Comparison study between two modification conditions of rosmarinic acid and amino acids- Identification of formed adducts using FI-ESI-QTOF-mass spectrometry

Mostafa Ali[1]*and Karin Schwarz[2]

[1]*Department of Food Technology, Faculty of Agriculture, KafrelsheikhUniversity, Kafrelsheikh, Egypt.*
[2]*Division of Food Technology, Institute of Human Nutrition and Food Science, Kiel University, Germany.*
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Abstract

This study was designed to compare and identify the adducts formed between rosmarinic acid and amino acids at two different modification conditions. Covalent interactions between different amino acids, including [L-Lysine (L-Lys) and its derivative,tert-butyloxycarbonyl-L-lysine (N-Boc-Lys) and L- cysteine (L-Cys)] and rosmarinic acid (Ros) were conducted with two different methods; alkaline (pH 9) and enzymatic [in the presence of tyrosinase (polyphenoloxidase , PPO)]. The formed adducts were identified and characterized using flow injection electrospray ionization - quadrupole time of flight (FI- ESI-QTOF) mass spectrometry. Many adducts were identified for the first time. The obtained data exhibited slight differences between the adducts formed after alkaline and enzymatic treatment. In case of the incubation of Ros with both methods without amino acids, numerous adducts, such as Ros quinone, Ros dimer and decarboxylated Ros at [M−H] - with an m/z of 359.08, 719.17 and 313.1 Da, respectively, were identified .Otherwise, in the presence of amino acids, the monomer and dimer of Ros were covalently added to the side chains of selected amino acids. Additionally, the covalent attachment between oxidized Ros and the side chains of two molecules of amino acids was also identified. Finally, L- Cys was the most susceptible amino acid to react with oxidized Ros compared to L- Lys and N-Boc-Lys. It could be concluded that, the results show some similarities between the adducts formed at both modification conditions. Moreover, the covalent interactions between Ros and the side chains of molecules of lysine or cysteine may also be expected to occur in proteins.

Keywords: Covalent interactions,tyrosinase, L-Lysine and L-Cysteine, rosmarinic acid, FI-ESI- QTOF-MS

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1. Introduction

Phenolic compounds as food components representthe largest group of secondary plant metabolites, which are distributed incrops, fruits, vegetables, and edible natural plants andhave many interesting biological activities including antibacterial, antioxidant, antiviral, antidiabetic, anti-inflammatoryandanticancer agents [1, 2]**.** They are characterized by a large range of structures and functions, but generally possessing an aromatic ring bearing one or more hydroxyl substituents [3, 4].

Rosmarinic acid (Ros)is an ester ofcaffeic acid and 3,4-dihydroxyphenyllactic acid (*Figure 1*). It is widelydistributed in Lamiaceae herbs, e.g. sageandrosemary, which are used as food additives in many food products [5-7].

As shown in Figure 1, the rosmarinic acidhas two catechol moieties: one of them is in the 2-oxyphenylpropanoyl moiety and the other one is in the caffeoyl moiety, which is susceptible to both enzymatic and non-enzymatic (pH 9) oxidation process in the presence of oxygen. Oxidation of one or two-electron of catechol leads to form

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Corresponding author: *e-mail:* mostafa.ali@agr.kfs.edu.eg

semiquinoneand quinonemetabolites, respectively. Formedreactive radicals are Michael acceptors, which have electrophilic properties. Theyare capable of undergoing a nucleophilic addition to amino acids or proteins and forming colored products [8-12], *Figure 1*. Resultingcolored products could be a cause of unacceptable organoleptic properties of the product. Moreover, this may havenegative effect on its nutritional value because this color indicates to the covalent interaction between quinone and essential amino acid e.g. lysine [13-15].
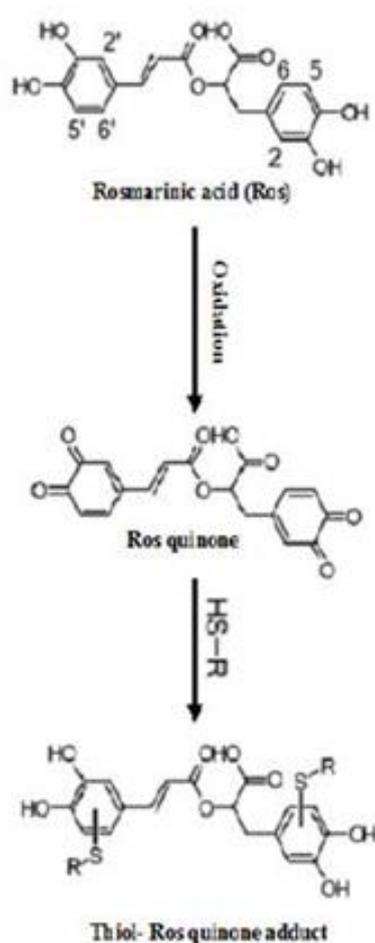

Figure 1. The proposed mechanism of interaction between Rosand L-Cys, modified from [16]

Polyphenol oxidases and peroxidases are the main oxidases enzymes in this process. Polyphenol oxidases (PPOs) are copper-containing two differentenzymes, which catalyze two different oxidative reactions; the first one is monophenolase and the second one is *o*-diphenolase.The first one hydroxylates the monophenols to *o*-diphenols, by inserting oxygen in a position ortho - to an existing hydroxyl group in an aromatic ring followed by the

second one, which oxidizes the *o*-diphenol to the corresponding quinone.

Quinone is unstable and very reactive compound, so sharein further interactions, by covalent attachment with nucleophilic molecules such asamino group of lysine and the thiol group ofcysteine, which are stated to be the favored sites of this interaction, or proteins [9, 10, 15, 17-22]. Quinone of rosmarinic acid reacted easily with compounds containing thiol groups, forming a thiol−rosmarinicquinone adduct via the 1,4-Michael addition reaction (Figure 1) [7, 16].

Although, the interactions of phenolic compounds with amino acids have been studied for a long time, the data about the formed reaction products still limited. In addition, according to our knowledge, both enzymatic and non-enzymatic (alkaline) modifications of amino acids with rosmarinic acid have not been reported yet. Consequently, the objective of this investigation was to increase the knowledge on the interactions of rosmarinic acid with amino acids including, L-Lysine, Boc-Lysine and L-Cysteine, at two different conditions, (alkaline, pH 9) and enzymatic (in the presence of tyrosinase), to identify the formed adducts, using flow injection electrospray ionization - quadrupole time-of-flight - mass spectrometry system (FI-ESI-QTOF- MS).

2. Materials and Methods

Modification of amino acids with rosmarinic acid: Two modification conditions, alkaline and enzymatic, of amino acids (L-Lys and L-Cys) and L-Lys derivative (N-Boc-Lys), with Ros, were conducted based on the models previously described [15, 19], with some modifications. For alkaline modification, 112 mM of individual amino acids, L-Lys, L-Cys and N-Boc-Lys as well as a 28 mM Ros were dissolved in deionized water and mixed at a ratio of (1/1, v/v, Ros / amino acids). The mixtures were adjusted to pH 9 with 0.1M NaOH and stirred in open flasks for 2h at room temperature. 28 mM solution of Ros was mixed with deionized water at pH 9 (1/1, v/v) and used as a control. While, for enzymatic modification, amino acids (L-Cys and N-Boc-Lys, 112 mM) and Ros (28mM L^{-1}) were dissolved in 0.5 mM McIlvaine buffer (pH 6.5) and mixed at a ratio of 1/1 (v/v) and PPO (20 U mL^{-1}) was added. Mixtures were stirred in open tubes for 2 h at 25 °C. 28 mM solution of Ros was mixed with

McIlvaine buffer (1/1, v/v) and PPO (20 U mL^{-1}) was added and used as a control. All mixtures were immediately evaluated using FI-ESI-QTOF mass spectrometer after similar reaction times.

Flow injection electrospray ionization - quadrupole time of flight - mass spectrometry (FI-ESI-QTOF-MS/MS) Analysis: The formed adducts during both interaction conditions were identified using an ESI-QTOF mass spectrometer (micrOTOF-QII, Bruker Daltonics, Bremen, Germany). Samples were injected via a syringe pump. For the ionization, an electrospray source in the negative mode was used. The method was optimized to the following conditions: source voltage at 4500 V, nitrogen as drying: 180°C with 4 L/min and as nebulizing gas: 0.4 bar. Funnel RF 1 & 2: 200 Vpp, Hexapole RF: 90 Vpp, Collision RF:

150 Vpp, Transfer Time: 60 µs and Pre-Pulse-Storage: five µs. The data were acquired each second with a rolling average factor of two. Full scan data were stored from 75 to 1000 m/z for MS and MSn. The method was calibrated each morning and in between of the measuring cycles with a lithium formate solution (Sigma - Aldrich, St. Louis, USA).The fragmentation was conducted with the multi-reaction-monitoring method. Therefore, nitrogen gas was used as collision gas. The collision energies were alternated between 0 eV (no fragmentation), 10 eV and - 20 eV. The data evaluation was conducted with Data Analysis 4.2 (Bruker Daltonics, Bremen, Germany). All expected and known compounds were identified by means of the SmartFormula algorithm of Data Analysis (Bruker Daltonics, Bremen, Germany). The mass tolerance was set to six mDa, minimum H/C ratio of 0.3 and a maximum of 2.3 and an mSigma value below 50.The mSigma value shows the possibility of a calculated sum formula to the measured mass, based on the different isotopes.

3. Results and discussion

Coloration of adducts formed from incubation of Ros with and without amino acids: Phenolic compounds at pH 9 or in the presence of PPO oxidizes to large amount of quinones, which polymerize and form melanins with a dark color [15]. Incubation of Ros with and without studied amino acids at alkaline and enzymatic conditions resulted adducts with dark green and brown colors except in case of L-Cys (no color is formed)**.**Where,

oxidation of Ros without adding any amino acids formed the same brown color as the reaction mixture of Ros and L-Lys, but the color was darker at alkaline conditions compared to enzymatic conditions. Moreover, the results showed that incubation of Ros with L-Cys at alkaline conditions did not form any color, which may be due to the highly nucleophilic character of L-Cys**.** This observation was reported previously by others [13, 15] who found that , no color formed during the interaction of chlorogenic acid with cysteine at alkaline conditions . Moreover, the color changes occurring during the reaction of amino groups with oxidized Ros maybe indicative to covalent attachment of Ros to the amino group.

Identification of the oxidized Ros adducts formed at alkaline and enzymatic conditions: Prior to the identification of covalent products formed between oxidized Ros and studied amino acids (L-Lys and its derivative (N-Boc-Lys) and L-Cys), the masses of the oxidized and fragmented adducts (colored products) of Ros formed at both oxidation conditions, alkaline and enzymatic, were identified using the QTOF mass spectrometer. The data were checked with the Smart Formula algorithm (Bruker Daltonics , Bremen , Germany), which calculated sum formula with the highest possibility and compare them with the measured masses. The sum formula with the highest score was compared to the expected sum formula to prove the results. The results are summarized in Table 1 and figures 2 (A and B) and Figures 3 (A, B, and C). Additionally, the proposed fragmentation pathway of the rosmarinic acid dimer is given in Figure 4. The results obtained through the incubation of Ros at pH 9 and in the presence of tyrosinase at pH 6.5 for 2 h, revealed that numerous adducts were formed **.** As discussed above, the observed dimers of Ros were identified as the main adducts of both modification conditions (Figures 2), such dimers had [M−H]⁻ ions at *m/z* 719.17 Da, which is in well agreement with literature [23-25]. Moreover, the oxidation of Ros produced the Ros quinone [M−H]⁻ ions with m/z 359 .08 and 313 .1, corresponding to loss of 46 Da from the Ros.

The formation of quinones and dimers of phenolic compounds were also observed from the oxidation of CQA and Ros at closely conditions [10, 15, 26, 27]. It is also noteworthy to point out that a new

compound [M−H]⁻ ion at *m/z* Da (corresponding to Ros dimer minus 262 Da) was only formed at enzymatic conditions Figure 2 (B).

Table 1. Mass spectrometric characteristics of the major oxidized rosmarinic acid (Ros) adductsformed at alkaline and enzymatic conditions without amino acids

Identified Masses (m/z)	Oxidation conditions		proposedadducts
	Alkaline	Enzymatic	
719.17	√	√	Rosmarinic acid dimer
457.05	×	√	Ros dimer minus 262 Da
359.08	√	√	Rosmarinic acid quinone
313.10	√	√	Decarboxylated rosmarinic acid
179.03	√	√	Caffeic acid
197.04	√	√	2-hydroxy derivative of hydrocaffeic acid
161.02	√	√	Caffeic acid minus 18 Da(deprotonated caffeoyl residue)
135.04	√	√	Caffeic acid minus 44 Da (a carboxylic acid moiety)

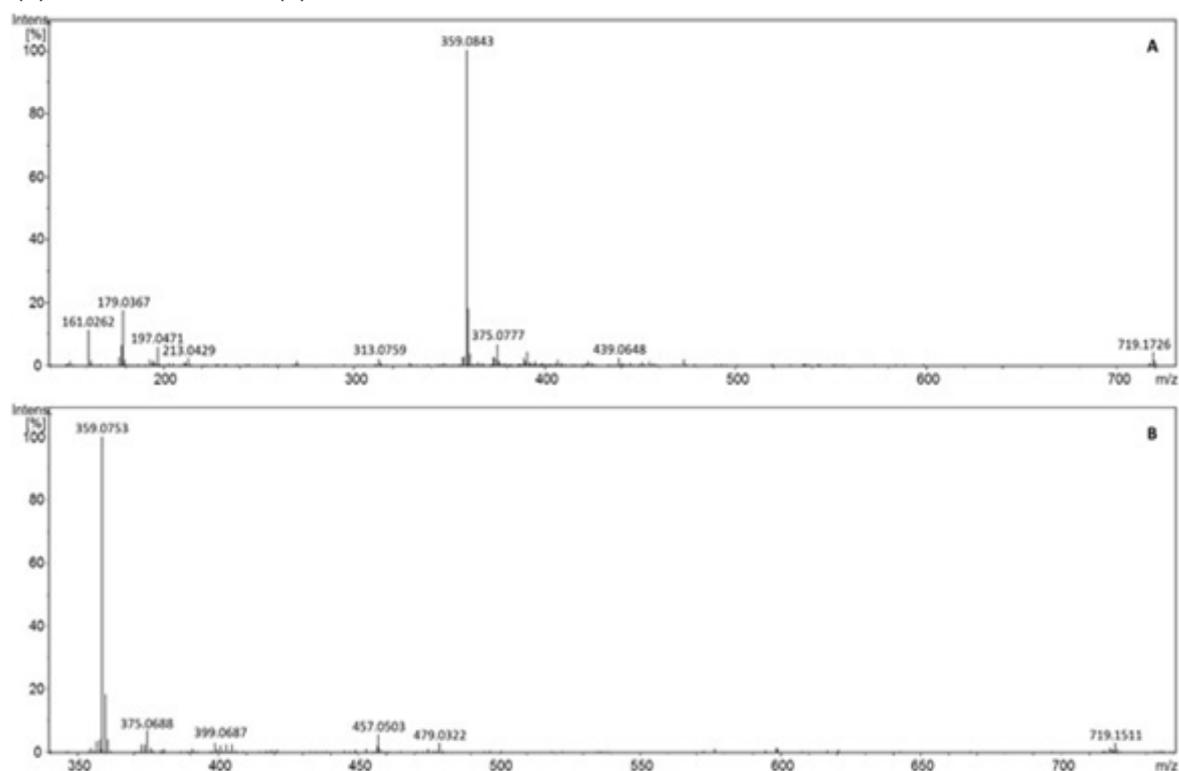**(×) = Not detected and (√) = detected**

Figure 2. Exemplary of electrospray ionization mass spectrum of oxidized rosmarinic acid adducts formed at alkaline (A) and enzymatic (B) conditions
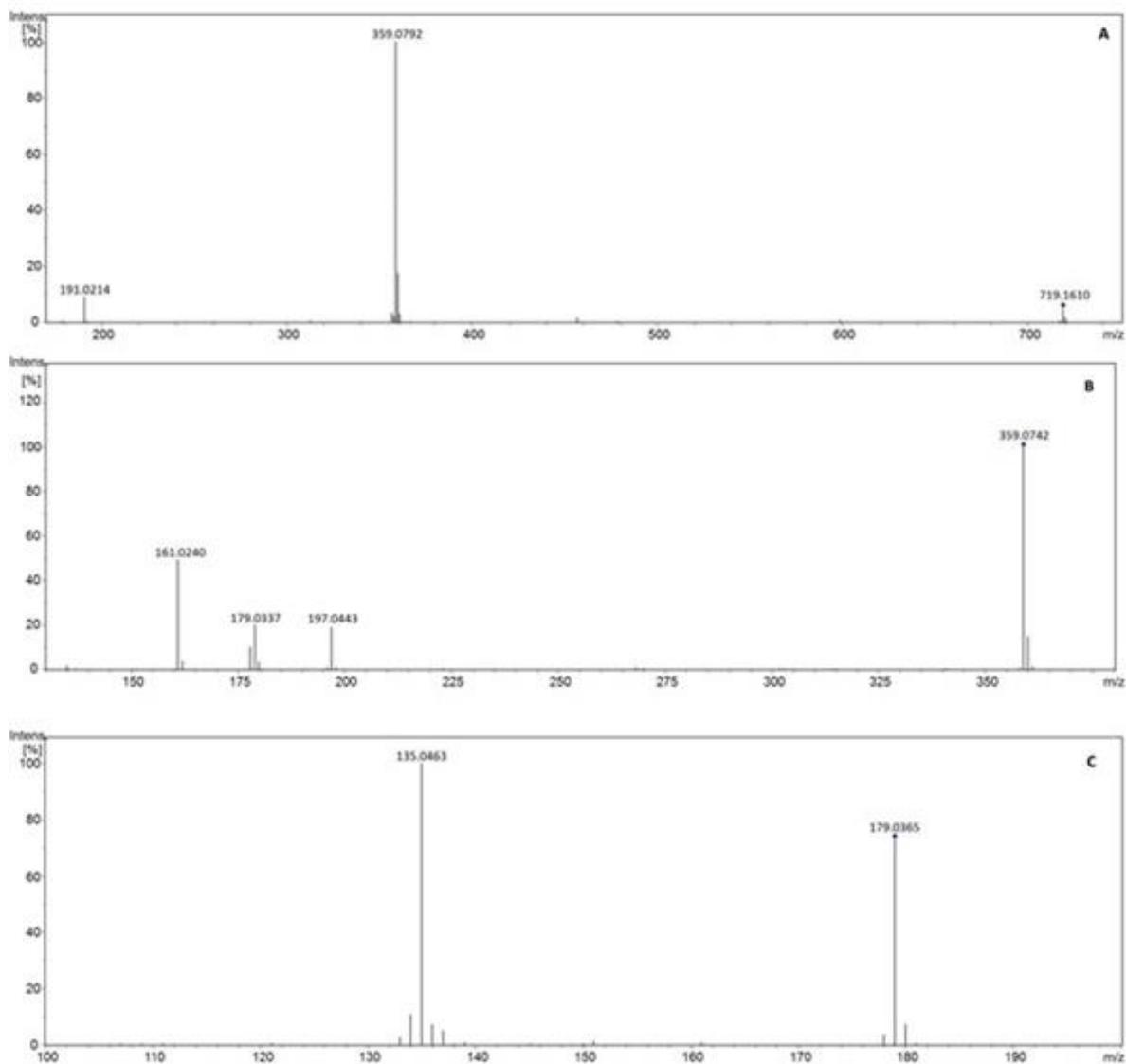
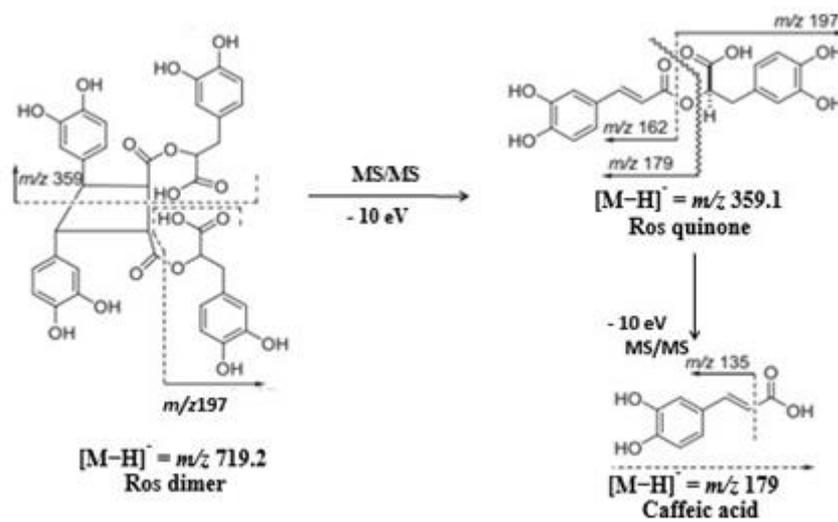*Figure 3*. ESI-MS2 spectrum of the fragmentation of rosmarinic acid dimer

Figure 4. Figure 4. Proposed fragmentation pathway of the rosmarinic acid dimer, adapted from *Barros et al*. [23]

In addition, the fragmentation of dimers formed at enzymatic and alkaline treatment was done at different energies and the results showed the identified oxidized monomers (Table 1 and Figures 3). As can be noticed from the MS^2 spectra of the oxidized Ros monomer,several degradation products were identified. [M−H]⁻ ions with m/z 197.04 Da (2- hydroxy derivative of hydrocaffeic acid formed by the loss of 162 Da from rosmarinic acid), m/z 179.03 Da (caffeic acid)and m /z 161. 02 Da(caffeic acid minus 18 Da or deprotonated caffeoyl residue) were identified (Figure 3 B.)

In addition to these fragments, a [M−H]⁻ ion with the mass 135.04 Da (corresponding to loss of 44 Da, a carboxylic acid moiety, from the caffeic acid) was formed from fragmentation of caffeic acid with 10 eV energy (Figure 3C). These results are consistent with those reportedpreviously in other studies [23, 24, 28, 29].

Identification of adducts formed between Ros and different amino acids at alkaline and enzymatic conditions: As mentioned above,upon oxidation at alkaline and enzymatic conditions, rosmarinic acid oxidizes to quinones,then two quinones polymerize together to form a dimer, whichis very reactive towardsamino acids to finally form benzacridineconjugates.In the current study, N-Boc-Lys, L-Lys and L-Cys, were usedin two models of modifications to study thenucleophilic addition of their aminoand thiol side chains to alkaline and enzymatically generated Rosquinone and to characterize the adducts formed via QTOF-MS.

The formed adduct masses (m/z values) in the reaction mixture of oxidized Ros with studied amino acids, at alkaline and enzymatic conditions, were identified and the results are presented in *Table 2*.

Identification of adducts formed between Ros and L-Cys at alkaline and enzymatic conditions: *Table (2)* shows that the incubation of Ros with L-Cys at alkaline conditions formed six different adducts compared to only three adducts at enzymatic conditions. In addition to Ros dimer formation, the interaction of L-Cys with Rosquinone produced from two hours incubation at alkaline and enzymatic conditions, presented many new products. Two of these products could be indeed identified as the adducts of one and two L-Cys with monomer of oxidized Ros. MS identified [M-H]⁻ ions with masses of 480.1 Da and 599.1 Da, which may correspond to L-Cys / Ros monomer quinone and 2L-Cys / Ros monomer quinone (*Table 2 and Figure 5*).

These results are in the line with *Tang etal*. [16], who studied the interaction between quinone formed from rosmarinic acid (RosA) and thiol group (cysteine) in the presence of H2O2 and FeSO4, at pH 5. 8 and 30 °C with stirring for 5 h in the dark and they identified two adducts: RosA quinone /Cys and RosA quinone /2Cys. These two adducts were found after both modification conditions in the present study. The mole masses of adducts were identified using ESI-MS spectra and the fragmentation was conducted for further examination. In the fragmentation process, the loss of a 121 Da fragment was detected, which is resulting from cleavage of the L-Cys moiety from L-Cys / Ros monomer quinone adduct (*Figure 6*).
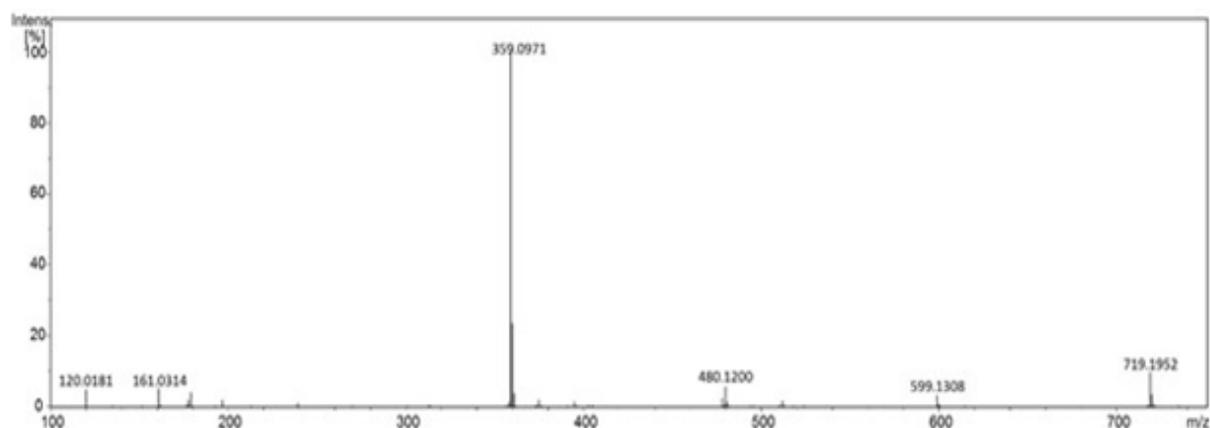

Figure 5. Exemplary of the electrospray ionization mass spectrum of the oxidized rosmarinic acid (Ros)and L-Cysteine (L-Cys) incubated at alkaline conditions

Table 2. Mass spectrometric characteristics of adducts formed betweenL-Cysteine (L-Cys) and tert-butyloxycarbonyl-L-lysine (N-Boc-Lys) amino acids and oxidized rosmarinic acid at alkaline and enzymatic conditions

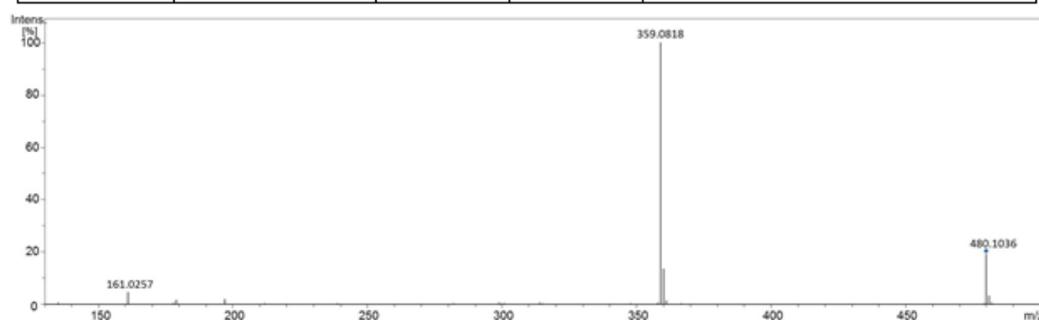| Amino acids	Identified masses (m/z)	Modification conditions		Hypothetical adducts
		Alkaline	Enzymatic	
L-Cys	719.19	√	√	Ros dimer
	480.11	√	√	L-Cys + Ros monomer
	599.13	√	√	2L-Cys + Ros monomer
	840.20	√	×	L-Cys + Ros dimer
	961.20	√	×	2L-Cys + Ros dimer
N-Boc-Lys	719.17	√	√	Ros dimer
	491.31	√	√	N-Boc-Lys dimer
	605.24	√	√	N-Boc-Lys + Ros monomer
	773.40	√	×	N-Boc-Lys + Ros dimer minus 192 Da

Figure 6. The ESI-MS2 spectrum of the fragmentation of identified L-Cysteine- rosmarinic acid quinone adduct (L- Cys-Ros) (mass 480.1037 Da)
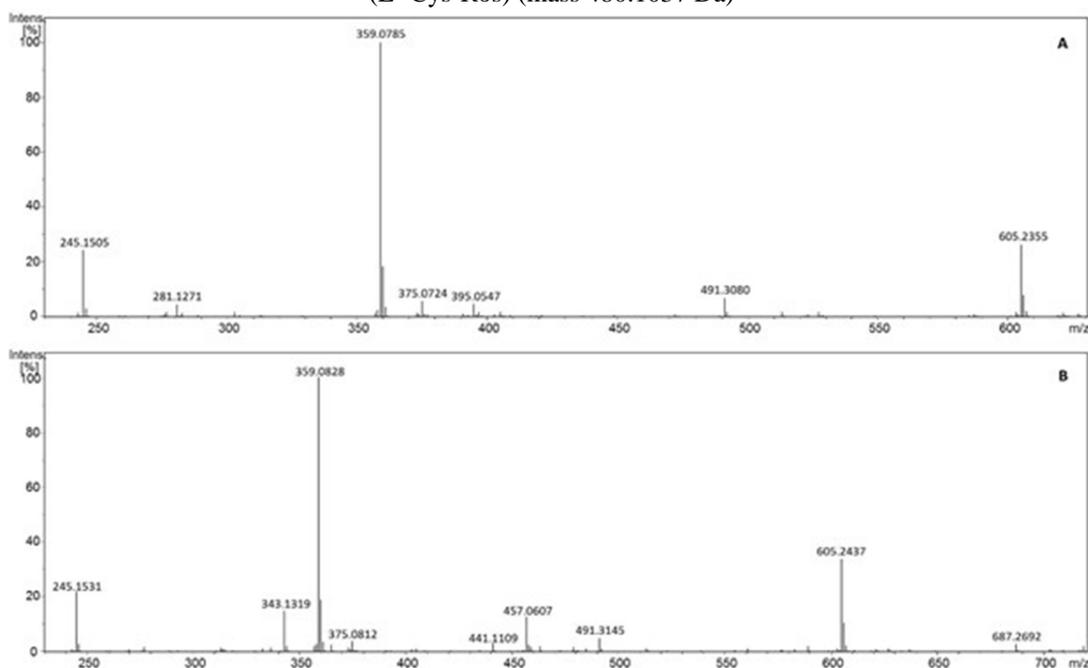

Figure 7. Exemplary of theelectrospray ionization mass spectrum of the oxidized rosmarinic acid and tert-butyloxycarbonyl-L-lysine (N-Boc-Lys) incubated at alkaline (A) and enzymatic (B) conditions.

As also mentioned in *Table 2*, another two adducts named 2L-Cys / Ros monomer and 2L-Cys / Ros dimer with *m/z* values of 840.2 and 961.2 Da respectively were observed during the interaction of L-Cys with Ros at alkaline conditions and not found at enzymatic conditions. This means that the reaction between Ros and L-Cys at alkaline condition is stronger compared to enzymatic conditions. Additionally, at pH 9, cross-linking between two cysteine molecules can happen. The explanation of forming the adducts between L-Cys and Rosquinone was described by [16], whoreported that, in case of L-Cys / Ros monomer quinone adduct, the L-Cys molecule was attached to the caffeoyl moiety of Rosquinone, while in 2L-Cys / Ros monomer quinone, two L-Cys molecules could be attached to the 2- and 2′-positions of Rosquinone, depending on the 1,4-Michael addition reaction (*Figure 1*).

Identification of adducts formed between Ros and N-Boc-Lys at alkaline and enzymatic conditions: *Table 2* and *Figures 7* (A and B) reveal the formed adducts between Ros and N-Boc-Lys at alkaline and enzymatic conditions. A [M-H]⁻ compound with an*m/z* value of 491.3 Da, corresponding to cross-linking between two N-Boc-Lys moieties, was detected at alkaline conditions and not found at enzymatic conditions. In addition to this *m/z* value, another new adduct with *m/z* value of 605.2 Da was identified in the reaction mixtures formed at both modification conditions. The detected mass may correspond to N-Boc-Lys covalently modified with one Ros monomer at the epsilon amine group. According to previous studies [15, 20], the covalent interactions between phenolic compounds and amino acids or proteins at alkaline conditions are stronger than at enzymatic conditions, probably because the alkaline modification occurs at pH 9 where most of the amine groups are deprotonated. In addition, an adduct with *m/z* value of 773.4 Da, which may correspond to the *m/z* value of N-Boc-Lys / Ros dimer minus 192 Da was only identifiedat alkaline conditions (*Table 2*). According to our knowledge, this is the first study on the covalent interactions between rosmarinic acid and N-Boc-Lys at alkaline and enzymatic conditions.

Comparison between the adducts formed between Ros and N-Boc-Lys and L-Lys atalkaline conditions: For the first time, the current study showed the formation of adducts from the covalent interactions between rosmarinic acid and L-Lys at alkaline conditions. Incubation of Ros with L-Lys at

alkaline conditions gave rise to form colored adducts which may be anindicationofthe covalent bound of Ros dimers to the NH₂ group of L-Lys. Mass spectra of L-Lys incubated with oxidized Ros were searched for the presence of masses (*m/z* values) that would confirm the presence of reaction products between Ros monomers or dimers and L-Lys. The *m/z* values detected in the reaction mixture of oxidized Ros and L-Lys are shown in *Table 3*. There were three new adducts, L-Lys / Ros monomer with m/z value 505.21 and L-Lys / Ros dimer with *m/z* value 865.3 Da. Moreover, an [M-H]⁻ compound with an*m/z* value of 792.25 Da, corresponding to L-Lys / Ros dimer minus 44 Da + 28 Da was detected. In arecent study by [15], the adducts formed between L-Lys and chlorogenic acid (CQA) at alkaline condition were characterized and the L-Lys and CQA quinone adduct wasnot found but the adductformed between the L-Lys and CQA dimer was observed. When comparing the hypothetical adducts formed with L-Boc-Lys and those with Lys there is not much difference between of them. Because of the similarities between L-Lys and L-Boc-Lys, the main binding site for Ros seems to be the epsilon amine group: in the L-Boc-Lysine (*Table 2*), theα-NH₂ group is protected, but the ε-NH₂ is available for reactions.

Table 3. Mass spectrometric characteristics of adducts formed between L-Lysine (L-Lys) and the oxidized rosmarinic acid (Ros) at alkaline conditions

Identified masses (m/z)	Hypothetical adducts
719.19	Ros dimer
505.21	L-Lys + Ros monomer
865.31	L-Lys + Ros dimer
792.25	L-Lys + Ros dimerminus 44 Da + 28 Da

4. Conclusion

In this study,the covalent modification of amino acids,N-Boc-Lys, L-Lys and L-Cys, with Ros were conducted under two different conditions (alkaline versus enzymatic treatment). The characterization of Ros monomers and dimers and its adducts with amino acidswere studied using flow injection electrospray ionization - quadrupole time-of-flight - mass spectrometry (ESI-QTOF - MS) via a syringe pump. There were more different adducts formed at alkaline modification than observed after enzymatic treatment. In addition, the L-Cys was more reactive towards Ros compared to L-Lys and L-Boc-Lys.

There was no significant difference between L-Lys and L-Boc-Lys, giving evidence that the epsilon amine group is the primary reaction site of the amino acids. According to our knowledge, thisis the first study about the identification and characterization of adducts formed from the covalent interactions of Ros with different amino acids at alkaline and enzymatic conditions. At the end, we think that the results of this work could be interesting because a new compound formed between amino acids and rosmarinic acid may have a better solubility in aqueous solutions furthermore, can keep somepositive properties of phenolic compounds (rosmarinic acid). Taking together, it can be concluded that covalent interactions between Ros and the side chains of molecules of lysine or cysteine may also be expected to occur in proteins. However, more workshould be done to know thefate of such adducts in thebody. For example, their metabolism andexcretion still needs to be studied.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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