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Evaluation of free amino acids in *Cirsium vulgare* (Bull thistle) plant materials using gas chromatography–mass spectrometry

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Amino acids are one of the main particles in a living organism. They perform many different functions, such as structural, receptor, energetic and enzymatic functions [1]. The aim of the investigation was to identify and evaluate the qualitative composition of free amino acids in samples of *Cirsium vulgare* material using gas chromatography–mass spectrometry (GC-MS). Our objectives were to apply the gas chromatography–mass spectrometry (GC-MS) method for free amino acids from *Cirsium vulgare* raw materials grown in Lithuania and determine the qualitative composition of free amino acids from extracts of *Cirsium vulgare* raw materials: roots, leaves, flowers and seeds. MTBSTFA (N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide) was selected for derivatisation in free amino acid analysis. Applying gas chromatography–mass spectrometry with compound derivatisation and based on the mass spectral database free amino acids were identified in bull thistle raw material: L-alanine, glycine, L-valine, L-leucine, L-isoleucine, L-proline, L-serine, L-threonine, L-phenylalanine, L-lysine, L-tyrosine, L-aspartic acid and L-glutamic acid. Studies have shown that the qualitative composition of free amino acids depends on the plant vegetation phase and plant parts.

Keywords: *Cirsium vulgare*, bull thistle, amino acids, gass chromatography–mass spectrometry, GC-MS

INTRODUCTION

Amino acids (AR) are molecules that combine to form proteins. Amino acids and proteins are the building blocks of life [1]. In plant life amino acids are important for many reasons. Amino acids are responsible for affecting plant growth velocity and activating chlorophyll formation. They activate photosynthesis and raise its efficiency as it enhances chlorophyll formation and encourages vegetative growth as well as it has a role in pollination and fruitfulness. Amino acids increase tolerance to the hard conditions, activate pollen grain germination, organise osmosis potential, maintain the colloidal properties of the cell protoplasm, and remove the negative effect of free radicals. They also increase plant tolerance to diseases, activate chlorophyll and improve plant cells and embryo formation [2]. When proteins are digested or broken down, amino acids are left. The human body uses amino acids to make proteins to help the body [1]. The release of amino acids from muscle helps maintain fasting blood glucose [3, 4]. Also, it is important for disease prevention and health – reduced muscle mass impairs the body's ability to respond to stress and chronic illness [5]. They also

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act as an antioxidant – protect cells from oxidative stress caused by free radicals [6]. All amino acids have been shown to be involved in the regulation of the body's immune system [7]. The transmission of nerve impulses is also affected by amino acids. Amino acids are mediators or precursors of low molecular weight in the biosynthesis of neurotransmitters [8]. Tyrosine is a major precursor to the synthesised catecholamines (dopamine, norepinephrine and epinephrine). Upon hydroxylation, tyrosine is converted to DOPA, which is converted to dopamine during decarboxylation, and norepinephrine is synthesised from dopamine and then epinephrine [9]. With the increasing demand for amino acids, a number of studies are being performed to detect amino acids in the raw material.

Cirsium vulgare (Savi) Ten., also known as bull, is a species of the Asteraceae, genus *Cirsium*. This plant is known as biennial native in most of Europe, Western Asia and Northwestern Africa. Bull thistle is an intrusive weed that grows in meadows, orchards and roadsides, among cereals [10]. Studies show that the main active compounds in *Cirsium* genus plant material are flavonoids. It is known that *Cirsium vulgare* contains secondary metabolites such as sterols and triterpenes, aliphatic aldehydes and phenolic acids [11]. One of the accumulating chemicals in *Cirsium vulgare* is free amino acids. Thanks to these organic compounds, the input, transport and consumption of substances in the plant are accelerated. Plants easily absorb only free amino acids [12]. Fourteen amino acids were identified, among which amino-n-butyric acid, aspartic acid and proline were present in almost all pollen samples of *Asteraceae* species. The other major amino acids present in a free form included arginine, cystine, glutamic acid, glycine, isoleucine, leucine, methionine, ornithine, tryptophan and tyrosine [13]. Other researches also identify free forms of amino acids in *Asteraceae* family plants. For example, 13 amino acids were identified, among them n-butyric acid, proline and aspartic acid were present in almost all different pollen samples. The other major amino acids present in a free form included alanine, cystine, arginine, glutamic acid, isoleucine, glycine, leucine, methonine, tryptophan and tyrosine [14]. A number of chemicals analyses have been performed on the free amino acids, which must also be analysed by gas chromatography (GC). Specific properties of the preparation are required for the GC-MS test. Only volatile compounds can be analysed by gas chromatography and no amino acids are present, so the effects on the compounds can be combined to provide volatility in this particular case [15]. Therefore, derivatisation of compounds is required for the study. In the analysis of free amino acids by gas chromatography–mass spectrometry, derivatisation of compounds is performed, which improves the stability of compounds and their determination in gas chromatography. Derivatisation converts highly polar compounds to volatiles and allows the process to be performed at normal temperatures without thermal decomposition or molecular transformation [16].

EXPERIMENTAL

Plant materials

Bull thistle samples were collected at the Šiauliai Academy Botanical Garden. The phenological stage of the plant and the part of the selected raw material are indicated in Table 2. The object of the research was bull thistle leaves, flowers, roots and seeds, which were collected during the phenological period from 18 June (mass regrowth) to 10 September 2021 (seed maturity). Different parts of the plant were separately dried at 40°C in a drying chamber. Bull thistle parts were grounded to a fine powder using an Ultra Centrifugal Mill ZM 200 (Retsh, Hann, Germany). Grinding

Table 1. **The phenological stage of the plant material and the proportion of the plant material**

Raw material collection date	Phenological stage	Collected part of raw material
02.07.2021	Butonisation	Leaves, roots, flowers
16.07.2021	Beginning of flowering	Leaves, roots, flowers
30.07.2021	Mass flowering	Leaves, roots, flowers, seeds
13.08.2021	Mass flowering	Leaves, roots, flowers, seeds
27.08.2021	End of flowering	Leaves, roots, flowers, seeds
10.09.2021	Seed maturity	Seeds

was performed using a 0.5 mm trapezoid hole sieve. The final moisture content of different parts of the herb was 7.37 ± 0.3%.

Solvents and reagents

Ethanol (96%) (Vilniaus degtinė, Vilnius, Lithuania). The water used for sample preparation was produced using a Super Purity Water System (Millipore, USA). MTBSTFA derivatisator (>97%) (Sigma-Aldrich, Germany) and a standard mixture of amino acids (2.5 μmol/ml each amino acid) (Sigma-Aldrich, Germany) were used.

Reference solutions

The reference solution was prepared by taking 100 μL of the mixture of amino acid standards and drying under a stream of nitrogen to dryness. The dry pellet was mixed with 100 μL of acetonitrile and 100 μL of the MTBSTFA derivatizer. The resulting solution was heated at 100°C in a glycerol bath for 2.5 h.

Heat reflux extraction

0.1 ± 0.001 g of dried and milled leaves, flowers, roots and seeds were taken and mixed with 50% ethanol in a 250 mL round bottom flask that was placed under reflux for 1.5 h at a temperature of 90°C. The obtained extract was filtered through 0.22 μm PVDF syringe filters. 1 ml was evaporated under a stream of nitrogen to dryness and the residue was mixed with 100 μL of acetonitrile and 100 μL of the MTBSTFA derivatizer. This solution was heated at 100°C for 2.5 h in a glycerol bath.

GC-MS conditions

Analyses were carried out using a SHIMADZU GC/MS-QP2010nc Ultra chromatography system (coupled to an Electron Ionization (EI) ion source and a single quadrupole MS (Shimadzu Technologies, Kyoto, Japan)). A robotic autosampler and a split/splitless injection port were used. The injection port temperature at 250°C was kept until the end of the analysis. The separation of analytes was carried out on a Rxi-5 ms (Restek Corporation, Bellefonte, PA, USA capillary column (30 m long, 0.25 mm outer diameter and 0.25 μm liquid stationary phase thickness)) with a liquid stationary phase of 5% diphenyl and 95% polydimethylsiloxane and with helium at a purity of 99.999% as the carrier gas in a constant flow of 1.49 mL/min. The oven temperature was programmed at 75°C for 5 min, then increased to 290°C at 10°C/min, and increased to 320°C at 20°C/min and kept for 10 min. The total time was 41 min. The temperatures of the MS interface and ion source were set at 280 and 200°C, respectively. The MS was operated in a positive mode (electron energy 70 eV). The full-scan acquisition was performed with the mass detection range (35–500 m/z) to determine retention times of analytes, optimise the oven temperature gradient, and to observe characteristic mass fragments for each compound. Data acquisition and analysis were executed by LabSolution GC/MS (version 5.71) (Shimadzu Corporation). The experiments were repeated 3 times.

RESULTS AND DISCUSSION

In the analysis of the plant raw material, it was found that the plant accumulates different free amino acids. Some of them are nonessential amino acids and others are essential amino acids. In any case, they all play an important role in the process of plant growth. The following amino acids have been found to accumulate in different parts of the plant, including the entire collection period: L-alanine, L-glycine, L-valine, L-leucine, L-isoleucine, L-proline, L-serine, L-threonine, L-phenylalanine, L-aspartic acid, L-glutamic acid, lysine and L-tyrosine (Figure). Based on the results in previous studies the Asteraceae family (*C. intybus* L., *Ch. Recutita* L. and *A. millefolium* L. flowers) accumulates 13 free amino acids such as arginine, lysine, tyrosine, phenylalanine, histidine, leucine, isoleucine, methionine, valine, proline, threonine, serine, alanine and glycine [17]. Comparing free amino acids identificated in *Cirsium vulgare*, methionine, histidine and cystine were not detected. It can be said that the plants in *Asteraceae* have a great diversity in the case of amino acid accumulation. Some studies show that Asteraceae family plants can also contain even more amino acids. For example, authors who studied amino acids in *Calendula officinalis* found main 15 free amino acids such as alanine, arginine, aspartic acid, aspargine, valine, histidine, glutamic acid, leucine, lysine, proline, serine, tyrosine, threonine, methionine and phenylalanine [18]. The researchers also studied amino acids accumulated in the plant

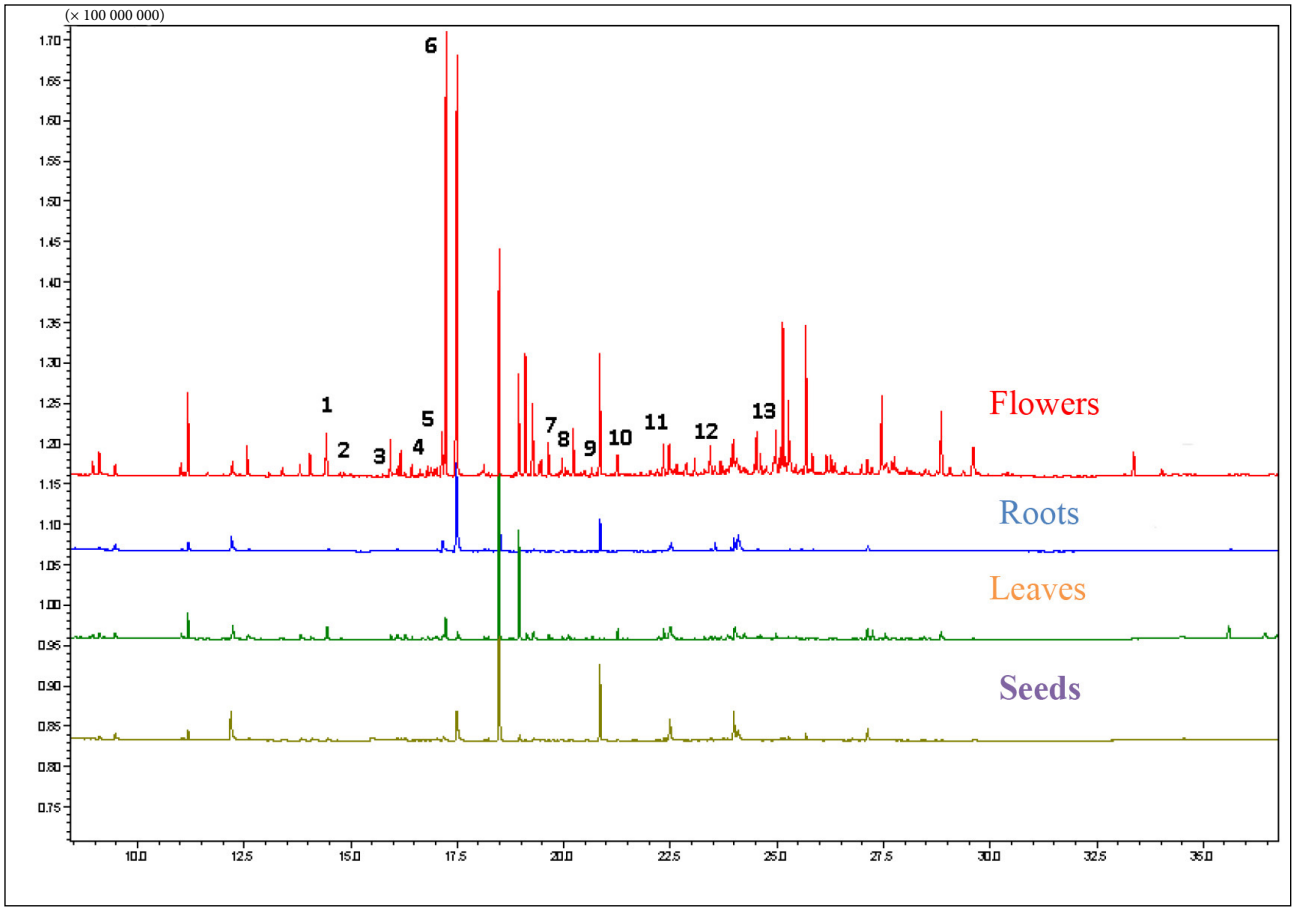

Figure. Amino acids identified in *Cirsium vulgare* extracts: 1 – L-alanine, 2TBDMS derivative, 2 – glycine, 2TBDMS derivative, 6 – L-proline, 2TBDMS derivative, 7 – L-serine, 3TBDMS derivative, 10 – L-aspartic acid, 3TBDMS derivative, 11 – L-glutamic acid, 3TBDMS derivative, 13 – L-tyrosine, 3TBDMS derivative – nonessential amino acids; 3 – L-valine, 2TBDMS derivative, 4 – L-leucine, 2TBDMS derivative, 5 – L-isoleucine, 2TBDMS derivative, 8 – L-threonine, 3TBDMS derivative, 9 – L-phenylalanine, 2TBDMS derivative, 12 – L-lysine, 3TBDMS derivative – essential amino acids

Cirsium arvense. Unlike the *Cirsium vulgare* that we studied, the *Cirsium arvense* in question accumulated only 5 amino acids. This is a significantly lower amount than *Cirsium vulgare* in the raw material [19]. The authors studied *Cirsium japonicum* and found that this plant accumulates all free amino acids studied. In total, 75 primary and secondary metabolites were determined: 19 amino acids, 10 organic acids, 12 sugars, 9 policosanols, 3 tocopherols, 4 phytosterols, 2 amyrins, 4 flavones, 2 flavanols, 3 flavonols, 5 hydroxycinnamic acids and 2 hydroxybenzoic acids [20].

During the growth phase of the plant, i.e. the time of collection of the raw material and the phenological stage, different amino acids accumulated (Table 1). The widest variety of amino acids was observed in the plant raw material collected during flowering: at the beginning of flowering, during mass flowering and at the end of flowering. The authors studied the plant *Artemisia mesatlantica* of the Asteraceae family and also found that this plant accumulates all the active substances studied, including amino acids, during flowering [21].

The parts of the plant that had the highest diversity of accumulated amino acids were plant flowers and the roots of the plant had the lowest diversity of accumulated amino acids, respectively (Table 2). The authors who studied *Cirsium japonicum* also found that flowers contained higher levels of sugars, pyruvic acid and amino acids than the other organs. Comparing these two *Cirsium* genus plants, it is seen that plants of this genus accumulate most of the amino acids in the flowers of the plant [20]. The study, that was done by B. P. Muley et al., 2009, found that the content of amino acids in leaves is about 5% and in flowers 4.5%. This study also used ethanolic extracts for amino acids determination and evaluation with

Table 2. **Amino acids diversity of *Cirsium vulgare* raw materials in different phenological stages**

Plant phenological stage	Collected part of raw material	L-Alanine	Glycine	L-Valine	L-Leucine	L-Isoleucine	L-Proline	L-Serine	L-Threonine	L-Phenylanine	L-Aspartic acid	L-Glutamic acid	L-Lysine	L-Tyrosine
Butonisation	Leaves	+	–	+	+	+	+	+	+	–	+	+	–	–
	Roots	+	+	+	+	+	+	+	–	–	+	–	–	–
	Flowers	+	+	+	+	+	+	+	+	+	+	+	+	+
Beginning of flowering	Leaves	+	–	+	+	+	+	+	+	–	+	+	–	–
	Roots	+	–	+	+	+	+	–	–	–	+	+	–	–
	Flowers	+	+	+	+	+	+	+	+	+	+	+	+	+
Mass flowering	Leaves	+	+	+	+	+	+	+	+	–	–	–	–	+
	Roots	+	–	+	+	+	+	–	–	–	+	+	–	–
	Flowers	+	+	+	+	+	+	+	+	+	+	+	–	–
	Seeds	+	+	+	+	+	+	+	+	+	+	+	+	+
Mass flowering	Leaves	+	+	+	+	+	+	+	+	+	+	–	–	+
	Roots	+	–	+	+	+	+	+	–	–	–	+	–	–
	Seeds	+	+	+	+	+	+	+	+	+	+	+	–	+
End of flowering	Leaves	+	+	+	+	+	+	+	–	–	+	+	–	+
	Roots	+	–	+	+	+	+	+	+	–	–	–	–	–
	Flowers	+	+	+	+	+	+	+	+	+	+	+	–	+
	Seeds	+	+	+	+	+	+	+	+	+	+	+	–	+
Seed maturity	Seeds	+	+	+	+	+	+	+	+	+	+	+	+	+

Calendula officinalis raw materials [18]. These results are slightly different than ours.

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DUJŲ CHROMATOGRAFIJOS-MASIŲ SPEKTROMETRIJOS METODO PRITAIKYMAS KOKYBINIAM LAISVŲJŲ AMINORŪGŠČIŲ NUSTATYMUI *CIRSIUM VULGARE* (DYGIOSIOS USNIES) AUGALINĖJE ŽALIAVOJE

S a n t r a u k a

Aminorūgštys yra viena pagrindinių gyvo organizmo dalelių. Jos atlieka daug įvairių funkcijų – struktūrinę, receptorinę, energetinę, fermentinę ir kitas. Tyrimo tikslas – dujų chromatografijos-masių spektrometrijos (DC-MS) metodu nustatyti ir įvertinti laisvųjų aminorūgščių kokybinę sudėtį *Cirsium vulgare* augalo žaliavos mėginiuose. Tyrimo metu buvo svarbu parinkti dygiosios usnies žaliavos ekstrahavimo sąlygas, pritaikyti dujų chromatografijos-masių spektrometrijos (DC-MS) metodą laisvosioms aminorūgštims aptikti iš Lietuvoje užaugintos *Cirsium vulgare* žaliavos bei nustatyti laisvųjų aminorūgščių kokybinę sudėtį iš *Cirsium vulgare* žaliavos ekstraktų, pagamintų iš augalo šaknų, lapų, žiedų ir sėklų. MTBSTFA (N-metil-N-(tret-butildimetilsilil)trifluoracetamidas) buvo atrinktas derivatizacijai atliekant laisvųjų aminorūgščių analizę. Taikant dujų chromatografiją-masių spektrometriją su junginių dariniais ir remiantis masių spektrine duomenų baze, dygiosios usnies žaliavoje buvo identifikuotos laisvosios aminorūgštys: L-alaninas, glicinas, L-valinas, L-leucinas, izoleucinas, L-prolinas, L-serinas, L-treoninas, L-fenilalaninas, L-lizinas, L-tirozinas, L-asparto rūgštis ir L-glutamo rūgštis. Tyrimai parodė, kad laisvųjų aminorūgščių kokybinė sudėtis priklauso nuo augalo vegetacijos fazės ir augalų dalies.