Aus dem Department für
der Veterinärmedizinischen Universität Wien

Technologie Plattform VetCore

Arbeitsgruppe Proteomics

Comparison of different protein extraction methods from *Matricaria chamomilla*

Bachelorarbeit

Veterinärmedizinische Universität

vorgelegt von

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Wien, im Juli 2021

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1. Abstract

Chamomile (*Matricaria chamomilla*) is an important and frequently used medicinal plant in Traditional European Medicine (TEM). Its pharmaceutical value has been proven through years of scientific research. It has been indicated that *M. chamomilla* possesses many health benefits, including analgesic, anti-allergic, antispasmodic, antibacterial, anti-inflammatory and sedative properties. Additionally, attention for research work has been paid to the flavonoid compounds in the extracts. Especially flavones and flavonols have been found to be spasmolytic and anti-phlogistic as well as to have antioxidant activities. Even though chamomile is a widespread used medicinal plant and its flavones are being investigated, there is actually no information on proteins expressed in the plant or their interactions with flavonoids.

The aim of this study was to find an appropriate protein extraction method for *Matricaria chamomilla* flower tissue. In order to determine, whether the protein extraction method was successful, the protein extracts were analysed by SDS-PAGE.

Pharmaceutical-grade dried chamomile blossoms were ground into fine powder in a mortar by adding liquid nitrogen. Pre-prepared dry extract was tested along the fresh material. Different extraction strategies were applied including addition of distilled water (25°C), water (100°C), 50 % methanol:water, 100 % methanol, urea and SDS buffer to the ground chamomile powder. After filtration, the protein concentration of extracts was measured. Since the concentration of extracted protein was extremely low, various precipitation methods were applied in order to enrich the proteins. In a first approach trichloroacetic acid and acetone solution (20 %) were added directly to ground chamomile powder. In a second method, SDS solution (12 %) was used in combination with trichloroacetic acid and acetone. The third method used a methanol/chloroform precipitation. Additionally, ultrafiltration was performed in order to enhance protein concentration in solution and to eliminate the interfering yellow colour. Finally, the samples were air dried, solved in lysis buffer and analysed by SDS-PAGE and silver staining. The direct precipitation with 20% trichloroacetic acid and acetone has been shown to be the most effective method for extraction of proteins. The SDS-PAGE gels showed the clearest bands, less smearing along the lanes and good reproducibility. However, further experiments are needed to improve the direct precipitation method.

2. Zusammenfassung

Die Kamille (*Matricaria chamomilla*) ist eine sehr wichtige und häufig verwendete Heilpflanze in der Traditionellen Europäischen Medizin, deren pharmazeutischer Wert als Heilpflanze durch Jahre an wissenschaftlicher Forschung bestätigt wurde. *M. chamomilla* werden viele gesundheitliche Nutzen zu geschrieben, besonders schmerzlindernde, anti-allergische, krampflösende, antibakterielle, entzündungslindernde und beruhigende Eigenschaften. Der Fokus der wissenschaftlichen Arbeit über *M.chamomilla* in den letzten Jahren lag besonders bei der Erforschung der enthaltenen Flavonoide und deren Glykoside. Diesen werden auch die vorherig erwähnten pharmazeutischen Wirkungen zugesprochen. Obwohl die Kamille eine weltweit verbreitete Pflanze ist und deren Flavonoide intensiv untersucht werden, gibt es keine Informationen über die Proteine oder über Interaktion von Proteinen und Flavonoid in der Kamille.

Das Ziel dieser Studie war es eine geeignete Methode zur Proteinextraktion aus *M. chamomilla* zu finden. Um zu bewerten, ob die Extraktionsmethoden erfolgreich waren, wurden die Extrakte mittels SDS-PAGE analysiert.

Die, in einer Apotheke erhaltenen, getrockneten Kamillenblüten wurden in einen feinen Puder mithilfe von einem Mörser und Stößel unter Zugabe von flüssigem Stickstoff gerieben. Kommerziell erhaltbarer Kamilletrockenextrakt wurde ebenfalls untersucht. Die unterschiedlichen Extraktionsmethoden wurden durch Zugabe von Wasser (25 °C), Wasser (100 °C), Methanol (50 %), Methanol (100 %), Urea (8 M) und SDS (0,4 %) durchgeführt. Nach der Filtration wurden die Proteinkonzentrationen gemessen. Aufgrund zu geringer Konzentration, wurden mehrere Präzipitationsmethoden getestet, um die Proteine anzureichern. Die erste Methode beinhaltete das direkte Hinzufügen von einer 20%igen TCA/Acetone-Lösung zu geriebenem Kamillenblütenpulver. Die zweite Methode war eine leichte Modifikation der ersten, bei der Kamillenblütenpulver vor der 20% TCA/Acetone Fällung mit SDS behandelt wurde. Bei der dritten Methode wurde eine Methanol/Chloroform Präzipitation angewendet. Eine weitere Methode basierend auf Ultrafiltration, wurde verwendet um die Proteinkonzentration zu erhöhen und gleichzeitig Flavonoide zu trennen. Die Proben wurden nach der Fällung in Lysispuffer gelöst und mit SDS-PAGE aufgetrennt und mittels Silberfärbung sichtbar gemacht. Dabei hat sich gezeigt, dass die TCA/Acetone Präzipitation am

besten geeignet war für die Proteinextraktion aus Kamillenblüten. Die SDS-PAGE Gele dieser Methode haben die klarsten Banden sowie den geringsten Schmiereffekt gezeigt und die Methode hatte eine gute Reproduzierbarkeit. Es sind jedoch noch weitere Experimente und Verbesserung notwendig, um Proteine aus Kamillenblüten genauer zu untersuchen.

3. Introduction and Hypothesis

3.1. Chamomile as a medicinal plant

Chamomile recutita, also known as Common Chamomile, German Chamomile or True Chamomile, is an important medicinal plant and one of the most frequently consumed tea plants (Ruzicka et al., 2021). The species is native to northern and central Europe with especially abundant crops in Eastern Europe. It is cultivated in many countries in western Asia, the Mediterranean region of northern Africa, and the United States of America(WHO, 2010). According to a market analysis from the *Fachagentur Nachwachsender Rohstoffe Deutschland*, Chamomile was the second most produced herbal medicine in Germany in the year 2011, standing at 4500 tons (Fachagentur Nachwachsender Rohstoffe Deutschland, 2014).

Matricaria chamomilla is well known for its pharmaceutical properties including; anti-inflammatory, arcaricadal property, anti-cancer activity, antipruritic effect, immunomodulatory activity, treatment of oral mucositis, intracanal irrigant, treatment of infant botulism, wound healing property, treatment of gastrointestinal disorders, antimicrobial activity, antiulcer activity, treatment of stress and, anti-allergic activity, antisolar agent, inhibition of poliovirus replication, anxiolytic agent, preventing of osteoporosis(Shakeri et al., 2012). The Committee on Herbal Medicinal Products concludes in a report on *Matricaria chamomilla*, that herbal preparations, like tea, essential oils and liquid extracts of *Matricariae flos*, can be used for relieving the symptoms of common cold, treating the symptoms of minor gastro-intestinal complaints such as bloating and minor spasms, treating minor ulcers and inflammations of the mouth and throat, treating minor inflammation of the skin and superficial wounds and small boils, as add-on treatment of irritation of the skin and areas around the anus and genitals (Neubeck, 2014). The use of herbal preparations of *Matricariae flos* is based on their 'traditional use' in these conditions. This means that, even though there is no evidence from clinical trials, the effectiveness of these herbal treatments is plausible and there is evidence that they have been used safely in this way for at least 30 years (Neubeck, 2014).

These health benefits of *M.chamomilla L.* are associated with several groups of active components, including phenolic compounds (Petronilho et al., 2012). In chamomile flowers

over 120 constituents have been identified. The main constituents of the oil include the terpenoids α-bisabolol and its oxides (≤ 78 %) and azulenes, including chamazulene (1-15 %). In chamomile extract, eleven bioactive phenolic compounds are found, mainly the flavonoids apigenin, quercetin, patuletin, luteolin and their glucosides. (Vikas Gupta et al., 2010) A considerable amount of epidemiologic evidence has shown that flavonoid-rich diets are associated with a lower incidence of chronic diseases, such as cardiovascular diseases (CVDs), type II diabetes, neurodegenerative diseases, and possibly cancers. (Lu et al., 2013)

3.2. Flavonoid and Protein Interaction

Recently there has been more attention on research on flavonoid pathways in certain plants like cotton, *Arabidopsis* and *Phyllanthus acuminatus*. This topic is particularly relevant for strategies aimed at engineering of flavonoid content in crops, pastures and bioenergy plants. Flavones have taken on very specific functions in controlling plant development through their action in cell wall synthesis. An important aspect of flavonoid biochemistry is their structural diversity, which allows an interaction with a variety of different biomolecules for example proteins. These interactions are directly responsible for their effects on human health, which have been discussed in the chapter above (Mathesius, 2018). Interaction between flavonoids and proteins in plants was specifically documented in the auxin-pathway, which plays an important role in plant growth. Flavonoids have been shown to act as a negative regulator of auxin efflux by binding with auxin transporter proteins, like plasma membrane 1-naphthylphthalmic (NPA) binding protein (NBP) (Pourcel & Grotewold, 2009). Furthermore, flavonoid oxidation produces semiquinones and quinones, which are highly reactive species that can react with phenolic compounds or proteins. These oxidative products may reinforce the testa structure by crosslinking with proteins and carbohydrates of the cell wall (Pourcel & Grotewold, 2009). All this research leaves little doubt of the vast amount of interaction with proteins, that flavonoids are capable of in plants as well as in humans. However this topic has been investigated in a variety of other plants, notably in *Arabidopsis* but not in *Matricaria chamomilla*. (Pourcel & Grotewold, 2009).

3.3. Protein extraction of plant tissue

There is a vast amount of investigations focused on chamomile and its phenolic compounds, however only little to no research regarding the protein content of chamomile plant tissue. A literature search on the topic of chamomile proteins was conducted and no published studies were found. This might be due to the fact, that protein extraction of plant tissue goes along with many challenges like adequate tissue disruption, inhibitory secondary metabolites and their removal and protein solubilization. (Wang et al., 2008) Plant cells consist of outermost walls which are made of a complex assembly of polysaccharides (hemi-cellulose, cellulose) and other compounds like lignin and are difficult to disrupt. A standard plant tissue disruption method is pulverizing materials in mortar and pestle with liquid nitrogen. This practice can also help to minimize proteolysis and other kinds of protein degradation occurring during tissue disruption. Using finely ground tissue powder as starting materials has become a common practice in sample preparation of plant proteomics (Wang et al., 2008). Beside the complex cell wall, plant cells have relatively low protein content, and are also rich in proteases as well as oxidative enzymes (Sheoran et al., 2009). Additionally, it has been demonstrated that the protein content can significantly differ within individual fruits and batches of one single cultivar. This phenomenon called 'phenotypic plasticity' represents the adaptation of individual plants to environmental changes (Marzban et al., 2008). Furthermore, while plant tissue has relative low protein concentrations, it is often rich in compounds that strongly interfere with downstream protein analysis, including cell wall and storage polysaccharides, lipids, phenolic compounds and a broad array of secondary metabolites (Saravanan & Rose, 2004). The common interfering compounds include terpenes, pigments, organic acids, proteolytic and oxidative enzymes, ions and nucleic acids. In particular, plant phenolics include approximately 8000 types of naturally occurring compounds, such as phenols, flavonoids, polyphenols, tannins, and lignins (Wu et al., 2014). An ideal extraction protocol would reproducibly capture and solubilize the full complement of proteins in a given sample, whilst minimizing post-extraction artifacts and non-proteinaceous contaminants (Rose et al., 2004). While a number of protocols have been developed to improve extraction of plant proteins, most proteomic studies of entire plant tissues use a basic strategy of protein precipitation with TCA and acetone followed by resolubilization in an IEF buffer containing chaotropes and detergents. This approach increases the protein

concentration and helps to remove contaminants, although some polymeric contaminants are often coextracted (Saravanan & Rose, 2004). TCA/acetone precipitation and phenol extraction followed by methanol precipitation are the most frequently used techniques for total protein extraction in plant proteomics (Wu et al., 2014). The TCA/acetone precipitation method allows the efficient extraction of total proteins for a large variety of plant tissues, especially young tissues. Since it is less time consuming and easier to perform than the phenol-based protocols, TCA/acetone precipitation is recommended as an appropriate protocol for plant proteomic analyses (Wu et al., 2014).

3.4. Hypothesis

In previous experiments, where chamomile extracts have been analysed by SDS-PAGE, the observation was made that the yellow colour caused by flavonoids migrated together with the proteins. This led to the question whether this yellow colour could be eliminated by separation of flavonoids and proteins. Therefore, the aim of the study was to compare four different methods of protein extraction of chamomile flower tissue. The methods were assessed based on the amount and quality of extracted proteins and the ability to remove secondary metabolites.

4. Materials and Methods

4.1. List of Buffer Receipts in Alphabetical Order

Lysis buffer	**Molarity**
Urea (Carl Roth, Karlsruhe Germany)	7 M
Thiourea (Sigma Aldrich, St.Louis USA)	2 M
CHAPS (Carl Roth, Karlsruhe Germany)	4 %
DTT (Carl Roth, Karlsruhe Germany)	1 %
TRIS pufferan (Carl Roth, Karlsruhe Germany) pH = 8,0	30 mM
Sample loading buffer	**Molarity**
TRIS pufferan (Carl Roth, Karlsruhe Germany) pH = 6.8	0.5 M
Glycerol (Carl Roth, Karlsruhe Germany)	25 %
SDS (Carl Roth, Karlsruhe Germany)	10 %
mQ H_2O	
Bromophenol blue (Sigma Aldrich, St.Louis USA)	
SDS-PAGE electrophoresis buffer	**Molarity**
TRIS pufferan (Carl Roth, Karlsruhe Germany)	25 mM
Glycine (Carl Roth, Karlsruhe Germany)	192 mM
SDS (Carl Roth, Karlsruhe Germany)	0.1 %
SDS buffer	**Molarity**
TRIS pufferan (Carl Roth, Karlsruhe Germany)	0.5 M
SDS (Carl Roth, Karlsruhe Germany)	0.4 %
HCl (Honeywell Fluka, New Jersey USA)	

Table 1: List of buffers used for sample analysis

4.2. List of Chemicals used

Chemicals	**Company**
Trichloroacetic acid	Sigma Aldrich, St.Louis, USA
Acetone	Fisher Scientific, New Hampshire, USA
Methanol	Honeywell Fluka, New Jersey USA
Urea	Carl Roth, Karlsruhe Germany
Ethanol	Fisher Chemical, Massachusetts, USA
Acetic acid	VWR Chemicals, Pennsylvania, USA
Coomassie Quick Stain	SERVA Electrophoresis, Heidelberg, Germany
Pierce 660nm assay reagent	Thermo Scientific, Massachusetts, USA
Acrylamide/Bisacrylamide (37.5:1)	Carl Roth, Karlsruhe Germany
TEMED	Merck, Darmstadt Germany
Ammonium persulfate	Carl Roth, Karlsruhe Germany
Sodium Thiosulfate	Carl Roth, Karlsruhe Germany
Silbernitrate	Carl Roth, Karlsruhe Germany
Sodiumcarbonate	Carl Roth, Karlsruhe Germany
Formaldehyde	Sigma Aldrich, St. Louis USA

Table 2: List of chemicals used for sample analysis

4.3. Extraction and Analysis of Flavonoids and Proteins

The first aim was to compare six different methods to find out the appropriate method for extracting different flavonoids. The chamomile flowers of *Matricaria chamomilla* (Ch.Nr.: 024/0620) was bought at Donaufelder Apotheke, located in 1210 Vienna, Austria. Parallelly a spray-dried powder of *Matricaria chamomilla* (unkown origin) was analysed to find out if any flavonoids are left in the sample. The dried pharmaceutical-grade plant material were ground into a fine powder and divided into six different parts with 0.2 g chamomile powder each. Different extraction solutions were used: water (100 °C), water (25 °C), methanol 50 %, methanol 100 %, urea (8 M) and SDS buffer (0.4 %). In Erlenmeyer flasks 20 mL of the different extraction solutions were added to 0.2 g chamomile powder. These mixtures were put in a shaking water bath at 25°C for one hour at 250 rpm. Afterwards the mixtures were poured into 50 mL centrifuge tubes and centrifuged for 10 min at 9000 rpm. The supernatant was aliquoted for further analysis. The pellets were resuspended with the same solutions and put in the shaking water bath with the same conditions for a second extraction to gather any flavonoids that did not dissolve during the first time. Once more, the mixtures were poured into 50 mL centrifuge tubes and centrifuged for 10 min at 9000 rpm. The supernatant was aliquoted again and the pellet was discarded. The same process was done for the spray-dried chamomile powder except that the mixtures were centrifuged for only 5 min instead of 10 min. The supernatant of both extractions was aliquoted and stored at -20 °C for further testing. The samples were analysed with high performance liquid chromatography.

4.4. High Performance Liquid Chromatography

For the analysis of flavonoids in the chamomile extracts, a high-performance liquid chromatography system consisted of components listed in **Table 3**. The programme and the gradient settings are described in **Table 4** and **Table 5**.

Component	Model
Controller	SCL – 40
Pump	LC20 AC
Degasser	DGU – 20A 5R
Autosampler	SIL – 20 AXR
Detector	SPD – M 20A, Dioden-Array
Oven	CTO – 20 AC
Software	Lab Solution, V 5.97
Column	Waters, X-Bridge Shield RP18, 3.5µm, 4.8x150 mm

Table 3: Components of HPLC system used for flavonoid analysis

Parameter	Setting
Flow	0.5 ml/min
Column oven	35 °C
Injection volume	20 µl
Wavelength	340nm

Table 4: Conditions of HPLC system used for flavonoid analysis

Time	A= Acetonitrile	B= 2% Acetic Acid in Water
2 min	99 %	1 %
80 min	30 %	70 %
83 min	95 %	5 %
90 min	95 %	5 %
91 min	99 %	1 %
95 min	99 %	1 %

Table 5: Gradient settings of HPLC system used for flavonoid analysis

4.5. Protein Purification from Plant Extracts

The stored pellets from the extraction and analysis of flavonoids detailed in chapter 4.3, were used for protein extraction. The samples, which derived from methanol (50 %) and methanol (100 %) extraction, were again washed with 20 ml 100 % methanol to remove the yellow colour from the sample. The mixture was vortexed for 4 min and left for one hour at room temperature. Afterwards the sample was centrifuged at 3000 g for 10 min. The supernatant was discarded. This washing procedure was repeated for three times until the yellow colour from the supernatant was removed. The pellet was solved in 10 ml SDS and stored at 4 °C.

4.6. TCA/Acetone Precipitation as "Classical Method"

One of the used methods for the comparison of different extraction methods was TCA/acetone protein precipitation. The ground chamomile flower was mixed with TCA/acetone (20 % TCA) and stored overnight as seen on **Figure 1**. After centrifugation, the pellet was washed repeatedly with cold acetone to remove the trichloroacetic acid. The pH of the supernatant was measured with pH paper (Macherey-Nagel GmbH, Düren Germany). The pellet was air dried, and 2.5 ml lysis buffer was added to the pellet to solve the extracted proteins and left for at least one hour at 4 °C.

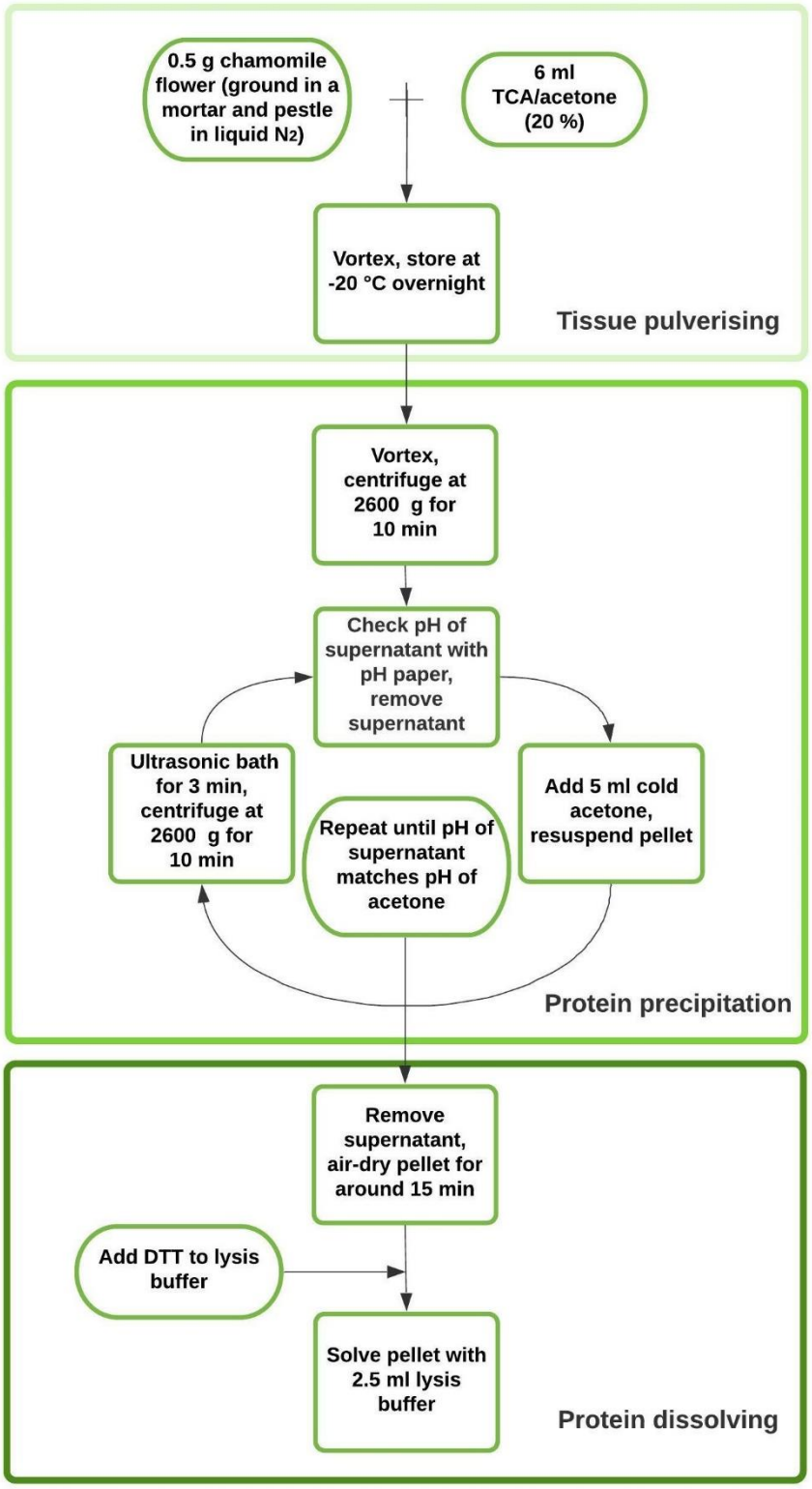

Figure 1: *Flowchart of TCA/acetone "classical" method*

4.7. Modification of the TCA/Acetone Precipitation

The second method was based on TCA/acetone precipitation with minor changes to the "classical method". The dried chamomile flower tissue was ground and 3 ml SDS buffer was added. After the mixture was homogenized, it was transferred into two 1,5 ml Eppendorf tubes. The tubes were centrifuged and 20 % TCA / Acetone (1:1) was added to the collected supernatant to precipitate the proteins. The mixture was left for five minutes and then centrifuged again. The supernatant was discarded, and the pellet was washed with acetone until the trichloroacetic acid was removed. The pellet was air dried and solved in 200 µl lysis buffer.

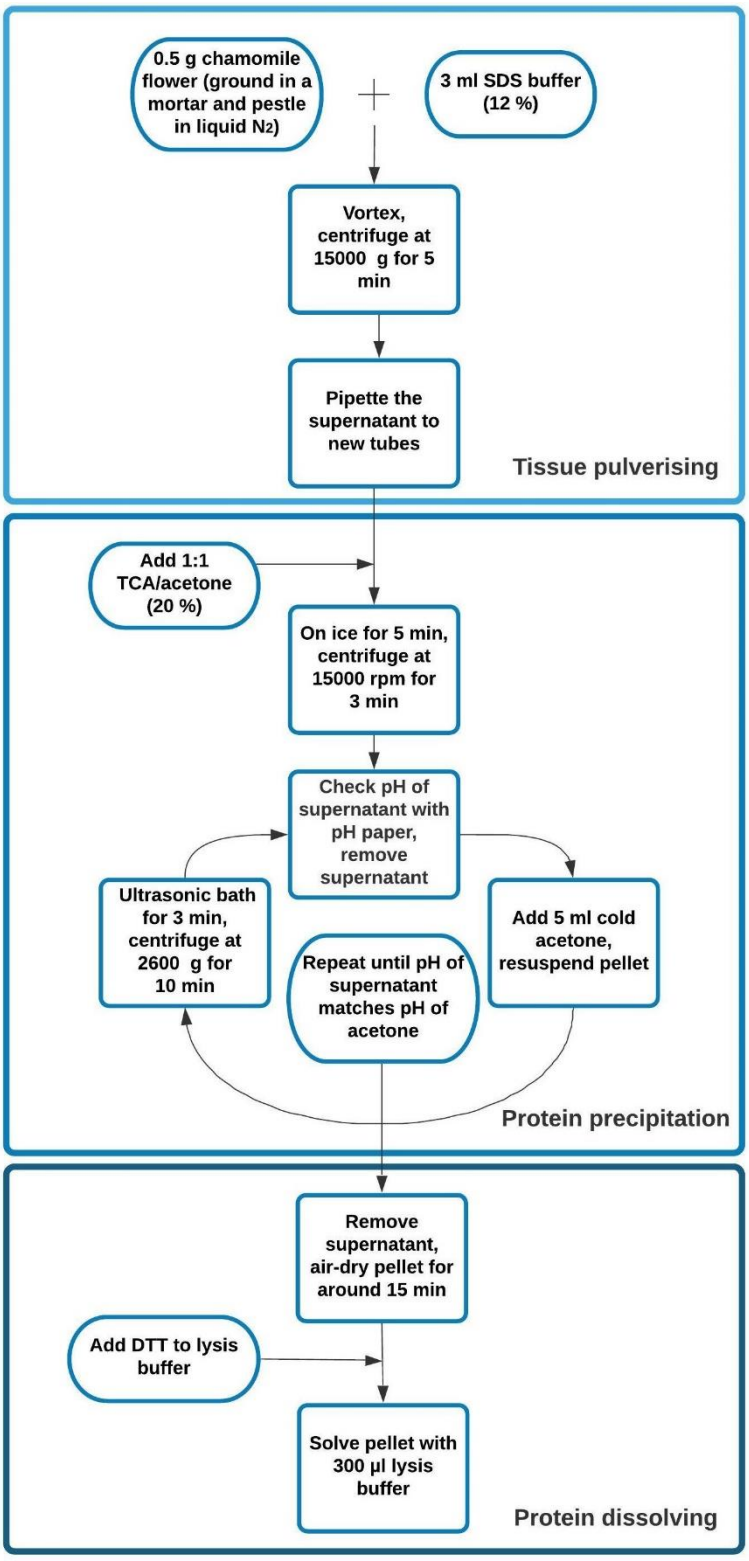

Figure 2: *Flowchart of TCA/acetone "modified" method*

4.8. Methanol/Chloroform Method

For the methanol/chloroform precipitation method, chamomile powder was ground and 200 µl of lysis buffer with protease inhibitor (cOmplete Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics Mannheim Germany) was added. This mixture was separated into four different 1.5 ml Eppendorf tubes and then centrifuged. The supernatant was collected and pooled into two 1.5 ml Eppendorf tubes with 155 µl supernatant each. Afterwards methanol (100 %) and chloroform were added and mixed by vortexing. After that, Milli-Q water was added, and the sample was again mixed by vortexing. The mixture was centrifuged, and the upper aqueous layer was carefully discarded. To sediment the protein layer, methanol (100 %) was added, and the sample was centrifuged again. The supernatant was carefully discarded, and the protein pellet was air-dried and solved in 200 µl lysis buffer.

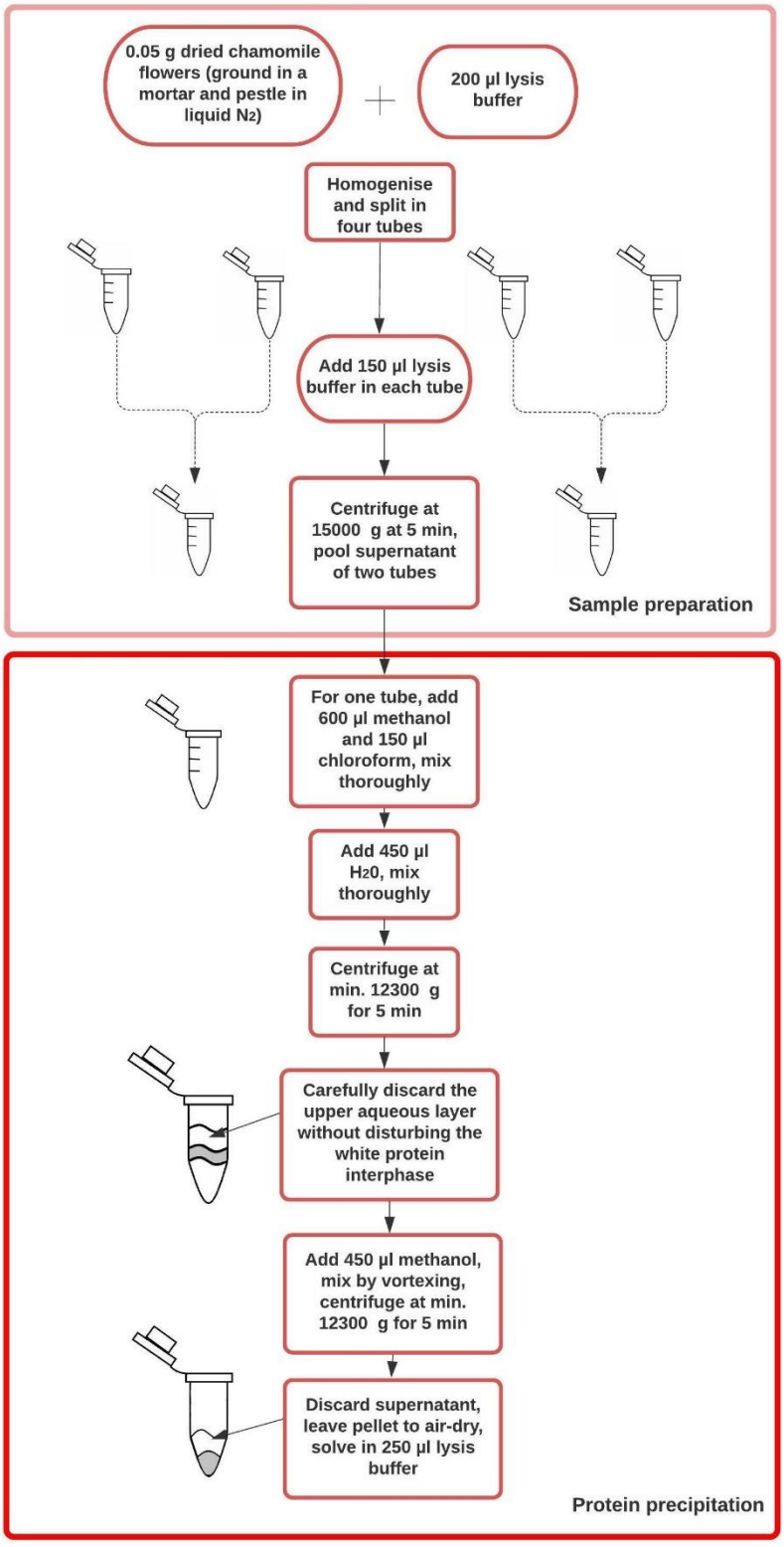

Figure 3: *Flowchart of methanol/chloroform method*

4.9. Ultrafiltration

For the ultrafiltration method, an Amicon® Ultra-15 3K centrifugal filter device (Merck KGaA, Darmstadt, Germany) was used to increase protein concentration. Lysis buffer was added to dried chamomile flowers and stored overnight before centrifugation as seen in **Figure 4** and **Figure 5**. The supernatant was collected. For further protein extraction from the pellet, this procedure was repeated twice. To remove the remaining unsolved plant material, the combined supernatant was centrifuged before being added on top of the membrane of the ultrafiltration centrifugal filter device. After each of the three following centrifugation steps, lysis buffer was added (5, 10, 10 ml) in order to wash the sample. The centrifugation was performed until the retentate was reduced to 500 µl. The liquid phase of the retentate was carefully transferred to an Eppendorf tube. The unsolved chamomile tissue that remained on the membrane was diluted in 2.5 ml 2x sample loading buffer.

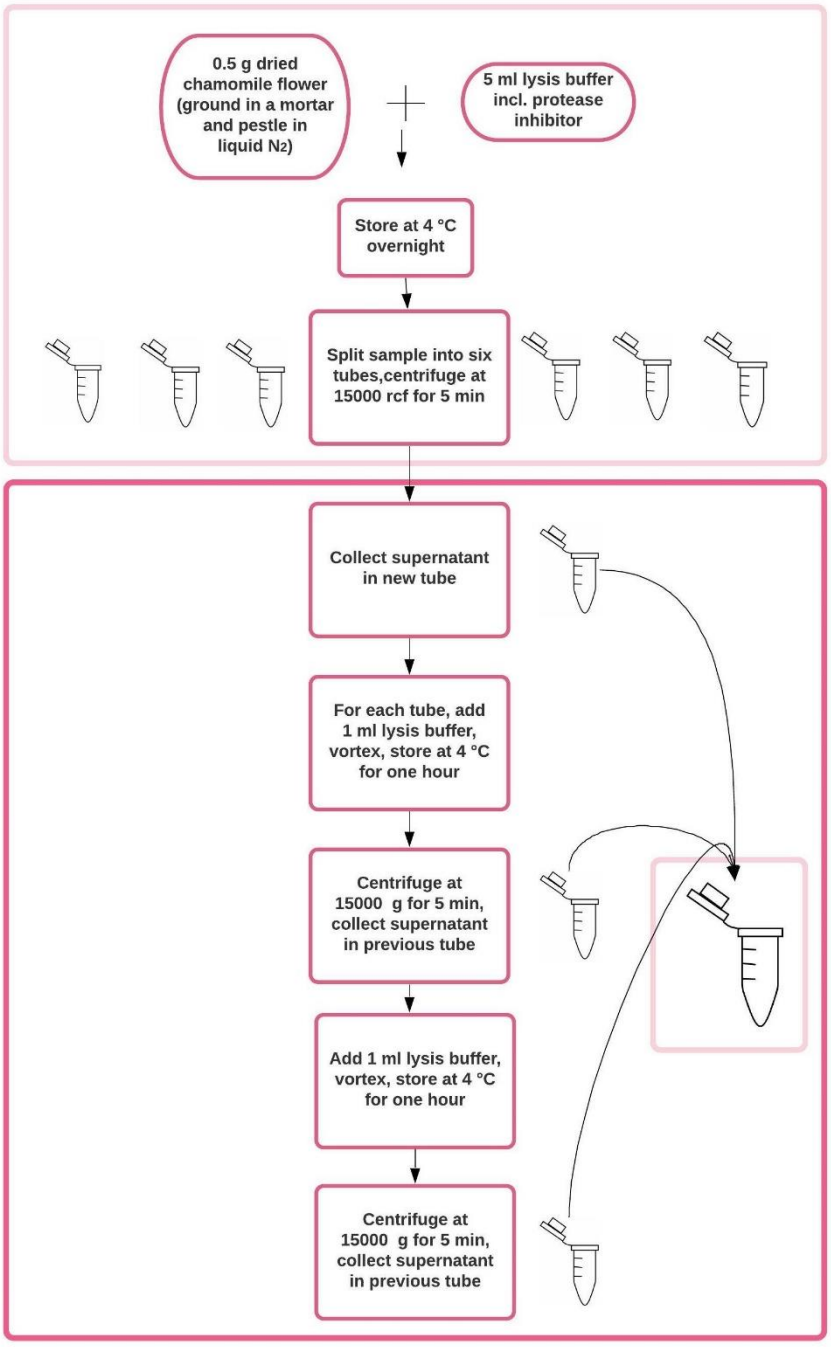

Figure 4: *Flowchart of ultrafiltration method – part 1*

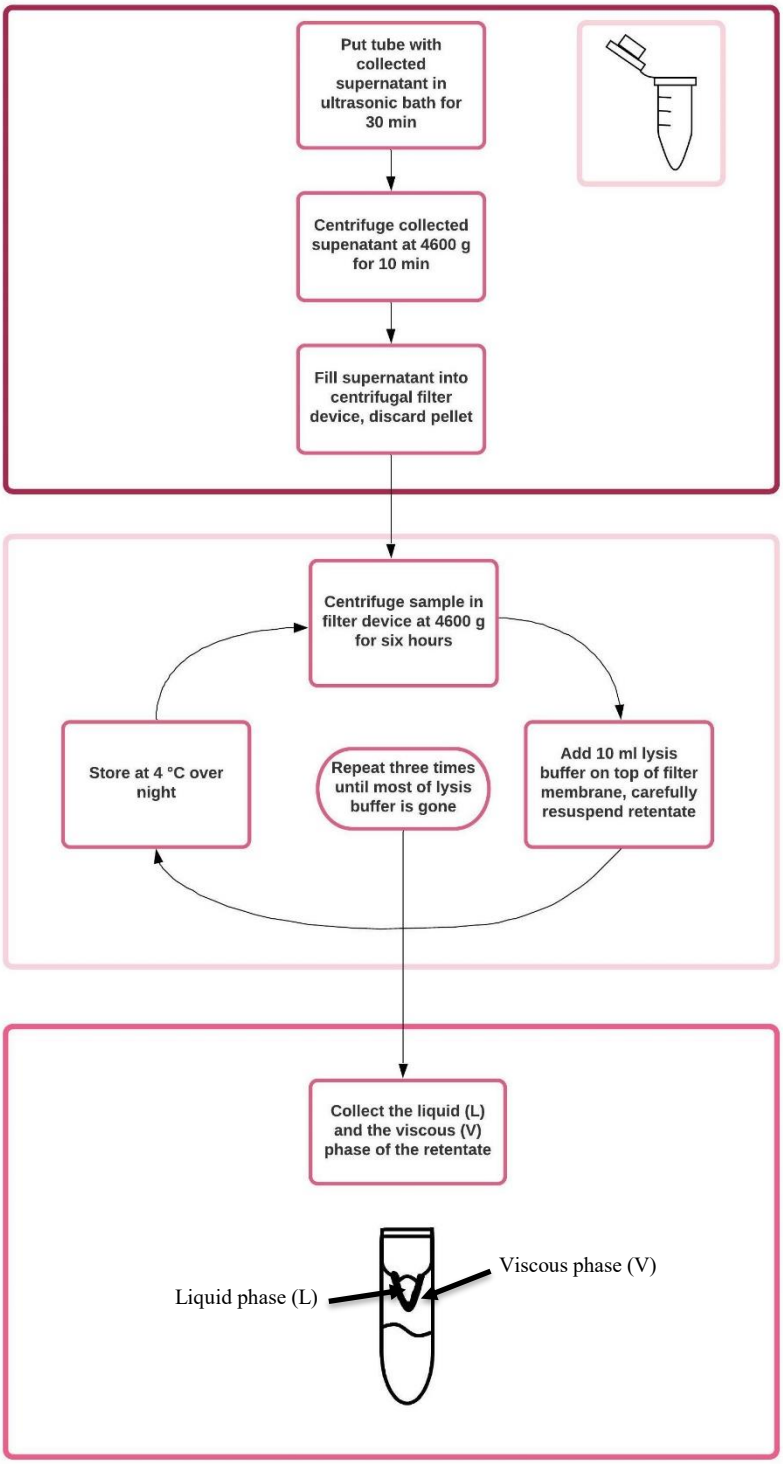

Figure 5:*Flowchart of ultrafiltration method – part 2*

4.10. SDS-PAGE

The efficacy of the extraction methods was investigated by SDS-PAGE. The gels were hand cast with the running gel poured first and the stacking gel poured after. The composition of the running gel is shown in **Table 6**.

Component	Volume
1,5 M Tris-HCl pH = 8,8	2.5 ml
10 % SDS	0.1 ml
30 % Acrylamide Bisacrylamide	4 ml
mQ H_20	3.3 ml
TEMED	4 µl
10 % APS	0.1 ml

Table 6: Composition of running gel, SDS-PAGE

After the glass plates were pinned adequality and checked for leakage, the separating gel was poured and left to polymerize for around 15 minutes. Meanwhile the stacking gel was prepared using the protocol shown in **Table 7**.

Component	Volume
0,5 M Tris-HCl pH = 6,8	0.63 ml
10 % SDS	0.05 ml
30 % Acrylamide Bisacrylamide	0.83 ml
mQ H_20	3.4 ml
TEMED	5 µl
10 % APS	0.05 ml

Table 7: Composition of stacking gel, SDS-PAGE

Immediately after, a 10 well comb was inserted into the 1.5 mm wide gel. Before loading the samples, 20 µl of sample was mixed 1:2 with 5x sample loading buffer unless stated otherwise. To compare molecular weight the protein standard SERVA Dual Color Protein Standard III (SERVA, Heidelberg Germany) was used. The polymerized gels were placed into the Mini-

PROTEAN Tetra Cell (BIO-RAD, USA) and filled with SDS-PAGE electrophoresis buffer. The SDS-PAGE was run for approximately 20 minutes at 100 V and then for an hour at 150 V until the visible sample front reached the end of the gel.

4.11. Silver Staining

After the SDS-PAGE, the gel was stained with silver staining. The first step was incubating it in fixing solution (40 % ethanol, 10 % acetic acid, 50 % Milli-Q H_20) for a minimum of 60 min. Then the gel was washed with Milli-Q H_20 three times for 20 min with a change of Milli-Q H_20 each time. Afterwards, it was then placed in freshly made sensitizer solution (0.02 % sodium thiosulfate) for 1 min and washed again with Milli-Q H_20 two times for 20 seconds each. The gel was rinsed with a silver solution (0.2 % silver nitrate, 0.02 % of 37 % formaldehyde) once and then incubated in the dark for 30 min. After it was rinsed with Milli-Q H_20 again two times for 20 seconds each, the gel was then placed in developer solution (6 % sodium carbonate, 0.1 % of 37 % formaldehyde, 0.0005 % (a spatula tip) of sodium thiosulfate) and incubated until protein bands were visible. After around three to ten minutes, the staining process was stopped using stop solution. For the stop solution 5 % acetic acid or 10 % glycine solution were used. The gel was then stored in Milli-Q H_20 at 4 °C.

5. Results

5.1. HPLC Data: Analysis of Flavonoids

The initial purpose of this thesis was to discover whether flavonoids and proteins bind to each other. Therefore, the first step was to find out which solution extracts contains the most flavonoids from dried chamomile flowers. The process of extraction is described in chapter 4.3. **Figure 6** and **Figure *7*** show the HPLC chromatograms of the six different extraction solutions. Regarding the number and the intensity of the peaks, the extraction solutions urea (8 M) and SDS (0.4 %) performed best in comparison to the other extraction solutions. Additionally, the extraction with water (25° C) showed a significantly smaller amount of phenolic acids than the infusion with 100° C water. The extraction with methanol (50 %) shows a higher variety of flavonoid and phenolic acid derivates and a higher intensity for each peak than the extraction with methanol (100 %) with the exception of a chlorogenic acid derivate at the 88 min mark of retention time. The extraction with methanol (100 %) exhibits the least amount of variety of flavonoids and phenolic acids.

In addition, differences between dried chamomile flowers and commercially available spray-dried chamomile powder were examined, particularly for the extraction solutions urea (8 M) and SDS (0.4 %). The results are presented in **Figure *8***. For the extraction solution urea (8 M), the chromatogram showed a notable difference between the dried chamomile flowers and the spray-dried chamomile powder. The spray-dried chamomile powder exhibited a larger number of flavonoids and a higher intensity for each peak. For the extraction solution SDS (0.4 %), the differences are more obvious. Not only are additional peaks present, but they also vary in intensity. Comparing all six extraction methods, the spray-dried powder extracts showed a higher diversity of flavonoids, as well as a higher concentration for each flavonoid compared to the dried chamomile flower extracts (data not shown).

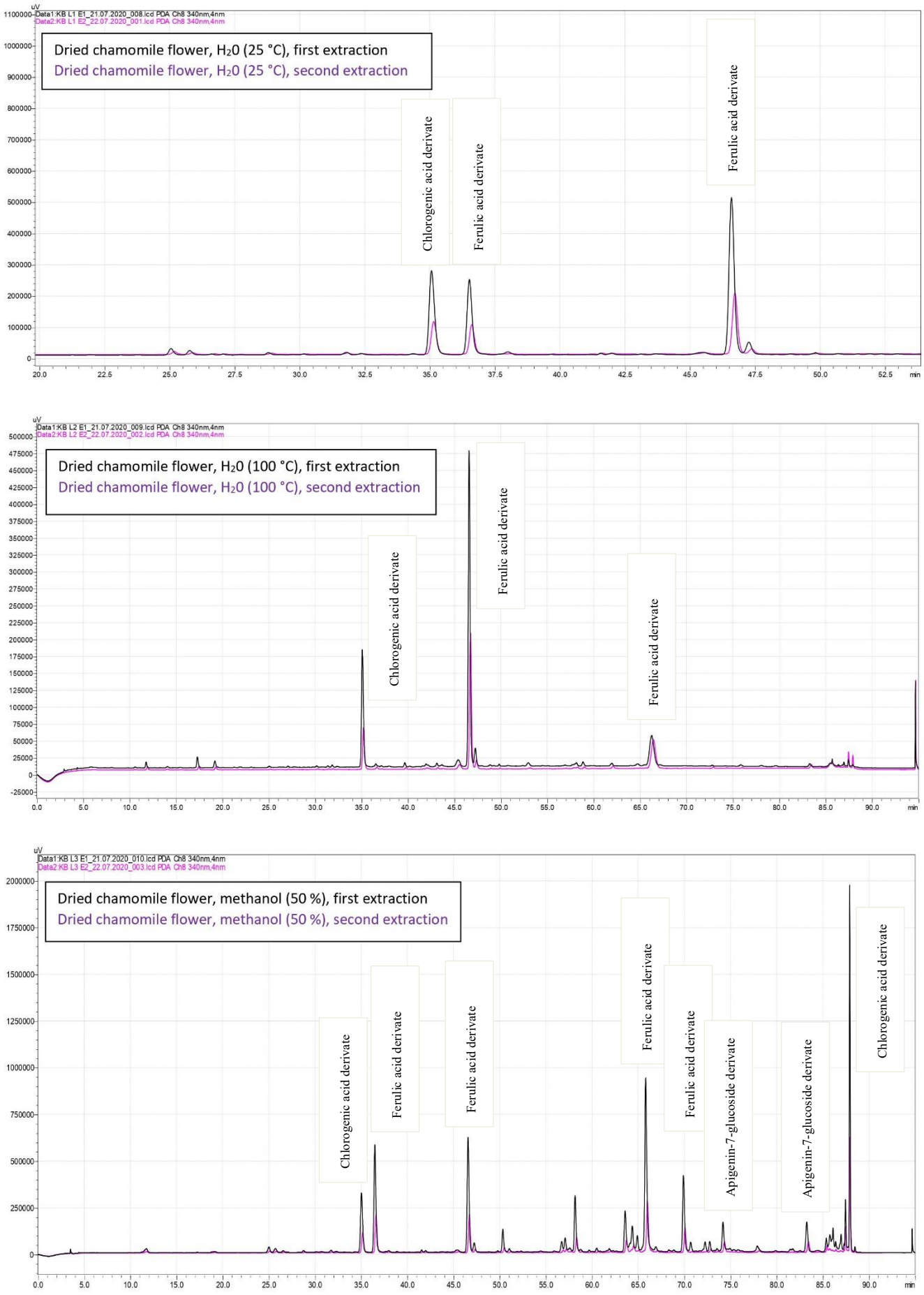

Figure 6: *HPLC chromatograms of different extracts (water 25 °C, water 100 °C, methanol 50%)*

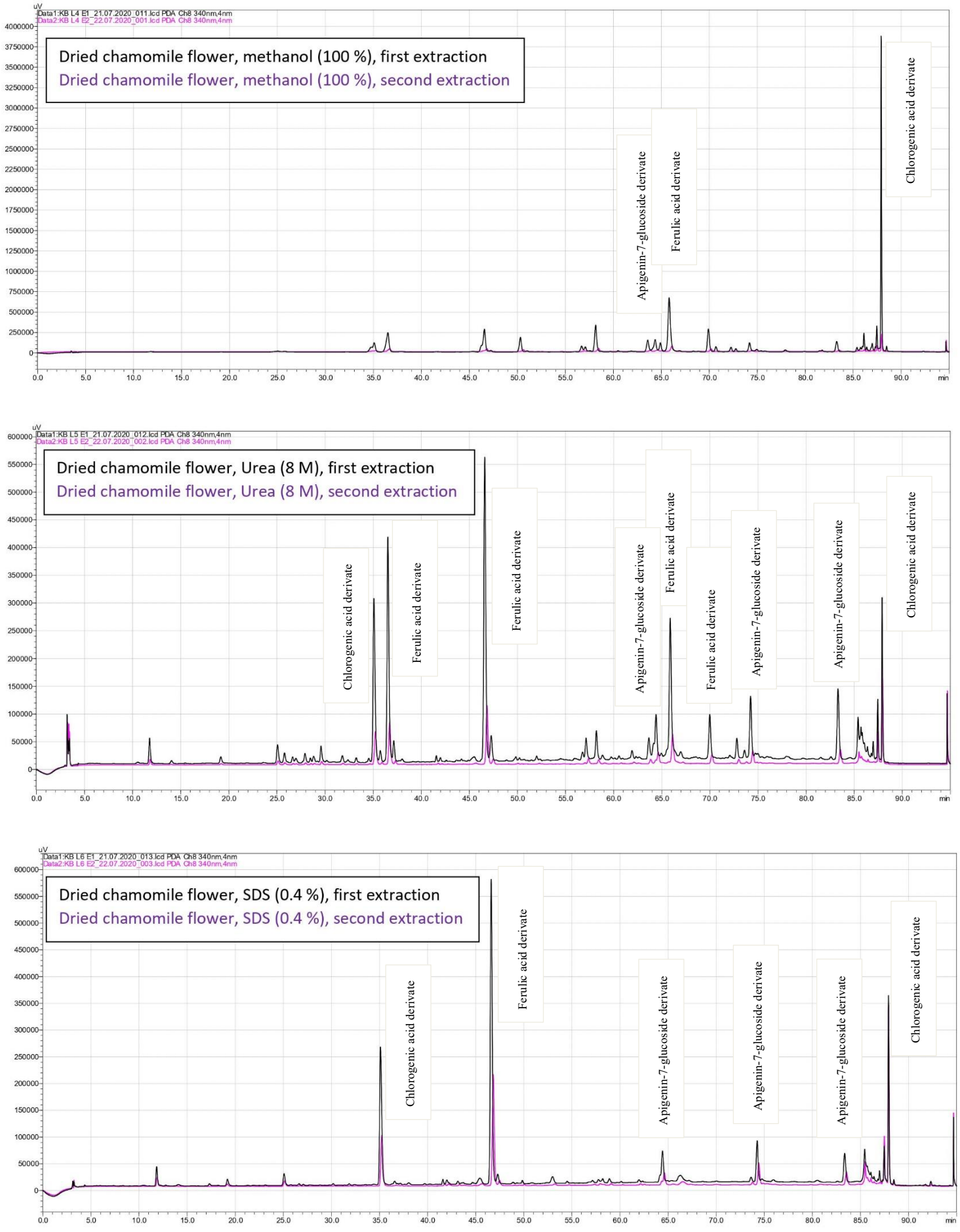

Figure 7*:* HPLC chromatograms of different extracts (methanol 100%, urea 8 M, SDS 0.4 %)

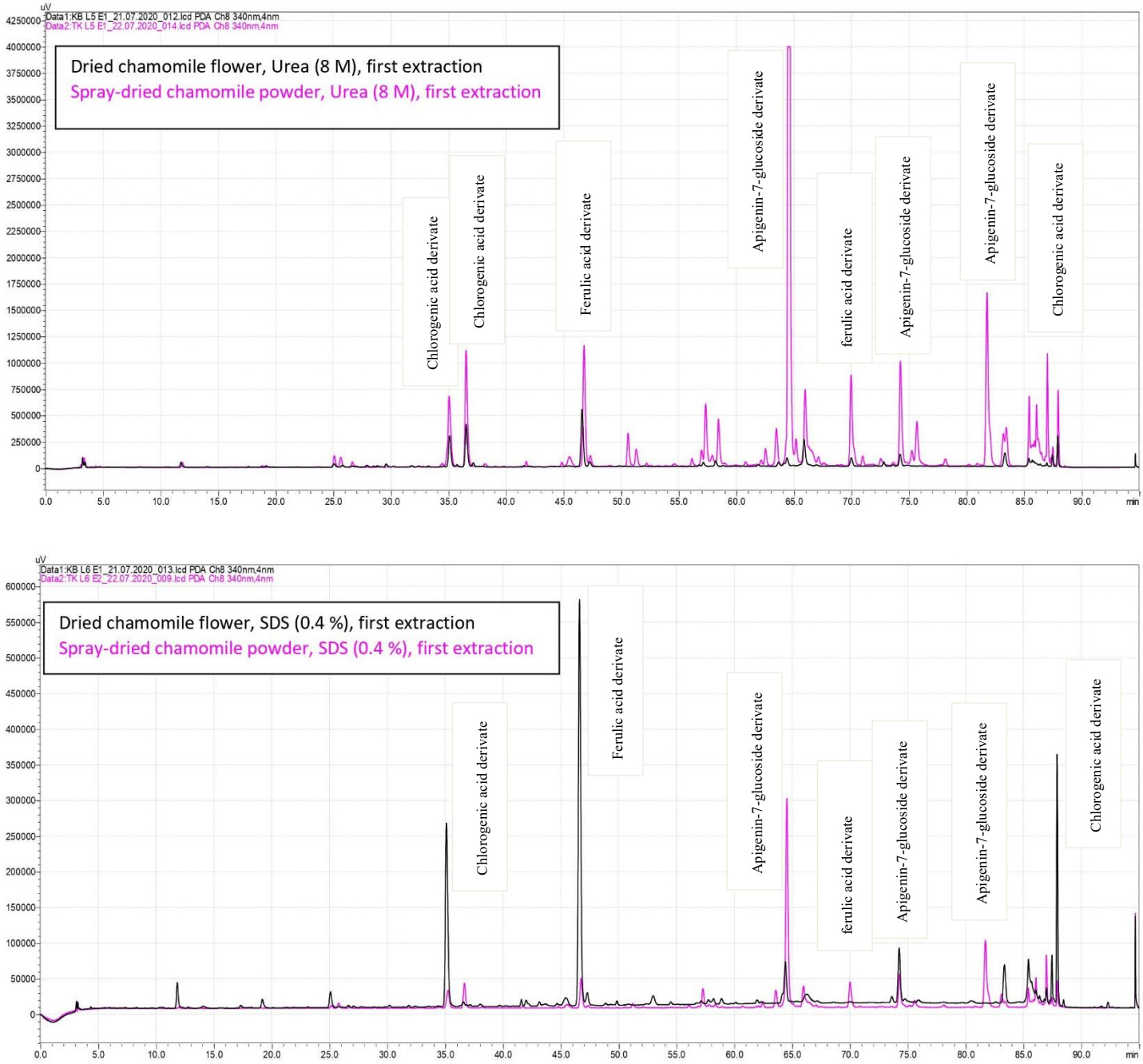

Figure 8*: HPLC chromatograms of different extracts from dried chamomile flowers vs. spray-dried chamomile powder*

5.2. Protein Extraction from Plant Material

Flavonoids are responsible for the yellow colour of the sample and can negatively interfere with the measurement of protein concentration and the gel staining process. The first step was to separate flavonoids and proteins in the extraction samples. The dried chamomile flower samples that were extracted with 50 % and 100 % methanol as described in chapter 4.3, were washed with methanol (chapter 4.5). After three washing steps, the pellet was solved in 6 ml SDS and stored at 4 °C overnight. This method did not include protein precipitation. The samples were loaded on an SDS-PAGE gel. Protein concentration could not be measured because the methanol interferes with the Pierce 660 nm assay. After gel electrophoresis, the gel was stained with Coomassie Quick Stain Solution.

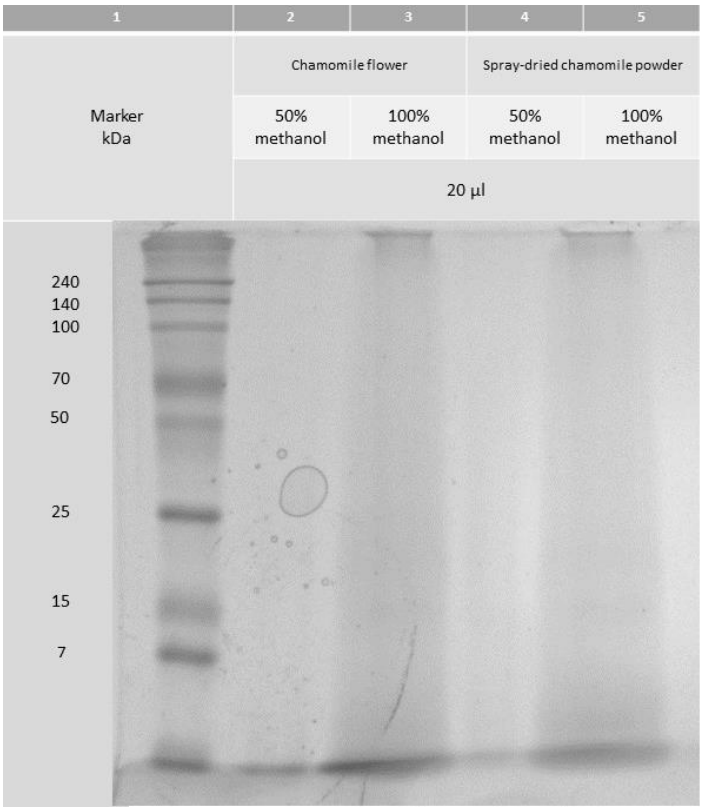

Figure 9*: SDS-PAGE gel of methanol-washed samples*

As can be seen in **Figure *9***, the gel did not show any protein bands. Therefore, it can be assumed that the samples did not contain an adequate amount of protein. To increase the protein concentration, four different extraction methods were tested and compared.

5.3. Modified Method of TCA/Acetone Precipitation

After TCA/acetone precipitation (chapter 4.7), the samples were loaded on an SDS-PAGE gel and stained with silver staining. This method of precipitation was performed in three replicates as seen in **Figure *10***.

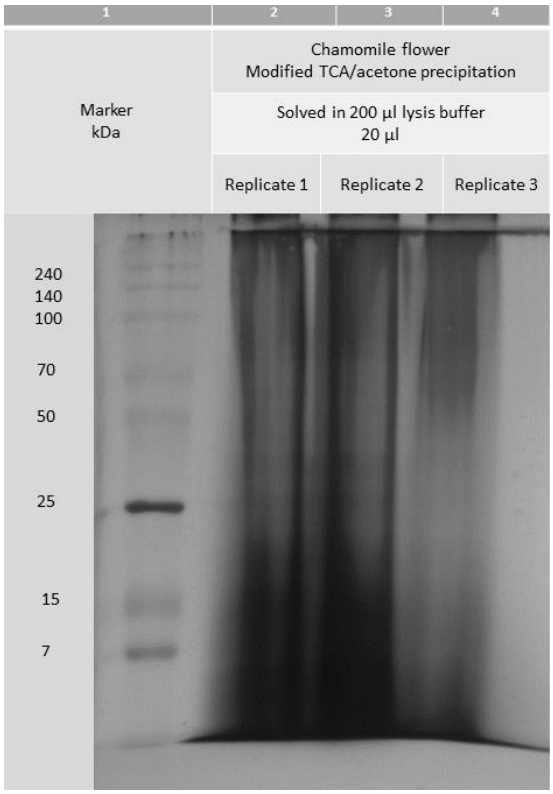

Figure 10: *SDS-PAGE of modified TCA/acetone precipitated samples*

The samples show a massive smearing effect and only few protein bands are visible. These do not have clear edges and are mostly obscured due to the strong smearing towards the end of the lanes.

5.4. Methanol/Chloroform Method

By using the methanol/chloroform precipitation method, the protein bands were only visible with Coomassie quick stain solution but not with silver staining (**Figure *11***). Still, just a few bands were barely visible while the rest was covered by the heavy smearing along the lanes.

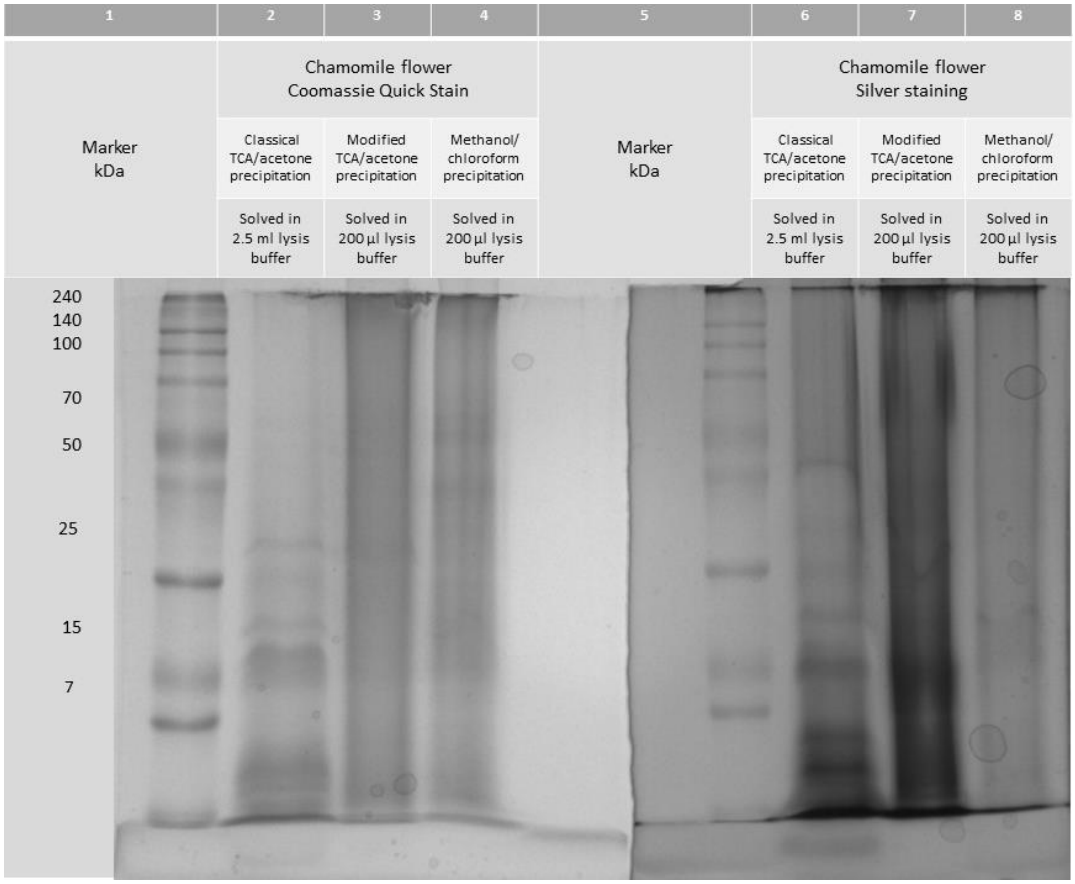

Figure 11: *SDS-PAGE of classical, modified TCA/acetone precipitation and methanol/chloroform precipitation with different staining methods*

5.5. TCA/Acetone Precipitation as "Classical Method"

After the protein extraction without precipitation by washing the samples with methanol did not show any protein bands, direct precipitation was used to increase protein concentration. A 20 % TCA/acetone was added to finely ground chamomile powder as described in chapter 4.6, and the pellet was solved in 2 ml lysis buffer with protease inhibitor. As can be seen on **Figure *12***, the lane on the far-right side shows faint protein bands with the least amount of smearing.

To ensure reproducibility, the precipitation as described in chapter 4.6 was repeated for five times. The resulting gel is shown in **Figure *13***. The gel shows visible protein bands with minimal smearing across the lanes. All five samples have a similar protein band pattern, which shows good reproducibility for this method. However, this precipitation method could be

improved since the protein bands do not have sharp edges due to the prevalent amount of background staining.

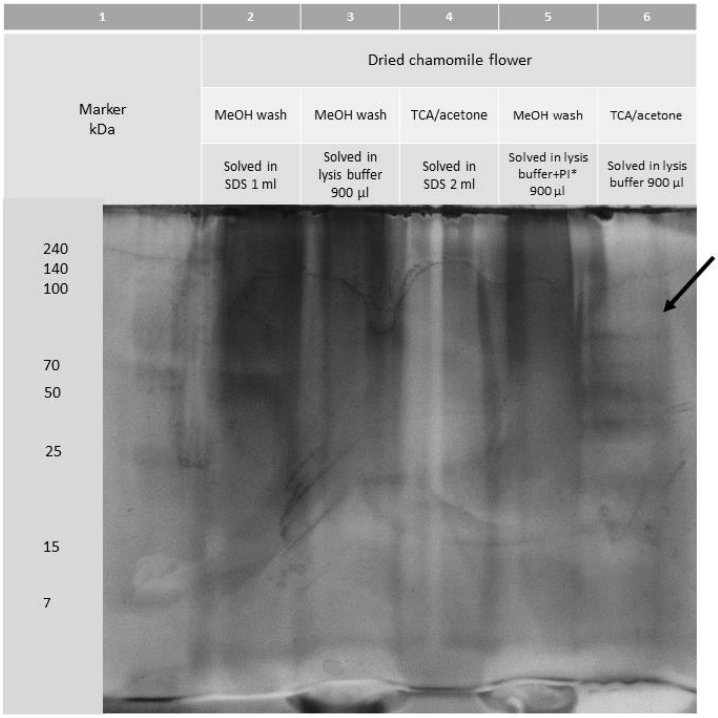

Figure 12*: SDS-PAGE, comparison of different extraction methods*

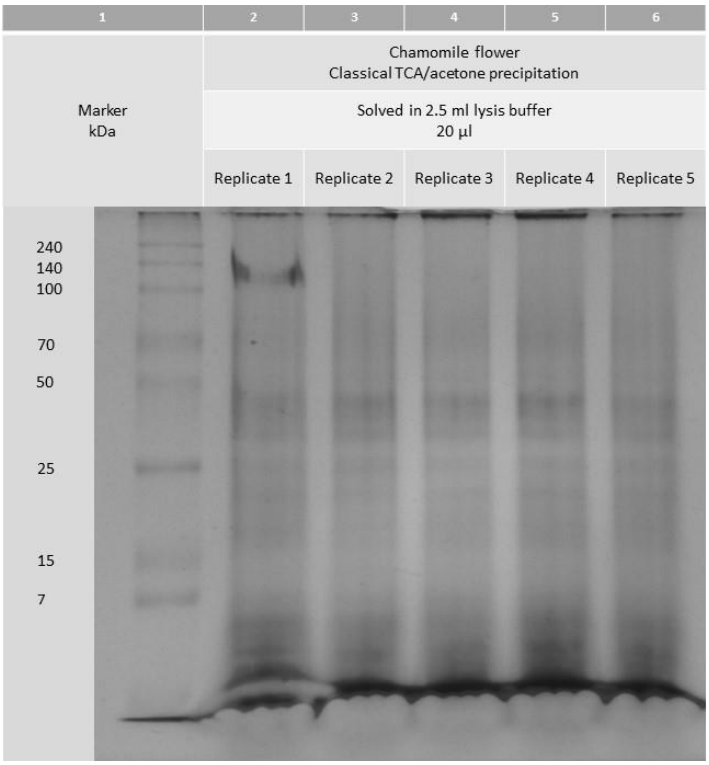

Figure 13*: SDS-PAGE of classical TCA/acetone precipitated sample*

5.6. Ultrafiltration

As an alternative to the precipitation methods, ultrafiltration, as described in chapter 4.9, was used to increase protein concentration and eventually eliminate the yellow colour caused by flavonoids. As seen in **Figure *14*** the filtrate sample shows one prominent protein band at 120 kDa, while the rest of the lane does not contain clearly visible protein bands. The filter cake does not show any protein bands, but a strong smearing effect.

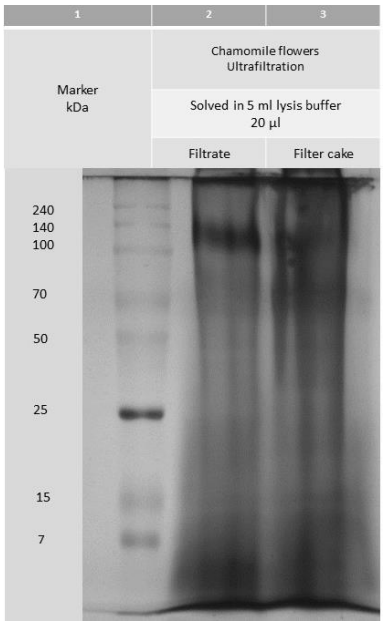

Figure 14: *SDS-PAGE of ultrafiltration samples*

6. Discussion

In contrast to protein content, the flavonoid content of *M. chamomilla* is well investigated. Apigenin and later apigenin-7-glucoside were the first flavonoid compounds isolated from Chamomile with the following flavonoids identified later on, like isorhamnetin, luteolin, quercetin, apigenin, patuletin and others. The phenolic fraction might further contain phenolic acids like chlorogenic acid, caffeic acid, vanillic acid, syringic acid, anisic acid and coumarins like umbelliferone and herniarin as well (Nováková et al., 2010). In the HPLC analysis, results shown in Chapter 5.1, multiple derivatives from four different phenolic compounds have been identified, being chlorogenic acid, ferulic acid, apigenin and apigenin-7-glucoside, which show a variety of unknown derivatives. These results are largely congruent with the current available literature, although the number of different flavonoids present in the samples is significantly lower than usually reported. This could be due to the extraction methods used.

In this study four methods of protein extraction for *Matricaria chamomilla* flower tissue were examined using SDS-PAGE. The suitability of each extraction method for chamomile flower are discussed in the following paragraph. Since protein extraction and analysis of chamomile flower tissue is relatively unknown, the results will be compared with different types of plant tissue.

The methanol/chloroform extraction method has been reported to be suitable for extraction of plant proteins, especially hydrophobic proteins, with dilute samples and in the presence of detergents (Wessel & Flügge, 1984). Regarding protein extraction from *M. chamomilla* tissue, the methanol/chloroform extraction did not yield appropriate results when compared to other methods. Additionally, it is the only extraction method, where a clear difference between gel staining methods is visible. The Coomassie Blue Quick staining method showed faint protein bands, while the gel stained with silver solution did not show any protein bands. Apart from the faint protein bands visible on the Coomassie Blue Quick stained gel, the lane shows a major smearing effect, which indicates that this method has not sufficiently removed secondary metabolites. In a study from Van Nguyen et al., 2021 the phenol-methanol/chloroform extraction method of different types of Korean ginseng tissue produced the least favourable gel profile due to pronounced smearing effects. Another publication from Ferro et al., (2000) analysed hydrophobic chloroplast membrane proteins from *Spinacia oleracea L.* by comparing

different chloroform/methanol ratios. Similarly, Vertommen et al., 2010 evaluated individual chloroform/methanol ratios in combination with gel electrophoresis to study membrane proteins in *Arabidopsis thaliana* and banana plants. While the analysed ratio of chloroform to methanol ranged from 0/9 to 8/1, both studies concluded that the ideal chloroform/methanol ratio is around 5/4. In the chloroform/methanol extraction protocol used in Chapter 4.8, the chloroform/methanol ratio was 1/4, which did not show sufficient results in this work as well as the mentioned publications. This opens a future possibility to analyse protein extraction of chamomile flower tissue with different chloroform/methanol ratios closer to 5/4 to find out if the gel electrophoresis results could be improved.

For plant proteome analysis, one of the most commonly reported protein extraction method involves protein precipitation with trichloroacetic acid and acetone (Rose et al., 2004). The TCA/acetone extraction method is especially successful for removing contaminants while increasing protein concentration. Although, polymeric contaminants are often co-extracted in tissues with high amounts of compounds like soluble cell wall polysaccharides and polyphenols (Saravanan & Rose, 2004). This method is based on protein denaturising under acidic and/or hydrophobic conditions that also inhibit the activity of proteases, phenoloxidases and peroxidases, which are commonly found in plant tissues and can cause significant loss of proteins (Wang et al., 2008). In this work, the TCA/acetone protein extraction shows the best results in comparison to the other extraction methods. The samples show clear protein bands on silver stained SDS-PAGE with minimal smearing effects. However, this precipitation method clearly favours smaller proteins under 50 kDa, which is also shown on **Figure *13***. Similar findings are reported for protein extraction of cotton seedling tissue, where protein bands of more than 50 kDa were barely observed for TCA/acetone precipitated samples (Xie et al., 2009). Most publications compare TCA/acetone precipitation with a phenol-based precipitation, which is not featured in this work. The protocol that works better for plant proteomics between the two mentioned depends strongly on the type of plant tissue used. Xu, Xu and Huang (2008) compared four methods of protein extraction for turfgrass plant tissue, including TCA/acetone and phenol-based precipitation, and found that TCA/acetone showed the highest protein yield among all methods. Additionally, TCA/acetone precipitation gave the best results overall for protein separation for leaf tissue in turfgrass plants. Similar findings

have been reported by Vilhena et al., (2015), where TCA/acetone precipitation was the most efficient protein extraction method for both tomato leaves and roots.

Solvent-based protein precipitation like the modified TCA/acetone precipitation used in this study (chapter 4.7) are not as common as the classical TCA/acetone precipitation previously described. Most used solvents for protein extraction of plant tissues are Tris buffer (Sheoran et al., 2009), sucrose extraction buffer (He et al., 2005) and SDS buffer (Niu et al., 2018). Niu et al. (2018) report that the modified method, in which proteins are dissolved in SDS buffer prior to TCA/acetone precipitation, yields equal or better protein extraction results, while being faster and easier than regular TCA/acetone precipitation. However, this was not the case in this study. The modified method did not result in good protein bands on SDS-PAGE, while also showing an intense smearing effect. Therefore, this method seems to be not well suitable for plant tissues with high amounts of secondary metabolites. In a study published by Sheoran et al. (2009), the solvent based method with Tris buffer also performed poorly compared with other extraction methods, like phenol-based extraction and TCA/acetone precipitation in Tomato pollen grain tissue.

As mentioned before, the TCA/acetone precipitation method favours smaller proteins under 50 kDa, while proteins with a higher molecular weight are lost due to either incomplete precipitation or solubilisation. Therefore, an additional extraction method was incorporated that did not depend on precipitation. Ultrafiltration as a protein purification method is not as commonly used as precipitation, due to several unfavourable factors involving this technique. The main problem has been the low flow rate and the plugging of the membrane, either due to remaining plant tissue or increasing protein concentrations (Gueguen, 1983). The plugging of the membrane was a persistent problem during the ultrafiltration process with *M. chamomilla* plant tissue, which increased centrifugation time significantly up to nearly 16 hours. The gels show a pronounced smearing effect with only one protein band at the 140 kDa mark. This protein band is not present with any other precipitation method. It could be theorized that these proteins are lost in the precipitation process but preserved with ultrafiltration since the membrane filter cut-off is at 3 kDa. This would proof the protein loss of proteins with higher molecular weight in *M. chamomilla* tissue as well. However, the results shown in Chapter 5.6 are not as satisfactory as the results acquired from TCA/acetone precipitation.

In conclusion, the method that performed best for *M. chamomilla* flower tissue was the TCA/acetone precipitation. The gel results showed the most consistently clear protein bands with minimal smearing effect across the lanes. However, this process still needs to be improved to be suitable for further protein investigation when using. mass spectrometry.

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