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Article

Structural Study of Phenolic Acids by Triple Quadrupole Mass Spectrometry with Electrospray Ionization in Negative Mode and H/D Isotopic Exchange

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This work reports the theoretical and experimental study of fragmentation reactions in the gas phase of five phenolic acids using triple quadrupole mass spectrometry by electrospray ionization in negative ionic mode, as well as the isotope exchange experiments. MS/MS spectra were analyzed to suggest the fragmentation mechanisms, while theoretical calculations at the theory level B3LYP/6-311+G** were performed to expose the proposed mechanisms viability for this class of compounds. As expected, compounds with aromatic methoxy substitution presented $\bullet CH_3$ radical elimination as the principal fragmentation pathway, forming dystonic ions. Compounds without methoxy substituents dissociate with higher energies losing the CO_2, CO and H_2O. The isotopic marking experiments indicated the exchange of hydrogens by deuterases in the hydroxyl protons, which corroborates with the proposed mechanisms.

Keywords: mass spectrometry, fragmentation, phenolic, structural study, CID

Introduction

Phenolic acids are substances belonging to the phenolic compounds group. It is extensively distributed in nature, being present in plants as free form or attached to sugars and proteins.[1]

Its structure is characterized by a benzene ring, a carboxyl group and one or more hydroxyl and/or methoxyl group. The major classes of the phenolic acids observed in the plant kingdom are C6-C1 and C6-C3. The phenolic acids of class C6-C3 have a structure derived from the hydroxybenzoic acid including gallic, protocatechic, vanillic, seringic and genetal acids. On the other hand, C6-C3 phenolic acids present a hydroxynamic acid backbone including caffeic, *p*-coumaric, ferulic and synapic acids. The difference between these compounds is the groups variation at the positions C3, C4, and C5.[2] Its general formulas are represented in Figure 1.

Phenolic compounds presence in plants has been frequently studied due to its health potential benefits. Phenolic acids are recognized to have antibacterial, antiviral, anti-carcinogenic, anti-inflammatory and vasodilatory activities.[3-8]

Due to commercial value of these compounds for cosmetic and pharmaceutical industries, the structural characteristics study is essential in order to develop rapid analysis methods using precursor ion scanning and/or neutral losses in processes of dereplication in plant matrices through mass spectrometry.[9,10]

Electrospray ionization mass spectrometry (ESI-MS) analysis is an important technique to identify and quantify compounds in complex matrices. Studies report that fragmentation reactions using ESI-MS are revealed as a significant tool for structural characterization and

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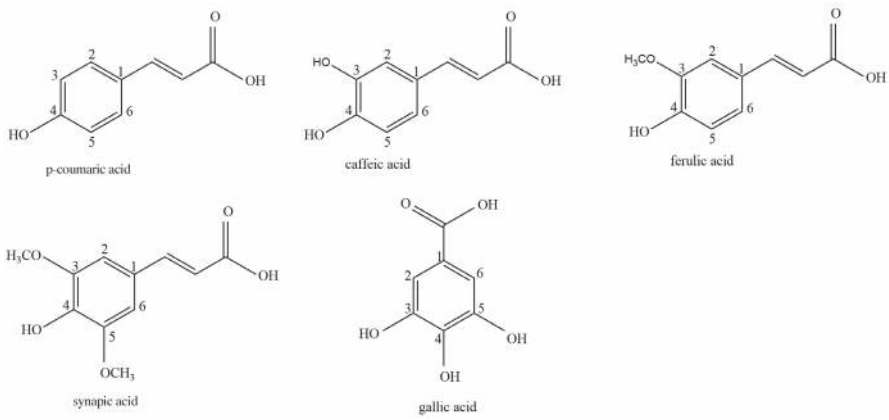


Figure 1. Structure of phenolic acids studied.

elucidation of synthetic and natural compounds.[10-13] The technique has been extensively used for phenolic acids analysis, though studies dedicated to these compounds' fragmentation are still scarce. Martens *et al.*[14] studied the ferulic acid fragmentation by infrared multiple-photon dissociation spectroscopy (IRMPD) and identified that protonated ferulic acid produces three ionic fragments (*m/z* 177, 149 and 163) and exhibit characteristic losses of CO and H_2O. Kuhnert *et al.*[15] report the use of liquid chromatography coupled in tandem to the mass spectrometer (LC-MS/MS) to easily distinguish eight regioisomers of ferulic acid by direct spectra comparison or rational fragmented ions probing.

The studies available in the literature discuss the fragmentation mechanisms only for some specific phenolic acids, however, this work reports the use of electrospray ionization mass spectrometry (ESI-MS/MS) to investigate the pattern fragmentation of five phenolic acids: *p*-coumaric acid, gallic acid, caffeic acid, ferulic acid and synapic acid, under low energy collision induced dissociation (CID) associated with theoretical calculations and isotopic labeling experience.

Experimental

p-Coumaric, gallic, caffeic, ferulic, and synapic phenolic acids standards were purchased from Sigma Aldrich (St. Louis, MO, USA) and diluted in UPLC grade methanol purchased from Merck (Darmstadt, Germany) to produce solutions with 1 μg mL^{-1} containing 0.1% of ammonium hydroxide. For the exchange marking of hydrogen by deuterium (H/D), the standards dilution in methanol-d_4 (99% D) of Sigma Aldrich (St Louis, MO, USA) was performed.[9]

The experiments were performed in Premier XE triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with electrospray ionization (ESI) operating in negative mode. The source ESI(−) parameters were: 2 kV capillary, 20 V cone with quadrupole adjusted for unit resolution.

The deprotonated molecules were fragmented with collision energy in the range of 5 to 25 eV and argon pressure of 1×10^{-3} Torr. Total energies of the optimized geometries based on the density functional theory (DFT) were obtained by the B3LYP method with base set 6-311+G** through Spartan'18 software and expressed in relative potential energy in kcal mol^{-1}.[16]

Fragmentation mechanisms were proposed based on the pathways energetic profile suggested by MS/MS studies.

Results and Discussion

Five phenolic acids (*p*-coumaric, gallic, caffeic, ferulic, and synapic) were analyzed for its fragmentation patterns. Structures of these compounds are characterized by having a benzene ring, a carboxyl group and one or more hydroxyl and/or methoxyl groups. The differences between these structures are: *p*-coumaric acid has a C4 hydroxyl group, caffeic acid has two hydroxyl groups at the C3 and C4 positions, ferulic acid has a C4 hydroxyl group and a C3 methoxyl group, synapic acid has a C4 hydroxyl group and two methoxyl groups at the C3 and C4 positions, and gallic acid has a double bond between the aromatic group and the carboxylic acid function, plus three hydroxyl groups in the C3, C4 and C5 positions (Figure 1).

All the compounds presented deprotonation site in the carboxylic hydrogen due to its higher acidity. ESI-MS/MS spectra with induced dissociation by collision revealed the following deprotonated ions: *p*-coumaric *m/z* 163, caffeic *m/z* 179, ferulic *m/z* 193, synapic *m/z* 223 and gallic *m/z* 169 (Figure 2). From these ions, the fragmentation mechanisms were suggested considering the results obtained by MS/MS experiments.

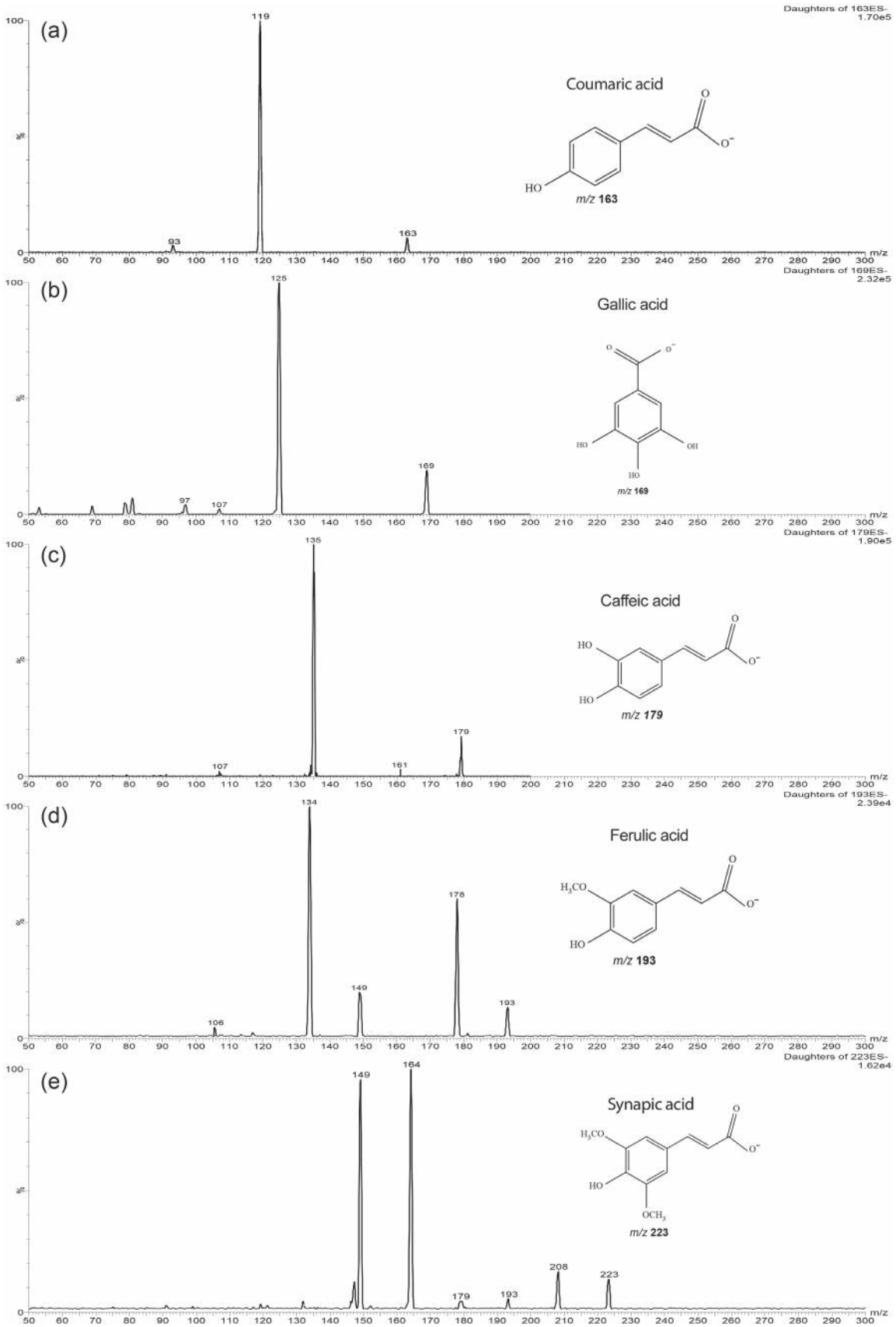


Figure 2. ESI(−)-MS/MS of phenolic acids. (a) *p*-Coumaric acid *m/z* 163, (b) gallic acid *m/z* 169, (c) caffeic acid *m/z* 179, (d) ferric acid *m/z* 193 and (e) synapic acid *m/z* 223.

The laboratory energy experiment (E_{lab}) exposes low stability for deprotonated compounds, suggesting low collision energies in the CID process. The most intense fragmentation could be observed after 15 eV (Figure 3).

The first fragments observed in *p*-coumaric, gallic and caffeic acids were: *m/z* 119, *m/z* 125 and *m/z* 135, respectively, related to the CO_2 (44 Da) neutral loss due to the α mechanism elimination which is a typical charge migration fragmentation (CMF) in deprotonated compounds. In ferulic and synapic acids, the first cleavage occurs by charge retention fragmentation (CRF) through the $\bullet CH_3$ (15 Da) elimination. The radical species formation occurs in E_{lab} of 5 eV and the energies calculated for $\bullet CH_3$ elimination were 39.4 and 46.6 kcal mol^{-1} lower than the energies presented for CO_2 loss (53.5 and 53.2 kcal mol^{-1}). These observations confirm that the radical fragmentation is a minor energy process (Scheme S1, Supplementary Information (SI) section).

The less energetic fragmentation pathway obtained for synapic acid confirmed with the theoretical calculations, as well as the laboratory energy study. It follows through the CO_2 (44 Da) elimination, generating the fragment *m/z* 164. The same occurs with the ferulic acid fragment formation *m/z* 134, followed by another CO (28 Da) neutral loss by the ring contraction. The same was observed by Kuhnert *et al.*,[15] exposing that ferulic acid preferentially loses CH_3 followed by loss of CO_2, the initial loss of CO_2 to form the *m/z* 149 fragment shows to be thermodynamically unfavorable.

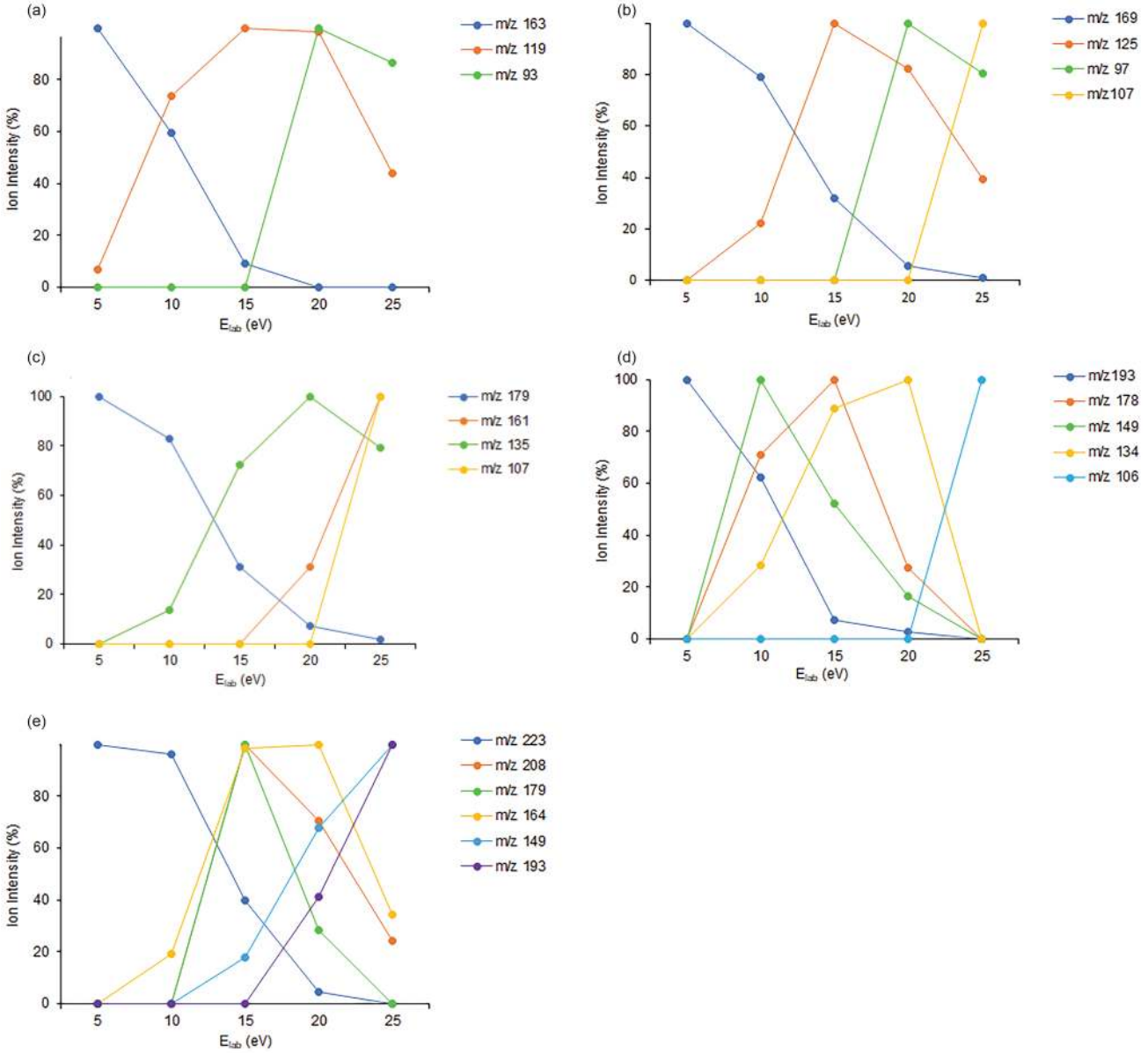
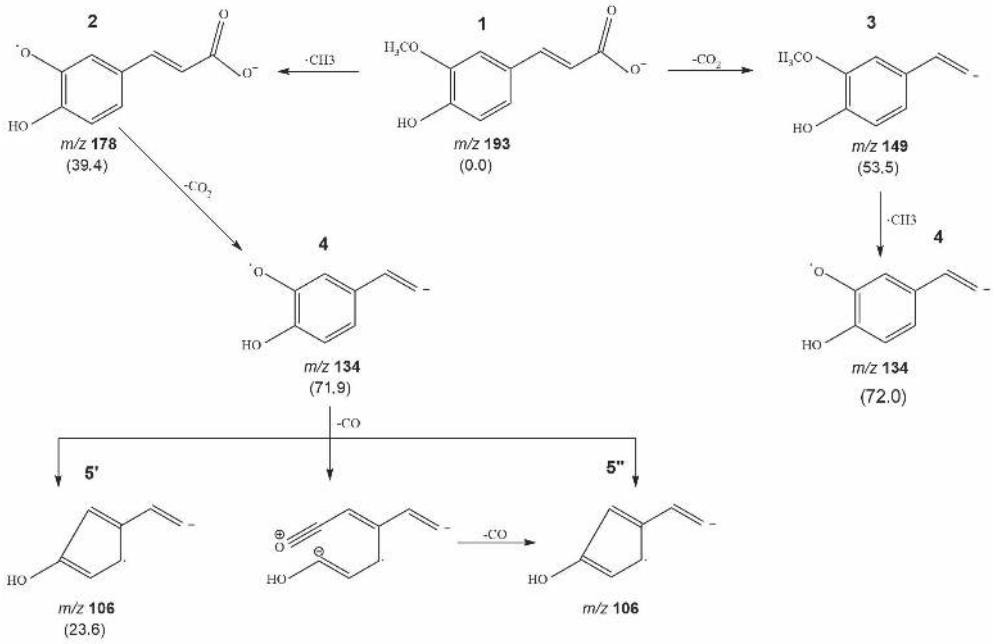


Figure 3. Fragmentation analysis of phenolic acids by ESI(−)-MS/MS in the E_{lab} (eV) variation obtained in the triple quadrupole (QqQ) analyzer. (a) *p*-Coumaric acid, (b) gallic acid, (c) caffeic acid, (d) ferulic acid and (e) synapic acid.

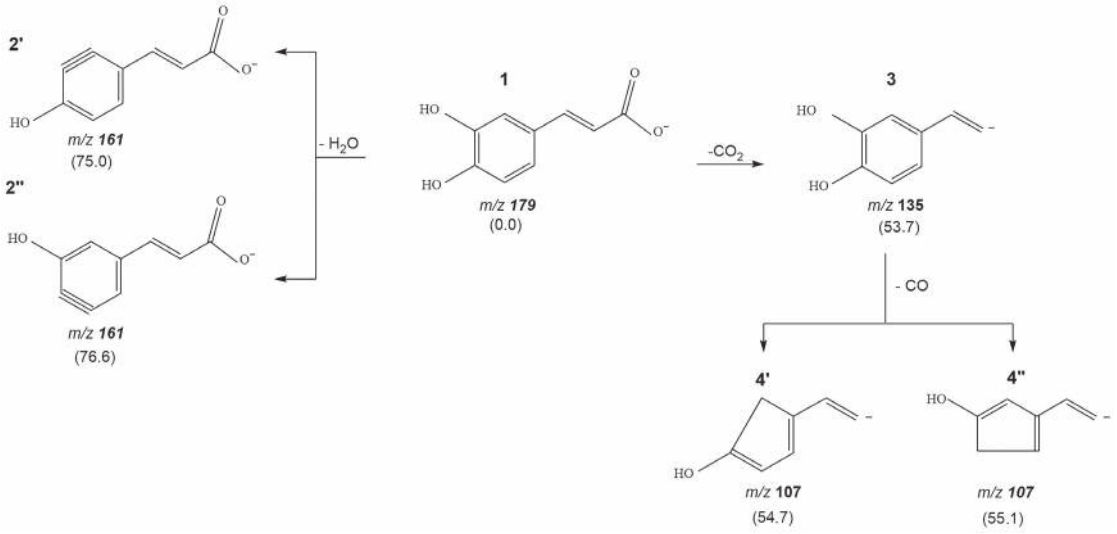
CO elimination in cyclic compounds can follow two possible mechanisms; through a single-step involving connections ruptures and formation, or through a reaction involving equilibrium between cyclic and open structures, followed by an internal attack.[17] The single-step mechanism is assumed to be the most favorable for this compound class, presenting energy of 23.6 kcal mol^{-1}, once the mechanism comprising the equilibrium between the cyclic and open structures does not present theoretical indications to support it (Scheme 1).

p-Coumaric acid fragmentation under the same conditions indicated that after the CO_2 removal from the ion precursor resulting in the fragment *m/z* 119, C1-C7 bonds rupture to form *m/z* 93 by acetyl loss (26 Da) that occurs in an E_{lab} of 15 eV (Scheme S2, SI section).

Scheme 2 displays the proposed fragmentation mechanism for caffeic acid. The thermodynamically most favorable pathway starts with CO_2 elimination and consecutive loss of CO in C3 with energy of 54.7 kcal mol^{-1}. The CO_2 neutral loss fragmentation channel presents lower energy in comparison to the H_2O loss (75.0 and 76.6 kcal mol^{-1}), generating the fragment *m/z* 161 through the triple bond formation (C2 or C4) by remote hydrogen rearrangement, very



Scheme 1. Fragmentation mechanisms for deprotonated ferulic acid. Values between parentheses are relative potential energies in kcal mol^{-1}, obtained at the theory level B3LYP/6-311+G**.



Scheme 2. Fragmentation mechanisms for deprotonated caffeic acid. Values between parentheses are relative potential energies in kcal mol^{-1}, obtained at the theory level B3LYP/6-311+G**.

usual in compounds containing hydroxyl groups in its structure.[17]

The proposed fragmentation mechanism for gallic acid initiates with the formation of ion *m/z* 125 generated from the CO_2 removal (44 Da) from the precursor ion *m/z* 169 followed by a second fragmentation compatible with H_2O (18 Da) neutral loss. Another fragmentation pathway is the CO elimination causing a ring contraction providing the fragment *m/z* 97 which is thermodynamically more favored than the H_2O neutral loss (Scheme S3, SI section).

The isotope marking experiment of the deuterated ions of of *m/z* 164 (*p*-coumaric-deuterated), *m/z* 172 (gallic-deuterated), *m/z* 181 (caffeic-deuterate), *m/z* 194 (ferulic-deuterated), *m/z* 224 (synapic-deuterated) was performed to evaluate the proposed mechanisms. The H/D exchange resulted in the formation of deuterated hydroxyl groups.

Figure 4 shows a direct comparison of the mass spectra of ferulic acid and ferulic-deuterated acid by MS/MS CID. The ferulic-deuterated acid (*m/z* 194), as well as *p*-coumaric-deuterated (*m/z* 165) and synapic-deuterated (*m/z* 224) acids presented all the observed fragments displaced by 1 Da in relation to its original structures (Figures S1-S2, SI section). Caffeic-deuterated (*m/z* 181) and gallic-deuterated (*m/z* 172) acids exposed identical fragmentation behavior in comparison to its non-deuterated form (Figures S3-S4, SI section).

All observed H/D exchange results for the five compounds studied corroborate with the results shown for theoretical calculations (B3LYP/6-311+G**) and also for laboratory energy, proving that phenolic acids with methoxyl substituents (ferulic and synapic acids) start to fragment at lower energies in comparison to those without methoxyl groups (gallic, *p*-coumaric and caffeic acids), which present a first fragment corresponding to CO_2 neutral loss. CO_2 and CO neutral losses occur in superior number for this compounds class, with potential to be applied for phenolic acids research and confirmation by neutral loss experiments.

Conclusions

The mechanisms of fragmentation were proposed through the MS/MS experiments of phenolic acids and phenolic acids deuterated. The study was conducted in conjunction with thermochemical data estimated by computational chemistry, laboratory energy experiment and isotope exchange. The fragmentation pattern reinforces that methoxy groups require lower energies and are preferably cleaved. The compounds without methoxy substituent follow the fragmentation pathways by CO_2, CO and H_2O multiple losses. The fragmentation profile reported in this work can be employed in neutral loss experiments to identify phenolic acids in plant matrices.

Supplementary Information

Supplementary information (Figures S1-S4 and Schemes S1-S3) are available free of charge at http://jbcs.sbq.org.br as a PDF file.

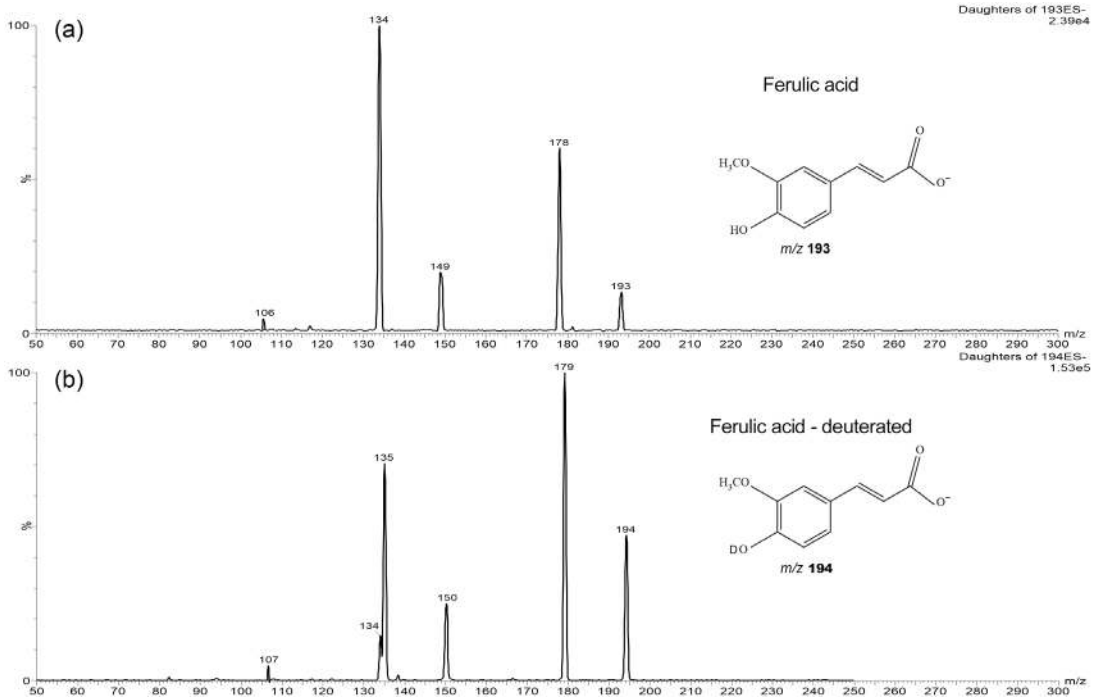


Figure 4. ESI(−)-MS/MS spectrum of (a) ferulic acid of *m/z* 193 and (b) ferulic-deuterated acid of *m/z* 194.

Acknowledgments

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