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ARTICLE

Detoxification of Cytotoxic Alachlor by Glutathione: Characterization of Conjugated Adducts by Electrospray Ionization Tandem Mass Spectrometry

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The most commonly used chloroacetamide herbicide, alachlor, and its conjugated adducts have been characterized by electrospray ionization mass spectrometry (ESI−MS). The reactivity of glutathione toward alachlor has been evaluated by changing experimental parameters, such as pH, temperature, and tube lens offset voltage (TLOV) in aqueous methanol, and the products were subjected to collision-induced dissociation (CID) for further characterization. In the positive mode, CID proves the formation of cyclic species by elimination of glycine and NH_3 moiety, which is similar to protonated cysteine. The results confirm that, only under basic conditions, glutathione is able to detoxify alachlor.

KEYWORDS: Alachlor; detoxification; glutathione; electrospray ionization

INTRODUCTION

Agricultural pesticides are primarily applied directly to the soil or indirectly through postemergence methods. Most detoxification processes of pesticides in the soil are coupled with degradation (*1*); therefore, the ability of the soil to degrade these pesticides affects their toxic and environmental impacts. While abiotic processes of degradation were suggested for some pesticides, soil microorganisms have long been considered the principal pathway for pesticide degradation (*2*). Alachlor (2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide) is one of the chloroacetamide herbicides that is most commonly used both as selective pre- and postemergence herbicides on a variety of agricultural crops, in the United States, particularly in the midwest (*3*). Although alachlor usage has declined recently, it is being detected in many agriculturally impacted surface and ground waters (streams, rivers, or reservoirs) and soil (*4*). The observance of alachlor in water and soil is significant because alachlor has been classified by the United States Environmental Protection Agency (U.S. EPA) as a class B2 or probable human carcinogen and a suspected endocrine disrupter.

Several potential sites on the alachlor molecule are available for biochemical attacks in the presence of appropriate enzymes. Therefore, many researchers have investigated the major mechanisms of carcinogenicity in alachlor (*5*, *6*). Alachlor is an *in vitro* clastogen in Chinese hamster ovary cells (*7*) and human blood lymphocytes (*6*, *8*, *9*). *In vivo* studies with Wistar rats exhibited a dose-related increase in chromosomal aberrations in bone marrow following a single intraperitoneal injection of alachlor (*8*). Alan et al. observed that the metabolism of alachlor in the liver and nasal epithelium yields a quinone-imine that binds to sulfhydryl groups of cysteine residues in nasal proteins (*10*).

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Because chemical pesticides have electrophilic moieties or can be metabolically bioactivated to electrophilic intermediates, the interaction of pesticide molecules with DNA can be a possible output. A conjugation reaction between electrophilic compounds and glutathione, a biological thiol compound, is a detoxification reaction common in the defense of mammals, fish, insects, higher plants, and microorganisms against oxidative stress (*11*). In addition, data on genotoxicity and mutagenicity are routinely collected on pest control agents as part of the regulatory and approval process for the agricultural use of pesticide formulations (*12*). Adducts of pesticide molecules with DNA bases have been detected and isolated, principally in mammalian tissues (*13*). Mammalian cell cytotoxicity of four commonly used agricultural chemicals (2,4-D, alachlor, dicamba, and oxamyl) was assessed after exposure to either reduced or oxidized ferruginous smectite (SWa-1). Results revealed that treatment with reduced smectite produced differential effects on mammalian cell viability, depending upon the pesticide. Oxamyl and alachlor reacted with reduced SWa-1 showed a significant decrease in their overall cytotoxic potential (*14*).

Glutathione (GSH), the major antioxidant and detoxifier of the body, is a tripeptide amino acid produced in the liver primarily from cysteine. It acts as a cellular antioxidant by inhibiting free-radical proliferation. GSH levels cannot be increased to a clinically beneficial extent by orally ingesting a single dose of GSH (*15*). This is because GSH is manufactured inside the cell, from its precursor amino acids, glycine, glutamate, and cysteine. Food sources or supplements that increase GSH must either provide the precursors of GSH or enhance its production by some other means. Asparagus is a leading source of GSH. GSH is by far the most important antioxidant and cellular protection that we have; therefore, consuming foods rich in sulfur-containing amino acids can help to boost glutathione levels.

Foods such as broccoli (*16*), avocado, and spinach are also known to boost GSH levels. Raw eggs, garlic, and fresh unprocessed

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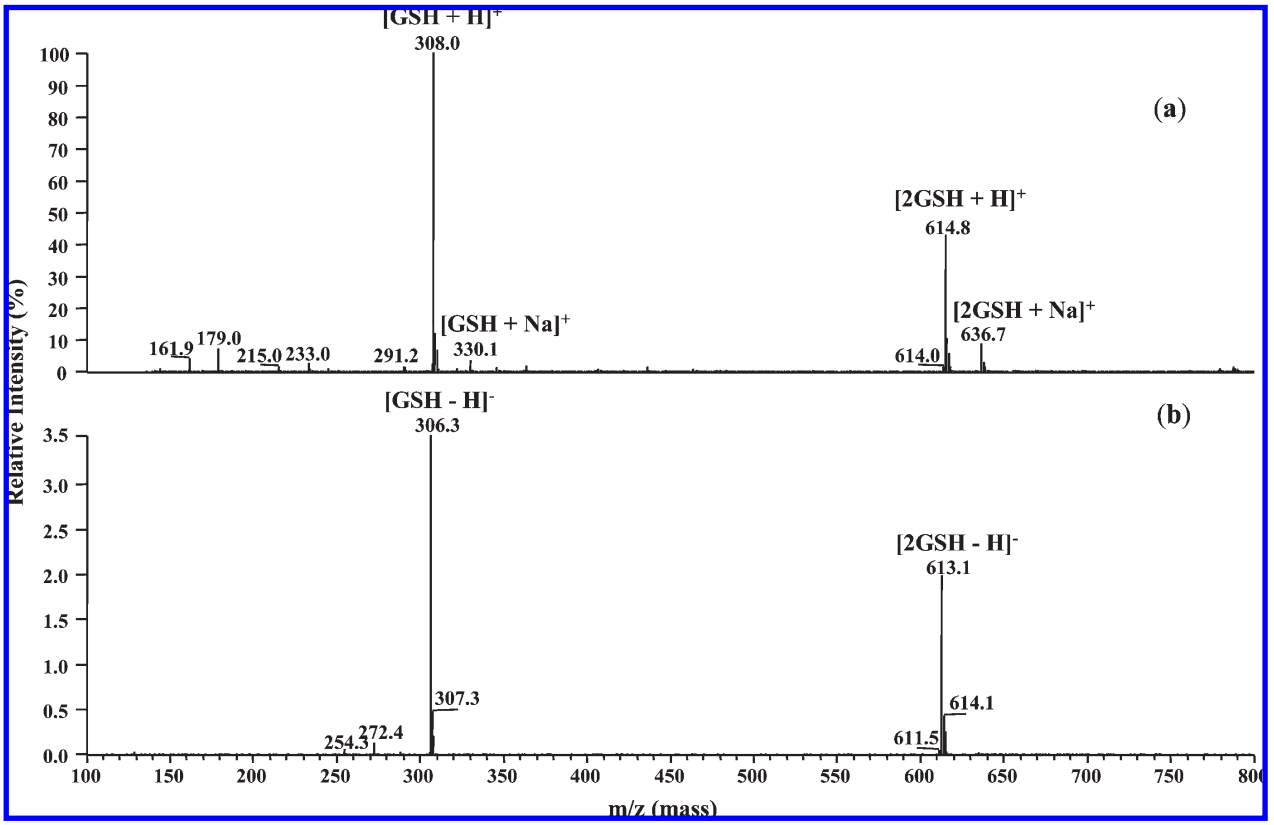


Figure 1. ESI (a) positive and (b) negative mass spectrum in aqueous methanol (50/50%) solution containing 5.0×10^{-4} M GSH.

meats contain high levels of sulfur-containing amino acids and help to maintain optimal GSH levels. Treatment of brain cells called astrocytes, with the Indian curry spice, curcumin (turmeric), has been found to increase expression of the GSH-*S*-transferase and protect neurons exposed to oxidant stress (*17*). Changkil saponins (CKSs) isolated from the roots of the Chinese herbal medicine, *Platycodon grandiflorum* A. DC (Campanulaceae), commonly called balloon flower root or jie geng, have been found to increase intracellular GSH content, significantly reduce oxidative injury to liver cells, and minimize cell death and lipid peroxidation (*18*).

GSH plays an important role in many cellular processes (*19*), and chloroacetamide and chloroacetanilide herbicides are detoxified by enzymatic and non-enzymatic conjugation with GSH (*20*). Alachlor is also detoxified rapidly in nonsensitive plants via conjugation with GSH and/or homoglutathione (*21*). However, the mechanism for detoxification of alachlor has not been elucidated; it has been proposed that alachlor can covalently bind to sulfhydryl groups of the GSH via HCl elimination to form alachlor−GSH conjugate adducts (*22*).

Electrospray ionization (ESI) constitutes a valuable means to investigate the native chemical system in solution phase, thanks to its soft ionization nature, which can produce singly or multiply charged ions directly from solution. ESI combined with mass spectrometry, ESI−MS, has been well-established as a useful tool for the structure characterization of nonvolatile compounds and employed to characterize a number of metal complexation chemistries with organic and bio-organic ligands.

In the present paper, we herein report the reactivity of GSH toward alachlor by the pH, temperature, and tube lens offset voltage (TLOV) changes. Furthermore, we describe characterization of the reaction products by ESI−MS/MS.

EXPERIMENTAL SECTION

All experiments were performed using a LCQ-Advantage ion-trap mass spectrometer (ThermoFinnigan Co., San Jose, CA) equipped with an electrospray interface. Operation conditions were as follows: spray voltage, 5.00 kV; capillary voltage, 40 V; heated capillary temperature, 150−280 °C; TLOV, from −120 to +120 V; sheath gas, N_2; 15 μL/min. Helium gas, admitted directly into the ion trap, was used as the buffer gas to improve trapping efficiency and as the collision gas for collision-induced dissociation (CID) experiments. TLOVs were set using a tune file created by auto tuning the LCQ on the ion signal of interest if not specified. CID experiments were performed by setting the isolation width between 5 and 10 mass units depending upon the species of focus and the activation amplitude at 5−25% of 5 V peak-to-peaks in resonance excitation RF voltage. All mass spectra were recorded at the average of 20 consecutive scans. Gas-phase complexes between alachlor and GSH were generated by electro spraying an aqueous methanol (50/50%) solution containing a mixture of alachlor (5.0×10^{-4} M) and GSH (5.0×10^{-4} M). Samples were infused using a syringe pump at a flow rate of 5 μL/min. To examine the effect of nominal pH on the reactivity of GSH toward alachlor, solutions composed of 1:1 alachlor/GSH or 1:1:1 alachlor/GSH/5% glacial acetic acid or 5% concentrated ammonium hydroxide were analyzed. The alachlor and GSH were purchased from Sigma (South Korea) and used as obtained. Other chemicals and solvents were purchased from Aldrich (South Korea and U.S.A.) and Merck (South Korea) and used without further purification.

RESULTS AND DISCUSSION

MS Spectra of GSH. To establish a reference point, we discuss briefly the ESI−mass spectra (at positive and negative conditions) of GSH itself. Under the positive conditions (**Figure 1a**), ESI of GSH gives $[GSH + H]^+$, $[GSH + Na]^+$ and $[2GSH + H]^+$, $[2GSH + Na]^+$ ions of *m*/*z* 308, 330 and 615, 637, respectively. CID of $[2GSH + H]^+$ ion gives the $[GSH + H]^+$ ion, and the

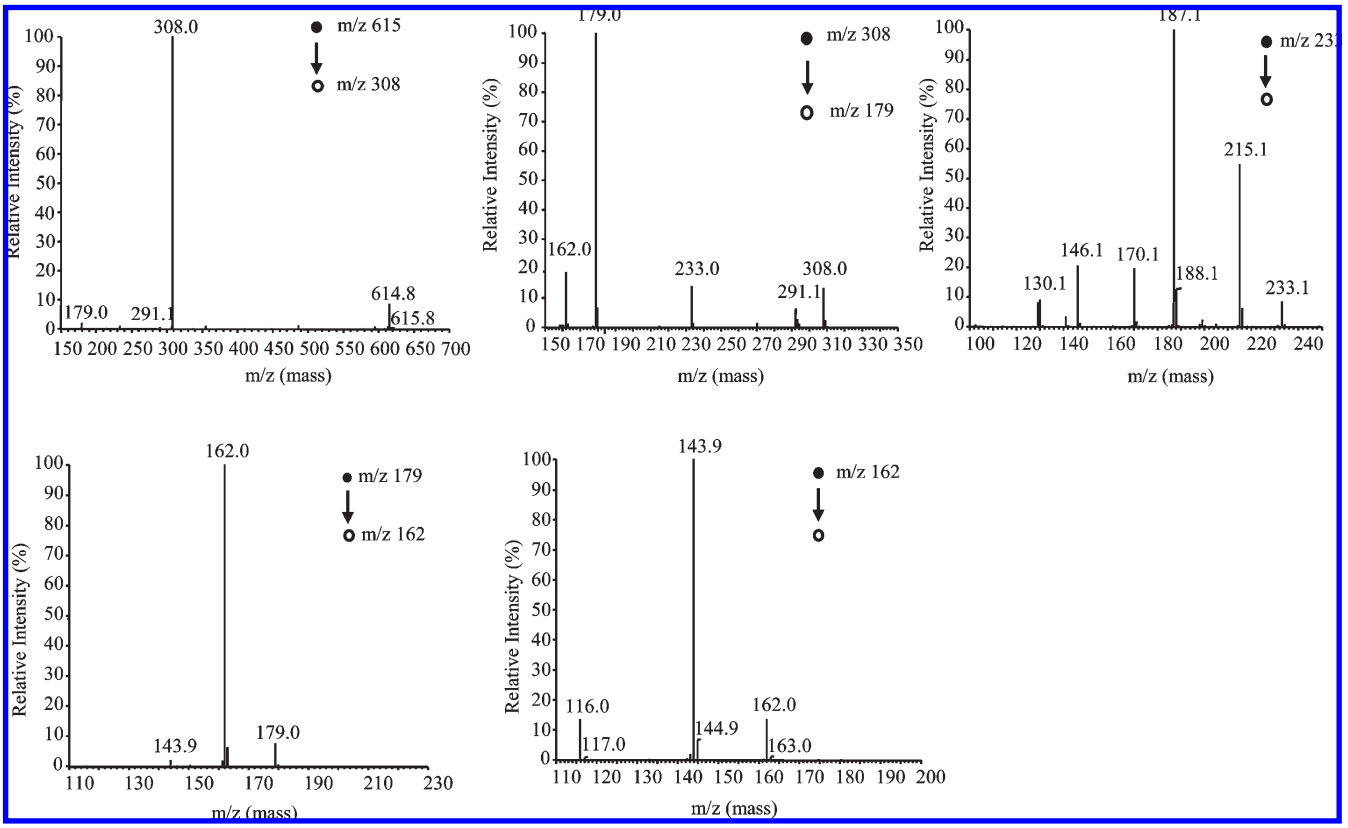


Figure 2. MS^n ($n = 2-5$) mass spectra of typical positive ions of protonation glutathione. Molecular ion at *m/z* 308.

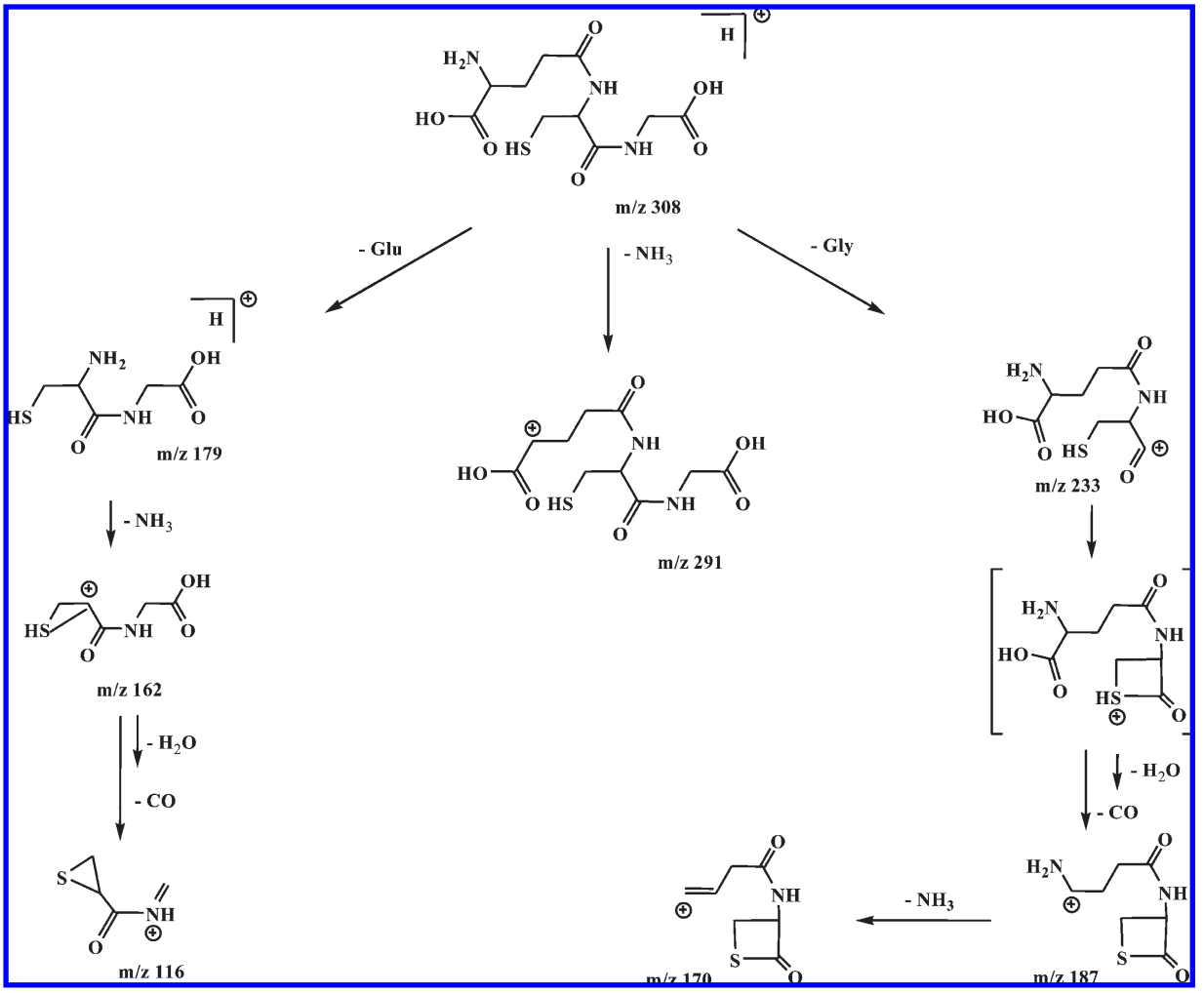
$[GSH + H]^+$ ion dissociates to the expected *m/z* 291, 233, and 179 ions (**Figure 2**), where the *m/z* 233 and 179 ions involve the formation of cyclic species by elimination of glycine and NH_3 moiety, respectively. The *m/z* 233 ion dissociates to an ion at *m/z* 215, corresponding to the loss of H_2O and to *m/z* 187 because of the successive loss of CO. The *m/z* 179 ion dissociates to *m/z* 162 because of the loss of NH_3, and *m/z* 162 ion dissociates to an ion at *m/z* 144, corresponding to the loss of H_2O and to *m/z* 116 because of the successive loss of CO. The proposed mechanism (**Scheme 1**) is similar to that described for the fragmentation of protonated cysteine (*23*).

At the negative conditions (**Figure 1b**), ESI of GSH gives $[GSH - H]^-$ and $[2GSH - H]^-$ ions of *m/z* 306 and 613, respectively. CID of the $[2GSH - H]^-$ ion gives the $[GSH - H]^-$ ion, and the $[GSH - H]^-$ ion dissociates to the expected *m/z* 288 and 272 ions by the loss of H_2O and H_2S, respectively. The *m/z* 288 ion dissociates to an ion at *m/z* 254 corresponding to the loss of H_2S, and also, the *m/z* 272 ion dissociates to the *m/z* 254 ion because of the loss of H_2O. Namely, we confirmed that H_2O and H_2S losses are competitive reactions. The *m/z* 254 ion dissociates to an ion at *m/z* 210, corresponding to the loss of CO_2 and to *m/z* 179 ion because of the loss of deprotonated glycine, involving the formation of cyclic species. The proposed fragmentation pathways mechanism is described in **Scheme 2**.

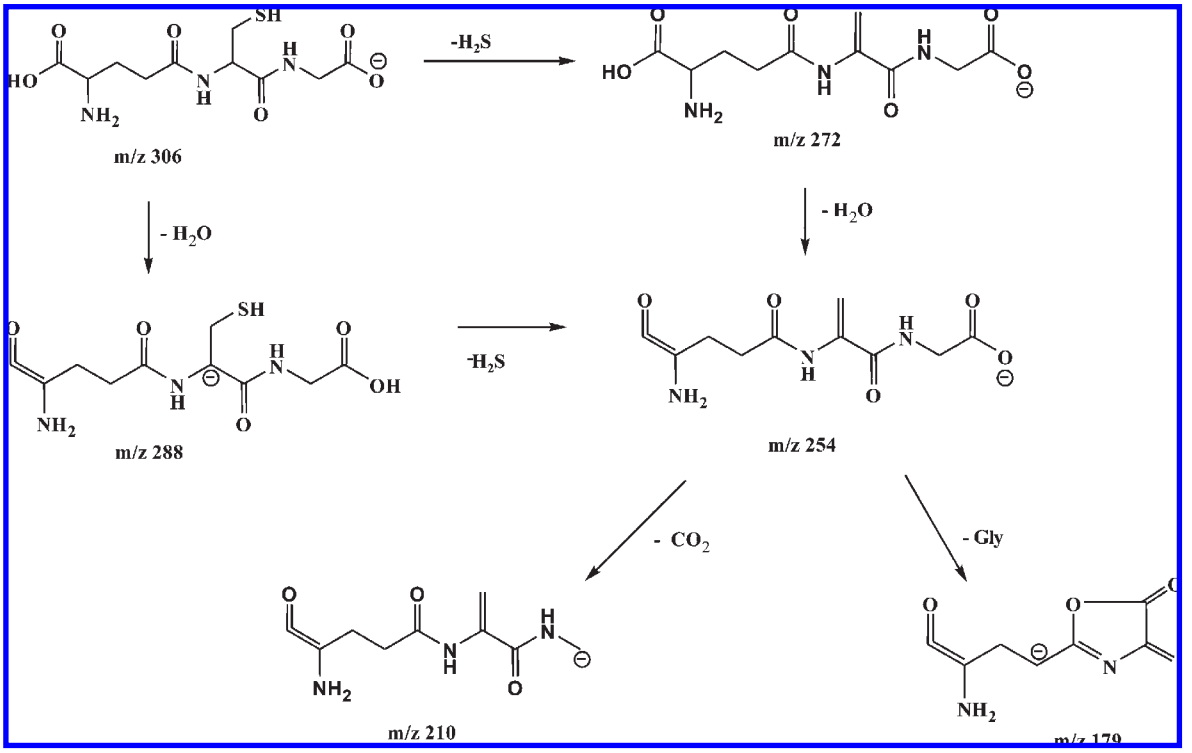
Reactivity of GSH toward Alachlor According to the Influence of pH. We evaluated the reactivity of GSH toward alachlor according to the influence of pH in aqueous methanol solution, where the acid and the base conditions were adjusted by acetic acid and ammonia solution, respectively (**Figures 3** and **4**). As shown in **Figure 3**, GSH is able to react toward alachlor (*m/z* 541) at base conditions. At acid and neutral conditions, the *m/z* 556, 540, and 287 ions are newly revealed in the spectra. In the MS/MS experiment, the *m/z* 556 ion give rise to the formation of fragment ions at *m/z* 539, 287 and 270, 238. The *m/z* 556 ion is probably produced from a dimer composed of two alachlor molecules with ammonium ion, where this ammonium ion is revealed from ammonia detached from GSH. To confirm this result, we investigated alachlor with ammonia solution, and this result was very similar to the neutral and acid conditions. At the lower CID voltage, the *m/z* 556 ion dissociated to *m/z* 539 of the $[2Ala + H]^+$ ion, corresponding to the loss of NH_3 and to *m/z* 287 of the $[Ala + NH_3 + H]^+$ ion because of the loss of alachlor. *m/z* 539 of $[2Ala + H]^+$ ion dissociated to *m/z* 507 of the $[Ala + (Ala—CH_3OH) + H]^+$ ion by the loss of CH_3OH and to *m/z* 270 of the $[Ala + H]^+$ ion, corresponding to the loss of alachlor. *m/z* 287 of the $[Ala + NH_3 + H]^+$ ion dissociated to *m/z* 270 of the $[Ala + H]^+$ ion, corresponding to the loss of NH_3. These results also indicate that the *m/z* 556 ion is produced from the hydrogen bond with ammonium ion. The proposed mechanism is revealed from several compounds under ESI−MS (*24*, *25*).

According to TLOV and Temperature, Reactivity of GSH toward Alachlor at Basic Conditions. At basic conditions, the GSH can quench alachlor by elimination of hydrogen chloride. Alachlor is ionized to protonated and deprotonated ions, leading to the formation of *m/z* 541 $[M + H]^+$ and *m/z* 539 $[M - H]^-$ ions at the positive and negative conditions, respectively. **Figure 5** indicates the reactivity of GSH toward alachlor according to the influence of temperature and TLOV in an aqueous methanol solution. **Figure 5a** shows dramatically different ESI mass spectra of the alachlor/GSH sample solution, recorded at TLOV from 0 to 120 V, at 180 °C, and in basic pH conditions. In this spectra, the base peak is *m/z* 541, corresponding to the formation of alachlor−GSH conjugate by elimination of hydrogen chloride. At low TLOV, the spectrum includes protonated GSH at *m/z* 308 and protonated alachlor at *m/z* 270. However, according to the increase of TLOV, the protonated alachlor and GSH are decreased in relative abundance in the spectrum but *m/z* 541 of

Scheme 1. Proposed Fragmentation Pathways of Glutathione in Aqueous Methanol at Positive Mode



Scheme 2. Proposed Fragmentation Pathways of Glutathione in Aqueous Methanol at Negative Mode



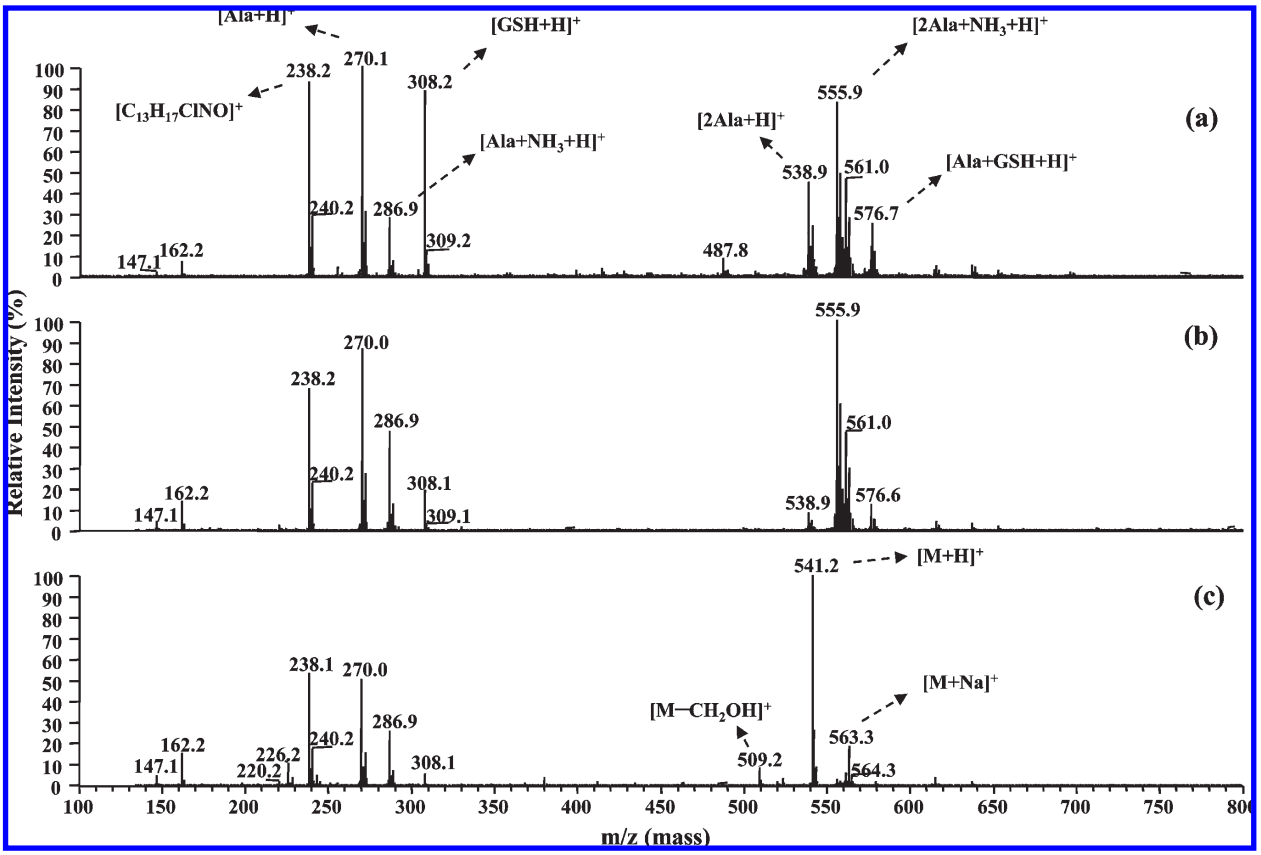


Figure 3. According to pH conditions, reactivity of glutathione toward alachlor at the positive conditions: (a) acid, (b) neutral, and (c) base.

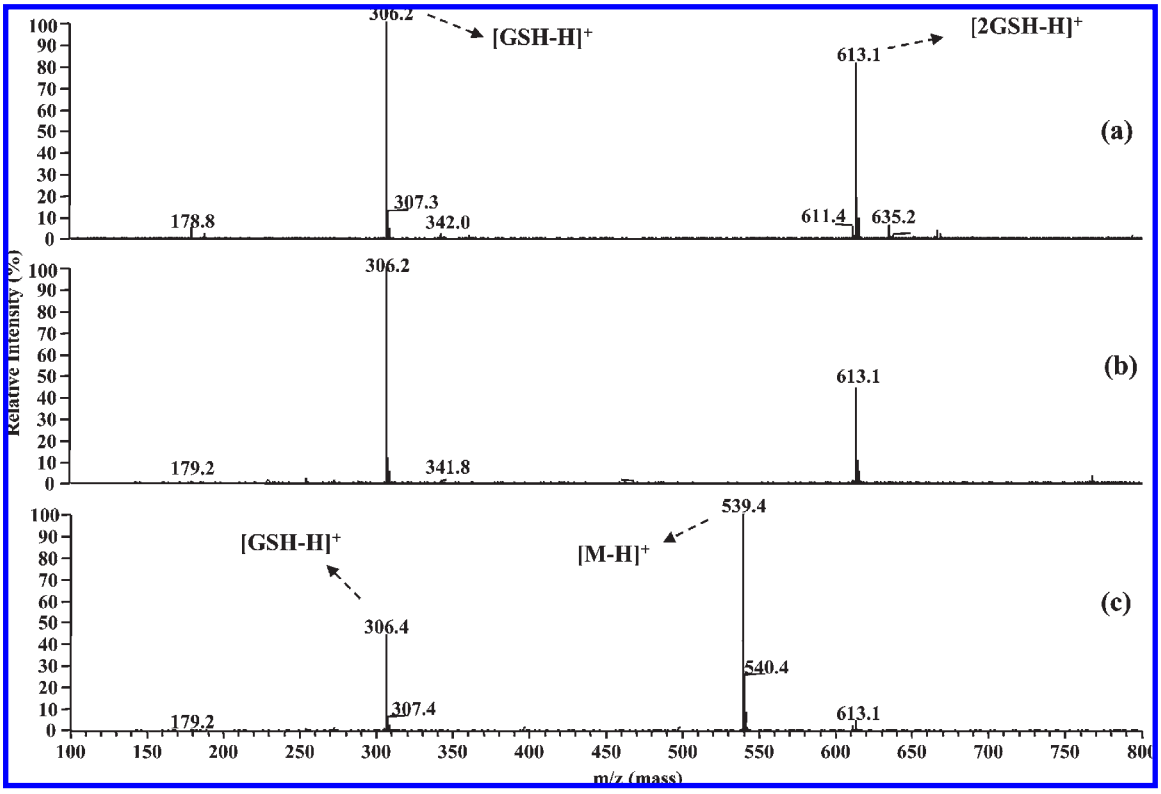


Figure 4. According to pH conditions, reactivity of glutathione toward alachlor at the negative conditions: (a) acid, (b) neutral, and (c) base.

$[M + H]^+$, m/z 563 of $[M + Na]^+$, and m/z 509 of $[M - CH_3OH]^+$ ions produced from alachlor−GSH conjugate are increased in relative abundance in the spectrum. The mass spectra investigated about the influence of TLOV are informative with respect to m/z 541, indicating that m/z 541 of the $[M + H]^+$ ion is easily produced under high TLOV. The high tube lens potential

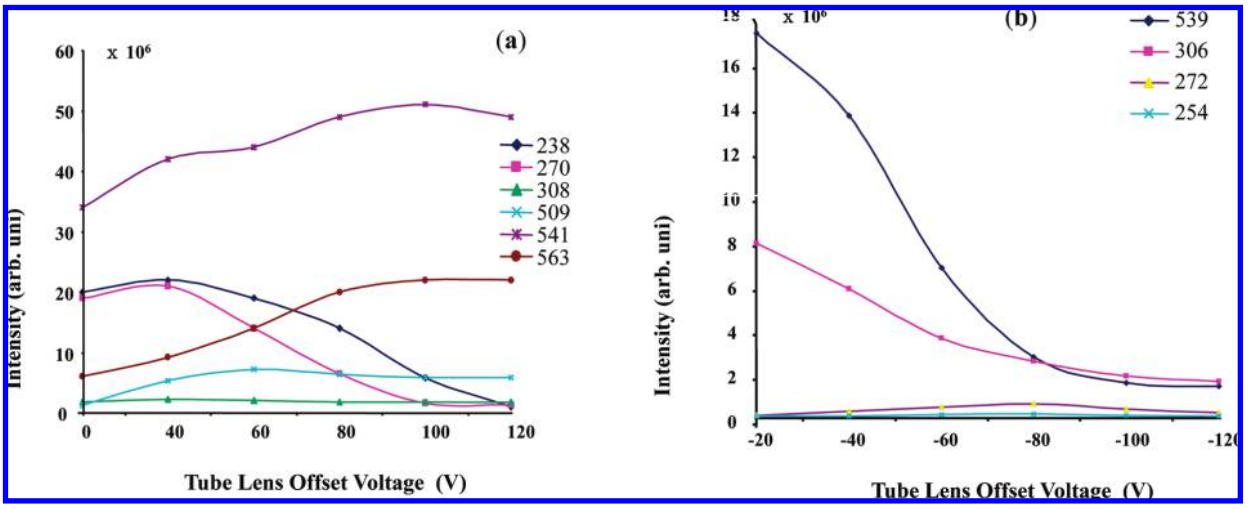


Figure 5. Breakdown curves for more significant ions as a function of the TLOV: (a) positive and (b) negative.

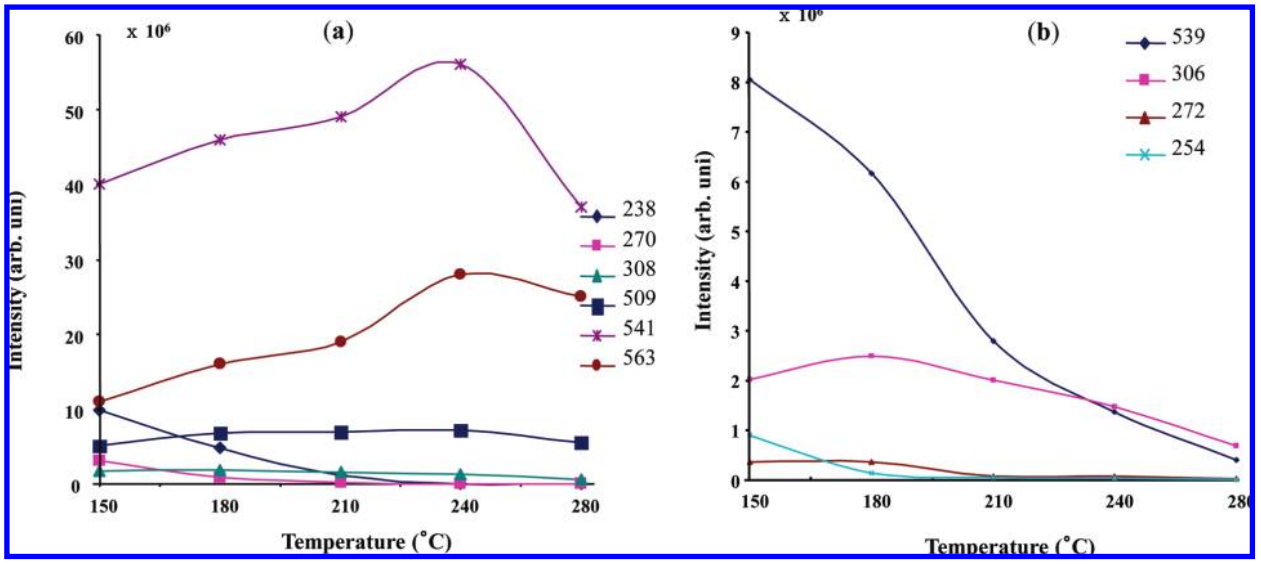


Figure 6. Breakdown curves for more significant ions as a function of the capillary temperature: (a) positive and (b) negative.

accelerates a significant quantity of GSH ions into alachlor before they pass through the skimmer. However, at the negative mode (**Figure 5b**), the TLOV shows greatly different results when it is compared to the positive mode. The *m*/*z* 539 ion produced from deprotonated alachlor−GSH conjugate is decreased in relative abundance according to the increase of TLOV. This result is probably due to the collision probability of deprontonated GSH into alachlor.

To confirm the temperature effect of GSH reactivity, which is known to affect the antioxidant defense within the tissues of rats (*26*), we changed the temperature from 150 to 280 °C. According to the change of temperature, at 60 V TLOV and in basic pH conditions, **Figure 6** shows dramatically different ESI mass spectra of the alachlor/GSH sample solution. From 150 to 240 °C, *m*/*z* 541 of $[M + H]^+$ and *m*/*z* 563 of $[M + Na]^+$ ions are increased but *m*/*z* 270 of $[Ala + H]^+$ and *m*/*z* 308 of $[GSH + H]^+$ are decreased in relative abundance in the ESI spectrum. Above 240 °C, the *m*/*z* 541 ion is decreased in relative abundance because of thermal decomposition at the high temperatures.

CID of Alachlor−GSH Conjugate Ion Studies. Under basic conditions (**Figures 3c** and **4c**), the alachlor−GSH was analyzed in positive and negative ionization modes, yielding protonated and deprotonated ion at *m*/*z* 541 and 539, respectively. As shown in **Figure 3c**, assuming that the initial ionization process involved the addition of a proton at the alkyl group (the methoxymethyl group) position on alachlor, the other protonated species, such as *m*/*z* 466 and 412, were formed by proton transfer from the alkyl-protonated species. To elucidate some fragmentation processes and to provide more structural information of alachlor−GSH conjugate fragment ions, MS^n experiments were carried out on the alachlor−GSH conjugate. In the MS/MS experiment (**Figure 7**), *m*/*z* 541 gave rise to the formation of fragment ions at *m*/*z* 509, 466, and 434 and 412, 380, and 368. This result indicates that the protonation process can occur at various sites of the molecule through proton transfer but favored the alkyl group (the methoxymethyl group) position on alachlor. The *m*/*z* 509 ion is produced from *m*/*z* 541 by the loss of CH_3OH. At this time, the sulfide group probably makes a four-cycle ring by attacking the alkyl group (the methoxymethyl group) position on alachlor. This proposed mechanism is analogous to cysteine and its peptides because the reactive thiol side chain can act as both an inter- (*26*) and intramolecular (*27*) nucleophile. The *m*/*z* 509 ion dissociates to the expected *m*/*z* 434, 380, and 368 by losing glycine, glutamic acid, and 141, respectively, and to the *m*/*z* 492 ion corresponding to the loss of NH_3. From the MS/MS spectrum of *m*/*z* 509, we can confirm that the CID fragmentation pattern

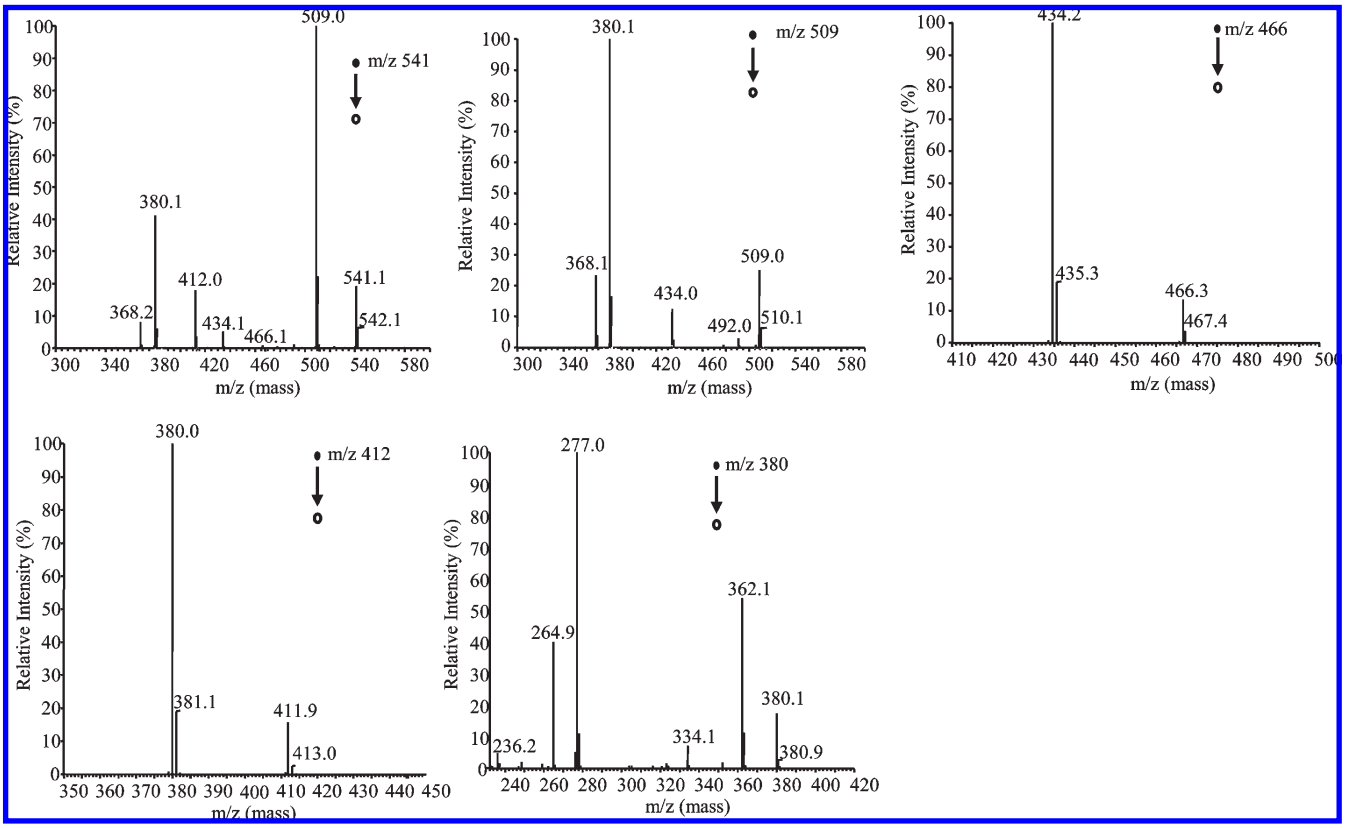


Figure 7. ESI mass spectra of glutathione in aqueous methanol with alachlor at positive mode (base condition). The *m/z* 541 ion is produced by elimination of hydrogen chloride through attack of the sulfide group of GSH.

of the *m/z* 509 ion is corresponded with the CID fragmentation pattern of the *m/z* 308 (protonated GSH) ion, except for the *m/z* 368 ion. The *m/z* 541 ion also produces *m/z* 466 and 412 ions of low abundance corresponding to the loss of glycine and glutamic acid, respectively. The *m/z* 466 and 412 ions again dissociate to the expected *m/z* 434 and 380 ions by the loss of CH_3OH. These results show that the protonation process of the alkyl group (the methoxymethyl group) happens more easily than other sites. The *m/z* 380 ion dissociates to the expected *m/z* 362, 334, 277, and 236 ions by the loss of H_2O, HCOOH, $C_3H_4NO_3$, and $C_5H_8N_2O_3$, respectively (**Scheme 3**).

The deprotonated alachlor−GSH conjugated ion can produce different sites from GSH moiety containing several labile hydrogen atoms, therefore leading to the formation of different deprotonated species. However, it must be favored at the carboxylate positions, leading to two possible carboxyl-deprotonated species. Other deprotonated forms, such as amide-deprotonated species and enolate anions, resulting from a hydrogen removal from the CH groups adjacent to the carbonyl and nitrogen of an amide moiety, can occur either directly in the ionization process or by proton transfer in a carboxyl-deprotonated species (*28*). To clarify some decomposition pathways and to provide more structural information on the various fragment ions, MS^n experiments were carried out on the alachlor−GSH conjugate ion. In the MS/MS experiment, the *m/z* 539 ion involved in the removal of a proton from a carboxylic moiety of the alachlor−GSH conjugate produces the *m/z* 272 and 266 ions by cleavage of the C−S linkage. The higher abundance of the *m/z* 272 ion (**Figure 8**) could be explained by the higher acidity of the GSH moiety containing two carboxylic acid functions relative to that of alachlor moiety. The MS^4 of the *m/z* 272 ion could competitively produce the *m/z* 254, 143, and 128 ions by elimination of H_2O, $C_5H_6NO_3H$, and $C_5H_8N_2O_3$, respectively. Further, the MS^4 experiment carried out on the *m/z* 254 ion produced the *m/z* 210 and 179 fragment ions. The *m/z* 210 ion was produced by the loss of CO_2 from the *m/z* 254 ion. The *m/z* 254 ion also produced the *m/z* 179 ion, involving the formation of a cyclic species (**Scheme 4**), by elimination of the glycine moiety (*28*).

The competitive decomposition of the *m/z* 272 ion is the *m/z* 521 ion. The *m/z* 521 ion is produced by the loss of water from the *m/z* 539 ion. The MS^3 of the *m/z* 521 ion produces the *m/z* 254 and 266 ions by cleavage of the C−S linkage. The *m/z* 521 ion forms 446, also involving the formation of cyclic species by elimination of the glycine moiety. The MS^4 experiment of the *m/z* 466 ion produces the *m/z* 266 and 179 ions by cleavage of the C−S linkage.

GSH conjugates are usually involved in the detoxification systems of living organisms, which are responsible for cell detoxification (*29*). Its other physiological roles include storage and transport of cysteine plus a co-enzymatic role in several reactions with foreign compounds (*30*). The recent evidence suggests that GSH and several of its dependent enzymes are central to detoxifying reactive low-molecular-weight organic compounds of xenobiotic or endogenous origins. In the present analysis, the endogenous or exogenous toxic compound to be eliminated was conjugated with GSH by glutathione transferase (GSTs). Plant and mammalian GSTs share a considerable structural similarity, especially within the amino-terminal domain, which forms the GSH-binding site (G site). GSH was broken down by γ-glutamyltranspeptidase, which eliminates glutamic acid, and a glycine, thus forming a cysteine conjugate (*31*) (**Schemes 1** and **2**). Cysteine and GSH delivery compounds have been used to protect normal cells from antitumor agents and radiation (*32*). Scanning for precursors of negative ions, such as *m/z* 254 or 272, should represent a potentially useful approach for the unbiased detection of GSH conjugates in

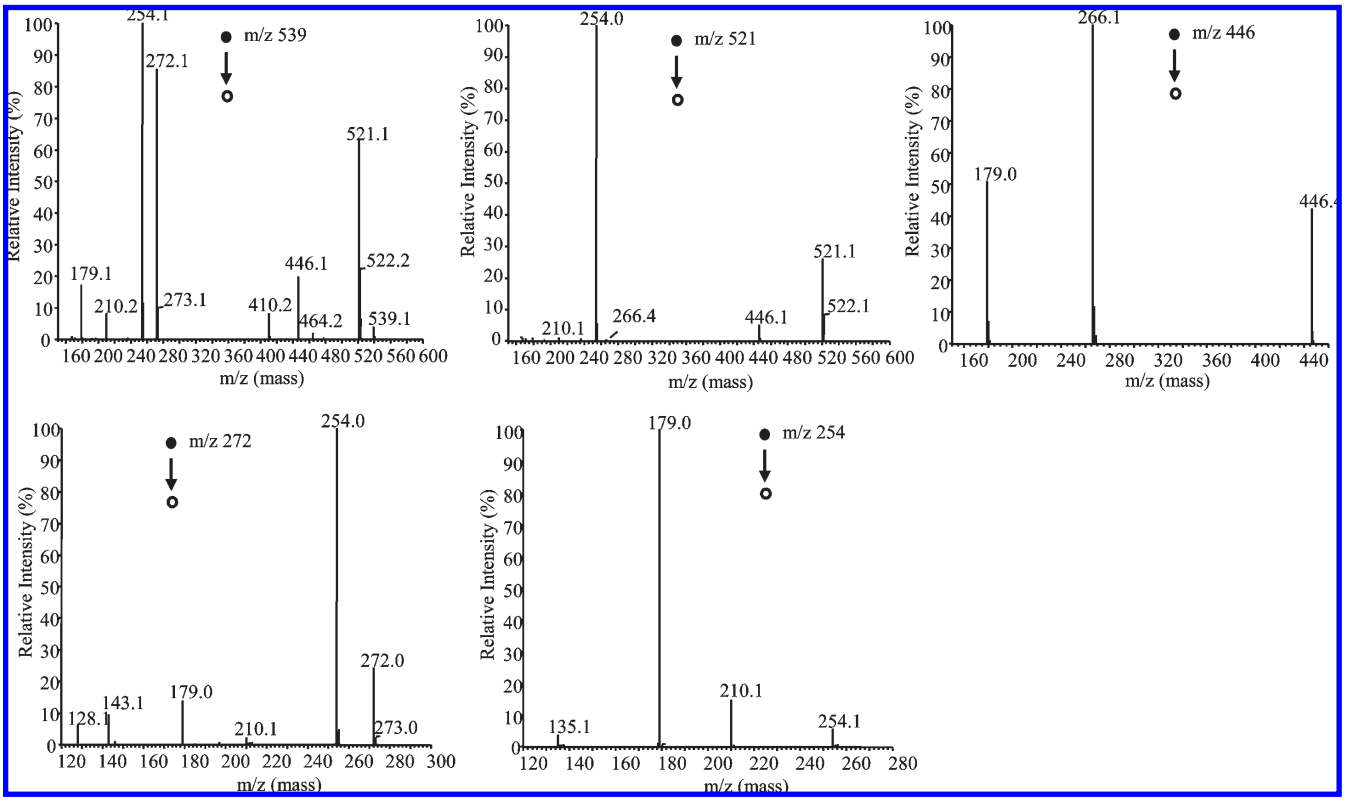
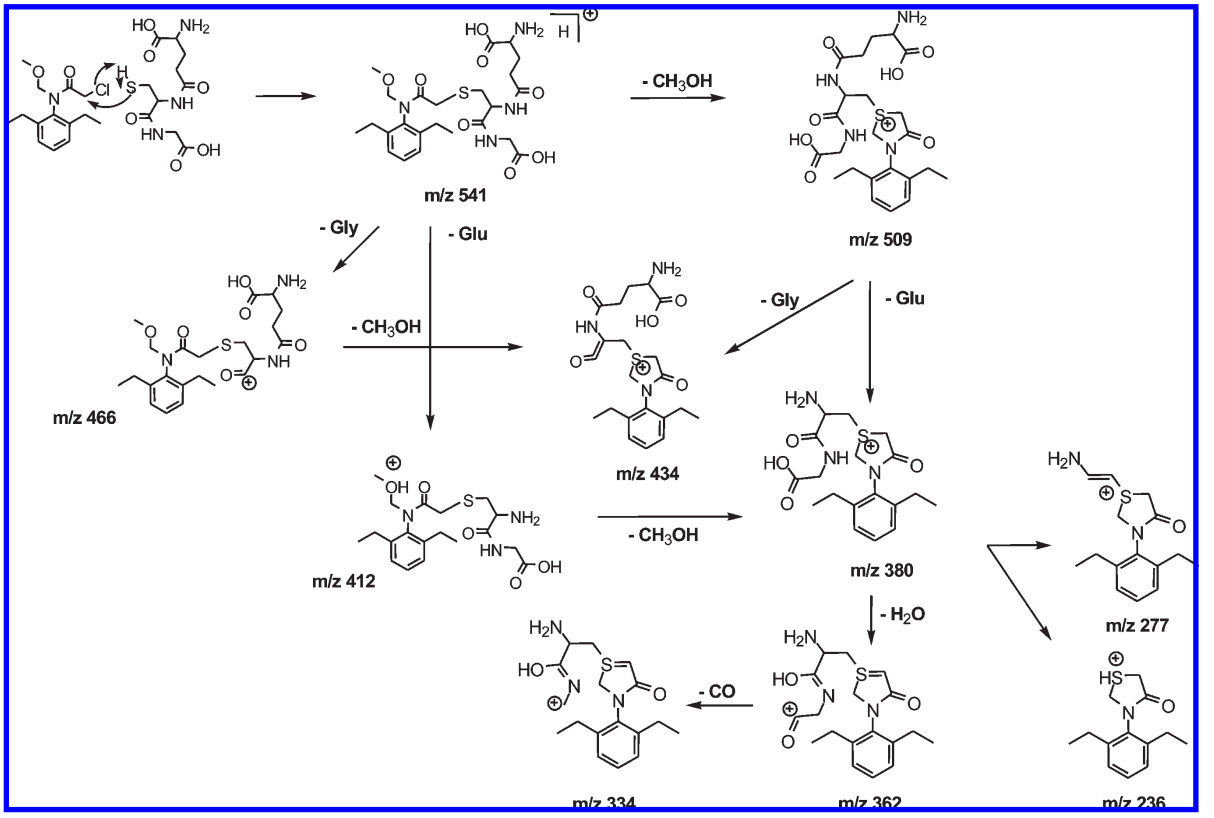


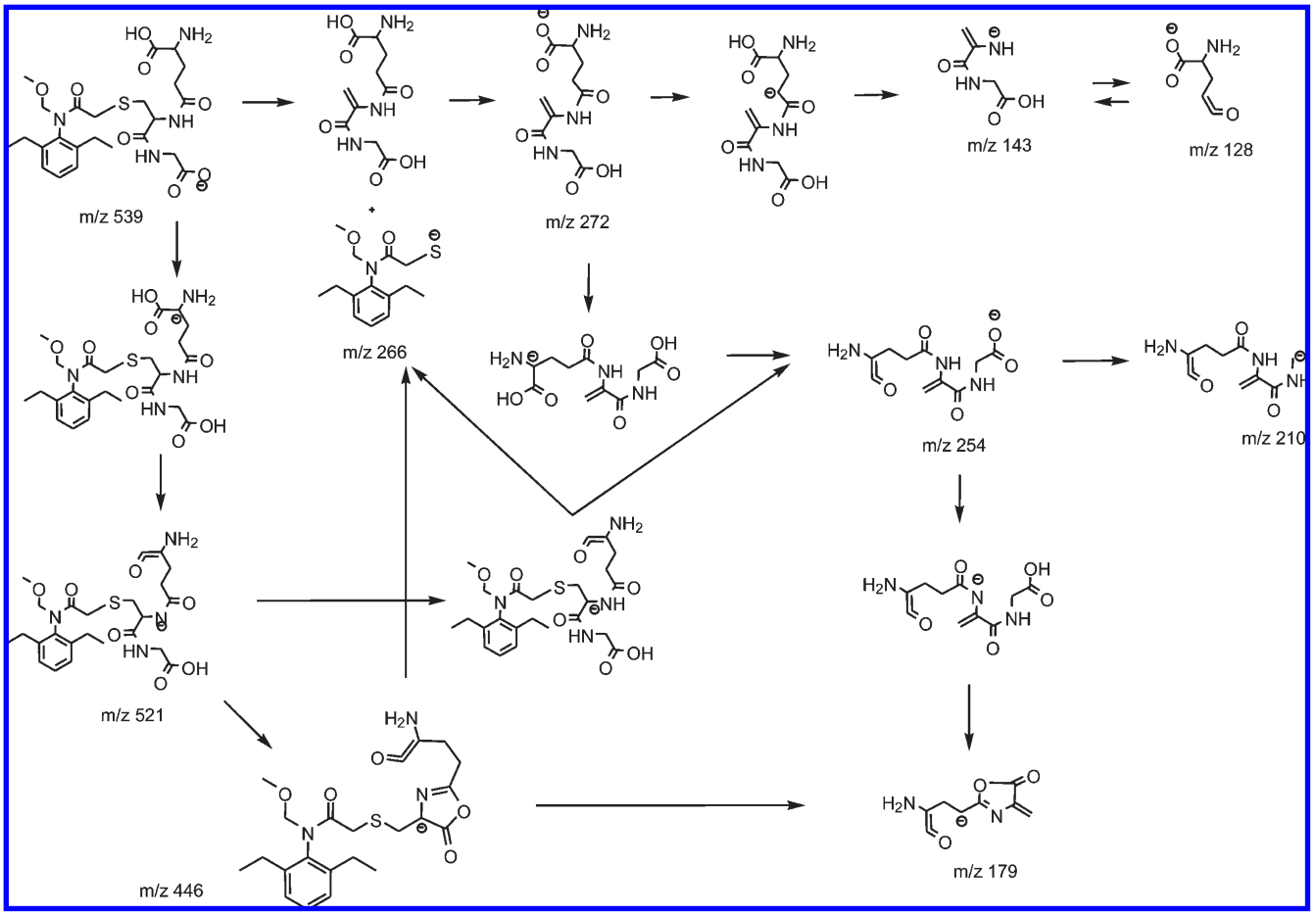
Figure 8. ESI mass spectra of glutathione in aqueous methanol with alachlor at negative mode (base condition). The *m/z* 541 ion is produced by elimination of hydrogen chloride through attack of the sulfide group of GSH.

Scheme 3. Proposed Fragmentation Pathways of the *m/z* 541 Ion Aqueous Methanol at Positive Mode



biological samples. 4-Hydroxy-2-nonenal (HNE) (**Schemes 3** and **4**) have been proposed as possible molecular mediators responsible for the close association between lipid peroxidation and enhanced deposition of the extracellular matrix reported *in vivo* and in cell culture systems (*33*). Biological effects of HNE strictly depend upon its ability to form aldehyde−protein adducts by

Scheme 4. Proposed Fragmentation Pathways of the *m/z* 541 Ion Aqueous Methanol at Negative Mode



interacting with sulfhydryl or amino groups of lysine and histidine. Biological thiols, found in plants, such as GSH, cysteine, etc., have strong antioxidant properties that allow them to protect cells from oxidative stress. Besides being potent antioxidants, these thiols act as therapeutic agents and indicators of diseases (*34*). Recent studies have reported that the GSH contained in various foods may increase the elimination of oxidized lipids by either enhancing their excretion or decreasing their absorption. Moreover, consumption of foods with a high GSH content has been shown to decrease the risk of having pharyngeal and oral cancers by 50% (*35*).

In the present work, reactivity of glutathione toward alachlor has been investigated using ESI−MS in combination with CID experiments. ESI−MS of GSH, in both positive and negative conditions, involves the formation of cyclic species. At the acidic and neutral conditions, we could not confirm the reactivity, but in basic conditions and at low CID voltage, reactivity was confirmed by the formation of the hydrogen bond with the ammonium ion. A change of temperature and TLOV leads to formation of the alachlor−GSH conjugate, and the relative abundance may increase or decrease according to the mode of action. Alachlor−GSH conjugate ion studies under basic conditions yielding protonated and deprotonated ions indicate that the initial ionization process involved the addition of a proton at the alkyl group. To clarify the decomposition pathway and to provide more structural information on various fragment ions, MS^n experiments were carried out. The results of this study unequivocally confirm the ability of GSH to react with the carcinogenic alachlor through the formation of the alachlor−GSH product, whose structure and decomposition pathways are elucidated by ESI−MS/MS analysis. One of the most important findings of this work is that GSH is able to detoxify alachlor only at basic conditions.

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