HPLC-FLD analysis of amino acids content in Chrysanthemum morifolium

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Abstract

Chrysanthemum morifolium (Asteraceae family) have long been used as a tonic, antioxidant, antipyretic, analgesic, sedative, antitumor, neuroprotector, hepatoprotector and cardioprotector agent. This species should be reconsidered as possible sources of many biocompounds, especially amino acids. Thus, the aim of this study was to validate the chromatographic method for detection of amino acids and their identification in flowers and leaves of Ch. Morifolium of variant Pectoral. HPLC-FLD method was evaluated in terms of linearity, precision, repeatability, accuracy, LOD and LOQ. The calibration curves of all analytical standards of amino acids were linear (R^2 > 0.99) over the range of 0.015–0.625 μmol/mL, the LODs and the LOQs were in the range of 0.001–0.096 µg/mL and 0.004–0.321 µg/mL, respectively. During the HPLC-FLD assay ten amino acids in free form and fifteen amino acids after hydrolysis in Ch. Morifolium flowers were identified. Besides, twelve amino acids were detected in free form and fourteen amino acids after hydrolysis in Ch. morifolium leaves. The results of HPLC-FLD analysis showed that the predominant amino acid was L-proline in both types of herbal raw materials. Its total content was 31.67±0.02 μg/mg in Ch. morifolium flowers and 18.56±0.02 μg/mg in Ch. morifolium leaves. This phytochemical study confirms that flowers and leaves of Ch. Morifolium (Pectoral) are rich sources of amino acids and can exhibit a wide range of pharmacological activities.

Keywords

Chrysanthemum morifolium, amino acids, high-performance liquid chromatography-fluorescence detector, L-proline

Introduction

Chrysanthemum (Chrysanthemum morifolium Ramat.) – a genus of flowering plants of Asteraceae family, which has more than 200 species of annual and perennial herbaceous plants or shrubs that grow in temperate subtropical areas of Southeast China and Japan (Shahrajabian 2019). The diversity and heterogeneity of Asteraceae family justifies the great importance of its individual members, which are known and used from ancient times, not only as food sources or as spices, but also for medicinal purposes. Several classes of compounds from Asteraceae family and Chrysanthemum species were studied and tested for different bio-activities and were reported as having medicinal potential (Marchyshyn et al. 2020; Youssef et al. 2020). Among these compounds, a special attention has been given to amino acids, which provide for these species important uses in the pharmaceutical and food industry, that are due to their important medicinal properties. They play an integral role in protein synthesis, hormone and enzyme production, help stimulate muscle growth and regeneration, are involved in energy production, play a role in fat metabolism and immune function, have...
the detoxification activity, help regulate blood sugar levels, stimulate wound healing (Savych 2021; Savych and Mazur 2021; Savych and Milian 2021; Savych and Siniggenko 2021). Thus, the essential amino acids are at the core of many vital processes. In this context and taking into consideration the fact that in the last decades these compounds have shown a significant importance in the field of medicinal compounds (Savych and Marchyshyn 2021a, b; Savych et al. 2021a, d, f, g, h), the Asteraceae family and Chrysanthemum species should be reconsidered as possible sources of amino acids.

Many species and varieties of Chrysanthemum are valuable ornamental plants that are widely used in landscaping in Europe and Asia. Chrysanthemums are now mainly grown as an ornamental plant but some species are used as medicinal (Marchyshyn et al. 2020; Youssef et al. 2020). In the folk medicine of Japan and China, the raw materials of Ch. morifolium have long been used as a tonic, detoxification, antipyretic, analgesic and sedative. Pharmacological studies of Ch. morifolium have shown that it influences on lipid and carbohydrate metabolism, has antioxidant, antitumor, neuroprotective, hepatoprotective and cardioprotective activities (Yang et al. 2017; Ryu et al. 2019; Mekapogu et al. 2020).

Today, chrysanthemums are grown mainly as ornamental plants, only certain species are used for medicinal purposes, but the phytochemical composition and mechanism of action on the human body is still poorly understood. That is why a detailed study of the genus of Ch. Morifolium and its biologically active substances that can have a certain pharmacological effect is an important task in pharmacy, because it will expand the potential sources for the manufacture of herbal medicines.

In addition it is important for medicine and pharmacy to study new promising plant species, as they can be a source of new phytodrugs that can have a numerous of advantages over synthetic agents, namely, they are low-toxic (Savych and Mala 2021), have a mild pharmacological effect and possibility to be used for long periods of time without significant side effects, have a complex activity through a numerous of biologically active compounds (Savych and Basaraba 2021; Savych and Milian 2021; Savych et al. 2021b, c, i, e).

Aim of the research

Thus, the aim of this study was to validate the chromatographic method for detection of amino acids and their identification in the herbal raw materials of Ch. morifolium of variant Pectoral.

Materials and methods
(experimental part)

Plant materials

The herbal raw material, such as flowers and leaves of Ch. morifolium (Pectoral), cultivated on experimental area as of the National M. M. Hryshko Botanic Gardens of the National Academy of Sciences of Ukraine were used. Aerial parts of Ch. morifolium were harvested during a mass flowering period in 2019. The raw materials were dried, crushed and stored according to the general GACP requirements (WHO 2003). Plants were authenticated by prof. Svitlana Marchyshyn, Department of Pharmacognosy with Medical Botany, Ivan Horbachevsky Ternopil National Medical University, Ternopil, Ukraine. A voucher specimen No. 324 was kept in departmental herbarium for future record.

Chemicals and standards

Standard mix of 17 amino acids AAS18 Supelco containing L-aspartic acid 2.5 μmol/mL, L-serine 2.5 μmol/mL, L-glutamic acid 2.5 μmol/mL, L-histidine 2.5 μmol/mL, glycine 2.5 μmol/mL, L-threonine 2.5 μmol/mL, L-arginine 2.5 μmol/mL, L-alanine 2.5 μmol/mL, L-lysine 2.5 μmol/mL, L-cysteine 1.25 μmol/mL, L-valine 2.5 μmol/mL, L-methionine 2.5 μmol/mL, L-phenylalanine 2.5 μmol/mL, L-isoleucine 2.5 μmol/mL, L-lysine 2.5 μmol/mL, L-leucine 2.5 μmol/mL, L-proline 2.5 μmol/mL of analytical standard grade was used and was purchased from Sigma-Aldrich Chemical Co. (USA) o-Phthalaldehyde (OPA) (≥ 99.0% purity HPLC); 9-fluorenylmethyl chloroformate (FMOC) (≥ 99.0% purity HPLC); acetonitrile (ACN) (≥ 99.0% purity HPLC); methanol (CH3OH) (≥ 99.9% purity HPLC); hydrochloric acid (HCl) (ACS reagent, 37%); disodium hydrogen phosphate (Na2HPO4) (ACS reagent, ≥ 99.0%); sodium hydroxide (NaOH) (ACS reagent, ≥ 97.0%); sodium tetraborate decahydrate (Na2B4O7 · 10 H2O) (ACS reagent, ≥ 99.5%) were also used. The used in the studies water was produced by MilliQ Gradient water deionization system (USA).

Extraction of amino acids

For the extraction of free amino acids the samples of the herbal raw material were grinded into a powder by laboratory mill, then about 0.1 g (accurately weighed) was selected and placed into flask with 2.0 mL of 0.1 N HCl. The extractions were carried out in the ultrasonic water bath at 50 °C for 3 hours.

Extraction of bound amino acids was carried out by adding 2 mL of 6 N HCl to 0.3 g (accurately weighed) of powdered herbal raw materials. Hydrolysis was conducted for 24 hours in a thermostat at 110 °C.

The resulting extracts were centrifuged at 3000 rpm and 0.5 mL of supernatants were evaporated to dryness on a rotary evaporator washing three times with distilled water to remove HCl. Then, resulting product was resuspended in 0.5 mL water and filtered through disposable membrane filters with pores of 0.22 μm (Savych and Nakonechna 2021; Savych et al. 2022).

Pre-column derivatisation

The pre-column derivatization was conducted with a help of an automatic programmable regulations using derivatization reagents, which contained borate buffer 0.4 M
in water (pH 10.2), FMOC 2.5 mg/mL in ACN and OPA 10.0 mg/mL in 0.4 M borate buffer. A fluorescence detector (FLD) was used for identification of the derivatized amino acids.

**Instrumentation and conditions of liquid chromatography-mass spectrometry**

The amino acids composition in the herbal raw materials was studied by high-performance liquid chromatography with fluorescence detector (HPLC-FLD) using the liquid chromatograph Agilent 1200 (Agilent Technologies, USA) equipped with a G1313A autosampler, a G1311A quaternary pump, a G1316A thermostat column and a G1315A fluorescence detector. The separation was performed on a Zorbax Eclipse-AAA chromatographic column (4.6 mm ± 150 mm, 3.5 μm) (Agilent Technologies, USA).

**Table 1.** Chromatographic conditions.

<table>
<thead>
<tr>
<th>Mobile phase A</th>
<th>40 mM Na₂HPO₄ pH 7.8 [5.5 g NaH₂PO₄\text{·}12H₂O monohydrate + 1 L of water, adjust to pH 7.8 with 10 N NaOH]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase B</td>
<td>ACN : CH₃OH : H₂O (45:45:10, v/v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>2 mL/min</td>
</tr>
<tr>
<td>Column temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Stop time</td>
<td>26 min</td>
</tr>
</tbody>
</table>

**Table 2.** Gradient mode.

<table>
<thead>
<tr>
<th>Chromatography time, min</th>
<th>Mobile phase A, %</th>
<th>Mobile phase B, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:00</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2:00</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>18:00</td>
<td>43</td>
<td>57</td>
</tr>
<tr>
<td>19:00</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>23:00</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>26:00</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**Identification and calculation by HPLC-FLD**

Amino acid identification in the herbal raw materials was performed by comparing the retention times (tₘ) of amino acid standards. The content of bound amino acids was determined by subtracting the content of free amino acids from their total content (Savych and Nakonechna 2021; Savych et al. 2022).

**Method validation**

HPLC-FLD method to quantify of amino acids was validated for linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and repeatability according to the International Conference on Harmonization (ICH) guidelines. The standard mix of amino acids was used as a stock solution. Amino acids in this standard was 2.5 μmol/mL in 0.1 N HCl, except L-cystine at 1.25 μmol/mL. To obtained standard calibration solutions, the stock solution was dissolved in 0.1 N HCl to give concentrations in range of 0.015–0.625 μmol/mL. Linearity was performed by injecting a series of standard solutions with a threefold derivatization procedure and a single injection for standard of amino acids. The mean value and standard deviation, as well as regression analysis were calculated using Microsoft Excel software package 2016 (USA). The values for LOD and LOQ were calculated based on the data obtained during linearity testing in the low concentration range of the working in the test solution, using the following formulas: LOD = 3.3 * s / Slope; LOQ = 10 * s / Slope. Linearity testing was repeated with the same samples after a complete restart of the system with removal and re-installation of the column. Repeatability precision was determined by six-fold injection of the same sample in a row. For the resulting relative peak area of the quantifiers the relative standard deviation (RSD) was calculated. To determine intra-day precision, six injection of amino acids reaction mixtures with the same concentration were single injected and the resulting relative peak areas were used to calculate the RSD. Inter-day precision for the day of sample preparation and the two following days was specified by injecting six standard sample of amino acids reaction mixtures once each on all three days. The RSD of the samples on that day together with the previous samples were calculated as above (Wang et. al 2020).

**Results and discussion**

The analytical procedure has been validated to confirm its reliability. All the peaks of analytical standard of amino acids showed good linearity (R² > 0.99) in a wide concentration range (0.015–0.625 μmol/mL). The results showed that the LODs and the LOQs of amino acids were in the range of 0.001–0.096 μg/mL and 0.004–0.321 μg/mL, respectively, indicating that the sensitivity of the method was satisfactory (Table 2). The repeatability of the subsequent derivatization and HPLC-FLD measurement of six standard samples with the same concentration resulted in precision values for the derivatization procedure. For intra- and inter-day precision, the RSD was in a range of 1.17% to 5.11%, which is acceptable.

According to the results of the HPLC-FLD assay, it was identified ten amino acids in free form in flowers and leaves of *Ch. morifolium* (Pectoral) and twelve amino acids in leaves of *Ch. morifolium* (Fig. 3). HPLC analysis of amino acids after hydrolysis showed that *Ch. morifolium* flowers contained fifteen species of these compounds (Fig. 2) and *Ch. morifolium* leaves – fourteen (Fig. 4).

The results of quantitative study showed that the predominant amino acid in free form was L-proline in flowers and leaves of *Ch. morifolium* (Pectoral), its content was 2.55±0.01 μg/mg and 3.79±0.01 μg/mg, respectively. As for amino acids after hydrolysis, the predominant compound was L-proline in both types of herbal raw materials too. Its content was 29.12±0.02 μg/mg in *Ch. morifolium* leaves and 14.76±0.02 μg/mg in *Ch. morifolium* flowers (Table 4). In plants, L-proline accumulation is a common physiological response to various stresses but is also part of the developmental
Proline – nonessential amino acid that have vital role in the structure of proteins and, also, exhibits significant hypoglycemic activity, which is due to a decrease in hepatic glucose production owing to inhibition of glycogenolysis, gluconeogenesis and glucose-6-phosphatase activity (Alqudah et al. 2021; Patriarca et al. 2021; Savych and Polonets 2021).

In addition, a high content of L-serine in bound form was detected in flowers and leaves of *Ch. morifolium* (Pectoral), its content was 11.81±0.02 μg/mg and 6.36±0.02 μg/mg, respectively (Table 4). Serine, as essential amino acid, is necessary for the full construction of DNA and RNA, as well as to produce muscle tissue and, accordingly, muscle growth (Šponer et al. 2018). It is also used by the body to produce the hemoglobin molecule. This amino acid is important for the full functioning of our immunity, promotes the production of immunoglobulins and antibodies. It is the starting product for the formation of other essential amino
Table 4. The results of the HPLC-FLD analysis of amino acids in the herbal raw materials of *Ch. morifolium*.

<table>
<thead>
<tr>
<th>Identified substance</th>
<th>Flowers</th>
<th>Content in <em>Ch. morifolium</em> (Pectoral), μg/mg</th>
<th>Leaves</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
<td>Bound</td>
<td>Total</td>
<td>Free</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>n/d</td>
<td>10.35±0.02</td>
<td>10.35±0.02</td>
<td>n/d</td>
</tr>
<tr>
<td>L-serine</td>
<td>0.04±0.01</td>
<td>11.81±0.02</td>
<td>11.86±0.01</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>0.14±0.01</td>
<td>7.14±0.02</td>
<td>7.27±0.02</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>L-histidine*</td>
<td>n/d</td>
<td>3.32±0.01</td>
<td>3.32±0.01</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.44±0.02</td>
<td>4.21±0.02</td>
<td>5.65±0.02</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>L-threonine*</td>
<td>0.11±0.01</td>
<td>5.44±0.01</td>
<td>5.55±0.01</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>L-arginine</td>
<td>n/d</td>
<td>6.40±0.02</td>
<td>6.40±0.02</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td>L-alanine</td>
<td>0.05±0.01</td>
<td>6.17±0.02</td>
<td>6.21±0.01</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>n/d</td>
<td>3.30±0.01</td>
<td>3.30±0.01</td>
<td>n/d</td>
</tr>
<tr>
<td>L-cystine</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>L-valine*</td>
<td>0.10±0.01</td>
<td>5.34±0.01</td>
<td>5.45±0.01</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td>L-methionine*</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>L-phenylalanine*</td>
<td>0.11±0.01</td>
<td>6.62±0.02</td>
<td>6.73±0.02</td>
<td>0.22±0.01</td>
</tr>
<tr>
<td>L-isoleucine*</td>
<td>0.04±0.01</td>
<td>5.07±0.01</td>
<td>5.10±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>L-lysine*</td>
<td>0.05±0.01</td>
<td>12.05±0.02</td>
<td>12.10±0.02</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>L-leucine*</td>
<td>n/d</td>
<td>5.64±0.02</td>
<td>5.64±0.02</td>
<td>n/d</td>
</tr>
<tr>
<td>L-proline</td>
<td>2.55±0.01</td>
<td>29.12±0.02</td>
<td>31.67±0.02</td>
<td>3.79±0.01</td>
</tr>
</tbody>
</table>

Note: 1. * – essential amino acid; 2. n/d – not detected; 3. Values are expressed as mean ± SD (n=6).
acids, in particular tryptophan, glycine, cysteine, and methionine. The amino acid – serine is a constituent of choline, ethanolamine, sarcosine, and phospholipids. When serine breaks down, pyruvate is formed, which is necessary for the conversion of glycogen into glucose (Akashi et al. 2018; Okamura et al. 2021).

Besides, another essential amino acid was identified in large quantities – L-lysine with content 12.05±0.02 μg/mg in Ch. morifolium flowers and 6.62±0.02 μg/mg in Ch. morifolium leaves (Table 4). Lysine has a wide range of biological effects, and above all, lysine is vital as a component of body proteins. This amino acid was found in large quantities in collagen, which provides strength to muscles, cartilage, ligaments and tendons. Indirectly, lysine strengthens bones, as it promotes the absorption of calcium from the intestines, with its deficiency, osteoporosis (increased bone fragility) can develop. Lysine plays an important role in the immune system, as it is needed in large quantities to produce antibodies (immunoglobulin). Lysine is part of the hormones and enzymes that regulate the body’s metabolic processes (Min et al. 2018; Green and Lamming 2019; Severanova et al. 2019).

This phytochemical study confirms that flowers and leaves of Ch. morifolium (Pectoral) are a rich sources of amino acids and these herbal raw materials can exhibit a wide range of pharmacological activities. The authors have no funding to report. The authors have declared that no competing interests exist. The authors have no support to report.

**Conclusion**

The method was validated in terms of linearity, precision, repeatability, LOD and LOQ. HPLC-FLD assay of amino acids revealed that flowers and leaves of Ch. morifolium (Pectoral) represent important sources of bioactive compounds with a wide range of pharmacological activities. Ten amino acids in free form and fifteen amino acids after hydrolysis in Ch. morifolium flowers were identified. Twelve amino acids in free form and fourteen amino acids after hydrolysis were detected in Ch. morifolium leaves. The results of HPLC-FLD analysis showed that the predominant amino acid in free and bound form was L-proline in both types of herbal raw materials. Its content was 2.55±0.01 μg/mg and 29.12±0.02 μg/mg in Ch. morifolium flowers and in Ch. morifolium leaves it was 29.12±0.02 μg/mg and 14.76±0.02 μg/mg, respectively.

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