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HPLC-HIGH RESOLUTION MASS SPECTROMETRY ANALYSIS OF *IN VITRO* AND *IN VIVO* METABOLISM OF SCOPARONE

Diploma thesis

Supervisor: Ing. Petra Matoušková, Ph.D.

Consultant: prof. Seppo Auriola

Hradec Králové 2018

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UNIVERZITA KARLOVA

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra biochemických věd

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IN VITRO A *IN VIVO* ANALÝZA METABOLISMU SKOPARONU POMOCÍ HPLC A HMOTNOSTNÍ SPEKTROMETRIE S VYSOKÝM ROZLIŠENÍM

Diplomová práce

Vedoucí diplomové práce: Ing. Petra Matoušková, Ph.D.

Konzultant: prof. Seppo Auriola

Hradec Králové 2018 Filip Novák

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„I declare that this thesis is my original author work. All literature and other sources I used during the processing are listed in the list of used literature and are properly cited throughout my work. The thesis has not been used to obtain different or equal degree.“

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Abstract

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Title of Diploma thesis: HPLC-high resolution mass spectrometry analysis of *in vitro* and *in vivo* metabolism of scoparone

Scoparone is an active ingredient of *Artemisia scoparia*, a medicinal plant used in traditional Chinese medicine. It has been studied for various pharmacological effects such as upregulation of conjugation enzymes included in excretion of bilirubin, reduction of proinflammatory cytokines, lowering of plasma lipids levels and inhibition of platelet aggregation. In this thesis, metabolism of scoparone was studied by LC-MS method using Q-ToF device. Scoparone was incubated with liver microsomes obtained from 6 different mammal species to study *in vitro* oxidation. In total, six metabolites were detected in the incubation samples. Scopoletin and isoscopoletin were identified as major metabolites in every species, however, the rates of scoparone oxidation as well as a ratio of formed isoscopoletin and scopoletin varied. Furthermore, *in vivo* metabolites in human were studied in urine samples obtained from two healthy volunteers after oral administration of scoparone. Nine metabolites were detected in the urine samples in total, major metabolites being glucuronide and sulphate conjugates. The highest levels of metabolites were detected in the urine samples taken three hours after scoparone administration suggesting rapid elimination. Unfortunately, conjugation metabolites could not have been fully identified during this study, however, it has been proved that this LC-MS method is suitable for further research of scoparone metabolism both *in vivo* and *in vitro*.

Abstrakt

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Název diplomové práce: *In vitro* a *in vivo* analýza metabolismu skoparonu pomocí HPLC a hmotnostní spektrometrie s vysokým rozlišením

Skoparon je účinnou složkou v rostlině *Artemisia scoparia* používané v tradiční čínské medicíně. Byl předmětem studií pro nejrůznější farmakologické účinky jako upregulace konjugačních enzymů zodpovědných za exkreci bilirubinu, snížení hladin prozánětlivých cytokinů, snížení hladin plasmatických lipidů nebo inhibice agregace krevních destiček. V této práci byl studován metabolismus skoparonu pomocí LC-MS metody využívající Q-ToF analysátor. *In vitro* oxidace skoparonu byla studována po inkubaci s mikrosomy připravenými ze vzorků jater ze šesti různých savčích druhů. Celkem bylo v *in vitro* vzorcích identifikováno 6 metabolitů. Skopoletin a isoskopoletin byly identifikovány jako hlavní metabolity u každého z vybraných druhů, nicméně celková míra oxidace skoparonu i poměr vznikajícího isoskopoletinu a skopoletinu se lišily. Dále byly studovány *in vivo* metabolity skoparonu ve vzorcích moči získaných od dvou zdravých dobrovolníků po aplikaci skoparonu *per os*. Celkem bylo detekováno devět metabolitů, z nichž nejvýznamnějšími byly glukuronidy a sulfáty. Nejvyšší hladiny metabolitů byly detekovány ve vzorcích odebraných 3 hodiny po podání, což svědčí o rychlé eliminaci skoparonu z těla. Přestože se nepodařilo identifikovat strukturu všech metabolitů, můžeme říci, že tato LC-MS metoda je vhodná pro další studium metabolismu skoparonu *in vitro* i *in vivo.*

1. Introduction

Scoparone is a coumarine derivate naturally occurring in plants. It has been studied for various pharmacological activities including anti-inflammatory, anticoagulant and antioxidant. Since it is a promising structure for further studies, there is a need for information concerning its metabolism. As metabolism is a complex process, and drug metabolites can have various activities (both therapeutic and toxic), it is a crucial part of drug development.

Drug metabolism can be divided into two phases. Oxidation reactions are important during phase I of drug metabolism, whereas conjugation reactions are important in phase II. During phase I many drugs are oxidized by cytochrome P450 enzymes. Although metabolism of scoparone has been studied before, there is still shortage of information, mainly regarding phase II metabolites.

High performance liquid chromatography coupled to mass spectrometry is a valuable tool not only in drug research. It can be used for both qualitative and quantitative studies of metabolites. Furthermore, due to possibility of tandem mass spectrometry, it provides us information on structure of unknown metabolites. Quadruple Time-of-flight mass analyser has benefits of high scanning speed and high sensitivity, therefore is suitable in drug research.

In this study, *in vitro* and *in vivo* metabolites of scoparone were studied. *In vitro* metabolites of scoparone oxidation were studied in incubation samples with mammal liver microsomes. *In vivo* metabolites in human were studied in urine samples collected from two healthy volunteers.

2. Theoretical part

2.1. Drug metabolism and its role in drug research

2.1.1. Introduction

Understanding pharmacokinetics and pharmacodynamics of new chemical entities is crucial for a successful drug development and selection of therapeutic herbal products. Pharmacodynamics can be described as “what the drug does to the biological system”, therefore we can talk about its bioactivity and toxicity. Description of pharmacokinetics is similar but reverse, “what the biological system does to the drug”, and it consists of four basic processes: absorption, distribution, metabolism and excretion (Kirchmair 2014). Two last mentioned can be summed up as an elimination. The process of metabolism can be further divided into two phases, functionalization (phase I) and conjugation (phase II). During the phase I, new functional groups such as hydroxyl, thiol, amino or carboxylic acid are created in the structure of drug or existing ones are modified. Reactions creating them are oxidation, reduction and hydrolysis. Whereas during the phase II, the functional groups of drugs are conjugated to endogenous compounds of glucuronic acid, sulfone, acetyl, methyl, amino acid or glutathione (Goodman et al. 2011).

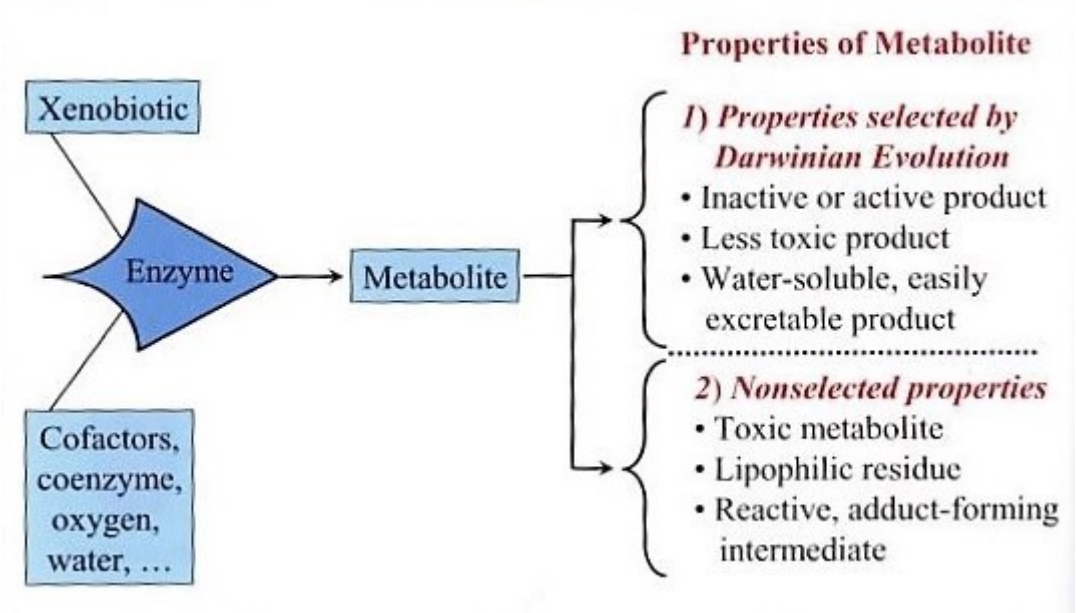


Figure 1 Summary of metabolism of xenobiotics (Testa and Krämer 2008).

The main result of the drug metabolism is to end the effects of a drug in the organism by creating inactive and more water soluble metabolites, therefore facilitating excretion to urine or bile. However, as known from many examples, the process of metabolism is not this straightforward, because sometimes an inactive or less active compound is metabolised to an active compound *(Figure 1).* This is turned to an advantage with pharmaceuticals called prodrugs, which are metabolized to effective metabolites in the body. Often prodrugs are inactive lipophilic esters, which are hydrolysed to active carboxylic acids or alcohols (Kirchmair 2014).

2.1.2. Cytochromes P450

One of the most important reactions in the drug metabolism are redox reactions, in which the parent drug is oxidized or reduced and co-substrate (cofactor) reduced or oxidized, respectively. Although, there are various enzymes with redox activities, majority of reactions are catalysed by cytochrome P450 enzymes (CYP). CYPs are a superfamily of evolutionary old enzymes present in almost every organism (Goodman et al. 2011, Testa and Krämer 2008).

The main mechanism of CYP redox reaction is a mono-oxygenation meaning that one atom of oxygen is used to oxidize the parent compound e.g. to hydroxyl metabolite and another oxygen is reduced to water. The reaction's catalytic cycle involves several steps during which molecular oxygen 0_2 is reduced and substrate is oxidized. For the reaction, one electron is donated from NADPH and second electron is donated from either NADPH or NADH via the coenzyme NADPH cytochrome P450 reductase or cytochrome b5. An activated atom of oxygen is then transferred to the substrate and a molecule of water is formed from the second oxygen atom. Based on the reaction mechanism, CYPs can conduct number of reaction types, most common are aliphatic and aromatic hydroxylations, N-dealkylation, O-dealkylation, N-oxidation, S-oxidation, deamination and dehalogenation (Testa and Krämer 2008).

Structurally, CYPs consist of a variable amino acid sequence part that is responsible for substrate specificity and a coenzyme part *(Figure 2).* As the coenzyme part is iron-protoporphyrin IX or shortly a heme, CYPs can be also referred to as hemoproteins. On a cellular level, CYPs are located on an outer membrane of an endoplasmatic reticulum, exceptionally few CYPs can be found in mitochondria. They are coupled with electron donating enzymes NADPH-cytochrome P450 reductase, cytochrome b_5 and NADH-cytochrome b_5 reductase which are also located on the membrane (Testa and Krämer 2008).

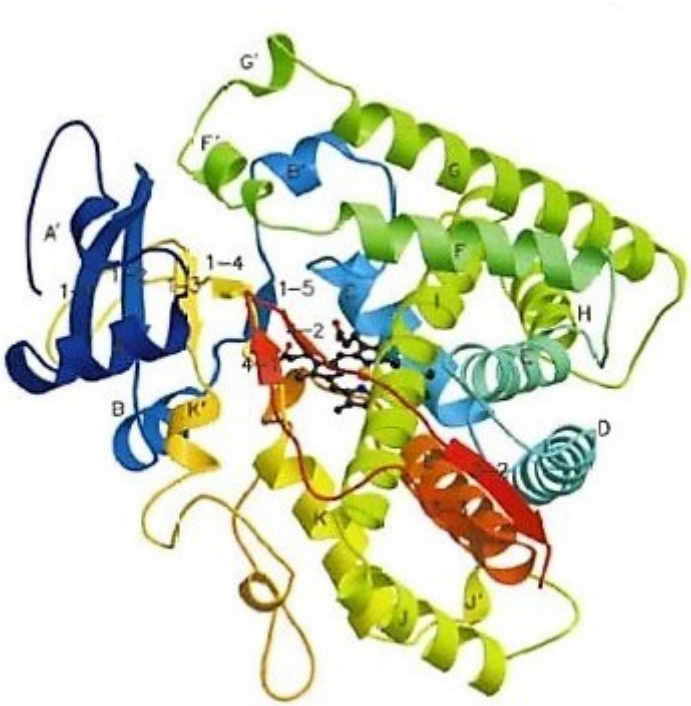


Figure 2 Structure of cytochrome P450 (Testa and Krämer 2008).

Most xenobiotic metabolising CYPs are located in liver, however, CYPs can be found in lower amounts in lungs, kidney, gastrointestinal tract and many other tissues. The first pass metabolism reffers to an extensive biotransformation in liver after oral administration, as the compounds absorbed from gastrointestinal tract to blood pass through liver via portal vein (Goodman et al. 2011).

CYP enzymes form superfamily, from which over 50 isoforms exist in humans. Individual enzymes of CYP super family are divided into families and subfamilies based on amino acid and gene sequence similarity *(Figure 3)*.

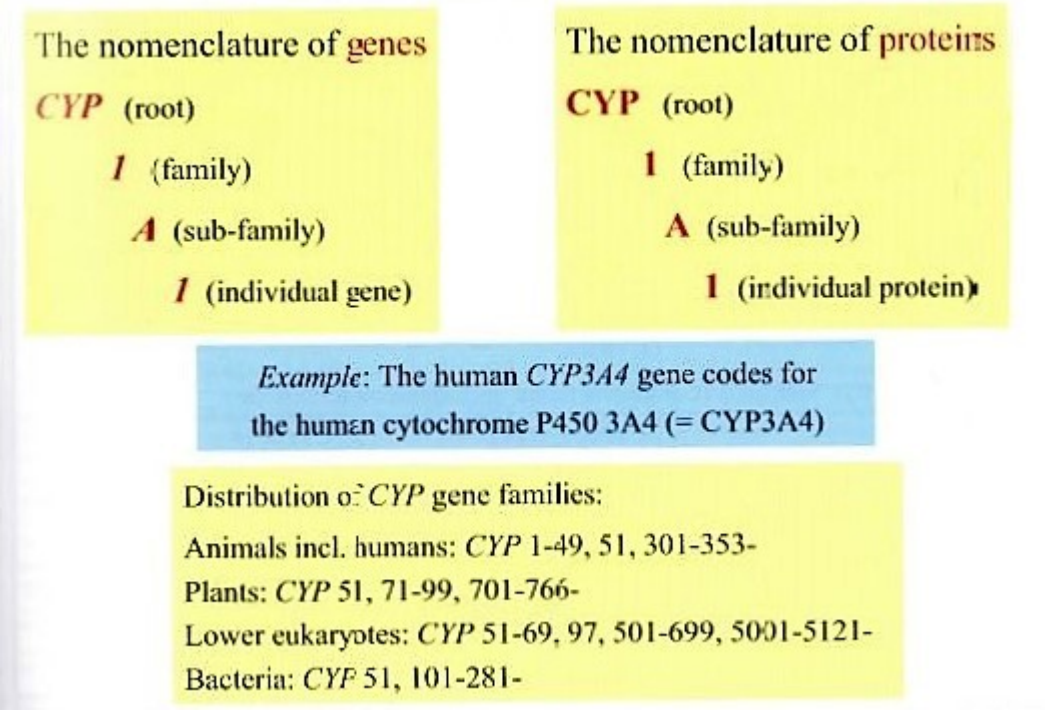


Figure 3 Summary of CYP nomenclature (Testa and Krämer 2008).

It is important to mention that CYPs do not only metabolise xenobiotics, but also many endogenous substances are substrates of CYPs. For example, fatty acids are oxidized mainly by enzymes of CYP4 family, prostaglandins are synthetized by families 5 and 8, whereas families 11, 17, 19 and 21 are responsible for steroid hormones synthesis. Families 1, 2 and 3 are abundantly expressed in liver and are involved mainly in the metabolism of xenobiotics. Isoforms 1A1, 1A2, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 are considered the most important in the drug metabolism in humans. Furthermore, CYP3A4 is responsible for metabolism of more than 50% of clinically used pharmaceuticals (Testa and Krämer 2008).

In comparison to enzymes catalysing endogenous substrates, CYPs with mainly xenobiotic activity show higher promiscuity – less substrate specificity and slower rates. This means that one CYP can metabolise number of substrates, although at slower rate. From a different point of view, one substrate can be metabolised by different CYPs leading to different metabolites (Goodman et al. 2011).

2.1.3. Glucuronidation and sulfatation enzymes

As mentioned before, conjugation reactions are part of the second phase of metabolism of drugs. Similarly to CYP enzymes, conjugation enzymes, i.e. transferases, are involved in metabolism of both endogenous and exogenous substances. The products, i.e. conjugates, are in general less lipophilic and less toxic, however, exceptions can be found (Testa and Krämer 2010). Only glucuronidation and sulphatation reactions will be briefly decribed, as these are relevant in the conditions of this work.

Glucuronidation reactions play an important role in metabolism due to diversity of potential substrates. These include following functional groups: phenols, alcohols, hydroxylamines, carboxylic acid, amines, thiols. With respect to substrate, *O*-, *N*-, *S*- or *C*-glucuronides are formed. During the reaction a molecule of glucuronic acid is provided by cofactor of uridine-5’-diphospho-α-D-glucuronic acid. The enzymes performing the reaction are called uridine 5'-diphospho-glucuronosyltransferases (UGTs) and are divided into families and subfamilies in the same way as CYPs (Testa and Krämer 2010).

The products of sulfatation reactions are called sulfates, containing sulfonate group tranfered from cofactor of 3’-phosphoadenylyl sulfate by enzymes called sulfotranferases (SULTs). Similarly to UGTs, sulfonyltranferases are involved in metabolism of phenols, alcohols, hydroxylamines and amines (Testa & Krämer 2010).

Both SULTs and UGTs are able to metabolise phenolic compounds. Although, sulphatation is usually faster than glucuronidation in low concentrations, the cofactor PAPS is rapidly depleted. Therefore, sulfataion is also characterised by lower capacity compared to glucuronidation (Testa and Krämer 2010).

2.1.4. Species differences in drug metabolism

There are various influencing factors affecting drug metabolism. Inter-individual factors such as species differences and genetic polymorphism are determined by genome and remain unchanged during a life time. On the other hand, intra-individual factors describe physiological, pathological, tissue specific and age-dependent changes in drug metabolism including drug-drug and food-drug interactions. These factors change during a life time (Testa and Krämer 2010).

Species differences in drug metabolism play an important role in drug development. Regarding the CYPs for instance, despite the similarities in gene sequence among the species, there are changes in enzyme activities. For example, *CYP2E1* and *CYPs 1A1* and *1A2* are considered to be well conserved genes, however, the first mentioned poses similar metabolic activities, *CYPs 1A1* and *1A2* code enzymes with different activities in various species. Furthermore, there are differences in CYP distribution and rate of expression, also the enzymes may response differently to inducers and inhibitors (Testa and Krämer 2010).

Therefore, factors such as bioavailibility, prodrug activation, biological half-life of tested molecule can vary between tested species and human. It is important to bear in mind those differences as it generally cannot be said which species posses the most similarities to human regarding the drug metabolism. Moreover, these differences explain usage of human liver samples in investigating drug metabolism (Testa and Krämer 2010).

2.2. LC-MS

Liquid chromatography - mass spectrometry (LC-MS) is an analytical method combining high-performance liquid chromatography (HPLC) and mass spectrometry (MS). It combines separation power of HPLC and uses MS for quantification and identification of compounds in a sample.

2.2.1. HPLC

HPLC is one of the most frequently used analytical methods in drug metabolism research. As a chromatographic method, it is based on a separation of compounds, so called analytes, in samples such as incubation mixtures or urine. During the separation process, an equilibria is set between the amount of molecules of analytes distributed in two phases – mobile and stationary phase. Therefore metabolites are usually well separated from parent substrate by HPLC. In HPLC, liquid mobile phase with analysed sample is pumped under pressure through a column filled with small porous particles coated with stationary phase (Braithwaite and Smith 1999).

Analytes with higher affinity to stationary phase are retained in a column for a longer period of time than analytes with an equilibrium set in favour of mobile phase *(Figure 4).* Thus, in successful separation, every analyte is eluted from the column in different time – called retention time (t_R) (Moldoveanu and David 2013).

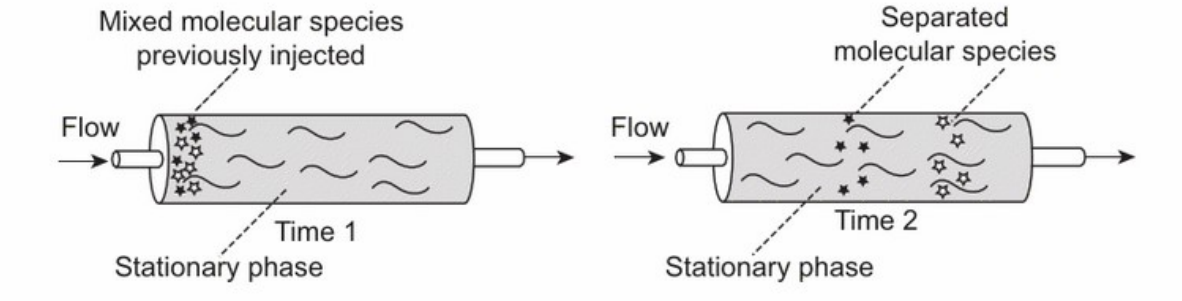


Figure 4 Simplified illustration of process of separation during HPLC analysis (Moldoveanu and David 2013).

There are many HPLC methods suitable for wide range of compounds from small molecules to protein structures. These methods differ in characteristics of both stationary and mobile phases, therefore there is a number of mechanisms of equilibrium formation explaining the separation process. It is important to state the fact that more than one mechanism might be responsible for the separation (Moldoveanu and David 2013).

In reversed phase HPLC, a most widely used method, the main mechanism of separation is partition equilibrium *(Figure 5).* An analyte is distributed between two liquids based on principle similar to liquid-liquid extraction. One liquid is obviously mobile phase, composed of polar organic solvents such as acetonitrile or methanol in a mixture with water or buffer, the other liquid is stationary phase. Chemically bonded hydrocarbon chains, most commonly C18 and C8, are considered as liquid phase for these purposes. On molecular level hydrophobic interactions take place. A molecule with larger hydrophobic area in molecule is retained in column for a longer period of time than a molecule with less hydrophobic structure. Therefore, the retention time of water soluble metabolites are shorter than of the lipophilic parent substrates of CYPs (Moldoveanu and David 2013).

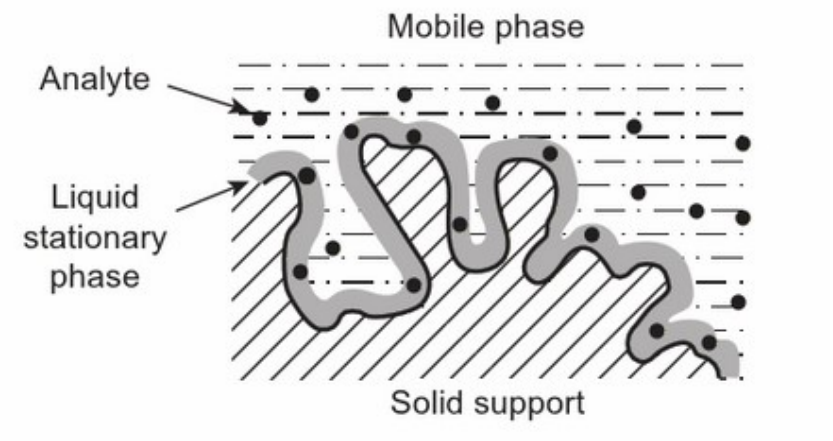


Figure 5 Illustration of partition equilibrium (Moldoveanu and David 2013).

Furthermore, an elution can be either isocratic or gradient. During the process of isocratic elution the composition of mobile phase remains constant. Although the analytes might be well separated, it prolonges the overall time of the analysis. Therefore, wisely chosen gradient of mobile phase can improve the overall time of analysis while keeping the analytes well separated (Moldoveanu and David 2013).

As there is number of factors affecting the separation such as pressure and composition of mobile phase, temperature, type of used detector and other, relatively complex instrumentation is needed to maintain stable conditions. Main parts of HPLC and basic aspects of the process of analysis are briefly described *(Figure 6)* (Moldoveanu and David 2013).

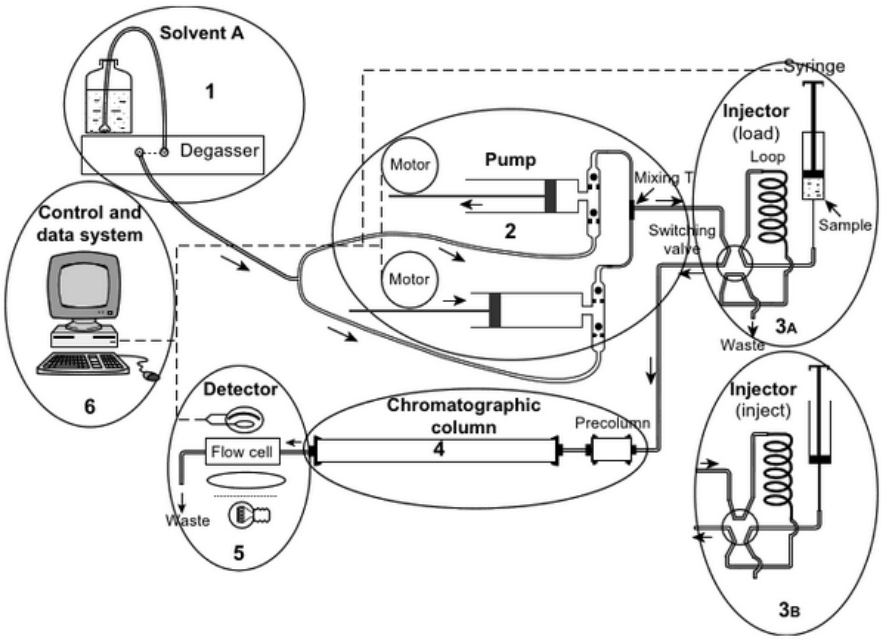


Figure 6 Simplified illustration of HPLC system (Moldoveanu and David 2013).

Prior to the injection of a sample, mobile phase passes through degassing and pumping systems. Although, only solvents of high purity are used, at HPLC grade or filtrated through 0.45 μm filters, dissolved gasses, mainly oxygen, have to be eliminated in degasser. To overcome a resistance of the column, mobile phase must be pumped under high pressure. To maintain stable flow and pressure with minimalized fluctuations, a set of reciprocating pumps is used. The sample is introduced to mobile phase flow via injector and switching valve. Typical injectors are capable of injecting volumes of 1-100 μl (Moldoveanu and David 2013).

The separation process takes place in the column. It is a metal or plastic tube of 1-10 mm in diameter, 3-25 cm long filled with small porous solid particles of 3-5 μm in diameter. For reverse phase-HPLC these particles are coated with hydrocarbon chains. For other types of analysis, monolithic materials can be used (Moldoveanu and David 2013).

When the analyte leaves the column, a detector is needed in order to create data output for quantification and/or identification. There are various types of detectors using different physical or chemical characteristics of analytes to create a signal. Selection of used detector depends on characteristics of an analysed compound, desired sensitivity and selectivity of the detector. Data obtained from an analysis form a chromatogram - a graph showing detector signal dependent on analysis time (Braithwaite and Smith 1999).

Main advantages of HPLC methods are full automatization of the process and an online connection to other instruments. Various changes of instrumentation, usage of different mobile phases and reusable columns offer to analyse wide range of compounds in many fields of scientific research. Thanks to these advantages, HPLC has become one of the most widely used analytical methods (Braithwaite and Smith 1999).

2.2.2. Mass spectrometry

Mass spectrometer as a detector in HPLC methods offers advantages of a high sensitivity, selectivity and thanks to multiple MS option of identification of unknown compounds. For purposes of metabolite analysis, high resolution devices are used, such as time-of-flight analysers or analysers based on Fourier transformation (Niessen 2006).

Mass analysis process can be described in five basic steps: sample introduction, ionization, mass analysis, ion detection and data handling (Niessen 2006).

Prior to mass separation, molecules introduced to mass spectrometer are ionizated. There is a number of ionization techniques each bearing its advantages and disadvantages. Electrospray ionization, atmospheric pressure chemical ionization, atmospheric pressure photo-ionization, atmospheric pressure laser ionization and desorption electrospray ionization are suitable options for LC-MS methods (Lee 2014).

As mentioned before, electrospray ionization (ESI) is one of the ionization techniques suitable for LC-MS analysis. As molecular ions are created, it is a type of soft ionization methods. As a solution from HPLC passes through a nebuliser charged with high voltage, analyte gains either a positive or a negative charge – regarding the polarity of nebuliser to a counter electrode *(Figure 7).* Solution emerging from a needle of the charger nebuliser forms so called Taylor cone, an elongation of analyte solution due to the voltage. Small droplets exit the Taylor cone and a flow of heated counter current gas, usually nitrogen, facilitates the evaporation of the liquid phase. During the process of evaporation, two forces are present within the droplets. Liquid surface tension force holds droplet in one

piece. Coulumbic repulsion forces of charged ions act contrariwise. Once the repulsion force overcome surface tension, smaller droplets are created, and the process starts again. In the end, only charged ions without any liquid are attracted to mass analyser entrance capillary. The process of ESI takes a place in a normal pressure (Downard 2004).

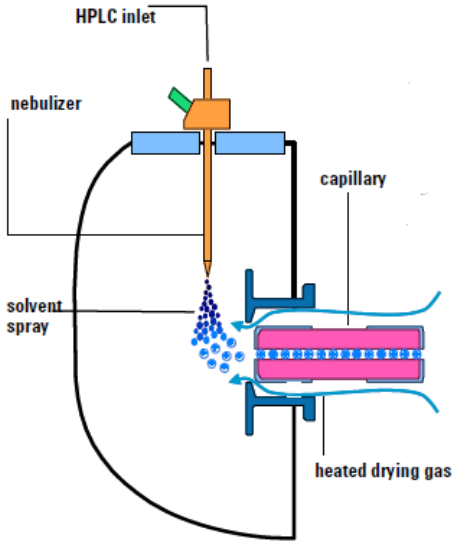


Figure 7 Schematic illustration of electrospray ionization (Agilent Technologies 2011).

Mass separation process is conducted in high vacuum. Ionizated molecules having entered the analyser are separated according to their mass to charge ratio (*m/z*). Various mass analysers operate on different principles, however based on electromagnetic forces. Separated ions have to be detected in order to create data output (Downard 2004).

Quadruple Time-of-flight (Q-TOF) mass analyse is a type of tandem mass spectrometer *(Figure 8).* It provides information on a molecular structure of analysed compounds. Simply said, it consists of two mass spectrometers joined together with a collision cell in between. A precursor ion, selected by first MS, is fragmented in the collision cell to fragment ions. Fragment ions are then separated according to *m/z* in second mass analyser. Q-TOF mass analyser offers high scanning speed and high sensitivity in full spectra, therefore is often coupled to HPLC as a detector (Downard 2004, Lee 2014).

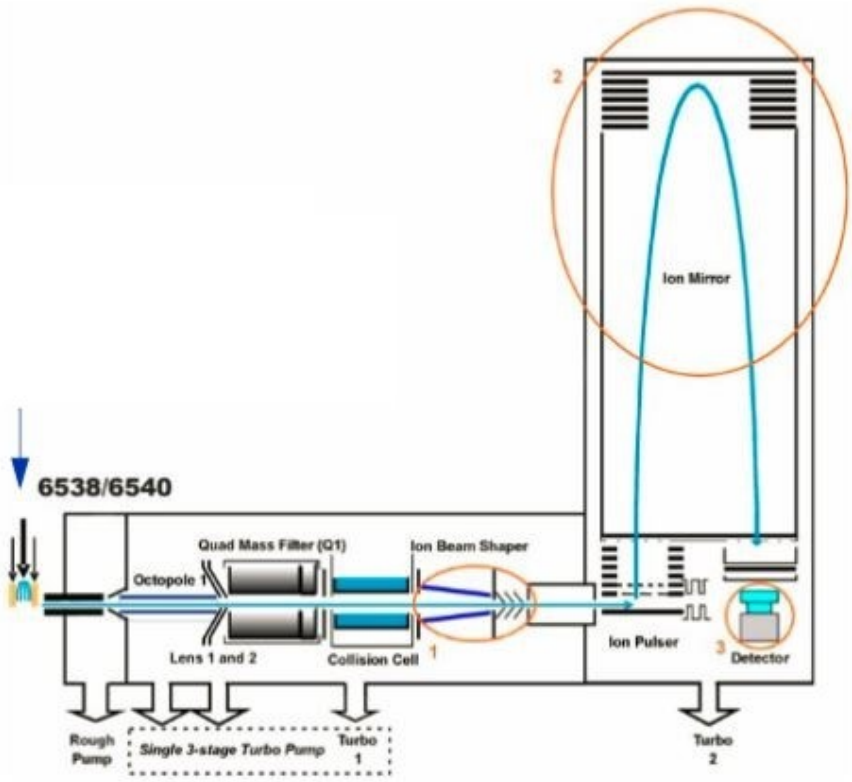


Figure 8 Illustration of Q-TOF mass analyser (Agilent Technologies 2011).

During the separation in quadruple, ions enter the space between four parallel electrodes in a shape of poles, therefore quadrupole. The trajectory of the ion depends on direct current voltage and radiofrequency applied to the electrodes. Based on the *m/z* ratio, the ions oscillate either on a stable trajectory, therefore can reach the detector and create a signal, or an unstable (collision) trajectory. Ions oscillating on collision trajectory collide with the electrodes, or exit the space between the poles, therefore do not reach the detector (Niessen 2006).

In tandem Q-TOF devices, quadrupole selects ions for a further fragmentation in the collision cell. In collision cell ions are accelerated by a hexapole and collide with nitrogen atoms resulting in a creation of fragment ions (Niessen 2006).

During the separation in TOF analyser, ions are accelerated by a pulse of high voltage and a time of the flight through a tube of a certain length is measured. The time depends on the mass and charge ratio. In order to double the travelled distance, reflecting mirrors are used resulting in a higher resolution (Downard 2004).

To create a data output from the mass analysis, a detector is needed. For time-of-flight devices, microchannel plate electron multiplier is a suitable detector as the ions from the analyser are not focused into one place. It is a thin plate with tiny channels in it. When an analysed ion hits a wall of the channel, a multiplying cascade of electrons is created. Freed electrons are focused on a scintillator which produces light that creates an electrical signal in a photomultiplier tube. The electrical signal is then processed into a chromatogram (Downard 2004).

2.2.3. Mass spectrum and its interpretation

A signal collected by the detector forms a mass spectrum. Mass spectrum is represented by a graph of relative abundance plotted against *m/z*. The signals shown in a mass spectrum are called peaks (Gross 2011).

All ion signals collected during the LC-MS analysis, plotted as ion intensity against time, form a total ion chromatogram (TIC). In other words, a TIC describes intensity of all ions detected at certain time of the analysis. While searching for an exact compound extracted ion chromatogram (EIC) can be used. It shows ion intensity of exact *m/z* or range of *m/z* plotted against time (Gross 2011).

Mass spectrometry provides information on molecular weight and afterwards on molecular formula. As ESI is a soft ionization technique, molecular ions, either $[M+H]^+$ or $[M-H]^-$ are created in positive or negative ionization mode respectively. Furthermore, adduct ions e.g. $[M+NH_4]^+$ $[M+Na]^+$ can be observed. Searching for a mass shift can be used in the studies of metabolites. For instance, mass shift of -14 suggests demethylation, mass shift of +176 indicates glucuronidation (Lee 2014).

Furthermore, tandem mass (MS/MS) spectrometry offers information on the structure of studied metabolites. Difference in *m/z* of precursor and fragment ion can be used for identifying functional groups. For instance, loss of 46 Da suggests presence of carboxylic functional group in the structure. Unfortunately, understanding the structure is often complicated by various rearrangements and possibilities of both homolytic and heterolytic bond cleavage (Lee 2014).

2.3. Coumarins and scoparone

2.3.1. Coumarins

Coumarins are secondary plant metabolites with a common structural feature of a benzopyran-2-on nucleus *(Figure 9).* They naturally appear in more than 150 plant species from more than 30 families, mainly from Apiaceae, Rutaceae and Ficaceae. Many coumarins are present in a form of glycosides. The highest levels of coumarins can be found in fruits and seeds. The function of coumarins in plants is partially unclear, however effects on growth regulation and bacteriostatic and fungistatic function have been proposed (Venugopala et al. 2013).

Similar to the structure of coumarins, other plant metabolites can be found in nature, such as isocoumarins, furanocoumarins and pyranocoumarins. Furanocoumarins are well known for their photosensitizing effects. The most common example of coumarin derivate is warfarin, a vitamin K antagonist widely used as an anticoagulant. Coumarin itself, the simplest of coumarins, has been studied for an antiedema effect both in animals and human (Hoult and Payá 1996).

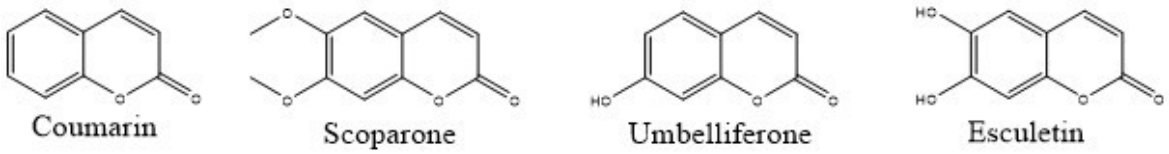


Figure 9 Structures of common coumarins

In plants, coumarins are synthetized via shikimate pathway from L-phenylalanine with a cinnamic acid and umbeliferone as the intermediate products. Umbeliferone can be further alkylated to furano and pyranocoumarins or hydroxylated or methylated to polyoxygenated coumarins. Most common polyoxygenated coumarines are esculetin, daphnegin, scopoletin and scoparone *(Figure 9)* (Kumar and Chopra 2005).

Coumarins poses various pharmaceutical activities such as anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant and neuroprotective (Hoult and Payá 1996, Venugopala et al. 2013).

2.3.2. Scoparone

Scoparone is a simple coumarin derivate systematically known as 6,7-dimethoxycoumarin *(Figure 9).*

It has attracted an immerse interest of scientists for being an active ingredient of *Artemisia capillaris* Thunb. a medicinal herb widely used in traditional Chinese medicine. It is referred to as Yin Chen or Yin Chen Hao in Chinese Pharmacopoeia. Along with *Gardenia jasminoides* and *Rheum rhabarbarum* it is a part of traditional herbal formula called Yin Chen Hao decoction. This medicine has been used for treating hepatic diseases for centuries. Both *Artemisia capillaris* and the herbal mixture have been studied for various pharmacological activities including effects on hepatic disorders, diabetes, lipid metabolism, skin inflammation, myorelaxation, fibrosis (Li et al. 2017, Hung and Kuo 2013).

Simirarly, scoparone itself was studied for various pharmacological effects such as upregulation of conjugation enzymes included in excretion of bilirubin, reduction of proinflammatory cytokines, lowering of plasma lipids levels and inhibition of platelet aggregation (Hung and Kuo 2013).

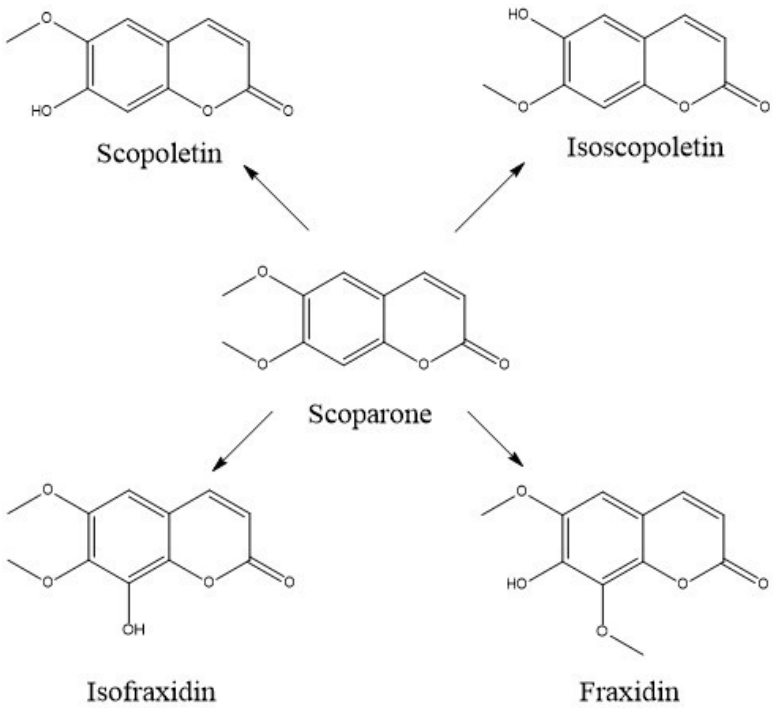


Figure 10 Scoparone metabolism in rat as proposed by Wang et al. (2007).

Furthermore, metabolism of scoparone in rat has been studied before. Scopoletin, isoscopoletin, isofraxidin and fraxidin have been identified as main phase I metabolites in rat using UPLC/ESI-QToF-MS method *(Figure 10).* Fragmentation pathways of metabolites in MS/MS have been identified (Wang et al. 2007).

Scoparone is absorbed rapidly after oral administration in rat with the most significant distribution to liver, kidney and spleen. It is not detectable in brain presumably due to presence of blood-brain barrier. Furthermore, it is rapidly eliminated from plasma, no long-term accumulation has been found (Yin et al. 2012).

As scopoletin, one of scoparone metabolites, is fluorescent, 7-O-demethylation of scoparone to scopoletin, was studied using fluorescence-based assay. When scoparone was incubated with microsomes from five mammalian species including human, differences in rate of 7-O-demethylation between species were found. The demethylation rate was significantly higher in mouse, pig and rabbit microsomes than in human and rat. Furthermore, CYP1A1 and 2A13 were identified as the most efficient CYPs in human (Fayyaz et al. 2018).

3. Aims of study

To identify *in vitro* metabolites of scoparone oxidation from hepatic microsomal incubation of six different mammal species including human.

To desribe main species differences of scoparone oxidation.

To determine scoparone and its metabolites in human urine of persons exposed to scoparone.

4. Materials and methods

4.1. Materials

4.1.1. Chemicals

All used chemicals are listed in *Table 1.*

Table 1 List of used chemicals.

Chemical	Additional information	Manufacturer
Acetonitrile	Ultra Gradient HPLC Grade	J.T. Baker
KCl	≥99% purity	J.T. Baker
$MgCl_2$	≥99% purity	J.T. Baker
Scoparone	=6,7-dimethoxycoumarin, 98% purity	Sigma-Aldrich
Scopoletin	≥99% purity	Sigma-Aldrich
Esculetin	=6,7-dihydroxycoumarin, 98% purity	Sigma-Aldrich
Isocitric acid	≥93% purity	Sigma-Aldrich
$MnCl_2$	≥99% purity	Sigma-Aldrich
Tris-HCl	reagent grade, ≥99% purity	Sigma-Aldrich
Isocitric acid dehydrogenase	from porcine heart, type IV, buffered aqueous glycerol solution, 3-20 units/mg protein	Sigma-Aldrich
NADP	98% purity	Roche Diagnostics GmbH
Isoscopoletin	95% purity	ABCR GmbH & Co. KG
Tris	ultra pure, ≥99.9 %	MP biochemical
Methanol	LC-MS Ultra CHROMASOLV tested for UHPLC-MS	Honeywell Riedel-de Haën
Formic acid	Eluent additive for LC-MS	Honeywell Fluka

4.1.2. Microsomes

Human and animal liver microsomes were obtained from the sample bank of the School of Pharmacy, University of Eastern Finland.

One human liver sample was prepared from a tissue surplus from an organ transplantation surgery at the University of Oulu Hostipital. The collection of the surplus was approved by the Ethics Committee of Medical Faculty of the University of Oulu (January 21, 1986). Furthermore, commercial human liver microsomal pools were purchased from BD Gentest, Bedford, MA, USA.

Pig liver samples were obtained from 8 months old female pigs used for practicing surgical procedures at the University of Kuopio.

Wistar rats and DBA/2N/Kuo mice were acquired from National Laboratory Animal Centre of the University of Kuopio. Liver microsomes obtained from control groups of mice and rats from the previous experiments were used in this study. Mice were given intraperitoneally 0.1 ml 0.9% saline or 0.1 ml olive oil per 10 g per day for 4 days. Rats were given 1% acetone in drinking water for 7 days or drinking water for 7 days.

Dog liver samples were obtained from beagle dogs previously used for pharmacokinetic studies. The age and weight of the animals were as following: 31 to 35 months, 7 to 14 kg. The samples were collected at F. Hoffmann-La Roche Ltd. (Nutley, NJ)

Rabbit liver microsomes were prepared from leftover tissue of control group rabbits from previous experiment.

The Ethics Committee for Animal Experiments, University of Kuopio, approved the liver samples.

4.1.3. *In vivo* experiment volunteers

Two academic researchers of the University of Eastern Finland were volunteers for *in vivo* scoparone metabolism.

4.1.4. Instrumentation and software

Agilent 1290 Infinity LC System, UHPLC (Agilent Technologies, Santa Clara, CA, USA)

Agilent 6540 Q-TOF LC-MS System (Agilent Technologies, Santa Clara, CA, USA)

USF ELGA Maxima Ultra pure water purifier

Milli-Q system – PURELAB Ultra Water Purification Systems, Model ULXXXANM2, ELGA LabWater (Millipore, USA)

Heidolph Incubator 1 000 Titramax 1 000 incubator system (Germany)

Eppendorf AG Centrifuge 5415D (Eppendorf AG, Hamburg, Germany)

Vortex – Genie 2, Bohemia N.Y. 11716 (Scientific Industries, Inc., USA)

4.2. Methods

4.2.1. Preparation of microsomes

Preparation of microsomes was done by a technician working in the School of Pharmacy, University of Eastern Finland.

The frozen liver samples were cut into 3-4 g pieces and buffer (100 mM Tris-HCL pH 7.4; 1 mM K_2-EDTA) of volume (ml) of four times the weight (g) was added to melt the samples. Melted tissue was cut into smaller pieces with scalpel. The samples were homogenized using Ultraturrax homogenizer twice for 5 seconds and afterward using Potter-Elvehjem homogenizer 10 times. The homogenate was centrifuged (15 min, JA25,50, 9092 rpm, 4°C) and the supernatant was further centrifuged (1 h, T-1250, 33 200 rpm, 4°C). The pellets were washed with 1 ml storage buffer (100 mM Tris-HCL pH 7.4; 0.1 mM EDTA; 20% glycerol). Microsomes were created from the pellets by adding the storage buffer (0.7 ml per g of liver piece) and homogenizing with Potter-Elvehjem homogenizer 10 times. The samples were stored at -80°C.

The microsomal protein levels were determined using Bradford method.

4.2.2. Incubation samples and standard solutions

The 100 µL incubation samples contained 100 mM Tris-HCl pH 7.4, 10 µM scoparone, 1 % liver microsomes and 20 % NADPH regenerating system. The samples were prepared in the Eppendorf tubes. Additional 1 µl of deionized water instead of liver microsomes was used for the blank samples. Samples were preheated at 37 °C for 10 minutes. Reaction was started by adding NADPH regenerating system. The reaction was stopped by adding 300 µl of acetonitrile after 30 minutes of incubation at 37°C. The samples were put on ice and centrifuged (10 000 rpm, 5 min, 4°C) and 300 µl of the supernatant was taken into a new eppendorf tube and stored at -80°C before LC-MS analysis.

200 ml of NADPH regenerating system contained 178.5 mg NADP, 645 mg isocitric acid, 340 mg KCl, 240 mg $MgCl_2$, 0.32g MnCl2 and 15 U isocitric acid dehydrogenase.

10 µM standard solutions were prepared by diluting of 10 mM stock solutions at the same time with incubation samples. DMSO was used for preparing a zero standard solution instead of the stock solutions. 300 µl of acetonitrile were added to 100 µl of 10 µM standard solution, the mixture was centrifuged (10 000 rpm, 5 min, 4°C). 300 µl of the supernatant was taken into a new eppendorf tube and stored at -80°C before LC-MS analysis.

Every incubation sample and standard solution was prepared in duplicate, four blank solutions were prepared.

Prior to the LC-MS analysis the samples were filtered through PALL acrodisc CR 13 mm syringe filter with 0.2 µm PTFE membrane.

The incubation samples were measured in full scan mode, positive ionization mode. One sample representing each species was selected for MS/MS analysis, collision energy of 20 V was used.

4.2.3. Urine samples

Scoparone solution for *in vivo* experiment was prepared mixing 5 mg of scoparone in 200 ml of water. Both volunteers drank the solution and the urine was collected in the tubes for the period of 24 hours. Prior to the administration of the scoparone solution, a urine sample was collected and used as a blank sample.

The urine samples were handled as following. An aliquot of 1 ml was centrifuged (14 000 rpm, 5 min, 4°C) and 100 µl of the supernatant was diluted 1:5 in methanol. 100 µl of every sample for one person was taken into a new tube creating a mixture for MS/MS analysis. The samples were filtrated through PALL acrodisc CR 13 mm syringe filter with 0.2 µm PTFE membrane.

All the urine samples were measured in full scan mode, in both positive and negative ionization mode. The mixtures of a person samples were analysed in MS/MS using 20 V and 40 V collision energy.

4.2.4. LC-MS analysis

LC-MS analysis of the samples was conducted on Agilent technologies 1290 Infinity LC system connected to 6540 UHD Accurate Mass Q-ToF.

2 µl of samples were injected on Agilent Zorbax Eclipse XDB-C18 Rapid Resolution HD 1.8 µm, 2.1x100 mm column. The temperature was set on 50 °C. 0.1 % formic acid in water and 0.1 % formic acid in methanol were used as mobile phase A and B, respectively. The flow rate was set on 0.4 ml/min. The gradient conditions were as following: 0-10 min – 98% A, 2% B; 10-14.5 min 0% A, 100 % B; 14.51 – 16.5 min 98% A, 2 % B.

Conditions of mass analysis are shown in Table 2. The data were analysed using Agilent MassHunter, Qualitative Analysis, version B.06.00

Table 2 Characteristics of MS method.

Parameter	Value
Gas Temperature (nitrogen)	325 °C
Gas Flow	10 l/min
Nebulizer Pressure	45 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 l/min
Capillary Voltage	3500 V
Nozzle Voltage	1000 V
Fragmentator Voltage	100 V

5. Results

5.1. *In vitro* experiment

5.1.1. Introduction

Scoparone was incubated with human, mouse, rat, pig, rabbit and dog liver microsomes in the presence of NADPH and incubation mixture was analysed by LC-MS. In total, scoparone and six metabolites were found in the incubation samples. Scopoletin, isoscopoletin and esculetin were identified as their retention time and fragmentation were similar with standards. MS/MS data were used to identify three unknown metabolites based on knowledge from previous studies and fragmentation patterns. Main differences in scoparone oxidation between the species were stated.

5.1.2. Identification of scoparone and its metabolites

Scoparone, scopoletin, isoscopoletin and esculetin were identified based on *m/z* and retention time obtained from prepared standard solutions *(Figure 11).* The effect of the hydroxyl groups in the structure affected retention time. Scoparone, the structure containing two methylated hydroxyl groups, was eluted last, whereas esculetin containing two hydroxyl groups was eluted firstly. Isoscopoletin and scopoletin, two isomers with different position of methoxy group in structure, were completely separated during HPLC.

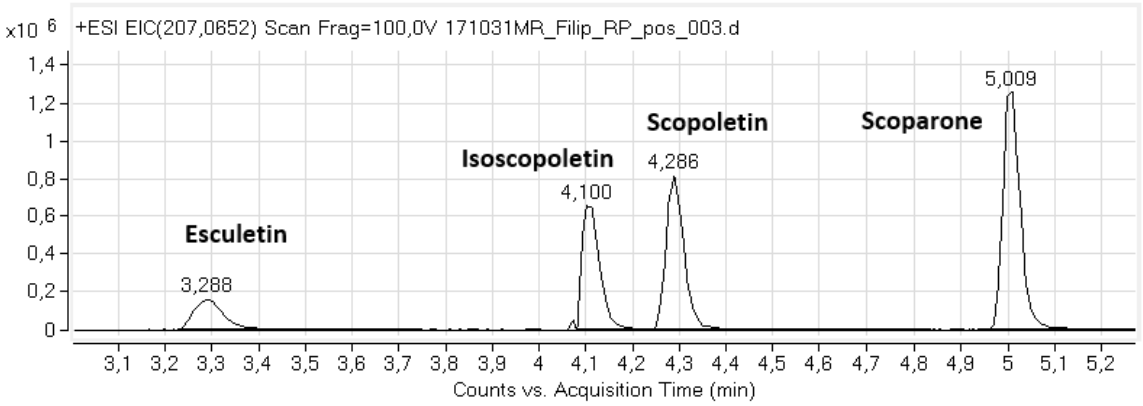


Figure 11 Elution of esculetin, isoscopoletin, scopoletin and scoparone on C18-column. 10 µM standard sample solution solutions were analysed. Four EIC combined in one chromatogram. Retention times (min) are shown above the peaks.

Fragmentation spectra of scopoletin, isoscopoletin, scoparone and esculetin differed *(Figure 12, Table 3).* Results were compared to the previously published fragmentation patterns (Wang et al. 2007). Loss of 15 Da indicating methyl group in the structure was

observed among methylated coumarins. Furthermore, a loss of 28 Da was present as coumarin structures tended to lose carbonyl group.

The same fragment ions were observed for isoscopoletin and scopoletin. Although there were differences in relative abundances between main fragment ions, isoscopoletin and scopoletin could not have been distinguished based only on fragmentation patterns in conditions of this study. Fortunately, standard solutions for isoscopoletin and scopoletin made the identification possible based on the retention times.

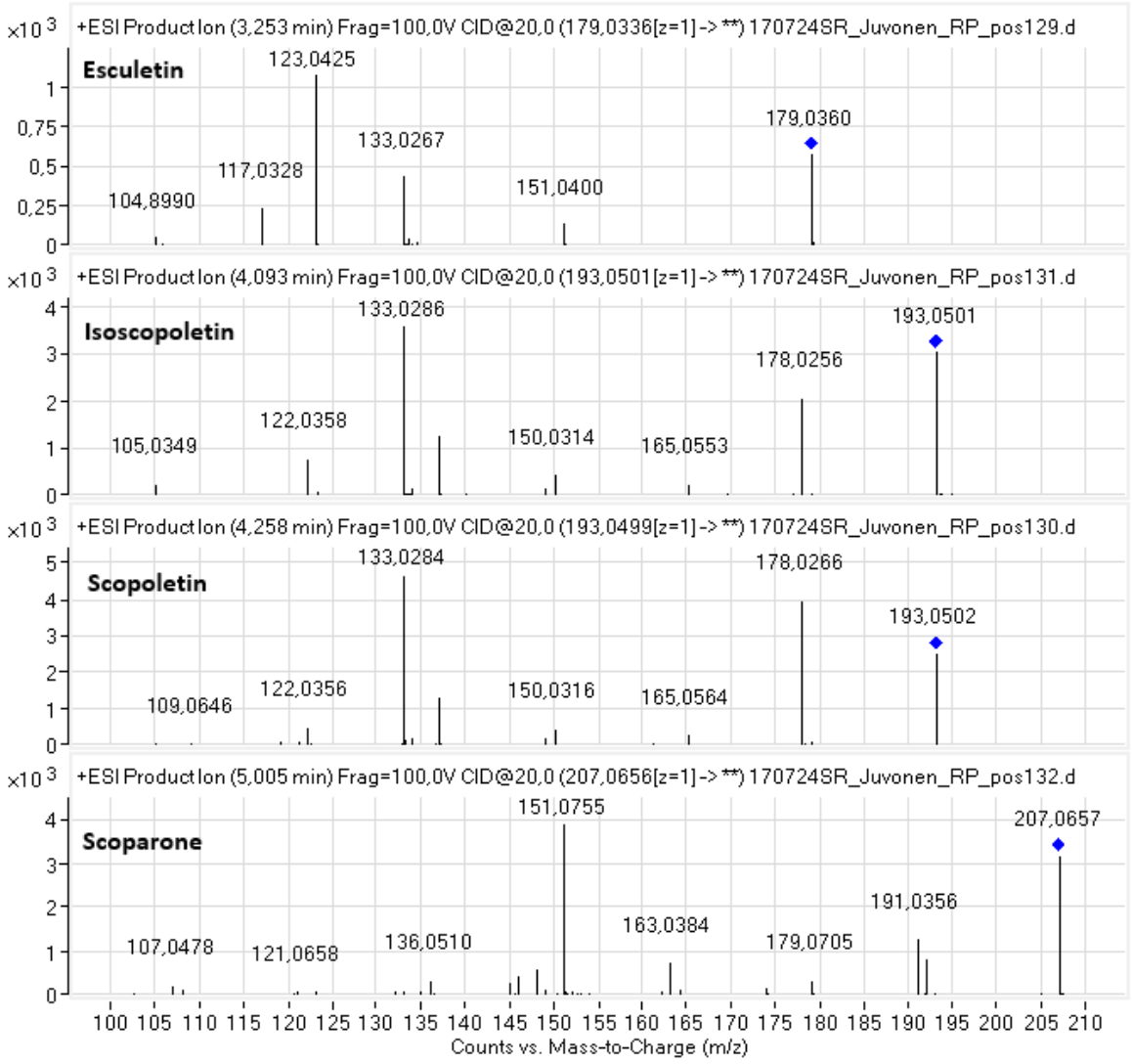


Figure 12 Fragmentation spectra of standards. Samples were measured in positive ionization mode, collision energy 20 V.

Identification of other found metabolites is described below. A typical combined EIC containing all the metbaloites is shown (*Figure 13*). Proposed structures and metabolic pathways are shown *(Figure 14).*

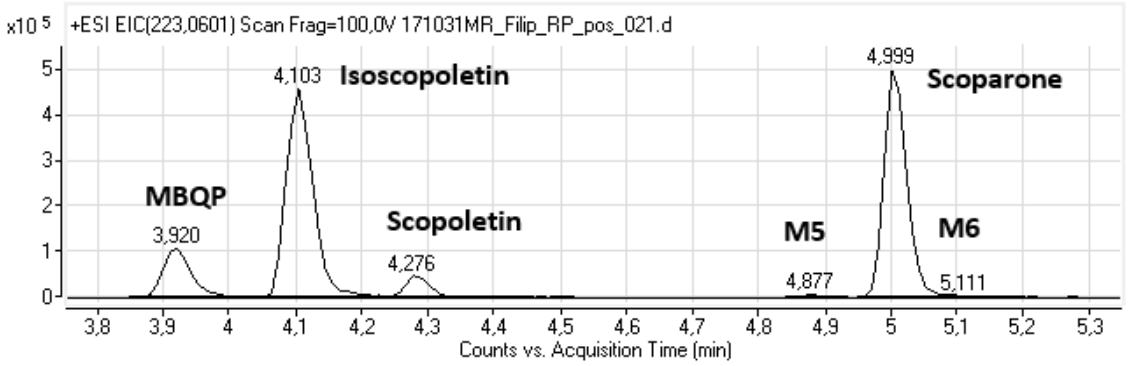


Figure 13 Elution of scoparone and five metabolites formed in pig samples on C18 column. Retention times (min) are shown above the peaks.

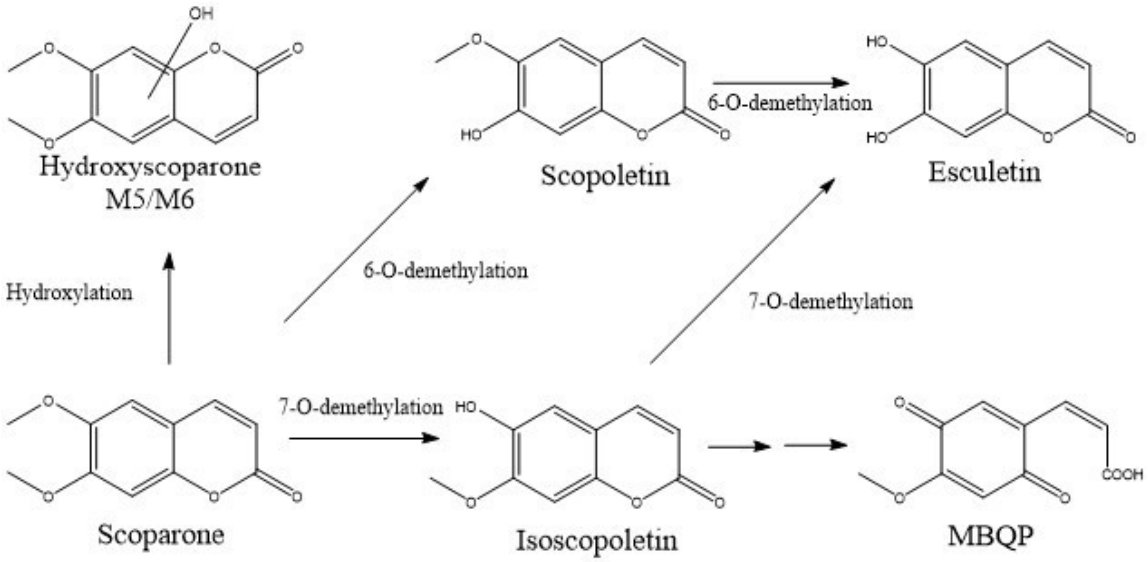


Figure 14 Summary of proposed in vitro metabolism of scoparone.

A compound of *m/z* 209.0450, t_R 3.920 min in positive ionization mode was found in the incubation samples *(Table 3, Figure 15)*. The proposed formula of $C_{10}H_8O_5$ correlates to two possible scoparone oxidation metabolites - monodemethylated hydroxyscoparone and 3-[4-methoxy-p-(3,6)-benzoquinone]-2-propenoic acid (MBQP) *(Figure 14).*

Its most abundant fragment ion *m/z 194.0220* corresponds to a loss of methyl radical (15 Da) in the MS/MS spectrum measured in positive ionization mode. In comparison to fragment spectra of scopoletin, isoscopoletin, scoparone and esculetin, further fragmentation (eg. loss of carbonyl) is not common, thus indicating a different core structure.

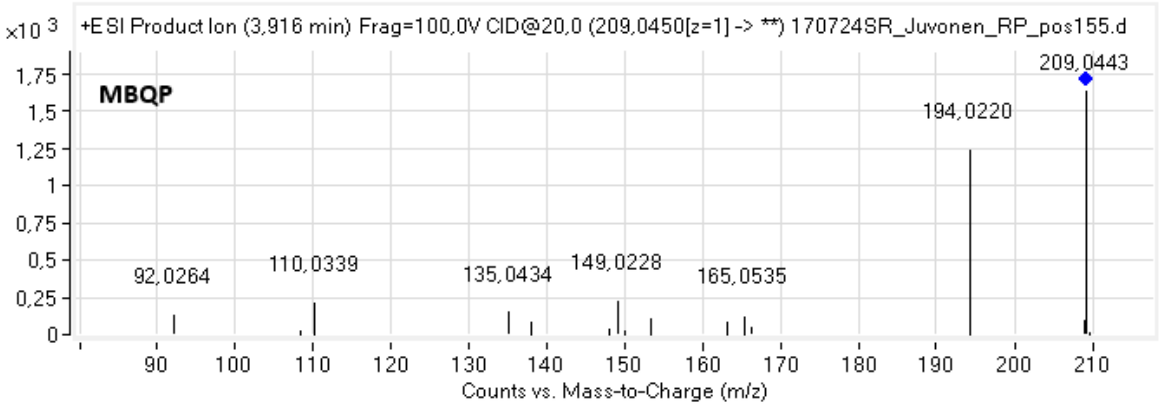


Figure 15 Fragmentation spectrum of MBQP in positive ionization mode, collision energy 20 V.

This compound was identified as MBQP, previously proposed as scoparone oxidation metabolite (Meyer et al. 2001). The different fragmentation can be explained by the effect of the carboxyl group on the stability of the structure after the loss of the methyl group.

Two compounds of *m/z* 223.0607, t_R 4.878 min and 5.110 min in positive ionization mode (M5 and M6) were found in the incubation samples *(Table 3, Figure 16)*. Their proposed mass matches with the mass of fraxidin or its isomer isofraxidin, a hydroxylated scoparone metabolites previously proposed *(Figure 10)* (Wang et al. 2007). Their HPLC peaks were close, but had different retention times. They both were found only in the pig samples suggesting two isomers. As there was no methylation possible during the incubation, isofraxidin (7-hydroxy-6,8-dimethoxycoumarin) was not possible to be formed. Therefore, one of these two metabolites can be assumed to be fraxidin (8-hydroxy-6,7-dimethoxycoumarin), whereas the other one could have the new hydroxyl substitution in position 3, 4 or 5 *(Figure 14).*

The signal of these compounds was low and therefore no fragmentation of the minor isomer M6 was detected. Because of insufficient information on structure, these isomer compounds were not fully structurally identified.

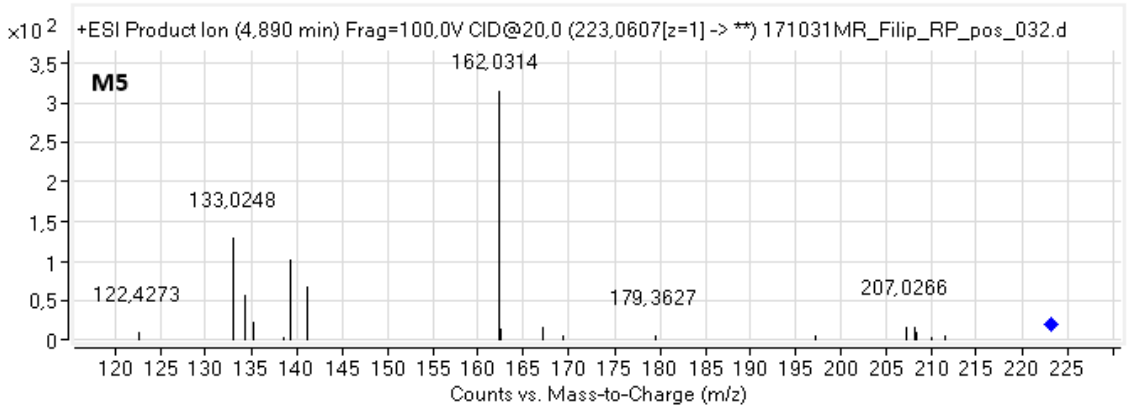


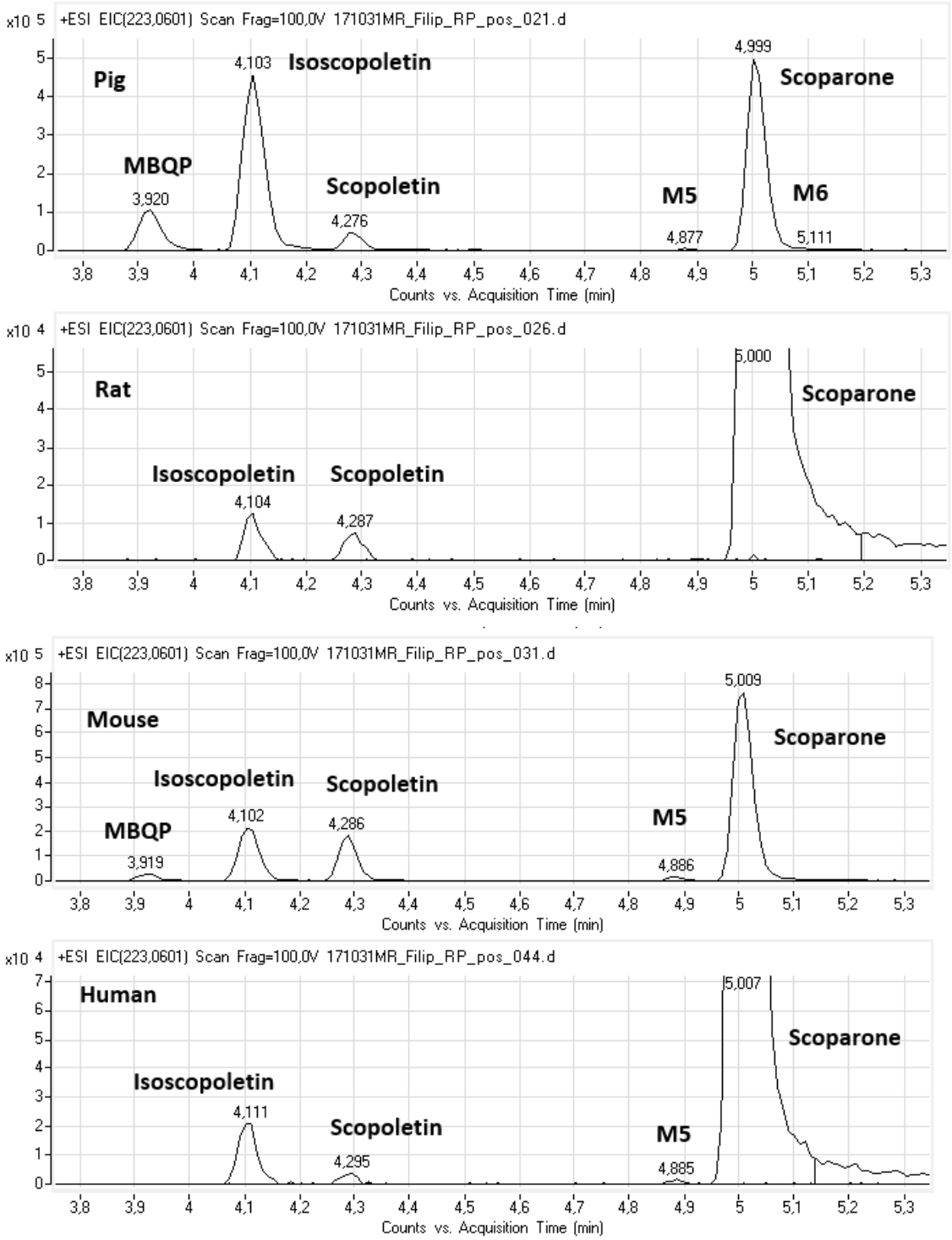
Figure 16 Fragmentation of M5 in positive ionization mode, collision energy 20 V.

Table 3 MS characteristics of scoparone and its oxidation metabolites in the incubation samples.

Name	Standard	Calculated Mass	Molecular Formula	Retention time (min)	Fragment ions		Loss from parent ion
Scoparone	Yes	206.0579	$C_{11}H_{10}O_4$	5.009	207.0657	Parent ion	
					191.0356	- CH_4 [1]	16
					163.0384	- CH_3 - CO [1]	16 + 28
					179.0705	- CO	28
					151.0755	- CO - CO	28 + 28
Isoscopoletin	Yes	192.0423	$C_{10}H_8O_4$	4.100	193.0501	Parent ion	
					178.0256	- $\cdot CH_3$ [1]	15
					150.0314	- $\cdot CH_3$ - CO [1]	15 + 28
					133.0286	- $\cdot CH_3$ - CO - OH [1]	15 + 28 +17
Scopoletin	Yes	192.0423	$C_{10}H_8O_4$	4.286	193.0502	Parent ion	
					178.0266	- $\cdot CH_3$ [1]	15
					150.0316	- $\cdot CH_3$ - CO [1]	15 + 28
					133.0284	- $\cdot CH_3$ - CO - OH [1]	15 + 28 +17
Esculetin	Yes	178.0266	$C_9H_6O_4$	3.288	179.0360	Parent ion	
					151.0400	- CO	28
					123.0425	- CO - CO	28 + 28
					133.0267	- CO - H_2O	28 + 18
MBQP	No	208.0372	$C_{10}H_8O_5$	3.911	209.0443	Parent ion	
					194.0220	- $\cdot CH_3$ [2]	15
M5	No	222.0528	$C_{11}H_{10}O_5$	5.110	223.0607	Parent ion	
					207.0266	- CH_3 [1]	16
					162.0314	- CH_3 - OH - CO [1]	16 + 17 + 28
M6	No	222.0528	$C_{11}H_{10}O_5$	4.878	No fragmentation due to low signal		

[1] Mentioned by Wang et al. (2007).
[2] Mentioned by Meyer et al. (2001).

5.1.3. Activities in different liver microsomes



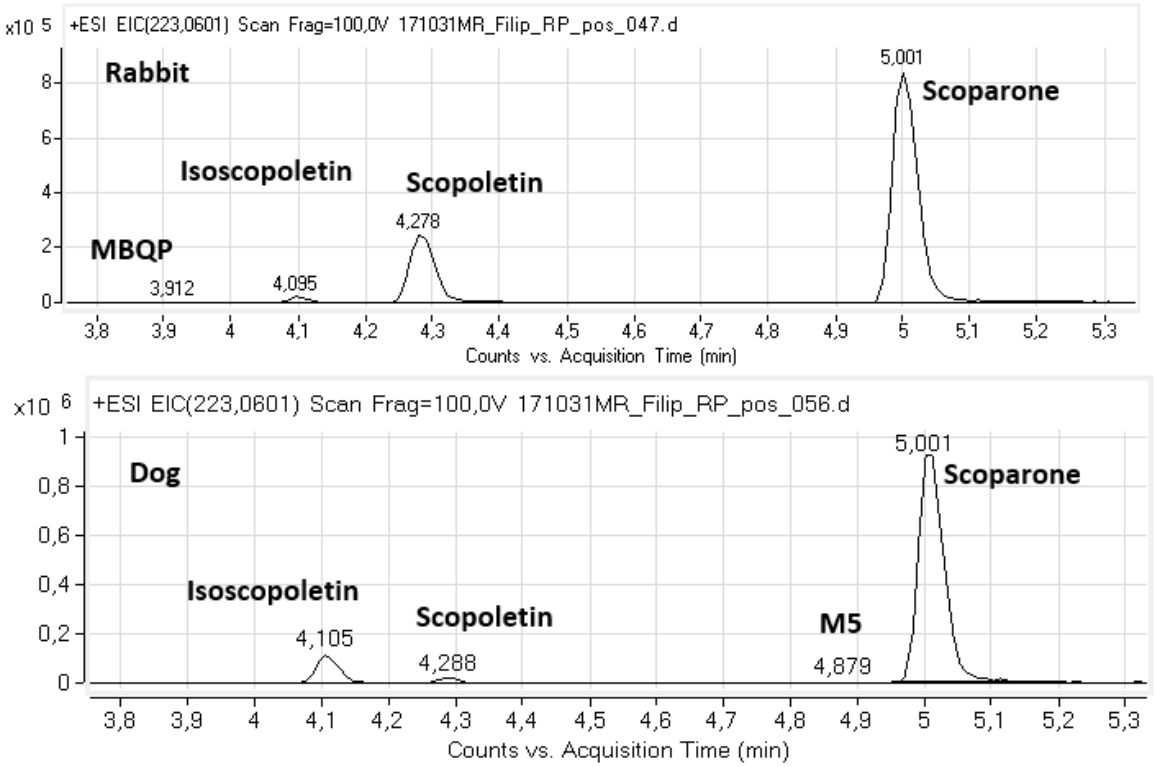


Figure 17 Elution of scoparone and its metabolites formed in samples of different species on C18 column. Retention times (min) are shown above the peaks.

Differences in the scoparone metabolism in the species can be seen in the chromatograms *(Figure 17)*. Both scopoletin and isoscopoletin were identified as major metabolites in every sample. In the pig, rat, mouse, human and dog samples, scoparone was metabolised mainly to isoscopoletin, whereas, scopoletin was a dominant metabolite in the rabbit samples.

Esculetin was found in very low amounts only in some of the pig and mouse samples. MBQP was found in the pig and mouse samples. It was also present in one of the rabbit samples, although in very low amount. M5 was found in every sample of the pig and mouse samples. It was not detected in the rat samples at all. In the human, rabbit and dog sample series, M5 was found in some samples, although in very low amounts. M6 was found only in pig samples, although in very low amounts.

As the standard solutions of scoparone, scopoletin, isoscopoletin and esculetin were measured, rate of scoparone oxidation and scopoletin, isoscopoletin and esculetin formation rate (nmol per minute per mg of protein) were calculated. The balance of formed isoscopoletin and scopoletin can be expressed as isoscopoletin/scopoletin ratio (I/S) *(Figure 18).* The difference in rates of scoparone demethylation between the species is shown in the *Figure 18.* As the primary aim of this study was the identification not quantification of the metabolites, no calibration curve of the standard was made. Therefore, these calculations do not provide enough information.

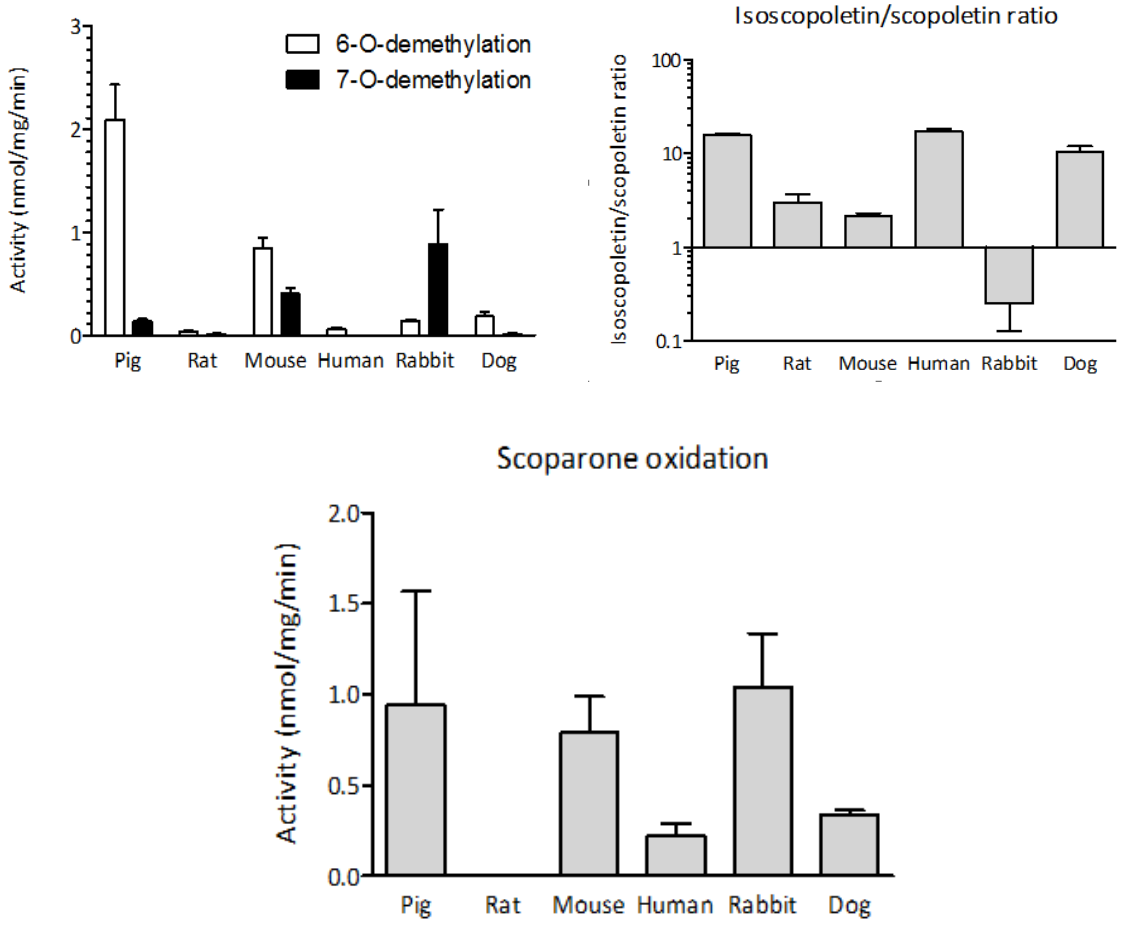


Figure 18: Oxidation rate of scoparone, its 6-O-demethylation and 7-O-demethylation in different species.
Top left: Comparison of formation of scopoletin and isoscopoletin shown in nmol/mg/min.
Top right: Comparison of formation of scopoletin and scopoletin shown as I/S
Bottom: Comparison of total rate of scoparone oxidation.

There were no standard solutions of MBQP, M5 and M6, therefore no activities could have been calculated. Furthermore, the rabbit, mouse and pig samples showed the highest rate of scoparone oxidation, whereas the rat samples showed virtually none *(Figure 18).* However, in the rat samples, scopoletin and isoscopoletin were found, although in low amounts.

5.2. *In vivo* experiment

5.2.1. Introduction

As opposed to the incubation samples, phase II metabolites were expected to be major metabolites in the urine samples. Glucuronides and sulphates were anticipated to be formed due to presence of hydroxyl groups in the structure of scoparone metabolites.

Scopoletin and isoscopoletin as observed in the human incubation samples were found also in the urine samples. Furthermore, MBQP was also detected, even though in low amounts.

Nine metabolites of scoparone were detected in urine samples of both study persons but no scoparone *(Table 4).* Scopoletin and isoscopoletin were identified based on the comparison to standard solutions. Conjugation metabolites were partially identified based on their *m/z.* Furthermore, urine samples were measured both in negative and positive ionization mode *(Figure 23).* Negative ionization mode allowed us to identify sulphate conjugates. On the other hand, scopoletin and isoscopoletin were detected only in positive ionization mode.

5.2.2. Identification of scoparone and its metabolites

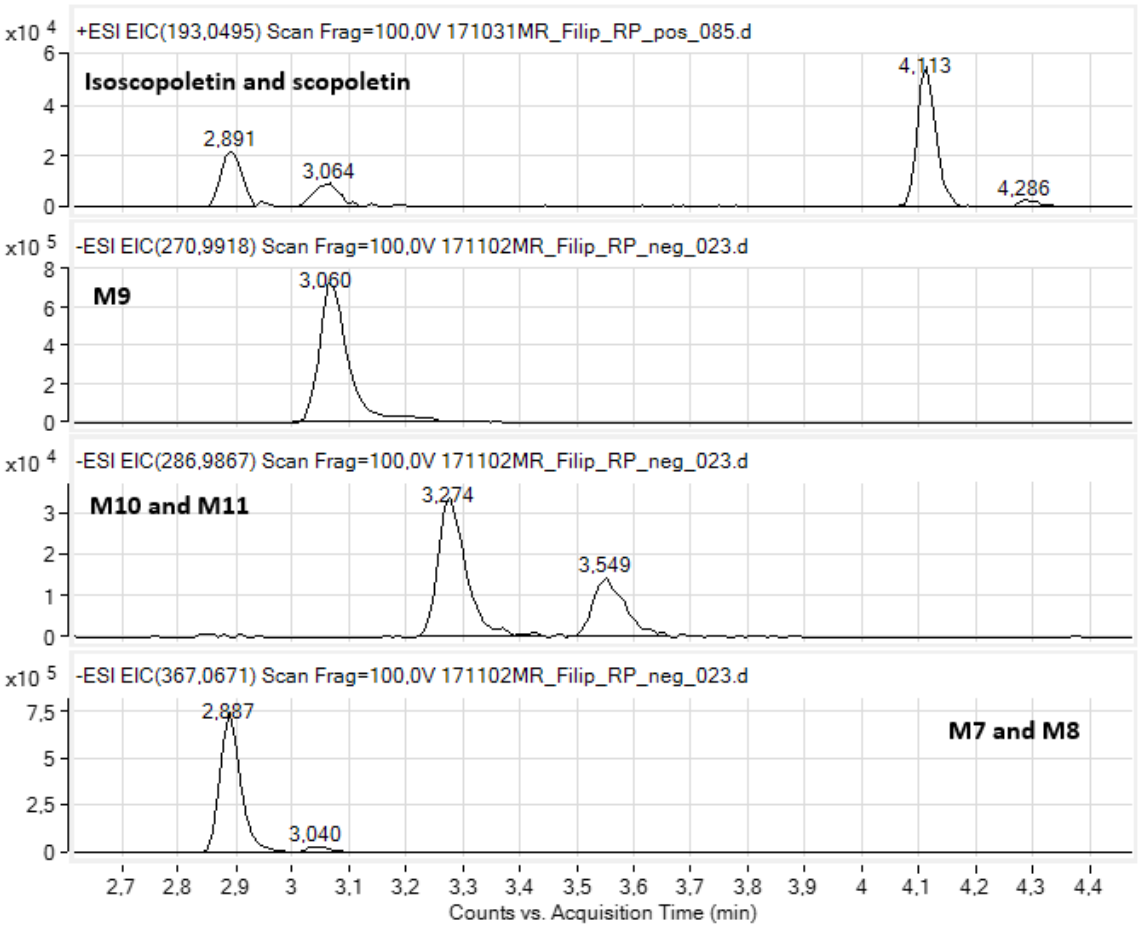


Figure 19 C18-Chromatographic elution of scoparone's metabolites of urine. EIC of selected metabolites found in the urine samples. Retention times (min) are shown shown above the peaks.

There were four peaks visible for mass of isoscopoletin and scopoletin (*Figure 19)*. Peaks in retention times 4.113 and 4.286 correlate with retention times of standard solutions of isoscopoletin and scopoletin, respectively. Peaks in retention times 2.891 and 3.064 were identified as fragments of glucuronide and sulphate conjugates, respectively. The glycosidic bond between glucuronic acid and scoparone metabolite hydroxyl group as well as the bond to sulphate group was probably broken during electrospray ionization.

In total, six conjugate metabolites were found in the urine samples *(Table 4).*

Three compounds M7, M8 and M9 were identified as isoscopoletin or scopoletin metabolites based on their mass. M9 was sulphate conjugate and M7 and M8 were glucuronides *(Figure 20)*. The sulphate conjugate M9 and the more abundant glucuronide conjugate M7 provided MS/MS spectra, proving the structure of the conjugate of monodemethylated scoparone *(Figure 21, Table 4)*. However, the fragmentation of these conjugates did not provide enough information to distinguish the isoscopoletin and the scopoletin conjugate.

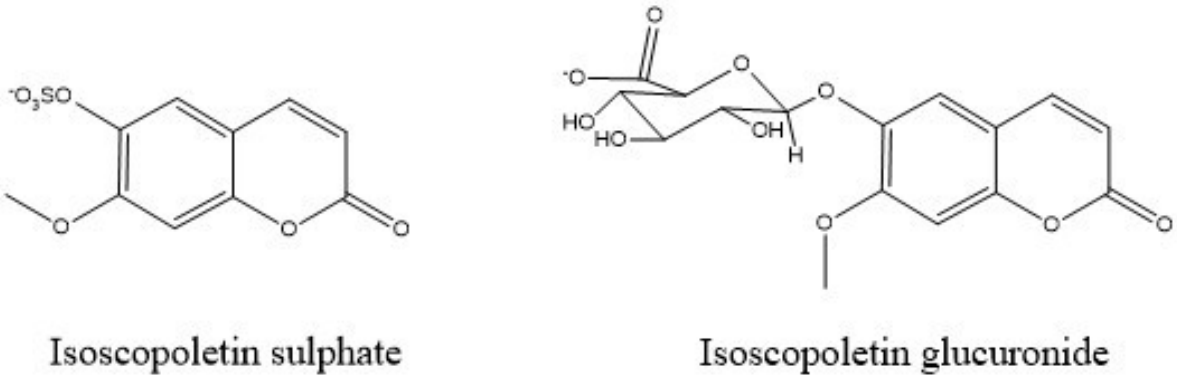


Figure 20 Structures of isoscopoletin conjugates.

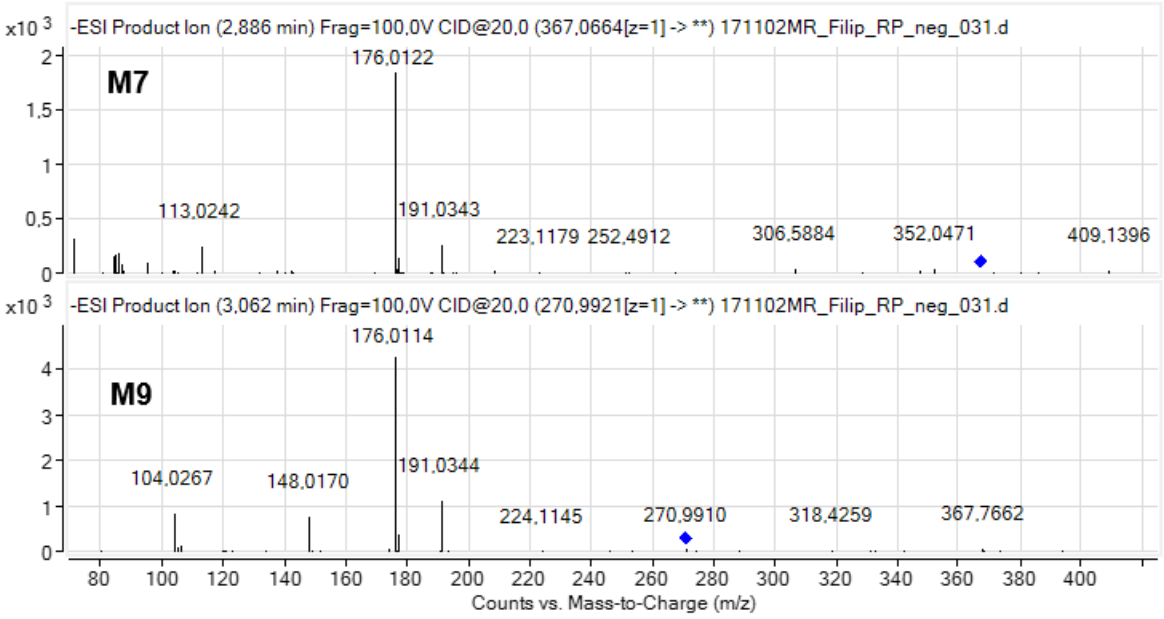


Figure 21 MS/MS spectra of the major metabolites M7 and M9 of the urine samples.

Fragmentation spectra of the major metabolies M7 and M9 in negative ionization mode, collision energy of 20 V are shown in *Figure 21.* Both compounds produce fragment ions with *m/z* corresponding to mass of isoscopoletin or scopoletin ion (191.0343, 191.0344). Furthermore, these fragment ions tend to lose 15 Da (methyl radical). Using higher collision energy of 40 V, virtually the same fragment ions were acquired.

Masses of compounds M10, M11 and M12 corresponded to monodemethylated hydroxyscoparone sulphates or glucuronide *(Figure 22).* Two separate peaks of M10 and M11 for sulphate conjugates were present indicating two isomers *(Figure 19).* Size of the peak of M12 corresponding to monodemethylated hydroxyscoaprone glucuronide indicated very low amount. As there were no MS/MS spectra due to the low signal, the structure of those compounds could not have been verified.

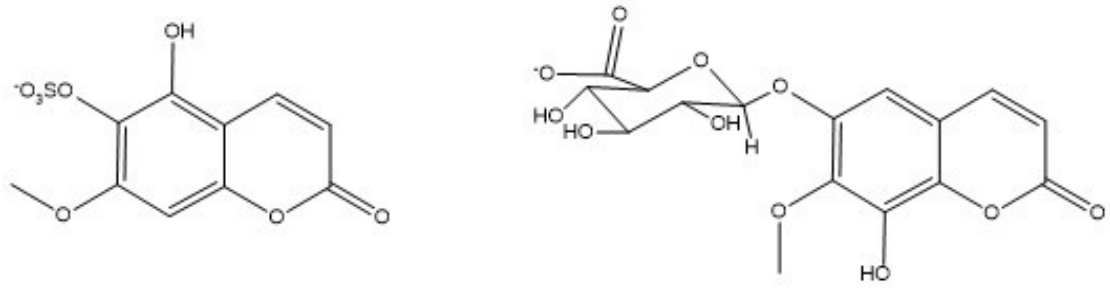


Figure 202 Possible structures of unknown conjugates M10, M11 and M12.

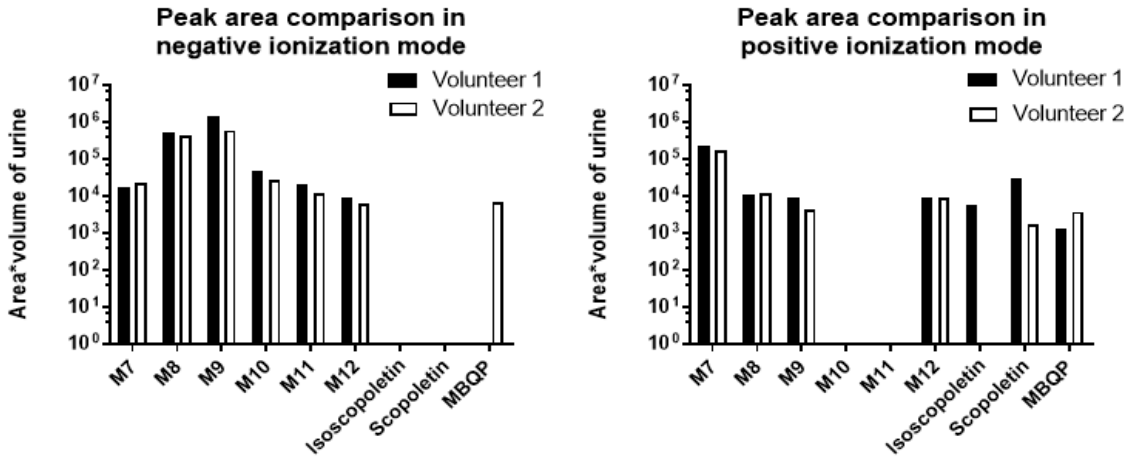


Figure 23 Semiquantitative abundance comparison of scoparone metabolites in the urine.

Table 4 Metabolites of scoparone in the urine.

Name	Molecular formula	Retention time (min)	Fragment ions in negative ionization mode	Fragment ions in positive ionization mode	Loss from parent ion
Isoscopoletin	$C_{10}H_8O_4$	4.113	No fragmentation due to low signal		
Scopoletin	$C_{10}H_8O_4$	4.286	No fragmentation due to low signal		
MBQP	$C_{10}H_8O_5$	3.928	No fragmentation due to low signal		
M7	$C_{16}H_{16}O_{10}$	2.889	191.0343		GA[1]
			176.0122		GA[1] + $\cdot CH_3$
				193.0482	GA[1]
				178.0285	GA[1] + $\cdot CH_3$
M8	$C_{16}H_{16}O_{10}$	3.042	No fragmentation due to low signal		
M9	$C_{10}H_8O_7S$	3.062	191.0344		sulphate
			176.0114		SO_3 + $\cdot CH_3$
			148.0170		SO_3 + $\cdot CH_3$ + CO
M10	$C_{10}H_8O_8S$	3.267	No fragmentation due to low signal		
M11	$C_{10}H_8O_8S$	3.552	No fragmentation due to low signal		
M12	$C_{16}H_{16}O_{11}$	3.539	No fragmentation due to low signal		

[1]Glucuronic acid

5.2.3. Time decrease pattern of scoparone metabolites

All found metabolites showed similar time dependent decrease pattern in the urine samples as the major metabolites *(Figure 24)*. The highest levels of the metabolites were in the first samples indicating rapid excretion during the first three hours after the administration. The minor metabolites were detected only in the first and second samples indicating absense after 8 - 9 hours after the administration.

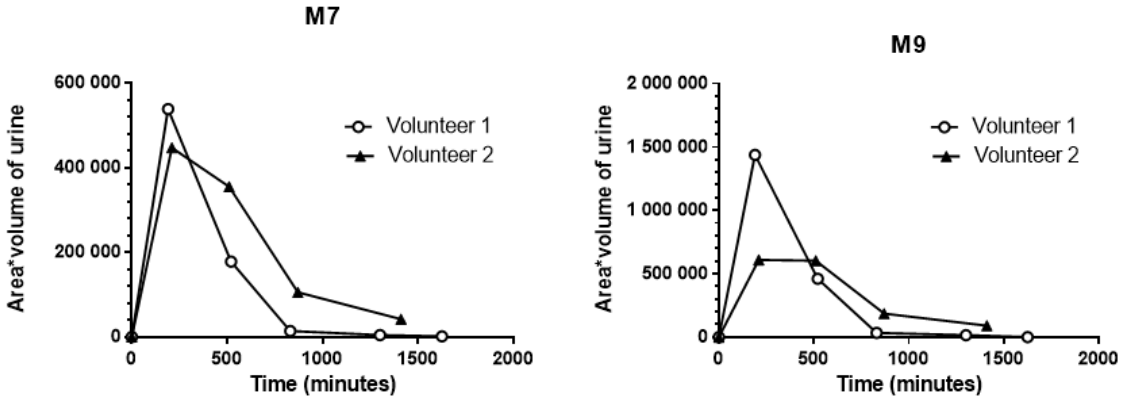


Figure 24 Excretion of M7 and M9 in the urine

As there were no standard solutions for the conjugates area per volume of the urine sample was used for quantification. However, this semiquantitave estimation of the amount of metabolites did not allow us to compare the metabolites as the ionization potency differs between them.

6. Discussion

Scoparone has attracted great interest of scientists in recent years. It is an active ingredient of *Artemisia scoparia* which is an important medicinal herb in traditional Chinese medicine. It has also shown to have various pharmacological effects (Hung and Kuo 2013). Although the pharmacokinetics of scoparone has been studied before, there is still shortage of knowledge of scoparone metabolism (Fayyaz et al. 2018, Yin et al. 2012).

This work describes a LC-MS method to study scoparone metabolism both *in vitro* and *in vivo.* Novel metabolites of scoparone were identified from urine using high resolution mass spectrometry. Described LC-MS method offers a valuable tool for further research.

In total, six metabolites were identified in the scoaprone incubation with hepatic microsomes and NADPH. Isoscopoletin was the major metabolites of *in vitro* scoparone oxidation in human, mice, rat, dog and pig and scopoletin in rabbit. Esculetin was found in low levels only in pig and mice. MBQP was also detected in the same species as proposed previously (Meyer et al. 2001). Two isomers of hydroxylated scoparone were found in the samples, one of them being described for the first time.

MBQP was identified based on accurate mass and fragmentation pattern in MS/MS and compared to fragmentation pattern proposed by Meyer et al. (2001). However, to verify the structure, a comparison to a standard would be necessary. To identify the precise structure of minor metabolites M5 and M6 (i.e. hydroxyscoparone) a further study would be needed.

Interestingly, further demethylation of isoscopoletin or scopoletin, i.e. formation of esculetin, was found to be inefficient. Esculetin was not identified as a significant metabolite in either *in vitro* or *in vivo* samples.

These results correspond to the previously published studies. Scopoletin and isoscopoletin have been known as major metabolites of scoparone oxidation for a long time. The changes of scoparone metabolism in favor of either scopoletin or isoscopoletin after introducing various enzyme inhibitors or inducers were described before and proposed as a tool for the research of impact of such inhibitors and inducers (Mennes et al. 1991, Witkamp et al. 1993).

Regarding the species differences in drug metabolism, a perfect model animal cannot be selected in general. Although, mouse liver microsomes show the most resemblance in overall profile of microsomal metabolism, specific activities of CYP isoforms vary (Turpeinen et al. 2008). In the conditions of this study and out of selected species, dog microsomes showed the highest resemblance to human microsomes. Firstly, the similarities were found in the identified metabolites, as I/S ratio was similar. Secondly, overall activity of scoparone oxidation was equal between dog and human liver microsomes. Furthermore, similar I/S ratio was observed in the pig samples, however, the overall rate of scoparone oxidation in pig samples was higher than in human.

Interestigly, the rabbit samples showed less than one I/S ratio indicating that scopoletin was the major oxidative metabolite of scoparone. Nevertheless, the overall scoparone oxidation was higher than in human samples.

Previous study using recombinant enzymes and fluorescent assay as a method of detection revealed the role of CYP isoforms in formation of scopoletin (Fayyaz et al. 2018). CYP1A1 and 2A13 were identified as the most efficient CYP catalysts in human. These enzymes are extrahepatic, mainly expressed in lungs, mammary glands and intestine. CYP1A2 showed the highest activity of human hepatic enzymes, but less than extrahepatic CYP1A1 and CYP2A13. However, this fluorescence based method is only capable to determine the formation of scopoletin. Similar incubation experiment using recombinat enzymes evaluated by LC-MS method used in this study, would reveal what CYPs are able to oxidize scoparone to isoscopoletin in human.

In total, nine metabolites were found in the urine samples. Isoscopoletin, scopoletin and MBQP were present both in the human urine and microsome incubation samples. Glucuronides and sulphates of monodemethylated scoparone were identified as the major metabolites.

Unfortunately, it was not possible to differentiate the isomers of conjugates. Regarding the fact that isoscopoletin is the major metabolite according to *in vitro* incubation samples, it is most probable that the major glucuronide and suplhate metabolites are conjugates of isoscopoletin. To confirm this statement, an incubation experiment of isoscopoletin and scopoletin in glucuronidation and sulphonation conditions could be conducted in the future. Identification based on *m/z* and retention time could differentiate the isomers.

Furthermore, three conjugates of monodemethylated hydroxyscoparone were present in the urine samples. However, these metabolites were present in so low levels

that no MS/MS data were obtained. The proposed structure of these metabolites could not have been verified due to lack of fragmentation data. Interestingly, the stucture of monodemethylated hydroxyscoparone was not found in the human *in vitro* incubation samples, suggesting it was a minor metabolite or extrahepatal formation.

The time dependant decrease pattern of scoparone metabolites in the urine corresponds to the previous findings that scoparone is eliminated rapidly from the organism (Yin et al. 2012). These results are also comparable to the elimination of coumarin, a closely related structure. Coumarin is hydroxylated at position 7 and conjugated to glucuronic acid, which is excreted into the urine (Ratio et al. 1992). However, the elimination of coumarin seems to be faster than the elimination of scoparone.

7. Conclusion

An incubation experiment and analysis of human urine was used to study scoparone metabolism *in vitro* and *in vivo*. HPLC-high resolution mass spectrometry was used to identify the metabolites of scoparone. Known metabolites of isoscopoletin and scopoletin and novel metabolites were detected. Although, it was not possible to determine structure of every metabolite, it has been proved that LC-MS analysis is a suitable method for further study of scoparone metabolism.

8. List of abbreviations

CYP	Cytochrome P450
EIC	Extracted Ion Chromatogram
ESI	Electrospray Ionization
HPLC	High Performance Liquid Chromatography
I/S	Isoscopoletin, scopoletin ratio
LC-MS	Liquid Chromatography – Mass Spectrometry
MBQP	3-[4-methoxy-p-(3,6)-benzoquinone]-2-propenoic acid
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	Mass to Charge Ratio
Q-ToF	Quadruple Time of Flight
SULT	Sulfotransferase
TIC	Total ion chromatogram
t_R	Retention time
UGT	uridine 5'-diphospho-glucuronosyltransferases

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10. Contents