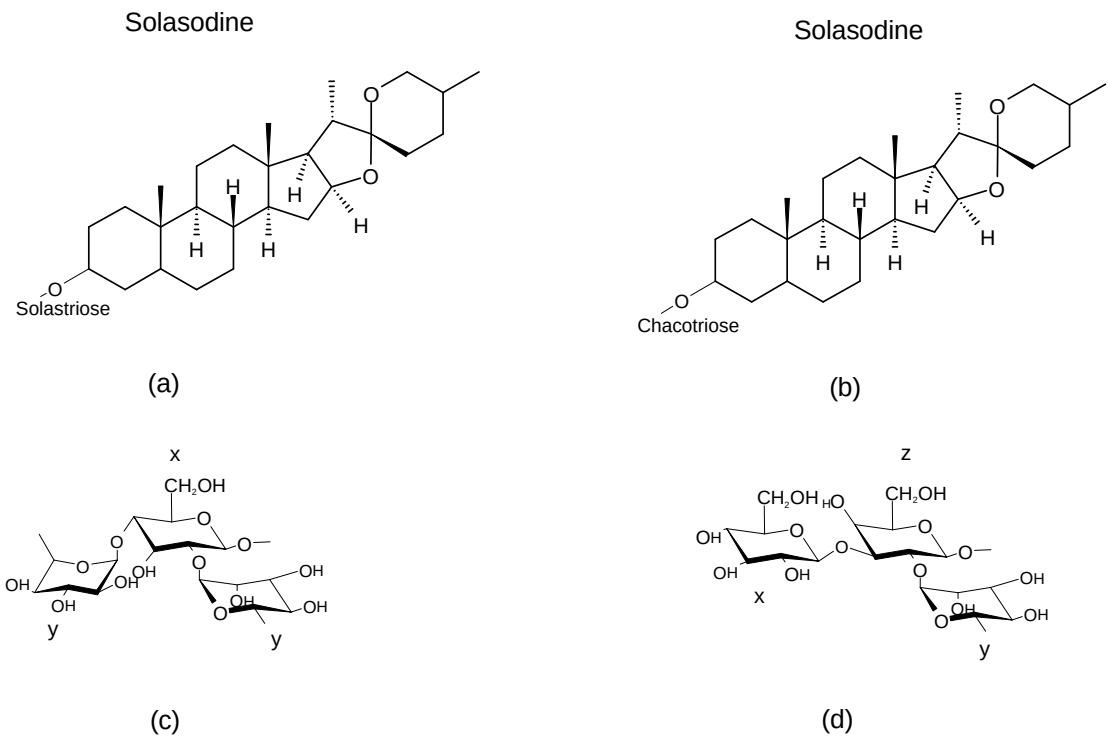


Figure 1.2: Structures of *Solanum* potato glycoalkaloids (a) solasonine and (b) solamargine showing different sugar side chains in (c) chacotriose and (d) solatriose where x is β-D-glucose, y is α-L-rhamnose and z is β-D-galactose

A variety of mechanisms on the mode of action of phytochemicals on pathogens have been suggested. These include interference with some metabolic processes or modulation of gene expression and signal transduction pathways (Friedman et al., 2015; Nepal et al., 2019; He et al., 2020). Glycoalkaloids have also been studied to disrupt cell membranes by affecting ion transport across cell membranes through alteration of cell potentials (Sucha et al., 2016; Nepal et al., 2019). Other mechanisms of action include the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Chowański et al., 2016; He et al., 2020). Shi (2013) reported that steroidal alkaloids such α-tomatine inhibited the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK 1, 2) and the serine-threonine kinase (Akt). These kinases (ERK 1, 2) were reported to be involved in metastasis and the progression of cancer in the human breast (Shi et al., 2013; Sucha et al., 2016).

In as much as natural products have been an integral part of both ancient and modern civilization, so has the extraction methods for acquiring useful phytochemicals. Extraction of phytochemicals

from plants dates to the Sumerian and the Akkadian civilizations in about the third millennium BC (Doughari et al., 2012). Ancient methods often involved the use of boiling water as a solvent to extract phytochemicals; other methods included fermentation where a crude extract is soaked in water for a period to allow for break-down of large organic compounds into simpler substances such as carbohydrates and alcohols. To cope with an ever-increasing demand for therapeutic drugs with high potency and efficacy, more advanced and greener extraction methods are constantly being developed to achieve high phytoconstituent yield and maintain the structural integrity of the extracted phytochemicals. Modern techniques such as pressurized hot water extraction (PHWE) and aqueous two-phase extraction (ATPE) are derivations of traditional methods where hot water and fermentation is used for extraction, respectively. ATPE has been recognized as a versatile technique for the downstream processing of biomolecules (Xie et al., 2017; Chong et al., 2020). Additionally, ATPE has a potential to achieve the desired purification and concentration of the product in a single step. The major advantages of ATPE are its high capacity, biocompatible environment, low interfacial tension of phases, high yields, low process time and energy (Jiang et al., 2019; Mittal et al., 2019; Mokgehle et al., 2021). Additionally, ATPE has a potential to achieve the desired purification and concentration of the product in a single step (Hua et al., 2013).

Another attractive and eco-friendly extraction technique is PHWE. This method is a simple, rapid "green extraction" alternative for recovery of analytes without the need for "clean-up". Therefore, the extracted compounds can be safely and immediately consumed or further used in the manufactured foods (Ameer, et al., 2017). PHWE is performed at elevated temperatures and pressures, so the solvent is kept in the liquid state, thus enhancing the extraction by improved mass transfer and stability. The principle behind the extraction ability of water at its critical point is its lowered dielectric constant (from $\varepsilon = 80$ at 25^{o}C at 1 bar to $\varepsilon = 27$) which is comparable to many organic solvents, permitting it to dissolve a wide range of compounds with low to medium polarity (Gbashi et al., 2016; Kovačević et al., 2018). This technique has been extensively explored in the pharmaceutical (McQueen et al., 2019), food (Jiang et al., 2019) and environmental (Li et al., 2020) industries. It has been successfully utilized for the extraction of nutritional constituents, pharmacoactive compounds and organic pollutants from vegetal tissues, food products, soil sediments and other ecological biomasses (Gbashi et al., 2016). Matshediso (2015) optimized the extraction of three flavanols i.e., kaempferol, quercetin and myricetin and investigated the total phenolic content in Moringa leaf powder using PHWE. Additionally, this technique was also

applied in the isolation of pharmacologically important metabolites from leaves (Nuapia et al., 2020), flavonoids from *Citrus unshiu* peel (Kim et al., 2020) and polyphenols from *Stevia rebaudiana Bertoni* leaves (Kovačević et al., 2018).

One technique that is dependent on electromagnetic waves for extraction of phytocompounds is microwave assisted extraction (MAE). This was first reported by Ganzler et al. (1986). This method is a relatively easy technique compared to tedious traditional methods. MAE is also renowned for its environment friendliness and is an economical technique for the extraction of biologically active compounds from different plant materials (Hemwimon et al., 2007; Vinatoru et al., 2017; Kaderides et al., 2019). Application of microwaves is quick, as it can heat the whole sample simultaneously (Horuz et al., 2017). Besides the application of heat during microwave radiation, there are non-thermal effects that play a role in the chemistry of phytochemicals (Liu et al., 2021; Bichot et al., 2020). This lies in the effect of the electric field brought about by the electromagnetic properties of microwaves to polarize the charge of the materials. Dielectric polarization occurs in the following manner as highlighted by Al-Harahsheh (2004); electron polarization due to the change of electron position around the nucleus; atomic polarization caused by positional shifts of the nucleus due to the non-uniform distribution of the charge within the molecule; orientation polarization caused by the reorientation of the permanent dipoles due to the influence of electric field and spatial charge polarization observed when material contains free electrons whose distribution is limited by the grain surface. This has advantages such as lowering the activation energy (Pourebrahimi and Kazemeini, 2018; Yu et al., 2018).

This entire study was directed at the application of modern extraction techniques such as PHWE, ATPE and MAE on the extraction of a toxic class of metabolites, alkaloids and glycoalkaloids that are predominant within the *Solanum* genus. Though glycoalkaloids are allelopathic defenders, toxic to other species, these are bioactivities metabolites are renowned for their anti-cancer characteristics (Sinani et al., 2017; Fekry et al., 2019; Carvalho et al., 2019, Nguenang et al., 2020, Hassan et al., 2020). Considering the grim milestone of approximately 5.02 million deaths in 2020 due to cancer related illnesses (WHO, 2021) and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) being responsible for over 200 million cases and 4.2 million deaths worldwide from 2020 to the first half of 2021, as reported by the John Hopkins Coronavirus Resource Centre, extraction of glycoalkaloids from natural products using green extraction techniques is even more worthwhile. One of the extraction approaches that was investigated was

the application of various chaotropes and kosmotropes salts in an ethanol/salt ATPE setup for obtaining polyphenols and glycoalkaloids (**Paper I**). Hyphenation involving microwave assisted aqueous two-phase extraction (MA-ATPE) was developed for the enrichment of solamargine and solasonine from leaves of *Solanum mauritianum* and compared to ATPE and MAE+ATPE (**Paper II**). Quantifications studies were also done using a highly sensitive approach on the ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-qTOF-MS) based on multiple reactions monitoring (MRM) approach. The UHPLC-qTOF-MS technique is an attractive approach due to its ability to offer fast, high resolution chromatographic separation from samples containing complex mixtures of compounds. The qTOF offers refined chromatographic peak separation and is hence the most widely used tool in the profiling of metabolites in crude samples and can identify elemental composition for both parent and fragment ions (Jin et al., 2018; Velamuri et al., 2020). Furthermore, the integration of quantitative and qualitative analysis is one of the important applications of this technique. One such technique that couples both the qualitative and the quantitative aspect of UPLC-qTOF-MS includes multiple reaction monitoring (MRM) window of selected mass range, can be used for structural elucidation of metabolites. The qTOF-MS is helpful in the structure elucidation and identification of fragmentation patterns of the compounds. Other advantage of MRM includes reduced chemical noise and contaminants from ion source, thereby allowing thorough quantification of the analyte. The MRM technique was applied for MA-ATPE from leaves of *Solanum retroflexum* (**Paper III**) and ATPE on leaves of *Solanum mauritianum* (**Paper IV**). Furthermore, ATPE was applied with PHWE on leaves of *Solanum mauritianum* (**Paper V**).

1.2 Problem statement

Sub-Saharan Africa with over 10% of the world's population has the highest occurrence of under-nutrition in the world (FAO, 2014), with over 70% of rural population who depend mainly on self-produced foods. Incidences of diet-related diseases like various cancers and cardiovascular diseases are also increasing in these communities (Sirma et al, 2018; Naja et al., 2019). The modern system of medicine which comprise of drugs from synthetic origin suffers a drawback of adverse effects and exorbitant costs which propelled indigenous communities towards ethnopharmacognosy. A practical and sustainable option for addressing this burden of malnutrition in such communities is by exploiting the potential of local biodiversity especially regarding vegetables. Indigenous African leafy vegetables have been shown to possess anti-oxidative properties and thus have the potential as natural sources for reducing cellular oxidative damage, and suppression of various cancers and cardiovascular diseases (Neugart et al., 2017; Arslan et al., 2018; Ezekwe et al., 2020). It is therefore common nowadays to find in restaurants, hotels and public canteens, vegetable dishes based on African nightshade (*Solanum scabrum*) and *Solanum retroflexum* (Neugart et al., 2017).

The Solanaceae family contains a variety of nutraceutical species and have captured the attention of many researchers around the world primarily due to the bioactivity of its metabolites. Despite this, *Solanum* plants are notorious for their poisonous effects and a metabolic class responsible for this toxicity are glycoalkaloids (Sinani et al., 2019; Mokgehle et al., 2021). Glycoalkaloids derived from *Solanum* plants are highly diverse and are amphiphilic composed of an aglycone (solanidanes or spirosolanes or epiminocholestanes or epiminocyclohemiketals or 3-aminospirostanes) which is glycosylated to a carbohydrate side chain, of which the individual monosaccharides such as xylose, glucose, galactose and rhamnose differ by number, nature, and arrangement. Researchers, have, therefore, exploited the toxic profile of glycoalkaloids and applied them as anticancer, antibacterial, and antifungal agents (Morillo et al., 2020; Pelo et al., 2021). For instance, solamargine, solasonine and α-tomatine exhibited toxic effects by reducing larval growth of the red flour beetle *Tribolium castaneum* and α-tomatine also showed inhibitory activity on tobacco hornworm, *Manduca sexta* (Weissenberg et al., 1998; Ventrella et al., 2016). Moreover, potato derived glycoalkaloids exhibited ovicidal effect and repellent activity against *Spodoptera exigua* moths (Adamski et al., 2009; Ventrella et al., 2016) and decreased the frequency of the *Zophobas atratus* heart contractions causing irreversible or fast and reversible cardiac arrests (Ventrella et

al., 2015). Notably. α-Solanine was reported to disturb *Galleria mellonella* development, fecundity and fertility, and disturbed prooxidant-antioxidant balance (Büyükgüzel et al., 2013; Ventrella et al., 2016). According to Smith et al. (2008), a 52-year-old woman who consumed *Solanum torvum* berries experienced vomiting, diarrhoea, blurry vision, ataxia, slurred speech, and weakness. Clinical evaluation of the patient revealed ptosis, muscle fasciculations, diaphoresis, dyspnea and urinary incontinence. Another study by Glover et al. (2016) reported on a case of poisoning of a 54 year old woman after intake of a glycoalkaloid (solasonine). Furthermore, the Centre for Food Safety (2015) reported on poisoning of patients after consumption of cooked potatoes, subsequent investigations revealed that the poisoning was due to the glycoalkaloid solanine.

Despite the toxicological effects of glycoalkaloids, these metabolites are also known for their pharmacological effectiveness towards human health such as antidiabetic (Al-Ashaala et al. 2018), antifungal (Sanchez-Maldonado et al., 2016), antiparacetic (Anwar et al., 2020), antimicrobial (Kalimuthu et al., 2018) and anticancer (Arsaln et al., 2018). Therefore, there is an ever-growing appetite for these compounds in nuetraceuticals. Acquiring biologically active compounds from plant resources is a multistep process and generally entails, extraction, isolation, analytical characterization, and clinical evaluation of bioactive compounds. The extraction stage is the most critical; however traditional extraction techniques are accompanied by many setbacks which include high costs associated with buying toxic organic solvents, large volumes of required organic solvents, long extraction times, low yields of bioactive phytoconstituents, poor reproducibility and requirement for high extraction temperatures (Azwanida et al., 2015; Verma, 2016; Zhang et al., 2018). Another drawback is that traditional extraction techniques require tedious multiple steps often yielding a limited number of metabolites (Zhan et al., 2020). Hence, there is a dire need for effective, eco-friendly, single-step extraction techniques, that are efficient in the isolation of targeted bioactive compounds which is often embedded in a complex plant matrix system.

Chromatographic applications involving silica gel, have therefore been applied for purification of plant metabolites. However, this technique is limited as it is dependent on the pH of the mobile phase, is time-consuming, requires large amounts of organic solvents, making it costly and demands careful consideration of solvent mixtures to enable the mobile phase to elute with the desired metabolites (Liu et al., 2020). A traditional practice following silica gel based column chromatography, would be to characterize the eluted samples using nuclear magnetic resonance

(NMR), which to some extent may elucidate the metabolic structure (Mediani et al., 2017). However, considering the diverse nature of plant secondary metabolites which can be in their tens of thousands in a single sample, NMR analysis is an inefficient technique for distinguishing each metabolite. More advanced technologies such as high-resolution chromatographic separation techniques such as ultra-performance liquid chromatography hyphenated to Quadrupole-Time-of-Flight Mass Spectrometry (UPLC-qTOF-MS), allows for the physical separation of thousands of metabolites in a single run and improve the ability to isolate complex phytochemical structures, thereby facilitating opportunities for unambiguous identification of the unknown metabolites.

Hence, this work was directed at applying environmentally friendly extraction methods (PHWE, ATPE and MAE), which use water or ethanol as extraction solvents, in optimizing extraction of toxic metabolites in *Solanum mauritianum* and *Solanum retroflexum*. This was achieved by applying MS/MS and metabolomics data to distinguish closely related isobaric and isomeric metabolites based on fragmentation patterns. This allowed for structural elucidation of metabolites of interest. Thereafter, targeted quantification of toxic metabolites (glycoalkaloids) using optimal multiple reaction monitoring (MRM) transitions and product ion scan (PIS) on the UPLC-qTOF-MS was performed. Optimization of the extracted metabolites was done through chemometric techniques for each extraction method through factorial designs, and the output viewed through response surface models.

1.3 Aim and objectives

1.3.1 Aim

To optimize extraction methods PHWE, ATPE and MAE for characterization toxic phytoconstituents such as glycoalkaloids in crude extracts obtained from *Solanum retroflexum* and *Solanum mauritianum*.

1.3.2 Objectives

- Sampling of *Solanum retroflexum* and *Solanum mauritianum* plant species located in the Vhembe district in South Africa.
- Metabolic profiling of toxic metabolites in *Solanum retroflexum*, extracted using kosmotropes or chaotropes during ATPE (**paper I**)

- Application of optimized hyphenated modern extraction techniques (microwave assisted aqueous two phase extraction) for extraction of glycoalkaloids from *Solanum mauritianum* (**paper II**)

- Optimization of modern extraction methods for targeted toxic metabolites based multivariate chemometric approaches involving central composite design (CCD) and quantification through optimal MRM transitions and MRM – product ion scan with the aid of UHPLC-qTOF-MS:

    - ❖ Evaluation of the effect of mass of plant powder, microwave power and irradiation time during (MA-ATPE) of solanine from *Solanum retroflexum* **(paper III)**

    - ❖ Assess the influence of mass of plant powder and extraction time during ATPE of solasodine from *Solanum mauritianum* **(paper IV)**

    - ❖ Determine the effect of temperature and percentage concentration of a kosmotrope and chaotrope in the extraction of solasodine from during (pressurized hot water extraction and aqueous two phase extraction (PHWE-ATPE) **(paper V**)

Chapter 2- Literature review

This chapter gives a literature scan of the toxic metabolites reported in *Solanum* species. It also highlights factors contributing to the synthesis of these toxic metabolites as well as their mode of action in plant defence. The application of *Solanum* derived toxic compounds in medicine as well as the evolution of extraction methods (ATPE, MAE and PHWE) and its mechanisms in obtaining pharmacologically active phytocompounds, is summarized.

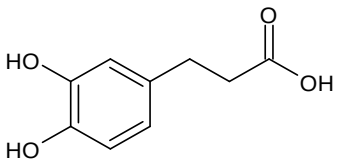
2.1 The general importance and variety of *Solanum* plants (Solanaceae)

The Solanaceae family comprises 90 genera and about 2300 species (Sharma et al., 2017; Chirini et al., 2018). The genus *Solanum* is the largest and most diverse of the genera consisting of over 1500 species (Alajmi et al., 2018; Zuluaga et al., 2020). The *Solanum* genus contains many food crops important to agriculture, food security, human nutrition, and health. For instance, edible plants such as potato (*Solanum tuberosum L.*), eggplant (*Solanum melongena L.*), naranjilla (*Solanum quitoense Lam.*) and tomato (*Solanum lycopersicum*) are common vegetables in supermarkets. Some of the plants derived from the genus *Solanum* have been applied as insecticides which include the winter cherry (*Solanum pseudocapsicum L.)* (Jeyasankar et al., 2017). Pharmacologically important plants such as bittersweet (*Solanum dulcamara L.*) and *Solanum viarum Dun.*, are rich sources of corticosteroids, among which includes dexamethasone, a drug reported to have reduced mortality among SARS-CoV-2 patients (Sterne et al., 2020). Other genera within Solanaceae include *Lycianthes, Cestrum, Nolana, Physalis, Lycium, Nicotiana, Brunfelsia, Atropa* with approximately 200, 150, 89, 85, 85, 76, 45 and 6 species, respectively. For instance, the *Nicotiana* genus (tobacco plants) contains a toxic alkaloid nicotine, which in small doses is an acetylcholine agonist resulting in muscle activation, however, excessive amounts may lead to heart attacks (Wang et al., 2015; Martinez et al., 2020).

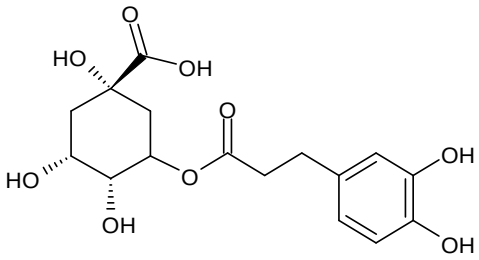
2.2 Genera within solanacae

2.2.1 *Solanum L.* and its nightshade species

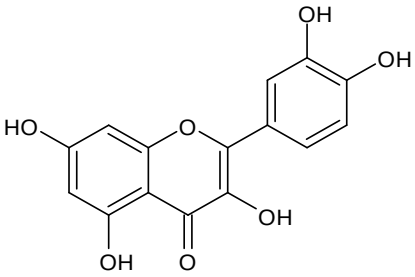
A vast majority of the plants within the *Solanum* genus consist of nightshade species. The edible nightshades consist of about 30 species of botanically and genetically related plants within the *Solanum* genus of the Solanaceae family (Yang et al., 2013). Some of the nightshade species of *Solanum* include vegetables such as *Solanum nigrum, Solanum lycopersicum. Solanum melongena and Solanum retroflexum.* Nightshade species within sub-Saharan Africa are consumed as vital nutrient-rich foods and applied for their medicinal qualities; hence most nightshade species are regarded as nutraceutical plants. The nutraceutical properties of *Solanum* species arise due to its metabolite composition. These metabolites include amongst others, polyphenols such as phenolic acids (Sánchez-Maldonado et al., 2016; Kaushik, 2019) chlorogenic acids (Głosek-Sobieraj et al., 2019; Joly et al., 2021) and flavonoids (Daji et al., 2018; Mokgehle et al., 2021) (Figure 2.1).



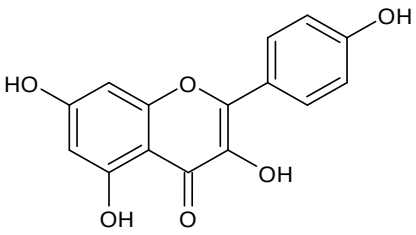
Caffeic acid



Chlorogenic acid



Quercetin



Kaempferol

Figure 2.1: Examples of some common metabolites in species of the *Solanum* genus; phenolic acid (caffeic acid), chlorogenic acids and flavonoids (quercetin and kaempferol)

2.2.1.1 Toxic metabolites (glycoalkaloids) in *Solanum* plants and factors influencing their synthesis

In addition to polyphenols, *Solanum* plants have been shown to produce a toxic class of metabolites known as steroidal glycoalkaloids (SGAs) (Fogelman et al., 2019; Mokgehle et al., 2020). SGAs are N-containing secondary metabolites found in plants of Solanaceae (Nightshade family), and have attracted immense attention from researchers, especially from a human health perspective (Fogelman et al., 2019; Henessesy et al., 2020). These toxic compounds are widely distributed across *Solanum* plants such as potato (*Solanum tuberosum L.*), sweet pepper (*Capsicum annum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), black nightshade (*Solanum nigrum*) and thorn apple (*Solanum incanum*). *Solanum* plants have evolved secondary biochemical pathways that allow them to synthesize SGAs, as a response mechanism to specific environmental stimuli, such as herbivore-induced damage (Silva et al., 2021) and pathogen attacks (Paudel et al., 2017; Al-Maawali et al., 2021). These secondary metabolites can be characteristic to species or genera and do not play any role in the plants' primary metabolic requirements but enhance the plant's ability to survive environmental difficulties. Some of the protective roles of SGAs against microorganism, bacteria or viruses involve antioxidant (Al-Ashaala et al., 2018), free radical-scavenging (Al-Hay Al-Ashaal, 2017), UV-light absorbing (Yuan et al., 2019), and antiproliferative activity (Sinani et al., 2017; Ali et al., 2020; Zhao et al., 2021). Glycoalkaloids also manage inter-plant relationships, acting as allelopathic defenders against competitor plants (Sinani et al., 2017; Sołtys-Kalina et al., 2019). They provide feeding deterrence, as many phytochemicals are bitter and/or toxic to potential herbivores, with their toxicity often affecting the herbivore's central and peripheral nervous systems (Figure 2.2). Some of the steroidal glycoalkaloids obtained from the *Solanum* genus are listed in Table 2.1.

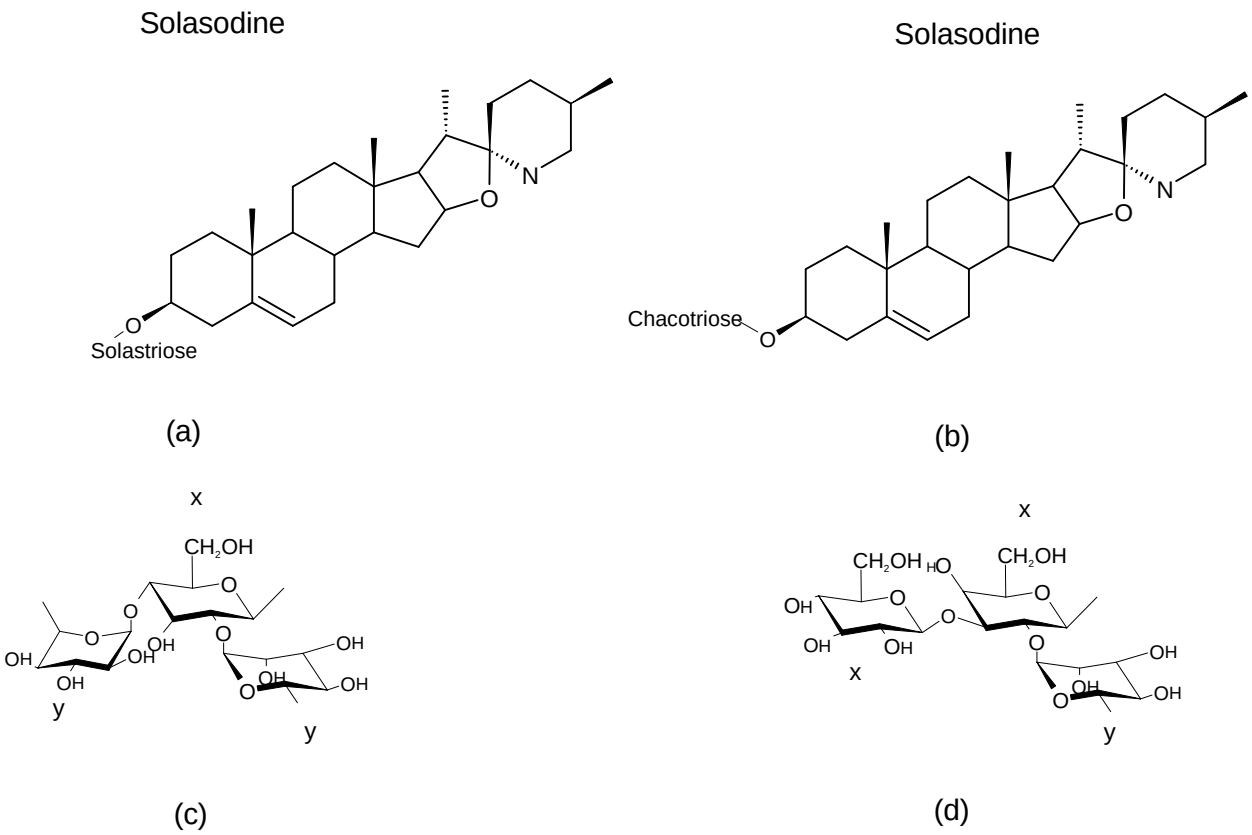


Figure 2.2: Glycoalkaloids contained in species of the *Solanum* genus where (a) + (c) gives solasonine and (b) + (d) gives solamargine

Table 2.1: Breakdown of some common glycoalkaloids within the *Solanum* genus

Glycoalkaloid	Aglycone	Carbohydrate unit
α-solanine	Solanidine	Solatriose: galactose + glucose + rhamnose
β-solanine	Solanidine	Solabiose: galactose + glucose
ɣ-solanine	Solanidine	Galactose
α-chocanine	Solanidine	Chacotriose: glucose + rhamnose + rhamnose
β-chocanine	Solanidine	Chacobiose: glucose + rhamnose
ɣ-chocanine	Solanidine	Glucose
Solamargine	Solasodine	Chacotriose: glucose + rhamnose + rhamnose
Solasonine	Solasodine	Solatriose: galactose + glucose + rhamnose
α-solmarine	Tomadidenol	Solatriose: galactose + glucose + rhamnose
β -solmarine	Tomatidenol	Glucose + rhamnose + rhamnose

2.2.1.2 Biosynthesis and degradation of toxic compounds (glycoalkaloids) in *Solanum* plants

The general pathway begins with steroid biosynthesis which involves the reaction of acetate with acetyl-coenzyme A and then follows through the intermediates of mevalonic acid, squalene, lanosterol, and cycloartenol to cholesterol. Two possible pathways for glycoalkaloid synthesis from cholesterol have been proposed by Milner et al. (2011) where solasodine and soladulcidine are formed from cholesterol, while tomatidenol, tomatidine, solanidine and demissidine from saturated cholesteranol. The next step in the biosynthesis after aglycone formation is glycosylation. Many studies have shown that, after their formation, aglycones are rapidly enzymatically glycosylated by sugar to the α-form of glycoalkaloids (Sinani et al., 2017; Lobo et al., 2018).

The concentration of glycoalkaloids in *Solanum* plants is dependent on various factors which include seasonal variations, UV-light and maturity of the plant. Naturally occurring glycoalkaloids are called α-compounds. The sugar chains are subject to hydrolytic cleavage by chemical or enzymatic means (Milner et al., 2011). Stepwise cleavage of the glycoside side chain leads to β- and γ-compounds in trisaccharides and β-, γ-, and δ-compounds in the case of tetrasaccharides (Sinani et al., 2017). Degradative enzymes and substrates are in different compartments within cells; thus, enzymes are activated after tissues are disrupted. In tomato, enzymatic degradation of glycoalkaloids occurs when tomatoes ripen (Friedman et al., 1993; Dzakovich et al., 2021).

Most nightshade species are consumed for their nutritious leafy vegetables compared to the fruits as the leaves are relatively less toxic diet (Sivakumar et al., 2020). Collected or cultivated nightshade species due to their demand are consumed or sold in local markets, generating an economic opportunity for small-scale farmers, particularly in poverty-stricken areas (Weinberger et al., 2004; Frison, 2016; Yuan et al., 2018). Furthermore, these localized grown nightshade species provide a more sustainable option rather than dependence on expensive imported as European vegetables (Yuan et al., 2018). Besides being used as a vegetable in Africa, *Solanum nigrum* has been reported in treatment of gastric ulcers (Zaghloul et al., 2020; Mureithi et al., 2020). Besides *Solanum* species being edible, these plants have a wide range of pharmacological applications due to the metabolites they produce which include glycoalkaloids and polyphenols (flavonoids) as listed in Table 2.1 and Table 2.2, respectively. A range of cinnamic acids have been reported by Singh et al. (2020) to have antidiabetic and hepatoprotective effects and include ferulic acid, chlorogenic acid caffeic acids. Flavonoids have been reported in *Solanum macrocarpon* (Ogunsuyi et al., 2020), *Solanum nigrum* (Ogunsuyi et al., 2020), *Solanum incanum* (Sbathu et al., 2020), *Solanum* terminale (Kingo et al., 2020) and *Solanum sisymbriifolium* (More et al., 2020).

Table 2.2: Some of the metabolites found within the *Solanum* genus and the respective biological activities

Solanum species	Metabolite	Biological activity	Reference
S. macrocarpon	Phenols, flavonoids, alkaloids	Inhibition of MAO, AChE	Ogunsuyi et al. (2020)
S. nigrum	Phenols, flavonoids, alkaloids	Inhibition of MAO, AChE	Ogunsuyi et al. (2020)
S. elaeagnifolium Cav.	Quercetin, kaempferol	Inhibition of perenial weeds	Balah et al. (2020)
S. nigrum	Polyphenols	Reduction of body fat	Peng et al. (2020)
S. gilo Raddi	Polyphenols	Antioxidant	Maimoto et al. (2020)
S. incanum	Alkaloids, saponins, flavonoids	Antimicrobial	Sbhatu et al. (2020)
S. lycopersicum	Rutin	-	Mokgehle et al. (2021)
S. tuberosum peels	Chlorogenic, caffeic and ferulic acids	Antidiabetic, hepatoprotective	Singh et al. (2020)
S. torvum	4-O-CQA, 3-O-CQA, 3,5-diCQA,	Inhibition of breast cancer	Helilusiatiningsih et al. (2020)
S. melongena	Afidopyropen	Insecticide	Chawla et al. (2020)
S. terminale	Alkaloids, steroids, flavonoids	Diabetes and hypertension	Kingo et al. (2020)
S. sisymbriifolium	Alkaloids, phenols, and flavonoids	Suppression of macrophage cells	More et al. (2020)
S. villosum	-	Antimicrobial	Abdelgawwad et al. (2020)

CQA-caffeouyl qunic acids, AChE- Acetylcholinesterase, MAO- Monoamine oxidase inhibitors

2.3 Protective mechanisms of Solanaceae alkaloids

Solanaceae species have been studied to produce to predominantly produce tropane (nitrogenous bicyclic compounds) based alkaloids. Plant derived tropanes have for centuries been used as poisons. Furthermore, tropanes have proven to have invaluable pharmacological properties due to their toxicity. Examples of toxic tropane alkaloids include scopolamine (*Atropa*), atropine (*Atropa*), hyoscyamine (*Atropa*), nicotine (*Nicotiana* and *Cestrum*) and glycoalkaloids such solanine and solamargine which are mainly reported in *Solanum* species. Glycoalkaloids have been reported to exhibit its toxic effects in pathogens, viruses, bacteria, or herbivores by disrupting cell-membrane function through complexation reactions with membrane 3 β-hydroxysterols, forming aggregates, eventually damaging the integrity of the cell membrane. The active components responsible for complexation of glycoalkaloids to cell membranes have been reported by Sinani et al. (2017) to be carbohydrate residues attached to the 3-OH position of the aglycone unit. Additionally, Rayburn et al. (1994) evaluated the toxic effects of modifying monosaccharides chains in glycoalkaloids on frog embryos, and it was observed that solasodine had significantly less biological activity when compared with its glycosidic form. The same researchers reported this trend across various cell lines including HT29 (colonic adenocarcinoma), HeLa (cervical carcinoma) and MCF-7 (breast adenocarcinoma) and agrees with what was reported by Friedman et al. (2018) and Beaulieu et al. (2018). The composition of the carbohydrate side chain is equally important in determining the extent and type of membrane – glycoalkaloid interaction within the cancerous cell. For instance, glycoalkaloids such as α-solamargine and α-solasonine share a common aglycone yet have different sugars; and α-solamargine consistently displayed higher activities in biological systems than α-solasonine for anticancer and membrane-disrupting properties in frog embryos (Nepal et al., 2019).

Glycoalkaloids have also been examined for their anti-acetylcholinesterase activity on the central nervous system. According to Sinani et al. (2017) and Kiełczewska et al. (2021), solasodine based metabolites such as α-solasonine and α-solamargine were observed to have limited acetylcholinesterase inhibition compared to solanidine-based glycoalkaloids. This highlighted that the structure of the aglycone unit determined the extent of inhibition of acetylcholinesterase (Sinani et al., 2017). In a separate study by Yelken et al. (2017), another glycoalkaloid, α-tomatine, was observed to inhibit cell proliferation of human breast cancer cells. The authors suggested that

α-tomatine−cholesterol interactions within the cell membrane of breast cancer cells, played a vital role in the anticarcinogenic effect of α-tomatine.

Glycoalkaloids have also been reported to disrupt active transport of ions through membranes, proceeding to disorders in general body metabolism (Väänänen, 2007; Nielsen et al., 2020; Schmidt et al., 2020; Nguenang et al., 2020). As part of an effort to establish the mechanism of action of glycoalkaloids in cells, Blankemeyer et al. (1997) evaluated the effect in exposure of varying concentrations of α-tomatine and tomatidine (glycoalkaloids predominantly found in *Solanum lycopersicum*) to frog embryos and their skin. α-Tomatine increased the fluorescence-measured membrane permeability of frog embryos by about 600% compared with control values; the corresponding value for tomatidine was about 150%. This indicated that the four carbohydrate residues attached to the 3-OH group in α-tomatine enhanced membrane permeability compared to the tomatidine aglycone which had no sugar chains attached. The increased membrane permeability of α-tomatine permitted for diminished sodium-active transport in frog skin by about 16% compared with control values, as estimated from the change in the interstitial short-circuit current. Similarly, a key mechanism of glycoalkaloids in malignant cell apoptosis, involves the ease of permeability through the infected cell membrane and subsequent changes in ion flux and interstitial currents between neighboring cells. The carbohydrate side chain (L-rhamnopyranosyl-(1 → 2) moiety) in another glycoalkaloid, solamargine, was reported by Kuo et al. (2000) and Fekry et al. (2019) to have anticarcinogenic activity in hepatoma cells. The main mechanism for solamargine involved upregulation of TNF receptor I and II in the hepatoma cells, which led to cell apoptosis.

Generally, tumeric cells have been reported to have multidrug resistance, making cancer therapy almost impossible. Besides the complexation ability of α-tomatine with cholesterol in the cell membrane, its aglycone unit tomatidine was reported to be an efficient chemosensitizer for human adenocarcinoma cells, enabling chemotherapy to occur more efficiently and inhibiting multidrug resistance (Hsieh et al., 2020). Similarly, according to Chen et al. (2017), solamargine inhibited the growth of multiple lung cancer cell lines. Solamargine initiated down-regulation of P-glycoprotein, a drug transport system responsible for excreting anticancer drugs out of cells (Burger et al., 2018). Moreover, solamargine inhibited the action of HER2, a gene involved in the development of human breast cancer, which is responsible for drug resistance (Xie et al., 2019;

Kalalinia et al., 2017). The presence of solamargine further in cell lines containing the HER2 gene triggered apoptosis and increased susceptibility of these cell lines to some common anticancer drugs which include methotrexate, 5-fluorouracil, cisplatin, and epirubicin (Xie et al., 2019; Kalalinia et al., 2017).

Reddivari et al. (2010) observed that micro-range concentrations of α-chaconine (≈ 5 μg mL^{-1}) obtained from *Solanum tuberosum,* exhibited potent antiproliferative properties in prostate cancer cells. The activity of α-chaconine was based on increased levels of cyclin-dependent kinase inhibitor p27 levels in two prostate cancer cell lines which included LNCaP and PC3. This resulted in cleavage of poly (adenosine diphosphate (ADP) ribose polymerase, a response that was essential in the induction of caspase-dependent apoptosis in LNCaP cells. The observations by Reddivari et al. (2010) indicated that apoptosis due to potato extracts in prostate.

Glycoalkaloids have also been studied to exhibit other forms of human cancer cell cytoxicity and this includes its interaction with membrane-based receptors such as endogenous endocytic lectins (EELs). The carbohydrate moieties of the solasodine glycosides (such as solamargine and solasonine in Table 2.1) may interact with EELs as shown in Figure 2.3 (Cham et al., 2017; Sinani et al., 2017). Once the steroidal glycoalkaloids bind to the EELs, the complex is absorbed into the cytoplasm of the infected cell where it merges with endosomes containing cell organelles such as mitochondria (Figure 2.3), eventually localizing in the lysosomes. Thereafter, the solasodine glycosides contained in the lysosomes are then hydrolyzed by enzymes within the lysosomes into solasodine (Cham et al., 2017; Sinani et al., 2017). Thereafter, solasodine performs its turmorcidal activity by binding to mitochondrial enzymes that generate ATP, chemical energy needed for biochemical reactions within an infected cell, activating the process of apoptosis (Figure 2.3). Subsequently, the contents of lysosomes, made of many hydrolytic enzymes are spilled into the cytoplasm of the affected cell leading to sudden death of the cancer cells by apoptosis (Serrano-Puebla et al., 2018). This indicates that the carbohydrate and the aglycone unit of the glycoalkaloid have distinct functions where the carbohydrates unlock the infected cell through interaction with receptors, while the aglycone unit is responsible for the exhibition of toxicity to the viruses the cell, resulting in apoptosis.

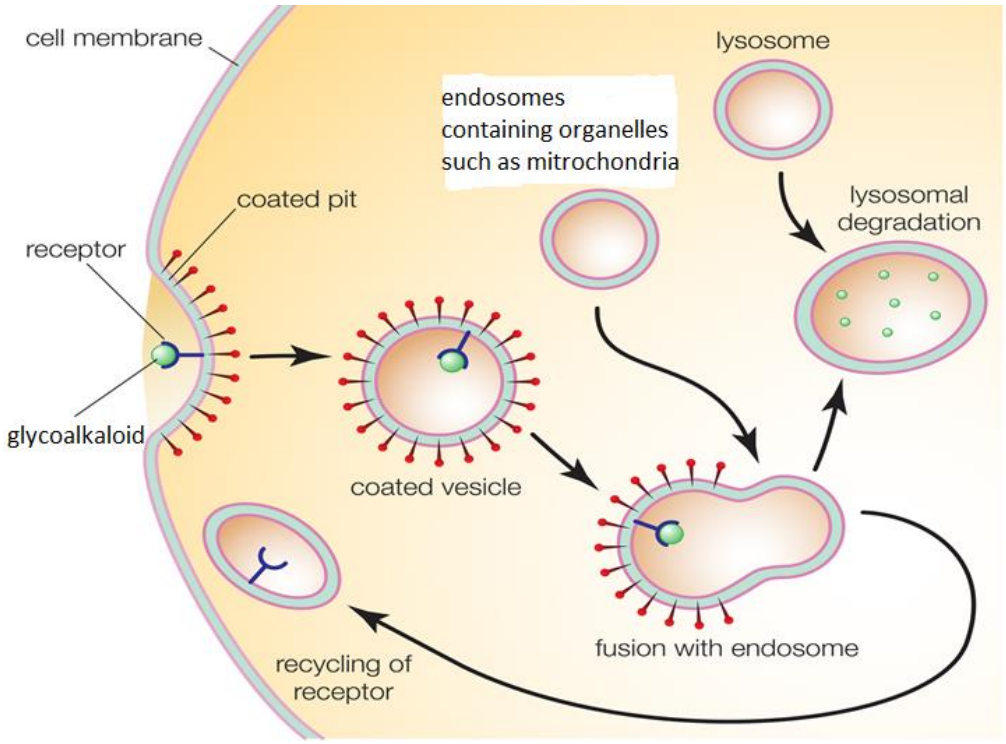


Figure 2.3: Interaction of glycoalkaloids through receptor-mediated endocytosis with endogenous endocytic lectins (EELs) located on the cell membrane of the infected cell, injestion into coated (Sinani et al., 2017)

2.4 Modern extraction methods for *Solanum* derived compounds

2.4.1 Microwave assisted extraction

2.4.1.1 Principle of microwave assisted extraction

Microwave assisted extraction (MAE) involves the application of microwave energy resulting in heat transfer to the sample and subsequent increase in mass transfer rate of the solutes from the sample matrix into the solvent. Microwave based extraction is guided by thermal and non-thermal effects. Thermal effects are responsible for the heating of solvents, which result in the enrichment of targeted metabolites. However, the mechanism of thermal effects during microwave dielectric heating and conventional heating differs. Microwave heating uses the ability of some compounds (liquids or solids) to transform electromagnetic energy into heat. Energy transmission is produced by dielectric losses within the solvent, which contrasts with conduction and convection processes observed in conventional heating. The magnitude of heating depends on the dielectric properties of the molecules that make the extraction solvent, also in contrast to conventional heating (Diaz-Ortiz et al., 2019; Grillo et al., 2021). These characteristics mean that absorption of the radiation and heating may be performed selectively. The dielectric properties of molecules with the sample dictate how rapid microwave irradiation can take place, often resulting in the whole material being heated simultaneously. In contrast, conventional heating is slow and is introduced into the sample from the surface (Grillo et al., 2021). The thermal effects observed under microwave irradiation conditions are a consequence of the inverted heat transfer, the inhomogeneities of the microwave field within the sample and the selective absorption of the radiation by polar compounds (Bichot et al., 2020). These effects can be used efficiently to improve processes (Bichot et al., 2020; Cavalcante et al., 2021). The thermal effect is manifested as a rise in temperature of the irradiated system and is accompanied by physiological responses such as cell rupture depending on the frequency and duration of the field (Bichot et al., 2020; Cavalcante et al., 2021).

Non-thermal effects

The existence of non-thermal effects during microwave irradiation is a controversial matter. According to Guo et al. (2020), non thermal effects are described as the direct interaction of the alternating electromagnetic (EM) fields with specific (polar) molecules and ions in the reaction medium that is not related to a macroscopic temperature effect. Additionally, Arjmandi et al. (2017) reported that non-thermal effects of microwaves cannot only enhance the inactivation of bacteria and enzymes, but also affect the integrity of cell membrane and release of intracellular constituents. Besides this, there were several studies that opposed the existence of 'non-thermal effects' of microwaves and therefore controversial (Stratakos et al., 2016; Bahari et al., 2017).

2.4.1.2 Mechanism of microwave assisted extraction

The principle behind MAE is that the sample must be moisture containing. In microwave assisted extraction, the objective of heating in case of dried plant material is heating that minute amount of moisture present in a plant cell. The warming up of this moisture within the plant cell due to microwaves causes evaporation and creates a huge pressure on the cell wall and subsequent plant cell expansion. The cell wall weakens from inside due to this pressure and breaks. In this way, the exudation of potential constituents from the ruptured cell happens, consequently, it helps developing extraction yield of phytoconstituents (Mirzadeh et al., 2020). The schematic for cell rupture event is in case of MAE methodology at progressive level is depicted in Figure 2.4. The higher extraction yield can be achieved further by increasing the temperature, which leads to quicker penetration of solvent into the cell wall of plant matrix (Azmir et al., 2013; Pandey et al., 2018). Microwave assisted extraction was also applies on *Solanum* species for the extraction of polyphenols (Salamutallah et al., 2018; Gu et al., 2019) and a glycoalkaloid (solasodine) (Lin et al., 2019). Microwave assisted extraction of metabolites from *Solanum* and other plant species are summarized in Table 2.3.

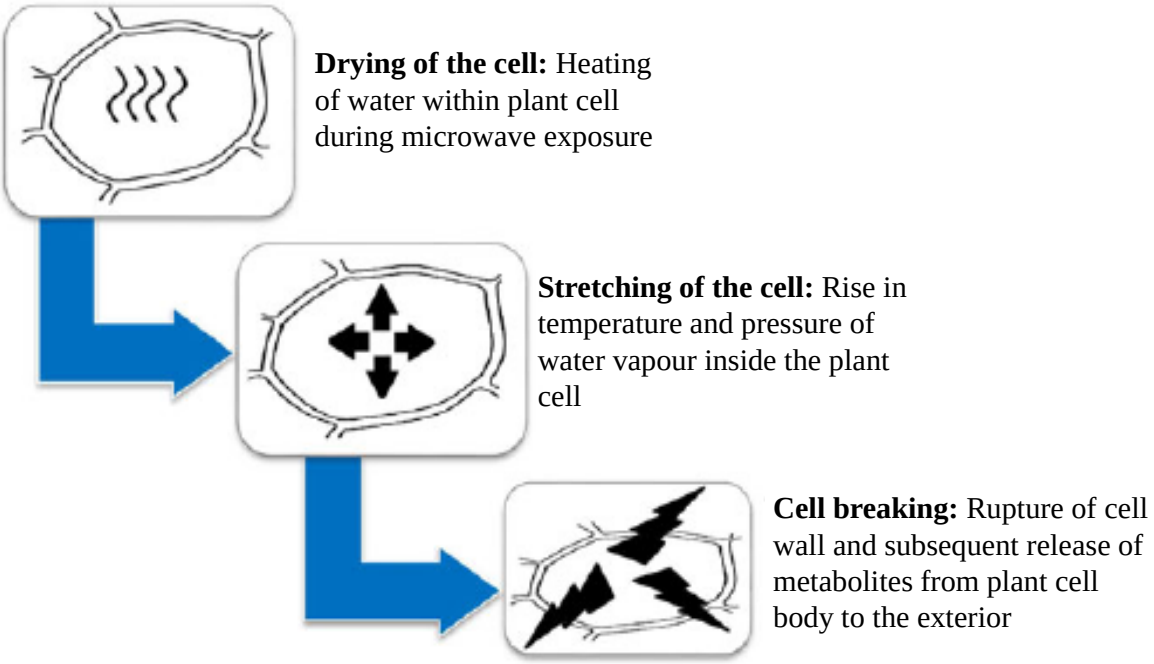


Figure 2.4: Progressive cell rupture event brought about by exposure to microwaves (Li et al., 2013)

Table 2.3: Some of the classes of metabolites obtained following microwave assisted extraction

Microwave assisted extraction technique	Extracted metabolite	Extracted from	Reference
MAE	Flavonoids, polyphenols	*Ziziphus spina-christi*	Keshavarz et al. (2020)
MAE	Flavonoids	*Rosa*	Zhou et al. (2010)
MAE	Flavonoids	*Vernonia amygdalina*	Alara et al. (2018)
Ionic liquid based assisted extraction	Verbascoside	*Rehmanie root*	Fan et al. (2018)
Vacuum microwave assisted extraction	Myricetin, quercetin	*Capsicum annuum*	Xiao et al. (2009)
Microwave hydrodiffusion and gravity	Polyphenols	*Malus domestica Borkh.*	Fernandes et al. (2020)
Microwave hydrodiffusion and gravity	Polyphenols	*Rosmarinus officinalis L.*	Ferreira et al. (2020)
MAE	Polyphenols	*Solanum melongena L.*	Salamatullah et al. (2018)
Microwave dry-diffusion and gravity method	Quercetin	*Allium cepa*	Tehrani et al. (2019)
Vacuum solvent-free microwave extraction	Polyphenols	*Clinacanthus nutans Lindau*	Othman et al. (2020)
MAE	Glycoalkaloids	*Solanum tuberosum*	Kondamudi et al. (2017)
Synergetic Microwave & Ultrasound Energy	Phenols	*Rosa*	Patrascu et al. (2016)
MAE	Flavonoids	*Solanum lycopersicum*	Mahieddine et al. (2018)
MAATPE	Solasodine	*Solanum nigrum*	Lin et al. (2019)
MAE	Polyphenols	*Solanum melongena*	Gu et al. (2019)
MAE	Polyphenols	*Solanum melongena*	Sivanathan et al. (2018)

2.4.2 Pressurized hot water extraction

2.4.2.1 Principle of pressurized hot water extraction

Pressurized hot water extraction (PHWE) is an extraction technique that uses liquid water as extractant (extraction solvent) at temperatures above the atmospheric boiling point of water (100°C/273 K, 101 kPa), but below the critical point of water (374°C/647 K, 22000 kPa) as shown in Figure 2.5. This technique is classified as a green approach as extraction is carried out entirely by water (Jin et al., 2020). The principle of PHWE is guided by the physico-chemical properties of water. Water is highly polar with a high dielectric constant (ε) of 80 at room temperature and atmospheric pressure, due to its extensive hydrogen-bonded structure (Mao et al., 2020; Jin et al., 2020). Traditionally, water is not known to dissolve non-polar compounds at room temperature. However, as the temperature of water is increased, there is a resultant decrease in its permittivity, viscosity, and surface tension but an increase in its diffusivity characteristics. Similarly, at elevated temperatures, the dielectric constant of water decreases from ε = 80 at 25˚C to ε = 27 at 250˚C and 50 bar. Under these conditions water has a dielectric constant comparable to other organic solvents, such as methanol (ε = 33) and ethanol (ε = 24) at 25˚C. Additionally, water is then able to dissolve a wide range of medium and low polarity analytes. For instance, Liau et al. (2017) obtained kaempferol glycosides (flavonoids) by applying PHWE on seeds of *Camellia oleifera.* Similarly, Gil-Ramırez et al. (2018) obtained saponins from *Chenopodium quinoa Wild*.) while Salplachta and Hohnova´ (2017) obtained proteins from branches of *Sambucus nigra L.* More examples on the application of PHWE for the enrichment metabolites derived from plants and food is tabulated in Table 2.4.

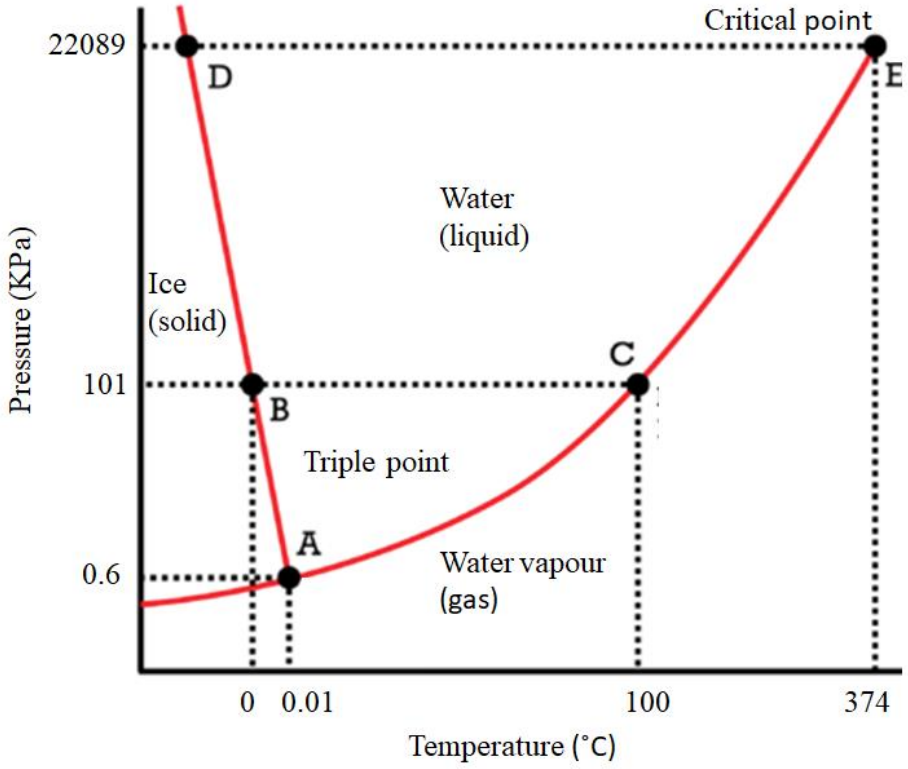


Figure 2.5: Phase diagram of water indicating its critical point

Table 2.4: Application of PHWE for the extraction of metabolites from food and plants

Metabolite	Source	Temperature (°C)	Pressure (MPa)	Static/Dynamic	Period (min)	Reference
Phenols	*Melissa officinalis*	150	10.30	Static	10	Miron et al. (2013)
Steviol glycosides	*Stevia rebaudiana leaves*	160	10.34	Static	10	Kovačević et al. (2018)
Phenols	*Tagetes*	220	6.00	Static	45	Xu et al. (2015)
Flavonols	*Malus domestica*	125	10.30	Static	3	Plaza et al. (2015)
Flavonols	*Moringa oleifera* leaves	100	-	Dynamic	20	Matshediso et al. (2015)
Phenols.	*Hordeum vulgare*	150	15.00	Static	15	Sarkar et al. (2014)
Flavonols	*Stevia rebaudiana* leaves	130	10.34	Static	10	Sandra et al. (2019)
Flavones	*Stevia rebaudiana* leaves	160	10.34	Static	10	Sandra et al. (2019)
Flavonoid glycosides	*Camellia oleifera*	140	4.13	Static	10	Liau et al. (2017)
Phenols	*Bertoni* leaves	160	10.34	Static	10	Kovačević et al. (2018)
Chlorogenic acid	*Sambucus nigra L.*	100	15.00	Static	5	Hohnova et al. (2017)
Rutin	*Sambucus nigra L.*	100	15.00	Static	5	Hohnova et al. (2017)

2.4.2.2 Mechanism of PHWE

The extraction mechanism in the extraction cell of a PHWE system is generally composed of three sequential steps. Firstly, desorption of solutes from active sites in the sample matrix under the pressurized and elevated temperature conditions occurs. This is followed by the diffusion of extraction fluid (water) into the matrix. And finally, depending on the sample matrix, the solutes partition themselves from the sample matrix into the extraction fluid before being chromatographically eluted out of the extraction cell (Jokić et al., 2018; Plaza et al., 2019). According to Ong et al. (2006), the enhancement on the extraction efficiency of PHWE can be due to an improvement in the solubility and mass transfer of the solute and an increased disruption of surface equilibria. As discussed in *Section 2.4.2.1,* the physicochemical properties of water at elevated temperatures, change drastically. The lowered viscosity and dielectric constant and improved diffusivity of water, allows for better penetration through the matrix particles. If fresh water is continuously introduced during a dynamic extraction in PHWE, it improves the mass transfer and hence, increases extraction rate. Both the high temperatures and pressures could disrupt the surface equilibria (Plaza et al., 2019). The same authors highlighted that increased temperature in PHWE plays an important role of overcoming the solute-matrix interaction caused by van der Waals forces, hydrogen bonding and dipole attraction. Therefore, the thermal energy supplied can disrupt cohesive (solute–solute) and adhesive (solute–matrix) interaction by decreasing the activation energy required for desorption process (Jokić et al., 2018). The transfer of the analytes from matrix to pressurized hot water is achieved by the diffusion and convection processes (Dias et al., 2020). Similar, to temperature, pressure plays an equally important role by providing the sufficient driving force to elute thermally labile compounds from the matrix (Dias et al., 2020). Pressure also facilitates extraction from samples where analytes are trapped in the matrix pores and drives the extraction fluid into ‘hard to reach’ matrices which are not normally covered if water at atmospheric pressure is used (Dias et al., 2020).

2.4.3 Aqueous two phase extraction

2.4.3.1 Aqueous two phase extraction principle

Aqueous two phase extraction is a liquid–liquid fractionation technique. The principle of this method is embedded on incompatibility of two aqueous solutions such as a polymer/ salt system, a polymer/polymer system (Kaplanow et al., 2018; Castro et al., 2020), an ionic liquid (IL) and a salt system, or a low molecular weight alcohol and a salt system (Li et al., 2020; Chong et al., 2020). Figure 2.6 is an illustration of two immiscible systems resulting in the separation of hydrophobic and hydrophilic compounds in an ethanol/salt/polymer setting.

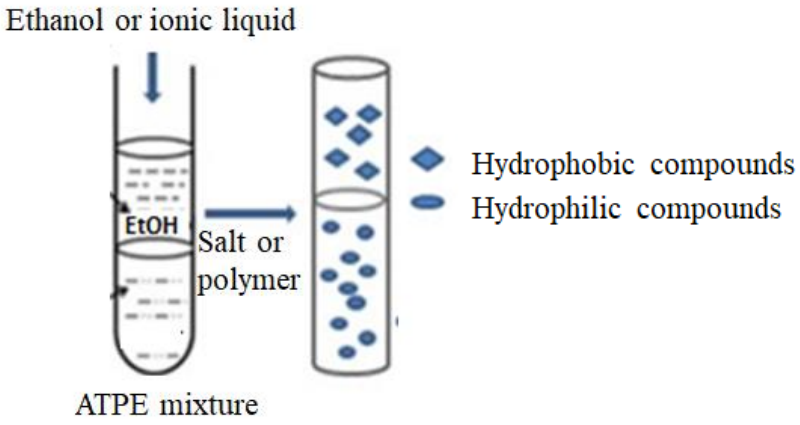


Figure 2.6: Separation of hyrophophic and hydrophilic compounds in an ATPE system

2.4.3.2 Ethanol/salt aqueous two phase extraction systems

Aqueous two-phase extraction (ATPE) has attracted increasing attention due to biphasic extraction capacity and selectivity, resulting in the achievement of target constituents that could be extracted either in the top or bottom phase (Xie et al., 2017; Xie et al., 2021). More importantly, it is a green and efficient pre-treatment solution for separation and purification of compounds from natural products (Xie et al., 2017; Li et al., 2020). Over the past decades, polymer/salt and polymer/polymer aqueous two-phase systems (ATPS) have been utilized in the purification of biomolecules (Wessner et al., 2020; Chikari et al., 2020). However, industrial upscaling of these systems remains a major setback due to the exorbitant cost of polymers and difficulties associated

with the separation of extracted molecules through back extraction processes (Zhang et al., 2020; Chong et al., 2021). This has resulted in the proliferation of studies around the alcohol/salt ATPE which have fast become key extracting systems for metabolite extraction and purification, due to their low viscosity, simple recovery mechanism, and cost-effectiveness (Dumas et al., 2020). Introduction of salts in aqueous systems result in structural changes in water, because of entropic alterations (Zangi et al., 2010). Entropic changes in aqueous systems can be explained based on the arrangement of water molecules surrounding a solute (Timson et al., 2020). For instance, the presence of some salts in aqueous environments give rise to a more ordered (quasi-crystalline) arrangement of water molecules surrounding a solute relative to the water molecules in the bulk solution (Zangi et al., 2010). This led to the classification of salts as either kosmotropes, structure-makers (Greek *kosmos* meaning order), or chaotropes, structure-breakers (in Greek *chao* means disorder) (Russo et al., 2008; Deary et al., 2014; Assaf et al., 2018 Mokgehle et al., 2021). This indicated that kosmotropic and chaotropic salts can promote and disturb hydrogen bond interaction between water molecules surrounding the solute, respectively. Similarly, Assaf et al. (2018) and Wang et al. (2021) reported that multivalent ions of high charge density (kosmotropes) bind water molecules strongly, however, large monovalent ions of low charge density (chaotropes) bind water molecules weakly relative to the strength of water-water interactions in bulk solution. Subsequently, this leads to the precipitation (salting-out) of the solute from the aqueous solution in the presence of kosmotropes and or salting-in as with chaotropes. Some salts with kosmotropic behaviour have been studied for extraction of metabolites. For instance, Chong et al. (2020) evaluated an Ethanol/NaH_2PO_4 system for the extraction of chlorogenic acids in *Lonicera caerula* with a yield of 96.8%. Similarly, Mokgehle et al. (2021) studied Na_2CO_3 for the extraction of glycoalkaloids in *Solanum retroflexum*. Additional examples of ethanol/salt ATPE systems are tabulated in Table 2.5. Furthermore, kosmotropes and chaotropes have been reported to be essential in maintaining osmotic balances in biological systems through ion-channels (Zajc et al., 2014; Xia et al., 2020).

.

Table 2.5: Application of ATPE for enrichment of metabolites from plant samples

Type of ATPE	Recovery (%)	Recovery (mg g^{-1})	Metabolite (plant species)	Reference
Ethanol/Na and K salt	-	-	Polysacharrides (*G. scabra*)	Cheng et al. (2017)
Ethanol/$(NH_4)_2SO_4$	94-105	-	Flavonoids (*C. sessiliflora*)	Xie et al. (2017)
Ethanol/Na_2CO_3	-	-	Glycoalkaloids (*S. retrofexum*)	Mokgehle et al. (2021)
Ultrasonication ATPE	-	9.12	β-carotene (okra leaves)	Li et al. (2021)
Ethanol/Na salts	85.6	-	Capsaicin (*C. chinese var*.)	Cienfuegos et al. (2017)
Ethanol/$(NH_4)_2SO_4$	94	-	Allicin (*A. sativum L*.)	Li et al. (2017)
Deep eutectic solvents	77	-	Flavonoids (*G. biloba*)	Cao et al. (2018)
Ethanol/NaH_2PO_4	96.8	-	Chlorogenic acid (*L. caerula*)	Chong et al. (2020)
Ethanol/Na-citrate	94	-	Phenols (*H. sabdariffa*)	Rodriguez-Salazar et al. (2019)
EOPO/DES	86	-	Polysaccharides (*C. oleifera*)	Gao et al. (2020)
Ethanol/K_2CO_3	-	7.39	Naringin (*C. aurantium L*.)	Yan et al. (2020)
UAE-ionic liquids	-	10.4	Lignans (*S. chinensis*)	Li et al. (2019)

Chapter 3 – Materials and methods

The chapter summarizes the experimental conditions applied during MAE, ATPE and PHWE for profiling and quantification of toxic metabolites (glycoalkaloids).

3.1 Materials

The salts, extraction solvent and the conductivity of the aqueous solvent used during ATPE based extraction are spelled out in **papers I** – **V**. The instruments used to aid MAE are described in **papers II - III** while for PHWE is included in **paper V**.

3.2 Methods

Sample collection and preparation, prior to ATPE, MAE and PHWE is outlined in **papers I** – **V.** Figure 3.1 is a summary of the extraction methods undertaken to obtained toxic metabolites from *Solanum retroflexum* and *Solanum mauritianum*. This involved using chaotropes and kosmotropes during ATPE for qualitative studies involving metabolic profiling of *Solanum retroflexum* (**Paper I**) through structural elucidation techniques. Quantitative studies were then done involving the chaotrope and kosmotrope which best extracted the glycoalkaloids from **paper I**. These quantitative studies which involved optimization are detailed in **papers II - V**. In **papers III - V** experimental design was conducted using central composite design (CCD) software which included 2 and 3 factorial inputs. Thereafter, quantification of the targeted toxic metabolites was done based on multiple reaction monitoring (MRM). Response surface models (RSM), which is a resultant fit of the predicted and experimental values, were generated. The chaotropes and kosmotropes were compared based on RSM for extraction of glycoalkaloids during the various extraction methods studied (**papers III** – **V**).

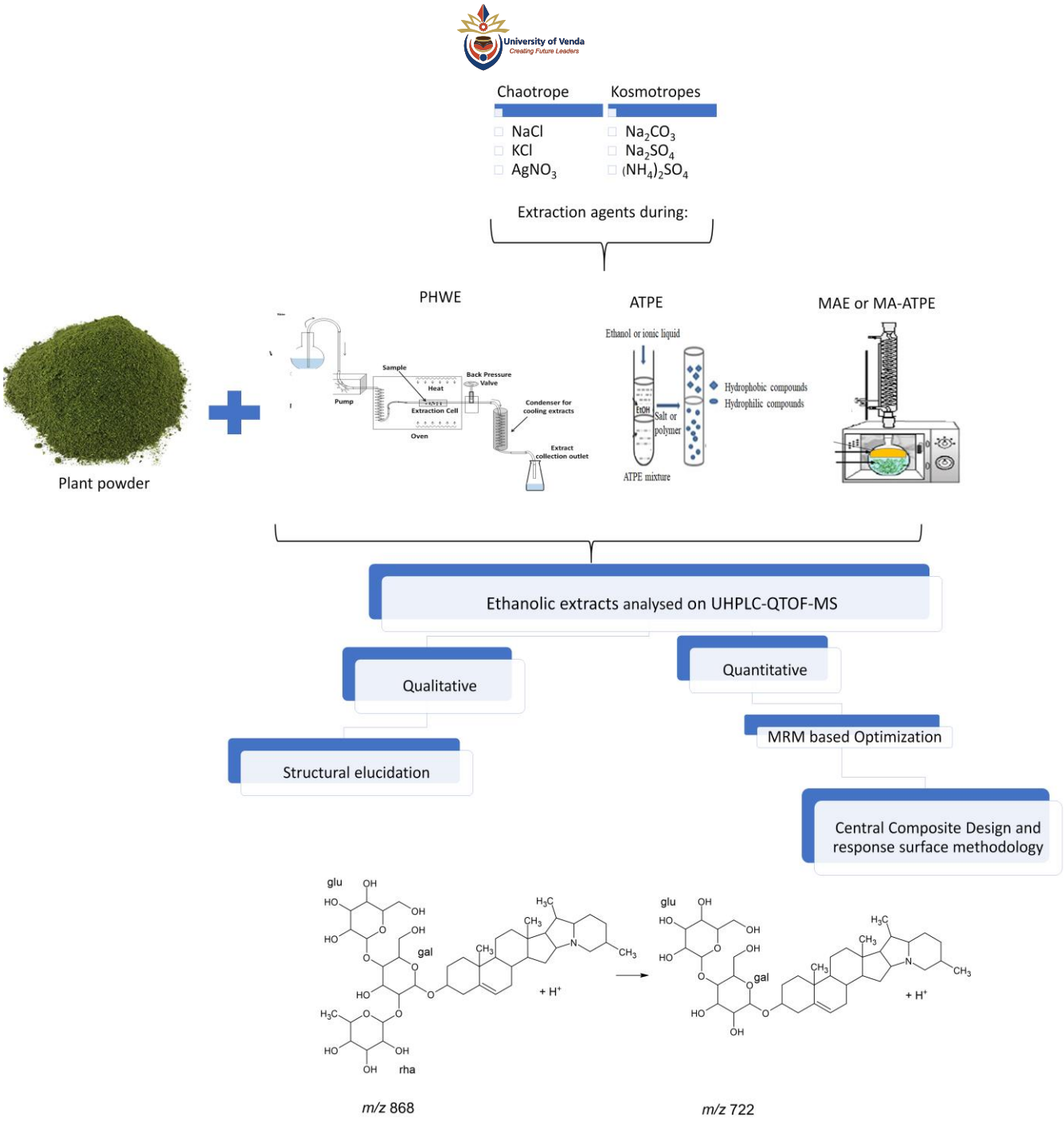


Figure 3.1: Summary of the methods used for obtaining toxic metabolites from selected *Solanum* species and subsequent quantification via statistical optimization softwares

3.3 Operation of UHPLC-qTOF-MS

The qTOF-MS is a hyphenated instrument consisting of an LC (Figure 3.2 (a)) and a time of flight mass spectrometer (Figure 3.2 (b). The qTOF-MS is almost a replica of the QQQ-MS with the only difference being the replacement of the third quadrupole with a time-of-flight tube. Introduction of the sample occurs through electron spray ionization (ESI), where the sample was sprayed into the ionization chamber with the aid of a nebulizing gas (N_2), resulting in droplets (Figure 3.2 (c-d)). At ESI (+), the analyte is sprayed at a low pH while at ESI (-), the analyte is at a pH above the molecule's isoelectric point. The droplets are then ionized in the presence of a high voltage power supply and desolvated due to the heat of the desolvation gas. Following ionization, the charged species to the first quadrupole (Q1) which is responsible for the selection of specific ions based on their mass-to-charge ratio (*m/z*), resulting in a precursor ion (Figure 3.2 (d)) (Allen and Whitney, 2019). The precursor ion is then introduced to a second quadrupole (Q2) which is collision cell where ions are bombarded by neutral gas molecules such as nitrogen or argon, resulting in fragmentation of the ions also described as collision induced dissociation (CID) (Allen and Whitney, 2019). After leaving the quadrupole (Q2) the fragmented ions (product ions) are reaccelerated into the ion modulator region of the time-of-flight analyser where they are pulsed by an electric field and accelerated perpendicularly to their original direction (Figure 3.2 (c)). All ions with the same kinetic energy enter the flight tube which is a field free drift region where mass separation occurs based on the velocity of the product ion. Ions exhibiting a lighter mass will have a shorter time of flight, whereas heavier ions will take longer to traverse the flight path towards the detector (Ingvarsson, 2020).

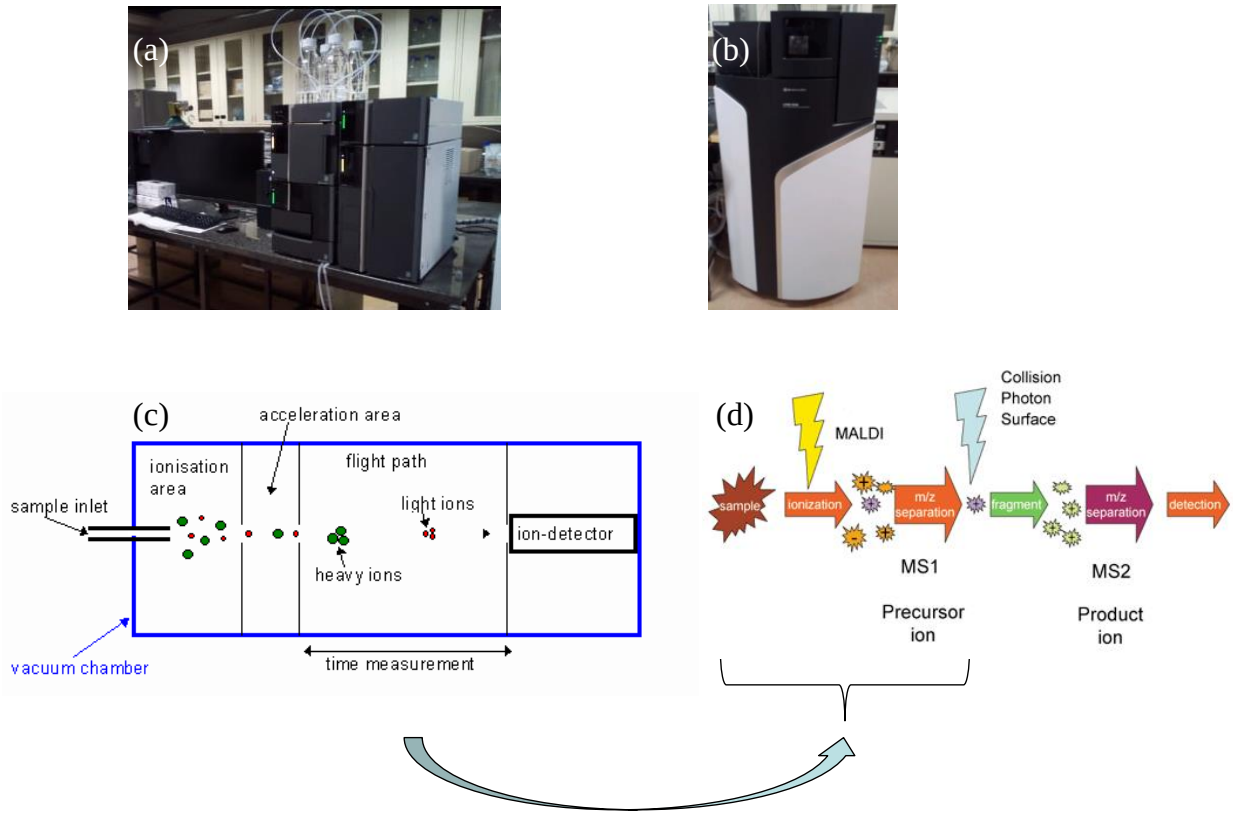


Figure 3.2: (a) The LC compartment (b) the mass ionizer (c) TOF ionization chamber (d) process of ionization from a precursor ion to a product ion

Chapter 4 – List of publications

This chapter gives the publications that were done during the duration of the PhD programme.

Paper 1

This work assessed the application of a range of chaotropes and kosmotropes to aid the ATPE extraction of metabolites from *Solanum retroflexum*, and analysed via UHPLC-qTOF-MS.

Mokgehle *et al. Appl Biol Chem* (2021) 64:28
https://doi.org/10.1186/s13765-021-00603-8

KSABC
The Korean Society for Applied Biological Chemistry

ARTICLE Open Access

Deciphering the effects of kosmotrope and chaotrope salts during aqueous two phase extraction (ATPE) of polyphenolic compounds and glycoalkaloids from the leaves of a nutraceutical plant, *Solanum retroflexum*, with the aid of UPLC-QTOF-MS

Tebogo Mokgehle[1], Ntakadzeni Madala[2*], Wilson Gitari[3] and Nikita Tavengwa[1*]

Abstract

Solanum plants (Solanaceae) are renowned source of nutraceuticals and have widely been explored for their phytochemical constituents. This work investigated the effects of kosmotropic and chaotropic salts on the number of phytochemicals extracted from the leaves of a nutraceutical plant, *Solanum retroflexum*, and analyzed on the ultra-performance liquid chromatography hyphenated to a quadrupole time of flight mass spectrometer (UPLC-QTOF-MS) detector. Here, a total of 20 different compounds were putatively characterized. The majority of the identified compounds were polyphenols and glycoalkaloids. Another compound, caffeoyl malate was identified for the first time in this plant. Glycoalkaloids such as solanelagnin, solamargine, solasonine, β-solanine (I) and β-solanine (II) were found to be extracted by almost all the salts used herein. Kosmotrope salts, overall, were more efficient in extracting polar compounds with 4 more polyphenolic compounds extracted compared to the chaotropes. Chaotropes were generally more selective for the extraction of less polar compounds (glycoalkaloids) with 3 more extracted than the kosmotropes. The chaotrope and the kosmotrope that extracted the most metabolites were NaCl and Na_2SO_4, respectively, with 12 metabolites extracted for each salt. This work demonstrated that a comprehensive metabolome of *S. retroflexum*, more than what was previously reported on the same plant, can be achieved by application of kosmotropes and chaotropes as extractants with the aid of the Aqueous Two Phase Extraction approach. The best-performing salts, Na_2SO_4 or NaCl, could potentially be applied on a commercial scale, to meet the ever-growing demand of the studied metabolites. The Aqueous Two Phase Extraction technique was found to be efficient in simultaneous extraction of multiple metabolites which can be applied in metabolomics.

Keywords: Aqueous Two Phase Extraction, Chaotropes, Kosmotropes, *Solanum retroflexum*, Extraction

Introduction

Solanum plants (Solanaceae) have been widely explored for their phytochemical constituents. These bioactive compounds have been isolated from various parts of plants such as leaves, fruits and roots [1–3]. The presence of various phytochemicals in *Solanum* plants has allowed for their use in medicine, food and dietary supplements

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[4]. The type of phytochemical compounds found in *Solanum* plants vary with species, plant part and the extraction method.

In view of the rich diversity of *Solanum* phytochemicals, these substances have recently become of great interest owing to their versatile applications as basic raw materials for indigenous pharmaceuticals. These phytochemicals are a rich bio-resource of drugs for traditional systems of medicine, modern medicines, nutraceuticals, pharmaceutical intermediates and chemical entities for synthetic drugs [5, 6]. Examples of phytochemicals include polyphenolic flavonoid compounds which are widely distributed in plants and have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory and anti-carcinogenic characteristics [3, 7]. Glycoalkaloids, which are common alkaloids within the Solanaceae family have been reported to play a protective role in species such as *S. tuberosum*, *S. melongena* and *S. lycopersium* [8–10]. The defensive role of glycoalkaloids include redox imbalance, disruption of biological membranes, disturbed metabolism, inhibition of cholinesterase, reproductive toxicity and disturbed development [11].

Patel et al. [12] reported on phytochemicals such as carbohydrates, saponins, tannins, alkaloids and triterpenoids in *S. dubium* roots. Piana et al. [1] reported on the presence of phenolic alkaloids and flavonoids compounds from ethanol extracts in *S. corymbiflorum* leaves. Upreti et al. [13] studied the phytochemical constituents of *S. xanthocarpum* fruits, obtained through methanolic extraction, and found that it contained alkaloids and glycosides. Daji et al. [14] investigated the phytochemical profile of *S. retroflexum* leaves using methanolic extracts and found a rage of cinnamic acids, polyphenols and alkaloids.

In light of the wide range of bioactive roles that phytochemicals display, extraction of these essential compounds is all the more worthwhile. Extraction of phytoconstituents from plants is dependent on a variety of factors, among which includes the use of chaotropic and kosmotropic salts. Chaotropes are salts that disrupt hydrophobic interactions of plant derived compounds in water, hence allowing for dissolution of non-polar compounds in water. They are also weakly hydrated compounds and generally consist of large singly charged ions. On the contrary, kosmotropes do not interfere with hydrophobic interactions and are strongly hydrated as a result of their structural design, which consists of small multiply charged ions.

Aqueous two-phase system (ATPE) is a liquid–liquid partitioning method where one layer is composed of a bottom salt-saturated aqueous layer and an upper organic extraction solvent for separation, purification and enrichment of metabolites [15]. Aqueous two-phase extraction has been recognized as a versatile technique for the downstream processing of biomolecules [16, 17]. The major advantages of ATPE are high capacity, biocompatible environment, low interfacial tension of phases, high yields and low process time [16, 18]. Additionally, this technique uses salts that allow for partitioning of ethanol (green solvent) from water, where the ethanol layer is enriched with metabolites. This extraction method is renowned for its ability to extract, separate, purify and enrich proteins, viruses and membranes, resulting in decent yields [18, 19]. Recently, researchers have turned their attention to an improved version of ATPE, salting-out assisted liquid–liquid extraction (SALLE) technique, which facilitates enhanced extraction of metabolites from complex matrices. This method uses solvents such as ethanol (EtOH) or acetonitrile (MeCN), as extraction solvents because water-solubility is minimized when salts are added to samples and extraction media [20, 21]. The SALLE procedure has been widely applied for extraction of compounds from various matrices because of its simplicity and effectiveness [21–26].

To the best of our knowledge, information on extraction of phytochemicals using chaotropes and kosmotropes is scarce. Hence, the aim of this work was to determine if ATPE using the salting-out technique was efficient in obtaining a comprehensive metabolome of *S. retroflexum*. If the study were to be successful, it would be the first of its kind. The efficiency of the salting-out technique was determined based on the number of metabolites that would have been extracted from the aqueous solution into the extractant phase using a group of kosmotropic and chaotropic salts. Analysis and characterization of the extracted metabolites was performed on the UPLC-QTOF-MS.

Experimental

Chemicals and reagents

All salts; KNO_3 (analytical grade ≥ 99% purity), Na_2SO_4 (analytical grade ≥ 99% purity), $BaCl_2{\cdot}2H_2O$ (ACS reagent ≥ 99% purity), KBr (anhydrous ≥ 99% purity), $MgCl_2{\cdot}6H_2O$ (anhydrous ≥ 99% purity), Na_2HPO_4 (anhydrous ≥ 99% purity), NaCl (anhydrous ≥ 99% purity), $NaH_2PO_4{\cdot}2H_2O$ (anhydrous ≥ 99% purity), $(NH_4)_2SO_4$ (anhydrous ≥ 99% purity), Na_2CO_3 (anhydrous ≥ 99% purity), KCl (anhydrous ≥ 99% purity), $AgNO_3$ (Analytical reagent ≥ 99% purity) and Ethanol (99% CP) were purchased from Associated Chemical Enterprises (Johannesburg, South Africa) and Sigma-Aldrich (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) was used for the preparation of the salt solutions. Chromatographic separation was conducted using an Acquity UHPLC (Ultra high performance liquid chromatography)

instrument. The UPLC was connected to a Synapt G1 qTOF-MS detector (Waters Corporation, MA, USA). The solvents used for the chromatographic runs were acetonitrile and formic acid, which were purchased from Romil Pure Chemistry (Cambridge, UK).

Sample collection, preparation and ATPE

The leaves of *S. retroflexum* were obtained from a street vendor within the Thulamela District in Thohoyandou, South Africa. The plants were air dried until a constant weight was obtained, and the leaves were ground into a fine powder with a blender at 2000 rpm and stored in glass containers. The containers were covered in paper bags to prevent light penetration. The powdered leaves (2.00 g) were placed in 50 mL centrifuge tubes. Thereafter, saturated salt concentrations of 30% (w/v) involving kosmotropes ($BaCl_2{\cdot}2H_2O$, Na_2SO_4, Na_2CO_3, $(NH_4)_2SO_4$, Na_2HPO_4 and $MgCl_2{\cdot}6H_2O$) and chaotropes ($AgNO_3$, KBr, KCl, KNO_3, $NaH_2PO_4{\cdot}2H_2O$ and NaCl which were prepared by weighing 15 g of salt in 50 mL of water, were added to the powdered leaves. The mixture was then shaken on the dragon shaker at 70 rpm for 12 h at 25 °C for the extraction of the metabolites. The ethanol extraction solvent (20 mL) was then added to the mixture for enrichment of the salt extracted metabolites that were initially in the aqueous phase, resulting in ATPE. The ATPE extractions were done in duplicates. The bottom layer contained the saturated salt while the top layer contained 99% ethanol extracting solvent. The metabolites contained in the extracts were then analysed on a UPLC-QTOF-MS.

Analysis on the UPLC-QTOF-MS

Chromatographic separation was conducted on an Acquity HSS T3 C18 column (150 mm × 2.1 mm with particle size of 1.7 µm) using a mobile phase which consisted of formic acid (0.1%) in deionised water (solvent A) and acetonitrile with 0.1% formic acid (solvent B) at a column temperature of 40 °C. Chromatographic separation was achieved using a 20 min gradient elution method consisting of the following settings: the initial conditions were 98% solvent A at a flow rate of 0.4 mL min^{-1}. The conditions were kept constant for 1 min. Conditions were changed to 98% solvent A at 1 min, sharply reduced to 5% solvent A at 6 min, held for 2 min, and then changed to 98% solvent A and maintained at 8 min for the next 2 min. Elution was monitored using a photodiode-array detector (PDA) collecting 20 spectra per second between the 200 and 500 nm range.

For mass spectrometry, the acquisition parameters discussed by Ramabulana et al. [27] were followed. Briefly, MS data were acquired using positive and negative electron spray ionization (ESI) modes. The MS was configured to scan the range of 100–1000 Da with a scan time of 0.2 s. After a series of optimization, the following settings were found to be optimal: capillary voltage of 2.5 kV, sample cone potential of 30 V, source temperature of 120 °C, desolvation temperature of 450 °C, cone gas flow of 50 L h^{-1}, desolvation gas flow of 550 L h^{-1}, and multichannel plate detector potential of 1600 V. In order to achieve efficient fragmentation to aid during identification, the mass spectrometry data were collected using a collision energy ramp of 10–30 eV. Structural elucidation was done using KNapSAck online metabolite database.

Results and discussion

LC–MS analysis of metabolites obtained from leaves of *S. retroflexum* using ethanol extracts from the ATPE system

LC–MS analysis of chlorogenic acids and related esters

Generally, approximately 15 polyphenolic compounds and 5 glycoalkaloids were identified, as presented in Fig. 1a, b. Peaks at *m/z* 353 which indicated caffeoylquinic acids (CQAs) when kosmotropes and chaotropes were used are shown in Fig. 1a and b. Madala et al. [28], Deshpande et al. [29] and Mhlongo et al. [30] have reported CQA at *m/z* 353. Chlorogenic acids are a family of esters that result from the esterification reaction of quinic acid (QA) and cinnamic acid (CA) derivatives comprised of either caffeic or coumaroyl or ferulic acid [31]. Kosmotrope salts such as $MgCl_2{\cdot}6H_2O$, Na_2HPO_4 and Na_2SO_4 extracted CQAs as identified in the chromatograms (Fig. 1a and b). As for the chaotropes, CQAs were also identified for NaCl, $NaH_2PO_4{\cdot}2H_2O$ and KCl (Fig. 1b). Multiple peaks at *m/z* 353 with retention times of 2.60, 3.51 and 3.53 min in Fig. 1a and b were observed and indicated isomers of CQA. Daji et al. [14] reported a similar observation when methanol extracts were screened on the UPLC-qTOF-MS. A precursor ion at *m/z* 295 with a retention time of 4.32 min was observed for all the kosmotropes and chaotropes except for $AgNO_3$, KNO_3 and Na_2CO_3. Interestingly, the precursor ion at *m/z* 295 and 4.32 min was not reported by Daji et al. [14] in the methanol extracts they analyzed on the UPLC-qTOF-MS, probably indicating that this was a unique compound.

LC–MS analysis of flavonoids

recursor ions at *m/z* 609 and *m/z* 593 appeared at 5.10 min and 5.51 min, respectively and were observed for most of the kosmotropic salts and chaotropic salts (Fig. 1a and b). Similarly, Daji et al. [14], Ramabulana et al. [27] and Pinela et al. [32] reported on precursor ions at *m/z* 609 and *m/z* 593 as flavonoids.

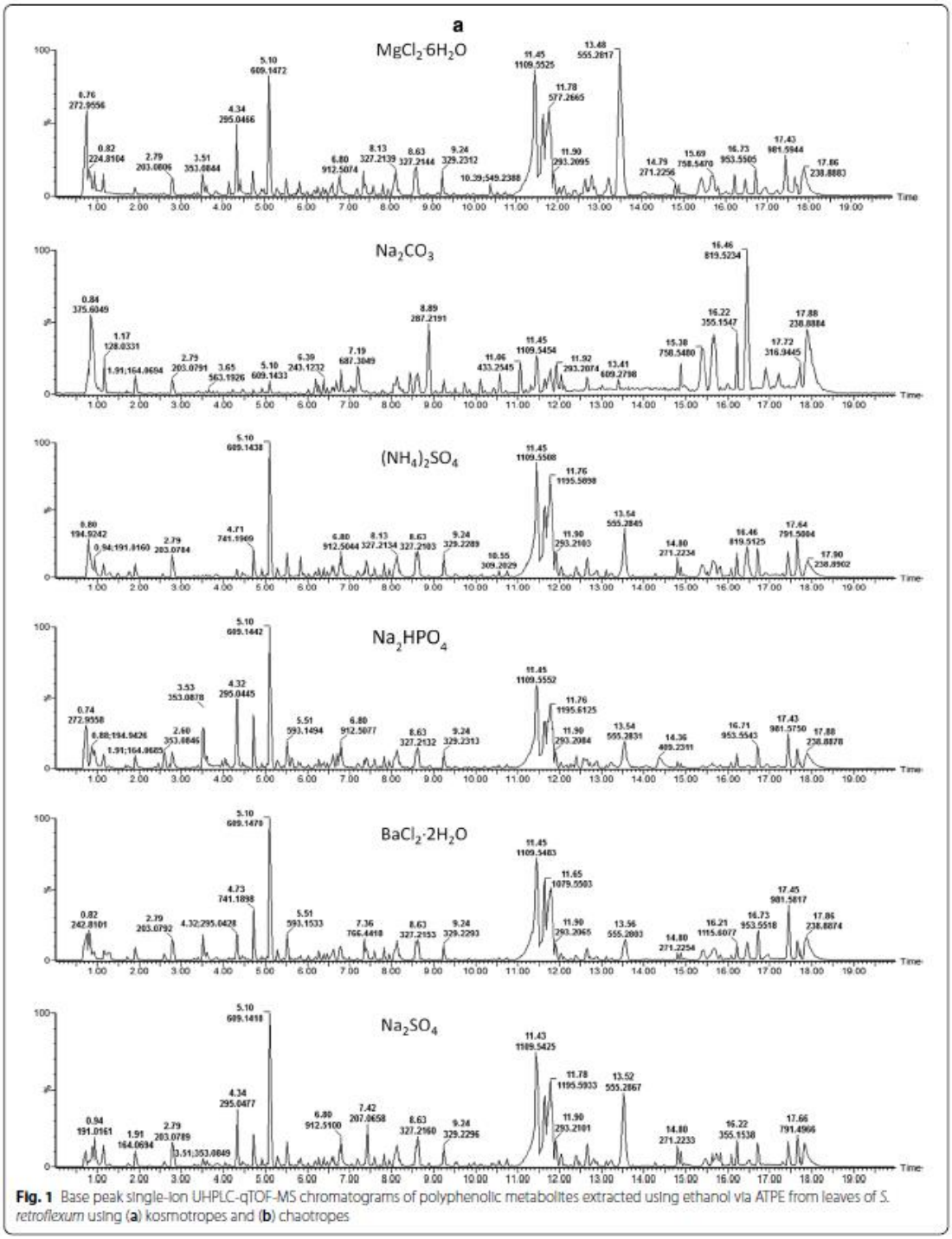

Fig. 1 Base peak single-ion UHPLC-qTOF-MS chromatograms of polyphenolic metabolites extracted using ethanol via ATPE from leaves of *S. retroflexum* using (**a**) kosmotropes and (**b**) chaotropes

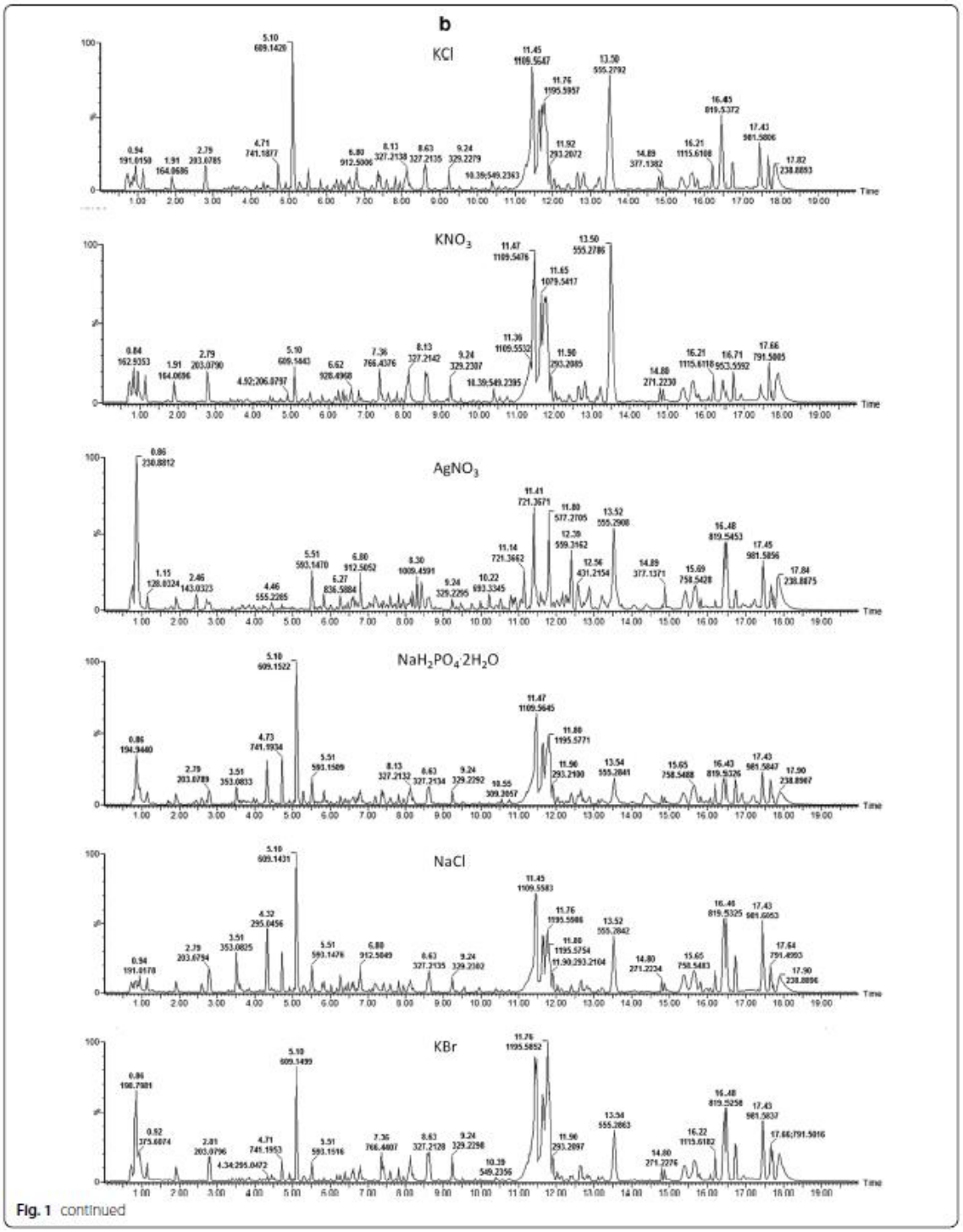

Fig. 1 continued

Table 1 List of compounds which were extracted with different kosmotropes from *S. retroflexum* leaves using aqueous ethanol extracts, under negative ESI

Metabolite	$[M-H]^-$	Diagnostic *m/z* ions	*t_R (min)	Na_2HPO_4	Na_2CO_3	$(NH_4)_2SO_4$	Na_2SO_4	$MgCl_2{\cdot}6H_2O$	$BaCl_2$	References
Quinolinic acid	164.0085	72, 116, 147	1.91	✓	✓	✓	✓	✓	✓	[49]
Quinic acid	191.0160	111, 115, 133	0.94	–	–	✓	✓	–	–	[40]
Caffeouyl malate	295.0462	133, 179	4.32	✓	–	✓	✓	✓	✓	This work
Trans-5-CQA	353.0846	135, 191	2.60	✓	–	✓	–	✓	✓	[14]
Cis-5-CQA	353.0849	135, 191	3.51	–	–	–	✓	✓	✓	[14]
4-CQA	353.0876	135, 173, 179, 191	3.53	✓	–	–	–	–	–	[14]
Hibcetin 3,7,4 trimethylether	375.6049	183, 295, 372	0.84	–	✓	–	–	–	–	[43]
Kaempferol 3-rutinoside	593.1516	285	5.51	✓	–	✓	✓	✓	✓	[43]
Quercetin 3-rutinoside	609.1431	301	5.1	✓	✓	✓	✓	✓	✓	[32]
Quercetin 3(2G-apiosyl)rutinoside)	741.1883	301, 463, 609	4.71	✓	–	✓	✓	✓	✓	[44]

CQA-Caffeoylquinic acid, CQM-Caffeoylquinic malate, *t_R Retention time, ✓, present, –, not present

Table 2 List of compounds which were extracted with different chaotropes from *S. retroflexum* leaves using aqueous ethanol extracts, under negative ESI

Metabolite	$[M-H]^-$	Diagnostic *m/z* ions	$^*t_R$ (min)	NaCl	Na.X	$AgNO_3$	KBr	KCl	KNO_3	References
Quinolic acid	164.0685	72, 116, 147	1.91	✓	✓	✓	✓	✓	✓ ✓	[49]
Quinic acid	191.0160	111, 115, 133	0.94	✓	–	–	–	✓	✓	[40]
CQM	295.0462	133, 179	4.32	✓	✓	–	✓	✓	–	This work
Trans-5-CQA	353.0846	135, 191	2.60	✓	✓	–	–	–	–	[14]
Cis-5-CQA	353.0849	135, 191	3.51	✓	✓	–	–	✓	–	[14]
4-CQA	353.0876	135, 173, 179, 191	3.53	–	–	–	–	–	–	[14]
Hibcetin. 3,7,4 trimethylether	375.6049	183, 295, 372	0.84	–	–	–	–	–	✓	[43]
Kaempferol-3 rutinoside	593.1516	285	5.51	✓	✓	✓	✓	✓	✓	[43]
Quercetin-3 rutinoside	609.1431	301	5.1	✓	✓	–	✓	✓ ✓	✓	[32]
Quercetin.3(2G-aplosylrutinoside)	741.1883	301, 463, 609	4.71	✓	✓	–	✓	✓	–	[44]

CQA, Caffeoyl qunic acid, CQM, Caffeoyl quinic malate, Na.X—$NaH_2PO_4.2H_2O$, present, –, not present, $^*t_R$, Retention time

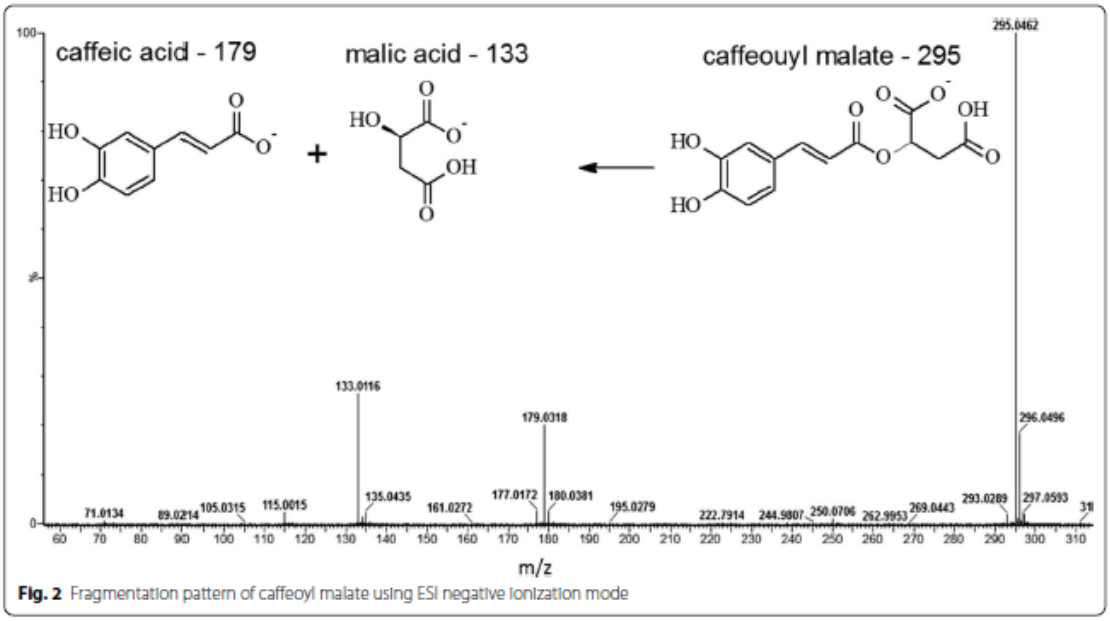

Fig. 2 Fragmentation pattern of caffeoyl malate using ESI negative ionization mode

Mass spectra of alkaloid derivatives

Two isomeric compounds at *m/z* 868 at retention times of 6.59 and 6.81 min were extracted for all kosmotropes and chaotropes, with the exception of $MgCl_2{\cdot}6H_2O$. The fragmentation patterns of both isomers were very similar with each isomer consisting of daughter ions at *m/z* 414 and 722 as shown in Additional file 1: Figs. S1 and S2. The chemical formulae of the two isomers at *m/z* 868 were found to be identical ($C_{45}H_{74}NO_{15}$) indicating that both compounds contained the same aglycone unit with the same sugar side chain of uniquely positionally arranged monosaccharides. The occurrence of the same aglycone unit is substantiated by the presence of the fragment *m/z* 414 for both isomers in Additional file 1: Figs. S1 and S2, which was identified as solasodine ($C_{27}H_{43}NO_2$). From the KNapSAcK metabolite database, only two metabolites at *m/z* 868 matched the formula $C_{45}H_{74}NO_{15}$ and were identified as solanelagnin and solamargine. Both solanelagnin and solamargine contained the same aglycone unit (solasodine) and the same chacotriose sugar

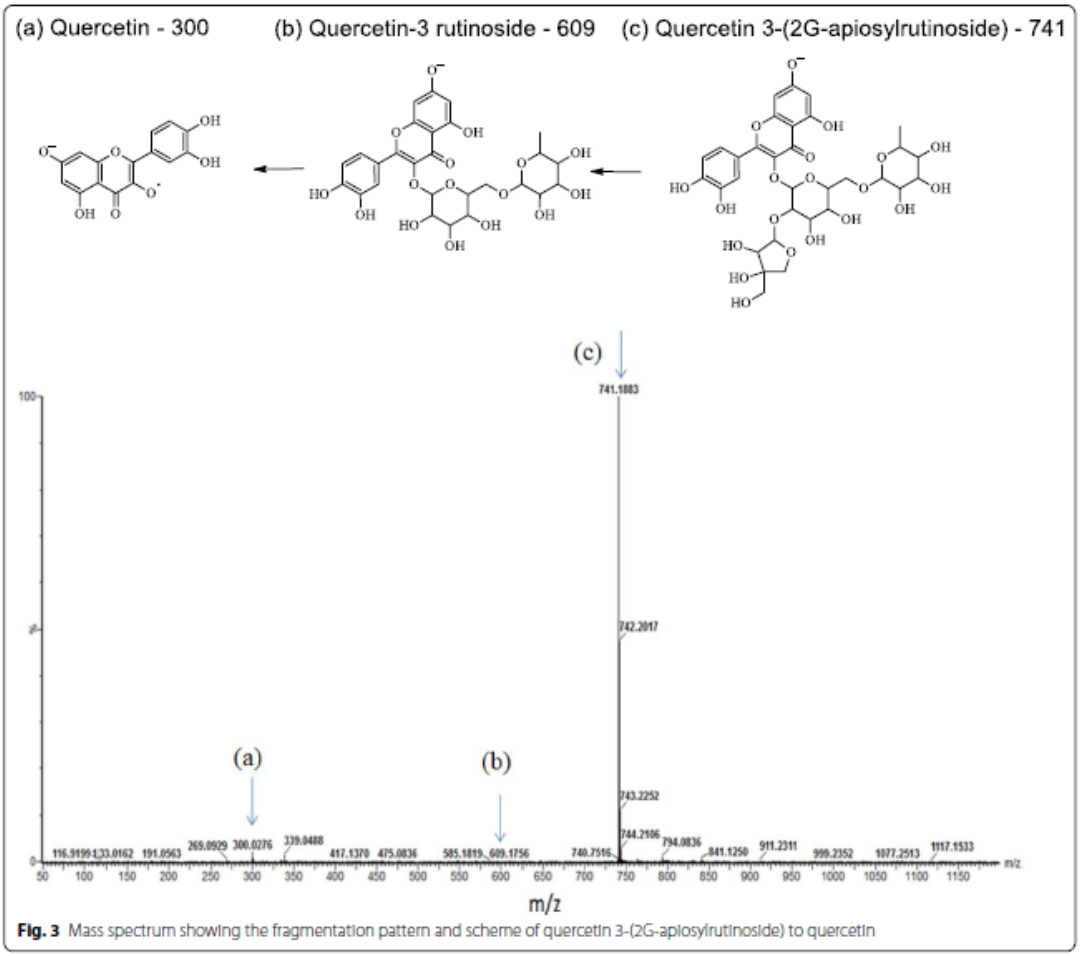

Fig. 3 Mass spectrum showing the fragmentation pattern and scheme of quercetin 3-(2G-apiosylrutinoside) to quercetin

side chain, which was composed of glucose and two rhamnose sugars. Chacotriose was glycosylated to solasodine through glucose, in both isomers. The only difference between the two isomers was the positional glycosylation of the rhamnose sugars to glucose. In solanelagnin, the rhamnose sugars were glycosylated at position 3 and 4 of glucose while in solamargine, glycosylation occurred at position 2 and 4 (Additional file 1: Figs. S1 and S2). Solanelagnin, due to the close proximity of the rhamnose sugars in comparison to solamargine, was observed to be the more polar, and hence eluted earlier at 6.59 min while solamargine followed at 6.81 min (Additional file 1: Fig. S3). Solamargine has been identified in other *Solanum* species such as in leaves of *S. incanum, S. nigrum* and *S. retroflexum* while solanelagnin was reported in *S. elaeagnifolium* [14, 50, 51]. Solamargine is highly toxic, intake at low doses can cause vomiting and diarrhea while high dosage intake can lead to death [14, 52]. Solanelagnin was reported to exhibit hepatoprotection against paracetamol induced liver injury in mice [53]. The toxicity of glycoalkaloids is due to its aglycone unit which is toxic and non-polar [27, 33].

Two other isomeric compounds at *m/z* 722 with retention times of 6.87 and 7.38 min were extracted for most kosmotropes and chaotropes. The fragmentation patterns of both isomers were similar with both isomers exhibiting daughter ions at *m/z* 397 and 559 as shown in Additional file 1: Fig. S4a and b, similarly to what was observed for the isomers at *m/z* 868. The chemical formulae of the two isomers at *m/z* 772 were determined to be identical

Table 3 Compounds identified with UPLC-QTOF-MS in *S. retroflexum* leaf aqueous ethanol extracts, under positive ESI, after the application of kosmotropes for extraction of metabolites

Metabolite	$[M+H]^+$	Diagnostic *m/z* ions	$^*t_R$ = (min)	Na_2CO_3	Na_2SO_4	$MgCl_2{\cdot}6H_2O$	$BaCl_2$	Na_2HPO_4	$(NH_4)_2SO_4$	References
Solanelagnin	868.5076	414, 576, 722	6.59	✓	✓	–	✓	✓	✓	[55]
Solamargine	868.5101	414, 559, 722	6.81	✓	✓	✓	✓ ✓	✓	✓	[14]
β-solanine (I)	722.4584	398, 576	6.87	✓	✓	–	–	–	–	[33]
β-solanine (II)	722.4514	398, 576	7.38	✓	✓	–	✓	✓	✓	[33]
Solasorine	884.4929	414, 722, 868	10.6	✓	✓	–	✓	–	–	[14]

✓, present, – not present, $^*t_R$ retention time

($C_{39}H_{64}NO_{11}$) indicating that both compounds contained the same aglycone unit with the same sugar side chain of uniquely positionally arranged monosaccharides. The occurrence of the same aglycone unit was due to the presence of the fragment at *m/z* 397 for both isomers in Additional file 1: Fig. S4a and b, which was identified as solanidine ($C_{27}H_{43}NO$). Structural elucidation of the isomers at *m/z* 722 ($C_{39}H_{64}NO_{11}$) using KNapSAcK metabolite database indicated that the compounds were isomers of β-solanine. β-solanine (I) and β-solanine (II) had the same aglycone unit, solanidine, and the same carbohydrate sugar side chain, solabiose, which both contained epimers glucose and galactose. Solabiose was glycosylated to solanidine through galactose in both isomers. In β-solanine (I) glycosylation of glucose to galactose occurred at position 4 while in β-solanine (II) glycosylation of glucose occurred at position 3. β-solanine (I) was shown to be glycosylated at position 4 which made glucose and galactose appear in close proximity to each other than β-solanine (II). Therefore, β-solanine (I) had a greater dipole moment than β-solanine (II), and hence eluted earlier at 6.87 min while solamargine followed at 7.38 min (Additional file 1: Fig. S5). Tata et al. [33] and Jia et al. [54] reported on the presence of β-solanine in sprouts of *S. tuberosum*. β-solanine was also reported by Filho et al. [41] to be less toxic than solamargine.

Kosmotropic salts such as Na_2CO_3, Na_2SO_4 and $BaCl_2$ were observed to extract solasonine while the same metabolite was not extracted for any of the chaotropes (Tables 3 and 4). This suggested the solasonine extraction is probably favored by the presence of multiply charged ions. The chaotrope salts were observed to extract a number of glycoalkaloids (Table 4). This indicated that generally chaotropes tend to have a greater affinity than kosmotropes for extraction compounds of lower polarity such as glycoalkaloids as compared to polyphenols. Identification of glycoalkaloids in this work further emphasized the prevalence of these toxic compounds among *Solanum* species that play a defensive role against microorganisms and competing plants [8–10]. The glycoalkaloids obtained from the kosmotropes and chaotropes are shown in Tables 3 and 4.

Figure 4 is a general reaction mechanism illustrating the reaction of chaotropes and kosmotropes with a plant metabolite in the aqueous phase of the ATPE system. A general observation from the extraction of polyphenols and glycoalkaloids was that kosmotropes were more efficient in extracting polar compounds such as polyphenols while the chaotropes were better off in extraction of less polar compounds such as glycoalkaloids. The mechanistics of this observation can be explained by the manner in which kosmotropes and chaotropes interact with water in the aqueous phase based on the salting-out effect. Salting-out is an effective pre-concentration method which has been studied for the extraction of Vitamin D3 in milk samples [26] partitioning of biomolecules [56] and determination of 5-nitroimidazolesin in fish [21]. Salting-out is dependent on the nature of the salt involved. Kosmotropes have been studied to form strong hydrogen bonds with water as opposed to chaotropes [57–60]. In the aqueous phase, *S. retroflexum* metabolites interacted with the water molecules through mainly, hydrogen bonds followed by weaker interactions such as electrostatic forces, dipole–dipole forces and van der wall forces. Introduction of salts in the aqueous phase resulted in the salting-out effect, whereby interactions between the water and the metabolites were disturbed. As a result, the miscibility of the phytochemicals for the water solvent was reduced resulting in precipitation of metabolites from the aqueous phase. The extent of precipitation was dependent on the nature of the salt (kosmotrope or chaotrope) and the polarity of the precipitated compounds. The ethanol extraction solvent that was introduced, removed the precipitated phytochemicals from the aqueous phase. Similarly, as shown in Fig. 4, the kosmotrope (Na_2SO_4) formed strong hydrogen bonds with the water molecules surrounding the metabolite (chlorogenic acid) as opposed to chaotropes. This led to the precipitation of the resultant metabolite from the aqueous phase, of which was eventually removed by the ethanol extractant solution. Hence, generally, a greater number of polyphenolic metabolites in this study were observed for kosmotropes as opposed to chaotropes (Tables 1, 2, 3 and 4).

For a chaotrope such as $AgNO_3$, which had the least amount of polyphenols extracted, while extracting all the glycoalkaloids present, can be explained based on salting-out effect. Additionally, silver has a destructive effect, as it has been reported to be persistent, bioaccumulative, and toxic to both aquatic species and plants. For instance, silver ions were shown to inhibit seed germination, biomass accumulation, and root and shoot growth in *Arabidopsis* [61], *B. nigra* [62], *Lemna* [63], *P. radiatus* [64] and *Lupinus termis L.* [65]. Similarly, the silver ions could have reacted directly with the metabolites (polyphenols in particular), as a result changing the polarity of the metabolite, subsequently reducing its precipitation from the aqueous phase. Hence, only two polyphenolic metabolites were observed for $AgNO_3$ as seen in Table 2.

Most ATPE techniques have been limited to a particular number of compounds, for instance Nainegali et al. [66] obtained four bioactive compounds from *Garcinia indica*. Other studies on ATPE were limited to a particular kind of bioactive compound. For example, Zhang et al. [67] used sodium citrate/sodium tartate ATPE system for extraction of glycyrrhizic acid from licorice while Santos et al. [68] extracted betanin from a

Table 4 Compounds identified with UPLC-QTOF-MS in *S. retroflexum* leaf aqueous ethanol extracts, under positive ESI, after the application of chaotropes for extraction of metabolites

Metabolite	$[M+H]^+$	Diagnostic *m/z* ions	$^*t_R$ = (min)	KCl	$AgNO_3$	KNO_3	KBr	NaCl	$NaH_2PO_4 \cdot 2H_2O$	References
Solanelagnin	868.5076	414, 576, 722	6.59	✓	✓	✓	✓	✓	✓	[55]
Solamargine	868.5101	414, 559, 722	6.81	✓	✓	✓	✓	✓	✓	[14]
β-solanine (I)	722.4584	398, 576	6.87	✓	✓	✓	✓	✓	✓	[33]
β-solanine (II)	722.4514	398, 576	7.39	✓	✓	✓	✓	✓	✓	[33]
Solasonine	884.4929	414, 722, 868	10.6	–	–	–	–	–	–	[14]

✓, present, –, not present, $^*t_R$, retention time

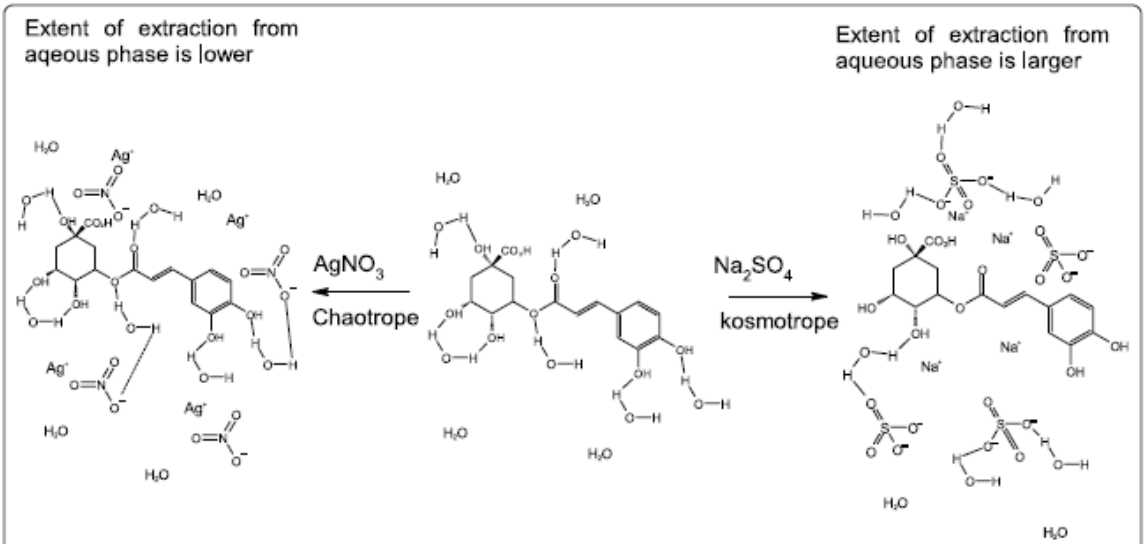

Fig. 4 General reaction mechanism of salting-out effect of one of the chaotropes (KNO_3) and kosmotropes (Na_2SO_4) on a metabolite (chlorogenic acid) in the aqueous phase of an ATPE system

THF/sodium salt system. This study, however, exploited the versatility of the salts involved in ATPE, where the chaotrope and the kosmotrope that extracted the most metabolites was NaCl and Na_2SO_4, with 12 metabolites extracted for each salt which included polyphenols and glycoalkaloids from leaves of *S. retroflexum*. The chaotrope (Na_2SO_4) aided in the extraction of 7 polyphenolic compounds (Table 1) and 5 glycoalkaloids (Table 3) while the kosmotrope (NaCl) facilitated the extraction of 8 polyphenolic compounds (Table 1) and 4 glycoalkaloids (Table 3). This indicated that a range of bioactive compounds can be simultaneously extracted using a single salt, making the extraction process less tedious. The robustness of single salts in ATPE can be further exploited with an intension to be applied in advanced scientific disciplines such as metabolomics.

Conclusion

The ATPE technique based on the salting-out method was shown to be an efficient approach for simultaneous extraction of multiple metabolites from *S. retroflexum*, more than what was previously reported on the same plant. Approximately, 20 different compounds were putatively identified. From the polyphenols, caffeoyl malate was obtained at *m/z* 295, and to the best of our knowledge, it has not been reported in other species of *Solanum* plants. Five glycoalkaloids were identified in this study which included two pairs of isomeric compounds at *m/z* 868 solanelagnin and solamargine, at *m/z* 722 β-solanine (I) and β-solanine (II) and at *m/z* 884 solasonine. Herein, kosmotropes were generally more efficient in the extraction of polar compounds, 38 polyphenols overall (Table 1) compared to

the 34 identified for the chaotropes (Table 2). On the other hand chaotropes were better extractants of less polar compounds such as glycoalkaloids with an overall amount of 24 being identified (Table 4) in comparison to 21 obtained for the komostropes (Table 3). The chaotrope and the kosmotrope that extracted the most metabolites was NaCl and Na_2SO_4, with 12 metabolites extracted for each salt. The chaotrope (Na_2SO_4) aided in the extraction of 7 polyphenolic compounds (Table 1) and 5 glycoalkaloids (Table 3) while the kosmotrope (NaCl) facilitated the extraction of 8 polyphenolic compounds (Table 1) and 4 glycoalkaloids (Table 3). Some of the metabolites obtained in this work were not found in literature reporting on the extraction phytochemicals from *S. retroflexum* leaves. This indicated that komotropes and chaotropes were better extractants of metabolites that could potentially have nutraceutical applications. Though the scope of this study was focused on qualitative determination of compounds extracted from *S. retroflexum* using ATPE, more work needs to be directed at quantification and optimization of the metabolites obtained, particularly glycoalkaloids owing to their potential pharmacological applications. For instance, studies can be directed at examining the biological properties of isomeric glycoalkaloid compounds. Additionally, studies involving hepatoprotection by isomeric glycoalkaloids obtained from *S. retroflexum* can also be conducted. The chaotropes and kosmotropes applied in this study, which had extracted most of the nutraceuticals, can potentially be applied on a commercial scale, to meet the ever-growing demand of the studied metabolites.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-021-00603-8.

Additional file 1: Fig. S1. Fragmentation pattern of solanelagnin (t_R = 6.59 min) yielding fragments of m/z 722, 575 and 414. **Fig. S2.** Fragmentation pattern of solamargine (t_R = 6.81 min) yielding fragments of *m/z*; 722, 575 and 414. **Fig. S3.** Two glycoalkaloids isomers, solanelagnin which eluted at 6.59 min and solamargine at 6.81 min. **Fig. S4.** Mass spectra showing fragmentation pattern of (a) β-solanine (t_R = 6.87 min) and (b) β-solanine (II) (t_R = 7.38 min. **Fig. S5.** Chromatogram of two isomers, β-solanine (I) at 6.87 min and β-solanine (II) 7.38 min

Acknowledgements
The authors would also like to express their gratitude to the Council for Scientific and Industrial Research studies for their technical support. The University of Venda is also thanked for financial support.

Authors' contributions
TM, NT and NEM conceived the study, TM and NEM conducted the experiments and data analyses. NT, NM and WG supervised the project. WG helped to draft the manuscript. All authors read and approved the final manuscript.

Funding
The national research Foundation (NRF) of South Africa and the University of Venda are thanked for financial support.

Availability of data and materials
Raw data will be provided upon request.

Declarations

Competing interests
None.

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Received: 17 September 2020 Accepted: 26 February 2021
Published online: 08 March 2021

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Publisher's Note

Paper 2

The optimization of microwave and aqueous two phase-based extraction techniques which involved MAE, ATPE + MAE and MA-ATPE for the extraction of solasonine and solamargine from leaves of *Solanum mauritianum* was evaluated.

Hyphenation of aqueous two phase and microwave extraction of solasonine and solamargine from *Solanum mauritianum* leaves, analysis via UHPLC-qTOF-MS

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Abstract

The biomass *Solanum mauritianum (S. mauritianum)* is an invasive weed specie, however, it is a source of medicinally metabolites, as reported in literature, such as solasonine and solamargine. The study was directed at the optimization of microwave and aqueous two phase based extraction techniques which involved microwave assisted extraction (MAE), (aqueous two phase extraction followed by microwave assisted extraction) ATPE + MAE and the 'one - pot' (microwave assisted aqueous two phase extraction) MA-ATPE for extraction of solasonine and solamargine from

leaves of *Solanum mauritianum (S. mauritianum)* was evaluated. The microwave-assisted extraction of solasonine and solamargine yielded optimums at 5.00 min, microwave power of 270 W, solid/liquid of 0.1 g L^{-1} at an ethanol concentration of 60%. Application of a two-stage extraction (MAE + ATPE) in CaO dried alcohol resulted in decreased amounts of solasonine and solamargine extracted. The best yields of solasonine and solamargine were achieved in the MA-ATPE method. Extraction of solamargine and solasonine using Na_2CO_3 in CaO dried ethanol during MA-ATPE was approximately three-fold and two-fold greater than that of MAE + ATPE, respectively. Furthermore, extraction of solamargine and solasonine using NaCl in CaO dried ethanol during MA-ATPE was approximately two-fold greater than that of MAE + ATPE. The synergy of microwaves and salting-out in the 'one-pot' MA-ATPE technique was shown to be a contributing factor for enhanced extraction of solamargine and solasonine from leaves of *S. mauritianum*. Application of this time and energy efficient extraction method could potentially be expanded for enrichment of nutraceutical compounds from biomass of other medicinal plants.

Keywords: Microwave-assisted extraction; toxic phytochemicals; *Solanum mauritianum*; aqueous two phase extraction

1 Introduction

Plant reaction to damage is an inherent character and occurs through exhibition of defence mechanisms against herbivores and piercing-sucking insects such as whiteflies and bacterial pathogens [1-3]. One of the modes of operation of bacterial pathogens involves the release of reactive oxygen species such as singlet oxygen and peroxide radicals which result in oxidative stress leading to cellular damage in the host plants [1]. Other anti-pathogenic modes of plant include inhibition of glioma growth [4] and apoptosis of human chordoma cells [5]. Defence mechanisms against biotic stressors also involve the accumulation of toxic secondary metabolites, such as alkaloids which directly reduce the fitness of the invader [3,6-7]. One special class of alkaloids are steroidal glycoalkaloids found in numerous members of the Solanaceae family. They are composed of nitrogen containing alkaloid groups and carbohydrate sugar side chains [3]. Extraction of toxic phytochemicals from nutraceutical plant sources, in this case *Solanum* plants, is essential as they are the richest bio-resource of drugs for medicinal applications [6]. Glycoalkaloids have been known for their pharmacological effectiveness towards human health such as being antidiabetic [8], antifungal [9], antiparacetic [10] and anticancer [11]. Hence, extraction of these invaluable natural derived compounds (glycoalkaloids) is worthwhile.

Microwave-assisted extraction (MAE) is a simple environmentally friendly and economical technique for the extraction of biologically active compounds from different plant materials [12-13]. The advantage of this technique includes shorter extraction time, lesser solvent requirement, improved purity of the extract, low cost, and better extraction yield in comparison to Soxhlet extraction. This extraction method is a quick and highly effective technique for obtaining extracts under mild conditions, therefore it has been considered as a potential alternative to traditional methods [14-16]. Aqueous two phase extraction (ATPE) is desired for its environmental

compatibility, low interfacial tension of phases, high yields and low process time [17-18]. Recently, researchers have turned their attention to an improved version of ATPE, salting-out assisted liquid-liquid extraction (SALLE) technique, which facilitates extraction of metabolites from complex matrices [19-21].

Species within the *Solanum* genus are generally known to contain toxic metabolites (glycoalkaloids); hence this work was directed at optimization involving microwaves in a binary solvent system by means of an aqueous two phase extraction for enrichment of solasonine and solamargine from a medicinal plant, *S. mauritianum*. This work also aimed to explore conventional extraction methods such as MAE, aqueous two phase extraction followed by microwave assisted extraction (MAE + ATPE) and microwave assisted aqueous two phase extraction (MA-ATPE) for enrichment of glycoalkaloids from *S. mauritianum*. To the best of our knowledge, *S. mauritianum* has been underexplored with regards to its metabolites. Though known to be an invasive weed species from the Solanaceae family, the plant has been studied to be an essential ingredient for South African traditional medicine for treatment of menorrhagia [22] dysentery, diarrhea [23] and infertility [24] due to its metabolite composition, which is comprised of a bioactive class of compounds, glycoalkaloids. Hence, the need arises to obtain these medicinally important glycoalkaloid metabolites such as solasonine and solamargine from *S. mauritianum* using environmentally friendly extraction methods. The application of microwaves or aqueous two phase systems in conjunction with a green extraction solvent (ethanol), could potentially pave the way for more reliable means of obtaining these compounds and its sustained use in metabolomics. Furthermore, application of plant based phytocompounds in medicine could likely eliminate the need for metabolic compounds synthesized in the lab, of which are often laborious and expensive.

2 Materials and methods

2.1 Sample collection

The leaves of *S. mauritianum* were obtained from Phiphidi, Limpopo in October 2019, South Africa. The plants were air dried until a constant weight was obtained, and the leaves were ground with a rotating blade blender into a fine powder with particle sizes ranging from 100 – 300 µm. Thereafter, this powder was stored in glass containers and covered to prevent light penetration.

2.2 Chemicals and materials

Absolute ethanol (99.9% CP), which was used as an extraction solvent, was purchased from Associated Chemical Enterprises (Johannesburg, South Africa). A modified microwave oven (DM 350, Defy, Polokwane, South Africa) of 28 L capacity, working at a frequency of 2450 MHz was used for microwave-assisted extraction. The salts for ATPE NaCl (anhydrous > 99% purity) and Na_2CO_3 (anhydrous > 99% purity) and the drying agent CaO (reagent grade > 99% purity), were all purchased from Associated Chemical Enterprises (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) was applied for the dissolution of salts studied. Whatman Grade 1 filter papers were purchased from Sigma Aldrich (Johannesburg, South Africa).

2.3 Extraction procedure

2.3.1 MAE

Ground leaves of *S. mauritianum* plant powder (mass: 0.6 – 1.4 g) was immersed in hydroalcoholic solutions with various concentrations (20-60%) contained in a 1-necked 250 mL round-bottomed flask at irradiation time (1 - 13 min) with power varied from 90 to 900 W. The method is illustrated in the appendix (A1: (a))

2.3.2 MAE + ATPE

The optimized parameters following chromatographic analysis (details are included in section 2.4) for extraction of solasonine and solamargine during MAE was a 60% ethanol extraction solvent, irradiation time of 5 min at a power of 270 W. A ground *S. retroflexum* biomass powder with a mass of 1.0 g was immersed in 30% (w/v) of NaCl (chaotrope) or Na_2CO_3 (kosmotrope). Thereafter, the extract (5 mL) obtained from the optimized conditions during MAE was mixed with either 30% (w/v) of NaCl (5 mL) or 30% (w/v) of Na_2CO_3 (5 mL) followed by the addition of the extraction solvents-CaO dried ethanol or 99% ethanol (10 mL), resulted in an aqueous two phase system. The spontaneous formation of ATPE under the conditions stated above was also reported by Mokgehle et al. [3]. CaO dried ethanol was prepared by adding 25 g of CaO drying agent to 100 mL of 99% ethanol. The mixture was stirred for 10 minutes before filtration with a Whatman Grade 1 filter paper. The method is illustrated in the appendix (A1: (b))

2.3.3 MA-ATPE

In this ‘one pot’ extraction the optimized results from MAE was applied on the ATPE solution which consisted of ground plant powder mass: 1 g, saturated salt concentrations of 30% (w/v) of NaCl (chaotrope) or Na_2CO_3 (kosmotrope) which formed the bottom phase while the upper phase extraction solvent consisted of CaO dried or 99% ethanol (10 mL). In MA-ATPE, both the saturated salt solution 30% (w/v), consisting of either NaCl or Na_2CO_3 and the ethanol extraction solvent were contained in the round-bottomed flask, all in the microwave oven. The spontaneous formation of ATPE under the conditions stated above was also reported by Mokgehle et al. [3]. In all the three extraction methods conducted, there was no agitation of the sample solution prior to or following extraction. Furthermore, the volumes of the solvents after the extraction period remained unaltered. The set-up of the MA-ATPE systems is shown in the appendix (A1: (c)).

2.4 Analysis on the UPLC-QTOF-MS

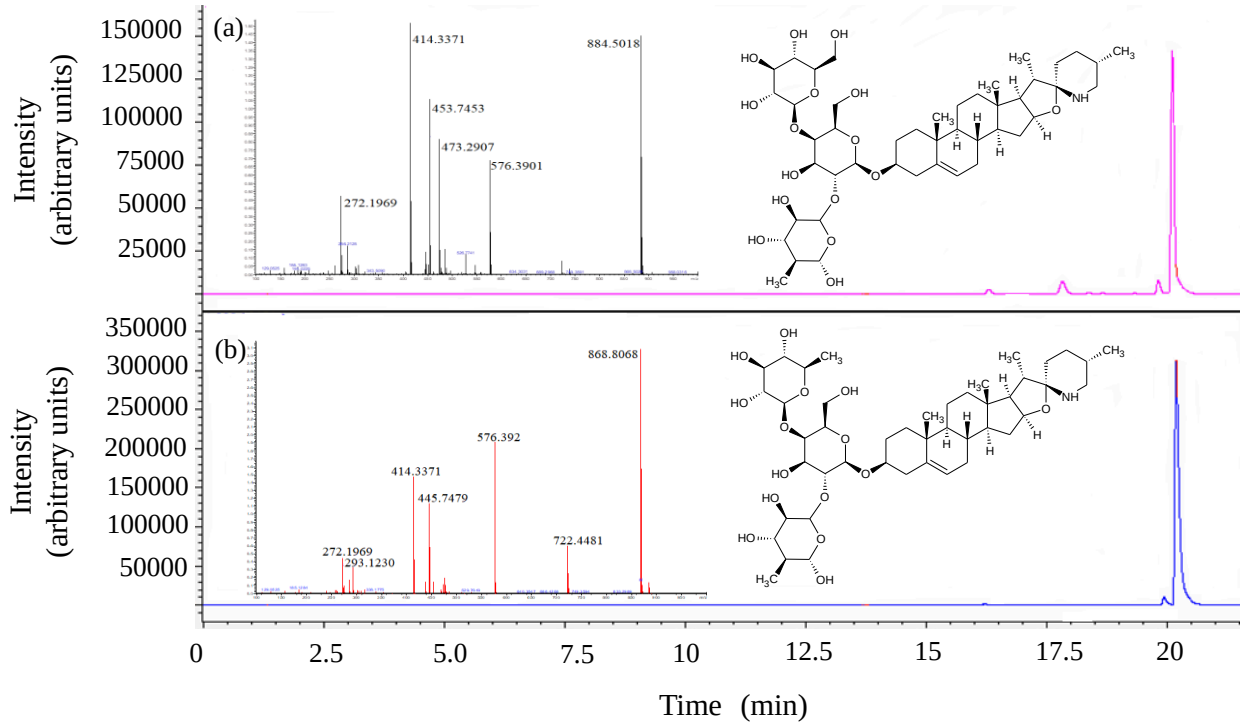
Chromatographic separation was conducted on a LC-Q-TOF/MS 9030 mass spectrometer (Shimadzu, Japan) containing a Shimpack C_{18}, 2.1 x 100 mm, 2.7 µm column from Shimadzu (Honeydew, South Africa) where the mobile phase consisted of formic acid (0.1%) in deionized water (solvent A) and methanol with 0.1% formic acid (solvent B). Chromatographic separation was achieved using a 30 min gradient elution method consisting of the following settings: the initial conditions were 5% solvent B at a flow rate of 0.4 mL min^{-1} and held constant for 3 min. Conditions were then changed to 45% solvent B at 9 min, increased slightly to 50% solvent A at 21 min and then quickly ramped up to 90% solvent B at 22 min and kept constant for 3 min. Conditions were changed to 5% solvent B at 27 min and kept for 3 min to allow re-equilibration before the next run.

For mass spectrometry, the acquisition parameters as discussed by Ramabulana et al. [25] were used. Briefly, MS data were acquired using positive electron spray ionization (ESI) modes. The MS was configured to scan the range of 100–1000 Da with a scan time of 0.2 s. After a series of optimization, the following settings were found to be optimal: capillary voltage of 4.5 eV, sample cone potential of 30 V, source temperature of 120°C, desolvation temperature of 450°C, desolvation gas flow of 550 L h^{-1}, and multichannel plate detector potential of 1600 V. In order to achieve efficient fragmentation to aid during identification, the mass spectrometry data were collected using a collision energy ramp of 10–30 eV and, when necessary, a higher collision energy ramp of 60-165 eV was also used. Structural elucidation was done using KNapSAck online metabolite database. Chemical identification was done using KNapSAck Core System online metabolite database (Version 1.200.03) [26].

3 Results and discussion

3.1 Chromatographic profile of solasonine and solamargine

In Figure 1 is shown the mass spectrometry and elution profile of two glycoalkaloid isomers, where Figure 1 (a) indicates solasonine *m/z* 884 and (b) solamargine *m/z* 868. Both contain the same aglycone unit solasodine, yet only differ in monosaccharides glycosylated to the aglycone unit. For instance, solamargine contains two rhamnose monosaccharides and glucose while solasonine is composed of glucose, rhamnose and galactose, which account for the 16 mass unit difference between the two compounds (Figure 1 (a) and 1 (b)). Figure 1 (c) shows a 30 min base peak chromatogram of a 60% ethanolic MAE extract from *S. mauritianum*, which also shows the elution order of two glycoalkaloids with the relatively more polar solasonine eluting at 19.56 min and solamargine at 19.76 min from the reversed phase column. Furthermore, Munari et al. [27] observed a similar trend in the extracts of *Solanum lycopersicum* on as Zorbax SB-C18 column where solasonine eluted before solamargine which indicated the relatively higher polarity of the former. In another study, Chester et al. [28] quantified solasonine and solamargine obtained from extracts of *Solanum nigrum L.* based on retention factors (R_f) on the HPTLC chromatogram on the UPLC-ESI-MS/MS. In the same work, solasonine had a lower R_f than solamargine due to its relatively higher polarity. Hence, the presence of galactose is a major contributor to the polarity of solasonine and its relatively higher affinity for the CaO dried ethanol extraction solvent.



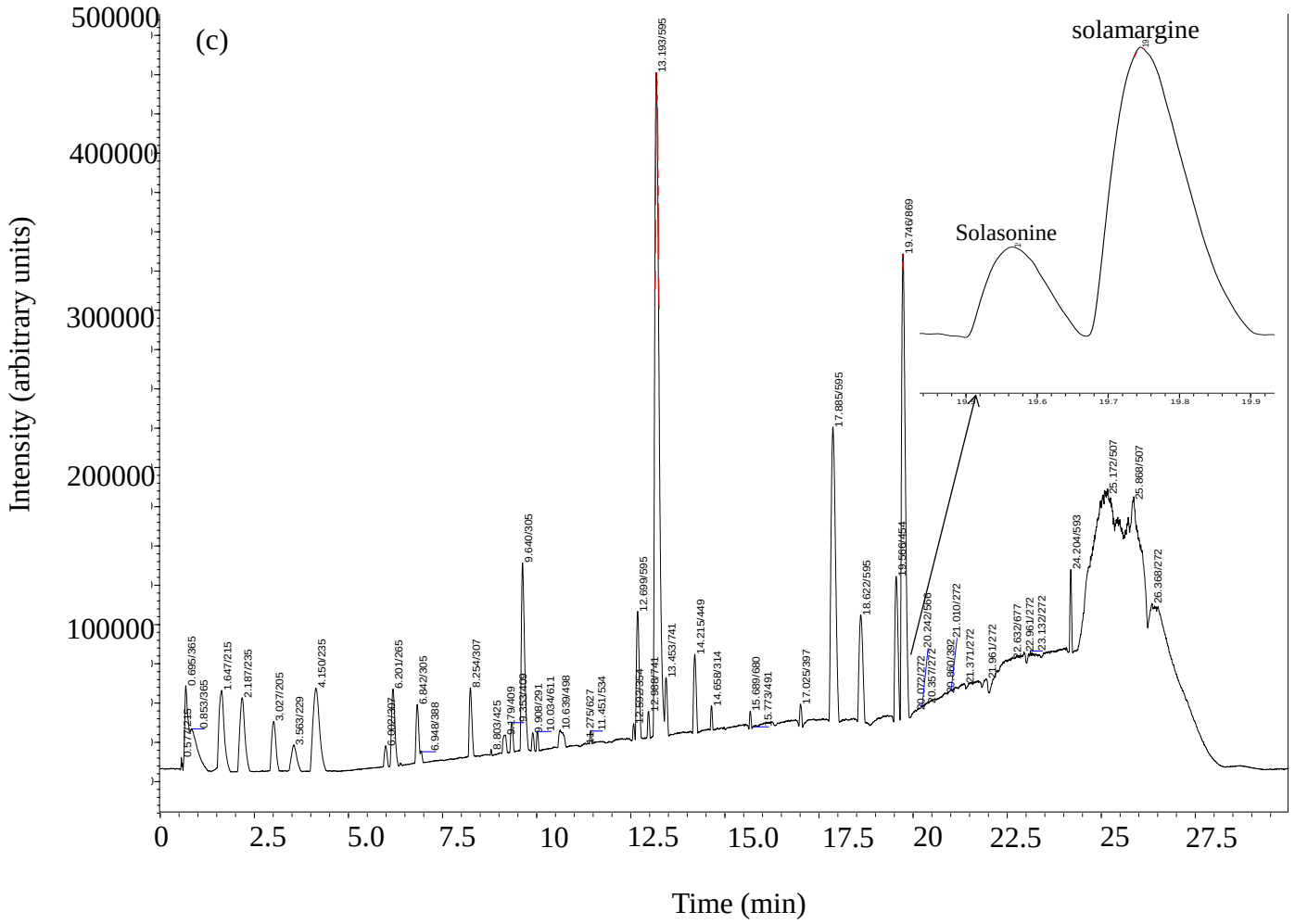


Fig. 1 Elution and mass spectrometry profile two closely related glycoalkaloid isomers (a) solasonine (t_R (min) -19.56) and (b) solamargine ((t_R (min) -19.76) (c) 30 min base peak chromatogram (BPC) of a crude (60% ethanolic extract) of *S. mauritianum*

3.1.1. Effect of concentration of ethanol on MAE

A study investigating the effect of ethanol percentage on the extraction of solasonine and solamargine was conducted as shown in Figure 2. As the concentration of ethanol was increased, the intensities of both solasonine and solamargine also increased. The higher intensities which correlated with the higher yield can be attributed to the higher proportion of ethanol and the lower level of water in the extraction solvent. The ethanol percentage in water was one of the driving factors influencing the MAE of compounds as it affected the solubility of metabolites, penetration of solvent into the cells of plants, interaction of solvent with matrix, and the absorption of microwave energy [29]. Increasing the water concentration in the solvent as highlighted by Veggi et al. [30] and Zhang et al. [31] has been reported to influence selectivity during extraction, which resulted in a greater affinity towards proteins and carbohydrates rather than bioactive plant metabolites. It was also observed that the relative intensities of solamargine:solasonine occurred on a general ratio of 2:1, which probably indicated the greater relative abundance of solamargine compared to solasonine in the leaves of *S. mauritianum*. Hence, the optimum extraction solvent was 60% ethanol and was then used in the following study.

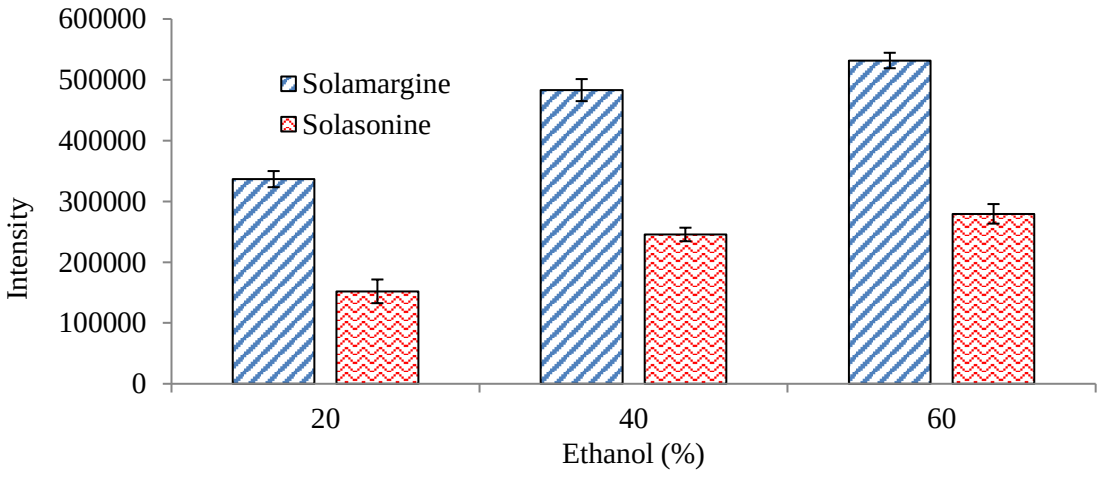


Fig. 2 Effect of (%) ethanol for MAE of solasonine and solamargine. Conditions: Irradiation time: 5 min, power: 270 W, mass of plant: 1.00 g (n = 3, RSD).

3.1.2. Effect of solid/ liquid ratio on MAE

The effect of solid/ liquid ratio was evaluated where 0.6 g, 1 g, 1.4 g were evaluated in 10 mL 60% ethanol and corresponded to solid/liquid ratios of 0.06 g L^{-1}, 0.1 g L^{-1}, 0.14 g L^{-1} respectively (Figure 3). The optimal extraction of solasonine and solamargine was observed at 0.1 g L^{-1}. The lower intensities of extracted solamargine and solasonine at 0.06 g L^{-1} could be due to the relatively lower amount of plant material used. Increased solvent volumes have also been reported to reduce the heating efficiency, limit the breakage of solid cell walls, and inhibit the driving force for the mass transfer of compounds [29]. Similarly, Alara et al. [32] reported that larger volumes of solvent required more energy and time to maximize extraction of analytes from the plant matrix. It was also noted that with larger solid/liquid ratios, reduced extraction of solasonine and solamargine was observed. This could be as a result of lumping of the powdered plant material, limiting access for the extraction solvent to penetrate through the cell walls. Similarly, Sajid et al. [33] reported on clogging during solid phase micro-extraction. The optimum mass for extraction of solasonine and solamargine was 1 g, solid/liquid ratio 0.1 g L^{-1}.

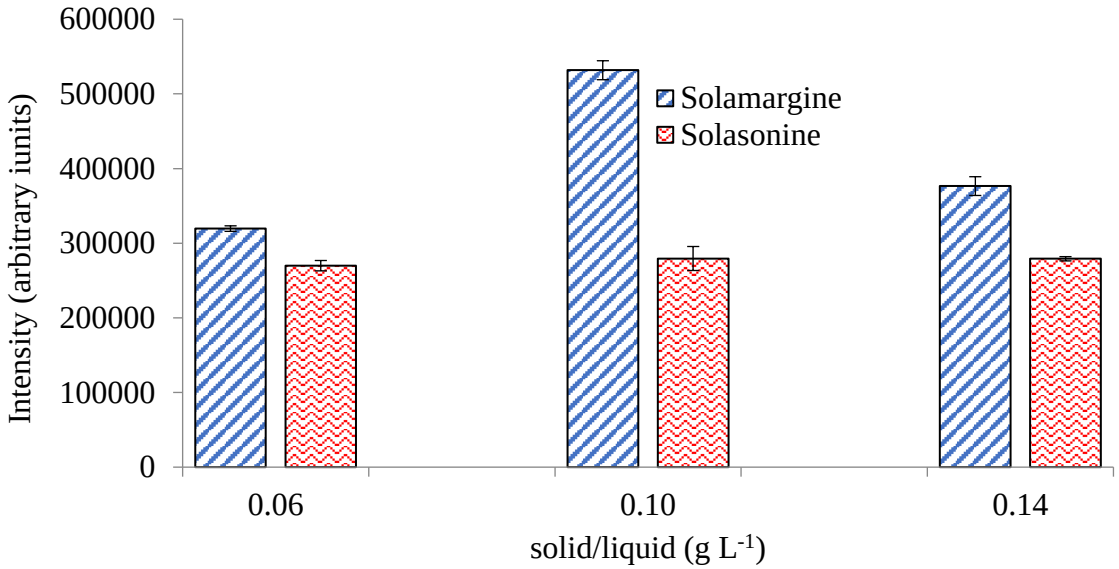


Fig. 3 Effect of solid/liquid ratio for MAE of solasonine and solamargine. Conditions: Irradiation time: 5 min, power: 270 W, solvent: 60% ethanol (n = 3, RSD).

3.1.3. Effect of irradiation time on MAE

A study evaluating the effect of irradiation time on extraction of solasonine and solamargine was performed as shown in Figure 4. As time was increased from 1 to 5 min, a gradual increase in the extraction of solasonine and solamargine was noted. In general, higher extraction time tends to increase the yield of extraction. In addition, the dielectric properties of solvents used in MAE may have significant impacts on the extraction time [30,34]. For instance, at room temperature, water has a dielectric constant of 80 however, the addition of ethanol in the aqueous mixture reduces this constant greatly allowing it to easily dissolve a wide range of less polar metabolites, hence in this instance it only took 5 mins to achieve optimal extraction of both solasonine and solamargine, whereas if only water was used as an extractant optimal extraction of both glycoalkaloids would have most likely taken longer than 5 mins [30,34]. Furthermore, extraction of metabolites from the plant matrix is not an instantaneous process, there are multiple phases occurring which involve removal of compounds from the outer surface of plant matrix, a transition state consisting of intermolecular forces between the metabolites and the plant matrix inhibiting mass transfer brought about by the extraction solvent. After the 5 min irradiation time, a steady decrease in the intensities of both glycoalkaloids was observed (Figure 4). This steady decrease at longer times was increased could be associated with the increased degradation of thermolabile metabolites, solasonine and solamargine, during this period. This is in agreement with what was reported by Doulabi et al. [29] and Veggi et al. [30]. The optimal extraction time to be used in the following studies was 5 min.

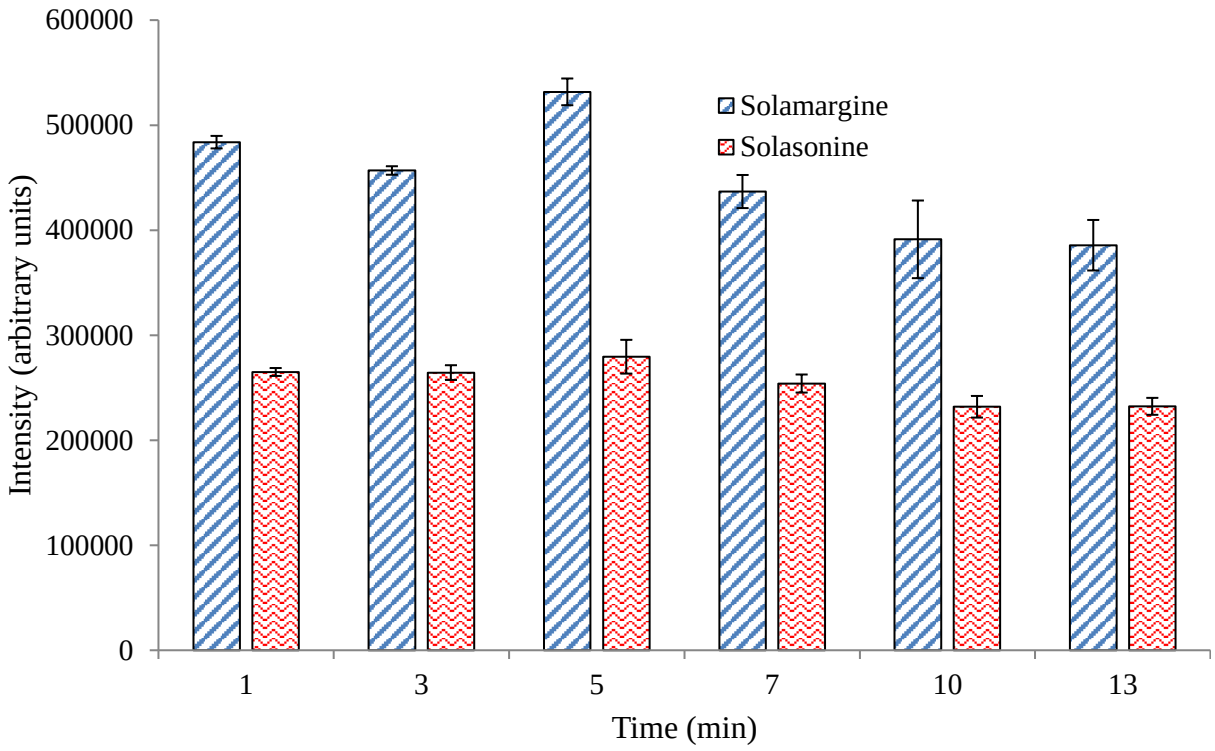


Fig. 4 The influence of irradiation time on the MAE of solasonine and solamargine.

Conditions: Mass of plant: 1.00 g, power: 270 W, solvent: 60% ethanol (n = 3; RSD).

3.1.4. Effect of power (W) on MAE

Power studies were conducted to examine its effect on the extraction of solasonine and solamargine as shown in Figure 5. From 90 to 270 W an increase in extraction of both glycoalkaloids was observed (Figure 5). Generally, an increase in microwave power can improve the penetration of solvent into plant matrix, resulting in rapid delivery of microwave energy to both solvent and plant matrix [29]. Accordingly, from 90 to 270 W microwave power, dissolution of solasonine and solamargine occurred due to the increased temperature of the extraction solvent. However, from 270 - 900 W, a steady decrease was observed (Figure 5). This could be because of excessive microwave irradiation energy degrading both solasonine and solamargine [34-35]. Therefore, the optimal extraction power was observed at 270 W.

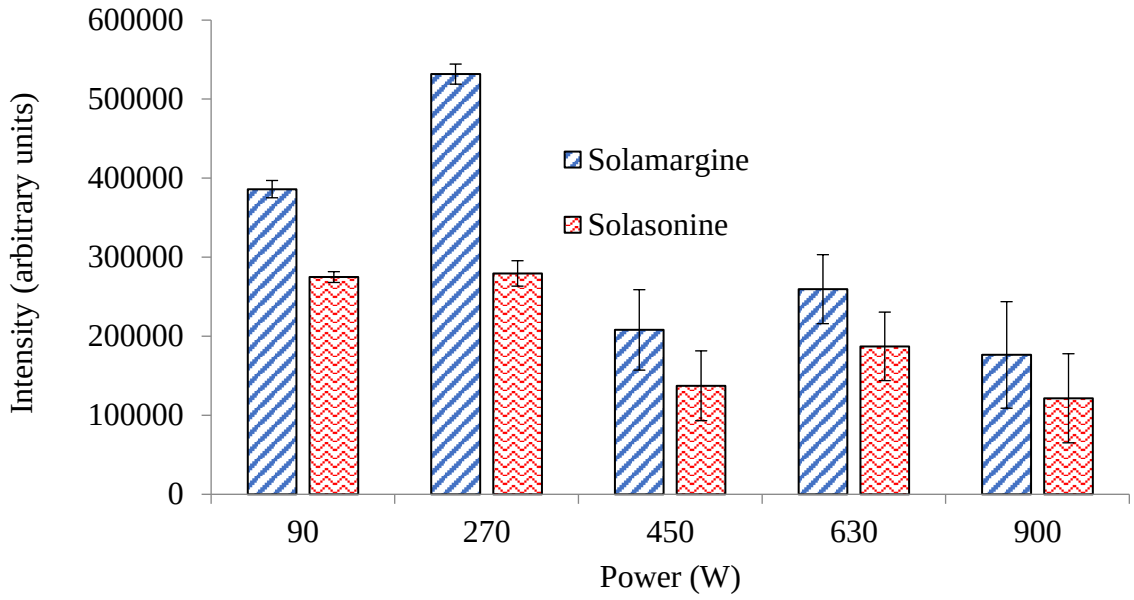


Fig. 5 Evaluation of the effect of microwave power on the MAE of solamargine and solasonine. Conditions: Mass of plant: 1.00 g, solvent: 60% ethanol, time: 5 min (n =3, RSD).

3.2 MAE +ATPE

Following the optimized result obtained for MAE (section 3.1) which included: 60% ethanol extraction solvent, solid/liquid ratio of 0.1 g L^{-1}, irradiation time of 5 min and power of 270 W, the obtained conditions were applied for MAE +ATPE to improve the extraction of solasonine and solamargine. Besides the inclusion of ATPE, the 99% ethanol extraction solvent was dried with CaO drying agent with the aim of enhancing the extraction of solasonine and solamargine. Figure 6 shows the extraction profile for solasonine and solamargine using an extraction solvent of 99% ethanol and CaO dried ethanol when the chaotrope and kosmotrope NaCl and Na_2CO_3 was used, respectively. In general, no differences were observed in intensities of solasonine and solamargine when CaO dried ethanol was used as an extraction solvent compared to 99% ethanol for both Na_2CO_3 and NaCl (Figure 6). Though, CaO was reported as an efficient drying agent by Danish et al. [36] and Jia et al. [37] in sewage sludge and mortar, respectively, its application in drying extraction solvents for the improving extraction of solasonine and solamargine, was limited.

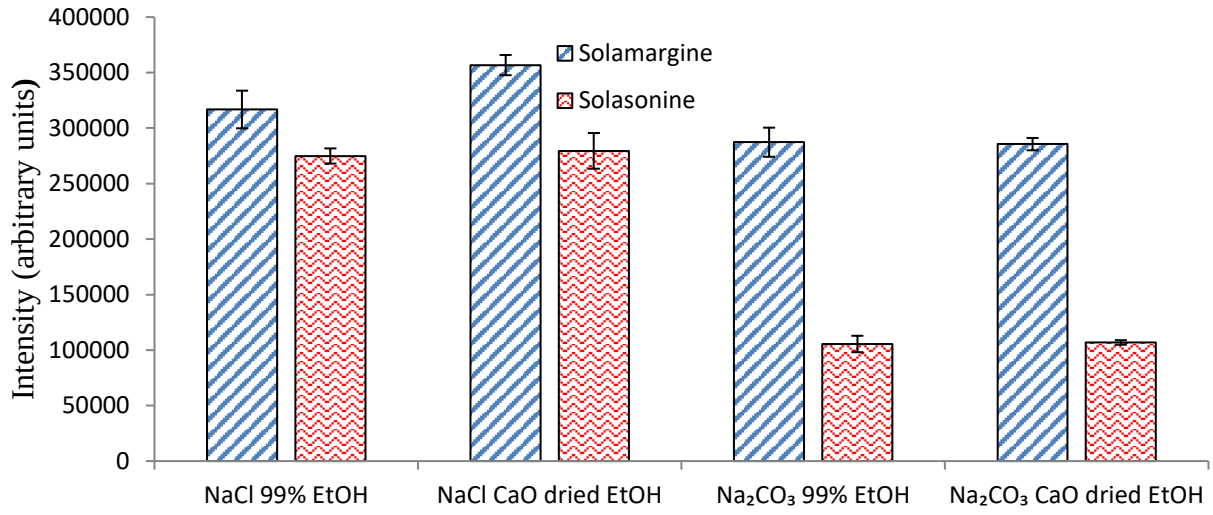


Fig. 6 Comparison on MAE + ATPE of solasonine and solamargine when 99% absolute ethanol and absolute ethanol dried with CaO was used in the presence of the chaotrope NaCl and kosmotrope Na_2CO_3 (n = 3, RSD).

3.3 MA-ATPE

With the aim of further improving the extraction of solamargine and solasonine from *S. mauritianum*, a hyphenated MA-ATPE was applied as shown in Figure 7. In both instances where the extraction solvent was 99% ethanol and CaO dried ethanol, Na_2CO_3 was generally observed to be a better extractant of solasonine and solamargine than NaCl. The opposite effect was observed for the two-stage extraction process MAE + ATPE (Figure 6), which indicated that the extraction method influenced the salting-out efficiency of solamargine and solasonine from the aqueous solution in the presence of NaCl or Na_2CO_3. The effect of extractions conditions on salting-out was also reported by Tajeda-Casado et al. [20] and Sazali et al. [21]. The doubly charged carbonate ions from Na_2CO_3 interacted with the hydration sphere surrounding the solute (solamargine and solasonine) to a greater extent than singly charged chloride ions from NaCl, forming carbonic acid. Thereafter, this led to the precipitation (salting-out) of the solute in the aqueous phase and resultant extraction by ethanol. Salting-out

effect has been reported to aid the extraction of Vitamin D3 from milk samples [21] and in the determination of 5-nitroimidazolesin in fish [19].

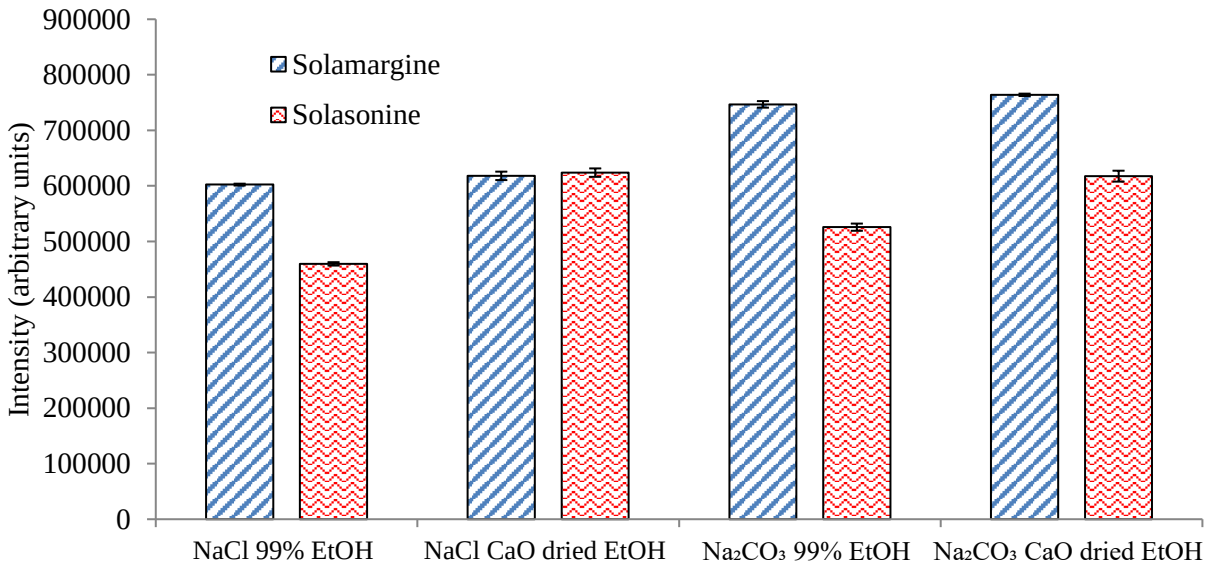


Fig. 7 Comparative MA-ATPE of solasonine and solamargine when 99% ethanol and ethanol dried with CaO was used in the presence of the chaotrope NaCl and kosmotrope Na_2CO_3 (n = 3, RSD).

3.4 Comparison of the extraction efficiency of MAE, MAE + ATPE, MA-ATPE

Enrichment of solasonine and solamargine was considerably greater for MA-ATPE in comparison to MAE+ATPE and MAE. For instance, extraction of solamargine and solasonine using Na_2CO_3 in CaO dried ethanol during MA-ATPE (Figure 7) was approximately three-fold and two-fold greater than that of MAE + ATPE, respectively (Figure 6). Similarly, extraction of solamargine and solasonine using NaCl in CaO dried ethanol during MA-ATPE (Figure 7) was approximately two-fold greater than that of MAE + ATPE (Figure 6, Table 1). This indicated that in MA-ATPE, the synergistic effect of microwaves and salting-out occurred simultaneously. In this case, extraction was assisted by microwaves which resulted in cell wall

rapture and subsequent mass transfer of solasonine and solamargine into the aqueous solution. In addition to microwaves the salting-out effect prompted the precipitation of solasonine and solamargine from the hydration sphere into the ethanol extraction phase during MA-ATPE. The two-step extraction method MAE + ATPE, was observed to have the lowest extraction of metabolites (Figure 6). This could be attributed to the inter-step loss of metabolites during MAE-ATPE. Furthermore, Gardernar et al. [36] highlighted some disadvantages associated with two step extractions which involved the requirement for a substantial amount of specimen to prevent analyte loses. It was also observed that MA-ATPE improved extraction of solasonine and solamargine compared to MAE (Table 1). This suggested that the chaotrope and kosmotrope were influential in aiding extraction of solasonine and solamargine in MA-ATPE compared to MAE, from which the extraction did not include salts. Hence, the simultaneous application of microwaves assisted the extraction of solasonine and solamargine in the aqueous two phase system based on salting-out, as seen with MA-ATPE, and was influential in enhancing the extraction of solasonine and solamargine in comparison to MAE and MAE + ATPE.

Table 1 Intensities of solasonine and solamargine under MAE, MAE+ATPE and MA-ATPE

	salt	Intensity (arbitrary units)			
		Solasonine		Solamargine	
		CaO dried EtOH	EtOH 99.9%	CaO dried EtOH	EtOH 99.9%
MAE			279507		531704
MAE+ATPE	NaCl	279507	274890	356772	316784
	Na_2CO_3	106899	105495	285572	287307
MA-ATPE	NaCl	623912	459642	618115	602356
	Na_2CO_3	617550	525691	763974	746758

4 Conclusions

In this study, the optimization of microwave and aqueous two phase based extraction techniques which involved MAE, ATPE + MAE and MA-ATPE for extraction of solasonine and solamargine from leaves of *S. mauritianum*, was evaluated. The first technique which only involved microwaves, MAE, maximal extraction of solasonine and solamargine was achieved when extraction was conducted for 5 min, microwave power of 270 W, solid/liquid of 0.1 g L^{-1} and an ethanol concentration of 60%. Efforts were then made to improve extraction of both solasonine and solamargine by applying drying agents such as CaO in the ethanol extraction solvent. Dried ethanol was applied in two-stage extraction (MAE + ATPE). However, the yields of solasonine and solamargine were observed to decrease due to possible analyte loss of metabolites during inter-step transfer between MAE and ATPE. Application of CaO dried ethanol in conjunction with the 'one-pot' MA-ATPE was shown to considerably enhance extraction of both glycoalkaloids relative to MAE and MAE-ATPE. For instance, extraction of solamargine and solasonine using Na_2CO_3 in CaO dried ethanol during MA-ATPE was approximately three-fold and two-fold greater than that of MAE + ATPE, respectively. Furthermore, extraction of solamargine and solasonine using NaCl in CaO dried ethanol during MA-ATPE was approximately two-fold greater than that of MAE + ATPE. Hence, the kosmotrope (Na_2CO_3) was shown to be a relatively better extractor of solamargine and solasonine in comparison to the chaotrope (NaCl) due to its superior salting-out capacity in MA-ATPE. The results suggested that MA-ATPE, a technique propelled by the synergy of microwaves and salting-out, is a promising time and energy efficient method for enrichment of solamargine and solasonine from leaves of *S. mauritianum.*

Declarations

Funding

The authors would also like to express their gratitude to the National Research Foundation and Sasol Inzalo for financial support. The University of Venda is also thanked.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material (data transparency)

Not applicable

Code availability (software application or custom code)

Not Applicable

Authors' contributions

TMM, NTT and NEM conceived the study, TMM and NEM conducted the experiments and data analyses. NTT, NEM and WMG supervised the project. WMG helped to draft the manuscript. All authors read and approved the final manuscript.

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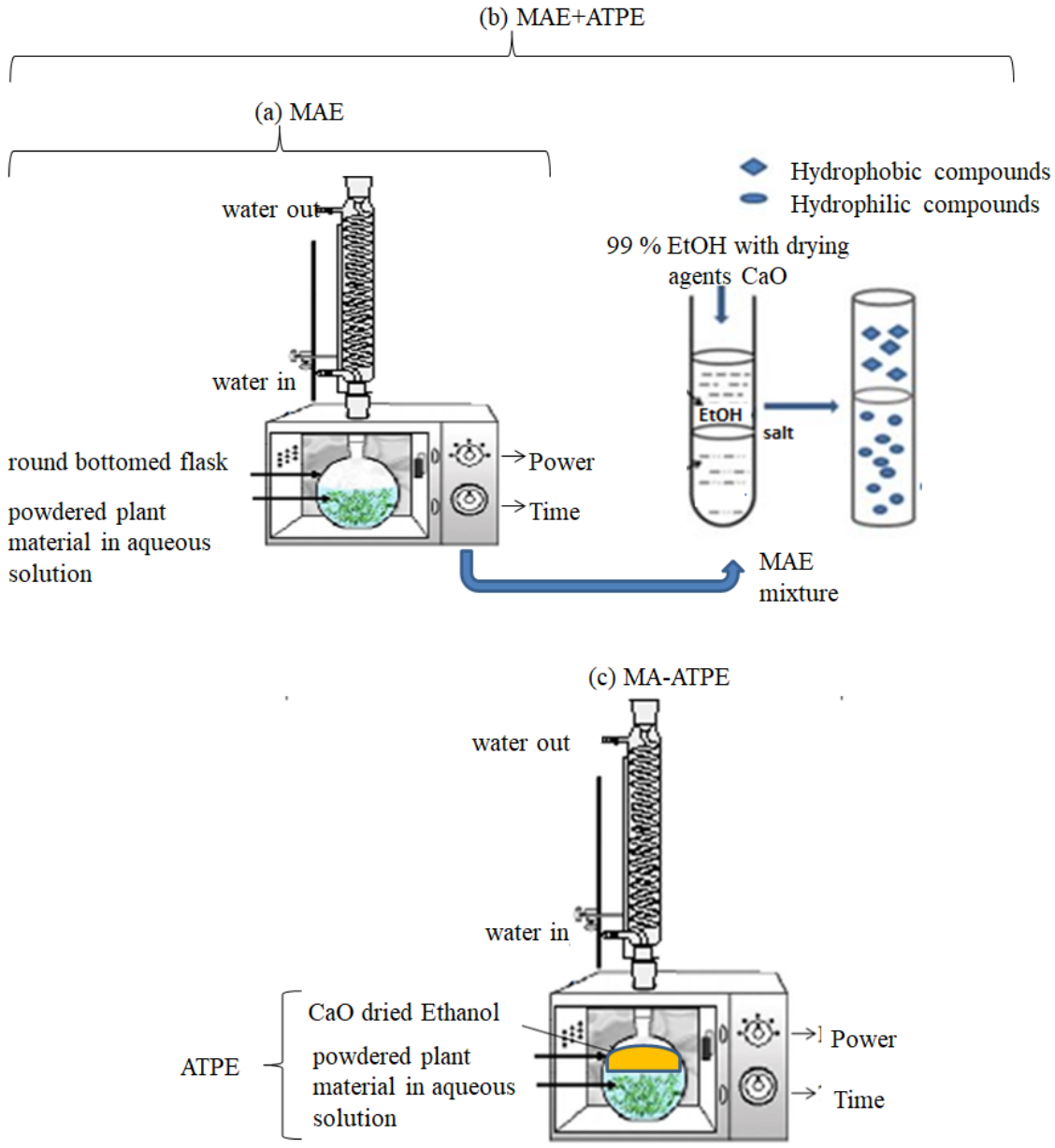

A1: Experimental set-up of (a) MAE, (b) MAE-ATPE and (c) MA-ATPE

Paper 3

A multivariate analysis examining the effect of mass of plant powder, extraction time and microwave power for optimization of MA-ATPE of α-solanine from *Solanum retroflexum*, aided by the kosmotrope (Na_2CO_3) or chaotrope (NaCl), was evaluated.

Effect of microwave assisted aqueous two phase extraction of α-solanine from *S. retroflexum* and analysis on UHPLC-qTOF-MS

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Abstract

A hyphenated microwave assisted aqueous two phase extraction (MA-ATPE) was applied in the extraction of α-solanine from *Solanum retroflexum*. Central composite design (CCD) was performed which included numerical parameters such as time, mass of plant powder and microwave power. The categorical factors included the chaotrope - NaCl or the kosmotrope - Na_2CO_3. Fitting the central composite design response surface model to the data generated a quadratic model with a good fit ($R^2 = 0.920$). The statistically significant ($p < 0.05$) parameters such as time and mass of plant powder were influential in the extraction of α-solanine. Quantification of α-solanine was achieved using a robust and sensitive feature of ultra high performance quadrupole time of flight mass spectrometer (UHPLC-qTOF-MS), multiple reaction monitoring (MRM). The optimized condition for the extraction of α-solanine in the presence of NaCl and Na_2CO_3 was a period of 1 min at a mass of 1.2 g using a microwave power of 40%. Maximal extraction of α-solanine was 93.50 mg kg^{-1} and 72.16 mg kg^{-1} for Na_2CO_3 and NaCl respectively. The synergistic effect of salting-out and microwave extraction was influential in extraction of α-solanine. Furthermore, the higher negative charge density of the kosmotrope (Na_2CO_3) was responsible for its greater extraction of α-solanine than chaotrope (NaCl). The shorter optimal extraction times of MA-ATPE make it a potential technique that could meet market demand as it is a quick, green, and efficient method for removal of toxic metabolites in nutraceuticals.

Keywords: α-solanine, *S. retroflexum*, response surface methodology, MA-ATPE, UHPL-qTOF-MS

Introduction

Solanum retroflexum is one of many species within the *Solanaceae* family widely distributed and boasting over 3000 species of trees, shrubs, and herbs. The consumption of *Solanum retroflexum* remains controversial in different cultural practices (Averbeke et al., 2007; Managa et al., 2020). In South Africa, *Solanum retroflexum* is an exclusively produced and consumed vegetable by African people, and due to its high nutrient composition in its leaves, its affordable means to alleviate malnutrition among poor rural based South Africans (Averbeke et al., 2007). On the contrary, *Solanum retroflexum* is an inedible plant and persistent weed in Europe and America, as it is perceived to be toxic (Karabegović et al., 2018).

The edibility or inedibility of *Solanum retroflexum* is due to its metabolic composition. Some of the classes of secondary metabolites derived from *Solanum* plants include polyphenols such as flavonoids, widely renowned for its antioxidant activities (Uchida et al., 2017; Mahieddine et al., 2018; Fratianni et al., 2020) and steroidal alkaloids widely reported for its toxic effects. The surge in concentration levels of steriodal alkaloids (glycoalkaloids) is triggered by the exposure of *Solanum* plants to the sun's uv-light or because of mechanical injury including peeling and slicing (Dao and Friedman, 1994; Kasnak et al., 2018). Some of the toxic effects of glycoalkaloids are due to anticholinesterase effects on the central nervous system (Caprioli et al., 2014; Lelario et al., 2019) and disruption of cell membranes (Blankemeyer et al., 1998; Nepal et al., 2019). Symptoms of glycoalkaloid poisoning in humans include colic pain in the abdomen and stomach, diarrhea, vomiting, burning sensation about the lips and mouth, fever, rapid pulse, and headache (Uluwaduge et al., 2018; Deng et al., 2021). Other destructive effects of glycoalkaloids include craniofacial malformations in hamsters (Garfield and Keeler, 1996; Ni et al., 2018; Kumar et al., 2019) and a variety of organ malformations in frog embryos and mealworms (Friedman et al., 1991, Chen et al., 2021). Furthermore, the Centre for Food Safety (2015) reported on poisoning of patients after consumption of cooked potatoes, subsequent investigations revealed that the poisoning was due to the glycoalkaloid α-solanine. As a result, regulatory bodies such as the Commission for Food and Agricultural Organization (FAO) and the World Health Organization (WHO) have established regulations for maximum permissible concentrations of glycoalkaloids, which currently stands at 200 mg kg^{-1} for fresh potatoes (*Solanum lycopersicum*) sold in supermarkets.

In view of the toxic potential of glycoalkaloids contained in *Solanum* vegetables, food toxicology regulating bodies have come-up with maximum allowable limits of these metabolites. Besides these policy frameworks, more still need to be done to improve the nutritional value and safety of some plant foods, especially those consumed by humans. This could come in the form of environmentally friendly extraction techniques that target these poisonous glycoalkaloids in food.

In this study, the one-pot extraction (MA-ATPE) of a toxic metabolite, α-solanine, from *Solanum retroflexum* was investigated and optimization was based on the application of central composite design (CCD) and response surface methodology (RSM). The CCD and RSM approach are useful as it reduces the number of experiments, making it less laborious and time efficient (Silva et al., 2019). Application of MA-ATPE could be a fast, environmentally friendly, and efficient method for extraction of α-solanine that could be vital by reducing toxicity of a popular vegetable, *Solanum retroflexum*, making it safe for consumption. Furthermore, this hyphenated environmentally friendly extraction technique could potentially be utilized on a commercial scale.

Materials and methods

Chemicals and reagents

The salts NaCl (anhydrous ≥ 99% purity), Na_2CO_3 (anhydrous ≥ 99% purity) and ethanol (99% CP) were purchased from Associated Chemical Enterprises (Johannesburg, South Africa) and Sigma-Aldrich (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) using a Direct-Q 5UV distiller (Massachusetts, United States of America) was applied for the preparation of the salt solutions. A modified microwave oven (DM 350, Defy, Polokwane, South Africa) of 28 L capacity, working at a frequency of 2450 MHz was used for microwave assisted extraction. Chromatographic separation of the metabolites in the extracts was done using a reverse phase Shim-pack Velox C_{18}, 2.1 x 100 mm, 2.7 µm with a serial number 227-32009-03 (Columbia, USA). The UPLC was connected to a Shimadzu 9030 LC, qTOF-MS detector (Shimadzu, Kyoto). The solvents used for the chromatographic runs were methanol and formic acid, which were purchased from Romil Pure Chemistry (Cambridge, UK).

Sample collection and preparation

The leaves of *Solanum retroflexum* were obtained from a street vendor within the Thulamela District in Thohoyandou, South Africa. The plants were air dried until a constant weight was obtained, and the leaves were ground into a fine powder with a blender at 2000 rpm and stored in glass containers. The containers were covered in paper bags to prevent light penetration. The MA-ATPE method consisted of powdered leaves placed in a 250 mL round bottomed flask (Fig.1). Thereafter, saturated salt concentrations (5 mL) of 30% (w/v) involving Na_2CO_3 (kosmotrope) and NaCl (chaotrope) were prepared by weighing 15 g of salt in 50 mL of water, were added to the powdered leaves in the round bottomed flask. The 99% ethanol extraction solvent (5 mL) as a top layer and the saturated salt solution containing the powdered leaves resulted in an ATPE system (Fig. 1). The ethanol extraction solvent was dried in CaO 25% (w/v) prior to it being used for extraction. The ATPE solutions were then exposed to microwaves for different periods (1-10 min) at different microwave power (30-100%). Thereafter, the extracts obtained were then analysed on the UPLC-qTOF-MS for α-solanine.

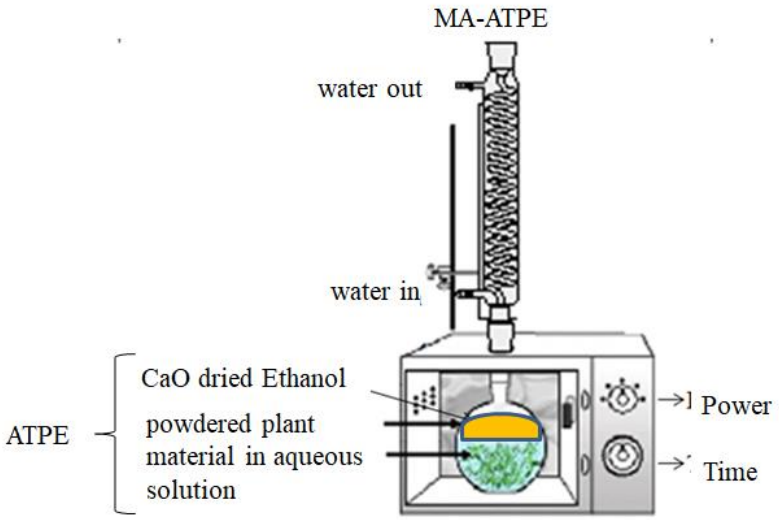


Fig.1 MA-ATPE setup containing the powdered *Solanum mauritianum* plant from which α-solanine was extracted.

Chromatographic and mass spectrometry conditions

α-Solanine was separated using the column stated in section 2.1. The column was maintained at 40 ºC at a flow rate of 0.4 mL min^{-1} and the injection volume was 5 µL. Mobile phase A was 0.1% formic acid in ultrahigh purity water (v/v) and mobile phase B was 0.1% (v/v) formic acid in methanol.

The UHPLC-qTOF-MS 9030 mass spectrometer was equipped with an electrospray interface (ESI) operating in positive mode. The ESI parameters were optimized for α-solanine by direct infusion of standard solutions into the mass spectrometer. The mass spectrometer was operated in the multi reaction monitoring (MRM) mode to confirm the identity of α-solanine. High-purity nitrogen (N_2) was used as the nebulizing and drying gas. The optimum parameters were as follows: drying gas temperature, 250°C; drying gas flow, 10 L min^{-1} and collision energy, 50 - 80V. Lab solutions software was used to run the LC-MS/MS instrument for data acquisition and the mass range used was *m/z* 100-1000.

Preparation of standards and quantification of samples

The stock α-solanine standard solution was prepared in methanol at a concentration of 1000 µg L^{-1}. The stock standard solution was stored at 4°C in amber volumetric flasks. A series of nine working standard solutions at the concentration values of 15 to 1000 µg L^{-1} were prepared from the stock standard solution by diluting with HPLC grade methanol. The α-solanine standards were quantified based on scheduled multiple reaction monitoring (MRM) where one *m/z* transition, from the precursor ion to the product ion, for α-solanine (868 → 722) was explored. The regression equation was y = 52.1677x + 624.135, the limit of detection (LOD) and limit of quantification (LOQ) were 0.3169 and 0.9509, respectively. The above mentioned transition was then applied for quantification of α-solanine from the ground leaves of *Solanum retroflexum* following MA-ATPE extraction. The parameters evaluated for optimization of MA-ATPE of α-solanine were time, mass of plant powder, microwave power and the salt type (kosmotrope or chaotrope)

Statistical analysis

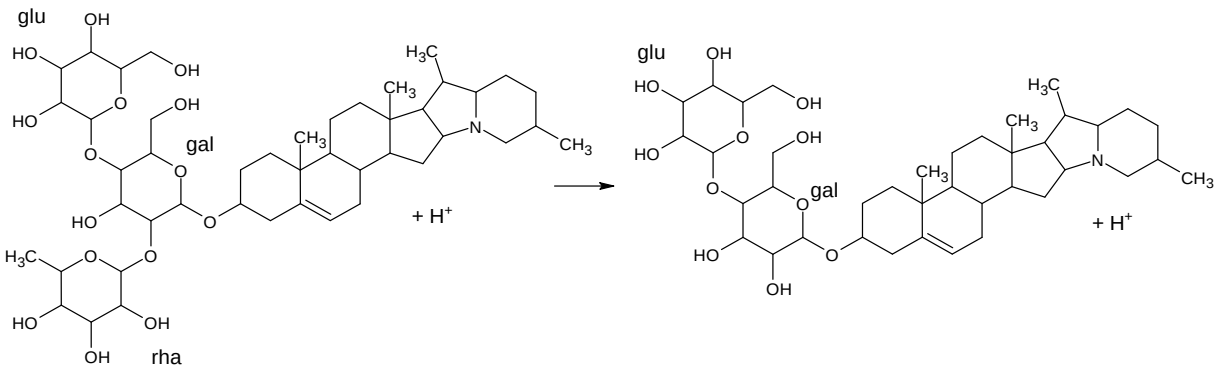
The central composite design response surface model (CCD RSM) was fitted to experimental data in order to obtain the relationship between factors and optimize the response of Z (α-solanine yield) in relation to A (time), B (plant mass) using Design Expert 11 (Minneapolis, USA). By using CCD, a total of 36 experimental runs, done in duplicate, were designed which included 3 numerical factor levels for time (1 min, 5 min 30 sec and 10 min) 3 factor numerical levels for mass of plant powder (0.2, 0.7, 1.2 g), 3 numerical factor levels for power (40, 70, 100%) and 2 categorical factor levels for salts which included the chaotrope (NaCl) and kosmotrope (Na_2CO_3).

The interaction between the various parameters studied and its resultant effected on the extraction of α-solanine (mg kg^{-1}) was fitted to experimental data by using a statistical multiple regression approach method of least square (MLS) and resulted in the lowest possible residual (Bas et al., 2007). Model parameters and model significance were determined at $p < 0.05$. The fitness of the model was determined by evaluating the coefficient of regression (R^2) obtained from the analysis of variance (ANOVA). The model fit generated the response surface that defined the behaviour of the response variable. By means of these plots, the optimized ranges for each factor that led to the highest response (i.e concentration of α-solanine) that can be extracted (Bas et al., 2007; Arteaga-Crespo et al., 2020).

Results and discussion

MRM quantification of α-solanine based on the 868→722 transition

In this study, the extraction of α-solanine was performed of which was reported to be contained in *Solanum retroflexum*, using a hyphenated MA-ATPE approach (Daji et al., 2018; Mokgehle et al., 2021). The MA-ATPE was modified using different factors as shown in Table 1, on the recovery of target metabolite α-solanine. The presence of α-solanine has been reported in *Solanum retroflexum* and other species within the *Solanum* genus (Daji et al., 2018; Mokgehle et al., 2021). Using a sensitive and robust tandem MS approach (UHPLC-qTOF-MS) with settings presented elsewhere (Gbashi et al., 2016; Mokgehle 2021) it was possible to efficiently fingerprint these α-solanine as shown in Fig. 2 based on *m/z* 722 product ion. Thereafter, based on the 868→722 transition within the MRM method, α-solanine was quantified as a function of the various factors shown in Table 1.



m/z 868 *m/z* 722

Fig. 2 Molecular transition of α-solanine (m/z = 868) to ß-solanine (m/z = 722)

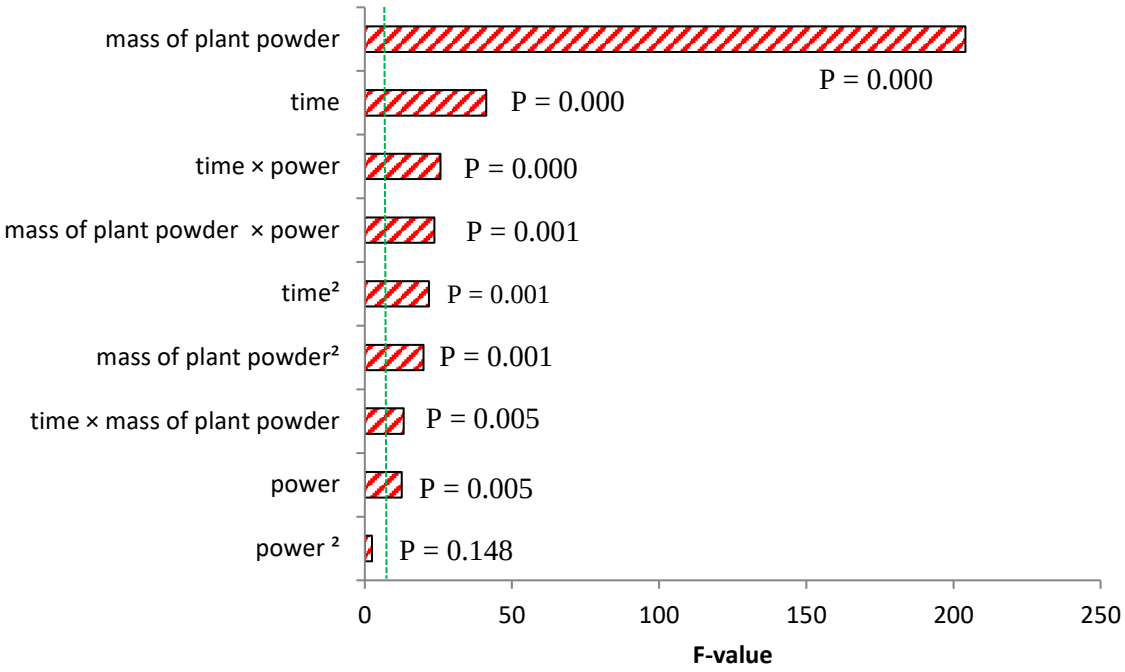
Table 1 Design layout from input variables in the central composite design and responses

Run	Factor 1: Time (min)	Factor 2: mass of plant powder(g)	Factor 3: Power (%)	Factor 4: Salt	Responses Run 1	Run 2	Mean ± SD	Predicted
1	1	0.2	100	NaCl	32.07	36.19	34.13±2.9	35.79
2	1	0.2	40	Na_2CO_3	27.34	71.32	49.33±31	45.89
3	1	1.2	40	Na_2CO_3	72.00	115.0	93.00±30	88.57
4	1	0.2	100	Na_2CO_3	30.21	46.73	38.47±11	34.3
5	1	0.2	40	NaCl	20.22	24.01	22.12±2.7	23.27
6	1	1.2	40	NaCl	64.32	80.00	72.16±11	73.36
7	1	1.2	100	NaCl	51.10	84.00	67.55±23	67.39
8	1	0.7	70	NaCl	42.03	65.39	53.71±16	58.46
9	1	1.2	100	Na_2CO_3	45.28	71.37	58.32±25	58.46
10	1	0.7	70	Na_2CO_3	51.42	72.00	61.71±15	65.31
11	5.5	0.7	70	Na_2CO_3	15.16	100.0	57.58±59	41.66
12	5.5	0.7	70	Na_2CO_3	15.41	40.07	27.74±17	41.66
13	5.5	0.7	40	Na_2CO_3	32.55	62.03	47.29±21	56.86
14	5.5	0.7	100	Na_2CO_3	20.78	29.14	24.96±6	28.72
15	5.5	0.7	70	NaCl	53.41	69.01	61.21±11	57.23
16	5.5	0.7	100	NaCl	50.67	67.18	58.93±12	56.35
17	5.5	1.2	70	Na_2CO_3	34.28	53.55	43.92±14	47.18
18	5.5	0.2	70	NaCl	22.69	32.57	27.63±6.9	28.69
19	5.5	0.7	70	NaCl	62.90	65.14	64.02±1.5	57.23
20	5.5	0.7	40	NaCl	50.73	73.00	61.86±16	60.38
21	5.5	1.2	70	NaCl	59.35	73.00	66.17±9.6	66.48
22	5.5	0.7	70	Na_2CO_3	36.47	48.10	42.28±8.2	-
23	5.5	0.2	70	Na_2CO_3	10.82	13.63	12.22±1.9	16.84
24	5.5	0.7	70	NaCl	51.12	70.92	61.02±14	57.23
25	5.5	0.7	70	Na_2CO_3	57.35	30.16	43.75±19	41.66
26	5.5	0.7	70	NaCl	48.55	65.14	56.85±12	-
27	10	1.2	40	Na_2CO_3	34.45	42.71	38.58±5.8	37.01
28	10	0.7	70	NaCl	30.09	49.86	39.97±13	47.53
29	10	0.2	40	Na_2CO_3	13.65	67.28	40.46±37	-
30	10	0.2	100	Na_2CO_3	-	-	-	-
31	10	0.2	100	NaCl	12.95	25.47	19.21±8.8	20.61
32	10	1.2	40	NaCl	48.38	81.00	64.69±23	66.66
33	10	0.7	70	Na_2CO_3	-	33.25	33.24	9.54
34	10	1.2	100	NaCl	4.739	44.78	24.76±28	-
35	10	1.2	100	Na_2CO_3	16.65	40.01	28.33±16	-
36	10	0.2	40	NaCl	23.26	26.59	24.93±2.3	22.69

Fit statistics of experimental and predicted data

The model fitted to the data was observed to have a quadratic fit P-values less than 0.001 indicate model terms are significant. The following terms: mass of plant powder, time, and power, were found to be significant ($P < 0.05$) while power^2 was found to be insignificant when both the chaotrope and kosmotrope salts were applied during extraction (Fig 3. (a) and Fig 3. (b)). This indicated that the linear terms were adequate predictors of the experimental values obtained. The quadratic effect of the terms studied was found to be insignificant for Na_2CO_3 in particular, Fig. 3 (b). The linear effect of a variable indicates that the variable correlates in a directly proportional manner to the response variable (α-solanine), whereas the quadratic effect of a variable implies that the response variable is correlated with the square of that variable (Gbashi et al., 2016). In the same work, the authors highlighted that a significant linear effect of a variable ($p < 0.05$) means that the optimal level of the response falls out of the range of the experimental values for that variable, similarly, this was observed for Na_2CO_3 (Fig. 3 (b)). The F-value was observed to be 0.37 which indicated that it was not significant relative to the absolute error. The non-significant lack of fit was desirable. The goodness of fit between the experimental and the predicted values was $R^2 = 0.920$. Furthermore, the predicted R^2 of 0.7936 was in reasonable agreement with the adjusted R^2 of 0.8594; i.e., the difference was less than 0.2.

(a)



(b)

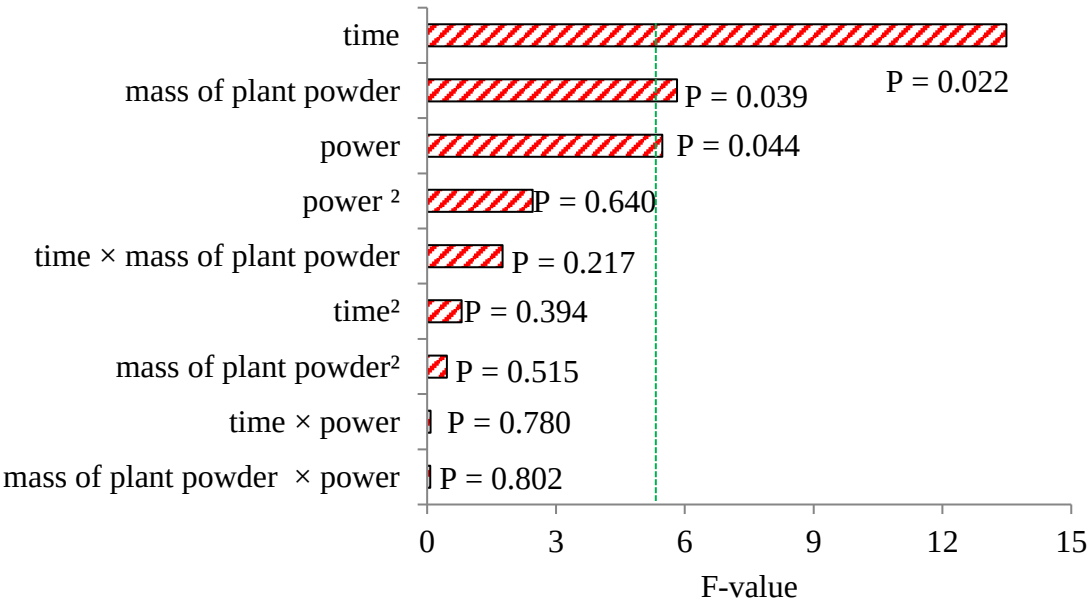


Fig.3 Pareto chart of standardized effects of time, mass of plant powder and power on the extraction α-solanine at 868→722 (a) NaCl and (b) Na_2CO_3

Box plots evaluating the effect of time, mass of plant powder and power on α-solanine extraction

In Fig. 4 and 5 are the box-and-whiskers plots of the effect of time, mass, and power on the MA-ATPE extractability of α-solanine from leaves of *Solanum retroflexum*. From these plots a proportional increase in α-solanine was observed with an increase in the mass of plant powder when NaCl and Na_2CO_3 were applied during extraction (Fig.4 (a) and 5 (a)). This indicated that the mass of the plant powder played a key role in the recovery α-solanine. The increased enrichment of α-solanine was more notable when Na_2CO_3 was used compared to NaCl. For instance, the concentration of α-solanine extracted increased from approximately 14.286 mg kg^{-1} (0.2 g) to 72.16 mg kg^{-1} (1.2 g) which equated to a five fold increase (Fig. 5 (a)). The observed enhancement in the yield of α-solanine with an increase in mass can be attributed to the increased mass transfer of metabolites from the plant matrix to the solvent when larger weights of the plant material were used (Doulabi et al., 2020). Additionally, the high α-solanine extractions was most likely due to the low solvent to mass ratio, i.e., 0.12 (m/v) when 1.2 g was used compared to 0.02 (m/v) with 0.2 g in a 10 mL mixture, which generally contributed to high microwave energy absorption of the plant material as the solvent absorbed most of the microwave energy (Doulabi et al., 2020). Conversely, increased solvent volumes have been reported to reduce the heating efficiency in microwave extraction, limit the breakage of solid cell walls, and inhibit the driving force for the mass transfer of compounds (Doulabi et al., 2020). Similarly, Alara et al. (2019) reported that larger volumes of solvent required more energy and time to maximize extraction of analytes from the plant matrix.

It was also observed from Fig. 4 (b) and 4 (c), 5 (b) and 5 (c) that an increase time and power led to a general decrease in the extraction of α-solanine. This highest extraction of α-solanine was observed at 40% microwave power, indicating that power had an influence on extraction. Microwave power was reported by Kuhnert (2002) and Khan et al. (2018) to cause superheated solvents. Additionally, an increase in microwave power resulted in the superheated extraction by water, resulting in quicker and easier penetration of solvent into the plant matrix. Furthermore, the thermal energy supplied by varying microwave power can overcome cohesive (solute – solute) and adhesive (solute–matrix) forces by reducing the activation energy needed for the desorption process, as seen at 40% power (Vergara-Salinas et al., 2013; Gbashi et al., 2016). However, at microwave powers greater than 40%, a steady decrease was observed due to excessive microwave irradiation energy degrading the α-solanine analyte (Routray and Orsat, 2012; Valdés et al. 2015). Therefore, the optimal extraction power was observed at 40%.

Moreover, according to the box-and-whiskers plots, the best extraction was generally observed at shorter times, this could probably be due to the synergistic effect of extraction time and microwave energy. A similar observation was reported by Martino et al. (2006) and Kaderides et al. (2019).

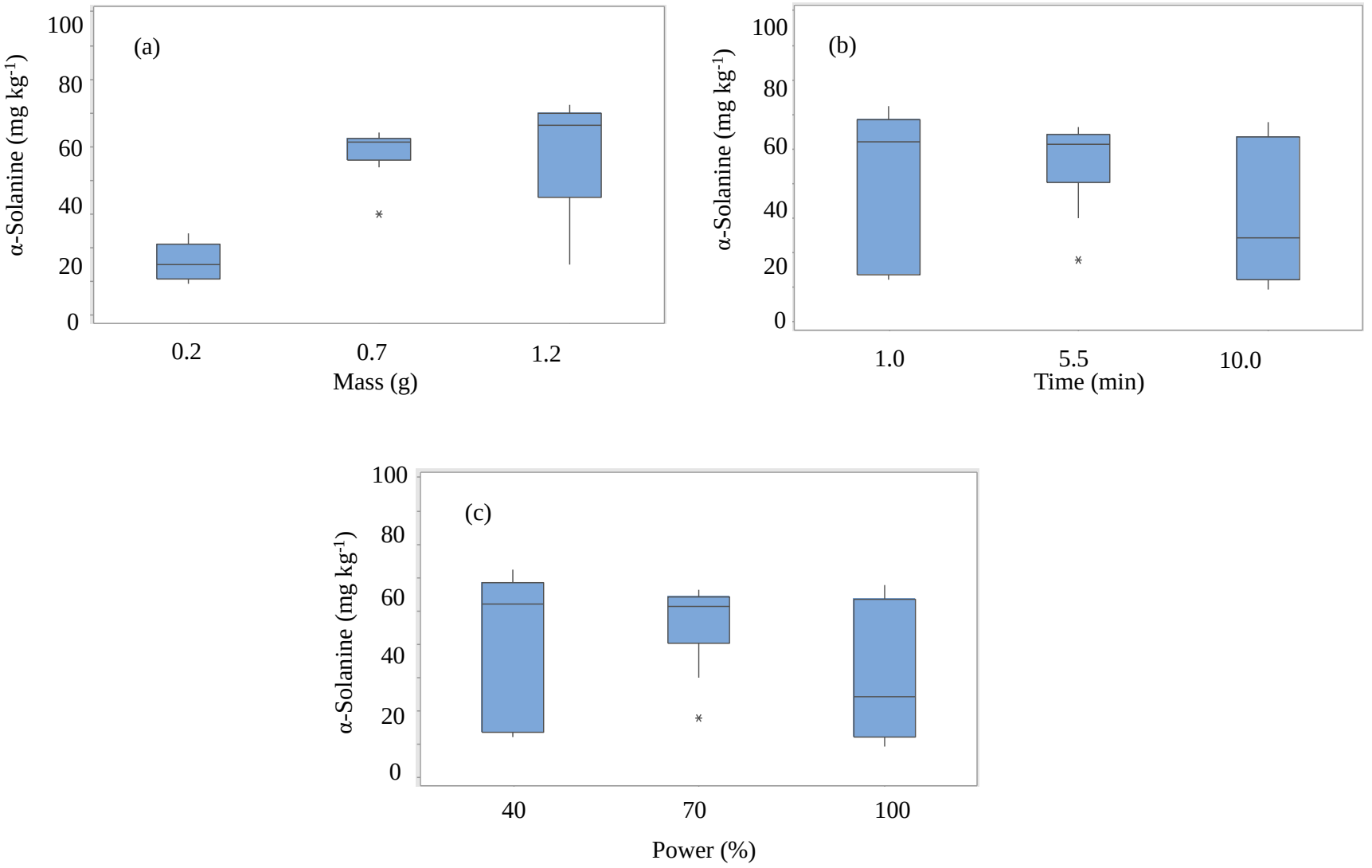


Fig. 4 *Box*-and-*whiskers* plots evaluating (a) time (b) mass (c) power on the enrichment of α-solanine when NaCl was used to aid extraction

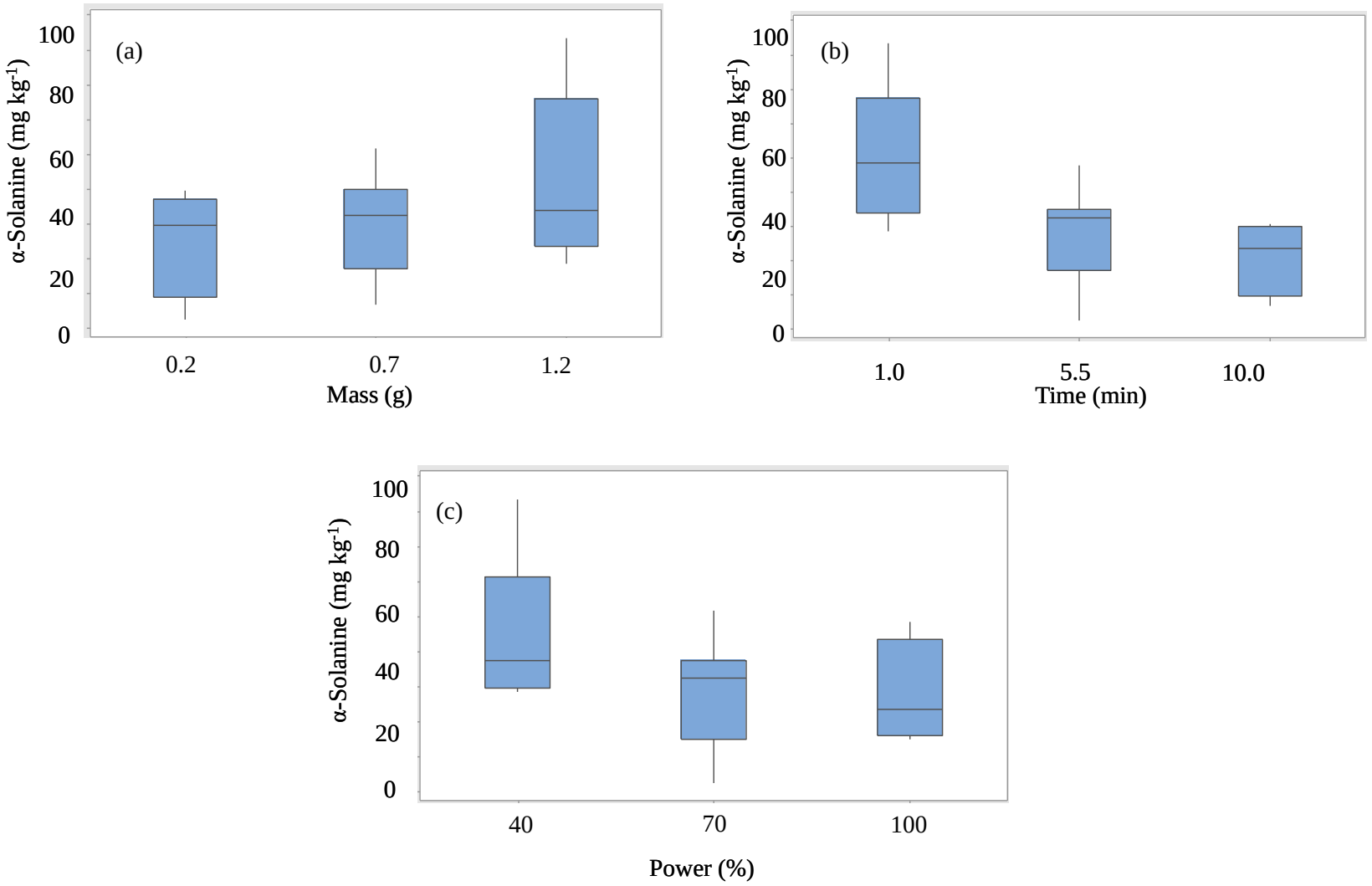


Fig. 5 *Box*-and-*whiskers* plots evaluating (a) time (b) mass (c) power on the enrichment of α-solanine when Na_2CO_3 was used to aid extraction.

Chromatographic profile of MRM based quantification of α-solanine

Chromatograms depicting the highest and lowest concentrations of α-solanine (mg kg^{-1}) obtained when NaCl and Na_2CO_3 was applied, is included in Fig. 6 (a) - (d). As seen in the chromatogram, the MRM transition of α-solanine 868 → 722 is observed at a retention time of 3.80 min (Fig. 6 (a), (b), (c) and (d)). The fragmentation profiles showing the product ions of α-solanine are also included in Fig. 6 (f). As seen in Fig. 2, α-solanine is composed of the solanidine aglycone unit glycosylated to solatriose. Solatriose is a trisaccharide composed of glucose, rhamnose and galactose monosaccharides. Of interest in this study was the 868→722 transition which was due to the loss of rhamnose at collision energy of 65 eV (Fig. 2 and Fig. 6 (f)). Other product ions of α-solanine were also observed which included *m/z* 576, 445 and 414 which were due to losses of glucose, loss of the $[Glu + H - H_2O - CO]^+$ adduct (*m/z* 131) and galactose, respectively (Kuuranne et al., 2000; Yuan et al., 2018).

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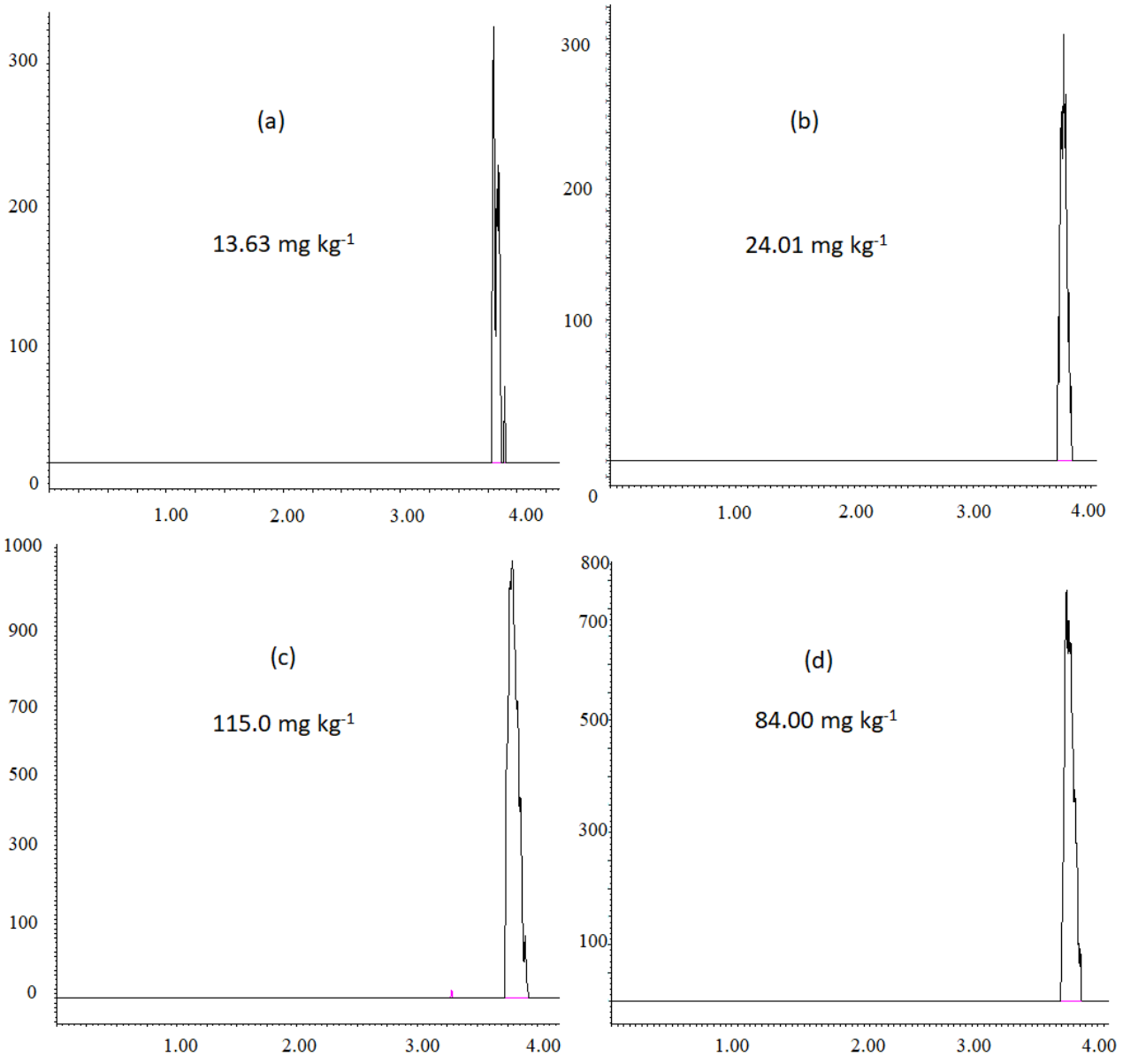

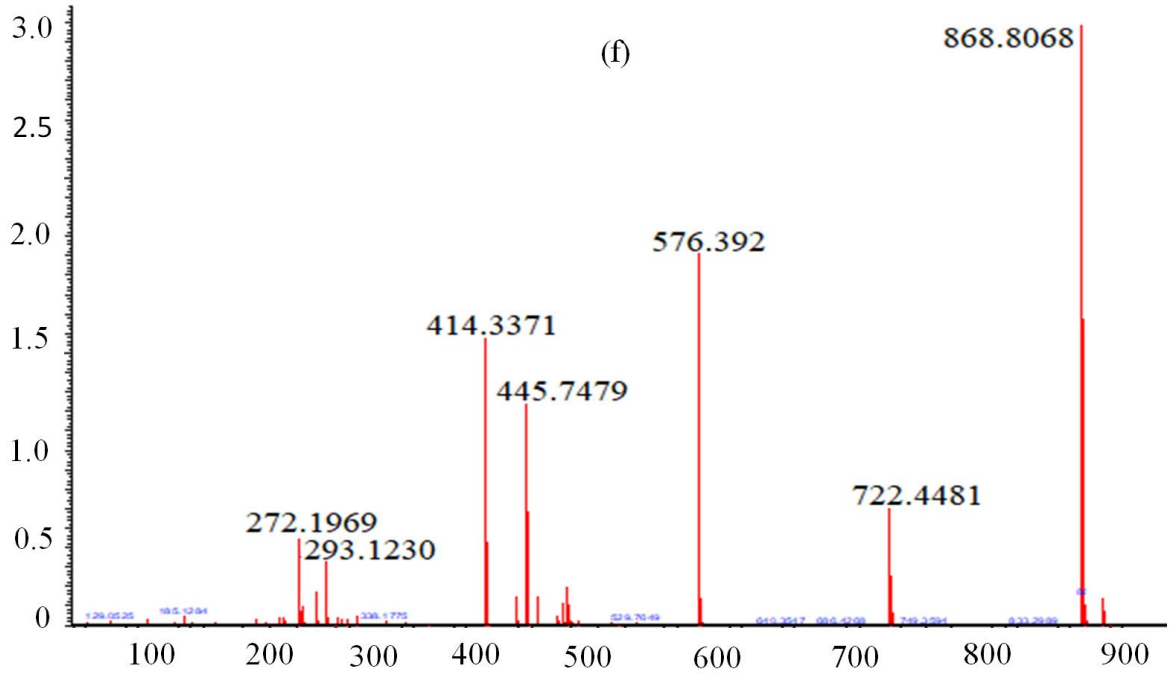

Fig. 6 Chromatogram of the lowest (a) Na_2CO_3, (b) NaCl and highest (c) Na_2CO_3, (d) NaCl concentration of α-solanine (mg kg^{-1}) and (f) mass spec of α-solanine

Response surface equations and corresponding for NaCl and Na_2CO_3 and the resultant optima

Response equations, Eqs 1 and 2, corresponding to NaCl and Na_2CO_3, respectively, and the resultant response surfaces evaluating the multivariate interaction between the mass of plant powder, power and time are shown in Fig. 7 and Fig. 8. Equations 1 – 3 and 4 - 6 represent the response surface equations for NaCl (Fig. 7 (a)- (c)) and Na_2CO_3 (Fig. 8 (a)-(c)), respectively, where A = time; B = mass of plant powder, C = power and Z = extraction yield (mg kg^{-1})

$Z = -10.5 + 197.7\ B + 0.31\ C - 77.9\ B^2 – 0.00063\ C^2 - 0.596\ BC$……...….(1)

$Z = 47.7 + 13.27\ A + 0.24\ C – 0.981\ A^2 – 0.0004\ C^2 – 0.0689\ AC$……..…..….(2)

$Z = 7.4 + 6.67\ A + 131.3\ B – 0.631\ A^2 - 48.6\ B^2 – 2.96\ AB$…….……..…..(3)

$Z = 52.1 – 7.26\ A + 75.3\ B + 0.571\ A^2 – 14.9\ B^2 – 5.17\ AB$……..(4)

$Z = 145.2 – 10.03\ A – 1.00\ C + 0.331\ A^2 + 0.0030\ C^2 + 0.0223\ AC$……..…… (5)

$Z = 98.4 + 57.3\ B – 1.64\ C – 8.9\ B^2 + 0.0101\ C^2 – 0.269\ BC$…… (6)

As the mass of plant powder was increased, in the presence of NaCl and Na_2CO_3 a proportional increase in the yield of α-solanine was obtained (Fig.7 (a) and Fig. 8 (a)). The observed increment in the concentration of α-solanine with an increase in mass can be attributed to the increased mass transfer of metabolites from the plant matrix to the extraction solvent (Doulabi et al., 2020). This concurs with observations from the pareto chart, Fig. 3 (a) and (b), which indicates the significant linear effect ($P < 0.05$) of mass of plant powder on the extraction of α-solanine. In Fig. 9 (a) and (b) the predicted optimal extraction of α-solanine in the presence of Na_2CO_3 and NaCl was 88.57 mg kg^{-1} and 73.36 mg kg^{-1}, with a desirability score of 0.804 and 0.868 respectively. The high (> 0.8) desirability score of Na_2CO_3 and NaCl indicated its closeness to the target requirement of 1, and hence the greater reliability of this optimum for maximal enrichment of α-solanine. Additionally, comparisons of the concentrations of α-solanine obtained in Table 1 and (Fig. 7 (a) – (c)) and (Fig. 8 (a) - (c)), indicated that more of α-solanine was extracted for Na_2CO_3 compared to NaCl. This suggested that, generally, α-solanine extraction was probably favoured by the presence of multiply charged ions (kosmotropes), Na_2CO_3 in this case, rather than NaCl. The doubly charged carbonate ions from Na_2CO_3, probably formed stronger hydrogen bonds with the solvation sphere surrounding α-solanine than singly charged chloride ions, enhancing the extent of its precipitation (salting-out) from the hydration sphere and its subsequent extraction by ethanol. This observation is correlated with the Hoffmeister series as narrated by Kang et al. (2020), Dogra et al. (2020) and Wang et al. (2021). Similarly, the salting-out effect was also reported by Sazali et al. (2019) and Mokgehle et al. (2021). Hence, α-solanine extraction is better achieved with the divalent Na_2CO_3 rather than monovalent NaCl.

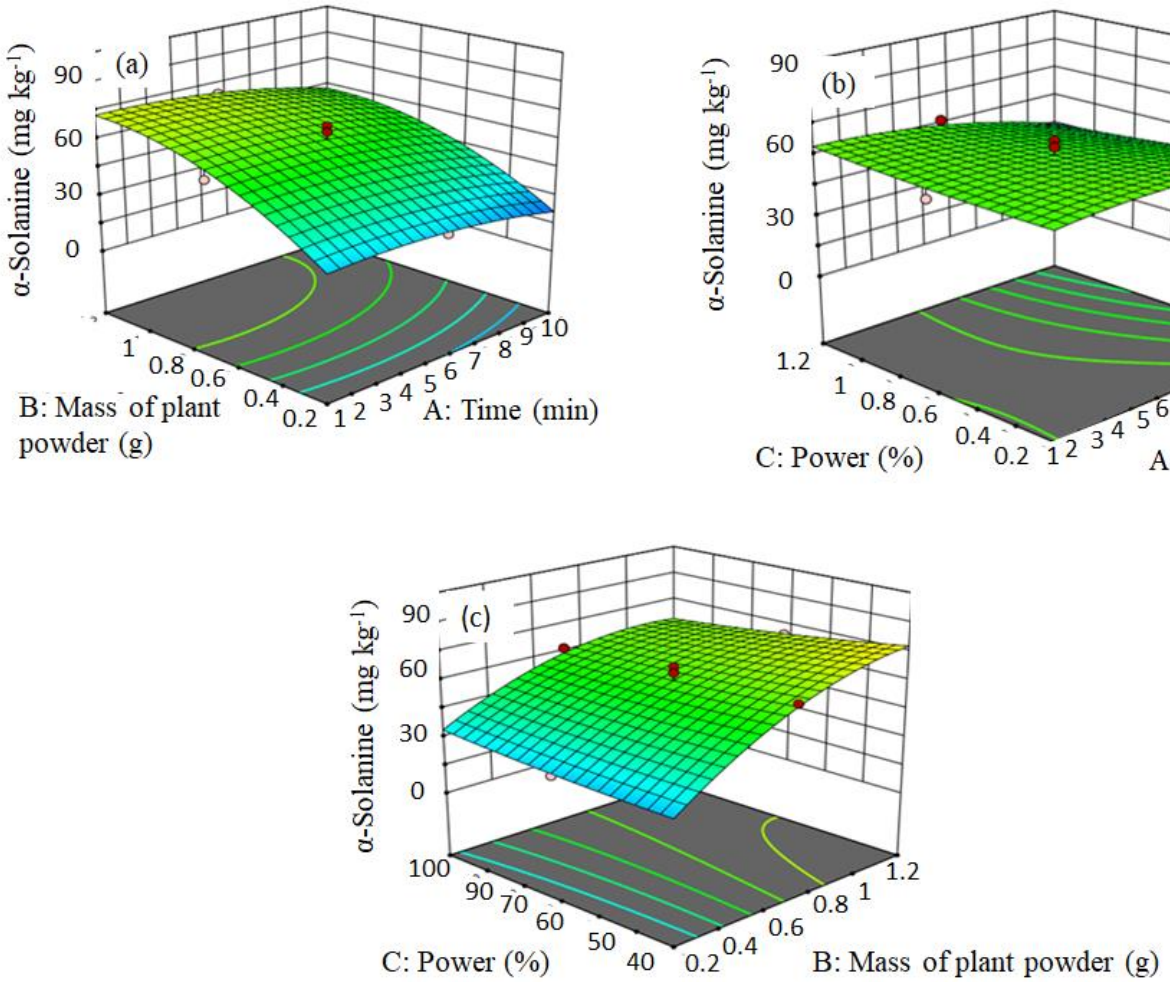

Fig. 7 Surface plots showing the interaction between the parameters studied during extraction of α-solanine in the presence of NaCl

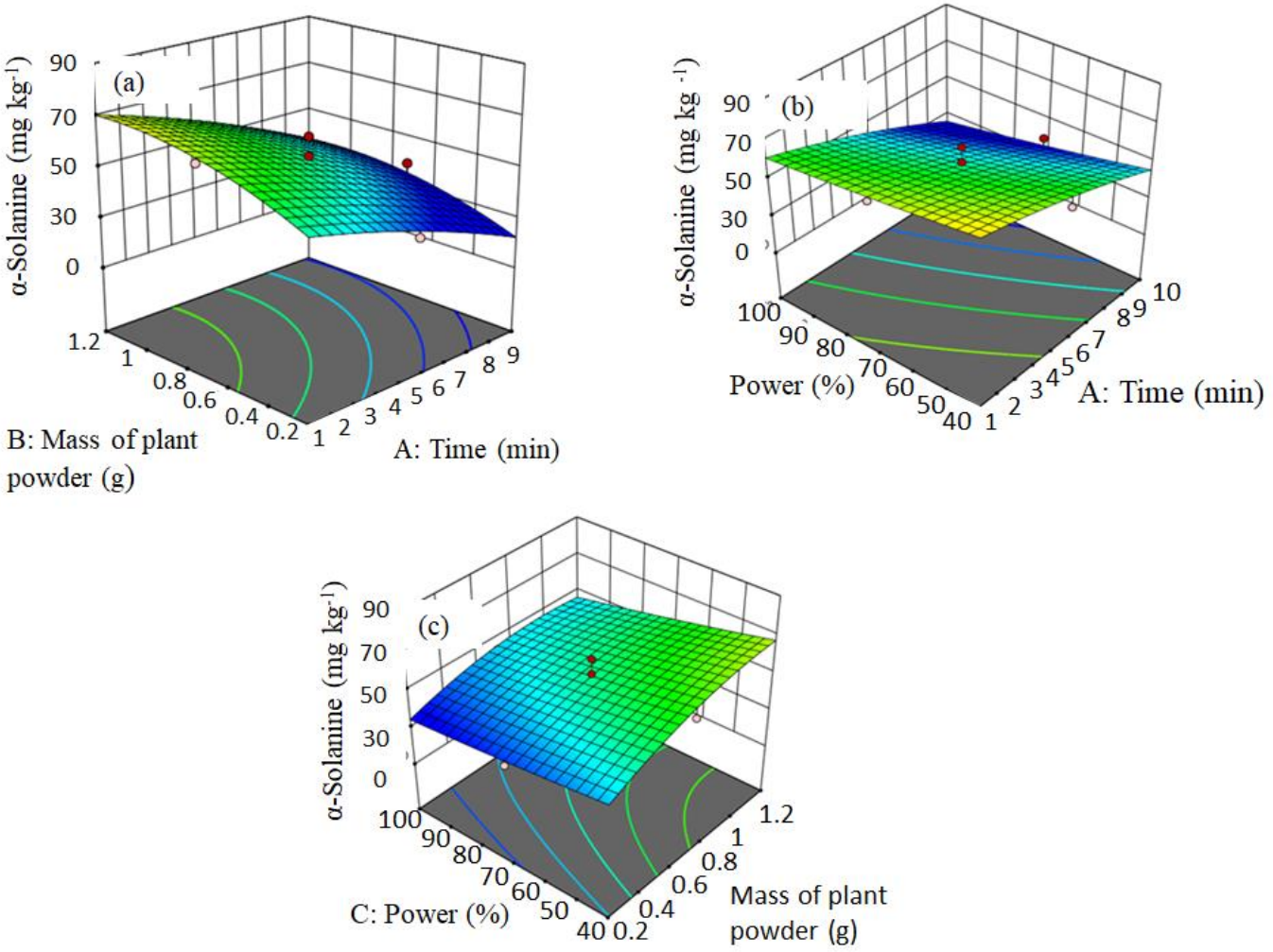

Fig. 8 Surface plots showing the AB, AC and BC on the extraction concentration of α-solanine with Na_2CO_3

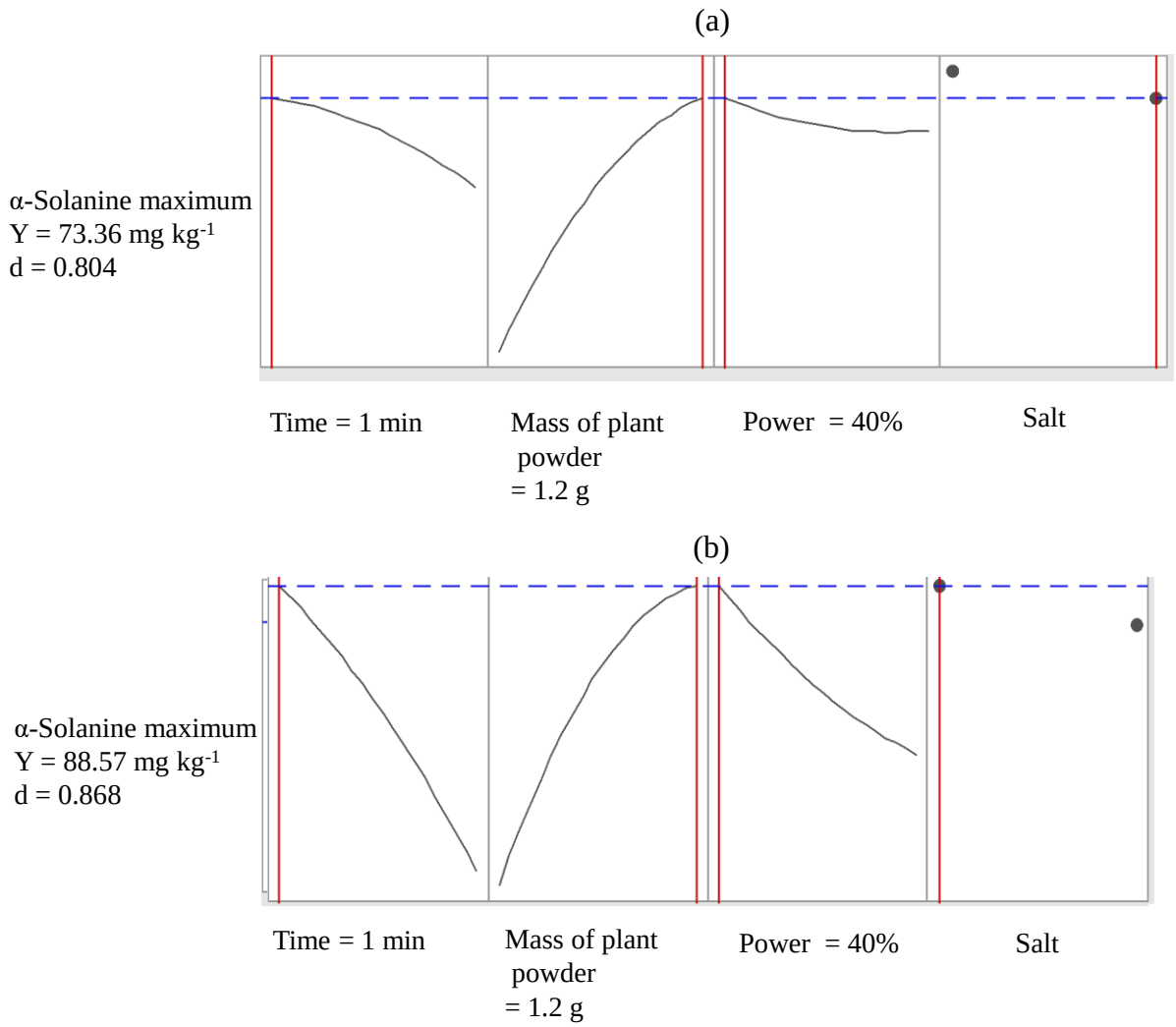

Fig. 9 Optimal conditions for extraction of α-solanine in the presence of (a) NaCl and (b) Na_2CO_3

Comparison of MA-ATPE to MAE and ATPE

Studies evaluating the extraction of glycoalkaloids involving either MAE or ATPE have been reported. For instance, Kondamaudi et al. (2017) examined MAE for obtaining glycoalkaloids from *Solanum tuberosum*. In that study, the concentrations of α-solanine extracted ranged from 15.40 – 28.12 mg kg^{-1} at an optimal extraction time of 10 minutes. In another study, Maldonado et al. (2014) examined ATPE systems for extraction of α-solanine from *Solanum tubersosum* peels and obtained concentrations of 71 mg kg^{-1}. However, this study has shown that hyphenation of microwave extraction and salting out through MA-ATPE, in the presence of kosmotrope-Na_2CO_3, can significantly reduce the extraction period (1 min) for α-solanine

while simultaneously obtaining greater concentrations (93.50 mg kg^{-1}) than what was reported by Kondamaudi et al. (2017) and Maldonado et al. (2014). In this view, MA-ATPE is an economical and efficient extraction method for α-solanine.

Conclusions

The application of MA-ATPE, a synergy of microwaves and salting-out, has demonstrated to be an energy efficient and time-saving method for enrichment of α-solanine from *Solanum retroflexum*. This is evident in the lower times (1 min) and microwave power (40%) required by MA-ATPE for maximal extraction of α-solanine. The maximal amount of α-solanine extracted was 93.50 mg kg^{-1} and 72.16 mg kg^{-1} for Na_2CO_3 and NaCl, respectively. Fitting the central composite design response surface model to the data generated a quadratic model with a good fit (R^2 = 0.92). It was statistically deduced that time and mass of plant powder had a significant effect ($p < 0.05$) on the extraction of α-solanine in MA-ATPE. The effect of microwave power was determined to be insignificant. The application of multiply charged salts such as the kosmotrope-Na_2CO_3 was shown to be a comparably better extractant of α-solanine than the chaotrope-NaCl and agrees with the Hoffmeister effect. Therefore, this cost-cutting technique, MA-ATPE, can potentially be escalated to be applied as a reliable means to minimize the concentrations of toxic compounds in other food sources.

Declarations

Acknowledgements

The authors would also like to express their gratitude to the Council for Scientific and Industrial Research studies for their technical support. The University of Venda is also thanked for financial support.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material (data transparency)

Not applicable

Code availability (software application or custom code)

Not Applicable

Contributions

TM, NT and NEM conceived the study, TM and NEM conducted the experiments and data analyses. NT, NM and WG supervised the project. WG helped to draft the manuscript. All authors read and approved the final manuscript.

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Paper 4

Optimization via CCD on the ATPE of solasodine from *Solanum mauritianum*, was investigated.

Optimization in the aqueous two phase extraction of a toxic metabolite, solasodine, from *S. mauritianum* and analysis via UHPLC-qTOF-MS

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Abstract

Aqueous two phase extraction (ATPE) was applied in the extraction of an allelochemical, solasodine, from an invasive plant, *Solanum mauritianum*. Central composite design was performed which included numerical parameters such as time and mass of plant powder. The categorical factors included the type of salt used in aiding extraction such as the chaotrope (NaCl) and kosmotrope (Na_2CO_3). Fitting the central composite design response surface model to the experimental data generated a quadratic model with a good fit (R^2 = 0.925). The linear effect of mass of plant powder was a statistically significant ($p < 0.05$) parameter for solasodine extraction. The optimized conditions for the extraction of solasodine in the presence of NaCl or Na_2CO_3 were time: 10 min and mass of plant powder: 1.2 g. Corresponding to these conditions, the maximal mean extraction based on multiple reaction monitoring (MRM) transition of solasodine (m/z 414 → 396) on the UHPLC-qTOF-MS was 233.65 mg kg^{-1} and 413.50 mg kg^{-1} for NaCl and Na_2CO_3, respectively. The greater extraction ability of the kosmotrope was due to the higher negative charge density of the carbonate ion during salting-out. Furthermore, the synergistic effect of mass of plant powder and salting-out was shown to enhance extraction of solasodine compared to the chaotrope. The kosmotrope assisted solasodine ATPE extracts from *Solanum mauritianum*, can potentially be applied as

antipathogenic agents in medicine while simultaneously limiting the allelopathic impact of *Solanum mauritianum.*

Keywords: Response surface methodology, solasodine, optimization, central composite design, multiple reaction monitoring

Solanum mauritianum Scopoli (Solanaceae), also known as the Bugweed, Tobacco tree or Woolly Nightshade, is an invasive tree of global significance. As an invasive alien plant species in South Africa, for more than a century, it has shown to have harmful impacts on organic matter content and on ecosystem services, thus degrading the lands productive potential (Lottering et al. 2020). The source of the devastating impact of *Solanum mauritianum* to its surroundings is its toxic metabolic composition. *Solanum mauritianum* produces toxic alkaloid allelochemicals that alter biogeochemical cycles, which constrain the growth rates of surrounding forest vegetation (Chornesky et al. 2005; Lottering et al. 2020) Despite the poisonous metabolic composition of *Solanum mauritianum*, tribes however, have used the plant to cure skin borne disorders (Jayakumar et al. 2017). A variety of *Solanum mauritianum* alkaloid secondary metabolites were isolated from herbals and were reported to exhibit antiproliferation and antimetastasis effects on diverse types of cancers both under in vitro and in vivo conditions (Jayakumar et al. 2017).

One class of alkaloid secondary metabolites are steroidal alkaloids (SA), which are nitrogen containing compounds prevalent in potatoes (*Solanum tuberosum*), tomatoes (*Solanum lycopersicum*) and eggplants (*Solanum melongena*). These metabolites include glycoalkaloids (containing mono/polysacharrides) and its hydrolysis products aglycones (without sugar moieties) (Mokgehle et al. 2021). In solanaceous plants, solasonine and solamargine are the most important glycoalkaloids of which the principal aglycone is solasodine (Neves et al. 2012). As a result, solasodine has been employed as a steroidal precursor within the steroid drug trade for the manufacturing of corticosteroids and antifertility drugs (Kumar et al. 2019. Solasodine which is an aglycone unit of glycoalkaloids such as solasonine and solamargine has been reported to play an important role in the apoptosis of cancer cells (Kumar et al. 2019; Bhattacharya et al. 2013; Jayakumar et al. 2016; Jayakumar et al. 2017). Solasodine has been reported to rupture lysosomes, resulting in lysosomal contents (mainly hydrolytic enzymes) being released into the cytosol causing apoptosis of cancer cells. Solasodine based rhamnosyl glycosides in combination with cisplatin proved more effective against cisplatin-resistant tumour cells, including lung and breast cancer cells (Cham et al. 2008; Jayakumar et al. 2017).

Despite the useful properties of solasodine, it remains a highly toxic compound. For instance, glycoalkaloids containing the solasodine backbone were reported for reducing larval growth of the red flour beetle *Tribolium castaneum* (Weissenberg et al., 1998; Ventrella et al., 2016). Furthermore, according to Smith et al. (2008) a 52-year-old woman who consumed *Solanum torvum* berries experienced vomiting, diarrhoea, blurry vision, ataxia, slurred speech, ptosis, muscle fasciculations, diaphoresis, dyspnea and urinary incontinence. Another study by Glover et al. (2016) reported on a case of poisoning of a 54-year-old woman after intake of a *Solanum torvum*. In both cases glycoalkaloids containing solasodine were responsible for poisoning.

Considering the toxic nature of solasodine, this project was directed at optimizing the aqueous two phase extraction (ATPE) of solasodine from the weed, *Solanum mauritianum*. A multivariate optimization approach based on central composite design (CCD) was performed and the responses viewed via response surface methodology (RSM). The CCD and RSM approach are useful as it reduces the number of optimization experiments, making it less laborious and time efficient (Silva et al. 2019). Two variables were investigated which included extraction time and mass of plant powder. Quantification was based on scheduled multiple reaction monitoring (MRM) on the ultra high performance liquid chromatography time of flight mass spectrometer (UHPLC-qTOF-MS), where an m/z transition, from the precursor ion to the product ion, for solasodine (414→396) was explored. Pareto charts were also examined to determine which of the parameters had the greatest influence on extraction of solasodine.

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Experimental

Chemicals and reagents

The salts NaCl (anhydrous ≥ 99% purity), Na_2CO_3 (anhydrous ≥ 99% purity) and ethanol (99% CP) were purchased from Associated Chemical Enterprises (Johannesburg, South Africa) and Sigma-Aldrich (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) using a Direct-Q 5UV distiller (Massachusetts, United States of America) was applied for the preparation of the salt solutions. The extraction was performed on a DIAB MX-RL-Pro dragon shaker. Chromatographic separation of the metabolites in the extracts was done using a reverse phase Shim-pack Velox C_{18}, 2.1 x 100 mm, 2.7 µm with a serial number 227-32009-03 (Columbia, USA). The UPLC was connected to a Shimadzu 9030 LC, qTOF-MS detector (Shimadzu, Kyoto). The solvents used for the chromatographic runs were methanol and formic acid, which were purchased from Romil Pure Chemistry (Cambridge, UK).

Sample collection, preparation and ATPE

The leaves of *S. mauritianum* were obtained from a street vendor within the Thulamela District in Thohoyandou, South Africa. The plants were air dried until a constant weight was obtained, and the leaves were ground into a fine powder with a blender at 2000 rpm and stored in glass containers. The containers were covered in paper bags to prevent light penetration. The powdered leaves were placed in a 250 mL in a centrifuge tube (50 mL). Thereafter, saturated salt concentrations (20 mL) of 30% (w/v) involving Na_2CO_3 (kosmotrope) and NaCl (chaotrope) which were prepared by weighing 15 g of salt in 50 mL of water, were added to the powdered leaves contained in the centrifuge tube (50 mL). The aqueous solution was placed over the dragon shaker for (1 - 10 min) and the range of the mass of plant powder studied included (0.2 - 1.2 g). Thereafter, 99.9% ethanol extraction solvent (20 mL) was added and resulted in an ATPE system. Following this, the extracts obtained were then analysed on a UPLC-QTOF-MS for detection of solasodine extracted.

Chromatographic and mass spectrometry conditions

Solasodine were separated using a Shimpack C_{18}, 2.1 x 100 mm, 2.7 µm column from Shimadzu (Honeydew, South Africa). The column was maintained at 40 ºC at a flow rate of 0.4 mL min^{-1} and the injection volume was 5 µL. Mobile phase A was 0.1% formic acid in ultrahigh purity water (v/v) and mobile phase B was 0.1% (v/v) formic acid in methanol.

An UPLC-QTOF-MS 9030 mass spectrometer (Shimadzu, Japan) was used for all mass spectral measurements. The mass spectrometer was equipped with an electrospray interface (ESI) operating in positive mode. ESI parameters were optimized for solasodine by direct infusion of standard solutions into the mass spectrometer. The mass spectrometer was operated in the multi reaction monitoring (MRM) mode to confirm the identity of solasodine. This was achieved by selecting specific precursor to product ion transitions for each solasodine based on MRM transitions. High-purity nitrogen (N_2) was used as the nebulizing and drying gas. The optimum parameters were as follows: drying gas temperature, 250 °C; drying gas flow, 10 L min^{-1} and collision energy, 30 - 60V. For chromatographic separation, a Shimadzu 9030 LC instrument (Shimadzu, Japan) was used. The instrument consisted of an autosampler, thermostated column compartment and a binary pump. Lab solutions software was used to control the LC-MS/MS instrument and for data acquisition and the mass range was *m/z* 100-1000.

Preparation of standards

The stock standard solution was prepared in methanol at a concentration of 1000 µg L^{-1}. The stock standard solution was stored at 4°C in amber volumetric flasks. A series of nine working standard solutions at the concentration values of 15 to 1000 µg L^{-1} were prepared from the stock standard solution by diluting with HPLC grade methanol. The solanine standards were quantified based on scheduled multiple reaction monitoring (MRM) where one *m/z* transition, from the precursor ion to the product ion, for solanine (414 → 396) was explored. The regression equation was y = 537.484x + 41.893, limit of detection (LOD) and limit of quantification (LOQ) were 0.078 and 0.236, respectively. The above mentioned transition was then applied for quantification of solasodine from the ground leaves of *Solanum mauritianum* following ATPE extraction. The parameters evaluated for optimization of solasodine from ATPE were time, mass of plant powder and the salt type (kosmotrope or chaotrope).

Statistical analysis

The central composite design response surface model (CCD RSM) was fitted to experimental data to obtain the relationship between factors and optimize the response of Z (solasodine yield) in relation to A (time), B (mass of plant powder) using Minitab 17 (UK). A Two-level full factorial CCD was designed, a total of 26 experimental runs (including 2 repetitions) were designed. This included numerical factors such as time (1 min, 5 min 30 sec and 10 min), mass of plant powder (0.2, 0.7, 1.2 g) and 2 categorical factor levels for salts which included the chaotrope (NaCl) and kosmotrope (Na_2CO_3).

Model parameters and model significance were determined at $p < 0.05$. The fitness of the model was determined by evaluating the coefficient of regression (R^2) obtained from the analysis of variance (ANOVA). The model fit generated the response surface that defined the behaviour of the response variable. By means of these plots, the optimized ranges for each factor that led to the highest response (i.e concentration of solasodine) that can be extracted.

The interaction between the various parameters studied and its resultant effected on the extraction of solanine (mg kg^{-1}) was fitted to experimental data by using a statistical multiple regression approach method of least square (MLS), and resulted in the lowest possible residual (Bas et al. 2007). Model parameters and model significance were determined at $p < 0.05$. The fitness of the model was determined by evaluating the coefficient of regression (R^2) obtained from the analysis of variance (ANOVA). The model fit generated the response surface that

defined the behaviour of the response variable. By means of these plots, the optimized ranges for each factor that led to the highest response (i.e concentration of solanine) that can be extracted (Bas et al. 2007; Arteaga-Crespo et al. 2020).

Results and discussion

MRM quantification of solasodine based on the 414→396 transition

Solanum mauritianum, an invasive species, is an abundant source of anti-cancer and antifungal metabolites such as solasodine glycosides (Jayakumar et al. 2016; Jayakumar et al. 2017). In this study, we demonstrated the extraction of an aglycone unit of solasodine glycosides, solasodine. from *Solanum mauritianum*, using ATPE. The ATPE technique was performed by evaluating the different factors shown in Table 1 on the recovery of solasodine. The presence of solasodine was reported in *Solanum mauritianum* and other species within the *Solanum* genus (Bhattacharya et al. 2013; Jayakumar et al. 2016; Jayakumar et al. 2017). Using a sensitive and robust tandem MS approach (UHPLC-qTOF-MS) with settings presented (Mokgehle et al. 2021) it was possible to efficiently fingerprint these solasodine based on *m/z* 396 product ion (Fig. 1). Thereafter, based on the 414 → 396 transition within the MRM method, solasodine was quantified as shown in Table 1.

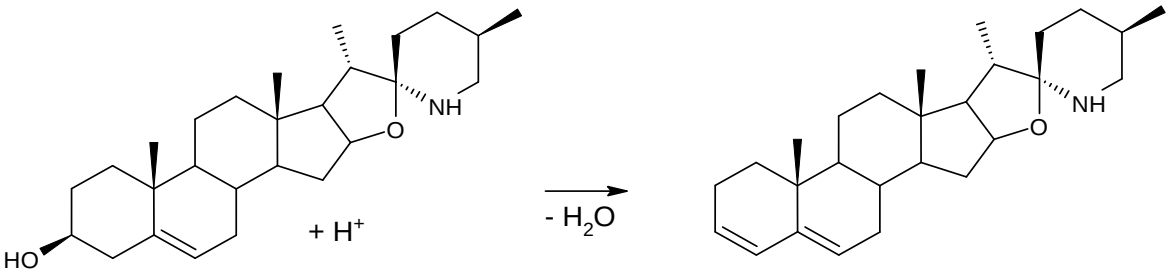


Fig. 1 Molecular transition of solasodine (*m/z* = 414) to [solasodine – H_2O] (*m/z* = 396) after the loss of water

Table 1 List of experiments using CCD for ATPE optimization, the response and predicted values.

Run	Factor 1: Time (min)	Factor 2: mass of plant powder (g)	Factor 3 Salt type	Solasodine (mg kg^{-1}) Run 1	Run 2	Mean ± SD	Predicted
1	1	0.2	Na_2CO_3	142.17	109.16	125.66+23	143.14
2	1	0.7	NaCl	287.55	286.09	286.82+1	242.66
3	1	1.2	Na_2CO_3	308.69	309.69	309.19+1	309.60
4	1	0.7	Na_2CO_3	232.21	300.07	266.14+48	248.24
5	1	1.2	NaCl	217.81	198.46	208.14+14	236.85
6	1	0.2	NaCl	137.62	158.38	148.00+15	163.42
7	5.5	0.7	NaCl	226.82	196.81	211.82+18	225.47
8	5.5	0.7	Na_2CO3	173.56	221.42	197.49+34	215.87
9	5.5	0.7	Na_2CO_3	206.84	172.81	189.83+24	215.87
10	5.5	0.7	NaCl	222.62	208.09	215.35+10	225.47
11	5.5	0.7	Na_2CO_3	196.11	261.55	228.83+46	215.87
12	5.5	1.2	Na_2CO_3	277.18	297.24	287.21+14	308.14
13	5.5	0.7	Na_2CO_3	273.98	251.05	262.52+16	215.87
14	5.5	0.7	NaCl	238.13	223.28	230.71+11	225.47
15	5.5	1.2	NaCl	265.45	288.52	276.99+16	240.13
16	5.5	0.7	Na_2CO_3	188.79	228.15	208.47+28	215.87
17	5.5	0.7	NaCl	209.45	193.59	201.52+11	225.47
18	5.5	0.2	NaCl	137.55	135.20	136.38+2	125.75
19	5.5	0.2	Na_2CO_3	106.62	79.50	93.06+19	79.875
20	5.5	0.7	NaCl	217.94	222.31	220.13+3	225.47
21	10	0.2	Na_2CO_3	104.28	60.35	82.32+31	78.02
22	10	1.2	Na_2CO_3	402.78	424.22	413.50+15	368.10
23	10	0.2	NaCl	88.87	94.34	91.60+4	86.79
24	10	0.7	Na_2CO_3	226.53	212.07	219.30+10	244.93
25	10	0.7	NaCl	221.03	200.30	210.66+15	206.99
26	10	1.2	NaCl	275.69	191.62	233.65+59	242.13

Fit statistics of experimental and predicted data

The model fitted to the data was observed to have a quadratic fit P-values less than 0.001 indicate model terms are significant. In this case mass of plant powder, mass of plant $powder^2$ (when NaCl was applied) were significant ($p < 0.05$) model terms and were adequate predictors of the experimental values obtained. The other terms such as time × mass of plant powder, $time^2$ and time were insignificant ($p > 0.05$). The lack of fit of F-value was observed to be 1.70 which indicated that the lack of fit was not significant relative to the pure error. The non-

significant lack of fit was desirable. The goodness of fit between the experimental and the predicted values was $R^2 = 0.925$

The Pareto chart of parameter main effects and their interactions produced from ANOVA and resultant box plots

In Fig. 2 (a) and (b) are the pareto charts showing the influences of the parameters time, mass of plant powder, the square of each model and the product of the two parameters. The model term mass of plant powder was shown to be statistically significant ($p < 0.05$) when both NaCl and Na_2CO_3 were applied, showing a linear effect on the yield of solasodine (Fig. 2 (a) and (b). In Fig. 3 are the box-and-whiskers plots of the effect of time and mass of plant powder on the ATPE extraction of solasodine from leaves of *Solanum mauritianum.* From these plots a proportional increase in solasodine was observed with an increase in the mass of plant powder when NaCl and Na_2CO_3 were applied during extraction (Fig. 3 (a) and (b)). This indicated that the mass of the plant powder played a key role in the recovery solasodine. The increased enrichment of solasodine was more notable when Na_2CO_3 was used compared to NaCl. For instance, the extracted solasodine increased from approximately 85 mg kg^{-1} (0.2 g) to 383 mg kg^{-1} (1.2 g) which equated to almost a five fold increase for Na_2CO_3. The observed increment in the yield of solasodine with increase in mass can be attributed to the increased mass transfer of metabolites from the plant matrix to the solvent when larger weights of the plant material were used. This observation agrees with what was reported by Doulabi et al (2020).

It was also observed from Fig. 3 (c) and (d) that an increase time generally had no impact on the extraction of solasodine. For instance, the median for extraction of solasodine settled at approximately 223 mg kg^{-1} as time was increased from 1 to 10 min, when NaCl was applied as to aid extraction, Fig. 3 (c). This correlates with the level of significance values for the linear effect of time when both NaCl and Na_2CO_3 were applied which indicated that time was statistically insignificant ($p > 0.05$) on the extraction of solasodine, as shown in Fig. 2 (a) and (b). Factors involving the quadratic effect of time as well as the product of time and mass of plant powder were also insignificant on the extraction of solasodine from *Solanum mauritianum.* Similar observations of the insignificance of time for extraction of metabolites based on a response surface methodology were reported by Pandey et al. (2018).

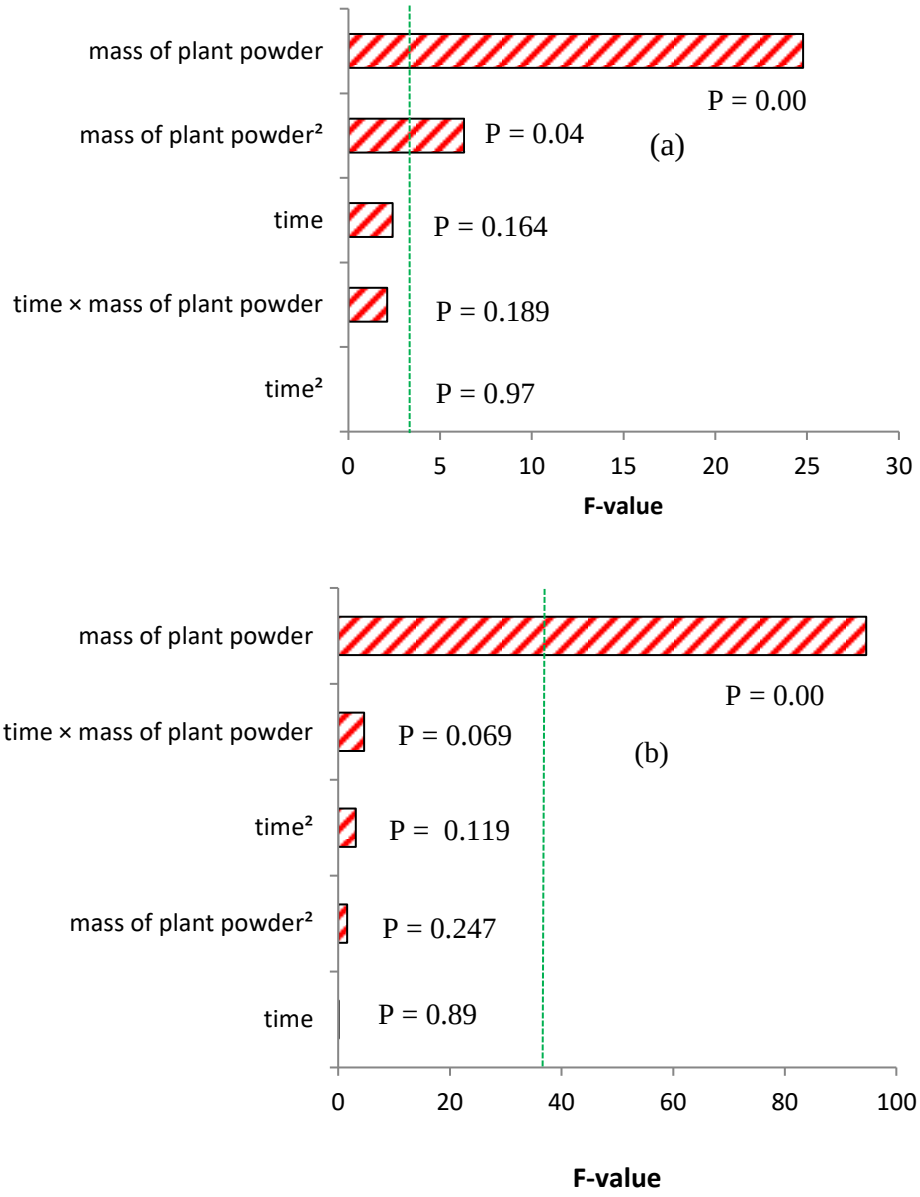
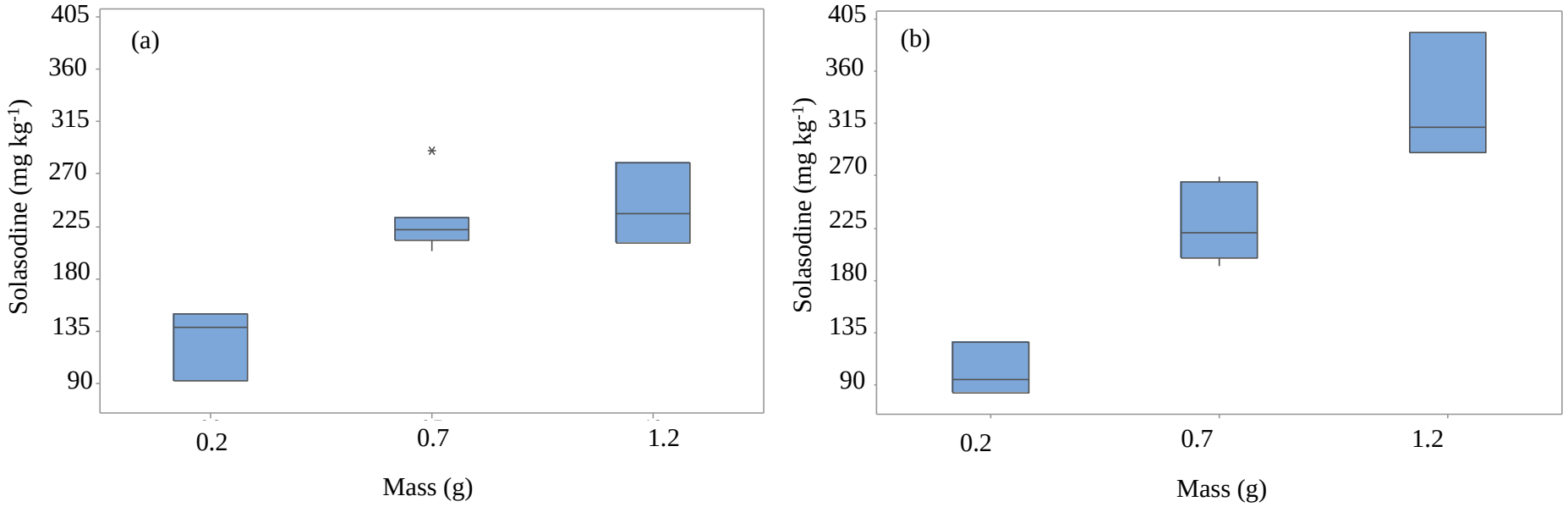


Fig. 2 Pareto chart of standardized effects of time, mass of plant powder and power on the extraction solasodine at 414 → 396 (a) NaCl and (b) Na_2CO_3



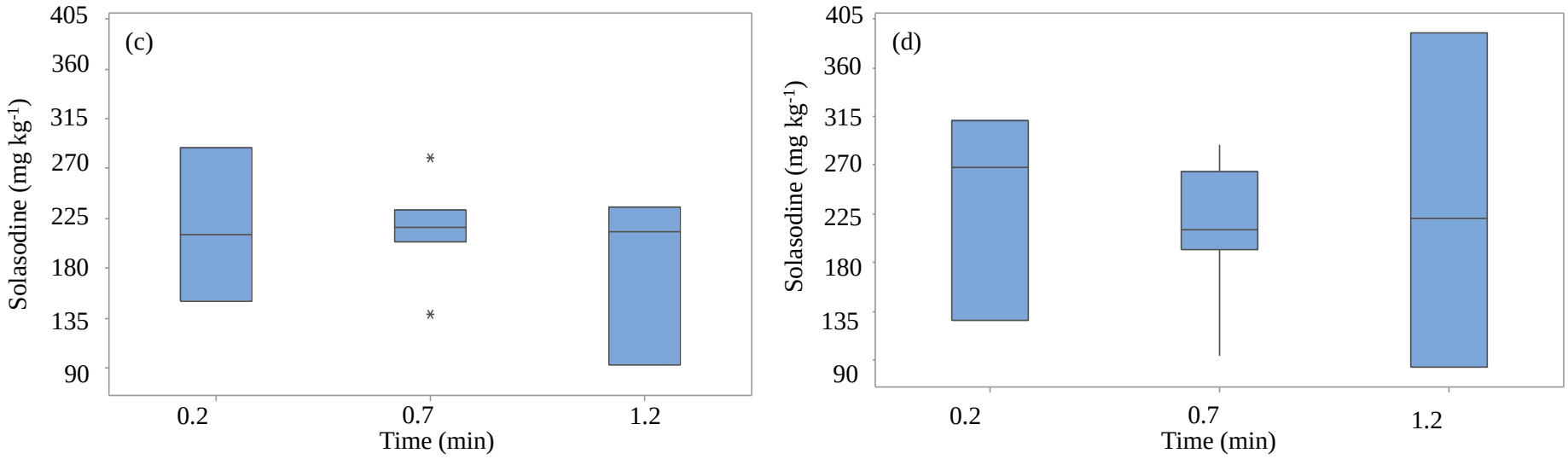
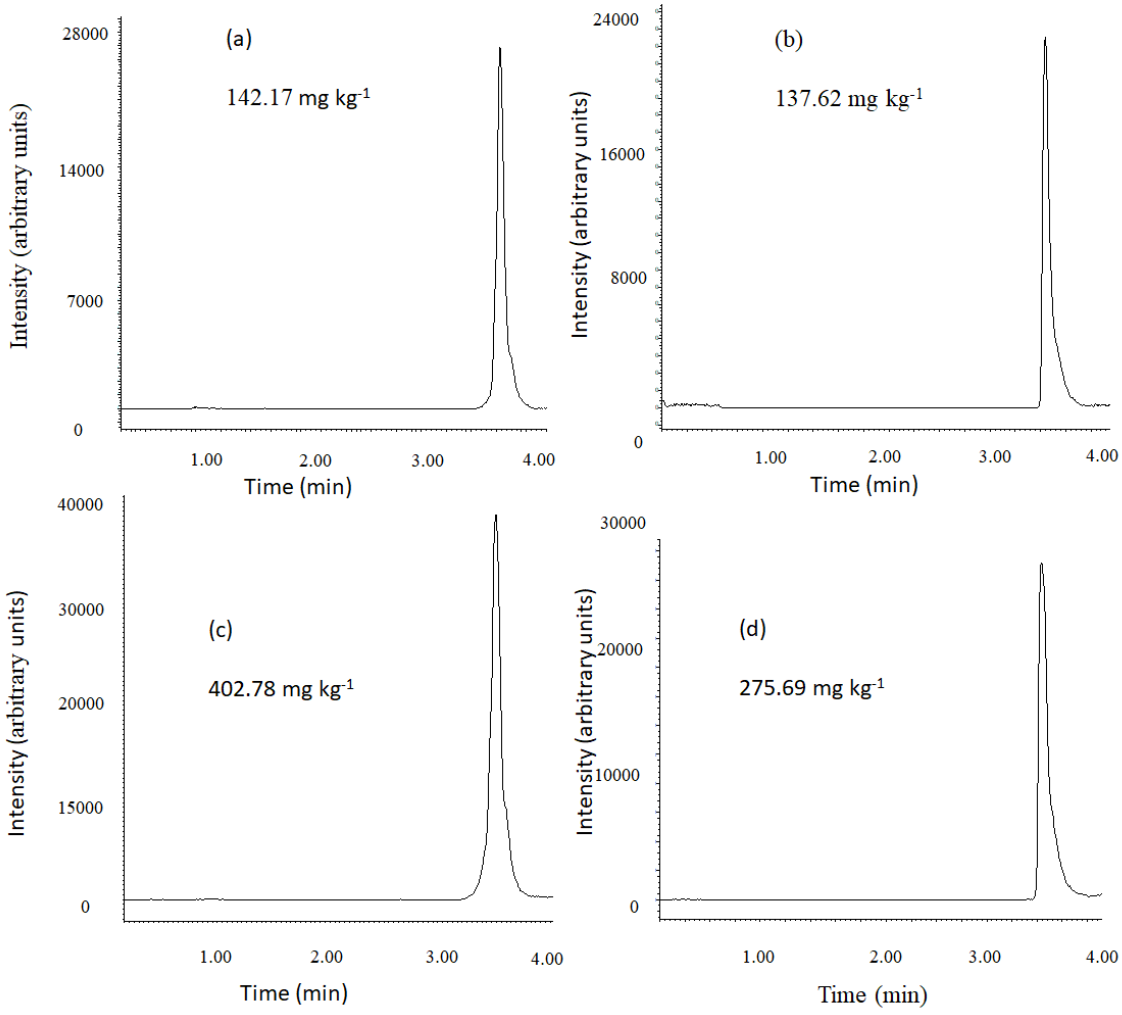


Fig. 3 *Box*-and-*whiskers* plots evaluating the effect of mass of plant powder on (a) NaCl (b) Na_2CO_3 and the effect of time on (c) NaCl and (d) Na_2CO_3 on the extraction of solasodine from leaves of *Solanum mauritianum*

Chromatographic profile of MRM based quantification of solasodine

Chromatograms depicting the highest and lowest concentrations of solasodine (mg kg^{-1}) obtained when NaCl and Na_2CO_3 was applied are shown in Fig. 4 (a), (b), (c) and (d). The chromatogram indicates an MRM transition of solasodine *m/z* 414 → 396 and is observed at a retention time of 3.825 min. The fragmentation profile showing the product ions of solasodine are also included in Fig. 4 (f). As seen in Fig. 1, solasodine underwent a dehydration reaction from the precursor ion *m/z* 414 to the product ion *m/z* 396. From the experimental results dehydration of solasodine seems to be more prominent at extraction parameters involving longer times in combination with larger masses of plant powder. Under such conditions, solasodine concentrations of 402.78 and 275.69 mg kg^{-1} for Na_2CO_3 and NaCl were obtained, respectively, as shown in Fig. 4 (c) and (d). This is also in agreement with the significant effect of mass of plant powder, as seen in the pareto charts in Fig. 2 (a) and 2(b), and the box plots in Fig. 3.



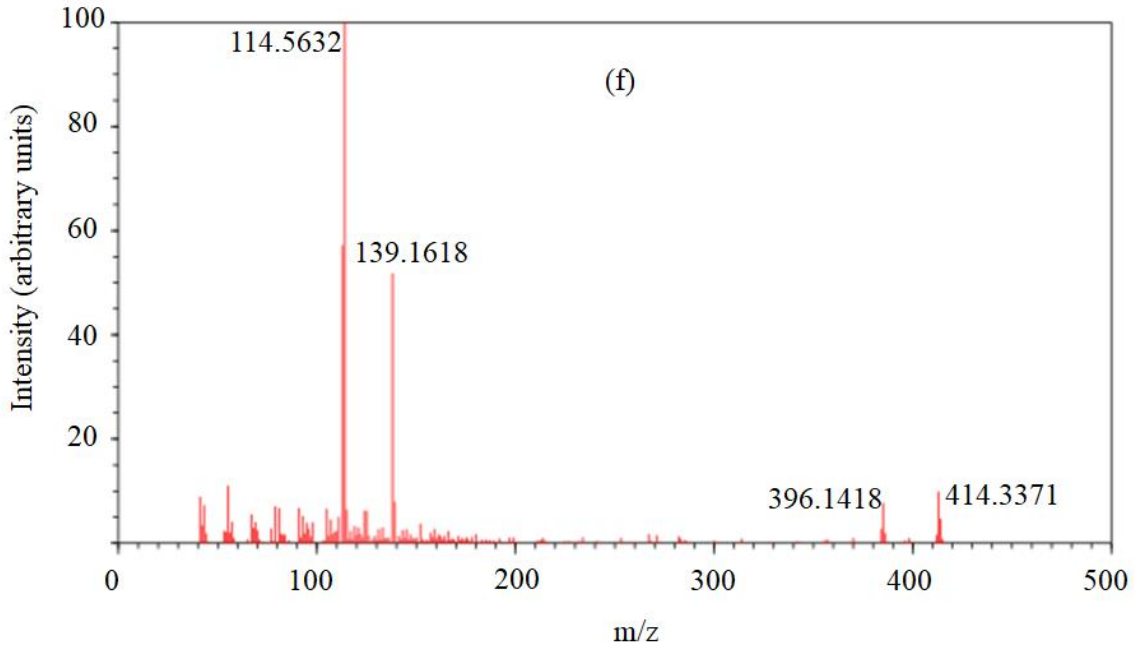


Fig. 4 Chromatogram of the lowest (a) Na_2CO_3 and (b) NaCl, and highest (c) Na_2CO_3 and (d) extraction yields of solasodine for run 1. (f) mass spec of solasodine

Response surface equations and corresponding for NaCl and Na_2CO_3 and the resultant optima

Response equations, Eqs 1 and 2, corresponding to NaCl and Na_2CO_3, respectively, and the resultant response surfaces evaluating the bivariate interaction between the time and mass of plant are shown in Fig. 5. In this case, Z was the dependent variable (solasodine concentration) and x (time) and y (mass of plant powder) the independent variables. From the quadratic fit of $r^2 = 0.925$, as reported earlier, Eqs 1 and 2 were obtained

$$z(x,y) = 132.09 - 11.18\,x + 338.74\,y - 0.035\,x^2 - 190.50\,y^2 + 10.19\,xy \quad \text{.....(1)}$$

$$z(x,y) = 127.68 - 29.86\,x + 308.22\,y + 1.69\,x^2 - 97.97\,y^2 + 15.38\,xy \quad \text{.....(2)}$$

As the mass of plant powder was increased, in the presence of NaCl and Na_2CO_3 a proportional increase in the yield of solasodine was obtained (Fig. 5 (a) and (b)). This concurs with observations from pareto chart, Fig. 3 (a) and (b), which indicates the significant linear effect ($P < 0.05$) of mass of plant powder. In Fig. 6 (a) and (b) the predicted optimal extraction of solasodine in the presence of NaCl and Na_2CO_3 was 261.75 mg kg^{-1} and 347.32 mg kg^{-1}, with a desirability score of 0.58 and 0.86 respectively. The higher desirability score of Na_2CO_3 compared to NaCl indicated its closeness to the target requirement of 1, and hence the greater

reliability of this optimum for maximal enrichment of solasodine. Additionally, comparisons of the concentrations of solasodine obtained in Table 1 and Fig. 6, indicated that more of solasodine was extracted for Na_2CO_3 compared to NaCl. This suggested that solasodine extraction during ATPE was probably favored by the presence of the multiply charged carbonate ion from Na_2CO_3 compared to the singly charged chloride ion in NaCl. The doubly charged carbonate ions from Na_2CO_3, probably formed strong hydrogen bonds with the water molecules surrounding the solasodine, weakening the solasodine-water interaction, and enhancing the extent of solasodine precipitation (salting-out) from the hydration sphere. The salted-out solasodine was subsequently extracted by ethanol into the organic phase. Hence, extraction of solasodine during ATPE occurred through the process of salting out. Furthermore, the extent of salting out was further driven by the increase in mass of plant powder due to mass transfer under the conditions studied. The salting-out effect in ATPE was also reported by Sazali et al. (2019) and Mokgehle et al. (2021). Additionally, the higher extraction of solasodine via Na_2CO_3 compared to NaCl can correlates with the Hoffmeister series. Larger multivalent anions have a propensity for salting out proteins than smaller monovalent anions (Kang et al., 2020; Dogra et al., 2020; Wang et al., 2021). Similarly, the Hoffmeister principle was evident in this study as the multivalent carbonate ion precipitated greater concentrations of solasodine under the optimized ATPE conditions of time: 10 min and mass of plant powder: 1.2 g.

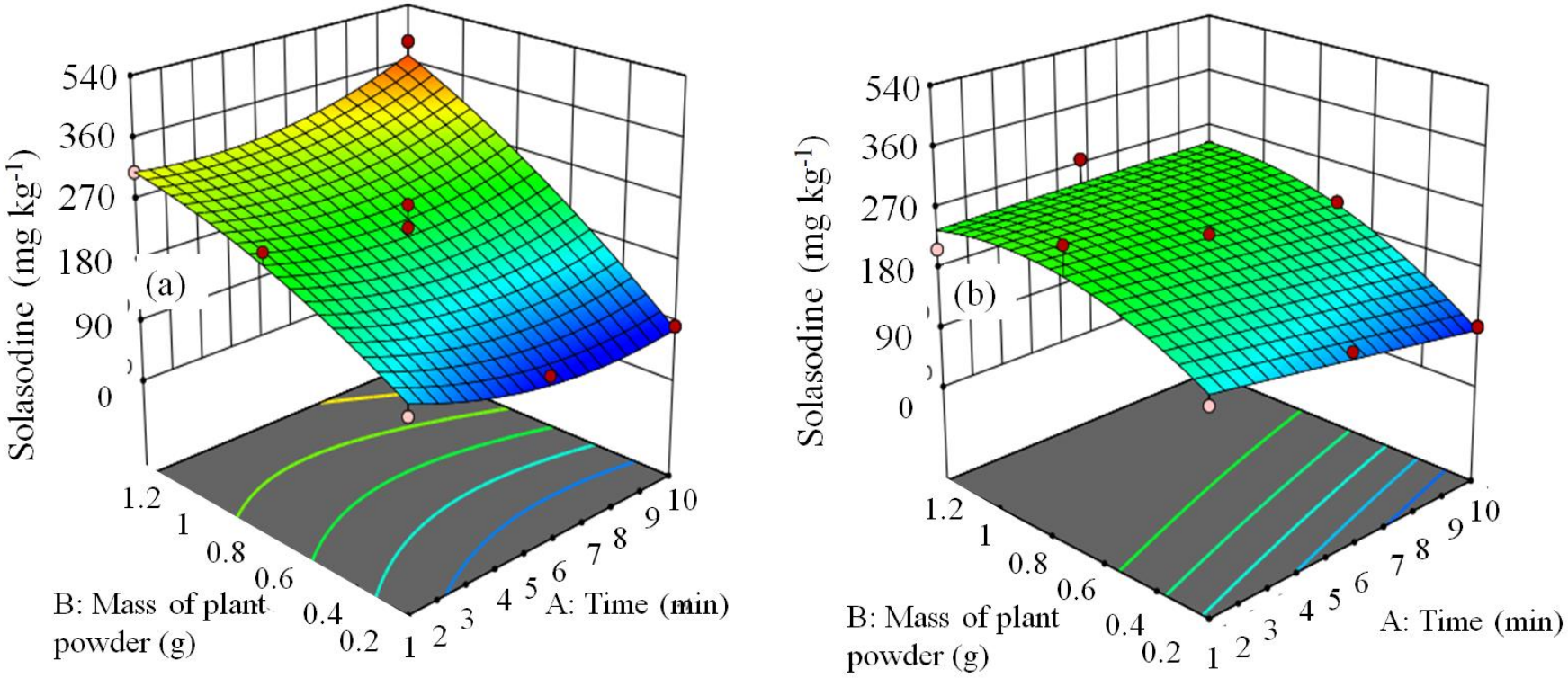


Fig. 5 Response surfaces plots evaluating the effect of time and mass of plant powder in the presence of (a) Na_2CO_3 and (b) NaCl on the extraction of solasodine

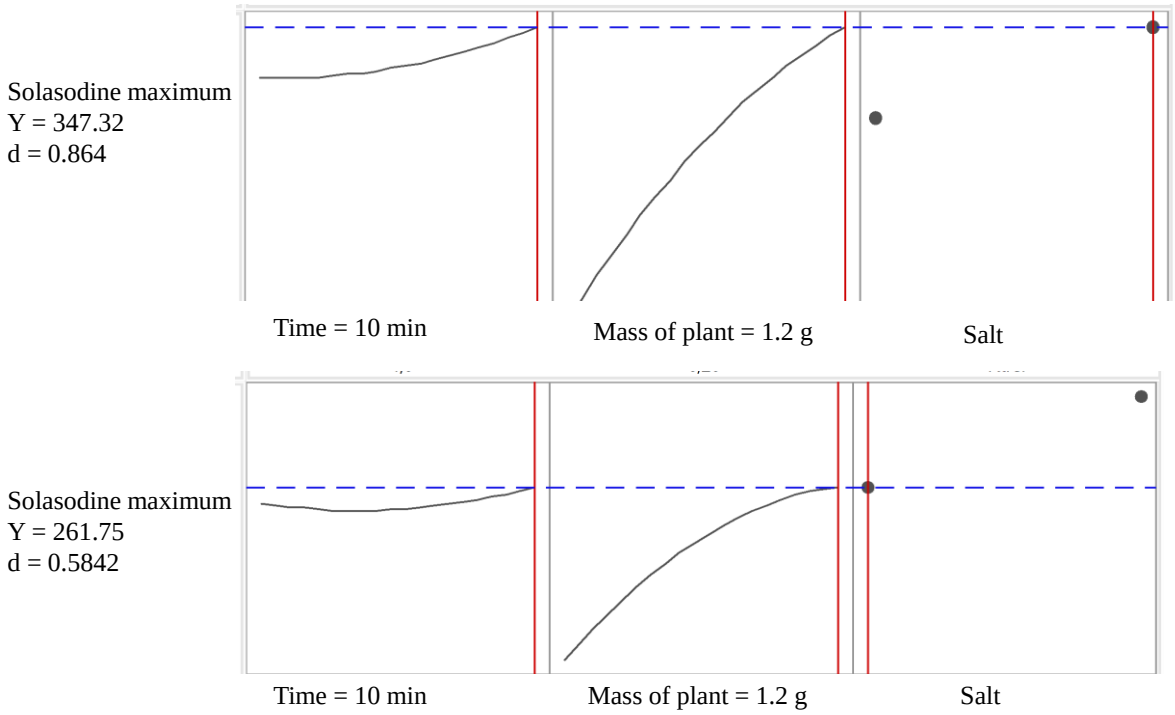


Fig 6 Optimal conditions for extraction of solasodine in the presence of (a) NaCl and (b) Na_2CO_3

Conclusions

The application of ATPE in the presence of a chaotrope and kosmotrope was shown to be a viable technique for extraction of an allelochemical, solasodine, from the weed, *Solanum mauritianum*. On average the maximal MRM transition based extraction of solasodine was 233.65 mg kg^{-1} and 413.50 mg kg^{-1} for NaCl and Na_2CO_3, respectively. This indicated that the higher charge density of the carbonate ion relative to the chloride ion was responsible for the greater extent of salting-out of solasodine. Furthermore, the application of ATPE using the kosmotrope was shown to enhance the extraction of solasodine compared to the chaotrope due to the synergistic effect of mass of plant powder, which was shown to be significant effect ($p < 0.05$), and salting-out. The effect of time and the paired factors were shown to have an insignificant effect ($p > 0.05$) on the enrichment of solasodine. In view of the better extraction performance of the kosmotrope in ATPE, this could possibly be extended to extraction of other toxic allelochemicals from other invasive plants which could better preserve the natural environment. Furthermore, the enriched solasodine ATPE extracts could potentially be a reliable source of antipathogenic agents in medicine.

Declarations

Acknowledgements

The authors would also like to express their gratitude to the Council for Scientific and Industrial Research studies for their technical support. The University of Venda is also thanked for financial support.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material (data transparency)

Not applicable

Code availability (software application or custom code)

Not Applicable

Contributions

TM, NT and NEM conceived the study, TM and NEM conducted the experiments and data analyses. NT, NM and WG supervised the project. WG helped to draft the manuscript. All authors read and approved the final manuscript.

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Paper 5

This multivariate optimization study evaluated the effect of salt concentration (%) and temperature for the PHWE-ATPE of solasodine from leaves of *Solanum mauritianum*.

Evaluation of a chaotrope and kosmotrope in the multivariate optimization of PHWE-ATPE of solasodine from leaves of *Solanum mauritianum*, UHPLC-qTOF-MS

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Abstract

A hyphenated microwave assisted pressurized hot water - aqueous two phase extraction (PHWE-ATPE) was applied in the extraction of solasodine from *Solanum mauritianum*. Central composite design (CCD) was performed which included numerical parameters such as percentage concentration of salt (NaCl or Na_2CO_3) and temperature. Fitting the central composite design response surface model for PHWE-ATPE to the data generated a model with a good quadratic fit ($R^2 = 0.901$). The statistically significant ($p < 0.05$) parameters such as the linear and quadratic effect of the concentration of salt (%) powder had a significant impact on the extraction of solasodine. The application of multiply charged salts such as the kosmotrope-Na_2CO_3 was shown to be a comparably better extractant of solasodine than the chaotrope-NaCl due to salting-out effect. The optimized condition for the extraction of solasodine in the presence of NaCl and Na_2CO_3 was a temperature of 80˚C at a salt concentration of 20%. Maximal experimental extraction of solasodine was 300.79 mg kg^{-1} and 162.34 mg kg^{-1} for Na_2CO_3 and NaCl respectively.

Keywords: solanine, *S. mauritianum*, response surface methodology, MA-ATPE, UHPL-qTOF-MS

1. Introduction

Over the past several decades, application of chemistry in industry has been directed at the use of environmentally friendly approaches (Shang et al., 2019; Dall'Acqua et al., 2020; Kuhn et al., 2021; Luo et al., 2020). The current adoption of environmentally friendly methods has been inspired by the 12 Principles of green Chemistry as initiated by Anastas and Warner (1991). This concept was aimed at revolutionizing chemistry into employing innovative scientific solutions to solve environmental dilemmas. Two of the principles dwelt on the use of safer solvents that are degradable. This is fundamental as the extent of environmental impact is affected by the type of solvent used. Furthermore, application of green solvents affects the way natural resources are harvested, energy usage, and emissions to air and water from the production and use of solvents, transportation, and disposal or recycling (Turner & Iba´n˜ez, 2011, Musarurwa and Tavengwa, 2020; Cederholm et al., 2020). Hence, the use of water as a potentially green extraction solvent, fits this category will as it is nontoxic to health and the environment and is the safest, abundant, and least expensive solvent.

PHWE (also called subcritical water extraction and superheated water extraction) is based on the use of water subjected to high enough temperatures (usually above its boiling point) and pressures to keep the water in the liquid state. Therefore, water in liquid state as a solvent at temperatures above its boiling point (100C, 0.1 MPa) and below its critical point (374˚C, 22.1 MPa) is employed in PHWE (Plaza & Turner, 2015; Gbashi et al., 2020; Nuapia et al., 2020). The principle of PHWE is guided by the physiochemical properties of water. Water is highly polar with a high dielectric constant (ε) of 80 at room temperature and atmospheric pressure because of its extensive hydrogen-bonded structure (Teo et al., 2010; Jin et al., 2020). Traditionally, water is not known to dissolve non-polar compounds at room temperature. However, as the temperature of water is increased, there is a resultant decrease in its permittivity, viscosity, and surface tension but an increase in its diffusivity characteristics. Similarly, at elevated temperatures, the dielectric constant of water decreases from ε = 80 at 25 ◦C to ε = 27 at 250 ◦C and 50 bar. Under these conditions water has a dielectric constant comparable to other organic solvents, such as methanol (ε = 33) and ethanol (ε = 24) at 25 ◦C. additionally, water is then able to dissolve a wide range of medium and low polarity analytes.

Lately, miniaturization for separation processes has become a crucial technique in various disciplines which includes biological engineering, pharmacy, environmental detection, and laboratory analysis (Cunha et al., 2020; Lendor et al., 2019). Some of the many advantages

miniaturized extraction offers, is improved heat and mass transfer which has been reported to result in enhanced separation efficiencies and (Ciceri et al., 2014; Fusari et al., 2019; Burato et al., 2020).

In view of the potential water has as an extraction solvent, this work was directed at the use PHWE for obtaining solasodine. This metabolite has attracted attention due to its impressive anticancer activity and insecticide property (Jacob and Malpathak, 2005; Marzouk et al., 2005, Fekry et al., 2019; Cham et al., 2020; Maddala et al., 2020). Moreover, this work was focused on the optimization of solasodine via PHWE-ATPE from *Solanum mauritianum* in the presence of doubly charged (Na_2CO_3-kosmotrope) and singly charged (NaCl-chaotrope) using central composite design (CCD) and response surface methodology (RSM). This statistical approach is beneficial as it reduces the number of experiments, making it less laborious and time efficient (Silva et al., 2019; Shokoohi et al., 2020). Furthermore, the work was also directed at the evaluation of the possible synergistic effect of salting-out due to varying salt concentration and temperature.

2 Experimental

Chemicals and reagents

The salts NaCl (anhydrous $\geq$ 99% purity), Na_2CO_3 (anhydrous $\geq$ 99% purity) and ethanol (99% CP) were purchased from Associated Chemical Enterprises (Johannesburg, South Africa) and Sigma-Aldrich (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) using a Direct-Q 5UV distiller (Massachusetts, United States of America) was applied for the preparation of the salt solutions. The extraction was performed on a DIAB MX-RL-Pro dragon shaker. Extraction of phytochemicals was achieved by a makeshift laboratory scale PHWE unit (Figure 1 (a)- (b)). The system consisted of a HPLC pump (Waters 6000 fluid controller, Waters Corporation, Manchester, UK), stainless steel extraction cell (70 × 30 mm and approximately 20 mL) fitted with a metal frit i.e. filter (3/8 in. diameter, 1/32 in. thickness and 2.0 µm pore size), refurbished GC 600 Vega Series 2 oven (Carlo Erba Instruments, Italy) with an automatic temperature controllable unit, stainless tubing (1.58 mm in outer dimension (OD) and 0.18 mm inner dimension (ID), back-pressure valve (Swagelok, Johannesburg, South Africa), and a collection flask. Chromatographic separation of the metabolites in the extracts was done using a reverse phase Shim-pack Velox C_{18}, 2.1 x 100 mm, 2.7 µm with a serial number 227-32009-03 (Columbia, USA). The UPLC was connected to a Shimadzu 9030 LC, qTOF-MS detector (Shimadzu, Kyoto). The solvents used for the chromatographic runs were

methanol and formic acid, which were purchased from Romil Pure Chemistry (Cambridge, UK)

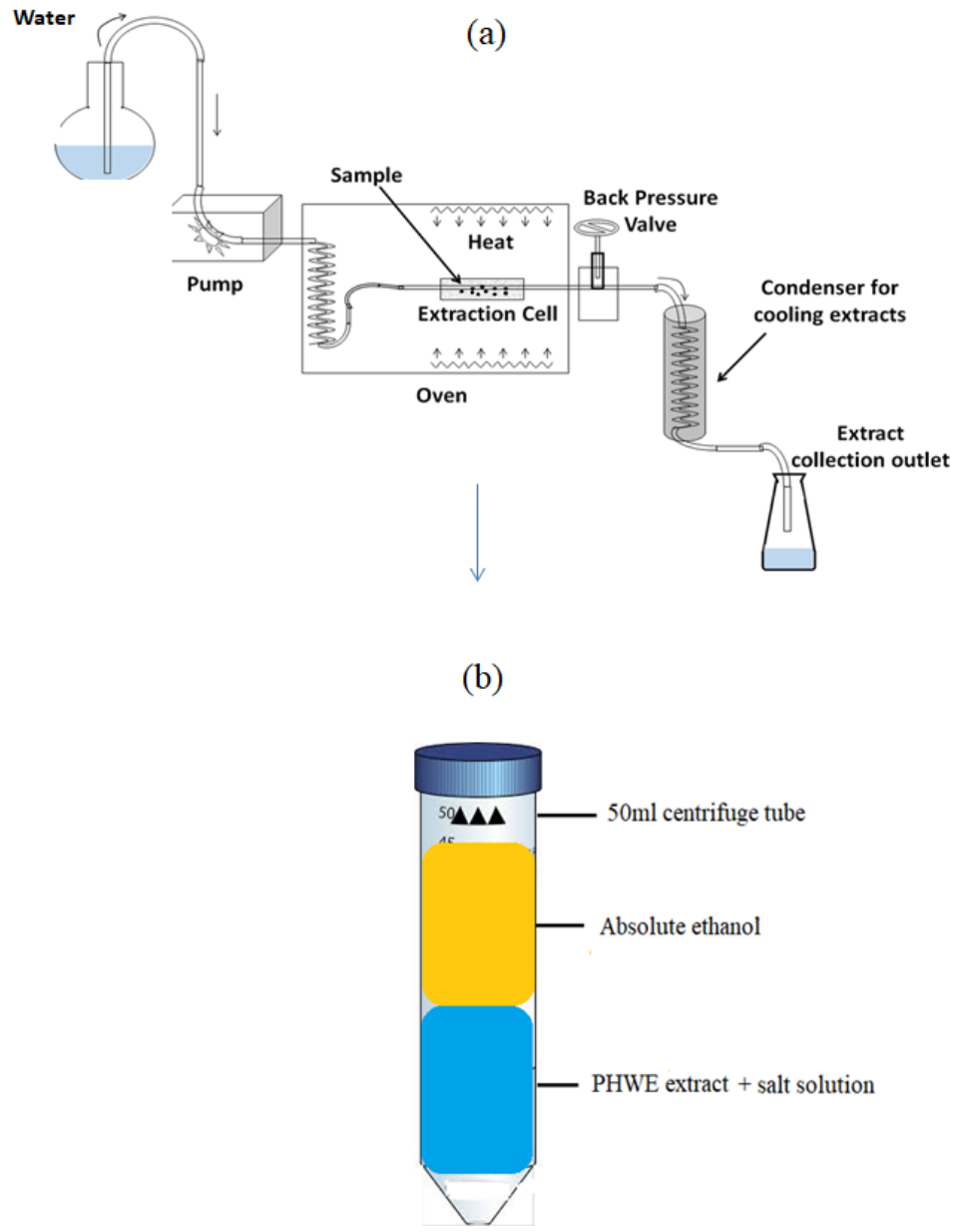


Figure 1: (a) A PHWE extraction system (Gbashi et al., 2016) consisting of the inflowing water propelled by a pump into the extraction cell. The metabolite containing water is then cooled within the condenser before being collected in the Erlenmeyer flask. (b) A PHWE-ATPE set-up

Sample collection, preparation and ATPE

The leaves of *S. mauritianum* were obtained from a street vendor within the Thulamela District in Thohoyandou, South Africa. The plants were air dried until a constant weight was obtained, and the leaves were ground into a fine powder with a blender at 2000 rpm and stored in glass containers. The containers were covered in paper bags to prevent light penetration. For the extraction, 3 g of ground leaves powder was mixed with 1.5 g of diatomaceous earth (Sigma, Munich, Germany), a dispersing agent and placed inside the extraction cell maintained at different oven temperatures of 80, 120 and 200 ± 1°C. The solvent was delivered at a constant flow rate of 10 mL min^{-1} and a pressure of 2500 ± 300 pa was maintained using the back-pressure valve. Extracts were collected in a falcon tube up to the 150 mL mark through an outlet coil immersed in a cooling water bath. The PHWE extracts (10 mL) were added to salt solutions containing 20, 35 and 50% (w/v) of NaCl (kosmotrope) or Na_2CO_3 (chaotrope). This solution was placed on the dragon shaker for 24 hours, rotating at 70 revolutions per minute (rpm). Thereafter, absolute ethanol (20 mL) was added, resulting in a PHWE-ATPE system (Figure 1 (b)). The extracts were filtered using a 0.22 µm nylon syringe filter into a 2 mL HPLC capped vial and preserved at -20 °C prior to analysis on the UPLC-QTOF-MS for detection of solasodine.

Chromatographic and mass spectrometry conditions

Solasodine were separated using a Shimpack C_{18}, 2.1 x 100 mm, 2.7 µm column from Shimadzu (Honeydew, South Africa). The column was maintained at 40 ºC at a flow rate of 0.4 mL min^{-1} and the injection volume was 5 µL. Mobile phase A was 0.1% formic acid in ultrahigh purity water (v/v) and mobile phase B was 0.1% (v/v) formic acid in methanol.

A UHPLC-qTOF-MS 9030 mass spectrometer (Shimadzu, Japan) was used for all mass spectral measurements. The mass spectrometer was equipped with an electrospray interface (ESI) operating in positive mode. ESI parameters were optimized for solasodine by direct infusion of standard solutions into the mass spectrometer. The mass spectrometer was operated in the multi reaction monitoring (MRM) mode to confirm the identity of solasodine. This was achieved by selecting specific precursor to product ion transitions for each solasodine based on the transitions shown in Figure 2. High-purity nitrogen (N_2) was used as the nebulizing and drying gas. The optimum parameters were as follows: drying gas temperature, 250 °C; drying gas flow, 10 L min^{-1} and collision energy, 30 - 60V. For the chromatographic separation and Shimadzu 9030 LC instrument (Shimadzu, Japan) was used. The instrument consisted of an

autosampler, thermostated column compartment and a binary pump. Lab solutions software was used to control the LC-MS/MS instrument and for data acquisition and the mass range was *m/z* 100-1000.

Preparation of standards

The stock standard solution was prepared in methanol at a concentration of 1000 µg L^{-1}. The stock standard solution was stored at 4°C in amber volumetric flasks. A series of nine working standard solutions at the concentration values of 15 to 1000 µg L^{-1} were prepared from the stock standard solution by diluting with HPLC grade methanol. The solanine standards were quantified based on scheduled multiple reaction monitoring (MRM) where one *m/z* transition, from the precursor ion to the product ion, for solanine (414 → 396) was explored. The regression equation was y = 537.484x + 41.893, limit of detection (LOD) and limit of quantification (LOQ) were 0.078 and 0.236, respectively. The above mentioned transition was then applied for quantification of solasodine from the ground leaves of *Solanum mauritianum* following ATPE extraction. The parameters evaluated for optimization of solasodine from ATPE were time, mass of plant powder and the salt type (kosmotrope or chaotrope).

Statistical analysis

The central composite design response surface model (CCD RSM) was fitted to experimental data to obtain the relationship between factors and optimize the response of Z (solasodine yield) in relation to A (time), B (mass of plant powder) using Minitab 17 (UK). A Two-level full factorial CCD was designed, a total of 36 experimental runs (including 2 repetitions) were designed, 3 factor numerical levels for concentration of salt (20, 35 and 50%), temperature (80, 140, 200˚C) and 2 categorical factor levels for salts which included the chaotrope (NaCl) and kosmotrope (Na_2CO_3).

Model parameters and model significance were determined at $p < 0.05$. The fitness of the model was determined by evaluating the coefficient of regression (R^2) obtained from the analysis of variance (ANOVA). The model fit generated the response surface that defined the behaviour of the response variable. By means of these plots, the optimized ranges for each factor that led to the highest response (i.e concentration of solasodine) that can be extracted

The interaction between the various parameters studied and its resultant effected on the extraction of solanine (mg kg^{-1}) was fitted to experimental data by using a statistical multiple regression approach method of least square (MLS), and resulted in the lowest possible residual

(Bas et al., 2007). Model parameters and model significance were determined at $p < 0.05$. The fitness of the model was determined by evaluating the coefficient of regression (R^2) obtained from the analysis of variance (ANOVA). The model fit generated the response surface that defined the behaviour of the response variable. By means of these plots, the optimized ranges for each factor that led to the highest response (i.e concentration of solanine) that can be extracted (Bas et al., 2007; Arteaga-Crespo et al., 2020).

3 Results and discussion

3.1 MRM quantification of solasodine based on the 414→396 transition

Solanum mauritianum, an invasive species, is an abundant source of anti-cancer and antifungal metabolites such as solasodine and solasodine glycosides (Jayakumar et al., 2017 (a); Jayakumar et al., 2017 (b)). In this study, we demonstrated the extraction of an aglycone unit of solasodine glycosides, solasodine. from *Solanum mauritianum*, using ATPE. The ATPE technique was performed by evaluating the different factors shown in Table 1 on the recovery of solasodine. The presence of solasodine was reported in *Solanum mauritianum* and other species within the *Solanum* genus have been reported in the literature (Bhattacharya et al., 2013; Jayakumar et al., 2017; Jayakumar et al., 2017). Using a sensitive and robust tandem MS approach (UHPLC-qTOF-MS) with settings presented (Mokgehle et al., 2021) it was possible to efficiently fingerprint these solasodine based on *m/z* 396 product ion (Figure 2). Thereafter, based on the 414 → 396 transition within the MRM method, solasodine was quantified as shown in Table 1.

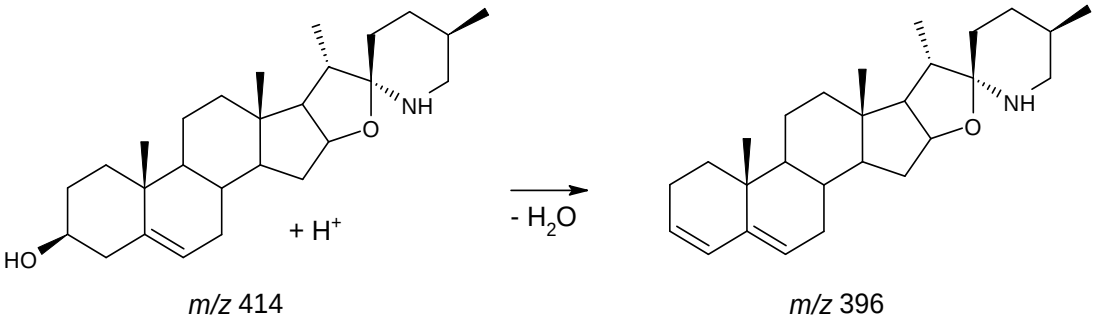


Figure 2: Molecular transition of solasodine (*m/z* = 414) to [solasodine – H_2O] (*m/z* = 396) after the loss of water.

Table 1: List of experiments using CCD for ATPE optimization, the response, and predicted values.

Run 1	Factor 1 % Salt	Factor 2 Temperature (˚C)	Factor 3 Salt type	Solasodine (mg kg^{-1}) Run 1	Run 2	Mean ± SD	Predicted
1	20	80	NaCl	149.421	175.273	162.34+18	144.95
2	35	80	NaCl	164.782	152.101	158.44+9	141.47
3	50	80	NaCl	167.337	173.475	170.40+4	152.15
4	20	80	Na_2CO_3	276.235	325.356	300.79+34	268.57
5	35	80	Na_2CO_3	137.631	242.474	190.05+74	169.69
6	50	80	Na_2CO_3	90.256	121.386	105.82+22	94.48
7	20	140	NaCl	145.755	147.766	146.76+1	131.04
8	35	140	NaCl	133.142	145.833	139.48+9	124.54
9	35	140	NaCl	141.55	185.129	163.33+31	145.84
10	35	140	NaCl	143.837	193.206	168.52+35	150.47
11	35	140	NaCl	153.087	193.257	173.17+28	154.62
12	35	140	NaCl	160.403	181.400	170.90+15	152.59
13	35	140	NaCl	147.844	189.184	168.51+29	150.46
14	35	140	NaCl	133.210	189.690	161.45+40	144.15
15	35	140	NaCl	146.783	199.122	172.95+37	154.42
16	35	140	NaCl	144.264	145.378	144.82+1	129.30
17	35	140	NaCl	135.107	156.155	145.63+15	130.03
18	50	140	NaCl	144.261	150.600	147.43+5	131.63
19	20	140	Na_2CO3	231.064	288.117	259.59+40	231.78
20	35	140	Na_2CO_3	132.953	148.042	140.49+11	125.44
21	35	140	Na_2CO_3	143.273	174.470	158.87+22	141.85
22	35	140	Na_2CO_3	140.993	178.487	159.74+27	142.63
23	35	140	Na_2CO_3	144.551	183.655	164.10+28	146.52
24	35	140	Na_2CO_3	160.268	135.875	148.07+17	132.21
25	35	140	Na_2CO_3	175.495	187.357	181.42+8	161.99
26	35	140	Na_2CO_3	182.617	177.587	180.10+4	160.81
27	35	140	Na_2CO_3	138.686	209.858	174.27+50.	155.60
28	35	140	Na_2CO_3	157.500	173.412	165.45+11	147.73
29	35	140	Na_2CO_3	161.165	191.737	176.45+21	157.55
30	50	140	Na_2CO_3	139.682	152.258	145.97+9	130.33
31	20	200	NaCl	183.968	110.836	147.40+52	131.61
32	35	200	NaCl	176.443	159.208	167.82+12	149.84
33	50	200	NaCl	178.835	157.696	168.26+15	150.24
34	20	200	Na_2CO_3	295.727	289.355	292.54+5	261.20
35	35	200	Na_2CO_3	175.636	200.448	188.04+18	167.89
36	50	200	Na_2CO_3	142.454	138.034	140.24+3	125.22

3.2 Fit statistics and Pareto chart of parameter main effects and their interactions produced from ANOVA and resultant box plots

The model fitted to the data was observed to have a quadratic fit P-values less than 0.0001 indicate model terms are significant. The probabilities for concentration of salt and temperature for NaCl and Na_2CO_3 were P = 0.269 and P = 0.799 and P = 0.000 and P = 0.55, respectively, as shown in the pareto charts in Figure 3 (a) and (b). This indicated that the linear effect of concentration of Na_2CO_3 was a significant ($P < 0.05$) model terms and was an adequate predictor of the experimental values obtained (Figure 3 (b)). Similarly, the quadratic effect of concentration of Na_2CO_3 was significant (P = 0.005) (Figure 3 (b)). The rest of the model terms for Na_2CO_3 and NaCl were insignificant ($P > 0.05$), which also included the linear and quadratic effect of temperature (Figure 3 (a) and (b)). Similar observations were reported by Gbashi et al. (2016) on the insignificance of temperature ($P > 0.05$) during PHWE of dicaffeoyl quinic acids from *Bidens Pilosa*. The lack of fit of F-value was observed to be 1.71 which indicated that the lack of fit was not significant relative to the pure error. The non-significant lack of fit was desirable. The goodness of fit between the experimental and the predicted values was $R^2 = 0.901$

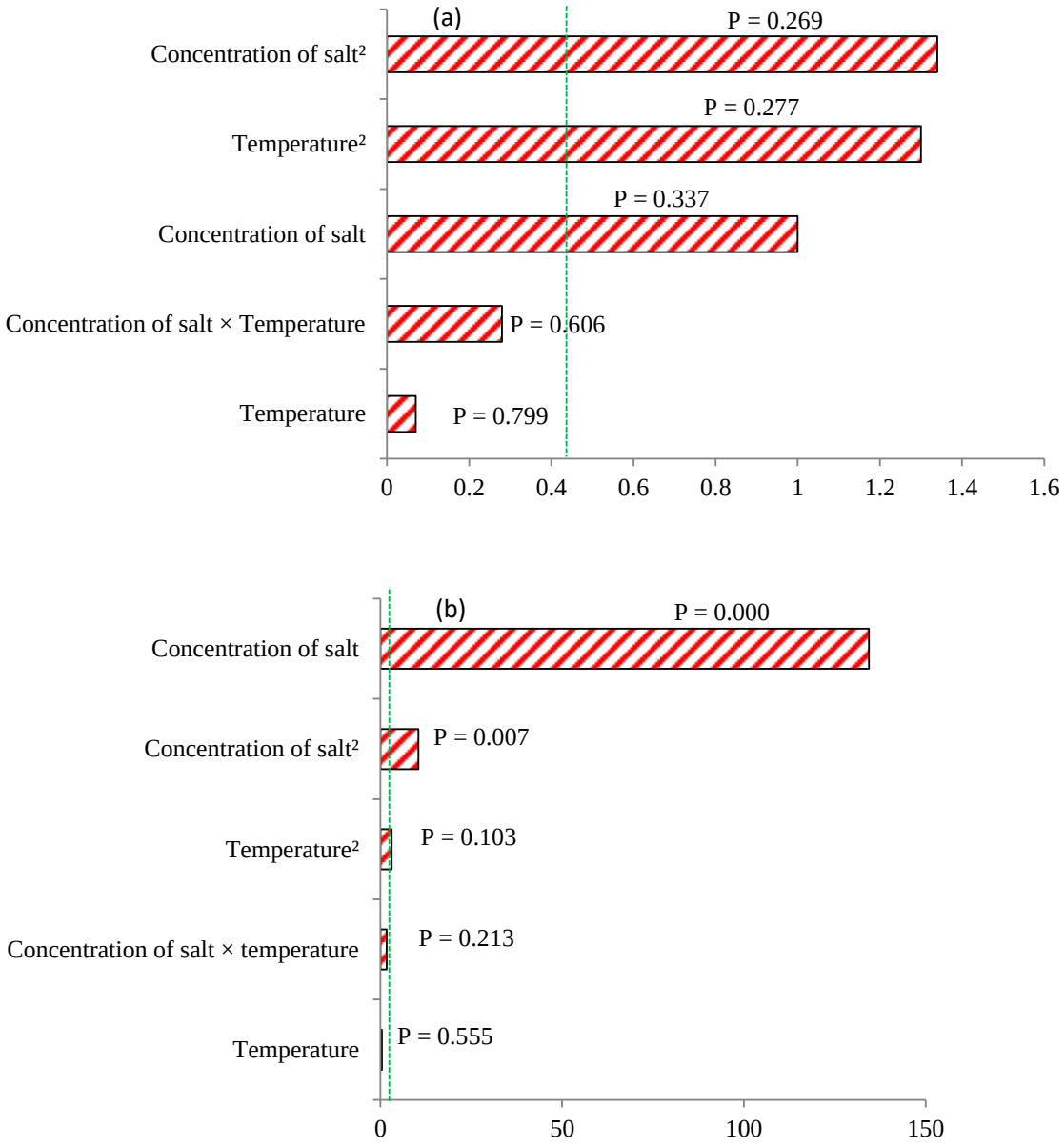


Figure 3: Pareto chart of standardized effects of time, mass of plant powder and power on the extraction solasodine at 414 → 396 (a) NaCl and (b) Na_2CO_3

Similarly, in Figure 4 (a) – (d), the box-and-whiskers plots of the effect of concentration and temperature on the PHWE-ATPE extraction of solasodine from leaves of *Solanum mauritianum*. From Figure 4 (a) –(d) as the % concentration of salt was increased for Na_2CO_3, a decrease in solasodine extracted was observed while there were no notable changes in solasodine (≈ 160 mg kg^{-1}) concentration as salt concentration was varied for NaCl (Figure 4 (a) and (b)). The highest extraction of solasodine with the variation of salt concentration was approximately 300 mg kg^{-1}, which indicated that a doubly charged anion, CO_3^{2-}, was more efficient than a singly charged ion, Cl^-, salting-out of solasodine at low % concentration (Figure

4 (b)) as most of the salt was dissolved in the solution. However, higher concentrations of salt led to a super-saturated solution which led to its precipitation from solution, reducing the salting-out efficiency at 50% salt concentration for Na_2CO_3 in particular. The relatively higher solasodine extraction capability of Na_2CO_3 in comparison to NaCl, can be explained using the Hoffmeister series where, CO_3^{2-} > Cl, and indicated that the divalent carbonate ion has a greater solute precipitation ability, due to its higher charge density, than the monovalent chloride ion (Figure 5) (Hyde et al., 2017; Tejada-Casado et al., 2018; Hernández-Mesa et al., 2018; Sazali et al., 2019). Similarly, Neves et al. (2018) reported on the better salting out capacity of SO_4^{2-} than Cl,

Generally, a variation in temperature did not have a significant effect on the solasodine obtained in the presence of NaCl. The kosmotrope Na_2CO_3 was more responsive to temperature changes with the highest extraction achieved at 80˚C. The probably implies that Na_2CO_3 does not require highest temperatures for efficient extraction of solasodine, but can be done at lower temperatures, which is recommended in green chemistry. Furthermore, the application of Na_2CO_3 in PHWE-ATPE demonstrated that extraction of solasodine was mainly driven by the salting-out process rather the temperature (Figure 3 (b)) while it was both factors seemed to be insignificant in the presence of NaCl.

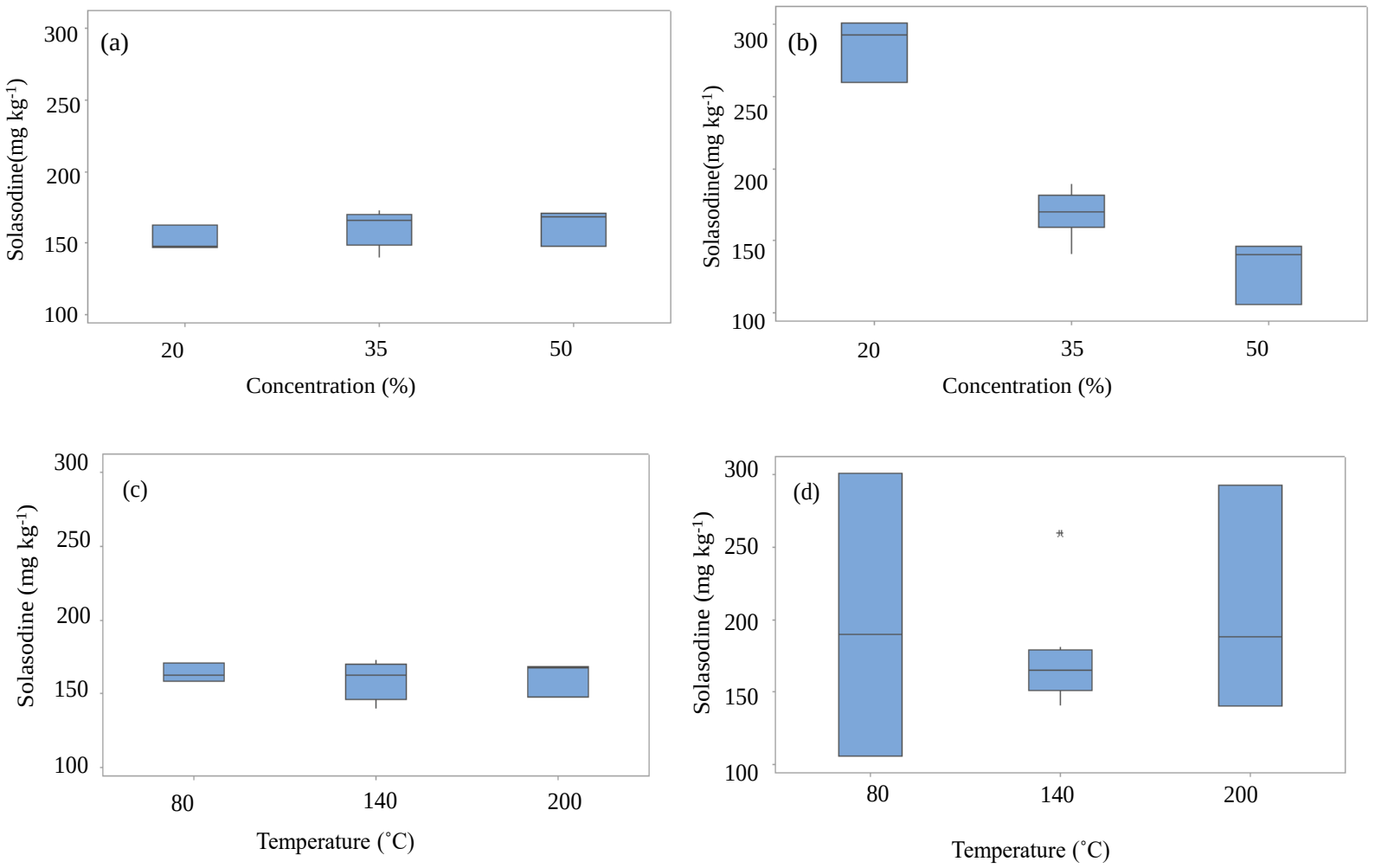


Figure 4: *Box*-and-*whiskers* plots evaluating the effect of 'Concentration of salt on (a) NaCl, (b) Na_2CO_3 and the effect of 'Temperature' on (c) NaCl and (d) Na_2CO_3 on the extraction of solasodine from leaves of *Solanum mauritianum*

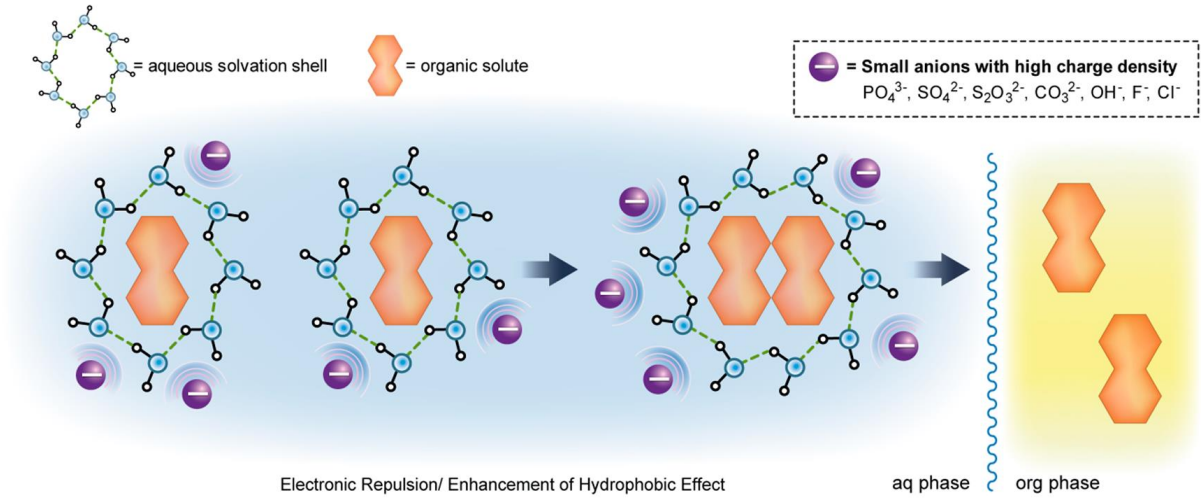
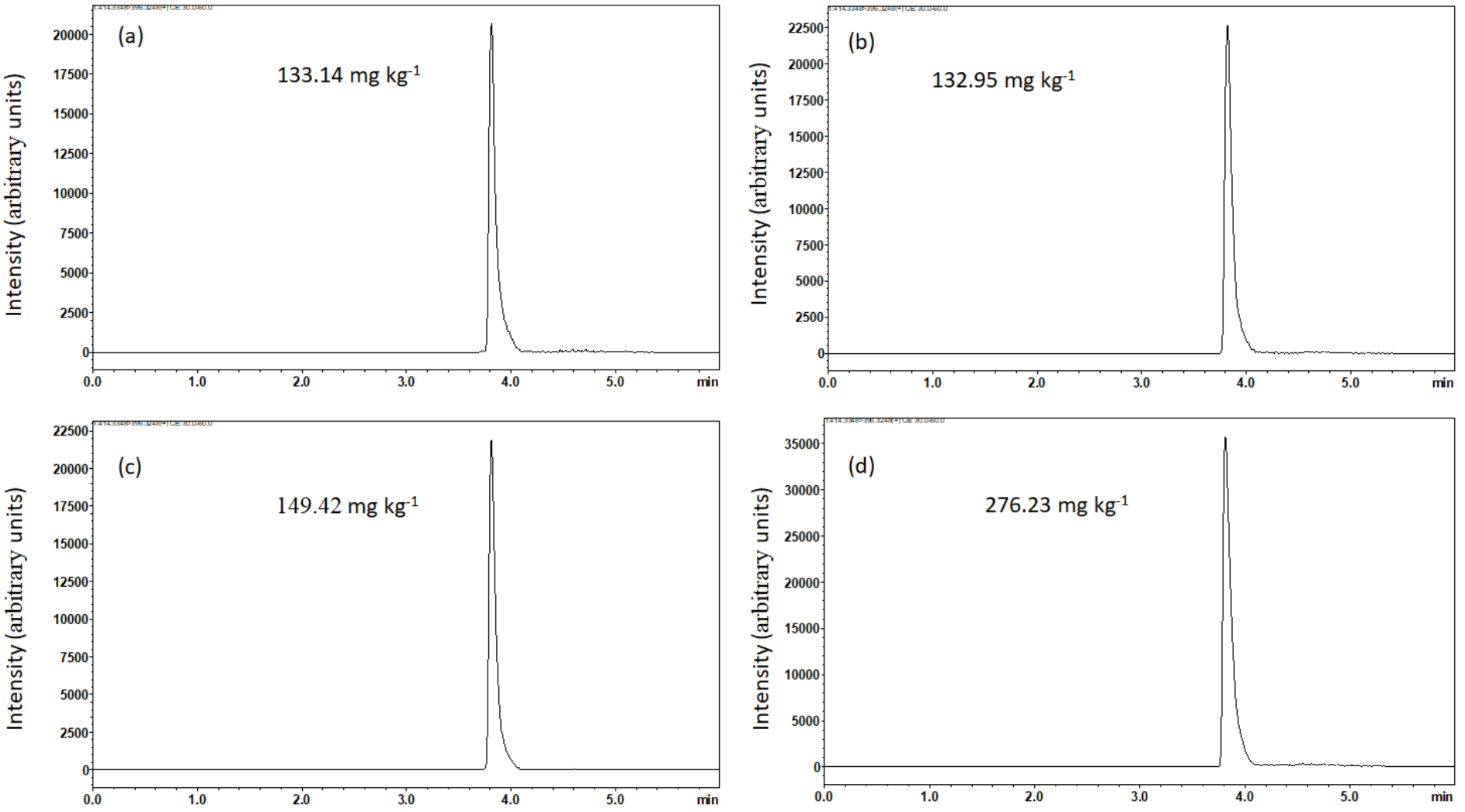


Figure 5: Role of intermolecular forces and charge density of anions involved in the salting out of organic solutes from the aqueous phase to the organic phase (Hyde et al., 2017)

3.3 Chromatographic profile of MRM based quantification of solasodine

Chromatograms depicting the highest and lowest concentrations of solasodine (mg kg^{-1}) obtained when NaCl and Na_2CO_3 was applied are shown in Figure 6 (a), (b), (c) and (d). The chromatogram indicates an MRM transition of solasodine *m/z* 414 → 396 and is observed at a retention time of 3.825 min. The fragmentation profiles showing the product ions of solasodine are also included in Figure 6 (f). As seen in Figure 2, solasodine underwent a dehydration reaction from the precursor ion *m/z* 414 to the product ion *m/z* 396. From the experimental results dehydration of solasodine generally seems to be favoured at extraction parameters involving lower concentrations of salt in combination with lower temperatures of salt which correspond to solasodine concentrations of 149.42 mg kg^{-1} and 276.23 mg kg^{-1} when both NaCl and Na_2CO_3 were applied to aid extraction as shown in Figure 6 (c) and (d), respectively. This is also in agreement with the significant effect of concentration of salt, in particular, Na_2CO_3, as seen in the pareto chart in Figure 3 (b), and the box plots in Figure 4.



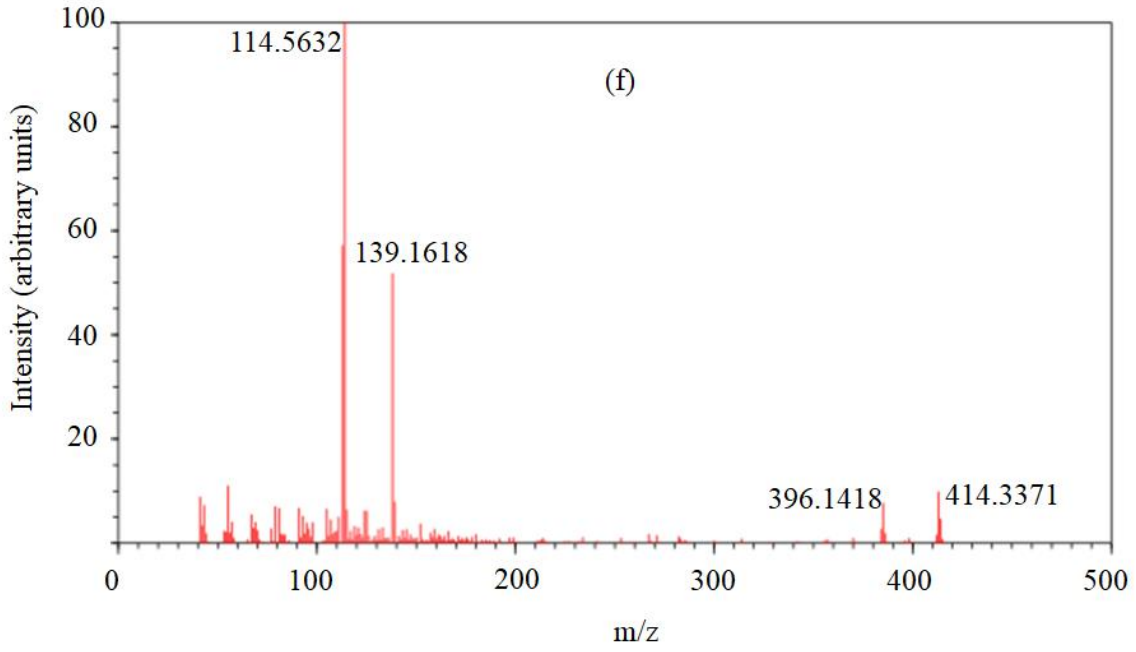


Figure 6: Chromatogram of the lowest extracted concentration of solasodine in (a) NaCl and (b) Na_2CO_3, and highest extracted concentration of solasodine (c) NaCl and (d) Na_2CO_3 for run 1. (f) mass spec of solasodine

3.4 Response surface equations and corresponding for NaCl and Na_2CO_3 and the resultant optima

Response equations, Eqs 1 and 2, corresponding to NaCl and Na_2CO_3, respectively, and the resultant response surfaces evaluating the bivariate interaction between the time and mass of plant are shown in Figure 7. In this case Z, concentration of solasodine, was the dependent variable (solasodine concentration) and x (concentration of salt (%)) and y (temperature (˚C)) the independent variables. From the quadratic fit of $r^2 = 0.901$, as reported in section 3.2, Eqs 1 and 2 were obtained

$$z(x,y) = 167.79 + 2.35x - 0.76y - 0.036x^2 + 0.0021y^2 + 0.0035xy \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$z(x,y) = 649.18 - 16.205x - 1.63y + 0.1346x^2 + 0.0046y^2 + 0.0118xy \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

The response surface plots in Figure 7 demonstrated that as the concentration of salt was increased, the yield of solasodine decreased in the presence of Na_2CO_3. This is consistent with the observations from the Box plots in (Figure 4 (b)). This also concurs with observations from pareto chart, Figure 3 (b), which indicates the significant linear effect of concentration of salt on extraction of solasodine. In Figure 8 (a) and (b) the predicted optimal extraction of

solasodine in the presence of NaCl and Na_2CO_3 was 178.69 mg kg^{-1} and 277.05 mg kg^{-1}, with a desirability score of 0.37 and 0.87, respectively. The higher desirability score of Na_2CO_3 compared to NaCl indicated its closeness to the target requirement of 1, and hence the greater reliability of this optimum for maximal enrichment of solasodine. Additionally, comparisons of the maximal concentrations of solasodine obtained in Table 1 and Figure 8, indicated that more of solasodine was extracted for Na_2CO_3 compared to NaCl. This suggested that solasodine extraction was influenced by the presence of multiply charged ions (kosmotropes), Na_2CO_3 in this case, rather than NaCl. The doubly charged carbonate ions from Na_2CO_3, probably formed strong hydrogen bonds with the water molecules surrounding the solasodine, enhancing the extent of its precipitation (salting-out) from the hydration sphere and its subsequent extraction by ethanol. (Sazali et al., 2019; Mokgehle et al., 2019).

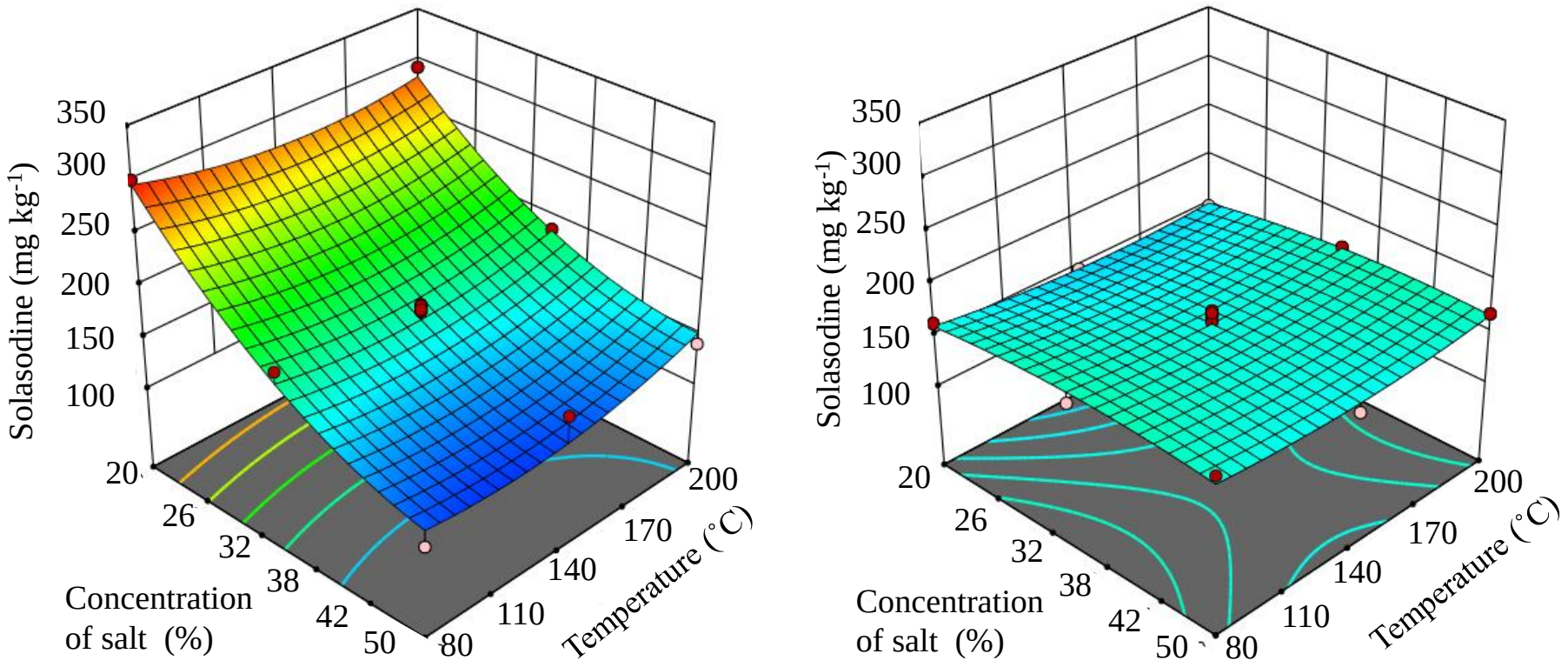


Figure 7: Response surfaces evaluating the effect of time and mass of plant powder in the presence of (a) Na_2CO_3 and (b) NaCl on the extraction of solasodine

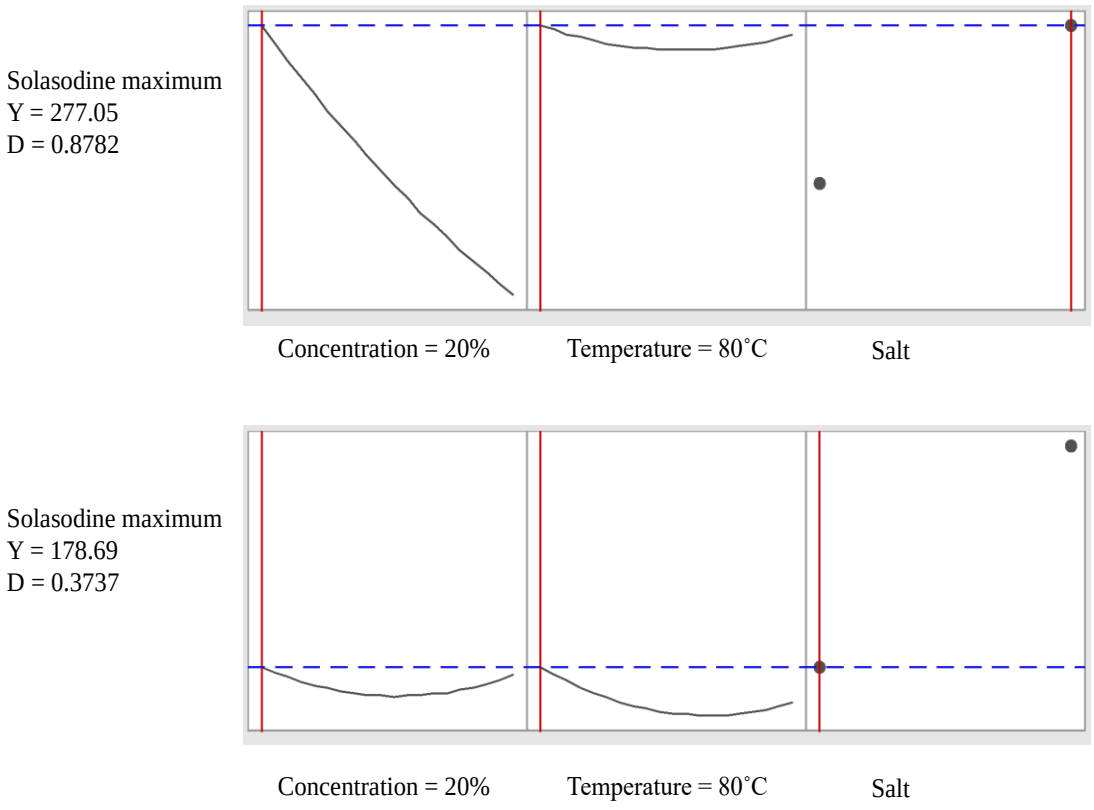


Figure 8: Optimal conditions for extraction of solasodine in the presence of (a) NaCl and (b) Na_2CO_3

4. Conclusions

The application of pressurized hot water assisted aqueous two phase extraction in the presence of a chaotrope and kosmotrope has proved to be a viable technique for extraction of a pharmaceutically important metabolite, solasodine, *Solanum mauritianum*. The optimized conditions for the extraction of solasodine in the presence of NaCl and Na_2CO_3 were a temperature of 80˚C at a salt concentration of 20%. Maximal experimental extraction of solasodine was 300.79 mg kg^{-1} and 162.34 mg kg^{-1} for Na_2CO_3 and NaCl respectively. It was statistically deduced that linear and quadratic effect of the concentration of salt (%), particularly for Na_2CO_3, had a significant effect ($p < 0.05$) on the extraction of solasodine during PHWE-ATPE. Temperature on the other hand and other paired factors were shown to have an insignificant effect ($p > 0.05$) on the enrichment of solasodine. The charge density on the CO_3^{2-} ion was responsible for the greater salting out ability of solasodine in comparison to the Cl-, making the kosmotrope-Na_2CO_3 a better

extractor. Extraction of solasodine from *Solanum mauritianum* could potentially improve through the application of miniaturized even greener solvents such as deep eutectic solvents (DES).

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Chapter 5 – Conclusions, recommendations, challenges, and future work

The chapter highlights the conclusions obtained from the experimental work conducted during the project. The prospects for this work are also included in this chapter.

5.1 Conclusions

Characterization of toxic compounds extracted *via* ATPE, MAE and PHWE contained in *Solanum retroflexum* and *Solanum mauritianum* was performed with the aid of UHPLC-qTOF-MS. The work involved the application of kosmotropes and chaotropes as precipitating agents in obtaining toxic metabolites such as alkaloids and glycoalkaloids. The kosmotrope (Na_2CO_3) and chaotrope (NaCl) was shown to extract glycoalkloids some of which included solanelagnin, solarmargine, solasonine, solasodine and solanine.

The application of ATPE was an efficient technique for obtaining multiple toxic metabolites from *Solanum* plants. This was observed in the ability of Na_2CO_3 and NaCl to simultaneously extract multiple glycoalkaloids in a single step. The ATPE method was also shown to be versatile as it could be hyphenated with MAE and PHWE. For instance, microwave assisted aqueous two-phase extraction (MA-ATPE) was quantitatively shown to be a better extractant of solasonine and solarmargine compared to MAE and MAE+ATPE. The synergy of microwaves and salting-out in the 'one-pot' MA-ATPE technique was a contributing factor for enhanced extraction of glycoalkaloids at shorter extraction periods.

This work also involved the application of central composite design during multivariate chemometric studies. MA-ATPE, ATPE and PHWE-ATPE methods were optimized for MRM quantification of solanine or solasodine. Comparison of ATPE and PHWE-ATPE for the extraction of solasodine from *Solanum mauritianum* indicated that ATPE was better extractor of solasodine by a factor of approximately 1.5. The effect of temperature in PHWE-ATPE was shown to be insignificant ($p > 0.05$) and could account for the lower extraction of solasodine compared to ATPE. Generally, the effect of mass of plant powder and % concentration salt during ATPE was a statistically significant parameter behind the enhanced extraction of solasodine.

In all the MRM based quantification studies conducted, the kosmotrope-Na_2CO_3 was a better extractant than the chaotrope-NaCl. This highlighted that the greater negative charge density of the carbonate ion played a crucial role during salting-out of the analyte (solanine or solasodine) by the formation of strong hydrogen bonds among water molecules surrounding the solute. This subsequently permitted for the precipitation of the solute from the aqueous phase into the extraction solvent.

The results of the current study demonstrated that the application of UHPLC-qTOF-MS was an efficient and feasible technique for putative characterization of toxic metabolites in *Solanum* plants. The implementation of a robust feature of qTOF-MS, MRM, was useful in the isolation and quantification of toxic metabolites such as solanine and solasodine from complex matrices of *Solanum* plants. The application of ATPE in conjunction with UHPLC-qTOF-MS was pivotal in room-temperature extraction of multiple alkaloids using a green extraction solvent (ethanol). This green and highly compatible technique (ATPE) in the presence of a kosmotropic salt (Na_2CO_3), makes it an attractive approach for enhanced enrichment and separation of pharmaceutically relevant phytocompounds which could potentially be applied as food supplements and pharmacology.

5.2 Challenges and Future work

Future endeavours need to be directed at further reduction in the number of organic solvents used in pre-concentration techniques involving, PHWE, ATPE and PHWE as it is quite expensive to run and is environmentally taxing. As a result, researchers need to invest efforts into upgrading the aspect of "greenness" of pre-concentrations techniques which could include deep eutectic solvent (DES) or ionic liquids.

To further improve the extraction of plant derived metabolites, hypenation of ATPE with miniaturized extraction methods such as membrane assisted extraction (MASE). Additionally, the MA-ATPE is currently non-specific to toxic compounds. To improve this, a combination of MA-ATPE and (molecularly imprinted polymers) MIPs may be explored to fish out toxic compounds. The more advanced preconcentration methods could potentially result in excellent yields of the targeted metabolites.

Chaotropes and kosmotropes were observed to simultaneously extract many metabolites from *Solanum retroflexum* and *Solanum mauritianum* during ATPE, MA-ATPE and PHWE-ATPE. The salts, especially kosmotropes can potentially be applied on a commercial scale, to meet the ever-growing demand of the bioactive compounds in metabolomics.

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Appendix

This section provides additional publications done during the PhD study as well as additional information that could not be included in the publications.

Paper 6

This review highlighted new trends in the development of biopolymers such as polysaccharides and proteins as adsorbents of nutraceutical compounds, with emphasis on metabolites derived from plants within the Solanaceae family.

Carbohydrate Polymers 264 (2021) 118049

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Review

Advances in the development of biopolymeric adsorbents for the extraction of metabolites from nutraceuticals with emphasis on Solanaceae and subsequent pharmacological applications

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ARTICLE INFO

Keywords:
Biologically derived polymers
Nutraceutical plants
Metabolites
Adsorbents
Pharmacology

ABSTRACT

Biopolymers are renowned for their sustainable, biodegradable, biocompatible and most of them have antitoxic characteristics. These versatile naturally derived compounds include proteins, polynucleotides (RNA and DNA) and polysaccharides. Cellulose and chitosan are the most abundant polysaccharides. Proteins and polysaccharides have been applied as emulsifiers. Additional applications of proteins and polysaccharides include cosmetics, food and wastewater treatment for adsorption of dyes and pesticides. However, more interesting applications of biopolymers are emerging, such as use in transport systems for delivery of plant derived nutraceuticals to sites of inflammation, due to its inherent ability to immobilize different biological and chemical systems. This review aims to give a summary on new trends and complement what is already known in the development of polysaccharides and proteins as adsorbents of nutraceutical compounds. The application of polysaccharides/protein containing the adsorbed *Solanum* derived nutraceutical compounds for drug deliveryis also reviewed.

1. Introduction

The Solanaceae genus belongs to one of the important plant taxa, boasting over 2000 species worldwide (Jayasinghe, Silva, & Karunaratne, 2017; Mandal, Dobhal, & Joshi, 2019). The majority of *Solanum* species are found as edible products in supermarkets such as *Solanum melongena* (eggplant) *Solanum lycopersicum* (tomato) and *Solanum tuberosum* (potatoes). Other species of *Solanum* have uses in medicine (e.g., deadly nightshade, jimson weed) or as drugs (e.g., tobacco) (Scholtz, MacMorris, Krogmann, & Auffarth, 2019; Xu et al., 2018). Solanaceae plants have an extensive potential to produce new nutraceuticals in treatment of various illnesses such as hyperglycaemia (Singh, Kaur, Ezekiel, & Singh Guraya, 2005; Tai et al., 2016), high blood pressure (Oluwagunwa, Alashi, & Aluko, 2019), endometrial cancer (Arslan & Yerer, 2018) and neurodegenerative diseases (Ogunsuyi, Ademiluyi, & Oboh, 2020). As a result, researchers have often looked for ways to extract nutraceutical compounds from plants. However, a challenge often faced by researchers during extraction of metabolites is the complex matrices of nutraceutical compounds in plants. Hence, a continuous need arises for the development of robust, friendly, highly selective and accurate analytical extraction techniques. Additionally, high sensitivity of analytical instruments is mandatory for determination of trace metabolites usually at concentrations in the range of nanograms per kilogram. For instance, *Solanum lycopersicum* contains trace amounts of fatty acids which play a pivotal role in heart disease prevention (Perveen et al., 2015). Sample preparation, a very important step in the overall analytical method, becomes crucial at these low concentrations. It essentially requires the pre-concentration of the target analytes by suitable adsorbents from the matrix before instrumental analysis (Dimpe & Nomngongo, 2016). Therefore, much attention has been paid to the development of new sorbents (Bernal, Nozal, Martín, Berna, & Ares, 2019; Grau, Benedé, Serrano, Segura, & Chisvert, 2019). Currently, green analytical chemistry has garnered a great deal of interest among chemists because of its eco-friendliness. The application of less toxic and preferably natural reagents during analysis is one of the recent trends in green analytical chemistry (Galuszka, Migaszewski, &

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https://doi.org/10.1016/j.carbpol.2021.118049
Received 30 October 2020; Received in revised form 31 March 2021; Accepted 3 April 2021
Available online 7 April 2021

Namieśnik, 2013). Therefore, the development of new highly selective sorbents using renewable resources for pollutants enrichment is desirable.

Biopolymers are plant, animal or microbial derived biomass. They may be polysaccharides, lipopolysaccharides, glycolipids, proteins, or polyhydroxyalkanoates and are suitable for environmental applications as sorbents (Palanisamy, Jeyaseelan, Murugesan, & Palanisamy, 2019). Among the biopolymers, cellulose and chitosan, the first and second most abundant biopolymers on earth have been preferred (Hermanutz, Vocht, & Buchmeiser, 2020; Olivera et al., 2016). Others such as lignin (Ge, Qin, & Li, 2016), tannin (Bacelo, Santos, & Botelho, 2016), polyisoprene, natural rubber, (Xie et al., 2020), inulin (Rahul, Kumar, Jha, & Sen, 2015), pectin (Sharma, Naushad, Pathania, & Kumar, 2015), starch (Sarmah & Karak, 2020; Yusof & Kadir, 2016), alginate (Swain, Patnaik, & Dey, 2013), agar (Saidi, Boudrahem, Yahiaoui, & Aissani-Benissad, 2019), xanthan gum (Moremedi et al., 2020), guar gum (Dinari, Shirani, Maleki, & Tabatabaeian, 2020) and polyhydroxyalkanoates (Goudarztalejerdi, Tabatabaei, Eskandari, Mowla, & Iraji, 2015; Mukheem et al., 2019) have also been utilized for a wide range of environmental applications as sorbents. Musarurwa and Tavengwa (2020) recently published a review on the use of of carboxymethyl polysaccharides as bio-sorbents for the sequestration of heavy metals in aquatic environments.

The use of biopolymers is not only restricted to the removal of dyes and heavy metals, but extends to a range of pollutants including nitrates (Rajeswari, Seenivasagan, Prabhakaran, Rajakumar, & Ayyasamy, 2016), phosphates (Pan et al., 2019), fluorides (Raghav, Nehra, & Kumar, 2019) and pesticides (Rissouli, Benicha, Chafik, & Chabbi, 2017). They also find their niche in other environmental applications. They serve as renewable substrates and biocatalysts in hydrogen production (Ren, Zhao, Liang, Ma, & Guo, 2017). They could be used as natural soil strengthening materials in the construction sector (Fatehi, Abtahi, Hashemolhosseini, & Hejazi, 2018). Over the past several decades, there has been a growing need for eco-friendly biosorbents to be used in the pharmacological sector. Uses of these natural materials may range from diagnosis tissue or bone repair (Iravani & Varma, 2019), drug delivery systems (de Oliveira Pedro, Goycoolea, Pereira, Schmitt, & Neumann, 2018), amongst others. Biopolymers have numerous advantages over synthetic polymers, as they exhibit biodegradability, biocompatibility, low immunogenicity and antibacterial activity (Ren et al., 2017).

This review seeks to complement earlier reviews on biopolymers as extractants. However, to the best of our knowledge, there is no review highlighting the application of biopolymers in the extraction of nutraceutical metabolites from plants such as *Solanum*. Hence, this work aims to provide collective information and enhance the readers' understanding on the paradigm shift from synthetic extractants to more eco-friendly biosorbents. This work will provide a general update on the applications of biopolymers in the extraction of phytocompounds and the subsequent usage of the obtained compounds in metabolomics, particularly in drug delivery. Thereafter, the challenges and recommendations are discussed to equip the reader on potential future applications of these biopolymers.

2. Importance of metabolites derived from natural products

The World Health Organization (WHO) has estimated that more than 80 % of the world's population is highly dependent on the use of metabolites derived from natural products for nutrition and treatment of a variety of ailments (WHO, 2008). With over 4000 known plant metabolites, researchers have not only aimed to distinguish these compounds, but incorporate them into food and pharmacological drugs (Sharma et al., 2019; Thakur, Ambwani, Ambwani, Ahmad, & Rawat, 2018). Metabolites possess discrete bio-activities with a variety of health benefits and are sometimes referred to as functional ingredients/nutraceutical compounds, some of which are identified as carotenoids, vitamins, minerals, fatty acids, peptides, proteins and secondary plant metabolites. Additional nutraceutical compounds abundant in fruits, vegetables, nuts, cereals and legumes include phenolics, terpenoids, glucosinolates, pollyacetylene, phytosterols, phytostanols and non-digestible carbohydrates. For instance, linoleic acid (fatty acid) found in protein rich food such as milk (O'Callaghan, Sugrue, Hill, Ross, & Stanton, 2019) and cheese are known for treatment of a variety of cancers (Mushtaq, Gani, & Masoodi, 2019). Phenolics contained in *Salvia rosmarinus*, *Fragaria* × *ananassa* and *Mentha* have been studied for anti-inflammatory (Miamoto et al., 2019), anti-cancer (Bonta, 2020) and antioxidant activities (Nwanna, Ibukun, & Oboh, 2019). flavonoids such as isorhamnetin, kaempferol, myricetin, quercetin in blue-berry (*Cyanococcus*), Broccoli (*Brassica oleracea*) and Spinach (*Spinacia oleracea*) have been applied in antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic studies (Ayaz et al., 2019). Terpenoids reported in *Salviadivinorum*, *cannabis*, *ginkgo biloba* are known for their anti-ulcer and anti-diuretic properties (Tlacomulco-Flores, Déciga-Campos, González-Trujano, Carballo-Villalobos, & Pellicer, 2019), while saponins from *Chenopodium quinoa* have been successfully evaluated for cholesterol degradation (Dong et al., 2020).

3. Metabolites derived from *Solanum* nutraceutical plants

One plant taxon that is arguably distinguished from *Spiniacia* and *Cyanococcus*, *Salviadivinorum*, in its widespread nutraceutical applications in the food industry and medicine, is the Solanaceae family. Boasting over 2000 species worldwide, Solanaceae plants have an enormous potential to deliver new chemicals for crop protection (Jayasinghe et al., 2017; Mandal et al., 2019). A diverse array of these compounds, or mixtures of these compounds, are being identified as pest control agents, especially against insects, fungi and mites (Ali, El-Tokhyorcid, El-Sherbini, Abdel-dayem, & Khpalwak, 2019; Nawaz et al., 2020) as summarized in Table 1. Solanaceae have wide applications in food production (e.g., tomatoes and potatoes), medicine (e.g., deadly nightshade and jimson weed) and drugs (e.g., tobacco) (Datir, Mirikar, & Kumar, 2019; Mabele & Ndong, 2019). Tomato (*Solanum lycopersicum*) have been reported to contain tomatine, carotenoids and guanosine which have been studied for cholesterol reduction, antioxidants and thromboembolic disorder, respectively (Table 1). Of the carotenoids in *Solanum lycopersicum*, 87 % is lycopene which is a natural antioxidant (Kristina et al., 2019; Soma, 2013). This nutraceutical compound (lycopene) has also been applied as food additives for modification of colour and functional characteristics (Li et al., 2018; Sipos et al., 2010). As a strong antioxidant, lycopene was reported by Shi and Maguer (2000) to exhibit a physical quenching rate constant double that of beta-carotene (caternoid). Other antioxidant compounds such as caffeoyl quinic acids and flavonoids have been reported in *Solanum melongena* and *Solanunm aethiopicum* (Table 1). In addition to antioxidant activity, Howes, Perry, Vásquez-Londoño, and Perry (2019) and Liu et al. (2020) highlighted that caffeoyl, dicaffeoyl and feruloyl-quinic acids played important roles in improved cognitive functions. Interestingly, some of these antioxidant compounds which include closely related compounds to caffeoyl quinic acids such as caffeic acid, p-coumaric and ferulic acid were reported to increase in concentration in *Solanum* lycopersicum when exposed to sunlight (Ray, Saha, Raychaudhuri, & Chakraborty, 2016). The majority of the compounds reported in Solanancae were flavonoids, phenols and polyphenols reported in *Solanum macropon L.*, *solanum Spp*, *Solanum torvum* and *Solanum tuberosum* associated with treatment of neurodegenerative and diabetes related ailments (Table 1).

In addition to the phenolics, flavonoids, terpenoids, reported in *Salvia rosmarinus*, *Brassica oleracea*, *Spinacia oleracea* and *Salviadivinorum*, *cannabis*, *Solanum* plants also contain a class of compounds, glycoalkaloids. Glycoalkaloids are toxic secondary metabolites consisting of a C27 cholestane aglycone unit with one or more, up to five, monosaccharide chains bonded to position 3 of the aglycone unit (Morillo,

Table 1
Metabolites obtained from their *Solanum* plants and their pharmacological activities.

Solanum species	Metabolite	Pharmacological activity	Reference
Solanum melongena	Caffeoyl quinic acids	Antioxidants	Wu et al. (2013)
Solanum lycopersicum	Tomatine	Cholesterol reduction	Friedman, Kozukue, & Harden (1998)
Solanum lycopersicum	Cartenoids	Antioxidants	Perveen et al. (2015)
Solanum lycopersicum	Guanosine	Thromboembolic disorder	Fuentes et al. (2013)
Solanum lycopersicum		Anti-cardiovascular dysfunction	Alam et al. (2018)
Solanum aethiopicum	Flavonoids	Antioxidants	Nwanna et al. (2019)
Solanum retroflexum	Quercetin	Antioxidant	Daji et al. (2018)
Solanum macrocarpon L.	Kaempferol-3-rutinoside	Diabetes mellitus	Ajiboye et al. (2018)
Solanum nigrum	Alkaloids	Erectile dysfunction	Omojokun, Famurewa, Jaiyeoba, Oboh, and Agbebi (2019)
Solanum macrocarpon	Rutin, caffeic acid, myricetin	Blood pressure and heart rate reduction	Oluwagunwa et al. (2019)
Solanum tuberosum peels	Polyphenols	DNA oxidation	Javed et al. (2019)
Solanum tuberosum peels	Polyphenols	Hyperglycemia	Singh et al. (2005)
Solanum gilo raddi	Polyphenols	Antioxidant	Miamoto et al. (2019)
Solanum quitoense	Spermidine	Obesity and hypertension	Betoret, Hinestroza, Seguí, and Barrera (2019)
Solanum kumba	Polyphenols	Type II diabetes	Nwanna et al. (2019)
Solanum Spp	Phenols, flavonoids, alkaloids	Neurodegenerarive diseases	Ogunsuyi et al. (2020)
Solanum macrocarpon L.	Phenols, flavonoids, alkaloids	Neurodegenerarive diseases	Ogunsuyi et al. (2020)
Solanum nigrum	Solanine	Anti-inflammatory	Zhao et al. (2018)
Solanum tuberosum	α-chaconine	Inhibition of endometrium cancer	Arslan and Yerer (2018)
	α-tomatine	Apoptosis of human glioblastoma cells	Wang, Dai, & Liu (2017)
	Solamargine	Apoptosis of human chordoma cells	Liu, Wang, Xu, Qi, and Zhang (2019)

Rojas, Lequart, Lamarti, & Martin, 2020) (Fig. 1). Considering a known total of up to nine monosaccharides, to date, they can bond to form disaccharides or even polysaccharides with unique chemical arrangements (Sonawane, Jozwiak, Panda, & Aharoni, 2020). Furthermore, these polysaccharides can be glycosylated to alkaloid aglycones units, resulting in a diverse range of glycoalkaloids (Sonawane et al., 2020). For instance, Lelario et al. (2019) reported on 19 different glycoalkaloids obtained from *Solanum melongena* extracts, and Tata, Perez, Hamid, Bayfield, and Ifa (2015) identified 10 different glycoalkaloids from *Solanum tuberosum*. Common vegetables such as Tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) were reported by Chowański et al. (2016) to contain steroidal glycoalkaloids. For instance, α-solanine and α-chaconine, solasonine and solamargine, as well as α-tomatine and dehydrotomatine are major steroidal glycoalkaloids in *Solanum tuberosum L.*, *Solanum melongena* L. and *Solanum lycopersicon*, respectively (Chowański et al., 2016) (Fig. 1). Glycoalkaloids synthesis, as a protective measure by *Solanum* plants, is triggered by mechanical stress, exposure to light and sprouting (Chowański et al., 2016). The toxic effects of glycoalkaloids include disruption of biological systems. Nepal and Stine (2019) reported on the ability of glycoalkaloids to form complexes with lipid bilayers of membranes such as 3β-hydroxysterols consequently leading to cell death. Fowsiya and Madhumitha (2020) reported on the destructive effects of plant derived alkaloids in the disruption of physiological and cellular systems in redox systems. In a separate study, Spochacz et al. (2018) reported on the reproductive toxicity of *Solanum* nigrum derived glycoalkaloids on a beetle (*Tenebrio molitor*). Bechtel et al. (2010) reported on poisoning, nausea and hypotention of a patient due to intake of glycoalkaloids from *Veratrum viride*. Another study by Glover et al. (2016) reported on a case of poisoning of a 54 year old woman after intake of a glycoalkaloid (solasonine). Furthermore, the Centre for Food Safety (2015) reported on poisoning of patients after consumption of cooked potatoes, subsequent investigations revealed that the poisoning was due to the glycoalkaloid solanine. As a result, regulatory bodies such as the Commission for Food and Agricultural Organization (FAO) and the World Health Organization (WHO) have established regulations for maximum permissible concentrations of glycoalkaloids, which currently stands at 200 mg kg^{-1} for fresh potatoes (*Solanum* lycopersicum) sold in super markets.

In spite of the generally known toxic attributes of *Solanum* derived glycoalkaloids, researchers have also found these compounds to be very useful. For instance, Ordonez-Vasquez, Aguirre-Arzola, De la Garza-Ramos, Urrutia-Baca, and Suarez-Obando (2019) reported on the anti-cancer activity of α-solanine, Arslan and Yerer (2018) communicated the inhibition efficiency of α-chocanine against malignant cells of the endometrium, while α-tomatine led to apoptosis of brain tumour cells. In another study, Zhao et al. (2018) outlined the activity of solamargine in prevention of malignant human chordoma cells (Table 1). Evaluative tests involving solamagine (Fig.1) have not only been limited to inhibition of cancer, but extended to parasites, for example, Moreira et al. (2013) obtained solamargine from crude ethanolic extracts of *Solanum palinacanthum* ethanolic extract and were shown to be active (IC_{50} = 15.3 μg mL^{-1}) against the parasite of Chagas' disease. Additionally, due to the same aglycone units (Fig. 1), both solamargine and α-solanine have had similar applications. For example, both glycoalkaloids have applications as contraceptives and anti-inflammatory agents (Al-Ashaal, 2019; Tiossi et al., 2012). Additionally, solamargine and α-solanine with solasonine, and solasodine were studied to show hepato-protection against D-galactos amine-induced hepatic fibrosis in rats (Chester et al., 2019). Despite the usefulness of these glycoalkaloids, a balance is often needed to be struck by researchers as to the adequate dosages for medical purposes to avoid toxicity. Though there is limited literature on the threshold dosages of glycoalkaloids for medical treatment, a pilot study evaluated toxic concentrations of potato derived α-chaconine and α-solanine with concentrations in the range 0.30–1.25 mg kg^{-1} body weight (BW), as conducted by Mensinga et al. (2005). The same study indicated that α-chaconine and α-solanine at 1.25 mg kg^{-1} was enough to cause nausea and vomiting. This suggests that α-chaconine and α-solanine concentrations below 1.25 mg kg^{-1} are deemed safe for intake by humans for medical treatment purposes. In view of the highly essential nutraceutical compounds obtained from *Solanum* plants and its applications in food additives and medicines, there is an ever growing need to extract these compounds. Traditional extraction techniques such as soxhlet presented challenges such poor extraction, need for exorbitant amount of toxic organic solvent and has been known to be time-consuming (Ghorbani, Aboonajmi, Ghorbani Javid, & Arabhosseini, 2017). Hence, a continuous need arises for the development of robust adsorbents.

4. Applications of synthetic adsorbents and its limitations

Over the past several decades, demand for nutraceuticals has increased substantially. Currently, increased health care costs and drug resistant bacteria or viral strains, has led to over 80 % of the world's 7.5

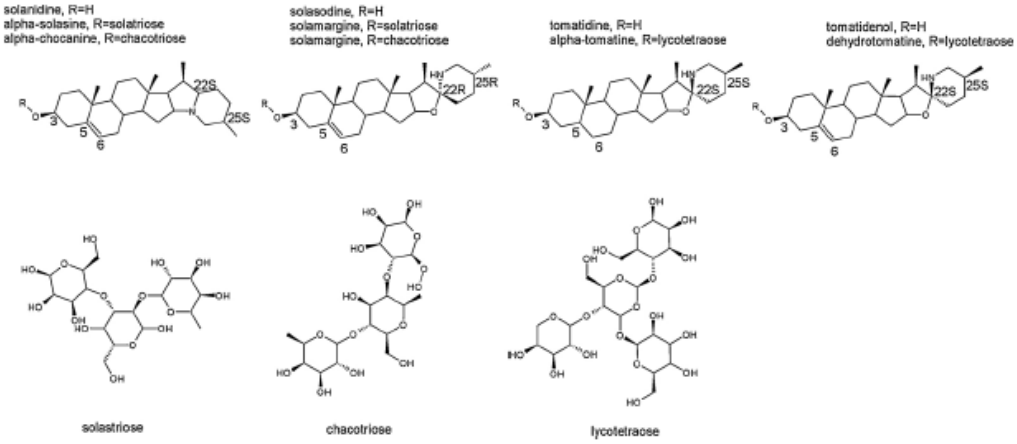


Fig. 1. Some of the glycoalkloids found in *Solanum melongena*, *Solanum tuberosum* and *Solanum lycopersicum*.

billion people being reliant on nutraceuticals for the betterment of their lives (Asif & Mohd, 2019; Gulati, Verma, Rai, & Ray, 2021). The most commonly employed method for the removal of organic pollutants or desired metabolites from water or matrices of plants are through adsorption. A range of synthetic polymeric adsorbents have been studied which include, synthetic polymers like chelating resins (Polowczyk et al., 2017), ion-exchange resins (De Abreu & da Fonseca, 2018), carbon black (Horikawa et al., 2017), biochar (Fan et al., 2017), zeolite (Hong et al., 2019), synthetic allophanes (Baldermann et al., 2018) and polystyrene (Ahmad, Chaojie, & Liu, 2019). Despite the diverse uses of synthetic polymers, the majority of these adsorbents are associated with high costs and regeneration challenges make this class of materials unattractive and tedious to work with. Additionally, these synthetic polymers often resulted in production of toxic by-products, making the technique inefficient (Mahir, Keya, Sarker, Nahiun, & Khan, 2019). Therefore, researchers have directed their efforts towards renewable,

Table 2
Extraction of common metabolites from Solanacae by biopolymers.

Biopolymer	Nutraceutical compound	Matrix	Extraction efficiency (%)	Adsorption capacity (mg g^{-1})	Reference
Silk fibroin	Rutin	Olea europaea L.	–	15	Altıok, Bayçın, Bayraktar, and Ülkü (2008)
Silk fibroin	Oleuropin	Olea europaea L.	–	96	Altıok et al. (2008)
Cross-linked collagen fibre	Kaempferol	–	18	–	Ding et al. (2012)
Cross-linked collagen fibre	Quercetin	–	45	–	Ding et al. (2012)
Cross-linked collagen fibre	Myricetin	–	65	–	Ding et al. (2012)
Calotropis gigantea fibre (milkweed)	Rapeseed oil	–	–	4–1700	Zheng, Zhu, Wang, and Hu (2016)
Collagen fibre	Baicalein	Scutellaria	83.42	–	Zhang, Li, Zhang, Liao, and Shi (2013)
Collagen fibre	Baicalin	Scutellaria	94.31	–	Zhang et al. (2013)
Lignocellulose	Catechins	tea extracts	–	209.409	Ye et al. (2009)
Cellulose coated $CoFe_2O_4$	Ferulic acid	vinegars	86–108	–	Minho et al. (2019)
Cellulose coated $CoFe_2O_4$	p-coumaric acid	vinegars	85–104	–	Minho et al. (2019)
Cellulose coated $CoFe_2O_4$	Chlorogenic acid	vinegars	87–108	–	Minho et al. (2019)
Tragacanth gum-based nanogel	Quercetin	–	–	173.45	Hemmati et al. (2016)
TG/poly(MMCA)-g-poly (caprolactone)	Quercetin	–	58	–	Hemmati et al. (2016)
TG-g-poly(DM)-poly (ε-caprolactone)-PEG	Quercetin	–	86.71	–	Hemmati and Ghaemy (2016)
TG-g-Poly(vinylimidazole)/ Fe_3O_4@SiO_2	Quercetin	–	75	–	Hemmati et al. (2016)
Rhein functionalized magnetic chitosan	Isoflavones	soymilk	–	–	Guo, Xue, Yao, Cai, and Qian (2017)
Chitosan	Salicylic acid	–	–	149.7 μmol g^{-1}	Rahangdale, Archana, and Kumar (2016)
Chitosan-4hydroxy benzoic acid MIP	Salicyclic acid	–	–	220 μmol g^{-1}	Rahangdale et al. (2016)
Corn silk	Flavonoids	–	–	5.32	Wu, Ye, and Wang (2017)
Cellulose	Ferulic acid	–	–	0.017	Phan et al. (2015)
Cellulose	chlorogenic acid	–	–	0.020	Phan et al. (2015)
Cellulose	Cyanidin-3-glucoside	–	–	0.057	Phan et al. (2015)
Chitosan reduced graphene	Catechins	–	–	173	Zhang, Chen, Feng, Yu, and Jiang (2019)
NP-F-AE*	Solamargine, solsonine	–	85	–	Miranda et al. (2020)

* NP-F-AE = glycoalkaloid extract loaded folate-targeted nanoparticles.

affordable, non-toxic, higly selective and biocompatible materials, biopolymeric extractants.

5. Biopolymers as adsorbents of nutraceuticals

5.1. Cellulose

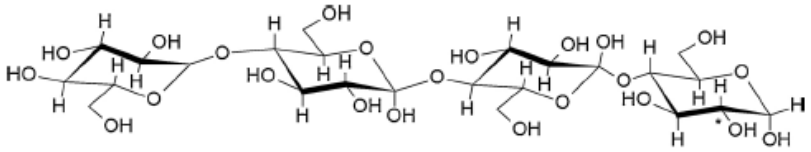
Not only have biopolymers been used in the extraction of organic pollutants such as dyes (Chen, Messing, Zheng, & Pullerits, 2019; Kumar, Dosanjh, & Singh, 2019) and pesticides (Durán, Bueno, Hermosín, Cox, & Gámiz, 2019), but has extended to extraction of nutraceuticals from plants (Table 2). Polysaccharides such as cellulose have been used quite extensively in the extraction of nutraceuticals (Table 2). Cellulose is composed of D-glucopyranose ring units in the chair configuration as shown in Fig. 2 (a), which exhibits the lowest energy conformation (Heinze, 2015; Rao, Sundararajan, & Ramakrishnan, 1967). Such units are linked by β-1,4-glycosidic bonds that results in an alternate turning of the cellulose chain axis by 180. Cellobiose with a length of 1.3 nm can be considered the repeating unit of cellulose (Heinze, 2015; Krässig, 1993). Three reactive hydroxyl groups exist in each anhydroglucose unit within the cellulose chain, a primary group at C6 and two secondary groups at C2 and C3 that are positioned in the plane of the ring. Phan et al. (2015) evaluated cellulose for the extraction of polyphenols such as ferulic acid, chlorogenic acid and cyanidin-3-glucoside with adsorption capacities of 17, 20 and 57 $\mu g\ g^{-1}$, respectively. These authors deduced that phenolic compounds having more aromatic rings in their molecular structure tended to form strong hydrogen bonds with cellulose than those compounds with a single aromatic ring, hence cyanidin-3-glucoside had a better adsorption capacity than ferulic acid and chlorogenic acid. This indicated that interaction of polyphenols with polysacharrides (cellulose) was mainly driven by hydrogen bonds and hydrophobic interactions. Lately, the majority of researchers turned their attention to the application cellulose nanoparticles with the aim of improving the surface area of the particles as determination of nutraceuticals at trace concentration levels often made quantification impossible due to possible matrix effects. One example is the study by Minho et al. (2019), where they evaluated cellulose coated $CoFe_2O_4$ naoparticles for adsorption of polyphenols such as ferulic acid, p-coumaric acid and chlorogenic acid, all the materials had extraction efficiencies of 85–108 %. Another class of polyphenols such as flavonoids have also been investigated for adsorption using biopolymers. Ding, Liao, and Shi (2012) obtained extraction efficiencies for kaempferol, quercetin and myricetin on glutaraldehyde cross- linked collagen fibre were reported as 18, 45, 65 %, respectively (Table 2). These authors deduced that the extent of adsorption of these flavonoids to the glutaraldehyde cross-linked collagen fibre was due to hydrogen bonding interaction. The hydrogen bonding interaction of flavonoids during adsorption as highlighted by Ding et al. (2012) was consistent with what was reported by Phan et al. (2015) and Bordenave, Hamaker, and Ferruzzi (2014). Despite polysaccharides such as chitosan, cellulose and cross-linked collagen fibres' being used for adsorption of nutraceuticals, most of these biopolymers had drawbacks particularly in drug delivery systems. Some of the disadvantages included, poor viscosity, uncontrolled rates of hydration and solubility, and contamination by micro-organisms of which attempts were made to minimize these negative effects via cross-linking as seen in the study by Ding et al. (2012).

5.2. Chitosan

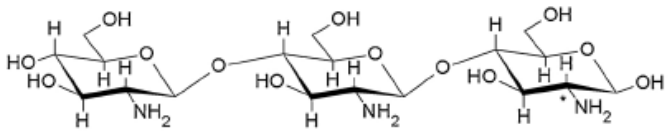
Chitosan is very similar in structure to cellulose as it contains approximately several thousand β-(1-4) linked D-glucose units (Bhuiyan, Shaid, & Khan, 2014) (Fig. 2 (b)). In chitosan, the hydroxyl at position carbon 2 (C-2) of cellulose is replaced by an acetamide group (Fig. 2 (b)). Zhang & Shi (2012) applied solid phase extraction of flavonoids from green tea using chitosan. The reported recoveries for the flavonoids which included quercetin, luteolin, kaempferol, and isorhamnetin were observed as 103.7, 101.3, 104.2 and 108.6 (%), respectively. Additionally, flavonoids such as quercetin and kaempferol have been reported in several *Solanum* species which include *Solanum retroflexum* (Daji, Steenkamp, Madala, & Dlamini, 2018), and *Solanum macrocarpon L* (Ajiboye et al., 2018) (Table 1). In another study, Soares et al. (2020) evaluated the adsorption of quercetin from butanol extracts of *Passiflora edulis* onto chitosan hydrogels. Though the concentration of the adsorbed quercetin was not resported in the same study, administration of chitosan hydrogels with the adsorbed quercetin onto wounds of male Wistar rats stimulated the antioxidant defense system. This indicated that chitosan containing adsorbed flavonoids such as quercetin, could be efficient agents in wound treatment

5.3. Gum tragacanth

One of several biopolymers that was shown to exhibit good metabolite adsorption characteristics for biomedical applications is gum tragacanth. As a result of the non-toxicity and safety of gum tragacanth for oral intake (Hemmati, Masoumi, & Ghaemy, 2016; Monjezi, Jamaledin, Ghaemy, Moeini, & Makvandi, 2018), biocompatibility and eco-friendliness, stability over wide pH range (Zare, Makvandi, & Tay, 2019), gum tragacanth was studied by Hemmati and Ghaemy (2016) for adsorption of quercetin. In their study, Hemmati and Ghaemy (2016)



(a)



* = Carbon 2 (C-2) (b)

Fig. 2. Representation of (a) cellulose and (b) chitosan.

observed that quercetin, a water-insoluble metabolite, adsorbed well to gum tragacanth improving its bioavailabilty, paving way for future applications in oral drug delivery systems.

Biopolymers as Solanancae nutraceutical carriers in drug delivery systems

A number of nutraceutical compounds derived from *Solanum* plants were evaluated for their ability to be loaded on biopolymeric drug delivery systems as shown in Table 3. The paradigm shift from synthetic drug carriers to biopolymers was influenced by lackluster solubility of the carried metabolites, poor intestinal adsorption, detrimental side effects and minimal therapeutic potency, amongst others (Jacob, Haponiuk, Thomas, & Gopi, 2018; Kukoyi, 2016).

5.4. Short linear chain gum arabic (SLG@GA)

Biopolymers as carriers for nutraceutical compounds have the following advantages which include; increased bioavailability, enhanced biodegradation, biocompatibility and reduced toxicity (Gopi, Amalraj, & Thomas, 2016; Jacob et al., 2018). For instance, some applications include the use of the polysaccharide short linear chain gum arabic (SLG@GA) for adsorption of ferulic acid and chlorogenic acid, with an encapsulation of 72 % and 92 % (Table 3), respectively, as reported by Li et al. (2017). The relatively higher encapsulating affinity for chlorogenic acid compared to ferulic acid was mainly due to hydrogen bonding with SLG@GA. Furthermore, the fewer phenolic hydroxyl and carboxyl groups in ferulic acid led to its hydrophobicity and reduced interaction with SLG@GA. Both ferulic acid which is also a precursor for cinnamic acids such as ferulic quinic acids, and chlorogenic acid were reported by Ray et al. (2018) in *Solanum lycopersicum* and Daji et al. (2018) in *Solanum retroflexum*.

5.5. Chitosan

Chitosan has also been studied as a carrier for quercetin, a flavonoid common in *Solanum* plants such as *Solanum torvum*, *Solanum aeithiopicum* and *Solanum macrocarpon* as communicated by Khatoon, Sharma, and Dubey (2018). Chitosan was studied by Pedro, Pereira, Goycoolea, Schmitt, and Neumann (2018) for delivery of quercetin for treatment of breast cancer. Besides encapsulation percentages of 78 % highlighted by these authors, most researchers were interested in further improving encapsulation capability of nutraceuticals, hence they turned to biopolymeric nanocomposites. For instance, Khan et al. (2019) reported on lipid-chitosan hybrid nanoparticles as a carrier for cisplatin in treatment of ovarian cancer and achieved relatively higher encapsulation efficiencies of 89 % compared to what was reported for chitosan only, by Pedro et al. (2018), though the carrier drug was different. The cytotoxic effects of cisplatin against multi drug resistant cancer cells was reported to be enhanced when used with aqueous extracts of *Solanum nigrum* as described by Tai et al. (2013) (Table 3). Other examples where biopolymeric nano-composites were used include lecithin/chitosan liposomal nanoparticles and lecithin-chitosan nanoparticles with entrapment capacities of 96 and 92 % for capsaicin and clobetasol propionate, respectively (Table 3). Capsaicin extracted from *Solanum mauense* was reported by Chirchir, Cheplogoi, Omolo, and Langat (2018) to be useful in treatment of cancer related illnesses. Chitosan has also been evaluated as a carrier for dopamine. For instance, Trapani et al. (2013) and De Giglio, Trapani, Cafagna, Sabbatini, and Cometa (2011) both examined chitosan nanoparticles as carriers for dopamine to the brain. However, transfer of dopamine to the brain was limited. As a result, the incorporation of dopamine containing nanoparticles with bio-friendly materials have captured the interests of many researchers. Considering that chitosan is insoluble in water, Ren et al. (2017) designed a quaternized chitosan system to improve its hydrophillicity and was highly regarded as nontoxic, biocompatible and efficient dopamine carriers for long term release into the brain for treatment of Parkinson's disease.

6. Challenges and future trends

Biopolymers have shown to be promising adsorbents of organic pollutants such as dyes and pesticides in wastewater. More importantly, the introduction of biopolymers has significantly improved the pharmokinetics and pharmodynamics of nutraceuticals in drug administration, addressing problems such as poor solubility, instability and low bioavailability. Biopolymers such as gum arabic and chitosan have successfully been applied in treatment of various illnesses which include ovarian cancer (Jaafar, 2019), Parkinson's disease (Ghamami, Golzani, & Lashgari, 2016) and diabetes (Babiker, Elmusharaf, Keogh, & Saeed, 2018). Despite the numerous advantages of biodegradable polymers in waste treatment and drug delivery, there is still room for improvement in the application of these materials. Improved performance can be achieved through modified biopolymers. In addition to physical and chemical modifications discussed, other applications involving nano scaled multilayer films, complexing agents or molecular imprinted

Table 3
Some biopolymers as drugs carriers containing nutraceutical compounds derived from *Solanum* plants.

Biopolymer	Nutracuetical drug carried	Encapsulating efficiency (%)	Loading capacity (%)	Medical application	Reference
Chitosan nanopartiles	Quercetin	78	–	Breast cancer	Pedro et al. (2018)
Short linear glucans@gum arabic (GA)	Ferulic acid	72.16 ± 1.52	24.05 ± 0.53	Antioxidant	Li et al. (2017)
Short linear glucans@gum arabic (GA)	Chlorogenic acid	92.03 ± 0.61	30.68 ± 0.23	Antioxidant	Li et al. (2017)
Zein nanoparticles	ß-carotene	91	–	Antioxidant	Wang et al. (2018)
NaCAS-CMC	Polyphenols	72	–	–	Estévez, Güell, De Lamo-Castellví, and Ferrando (2018)
NaCAS-GA	Polyphenols	78	–	–	Estévez et al. (2018)
Quaternized chitosan hydrogels	Dopamine	–	–	Parkinson's disease	Ren et al. (2017)
Zein-particle nanoparticles	Resveratol	97.6 ± 0.7	86.4 ± 3.5	Anti-cancer	Huang et al. (2017)
Lipid-chitosan hybrid nanoparticles	Cisplatin	89.2 ± 0.5	2.11 ± 0.7	Ovarian cancer	Khan et al. (2019)
Lecithin-chitosan nanoparticles	Piperine	52.91 ± 3.56	8.46 ± 0.57	MDR cancer therapy	Alkholief (2019)
Lecithin-chitosan nanoparticles	Doxorubicin	45.96 ± 2.63	8.93 ± 0.82	MDR cancer therapy	Alkholief (2019)
Lecithin/chitosan liposomal nanoparticles	Capsaicin	96.80 ± 0.59	96.0	Muscle pain	Terrón-Mejía et al. (2018)
Lecithin-chitosan nanoparticles	Beta-lapachone	52.4 ± 2.4	25.8 ± 7.4	Cutaneous leishmaniasis	Moreno et al. (2019)
Lecithin-chitosan nanoparticles	Clobetasol propionate	92.2 ± 0.5	10.9	Edema inhibition	Şenyiğit et al. (2016)

MDR-multi drug resistance.

polymers can also be explored to a greater depth. These diversified biopolymers can potentially function as efficient carriers of bioactive nutraceuticals compounds such as quercetin, kaempferol and myricetin of which have been widely applied as antioxidants at target sites with the aid of biopolymers (Daji et al., 2018; Miamoto et al., 2019; Nwanna et al., 2019; Wu et al., 2013). However, considering the vast range of alkaloid compounds in Solanacae, these metabolites can potentially be applied in pharmacology with unique therapeutic effects.

One of the most abundant and highly diverse and toxic alkaloids in Solanaceae include glycoalkaloids and tropane alkaloids. Glycoalkaloids such as solanine, chaconine and solamargine have been reported for the treatment of various cancers due to its toxicity (Burger et al., 2018; Chen et al., 2021). However, the efficacy of these drugs at the target sites have often been limited due to its poor bioavailability, instability and incompatibility synthetic drug carriers. To address this problem, the need arises for biodegradeable nanomaterials that can be flexible enough to permeate through difference regions of infected cells while maintaining the integrity of the delivered compound glycoalkaloids or tropane alkaloid.

The mostly studied mode of adsorption between the polysaccharide drug carrier and the drug being carried is hydrogen bonding, considering that the drug contains hydroxyl groups such as polyphenols which are generally hydrophilic (Bordenave et al., 2014; Ding et al., 2012; Phan et al., 2015). The challenge, however, arises during transportation of generally hydrophobic compounds such as aglycones, glycoalkloids and tropane alkaloids to target sites. Hence, multi-functional polysaccharides containing various functional groups are required that can accommodate a range of chemical interactions such as hydrogen, ionic, dipole-dipole or dispersion forces. Other researchers investigated hybrid biopolymeric nanomaterials to improve their adsorption characteristics (Alkholief, 2019; Khan et al., 2019). Furthermore, the inclusion of molecular imprinted polymers have also been looked into with the aim of improving drug delivery while minimizing the influence of external stimuli such as pH changes. Lastly, demand for recyclable nano-materials that can repetitively administer the intended drug to the target site is essential, as it is cost effective. For instance, Ge et al. (2016) observed that lignin microspheres could be used up to five times for removal of lead and Sirajudheen, Nikitha, Karthikeyan, and Meenakshi (2020) investigated magnetic Fe_3O_4 reinforced graphene oxide–carboxymethyl cellulose for adsorption of toxic azo dyes from water, with a maximum of 5 adsorption cycles. Other biopolymers that were shown to be recyclable include chitosan biopolymers derived from crab shells for the adsorption of an emerging pollutant, ketoprofen, with up to 10 adsorption cycles as studied by Rizzi et al. (2019).

7. Conclusions

Biopolymers are derived from living organisms such as plant cell wall structures and possess desirable characteristics such as being biodegradeable, biocompatible, affordable and sustainable in comparison to synthetic polymers. Common biopolymers include polysaccharides such as cellulose and chitosan. Besides having applications in wastewater treatment, polysaccharides have been widely studied as efficient biodegradeable drug carriers for treatment of cancer related illnesses, viral, bacterial or fungal infections. The narrative behind the success of these natural compounds is mainly due to the presence of functional groups namely, hydroxyl and amino groups which are responsible for formation of hydrogen bonding interactions with the drug being carried. Furthermore, the development of polysaccharide nanoparticle drug carriers improve the surface area to volume ratio for adsorption of nutraceuticals. Lately, the application of nano-composite biopolymers such as lechitin-chitosan, functionalized nano-biopolymeric materials and crosslinked nano-carriers are all modifications to improve the mechanical strength and stability of the natural compounds during drug administration. Additionally, lipid-derived biopolymers have been studied to improve the permeability through hydrophobic regions such as the lipid-bilayer of cells or blood brain barriers, permitting for increased efficacy at the target site. Some of the drugs that have been carried by polysacharrides for drug delivery purposes include *Solanum* derived polyphenolic nutraceuticals such as quercetin, kaempferol, mycetrin and chlorogenic acids, amongst others. Considering the over 2000 species Solanaceae boasts and the diverse range of toxic glycoalkaloids such as solamargine, solasonine and chaconine, polysacharride drug carriers still have a lot to offer as biocompatible transport systems for glycoalkaloids in treatment of cancer related illnesses.

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Paper 7

The study was directed at the use of UPLC-qTOF-MS for simultaneous extraction of HP-TLC fluorescent compounds obtained from *Solanum retroflexum*.

Application of TLC and UHPLC-QTOF-MS for identification of aqueous two phase extracted UV-fluorescent metabolites from *Solanum retroflexum*

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Abstract

This work aimed to identify UV-flourescent metabolites obtained via aqueous two phase extraction from *Solanum retroflexum* (*S. retroflexum*). Of the compounds simultaneously extracted via aqueous two phase extraction, nine UV-fluorescent compounds were identified. Using HPTLC and UPLC-qTOF-MS, two of the nine metabolites were identified as glycoalkaloids, ie., alpha-tomatine and gamma-solanine. To date, the glycoalkaloid alpha-tomatine has generally been limited to *S. lycopersicum*. However, this study revealed that alpha-tomatine can also be found in *S. retroflexum*. Though tomatine and gamma-solanine are known for host-plant resistance, these metabolites also exhibit beneficial health effects as food supplements and have been an essential diet in rural South African communities as an abundant and inexpensive source of nutracecutical compounds. The use of ATPE could possibly strike a balance in both maintaining bioactivities after extraction while simultaneously reducing toxicity in *S. retroflexum*. Furthermore, small scale extraction using ATPE could possibly be escalated to a competitive trade business.

Keywords ATPE •·metabolites • *S. retroflexum* • TLC • Flourescence

1 Introduction

Solanum species are renowned for their rich diversity of metabolites. Some of the many metabolites include flavonoids and glycoalkaloids which have been reported to exhibit antioxidant and protective roles, respectively [1]. *Daji et al.* [2] investigated the phytochemical profile of *Solanum retroflexum* leaves using methanolic extracts and found a range of cinnamic acids, polyphenols and alkaloids. Though methanolic extraction has been applied quite extensively [2–6], this approach is accompanied by setbacks such as the use of toxic organic solvent which often requires large volumes for extraction solvents, making it a costly exercise. Hence, there is a dire need for effective, eco-friendly, single-step extraction techniques.

Aqueous two-phase extraction (ATPE) is one such method that offers efficient, eco-friendly quick metabolite extraction. ATPE uses salts that allow for partitioning of a green extraction solvent, usually ethanol, from water, resulting in the extraction solvent being simultaneously enriched in the desired metabolites [7]. ATPE extracted phytocompounds were reported by *Mokgehle et al.* [8] with a range of cinnamic acids, polyphenols and alkaloids being obtained from *S. retroflexum*. Some of the ATPE extracted alkaloids include solanelagnin, solamargine, solasonine, β-solanine. The same authors also reported ATPE as being essential for the simultaneous extraction of multiple metabolites. Some studies have been directed at the use of thin-layer chromatography (TLC) methods for the isolation of potentially bioactive compounds from *Solanum* species. For instance, *Shamsaldin et al.* [9] applied TLC for the isolation of flavonoids from *Solanum villosum* Mill., *Patel et al.* [10] reported on the separation of alkaloids from *Solanum trilobatum* using TLC. In another study, *Jadesha et al.* [11] examined TLC for the isolation of phenolic compounds from *Solanum torvum*. Therefore, this work attempts to answer the question why a locally grown vegetable, *S. retroflexum*, in the Vhembe district (South Africa) has been a sought-after commodity for the health and well-being of communities in the area. The current work is the first attempt to separate and identify ATPE extracted ultraviolet (UV)–fluorescent metabolites present in *S. retroflexum* based on two independent chromatographic techniques, *i.e.*, as TLC and ultra-high performance liquid chromatography–quadrupole time-of-flight hyphenated to mass spectrometry (UHPLC–QTOF–MS).

2 Experimental

The leaves of *S. retroflexum* were air-dried and ground into a fine powder with a rotating blade blender and stored in covered glass containers. The powdered leaves (2.00 g) were placed in 50 mL polypropylene tubes. To each tube, a saturated salt solution of 25 mL was added. The saturated salt concentrations used were 30% (*w/V*) of $BaCl_2$, $MgSO_4{\cdot}7H_2O$, Na_2HPO_4, $MgCl_2{\cdot}6H_2O$, $AgNO_3$, KBr and KNO_3. Thereafter, 25 mL of absolute ethanol (99.9%) extract

and 25 mL of each salt solution containing the powdered leaves were mixed, resulting in ATPE. The spontaneous formation of ATPE under the conditions stated above was also reported by *Mokgehle et al.* [8]. Portions of 3 × 20 µL absolute ethanolic (99.9%) top-layer extractant solution were spotted on the TLC plate and developed using a mobile phase of chloroform–ethyl acetate–methanol (45:40:15, *V/V*). The separated phytocompounds were observed on the TLC plate using a UV lamp (365 nm), scraped (Figs. 1a and 1b), dissolved in ethanol and analyzed on a UHPLC–QTOF–MS. The metabolites were separated on an Acquity HSS T3 C18 column (150 mm × 2.1 mm with particle size of 1.7 µm; Waters Corporation, Milford, MA, USA) at an oven temperature of 40 °C. The UPLC was connected to a Synapt G1 QTOF–MS detector (Waters). An injection volume of 3 µL was used with solvent A: 0.1% formic acid in Milli-Q water and solvent B: acetonitrile with 0.1% formic acid. Chromatographic separation was achieved using a 10 min gradient elution: 2% B over 0.0–1.0 min, 2%–95% B over 1.0–6.0 min, from 6.0–7.0 min the conditions were maintained at 95% B, the column was washed with 95%–2% B over 7.0–8.0 min, re-equilibration with 2% B over a 2 min isocratic wash. The MS was configured to scan the range of 100–1000 Da with a scan time of 0.2 s. The MS data were acquired using positive electrospray ionization (ESI) mode. Chemical identification was done using KNapSAck Core System online metabolite database (Version 1.200.03) [12].

3 Results and discussion

Generally, an average of 6 bands (B1–B6) potentially corresponding to 6 compounds or more were observed on the TLC plate (Figs. 1a and 1b) when salts such as $MgCl_2{\cdot}6H_2O$, $BaCl_2$, $MgSO_4{\cdot}7H_2O$, Na_2HPO_4, KNO_3 and KBr were used during ATPE to aid the extraction of metabolites. Additionally, while the majority of the red fluorescent compounds were observed further up the TLC plate, blue fluorescent compounds were also observed at the top of the plate. *Nazir et al.* [13] also reported on fluorescent compounds in *S. lycopersicum* separated *via* TLC. The UV–fluorescent TLC extracted bands (B1–B6), after dissolution in ethanol, were run on the UPLC–QTOF–MS. A chromatographic base peak at *m/z* 435 and retention time (t_R) of 5.4 min were observed for B2 (Fig. 2 and Table 1) and another at *m/z* 457 (Fig. 2 and Table 1) with a retention time (t_R) of 7.31 min for B1. The base peak at *m/z* 457 was identified as oleanolic acid (OA) or its isomers such as ursolic acid (UA) or betulinic acid (BA) (Table 1) (pentacyclic triterpenoid) and was also reported to have been extracted from *Scutellaria barbata* D. [14]. The chromatographic base peak at *m/z* 435 detected for B2 had fragments at *m/z* 149, 277 and 303, and was identified as quercetin 3-O-β-D-xylofuranoside (Fig. 2 and Table 1). Quercetin-3-O-β-D-xylofuranoside was also extracted from potato (*Solanum* spp.) [15] and *Vernonia*

Schreb. [16]. A chromatographic appearance at retention time of 3.27 min with a base peak at *m/z* 415 was observed for B3 and B4 (Fig. 2 and Table 1). The fragments of the base peak at *m/z* 415 were at *m/z* 142 and 224, and hence identified as solanocapsine (Fig. 2 [B3] and Table 1). Solananocapsine was also extracted from *S. pseudocapsicum* (Jerusalem cherry) as reported by *Garcia et al.* [17].

Two chromatographic base peaks at *m/z* 413 were observed at B5 and indicated the presence of isomeric compounds (Fig. 2 and Table 1). The isomers had nominal masses of 413.2645 and 413.3223 and eluted at 7.46 and 7.83 min, each with fragments at *m/z* 133, 149, 301, 326 and at *m/z* 133, 327, 301, 369, and were identified as stigmasterol I and stigmasterol II, respectively (Fig. 3a and Table 1). The types (I or II) indicated isomers of stigmasterol. *Kaminski* [18] also reported on the presence of stigmasterol in *S. tuberosum.* The chromatographic appearance at 3.83 min with a base peak at *m/z* 1034 was observed in B4, furthermore the fragment of *m/z* 1034, *m/z* 578 was also observed as a chromatographic peak at the same retention time in B2 (Fig. 2 and Table 1).

The chromatographic base peak at *m/z* 1034 had fragments at *m/z* 263, 416, 528, 578 (Table 1). Similarly, the chromatographic base peak at *m/z* 578 (B2) also produced the same fragments as those of *m/z* 1034 (B4), and suggests that the two compounds are identical due to the same motif. In addition, the chromatograms in Fig. 2 show a richness in peaks which indicated that the richness peaks from the various zones on the TLC plate could be due to a common structural backbone present in multiple compounds with similar polarities. Additionally, the richness in peaks may be a result of the presence of isomeric metabolites. From the KNapSAcK metabolite database, the fragment at *m/z* 578 and the base peak ion at *m/z* 1034 were identified as tomatidine galactoside ($C_{33}H_{56}NO_7$) and alpha-tomatine ($C_{50}H_{83}NO_{21}$) (Fig. 3b and Table 1), respectively [21]. Alpha-tomatidine was composed of a tomatidine algycone unit glycosylated by four monosaccharides which included D-galactose, 2 × D-glucose and D-xylose, whereas tomatidine galactoside contained D-galactose as a saccharide [22]. Therefore, this indicated that the transition from *m/z* 1034 to *m/z* 578, which is alpha-tomatine to tomatidine galactoside, occurred with the loss of 3 monosaccharaides which consisted of 2 × D-glucose and D-xylose. A chromatographic appearance at 7.42 min with a base peak at *m/z* 560 that produced daughter ions at *m/z* 376 and 443 was observed as a blue-fluorescent band at B6. From Fig. 1a, the red fluorescent metabolites were dominant in zones B1–B5 while the blue fluorescent compounds were present at B6. Through KNapSAcK, the base peak at *m/z* 560 was identified as gamma-solanine. The diverse fluorescing behavior of the metabolites, for instance the blue and red fluorescent gamma solanine and solanocapsine, respectively, on the TLC in Fig. 1a, could be

due to the possibility of various structural moieties within the metabolites. Alpha-tomatine and gamma-solanine have been reported in *S. lycopersicum* (tomato) to be toxic for consumption particularly during the greening stage [21].

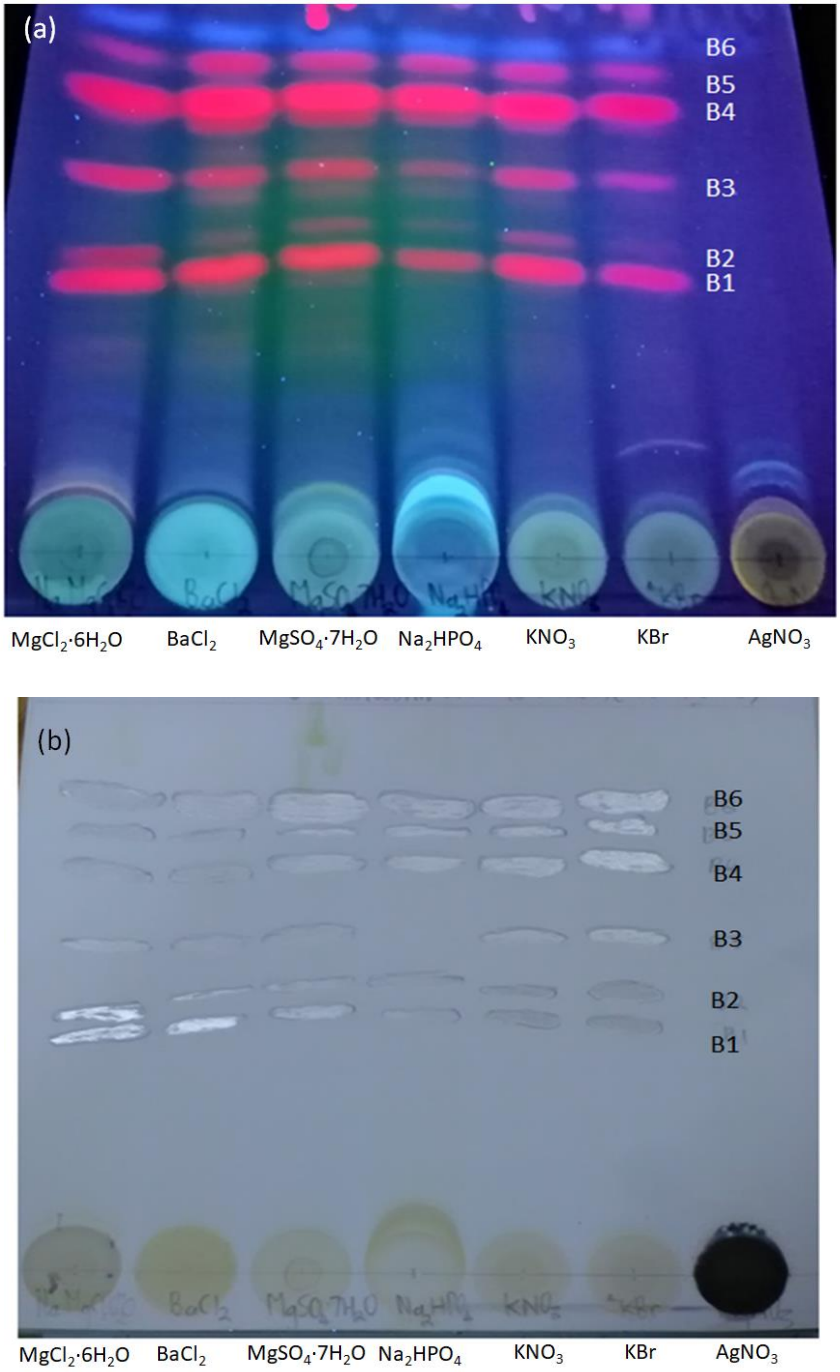


Fig. 1 a Flourescent spots on TLC pate when choroform–ethyl acetate–methanol (45:40:15, *V/V*) was as a developing solvent at 365 nm. **b** Scraped fluorescent spots from B1 to B6

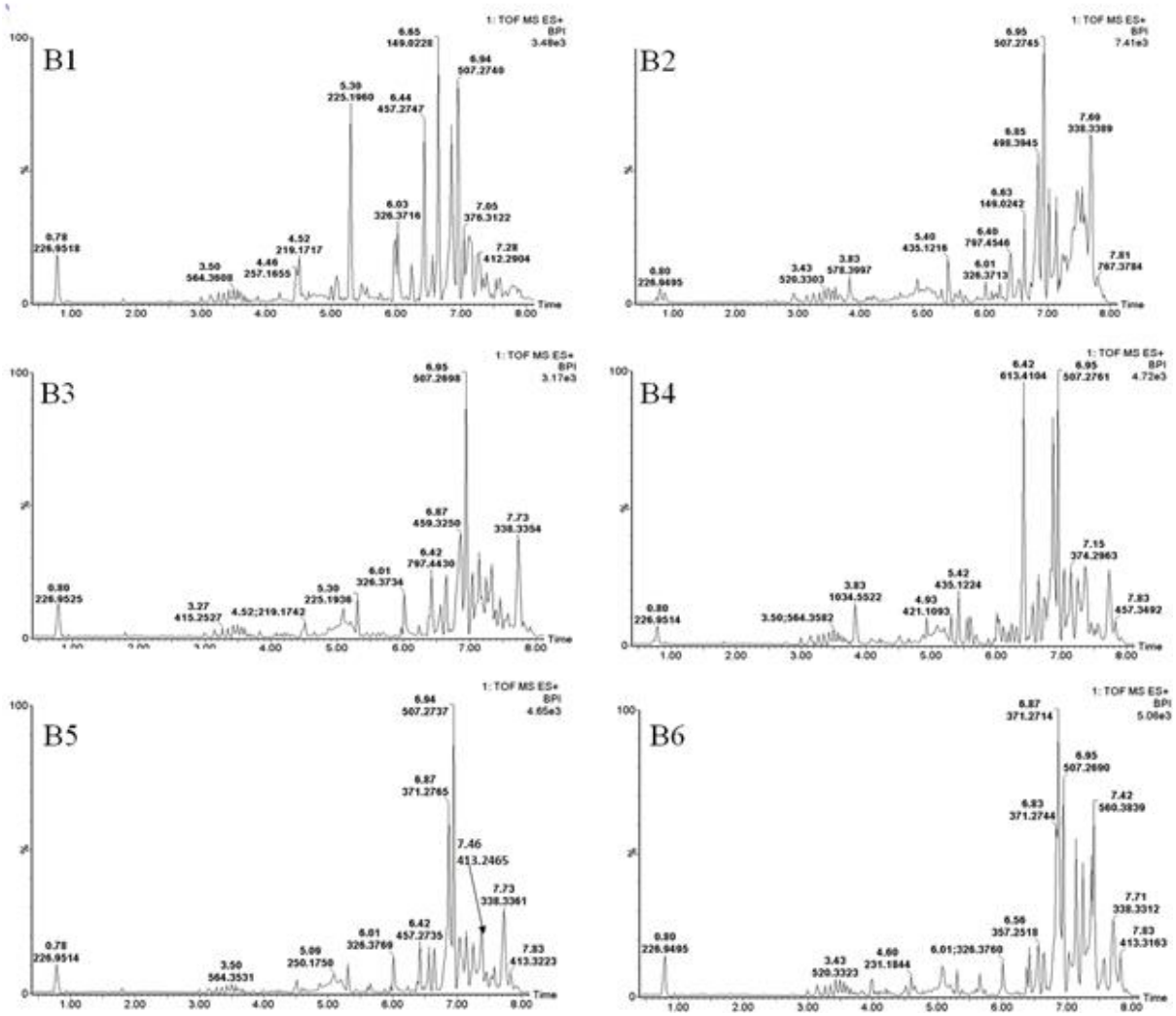
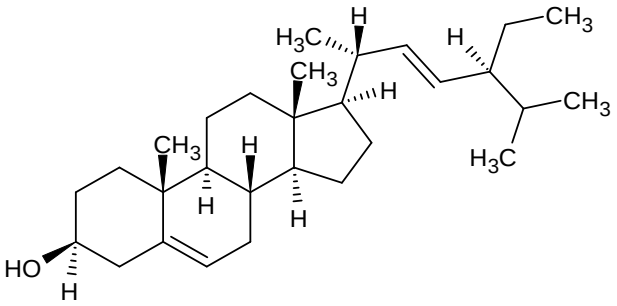


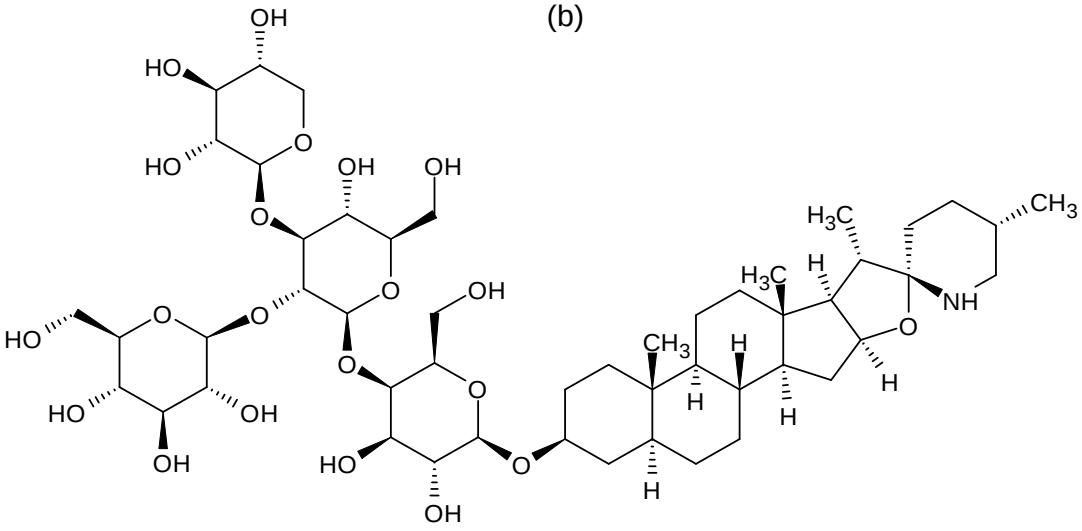
Fig. 2 Base peak single-ion UHPLC–QTOF–MS chromatograms of metabolites extracted using ethanol under positive ionization *via* ATPE from the leaves of *Solanum retroflexum*

(a)



m/z 413

(b)



m/z 1034

Fig. 3 Some of the structures detected from ATPE extracts of *Solanum retroflexum* **a** stigmasterol **b** alpha-tomatine

Table 1 Major compounds identified by UHPLC in *Solanum retroflexum* leaf aqueous methanol extracts

Band	Compound	Chemical formula	$[M+H]^+$	Diagnostic ions	t_R (min)	λmax (nm)	Plant species previously found in	Reference
B1	UO/OA/BO	$C_{30}H_{48}O_3$	457	374, 512	7.31	210	*Scutellaria barbata* D.	[14]
B2	Quercetin-X (reynoutrin)	$C_{20}H_{18}O_{11}$	435	149, 277, 303	5.40	352	*Solanum* spp.	[15]
B3	Solanocapsine	$C_{27}H_{46}N_2O$	415	142, 224	3.27	293	*S. capsicastrum*, *S. psuedocapsicum*	[16]
B4	Alpha-tomatine	$C_{50}H_{83}NO_{21}$	1034	263, 416, 528, 578	3.83	208	*S. lycopersicum*	[19]
B5	Stigmasterol I	$C_{29}H_{48}O$	413	133, 301, 326	7.46	257	*S. chacoense*, *S. tuberosum*	[20]
	Stigmasterol II	$C_{29}H_{48}O$	413	133, 301, 369	7.83	257	*S. chacoense*, *S. tuberosum*	[20]
B6	Gamma-solanine	$C_{33}H_{53}NO_6$	560	376, 443	7.42	325	*S. chacoense*, *S. tuberosum*	[21]

*X = 3-O-β-D-xylofuranoside, UO = ursolic acid, OA = oleanolic acid, BO = betulinic acid

4 Conclusion

The combined application of TLC and UPLC–QTOF–MS was shown to be useful in the identification of nine UV–fluorescent compounds from *S. retroflexum* for the first time, with the aid of the KNapSAcK metabolite database. Three UV–fluorescent alkaloids, solanocapsine (red fluorescent), alpha-tomatine (red fluorescent) and gamma-solanine (blue fluorescent) were simultaneously extracted *via* ATPE and subsequently isolated by chromatography-based methods. The diverse fluorescing behavior of the metabolites, under UV light, was possibly due to the variation in the structural moieties of the metabolites. To date, alpha-tomatidine and gamma-solanine have generally been limited to *S. lycopersicum*. However, this study has demonstrated that both glycoalkaloids can also be found in *S. retroflexum*. The use of an environmentally friendly method, ATPE, in conjunction with TLC and UPLC–QTOF–MS has shown to be an efficient method for the simultaneous extraction of UV–fluorescent metabolites from *S. retroflexum* and may prove vital for the well-being of man. Though this study was an untargeted approach with a tentative identification of UV–fluorescent metabolites from *S. retroflexum*, future studies may be directed at the isolation and comprehensive identification of isomers (ursolic acid, betulinic acid, oleanolic acid) by incorporation of derivatization agents.

Acknowledgements

The authors are grateful to the National Research Foundation of South Africa (NRF-SA) and the University of Venda for its financial support.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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