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Sincerely,

Bijay Kafle

Abstract

Introduction

Over the past decade, the worldwide consumption and sales of Traditional Chinese Medicine (TCM) herbs have grown enormously. Astragalus Radix (Chinese: Huang qi) (AR), the dried root of *Astragalus membranaceous* is a typical example. It is widely used in TCM to boost the body's immune system, to reinforce the vital energy ("Qi" in Chinese) and for the treatment of bronchitis, pneumonia, and fatigue. The bioactive compounds of AR are flavonoids, saponins, polysaccharides, amino acids, and trace elements.

In many countries (China, Japan, the USA, and Europe), Pharmacopeia's and monographs have been published, which describes the morphological characteristics and procedure for assays to test the quality and standardization of medicinal plants. The purpose of the study is to routinely investigate the quality of herbs, analysing the bioactive compounds in a highly accurate, reproducible, qualitative, and quantitative manner.

Objectives

1. Identification and quantification of isoflavonoids (formononetin, ononin, and calycosin-7-O-β-D glucoside) and saponins (astragaloside IV and cycloastragenol) in Astragalus Radix samples.
2. Technical comparison and use of improved methods for the quantitative determination of chemical components in AR samples and,
3. Comparison of commercial samples from different vendors.

Methods

Chemical standards were used for comparison and confirmation. Ultrasonication extraction was performed for a higher yield and sample preparation was optimized. Thin-layer chromatography (TLC) was used for detecting the presence of compounds. These were confirmed afterward when TLC plates were used in mass spectrometric (MS) detection. Fourier Transform Infrared Spectroscopy (FTIR) analysis was used to identify the functional groups of bioactive chemical compounds. The samples were further analysed using rapid and sensitive high-performance liquid chromatography–ultraviolet detector (HPLC-UV) and tandem mass spectrometric (LC-MS/MS) methods. The chromatographic conditions were optimized using the gradient elution of 0.2% formic acid in water and acetonitrile as mobile phases for HPLC-UV and methanol instead of acetonitrile in LC-MS/MS. The isoflavonoids were measured with a detection wavelength of 254 nm using a diode-array detector. The triple quadrupole tandem

mass spectrometer was operated using positive electron-ionization modes and monitored using multiple reaction monitoring (MRM). The method was validated for linearity, selectivity, accuracy, and precision. External calibration and standard addition were performed during LC-MS/MS analysis.

Results

TLC-MS analysis of Astragalus Radix samples showed four of the five compounds detected. Compared with the other bioactive compounds, cycloastragenol gave very low-intensity peaks. The FTIR spectra of the sample extracts were not good enough to make a baseline fingerprint, but some functional groups could still be detected. The three isoflavonoids could successfully be quantified by HPLC-UV in different extracts of AR samples, but not formononetin in hydrophilic concentration. The presence of the latter was confirmed by LC-MS/MS. Astragaloside IV and cycloastragenol could not be detected by the UV method, while all five standards of compounds were detected using tandem mass spectrometry. LC-MS/MS method is a more selective, sensitive, reliable, and accurate method than HPLC-UV for the analysis of bioactive compounds of AR. Compared to external calibrations in LC-MS/MS, the quantitative results were significantly improved by using standard addition performed by adding known concentrations of standards to the sample solutions. These improved results can be due to a reduction in matrix effects by dilutions and ion suppression. Cycloastragenol was not detectable in any of the Astragalus Radix samples. The concentration in sample extracts might be below the detection limit or be naturally absent. The peaks in both MRM channels in exact retention times were not seen. Samples from different vendors contain widely different concentrations of the bioactive compounds, indicating much lower quality of Astragalus Radix samples from certain vendors. The highest concentration of astragaloside IV (203 ± 6 µg/g) was present in granulate samples from one vendor, whereas capsules from another vendor contained more ononin and calycosin 7-O-β-D glucoside. Formononetin in the capsule sample was comparable with samples from different vendors. The lowest concentrations of all compounds were observed in tablets from one vendor. This is partly but not completely understandable, as the tablets contain raw herbs rather than granulates (granulates are on average 3-5 times more concentrated than raw powder).

Conclusions

In the absence of isotopic labelled internal standards, the accuracy of quantification of bioactive components in Astragalus Radix samples of LC-MS/MS can be improved by using

standard addition. The standard addition method was applied in diluted samples and quantification was independent of variations in signal response caused by matrix effects. LC-MS/MS was found to be significantly more sensitive and accurate than HPLC-UV for the measurement of essential bioactive compounds of AR. LC-MS/MS therefore should be used for Quality control of AR samples. This is probably also true for many other traditional Chinese medicine herbs, in the absence of isotope labelled internal standards. However, this should be further investigated. Another incredibly important research project would be to assess the pharmacokinetics of AR compounds in different persons, under various conditions, in blood, tissues, cells, and excretions (feces, urine, saliva). The results will assist the personalized dosing of AR herb in individual patients with different diseases.

Keywords: Astragalus Radix, Isoflavonoids, Saponins, HPLC-UV, LC-MS/MS

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Abbreviations

AG	Astragalosides
ATR	Attenuated Total Reflectance
AR	Astragalus Radix
CAG	Cycloastragenol
CMC	Chinese Medical Center
CV	Coefficient of Variance
CYP-450	Cytochrome-450 (enzyme)
ELSD	Evaporative Light Scattering Detector
EMA	European Medicines Agency
ESI	Electro Spray Ionization
EU	European Union
FTIR	Fourier Transform Infrared Spectroscopy
GLC	Glucose
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High-Performance Liquid Chromatography
IR	Infrared
ICH	International Council for Harmonization
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometer
MS/MS	Tandem Mass Spectrometer
MRM	Multiple Reaction Monitoring
NSCLC	Non-Small Cell Lung Cancer
QOL	Quality of Life
QDA	Single Quadrupole mass detector
RF	Retention Factor
SIM	Single Ion Monitoring
SF	Seven Forest
S/N	Signal to Noise ratio
SD	Standard Deviation

TCM	Traditional Chinese Medicine
TA	Total Astragalosides
TLC-MS	Thin Layer Chromatography-Mass Spectrometer
UV	Ultraviolet
UHPLC	Ultra-High Performance Liquid Chromatography
XYL	Xylose

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Introduction

Traditional Chinese Medicine (TCM) herbs are used worldwide to treat very many different diseases. Over the past decade, the demand for TCM herbs has increased greatly. In 2015, the total production value of the TCM pharma industry in China alone was over 110 billion USD. Due to the significantly increased worldwide interest in the use of TCM herbal medicines, it is expected that the global market value of TCM herbs will significantly increase in the coming years (Lin et al., 2018; Xu and Xia, 2019).

Herbs have been used for more than 2000 years for the treatment and prevention of diseases. Moreover, due to extensive studies of herbs, in modern advanced research laboratories in China, North America, Asia, and Europe, many new plant-based medicines have been and are currently being developed. These medicines are not only used for the cure but also prevention of diseases (Rajiv et al., 2016) and anti-aging. Biological, molecular, and chemical studies have also considerably increased the knowledge of how TCM herbs work.

In China and Chinese worldwide, the confidence and trust in TCM herbs are strong. Contrarily, most western medical doctors, scientists, and politicians are often utterly negative. When asked about their negativity, any or several of the following answers may follow as,

- Herbs are regarded as old-fashioned and of variable uncontrollable quality.
- Herbs often contain toxic by-products, such as pesticides and heavy metals.
- Herbs are dangerous and have potential side effects. They can cause life-threatening damage to humans.
- There is no scientific proof that TCM herbal medicines are effective in prospective randomized trials.
- The therapeutic effects of TCM herbs vary greatly and often they do not work. Even if they work, the effect of TCM herbs is very slow.
- In contrast, western chemical medicines can work very fast. Why to use the inferior old-fashioned stuff/rubbish?
- The quality of TCM herbs is very variable (due to variations in cultivation, the locality where it was grown and others), so as a medical doctor you do not know what you are prescribing.
- The quality of TCM herbs sold by different vendors varies.
- TCM herbs do not consist of one or two molecules but are a mixture of many chemical components and there is strong inter-patient variability in uptake and excretion (pharmacokinetics) of those herbs. As the effect in an individual patient is unknown

beforehand one must rely on an “average” dosage and cannot determine the optimal dosage in an individual patient.

The results of Network pharmacology and Deep Learning /Artificial Intelligence have not only confirmed well-known knowledge and insights but also resulted in exciting new knowledge. It is widely expected that these results will further increase the usage of TCM herbal medicines. Well reproducible and accurate methods are essential for standardization and adequate quality control of marketed herbal products. Moreover, effective international regulations to guarantee the quality of the herbs sold are required.

Quality of life

Diseases are caused by pathogens, which result in pathophysiological disorders in the body. Chronic diseases like cancer, diabetes mellitus, hypertension, and many autoimmune diseases are often life-long issues for patients and the (inter)national health care systems, as they can cause serious biological degenerations, with long-term psychological and somatic problems (Somrongthong et al., 2016). Diseases can have a serious negative impact on people’s health status and quality of life.

Herbal medicine is often associated with China and India. However, western medicine historically also mostly used herbs to cure diseases as is well known from historically famous medical doctors like Hippocrates and Boerhaave. Only after the development of modern chemistry in the second half of the 19th century, chemical analysis of herbs led to the identification of bioactive molecules. Typical examples are Acetyl-salicylic acid and Artemisinin.

A water extract (decoction, “tea”) of willow (*Salix alba)* (Figure 1) bark extracts have been used worldwide for thousands of years to treat pains and fever.

Figure 1. Willow (*Salix alba*) tree

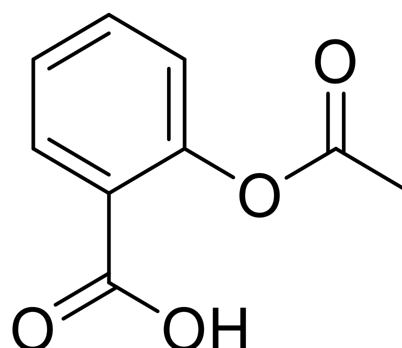


Figure 2. Acetyl Salicylic acid (Chemical structure)

Around 1880, German chemists analyzing the decoction discovered many chemicals in these extracts. Acetylsalicylic acid (Figure 2) was one of them and could easily be reproduced in chemical processes.

In 1899, the German dye company Bayer started to market chemically produced acetylsalicylic acid as Aspirin for pain and fever relief. Later it was also introduced and is now used worldwide as a thrombolytic agent.

Another famous "western" drug Artemisinin (Figure 3) was obtained from *Artemisia annua* (Figure 4), to treat malaria due to *Plasmodium falciparum.* The Chinese Dr. Tu Youyou (Figure 6) received The Nobel Prize in Physiology and Medicine 2015, for her wonderful discovery in 1972 from Chinese medicine prescriptions from 300AD.

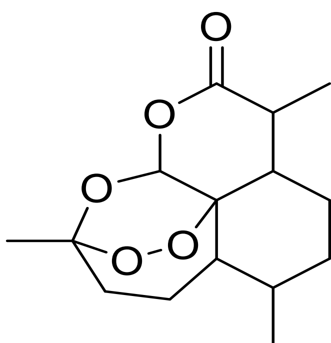


Figure 3. Chemical structure of Artemisinin.

Figure 4. *Artemisia annua.*

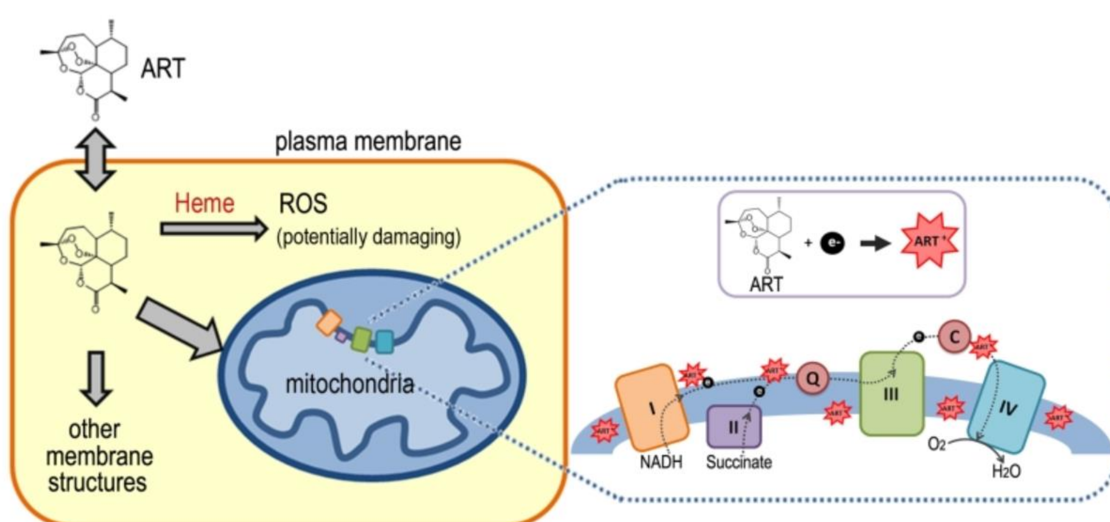


Figure 5: Diagrammatic representation of the mechanism of action of Artemisinin (ART) (Sun C. and Zhou B, 2016)

Figure 6: Dr. Tu Youyou, Nobel prize laureate 2015.

Western chemical treatments often work very quickly and are relatively inexpensive. On the other hand, they are well known for their serious side effects, different interactions and of course, inevitably sometimes therapeutic failure. The side effects of chemotherapy can diminish the patient's adherence to prescribed medications. Many patients choose treatments using Traditional Chinese Medicines as a supplement or alternative to regular western chemical medications. In fact, in China and among Chinese outside China (at least a quarter of the world population), TCM medicines are often the first rather than second choice, due to the trust persons have in TCM medicines. This is not only true for cancer and other serious diseases, but also as "anti-aging" and prevention of infectious viral diseases, such as recently with COVID-19. In fact, 'the Guideline for the Diagnosis and Treatment of Novel Coronavirus (2019-nCoV) Pneumonia (On Trials, the Fourth/Fifth/ Sixth/Seventh Edition)' issued by National Health Commission of the People's Republic of China and National Administration of traditional Chinese medicine encouraged the use of the TCM medical formula Lianhuaqingwen (LHQW) which was found effective in the treatment of COVID-19 (Runfeng et al., 2020).

Due to the development of Phytochemistry in the late 19th century, the use of the "total decoction of herbs" was slowly and increasingly rejected. Rather, one should rely increasingly on one chemically identified bioactive compound and leave out the "useless other parts of plants". Only concentrated tannins from tormentilla root, salicyl acetate from willow, morphine from opium, digitoxoside from foxglove, should be used. However, it was increasingly discovered that pure tannins had a nearly toxic effect when the protective effect of the softening mucoid substance from the original plants was left out. The same holds for salicyl acetate after

the other buffering bioactive compounds were left out, regularly causing severe gastric mucosal erosions and sometimes life-threatening gastric bleeding. In his splendid book "Handbook of medicinal plants", 6th edition, 2014, the author states: "Modern phytotherapy addresses again the *Totum* of the plant. Herbs, or extractions thereof, contain a "totum" of bioactive compounds, which harmoniously collaborate and result in a therapeutic effect, which is different from the sum of individual chemical compounds/molecules. This is called *Synergy* (Verhelst, 2014).

Contrary to chemical active compounds, ***plants cannot be patented***. Consequently, commercial (pharmaceutical) companies (understandably) do not spend large amounts on the research of total herbal decoctions. Compared to pharmaceutical chemical medicines, fundamental research of "totum" plant-based decoctions, therefore, has been rather limited.

On the other hand, many people can be afraid of the serious side effects of chemically isolated bioactive molecules and pharmaceutical chemical single molecular medicines. There is a worldwide increased interest in and search for "alternatives". As an overshoot, amongst these persons, herbs can sometimes erroneously be regarded as "*natural, that is always good*" (contrasting the "*chemical rubbish of pharmaceutical money wolves*"). This "*It is from Nature, so it is always good*" attitude, seems to forget that certain herbs and plants are simply extremely dangerous and can be life-threatening.

Amongst Chinese, TCM herbal treatments are famous as they can be very effective and have few side effects (apart from often not tasting nice!). Moreover, they can be used as additional therapy in combination with western conventional therapies (for example, chemotherapy, irradiation), to improve the Quality of Life, improve prognosis, remove or diminish side effects of toxic western therapies (without decreasing the prognosis improving effect) and help to strengthen and rejuvenate the body after chemotherapy and radiotherapy (Guo et al., 2017).

First-line therapies for metastatic cancer often are chemotherapy, radiation and sometimes selectively (due to the high costs) targeted therapy or immunotherapy. However infamous side effects like nausea and vomiting, hair loss, fatigue, and substantial toxicities have made many patients very reluctant to use them. Such side effects can be (partly, or largely) prevented and reduced by using additional TCM phytomedicines, thereby greatly improving the Quality of Life (Chen et al., 2018; Guo et al., 2017; Wu et al., 2017; Wu et al., 2016). TCM herbal treatment together with platinum-based chemotherapy has shown to significantly improve prognosis in patients with metastatic non-small cell lung cancer (NSCLC) (Baak et al., 2015; Guo et al., 2011; McCulloch et al., 2011b; McCulloch et al., 2006). Similar findings

were obtained in colorectal cancer, independent of the stage (McCulloch et al., 2011a; Tan et al., 2008). Interestingly, these results (in part) confirmed many decades of empirical experience that therapy with traditional Chinese medicines in addition to chemo- and radiotherapy dramatically decreases the side effects of the latter, strengthens the immune functions and improves the quality of life. There are very few adverse effects reported with the use of herbal medicines (Guo et al., 2017; Wu et al., 2017). This is also clear from the Norwegian nationwide registry of herbal medicines (The Norwegian Medicines Agency). This was set up to register the "worrisome" side effects of herbal medicines. Unfortunately, and strangely, the Registry does not distinguish between European Union approved herbs, and those lacking a certificate of authenticity (often bought cheaply on the internet or taken by the users from foreign countries) (nice, SOOO cheap!). This major shortcoming only undermines the fact that the side effects of EU-approved TCM herbs, which are sold by officially acknowledged pharmacies in the EU, are very low indeed. Publications in a Norwegian newspaper, classified the increase of side effects due to "(unspecified) herbal medicines" as "dramatic", although the total registered number in the whole of Norway was less. In a study, only 7 % of adverse effects were reported by using herbal medicines and western medicines together. There were many possible herb-drug interactions reported to be clinically harmful (Djuv et al., 2013).

Phytomedicine

Astragalus Radix (AR) (China: Huang qi) are roots of *Astragalus membranaceous (Fisch.) Bge. Or Astragalus membranaceous var. mongholicus (Bge.) Haiso* (Leguminosae family). As described briefly above, they are used in traditional Chinese medicine as a tonic, which is supposed to reinforce vital energy (Qi) or adaptogenic use (Wu et al., 2005). *Astragalus membranaceus* is also used in Korean and Japanese medicine and grows naturally in Shanxi and Gansu provinces in China, Mongolia, Kazakhstan, and the Russian Federation (Li et al., 2017). There are many other species found in different parts of the world, also in Scandinavian and other European countries. Species like *Astragalus alpinus* (Alpine Milk-vetch) are found in Norway (Olsen et al., 2013). It is not known if these have medicinal effects and/ or contain similar bioactive compounds as AR. It would be of interest to investigate this in the future.

Kingdom: Plantae

Family: Leguminosae

Genus: *Astragalus*

Species: *A. membranaceus*

Figure 7: *Astragalus membranaceus* plant and its roots

There are many claimed biological effects of Astragalus Root like immunopotentiation, anticancer, hepatoprotective, cardioprotective, anti-diabetic. They also reduce the side effects of chemotherapy (Tin et al., 2007). Modern chemical technologies have revealed the mystery of many chemicals present in the AR samples (Duan et al., 2016). Astragalus Radix contains biologically active compounds such as polysaccharides, flavonoids, saponins, alkaloids, glycosides, terpenes, tannins, phenolic compounds, and their derivatives (Chen et al., 2015; Maobe and Nyarango, 2013; Zu et al., 2009). Saponins, isoflavonoids and polysaccharides are targeted for their pharmacological activity. A single compound can show bioactivity, but it also can work in synergy with other compounds.

Astragalus Radix especially *A. membranaceous* is a rich source of astragalosides (AG) which are saponins distinctive of the astragalus species (Liu et al., 2020). There different types of astragalosides (I-VIII) compounds present in plant tissue, with astragaloside IV (AG-IV) being the most famous one for its high bioactivity (Zu et al., 2009). Astragaloside IV is a 9,19-cycloartane type major active triterpene glycoside (Figure 8).

Astragaloside IV increases T and B lymphocyte proliferation and production of antibodies. Astragaloside IV also acts as an anti-inflammatory agent, helps in wound healing,

used to treat renal injuries, protects from ischemic brain injuries, is immunomodulating and shows anti-scarring, angiogenesis, anti-photo aging, positive inotropic action, cardioprotective, osteogenesis, anti-fatigue, anti-coxsackie B virus activity, free radical scavenging activity and increases telomerase activity (Cheng and Wei, 2014; Yan and Guo, 2005; Zhang et al., 2005).

Cycloastragenol, the aglycone of astragaloside IV extends T-cell proliferation by increasing the telomerase activity, a vital process to delay cellular aging (Yu et al., 2018). Studies suggest that cycloastragenol is one of the metabolic products of astragaloside IV formed by intestinal bacterial conversion (Zhou et al., 2012). The synthesis of cycloastragenol is done by acid hydrolysis of astragaloside IV (Ip et al., 2014). It has been introduced on the market as a dietary supplement as a new anti-aging compound by T.A. Sciences (New York, USA) under the brand name TA-65® (Yu et al., 2018).

The major isoflavonoids such as formononetin, ononin, calycosin and their glycosides present in Astragalus Radix boost immune function and increase vitality, have a role in human nutrition and help in skin growth (Liu et al., 2016; Lv et al., 2011; Sun et al., 2014). They have a significant impact on glucose homeostasis, energy and lipid metabolism, and cardio-protection (Liu et al., 2018). Formononetin is supposed to act as a neuroprotective, anti-inflammatory, anti-oxidative, cardioprotective agent and is used in cancer treatment by inducing apoptosis and causes cell cycle arrest (Jiang et al., 2019; Tay et al., 2019). Calycosin showed angiogenesis in human endothelial cells and protects endothelial cells from hypoxia-induced barrier impairment whereas its glycoside showed anti-coxsackie virus activity and used as an anti-osteoarthritis agent (Lau et al., 2012; Thwe et al., 2012; Xiao et al., 2008). Polysaccharides were also studied for their anticancer properties.

Studying the pharmacokinetics of the bioactive compounds in the human body is very important in establishing the therapeutic dose required. The absorption, distribution, metabolism, and clearance of bioactive compounds in Astragalus Root are the parameters that determine the blood and tissue concentrations of each of the bioactive compounds. Naturally, these biological factors vary from patient to patient and perhaps also within the same patient under different conditions over time. Quantification of bioactive compounds present after a given dosage, in representative body samples or excretions (such as saliva) in an individual patient, should be studied, to adjust the optimal dosage required in each patient. This can be done at regular time intervals after AR administering. Such results will determine the pharmacological activity in individual patients. Pharmacokinetic studies of Astragalus Radix are rare and performed mostly in animal models (Liu et al., 2015; Sun et al., 2014). It is essential to study the pharmacokinetics of bioactive compounds present in AR samples in

different human samples. Some studies suggested that the maximum plasma concentration of formononetin was about 2.4 ng/ml after oral administration of a single dose of 30 g Astragalus Radix ultrafine granular powder (Rao et al., 2019). Similarly, the maximum plasma concentration of astragaloside IV after a single intravenous infusion of 18 mg Astragaloside IV was found to be 2.12 µg/ml in healthy Chinese volunteers (Xu et al., 2013).

However, it remains to be assessed what the best therapeutic concentrations of AR compounds are, in different persons and how long that concentration must remain in treating different diseases. The results of such studies will greatly help to validate the therapeutic effect of Astragalus root in patients with various diseases.

Test assays of Astragalus Radix and many other potent herbal plants are described in different Pharmacopeias in China, Japan, Europe, and the USA. Appropriate quality control measures must be taken for patients' belief in and adherence to such medicines.

The methods used to identify, detect, and quantify these bioactive chemical compounds present in AR samples must be highly sensitive and well reproducible. It is the goal of this master study to evaluate different methods and develop an optimal determination method. The second goal of this thesis is to compare the quality of Astragalus Root samples from different vendors

Quality analysis of compounds

Ethnomedicinal use of many herbal plants is the backbone in the development of new drugs that are based on plant metabolites. It is particularly important to have an accurate measurement of these bioactive compounds for quality control. The morphological characteristics and physical appearance are checked for the best quality Astragalus roots. The quality can be tested for raw samples, finished preparations, and marketed products of Astragalus Radix by modern technical analytical measures.

Thin-layer chromatography combined with mass spectrometry (TLC-MS) can be used as a semiquantitative method to detect the presence of marker compounds (Nikam et al., 2012). The plant extracts are run on TLC plates for chromatographic separation of compounds using different mobile phases. The retention factors can be compared with the standard compounds. There might be many other compounds that can elute together, therefore further confirmation can be made by subjecting the TLC plates for mass spectrometry (MS) detection. The MS detector confirms the presence of chemical compounds based upon their respective mass to charge ratio (m/z values).

The IR fingerprinting confirms the presence of chemical compounds when compared to their chemical standards. Fourier Transform Infrared (FTIR) spectrometer can help in the detection of functional groups of the compounds (Pakkirisamy et al., 2017; Wulandari et al., 2016). This is made feasible by a diamond crystal attenuated total reflection (ATR) cell. The sample extracts can be introduced directly onto ATR-FTIR in any form (Taiwan Herbal Pharmacopeia, 2016). However, sometimes it can be difficult to obtain a clean fingerprint FTIR spectrum for complex sample mixtures.

The compounds from plant extracts can be well separated using optimized chromatographic conditions, which can be coupled with different detector types based upon UV light absorption, light scattering properties, or mass to charge ratio of the compounds. The marker compounds from Astragalus Radix extracts are separated by using high-performance liquid chromatography. After complete separation, flavonoids can be detected using HPLC-UV (diode array detector) because of strong chromophores (Kwon and Park, 2012). It is difficult to quantify the saponins, i.e. astragalosides and Cycloastragenol using UV detectors because of non-chromophores where evaporative light scattering detector (ELSD) can be used instead. Since compound identification using HPLC-ELSD only takes account of retention times which can be the same for different compounds, the mass spectrometer offers additional selectivity and is therefore, a better detector for simultaneous quantification of all saponins as well as isoflavonoids (Huang et al., 2009).

It has recently been shown that liquid chromatography coupled with mass spectrometry (LC-MS) provides a more sensitive and convenient quantification of AG-IV than LC-ELSD (Zu et al., 2009). Most of the reported methods use a tandem mass spectrometer, which is a MS/MS system that can detect compounds based upon the mass to charge ratio (*m/z*) of parent ion (molecular ion) as well as fragment ion. Comprehensive characterization of phytochemicals in AR has been performed by using hybrid mass spectrometers with higher mass resolution, such as quadrupole time-of-flight (Q-TOF) (Duan et al., 2016). Higher sensitivity and throughput can be obtained by using a triple quadrupole mass spectrometer (QqQ) with multiple reaction monitoring (MRM) rather than single ion monitoring (Liu et al., 2018; Yan and Guo, 2005). The effectiveness of the LC-MS/MS measurements can suffer from different matrix effects and ion suppression which must be reduced to obtain an accurate quantification of compounds. Isotope labelled internal standards are applied to correct for ion suppression in the LC-MS analysis, to the best of our knowledge no such internal standards exist for AG-IV and CAG.

In the present paper, we have explored the use of different calibration techniques, as an alternative to isotope labelled internal standard calibration, for more accurate LC-MS/MS measurement of astragaloside IV and isoflavones in various Astragalus Radix preparations. Our results strongly suggest that the standard addition method will provide concentration measurements that are closer to the true levels in various AR preparations.

Rationale

Different identification parameters and quantification assays of herbal plants including traditional Chinese medicines are described in great detail in pharmacopeias, published in different countries. Herbal medicinal products, including TCM, are regulated in the European Union by the EU medicine directive 2001/83/EC which was amended by the 2004/24/EC directive to include herbal medicinal products. TCM products need approval by the European Medicines Agency (EMA), and there are guidelines (EMA, 2011) on testing, which include phytochemical characterization as a requirement. Both for quality control and to correlate pharmaceutical effects with consumption of TCM herbs, it is essential to gather information on their chemical composition, measure concentrations of their active components, and study of absorption patterns in patients.

Many pharmacopeias have stated that astragaloside IV present in Astragalus Radix samples should not be less than 0.04 % to pass the quality control. The methodology has used ammonia in the sample preparation method which is supposed to hydrolyze many other astragalosides to astragaloside IV. The traditional Chinese way to prepare Astragalus Radix decoction is by boiling roots in water, which is then consumed by patients and does not involve ammonia pre-treatment. The concentration of astragaloside IV obtained after ammonia treatment as stated in pharmacopeia does not correlate with the pharmacokinetics of AR samples ingested because only naturally available astragaloside IV in AR samples is available for systemic absorption. The naturally present concentration of astragaloside IV should be measured without any chemical treatment. Therefore, the pharmacopeial limits must be re-evaluated in terms of astragaloside IV present naturally and total astragaloside concentration obtained after treatment with ammonia or any other chemical treatment. Similar limits can be set for other bioactive compounds.

Astragalus Radix has a long history of traditional use in China, because of its health benefits: immunostimulant, hepatoprotective, cardioprotective, anti-oxidative, antiviral, antiperspirants, diuretic, antidiabetic, expectorant, growth of new tissue, induces cancer cell apoptosis and as an additional therapy for cancer (Wu et al., 2014). The market value of

Astragalus Radix is associated with its characteristics to improve the patient's quality of life. The dynamic study of Astragalus Radix is essential. On the other hand, adulterants available on the market as Astragalus Radix are a serious issue. A single genus *Astragalus* has many species and sub-species that are marketed as Huang Qi (Dong et al., 2011; Ma et al., 2002; Valant-Vetchera and Zyka, 2003). Similarly, the difference in chemical composition from sample to sample might be due to several factors such as the land of origin, when and where it was grown, harvesting period, processing and manufacturing processes, different batches, cultural manner, and the transformations during heating, storage and extraction conditions of the herbs (Chu et al., 2014; Huang et al., 2009; Jie et al., 2013). It is therefore essential for appropriate quantification of Astragalus Radix components using different analytical techniques because of its complex chemical compositions and plant species. All these challenges highlight the need for quality control of Astragalus Radix in terms of the presence of biologically active constituents and their quantification. This can be the basis for the rational prescription of Astragalus Radix formulations as well as future clinical trials.

Literature review

Traditional Chinese medicines are widely used nowadays. The whole plant, plant parts and extracts which contain potent bioactive compounds are used in traditional Chinese medicine for treating and curing illnesses and for nutritional purposes. The compounds act singly or in combination with multiple other compounds in a synergistic way to exert its pharmacological activity. The secondary metabolites such as flavonoids, phenolic compounds, steroids, polysaccharides, saponins, alkaloids, terpenes, tannins, glycosides, and many more compounds are responsible to exert the therapeutic activity (Maobe and Nyarango, 2013; Rajiv et al., 2016).

Astragalus Radix, Huang Qi in Chinese milk-vetch root in English and Ogi in Japanese, a famous herb, which has a long history of medicinal use in traditional Chinese medicine was first recorded in *Shen Nong Ben Cao Jing* (Liu et al., 2015). Many pharmacopeias such as Chinese, Japanese, Taiwanese, and European have identified Astragalus Radix as the dried roots of *Astragalus membranaceus (Fisch.) Bge.* or *Astragalus membranaceus var. mongolicus (Bge) Hsiao* from the Leguminosae family, which are used as an apoptogenic tonic to reinforce vital energy (Qi) (Tang et al., 2010).

Astragalus Radix consists of many secondary metabolites, phenolic compounds, isoflavonoids, triterpene saponins, polysaccharides, aminobutyric acids, and various trace elements which are medicinally important (Zu et al., 2009). The astragalus roots are used to

treat disorders of the hepatic, renal, cardiovascular, and immune system and as an antioxidative, an antiviral agent, to induce apoptosis of cancer cells and as an adjuvant to cancer treatment. It is also known to reduce the side effects of cytotoxic antineoplastic agents (Tin et al., 2007). There are many other therapeutic indications for Astragalus Radix, such as improved tissue growth, treatment of nephritis and diabetes, as an antiperspirant and anti-diuretic (Qi et al., 2008; Xiao et al., 2004) and prescribed to treat symptoms of common colds, shortness of breath, frequent sweating, fatigue, allergic rhinitis, asthma, atopic dermatitis (Kang et al., 2004; Kim et al., 2007). Moreover, water extracts are used to treat chronic disorders (Shi et al., 2015). A study by Sun et al.,(2019) showed that more than 200 pharmaceuticals prepared using Astragalus Radix are approved by China Food and Drug Administration and many more are marked in different continents. Astragalus Radix formulations are also marketed as food supplements (Zhang et al., 2013; Zhao et al., 2015). AR is warm in nature and sweet in taste with a pleasant smell (Wang and Ba, 2015). The isoflavonoids and saponins are considered to be the characteristic compounds for the standardization of Astragalus Radix (Kwon and Park, 2012; Song et al., 2008).

There are at least eight different astragalosides (I-VIII) and astragaloside derivatives which are cycloartane type triterpene saponins based upon aglycone cycloastragenol and differentiated by the number and positions of glucosyls present in C3, C6 and C25 positions (Monschein et al., 2014; Zu et al., 2009). But astragaloside VIII possesses an Oleanane-type soyasapogenol β as an aglycone astragenol whereas cycloastragenol is in contrast, a dammarane type (Monschein et al., 2014; Xu et al., 2007). Astragaloside IV and cycloastragenol are both thought to delay aging due to their telomerase activity and develop immune resistance (Liu et al., 2020). Astragalin is a flavonoid type of glycoside which is also present in Astragalus Radix which helps to reduce inflammation. These potent compounds are used for their anti-inflammatory, anti-renal injuries, anti-scaring, antioxidative, neuroprotective, inhibiting myocardial ischemia, and immune-modulating properties (Cheng and Wei, 2014; Zhang et al., 2007; Zhao et al., 2018). Astragaloside IV, a-3-O-β-D-xylopyranosyl-6-O-β-D glucopyranosyl-cycloastragenol, also helps to increase cell-mediated and humoral immunity and antibody production (Wu et al., 2005). Cycloastragenol is marketed as the new antiaging activity, however, the transformation of glycosides to their aglycone is difficult but can be achieved by some microbial transformations (Nartop et al., 2015b).

Flavonoids act as radical scavengers. Isoflavonoids are supposed to have potent α-glucosidase inhibition activity (delay the production or absorption of glucose) i.e. can maintain glucose balance, fat and glucose metabolism, and reduce cardiovascular risk factors (Liu et al.,

2018). Isoflavonoids such as formononetin, ononin, calycosin and their glycosides can boost energy, activate immune system, and helps to grow new tissues in skin (Liu et al., 2016). Calycosin showed formation of new blood vessels in human endothelial cells (Lau et al., 2012) whereas antiviral activity and alleviated osteoarthritis were noticed with its glycoside (Xiao et al., 2008).

Many chemical compounds have been identified in AR samples by using different identification and detection techniques. There are many different methods used for the qualitative and quantitative analysis of Astragalus Radix such as TLC, LC-UV, HPLC-ELSD, LC-MS, FTIR, and GC-MS (Kim et al., 2007; Zu et al., 2009). TLC is a semi-quantitative method for the identification and quantification of compounds. The samples are spotted on a stationary silica layer and then the mobile phase helps to elute the compounds based upon their polarity. Infrared (IR) spectroscopy is a technique based upon the molecular vibrations of atoms. It is non-destructive, fast, accurate, ecofriendly, only requires a very small sample and no reagents. The functional groups of compounds can be analyzed using IR in the mid-IR region of 4000 to 400 cm^{-1} (Sun et al., 2010; Wulandari et al., 2016). FTIR is a more advanced form of IR spectroscopy.

The chromatographic separation of compounds using HPLC followed by UV detection is used for the detection of flavonoids and many other chromophore containing compounds present in plant samples (Qi et al., 2006). The other technique is to use the evaporative light scattering detectors (ELSD) in which the chromatographic separation of compounds from the sample is followed by ELSD where the nebulization of samples into the temperature-controlled evaporator causes the evaporation of samples. The cloud is then directed towards a narrow beam of light where the light is scattered because of the microparticles in the cloud. The scattering pattern is measured with the help of a photodiode or photomultiplier detector. The response is plotted against the analyte concentration. (Li and Fitzloff, 2001).

Despite UV and ELSD detectors, mass spectrometric detectors are also commonly used to detect the presence of compounds in samples using respective mass to charge ratio. The HPLC coupled with tandem mass spectrometry (LC-MS/MS) is said to be more powerful than UV and ELSD for detection and quantification of multiple ingredients in the complex herbal preparations and biofluids. The LC-MS/MS has low detection limits, high separation and selectivity, less time consumption, multiple analysis properties, and advanced software. Nitrogen gas (95-98 %) and ultra-pure argon gas (99.999 %) are used for nebulization and collision-induced dissociation (CID), respectively. The linear regression lines are drawn using standard concentrations and peak areas (Kim et al., 2007).

The roots must be crushed to fine powders for efficient extraction using different techniques such as reflux extraction, soxhlet extraction, ultrasonication. Different solvents like ethanol, methanol, and water are used for extraction. After extraction, the impurities must be filtered or can be removed by centrifugation (Jie et al., 2013). The extracts can be dried by using a rotary evaporator connected with a high vacuum. The Astragalus Radix residue left after extraction with a suitable solvent and dried with a rotary evaporator, in some experiments, is treated with ammonia and butanol solution (Liu et al., 2020; Zhao et al., 2018). The samples are again centrifuged before analysis to remove impurities. There are many methods used for the qualitative and quantitative analysis of Astragalus Radix such as TLC, LC-UV, HPLC-ELSD, LC-MS, FTIR, and GC-MS (Zu et al., 2009).

The TLC plates coated with silica are used as the stationary phase. The plates are developed using a mixture of solvents as the mobile phase. In the TLC identification test stated in Taiwan pharmacopeia, the solution of n-butanol, 4N ammonia, and ethanol (5:2:1) is used (Taiwan Herbal Pharmacopeia, 2016). The eluted compounds are examined under UV light at wavelengths of 254 nm for flavonoids, however in the case of saponins, they are treated with dilute sulphuric acid and heated for 5 mins, the spots seen are tallied with the spots of the respective standards (Japanese Pharmacopeia, 2016).

During the IR analysis, dried extract powders can be analyzed, where about 10 mg of samples are incorporated into 100 mg of potassium bromide pellet to prepare a luminous disc (Pakkirisamy et al., 2017). FTIR is a more advanced and user-friendly technique where the samples can be analyzed directly in any form i.e. powder, crystal, liquid.

The HPLC-UV is used for the detection of compounds containing chromophores. The compounds are separated using reversed-phase C18 columns and guard columns. Solvents like water, methanol, 1% acetic acid, 2% formic acid and acetonitrile are used as a mobile phase. The reversed-phase liquid chromatography separation mechanism provides higher retention for non-polar components and less retention for polar and more water-soluble components. The gradient programming for the mobile phase was used to get the complete separation of the compounds. The flavonoids have chromophores and will absorb light with UV-wavelength of 254 nm. Saponins which do not have chromophores absorb UV light poorly (Jie et al., 2013) even at very low UV-wavelengths of 203-210 nm. The acidic mobile phases are typically used in the LC system to suppress silanol charge of the silica-based stationary phase and also for the neutralization of acidic analytes, to increase their retention. Previous work has demonstrated

baseline separation on a C18 reversed-phase column of six isoflavonoids from commercial AR by gradient elution, using acetonitrile in water as a mobile phase (Wu et al., 2005).

For all types of compounds with or without chromophores, chromatographic separation with ELSD detectors is used. Even the pharmacopeia stated the use of ELSD detectors for the determination of AR. Despite the characteristic analytical quality, LC-ELSD and UV methods show insufficiency regarding the accurate peak identification and are non-sensitive for the detection of low constituents of compounds. Mass spectrometry has nowadays become a protocol as the post-separation detection technique to analyze the medicinal herbal extracts, the application using LC-MS/MS can show better performance (Huang et al., 2009). This method is more powerful for analysis and rapid identification of compounds even from herbal extracts with complex mixtures of compounds with higher selectivity, low detection limits, and sensitivity (Kim et al., 2007).

The analytical method validations were performed by generating the linear calibration curves. The extraction recoveries can be calculated by adding the known concentrations of standards in the samples. The tuning of the LC-MS/MS instrument was also done to yield the maximum product ions of individual compounds (Liu et al., 2016). According to ICH guidelines Q2B, the limit of detection (LOD) and limit of quantification (LOQ) is defined as $3.3\sigma/s$ and $10\ \sigma/s$ respectively, where σ is the standard deviation of y-intercept and s is the slope of calibration curve of the regression line (Kwon and Park, 2012). The LOD and LOQ are also determined with the signal-to-noise ratio (S/N) of 3 and 10 respectively (Qi et al., 2008). The response of analytes was compared to responses of standards for the quantification of compounds.

The results reviewed are mostly based upon the MS detection of compounds after chromatographic separation. The mass spectrometric analysis of Astragalus Radix samples showed molecular and fragment ions with mass to charge ratio (*m/z*) of 431 $[M+H]^+$, 269 $[M+H\text{-}glc]^+$, 237 $[M+H\text{-}glc\text{-}CH_3OH]^+$ and 209 $[M+H\text{-}CO\text{-}CH_3OH]^+$ for ononin, 269 $[M+H]^+$, 237 $[M+H\text{-}CH_3OH]^+$ and 209 $[M+H\text{-}CO\text{-}CH_3OH]^+$ for formononetin and 807 $[M+Na]^+$, 645 $[M+Na\text{-}glc]^+$ and 627 $[M+Na\text{-}glc\text{-}H_2O]^+$ for astragaloside IV in positive ionization modes (Jie et al., 2013; Qi et al., 2008; Xiao et al., 2004). Similarly, MS analysis of astragaloside IV showed a strong signal of $[M+HCOOH\text{-}H]^-$ at *m/z* 829.4567 and isotope peaks of *m/z* 830.4609 and *m/z* 831.4634 whereas the MS/MS scan showed a peak at *m/z* 651.4076 $[M\text{-}C_6H_{11}O_5\text{-}OH+Na]^+$ which was due to the loss of xylose from C3 carbon (Cheng and Wei, 2014). Some studies showed that the *m/z* transitions were: 285.2 - 213.1 for calycosin, 447.0 – 285.0 for calycosin 7-O-β-D glucoside, 269.0 – 197.3 $[M\text{-}OH\text{-}CO\text{-}OCH_3+H]^+$ for formononetin and

807.1 – 627.2 and 785.5 – 143.2 for astragaloside IV (Sun et al., 2014; Yan and Guo, 2005). In one experiment, the mass to charge value of *m/z* 491 was reported for cycloastragenol. This might be due to the loss of sugar moieties attached in astragalosides (Zhang et al., 2013) and have fragment ions at *m/z* at 143 $[M-C_{22}H_{36}O_3+H]^+$. A study showed that 17 isoflavonoids and 12 astragalosides were identified using LC-MS with electrospray ionization (ESI) where the ultrasonication method was used for the extraction of compounds from the sample. The chromatograms showed parent and fragment ions for many compounds. For calycosin, *m/z* of 285 $[M+H]^+$ was seen whereas formononetin was characterized by a peak at *m/z* of 269 $[M+H]^+$, the ions at *m/z* of 254 and 237 were produced due to the loss of CH_3 and CH_3OH at C-4 carbon. Other peaks at *m/z* of 198 $[M+H-CH_3-2CO]^+$, 209 $[M+H-CH_3OH-2CO]^{+,}$ and 213 $[M+H-2CO]^+$ were identified as fragment peaks of formononetin. The parent ion $[M+H]^+$ at *m/z* of 447 for calycosin 7-O-β-D glucoside gave weak ion at *m/z* of 285 corresponds to direct loss of 162 Da as a hexose residue. Ononin and formononetin showed similar fragmentation patterns (Zhang et al., 2013). The studies also showed many fragment ions for astragaloside IV as well, at different *m/z* values as 653 $[M+H-xyl]^+$, 623 $[M+H-glc]^+$, 605 $[M+H-glc-H_2O]^+$, 491 $[M+H-xyl-glc]^+$, 473 $[M+H-xyl-glc-H_2O]^+$, 437 $[M+H-xyl-glc-3H_2O]^+$, 419 $[M+H-xyl-glc-4H_2O]^+$, 297 $[M+H-xyl-glc-3H_2O-(25\ hydroxy-20,24\ epoxy\ residue]^+$ and 1569 $[2M+H]^+$ ion (Huang et al., 2009; Zu et al., 2009). The compound Diadzein, a metabolic product of formononetin, was also found (Chu et al., 2014). There are studies which showed different fragmentation patterns and fragment ions, such as *m/z* of 190 $[M+H-OH-CH_3-COOH]^+$ for formononetin, *m/z* of 325 $[M-C_6HO_2]^+$ for ononin and *m/z* of 607 $[M+H-C_5H_9O_4-COOH]^+$, 499 $[M+H-C_5H_9O_4-C_9H_{13}O_2]^+$, 415 $[M+H-C_{11}H_{18}O_{10}-CH_3-COOH]^+$ and 324 $[M+H-C_{21}H_{33}O_{11}]^+$ for astragaloside IV (Du et al., 2014). The electrospray ionization in negative mode is also evaluated during the analysis (Xu et al., 2007).

Based upon the above-mentioned selective mass-to-charge ratios (*m/z*) of the parent as well as fragment ions, identification, detection, and quantification of compounds were performed. There was a sample to sample variations in Astragalus Radix. One of the studies showed that calycosin 7-O-β-D glucoside is more accumulated in roots. It was also stated that under some external stress such as salt stress there was variation in the concentration of the compounds ononin and astragaloside IV which tend to increase whereas other compounds like formononetin, calycosin and cycloastragenol were decreased (Liu et al., 2016). The concentration of compounds like astragalosides and isoflavonoids in older samples were found to be higher (Shin et al., 2009).

Many new plant-based medicines are derived from traditional medicinal herbs (Rajiv et al., 2016). The exact mechanisms of action of many traditional medicines are still to be fully explored (Tang et al., 2010). The traditional remedies of Astragalus Radix in Chinese medicine tonifies the Qi and *spleen Qi* deficiencies like fatigue, lack of appetite, diarrhea and raises the *Yang Qi* of stomach and spleen. The *lung Qi* deficiencies like sweating, shortness of breath and colds as well as soreness, numbness, ulcerations, paralysis of limbs, and edema are also treated with preparations of Astragalus Radix (Monograph, 2003).

The chemical compositions and hence the quality of Astragalus Radix might vary according to species, location of origin, age of plant, collection and harvesting period and manufacturing process (Huang et al., 2009; Jie et al., 2013). The natural distributions of Astragalus species are mostly found in the Shanxi and Gansu provinces of China, Mongolia, Kazakhstan, and Russian federations (Li et al., 2017). The different species and ages of Astragalus plants can contribute to different content and quantity of bioactive metabolites (Liu et al., 2018). Botanists recognize *A. membranaceus* (Menggu Huangqi) and *A. mongholicus* (Mojia Huangqi) as different species which are distinguished by the presence or absence of hair on the ovary and pod (Duan et al., 2012). In the local market, the root appearance is evaluated as longer and thicker having better quality for grading the Astragalus Radix (Xiao et al., 2011).

The extraction method must be optimized to extract all targeted compounds. The traditional extraction processes might be more time consuming, laborious, and require a lot of samples and solvent (Xiao et al., 2008). One experiment showed that a higher content of compounds was obtained after ultrasonication than in soxhlet or reflux extraction (Liu et al., 2016). During the sample preparations, many technical guidelines suggest treating the samples with an ammonia solution. It is believed that other saponins are hydrolyzed to astragaloside IV. Studies showed that other astragalosides seem to be major constituents but the peak of astragaloside IV is seen higher due to summing up of peaks. This may be due to the hydrolysis of other astragalosides (Monschein et al., 2014). Not only the mentioned parameters affect the composition but also the transformation of astragalosides during processing, such as heating, storage and extraction conditions might affect the efficacy and potency of herbs. Astragaloside IV is shown to be more stable even in acidic solutions (Chu et al., 2014)

The saponins, astragaloside IV and cycloastragenol are of great interest in the studies because they are supposed to enhance T cell and B cell proliferation and promote telomerase activities which result in a delay in onset of the cellular aging process and enhance the immune system (Nartop et al., 2015b). *Taiwan Herbal Pharmacopeia* states that AR samples contain

around 16% of ethanol-soluble compounds, not less than 17 percent of water-soluble compounds. The pharmacopeial limit of astragaloside IV is not less than 0.04 % (Liu et al., 2020). Similar limits have been set by other pharmacopeias like Chinese and European pharmacopeia.

Isoflavonoids and polysaccharides are also potent for pharmacological activities. Flavonoids are used to boost immune response and promote health status whereas polysaccharides are used together with chemotherapy to improve patient's quality of life in advanced non-small cell lung cancer (Liu et al., 2016; Wu et al., 2017). Saponins and flavonoids present in Astragalus Radix can prevent lipid peroxidation generated by UV rays, hydrogen peroxide and superoxide as well as prevents protein oxidative modification by copper (Monograph, 2003). Quantification of major bioactive compounds can be done by different analytical techniques.

Highly selective, sensitive, reliable, quantitative, and analytical methods are essential for quality control of herbal medicines. Infrared spectroscopy is useful to identify the functional groups of compounds. Most of the functional groups are observed in mid-infrared since the fundamental molecular vibrational resonance falls within the range of 4000 to 400 cm^{-1} excitation frequencies (Liu et al., 2020). The FTIR analysis was performed for the presence of polysaccharides in Astragalus Radix, but the IR spectra could not find the significant differences between different polysaccharides. However, the major functional groups were observed. The hydroxyl stretching vibrations were seen at bands at 3425 cm^{-1} and 3376 cm^{-1}, whereas bands at 2939 cm^{-1} and 2935 cm^{-1} showed the C-H stretching vibrations. The water-bound polysaccharides can be confirmed by the band at 1642 cm^{-1}. The bands between 1121 cm^{-1} and 1155 cm^{-1} showed the presence of pyran configuration and bands at 855 cm^{-1} and 931 cm^{-1} were of 1-3 α glucan (Zhu et al., 2011).

Nowadays, the ultra-high-performance liquid chromatography (UHPLC) is in use for better separation in the shortest run times (Duan et al., 2016). Higher throughput results, selectivity, and sensitivity were expected using UHPLC followed by triple quadrupole mass detection in multiple reaction monitoring (Liu et al., 2018). Most of the isoflavonoids were of similar structure so it was difficult to separate them. The problem was solved by optimization of the chromatographic conditions (Lv et al., 2011). Optimization not only gives better separation but also helps to reduce the influence of other interference, improves the signal response of analytes, increases peak shape, and gives a good separation within a short time.

Even though many mechanisms are used for the analysis of saponins, HPLC-UV could not detect the compounds due to lack of chromophores which sometimes can only be detected

in shorter wavelengths. HPLC-UV is a method of choice for flavonoids and many other compounds that have chromophores or UV absorption properties. ELSD is widely used to overcome the drawbacks of UV detectors, however, the identification is done only considering the retention time. ELSD can sometimes be non-reliable because many of the different compounds might have the same or very close retention times to distinguish them. Though the isoflavonoids are measured using UV detectors, it might not be suitable to determine isoflavonoids in the biological fluids like plasma matrices due to poor sensitivity and selectivity (Zhang et al., 2007). Also, the UV and ELSD methods are not reliable regarding the accuracy of peaks and have low sensitivity for compounds with very low quantities (Huang et al., 2009). More accurately, mass spectrometry can be used to determine the complex herbal preparations as well as for quantification of the compounds in biological fluids like plasma and urine in animal models (Zhang et al., 2005). The advanced form of LC-MS, tandem mass spectrometry is successfully applied for detection and quantification (Kwon and Park, 2012; Zu et al., 2009). This post-separation detection technique uses detectors like single quadrupole, triple quadrupole, ion trap, and time-of-flight (TOF) detectors. Both single ion monitoring (SIM) or multiple reaction monitoring (MRM) channels are used for analysis, but MRM channels are known for their selectivity and sensitivity compared to SIM mode (Yan and Guo, 2005). However, matrix effects and ion suppression are reported to show the great influence in LC-MS and have an important role in quantitation of desired compounds. This effect can increase or decrease the response when compared with standards dissolved in pure solvents. Recovery tests can be helpful to find out such effects (Qi et al., 2008). Similarly, in the MS/MS system the optimization can be done in ion spray voltage, cone voltage, de-cluster potential, and collision energy (Liu et al., 2016).

The LC-MS/MS gave parent and many fragment ions for astragaloside IV, where the characteristic ions at *m/z* of 473, 455, 437, 419, and 297 can reveal that all the astragalosides have a 9,19-cycloanostane aglycone. The sugar molecules attached to them can distinguish between different astragaloside types. Where there are no more fragment ions by losing sugar moieties it can be identified as Cycloastragenol compound which plays an important role in the biosynthesis of phytosterols (Huang et al., 2009).

Some studies showed that the metabolic studies of astragaloside IV are studied in biological samples of rats (Cheng and Wei, 2014). The bioavailability of astragaloside IV was found to be very low due to its complex structure, high lipophilicity, poor water solubility and it is not the substrate for p-glycoprotein (Gu et al., 2004). A study by Sun et al., (2019) suggested modifying the structure of chemical compounds which will be readily absorbed in

the stomach resulting in better plasma concentrations. Intestinal bacteria are supposed to improve the systemic absorption of saponins by biotransformation which can be suspected by the presence of sugar moieties (Sun et al., 2019). The astragaloside IV concentration was studied in human plasma as well in urine, which showed that the plasma half-life was around 2 hours whereas some of the astragaloside IV was detected in urine extractions in healthy Chinese volunteers (Xu et al., 2013). Similarly, the metabolic profiling of Astragalus Radix was seen in biological fluids of rat/pig and the metabolic products were results of glucuronidation, hydroxylation, sulfation, demethylation, and reduction of isoflavonoids. Formononetin is metabolized by the CYP450 enzyme to daidzein (Tay et al., 2019). The calycosin 7-O-β-D glucopyranoside was deglycosylated to calycosin, a typical aglycone in rat studies. The metabolic profiling of Astragalus Radix in humans is still to be explored (Zhao et al., 2015). The acid-labile cyclopropane ring in glycosides is supposed to prevent its transformation to the aglycone cycloastragenol (Nartop et al., 2015a). A study showed that astragaloside IV has been metabolized to cycloastragenol in rat models (Sun et al., 2019; Zhou et al., 2012). However, cycloastragenol is believed to be naturally absent in Astragalus Radix (Zhou et al., 2012).

The morphological resemblance *A. membranaceous* with many adulterant species without any medical effects makes it difficult for the quality control of Astragalus Radix (Dong et al., 2011). Studies showed that genus *Astragalus* has more hundreds of species so the possibilities of adulteration are high (Ma et al., 2002). Some other species used for adulteration are *A. floridus, A. chrysopterus, A. hoantchy, A. tongolensis, A. tribulifolius, A. aksuensis, A. ernestii, A. lehmannisnus* and even *Hedysarum polybotrys* (Dong et al., 2011; Valant-Vetchera and Zyka, 2003).

Aim and Objectives

Aim: To explore different analytical methods for accurate quantification of major bioactive components and quality control of Astragalus Radix samples.

Objectives: The objectives of the present study are to detect, identify, and accurately quantify the major bioactive compounds present in Astragalus Radix samples and compare the quality of AR samples from different vendors.

- To identify chemical compounds present in Astragalus Radix using TLC-MS
- To detect the functional groups of bioactive compounds in AR samples using FTIR analysis
- To perform quantitative analysis of three major isoflavonoids using HPLC-UV method
- To determine the concentration of three isoflavonoids (formononetin, ononin, and calycosin-7-O-β-D glucoside) and two saponins (astragaloside IV and cycloastragenol) using different calibration techniques in LC-MS/MS method
- To perform quality control on different Astragalus Radix samples from various vendors.

Materials and Methods

Chemicals and reagents

Chemical standards, Astragaloside IV (98%), Ononin (98%), and Calycosin 7-O-β-D glucoside (98%) were purchased from Chengdu Biopurify Phytochemicals Limited (Sichuan, China). Formononetin (≥ 98%) and Cycloastragenol (≥ 98%) were obtained from Sigma-Aldric Co. (St. Louis, USA). Acetonitrile, methanol, ammonia, MTBE, and formic acid of HPLC-LC MS grades were obtained from VWR Chemicals (Oslo, Norway). All other chemicals used were of analytical grades. Purified water was obtained from the Ultra purified water purification system (Pure Lab Options, Model: ELGA Purelab Flex).

Samples

The samples were raw dried herbs, granulate, tablets, capsules or hydrophilic concentration of RA, and were numbered as follows: Sample A, A1 and A2 (granulates of AR from Natuurapotheek, Pijnacker, the Netherlands), sample B and B1 (dried roots of AR from, Natuurapotheek, Pijnacker, the Netherlands), sample C (hydrophilic concentrate of AR from Natuurapotheek, Pijnacker, the Netherlands), sample D (powder of dried AR in capsules from Authentic Produce Limited, Jersey, UK), sample E (tablets, Seven Forests in Seattle, USA), sample F (granulates, Chinese Medical Centre, Amsterdam, the Netherlands). Dried AR samples and granulates were pulverized by electric pestle before extraction

Preparation of standard solutions

Pure analytical grade standard chemicals were weighed and dissolved in methanol to a concentration of 1 mg/mL. Ononin and formononetin were dissolved by adding 4-5% acetone in methanol and heating gently to 40°C. For HPLC-UV analysis, standards for formononetin, ononin, and calycosin 7-O-β-D glucoside were prepared in the following concentrations of 2.5, 5, 10, 20 µg/mL and 8, 40, 100, and 200 µg/mL. Similarly, different concentrations of chemical standards were prepared for LC-MS/MS. The stock solutions of each standard compound were prepared with a final concentration of 0.1 mg/mL in methanol. The standard dilutions of each compound were: 0.1565, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 µg/mL. The diluted samples were spiked to concentrations of 0, 0.5, 1, and 2 µg/mL of all compounds during standard addition.

Preparation of sample solutions

Table 1: Information on sample preparation

Sample ID	Sample name	Weight	Extraction conditions	Final Volume
A	RA Granules	5g	Ultrasonication	20 mL
B	Dried Roots	5g	Ultrasonication	20 mL
C	Hydrophilic concentrate	5g ~ 6.009 mL	Original form	6.009 mL
D	Dried root Capsules	5g	Ultrasonication	20 mL
E	RA Tablets, SF *	5g	Ultrasonication	20 mL
F	RA Granules, CMC	5g	Ultrasonication	20 mL
B1	Dried Roots	5g	Boiled in water	20 mL
A1	RA Granules	5g	Lukewarm water	20mL
A2	RA Granules	5g	Boiled in water	20 mL

* The SF tablets contain 12 % of astragalus roots per gram (w/w)

All samples were weighed using an analytical balance (Model: OHAUS AX224, UIS). Sample extraction was performed using 15 ml of 70% methanol using ultrasonication (Branson Ultrasonic Corporation, USA) for 30 mins twice at 40°C and finally washed with 15 ml of methanol and collected. All the samples were centrifuged at 4000 rpm for 10 mins twice to remove impurities (Eppendorf Centrifuge 5702, Germany). The extracts were made to powder form by drying it using a rotary evaporator (Model: IKA HB 10, VWR, UIS) under reduced pressure (40 degree Celsius, 260 rpm, pressure from reduced from 300 mbar down to 72 for water). The final test samples were prepared by reconstituting dried samples with methanol. The hydrophilic concentration (sample C) was used without extraction after centrifugation using a liquid density of 0.83 g/mL.

A very small portion of samples was dried again for FTIR analysis. For the rest of the samples they were used just after extraction, the extracts were centrifuged, and volume was adjusted to 20 ml using methanol. The sample C was used in its original form and applied to TLC plates as well as for FTIR analysis.

Ammonia treatment

After extraction, some of the samples were also tested after treating with 20% ammonia using 50/50 of ammonia solution and sample extract solution to see the effects of ammonia on sample extracts.

All the standards and samples prepared were centrifuged at 4000 rpm for 10 minutes using Eppendorf Centrifuge 5702 (Germany) before injection into LC-UV and LC-MS/MS.

TLC-MS analysis

Preparation of TLC plates

The analytical grade TLC Silica gel 60 F_{254} plates were obtained from Merck KGaA (Germany). The TLC plates were cut in sizes of 3*8 cm and marked with a line 1 cm above the base, where the samples were spotted. Five chemical standards and four samples were applied to different TLC plates with flavonoids and saponins on different plates. The mobile phase was prepared using acetonitrile and water (84:16 v/v and optimized to 90:10 v/v). A capillary tube was used to apply standards as well as samples. The TLC plates were run in the mobile phase (4 mL), and the distance travelled by the solvent was noted. Flavonoids have chromophores so were visible in UV light but the saponins were not. The separation of compounds was noticed in UV light at a wavelength of 254 nm taking reference to respective standards and the distance moved by each compound was noted. For non-chromophore compounds, the portion of TLC plates applied with standard as well as samples were cut down and oxidised using dilute sulphuric acid and heated for up to 5 mins at 105°C. The brownish-black spots were noted for each standard and sample.

The retention factor (RF) for each chemical was calculated as,

Rf = Distance travelled by chemicals/ Distance travelled by solvent

Fingerprint analysis using TLC-MS

The Advion Expression CMS (Model: Plate Expression, UIS) will take the samples directly from the spots on the TLC plates and then subject them to the mass spectrometer. The mass peaks can therefore, be identified for the standards and these fingerprints can be used to analyse the samples. However, the mobile phase for TLC MS was prepared using:

For flavonoids,

- Acetonitrile (CH_3CN): Water (95:5)
- 0.3 mM NH_4OAC
- 0.2% Formic Acid

To make a final volume of 500 mL.

For saponins,

- Acetonitrile: Water (95:5)
- 0.3 mM NH_4OAC
- 0.5% of Formic acid

To make a final volume of 500 mL.

Calculations: 0.3 mM NH_4OAC

Molecular Weight = 77.08 g/mol

Concentration = 0.3mM NH_4OAC

Final Volume = 500 mL

Mass (mg) = Concentration (Millimolar) * Volume (mL) * Molecular weight (g/mol)

= 0.3 * 500 * 77.08

= 11562 µg

= 11.562 mg

The mass spectrometer was calibrated using ESI Tuning Mix (Agilent Tech, USA) using both positive and negative ionization. The mass peaks for all the compounds were searched in the mass spectrum based upon the following mass of the compounds in both positive and negative ionization modes.

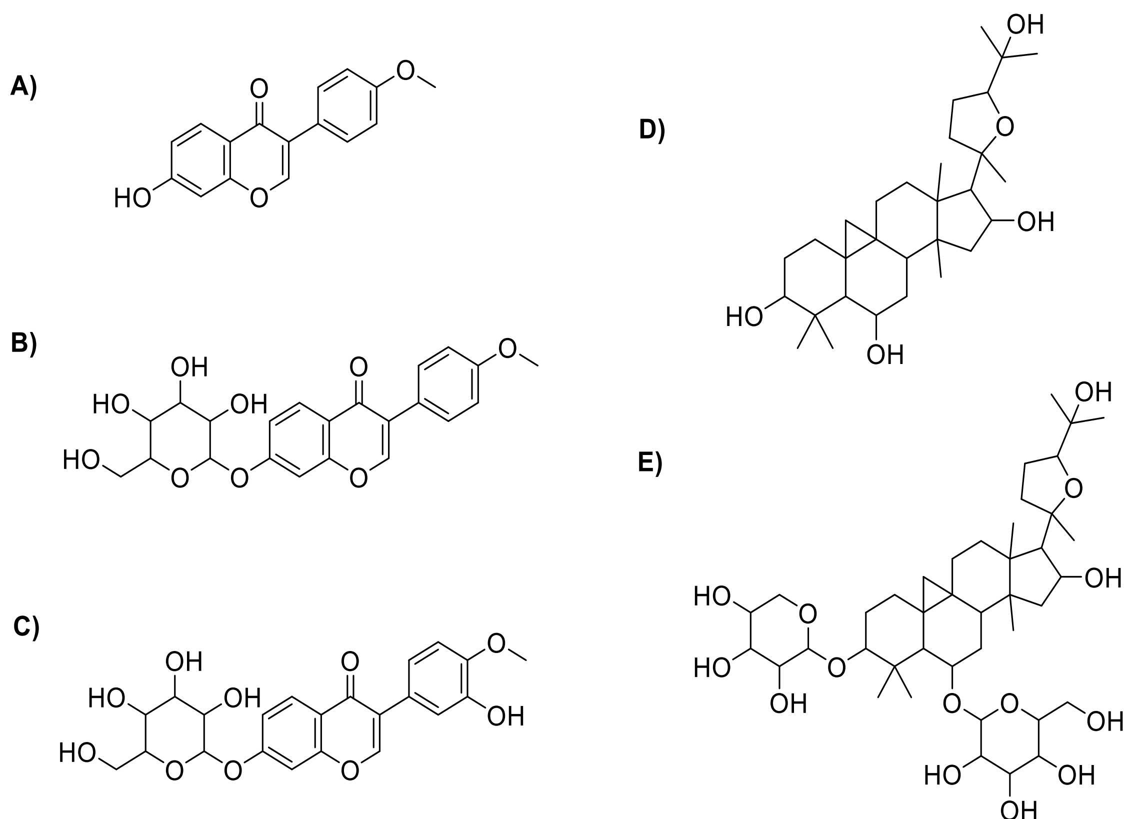


Figure 8. Molecular structures of A) Formononetin, B) Ononin, C) Calycosine 7-O-β-D-glucoside, D) Cycloastragenol and E) Astragaloside IV (*Chemdrug*)

-	Formononetin	($C_{16}H_{12}O_4$)	= 268.26 g/mol
-	Ononin	($C_{22}H_{22}O_9$)	= 430.4 g/mol
-	Calycosin 7-O-β-D-glucoside	($C_{22}H_{22}O_{10}$)	= 446.4 g/mol
-	Astragaloside IV	($C_{41}H_{68}O_{14}$)	= 785 g/mol
-	Cycloastragenol	($C_{30}H_{50}O_5$)	= 490.7 g/mol

The computerized software, Advion Che MS Express was used for obtaining the data.

FTIR analysis

The Fourier Transform Infrared Spectrometer (Cary 630, Agilent Technologies) was used for the analysis of samples. The instrument was fitted with diamond ATR (Attenuated total reflectance) crystal where the samples are directly introduced for analysis. Both dried samples as well as liquid samples of sample C were tested. The Functional groups were analysed using FTIR in the region of 4000 - 400 cm^{-1} with a resolution of 4 cm^{-1}. The FTIR software Microlab PC (Agilent Technologies, UIS) was used for the analysis of samples. The presence of water molecules could affect the reading; therefore, Toluene was used to remove

the water content during evaporation (Azeotroping), in sample A and the sample was subjected to a high vacuum to dry the samples.

HPLC-UV-DAD analysis

A high-performance liquid chromatography instrument was used for the separation of compounds from the sample as well as the elution of standards. The apparatus was an Alliance 2795XE high throughput LC system (Waters Corporation, Milford, MA) with automated sample injection and binary solvent management system connected to a PDA 996 diode array detector (Waters Corporation, Milford, MA). The C18 column, ACE AQ (50mm * 2.1 mm id, Advance Chromatography Technology ltd., Scotland) together with the guard column, Gemini C18 column (4mm x 2.1mm, Phenomenex) were used to separate the compounds. The column temperature was maintained at 40 °C. The mobile phases were assembled with 0.2% formic acid water (A) and Acetonitrile (B) with the gradient elution of 0-1 min, 5-25% B, 1-4 min, 25-50% B, 4-5 min, 50-90% B, 5-7 min, 90% B, 7-7.10 min, 5% B, 7.10-9 min, 5% B. The eluents were introduced to the diode array detector for data analysis. The flow rate was set to be 0.4 ml/min. The injection volume was 8 µl. The re-equilibrium time for the gradient elution was set to be 1 minute. The data obtained were analysed using computer software MassLynx (V4.1). Isoflavonoids in the sample and their standards available were analysed using HPLC UV analysis. The two different standard range of concentrations were prepared as 8, 40, 100, and 200 µg/ml and 2.5, 5, 10, and 20 µg/ml. The detection wavelengths were set at a wavelength of 254 nm. Astragaloside IV and cycloastragenol (saponins) were not detected even at lower wavelengths of 204 nm-210 nm using UV detectors.

LC-ESI-MS/MS analysis

An ultra-performance liquid chromatography system (Acquity UPLC, Waters) was used for the chromatographic separation of compounds from samples and standards. The system consists of a binary solvent management system with autosampler, automatic thermostatic column oven. The column used for separation was Acquity UPLC BEH C18, 1.7µm (2.1 * 100 mm column). The column temperature was set to 50 °C. The sample temperature was set to 10 °C. The UPLC system was connected to a Quattro Premier XE MS/MS detector (Waters) equipped with electrospray ionization in both positive and negative modes and computer software with MassLynx version 4.1. The flow rate was set to 0.5 ml/min and the injection volume was 8 µL. A mixture of 0.2% formic acid (A), Methanol (B), were used as a mobile phase and water and methanol were used as strong and weak wash solutions.

The gradient was set for 8 minutes as, 0-5 mins, 5-95% B, 5-6 mins, 95% B, 6-6.10 mins, 5% B, 6.10-8 mins 5% B. The detection was performed by mass spectrometer using MRM channels (*Appendix I*). Nitrogen (N_2) was used as a nebulizer gas and argon (Ar) was used as the collision gas. The mass calibration of the instrument was performed by using sodium formate prepared by a mixture of 1 ml of 20% Formic Acid and 1ml of 0.1M NaOH in methanol to make the final volume of 30ml and tested with Waters Test Kit (Diadzein Standard). The ionization chamber in the mass spectrometer was set at desolvation gas flow of 1002 L/hr, Cone gas flow of 50 L/hr, source temperature of 100 °C and desolvation temperature of 400°C. The collision cell pressure was maintained at $2.56e^{-003}$ m bar. The cone voltage and capillary voltage were optimized for both molecular ion and fragment ions. All the standards were tuned to yield the maximum product ions for each compound. The MS system uses a triple quadrupole mass detector for the detection of molecular and fragment ions.

The samples were analysed using external calibration methods for non-diluted samples as well as by diluting the samples (ten times). The experiment was more focused on analysing the samples using standard addition methods. The standard addition method is thought to resemble recovery testing and is also used when the matrix effects are not corrected by internal standards. The diluted samples were spiked with a known concentration of standards in a gradually increased concentration level. The response for both spiked and non-spiked samples is plotted in the y-axis and spiked concentration in the x-axis. The sample concentration is given by the cross-section in the x-axis where y is equal to zero. Standard addition corrects the variation of response in different sample materials (Figure 34, *Appendix II*).

Table 2: MRM transitions used in the LC-MS/MS analysis

Compound Name	Molecular formula	Parent ion adduct	Parent ion *m/z*	Daughter ion *m/z*	Cone (V)	Collision (V)
Formononetin	$C_{16}H_{12}O_4$	$[M+H]^+$	269.2	118.1*	50	28
				213.3	50	25
				237.3	50	25
Ononin	$C_{22}H_{22}O_9$	$[M+H]^+$	431.25	118.1	28	46
				237.3	28	44

				269.2*	28	22
Calycosin 7-O-β-D glucoside	$C_{22}H_{22}O_{10}$	$[M+H]^+$	447.25	270.3	28	38
				285.35*	28	20
Cycloastragenol	$C_{30}H_{50}O_5$	$[M+H]^+$	491.5	143	45	10
		$[M+Na]^+$	513.4	513.4 **	80	2
Astragaloside IV	$C_{41}H_{68}O_{14}$	$[M+H]^+$	785.45	143*	70	20
		$[M+Na]^+$	807.4	203.1	120	55
				627.5*	120	50

* Daughter ion used in the MRM transition for quantification

** Parent ion used in MRM transition for quantification (no viable fragment ion)

Table 3: Pharmacopeial limit of compounds to be present in Astragalus Radix

Pharmacopeia	Compounds	Limits	Detectors
Taiwan Herbal Pharmacopeia, 2nd ED. (2016)	Astragaloside IV	0.04 %	ELSD
Chinese Pharmacopeia (Anonymous, 2010	Astragaloside IV	0.04 %	ELSD
Chinese Pharmacopeia, 2010 (Monschein et al., 2014)	Calycosin 7-O-β-D glucoside	0.02 %	ELSD
European Pharmacopeia (Ph. Eur. 7.0; Anonymous, 2011)	Astragaloside IV	0.04 %	ELSD
Japanese Pharmacopeia, 17th Ed. 2016 (2016)	Astragaloside IV	Not specified	TLC

* All the samples tested with ELSD used ammonia for extraction

This suggests that an optimized sample preparation method is most essential for determining Astragaloside IV and the treatment with ammonia is essential, however, the quality of Astragalus Radix without treatment with ammonia still needs to be studied, which means the pharmacopeial assays must be re-evaluated.

Detecting bioactive compounds present in Astragalus Radix in human saliva/urine (method development)

The concentration of many bioactive compounds can be tested in biological fluids after oral or systemic administration of herbal medicines. Saliva and other biological samples contain a lot of proteins and precipitants. Therefore, it is necessary to freeze and then thaw the saliva and other samples and then centrifuge at 4000 rpm for 5 minutes. This process helps to remove all the cell debris and precipitated mucin proteins. If not done, there might be a risk of formation of the emulsion during the liquid-liquid extraction process.

Sample administered (self) = 5 gram of granules samples

The sample collection should be done at different time intervals.

Preparation of spiked saliva or urine samples and test samples

- Spiked saliva or urine:

  At least 30 ml of saliva or urine was collected in a large disposable polypropylene centrifugation tube. After freezing, thawing and centrifugation**,** 5 ml of saliva or urine samples were spiked to a final concentration of 0, 1, 2, 5, 10 and 20 ng/ml for all five compounds. Then the samples were introduced to liquid-liquid extraction.
- Saliva samples after Ingestion

  Granules samples were ingested orally. The saliva was collected in polypropylene centrifugation tubes at different time intervals after ingestion. Similarly, urine was collected in the same way. The samples were thawed after freezing and centrifuged at 4000 rpm for 5 minutes. The supernatant was transferred to separate tubes.

Sample preparation by liquid-liquid extraction (LLE)

- For all the spiked samples with increasing concentration and test samples collected at different time intervals, 500µL of saliva or urine samples were taken in a small 2 ml Eppendorf vials with screw cap.
- After that, 1000 µL of 10% butanol in Methyl-tert-Butyl-ether was added to the Eppendorf vials.
- The Eppendorf tubes were shaken and centrifuged to get a phase separation. Complete phase separation was observed, i.e. no emulsification.

- The top phase was pipetted off as much as possible (900 µL) into new Eppendorf vials, and subjected to evaporation to dryness in a speed vacuum centrifuge for almost 40 minutes at 60 °C.
- After drying, it was re-constituted in 50 µL of pure methanol which provided close to the theoretical 10x enrichment.
- The volume was transferred to special tapered micro-volume vials and then subjected to LC-MS/MS analysis.
- The blank samples, spiked samples, and test saliva samples or urine samples were tested.

Analytical method validation

Selectivity: The HPLC/UV and LC-MS/MS conditions were optimized and determined to obtain good peak separation of the marker compounds in the Astragalus Radix samples.

Linearity: The reference stock solutions made in different concentrations for three flavonoids (Formononetin, Ononin, and Calycosin 7-O-β-D glucoside) were analysed by HPLC/UV and all five compounds including Astragaloside IV and Cycloastragenol were analysed using LC-MS/MS to obtain the response with respect to concentrations. For the linearity, the regression equations were calculated as $y= ax + b$, where x and Y are the concentration of compounds and peak areas respectively.

The limit of detection (LOD) and limit of quantification (LOQ) were defined as 3.3 σ/s and the LOQ was 10 σ/s. The standard deviation of the y-intercepts of the regression lines used as 'σ' and the slope of the calibration curve was used as 's'.

The limit of detection (LOD) is defined as the concentration of standard solutions whose signal to noise ratio (S/N) ratio is 3:1 and limit of quantification (LOQ) is denoted by S/N ratio of 10:1.

Accuracy and precision

Intra and inter-day precision and accuracy were calculated for all the samples to be less than 20%.

Statistical analysis

The statistical analysis was performed using the MassLynx software (Ver.4.1) and Microsoft Excel sheets.

Results

Thin layer chromatography-mass spectrometry (TLC-MS)

The extraction yield of the sample extracts was calculated after the extracts were dried using a rotatory evaporator under reduced pressure. The yield extracted from each 5 g of raw samples were as,

- The net yield of sample A = 1.6 g
- The net yield of sample B = 1.2 g
- The net yield of sample C = 1.2 g

The dried samples obtained were reconstituted in methanol and water (70:30 v/v) to make a final volume of 20 mL. A little portion was dried again to get samples for FTIR analysis.

The retention factor (Rf) for each chemical was calculated as,

Rf = Distance travelled by chemicals/ Distance travelled by solvent

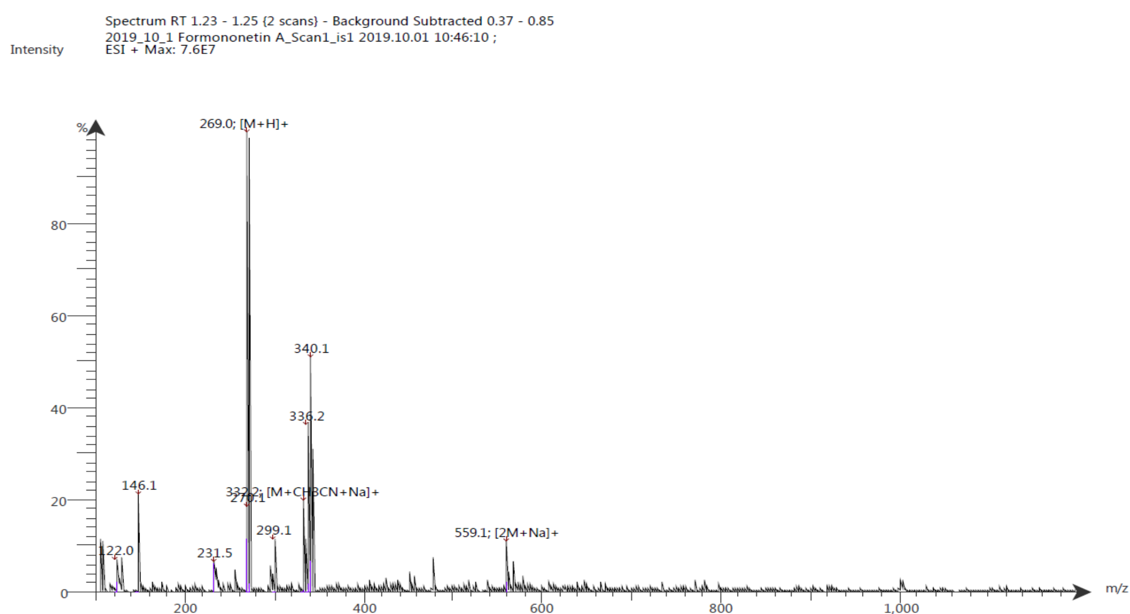
Table 4: Rf values of all five chemical standards

Chemicals	Mobile phase				Retention factor (Rf)
Acetonitrile: Water	84:16 v/v	90:10 v/v			90:10 v/v
	Distance Travelled by solvent				
	7.1	**7.3**	**7.8**	**7.4**	-
	Rf values				
Formononetin	0.94	0.95	0.94	0.95	0.94 ± 0.01
Ononin	0.66	0.60	0.49	0.62	0.57 ± 0.07
Calycosin 7-O-β-D-glucoside	0.62	0.55	0.41	0.55	0.50 ± 0.08
	Distance Travelled by solvent				
	7	**7.2**	**7**	**6.7**	-
	Rf values				
Astragaloside IV	0.26	0.21	0.23	0.19	0.21 ± 0.02
Cycloastragenol	0.76	0.76	0.77	0.76	0.76 ± 0.01

The retention factors of all five compounds are shown in Table 4. The thin layer chromatography uses acetonitrile and water as a mobile phase, with hydrophilic interaction

chromatography (HILIC) type of retention mechanism. The elution order in HILIC is like normal phase LC. Formononetin has the highest retention factor of 0.94 ± 0.01, cycloastragenol being the second highest Rf value of 0.94 ± 0.01, followed by Ononin with Rf value of 0.57 ± 0.07, Calycosin 7-O-β-D-glucoside with Rf values of 0.50 ± 0.08 and Astragaloside IV with the lowest Rf values of 0.21 ± 0.02. This reveals that formononetin is more non-polar than other compounds while astragaloside IV is the most polar compound of all five compounds. All the sample extracts were compared with the reference standards after running TLC.

TLC response and mass peak intensity



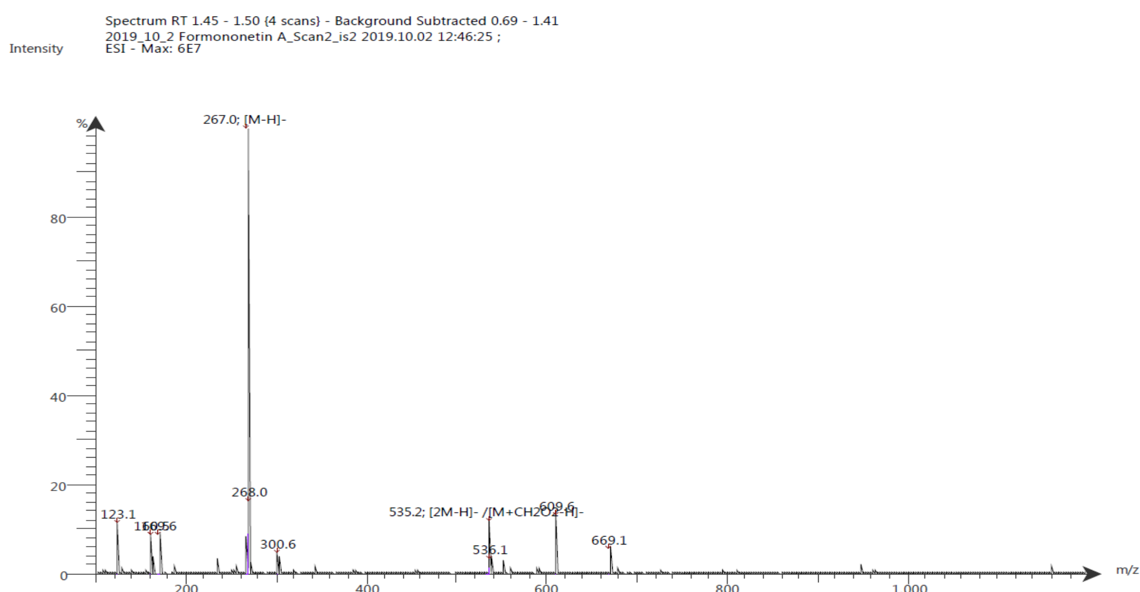


Figure 9. TLC-MS of Formononetin standard (Both positive and negative ionization modes)

Table 5: TLC response of samples together with mass peak intensity

S.N.	Sample name	Clear Spot (+)	Mass peak intensity (%)
1.	Reference Standards		
	Formononetin	+	100
	Ononin	+	28
	Calycosin 7-O-β-D glucoside	+	57
	Astragaloside IV	+	1.6
	Cycloastragenol	+	1.8
2.	Sample A		
	Formononetin	+	100
	Ononin	+	6
	Calycosin 7-O-β-D glucoside	+	16
	Astragaloside IV	+	0.5
	Cycloastragenol	-	6.2
3.	Sample B		
	Formononetin	+	100
	Ononin	+	100
	Calycosin 7-O-β-D glucoside	+	100
	Astragaloside IV	+	4.7
	Cycloastragenol	-	2
4.	Sample C		
	Formononetin	+	24
	Ononin	-	2.3
	Calycosin 7-O-β-D glucoside	-	5.2
	Astragaloside IV	-	-
	Cycloastragenol	-	2.5
5.	Sample B1		
	Formononetin	+	35
	Ononin	+	13
	Calycosin 7-O-β-D glucoside	+	100
	Astragaloside IV	+	1.4
	Cycloastragenol	-	2

Table 5 shows that there were clear spots in TLC plates in UV light or after oxidation of the plates with sulphuric acid and heating process for most of the compounds except cycloastragenol. It was also difficult to locate Ononin, Calycosin 7-O-β-D glucoside, and Astragaloside IV in sample C. The mass peak intensities in mass chromatograms are expected to show the relative abundance of compounds present in the samples. Hence, the lower mass peak intensities mean these compounds are present in lower concentrations and some other peaks are dominant over these peaks.

The mass spectrum of all compounds is given in *Appendix III***.**

Table 6: The mass peaks of compounds at given *m/z* values

Compounds	*m/z* values	Ions
Formononetin	269, 267	$[M+H]^+$, $[M-H]^-$
Ononin	431.1	$[M+H]^+$
Calycosin 7-O-β-D glucoside	447.2	$[M+H]^+$
Astragaloside IV	786.3	$[M+H]^+$
Cycloastragenol	491.1	$[M+H]^+$

Fourier Transform Infrared (FTIR) spectroscopy

FTIR is the most powerful tool for the detection of different types of functional groups and chemical bonds in a sample of phytochemicals. The standards and samples of chemicals and extracts of AR tested with FTIR are,

i. Formononetin standard
ii. Ononin standard
iii. Calycosin 7-O-β-D-glucoside standard
iv. Astragaloside IV standard
v. Cycloastragenol standard
vi. Sample A (Granules extract)
vii. Sample B (Root Powder extract)
viii. Sample C (Hydrophilic concentrate)
ix. Sample B1 (Boiled root extract)

The IR spectrum is given below for all the standards and samples:

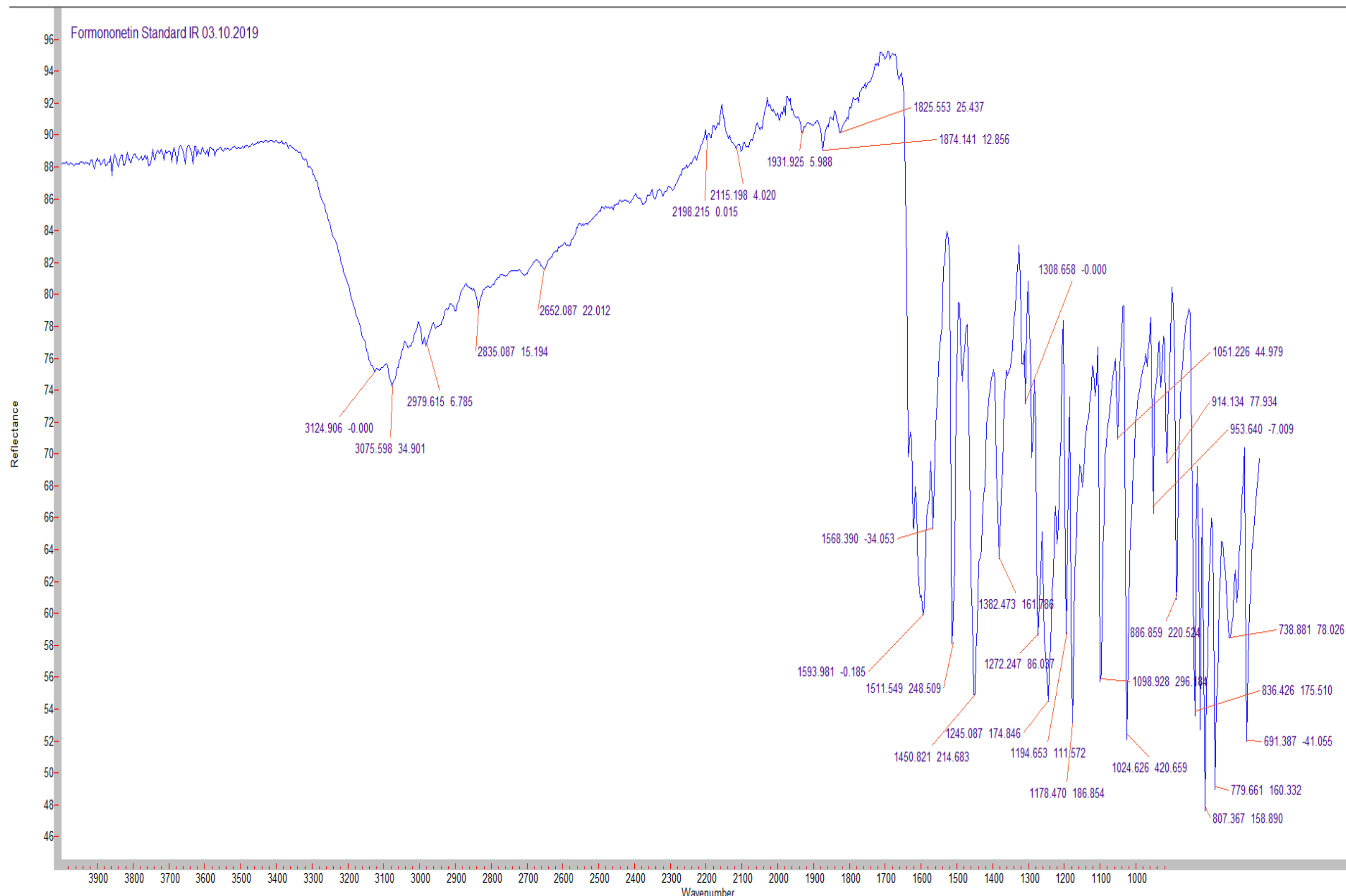


Figure 10. Formononetin reference standard

The fingerprint region of the IR spectrum of Formononetin reference standard gives the following peaks as:

S.N.	Wavenumber	Peak description
1.	3194	Bonded OH, alcohol and phenol
2.	3075	Presence of protons with Sp2 hybridization, CH2 stretch
3.	2979	Presence of protons with Sp3 hybridization, C-H stretch
4.	1593	Aromatic CH stretch, disubstituted benzene
5.	1450	O-CH3
6.	1245	C-O-H, Phenol

The conjugated ketone bond in the spectrum is not seen, might be overlapping.

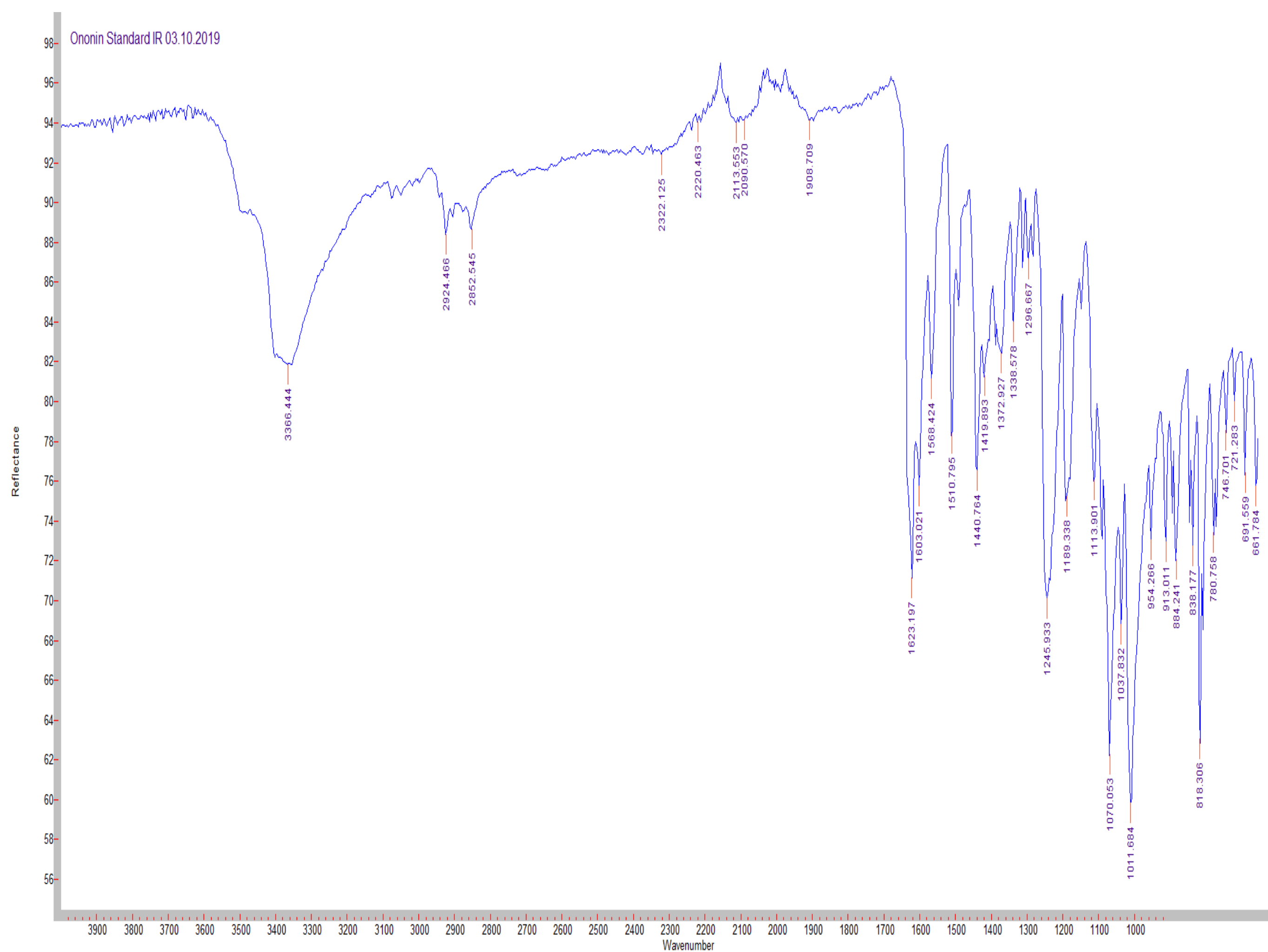


Figure 11. Ononin reference standard

The fingerprint region of the IR spectrum of ononin reference standard gives the following peaks as:

S.N.	Wavenumber	Peak description
1.	3366	Bonded OH, alcohol and phenol
2.	2924	Presence of protons with Sp3 hybridization, CH stretch
3.	1603	Doubly conjugated Ketone, C=O
4.	1510	Aromatic CH stretch, disubstituted benzene
5.	1440	O-CH3
6.	1245	C-O-H, Phenol

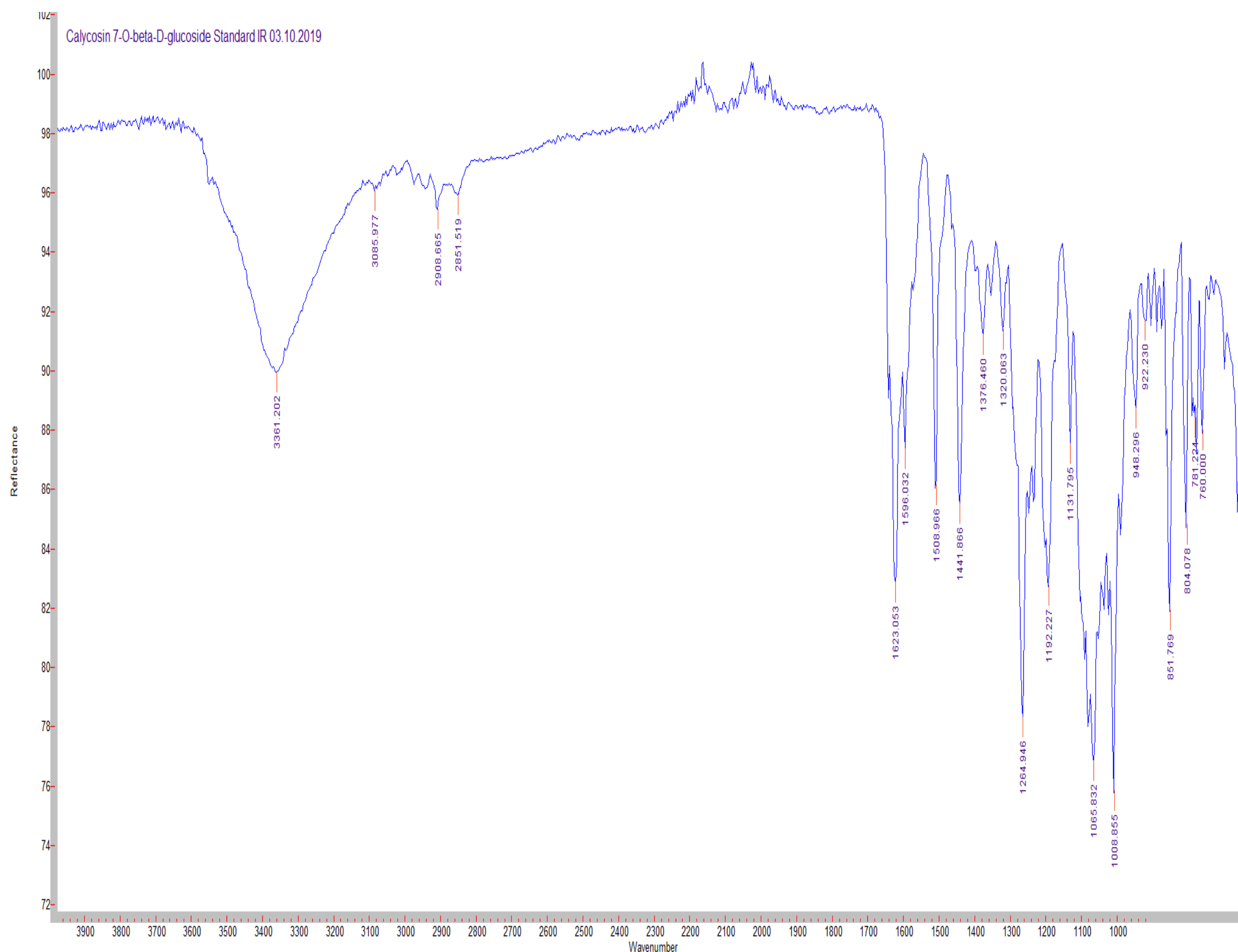


Figure 12. Calycosin 7-O-β-D-glucoside reference standard

The fingerprint region of the IR spectrum of calycosin 7-O-β-D glucoside reference standard gives the following peaks as:

S.N.	Wavenumber	Peak Description
1.	3361	Bonded OH, alcohol and phenol
2.	3085	Presence of protons with Sp2 hybridization, CH2 stretch
3.	2908	Presence of protons with Sp3 hybridization, C-H stretch
4.	1623	Conjugated Ketone
5.	1508	Aromatic CH stretch, trisubstituted benzene
6.	1441	O-CH3

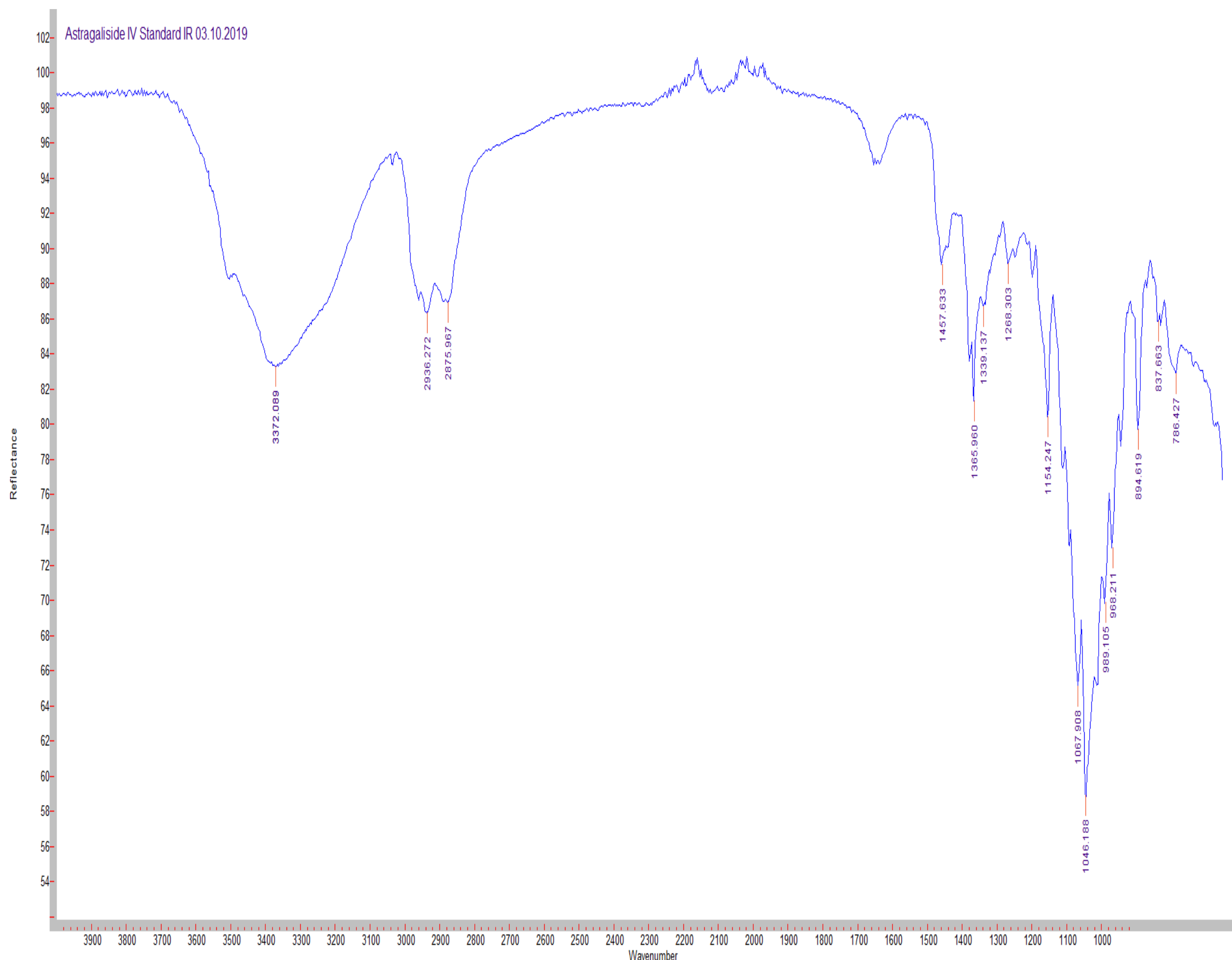


Figure 13. Astragaloside IV reference standard

The fingerprint region of the IR spectrum of astragaloside reference standard gives the following peaks as:

S.N.	Wavenumber	Peak description
1.	3372	Bonded OH, alcohol and phenol
2.	2936	Presence of protons with Sp3 hybridization, CH stretch
3.	2875	Presence of protons with Sp3 hybridization, CH stretch
4.	1154	Presence of C=O, Ketone?

The peak before 2936 might indicate the presence of cyclopropane.

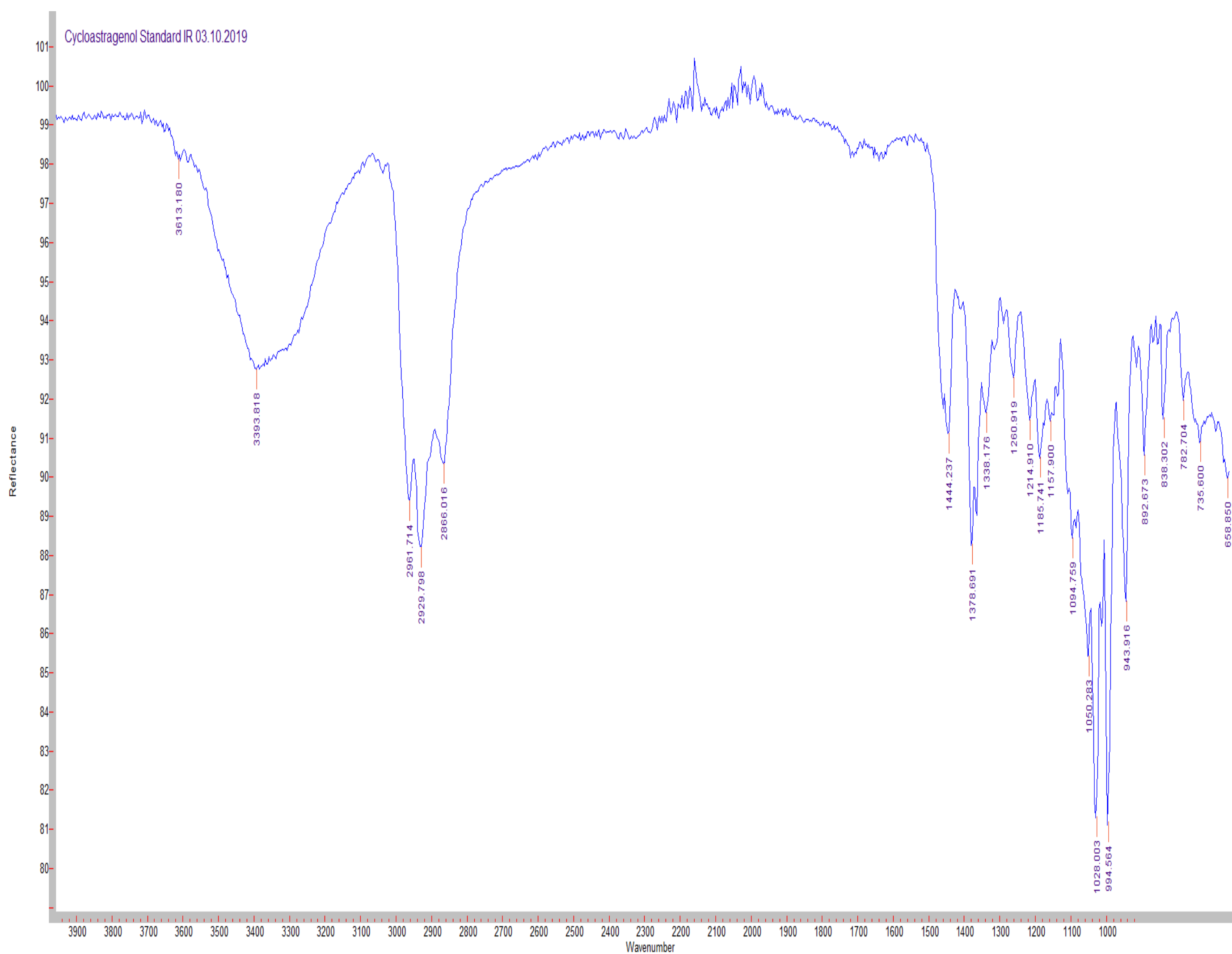


Figure 14. Cycloastragenol reference standard

The fingerprint region of the IR spectrum of cycloastragenol reference standard gives the following peaks as:

S.N.	Wavenumber	Peak description
1.	3393	Bonded OH, alcohol and phenol
2.	2961	Presence of Cyclopropane
3.	2929	Presence of Sp3, CH stretch
4.	1185	Presence of C=O, ketone

Figures 10-14. FTIR spectra of five standard compounds

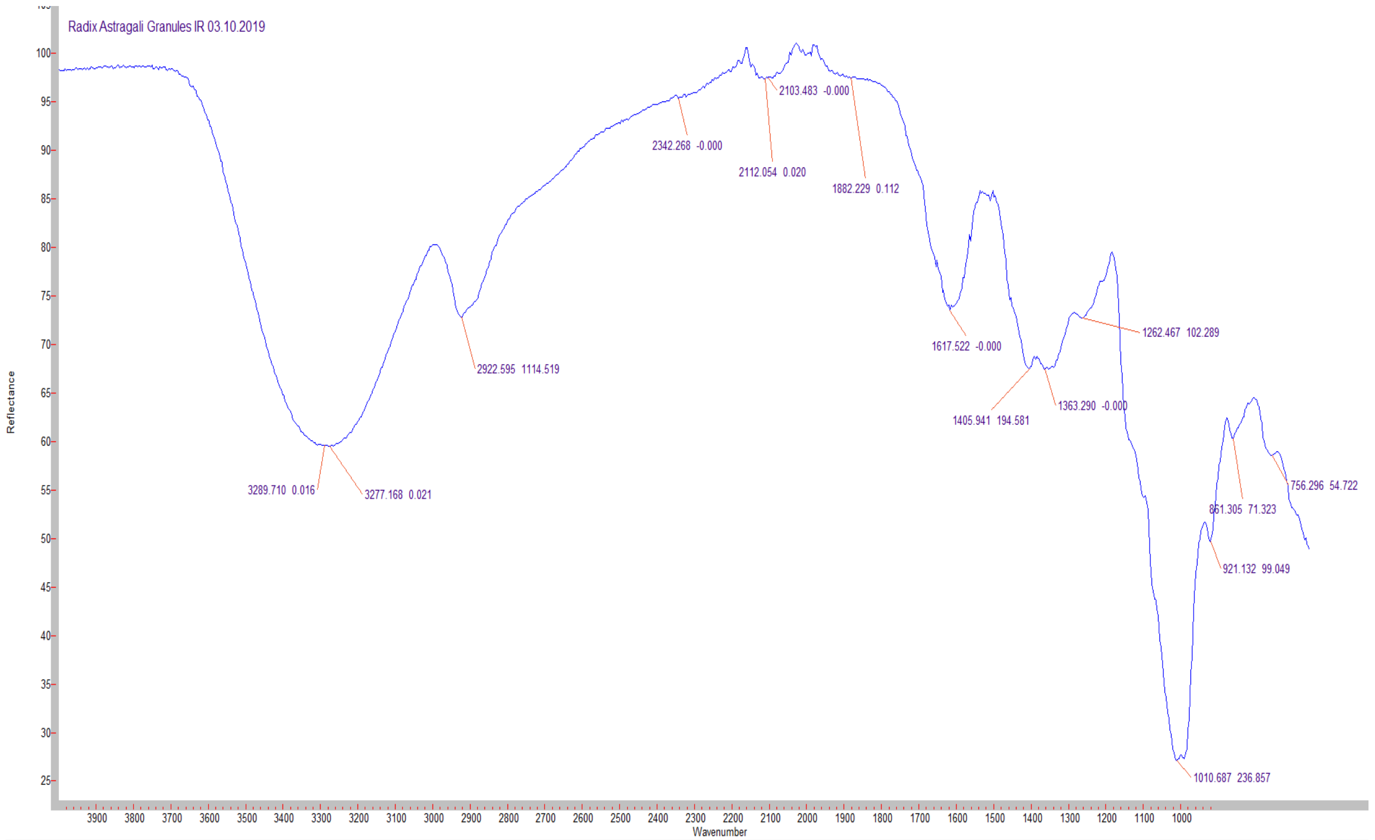


Figure 15. Sample A (RA Granulates)

Radix Astragali Root IR 03.10.2019

Reflectance

Wavenumber

1896.866 -0.000
2090.294 2.452
2115.367 0.415
2341.106 2.214
2924.189 1444.782
3232.089 -0.000
3248.831 0.556
1264.736 90.631
1602.171 1.022
1405.298 565.172
853.248 43.668
1103.308 378.932
921.838 321.482
1039.826 465.790
985.632 378.713

Figure 16. Sample B (Root Powder methanol extract)

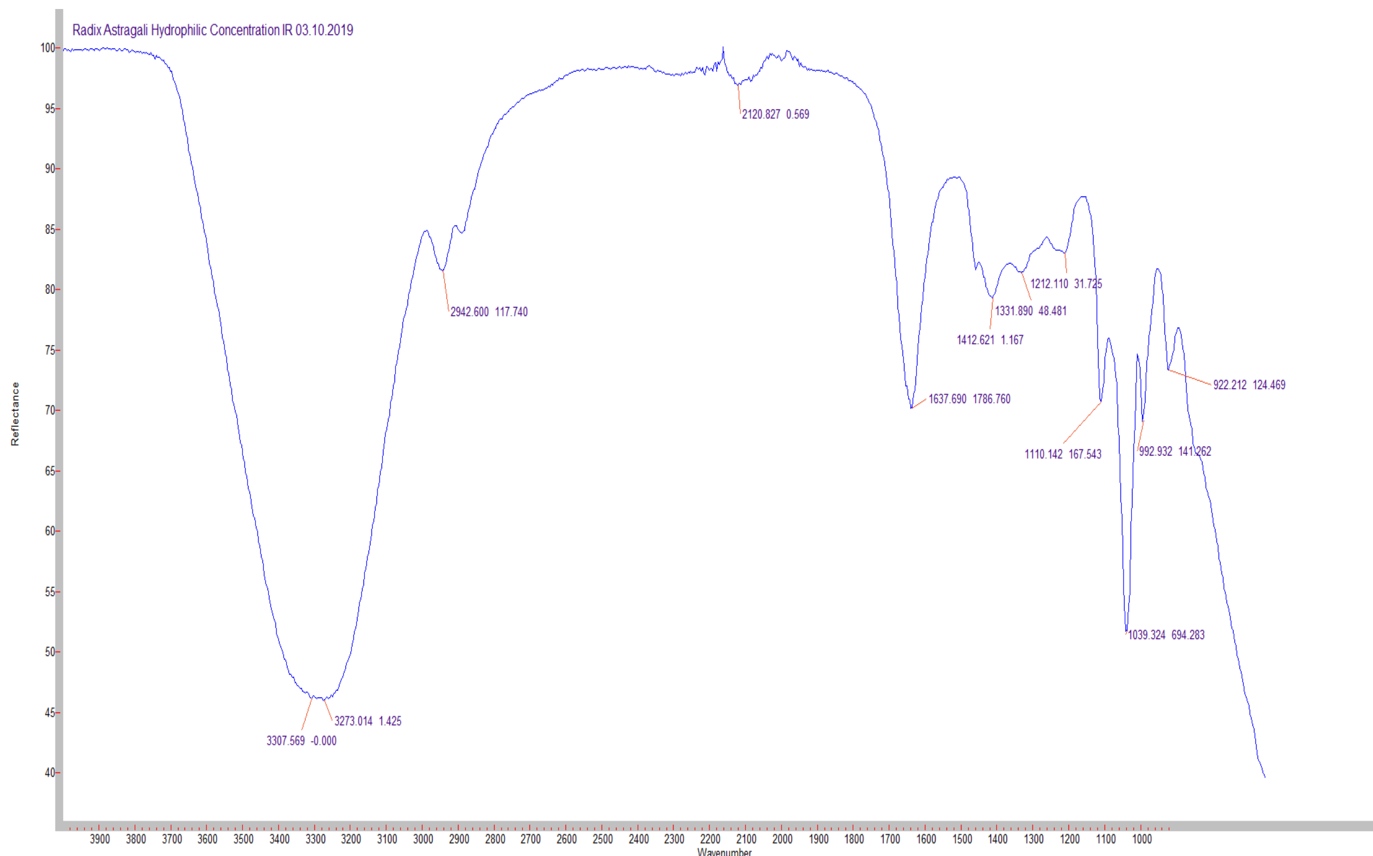


Figure 17. Sample C (Hydrophilic concentration of AR)

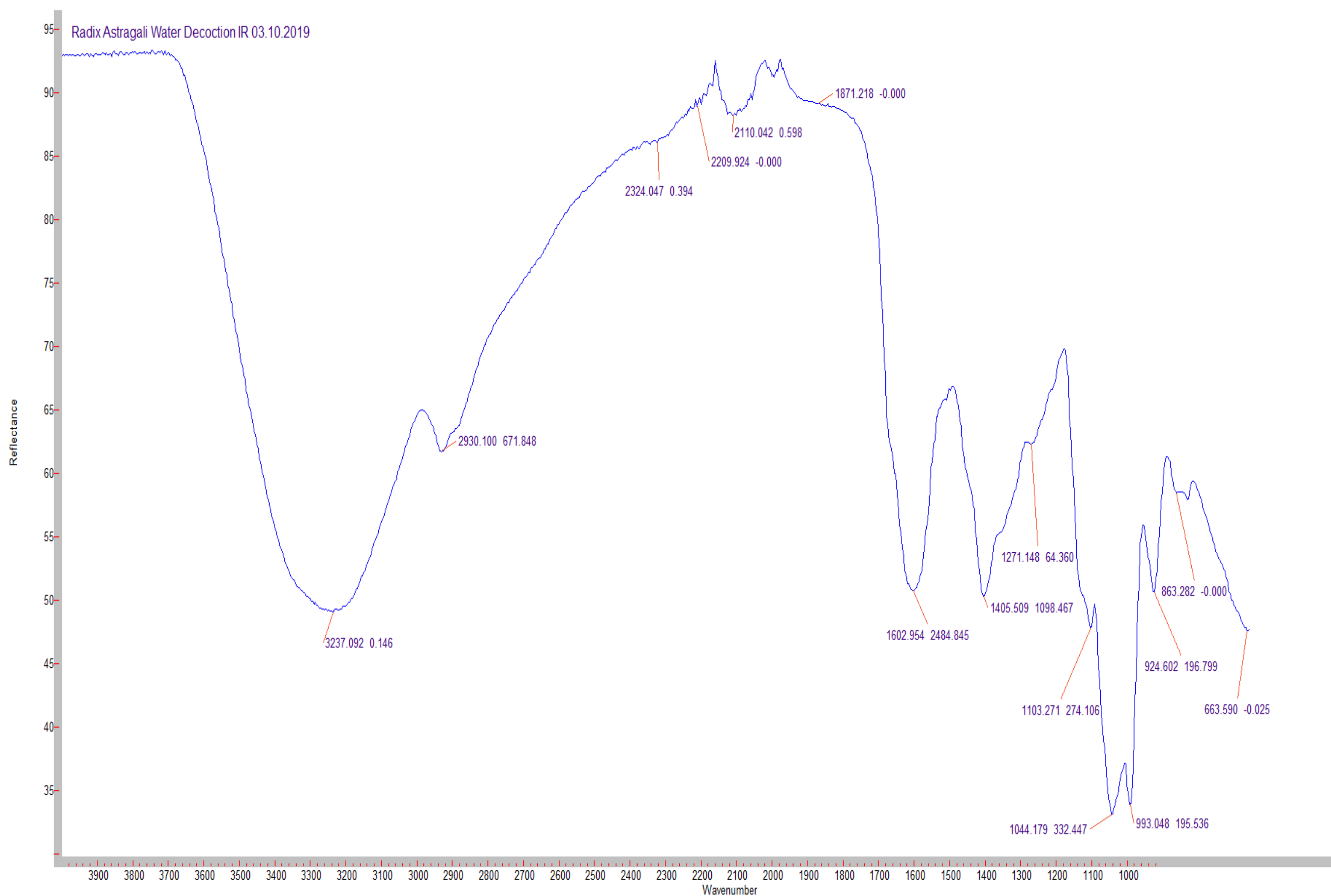


Figure 18. Sample B1 (Boiled root extracts)

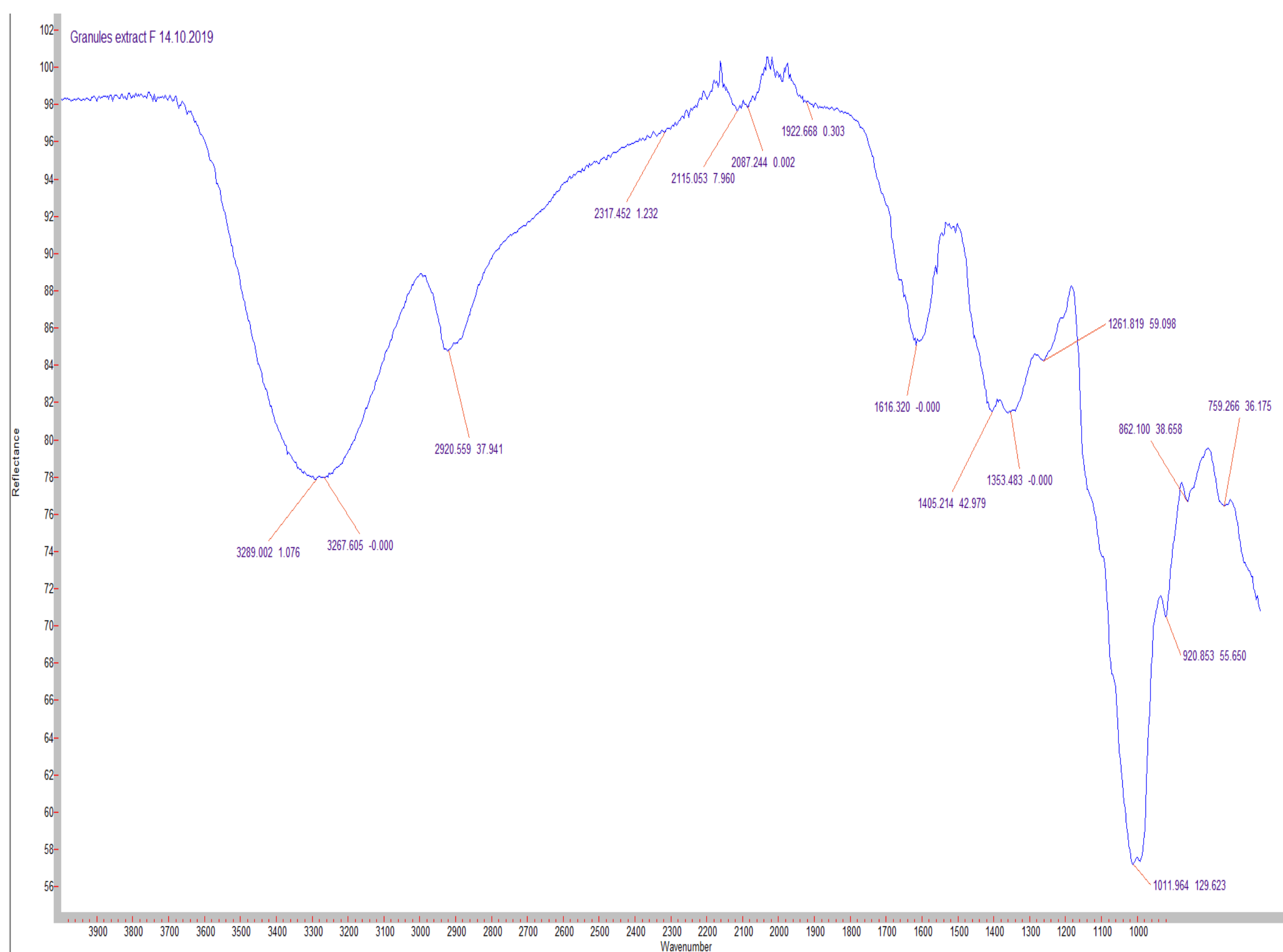


Figure 19. Sample A after treating with toluene showed no significant difference in the IR spectra.

Figures 15-19. FTIR spectra of four AR samples

The FTIR analyses of all Astragalus Radix samples were performed. The spectra obtained for all samples were not so clear. Specific IR absorbance peaks could not be well isolated due to the high complexity of the mixtures of many components in the AR extracts. Some of the functional groups were seen like OH group, CH stretched (Sp^3 hybridization). The large peak around 3300 cm^{-1} in hydrophilic concentrate samples of AR (sample C) might be due to the presence of glycerol in the preparations. All the samples showed some common peaks which might be 1600 cm^{-1} (Ketone), 1400 cm^{-1} ($O-CH_3$), 1200 cm^{-1} (C-O-H, Phenol), and 1010 cm^{-1}.

HPLC-UV and LC-MS/MS analysis

HPLC-UV

The chromatographic conditions were set to give the best resolution of peaks in the chromatogram within a shorter retention time. The peaks of the compounds in the sample were compared with the retention time and UV spectrum of standard compounds, which are supposed to be eluted in the same retention times in the given mobile phase. The UV light was set at a wavelength of 254 nm for all flavonoids. The wavelength was lowered to 203-210 nm to see if saponins could be detected. Since saponins did not possess chromophores they were not detected by the UV spectrophotometer.

Selectivity

All three isoflavonoids, formononetin, ononin, and calycosin 7-O-β-D glucoside standards were run and the respective retention time was noted as 4.42 min, 3.37 min and 2.83 min in a long gradient of total nine minutes. As expected, elution order on the reversed-phase LC column was according to analyte polarity, with formononetin being the most non-polar and thus eluting last.

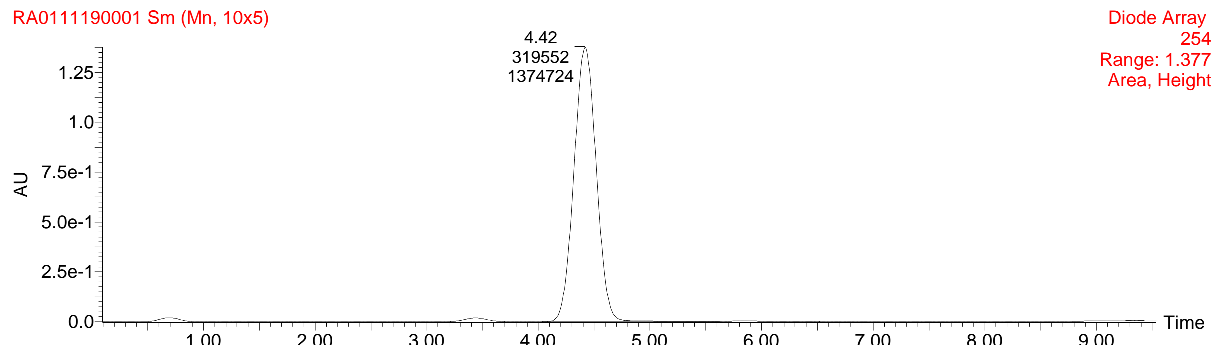


Figure 20. The retention time for formononetin standard

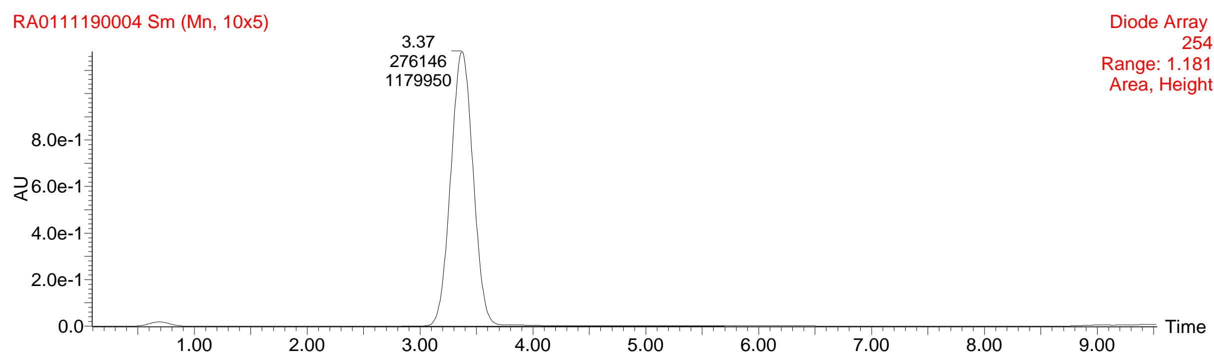


Figure 21. The retention time for ononin standard

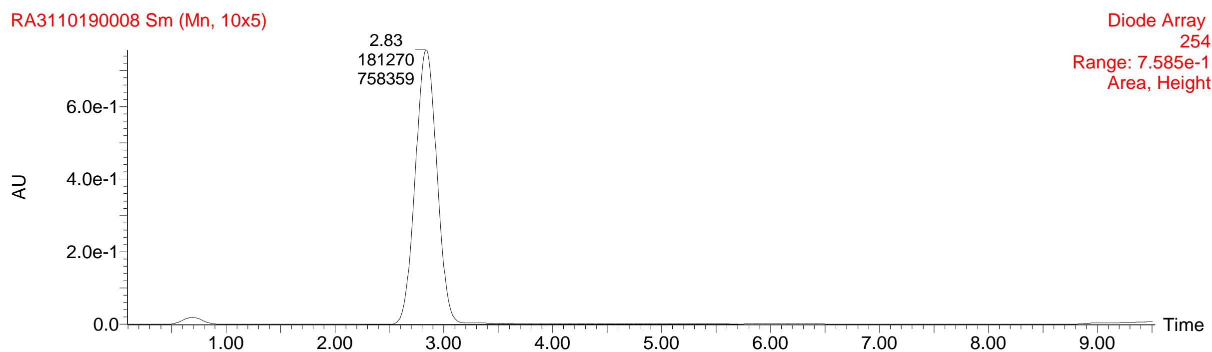


Figure 22. The retention time for calycosin 7-O-β-D glucoside standard

Linearity

The standard concentrations were plotted against the respective peak area to obtain the regression equation. The standard curves were found to be linear when plotted with concentrations of 2.5, 5, 10 and 20 µg/ml. The regression equations were calculated as,

Formononetin, $y = 1516.1\ x - 37.7$ ($R^2 = 0.99$)

Ononin, $y = 981.64\ x + 258.3$ ($R^2 = 0.99$)

Calycosin 7-O-β-D glucoside, $y = 672.94\ x + 12.95$ ($R^2 = 1$)

where y and x are peak area and compound concentrations respectively and determined by least square analysis.

LC-MS/MS

The LC-MS/MS analyses of AR samples were performed using the Acquity UPLC (Waters) system connected to a Quattro Premier XE MS/MS detector (Waters). The positive electrospray ionization mode was used for optimizing the conditions for ionization of compounds using a gradient mobile phase of 0.2% formic acid and methanol. The results showed that it was difficult to detect cycloastragenol in the AR samples, even with a detection limit as low as 0.4 ng/mL. Hence, none of the samples contained cycloastragenol above 1.6 ng/g sample. The molecular ions and fragments ions of formononetin, ononin, calycosin 7-O-β-D glucoside, astragaloside IV, and cycloastragenol were obtained using different cone and collision energy and the system was operated in different MRM channels as shown in table 2. The mass spectra were produced where mass peaks of $[M+H]^+$ for flavonoids and $[M+Na]^+$ peak for two of the saponins were observed. All the peaks observed were in good shape and all the compounds were eluted within the gradient of 8 minutes.

Selectivity

The retention time of compounds in AR extracts was as 2.38 min, 3.00 min, 3.79 min, 4.73 min, and 4.88 min for calycosin 7-O-β-D glucoside, ononin, formononetin, astragaloside IV and cycloastragenol respectively.

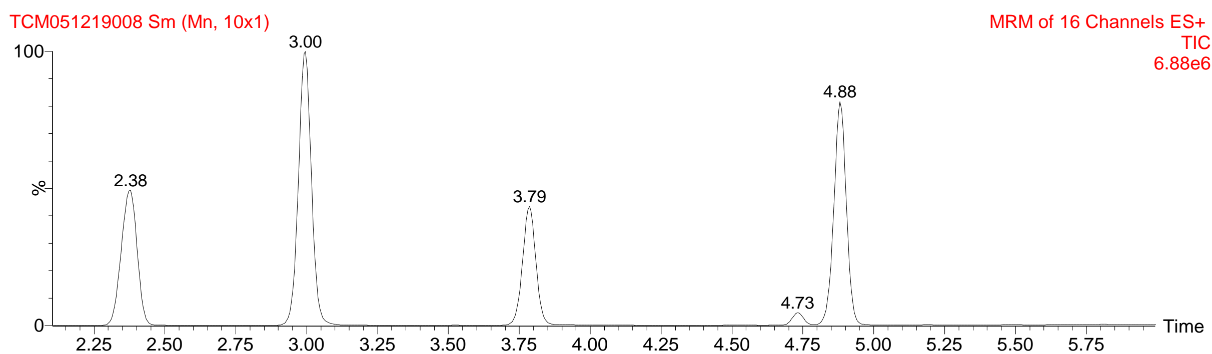
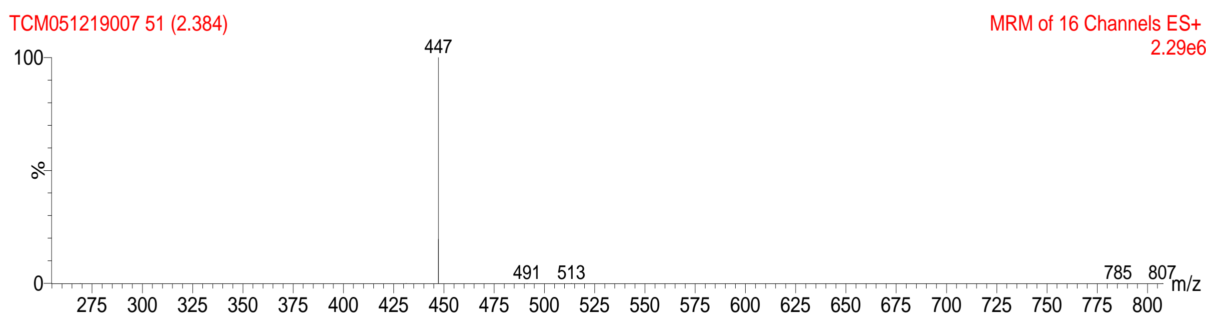


Figure 23. Retention times for calycosin 7-O-β-D glucoside, ononin, formononetin, astragaloside IV, and cycloastragenol in serials from right to left of the figure.

a)



b)

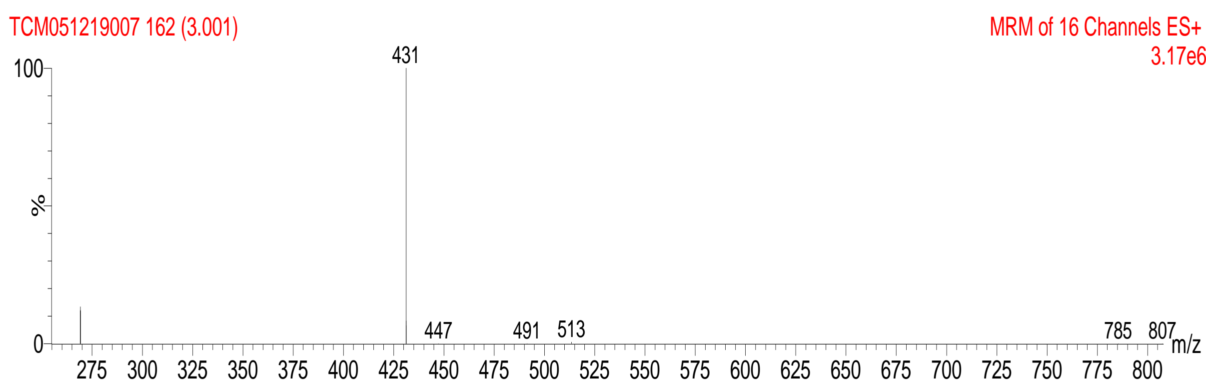


Figure 24. The spectrum of a) calycosin 7-O-β-D glucoside and b) ononin in their respective retention time

Linearity

Regression lines were used to test the linearity of the peak area ratio with respect to the concentration of the compounds. The high correlation showed good linearity with peak area and concentration given by the regression equations as,

- Astragaloside IV, $y = 9484.7x + 1475.8$ ($R^2 = 0.99$)
- Cycloastragenol, $y = 14935x - 8489.5$ ($R^2 = 0.99$)
- Formononetin, $y = 21157x + 5312.2$ ($R^2 = 0.99$)
- Ononin, $y = 79049x + 20692$ ($R^2 = 0.99$)
- Calycosin 7-O-β-D glucoside, $y = 47917x + 51042$ ($R^2 = 0.99$)

when measured using the standard concentrations of 2.5, 5, and 10 µg/ml where y denotes the measured peak area and x is the concentrations.

Limit of detection and limit of quantification

HPLC-UV

The limit of detection (LOD) and limit of quantification (LOQ) were defined as 3.3 σ/s and the LOQ was 10 σ/s. The standard deviation of the y-intercepts of the regression lines used was 'σ' and the slope of the calibration curve used was 's'. The standard error was calculated using excel and then multiplied by the square root of the number of samples to obtain the standard deviation of y-intercepts.

Table 7: The limit of detection (LOD) and limit of quantification (LOQ) of isoflavonoids using HPLC-UV

S.N.	Compound name	Regression equation	LOD (ng/ml)	LOQ (ng/ml)
1	Formononetin	$y = 1516.1x - 37.7$	643	1949
2	Ononin	$y = 981.64x + 258.3$	997	3022
3	Calycosin 7-O-β-D glucoside	$y = 672.94x + 12.95$	188	571

where y denotes the peak area and x denotes the concentrations.

The limit of detection was found to be 643 ng/ml, 997 ng/ml, and 188 ng/ml for formononetin, ononin, and calycosin 7-O-β-D glucoside and limit of quantification was found to be 1949 ng/ml, 3022 ng/ml and 571 ng/ml respectively.

LC-MS/MS

The limit of detection (LOD) is defined as the concentration of standard solutions whose signal to noise ratio (S/N) ratio is 3:1 and limit of quantification (LOQ) is denoted by S/N ratio of 10:1. The limit of detection and limit of quantification is calculated as,

LOD = Average (blank) + 3 x Standard deviation

LOQ = Average (blank) + 10 x Standard deviation

Putting the values in regression equations we get,

Table 8: The limit of detection (LOD) and limit of quantification (LOQ) of isoflavonoids and saponins using LC-MS/MS

S.N.	Compound name	Regression equation	LOD (ng/ml)	LOQ (ng/ml)
1	Formononetin	y = 22936.6x	3.8	9.9
2	Ononin	y = 90203.9x	0.4	1.1
3	Calycosin 7-O-β-D glucoside	y = 61108.6x	0.7	1.7
4	Astragaloside IV	y = 3709.04x	0.6	1.5
5	Cycloastragenol	y = 8503.22x	0.4	0.9

where y denotes the peak area and x denotes the concentrations.

Calibration curves were linear ($R^2 > 0.99$) from the LOQs up to the highest calibrator concentrations, which were either 200 or 20 µg/mL respectively for LC-UV and LC-MS/MS. LC-MS/MS provided LODs that were 2-3 orders of magnitude lower than LC-UV for the isoflavones. As expected, astragaloside IV and cycloastragenol were not detected by the LC-UV method even at the lower wavelength of 203 nm.

The intra-day (n=3) and inter-day (n=6) precision for measurement of the three isoflavones by LC-UV and all five analytes by LC-MS/MS were assessed by analysis of samples spiked to concentrations in the 2.5 – 10 µg/mL range (Table 9).

Table 9: Method repeatability and reproducibility for LC-UV and LC-MS/MS

	LC-UV		LC-MS/MS	
Analyte **Concentration level (µg/mL)**	**Intra-day** **CV%, (n=3)**	**Inter-day** **CV%, (n=6)**	**Intra-day** **CV%, (n=3)**	**Inter-day** **CV%, (n=6)**
Formononetin				
2.5	NA	NA	3.5	12
5	11	23	5.1	8.9
10	12	21	1.5	5.7
20	2.8	11	NA	NA
Ononin				
2.5	NA	NA	5.2	3.4
5	5.5	16	7.8	6.2
10	6.1	16	6.4	4.5
20	0.7	1.2	NA	NA
Calycosin 7-O-β-D glucoside				
2.5	NA	NA	1.5	3.9
5	28	33	2.7	5.4
10	9.2	12	1.0	4.8
20	2.7	3.5	NA	NA
Astragaloside IV				
2.5	NA/NP	NA/NP	2.1	5.7
5	NA/NP	NA/NP	1.4	5.2
10	NA/NP	NA/NP	0.9	1.2
Cycloastragenol				
2.5	NA/NP	NA/NP	4.0	5.3
5	NA/NP	NA/NP	4.3	3.7
10	NA/NP	NA/NP	4.6	3.9

NA = Not analyzed, NP = Not possible to detect

Quantitative analysis of compounds in the sample by HPLC-UV and LC-MS/MS

HPLC- UV

The standards of all three isoflavonoids as well as samples were analysed using HPLC UV analysis. Different concentrations of isoflavonoids in the samples were observed.

Table 10: The concentration of three flavonoids in Astragalus Radix samples (n = 4) determined by HPLC-UV

RA samples	**Standard Concentrations** 8, 40 , 200 (µg/ml)	**Standard Concentrations** 2.5, 5, 10, 20 (µg/ml)
	Concentration, µg/g **(Mean ± SD)**	**Concentration,** µg/g **(Mean ± SD)**
Sample A (Granulates)		
Formononetin	38.5 ± 22	23 ± 1.4
Ononin	25 ± 20	86 ± 6
Calycosin 7-O-β-D glucoside	339 ± 90	525 ± 33
Sample B (Root Powders)		
Formononetin	181 ± 10	59 ± 3
Ononin	150 ± 58	46 ± 3.5
Calycosin 7-O-β-D glucoside	536 ± 152	188 ± 10
Sample C (Hydrophilic Concentrate)		
Formononetin	ND	ND
Ononin	27 ± 16	27 ± 0.6
Calycosin 7-O-β-D glucoside	89 ± 26	94 ± 3
Sample B1 (Boiled Root)		
Formononetin	31 ± 23	7.4 ± 0.3
Ononin	45 ± 25	34 ± 2
Calycosin 7-O-β-D glucoside	427 ± 107	170 ± 7

* ND = Not Detected

The result showed that there are significant changes in the concentration of isoflavonoids when tested in the same sample using different calibration curves with different

standard concentrations. The results calculated with the lower concentration of standards seem to be more accurate due to small standard deviations. Table 10 above shows that the highest concentration of formononetin was found in root powder with the value of 59 ± 3 µg/g of samples, whereas in sample B1 (boiled root) it was found to be only 7.4 ± 0.3 µg/g. The reason might be that formononetin is less water-soluble i.e. non-polar**.** Sample A (granules) of AR comes in the second position with 23 ± 1.4 µg/g of formononetin. Interestingly, the concentration of formononetin in hydrophilic concentration (sample C) could not be determined which might be due to the interference present in the sample. A significant concentration of 86 ± 6 µg/g of ononin was found in granules which were more than other samples tested. Similarly, granules also contained significantly higher concentrations of calycosin 7-O-β-D glucoside (525 ± 33 µg/g) compared to other samples. The raw HPLC-UV chromatogram of the samples is given in *Appendix IV.*

There were significant differences in the results obtained when the same samples were measured with two different standard concentrations. Based upon the standard deviation, the results with low standard concentrations gave fewer standard deviations, hence it can be assumed to be the better option to use standard concentrations where the sample concentrations might fall under the limit of linearity. However, such large variation in results might affect the reliability of the method used.

HPLC-UV spectrum of sample C of Astragalus Radix

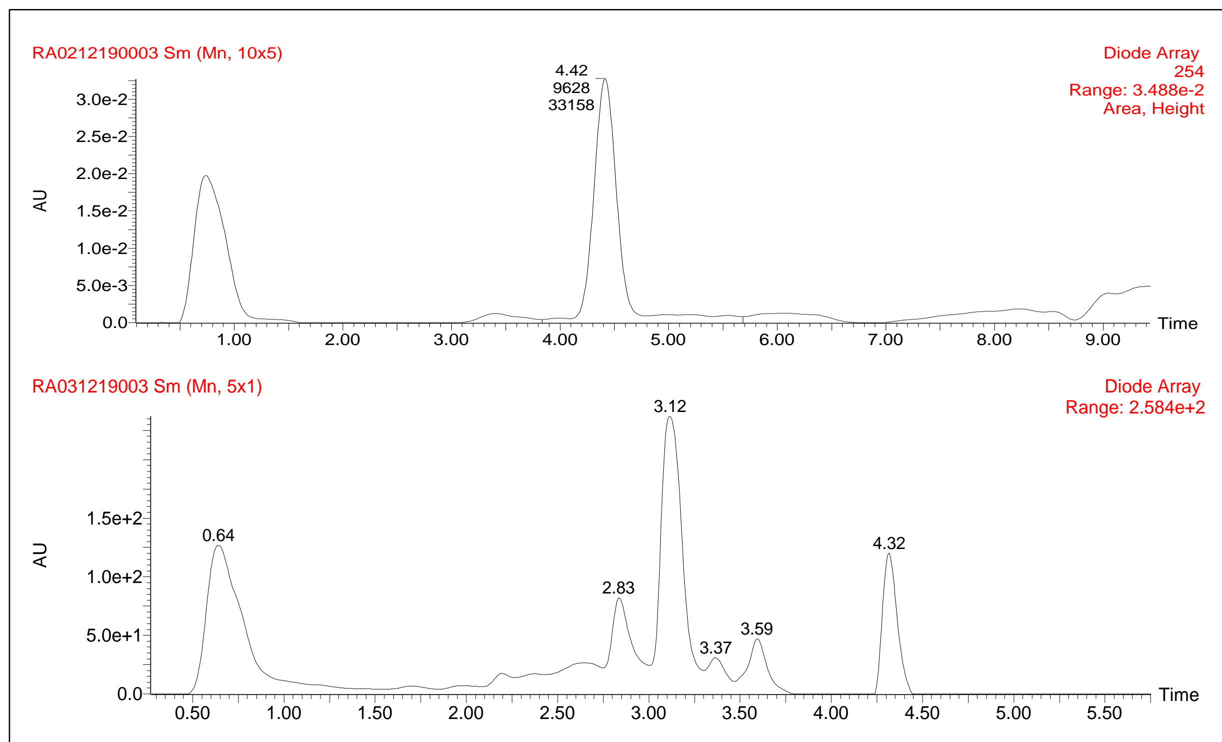


Figure 25. The HPLC-UV chromatogram of formononetin standard (top) and hydrophilic concentrate of AR (below) (this does not show a peak at the retention time of 4.42 mins for formononetin)

The LC-MSMS of sample C showed there is the presence of Formononetin in the sample, however, we could not detect the peak using HPLC-UV even after spiking it with known concentration. Therefore, during the analysis of the sample by HPLC-UV, the analyte eluate at retention times of around 4.32 min (retention time verified by standard) was collected and then spotted on a TLC plate. Thereafter, the sample was subjected to TLC-MS, and the result was obtained in only positive modes as below.

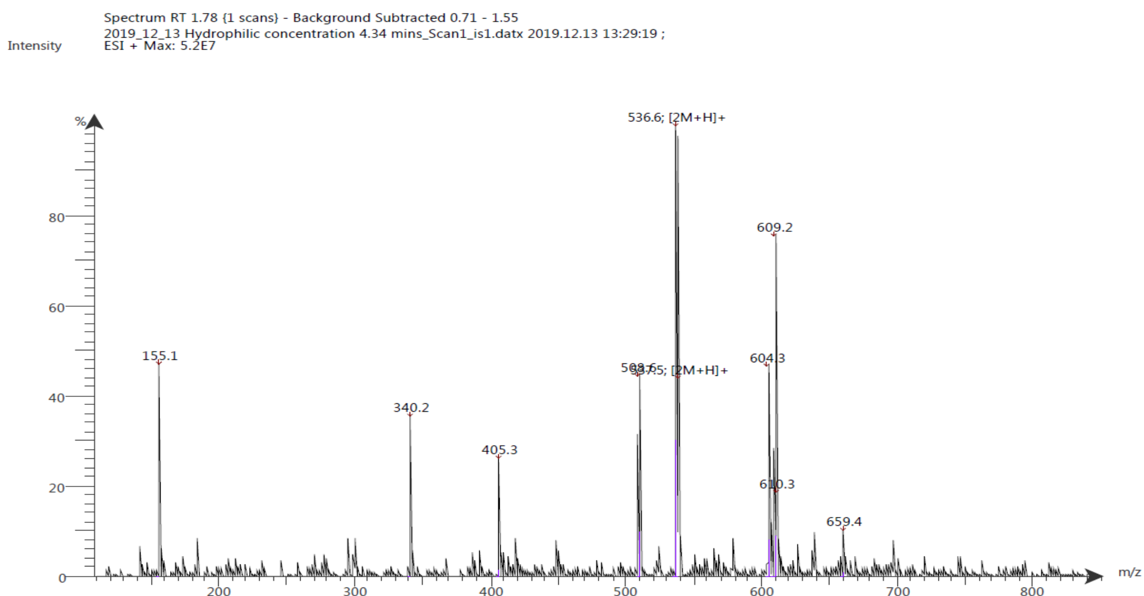


Figure 26. TLC-MS of hydrophilic concentration of Astragalus Radix showing peak for formononetin

Figure 26 showed that the *m/z* of 536.8 $[2M+H]^+$ which confirms that there is formononetin in the sample C. Molecular mass of Formononetin is 268.27 g/mol.

LC-MS/MS

The simultaneous determination of the concentration of three flavonoids, formononetin, ononin, and calycosin 7-O-β-D glucoside, as well as two of the saponins, astragaloside IV and cycloastragenol, were performed using the developed method for LC-MS/MS. In the experiment, the non-diluted samples were measured, similarly, different methods were developed as external calibration using dilutions and standard addition methods. The standard addition test was also performed in some sample extracts which were treated with 20% ammonia (50:50 v/v). The concentration of all compounds was measured as µg per gram of sample. Different regression equations were used to calculate the results. The data are presented in the table 11.

Table 11: Quantitative analysis of Astragalus Radix samples using LC-MS/MS

Sample Name	Normal Samples Mean ± SD	External Calibration (Diluted x10) Mean ± SD	Standard addition (Diluted x10) Mean ± SD	Standard Addition with 20% Ammonia Mean ± SD
Sample A (Granules)	μg/ g	μg/ g	μg/ g	μg/ g
Formononetin	19 ± 2	28 ± 3	21 ± 2	25 ± 5
Ononin	16 ± 2	35 ± 2	35 ± 3	13 ± 8
Calycosin 7-0-β-D glucoside	39 ± 5	125 ± 2	173 ± 41	121 ± 24
Astragaloside IV	6 ± 0.5	74 ± 2	203 ± 6	537 ± 178
Sample B (Root powder)				
Formononetin	40 ± 7	69 ± 4	65 ± 11	78 ± 14
Ononin	33 ± 12	59 ± 7	64 ± 12	36 ± 7.5
Calycosin 7-0-β-D glucoside	57 ± 16	163 ± 10.5	177 ± 34	216 ± 80
Astragaloside IV	1 ± 0.2	21 ± 2	37 ± 6.6	315 ± 136
Sample C (Hydrophilic concentrate)				
Formononetin	8 ± 0.4	13 ± 0.6	13 ± 1	21 ± 8
Ononin	10 ± 0.5	23 ± 1	21 ± 4	7 ± 0.1
Calycosin 7-0-β-D glucoside	18 ± 0.8	72 ± 3	60 ± 40	47 ± 11
Astragaloside IV	1.7 ± 0.1	17 ± 2	23 ± 5	104 ± 28
Sample D (Capsules)				
Formononetin	NA	NA	47 ± 2	57 ± 8
Ononin	NA	NA	109 ± 30	112 ± 0
Calycosin 7-0-β-D glucoside	NA	NA	336 ± 104	13 ± 2
Astragaloside IV	NA	NA	78 ± 11	369 ± 95
Sample E (SF tablets)				
Formononetin	NA	NA	5 ± 0.4	6 ± 0.4

Ononin	NA	NA	8 ± 1	31 ± 23
Calycosin 7-0-β-D glucoside	NA	NA	17 ± 2.3	14 ± 0.7
Astragaloside IV	NA	NA	9 ± 2	221 ± 67
Sample F (CMC granules)				
Formononetin	NA	NA	54 ± 1	84 ± 2
Ononin	NA	NA	25 ± 3	18 ± 4
Calycosin 7-0-β-D glucoside	NA	NA	120 ± 4	92 ± 11
Astragaloside IV	NA	NA	36 ± 3	306 ± 71
Sample B1 (Boiled Root)				
Formononetin	12.5 ± 5	28 ± 0	24 ± 5	NA
Ononin	19 ± 7	64 ± 3	76 ± 37	NA
Calycosin 7-0-β-D glucoside	38 ± 11	209 ± 7	65 ± 17	NA
Astragaloside IV	4 ± 0.6	47 ± 4	145 ± 8	NA
Sample A1 (Granules, lukewarm water extraction)				
Formononetin	NA	NA	21.5 ± 2	NA
Ononin	NA	NA	41 ± 2	NA
Calycosin 7-0-β-D glucoside	NA	NA	241 ± 53	NA
Astragaloside IV	NA	NA	200 ± 69	NA
Sample A2 (Granules, boiled water extraction)				
Formononetin	NA	NA	21.5 ± 2	NA
Ononin	NA	NA	33 ± 6	NA
Calycosin 7-0-β-D glucoside	NA	NA	197 ± 48	NA
Astragaloside IV	NA	NA	207 ± 0	NA

* NA = Not Analysed

The quantitative analysis of four of the Astragalus Radix samples was tested using external calibration with and without dilutions. There were significant differences in the quantity of compounds present in all samples. The diluted samples showed more exact values

than non-diluted samples which might be due to the reduction in matrix effects as well as interferences**.** For example, the concentration of astragaloside IV in non-diluted sample A was 6 ± 0.5 µg/g while after dilutions it was found to be 74 ± 2 µg/g. Moreover, the standard addition method was introduced with dilutions to determine a more reliable quantity of compounds, where the concentration of astragaloside IV in sample A was found to be 203 ± 6 µg/g which was surprisingly more interesting. The standard addition method is considered to give more accurate results regarding the analysis. Standard addition also resembles recovery testing and compensates for ion suppression. Similar effects were seen for other compounds in diluted samples and samples tested with standard addition.

Comparing the results of the standard addition method, the highest concentration of formononetin was obtained in 70% methanol extract of root powder (sample B) with a value of 65 ± 11 µg/g. Other isoflavonoids tested were ononin and calycosin 7-O-β-D glucoside, which were found more in capsule samples (sample D) of Astragalus Radix with the values of 109 ± 30 µg/g and 336 ± 104 µg/g respectively. The concentration of one of the major bioactive compounds astragaloside IV was found to be present in the highest concentration of 203 ± 6 µg/g in Sample A. The lowest concentration of all compounds was found in SF tablets (sample E) because one gram of SF tablet contained only 12 percent (*w/w*) of Astragalus root in it. The hydrophilic concentrate of AR samples did not prove to be of adequate quality compared to other samples having the lowest concentration of compounds compared to the total concentration of astragalus root present in the samples. The analysis of AR samples showed that cycloastragenol was either absent in all the sample extracts or its concentration was lower than the detection limit.

There was a significant difference in compound concentration from the same sample when extracted using different extraction methods. The root powder was extracted using 70 % methanol and by boiling in water. The concentration of astragaloside IV was increased by almost four-fold when boiled in water but the formononetin was decreased. However, there was not much significant difference in concentration of all compounds present between methanol extract or lukewarm water extract or boiled water extract (sample A or A1 or A2) of AR granulates. Though there were variations in the content of compounds when treated with ammonia, the concentration of astragaloside IV was significantly increased in all samples. This might be due to the hydrolysis of all other astragalosides to astragaloside IV. After ammonia treatment, AG-IV is the same in raw root samples, sample B and sample D (not significantly different), which indicates that the total concentration of astragalosides in raw roots might be similar. The raw LC-MS/MS chromatogram of samples are given in *Appendix V.*

Analysis of granulate samples (sample A) by LC-UV and LC-MS/MS

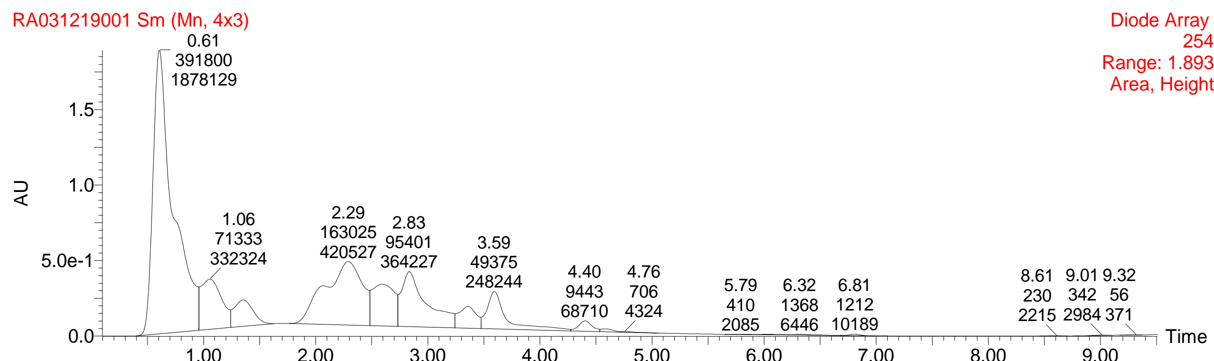


Figure 27. LC-UV of granulate samples (sample A)

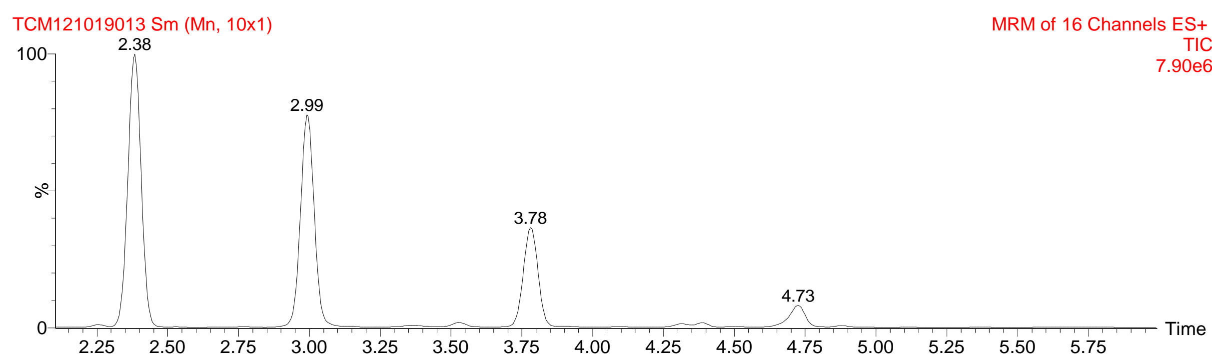


Figure 28. LC-MS/MS of granulate samples (sample A)

The LC-MS/MS of granulate samples clearly shows higher selectivity for calycosin 7-O-β-D glucoside (retention time of 2.38 mins). The lower selectivity might be the reason for the higher value observed during HPLC-UV analysis (retention time of 2.83 mins).

Ion ratios

When using two different MRM channels for each analyte, it is possible to compare ion ratios of the standard solutions with the sample extracts. The ion ratio comparison gives an additional identification parameter, together with peaks in both MRM channels at the correct retention time. The ratios of ions between MRM channels is measured for both standards and samples. The ion ratios of standards of formononetin, ononin, calycosin 7-0-β-D glucoside, astragaloside IV, and cycloastragenol are found to be 0.99, 17.6, 4.41, 4.8 and 0.06 respectively. The ion ratios of standards and sample extracts are shown in *Appendix* VI (Table 12)**.** The ion ratios of cycloastragenol in samples did not match with chemical standards.

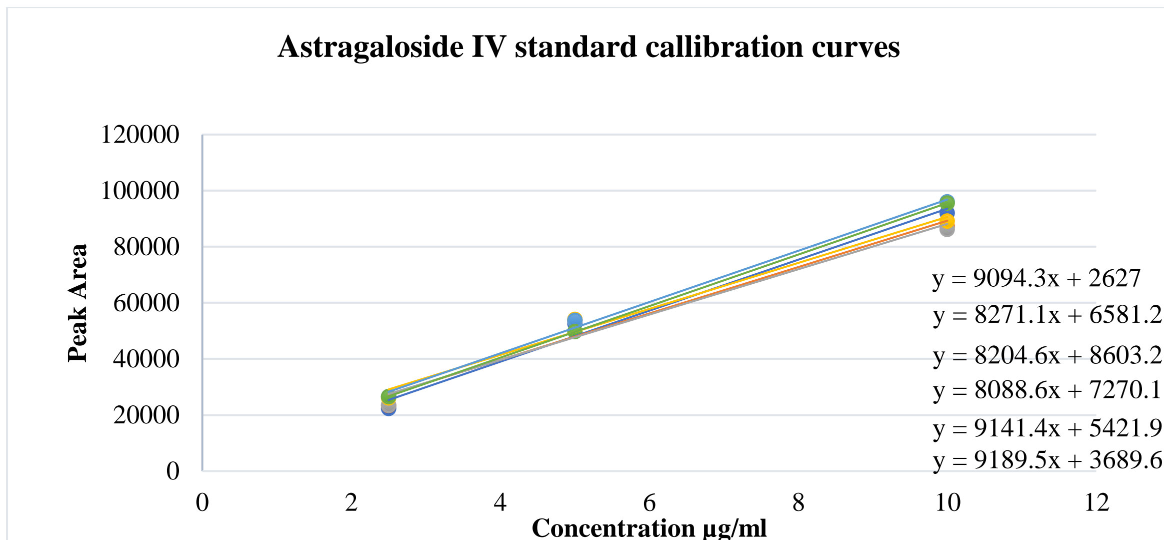


Figure 29. Standard addition curves for AG-IV standards

Figure 30. Standard addition curves for determination of AG-IV in sample extracts

The above figures, figure 29 and figure 30 showed that there are variations on slopes of astragaloside IV standards measured and astragaloside IV in the samples. Each of the samples showed closer concentration values of astragaloside IV during repetitions.

Testing of saliva and urine samples:

The peak areas of concentrations showed that the extraction with MTBE with 10% Butanol was more effective than with MTBE alone. This experiment was done as a trial by self-administration of a 5 grams sample of granules. The amount taken was safe for consumption. The presence of the compounds in saliva and urine were detected except for cycloastragenol. The full phase experiment was not performed but it was confirmed that the pharmacokinetic studies can be performed by testing the biological fluids from the human body (Figure 31, 32). Testing saliva samples is very easy for sample collection because it is a non-invasive procedure and it is thought to be a mirror image of blood samples. Furthermore, testing of plasma samples and correlation with saliva samples should be done for confirmation. The urine samples can be tested to detect the presence of compounds together with metabolites to see the clearance rate and formation of metabolites.

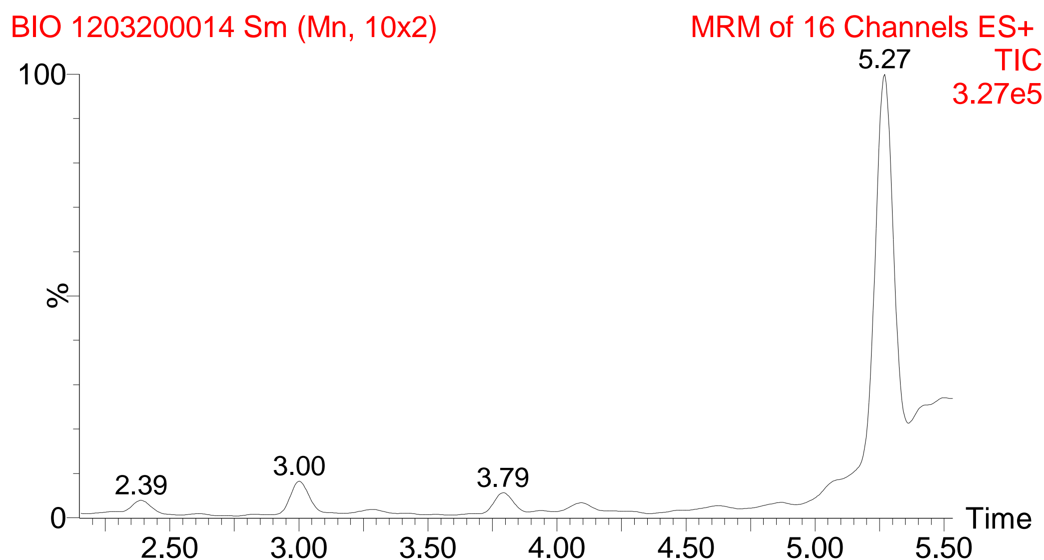


Figure 31. Chromatogram of saliva samples taken in 4 hours after sample ingestion

The chromatogram indicates that the isoflavonoids can be detected in saliva samples with the peaks observed in their respective retention times.

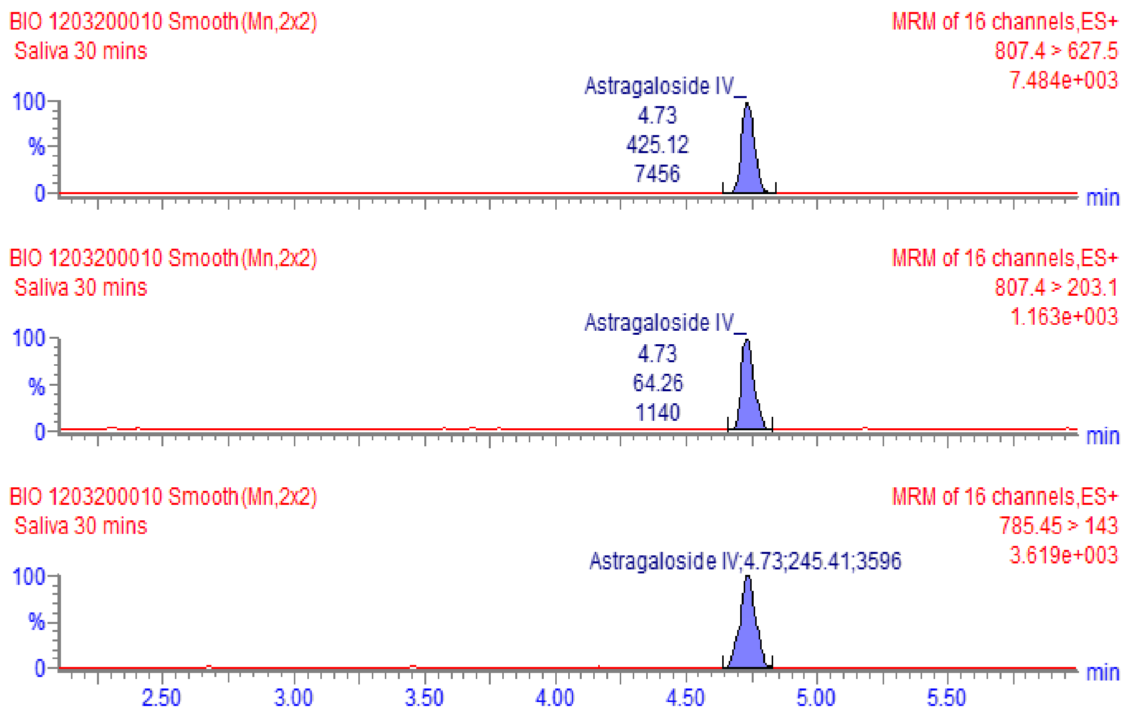


Figure 32. MRM channels of astragaloside IV in saliva samples collected 30 mins after ingestion of AR sample

Discussions

The samples of Astragalus Radix analyzed in this study are different preparations manufactured and marketed by different vendors. These samples consist of granules, tablets, capsules, dried roots, or hydrophilic concentrations of Astragalus Radix. Originally, thicker and longer roots were considered signs of better quality. Effective analytical measures have been used for identification, quantification, and quality control of bioactive compounds of AR samples.

The optimization method used during sample analysis can be done to obtain reliable results. Optimization can reduce the chromatographic run time, make sample preparation easy, improve sensitivity, selectivity, reduce sample size and overcome the effects caused by endogenous interferences (Shi et al., 2015; Yan and Guo, 2005). The reflux and soxhlet extraction methods are more old fashioned and require large quantities of samples and solvents (Song et al., 2007; Xiao et al., 2008). The optimization method can contribute to more yield (Qiu et al., 2015). Gradient elution is preferred above isocratic elution. The Selection of particular wavelength or retention time or mass to charge ratio is essential for optimized detection.

Thin-layer chromatography is a separation technique based on the elution of chemical compounds based upon polarity by using solvents as the mobile phase. Silica was used as the polar stationary phase. The TLC plates were further subjected to a mass spectrometer to confirm the compounds based upon the mass to charge ratio. The HILIC (hydrolysis-hydrophilic interaction liquid chromatography) was used because it is suitable for compounds which are polar molecules with plenty of OH groups. The mobile phase used in the HILIC system was a combination of acetonitrile and water (Hui et al., 2016). All five standard compounds were well separated. A higher retention factor indicated that the compounds are less polar. Formononetin and cycloastragenol are found to be less polar and astragaloside IV the most polar compound. Temperature, moisture, depth of mobile phase, sample size, the polarity of samples, the thickness of TLC plates, and other solvent parameters can affect the elution and separation. The more polar compounds tend to bind tightly with the stationary phase i.e. silica.

The spots of samples on TLC plates were compared to those of standards for analysis. The spots on the TLC plates were subjected to mass spectrometric analysis, where the compounds were detected based upon their respective mass to charge ratio in either of positive or negative ESI modes. The mass spectrometric analysis of all compounds on TLC plates

confirmed that those compounds are present in the samples. Therefore, TLC-MS is used for the confirmation of the presence of compounds in samples.

The FTIR spectroscopic analysis of all samples when compared with the standards, showed that the hydroxyl (OH) groups are present in the compounds at wavelengths between 3100 cm^{-1} and 3400 cm^{-1}. The peaks might be due to the presence of water molecules. The larger peak in hydrophilic concentrate might be due to glycerol used as a formulation base. The dried extracts of granules, root powder, and boiled roots also gave a similarly structured spectrum. There was a peak below 3000 cm^{-1} which is a proton with Sp3 hybridization and the peaks above 3000cm^{-1} indicating protons with Sp2 hybridizations. The peaks above 3000cm^{-1} were diagnostics for unsaturation. Similarly, the peak around 1600 is thought to be of C=C, however, it might be of the water moiety. It is seen that there are no peaks just above 3000 cm^{-1} in astragaloside IV and cycloastragenol which shows that those compounds are not aromatic. There must be a peak in ononin above 3000 cm^{-1} which is not seen in spectra, this might be due to overlap or is hidden. The common peaks around 1200-1250 cm^{-1} in all standards and samples might be of C-O-H (phenol) group. Similarly, the C=O bonds in between 1100-1200 cm^{-1} are present in all samples and isoflavonoids. Aromatic ether is present in wavelength range 1200-1300 cm^{-1}. Conjugated Ketones have an IR wavelength around 1650 cm^{-1} (Pavia et al., 2015)**.** Hereby, the presence of methanol or water moiety might influence the IR spectra. To confirm, and remove water molecules from the samples if present, Toluene was added to dried granule extracts (for the Azeotroping effect) and again dried using a rotary evaporator under reduced pressure. After the complete drying of the sample, it was again analysed under FTIR. Similar results were obtained which confirmed that there was no influence of water molecules present in the sample (Figure 19). Another challenge was to remove the Glycerol from sample C, but it seemed difficult. The IR spectra of all the samples were not nicely formed which might be due to the complex compound structures and samples containing mixtures of several compounds. Similar functional groups were found in another study of FTIR analysis of Astragalus Radix samples (Liu et al., 2020). The FTIR is fast, easy and needs no sample treatment so that we can subject samples in any form. The fingerprint which is generated can be used for further confirmation of compounds in samples by cross match. This is used for confirmation of functional groups present in the sample and quality control of herbal medicines.

In HPLC-UV analysis, the chromatograms showed that the three isoflavonoids have different retention times so they can be readily separated. The samples were eluted within 5 minutes in a total run time of 9 minutes and some have longer run times (Kim et al., 2007). The

C18 column was selected to capture peptides or small molecules together with the guard column. The temperature was well controlled to prevent the drift or shifting of peak retention times in the spectrum. Blank samples were run in between samples to prevent carryover.

Isoflavonoids present in AR can be well detected by UV detectors and variations were observed between samples which are also supported by a study done by Wu T. et al (2005). The linearity was tested by best fit in the regression line of standards. The lower concentration of standards (2.5, 5, 10, and 20 μg/ml) showed better results since they have small standard deviations. The saponins were not detected by UV detectors because of unsatisfactory detection limits due to lack of chromophores or conjugated systems even in low wavelengths of 203-210 nm. In some studies, pre-column derivatizations were done and ELSD detectors were used to detect astragaloside IV because of its weak UV-absorbance (Xu et al., 2007). The ELSD detectors are supposed to provide retention time as a single parameter for identification, and sometimes other co-existing compounds and solvents might interfere with the quantification with longer run times. (Li and Fitzloff, 2001; Zhang et al., 2013; Zu et al., 2009).

Interestingly, there was no peak detection for formononetin in sample C (hydrophilic concentration). For further confirmation, the portion of the sample was collected from the waste pipe that goes to waste collector from UV detector around 4.32 minutes. The sample was spotted on a TLC plate and tested using a mass spectrometer. The result showed the presence of formononetin with a peak of *m/z* at 536.6 $[2M+H]^+$. The presence of formononetin in the sample was also proven by LC-MS/MS analysis of sample C. This reveals that the LC-UV method is less sensitive and the different peak might be due to overlapping or ion addition or suppressions. The result also showed that the HPLC-UV method is less reliable and sensitive than the LC-MS/MS method.

As noticed, only isoflavonoids were detected and quantified using the HPLC-UV method but saponins were not detected. All five compounds were simply quantified with the use of UHPLC-MS/MS system. Tandem mass is the optimal analytical method for Quality Control at the moment as it is more selective, precise, reliable, and accurate compared to UV detection methods and even better than single MS detection systems (Shi et al., 2015). The precision was more than 20 % in the UV method, whereas less than 20 % in the LC-MS/MS within the acceptable range (Shi et al., 2015; Zhang et al., 2007). The limit of detection and limit of quantification in the latter method was almost 2-3 magnitudes lower than in the UV method expressed in nanograms indicating the LC-MS/MS method is super sensitive and has high detectability. The selectivity is very low in HPLC-UV chromatograms. The content of all three isoflavonoids was significantly lower when the same samples were tested with tandem

mass spectrometry. There was less effect on formononetin but the concentration of calycosin 7-O-β-D glucoside was drastically reduced from 525 ± 33 µg/g in sample A (granules) tested using UV method to 173 ± 41 µg/ g when tested by LC-MS/MS method. The higher value in samples detected with UV method might be due to matrix effect or possible interference with other compounds as shown in Figure 27. The UV detection showed much less selectivity for calycosin 7-O-β-D glucoside. Formononetin was not detected in sample C using the UV method (Figure 25). Therefore, LC-MS/MS methods can overcome LC-UV methods for quantitative and qualitative analysis of compounds.

The total chromatographic run time in LC-MS/MS was about 8 minutes considering equilibrium time, but all five compounds were eluted within five minutes with good separation as shown in figure 23. Some studies showed the chromatographic run time for more than 8 minutes for the same compounds using LC-MS/MS (Huang et al., 2009; Liu et al., 2015). The acidic mobile phase helps to provide a source of protons and better separation for compounds with OH groups by reducing the peak tailing and also can increase the MS signal intensity and peak shape (Zhang et al., 2013). They also reduce the signal/noise ratio and baseline drift of chromatograms (Wu et al., 2005). Hundreds of samples were studied in a single day as a single analysis was less time-consuming.

Many compounds can have the same intact mass so difficult to be identified by a single MS detector. The tandem mass spectrometer has a triple quadrupole mass detector. The first MS gives the parent mass whereas the second MS is more selective by providing unique fragments and can be the best method for accurate analysis. After the ionization using positive or negative ESI, the first quadrupole selects the parent mass based upon mass to charge ratio, then the fragmentation of compounds takes place in the second quadrupole, in presence of collision gas, which also acts as an ion guide. The daughter ions produced as a result of collision by inert gases were selected by the third quadrupole. Then both parent mass and fragment ions are monitored in combination using multiple reaction monitoring channels. The transition can be written as parent mass > fragment mass where MRM monitors selective fragment ions with respect to their parent mass. Quadrupole mass detectors are inexpensive and small mass spectrometer detectors when compared with quadrupole time of flight detector. Saponins have a high sensitivity when detected with QDA compared to ELSD (Zhao et al., 2018).

The multiple reaction monitoring (MRM) channels utilize many reactions at a time for selection and are sensitive compared to single ion monitoring (Yan and Guo, 2005). Most of the MRM channels in this study were similar to Chen et al., (2015). The molecular mass with the adducts like sodium, ammonia can also be used to select the compounds. The saponins have

shown molecular ions with sodium ions, $[M+Na]^+$ in the spectrum. All parent and daughter ions are noted in (Table 13, *Appendix VII*), any fragmentations were not seen for cycloastragenol below the *m/z* of 491 $[M+H]^+$. In some studies the higher *m/z* values for cycloastragenol were observed at *m/z* of 513$[M+Na]^+$, 981$[2M+H]^+$ and 1003 $[2M+Na]^+$ (Zhou et al., 2012).

The result presented in table 11 showed the varying concentrations of compounds in different samples. Comparing results for astragaloside IV, one of the major compounds of interest, granules (sample A) showed better quality. It also showed that the content of all isoflavonoids was high. The concentration of all three isoflavonoids, formononetin, ononin, and calycosin 7-O-β-D glucosides were higher in root powder samples (sample B and sample F) than any other preparations. There was no quantifiable concentration of cycloastragenol in any of the available samples which might be because the concentration was below the limit of quantification. Only peaks for sodium channels can be seen in MRM channels, so for the perfect detection, there must be a peak in both channels in the same retention time. One study showed that fragmentation of astragaloside IV produced cycloastragenol (Duan et al., 2016), but others suggest that cycloastragenol is not present naturally (Zhou et al., 2012). The concentration of cycloastragenol was found to be only 27.10 ng/g for the fresh weight of plant samples produced under some stress. The glycosylation process might decrease cycloastragenol to form astragaloside IV in plants (Liu et al., 2016).

Different analytical methods were explored for accurate quantification of biologically active compounds because of possible instrumental variations and endogenous interference like matrix effects and ion suppression which might result in wrong interpretation (Qiu et al., 2015). The identification of matrix effects can be done by comparing the sample response with the standards in neat solvents where the responses can vary (Qi et al., 2008). The samples were diluted to reduce the matrix effects and standard addition was performed by adding a known concentration of standard concentrations to reduce ion suppression and overcome such consequences that can lead to false results. There are no isotopic labelled internal standards available for astragaloside IV and biologically active compounds, as far as we know, making it difficult to quantify by using internal standard calibration to fully compensate for ion suppression. The results were surprising, the astragaloside IV present in granule samples were found to be 74 ± 2 µg/g using external calibration after dilution which was almost 12 times more than non-diluted samples and found to be 203 ± 6 µg/g using standard addition methods after dilution which was almost 34 times higher than non-diluted samples. This proved that the presence of matrix effects can be reduced by dilution of samples and ion suppression was

reduced using standard addition. Similar effects were seen with other bioactive compounds and in other AR samples. The standard addition is like recovery testing. After selecting standard addition as the best method, other samples such as samples D, E, and F were also tested. The standard addition is the more accurate method for quantification which can be supported by analysing the ion ratios of compounds and covering the range of matrix effects. It might not be possible to fully compensate the ion suppression because of the absence of isotopic labelled internal standards. Different compounds other than the isotopes of compounds present in Astragalus Radix have been used in other studies as internal standards, like Ginsenoside Rg1 (Zhao et al., 2018; Zu et al., 2009), digoxin (Yan and Guo, 2005; Zhang et al., 2005), erlotinib (Shi et al., 2015), mycophenolic acid (Sun et al., 2014).

The similar ion ratios of standards and samples give more trueness. The six different calibration curves of astragaloside IV standards plotted with the same concentrations gave different regression lines with different slopes. These variations might be due to the daily instrument variation or more interference or ion suppression (Figure 29). The signal response was significant with repeated experiments of the same samples, which showed a wide range of the matrix effects during analysis as shown in figure 30. Though the large variation in slope indicated the interference present during sample testing, the concentrations were found to be very close to each other for the same compound during repetitions. The closer results directed towards more accurate values. The reason behind the slope variation might be due to different concentrations of proton adducts and sodium adducts coming from the mobile phase. The glass bottles might be the source of sodium ions. Therefore, the internal standards calibration is needed in such instances.

In traditional Chinese medicine, the crude drugs are boiled in water and prepared as a water decoction before its application. The higher concentration of astragaloside IV of 135 ± 50 µg/g was found to be present in boiled decoction than ultrasonic extraction with 70% methanol extract of root powder samples with a value of only 37 ± 7 µg/g. But there were no significant changes in sample A1 (lukewarm) and sample A2 (boiled water) extractions of granules compared to sample A. The reason might be that the granules were processed samples. The extraction process and solvents used might vary the concentrations of saponins and isoflavonoids in AR samples.

There was a significant increase in the concentration of astragaloside IV when the samples were treated with an ammonia solution. The suggested reason is the hydrolysis of all other astragalosides present in the sample to astragaloside IV (Chu et al., 2014). Pharmacopeia and reports state that the total content astragaloside IV should not be less than 0.04% but it is

not specified if this is the total astragaloside IV naturally present in AR sample or astragaloside IV obtained after treatment with ammonia (Liu et al., 2020). Only granulate samples (sample A) in this study passed the limit after the sample extracts were treated with ammonia. It is essential to clearly state whether the pharmacopeial limit of 0.04 % for astragaloside IV for better quality is after treatment with ammonia or without any treatment. If treated with ammonia, then it can be accounted for total astragaloside content. In some cases, the sample is treated with ammonia to find the total concentrations of astragalosides but it does not seem very relevant (Monschein et al., 2014). A study followed European pharmacopeia and still found astragaloside I, II, and malonyl astragaloside I after treating with ammonia and butanol (Monschein et al., 2014).

Very few studies are done in the pharmacokinetics of Astragalus Radix samples in humans. The concentration of formononetin and astragaloside IV was detected in human plasma samples and pharmacokinetic studies were performed (Rao et al., 2019; Xu et al., 2013). One study revealed that astragaloside IV can be bio-transformed by intestinal bacteria, perhaps due to the presence of glucose and xylose moiety. This generates metabolites and cycloastragenol is one of them. This is thought to be clinically important because of its telomerase activating property (Zhou et al., 2012). The presence of compounds and their metabolites in plasma of animals can give indications to study the pharmacokinetics in humans (Liu et al., 2015; Sun et al., 2014). The pharmacokinetic study can be based upon the quantification of components in Astragalus Radix samples, and in fact, can be related to the dose-response relationship of bioactive components in patients.

The differences in contents of isoflavonoids and saponins in the present samples might be due to different manufacturing processes, location of collection, harvesting time, species used and other cultural manners and age (Huang et al., 2009; Ma et al., 2002; Zhang et al., 2013). Similarly, environmental conditions like climate, geology, environmental stress, and soil might contribute to variation in concentration and yield of chemical constituents from sample to sample (Qiu et al., 2015). Adulterants are easily available on the market (Dong et al., 2011) though species like *A. chrysopterus Bunge, A. floridus Benth,* and *A. tongolenis Ucbr* are permitted officially in Japan but not in China where the pharmacopeia specifies only two species *Astragalus membranaceous (Fisch.) Bge. Or Astragalus membranaceous var. mongholicus (Bge.) Haiso* as the major resources (Ma et al., 2002).

The present study found that standard addition using LC-MS/MS method can be widely used for quality control of AR samples where there is no availability of isotopic labelled internal standards. Many studies and guidelines have used different methods for quality testing

of Astragalus Radix which are less reliable and do not give accurate results. Secondly, the concentrations of compounds vary greatly between raw herbs and different preparations obtained from different vendors. None of the samples passed the pharmacopeial limit of not less than 0.04 % of astragaloside IV without ammonia treatment. Only the granulate samples (sample A) passed the pharmacopeial quality standard, and then only after treating the sample extracts with ammonia. Hence, the pharmacopeial standards must be re-evaluated in terms of actual concentrations of bioactive chemical components present in Astragalus Radix samples without any physical or chemical treatments. This can be correlated with bioavailability and their therapeutic mechanisms which can be helpful for the optimization of the dosage regimens as well as pharmacological and pharmacokinetic profiling of the compounds (Shi et al., 2015). The quality control strictly prohibits any adulterated and low-quality Astragali Radix on the market. Therefore, quality control of Astragalus Radix and many other herbal medicines using accurate test methods is essential.

Limitations of the study

The present study was limited to accurate quantification of bioactive compounds by standard addition using LC-MS/MS. The results obtained could further be compared with results using different compounds as internal standards since there are no available isotopic labelled internal standards of compounds present in Astragalus Radix e.g. astragaloside IV.

This study could have been further expanded to the in-vitro analysis of AR sample extracts and compounds in cancer cell line studies to determine anti-cancer properties and pre-testing of AR samples in biological fluids was limited due to COVID-19 situation.

The pharmacokinetic study of Astragalus Radix samples was not performed on animal or human models as this was outside the scope of the current master study.

Recommendations

The present study recommends using the optimized methods during the sample preparation as well as LC-MS/MS method for better yield and accurate detection. The standard addition can be used for more accurately determining the quantity of bioactive compounds present in Astragalus Radix samples. The present study can be used as a base to set the parameters for quality control concerning the chemical composition of Astragalus Radix. The pharmacopeia and guidelines could provide a prominent way for accurate determination and quantification of compounds present in traditional Chinese medicines. This study strongly recommends analyzing the full pharmacokinetic studies in human beings and comparing uptake of AR samples in different persons of different age groups.

In the absence of invasive testing, it is recommended to investigate whether a saliva test can be used to measure the uptake and bioavailability of compounds in individual persons. This could lead to the personalized adjustment of TCM herbal Astragalus Root treatment, which would be important to evaluate the effect of AR treatment on biological parameters, such as cancer regression, immune stimulation, and others.

Conclusions

Saponins and isoflavonoids are the major biologically active compounds present in Astragalus Radix. Different analytical methods, TLC-MS, FTIR, HPLC-UV, and LC-MS/MS, were explored for accurate quantification of these compounds and quality control of AR samples. The AR samples were extracted by ultrasonication using 70 % methanol and some samples were also extracted by boiling in water. The extraction and sample preparation methods were optimized. The TLC-MS of five compounds were presumed to be present in all samples as compared to respective standards. The TLC-MS confirmed the presence of four compounds in Astragalus Radix samples except for cycloastragenol. The FTIR spectroscopic analysis showed that some functional groups like OH, CH, C=O, and aromaticity of the compounds can be distinguished from the FTIR analysis of standards however the IR spectra of samples were not good enough.

The three isoflavonoids, formononetin, ononin, and calycosin 7-O-β-D glucoside were detected using UV detectors. There was no detection for astragaloside IV and cycloastragenol with this method. The formononetin content in hydrophilic concentrate of Astragalus Radix samples was also not detected using the UV method but was confirmed by using TLC-MS as well as LC-MS/MS method. The tandem mass spectrometric method was found to be more precise (<20%) and accurate compared to the UV method for all compounds. The LC-MS/MS was found to be more selective, sensitive, and rapid compared to the HPLC-UV method. The elution time for all five compounds was less than 5 minutes and had very low detection and quantification limits less than 2-3 magnitudes. There was a significant variation in results between the UV method and MS/MS method.

The concentration of astragaloside IV was observed to be different in the same root powder samples following different extraction processes, suggesting the need for optimization during sample extraction. The concentration of astragaloside IV was quite similar for granulates extracted with 70 % methanol, lukewarm water, or extracted by boiling in water because the granulates were processed samples.

There was a significant difference in concentration measured by LC-MS/MS between non-diluted, diluted, and diluted samples measured by standard addition. In granulate samples, the calycosin 7-O-β-D glucoside by UV method was found to be 339 ± 90 µg/ g which was reduced to 39 ± 5 µg/g by MS/MS method without dilutions and again corrected to 173 ± 41 µg/ g after standard addition. Similarly, the astragaloside IV present in sample A was found to be 6 ± 0.5 µg/g using external calibration without dilutions which tended to increase by 12

times its value after dilutions. This showed that there was interference present due to matrix effects. More interestingly, the astragaloside IV was found to be 203 ± 6 µg/g for the same sample using standard addition methods after dilution which was almost 34 times higher than non-diluted samples. Similar results were seen in all samples tested. Comparing the results of the standard addition method, granulate samples were found to be the better quality of all the samples, whereas hydrophilic concentrate and SF tablets were found to have lower qualities. The concentration of astragaloside IV was drastically increased after treatment with an ammonia solution. In SF tablets it was increased up to 221 ± 67 µg/g which was 24 times more than without sample extracts treated with ammonia. The study showed that the standard addition technique compensated for both matrix effects and ion suppression resulting in more accuracy in the values. Cycloastragenol was not quantified in any of the AR samples.

The present study suggests that the optimization of sample preparation and method is needed to get more yield and better results. The LC-MS/MS method is more sensitive, selective, and accurate compared to the HPLC-UV method which will provide great support for future studies. Standard addition techniques can be used for more accurate quantification and quality control of many traditional Chinese medicines and testing biochemicals when isotope labelled internal standards are unavailable.

Lastly, there were variations in the quantity of bioactive components between Astragalus samples obtained from different vendors in Europe. Most of the samples did not pass the pharmacopeial limit of astragaloside IV content in samples even after ammonia treatment. The quality control of such herbal medicines is very essential in Norway and many European countries.

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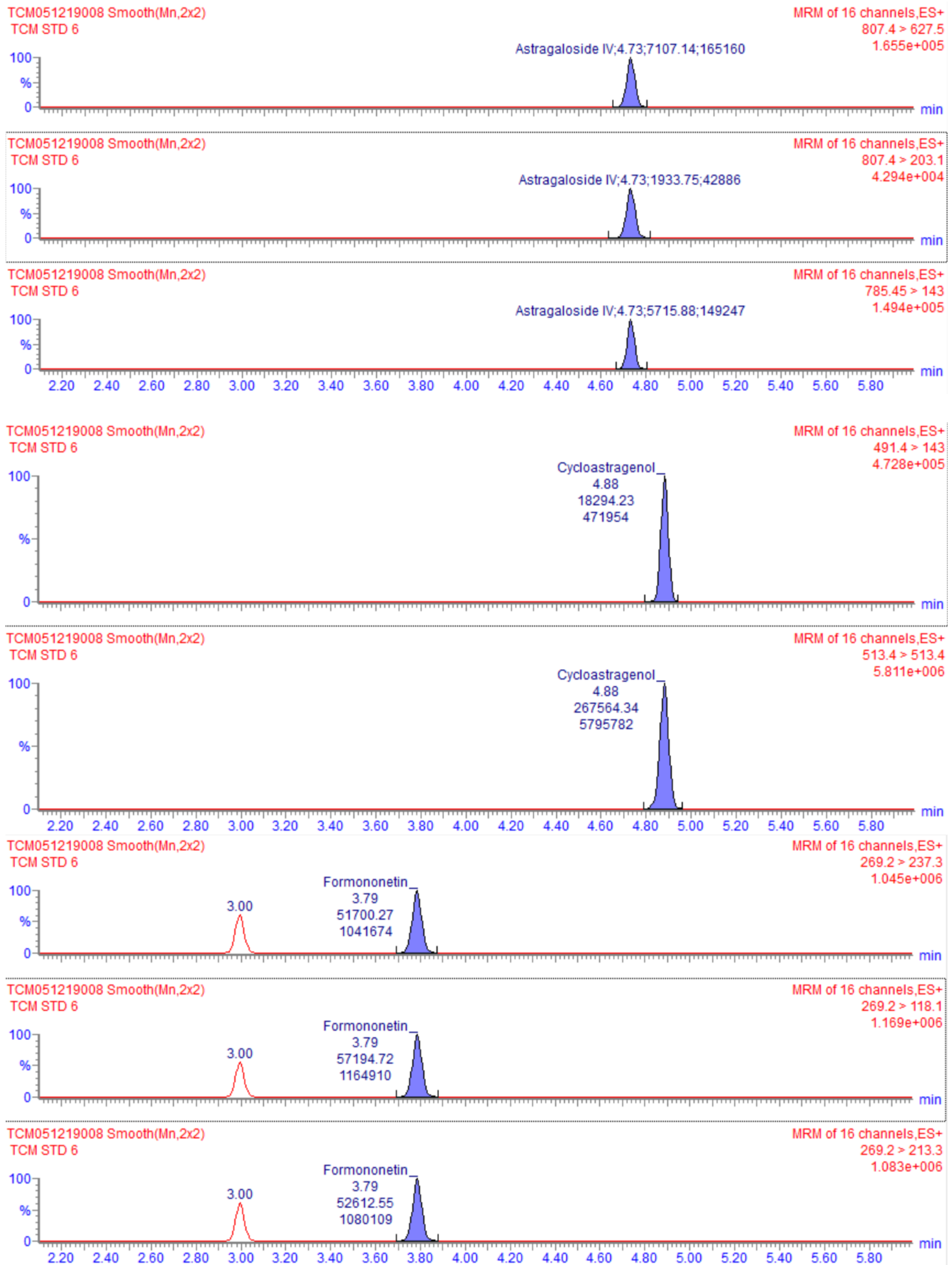
Appendices

Appendix I: MRM channels of all five compounds in LC-MS/MS



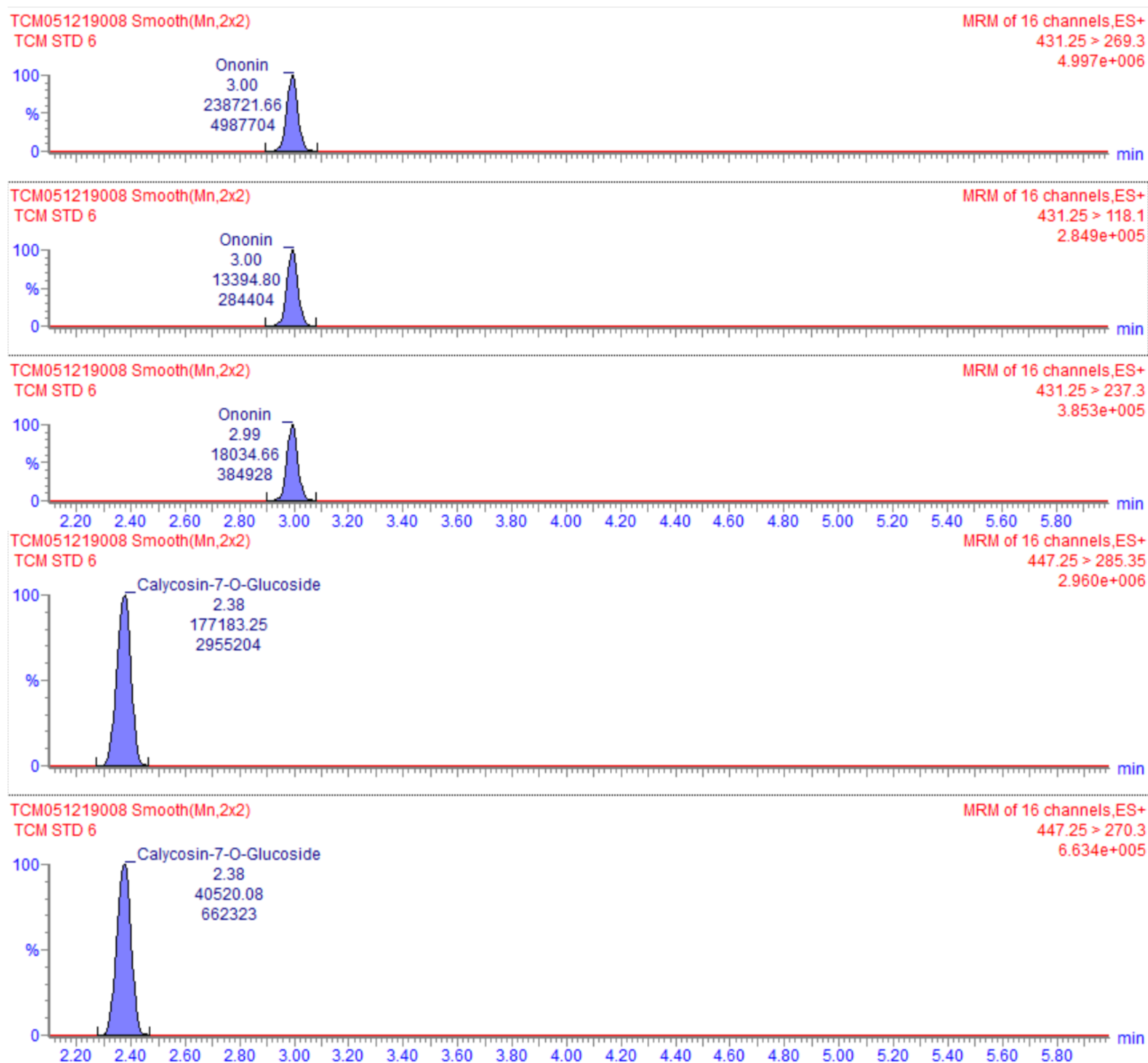


Figure 33: MRM channels of all five compounds using LC-MS/MS

Appendix II: Standard addition calibration curve

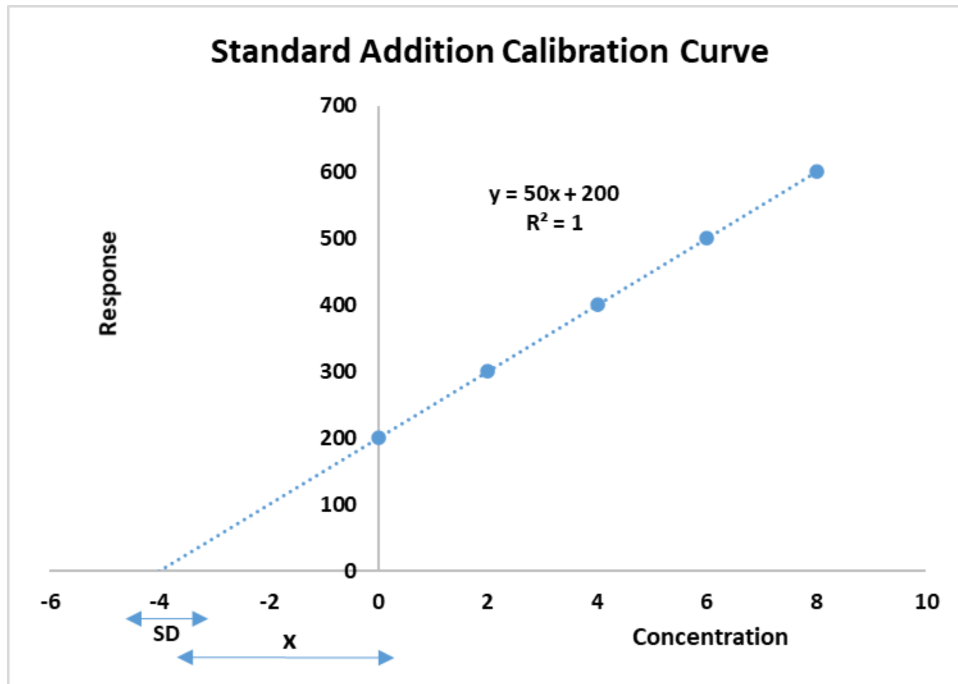


Figure 34: Standard addition calibration curve

When y is equal to zero **x** is calculated as,

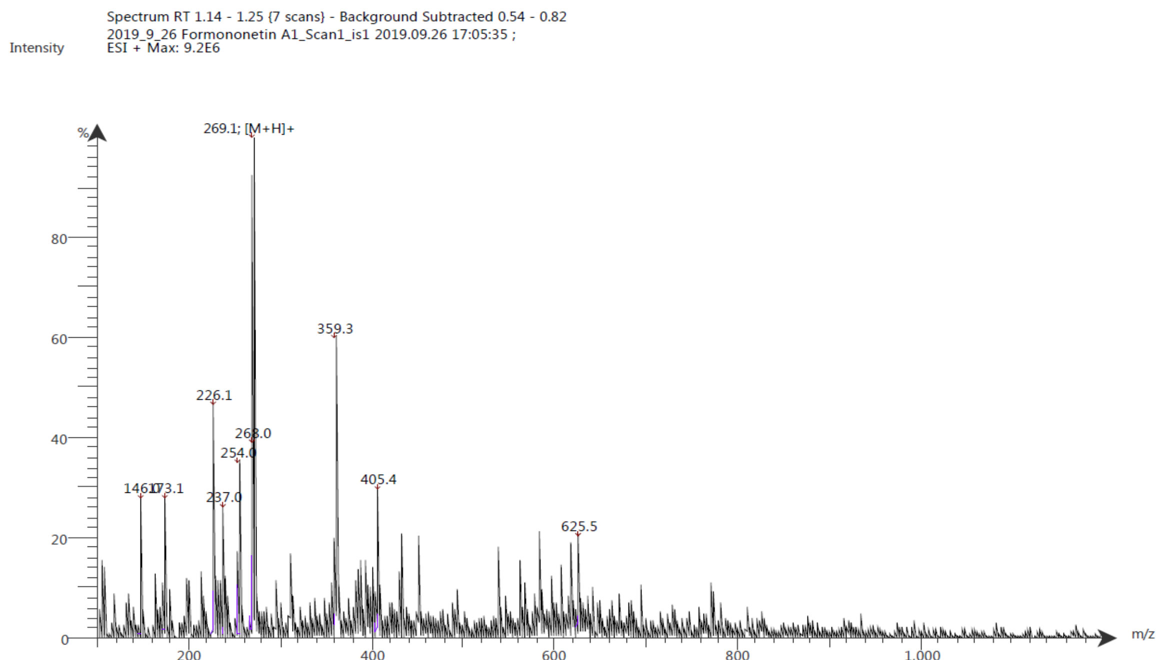
$$X = -200/50$$

$$= -4$$

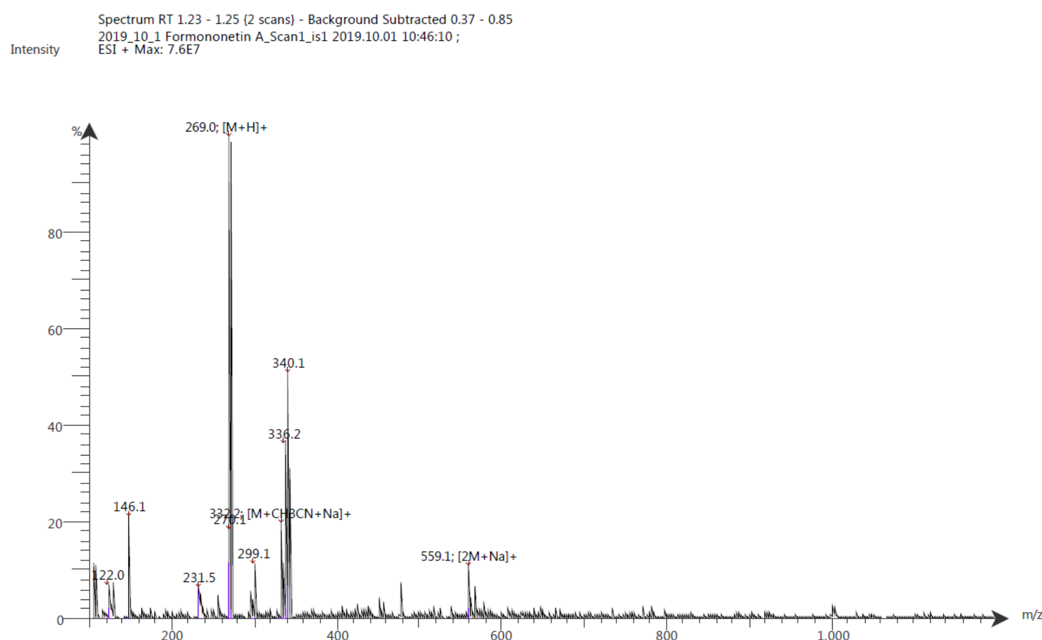
Therefore, the concentration is 4 units.

Appendix III: Mass chromatograms for all standards and samples using TLC-MS

1. Formononetin (268.26 g/mol)

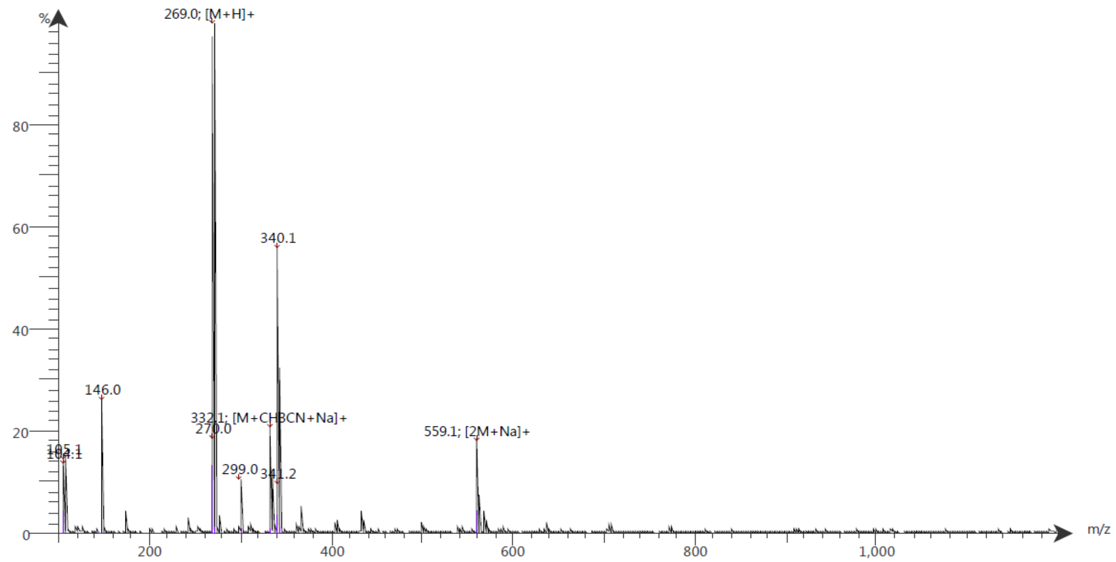


Tested on: *2019.09.26*



Tested on: *2019.10.01*

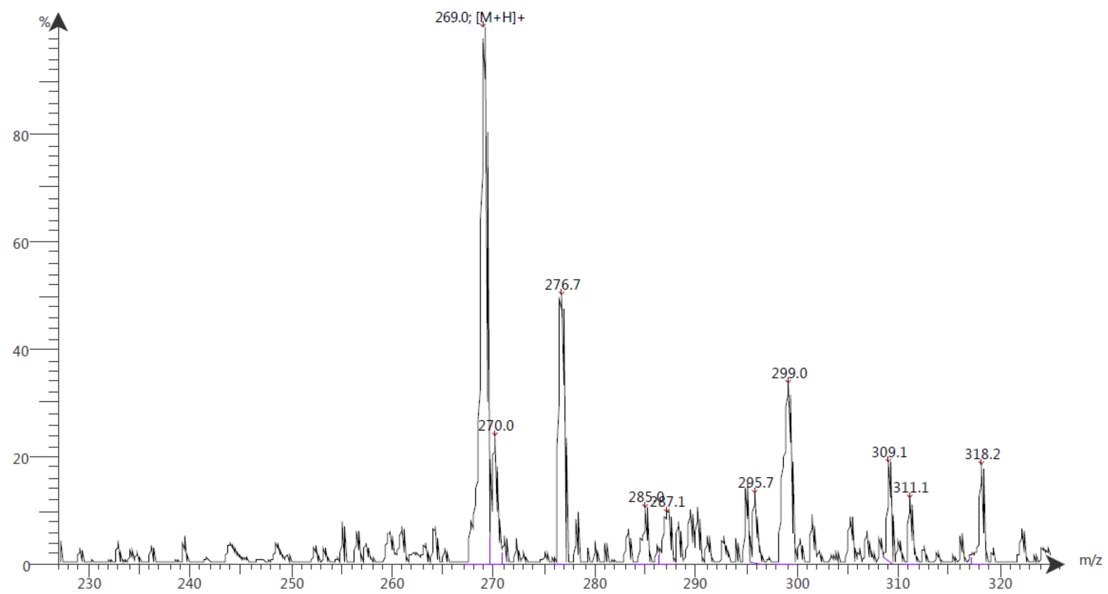
Spectrum RT 1.44 - 1.51 (5 scans) - Background Subtracted 0.46 - 1.37
2019_10_2 Formononetin A_Scan1_is1 2019.10.02 12:46:25 ;
Intensity ESI + Max: 6.1E7



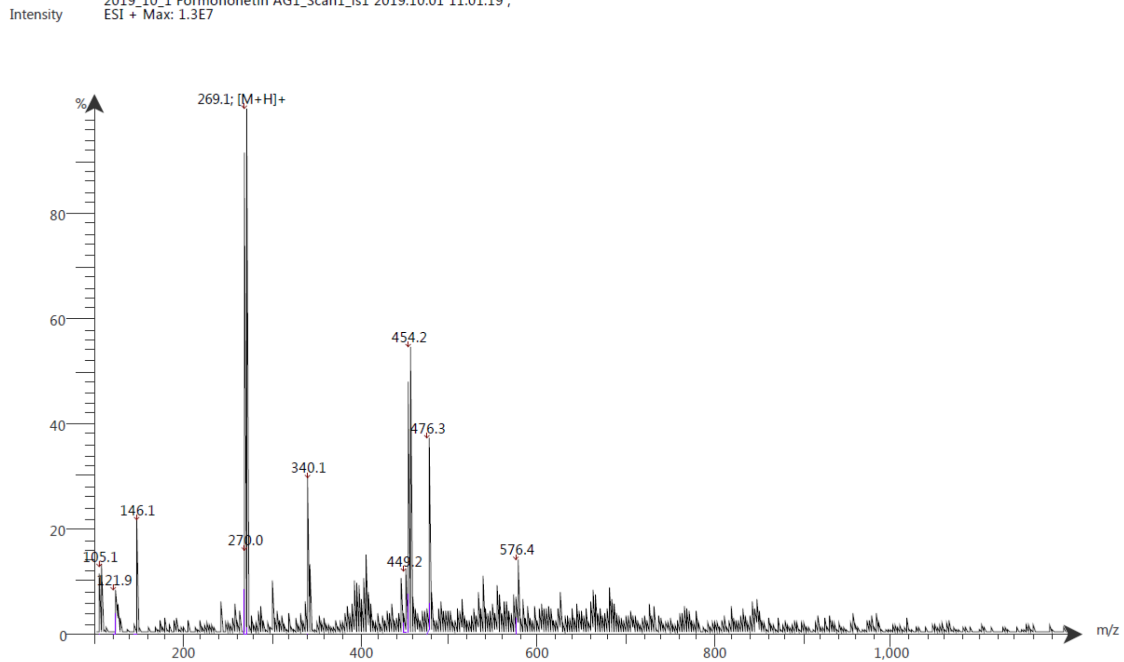
Tested on: *2019.10.02*

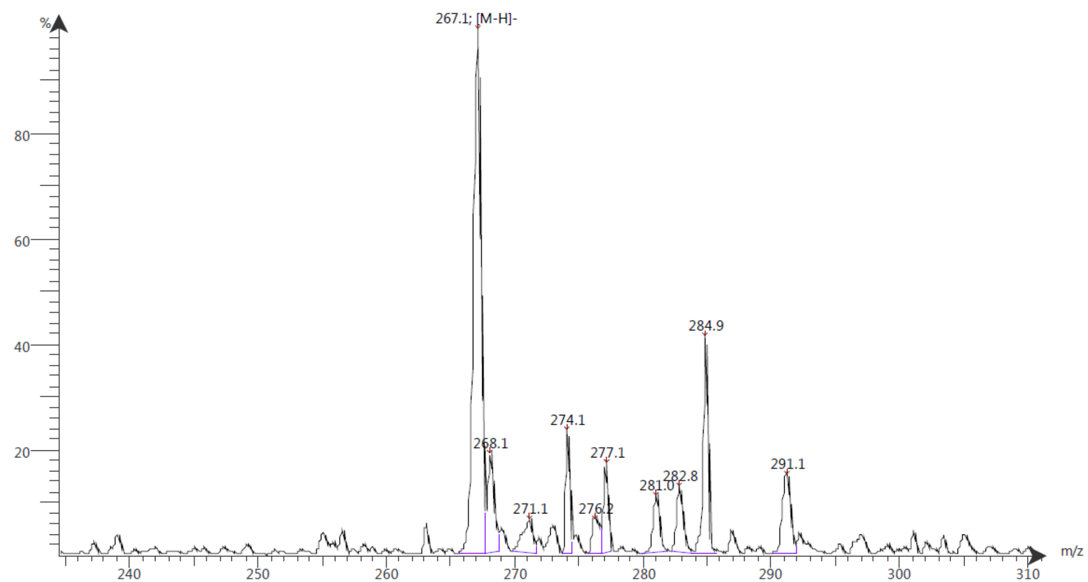
i. Formononetin standard mass spectrum tested on different dates (positive ionization modes)

Spectrum RT 1.12 - 1.16 (3 scans) - Background Subtracted 0.38 - 0.71
2019_10_1 Formononetin AF_Scan1_is1 2019.10.01 10:49:17 ;
Intensity ESI + Max: 7.1E6

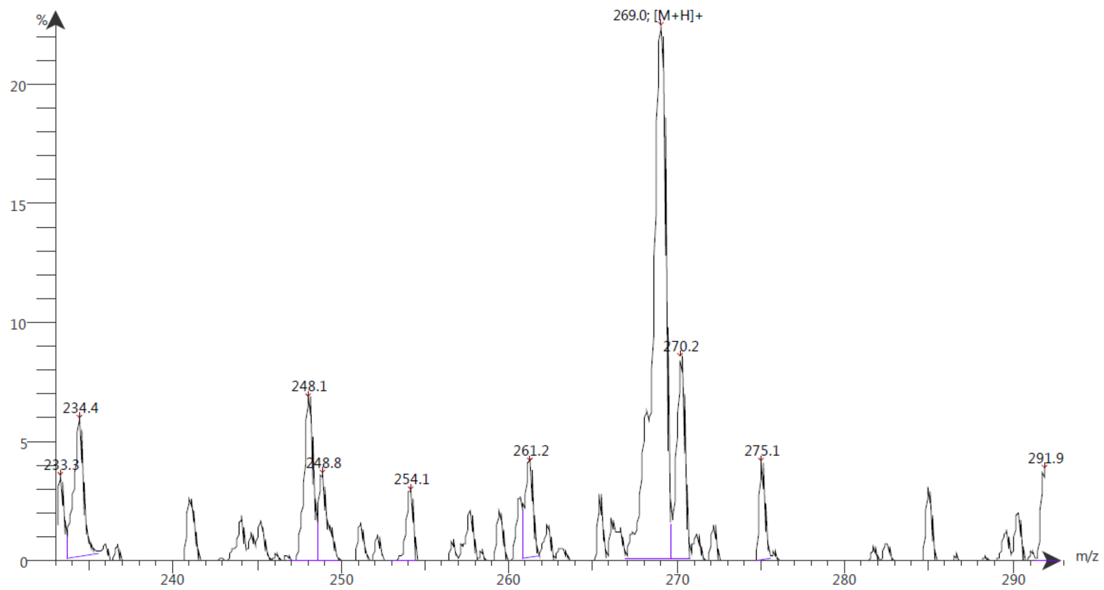


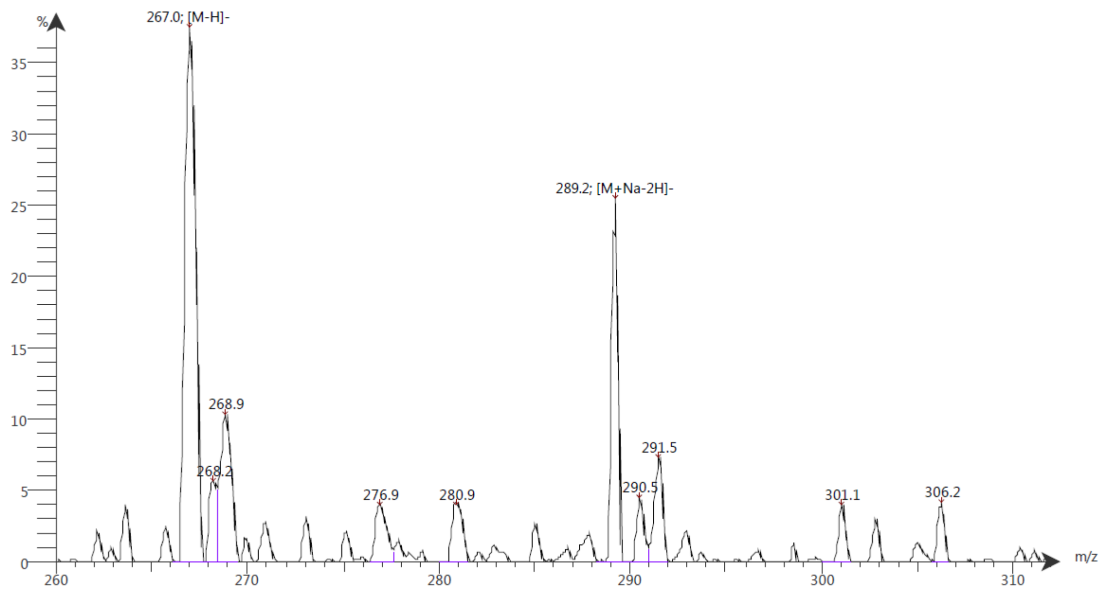
ii. Formononetin in Granules extract (positive ionization)



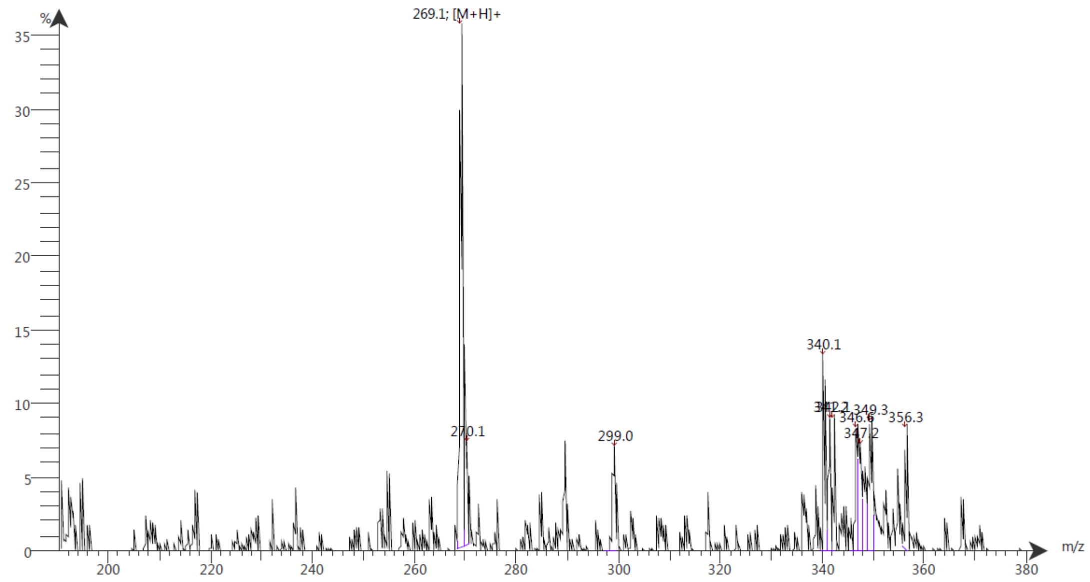


iii. Formononetin in Root Extracts in 70 % methanol (both positive and negative ionization)



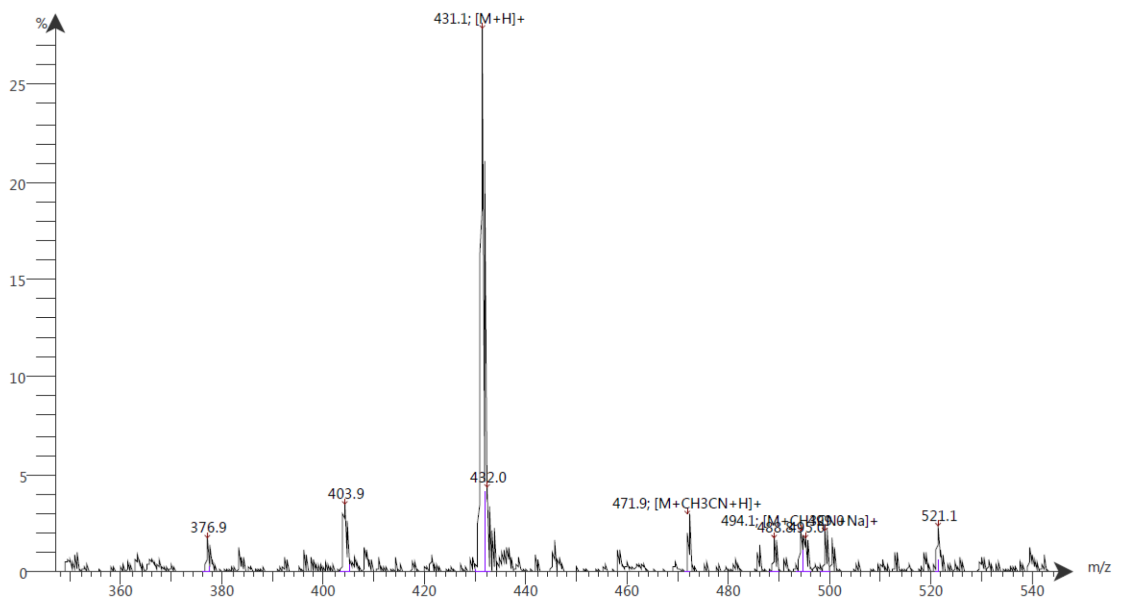


iv. Formononetin in Hydrophilic concentration (both modes)

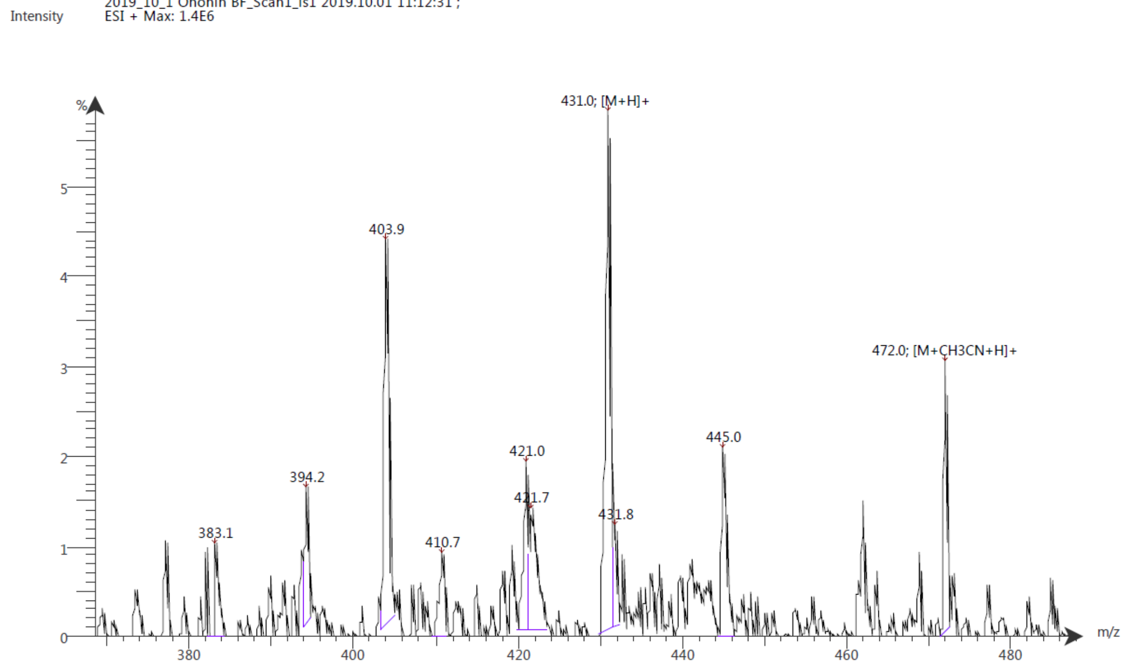


v. Formononetin in Boiled water decoction

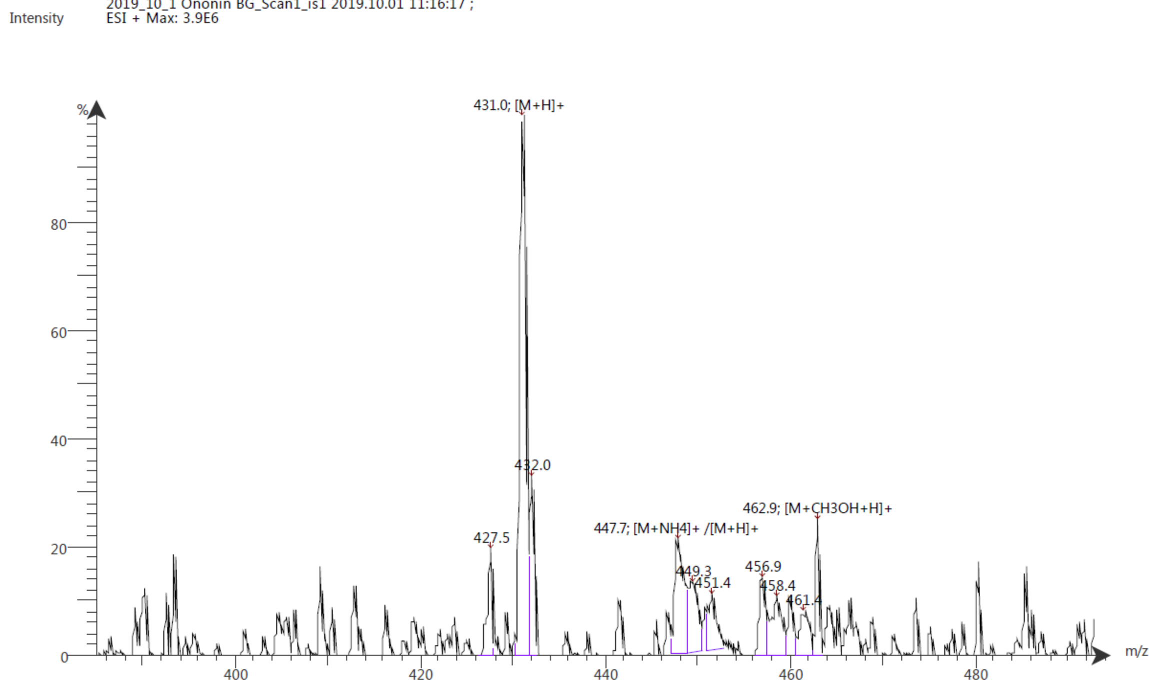
2. Ononin (430.4 g/mol)



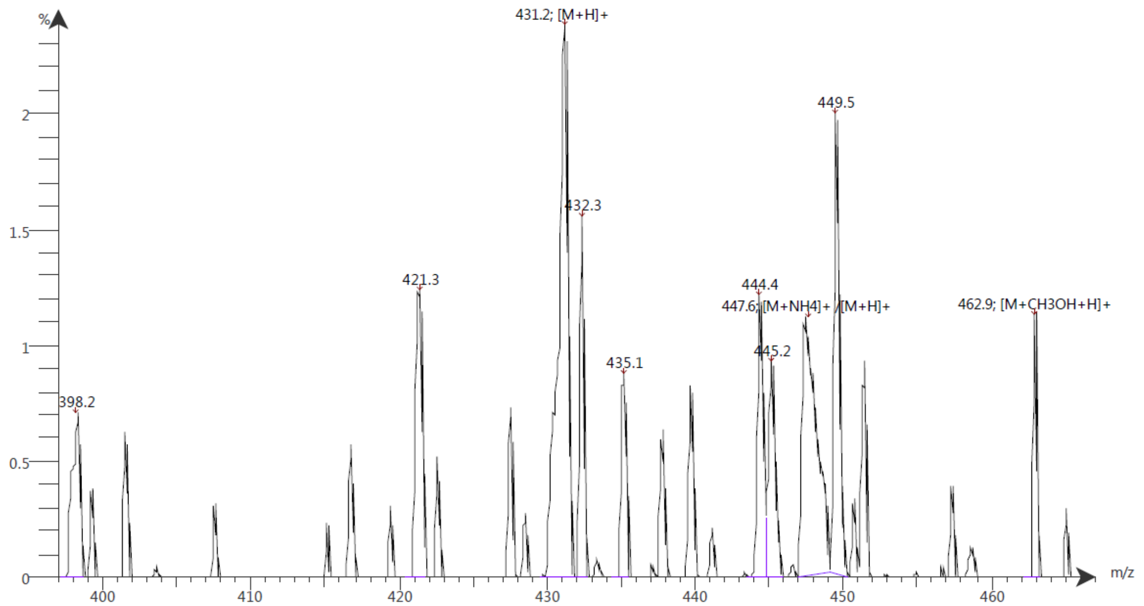
i. Ononin Standard



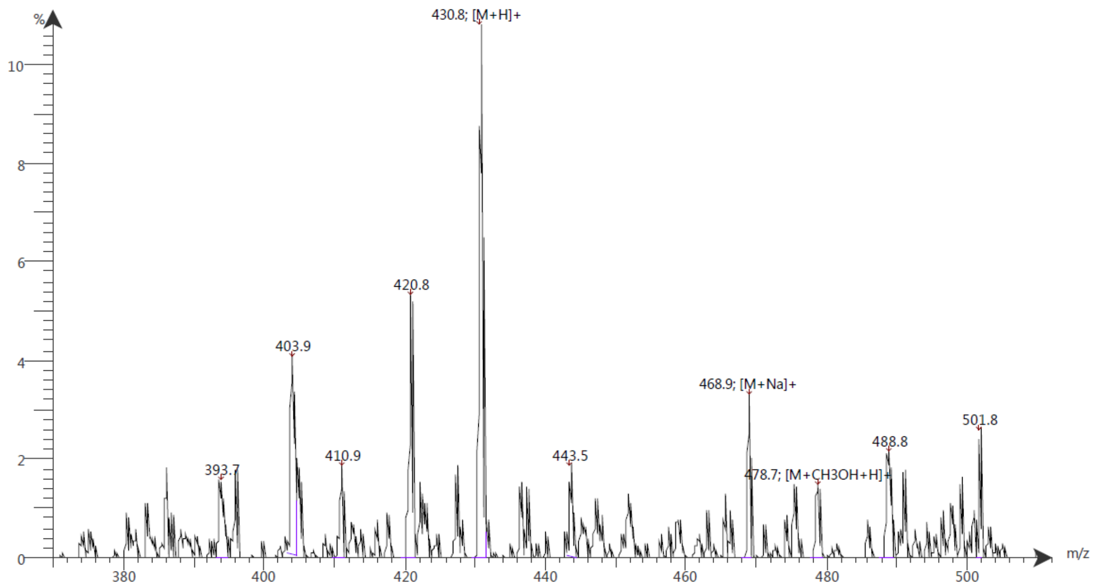
ii. Ononin in Granules extract



iii. Ononin in Root extract

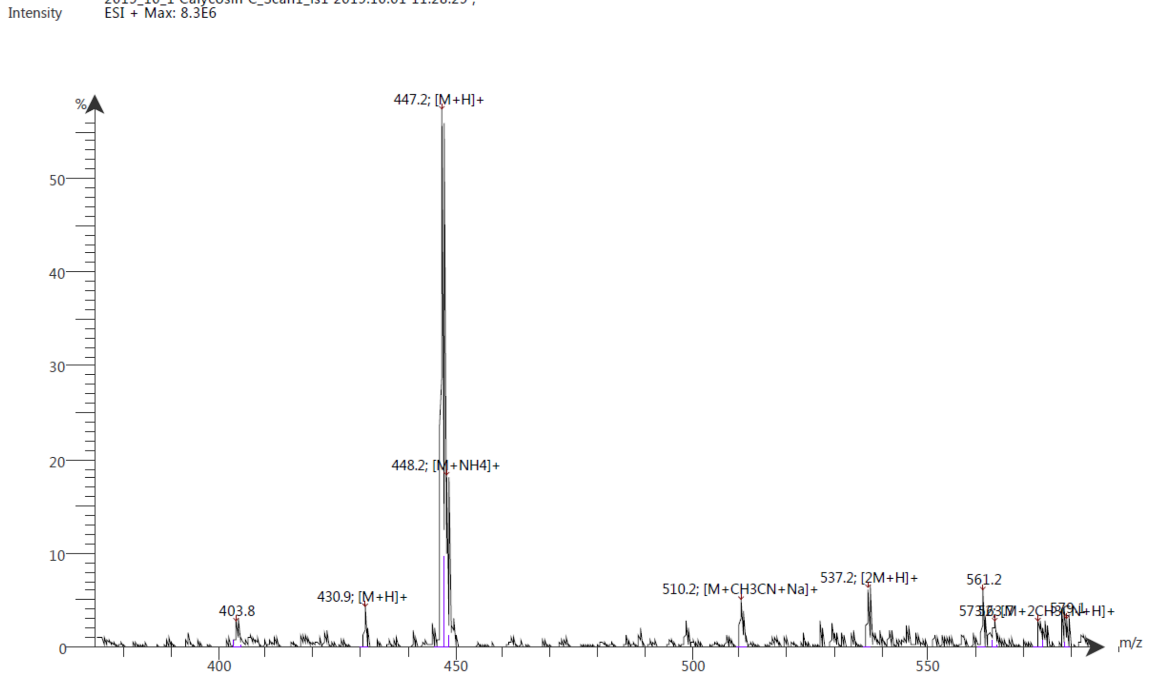


iv. Ononin in hydrophilic concentration

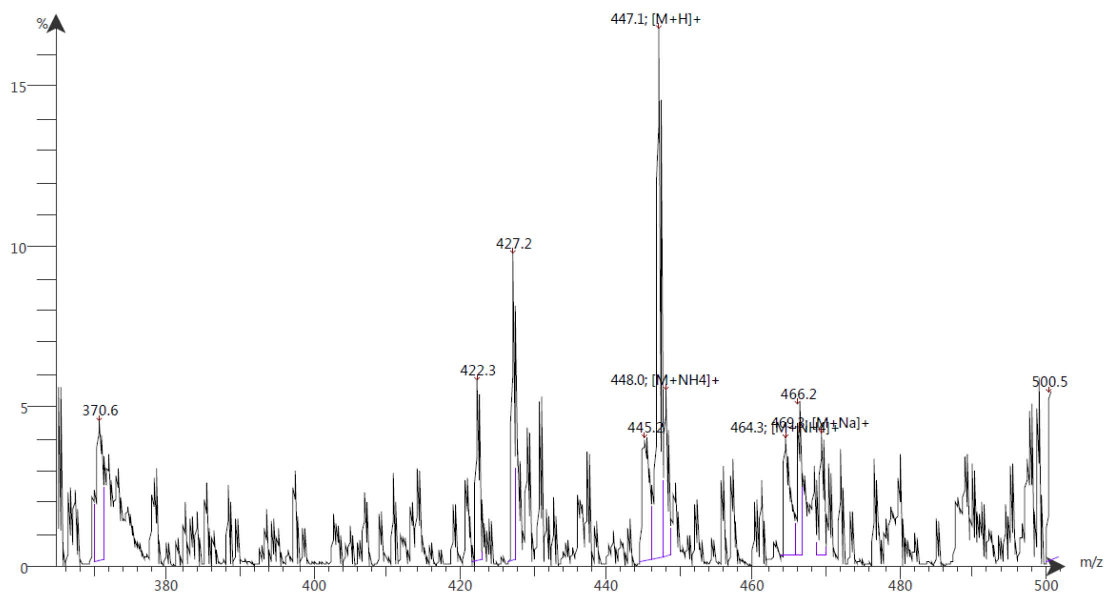


v. Ononin in boiled water decoction

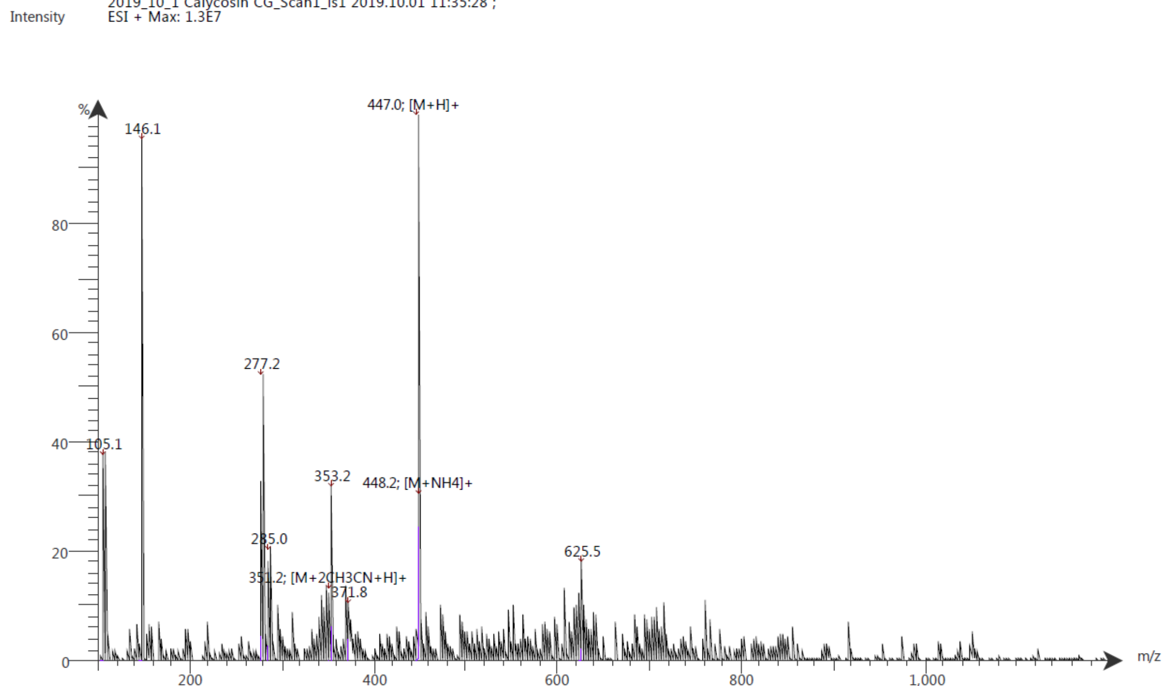
3. Calycosin 7-O-beta-D-glucoside (446.4 g/mol)



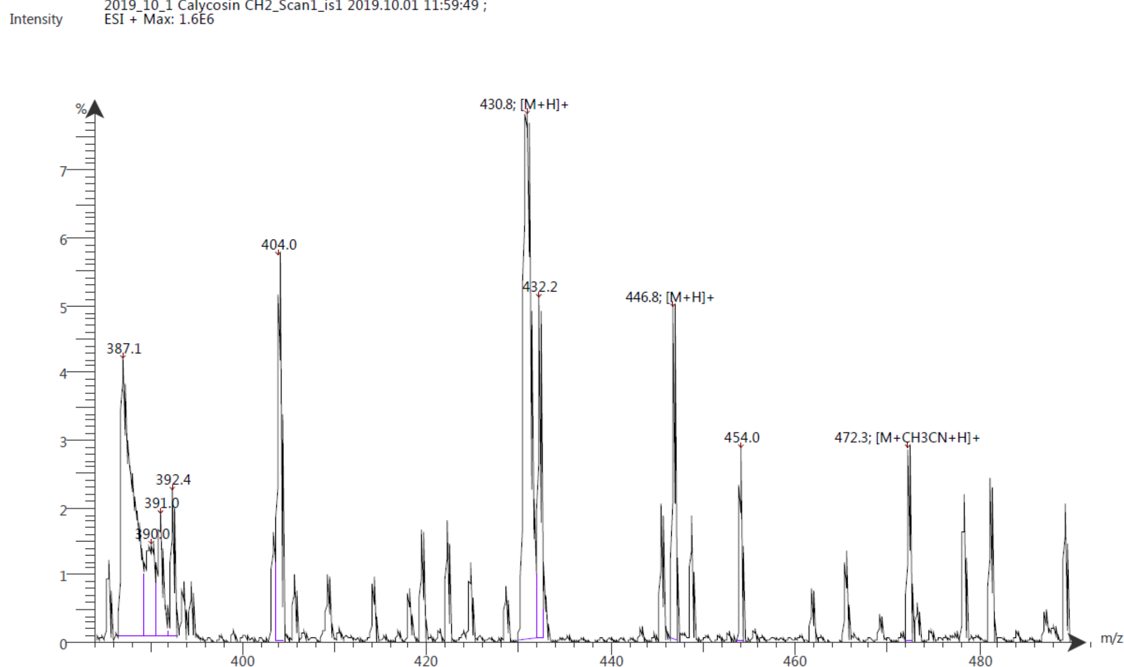
i. Calycosin Standard



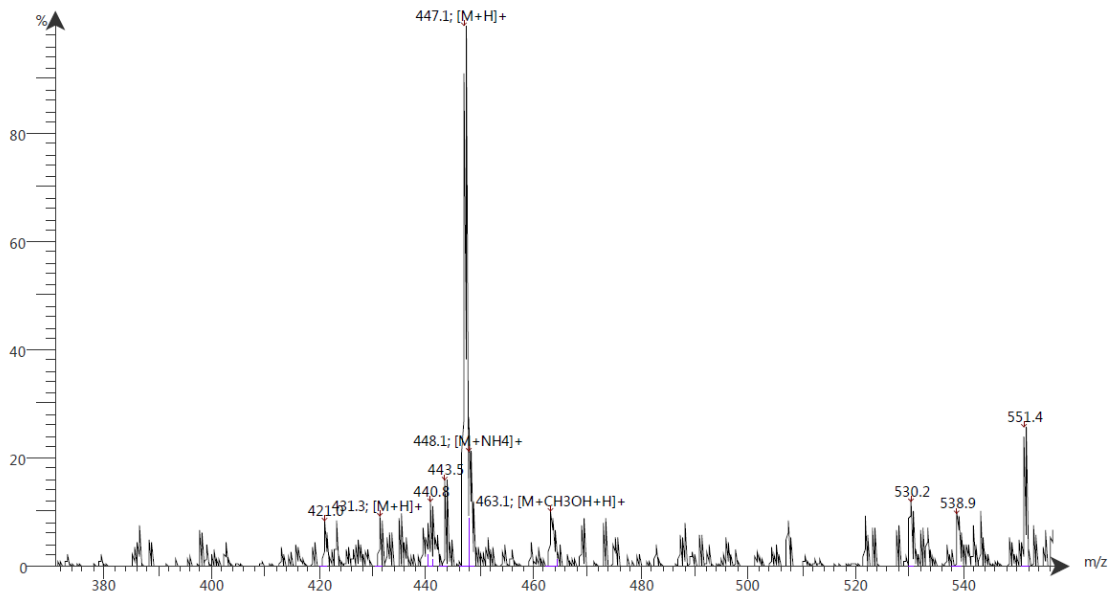
ii. Calycosin in Granule extract



iii. Calycosin in root extract using methanol

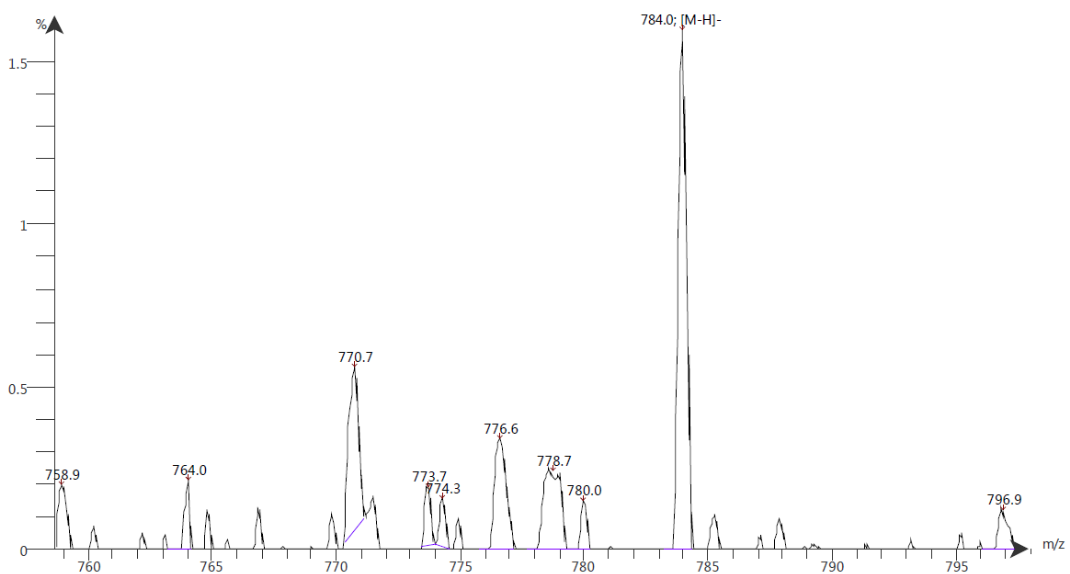


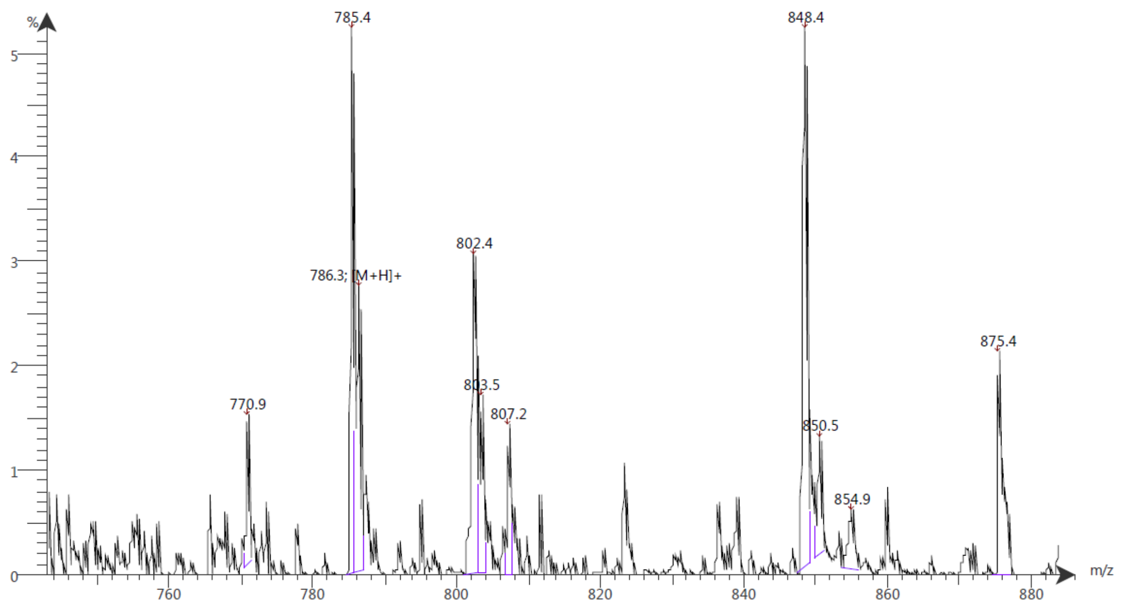
iv. Calycosin in hydrophilic concentration



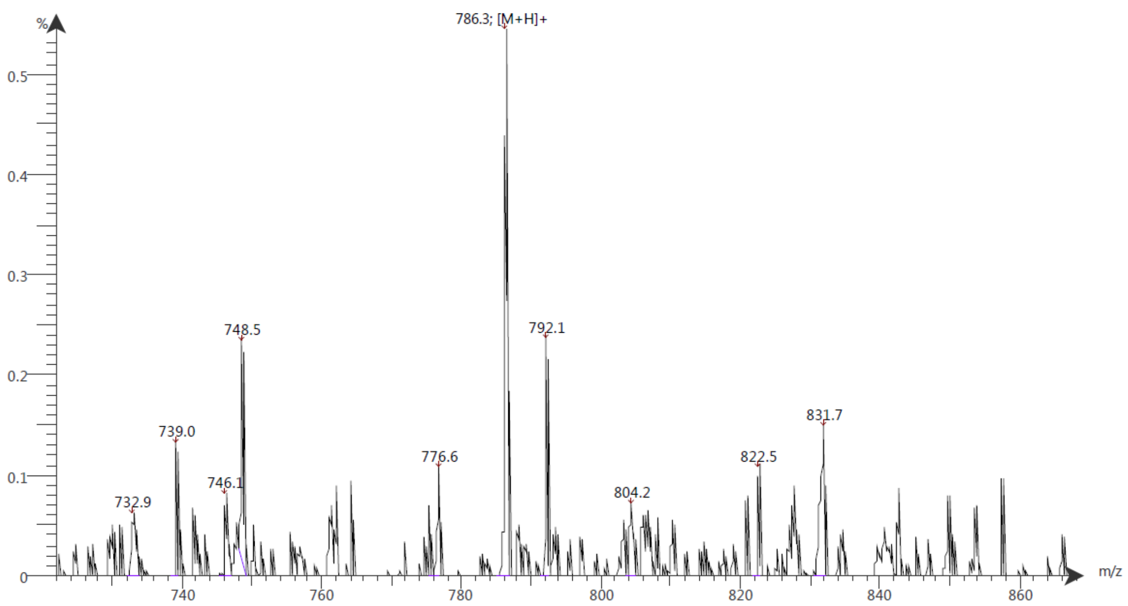
v. Calycosin in boiled water decoction

4. Astragaloside IV (785 g/mol)

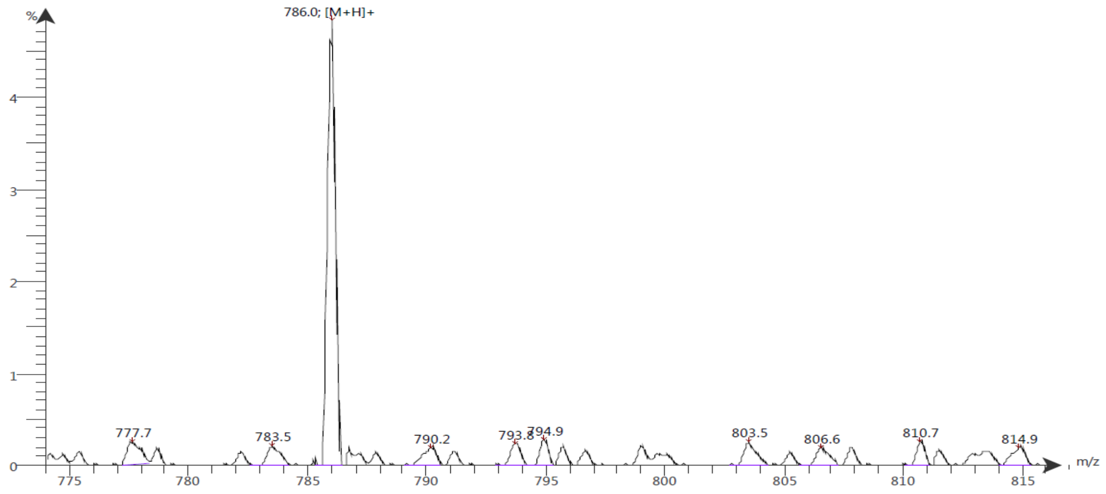


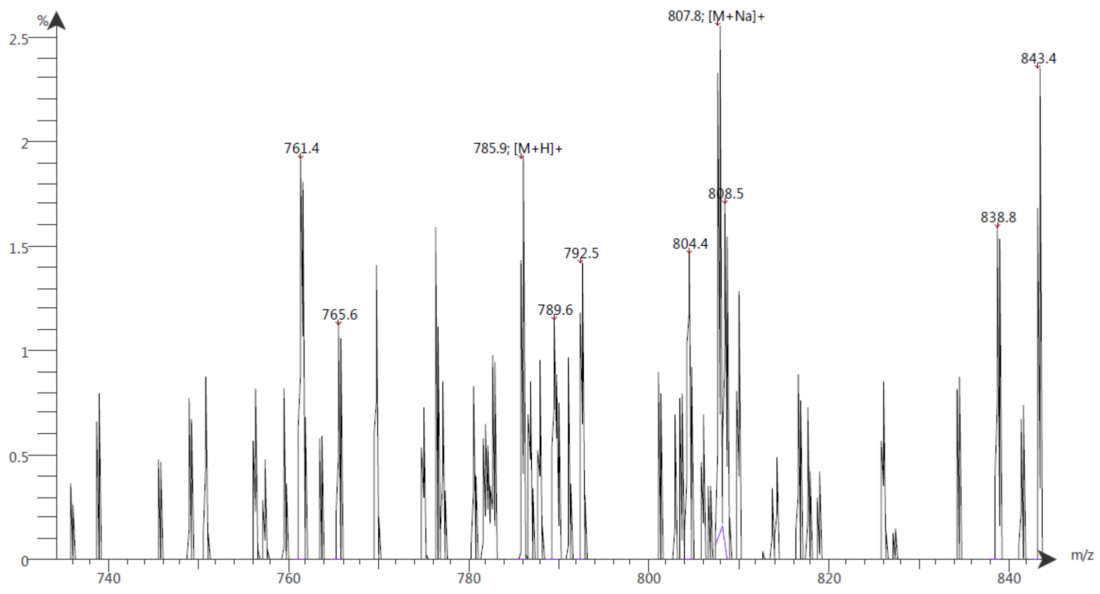


i. Astragaloside IV standard

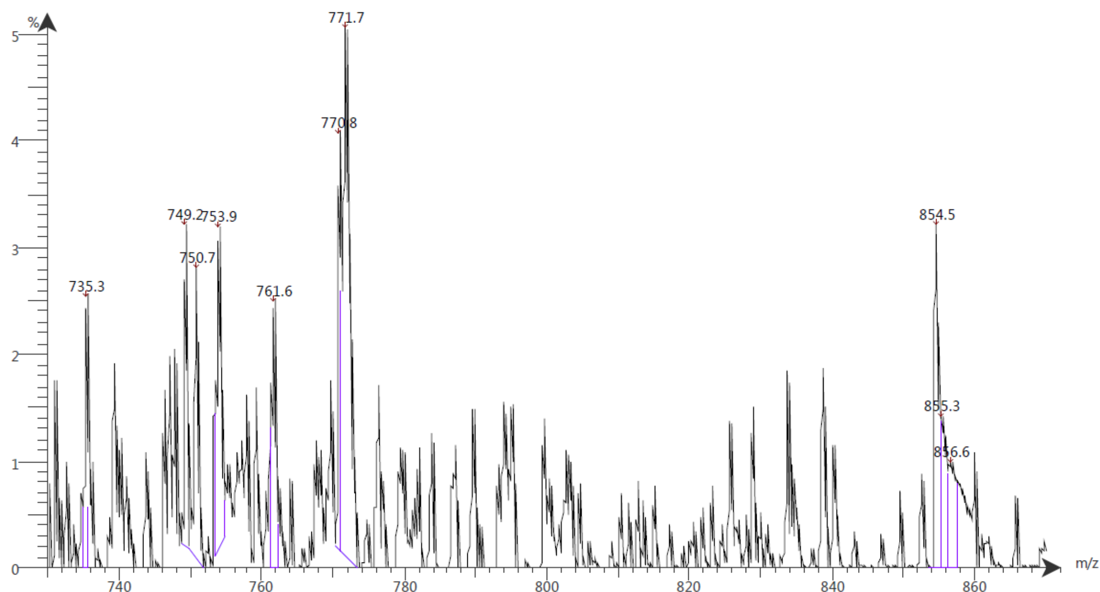


ii. Astragaloside IV granules extracts



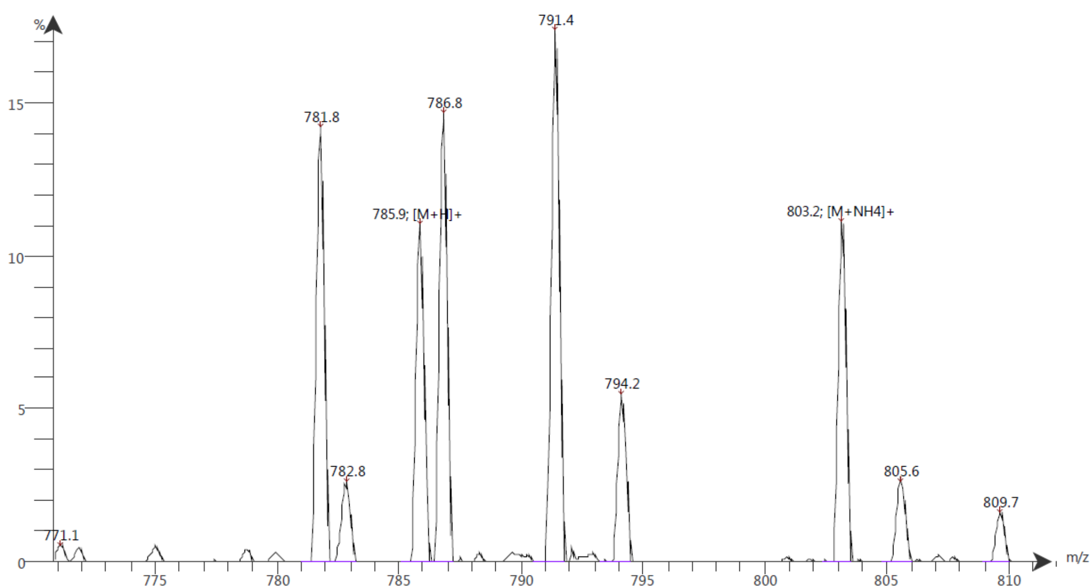


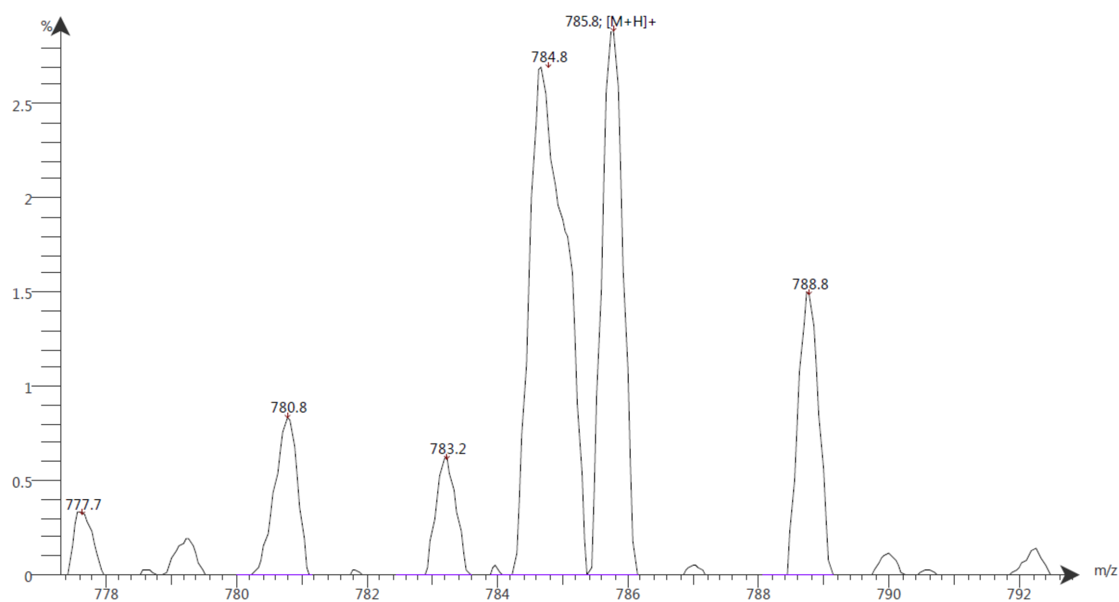
iii. Astragaloside IV root extract



iv. Astragaloside IV Hydrophilic concentration

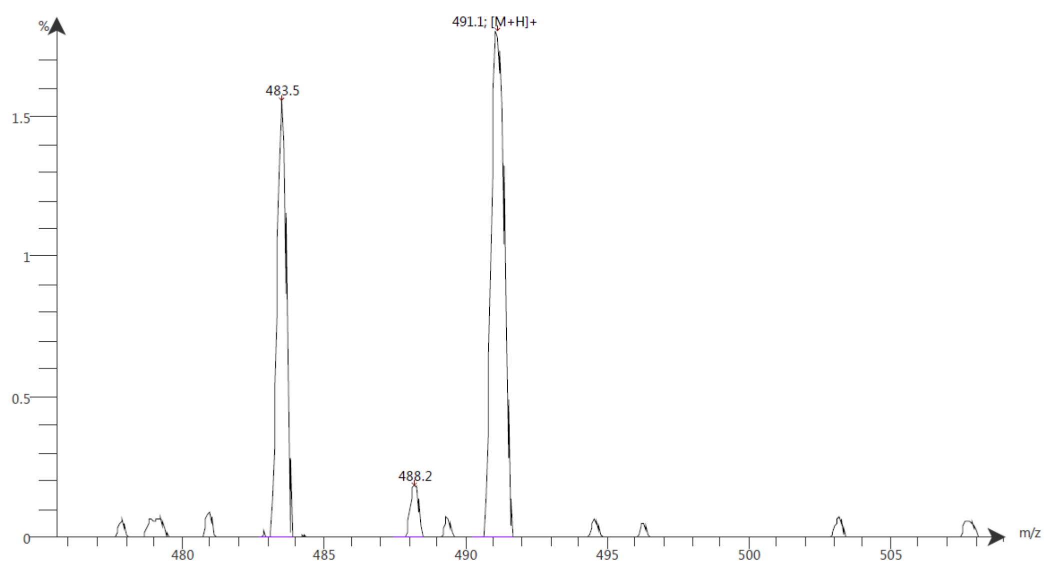
There is no mass peak for astragaloside IV, in the hydrophilic concentration of Astragalus Radix.

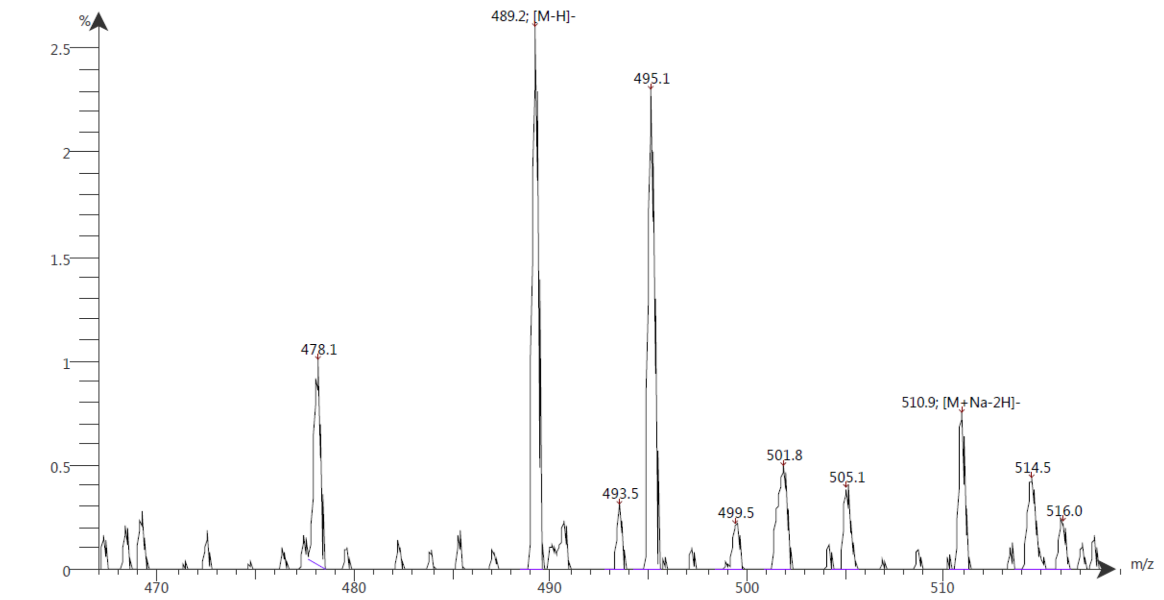




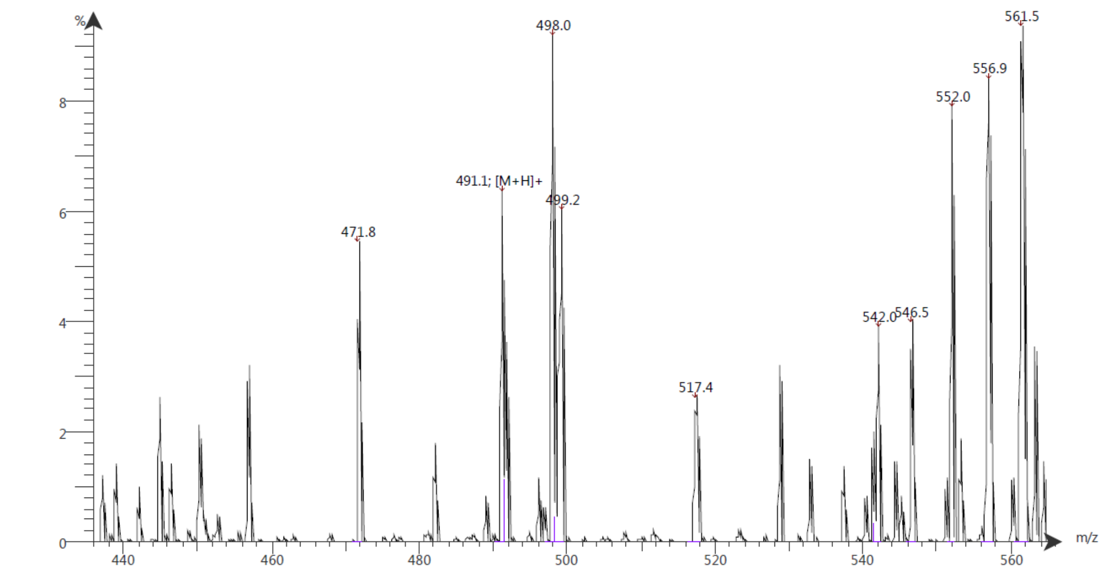
v. Astragaloside IV in boiled water decoction

5. Cycloastragenol (490.7 g/mol)





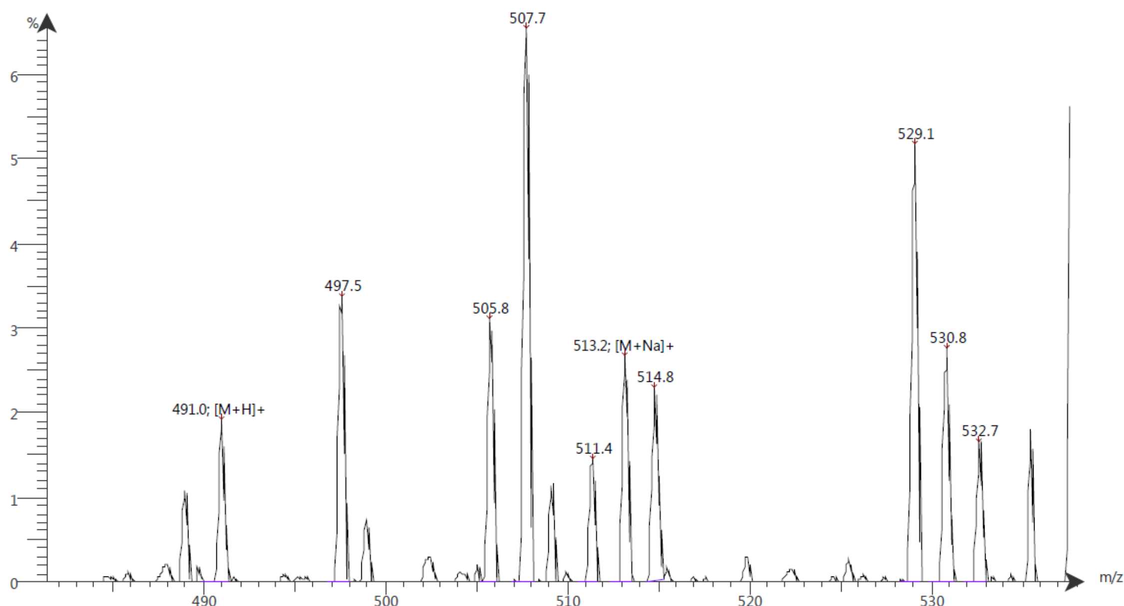
i. Cycloastragenol standard (both positive and negative ionization)



ii. Cycloastragenol in granule extract

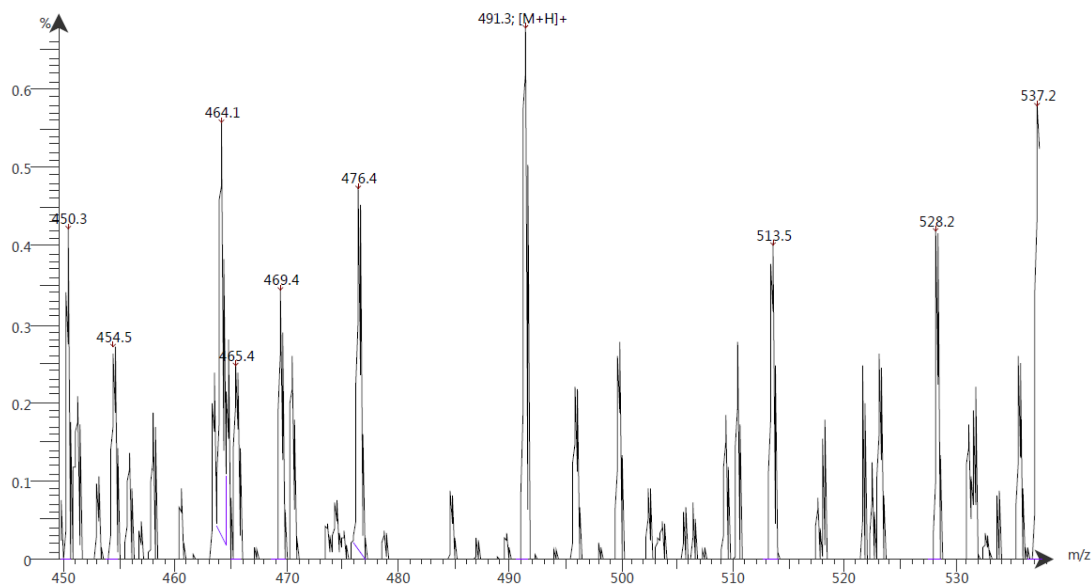
Intensity

Spectrum RT 1.25 (1 scans) - Background Subtracted 0.41 - 0.91
2019_10_2 Cycloastragenol EG_Scan1_is1 2019.10.02 14:47:10 ;
ESI + Max: 1.5E6



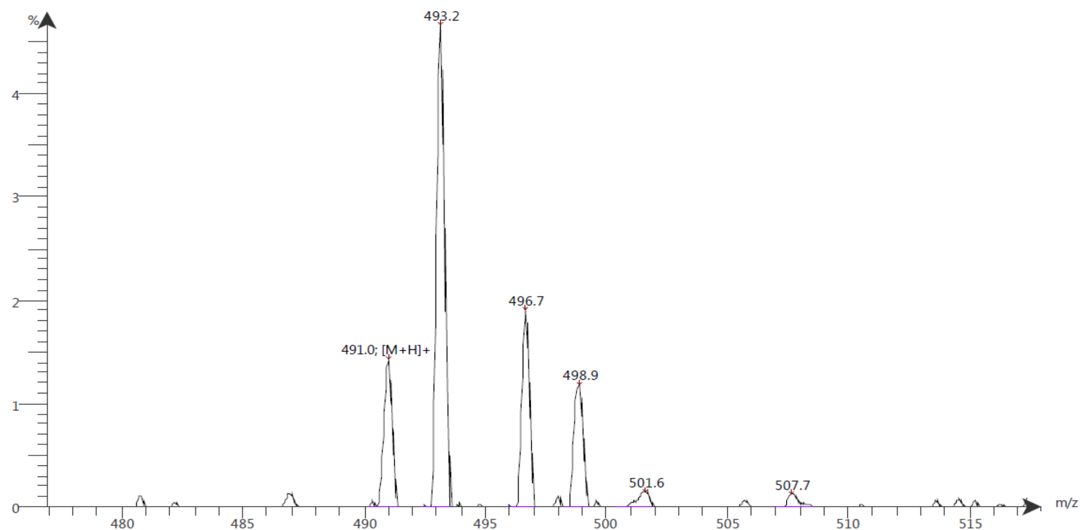
Intensity

Spectrum RT 0.89 - 1.05 (10 scans) - Background Subtracted 0.41 - 1.14
2019_10_1 Cycloastragenol EG_Scan1_is1 2019.10.01 14:34:27 ;
ESI + Max: 1.2E5

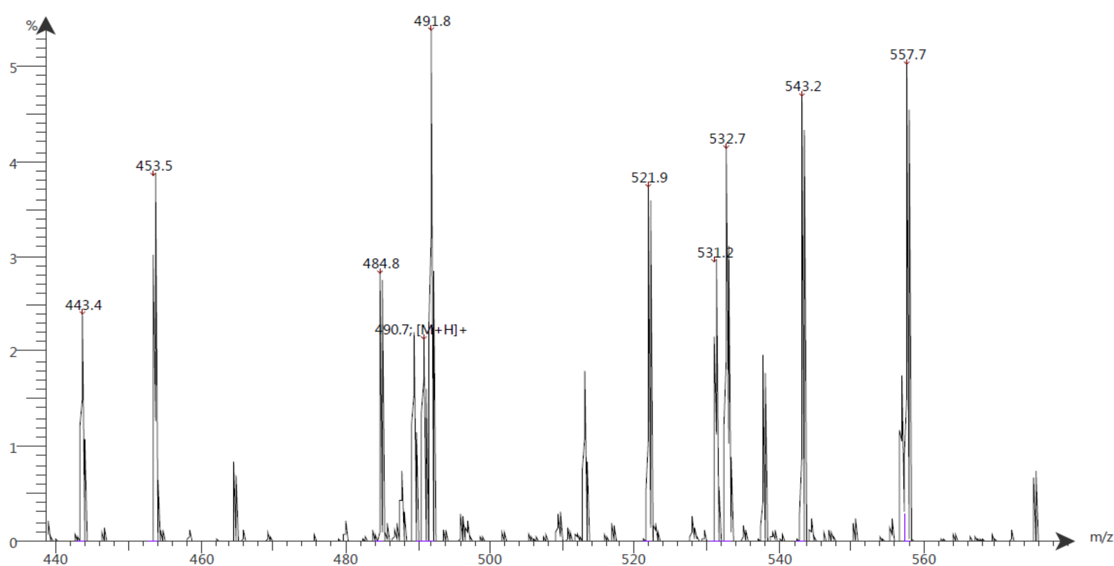


iii. Cycloastragenol in root extract

The two mass spectra are from different sample spots, the presence of cycloastragenol can be detected however there are many other dominant peaks at the spectra. Some peaks are shown as the mass of cycloastragenol plus mass of other compounds as well.



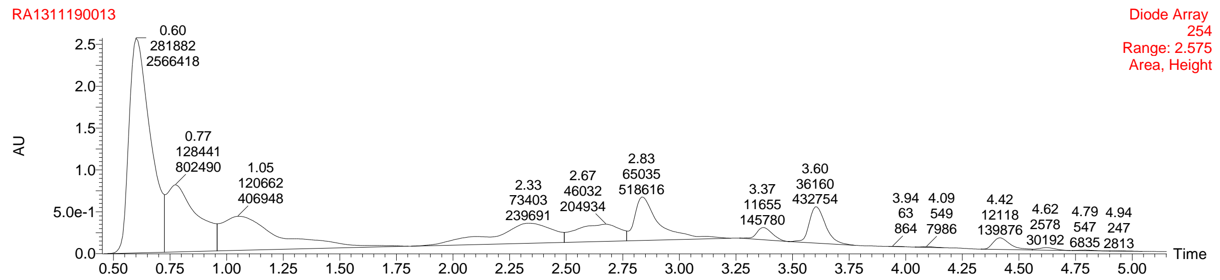
iv. Cycloastragenol in hydrophilic concentration



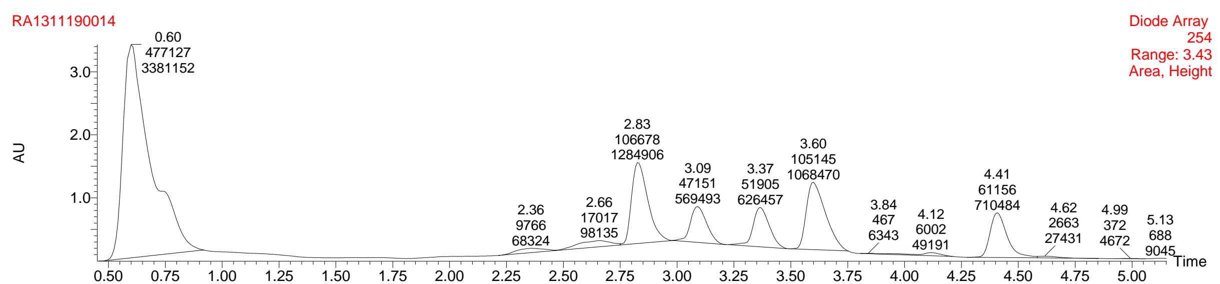
v. Cycloastragenol in boiled water decoction

There are low quality peaks obtained for chemical compounds during TLC-MS analysis of different AR samples, which are included, as a part of this study. However, they are not good enough to be discussed scientifically.

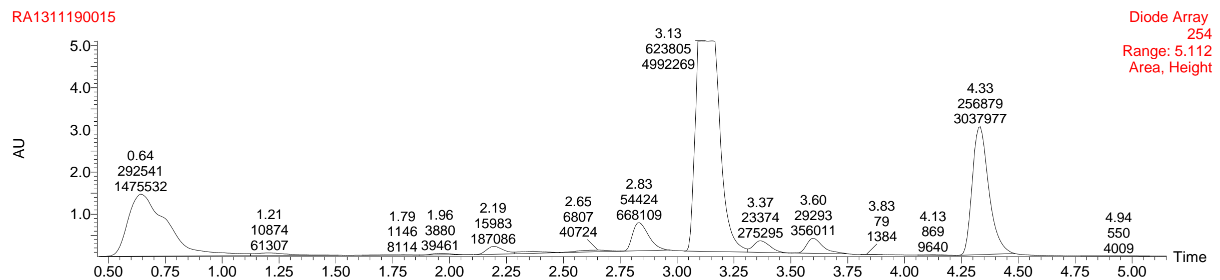
Appendix IV: HPLC-UV chromatogram of Astragalus Radix samples



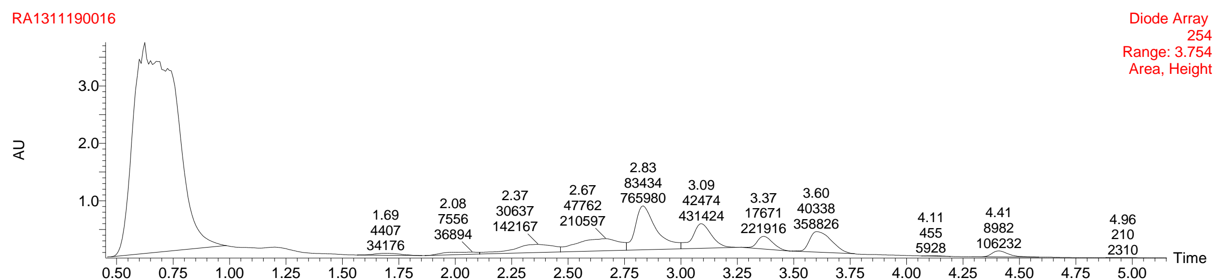
i. RA Granules samples (sample A)



ii. Root Powder samples (sample B)

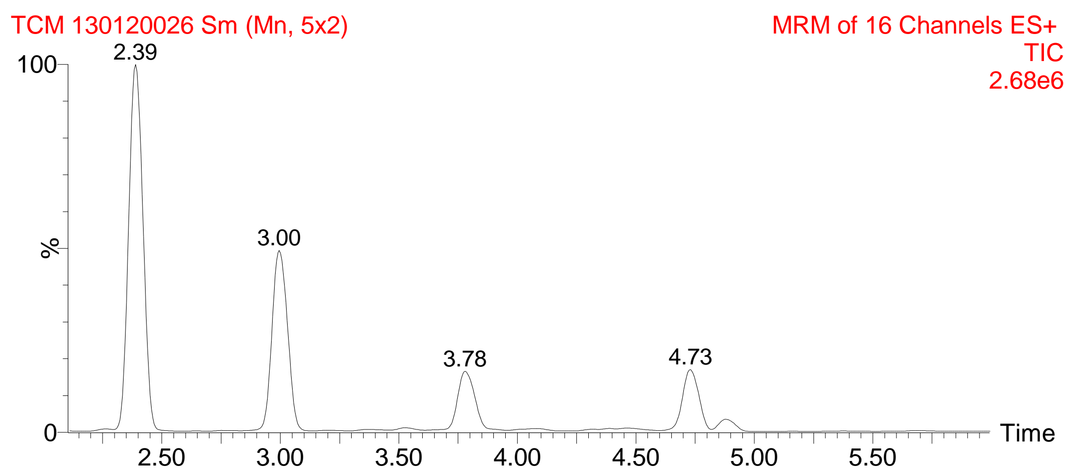


iii. Hydrophilic concentrate samples (sample C)

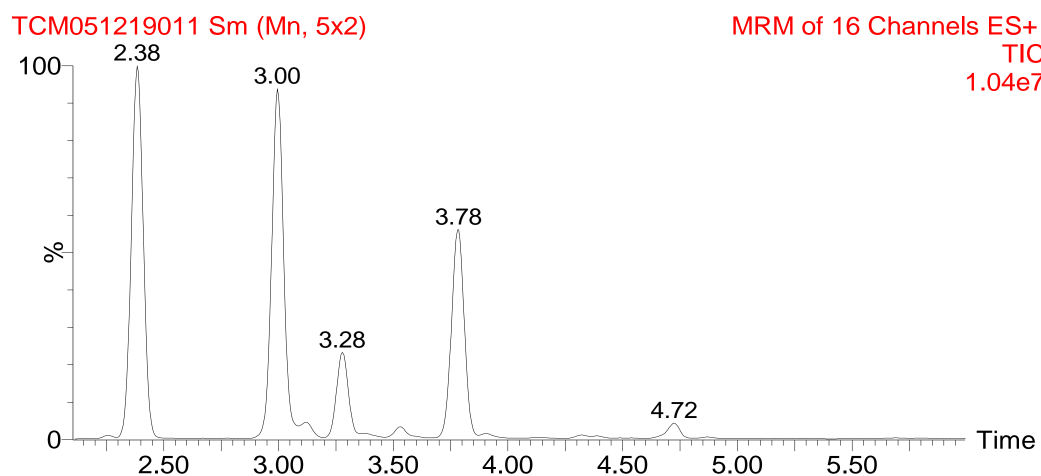


iv. Boiled root samples (sample B1)

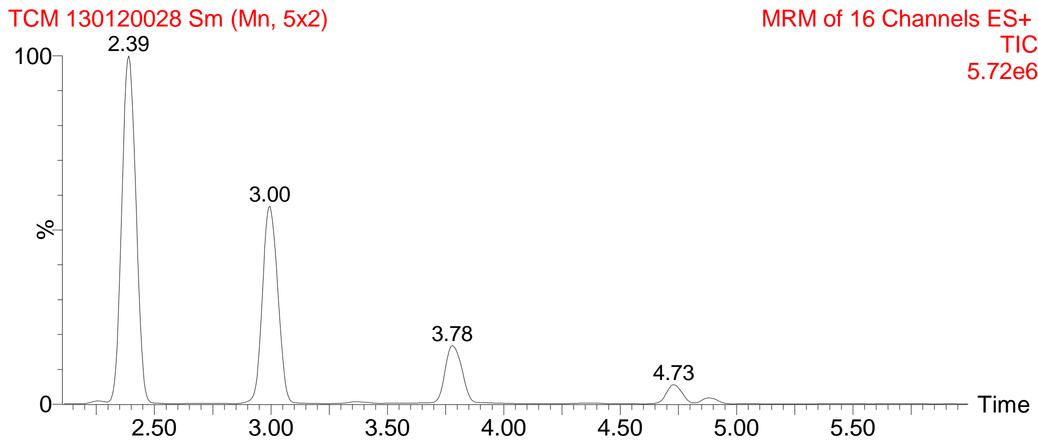
Appendix V: LC-MS/MS of Astragalus Radix samples



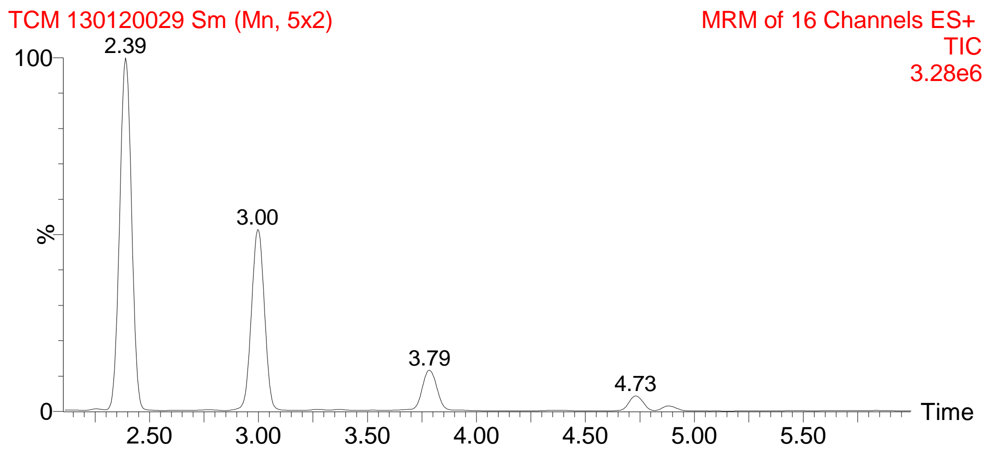
i. Granule samples (sample A)



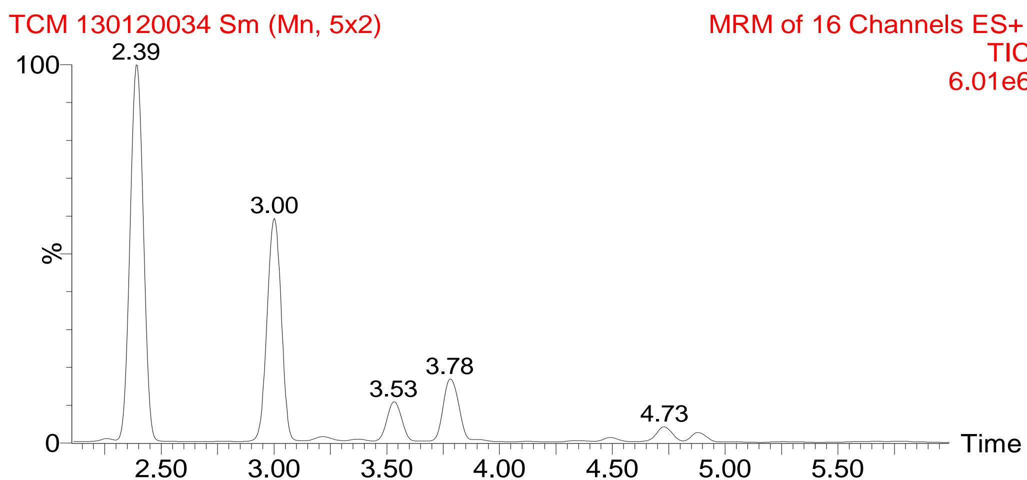
ii. Root powder samples (sample B)



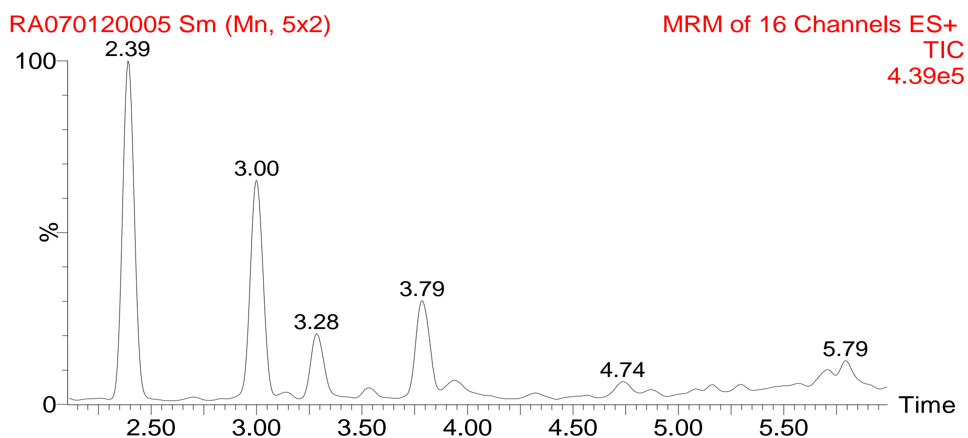
iii. Hydrophilic concentrate (sample C)



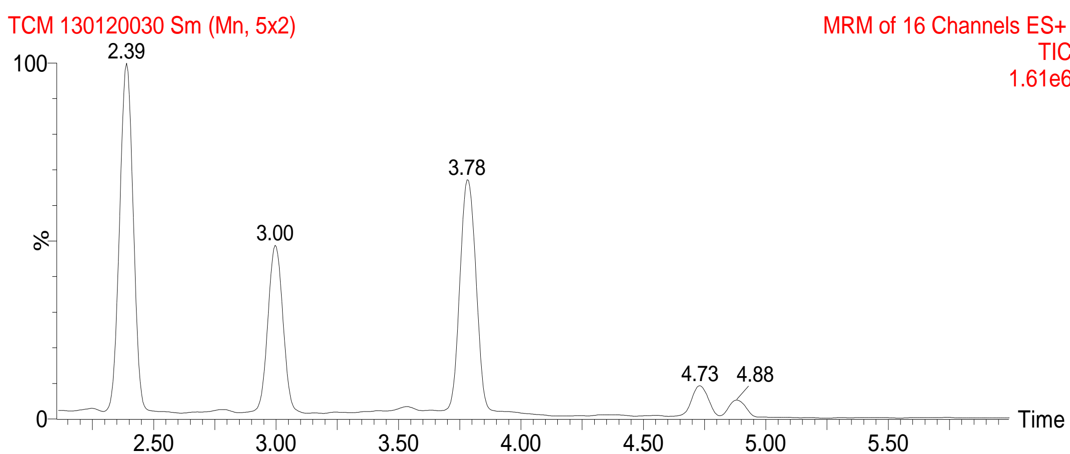
iv. Boiled Root powder samples (Sample B1)



v. Capsules samples (Sample D)



vi. SF table samples (Sample E)



vii. CMC granule sample (Sample F)

Appendix VI: Ion ratios of all five bioactive compounds

The ion ratios are the ratio of peak areas of parent as well as fragment ions. They should be almost the same or within the same range.

Table 12: The ion ratios of all five compounds standards and samples

	Ion ratios	
Chemical Standards		
Formononetin	0.99	-
Ononin	17.6	-
Calycosin 7-0-β-D glucoside	4.41	-
Astragaloside IV	4.8	-
Cycloastragenol	0.06	-
Samples	**Sample extracts**	**Diluted samples**
Sample A (Granulates)		
Formononetin	0.9	0.94
Ononin	13.38	15.32
Calycosin 7-0-β-D glucoside	4.4	4.39
Astragaloside IV	5.2	6.7
Cycloastragenol	0.11	0.04
Sample B (Dry root powder)		
Formononetin	0.89	0.96
Ononin	13.3	15.6
Calycosin 7-0-β-D glucoside	4.5	4.4
Astragaloside IV	3.58	6.34
Cycloastragenol	0.09	0.05
Sample C (Hydrophilic concentration)		
Formononetin	0.88	0.97
Ononin	14.73	14.84
Calycosin 7-0-β-D glucoside	4.51	4.43
Astragaloside IV	5.8	6.3
Cycloastragenol	0.04	0.11
Sample B1 (Boiled root extract)		

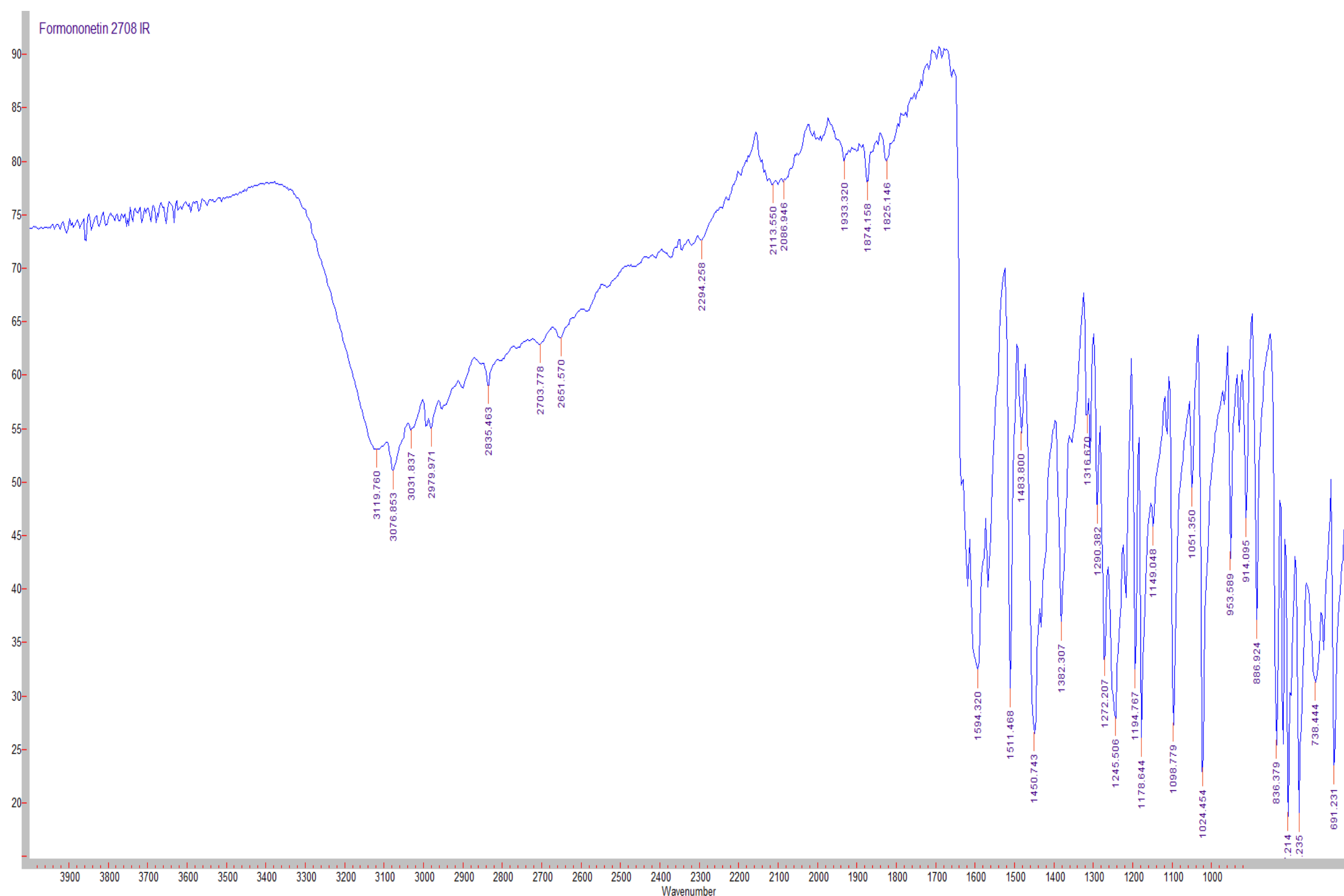
Formononetin	0.90	0.97
Ononin	13	14.3
Calycosin 7-0-β-D glucoside	4.2	4.6
Astragaloside IV	5.4	6.6
Cycloastragenol	0.09	0.03
Sample D (Capsules)		
Formononetin	-	0.98
Ononin	-	15.56
Calycosin 7-0-β-D glucoside	-	4.83
Astragaloside IV	-	6.8
Cycloastragenol	-	-
Sample E (SF tables)		
Formononetin	-	0.85
Ononin	-	16.10
Calycosin 7-0-β-D glucoside	-	4.73
Astragaloside IV	-	4.41
Cycloastragenol	-	0.006
Sample F (CMC, granulates)		
Formononetin	-	1.01
Ononin	-	14.22
Calycosin 7-0-β-D glucoside	-	4.60
Astragaloside IV	-	5.46
Cycloastragenol	-	-

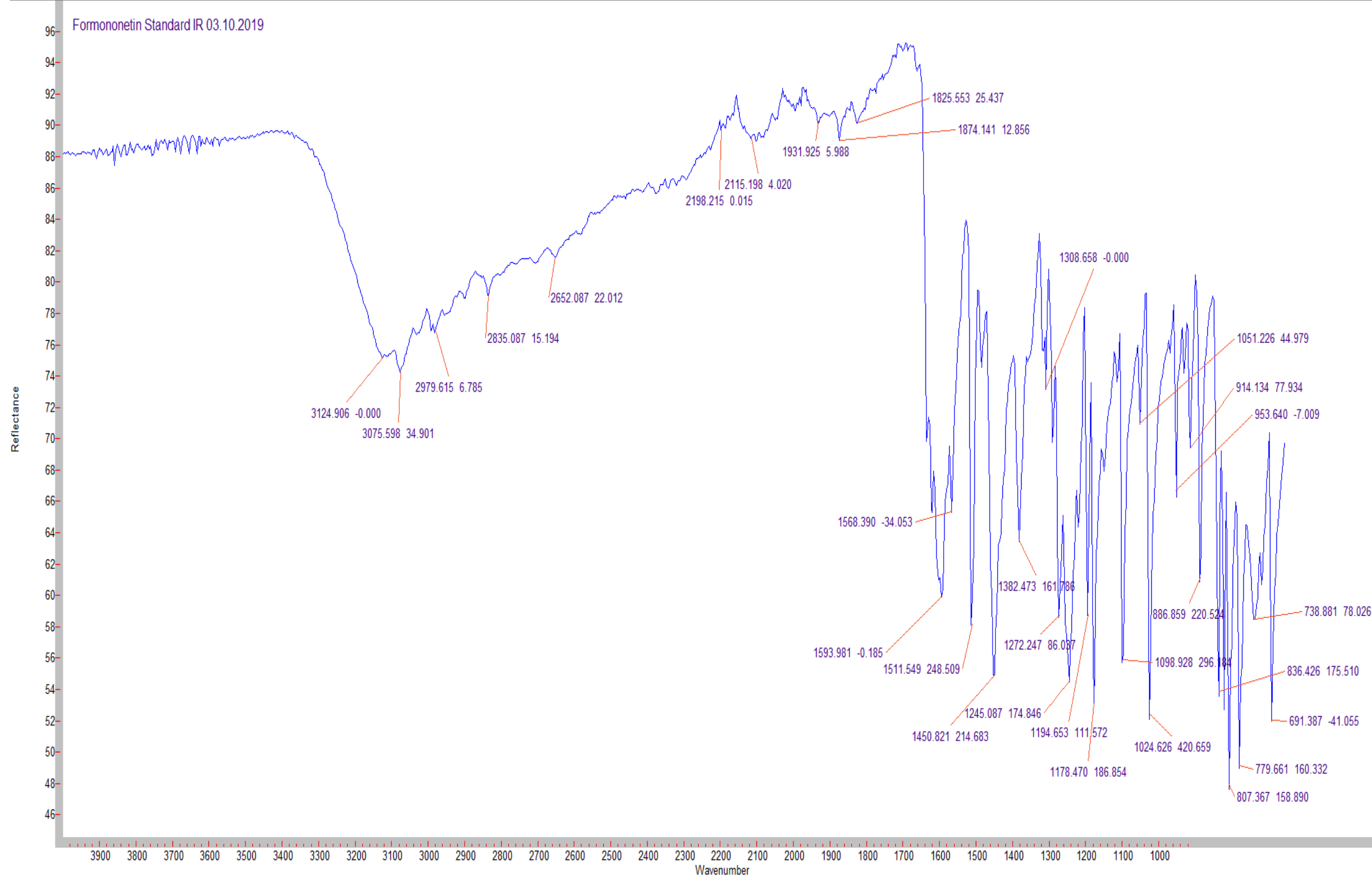
Appendix VII: Parent and daughter ions

Table 13: Parent and daughter ions for five different compounds (*Molecular weight calculator)*

S.N.	Compound name	Parent ions (*m/z*)	Daughter (*m/z*)	Daughter ions
1.	Formononetin, $C_{16}H_{12}O_4$	269.2, $[M+H]^+$	118.1	$[M-C_8H_7O_3+H]^+$
			213.3	$[M+H-2CO]^+$
			237.3	$[M+H-CH_3OH]^+$
2.	Ononin, $C_{22}H_{22}O_9$	431.25, $[M+H]^+$	118.1	$[M-Glc-C_8H_5O_2+H]^+$
			237.3	$[M-Glc-CH_3+H]^+$
			269.2	$[M+H-Glc+OH]^+$
7.	Calycosin 7-O-β-D glucoside, $C_{22}H_{22}O_{10}$	447.25, $[M+H]^+$	270.3	$[M+H-Glc]^+$
			285.35	$[M+H-Glc+CH_3]^+$
9	Cycloastragenol, $C_{30}H_{50}O_5$	491.5, $[M+H]^+$	143	$[M-C_{22}H_{36}O_3+H]^+$
		513.4, $[M+Na]^+$	513.4	$[M+Na]^+$
11	Astragaloside IV, $C_{41}H_{68}O_{14}$	785.45, $[M+H]^+$	143	$[M-Glc-Xyl-C_{22}H_{32}O+H]^+$
		807.4, $[M+Na]^+$	203.1	$[M-C_{32}H_{54}O_9+H]^+$
			627.5	$[M-Glc+Na]^+$

Appendix VIII: FTIR of Formononetin standard





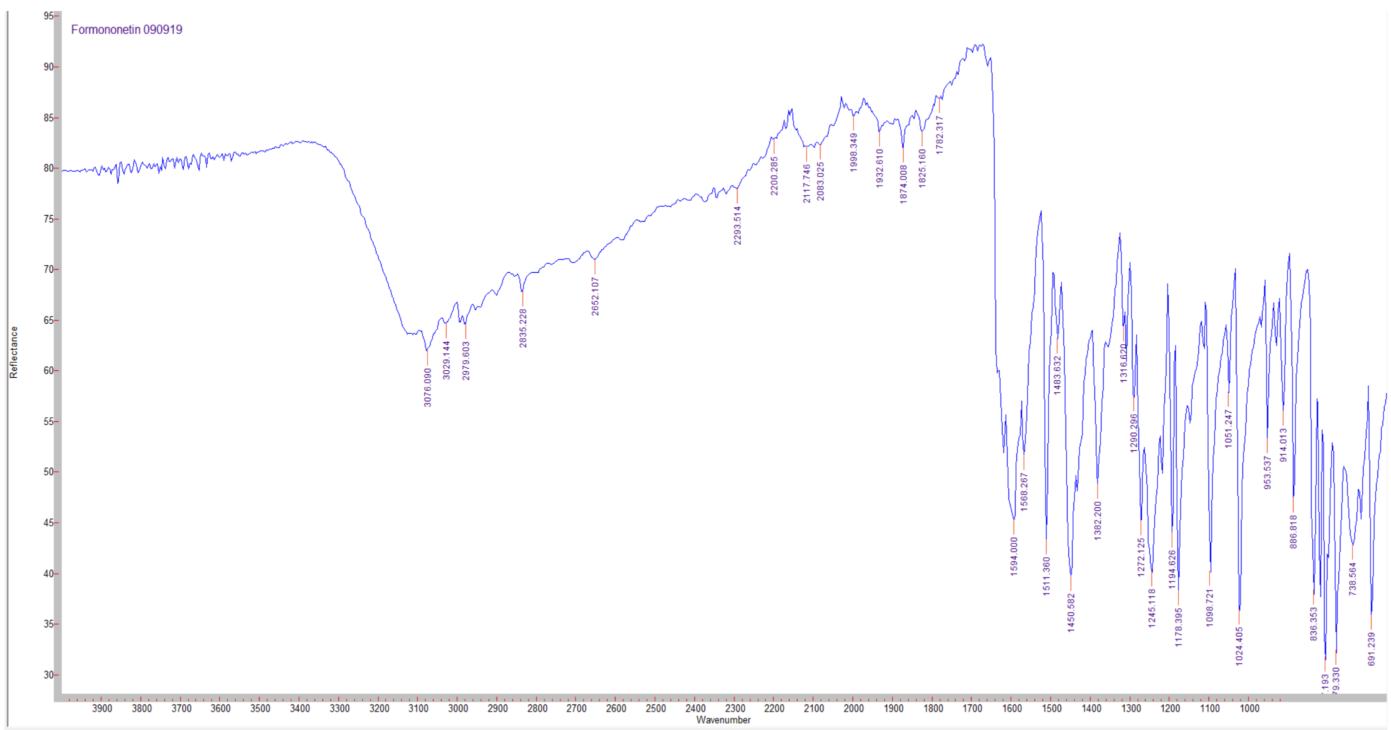


Figure 35. FTIR of Formononetin standard (repetitions)