



Scanning of chicoric acid in different parts of *Cichorium intybus* by high-performance thin-layer chromatography with quantitation by image analysis

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Abstract

Cichorium intybus, commonly known as chicory, is a widespread plant, valued for its medicinal properties and use as a coffee substitute, chicoric acid being one of its key constituents. This study investigates the variation in chicoric acid content across different parts of the chicory plant—flowers, leaves, root and aerial parts. Three extraction methods, namely maceration in 70% ethanol, ultrasound-assisted extraction (UAE), and enzyme-assisted UAE were compared. A new optimized high-performance thin-layer chromatography (HPTLC) method was used to determine the chicoric acid quantities. The chicoric acid was separated on HPTLC silica gel F₂₅₄ plates developed with a mixture of ethyl acetate–formic acid–acetic acid–water (8:0.2:2:1.8, v/v) as the mobile phase. The detection was performed in UV light at 366 nm after derivatization with natural product (NP) and polyethylene glycol solution (PEG). The quantification was performed by image analysis (IA) using ImageJ software, with the green color channel offering the most sensitive detection (LOD = 2 ng/band and LOQ = 6 ng/band). The analysis revealed that leaves contained the highest concentration of chicoric acid, while roots exhibited the lowest. Enzyme-assisted UAE proved to be the most effective method. Antioxidant activity was tested using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical revealing that the chicoric acid possesses this activity. These findings highlight chicory leaves as a valuable source of chicoric acid and emphasize the importance of choosing effective extraction methods to maximize the yield of bioactive compounds.

Keywords High-performance thin-layer chromatography–image analysis (HPTLC–IA) · Chicoric acid · *Cichorium intybus* · Extraction methods · Antioxidant activity

1 Introduction

Since ancient times, plants have been used for therapeutic purposes due to their healing properties. Although initially many were used based solely on traditional knowledge, later research revealed that they contain substances with

therapeutic functions. With the advancement of science, synthetic methods have been developed for many therapeutic substances, but some active principles from plants cannot be replaced by laboratory-synthesized compounds.

Plant extracts are commonly utilized because they are rich in compounds with therapeutic actions. These extracts have superior compatibility with living organisms and offer various health benefits such as antioxidant, anti-inflammatory, hypoglycemic effects, and protection for the hepatic, gastrointestinal, nervous, and cardiac systems [1].

Cichorium intybus L. is a herbaceous plant from the *Asteraceae* family, widely spread in Romania in lowland and mountain areas, along roadsides and rivers. In terms of appearance, it has a thick root, a tap rooted and upright stem, which is branched and can grow up to 2 m tall. The basal leaves are elongated and hollowed, while the stem leaves are pointed at the tip and encircle the stem. The flowers are blue, white, or pink and are located axially or terminally, grouped

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singly on the stem and lateral branches. Known since ancient times by its common name, chicory, this plant has been used both for its medicinal properties and in salads or as a coffee substitute. The roots are a source of vitamins A, B, C, E, and K, as well as minerals such as potassium, calcium, phosphorus, zinc, magnesium, and inulin. Consuming chicory root lowers cholesterol levels, boosts the immune system, and reduces the risk of intestinal diseases [2]. Chicory extracts are useful for improving digestion, combating coughs, and anemia [3]. Some of chicory's main health benefits include detoxifying the body, stimulating digestion, and promoting insulin secretion, which leads to a reduction in blood sugar levels [2, 3]. *C. intybus* contains sesquiterpene lactones, caffeic acid derivatives, inulin, sugars, proteins, flavonoids, oils, volatile compounds, alkaloids, steroids, terpenoids, coumarins, hydroxycoumarins, vitamins, and polyphenols [1–5].

Chicoric acid is a natural compound with several health benefits. Chemically, it is a caffeic acid diester of tartaric acid and belongs to the polyphenol class (Fig. 1). In organisms, after ingestion, their main activity is antioxidant, preventing the formation of free radicals [6]. Chicoric acid is found in a variety of plant species, such as chicory (*C. intybus* L.), echinacea (*Echinacea purpurea*), and dandelion (*Taraxacum officinale*), particularly within the *Asteraceae* family [7].

According to literature, chicoric acid was previously isolated and determined from leaves, aerial parts and seeds of *C. intybus* by chromatographic (mainly high-performance liquid chromatography) and spectrometric methods [2, 8–12].

This study aims to develop a new high-performance thin-layer chromatography (HPTLC) method for the quantification of chicoric acid to check whether this target compounds are present or not in different part of plant and to compare its content. Also, the developed method was used to compare various extraction methods of chicoric acid from different parts of the plant, namely maceration, ultrasound-assisted extraction (UAE), and enzyme-assisted UAE with cellulase (E.C. 3.2.1.4) as a biocatalyst. Furthermore, antioxidant

activity of the chicoric acid was evaluated in the prepared extracts.

2 Experiments

2.1 Reagents and chemicals

All reagents used in the study were of analytical grade, as specified by the suppliers. Ethanol (96%), glacial acetic acid ($\geq 99.7\%$), hydrochloric acid (37%), 1,1-diphenyl-2-picrylhydrazyl radical, (DPPH $\cdot$, $\geq 98.0\%$), polyethylene glycol 400 (macrogol, tested according to Ph. Eur.), 2-aminoethyl diphenylborinate (97.0%), and chicoric acid ($\geq 95.0\%$) were purchased from Merck (Darmstadt, Germany). Cellulase was purchased from Pangbo Enzyme (Guangxi, China). The solvents used for chromatographic analysis were purchased from Millipore (Bedford, MA, USA) with the following purities: methanol ($\geq 99.8\%$), ethyl acetate ($\geq 99.5\%$), and formic acid ($\geq 98.0\%$).

2.2 Plant material

The plant material was collected from the village of Strâmbu, Cluj County, during July and August of 2022 and 2023. It was certified at the Alexandru Borza Botanical Garden in Cluj-Napoca under identification number 61134. The fresh plants were air-dried in the dark at room temperature (22–24 °C) in a well-ventilated space. Afterward, the material was finely grounded for 5 min at 29 Hz to achieve a uniformly granulated powder using Retsch Mixer Mill MM 400.

2.3 Preparation of the extracts

The preparation of the extracts followed the same procedure for each extraction method. Separate extracts were prepared from roots, leaves, flowers, and aerial parts using each extraction method (Table 1).

The ratio of plant material to extraction solvent was consistent across all methods, with 1 g of plant material to 10 mL of solvent. The solvent used for maceration was 70% ethanol and the samples were left in the dark for 10 days and shaken daily. The UAE were performed in Emmi-20 HC (EMAG, Germany) ultrasonic bath at 30 °C temperature for 30 min using 70% ethanol as solvent. For enzyme-assisted UAE, the amount of cellulase added to each sample was calculated to represent 3% relative to plant material weight [13]. The extractions were performed in the ultrasonic bath at 55 °C for 40 min, using 55% ethanol as solvent.

After each extraction, the supernatants were separated from the solid residue by centrifugation using a Centurion Scientific centrifuge C2006 (Centurion Scientific Limited,

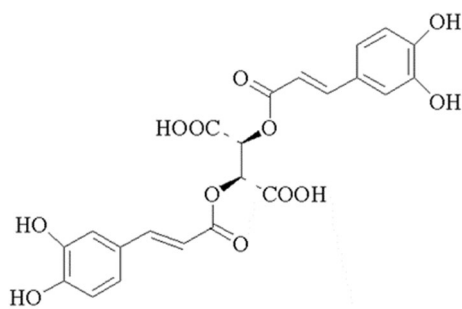


Fig. 1 Chicoric acid

Table 1 Plant materials, extraction methods, and codification

Extract	Extraction method	Part of the plant
M1	Maceration	Flowers
M2	Maceration	Leaves
M3	Maceration	Aerial parts
M4	Maceration	Root
U1	UAE	Flowers
U2	UAE	Leaves
U3	UAE	Aerial parts
U4	UAE	Root
EE1	Enzyme-assisted UAE	Flowers
EE2	Enzyme-assisted UAE	Leaves
EE3	Enzyme-assisted UAE	Aerial parts
EE4	Enzyme-assisted UAE	Root

Bosham, UK) and the extracts were kept at 4 °C for further analysis.

2.4 Quantification of chicoric acid by HPTLC–IA

For the quantitative analysis and determination using high-performance thin-layer chromatography, normal-phase chromatographic plates (HPTLC Silica Gel 60 F₂₅₄, 20 × 10 cm, 1,056,420,001) were used. To quantify chicoric acid in the extracts, a calibration curve was first established. The calibration curve was established on 0.1–3 µg/band domain by applying different volumes of standard ethanolic solution of chicoric acid (0.1 mg/mL) on the chromatographic plates as 8 mm bands with application speed of 70 nL/s. In the case of extracts, aliquots of 5 µL of flower and leaf extracts and 10 µL of root and aerial parts extracts were applied on plates as 8 mm bands with application speed of 70 nL/s. All bands were applied at 1.5 cm from the bottom on the plate using CAMAG Linomat 5 applicator (Muttenez, Switzerland). The optimum mobile phase is a mixture of ethyl acetate–formic acid–acetic acid–water (8:0.2:2:1.8, v/v). The plates were developed over a distance of 7.5 cm in a twin-through chromatographic chamber presaturated for 30 min with mobile phase. After developing with the mobile phase, to enhance fluorescence, the plates were immersed first in NP natural product (NP) reagent and then in polyethylene glycol solution (PEG). The NP reagent was prepared by weighing 1 g of 2-aminodiphenylborinate and dissolving it in 200 mL of ethyl acetate with agitation until fully dissolved. PEG was prepared by dissolving 10 g of polyethylene glycol in 200 mL of dichloromethane [14]. Before immersion, the plates were heated at 100 °C for 3 min and then the hot plates were immersed in NP solution. After drying, the plates were derivatized with PEG. Immersion was performed using an automatic immersion device (CAMAG) with a vertical speed of 35 mm/s, for 3 s. In all instances, the final treated plates

were documented at 366 nm with a TLC visualizer device (Digistore 2, CAMAG) and the images were stored as JPEG files. winCats software controlled all CAMAG instruments. The ImageJ software was used to create the densitograms and integrate the peaks [14].

The calibration curve was constructed by using ImageJ software to process the image of the original plate and after splitting in red, blue and green channels to obtain the peak areas after subtracting the background. The LODs and LOQs were calculated using the formulas:

$$\text{LOD} = 3\sigma/s \text{ and } \text{LOQ} = 10\sigma/s$$

where σ represents the standard deviation of the residuals and s denotes the value of the slope of the curve.

2.5 Screening for antioxidant activity: DPPH assay

To assess the antioxidant activity of chicoric acid separated by thin-layer chromatography from the extracts, the DPPH· radical was used. DPPH· is a stable organic radical with a violet color that turns yellow in the presence of antioxidant compounds. The DPPH· solution was prepared at a concentration of 0.03% by dissolving 0.0750 g of DPPH· in 250 mL of ethanol.

The chromatographic plates were immersed in the preprepared DPPH· solution using the automatic immersion device for 1 s. Afterward, the plates were dried for 30 min in the dark, resulting in plates with yellowish bands on violet–blue background, indicating the presence of antioxidant compounds [13, 14].

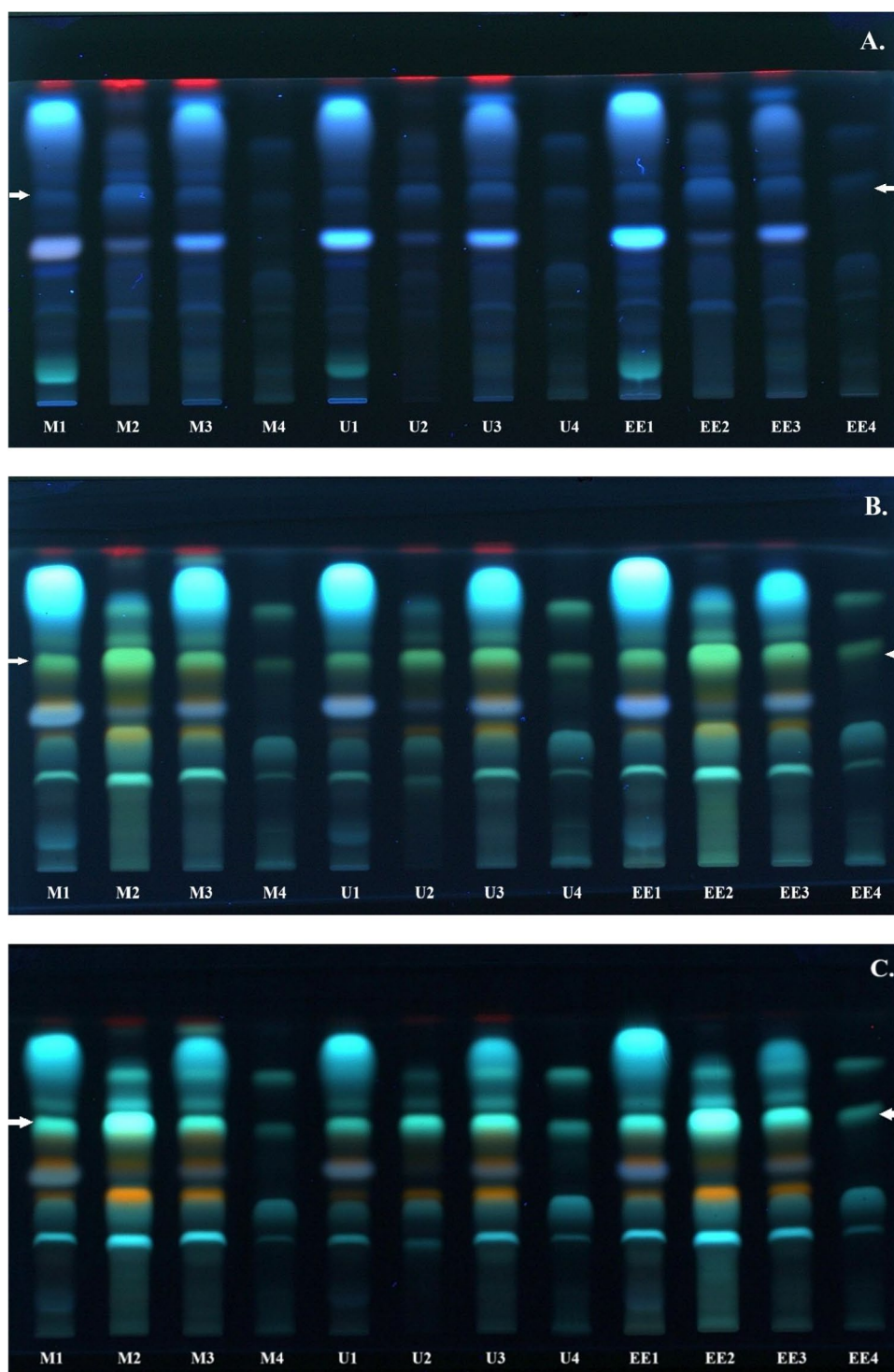
2.6 Statistics

Results for the calculated concentrations of chicoric acid in original, red, green, and blue channels were analyzed with a one-way analysis of variance (ANOVA) at a 95% confidence level with the statistical software for MS Excel. The difference between the methods is considered significant if the calculated p -value is lower than 0.05 and the F -value is higher than F critical.

3 Results and discussion

Multiple combinations of various solvents and ratios [15–18] were evaluated to create an effective method for analyzing chicoric acid. The following mobile phases were tested: formic acid–ethyl acetate–water (1:0.5:8.5, v/v), ethyl acetate–acetic acid–water (50:5.5:5.5, v/v), and ethyl acetate–formic acid–acetic acid–water (10:1.1:1.1:1.7, v/v), but chicoric acid separation was not effective. A reversed-phase plate using formic acid–acetonitrile (7:3, v/v) was

Fig. 2 The images of the chromatographic plate in UV light at 366 nm: **A** before derivatization, **B** after derivatization with NP, **C** after derivatization with PEG. The following codification is used: M, maceration; U, UAE; EE, enzyme-assisted UAE; 1, flowers; 2, leaves; 3, aerial parts; 4, root

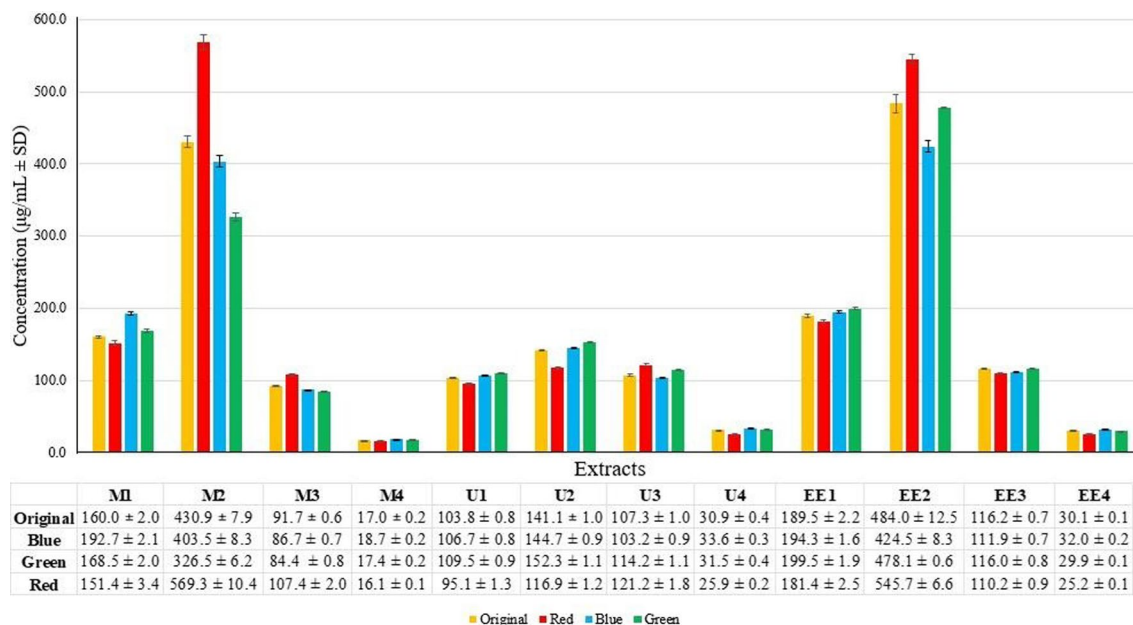
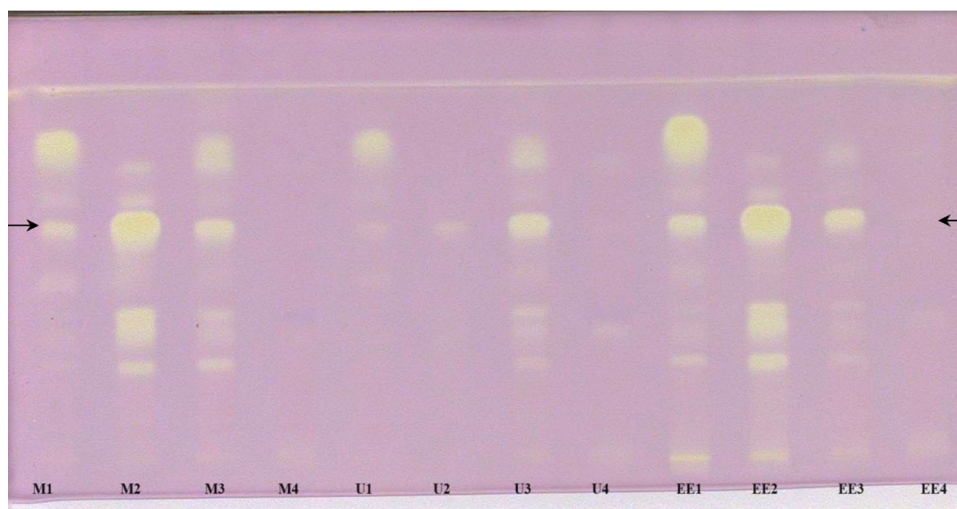


also tested, but this was ineffective as chicoric acid, a polar compound, did not interact with the stationary phase. A good separation was achieved using the mobile phase ethyl acetate–formic acid–acetic acid–water (10:1.1:1.1:1.7, *v/v*). To optimize the separation the solvent ratio is adjusted at ethyl acetate–formic acid–acetic acid–water (8:0.2:2:1.8,

v/v). This mobile phase was finally chosen because it provided the most favorable results, with the bands generated from the target compounds distributed at the R_F of 0.65 and effective separation of the target substance. Moreover, the specificity of the HPTLC method was determined by comparing the R_F value of the chicoric acid in a sample with that

Table 2 The characteristics of the calibration curves and the obtained limit of detection and limit of quantitation

Channel	Polynomial regression	Coefficient of determination	LOD (ng/band)	LOQ (ng/band)
Original plate	$y = -793.85x^2 + 4698.2x - 451.05$	$R^2 = 0.9996$	6	21
Blue	$y = -918.18x^2 + 5304.0x - 519.53$	$R^2 = 0.9993$	4	13
Green	$y = -1155.8x^2 + 6090.5x - 424.19$	$R^2 = 0.9970$	2	6
Red	$y = -181.83x^2 + 1739.7x - 192.73$	$R^2 = 0.9996$	3	9

**Fig. 3** Concentrations of chicoric acid ($\mu\text{g/mL} \pm \text{SD}$, $n = 3$) in the analyzed extracts. The following codification is used: M, maceration; U, UAE; EE, enzyme-assisted UAE; 1, flowers; 2, leaves; 3, aerial parts; 4, root**Fig. 4** Antioxidant activity evaluation of the samples. The following codification is used: M, maceration; U, UAE; EE, enzyme-assisted UAE; 1, flowers; 2, leaves; 3, aerial parts; 4, root

of the standard chicoric acid solution. NP/PEG derivatization was used to increase the sensitivity of detection for the compound of interest, making it more visible on the HPTLC

plate (Fig. 2). Additionally, no light green bands corresponding to the R_F value of chicoric acid were detected in the

samples, indicating that the other compounds present in the extracts did not affect the identification of chicoric acid.

The image of the HPTLC plate was analyzed using ImageJ. Calibration curves relating peak area to concentration were created from the original plates and after splitting the image of plate in red, green, and blue channels. The polynomial regression data indicated a robust relationship across the concentration range of 0.1–3 µg/spot for all approaches ($R^2 > 0.9970$) (Table 2).

The LOD and LOQ values (Table 2) revealed that the developed method shows a high sensitivity for the investigated compound (chicoric acid). The lowest LOD is obtained for the chromatographic plate in green channel (2 ng/band) and the highest for the original plate (6 ng/band).

The calculated concentrations of chicoric acid in µg/mL for all approaches (12 *C. intybus* extracts on the original plate and after splitting in color channels) determined by HPTLC–IA are shown in Fig. 3.

The ANOVA test generated the following results for F value: 0.00, blue/green; 0.01, original/green; 0.01, original/blue; 0.04, original/red; 0.07, red/blue; 0.09, red/green; and F_{crit} value was 4.30. The results show that there is no significant variance between the concentration of chicoric acid determined from the image of original plate and from splitting image at a confidence level of 95%. Concluding the determination of chicoric acid could be performed in any way obtaining results that do not differ significantly, but it is preferable to be determined from the green channel image, where the sensitivity of the determination is the lowest (lowest LOD and LOQ value).

It is noted that chicoric acid is present in the highest quantities in leaf samples; however, its concentration in the extracts depends on the extraction method used. In nearly all cases, the most effective extraction method is enzyme-assisted UAE, as the addition of enzymes facilitates the faster degradation of the cell wall compared to other extraction methods. It is noted that in the case of the extracts from leaves and flowers, UAE is less effective than maceration and enzyme-assisted UAE. From all extracts, root samples contain the least amount of chicoric acid and the extracts from leaves contain the highest.

To evaluate the antioxidant activity of chicoric acid in the samples, the chromatographic plate was dipped in a DPPH solution and the image of plate in visible light after 30 min is shown in Fig. 4.

It can be observed that where antioxidant-active compounds are present, the violet background changes to pale yellow. The intensity of the yellow coloration is directly proportional to the antioxidant activity of the compound or, in this study, to the amount of chicoric acid contained in the extract. In the case of leaf extracts obtained through maceration (M2) and enzyme-assisted UAE (EE2), the yellow color is more pronounced due to a higher amount of chicoric acid.

Additionally, yellow coloration is also observed in extracts from aerial parts (M3, U3, EE3) and flower extracts (M1, U1, EE1), while the root extracts show almost no color change since chicoric acid is present in very small quantities in that part of the plant.

4 Conclusions

The study succeeds to develop a sensitive, rapid, and cost-effective HPTLC–IA method for analysis of chicoric acid in different parts of *C. intybus* L. The results show that the most sensitive determination is the one in the green channel, but there is no significant difference between the results obtained from the original plate or from blue and red channels.

The results also highlight the effectiveness of the various extraction methods in maximizing chicoric acid recovery.

The experimental results revealed that the aerial parts of the plant are richer in chicoric acid compared to the root, and the leaves contain the highest quantity of the tested compound. The antioxidant activity of chicoric acid was assessed using the DPPH radical showing that the target compound exhibits antioxidant activity.

Author contributions Conceptualization: Claudia Cimpoiu. Methodology: Claudia Cimpoiu. Software: Julia Baumli and Alina-Ioana Mărinean. Validation: Claudia Cimpoiu and Julia Baumli. Formal analysis: Julia Baumli and Ioana-Alina Mărinean. Investigation: Claudia Cimpoiu, Ioana-Alina Mărinean, and Julia Baumli. Resources: Claudia Cimpoiu. Writing—original draft preparation: Claudia Cimpoiu and Julia Baumli. Writing—review and editing: Claudia Cimpoiu and Julia Baumli. Supervision: Claudia Cimpoiu. Project administration: Claudia Cimpoiu.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Competing interests The authors assert that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper. The corresponding author, Claudia Cimpoiu, is a member of the editorial board of the journal. Therefore, the submission was handled by a different member of the editorial board, and he did not take part in the review process in any capacity.

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