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Antioxidant Activity of Sulfate Metabolites of Chlorogenic Acid

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Abstract: This study aimed to determine the antioxidant properties of the sulfate monoesters of ferulic, caffeic, dihydroferulic and dihydrocaffeic acids, the main metabolites of chlorogenic acids. These compounds are not commercially available, so they were synthesized in the laboratory. The LC-MS/MS analysis allowed for the full characterization of these derivatives, which has made them reliable standards for further research. Purified metabolites including ferulic acid-4-*O*-sulfate, caffeic acid-4-*O*-sulfate and caffeic acid-3-*O*-sulfate, dihydrocaffeic acid-4-*O*-sulfate and caffeic acid-3-*O*-sulfate were examined for their antioxidant capacities and compared to their precursor compounds using Folin–Ciocalteu, CUPRAC (cupric ion—reducing) and DPPH• (2,2-diphenyl-1-picrylhydrazyl) methods. This study shows that hydrogenation of caffeic and ferulic acids into dihydrocaffeic and dihydroferulic acids has a positive influence on their reducing properties. Moreover, all synthesized sulfate monoesters exhibited very weak antioxidant properties compared to precursor compounds. The presented results show that the transformation of phenolic acids via sulfation leads to the inhibition of antioxidant properties due to the blockage of hydroxyl groups.

Keywords: phenolic acids; sulfate metabolites; antioxidant activity; LC-MS/MS

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Citation: Rogozinska, M.; Lisiecki, K.; Czarnocki, Z.; Biesaga, M. Antioxidant Activity of Sulfate Metabolites of Chlorogenic Acid. *Appl. Sci.* **2023**, *13*, 2192. https://doi.org/10.3390/app13042192

Academic Editor: Snezana Agatonovic-Kustrin

Received: 13 December 2022
Revised: 4 February 2023
Accepted: 6 February 2023
Published: 8 February 2023

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1. Introduction

Recently, a vast number of studies on free radicals and oxidative stress have been conducted due to their roles in many noncommunicable diseases, i.e., cardiovascular diseases, Alzheimer's disease, cancer and skin aging [1–4]. Studies have indicated that a diet rich in polyphenols has health-promoting properties and acts as a defensive shield in organisms fighting against degenerative processes in the human body. Phenolic acids are a group of polyphenols mainly occurring in honey, plants, food and beverages of plants origin [5–7]. The total intake of various polyphenols can be up to 1 g per diem, which is associated with a diet rich in fruits and vegetables [8,9]. The esters formed between quinic acid and cinnamic acids such as caffeic and ferulic acids are called chlorogenic acids (CGAs). These compounds are among the most common plant polyphenols found in the human diet [10]. Therefore, in recent years, their metabolism has become a popular subject of research. Both in vivo and in vitro studies proved that CGAs are extensively metabolized in the human body [11–15]. The metabolism of CGAs includes hydrolysis to quinic acid and different hydroxycinnamates, mainly caffeic and ferulic acids, making them available for further absorption [16–18]. Additionally, various conjugated metabolites, such as glucuronides and/or sulfates of caffeic and ferulic acids, have been detected in urine and plasma samples after CGA administration, while the precursor CGAs were found only in trace amounts [12,13,19,20]. Moreover, previous in vitro and in vivo data highlight that the main metabolites excreted in urine after CGA administration were free hydrogenated analogues of caffeic and ferulic acids and their sulfate conjugates [12,15,20–23]. For this reason, studies on biological responses cannot be carried out without taking into consideration that the metabolic processes of phenolic compounds may change their positive capacities. *O*-sulfation and *O*-glucuronidation reactions may significantly reduce or even inhibit antioxidant activity due to the blocking of hydroxyl groups, which are responsible for this property, in the phenolic structure [24–27]. Manach et al. [28] demonstrated that some

glucuronide and sulfate derivatives of quercetin retain antioxidant properties, although the magnitude of these properties is about half that measured for unconjugated quercetin. In addition, it was proven that the antioxidant activity of the purified CGA dimer was significantly enhanced after simulated digestion in vitro [29]. Piazzon et al. [30] proved that *O*-sulfation of caffeic and ferulic acids leads to the inhibition of their antioxidant properties. In parallel, changes in hydrophobicity could significantly influence the reducing capacity of these compounds. Information on the antioxidant activity of phenolic metabolites seems to be key to understanding their potential effects on human health.

In this paper, we aim at estimating the antioxidant properties of sulfate conjugates of caffeic and ferulic acids and, for the first time, the antioxidant properties of sulfate conjugates of dihydrocaffeic and dihydroferulic with regard to their precursor compounds. To our knowledge, there has never been a comprehensive screening study examining the antioxidant capacity of the major CGAs metabolites. As these compounds are not available as standards commercially, we synthesized them and purified the sulfate conjugates of the main metabolites of CGAs: ferulic, caffeic, dihydroferulic and dihydrocaffeic acids (Figure 1).

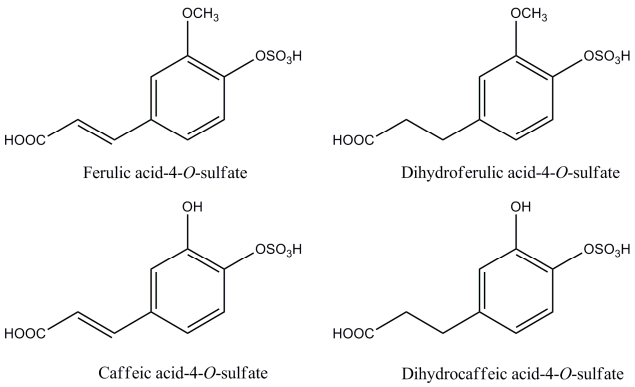


Figure 1. Chemical formulas of ferulic, caffeic, dihydroferulic and dihydrocaffeic acid sulfate metabolites.

2. Materials and Methods

2.1. Chemicals and Reagents

The commercial phenolic acid standards and the rest of the chemicals were purchased from Sigma (Steinheim, Germany), while methanol was purchased from Merck (Darmstadt, Germany). For all experiments reported in this paper, we used ultrapure water from the Milli-Q system with an electrical resistivity of 18 MΩ × cm (Millipore, Darmstadt, Germany). Moreover, we prepared stock solutions of phenolic acids into methanol and filtered all solutions through 0.45 μm membranes (Millipore) and degassed them prior to use.

2.2. LC-MS/MS Analysis

In this study, we applied the same separation method as that used in our previous works. It has been described and discussed in detail, i.e., [31]. LC-MS/MS analysis was performed using an LC20 liquid chromatograph (Shimadzu, Kyoto, Japan), which was coupled to a 3200 QTRAP mass spectrometer (Applied Biosystem/MDS SCIEX, Waltham, MA, USA). The chromatograph consisted of the following components: two LC20-AD high-pressure pumps, a DGU-20A5 degasser, a CTO-20AC thermostat and an SIL-20AC autosampler. A Chromolith Performance C18 Column (100 × 2 mm, Merck) was used to separate the studied compounds. The separation was carried out at 30 °C. The mobile phase (eluent A, HCOOH, pH 2.8, eluent B, MeOH) was delivered at 0.2 mL/min in gradient mode: 0–3 min 10% B, 20–25 min 50% B, 26–40 min 10% B. Injections were carried out with an autosampler maintained at 5 °C. Electrospray ionization (ESI) conditions were as follows: capillary temperature 450 °C, curtain and auxiliary nitrogen gas 0.3 MPa, negative ionization mode source voltage 4.5 kV. Standard solutions were infused into the electrospray source via a 50 μm i.d. PEEK capillary using a Harward Apparatus pump at

10 µL/min. Mass spectra were obtained by scanning m/z from 50 to 650. The compound-specific parametersfor MS/MS determination were optimized for each compound: entrance potential (EP), declustering potential (DP) and collision cell entrance potential (CEP) for deprotonated pseudomolecular ions. Optimal collision energy (CE) and collision cell exit potential (CXP) values were determined for the most intense fragment ions. Identification of compounds was carried out by comparing retention time and m/z values obtained by MS and MS2 (product ion scan mode) with the mass spectra of standards tested under the same conditions. For each compound, the optimum conditions of single reaction mode (SRM) were determined in infusion mode (Table 1). Additional information about the structure of synthesized compounds was obtained with MS3 experiments using linear ion trap (LIT).

Table 1. LC-MS/MS characteristics of phenolic compounds in the negative mode.

Compound	Retention Time, min	Q1 Mass	Q3 Mass	DP, V	CE, V
Dihydrocaffeic acid	8.4	181	137	−35	−10
Caffeic acid	10.5	179	135	−10	−24
Dihydroferulic acid	13.7	195	136	−55	−22
Ferulic acid	15.3	193	134	−30	−20
		193	178	−30	−16

2.3. Spectrophotometric Measurements

Spectrophotometric measurements were performed at room temperature with the use of a BIOSENS model VIS V-5800 spectrophotometer. For the proposed analysis, absorbance was measured in the visible range of wavelengths characteristic of DPPH• via CUPRAC and Folin–Ciocalteu (FC) assays with 0.2 nm resolution in 10 mm polystyrene cells. We used WinLab software v. 2.85.04 for the processing of the data.

2.3.1. DPPH• Assay

A DPPH• (2,2-diphenyl-1-picrylhydrazyl) assay was used to measure the antioxidant activity of pure phenolic acids and their sulfate conjugates. For this purpose, aliquots (0.1 mL) of analyzing samples (25 µg/mL in methanol) were added to 2.4 mL of DPPH• solution (3×10^{-5} mol/L) in methanol, and the change in absorbance at λ = 539 nm was measured over 20 min against the blank. Results are expressed as % inhibition of DPPH• according to the expression $[(A0 - At)/A0] \times 100$, where (A0) is the initial absorbance and (At) is the absorbance at time (t) as well as Trolox equivalent (TE).

2.3.2. Folin–Ciocalteu (FC) Assay

A 1 mL aliquot of the examined compound (25 µg/mL in methanol) was mixed in a tube with 0.1 mL of Folin–Ciocalteu reagent (FC) and 0.9 mL of water and left for 5 min. After that time had elapsed, we added 1 mL of Na_2CO_3 aqueous solution (70 g/L) and 0.4 mL of water. Afterward, we left the tube containing the prepared mixture for 30 min for stabilization. The absorbance against the blank at λ = 765 nm was measured. We used gallic acid as a reference compound. The antioxidant activity of the analyzed compound was expressed as a gallic acid equivalent (GAE).

2.3.3. CUPRAC Assay

A 1 mL aliquot of $CuCl_2$ (0.01 mol/L) was mixed with 1 mL of neocuproine methanol solution (0.0075 mol/L) and 1 mL of acetate buffer (1 mol/L, pH = 7). Then, 0.5 mL of the analyzed compound (25 µg/mL in methanol) and 0.6 mL of H_2O were added. The tube with the prepared mixture was incubated in a water bath at 50 °C for 20 min; afterward, we cooled it and analyzed it. The absorbance at λ = 450 nm was measured against the blank. We prepared the calibration curve using Trolox as a reference compound. The antioxidant activity of the examined phenolic acid/phenolic acid ester was expressed as Trolox equivalent (TE).

2.4. Synthesis, Purification and Identification of Sulfate Conjugates of Ferulic Acid, Dihydroferulic Acid, Caffeic Acid and Dihydrocaffeic Acid

We followed Todd et al.'s [32] method to synthesize esters. Briefly, $ClSO_3H$ (3 eq) was added dropwise to the required substrate (ferulic acid, dihydroferulic acid, caffeic acid, dihydrocaffeic acid) (1 eq) in pyridine (6 eq) with stirring at room temperature. After 30 min of stirring, we added ice-cold water and extracted the reaction mixture with diethyl ether (Et_2O), basified with Na_2CO_3. H_2O was removed under vacuum. The residue was triturated with H_2O, neutralized with HCl to pH = 7 and dried under vacuum. The final product was triturated in MeOH and filtrated. Purification and identification of MeOH-soluble residues were followed by HPLC with mass and UV detection.

3. Results

3.1. Characterization of Sulfate Derivatives of Ferulic, Caffeic, Dihydroferulic and Dihydrocaffeic Acids

Three main peaks were observed on the total ion chromatogram (TIC) obtained for the mixture after synthesis of the ferulic acid sulfate ester (Figure 2A). Peak 1 (P1), with a retention time of 12.8 min, produced a negatively charged pseudomolecular ion, $[M\text{-}H]^-$, at m/z = 273, corresponding to the value expected for ferulic acid-4-*O*-sulfate. The major fragment of the analyzed compound produced an ion at m/z = 193 characteristic of ferulic acid. A loss of 80 atomic mass units (amu) indicates cleavage of the sulfate moiety. Fragmentation ions produced in linear ion trap (MS3) with m/z 178 and 149 were characteristic for fragmentation pathway of a standard of ferulic acid (Figure 2C). The retention time (14.8 min) and SRM pairs of the second peak (P2) were characteristic of ferulic acid, which was the main substrate used for the synthesis. The last peak (P3) was identified as an impurity. During the purification process, only the P1 fraction (10–13 min) was collected and re-analyzed using HPLC with mass detection. A final LC-MS/MS analysis allowed us to obtain a pure compound with a retention time of 12.8 min and fragmentation spectra characteristic of ferulic acid-4-*O*-sulfate (Figure 2B).

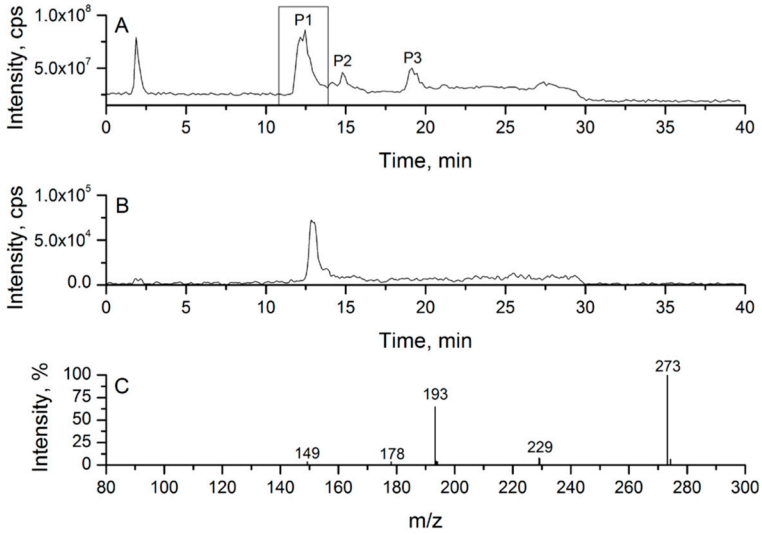


Figure 2. (**A**) TIC of mixture after synthesis of ferulic acid-4-*O*-sulfate; (**B**) chromatogram of ferulic acid-4-*O*-sulfate obtained after purification; (**C**) fragmentation spectra of ferulic acid-4-*O*-sulfate.

We identified dihydroferulic acid-4-*O*-sulfate using a similar procedure. The TIC presents six main peaks (Figure 3A). The fraction between 9 and 13 min (P3) had $[M\text{-}H]^-$ at m/z = 275, which is characteristic of the expected product. The resulting MS2 fragmentation ion at m/z = 175 indicates a loss of 80 amu and is characteristic of dihydroferulic acid standards. Further fragmentation in linear ion trap (MS3) resulted in the presence of ions at m/z 151, 135 of which are similar to that obtained for the substrate used. The fragmentation pathway of the synthesized product was in good agreement with previous studies and

expected fragmentation spectra (Figure 3C). The retention time (13.7 min), characteristic SRM pair and fragmentation pathway of peak 4 (P4) are characteristic of pure substrate-dihydroferulic acid, which was used in the synthesis. The rest peaks were identified as impurities. Collection of only the P3 fraction allowed us to obtain a pure compound with a retention time of 12.0 min and fragmentation spectra characteristic of dihydroferulic acid-4-*O*-sulfate (Figure 3B).

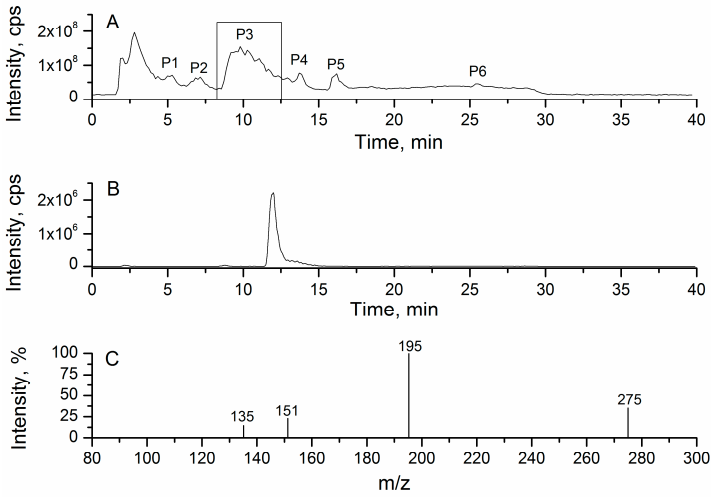


Figure 3. (**A**) TIC of mixture after synthesis of dihydroferulic acid-4-*O*-sulfate; (**B**) chromatogram of dihydroferulic acid-4-*O*-sulfate obtained after purification; (**C**) fragmentation spectra of dihydroferulic acid-4-*O*-sulfate.

Figure 4A presents the TIC of the mixture obtained after sulfation of caffeic acid. Peak 1 (P1) produced a negatively charged pseudomolecular ion $[M\text{-}H]^-$ at m/z = 259, which on MS2 produced fragmentation ion at m/z = 179 corresponding to the caffeic acid moiety. The ion produced on MS3 at m/z = 135 is characteristic of the fragmentation of caffeic acid. The fragmentation spectra of peak 1 (P1) correspond to the expected product, a sulfate ester of caffeic acid (Figure 4C). Two other peaks were identified as impurities. Contrary to the TIC obtained for mixtures after the synthesis of ferulic acid-4-*O*-sulfate and dihydroferulic acid-4-*O*-sulfate, we did not observe a peak corresponding to a caffeic acid substrate. The purification process consisted of the collection of only the fraction between 8 and 12 min (P1). Re-analysis of the collected fraction allowed us to identify two products with similar fragmentation pathways but different retention times: 10.97 and 11.80 min (Figure 4B). The loss of 80 amu, which is characteristic of sulfate moieties, and MS3 producing an ion at m/z = 135 indicate that both peaks are sulfate esters of caffeic acid. Therefore, the obtained products correspond to two monoesters of caffeic acid (caffeic acid-4-*O*-sulfate and caffeic acid-3-*O*-sulfate). However, disulfate esters of caffeic acid were not observed.

Synthesis of the dihydrocaffeic acid ester resulted in the presence of three main peaks on a chromatogram of the mixture after synthesis (Figure 5A). The peak 2 fraction (P2), with an elution time ranging between 7 and 10 min, had a pseudomolecular ion $[M\text{-}H]^-$ at m/z = 261 and yielded a fragmentation ion at 181 on MS2. The 80 amu difference between m/z = 261 and m/z = 181 is characteristic of the cleavage of sulfate moieties (Figure 5C). After matching the MS fragmentation with the expected fragmentation pathway and reference compound of dihydroferulic acid, peak 2 was identified as a sulfate ester of dihydroferulic acid. The rest of the peaks present on the TIC were identified as impurities. Similar to the synthesis of caffeic acid, the disulfate esters of dihydrocaffeic acid were not observed. Purification was achieved via the collection of only the P2 fraction. Contrary to caffeic acid sulfates, only one peak corresponding to one monoester of dihydrocaffeic acid was observed on the chromatogram obtained after purification (Figure 5B). These results indicate co-elution of two different monoesters in those conditions. This phenomenon is

associated with difficulties in the separation of isomeric compounds without a carbon–carbon double bond due to the free rotation around the single bond.

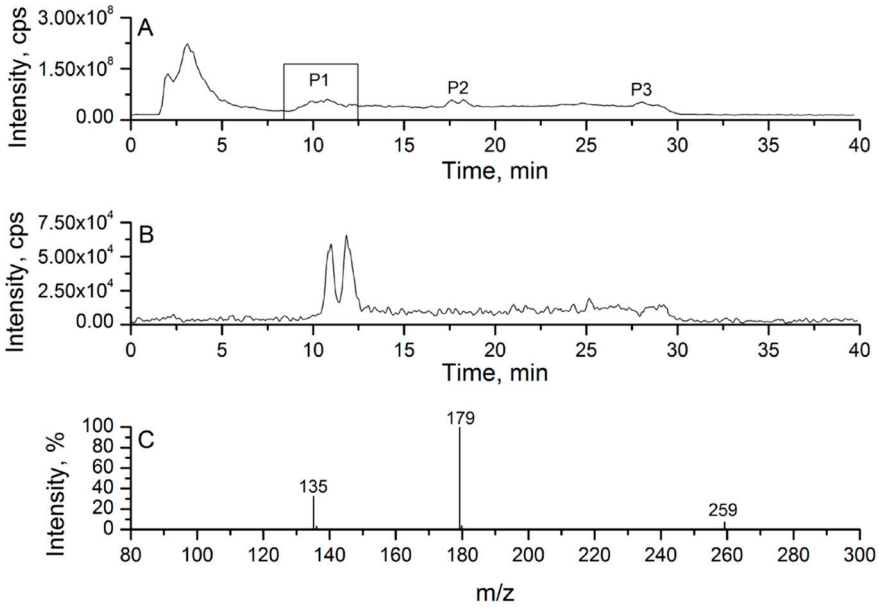


Figure 4. (**A**) TIC of mixture after synthesis of caffeic acid sulfate; (**B**) chromatogram of caffeic acid sulfate obtained after purification; (**C**) fragmentation spectra of caffeic acid sulfate.

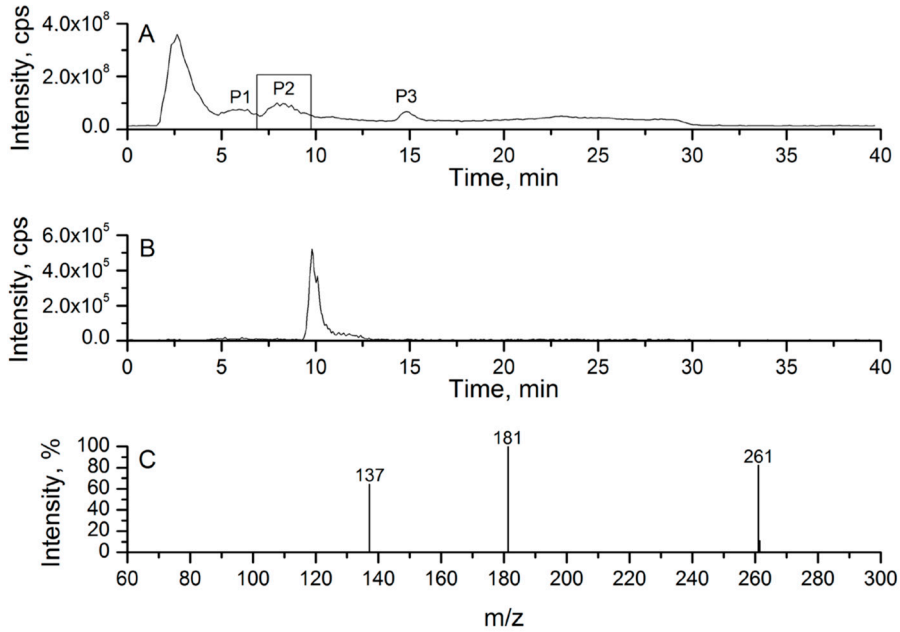


Figure 5. (**A**) TIC of mixture after synthesis of dihydrocaffeic acid sulfate; (**B**) chromatogram of dihydrocaffeic acid sulfate obtained after purification; (**C**) fragmentation spectra of dihydrocaffeic acid sulfate.

3.2. Characterization of Antioxidant Activity of Synthesized Metabolites

We were not able to fully examine the antioxidant activity of metabolites of phenolic acids because of the lack of commercially available standards. To evaluate the antioxidant properties of the synthesized compounds, we used FC and CUPRAC (summarized in Table 2) and DPPH• neutralization assays (summarized in Table 3). FC and CUPRAC assays are based on single-electron transfer (SET) and reflect the reducing capacity of the sample. As a reference compound, we used gallic acid and Trolox, while the antioxidant

activity of the analyzed compound was expressed as a GAE and TE. The higher the value of TE and GAE, the better the reducing capacity of the analyzed compound. The DPPH• test is based on the neutralization of a synthetic radical. This mechanism in a polar and hydrogen bonding solvent such as methanol is a two-step process involving the sequential loss of an acidic proton followed by an electron transfer (SPLET) [33]. The results are expressed as a scavenging effect (%) corresponding to the percentage of neutralized DPPH• as well as TE.

Table 2. Antioxidant capacities of phenolic acids and their sulfate metabolites measured with the CUPRAC (calculated as Trolox equivalent mM) and Folin–Ciocalteu (calculated as gallic acid equivalent ppm) assays.

Compound	CUPRAC TE (mM)	Folin–Ciocalteu GAC (ppm)
Caffeic acid	0.77 ± 0.02	86.87 ± 7.05
Caffeic acid monosulfate [1]	0	4.37 ± 0.22
Dihydrocaffeic acid	0.49 ± 0.02	72.07 ± 2.00
Dihydrocaffeic acid monosulfate [2]	0	2.19 ± 0.11
Ferulic acid	0.33 ± 0.04	80.01 ± 4.77
Ferulic acid-4-*O*-sulfate	0	14.29 ± 0.71
Dihydroferulic acid	0.39 ± 0.05	66.58 ± 2.48
Dihydroferulic acid-4-*O*-sulfate	0	15.20 ± 0.76

[1] Mixture of caffeic acid-3-*O*-sulfate and caffeic acid-4-*O*-sulfate at a ratio of 1:1. [2] Mixture of dihydrocaffeic acid-3-*O*-sulfate and dihydrocaffeic acid-4-*O*-sulfate at an unknown ratio.

Table 3. Scavenging effects of phenolic acid and its metabolites measured with the DPPH free radical assay.

Compound	Scavenging Effect (%)	Trolox Equivalent (mM)
Caffeic acid	20.99 ± 1.05	0.296 ± 0.015
Caffeic acid monosulfate [1]	2.97 ± 0.15	0.042 ± 0.002
Dihydrocaffeic acid	39.96 ± 2.00	0.563 ± 0.028
Dihydrocaffeic acid monosulfate [2]	3.61 ± 0.18	0.051 ± 0.003
Ferulic acid	13.56 ± 0.68	0.191 ± 0.010
Ferulic acid-4-*O*-sulfate	1.28 ± 0.068	0.018 ± 0.001
Dihydroferulic acid	23.15 ± 1.16	0.326 ± 0.055
Dihydroferulic acid-4-*O*-sulfate	2.76 ± 0.14	0.039 ± 0.009

[1] Mixture of caffeic acid-3-*O*-sulfate and caffeic acid-4-*O*-sulfate at a ratio of 1:1. [2] Mixture of dihydrocaffeic acid-3-*O*-sulfate and dihydrocaffeic acid-4-*O*-sulfate at an unknown ratio.

Table 2 shows the comparison of the reducing capacity of GCA metabolites according to CUPRAC and FC assays. As is shown in both assays, caffeic acid possesses a higher reducing capacity in comparison to ferulic acid. In the CUPRAC assay, caffeic acid and ferulic acid corresponded to 0.77 and 0.33 mM TE, respectively, and in the FC assay, 86 and 80 ppm GAE, respectively. Similar results were obtained with the DPPH• assay; caffeic and ferulic acids neutralized about 21% and 14% of DPPH•, respectively, corresponding to approximately 0.3 and 0.2 mM Trolox (Table 3). The hydrogenated analogue of caffeic acid—dihydrocaffeic acid—demonstrated lower reducing abilities in both the FC and CUPRAC assays than its precursor compound.

Surprisingly, in the FC test, ferulic acid and its hydrogenated analogue showed a similar reducing capacity, unlike in the CUPRAC assay, where ferulic acid showed a higher reducing power than dihydroferulic acid. Interestingly, the results obtained using the DPPH• assay are contradictory; the hydrogenated analogues of caffeic and ferulic acids were shown to have higher ability to neutralize DPPH• via their antioxidant activity. The antioxidant activity of dihydrocaffeic acid was 1.8 times higher than that of the precursor caffeic acid, while the antioxidant activity of dihydroferulic acid was 1.7 times higher compared to precursor ferulic acid. This shows that the reduction of carbon–carbon

double bonds in hydroxycinnamic acid derivatives considerably enhances the antioxidant activity compared to precursor compounds in a DPPH• assay. In all presented assays, *O*-sulfation of hydroxyl groups attached to aromatic ring resulted in a significant decrease or even inhibition of the reducing capacities of the measured samples. With regard to sulfate derivatives, the presented measurements show a significant decrease in antioxidant properties in comparison with precursor phenolic acids. In the assays based on the ET mechanism, the antioxidant properties of sulfate metabolites were significantly decreased or inhibited compared to those of precursor compounds. The same results were obtained using the DPPH• assay with the SPLET mechanism. The neutralization effect of the caffeic acid monosulfate is about seven-fold lower than that of caffeic acid, while the scavenging effect of ferulic acid-4-*O*-sulfate is eleven-fold lower in comparison with that of precursor ferulic acid. Similar results were obtained for dihydrocaffeic monosulfate and dihydroferulic-4-*O*-sulfate, with eight-fold and eleven-fold lower values, respectively. Overall, sulfate esters of cinnamic acid derivatives exhibit a much lower antioxidant activity compared to precursor phenolic acids and are comparable to blanks.

4. Discussion

Phenolic acids can be found in all plant-originating food, such as grains, coffee, tea, etc. They are one of the most common ingredients in the food we eat every day. Although the human diet was not originally heavily dependent on plant-originating food like nowadays, over centuries of evolution, our bodies have 'learned' how to efficiently metabolize phenolic acids. They circulate in plasma as metabolites, mainly as *O*-sulfates and *O*-glucoronates, rather than their native forms. However, surprisingly, in the literature on this subject, very little attention has been given to the antioxidant activity of polyphenol metabolites. The papers which do discuss it often report inconclusive results; for example, quercetin 3-*O*-sulfate was found to retain antioxidant properties, but less efficiently than unconjugated quercetin [34]. The *O*-methylation of catechol B-ring hydroxyls caused a decrease in the antioxidant activity with regard to the precursor compounds. However, at pH 7.4, they maintained significant radical neutralization activity [35,36]. Moreover, Botto et al. [37] reported that the phenolic metabolites of coffee provide an effective defense against DEP (diesel exhaust particle)-induced oxidative stress by supporting its antioxidant activities. On the other hand, Piazzon et al. [30] synthesized the metabolites of caffeic and ferulic acids (ferulic acid-4-*O*-sulfate, caffeic acid-4-*O*-sulfate and caffeic acid-3-*O*-sulfate) and showed that monosulfate derivatives of caffeic and ferulic acids exhibited very low antioxidant activity. Nevertheless, it is worth noting that the main metabolites circulating in plasma after CGA administration are dihydrocaffeic and dihydroferulic acids, as well as their *O*-sulfate conjugates [12,13,22]. To our knowledge, there are no studies that compare the antioxidant activity of their precursors, caffeic and ferulic acids, to their hydrogenated analogues, dihydrocaffeic and dihydroferulic acid, as well as their *O*-sulfate derivatives.

Herein, we followed Todd et al.'s [32] method and synthesized sulfate monoesters of caffeic, ferulic and their hydrogenated analogues according to their pathway. The LC-MS/MS analyses allowed for the full characterization of these derivatives, which makes them reliable standards for further research. The reaction of ferulic and dihydroferulic acids with $ClSO_3H$ resulted in the formation of two monoesters, ferulic acid-4-*O*-sulfate and dihydroferulic acid-4-*O*-sulfate, since they possess only one hydroxyl group at which sulfation may occur. Synthesis of sulfate conjugates of caffeic acid resulted in a mixture of two monoesters, caffeic acid-3-*O*-sulfate and caffeic acid-4-*O*-sulfate, at a ratio of 1:1, which were separated under chromatographic conditions. The results obtained for monoesters of dihydrocaffeic acid indicated co-elution of two different monoesters under these conditions: dihydrocaffeic acid-3-*O*-sulfate and dihydrocaffeic acid-4-*O*-sulfate. Although the *O*-sulfation of caffeic and dihydrocaffeic acids could occur at both hydroxyl groups simultaneously, disulfates were not observed.

Our results shed new light on the antioxidant capacity of the main metabolites of CGAs. Firstly, all assays used in the experiment showed that caffeic acid exhibited higher

antioxidant efficiency than ferulic acid. This phenomenon has already been presented in the literature and correlated with the substitution of the hydroxyl group in the third position of the aromatic ring (caffeic acid) with a less polar methoxyl group (ferulic acid) [26,38]. In CUPRAC and FC assays, dihydrocaffeic acid exhibited lower antioxidant activity than its precursor compound. Furthermore, the same results were observed for dihydroferulic acid in the FC assay, while in the CUPRAC assay, the antioxidant abilities of dihydroferulic acid and its precursor ferulic acid were comparable. However, these two assays use different chromogenic redox reagents as well as different conditions of measurement. As was mentioned before, the FC assay is based on the SET mechanism in a basic medium (adjusted with a sodium carbonate solution to pH∼10), while the CUPRAC assay is based on the SET mechanism in neutral pH (acetate buffer, pH = 7). The pH values have an important effect on the reducing capacity of antioxidants. In particular, at basic pH, proton dissociation of phenolic compounds may enhance their vulnerability to reduction [39]. The above studies highlighted the poor correlation of CUPRAC and FC assays due to the different reduction potentials, which are well known for CUPRAC, while the exact potential of the Folin reagent with a presumably higher potential is not definitely known. Consequently, the authors noted that the FC assay was originally thought to be specific for phenolic compounds and should oxidize samples more efficiently than the CUPRAC assay [40]. Interestingly, the DPPH• assay results show the opposite: hydrogenated analogues and dihydrocaffeic and dihydroferulic acids exhibited higher antioxidant activities than their precursor compounds. However, it has already been highlighted that due to the different mechanisms involved, analytes can react differently in different assays [28]. The results obtained during the radical neutralization experiment show that the reduction of the carbon–carbon double bond in hydroxycinnamic acid derivatives considerably enhanced the antioxidant activity with respect to the precursor compounds. Since changes in antioxidant structure result in changes in electron delocalization possibilities, the antioxidant activity might vary. Furthermore, the structure of cinnamic acid derivatives is characterized by a conjugated double-bond system, which is a more stable structure than that of their hydrogenated analogues. In parallel, changes in hydrophobicity could significantly influence the reducing capacity of compounds. Therefore, for a more precise estimation of the antioxidant activity of a given sample, one should use at least two distinct methods based on different reaction mechanisms. With regard to sulfate esters of caffeic and ferulic acids, our results are in line with the results reported by Piazzon et al. [30]. We provide evidence that the sulfation of hydroxyl groups results in inhibition of antioxidant properties with respect to precursor compounds [30]. Similar results were obtained for dihydrocaffeic and dihydroferulic acids and a significant decrease in the antioxidant properties of the sulfate esters was observed. It is generally believed that *O*-sulfation reduces or even inhibits reducing efficiency due to the modification of the hydroxyl groups which are responsible for the antioxidant activity of the phenolic compounds. Firstly, the *O*-glucuronidation and *O*-sulfation processes modify the hydrophobicity and the possibilities of electron delocalization, so the antioxidant activity of conjugates might be different from that of the precursor compounds. Secondly, considering the model of the aromatic sulfates (Figure 6A), the main reason for the reduced antioxidant activity of caffeic acid and dihydrocaffeic acid sulfates can be noted. The ortho-position of the sulfate moiety favors the intramolecular H bond and, in consequence, stabilizes the phenol (Figure 6B). This effect has been widely discussed in the literature [41]. Considering ferulic acid and dihydroferulic acid sulfates, the inhibition of antioxidant activity is caused by the lack of a free –OH group. This means that the reducing capacity of phenolic acids, after the metabolic process, decreases due the conjugation of hydroxyl groups. Consequently, our results highlight the importance of the presence of unsubstituted hydroxyl groups in the antioxidant activity of phenolic acids and their metabolites.

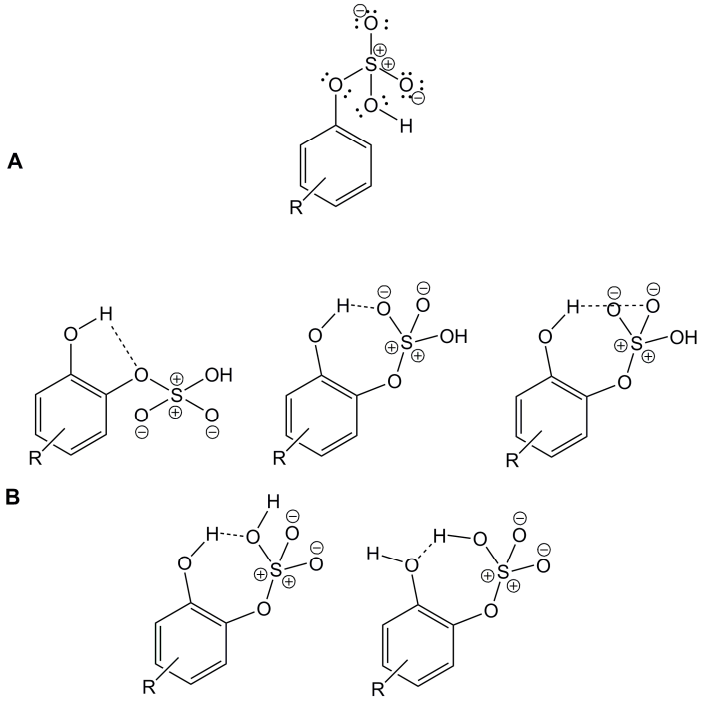


Figure 6. Model of structure of the aromatic sulfates (**A**) and possible intramolecular H bonds in phenols with sulfate group in ortho-position (**B**).

5. Conclusions

In summary, our results prove that, although CGAs are extensively metabolized in the human body, some metabolites retain antioxidant activity. Caffeic and ferulic acids formed upon the hydrolysis of chlorogenic acids are well-known antioxidants [26]. It appears that hydrogenation is a significant step in increasing the antioxidant activity of these compounds in the free radical neutralization reaction. Moreover, dihydrocaffeic acid is more cytotoxic to several cancer cell lines than healthy cells; therefore, a diet rich in CGAs should be included in cancer prevention routines and the treatment of oncological patients [42]. On the other hand, the conjugation of different hydoxycinnamic acids into their *O*-sulfate esters leads to the inhibition of antioxidant capabilities. However, our results should be considered carefully because the simultaneous presence of a high number of metabolites could produce some additional synergistic or antagonistic effects in vivo. Our results might be viewed as crucial for the elucidation of phenolic compounds' bioefficiency. That is because CGAs appear in almost all plant-originating food. Therefore, they are a key element of our diet. Consequently, additional studies that evaluate the role of other CGA metabolites and their mutual intersections are necessary. Finally, it must be emphasized that the assays used in the present study are based on chemical reactions in vitro; therefore, they cannot be simply translated to biological systems. It is of the utmost importance to apply more reliable biological models to study the correlation between the intake of high-potent antioxidants and the level of oxidative stress.

Author Contributions: Conceptualization, M.R. and M.B.; Methodology, M.R. and K.L.; Validation, M.R.; Formal analysis, M.R. and K.L.; Investigation, M.R. and K.L.; Writing—original draft, M.R. and M.B.; Writing—review and editing, M.R., Z.C. and M.B.; Supervision, M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data used in the study have been presented in the manuscript.

Conflicts of Interest: The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript or in the decision to publish the results.

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