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

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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 rosmarinicacid (Ros) were conductedwithtwo different methods;alkaline (pH 9) and enzymatic [in the presence of tyrosinase (polyphenoloxidase, PPO)].The formed adducts were identified and characterized usingflow injection electrospray ionization - quadrupole time of flight (FI-ESI-QTOF) mass spectrometry.Many adducts were identified for the first time. The obtained dataexhibitedslight differences between the adducts formed afteralkaline and enzymatic treatment.In case of the incubation of Roswith both methods without amino acids,numerousadducts, such as Rosquinone, Ros dimer anddecarboxylatedRos 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 monomerand dimerof Roswere covalently added to the side chainsof selected amino acids.Additionally, the covalent attachment between oxidized Rosand the side chains of twomolecules of amino acids was also identified.Finally, L-Cys was the most susceptible amino acid to react withoxidized Ros compared to L-Lys and N-Boc-Lys. It could be concluded that, the results show some similarities betweenthe adducts formed at both modification conditions. Moreover, thecovalent interactions between Rosand 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

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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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].

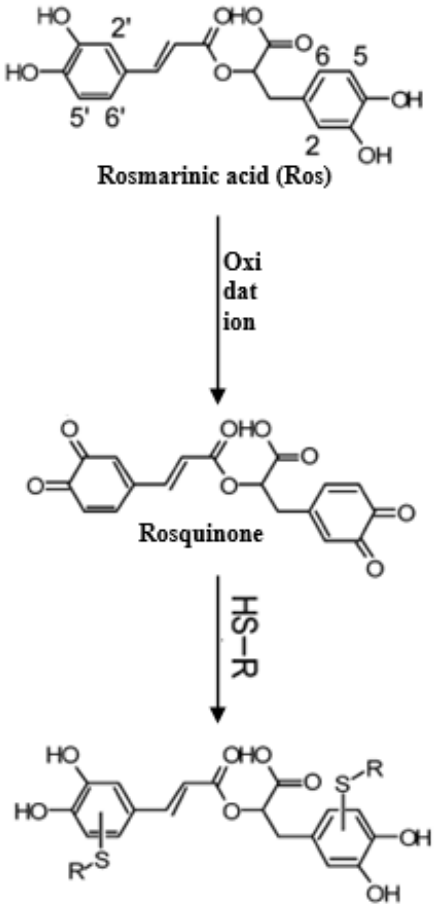

Figure 1. **T**he proposed mechanism of interaction between Rosand L-Cys, modified from [16]

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 productsstill 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 theknowledge on the interactions of rosmarinic acidwith 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

2.1. Materials

Rosmarinic acid (Ros; 96%; (R) – O - (3, 4-Dihydroxycinnamoyl) – 3 - (3, 4-dihydroxyphenyl) lactic acid, 3,4-Dihydroxycinnamic acid (R) – 1-carboxy-2-(3,4-dihydroxyphenyl)ethyl ester, MW 360.32 g/mol). Amino acids, L-Lysine (L-Lys, MW 146.19 g/mol) and its derivative, N-Boc-Lysine, tert – butyloxycarbonyl – L – lysine (N – Boc - Lys, MW 246.30 g/mol), and L-Cysteine (L-Cys, MW 121.16 g/mol) and tyrosinase T3824 from mushroom (PPO, with an activity of 2687 U mg^{-1}at 25 ˚C and pH 6.5) were obtained from Sigma-Aldrich (St. Louis, USA). All other reagents and solvents used were analytical or gradient grade.

2.1. Methods

Modification ofamino 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 modelspreviously described by [15, 19], with somemodifications.

For alkaline modification, 112 mM of individual amino acids, L-Lys, L-Cys and N-Boc-Lys as well as a 28mM Ros were dissolved in deionized waterand mixed at a ratio of (1/1, v/v, Ros/amino acids). The mixtures were adjusted to pH 9 with 0.1M NaO Hand stirred in open flasks for 2h at room temperature. 28 mM solution of Roswas mixed with deionized water at pH9 (1/1, v/v) and used as a control. While, for enzymatic modification, amino acids (L-Cysand N-Boc-Lys, 112 mM) and Ros (28mML^{-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 werestirred in open tubes for 2 h at 25 °C. 28mM solution of Roswasmixed 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 bothinteraction conditionswere identified using an ESI-QTOF mass spectrometer (micrOTOF-QII, Bruker Daltonics, Bremen, Germany). Samples wereinjectedvia a syringe pump. For the ionization, an electrospray source in thenegative 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 asnebulizing 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 MS_n. The method was calibiratedeach morning and in between of the measuring cycles with a lithium formate solution (Sigma - Aldrich, St. Louis, USA). The fragmentation wasconductedwith the multi-reaction-monitoring method. Therefore, nitrogen gas was used as collision gas. Thecollision energies were alternated between0 ev(no fragmentation), 10 eVand 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 adark color [15]. Incubation of Ros with and withoutstudied amino acids at alkaline and enzymatic conditionsresulted 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-Cysat 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 [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 Rosmay be indicativeto covalent attachment of Ros to the amino group.

Identification of theoxidized Rosadducts formed at alkaline and enzymatic conditions: Prior to the identification of covalent products formedbetween oxidized Rosand studied amino acids (L-Lysand its derivative (N-Boc-Lys) and L-Cys), the masses of the oxidized and fragmentedadducts(colored products) of Rosformed at both oxidation conditions,alkaline and enzymatic,wereidentified usingthe QTOF mass spectrometer. The data were checked with the SmartFormula 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 aresummarized in *Table 1* and *Figures 2* (A and B) and *Figures 3* (A, B, and C). Additionally, the proposed fragmentation pathway of therosmarinic acid dimer is given in *Figure 4*. The results obtained throughthe incubation of Rosat pH 9 and in the presence of tyrosinase at pH 6.5 for 2 h, revealedthat numerous adducts were formed**.** As discussed above, the observed dimers of Roswere

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 [23-25]. Moreover, theoxidation of Ros produced the Rosquinone $[M-H]^-$ ionswith 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* 457.05 Da (corresponding toRos 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

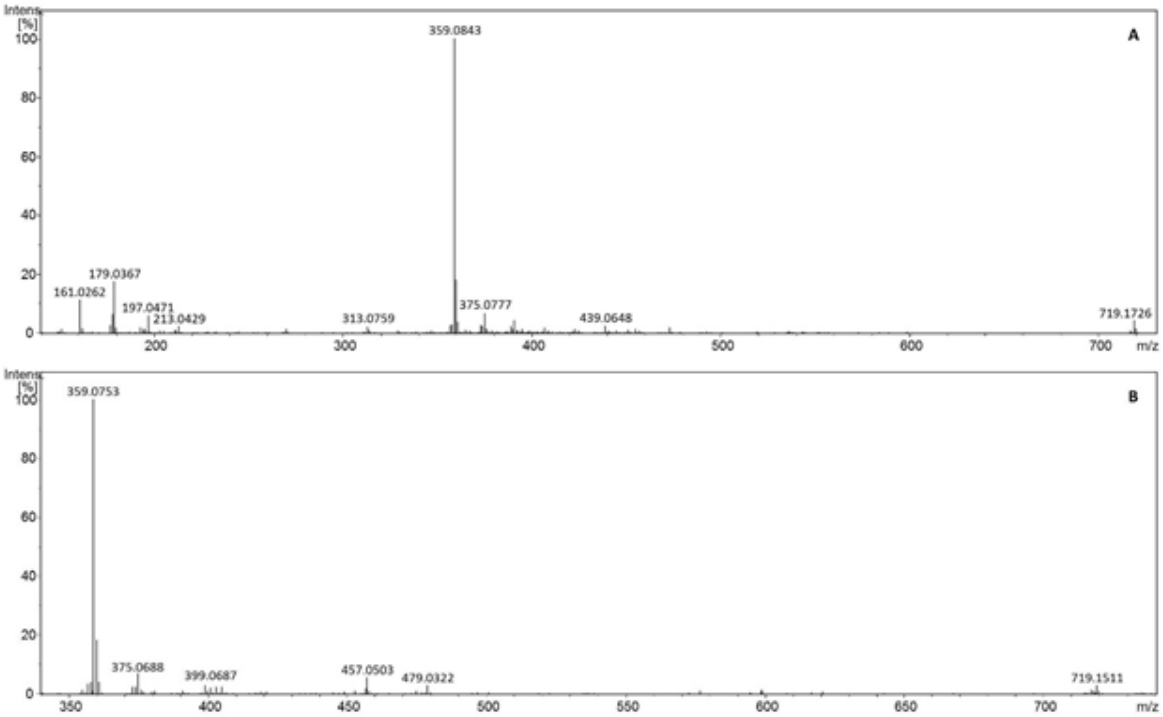

Figure 2. Exemplary of electrospray ionization mass spectrum of oxidized rosmarinic acid adducts formed at alkaline (A) and enzymatic (B) conditions

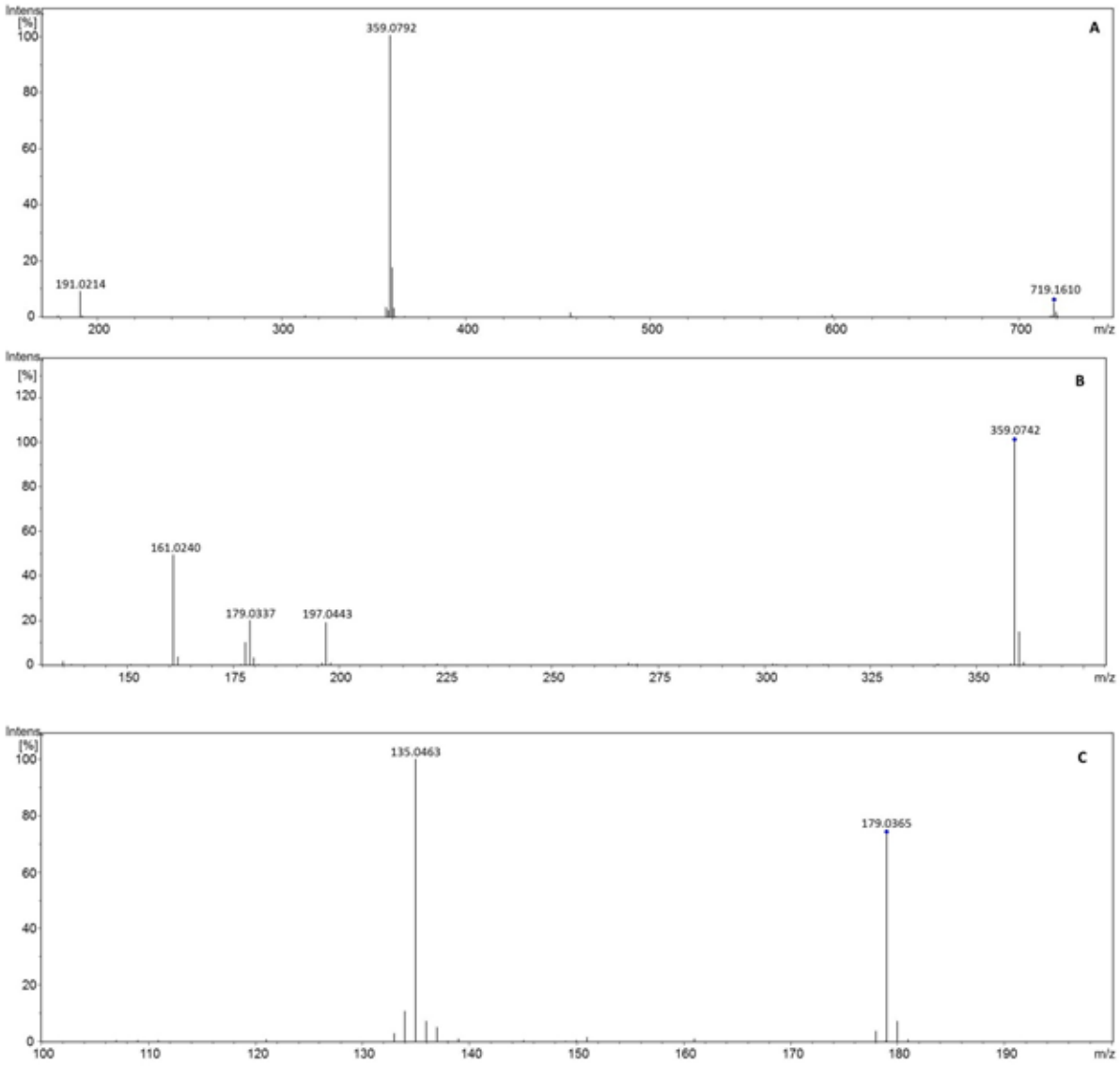

Figure 3. ESI-MS2 spectrum of the fragmentation of rosmarinic acid dimer

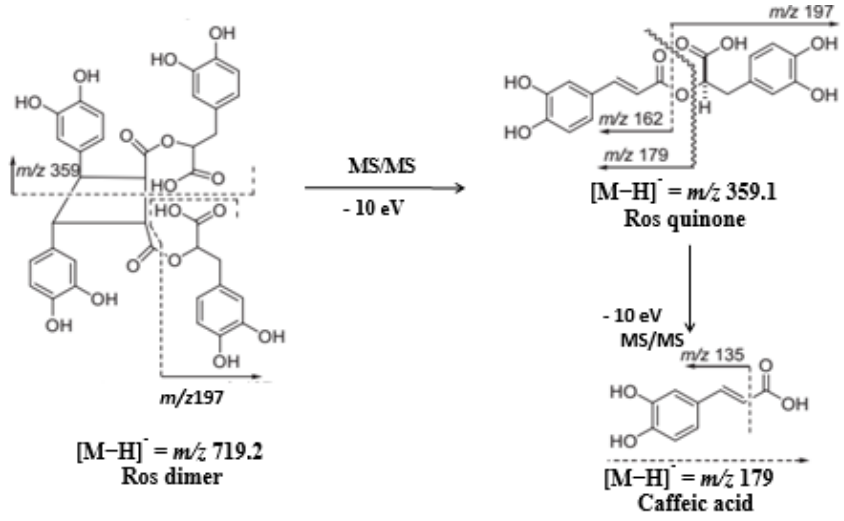

Figure 4. Proposed fragmentation pathway of the rosmarinic acid dimer, adapted from [23]

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 *Figure 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 bythe 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*).

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 3*). These results are consistent with those reported previously by [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 [16], who studied the interaction between quinone formed from rosmarinic acid (RosA) and thiol group (cysteine) inthe presence of H_2O_2 and $FeSO_4$, at pH 5.8 and 30 °C with stirring for 5 h in the dark and they identified two adducts: RosAquinone /Cys and RosAquinone /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*).

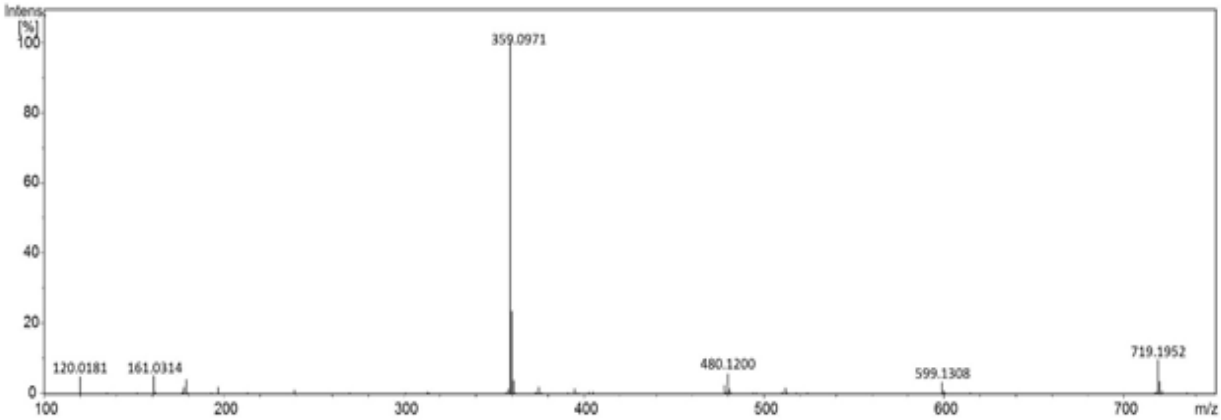

Figure 5. Exemplary of the electrospray ionization mass spectrum of the oxidized rosmarinic acid (Ros)and L-Cysteine (L-Cys) incubated at alkaline conditions

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

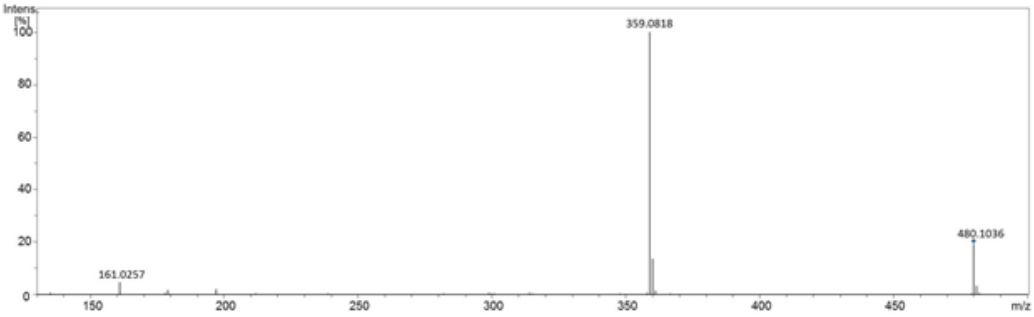

Figure 6. The ESI-MS2 spectrum of the fragmentation of identified L-Cysteine- rosmarinic acid quinone adduct (L- Cys-Ros) (mass 480.1037 Da)

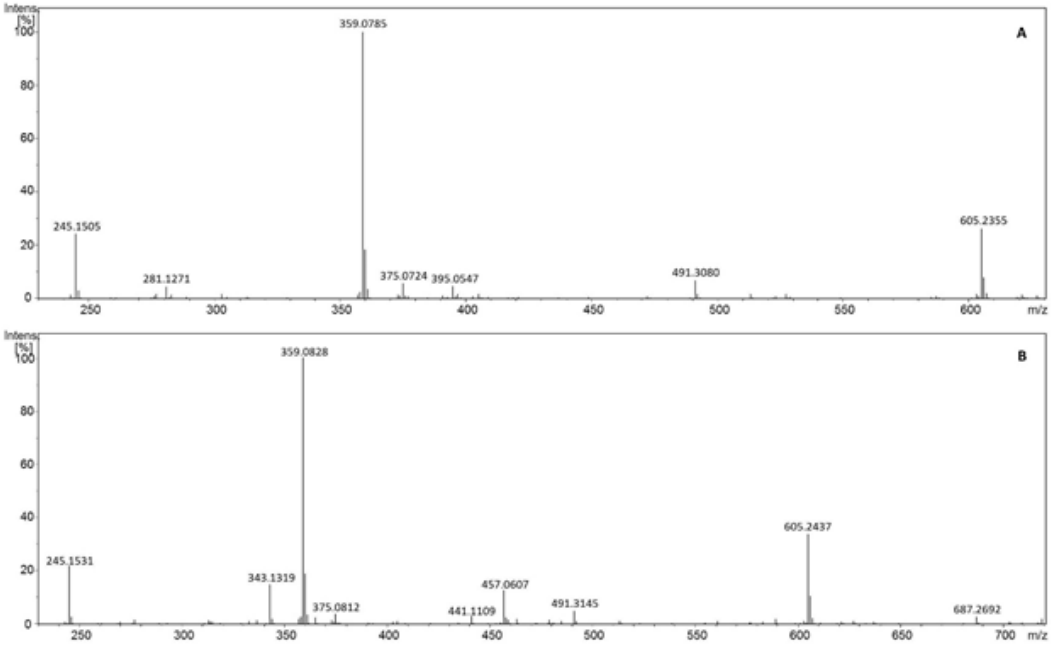

Figure 7. Exemplary of theelectrospray ionization mass spectrum of the oxidized rosmarinic acid and tert-butyloxycarbonyl-L-lysine (N-Boc-Lys) incubated at alkaline (A) and enzymatic (B) conditions.

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 descripted 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 NH_2 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-NH_2 group is protected, but the ε-NH_2 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.

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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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