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International Journal of Mass Spectrometry 231 (2004) 119–129
International Journal of Mass Spectrometry
www.elsevier.com/locate/ijms

Liquid chromatography coupled to negative electrospray/ion trap mass spectrometry for the identification of isomeric glutathione conjugates of catechol estrogens

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Received 4 July 2003; accepted 29 September 2003

Abstract

Conjugation to glutathione (GSH) represents an important detoxification pathway for preventing DNA damage due to oxidation products of catechol estrogens. In order to identify isomeric GSH conjugates of catechol estrogens, liquid chromatography coupled to electrospray/ion trap mass spectrometry was used. For this purpose, both positive and negative ion modes were applied, generating protonated and deprotonated species, respectively. Energy-resolved fragmentation of each isomeric quasi-molecular ion was achieved in two regions of the mass spectrometer: (i) the mass analyzer (ion trap mass spectrometer) and (ii) the interface region of the electrospray ionization source. The resonance excitation of $[M + H]^+$ protonated ions carried out into the ion trap did not show any isomeric differentiation. Although ion source fragmentation of these same species allowed identification of each isomer, this method requires a good chromatographic separation, making it inefficient for the analysis of low sample amounts from in vitro or in vivo sources. Conversely, using resonance excitation of deprotonated ions, isomer distinction could be achieved. Thus, this technique should yield the best data for the direct characterization of isomers of catechol estrogen–GSH conjugates from biological samples.

Keywords: Catechol estrogens; Glutathione conjugates; Electrospray ionization; Ion trap mass spectrometry; Energy resolved mass spectrometry

1. Introduction

One of the major significant pathways of estrogen metabolism in man and mammal is the aromatic hydroxylation of the primary estrogens at either the C2 or C4 position leading to the formation of catechol estrogens. These products have been shown to play a causative role in the initiation of breast and other cancers [1–4]. The C2 or C4 hydroxylated metabolites can undergo further enzymatic oxidation processes yielding highly electrophilic intermediates, such as the *o*-quinones, that are highly reactive and can damage DNA. These *o*-quinones are susceptible to adduction to nucleophilic groups via Michael addition [5,6]. They can covalently bind to DNA bases and lead to two types of adducts: stables adducts that may be removed from DNA by repair and depurinating adducts [1,3,7] generating apurinic sites by cleavage of the glycosidic bond. The presence of these apurinic sites may induce mutagenicity phenomena [8] that constitute a critical initiating event in carcinogenesis. A competitive mechanism preventing DNA damage by estrogen *o*-quinones is the detoxification of the *o*-quinones by conjugation with the tripeptide glutathione (GSH). GSH reacts very rapidly with the *o*-quinones via a Michael addition process similar to that involved with DNA bases, resulting in the formation of isomeric catechol estrogen-derived GSH. This process gives much more abundant products relatively to DNA adducts. GSH conjugates of catechol estrogens have been identified both in vitro and in vivo [2,7,9–13]. Their presence provides relevant information on the extent of enzymatic oxidation of catechol estrogens to their *o*-quinones, reactive electrophilic metabolites. Thus, the GSH conjugates can be considered as biomarkers to reveal the formation of the catechol estrogen *o*-quinones and then the possible susceptibility to estrogen-induced carcinogenesis [7,13]. Moreover, the characterization of GSH conjugates may allow determining the structure of the primary catechol estrogen. Indeed, two isomeric GSH adducts (i.e., 2OH-E-1SG and 2OH-E-4SG) can be formed from

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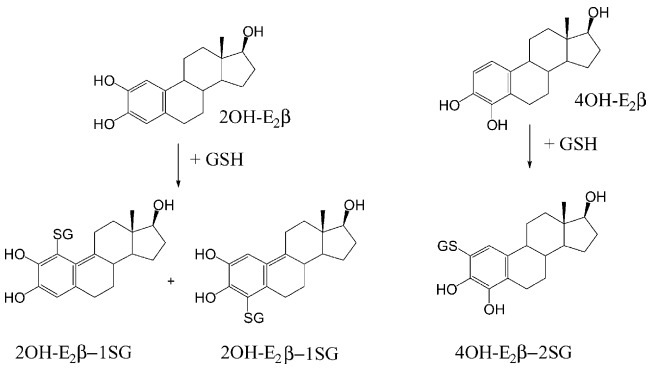
doi:10.1016/j.ijms.2003.09.016



Scheme 1. Formation of GSH conjugates from 2OH-$E_2\beta$ and 4OH-$E_2\beta$.

the reaction of GSH with estrogen 2,3-quinones, whereas the reaction of estrogen 3,4-quinone with GSH leads to 4OH-E-2SG, as the only adduct (Scheme 1).

Liquid chromatography coupled to electrochemical detection has been used to detect GSH conjugates [2,7,13,14]. Although this method possesses a high sensitivity and selectivity for the detection of catechols, it does not provide any structural information. Because of its sensitivity and specificity, mass spectrometry appears to be a powerful technique for the structural characterization of these conjugates [15–17]. The evaluation of mass spectrometric methods for isomeric differentiation of catechol estrogen–GSH conjugates has been achieved by the group of Gross using positive electrospray ionization [18]. The results showed that the isomers were much distinctive using high energy collision-induced dissociation (CID) from a tandem four sector instrument than using low energy CID from a triple quadrupole instrument or an ion trap mass spectrometer.

In this work, the potentiality of liquid chromatography coupled to electrospray/quadrupole ion trap mass spectrometry was examined in order to develop a methodology for the direct identification of isomeric catechol estrogen–GSH conjugates from biological samples. CID experiments, and particularly energy-resolved mass spectrometry experiments, are often used to distinguish isomeric compounds [19,20]. By varying the collision energy, various CID mass spectra of each isomeric quasi-molecular ion are obtained, leading to energy-resolved breakdown graphs. In this work, both positive and negative ionization modes were assessed allowing the examination of the behavior of both protonated and deprotonated isomeric quasi-molecular ions, respectively. For the three isomers, i.e., 2OH-$E_2\beta$-1SG, 2OH-$E_2\beta$-4SG and 4OH-$E_2\beta$-2SG, a comparison of the breakdown graphs established from energy-resolved CID data was studied.

2. Experimental

2.1. Mass spectrometry

All experiments were carried out using an electrospray/quadrupole ion trap mass spectrometer (Finnigan LCQ, Thermo Finnigan, Les Ulis, France). Both positive and negative ionization modes were used. The electrospray needle was set at 5 kV for the positive ion mode and at −4.5 kV for the negative ion mode. The heated capillary was maintained at 230 °C. CID studies were carried out in both the interface region (so-called ion source CID) and the mass analyzer. Energy-resolved experiments were achieved by varying the collision energy (offset voltage of the octapoles in the transfer region for ion source CID and peak-to-peak resonance excitation rf voltage for mass analyzer CID).

To clarify some decomposition processes, MS^n experiments were performed on the mass selected ion in the ion trap mass spectrometer using standard isolation and excitation procedures. Helium buffer gas also served as collision gas for these experiments. All spectra were acquired under automatic gain control (AGC) conditions.

2.2. Liquid chromatography

Liquid chromatography was used to separate the three isomeric GSH conjugates of catechol estrogens. This was achieved using a Thermo Separation P4000 pump (Thermo Quest, Les Ulis, France) fitted with a Rheodyne injector. The LC column was an Ultrabase 5 μm C18 column (250 mm × 2 mm) operated at a flow rate of 0.2 ml/min. The following gradient elution was used: 100% A for 5 min, 100% A to 80% A from 5 to 10 min, 80% A from 10 to 15 min, then 80% A to 40% A from 15 to 50 min, 40% A to 100% B from 50 to 55 min, and finally 100% B from 55 to 60 min, with A, $H_2O/CH_3CN/CH_3COOH$ (95:5:0.5 v/v/v) and B, $H_2O/CH_3CN/CH_3COOH$ (45:55:0.5 v/v/v).

2.3. Materials

The synthetic procedure used for the preparation of the studied isomeric GSH conjugates of catechol estrogens was described in the literature. The catechol estrogens, 2-OH-$E_2\beta$ and the 4-OH-$E_2\beta$ were prepared from commercial 17β-estradiol ($E_2\beta$) following the procedure of Gelbke et al. [21]. Oxidation of these catechols with activated manganese dioxide led to the corresponding quinones as described by Stack et al. [22], according to the method of Abul-Hajj [23]. The formed quinones were then conjugated with GSH as described by Cao et al. [11]. The reaction of 3,4-quinone with GSH produced 4OH-$E_2\beta$-2SG while 2,3-quinone gave a mixture of 2OH-$E_2\beta$-1SG and 2OH-$E_2\beta$-4SG (Scheme 1).

3. Results and discussion

3.1. Positive ionization

Under positive ionization conditions, the three isomeric catechol estrogen–GSH conjugates were protonated leading to the formation of the *m/z* 594 $[M + H]^+$ ions.

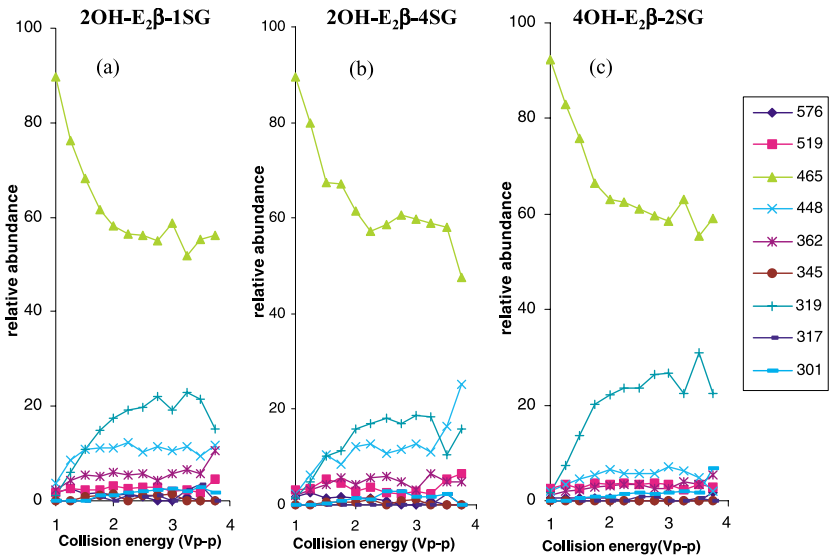


Fig. 1. Breakdown graphs of the *m/z* 594 $[M + H]^+$ ions from (a) 2OH-E_2β-1SG, (b) 2OH-E_2β-4SG and (c) 4OH-E_2β-2SG obtained by resonance excitation in an ion trap mass spectrometer.

Energy-resolved fragmentations of each isomeric quasi-molecular ion were achieved by both CID in an ion trap mass spectrometer using resonance excitation and in-source CID in the interface region of an electrospray/ion trap instrument.

3.1.1. CID studies in the mass analyzer

Fig. 1 shows the low energy CID spectra of the *m/z* 594 protonated $[M + H]^+$ ion of the three isomeric GSH conjugates as their breakdown graphs expressing the percent of product-ion abundance as a function of the collision energy. Whatever the selected protonated isomer, a similar trend was observed, showing dominant formation of the *m/z* 465 fragment ion resulting from the loss of a glutamic acid via cleavage of the amide bond of the GSH moiety. Other ions were observed in low abundance, and only for excitation voltages greater than 1 $V_{p–p}$. This could be explained by the high specificity of the mass analyzer due to the use of the resonance excitation [24]. Indeed, during the resonance excitation period, an excitation voltage is applied to the endcap electrodes of the ion trap, and thus the ion at the specified *m/z* ratio is selectively excited. In this case, the extent of deposited energy is limited compared to the non-resonance excitation process or to CID achieved in the collision cell of a triple quadupole instrument. Thus, after resonance excitation, ions formed by consecutive decomposition processes are generally not observed. However, when increasing the excitation voltage, the abundance of the *m/z* 465 ion was found to decrease whereas that of ions at lower *m/z* ratio was increased. This suggested that the ions detected at high collision energy correspond to the third generation fragment ions arising from consecutive decompositions of the *m/z* 465 fragment ion. Note that the results obtained from collision energies higher than 3 $V_{p–p}$ could not be considered because of irregularities observed in the breakdown graphs.

Breakdown graphs for 2OH-E_2β-1SG (Fig. 1a) and 2OH-E_2β-4SG (Fig. 1b) were almost identical and no isomer distinction could be made in this case. However, the breakdown graph obtained for 4OH-E_2β-2SG (Fig. 1c) presented a little difference, consisting of a lower abundance of the *m/z* 448 fragment ion formed by consecutive loss of NH_3 from the *m/z* 465 ion, compared to the two other isomers studied in this work. Because of its low abundance, the *m/z* 448 ion could not be used for an unambiguous distinction of the isomers. Hence, it was concluded that the characterization of isomeric catechol estrogen–GSH conjugates by using resonance excitation of protonated species in an ion trap was difficult. This result is in agreement with previously published data [18].

3.1.2. CID studies in the octapole region

Ion fragmentation can also be achieved in the interface region between the atmospheric pressure ion source and the mass analyzer [25]. This technique, so-called in-source CID, is non-specific. Indeed, competitive and consecutive processes can occur at the same time, leading to the formation of several fragment ions in high abundance. In addition, ion source CID generally leads to results very close to those obtained from low energy CID achieved in a triple quadrupole collision cell [26,27].

In the in-source CID experiments, energy-resolved mass spectra were obtained by varying at the same time the offset voltages of the two octapoles and the inter-octapole lens of the LCQ instrument, leading to the breakdown graphs reported in Fig. 2. These graphs were very similar to those obtained from a triple quadrupole collision cell for 2OH-E_2β-1SG and 4OH-E_2β-2SG [18]. Specific isomer

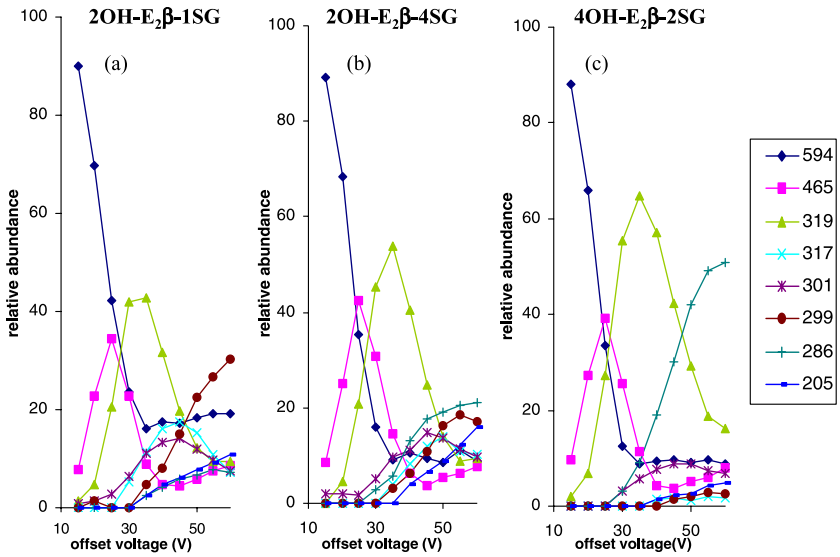


Fig. 2. Breakdown graphs of the *m/z* 594 $[M + H]^+$ ions from (a) 2OH-E_2β-1SG, (b) 2OH-E_2β-4SG and (c) 4OH-E_2β-2SG obtained by CID in the interface region of an electrospray/ion trap mass spectrometer.

fragment ions resulting from consecutive decomposition appeared at additional offset voltages higher than 35 V. In particular, the variation of abundance observed for the *m/z* 286, *m/z* 299 and *m/z* 317 fragment ions was different for each isomer. The formation of these fragment ions has already been discussed [18]. For 2OH-E_2β-1SG, the (*m/z* 286/*m/z* 299) ion abundance ratio was lower than 1 whereas for 2OH-E_2β-4SG this ratio was close to 1. In the case of 4OH-E_2β-2SG, the *m/z* 299 and *m/z* 317 fragment ions were not detected and this behavior could be considered as characteristic of this isomer.

These results showed that the in-source CID spectra obtained for offset voltages higher than 35 V allowed the differentiation of isomeric protonated GSH conjugates of catechol estrogens. In contrast with the CID experiments carried out in a triple quadrupole instrument, in-source fragmentation achieved in the interface region of an electrospray/ion trap mass spectrometer is non-specific. Indeed, in this case, no parent ion is selected and any ions present in the ion beam are fragmented. This should make difficult the identification of isomeric GSH conjugates since other compounds may co-elute during chromatographic runs. Furthermore, it should be noted that high octapole offset voltages had to be used in order to distinguish the isomeric compounds. This led to lower ion transmission efficiencies into the mass analyzer, and then to a lower sensitivity of detection.

3.2. Negative ionization

The three isomeric GSH conjugates of catechol estrogens were then analyzed in the negative ionization mode, yielding deprotonated $[M - H]^-$ ions at *m/z* 592. Energy-resolved fragmentations of each isomeric quasi-molecular ion were achieved using the resonance excitation into the ion trap device.

3.2.1. CID studies in the mass analyzer

By varying the resonance excitation voltage, CID mass spectra were obtained and plotted as breakdown graphs, as shown in Fig. 3. The examination of these graphs indicated that the three isomers mainly gave rise to the formation of a fragment ion at *m/z* 272, and that differences appeared for ions of lower abundance. By scrutinizing the portion of the previous breakdown graphs from 0 to 20% of product-ion abundance (Fig. 4), different behavior of the isomers could be evidenced for collision energies higher than 0.9 V_{p-p}. In particular, the relative abundance of the *m/z* 306 and *m/z* 319 fragment ions was characteristic of each isomer. For 2OH-E_2β-1SG, the *m/z* 306 ion intensity was always higher than that of the *m/z* 319 ion whereas for 2OH-E_2β-4SG the behavior was reverse. Finally, in the case of 4OH-E_2β-2SG, the *m/z* 306 and *m/z* 319 daughter ions were barely detectable. Note that consecutive decomposition processes were also observed at higher collision energies. For resonance excitation values greater than 1.1 V_{p-p}, the abundance of the *m/z* 574 ion decreased whereas that of the *m/z* 319 and *m/z* 254 ions increased at the same time. This indicated that the formation of these species could involve an ion–dipole intermediate (see further in the text). Thus, these results clearly showed that the resonance excitation of deprotonated ions into an ion trap can be a very useful method to distinguish isomeric GSH conjugates of catechol estrogens.

3.3. Formation of fragment ions

It is well known that under negative ionization conditions, the removed hydrogen must correspond to the most acidic site. However, the deprotonation process can competitively occur on different sites from compounds containing several labile hydrogen atoms, thus leading to the formation of different deprotonated species.

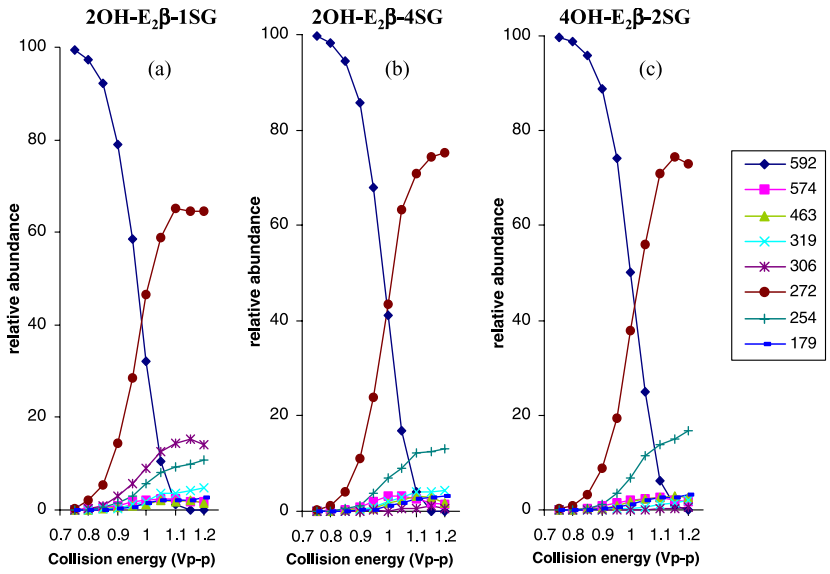


Fig. 3. Breakdown graphs of the *m/z* 592 $[M - H]^-$ ions from (a) 2OH-E_2β-1SG, (b) 2OH-E_2β-4SG and (c) 4OH-E_2β-2SG obtained by resonance excitation in an ion trap mass spectrometer.

The isomeric catechol estrogen–GSH conjugates studied in this work contain several labile hydrogen atoms located on both the steroid and the GSH tripeptide moieties. Thus, the deprotonation process can occur at various sites of the molecule but it must be favored at the carboxylate positions, leading to two possible carboxyl-deprotonated species, although other deprotonated forms cannot be ruled out. Particularly, amide-deprotonated species or enolate anions resulting from an hydrogen removal from the CH groups adjacent to the carbonyl and nitrogen of an amide function should also be taken into account. Furthermore, the formation of these species can occur either directly in the ionization process or by proton transfer in a carboxyl-deprotonated species (when deprotonation takes place at one of the carboxylic acid functions). This later process often precedes peptide-specific fragmentation through ion complexes [28–30]. Finally, the deprotonation of the steroid catechol functions has also to be considered.

In order to evidence the possible deprotonation sites, the two carboxylic acid functions of the GSH conjugates were methylated. Negative ionization of such compounds led to the formation of an ion at *m/z* 620, corresponding to the deprotonated $[M - H]^-$ species (data not shown). This indicated that in this case, an ionization process involving the removal of a hydrogen located on a site other than the carboxylate positions could occur. However, the ob-

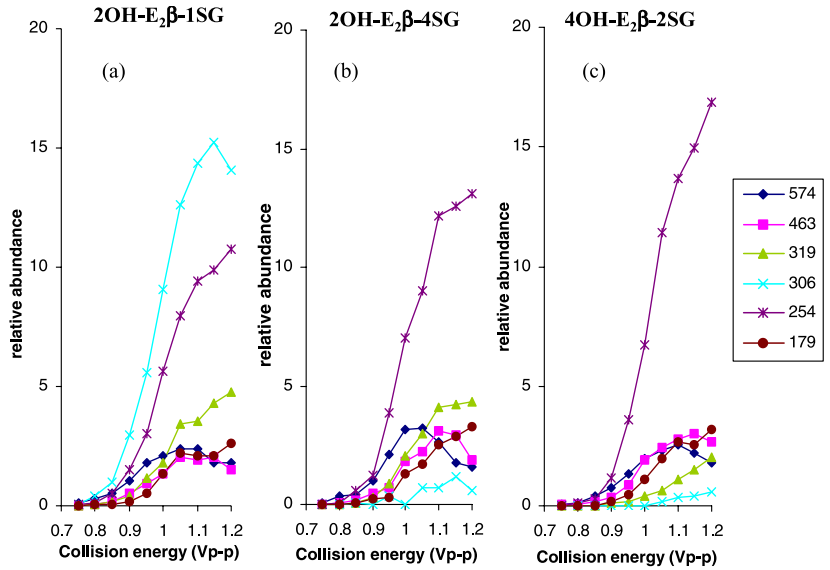


Fig. 4. Lower part (0–20% relative abundance) of the breakdown graphs of the *m/z* 592 $[M - H]^-$ ions from (a) 2OH-E_2β-1SG, (b) 2OH-E_2β-4SG and (c) 4OH-E_2β-2SG obtained by resonance excitation in an ion trap mass spectrometer.

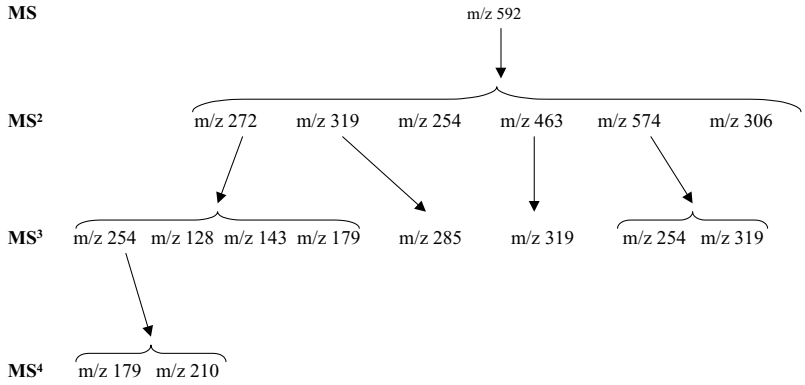


Fig. 5. Fragment ions observed from the decomposition of the *m/z* 592 $[M - H]^-$ ions from 2OH-E_2β-GSH conjugates.

served ionization efficiency was very low from the methylated compounds compared to the initial conjugates. Thus, it could be concluded that from catechol estrogen–GSH conjugates, deprotonation should mainly occur on one of the two carboxylic functions, leading to the carboxyl-deprotonated species and that ionization involving the removal of another proton should constitute a minor process.

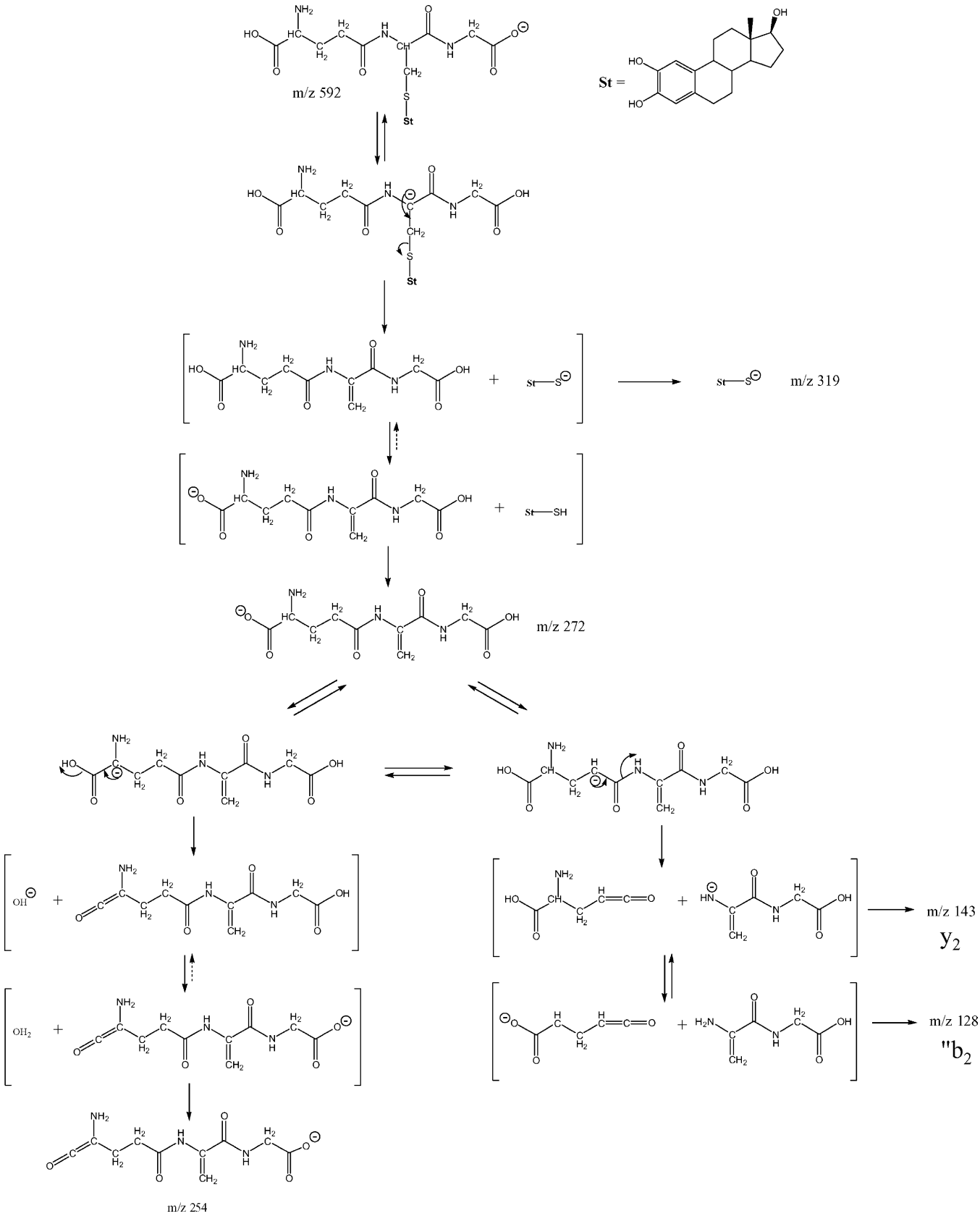
In order to clarify some decomposition processes and to provide more structural information on the various fragment ions, MS^n experiments were carried out on the three isomeric GSH conjugates. A schematic representation of the different fragment ions observed in the various MS^n steps is presented in Fig. 5.

Assuming that the initial ionization process involved the removal of a proton from a carboxylic function of the conjugates, and that the other deprotonated species were formed by proton transfer in the carboxyl-deprotonated species, some mechanisms could be proposed to explain the formation of fragment ions (Scheme 2). In the MS/MS experiment, the *m/z* 592 ions in the enolate anion form mainly underwent the cleavage of the C–S linkage leading to the formation of two complementary ions detected at *m/z* 319 and *m/z* 272, corresponding to the steroid bonded to a sulphur atom and the GSH moiety, respectively. The formation of these species could be rationalized considering a mechanism involving an ion–dipole complex (Scheme 2). The higher abundance of the *m/z* 272 ion (Fig. 3) could be explained by the higher acidity of the GSH moiety (containing two carboxylic acid functions) relatively to that of the steroid moiety. Furthermore, the decomposition of the $[M - H]^-$ *m/z* 620 species from methylated conjugates in MS^2 experiment (data not shown) yielded the *m/z* 319 ion as the main fragment species, which was also consistent with the formation of an ion–dipole complex. In this case, the acidity of the steroid linked to a sulfur atom became higher than that of the GSH moiety due to the methylation of the two carboxylic acid functions.

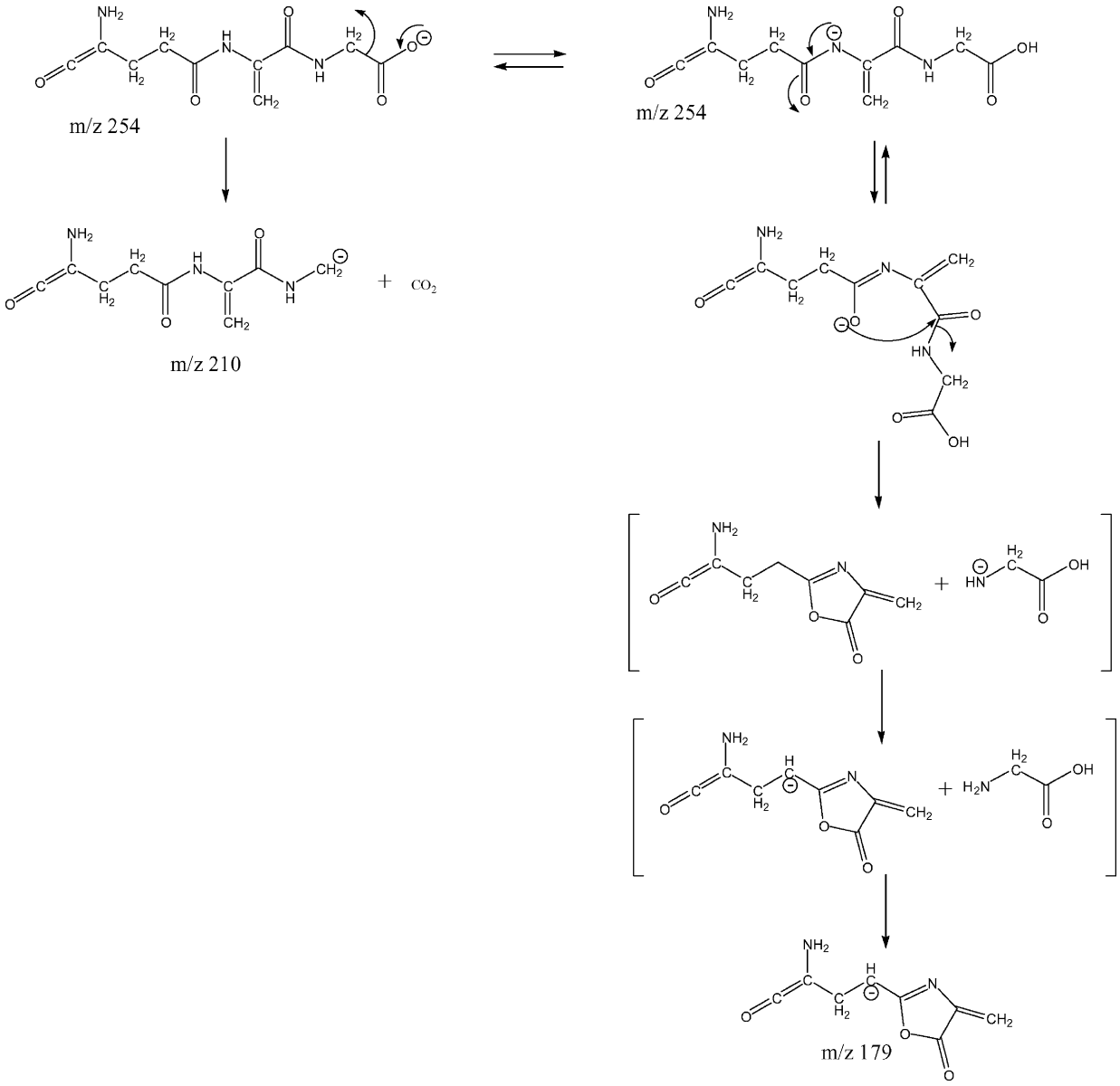
A similar mechanism involving an ion–dipole intermediate could also be proposed to explain the presence of two complementary ions at *m/z* 128 and *m/z* 143 in the MS^3 experiment carried out on the *m/z* 272 fragment ion. The proposed mechanism (Scheme 2) is similar to that described for the fragmentation of deprotonated peptides [29,30]. Thus, the *m/z* 128 and *m/z* 143 ions could be considered as b_2 and y_2 ions, respectively, according to the nomenclature adapted for the fragmentation of deprotonated peptides [28]. In addition, the selected *m/z* 272 ion could also eliminate a molecule of water, leading to the formation of the *m/z* 254 ion. Such an elimination has already been observed on peptides [30]. Further MS^4 experiment achieved on the third generation *m/z* 254 ion led to the *m/z* 210 and *m/z* 179 fragment ions (Fig. 5). The *m/z* 210 ion was formed by elimination of CO_2 from the *m/z* 254 ion (Scheme 3). Concerning the *m/z* 179 ion, an alternative process is proposed in Scheme 3, involving the formation of a cyclic species and resulting in the elimination of the glycine moiety. This process is analogous to that mentioned for the formation of protonated oxazolones from protonated peptides [31,32] and has also been reported for deprotonated peptides [29].

Similarly to the main fragmentation pathway of the *m/z* 592 ion, the *m/z* 574 ion formed by water loss from the *m/z* 592 ion underwent the same cleavage of the C–S linkage, yielding two complementary ions at *m/z* 319 and at *m/z* 254 (Fig. 5). These two fragment ions corresponded to the steroid and the dehydrated GSH moieties, respectively. The presence of the fragment ion at *m/z* 254 instead of that at *m/z* 272 suggested that water loss from the *m/z* 592 ion should take place at the GSH moiety. Furthermore, the loss of glycine observed in the MS^4 experiment carried out on the resulting *m/z* 254 ion indicated that the water elimination from the *m/z* 592 ion should occur on the acid function of the glutamic acid. From this information, a mechanism of formation and subsequent decomposition of the *m/z* 574 ion by sequential MS^n experiments could be proposed as shown in Scheme 4.

Concerning the formation of the most important isomer-specific *m/z* 306 ion, a phenolate $[M - H]^-$ ion had to be considered as reported in Scheme 5. From such a deprotonated species, a process involving an assisted 1,3-hydrogen migration could be proposed. In this case,



Scheme 2. Proposed mechanism for the collisionally activated decomposition of negative ESI-produced $[M-H]^-$ ions (*m*/*z* 592) from 2OH-E_2β-GSH conjugates.

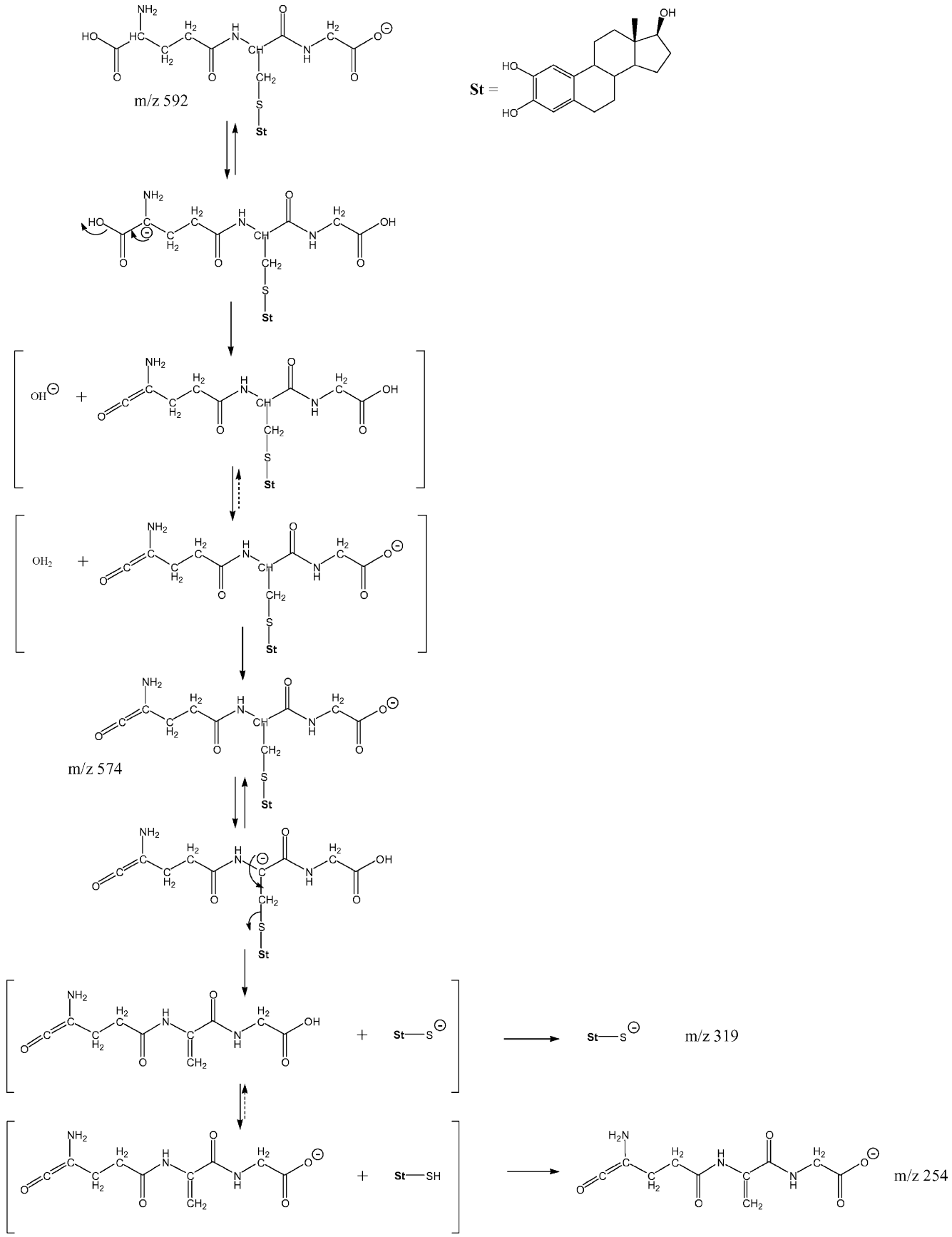


Scheme 3. Proposed mechanism for the formation of *m*/*z* 210 and *m*/*z* 179 fragment ions from the decomposition of the parent *m*/*z* 254 ion in an MS^4 experiment.

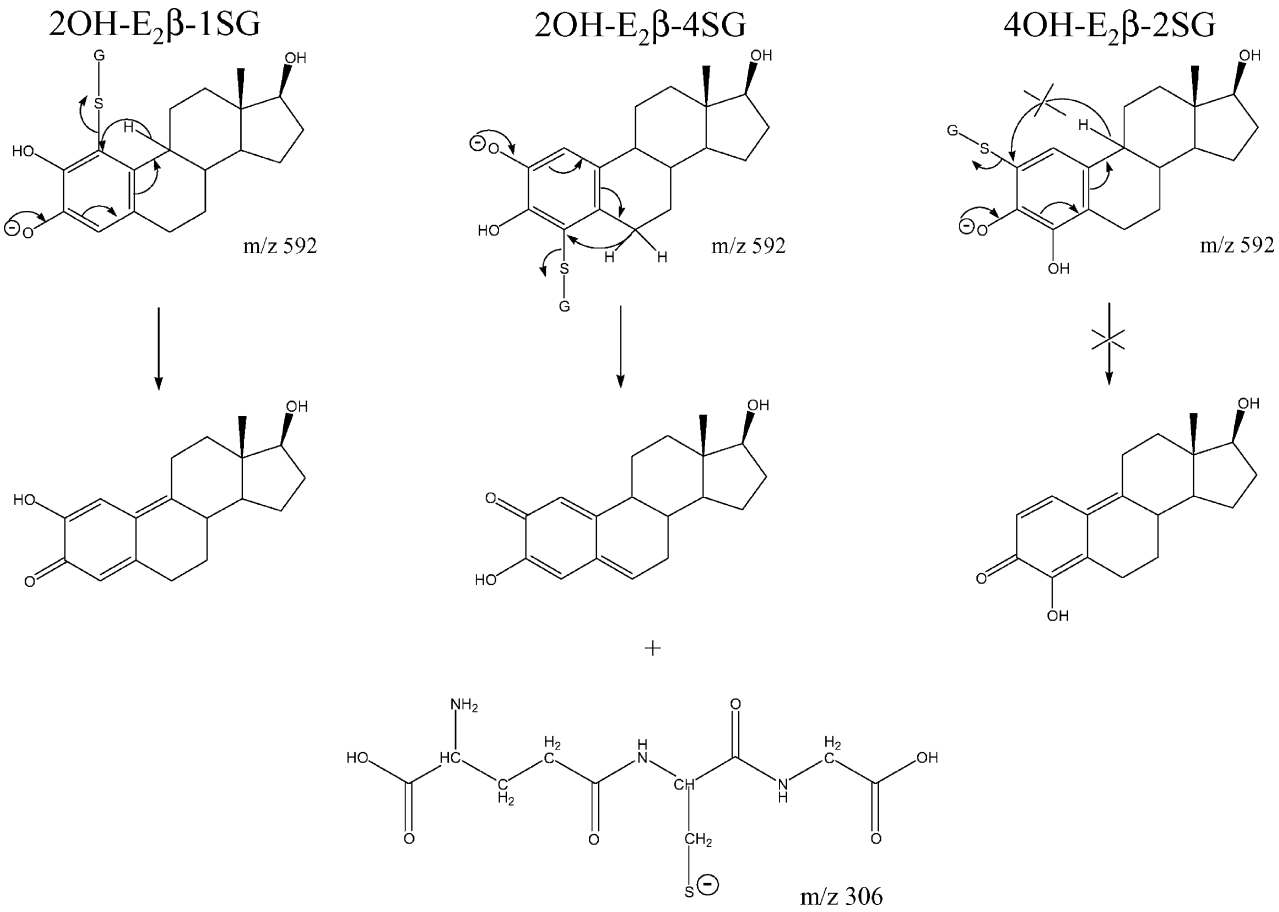
the delocalization of the negative charge may assist the 1,3 migration of one hydrogen atom situated at the γ position from the sulfur atom of the GSH moiety. The cleavage of the steroid–GSH linkage may lead to the charge retention on the GSH moiety since the GSH should be very likely much more acidic than the steroid. For 2OH-E_2β-1SG, the migration of the C9 hydrogen atom should be considered whereas in the case of 2OH-E_2β-4SG, the 1,3 migration process should involve one of the C6 hydrogen atoms of the steroid. On the other hand, no 1,3 migration process could occur for 4OH-E_2β-2SG. This observation was consistent with the absence of the *m*/*z* 306 ion on the CID spectra of 4OH-E_2β-2SG, since for this compound, the 1,4-hydrogen shift was hindered.

3.4. Identification of isomeric GSH conjugates from in vitro sample

The previous breakdown graphs (Fig. 4) allowed to determine the best collision energy for distinguishing negative CID spectra of the isomeric GSH conjugates. Isomeric differences were observed for collision energies higher than 0.9 V_{p-p}, and further increasing of the excitation energy led to a loss of ion intensities. Thus, a collison energy of 1 V_{p-p} was chosen to record tandem mass spectra. Using these conditions, the identification of isomeric GSH conjugates from in vitro samples (rat isolated hepatocytes [33]) could be achieved, as illustrated by the CID spectra presented in Fig. 6a and b. In this case, two isomeric catechol



Scheme 4. Proposed mechanism for the formation and subsequent decomposition of the *m/z* 574 ion by sequential MS^n experiments.



Scheme 5. Proposed mechanisms for the possible or hindered formation of the *m/z* 306 fragment ion from 2OH-E_2β-GSH and 4OH-E_2β-GSH conjugates.

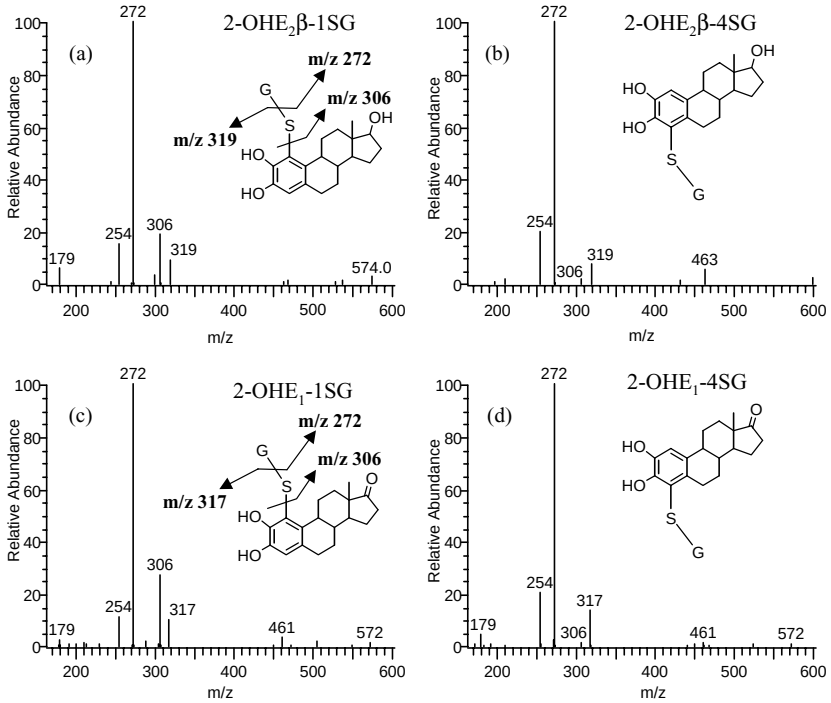


Fig. 6. Tandem mass spectra of $[M - H]^-$ ions from (a) 2OH-E_2β-1SG, (b) 2OH-E_2β-4SG, (c) 2OH-E_1-1SG and (d) 2OH-E_1-4SG formed in vitro by rat isolated hepatocytes.

estradiol–GSH conjugates were detected. The relative abundances of the *m*/*z* 306 and *m*/*z* 319 ions allowed to identify them as the 2OH-E_2β-1SG and 2OH-E_2β-4SG, respectively. In addition, two isomers of catechol estrone–GSH were also detected at *m*/*z* 590 from the supernatant of isolated rat hepatocytes. An analogous situation can be applied to the *m*/*z* 306 and *m*/*z* 317 fragment ions from the estrone isomers (Fig. 6c and d), and thus the two isomeric GSH conjugates were identified as 2OH-E_1-1SG and 2OH-E_1-4SG, respectively [33].

4. Conclusion

Identification of GSH conjugates to catechol estrogens should allow to evidence the formation and the structure of primary catechol estrogens that play a causative role in catechol-induced cancer.

Until now, only positive ionization mode has been used to analyze catechol estrogen–GSH conjugates [11,12,18] but no efficient identification of isomeric GSH conjugates could be made using MS/MS by resonance excitation in an ion trap mass spectrometer [18]. In this study, evaluation of the technique using an electrospray/ion trap instrument showed that isomer distinction could be achieved in both positive and negative ion modes. From protonated species only the ion source fragmentation allowed to distinguish isomers. However, the use of negative ionization and resonance excitation into an ion trap yielded the best data to characterize each isomer. When coupled to liquid chromatography, this technique appears to be a very powerful tool for direct identification of isomeric GSH conjugates from biological samples.

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