



# Determination of Selected Biologically Active Substances in Dietary Supplements and the Adaptogenic Herb *Rhaponticum carthamoides* Using HPLC–MS/MS

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## Abstract

*Rhaponticum carthamoides* (Willd.) Iljin, widely known as maral root, is an adaptogenic herb that has been used in traditional medicine for centuries. Several different classes of compounds were identified in *R. carthamoides*, including phytosteroids, flavonoids, and phenolic acids. Nowadays, *R. carthamoides* is available on the market in many forms, such as dried root, milled plant, capsules, and tinctures. This study presents the development and optimisation of four HPLC–MS/MS methods for the determination of 37 selected bioactive compounds in both plant and dietary supplements. Due to insufficient ionisation at the ion source, stigmasterol and  $\beta$ -sitosterol required derivatization, which was developed and optimised using 2-fluoro-1-methylpyridinium *p*-toluenesulfonate. The dried plant samples were extracted with 60% ethanol or *tert*-butyl methyl ether. The results showed that chlorogenic acid and 20-hydroxyecdysone were the most abundant. To reduce the dimensionality of the obtained data, factor analysis was applied. Based on the distribution of selected compounds, four clusters were observed: tinctures, seeds, root, and shoot systems of the herb. Using the developed methods, selected bioactive compounds in the herb and dietary supplements were successfully quantified, and their distribution in different organs of *R. carthamoides* was clarified.

**Keywords** *Rhaponticum carthamoides* · Adaptogen · HPLC–MS/MS · Polyphenols · Phytosteroids · Derivatization

## Introduction

*Rhaponticum carthamoides* (Willd.) Iljin is a perennial medicinal herb from the Asteraceae family, widely known as maral root. The herb is endemic to Siberia and Mongolia growing at altitudes ranging from 1200 to 2300 m above sea level (Głazowska et al. 2018). For millennia, *R. carthamoides* has been used in traditional medicine in cases of overstrain and weakness after illness. The first to notice the effects of *R. carthamoides* was Altai hunters. They observed *Cervus elaphus sibiricus* deers, also called maral deers, feeding on the roots of the herb and therefore being able to survive even harsh winters. Based on these observations, *R. carthamoides* was given its traditional name ‘maral

root’ (Kokořka and Janovská, 2009). Nowadays, *R. carthamoides* is classified as an adaptogenic herb. According to the definition first introduced by Dr. Nikolai Lazarev in 1947, adaptogens are substances with the ability to enhance non-specific resistance and adapt to exceptional stress factors in humans (Wagner et al. 1994). Adaptogens increase the ability of the organism to adjust to and prevent damage from environmental factors (Panossian et al. 1999).

Extracts of *R. carthamoides* have a wide range of biological activities, including anabolic (Roumanille et al. 2020), antioxidant (Skała et al. 2016), DNA repair-stimulating (Skała et al. 2016), antimicrobial (Jurkštienė et al. 2011), anticancer (Skała et al. 2018), hypolipidemic (Faizieva et al. 1999), and neuroprotective (Wu et al. 2017) effects. Several different classes of compounds are responsible for these beneficial properties, including phytosterols, flavonoids, phenolic acids, sesquiterpene lactones, and triterpenes (Kokořka and Janovská, 2009). The combination of the mentioned compounds contributes to and enhances the overall beneficial effect of *R. carthamoides*.

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*R. carthamoides* is available on the market in many forms, not only the whole plant and seeds for cultivation but also dried root, milled plant, capsules, and tinctures for consumption. *R. carthamoides* supplementation is recommended for stimulating both mental and physical sharpness and fortitude, for relief from overstrained muscles, fatigue, and weakness from illness. It is advertised as being helpful for those who suffer from anxiety, depression, and alcohol addiction. The herb is highlighted for its efficiency, excellent natural muscle-building properties, libido enhancement, and fitness recovery. One of the main compounds of *R. carthamoides* 20-hydroxyecdysone is available for purchase in isolated form and is marketed as a ‘natural anabolic agent’ for body-builders. Most of the products are recommended to be prepared as hot beverages by pouring boiling water over the dry plant material. *R. carthamoides* is often included in combined supplements containing more adaptogenic herbs such as *Withania somnifera* (Ashwagandha), *Schisandra chinensis*, *Rhodiola rosea*, *Eleutherococcus senticosus* (Siberian ginseng), and others. These adaptogenic products promote immunity, appetite, and productivity. Additionally, they ought to help with stress reduction, adaptation to extreme and stressful situations, or maintaining physical and mental abilities.

Nowadays, interest in traditional medicine is increasing. Many individuals seek adaptogenic herbs with other procedures as complementary and alternative to conventional medicine. With the high availability of dietary supplements comes the question of their quality. Each product, or even different batches of one product, may differ in the content of bioactive compounds depending on the plant’s origin, cultivation, or processing. For marketing purposes, some companies do laboratory testing of their products to determine the content of selected substances. One of the aims of this work was to analyse food supplements purchased on the Czech market and quantify selected biologically active substances. We developed four selective analytical methods and quantified 37 bioactive compounds in *R. carthamoides*. Based on these methods, we studied the content of each selected compound in different organs of *R. carthamoides* and the quality of dietary supplements.

## Materials and Methods

### Chemical and Reagents

Ethanol (Thermo Scientific, USA), methanol, and acetonitrile (Honeywell, Germany) were of LC–MS grade. Formic acid of LC–MS grade and *tert*-butyl methyl ether were obtained from Merck (Germany). Dimethyl sulfoxide p.a. was supplied by Lach-Ner (Czech Republic).

Derivatization agent 2-fluoro-1-methylpyridinium *p*-toluenesulfonate  $\geq 90\%$  was obtained from Sigma-Aldrich (USA). Triethylamine of high purity grade (VWR Life Science, USA) was dried on site.

Water was deionised and demineralised by 18.2 M  $\Omega$  cm Smart2Pure Barnstead (Thermo Scientific, USA).

Apigenin, apigenin-7-*O*-glucoside, gallic acid, isorhamnetin, isorhamnetin-3-*O*-glucoside, kaempferol, liquiritigenin, luteolin, luteolin-7-*O*-glucoside, naringenin-7-*O*-glucoside, and rutin were obtained from Extrasynthese (France). Biochanin A and prunetin were acquired from Fluka Chemistry (Switzerland). Salicylic acid and vanillic acid were obtained from Sigma-Aldrich (Germany). Daidzein, formononetin, genistein, genistin, naringin, puerarin, and scopoletin were supplied by Indofine Chemical Company (USA). Sophoricoside, tectoridin, and tectorigenin were purchased from Phytolab (Germany). Caffeic acid, hesperetin, chlorogenic acid, quercetin, and syringic acid were supplied from Sigma-Aldrich (China). Sinapic acid was acquired from Sigma-Aldrich (India), ferulic acid from Sigma-Aldrich (Switzerland), and naringenin from Sigma-Aldrich (UK). 20-Hydroxyecdysone, polypodine B, stigmasterol, and  $\beta$ -sitosterol were supplied from Sigma-Aldrich (USA).

Stigmasterol- $d_6$ ,  $\beta$ -sitosterol- $d_7$ , and vanillic acid- $d_5$  were acquired from Toronto Research Chemicals (Canada). Quercetin- $d_3$  and rutin- $d_3$  from Santa Cruz Biotechnology (USA). Daidzin- $^{13}C$ , formononetin- $^{13}C$ , genistein- $^{13}C$ , and genistin- $^{13}C$  were obtained from Dr. Nawaf al-Maharik (Al-Maharik and Botting 2010).

### Plant Materials and Dietary Supplements

Plant samples (S1; R1) were provided by the Botanical Garden of the Faculty of Science, Charles University. Seeds (D1–D2) were bought from two distinct suppliers.

Dietary supplements were purchased from different manufacturers on the Czech market. *R. carthamoides* is predominantly available as dried root (R2–R6) or encapsulated dried root (C1–C2) although dried shoot system (S2) and various tinctures (T1–T4) were also purchased. Notably, not all the manufacturers explicitly specify the herbal parts used in their supplements. Tincture compositions are occasionally labelled merely as ‘*Rhaponticum* in 40% alcohol’. Among the four purchased tinctures, two (T1; T4) do not provide information about the exact source of the plant material. Nevertheless, one of the tinctures we obtained was composed of leaf extract (T2) and another was derived from flower extract (T3).

Table 1 provides a summary and number of analysed samples, including their identifiers, types, and origins.

**Table 1** Summary of analysed samples

| Identifiers | Sample type       | Source             | Obtained from    |
|-------------|-------------------|--------------------|------------------|
| S1          | Shoot system      | Plant              | Botanical garden |
| S2          | Shoot system      | Dietary supplement | Brand 1          |
| R1          | Root system       | Plant              | Botanical garden |
| R2          | Chopped root      | Dietary supplement | Brand 2          |
| R3          | Chopped root      | Dietary supplement | Brand 3          |
| R4          | Tea bag (root)    | Dietary supplement | Brand 1          |
| R5          | Milled root       | Dietary supplement | Brand 4          |
| R6          | Milled root       | Dietary supplement | Brand 2          |
| C1          | Capsules (root)   | Dietary supplement | Brand 2          |
| C2          | Capsules (root)   | Dietary supplement | Brand 4          |
| T1          | Tincture (herb)   | Dietary supplement | Brand 5          |
| T2          | Tincture (leaf)   | Dietary supplement | Brand 6          |
| T3          | Tincture (flower) | Dietary supplement | Brand 7          |
| T4          | Tincture (herb)   | Dietary supplement | Brand 4          |
| D1          | Seed              | Plant              | Brand 8          |
| D2          | Seed              | Plant              | Brand 9          |

## Preparation of Standard and Stock Solutions

Pure analytical standards were accurately weighted and individually dissolved in methanol or ethanol to a concentration of  $500 \mu\text{g ml}^{-1}$ . In case of incomplete dissolution, dimethyl sulfoxide (DMSO) was added. For sinapic acid, DMSO was added to reach a final concentration of 20% (v/v). For other poorly soluble compounds—apigenin, apigenin-7-*O*-glucoside, biochanin A, daidzein, formononetin, genistein, genistin, hesperetin, isorhamnetin, isorhamnetin-3-*O*-glucoside, luteolin, luteolin-7-*O*-glucoside, naringin, prunetin, rutin, sophoricoside, tectoridin, stigmasterol, and stigmasterol- $\text{d}_6$  the final concentration of DMSO was adjusted to 10% (v/v). Certain standard solutions were mixed and further diluted with methanol to create a mixed stock solution at the desired concentration. All prepared solutions were stored at  $-20^\circ\text{C}$ .

## Sample Preparation

The plant's shoot (S1) and root systems (R1) were separated and dried for 1 month at laboratory temperature. Afterward, the dried herb parts (S1–S2; R1–R4) underwent homogenisation using an analytical mill, while seeds (D1–D2) were homogenised using pestle and mortar. Samples R5–R6 were already homogenised. Capsules (C1–C2) were opened, and their contents were emptied into the test tubes without homogenisation. Tinctures (T1–T4) were evaporated or diluted prior analysis. To address the variations in the levels of specific bioactive substances within the herb and dietary supplements, two sample dilutions were carried out for each class of compounds and sample types.

## Sample Preparation for 20-Hydroxyecdysone and Polypodine B Analysis

Homogenised plant parts (S1–S2; R1–R6), seeds (D1–D2), or content of capsules (C1–C2) (250 mg) were weighed in test tubes. 20-Hydroxyecdysone and polypodine B were extracted with 5 ml of 60% ethanol by continuous mechanical mixing on a tube rotator for 1 h. The samples underwent centrifugation (10 min, 3000 rpm,  $20^\circ\text{C}$ ). An aliquot (60  $\mu\text{l}$  or 3  $\mu\text{l}$ , respectively) of the supernatant was removed and diluted in a clean vial with 20% methanol to the total volume of 1 ml.

For tinctures (T1–T4), a pre-analysis dilution was performed by adding 10  $\mu\text{l}$  of tincture to a clean vial with 990  $\mu\text{l}$  of 20% methanol.

Calibration solutions of 20-hydroxyecdysone and polypodine B were prepared in 20% methanol at nine different concentrations in triplicate: 0.1, 0.5, 1, 5, 10, 50, 100, 500, and  $1000 \text{ ng ml}^{-1}$ .

## Sample Preparation for Polyphenol Analysis

Homogenised plant parts (S1–S2; R1–R6), seeds (D1–D2), or content of capsules (C1–C2) (250 mg) were weighed in test tubes. Polyphenols were extracted with 5 ml of 60% ethanol by continuous mechanical mixing on a tube rotator for 1 h. The samples were centrifuged (10 min, 3000 rpm,  $20^\circ\text{C}$ ). An aliquot (60  $\mu\text{l}$  or 3  $\mu\text{l}$ , respectively) of the supernatant was removed and transferred to a clean vial containing 40% methanol. If necessary, some samples were diluted (tenfold) to fit within the calibration curve range.

For seed samples (D1–D2), an additional sample preparation step was performed based on the hypothesis that polyphenols are present in higher concentrations in the aerial parts of the plant. A volume of 600  $\mu\text{l}$  of the prepared seed extract was evaporated under a stream of nitrogen, and the residue was subsequently reconstituted in 40% methanol.

For all samples, deuterated standards were added to the vial, so the final concentration of vanillic acid- $\text{d}_5$  was  $5000 \text{ ng ml}^{-1}$ , and daidzin- $^{13}\text{C}$ , formononetin- $^{13}\text{C}$ , genistein- $^{13}\text{C}$ , genistin- $^{13}\text{C}$ , quercetin- $\text{d}_3$ , and rutin- $\text{d}_3$  were  $100 \text{ ng ml}^{-1}$ . The total volume in the vial was 1 ml.

Tinctures (T1–T4) were diluted before analysis, and 60  $\mu\text{l}$  (respectively 3  $\mu\text{l}$ ) of tincture was added in a clean vial with 940  $\mu\text{l}$  (respectively 997  $\mu\text{l}$ ) of 40% methanol.

Calibration solutions of phenolic compounds were prepared in 40% methanol at nine different concentrations in triplicate: 0.1, 0.5, 1, 5, 10, 50, 100, 500, and  $1000 \text{ ng ml}^{-1}$ .

## Sample Preparation for Stigmasterol and $\beta$ -Sitosterol Analysis

Homogenised plant parts (S1–S2; R1–R6), seeds (D1–D2), or content of capsules (C1–C2) (250 mg) were weighed in

test tubes. Stigmasterol and  $\beta$ -sitosterol were extracted with 5 ml of *tert*-butyl methyl ether by continuous mechanical mixing on a tube rotator for 1 h. The samples were centrifuged (10 min, 3000 rpm, 20 °C). An aliquot (50  $\mu$ l or 250  $\mu$ l, respectively) of the supernatant was transferred into a clean test tube and evaporated under a nitrogen stream. After evaporation, derivatization reaction was performed.

The volume of 500  $\mu$ l (respectively 5  $\mu$ l) of tincture (T1–T4) was added to a clean test tube and evaporated under nitrogen steam. After evaporation, the derivatization reaction was performed.

For the quantification, the extract from *R. carthamoides* was spiked in triplicate at seven different levels of stigmasterol- $d_6$  and  $\beta$ -sitosterol- $d_7$  (100, 250, 500, 750, 1000, 1250, 1500 ng ml<sup>-1</sup>) and derivatised as described below and analysed.

### Manufacturer-Recommended Sample Preparation

The dietary supplements (S2; R2–R6) were also prepared according to the manufacturer's instructions without homogenisation in laboratory and extracted solely with water. Tea preparation procedures vary in amounts of herb material and water. Applied conditions are shown below.

**Chopped Root (R2–R4)** One litre of boiling water was poured over two teaspoons of chopped roots of sample R2. After 10 min, the extract was transferred into a clean test tube and centrifuged.

For root samples R3, boiling water (200 ml) was poured over 1 teaspoon of dried root. After 20 min, the extract was transferred into a clean test tube and centrifuged.

One tea bag of R4 was poured with 200 ml of boiling water. After 15 min, the extract was transferred into a clean test tube and centrifuged.

**Milled Root (R5–R6)** Supplements R5 and R6 were prepared by pouring 200 ml of boiling water over 1 teaspoon of milled roots and stirring. After 45 min, the extracts were transferred into a clean test tube and centrifuged.

**Shoot System (S2)** Half of the spoon of dietary supplement S2 containing shoot system was poured with 500 ml of boiling water. After 30 min, the extract was transferred into a clean test tube and centrifuged.

After centrifugation (10 min, 3000 rpm, 20 °C), supernatant aliquots of all samples were removed. For polyphenols and ecdysteroids analyses, aliquots (60  $\mu$ l or 3  $\mu$ l, respectively) were diluted in a clean vial with 40 or 20% methanol to the total volume of 1 ml. For stigmasterol and  $\beta$ -sitosterol, aliquots (50  $\mu$ l or 250  $\mu$ l, respectively) were transferred into clean test tubes and evaporated under a

nitrogen stream. After evaporation, the derivatization reaction was performed.

### Derivatization Procedure of Stigmasterol and $\beta$ -Sitosterol

The solution of the derivatization agent 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (25 mg ml<sup>-1</sup> in acetonitrile) was freshly prepared prior to the reaction. For each sample, 150  $\mu$ l of 2-fluoro-1-methylpyridinium *p*-toluenesulfonate solution, 6  $\mu$ l of triethylamine, and 350  $\mu$ l of acetonitrile were added to the evaporated standards or extracts. The mixture was ultrasonicated (10 s), vortexed (10 s), and then incubated (15 min, 50 °C). To stop the reaction, the sample was evaporated and then reconstituted using 1 ml of 20% methanol, centrifuged (10 min, 3000 rpm, 20 °C), and transferred to a vial.

### HPLC and MS Conditions

HPLC–MS/MS analyses were performed on an Infinity 1290 UHPLC system coupled with a 6460 triple quadrupole, both from Agilent Technologies. Four distinct HPLC–MS/MS methods were developed. Compound identification was based on their retention times compared to analytical standards, precursor ions  $[M + H]^+$ ,  $[M - H]^-$ ,  $[M - H_2O + H]^+$ , and specific multiple-reaction monitoring (MRM) fragmentation transitions.

### Analysis of 20-Hydroxyecdysone and Polypodine B

20-Hydroxyecdysone and polypodine B chromatographic separation was achieved within 8 min and conducted at 35 °C on a Zorbax Eclipse Plus C18 (2.1 mm  $\times$  50 mm, 1.8  $\mu$ m, Agilent Technologies) column with precolumn using a gradient elution of solvents A (water with 5 mM ammonium acetate and 0.35% acetic acid) and B (methanol). The gradient elution procedure was as follows: 0–2 min, 20–50% B; 2–5 min, 50–80% B; 5–5.5 min, 80–100% B; 5.5–6.5 min, 100% B; 6.5–6.7 min, 100–20% B; 6.7–8 min, 20% B using a flow rate of 0.3 ml min<sup>-1</sup>. The injection volume was 2  $\mu$ l.

Compounds were ionised in ESI+ mode and identified by dynamic multiple-reaction monitoring (dMRM) mode following transitions in Table 2. The MS conditions were optimised as follows: gas temperature 350 °C, gas flow 6 l min<sup>-1</sup>, nebulizer 35 psi, sheath gas temperature 350 °C, sheath gas flow 9 l min<sup>-1</sup>, capillary 3500 V, and nozzle voltage 1000 V. Based on linear regression analysis, calibration curves were constructed, and concentrations of analytes in dietary supplements and plant organs were calculated.

**Table 2** MRM transitions of 20-hydroxyecdysone and polypodine B (quantifier in bold)

| Compound           | ESI  | $t_R$<br>min | Precursor ion<br>$m/z$ | Product ion<br>$m/z$ | Fragmentor<br>V | Col-<br>lision<br>energy<br>eV |
|--------------------|------|--------------|------------------------|----------------------|-----------------|--------------------------------|
| 20-Hydroxyecdysone | ESI+ | 2.9          | 481.3                  | <b>445.3</b>         | 111             | 13                             |
|                    |      |              |                        | 371.2                | 105             | 13                             |
|                    |      |              |                        | 69.2                 | 105             | 49                             |
| Polypodine B       | ESI+ | 3.0          | 497.3                  | 91.1                 | 102             | 109                            |
|                    |      |              |                        | <b>81.1</b>          | 102             | 61                             |
|                    |      |              |                        | 69.2                 | 102             | 57                             |

### Analysis of Polyphenols

The chromatographic separation of polyphenols was achieved within 19 min and conducted at 35 °C on a Kinetex F5 (3.0 mm × 150 mm, 2.6 µm, Phenomenex) column with precolumn using a gradient elution of solvents A (water with 50 mM formic acid) and B (methanol). The gradient elution procedure was as follows: 0–10 min, 40–100% B; 10–14 min, 100% B; 14–15 min, 100–40% B; 15–19 min, 40% B using a flow rate of 0.5 ml min<sup>-1</sup>. The injection volume was 2 µl.

Polyphenols were determined using the same chromatographic conditions and two separate MS methods. Compounds were ionised in both ESI+ and ESI- modes and identified by dMRM modes following transitions as listed in Table 3. The MS conditions for ESI+ were optimised as follows: gas temperature 290 °C, gas flow 6 l min<sup>-1</sup>, nebulizer 35 psi, sheath gas temperature 400 °C, sheath gas flow 11 l min<sup>-1</sup>, capillary 5750 V, and nozzle voltage 2000 V. The MS conditions for ESI- were optimised as follows: gas temperature 290 °C, gas flow 4 l min<sup>-1</sup>, nebulizer 35 psi, sheath gas temperature 380 °C, sheath gas flow 10 l min<sup>-1</sup>, capillary 5000 V, and nozzle voltage 1800 V.

Using the peak-area ratios of the base peak ions of the quantifier ions versus the deuterated standard, calibration curves were constructed and concentrations of analytes in food supplements and plant organs were calculated.

### Analysis of Stigmasterol and β-Sitosterol

For stigmasterol and β-sitosterol, chromatographic separation was achieved within 13 min and conducted at 35 °C on a Kinetex XB-C18 (2.1 mm × 100 mm, 1.7 µm, Phenomenex) column with precolumn using a gradient elution of solvents A (water with 20 mM ammonium formate and 1% formic acid) and B (methanol with 20 mM ammonium formate and 1% formic acid). The gradient elution procedure was as follows: 0–6.5 min, 20–100% B; 6.5–10 min, 100% B; 10–10.5 min, 100–20% B; 10.5–13 min, 20% B using a flow rate of 0.3 ml min<sup>-1</sup>. The injection volume was 2 µl.

Compounds were ionised in ESI+ mode and identified by dMRM mode following transitions in Table 4. The MS conditions were optimised as follows: gas temperature 350 °C, gas flow 12 l min<sup>-1</sup>, nebulizer 50 psi, sheath gas temperature 400 °C, sheath gas flow 129 l min<sup>-1</sup>, capillary 400 V, and nozzle voltage 2000 V.

Based on linear regression analysis, calibration curves were constructed, and concentrations of analytes in dietary supplements and plant organs were calculated.

### Method Validation

The ICH guidelines (ICH 2005) were followed to validate the methods. Compounds were identified by peak retention time in MRM channels. For each analyte, a linear calibration curve was constructed. Linearity was evaluated by visual inspection of a plot of signals as a function of analyte concentration, and a regression line was calculated. The calibration curve was measured at the beginning of the run and at the end of the run. The limit of quantification (LOQ) is the lowest calibration standard, where the signal-to-noise ratio is 10. The limit of detection (LOD) is determined as the concentration of a standard where the signal-to-noise ratio is 3. Precision describes the closeness of repeated individual measures of analyte and is expressed as the relative standard deviation (RSD). Precision of the method was tested by intra-day (within-run; repeatability) and inter-day (between-run; intermediate precision) studies. Repeatability was assessed using ten injections at the highest level of the test concentration. For intermediate precision, inter-day experiments were performed by 12 injections in 3 consecutive days. The relative standard deviations of the retention times and peak areas were calculated. Accuracy describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte. For the newly validated compounds, stigmasterol, and β-sitosterol, accuracy was tested by analysing five replicates of standard samples from each of three concentrations: 500, 1000, 1500 ng ml<sup>-1</sup>. For the remaining compounds, accuracy was previously assessed by analysing five replicates at concentrations of 10 and 100 ng ml<sup>-1</sup>.

**Table 3** MRM transitions of polyphenols (quantifier in bold)

| Compound                          | ESI  | $t_R$<br>min | Precursor ion<br>$m/z$ | Product ion<br>$m/z$ | Fragmentor<br>V | Col-<br>lision<br>energy<br>eV |
|-----------------------------------|------|--------------|------------------------|----------------------|-----------------|--------------------------------|
| Apigenin                          | ESI- | 6.51         | 269.04                 | 151.1                | 108             | 17                             |
|                                   |      |              |                        | <b>117.0</b>         | 108             | 33                             |
|                                   |      |              |                        | 65.1                 | 108             | 37                             |
| Biochanin A                       | ESI- | 7.60         | 283.06                 | <b>268.0</b>         | 105             | 14                             |
|                                   |      |              |                        | 239.0                | 105             | 26                             |
|                                   |      |              |                        | 132.0                | 105             | 30                             |
| Caffeic acid                      | ESI- | 2.36         | 179.00                 | <b>135.1</b>         | 81              | 13                             |
|                                   |      |              |                        | 89.0                 | 81              | 30                             |
| Chlorogenic acid                  | ESI- | 1.93         | 353.09                 | 191.1                | 81              | 9                              |
| Daidzein                          | ESI- | 4.70         | 253.05                 | <b>132.1</b>         | 126             | 42                             |
|                                   |      |              |                        | 91.0                 | 126             | 34                             |
| Formononetin                      | ESI- | 6.36         | 267.06                 | <b>252.0</b>         | 108             | 13                             |
|                                   |      |              |                        | 223.0                | 108             | 25                             |
|                                   |      |              |                        | 132.0                | 108             | 29                             |
| Formononetin- $C_{13}$            | ESI- | 6.36         | 270.23                 | <b>255.1</b>         | 117             | 21                             |
|                                   |      |              |                        | 225.2                | 117             | 33                             |
| Gallic acid                       | ESI- | 1.55         | 169.01                 | <b>124.9</b>         | 75              | 9                              |
|                                   |      |              |                        | 112.9                | 75              | 5                              |
| Genistein                         | ESI- | 5.86         | 269.04                 | 171.0                | 123             | 26                             |
|                                   |      |              |                        | <b>133.0</b>         | 123             | 30                             |
|                                   |      |              |                        | 63.1                 | 123             | 34                             |
| Genistein- $C_{13}$               | ESI- | 5.86         | 272.00                 | 171.0                | 123             | 26                             |
|                                   |      |              |                        | 135.0                | 123             | 30                             |
|                                   |      |              |                        | <b>63.1</b>          | 123             | 34                             |
| Genistin- $C_{13}$                | ESI- | 3.23         | 434.50                 | <b>272.0</b>         | 162             | 10                             |
|                                   |      |              |                        | 241.0                | 162             | 46                             |
| Hesperetin                        | ESI- | 5.91         | 301.07                 | 286.0                | 108             | 9                              |
|                                   |      |              |                        | <b>164.0</b>         | 108             | 17                             |
| Liquiritigenin                    | ESI- | 4.63         | 255.06                 | 135.0                | 90              | 9                              |
|                                   |      |              |                        | <b>119.1</b>         | 90              | 21                             |
| Luteolin                          | ESI- | 5.85         | 285.04                 | 151.0                | 128             | 20                             |
|                                   |      |              |                        | <b>133.0</b>         | 128             | 33                             |
| Luteolin-7- <i>O</i> -glucoside   | ESI- | 3.54         | 447.09                 | <b>285.0</b>         | 151             | 25                             |
|                                   |      |              |                        | 284.1                | 151             | 33                             |
|                                   |      |              |                        | 133.0                | 151             | 20                             |
| Naringenin                        | ESI- | 5.66         | 271.06                 | 151.0                | 93              | 9                              |
|                                   |      |              |                        | <b>119.0</b>         | 93              | 21                             |
| Naringenin-7- <i>O</i> -glucoside | ESI- | 3.48         | 433.11                 | <b>271.1</b>         | 117             | 10                             |
|                                   |      |              |                        | 151.0                | 117             | 30                             |
|                                   |      |              |                        | 119.0                | 117             | 50                             |
| Naringin                          | ESI- | 3.34         | 579.17                 | 271.1                | 166             | 29                             |
|                                   |      |              |                        | <b>151.0</b>         | 166             | 49                             |
| Prunetin                          | ESI- | 7.36         | 283.06                 | <b>268.0</b>         | 111             | 13                             |
|                                   |      |              |                        | 239.0                | 111             | 25                             |
| Puerarin                          | ESI- | 1.96         | 415.10                 | 295.0                | 141             | 14                             |
|                                   |      |              |                        | <b>267.0</b>         | 141             | 30                             |
| Quercetin                         | ESI- | 5.53         | 301.03                 | <b>151.0</b>         | 106             | 13                             |
|                                   |      |              |                        | 121.1                | 106             | 24                             |

**Table 3** (continued)

| Compound                            | ESI  | $t_R$<br>min | Precursor ion<br>$m/z$ | Product ion<br>$m/z$ | Fragmentor<br>V | Col-<br>lision<br>energy<br>eV |
|-------------------------------------|------|--------------|------------------------|----------------------|-----------------|--------------------------------|
| Quercetin- $d_3$                    | ESI- | 5.53         | 304.05                 | <b>151.0</b>         | 124             | 21                             |
|                                     |      |              |                        | 65.2                 | 124             | 49                             |
| Rutin                               | ESI- | 3.48         | 609.14                 | 300.0                | 163             | 65                             |
|                                     |      |              |                        | <b>271.0</b>         | 163             | 61                             |
| Rutin- $d_3$                        | ESI- | 3.48         | 612.16                 | 326.9                | 206             | 69                             |
|                                     |      |              |                        | <b>303.1</b>         | 206             | 45                             |
| Salicylic acid                      | ESI- | 4.02         | 137.02                 | <b>93.1</b>          | 72              | 13                             |
|                                     |      |              |                        | 65.1                 | 72              | 29                             |
| Scopoletin                          | ESI- | 3.14         | 191.03                 | <b>176.0</b>         | 81              | 10                             |
|                                     |      |              |                        | 148.0                | 81              | 18                             |
|                                     |      |              |                        | 104.0                | 81              | 22                             |
| Sinapic acid                        | ESI- | 3.28         | 223.06                 | <b>208.1</b>         | 81              | 9                              |
|                                     |      |              |                        | 149.0                | 81              | 17                             |
|                                     |      |              |                        | 93.1                 | 81              | 37                             |
| Tectoridin                          | ESI- | 3.36         | 461.11                 | <b>299.0</b>         | 138             | 13                             |
|                                     |      |              |                        | 283.0                | 138             | 29                             |
| Tectorigenin                        | ESI- | 5.65         | 299.05                 | <b>284.0</b>         | 116             | 14                             |
|                                     |      |              |                        | 240.0                | 116             | 22                             |
| Apigenin-7- <i>O</i> -glucoside     | ESI+ | 4.10         | 433.12                 | <b>271.0</b>         | 109             | 13                             |
|                                     |      |              |                        | 153.0                | 93              | 60                             |
| Daidzin                             | ESI+ | 2.41         | 417.12                 | <b>255.0</b>         | 85              | 14                             |
|                                     |      |              |                        | 199.0                | 85              | 46                             |
|                                     |      |              |                        | 152.0                | 85              | 78                             |
| Daidzin- $C_{13}$                   | ESI+ | 2.41         | 420.37                 | <b>258.0</b>         | 88              | 13                             |
|                                     |      |              |                        | 200.0                | 88              | 45                             |
|                                     |      |              |                        | 138.0                | 88              | 53                             |
| Ferulic acid                        | ESI+ | 3.18         | 195.07                 | <b>177.0</b>         | 63              | 5                              |
|                                     |      |              |                        | 145.0                | 63              | 13                             |
| Homovanillic acid- $d_3$            | ESI+ | 2.28         | 188.00                 | <b>142.0</b>         | 47              | 9                              |
|                                     |      |              |                        | 127.1                | 47              | 25                             |
|                                     |      |              |                        | 99.2                 | 47              | 37                             |
| Isorhamnetin                        | ESI+ | 6.57         | 317.07                 | 191.9                | 95              | 53                             |
|                                     |      |              |                        | <b>153.0</b>         | 95              | 37                             |
|                                     |      |              |                        | 69.2                 | 95              | 97                             |
| Isorhamnetin-3- <i>O</i> -glucoside | ESI+ | 4.29         | 479.10                 | <b>317.0</b>         | 60              | 17                             |
|                                     |      |              |                        | 69.2                 | 60              | 101                            |
| Kaempferol                          | ESI+ | 6.30         | 287.06                 | <b>153.0</b>         | 161             | 41                             |
|                                     |      |              |                        | 69.1                 | 161             | 53                             |
| Quercetin-3- <i>O</i> -glucoside    | ESI+ | 3.62         | 465.10                 | <b>303.0</b>         | 60              | 17                             |
|                                     |      |              |                        | 259.8                | 60              | 129                            |
| Sophoricoside                       | ESI+ | 3.85         | 433.12                 | <b>271.0</b>         | 109             | 13                             |
|                                     |      |              |                        | 153.0                | 109             | 60                             |
| Syringic acid                       | ESI+ | 2.61         | 199.06                 | <b>140.1</b>         | 60              | 13                             |
|                                     |      |              |                        | 77.2                 | 60              | 25                             |
| Vanillic acid                       | ESI+ | 2.47         | 169.05                 | 125.1                | 78              | 5                              |
|                                     |      |              |                        | <b>65.2</b>          | 78              | 22                             |

**Table 4** MRM transitions of stigmasterol and  $\beta$ -sitosterol (quantifier in bold)

| Compound                   | ESI  | $t_R$<br>min | Precursor ion<br>$m/z$ | Product ion<br>$m/z$ | Fragmentor<br>V | Col-<br>lision<br>energy<br>eV |
|----------------------------|------|--------------|------------------------|----------------------|-----------------|--------------------------------|
| $\beta$ -Sitosterol        | ESI+ | 8.57         | 397.40                 | <b>81.2</b>          | 110             | 57                             |
|                            |      |              |                        | 67.2                 | 92              | 69                             |
|                            |      |              |                        | 55.3                 | 93              | 77                             |
| $\beta$ -Sitosterol- $d_7$ | ESI+ | 8.57         | 404.43                 | <b>81.2</b>          | 110             | 57                             |
|                            |      |              |                        | 67.3                 | 110             | 61                             |
|                            |      |              |                        | 55.3                 | 93              | 77                             |
| Stigmasterol               | ESI+ | 8.38         | 395.40                 | <b>55.3</b>          | 47              | 61                             |
|                            |      |              |                        | 91.1                 | 98              | 77                             |
|                            |      |              |                        | 81.2                 | 47              | 49                             |
| Stigmasterol- $d_6$        | ESI+ | 8.38         | 401.41                 | <b>55.3</b>          | 47              | 61                             |
|                            |      |              |                        | 91.1                 | 98              | 77                             |
|                            |      |              |                        | 81.2                 | 47              | 49                             |

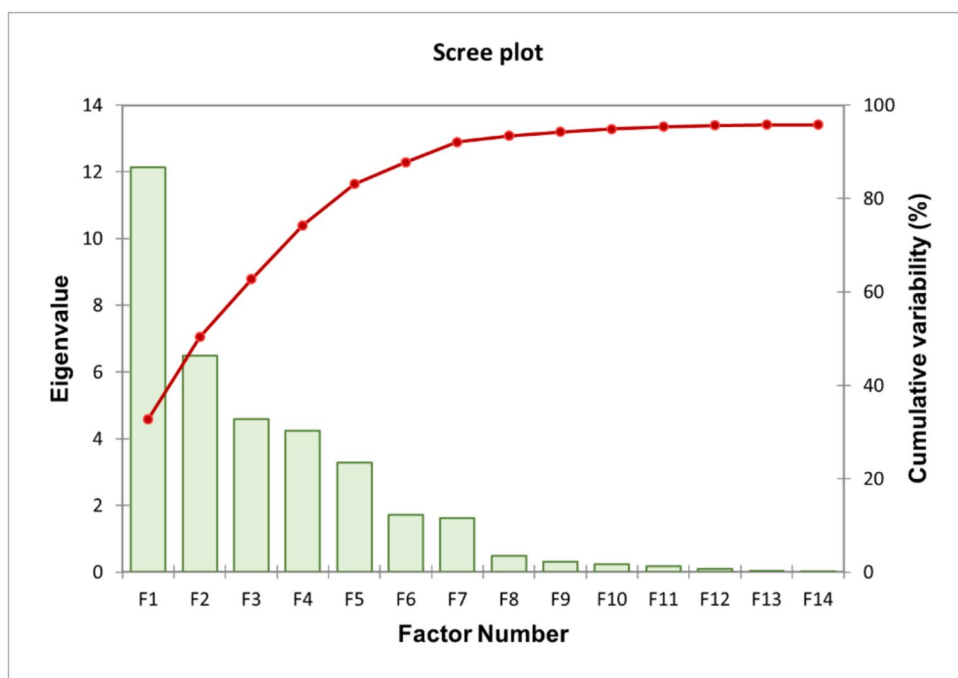
## Data Processing

To reduce the dimensionality of the data, factor analysis was applied using XLSTAT software. The Pearson ( $n$ ) correlation coefficient was used for correlation matrix calculation, indicating a statistically significant correlation. Principal component analysis (PCA) was utilised for factor extraction. Based on the Scree plot (Fig. 1), seven factors describing 92.1% of the cumulative variance (Table 5) were selected for the factor analysis itself.

## Results and Discussion

### Optimisation of the Sample Preparation, LC and MS Conditions

The extraction procedure was optimised to achieve the highest possible yield of analytes within a reasonable time frame. To determine the optimal extraction method, a variety of solvents for different compounds were tested. For 20-hydroxyecdysone, polypodine B and polyphenol extractions were performed using 50%, 60%, and 70% methanol, as well as 50%, 60%, and 70% ethanol, with extraction times of 30, 60,

**Fig. 1** Scree plot

**Table 5** Factor analysis

| Parameter/factor | F1   | F2   | F3   | F4   | F5   | F6   | F7   |
|------------------|------|------|------|------|------|------|------|
| Eigenvalue       | 12.1 | 6.5  | 4.6  | 4.2  | 3.3  | 1.7  | 1.6  |
| Variability (%)  | 32.8 | 17.5 | 12.4 | 11.5 | 8.9  | 4.7  | 4.4  |
| Cumulative %     | 32.8 | 50.3 | 62.7 | 74.2 | 83.1 | 87.7 | 92.1 |

and 120 min. Samples were ultrasonicated when extracted for 30 min only. The best results for 20-hydroxyecdysone, polypodine B, and polyphenols were obtained with extraction using 60% ethanol for 1 h.

Due to the lower polarity of stigmaterol and  $\beta$ -sitosterol, aforementioned conditions were not suitable for their extraction. Therefore, chloroform, dichloromethane, hexane, and *tert*-butyl methyl ether were tested. The extraction was carried out for an hour. While chloroform is commonly used for the extraction of stigmaterol (Oyedeji et al. 2021; Rajput and Rajput 2012), the poor ability of chloroform extracts to centrifuge and separate from the solid plant parts led to the selection of *tert*-butyl methyl ether, which proved more convenient with satisfactory yield.

Owing to the insufficient ionisation of stigmaterol and  $\beta$ -sitosterol in the electrospray ion source, the development of a derivatization reaction was necessary to enhance the ionisation. Various reagents, including dansyl chloride, picolinic acid, and 2-fluoro-1-methylpyridinium *p*-toluenesulfonate, were investigated. However, after extensive experiments under different conditions, derivatization using dansyl chloride and picolinic acid provided no product. This fact has been verified by thin-layer chromatography (TLC). Therefore, the 2-fluoro-1-methylpyridinium *p*-toluenesulfonate derivatization method was selected. Different conditions like concentrations of the derivatization agent (25–75 mg ml<sup>-1</sup>), environments (bicarbonate buffer, pH  $\approx$  10; acetonitrile), temperature (40–80 °C), and duration (10–20 min) of incubation were tested.

Based on previous experience, various mobile phase modifiers and columns were tested.

## Validation of Methods

The validation data of the HPLC–MS/MS methods are summarised in the Table 6. The analysis of polyphenols was carried out using modified methods previously described by Mikšátková et al. (Mikšátková, 2017; Mikšátková et al. 2014). For the construction of the calibration curves, a 1/x weighting was applied. Each calibration curve showed great linearity. Recoveries of polyphenols ranged from 88.4 to 101.1%, of stigmaterol and  $\beta$ -sitosterol from 85.4 to 103.8%, and for ecdysteroids from 89.3 to 102.5%. The repeatability of retention times of polyphenols was 0.01–0.31% (intraday) and 0.09–0.82% (interday), of stigmaterol and  $\beta$ -sitosterol was 0.04–0.05% (intraday) and 0.07–0.09% (interday), and

of ecdysteroids was 0.08–0.13% (intraday) and 0.07–0.14% (interday). The repeatability of peak area of polyphenols was 0.62–3.22% (intraday) and 1.52–6.25% (interday), of stigmaterol and  $\beta$ -sitosterol was 1.88–2.00% (intraday) and 3.80–4.46% (interday) of ecdysteroids was 1.58–1.93% (intraday) and 2.46–2.50% (interday). The accuracy of polyphenols ranged from -5.5 to 7.8% at 10 ng ml<sup>-1</sup> and -4.9 to 7.2% at 100 ng ml<sup>-1</sup>. For ecdysteroids, accuracy ranged from 1.4 to 2.2% at 10 ng ml<sup>-1</sup> and from 0.8 to 2.5% at 100 ng ml<sup>-1</sup>. For stigmaterol and  $\beta$ -sitosterol, accuracy values were 2.1% and -7.8% at 100 ng ml<sup>-1</sup>, 1.8% and 2.0% at 500 ng ml<sup>-1</sup>, and -1.4% and 0.1% at 1500 ng ml<sup>-1</sup>.

## Quantification of Selected Biologically Active Substances

The developed HPLC–MS/MS methods were successfully applied to the determination of bioactive substances. Overall, 37 bioactive compounds were quantified in samples of *R. carthamoides*. The contents of selected bioactive compounds, extracted using extraction solvent or prepared by manufacturer's instructions, were estimated as ng per gram of dry weight (ng g<sup>-1</sup>). The results are presented in the Table 7 and Table 8.

## Quantification of 20-Hydroxyecdysone and Polypodine B

Ecdysteroids have multifaceted roles in fundamental metabolic processes, including protein, carbohydrate, and lipid metabolism, alongside their neuroprotective properties (Wu et al. 2017). Moreover, their significant hypoglycaemic effects hint at potential applications in diabetes treatment (Alappat et al. 2010; Kutepova et al. 2001). Given these beneficial effects, quantifying ecdysteroids holds immense importance. In our investigation, we observed wide variation in ecdysteroid contents within a single gram sample, ranging from 208 ng to 5.4 mg. In dietary supplements, the levels averaged approximately half of those found in plants organs, possibly due to the various factors such as processing, age, climate (Martinussen et al. 2011), and other pertinent variables.

The predominant, most studied and analysed component of *R. carthamoides*, 20-hydroxyecdysone, is well-known in fitness communities for its ability to enhance physical performance. Interestingly, some studies even suggest that its anabolic effects may surpass those of certain

**Table 6** Summarized validation parameters

| Compound                            | $t_R$<br>min | Linearity<br>ng ml <sup>-1</sup> | Slope    | Y-intercept | $R$    | LOQ<br>ng ml <sup>-1</sup> | LOD<br>ng ml <sup>-1</sup> |
|-------------------------------------|--------------|----------------------------------|----------|-------------|--------|----------------------------|----------------------------|
| 20-Hydroxyecdysone                  | 2.96         | 0.1–1000                         | 2.35E+01 | 1.43E+01    | 0.9998 | 0.1                        | 0.03                       |
| Apigenin                            | 7.98         | 0.5–1000                         | 2.76E-02 | 5.26E-03    | 0.9979 | 0.5                        | 0.15                       |
| Apigenin-7- <i>O</i> -glucoside     | 5.54         | 0.1–1000                         | 9.07E-03 | -5.95E-04   | 0.9984 | 0.1                        | 0.03                       |
| β-Sitosterol                        | 8.68         | 100–1500                         | 7.75E-01 | 4.39E+01    | 0.9979 | 100                        | 30                         |
| Biochanin A                         | 8.97         | 0.1–1000                         | 2.26E-02 | 1.16E-03    | 0.9993 | 0.1                        | 0.03                       |
| Caffeic acid                        | 3.60         | 10–1000                          | 3.66E+01 | 2.47E+02    | 0.9988 | 10                         | 3                          |
| Daidzein                            | 6.01         | 0.1–1000                         | 1.53E-02 | 3.87E-03    | 0.9979 | 0.1                        | 0.03                       |
| Daidzin                             | 3.66         | 0.1–1000                         | 7.05E-03 | 3.48E-04    | 0.9996 | 0.1                        | 0.03                       |
| Ferulic acid                        | 4.65         | 1–1000                           | 9.99E-02 | -4.04E-02   | 0.9974 | 1                          | 0.3                        |
| Formononetin                        | 7.73         | 0.1–1000                         | 6.81E-03 | 1.00E-03    | 0.9993 | 0.1                        | 0.03                       |
| Gallic acid                         | 2.43         | 0.5–1000                         | 1.80E+01 | 9.37E+00    | 0.9988 | 0.5                        | 0.15                       |
| Genistein                           | 7.21         | 1–1000                           | 2.81E-02 | 1.48E-02    | 0.9988 | 1                          | 0.3                        |
| Hesperetin                          | 7.30         | 0.5–1000                         | 4.41E-04 | 3.90E-05    | 0.9995 | 0.5                        | 0.15                       |
| Chlorogenic acid                    | 3.01         | 0.1–1000                         | 2.43E+01 | 1.27E+01    | 0.9988 | 0.1                        | 0.03                       |
| Isorhamnetin                        | 6.97         | 5–1000                           | 7.80E-04 | -4.49E-03   | 0.9905 | 5                          | 1.5                        |
| Isorhamnetin-3- <i>O</i> -glucoside | 4.59         | 0.1–1000                         | 2.89E-03 | -3.19E-04   | 0.9910 | 0.1                        | 0.03                       |
| Kaempferol                          | 7.55         | 5–1000                           | 5.13E-04 | -2.51E-03   | 0.9952 | 5                          | 1.5                        |
| Liquiritigenin                      | 6.06         | 0.1–1000                         | 3.63E-02 | 6.86E-03    | 0.9943 | 0.1                        | 0.03                       |
| Luteolin                            | 7.23         | 5–1000                           | 3.67E-02 | -1.38E-01   | 0.9976 | 5                          | 1.5                        |
| Luteolin-7- <i>O</i> -glucoside     | 4.92         | 0.5–1000                         | 1.76E-02 | 1.09E-02    | 0.9952 | 0.5                        | 0.15                       |
| Naringenin                          | 7.05         | 0.5–1000                         | 7.63E-02 | 2.22E-02    | 0.9987 | 0.5                        | 0.15                       |
| Naringenin-7- <i>O</i> -glucoside   | 4.76         | 1–1000                           | 4.66E-03 | 8.13E-03    | 0.9965 | 1                          | 0.3                        |
| Naringin                            | 4.57         | 1–1000                           | 8.60E-03 | 6.10E-03    | 0.9980 | 1                          | 0.3                        |
| Polypodine B                        | 2.97         | 0.1–1000                         | 6.96E+00 | -6.96E+00   | 0.9978 | 0.1                        | 0.03                       |
| Prunetin                            | 9.10         | 0.1–1000                         | 2.29E-02 | 1.01E-03    | 0.9988 | 0.1                        | 0.03                       |
| Puerarin                            | 3.06         | 0.5–1000                         | 1.23E-02 | 2.47E-03    | 0.9985 | 0.5                        | 0.15                       |
| Quercetin                           | 6.70         | 5–1000                           | 1.38E-02 | 2.62E-02    | 0.9977 | 5                          | 1.5                        |
| Quercetin-3- <i>O</i> -glucoside    | 3.90         | 5–1000                           | 1.45E-03 | -7.33E-03   | 0.9912 | 5                          | 1.5                        |
| Rutin                               | 4.69         | 0.5–1000                         | 2.04E-03 | 4.34E-03    | 0.9972 | 0.5                        | 0.15                       |
| Salicylic acid                      | 4.29         | 0.5–1000                         | 4.39E+01 | 4.42E+01    | 0.9948 | 0.5                        | 0.15                       |
| Scopoletin                          | 4.84         | 1–1000                           | 1.40E+01 | 1.28E+01    | 0.9966 | 1                          | 0.3                        |
| Sinapic acid                        | 4.80         | 5–1000                           | 1.24E+00 | 3.02E+00    | 0.9830 | 5                          | 1.5                        |
| Sophoricoside                       | 5.45         | 0.5–1000                         | 1.08E-02 | -4.41E-03   | 0.9964 | 0.5                        | 0.15                       |
| Stigmasterol                        | 8.48         | 100–1500                         | 2.16E+00 | 3.06E+01    | 0.9996 | 100                        | 30                         |
| Syringic acid                       | 3.99         | 5–1000                           | 8.46E-03 | -2.10E-02   | 0.9965 | 5                          | 1.5                        |
| Tectoridin                          | 4.74         | 1–1000                           | 8.23E-03 | 6.64E-03    | 0.9976 | 1                          | 0.3                        |
| Tectorigenin                        | 7.00         | 0.1–1000                         | 1.00E-01 | -1.05E-02   | 0.9980 | 0.1                        | 0.03                       |
| Vanillic acid                       | 3.79         | 5–1000                           | 5.90E-03 | 5.42E-04    | 0.9984 | 5                          | 1.5                        |

anabolic–androgenic steroids (Chermnykh et al. 1988). 20-Hydroxyecdysone might potentially treat skeletal muscle atrophy by enhancing protein synthesis, promoting muscle fibre growth and regeneration of skeletal muscles (Muthita et al. 2016). In our study, 20-hydroxyecdysone was most abundant in root systems, constituting 0.45% of the sample, aligning closely with reported literature values ranging from 0.1 to 1% (Roumanille et al. 2020; Stránský et al. 1998). Conversely, in dried shoot system, the concentration was approximately 0.05%, lower than what was reported by

other researchers (Skała et al. 2015; Stránský et al. 1998). However, it is essential to acknowledge existing literature primarily focuses on 20-hydroxyecdysone levels in leaves, whereas our analysis encompasses the entire shoot system. The concentration of the lesser-known and less studied bioactive ecdysteroid of *R. carthamoides*, polypodine B, was on average exceeded by 20-hydroxyecdysone by sevenfold. Notably, the highest concentrations of both ecdysteroids were observed in milled plants or capsules filled with milled plant material, while tinctures exhibited the lowest levels.

**Table 7** The contents of bioactive compounds in the plant, shoot, and root supplements ( $n = 3$ ,  $\text{ng g}^{-1}$ )

| Sample type<br>Compound/sample       | Shoot system |              | Chopped root |             |             | Tea bag (root) |              |             | Milled root |             | Capsules (root) |  |
|--------------------------------------|--------------|--------------|--------------|-------------|-------------|----------------|--------------|-------------|-------------|-------------|-----------------|--|
|                                      | S1           | S2           | R1           | R2          | R3          | R4             | R5           | R6          | C1          | C2          |                 |  |
| 20-Hydroxyecdysone                   | 545,748.2    | 460,314.5    | 4,546,666.7  | 1,055,966.2 | 836,037.5   | 2,296,123.2    | 5,348,384.1  | 743,213.9   | 860,203.1   | 4,680,137.4 |                 |  |
| 20-Hydroxyecdysone*                  | –            | 618,545.2    | –            | 508,128.2   | 1,495,576.1 | 3,626,726.7    | 48,199.7     | 1,185,816.2 | –           | –           |                 |  |
| Apigenin                             | 1224.9       | 11,677.7     | 487.9        | 238.2       | 481.6       | 1472.8         | 1061.3       | 423.1       | 302.7       | 1043.7      |                 |  |
| Apigenin*                            | –            | ND           | –            | ND          | ND          | 329.2          | 891.6        | ND          | –           | –           |                 |  |
| Apigenin-7- <i>O</i> -glucoside      | 760.0        | 9758.9       | 276.5        | 161.5       | 127.3       | 742.2          | 1004.2       | 157.7       | 131.4       | 1247.4      |                 |  |
| Apigenin-7- <i>O</i> -glucoside*     | –            | 1361.3       | –            | ND          | ND          | 437.5          | 795.0        | ND          | –           | –           |                 |  |
| $\beta$ -Sitosterol                  | 34,369.9     | 94,674.6     | 39,903.2     | 50,880.2    | 35,918.8    | 80,598.9       | 36,349.2     | 9676.2      | 68,786.1    | 106,532.3   |                 |  |
| $\beta$ -Sitosterol*                 | –            | ND           | –            | ND          | ND          | ND             | ND           | ND          | –           | –           |                 |  |
| Biochanin A                          | ND           | ND           | ND           | ND          | ND          | ND             | ND           | ND          | ND          | ND          |                 |  |
| Biochanin A*                         | –            | ND           | –            | ND          | ND          | ND             | ND           | ND          | –           | –           |                 |  |
| Caffeic acid                         | 24,636.5     | 54,176.2     | 36,829.2     | 20,431.8    | 12,394.9    | 17,175.9       | 9254.9       | 18,237.0    | 18,320.6    | 65,856.5    |                 |  |
| Caffeic acid*                        | –            | 40,278.4     | –            | ND          | ND          | 15,189.7       | 12,338.2     | 13,095.6    | –           | –           |                 |  |
| Daidzein                             | ND           | ND           | ND           | ND          | ND          | ND             | ND           | ND          | ND          | ND          |                 |  |
| Daidzein*                            | –            | ND           | –            | ND          | ND          | ND             | ND           | ND          | –           | –           |                 |  |
| Daidzin                              | ND           | ND           | 9420.7       | 157.3       | 54.5        | 120.4          | ND           | 142.0       | 97.1        | ND          |                 |  |
| Daidzin*                             | –            | ND           | –            | ND          | ND          | ND             | ND           | ND          | –           | –           |                 |  |
| Ferulic acid                         | 1547.0       | 4226.7       | 3699.1       | 2822.2      | 1890.1      | 4848.9         | 8430.5       | 2668.9      | 2499.5      | 7397.4      |                 |  |
| Ferulic acid*                        | –            | 3652.4       | –            | ND          | 2042.9      | 4155.8         | 7065.4       | 2904.4      | –           | –           |                 |  |
| Formononetin                         | ND           | 10.7         | ND           | 231.7       | 44.6        | ND             | ND           | 127.8       | ND          | ND          |                 |  |
| Formononetin*                        | –            | ND           | –            | ND          | ND          | ND             | ND           | ND          | –           | –           |                 |  |
| Galic acid                           | 60,289.9     | 333,807.4    | ND           | 2430.7      | 4330.8      | 839.0          | 1497.4       | 2599.5      | 1907.4      | 3331.0      |                 |  |
| Galic acid*                          | –            | ND           | –            | ND          | ND          | ND             | ND           | ND          | –           | –           |                 |  |
| Hesperetin                           | 147.5        | 555.6        | 300.7        | 151.5       | ND          | 340.4          | 905.1        | 117.3       | ND          | 697.1       |                 |  |
| Hesperetin*                          | –            | ND           | –            | ND          | ND          | ND             | 552.0        | 550.3       | –           | –           |                 |  |
| Chlorogenic acid                     | 6,160,069.7  | 5,402,080.1  | 6,738,753.4  | 4,780,274.0 | 1,968,454.6 | 4,799,349.2    | 6,216,143.6  | 4,169,993.9 | 4,721,371.7 | 8,619,181.1 |                 |  |
| Chlorogenic acid*                    | –            | 11,043,685.7 | –            | 1,859,433.9 | 808,714.4   | 9,331,526.4    | 19,419,410.0 | 4,323,094.9 | –           | –           |                 |  |
| Isorhamnetin                         | 2841.7       | 5687.9       | 2739.1       | ND          | ND          | ND             | ND           | ND          | ND          | ND          |                 |  |
| Isorhamnetin*                        | –            | ND           | –            | ND          | ND          | ND             | ND           | ND          | –           | –           |                 |  |
| Isorhamnetin-3- <i>O</i> -glucoside  | ND           | ND           | 1124.5       | 4517.1      | 1731.9      | 1719.0         | 385.4        | 2439.2      | 2655.7      | 469.1       |                 |  |
| Isorhamnetin-3- <i>O</i> -glucoside* | –            | ND           | –            | ND          | 452.9       | 775.7          | 511.3        | 1012.3      | –           | –           |                 |  |
| Kaempferol                           | 5475.3       | 19,921.9     | ND           | 2382.4      | ND          | ND             | ND           | ND          | ND          | ND          |                 |  |
| Kaempferol*                          | –            | ND           | –            | ND          | ND          | ND             | ND           | ND          | –           | –           |                 |  |
| Liquiritigenin                       | ND           | ND           | ND           | 132.0       | ND          | ND             | ND           | 122.0       | 61.6        | ND          |                 |  |
| Liquiritigenin*                      | –            | 277.7        | –            | ND          | ND          | 569            | ND           | 673.5       | –           | –           |                 |  |
| Luteolin                             | 7041.2       | 9232.6       | 4628.1       | 5722.0      | 9599.1      | 8244.2         | 10,279.4     | 5226.0      | 5311.1      | 12,426.8    |                 |  |

Table 7 (continued)

| Sample type<br>Compound/sample | Shoot system |             | Chopped root |           |           | Tea bag (root) |           | Milled root |           | Capsules (root) |  |
|--------------------------------|--------------|-------------|--------------|-----------|-----------|----------------|-----------|-------------|-----------|-----------------|--|
|                                | S1           | S2          | R1           | R2        | R3        | R4             | R5        | R6          | C1        | C2              |  |
| Luteolin*                      | –            | 6852.8      | –            | ND        | 6917      | 6819           | 10,170    | ND          | –         | –               |  |
| Luteolin-7-O-glucoside         | 72,266.9     | 87,087.6    | 12,055.9     | 35,005.6  | 15,005.0  | 48,556.5       | 57,576.1  | 25,937.5    | 19,728.5  | 57,906.6        |  |
| Luteolin-7-O-glucoside*        | –            | 22,930.8    | –            | ND        | 1961      | 25,305         | 35,233    | 7955.4      | –         | –               |  |
| Naringenin                     | 6630.8       | 58,336.0    | 8869.3       | 1580.7    | 1220.6    | 2752.5         | 6153.5    | 928.7       | 724.0     | 12,385.7        |  |
| Naringenin*                    | –            | 44,161.4    | –            | ND        | ND        | 1053           | ND        | 1327.7      | –         | –               |  |
| Naringenin-7-O-glucoside       | 3,193,130.1  | 1,902,581.6 | 29,695.4     | 3601.0    | 1905.9    | 20,033.6       | 52,275.4  | 3074.0      | 2349.1    | 70,975.9        |  |
| Naringenin-7-O-glucoside*      | –            | 1,328,729.9 | –            | ND        | ND        | 11,993         | 39,896    | ND          | –         | –               |  |
| Naringin                       | 32,425.7     | 58,089.0    | 1242.9       | 1660.2    | 920.5     | 1149.2         | 1938.0    | 1426.5      | 1211.8    | 2757.0          |  |
| Naringin*                      | –            | 44,161.4    | –            | ND        | ND        | 1053           | ND        | 1327.7      | –         | –               |  |
| Polypodine B                   | 51,459.6     | 119,577.1   | 485,800.0    | 200,872.7 | 148,965.0 | 305,617.4      | 517,325.3 | 158,705.4   | 140,819.7 | 658,408.1       |  |
| Polypodine B*                  | –            | 150,805.3   | –            | 69,836.4  | 322,501.0 | 275,720.4      | 4662.1    | 155,971.5   | –         | –               |  |
| Prunetin                       | ND           | ND          | ND           | ND        | ND        | ND             | ND        | ND          | ND        | ND              |  |
| Prunetin*                      | –            | ND          | –            | ND        | ND        | ND             | ND        | ND          | –         | –               |  |
| Puerarin                       | ND           | ND          | ND           | ND        | ND        | ND             | ND        | ND          | ND        | ND              |  |
| Puerarin*                      | –            | ND          | –            | ND        | ND        | ND             | ND        | ND          | –         | –               |  |
| Quercetin                      | 7668.0       | 20,093.1    | 2111.4       | 8443.4    | 2719.3    | 1551.3         | ND        | 6428.7      | 4390.3    | ND              |  |
| Quercetin*                     | –            | ND          | –            | ND        | ND        | ND             | ND        | ND          | –         | –               |  |
| Quercetin-3-O-glucoside        | 3,565,032.8  | 522,192.2   | 32,094.5     | 24,196.8  | 8311.2    | 12,947.0       | ND        | 24,328.8    | 14,673.7  | ND              |  |
| Quercetin-3-O-glucoside*       | –            | 167,400.4   | –            | ND        | 9820.8    | ND             | ND        | 13,967.2    | –         | –               |  |
| Rutin                          | 46,934.5     | 32,647.2    | 1310.5       | 6480.3    | 4020.5    | 15,820.0       | 590.3     | 4979.3      | 5319.6    | 1191.0          |  |
| Rutin*                         | –            | 8004.2      | –            | ND        | ND        | 9089.5         | ND        | ND          | –         | –               |  |
| Salicylic acid                 | 2669.2       | 3728.1      | 97.3         | 1386.9    | 804.4     | 517.1          | 95.9      | 1382.8      | 1308.5    | ND              |  |
| Salicylic acid*                | –            | 756.2       | –            | ND        | ND        | ND             | ND        | ND          | –         | –               |  |
| Scopoletin                     | 1421.9       | ND          | ND           | ND        | ND        | ND             | ND        | ND          | ND        | ND              |  |
| Scopoletin*                    | –            | ND          | –            | ND        | ND        | ND             | ND        | ND          | –         | –               |  |
| Sinapic acid                   | ND           | ND          | ND           | ND        | ND        | ND             | ND        | ND          | ND        | ND              |  |
| Sinapic acid*                  | –            | ND          | –            | ND        | ND        | ND             | ND        | ND          | –         | –               |  |
| Sophoricoside                  | 270.3        | ND          | 206.2        | 283.6     | 206.2     | 337.1          | 435.7     | 238.0       | 280.9     | 586.8           |  |
| Sophoricoside*                 | –            | ND          | –            | ND        | ND        | 807.2          | 1324.2    | 431.3       | –         | –               |  |
| Stigmasterol                   | 60,823.5     | 19,952.1    | 47,557.2     | 37,242.8  | 3200.0    | 4543.7         | 3513.6    | 8296.7      | 30,938.5  | 4575.3          |  |
| Stigmasterol*                  | –            | ND          | –            | ND        | ND        | ND             | ND        | ND          | –         | –               |  |
| Syringic acid                  | 3979.4       | 5876.4      | 13,244.9     | 40,925.3  | 25,422.5  | 26,006.2       | 10,680.6  | 36,829.3    | 31,708.0  | 31,633.6        |  |
| Syringic acid*                 | –            | 9449.7      | –            | ND        | 26,807.6  | 26,323.0       | 13,313.6  | 21,376.3    | –         | –               |  |
| Tectoridin                     | 4167.1       | 8123.5      | ND           | 2742.2    | 840.9     | 828.5          | 196.0     | 2076.3      | 2007.9    | 557.9           |  |
| Tectoridin*                    | –            | 8438.6      | –            | ND        | ND        | 1130.1         | ND        | ND          | –         | –               |  |

Table 7 (continued)

| Sample type<br>Compound/sample | Shoot system |          | Chopped root |          |          | Tea bag (root) |        | Milled root |          | Capsules (root) |  |
|--------------------------------|--------------|----------|--------------|----------|----------|----------------|--------|-------------|----------|-----------------|--|
|                                | S1           | S2       | R1           | R2       | R3       | R4             | R5     | R6          | C1       | C2              |  |
| Tectorigenin                   | ND           | ND       | ND           | 74.9     | ND       | ND             | ND     | ND          | ND       | ND              |  |
| Tectorigenin*                  | –            | ND       | –            | ND       | ND       | ND             | ND     | ND          | –        | –               |  |
| Vanillic acid                  | 7798.6       | 37,662.1 | 8992.0       | 33,243.6 | 18,842.4 | 12,223.2       | 4009.0 | 31,279.6    | 29,116.2 | 8862.3          |  |
| Vanillic acid*                 | –            | 51,622.1 | –            | 23,841.7 | 25,367.5 | 14,531.2       | ND     | 32,875.3    | –        | –               |  |

Abbreviation: ND not detected

\*Compounds extracted by performing recommended preparation by manufacturer

– Samples without recommended preparation, or intended for direct consumption

Both 20-hydroxyecdysone and polypodine B were quantified in samples prepared by manufacturer's instructions. Averagely, the same amounts of ecdysteroids were extracted as in procedures using 60% ethanol as extraction solvent.

### Quantification of Polyphenols

Polyphenols are present in various herbs and plant-based foods, offering numerous medicinal properties and a wide range of therapeutic uses. Flavonoids demonstrate the ability to inhibit virus replication, with some showing higher antiviral activity compared to conventional drugs (Choi et al. 2009). They were found to have significant antioxidant activity and cytoprotective effect (Potapovich and Kostyuk 2003). Furthermore, flavonoids positively influence lipid metabolism, for example, by lowering the levels of cholesterol and triglycerides in the blood serum (Faizieva et al. 1999). Their cardioprotective properties contribute to preventing the progression of atherosclerosis and other related diseases such as stroke and heart attack (Salvamani et al. 2014). Foods rich in certain flavonoids may protect against some types of cancer (Le Marchand et al. 2000). Phenolic acids possess many biological effects, like antioxidant (Lodovici et al. 2001; Mudnic et al. 2010), DNA-protective (Lodovici et al. 2001), antimutagenic (Birošová et al. 2005), anti-cancer (Kampa et al. 2003), anti-inflammatory (Fernandez et al. 1998), vasodilatory (Mudnic et al. 2010), antibacterial (Sánchez-Maldonado et al. 2011), neuroprotective (Huang et al. 2008), and anti-diabetic (Jung et al. 2007). To find out the potential bioactivity of the *R. carthamoides* and its supplements based on the levels of polyphenols, the quantification is the key step.

The amounts of total phenols in *R. carthamoides* leaves following the literature were using spectrophotometry around 3.5 mg in 1 g of sample (Maslennikov, Chupakhina, Skrypnik 2014). Another team analysed roots using different spectrophotometric methods, the total phenolic content was 7 mg in 1 g of sample, and total flavonoid content was 3 mg in 1 g of sample (Petkova et al. 2021). Based on our study, the sum of quantified polyphenols in 1 g of a root or root samples was averagely 5.5 mg; in shoot system, the amounts were averagely 10 mg. The methods used in literature were aiming for quantification of total phenolic or flavonoid content, but in our study, we focused on quantification of selected compounds from these groups.

Based on our study, chlorogenic acid emerged as the most abundant compound, reaching a maximum amount of 8.6 mg and the average amount of 3.6 mg in 1 g of sample. The calculated amounts of each polyphenol are listed in Table 7 and Table 8. The quantification of some of the polyphenols was quite challenging. Biochanin A, daidzein, formononetin, liquiritigenin, prunetin, puerarin, scopoletin, sinapic acid, tectorigenin, isorhamnetin, and

**Table 8** The contents of bioactive compounds in tinctures ( $n = 3$ , ng ml<sup>-1</sup>) and seeds ( $n = 3$ , ng g<sup>-1</sup>)

| Sample type<br>Compound/sample      | Tinctures |             |          |             | Seeds       |           |
|-------------------------------------|-----------|-------------|----------|-------------|-------------|-----------|
|                                     | T1        | T2          | T3       | T4          | D1          | D2        |
| 20-Hydroxyecdysone                  | 1602.0    | 9845.1      | 5837.2   | 123,123.2   | 1,375,415.5 | 965,223.2 |
| Apigenin                            | 20.7      | 2658.0      | ND       | 2555.0      | 2251.5      | 11,781.3  |
| Apigenin-7- <i>O</i> -glucoside     | 5.6       | 564.9       | 1.7      | 258.7       | 139.5       | 470.5     |
| β-Sitosterol                        | ND        | ND          | ND       | ND          | ND          | ND        |
| Biochanin A                         | 35.3      | ND          | ND       | 201.4       | ND          | ND        |
| Caffeic acid                        | 2808.2    | 13,245.7    | 8262.1   | 39,385.8    | 2800.4      | 1532.5    |
| Daidzein                            | ND        | ND          | ND       | ND          | 54.1        | 3.0       |
| Daidzin                             | ND        | 5.3         | ND       | 1.2         | 1.7         | 1.4       |
| Ferulic acid                        | 336.1     | 336.0       | 44.0     | 2409.9      | 311.5       | 551.8     |
| Formononetin                        | ND        | ND          | ND       | 178.3       | ND          | ND        |
| Gallic acid                         | 459.0     | 30,275.8    | 639.1    | 10,301.9    | 388.2       | 381.6     |
| Hesperetin                          | ND        | 341.3       | ND       | 1778.0      | 14.2        | 35.4      |
| Chlorogenic acid                    | 4390.2    | 2,778,442.4 | 39,467.1 | 5,404,966.0 | 384,132.5   | 295,972.1 |
| Isorhamnetin                        | ND        | 775.7       | ND       | ND          | ND          | ND        |
| Isorhamnetin-3- <i>O</i> -glucoside | ND        | 2699.5      | 11.3     | 106.7       | 108.3       | 114.3     |
| Kaempferol                          | ND        | 3455.0      | ND       | ND          | ND          | ND        |
| Liquiritigenin                      | ND        | ND          | ND       | ND          | ND          | ND        |
| Luteolin                            | 93.8      | 2961.6      | 140.9    | 2691.7      | 1687.4      | 8049.8    |
| Luteolin-7- <i>O</i> -glucoside     | 7.9       | 3974.9      | 4.3      | 8468.2      | 4351.3      | 1995.1    |
| Naringenin                          | ND        | 6991.9      | 9.4      | 465.4       | 11.3        | 33.3      |
| Naringenin-7- <i>O</i> -glucoside   | 18.8      | 145,573.3   | 12.7     | 9059.8      | 360.3       | 659.6     |
| Naringin                            | 32.6      | 7774.2      | 45.0     | 461.4       | 16.7        | 55.1      |
| Polypodine B                        | 208.1     | 2245.6      | 472.1    | 14,285.2    | 377,291.6   | 308,416.3 |
| Prunetin                            | ND        | 81.6        | ND       | 200.7       | ND          | ND        |
| Puerarin                            | ND        | ND          | ND       | 7.0         | ND          | ND        |
| Quercetin                           | ND        | 6668.7      | ND       | 141.1       | ND          | ND        |
| Quercetin-3- <i>O</i> -glucoside    | ND        | 57,958.1    | 113.1    | ND          | 5249.4      | 3398.6    |
| Rutin                               | 315.6     | 1206.6      | 21.7     | 150.7       | 79.7        | ND        |
| Salicylic acid                      | 107.5     | 184.4       | ND       | 32.8        | 54.7        | 142.2     |
| Scopoletin                          | 24.7      | ND          | ND       | ND          | ND          | ND        |
| Sinapic acid                        | ND        | ND          | ND       | ND          | 160.1       | ND        |
| Sophoricoside                       | ND        | 57.3        | ND       | 40.0        | ND          | ND        |
| Stigmasterol                        | ND        | ND          | ND       | ND          | ND          | ND        |
| Syringic acid                       | 1427.4    | 583.0       | 205.5    | 4639.8      | 484.5       | 623.8     |
| Tectoridin                          | 2.8       | 516.0       | ND       | 199.2       | ND          | ND        |
| Tectorigenin                        | ND        | ND          | ND       | 42.3        | ND          | ND        |
| Vanillic acid                       | 2792.1    | 11,353.1    | 332.3    | 1721.1      | 982.2       | 1496.3    |

Abbreviation: *ND* not detected

kaempferol were quantified only in some of the samples. Following the manufacturer's recommended tea preparations and using water as an extraction solvent, 14 out of 33 polyphenols were not quantified in any sample. For those that were quantified, the amounts of most polyphenols in 1 g of the sample decreased, on average, 2 times lower compared to using 60% ethanol as extraction solvent. This suggests a notable decrease in the overall polyphenol content in teas prepared for consumption, despite the presence of these compounds in the herb and supplements.

### Quantification of Stigmasterol and β-Sitosterol

Stigmasterol and β-sitosterol play crucial roles in both food and pharmacy (Rashed 2020), exhibiting antioxidant (Panda et al. 2009; Paniagua-Pérez et al. 2008), anticancer (Bae et al. 2020; Park et al. 2007), anti-inflammatory (Gupta et al. 1980; Morgan et al. 2021), and cholesterol-lowering (Feng et al. 2018) properties. Stigmasterol serves as a precursor in hormone synthesis (for example progesterone) and has also shown immuno-modulatory (Alappat et al.

2010), neuroprotective (Karim et al. 2021), antinociceptive (Walker et al. 2017), and radioprotective (Hussein et al. 2022) effects.  $\beta$ -Sitosterol contributes to the stabilisation of cell membranes (Rashed 2020). Found across a wide array of plants, animals, and fungi, the quantification and comparison of these phytosterols in various herbs promise fascinating insights.

The amount of stigmasterol and  $\beta$ -sitosterol in 1 g of a sample ranged between 3.2  $\mu\text{g}$  and 1.1 mg (Table 7 and Table 8). On average,  $\beta$ -sitosterol was seven times more abundant than stigmasterol. Stigmasterol and  $\beta$ -sitosterol were not quantified in tinctures and seeds since tinctures are predominantly consist of ethanolic extracts, and both steroids require a less polar solvent for extraction from plant material. Additionally, when the supplements were prepared according to the manufacturer's instructions using only water, neither stigmasterol nor  $\beta$ -sitosterol was detected. Despite the detection of these steroids in the plant material or supplements when using a less polar solvent (*tert*-butyl methyl ether) for extraction, the intake of stigmasterol or  $\beta$ -sitosterol is not assured while drinking *R. carthamoides* teas without the herb material. This fact is caused by their poor ability to be extracted with polar solvents like water. However, if the herb is ingested, as in capsules containing the herb material, the outcome may differ due to the absorption in the human body. Therefore, purchasing other supplements rather than tinctures and teas is recommended when aiming for the consumption of stigmasterol and  $\beta$ -sitosterol.

In literature, stigmasterol and  $\beta$ -sitosterol were not quantified in dietary supplements containing *R. carthamoides*.

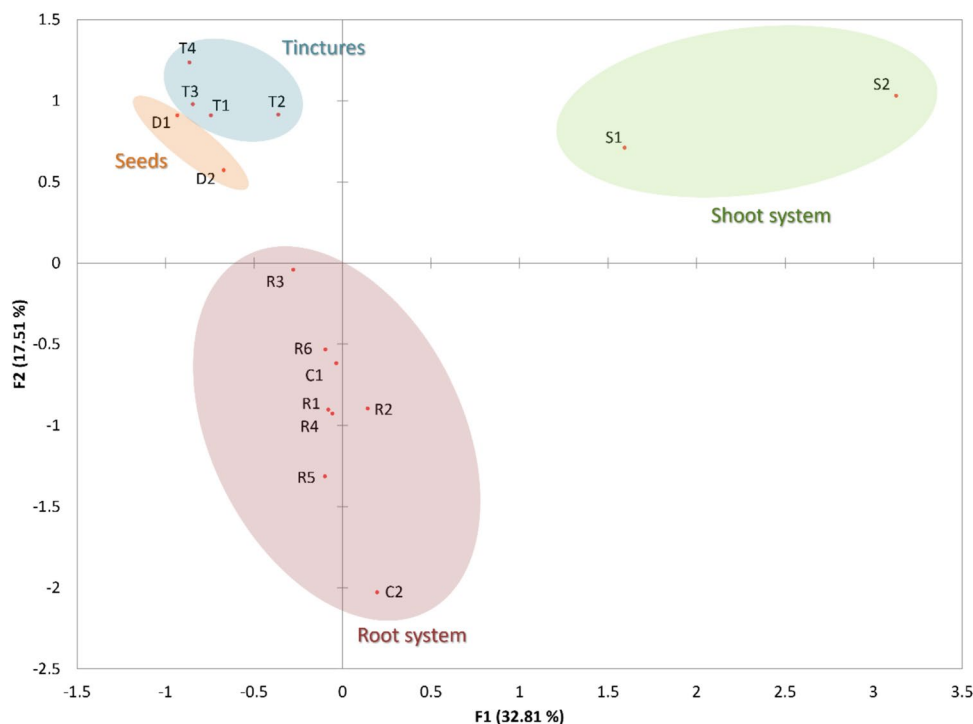
However, in other supplements containing herbs, the presence of these phytosterols was found to be at least 10  $\mu\text{g}$  per 1 g of sample (Sorenson and Sullivan 2019). Another study reported significantly higher levels of stigmasterol and  $\beta$ -sitosterol, averaging 100–1000 times more (Giammarioli et al. 2019). In our research, excluding tinctures, the average amounts of stigmasterol and  $\beta$ -sitosterol in plant and dietary supplements were 20  $\mu\text{g}$  and 55  $\mu\text{g}$  per 1 g of sample, respectively. The variability in the quantities of these phytosterols across different studies suggests a dependence on the specific plant or source material.

## Statistical Analysis

Due to the dimensionality of the data, the most effective way to interpret the results was to utilise multivariate statistical methods. Based on the hypothesis that unobserved latent variables influence cluster formation, and the group formation dependency on the type of supplement or product, factor analysis was applied. Only data obtained by the analysis of samples extracted with the extraction solvent (as presented in Table 7) were used for the factor analysis. The score plot (Fig. 2) generated by the comparison of Factor 1 (F1) (32.81%) and Factor 2 (F2) (17.51%) accounted for 50.32% of the total variance. Four distinct clusters (Fig. 2) were observed with great discrimination: tinctures, seeds, shoot system, and root system samples.

The superposition of the score and loading plots provided insight into the distribution of selected compounds in different organs of *R. carthamoides* and food supplements.

**Fig. 2** Score plot



Notably, chlorogenic acid emerged as the most abundant compound. With other phenolic acids, chlorogenic acid was spread throughout all samples (Table 7 and Table 8). Both the loading plot (Fig. 3) and length of sophoricoside's vector (marked as number 30) may indicate that the major component of the root system is sophoricoside itself. However, the length of the vector is affected by sophoricoside's occurrence predominantly in the root and its influence on the construction of the model using factor analysis. Indeed, the major components of the herb's root are 20-hydroxyecdysone and polypodine B (Table 7). Both ecdysteroids are responsible for anabolic properties and are the most characteristic compounds of *Rhaponticum carthamoides*. This corresponds to the fact that supplements targeted at bodybuilders contain the root of *R. carthamoides*.

In the shoot system, a high occurrence of flavonoid glycosides (Fig. 3) was distinctive, of which quercetin-3-*O*-glucoside and naringenin-7-*O*-glucoside being dominant (Table 7). Flavonoid glycosides are crucial components of leaves, and their content is affected by season and light levels due to their ability to protect against damage from solar radiation, particularly ultraviolet radiation (Groenbaek et al. 2019). Seeds and tinctures showed, as the only two groups, an increased isoflavonoid content (Fig. 3). However, the amount of all bioactive substances is much lower than in other samples (Table 8). Despite tinctures being produced by extracting different parts of the herb, they formed a single cluster (Fig. 2) regardless of the source material.

## Conclusion

In summary, the factor analysis provided insights into the distribution of selected bioactive compounds across different herb organs. The score plot revealed four distinct groups: tinctures, seeds, root, and shoot systems. Each supplement or organ group exhibited characteristic compound classes, with ecdysteroids prevailing in the root system, flavonoid glycosides in the shoot system and isoflavonoids in both tinctures and seeds. Phenolic acids were spread throughout all samples. The investigations unveiled significant variability in the contents of biologically active compounds in each dietary supplement obtained from the Czech market. Evaluating supplement quality proved challenging due to the dependence on various factors, including the type of supplement, daily dosage, origin of the plant material, collection location, season, climate, and other external conditions. Furthermore, the levels of bioactive compounds are influenced by the type of dosage administration, as several compounds remained undetected in teas prepared according to the manufacturer's guidelines, despite being quantified using less polar solvents for their extraction. On the contrary, the ingestion of milled supplements or capsules containing the herb may result in the further extraction of bioactive compounds within the digestive system. Simulating conditions within the human body, such as using gastric juices for compound extraction, could reveal the optimal way of preparing and consuming dietary supplements

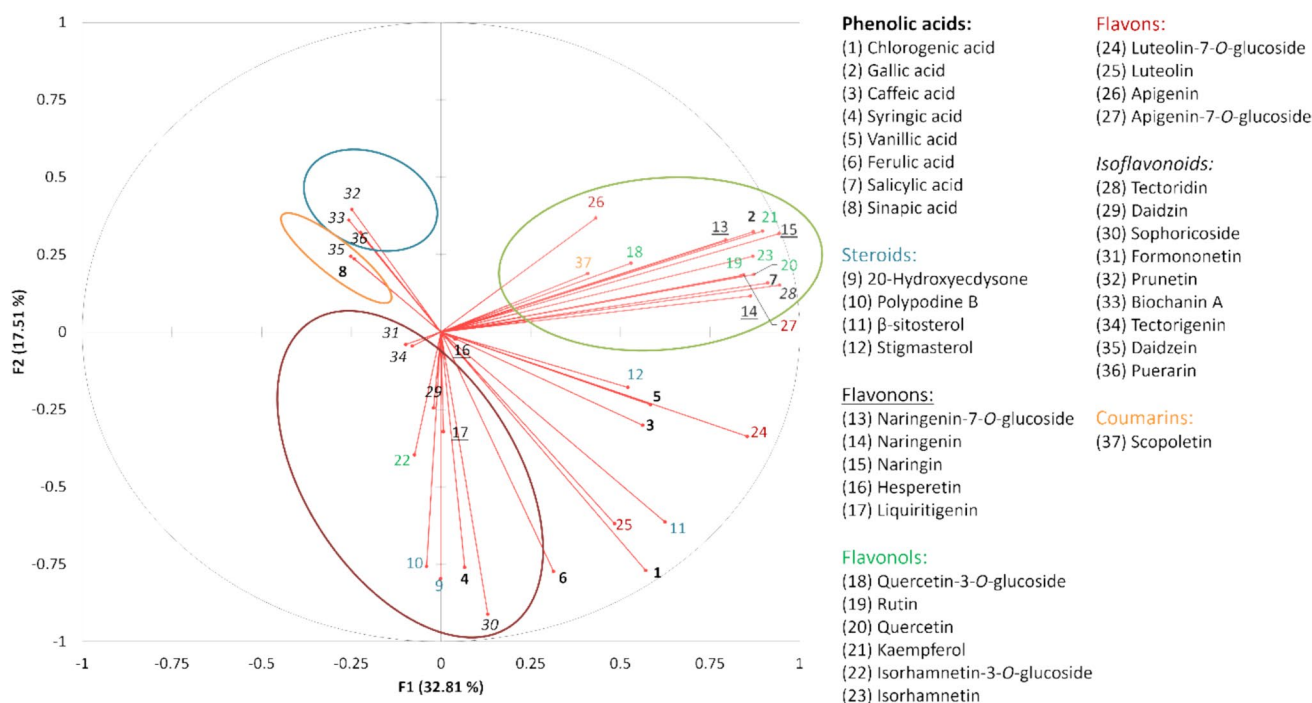


Fig. 3 Loading plot

to achieve the highest compound intake. Consumers aiming for a guaranteed intake of specific substances might find isolated products with standardized content to be the best solution. However, it is crucial to acknowledge that plants and plant extracts contain a complex of substances contributing to the supplement's biological activity. Moreover, the synergistic effect of various bioactive compounds can enhance the overall biological effect.

**Author Contribution** MK conceived the study idea and designed the research approach. KK and PC developed and optimized the HPLC–MS/MS analytical methods. KK conducted the market screening and home brew investigation. KK performed data analysis, including factor analysis. KK and MK performed data interpretation. KK and PC prepared the figures and tables. KK wrote the main manuscript text. All authors reviewed and approved the final manuscript.

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**Data Availability** The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

## Declarations

**Competing Interests** The authors declare no competing interests.

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