行政院國家科學委員會專題研究計畫　期中進度報告

1 溶劑及液相與固相微萃取結合電灑質譜及氣相層析化學游離串聯質譜於環境藥物生化的應用 2 自身離子分子反應 (2/3)

計畫類別：個別型計畫
計畫編號：NSC93-2113-M-032-014-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：淡江大學化學系

計畫主持人：吳慧芬

計畫參與人員：R. Sekar，林其賢,吳昭賢,嚴哲毅,秦偵哲，顏之浩，吳慧芬

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 5 月 30 日

中文摘要:
本計劃主要進行溶劑微萃取法、液相微萃取法與固相微萃取法結合電灑質譜及氣相層析質譜於環境,藥物的應用及離子/分子反應, 應用大氣壓基質輔助雷射脫附游離質譜法及電噴灑質譜法/離子阱串聯質譜儀及毛細管電泳儀進行藥物分析,蛋白質的分析,新型液相微萃取法的開發,奈米粒子在分析上之應用及一滴溶劑微萃取法的開發及應用。具體的研究內容主要包括下列：利用溶劑微萃取法結合氣相層析儀/化學游離法/串聯質譜鑑別及定量茴香醛結構異構物之混合物; 離子阱串聯質譜儀偵測二甲苯及同分異構物鑑別; 溶劑微萃取法連結質譜於農藥鑑別及定量的應用; 開發一滴溶劑微萃取法作為大氣壓基質輔助雷射脫附質譜法之濃縮探針以提高生化分子偵測的靈敏度, 一滴溶劑萃取一滴樣品之超微量萃取法連結大氣壓基質輔助雷射脫附質譜並作為藥物濃縮探針的開發, 大氣壓基質輔助雷射脫附游離質譜法及電噴灑質譜法於蛋白質的分析, 兩段式注射針鉗合中空纖維管之液相微萃取法的開發; 研究蛋白質和金屬之反應; 金屬和藥物之反應研究, 串聯質譜於藥物的結構分析; 一滴溶劑微萃取法結合大氣壓基質輔助雷射脫附質譜及毛細管電泳儀於藥物, 生化分析的應用; 開發一滴溶劑微萃取法連結毛細管電泳儀的技術; 一滴溶劑萃取一滴樣品之超微量萃取法的開發。

關鍵詞: 液相微萃取法,溶劑微萃取法, 氣相層析儀/離子阱質譜儀, 化學游離法, 電灑質譜, 串聯質譜,離子阱質譜儀，碰撞活化解離, 大氣壓基質輔助雷射脫附質譜

計劃成果自評:

This project is the second year of my three year of NSC project, we have published several SCI journals in this year. In addition, we also demonstrated some of our experimental results in the posters or oral reports in conferences within this year. All information in regards to publications and conference presentations is provided in the references below.

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本研究計劃的成果:

Analysis of silymarin Extracted from commercial dosage form by using electrospray tandem mass spectrometry, ESI/MS/MS in negative ion mode.

Silymarin, 3,5,7-trihydroxy-2- [3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxy-methyl)-1,4-benzodioxan-6-yl]-4-chromanone, is an important antihepatotoxic drug, which is obtained from the dried fruits of *Silybum marianum* and widely used for the treatment of hepatic disorders (1-4). The drug is officially listed in Martindale The Extra Pharmacopoeia (5).

The assay procedure listed in the monograph of the Italian pharmacopoeia describes the UV-Visible spectrophotometric method (6) for determination of drug in bulk and pharmaceutical formulations. Several other methods have been employed to determine silymarin in biological specimens and/or pharmaceutical formulations such as high performance liquid chromatography (7-20), thin layer chromatography (21-27), high performance thin layer chromatography (28), potentiometric titration (29), diffuse reflectance Fourier transform infrared spectroscopy (30) and UV spectrophotometry (31,32).

In the literature none of the method have been reported for the structure elucidation of silymarin by using Electrospray ionization mass spectrometry (ESI/MS).

Tandem mass spectrometry is a rapid and powerful analytical technique that can provide trace mixture analysis and structure elucidation. The ion trap mass spectrometer has been widely used since it became commercially available in1983.

In the present communication, prior to the application of an analytical methodology, silymarin in dosage forms are normally extracted with an appropriate procedure to

remove possible interfering materials. The extraction procedure involves the adjustment to an appropriate pH value of the sample solution, the mixing of chloroform (an organic solvent) with the sample dissolved in water, followed by the removal of the solvent for further purification or analysis.

This paper describes mass spectral fragmentation of silymarin in the negative ion mode investigated by electrospray with tandem mass spectrometry (ESI/MS/MS).

All mass spectrometric measurements were carried out by using the Finnigan MAT ion trap mass spectrometer (Finnigan LCQ-Advantage, San Jose, CA, USA) equipped with an electrospray source in the negative ion mode for detection of ions. Ions are generated at atmospheric pressure and are introduced to the electrospray interface through a 0.5mm i.d. stainless steel heated capillary (12 cm length; 15-20 V) at a flow rate of 5μL/min per minute. A high voltage of 3 or 4 kV was applied to the tip of capillary, which is situated within ionization source of mass spectrometer. A potential of 15-25 V was applied to a focusing stainless steel tube lens located at the end of the capillary. Ions from the source are guided into a ion trap mass analyzer. The ion trap was performed with the automatic gain control for all experiments. In this mode, the instrument can automatically select the best trapping parameters to keep the number of ions present in the trap to maintain at a constant value in order to avoid the space-charge effect. The ITMS is operated in the mass selective instability mode. An aliquot of 10 ppm of extracted solution of silymarin in chloroform was injected. The spectra scan range is from m/z 50 to 2000. All experiments were repeated at least 3 times.

The observed fragmentation modes of the mass spectra of silymarin, antihepatotoxic drug is proposed in scheme 1.In the full mass spectra of the silymarin (figure 1.), the two main

peaks corresponding to m/z 481.07 and m/z 962.73 was observed and were assigned for $[M-H]^-$ and $[M_2-H]^-$ (dimmers), the relative abundance of peak at m/z 962.73 is not high i.e., about 30%, by selecting peak at m/z 481.07, MS/MS experiment was performed to get three main peaks corresponding to m/z 463.06, 453.01, 301.05 and 257 were assigned as $[M-H_2O]^{-\cdot}$, $[M-CO]^{-\cdot}$ and $[M-C_8O_3H_8]^{-\cdot}$, $[M-C_{11}O_5H_{12}]^{-\cdot}$, respectively (Figure 2.).

In this study, ESI/MS/MS in negative mode was performed to study the fragmentation of drug after extracting it from commercial dosage forms via liquid-liquid extraction method. Previously pure compounds are used for the study of fragmentation pattern. This study reveals that tandem mass spectrometry can also be applied to the commercial dosage forms after carefully application of extraction procedure for the determination of molecular ion and their fragmentation pattern.

Acknowledgements

We acknowledge support from the National Science Council of the Republic of China and Tamkang University.

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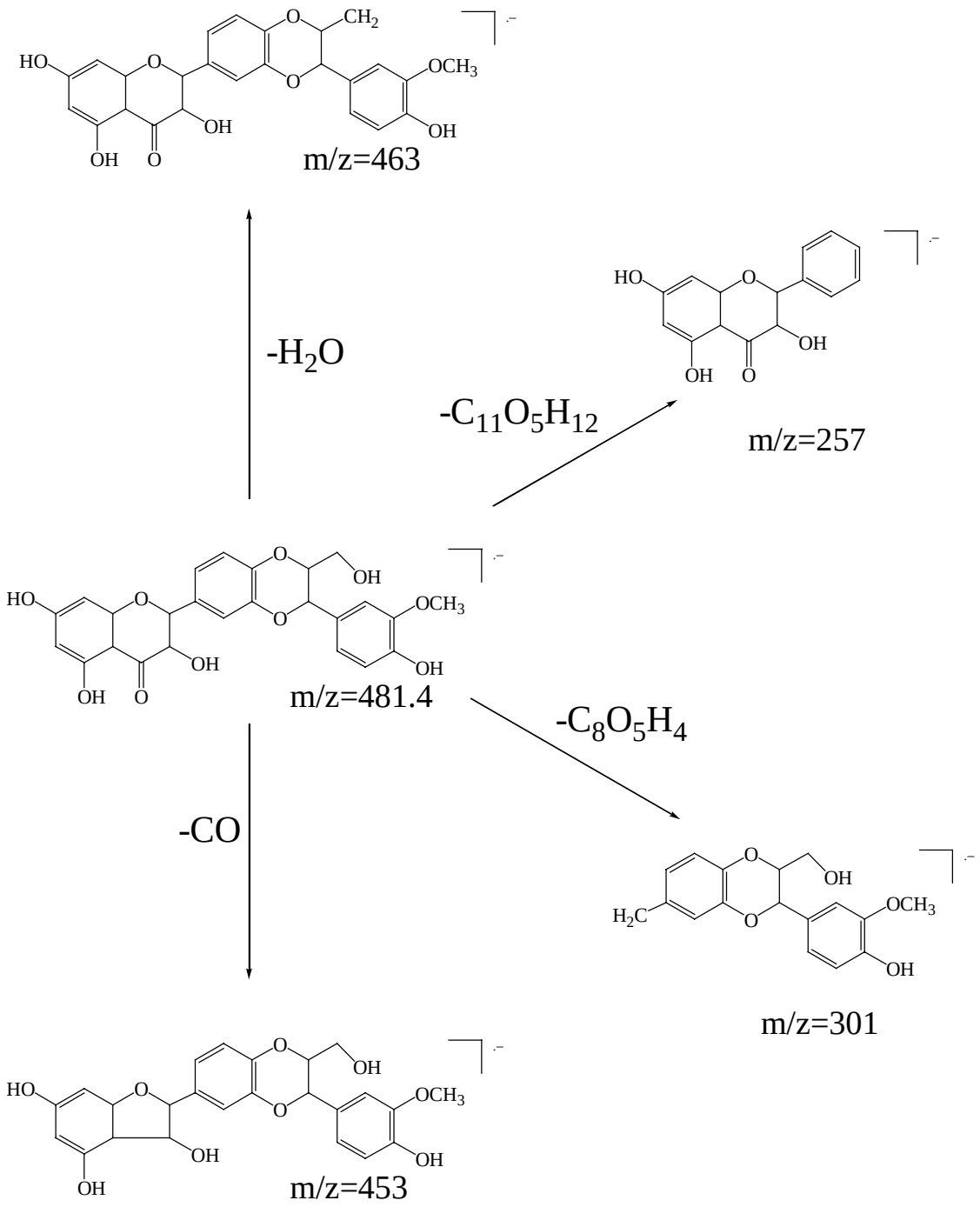
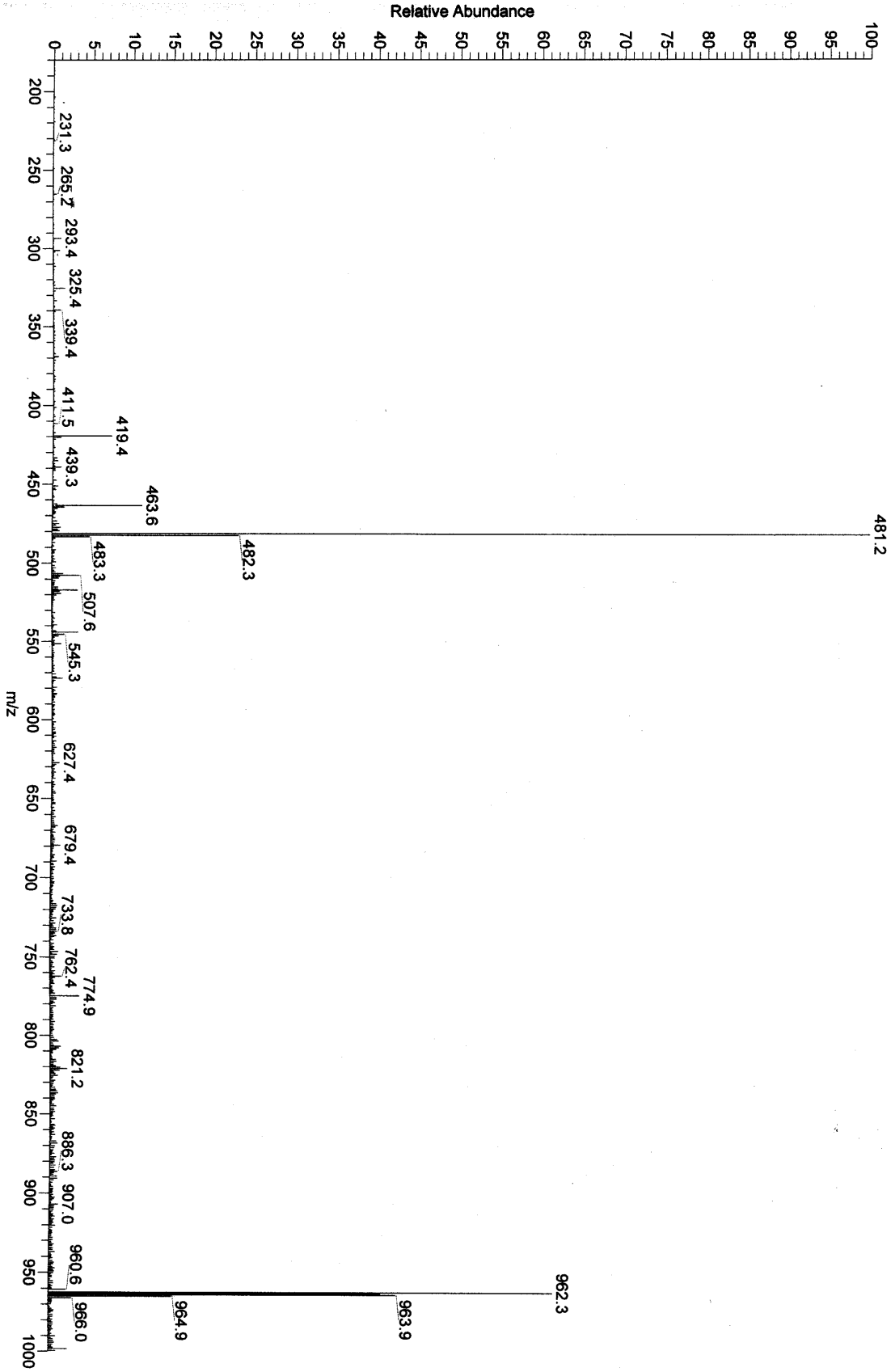


Scheme 1.


Relative Abundance
m/z
481.2
962.3
482.3
963.9
463.6
964.9
419.4
483.3
966.0
507.6
545.3
627.4
679.4
733.8
762.4
774.9
821.2
886.3
907.0
960.6
231.3
265.2
293.4
325.4
339.4
411.5
439.3

Probing the interaction of kojic acid antibiotics with iron by using electrospray tandem mass spectrometry

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ABSTRACT

Kojic acid, 5-hydroxy-2-(hydroxymethyl)-4H-pyran-one has been used extensively as iron chelating drug though the complexes of kojic acid and iron were poorly studied. Here, in this article we demonstrate the complexation of kojic acid with iron by using electrospray ionization mass spectrometry (ESI-MS). The ESI-MS analysis revealed different reactions between iron and kojic acid (M), and the product ion spectrum showed four complexes: $[Fe+2(M-H)]^+$, $[Fe+3(M-H)+H]^+$, $[Fe+(4M-5H)+FeOH+H_2O+H]^+$, and $[2Fe+(5M-6H)+H]^+$. Each complex species was subjected to collision-activated dissociation (CAD) / tandam mass spectrometry (MS^n). Fragmentation pathways for iron-kojic acid complexes and deprotonated structure of binucleated complex, $[2Fe+(5M-6H)+H]^+$ are proposed.

INTRODUCTION

Kojic acid, a fungal metabolic product, has been suggested to play an important role in iron-overload diseases, since it possesses iron-chelating activity[1,2]. The most familiar iron-overload genetic disorder known as hemochromatosis, affecting 1-in-200 people causes the body to absorb and store too much iron and causes several iron-overload diseaes[3-5]. The extra iron builds up in the organs (liver, heart, and pancreas) and damages them, sometimes leads to the malfunction of these organs[3]. Thus, it is of the essence to study iron-chelating agents to produce effective drugs against iron-overload diseases. Some studies[6] revealed, kojic acid might act as a model for the synthesis of new biologically active derivatives having the capability of chelating iron. Katho et al[7] were studied extensively on iron-chelating agents including kojic acid by using animal model. In hairless mice, it has already been demonstrated that kojic acid is having anti-wrinkling

activity: iron in the skin involved in wrinkling due to chronic photo damage[8]. But the complexes formed due to the interaction of iron and kojic acid are not established.

To understand specific activities and biomedical processes of pharmaceutical compounds, it is important to study drug interaction with metal ions including iron, as this metal has significant importance in biological studies. Some of the metal ions are able to activate or deactivate pharmaceutical compounds upon binding to drugs. Few drugs act, as metal chelators are useful to remove excess amount of metal ions from body. ESI-MS has great significance in the last few years in the field of identification and characterization of metal coordination complexes. Because of the 'softness' of ESI-MS, it is possible to study and decompose the coordination complexes[9,10]. Also this technique has been extensively used to study iron coordination complexes[9,11-13]. Thus, in this study we concentrate to report the noncovalent complexes of iron and kojic acid and elucidate the fragmentation routes by using ESI-MS in the gas phase.

EXPERIMENTAL

Kojic acid and iron(III) chloride ($FeCl_3$) were obtained from Avocado Research Chemicals Ltd (Heysham, Lancs) and Aldrich (St.Louis, USA) respectively.

In this study iron(III) chloride has been used as a source for iron. ESI-MS experiments were carried out by using the Finnigan MAT ion trap mass spectrometer (Finnigan LCQ-Advantage, San Jose, CA, USA). Kojic acid (40 ppm) was incubated with iron(III) chloride (15 ppm) for 10 min at 26±2 ^{o}C prior to the experimental measurements. Stock solutions were prepared by using water as a solvent for both kojic acid and iron(III) chloride. The spectral analysis was carried out in positive ion mode and the solutions were injected into the ESI source with a solvent flow rate of 5μL/min. The tip and spray chamber were held at 5000V and 250 ^{o}C respectively. Capillary voltage

equal to 10V was applied. Data was obtained across the mass range of 50 – 2000 Da. Fragmentation analyses were performed on each product ion by means of CAD and data was obtained across the mass range of 50-1000 Da. Each spectrum is combination of 10 - 20 individual scans, depending on the signal intensity.

RESUTLS AND DISCUSSION

Several studies[14-19] revealed that, based on the electronic state of metal center, various types of reactions are possible in gas phase. Figure 1 represents typical protonated ion from, $[M+H]^+$, m/z 143.04, of kojic acid. In order to ascertain the reactions of reactants, iron(III) chloride and kojic acid were admitted into the ion trap and product mass spectrum was examined. In the product mass spectrum displayed in Figure 2, four m/z main peaks, 338.14, 479.74, 710.75, and 816.78 were prominent. Relative abundances of these peaks are 30, 100, 47, and 76% respectively. The observed m/z of 338.14 and 479.74 are corresponding to the combination of two $[Fe+2(M-H)]^+$, and three $[Fe+3(M-H)+H]^+$ kojic acid molecules with iron respectively. Similarly different stoichiometries were observed in the study of iron and copper interaction with flavonoids[13]. The m/z of 710.74 could be originated due to the complexation of iron with both kojic acid and hydroxyl ion, $[Fe+(4M-5H)+FeOH+H_2O+H]^+$. The source of hydroxyl ions is considered to arise from water, used in this study as a solvent for both kojic acid iron(III) chloride. Gas phase complexation of hydroxyl ions with metals has been demonstrated previously[20].

The groupings of two iron molecules with five kojic acid molecules show the m/z of 816.78, $[2Fe+(5M-6H)+H]^+$. The proposed structure of deprotonated form of $[2Fe+(5M-6H)+H]^+$ has been shown in Figure 3. In this binuclear complex two iron

atoms are coordinated with five kojic acid molecules and one of the iron atoms show coordination number 4. Two iron atoms held together with one kojic acid molecule. The calculations of this chemical structure corresponding to energy minimum made by using CAChe Ab initio 5.0 software show that the heat of formation is –477.276 kcal/mol, which suggests that the binucleated complex formed between iron and kojic acid with 2:5 stoichiometry is relatively stable one.

All product complexes of iron and kojic acid were subjected to collision-activated dissociation (CAD) to evaluate the dissociation reactions (Table 1). These CAD fragmentation studies were supported by proposed schemes (1-4). Using HighChem Mass Frontier 1.0 software makes some of the schemes are proposed. The possibility of m/z of 816.78 is combination of both m/z 338.14 and 479.74 forms with the loss of one proton. But MS/MS spectrum of m/z 816.78 showed m/z of 674.83 peak, derive from loss of one kojic acid molecule (Scheme 1). MS^n was required to generate a complete series of fragment ions. Further CAD (MS^3) study leads to the elimination of a simple neutral loss mass 140 and m/z of 534.89 was observed (Scheme 1). The MS/MS study of m/z 710.75 and 479.74 exhibited m/z 338. The proposed scheme 2 shows the fragmentation pathway of m/z 710.75, where two fragment ions (m/z of 355.11 and 337.98) were observed with loss of one water molecule. In the MS/MS product spectrum, 100 and 28 % abundances were shown by m/z of 337.98 and 355.11 peaks respectively.

The fragment ion (m/z of 338.14) observed in the MS/MS spectrum of m/z of 479.74, arises due to the elimination of one kojic acid molecule (Scheme 3). This elimination process of kojic acid molecule is similar to the formation of m/z of 674.83 from m/z of 816.78. The proposed fragmentation pathways for m/z of 338.14 are

illustrated in scheme 4, suggest the stepwise elimination of kojic acid side chain molecules. In the MS/MS spectrum of m/z of 338.14, two kinds of fragment ion peaks (m/z of 308.88 and 320.86) were observed. The abundance of m/z of 308.88 and 320.86 are 95 and 28% respectively. Along with these two fragment ions some other peaks were also observed with less abundance. The fragment ions, m/z of 320.86 and 308.88 were formed due to the loss of OH and COH ions respectively from m/z of 338.14. The collision energy used for the dissociation of m/z of 338.14 is relatively higher than the energy used for the dissociation of other m/z complexes observed in the product mass spectrum. From the above CAD study it is clear that two kojic acid molecules can bind to one iron atom in a relatively stable form.

CONCLUSION

From the above study, we concluded that Kojic acid can complex with iron in a variety of combinations and complete fragmentation pathways are proposed for all the observed complexes. The results presented in this study, would be useful in biomedical research for identification of iron and kojic acid metabolites and derivatives produced in biochemical processes.

ACKNOWLEDGEMENT

Financial support from National Science Council of Taiwan, Department of Chemistry, Graduate Institute of Life Sciences, Tamkang University is greatly acknowledged.

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FIGURE CAPTIONS

Figure 1. The ESI-MS spectrum of protonated kojic acid, m/z 143.04

Figure 2. The ESI-MS product ion spectrum for reactions of iron with kojic acid

Figure 3. The proposed structure of iron and kojic acid with a stoichiometry of 2:5, in which one iron molecule is believed to show coordination number 4, bonded with four kojic acid molecules.

Scheme 1. Proposed fragmentation pathway showing the formation of m/z 674.83 and 534.89 fragment ions in the CAD study of m/z 816.78

Scheme 2. Proposed fragmentation pathway showing the formation of m/z 337.98 and 355.11 fragment ions in the CAD of m/z 710.75

Scheme 3. Proposed fragmentation pathway showing the formation of m/z 338.14 fragment ion in the CAD of m/z 479.74

Scheme 4. Proposed fragmentation pathway showing the formation of m/z 320.86 and 308.88 fragment ions in the CAD study of m/z 338.14.

Table 1. m/z observed, m/z calculated, product identity for the entire fragment ions observed in the product ion mass spectrum. The m/z fragment ions generated of corresponding product identity after CAD were also given in the Table.

Product m/z observed	Product m/z calculated	Product identity	CAD of observed m/z, $(MS)^n$
816.78	816.96	$[2Fe+(5M-6H)+H]^+$	674.83 $(MS)^2$, 534.89 $(MS)^3$
710.75	710.96	$[Fe+(4M-5H)+FeOH+H_2O+H]^+$	337.98, 355.11 $(MS)^2$
479.74	480	$[Fe+3(M-H)+H]^+$	338.14 $(MS)^2$
338.14	337.97	$[Fe+2(M-H)]^+$	320.86, 308.88 $(MS)^2$

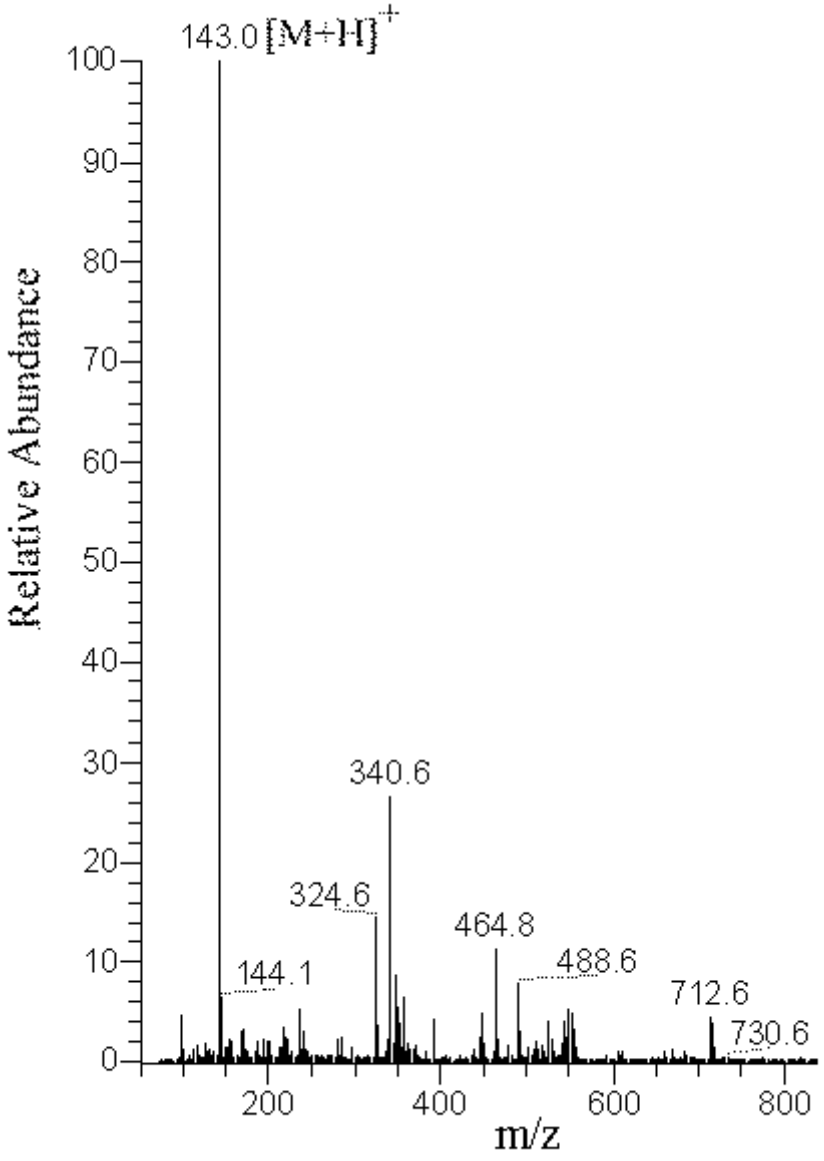
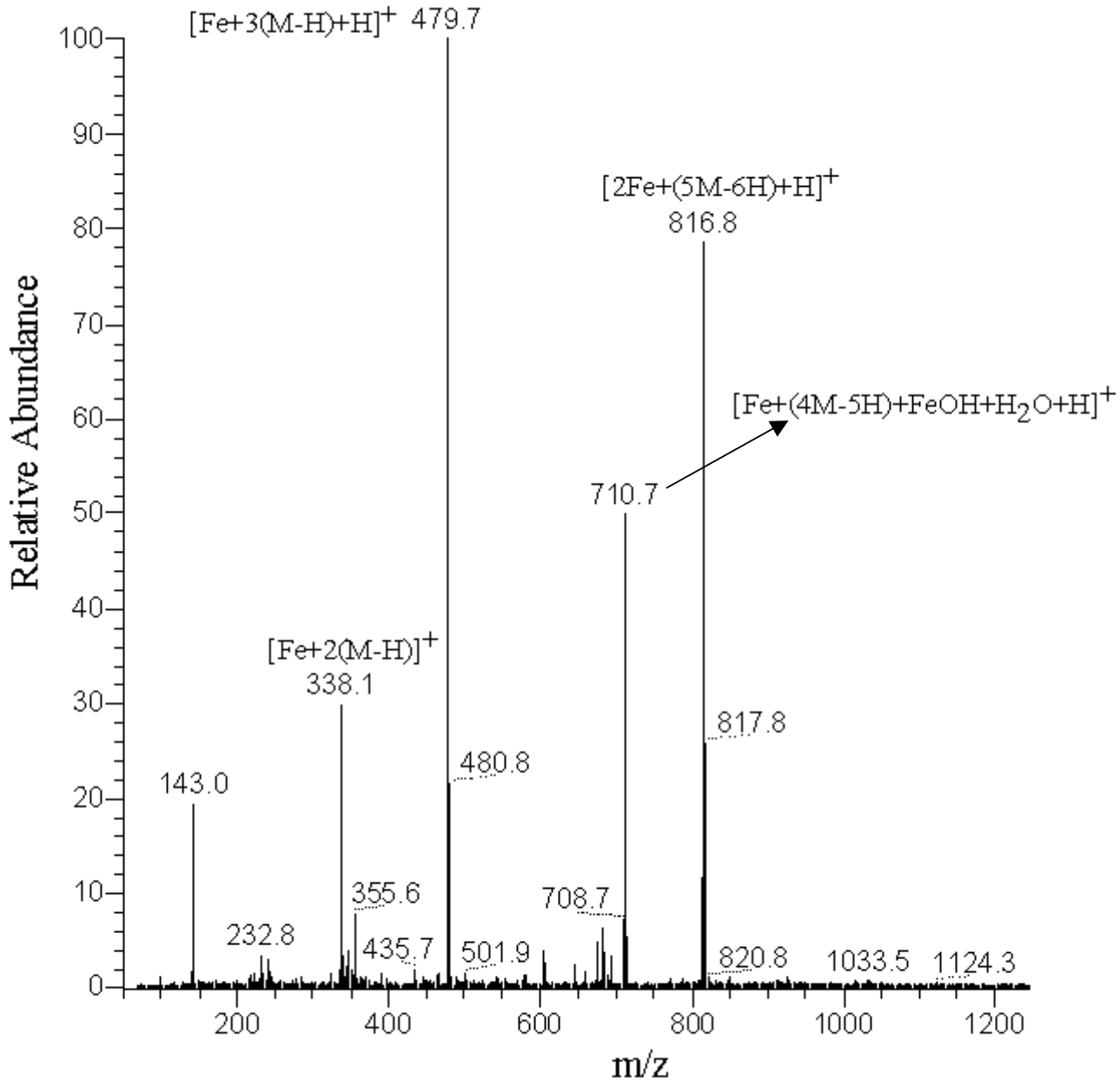

143.0 [M+H]+
Relative Abundance
100
90
80
70
60
50
40
30
20
10
0
340.6
324.6
464.8
488.6
144.1
712.6
730.6
200
400
600
800
m/z

Figure 1


[Fe+3(M-H)+H]+ 479.7
[2Fe+(5M-6H)+H]+
816.8
[Fe+(4M-5H)+FeOH+H2O+H]+
710.7
[Fe+2(M-H)]+
338.1
817.8
480.8
143.0
355.6
708.7
232.8
435.7
501.9
820.8
1033.5
1124.3
Relative Abundance
m/z
0
10
20
30
40
50
60
70
80
90
100
200
400
600
800
1000
1200

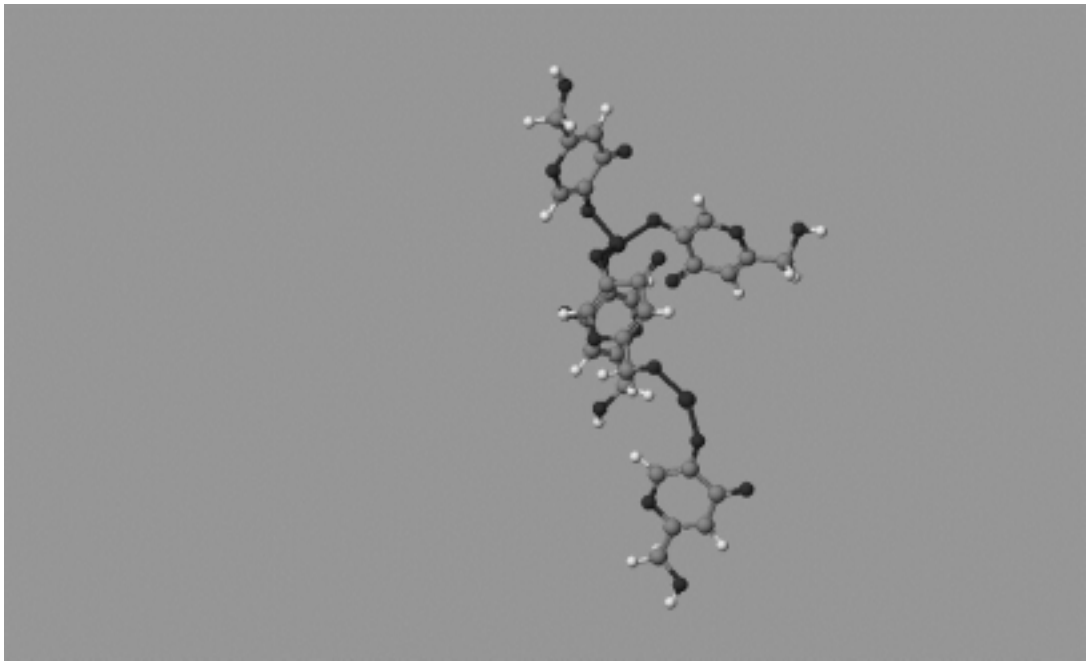
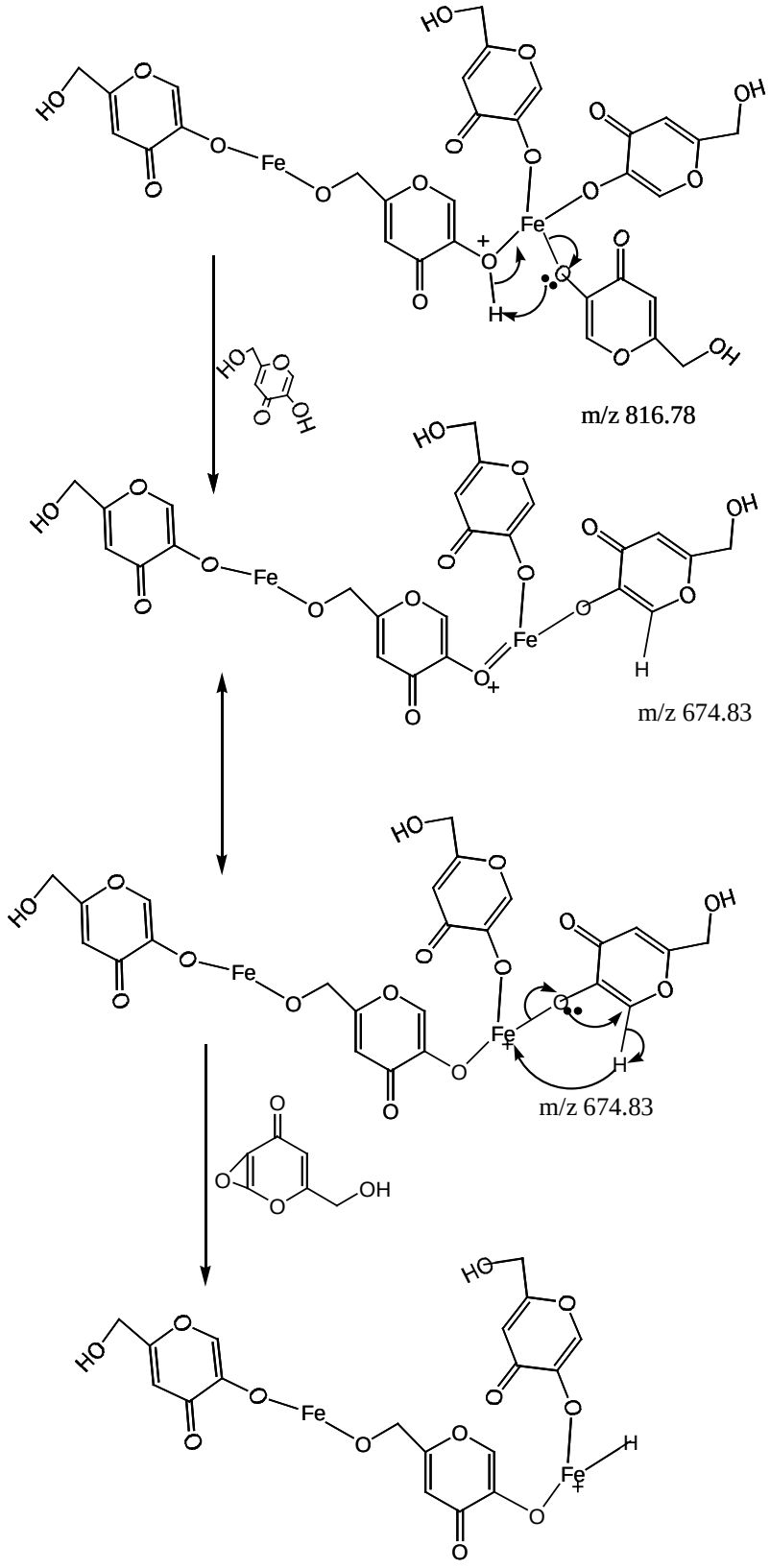
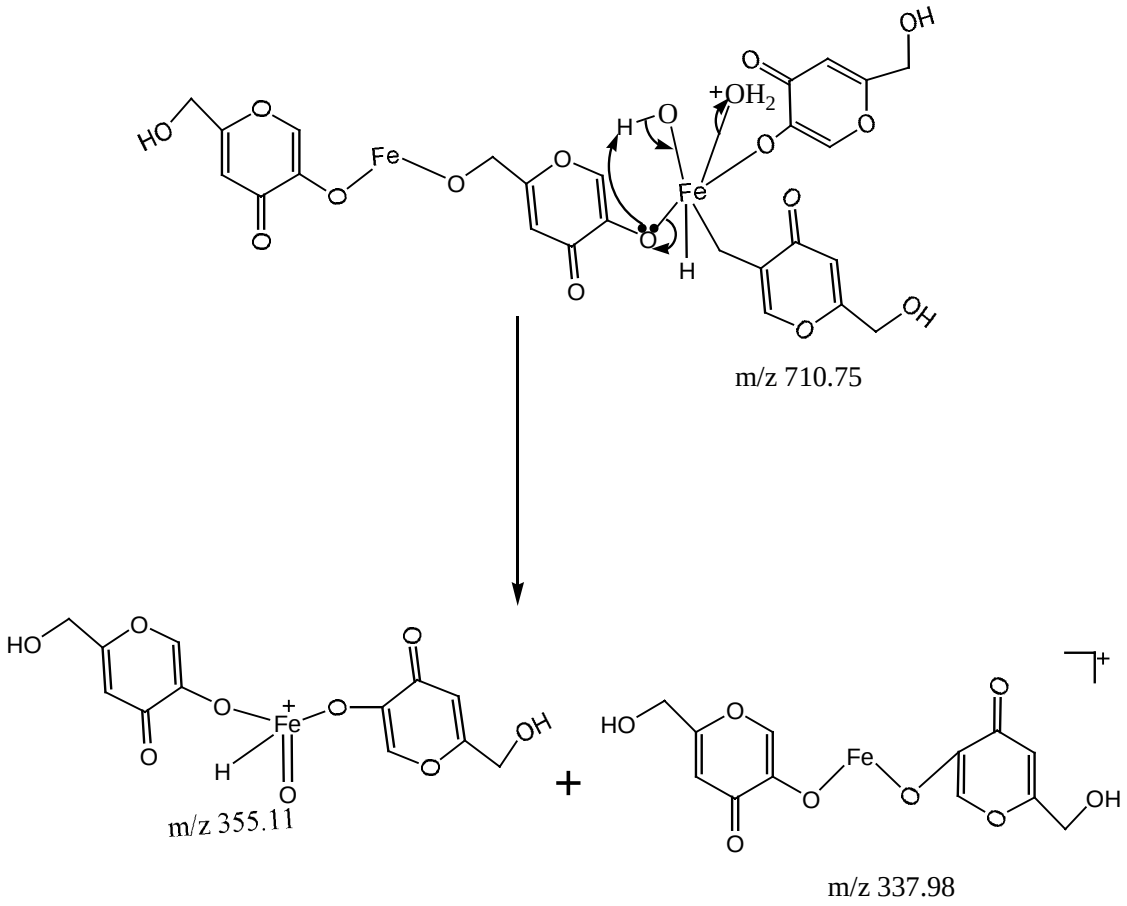


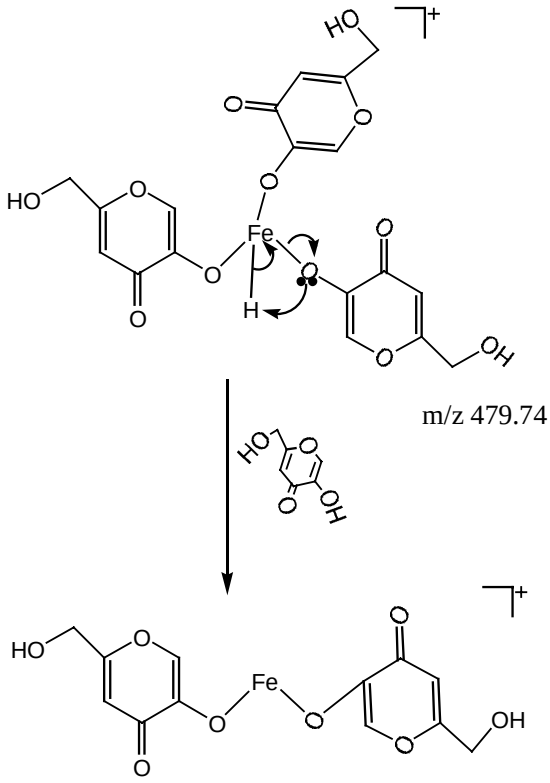
Figure 3



Scheme 1

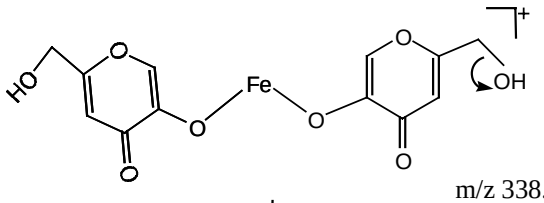


Scheme 2



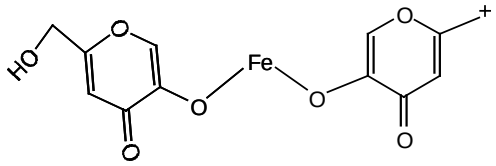
Scheme 3

A



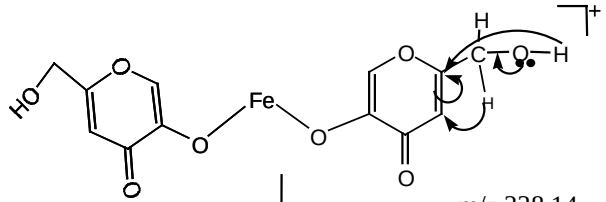
m/z 338.14

-OH

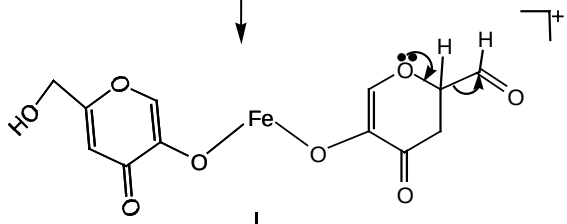


m/z 320.86

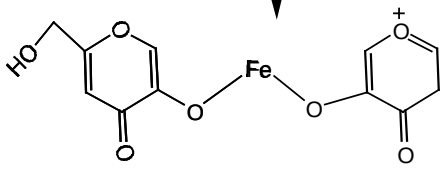
B



m/z 338.14



-COH



m/z 308.88

Scheme 4

A Liquid-Phase Microextraction Method by Combining a Dual Gauge Microsyringe with a Hollow Fiber Membrane (LPME/DGM-HF) for the Determination of Organochlorine Pesticides in Aqueous Solution by Gas Chromatography/Ion Trap Mass Spectrometry

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Abstract

A liquid-phase microextraction method (LPME) by combining a dual gauge microsyringe with a hollow fiber membrane (LPME/DGM-HF) for the extraction and determination of organochlorine pesticides (OCPs) in aqueous solution then followed detection by Gas Chromatography/Ion Trap Mass Spectrometry (GC/ITMS) has been demonstrated. The advantages of this technique include little solvent and sample consumption, simplicity, easy of use and rapid. Influence of parameters including extraction time, solvent selection, salt concentration and sample stirring rate have been investigated in order to optimize the extraction efficiency for this method. The viability of this methodology is evaluated by measuring the linearity and detection limit of the five OCPs in aqueous solution. The detection linearity for the OCPs has been obtained over a range of concentrations between 1 to 500 µg/l (r^2 > 0.930), with detection limit of 0.1 µg/l for each of OCPs.

Introduction

Organochlorine pesticides (OCPs) have been widely used in the environments. They can easily spread in environmental and biological matrices due to their volatility and poor biodegradability [1-3]. Most of them are associated with cancers in humans [4] and endocrine disruption in wildlife [5]. Thus, determination of accurate concentrations of OCPs in environmental and biological samples is extremely important. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [6,7] have been applied for the determination of OCPs [8,9]. With recent developments in sampling pretreatment techniques, the solid-phase microextraction (SPME) [10-12] and the solvent microextraction (SME) using single drop solvent [13-15] have been shown to be more

useful than these methods. However, the SPME requires expensive equipments [16]. While the SME encounters difficulty in maintaining the solvent drop at high sample stirring rate [13, 15]. The LPME using hollow fiber membrane (LPME-HF) has been developed [17-22]. It is an efficient sampling tool with many advantages such as simplicity, high sensitivity and low cost [18-22]. However, the traditional LPME-HF method is performed by combining a hollow fiber with a microsyringe needle. The disadvantage of this method is that the fiber is difficult to fit tightly with the needle. Since the outer diameter of the microsyringe needle is difficult to match with the internal diameter of the commercially available fibers. This factor seriously reduces the feasibility of this extraction method. It often occurs detachment of the hollow fiber from the needle during the extraction leading to loss of sample or causing other experimental difficulties. To overcome this problem, we have developed a novel LPME method by combining a dual gauge microsyringe with a hollow fiber (LPME/DGM-HF). We also demonstrated the effectiveness of this extraction method by applying to the extraction of OCPs from aqueous solution. Some parameters that would influence the extract efficiencies of extraction such as solvent selection, extraction time, sample stirring rate and salt concentration in sample solution were examined in order to prove the effectiveness of this method and to optimize the extraction procedures.

Experimental sections

Chemicals

All OCPs were purchased from Supelco Inc., Bellefonte, PA, USA. Fig. 1 displays the structures of these OCPs. Q3/2 Accurel KM polypropylene hollow fiber membrane (0.6 mm I.D., 0.2 mm thickness of the wall, and 0.64 μm pore size) obtained from

Membrana, Wuppertal, Germany was used for all LPME/DGM-HF experiments. Stock solutions were prepared by dissolving 100 mg of each compound in 100 ml of solvent and were stored in glass-stoppered bottle in dark at 20℃. Standards solutions, at various concentrations ranging from 2.5 ppm to 80 ppt were prepared by appropriating dilution of aliquots of the stock solutions in deionized water.

LPME/DGM-HF extraction apparatus

The extraction of OCPs was performed using a 10 µl microsyringe with dual gauge needle (0.64 mm and 0.47 mm O.D.). The utility of using a dual gauge needle is to fit the hollow fiber membrane easily and tightly with the microsyringe needle. The apparatus for LPME/DGM-HF is shown in Fig. 2. 3 µl of desired solvent was withdrawn into the microsyringe. The syringe needle was then tightly fitted with a 3 cm length of hollow fiber membrane and then immersed into 20 ml of sample solution in a 22 ml vial sealed with PTEF silicon septa (Supelco Inc., Bellefonte, PA, USA). The microsyringe plunger was depressed so that the hollow fiber was impregnated with 3 µl of solvent. The whole assembly was then subjected to magnetic stirring. Extraction was performed for desired time period. After the completion of extraction, the solvent in the hollow fiber was withdrawn into the dual gauge microsyringe. The hollow fiber assembly was then removed. The extract was injected into the GC/ITMS for analysis and detection.

GC/ITMS analysis and detection

A Varian GC 3800 gas chromatography coupled to a Varian Saturn 2000 ITMS was applied for analysis and detection for OCPs. The GC was equipped with a DB-5 fused silica capillary column (30 ㄨ 0.25 mm I.D., 1 µm film thickness) obtained from Supelco Inc., Bellefonte, PA, USA. Helium gas (purity 99.99%) was applied as the

carrier gas. The 3 µl sample was injected into the GC/ITMS. The injection temperature was maintained at 240°C. The GC/ITMS temperature program was set as below. Initial oven temperature was set at a 180°C, then increased by 5°C per min. to 200°C, then increased by 7°C per min. to 230°C and held for 5 min. The ITMS was operated in electron impact (EI) mode. The ionization energy was set at 70 eV. The detection range was from 40 – 650 Da. The selective ion monitoring (SIM) was used for quantitaion and detection.

Results and discussion

In the LPME/DGM-HF, analytes diffuse through the membrane and undergo mass transfer into the solvent. In order to optimize the extraction of OCPs, parameters that influence the extraction were investigated. The factors examined include extraction time, solvent types, sample agitation rate and salt content. Although increase the extraction temperature can speed up the time for equilibrium of extraction due to an increase in diffusion coefficient of analyte across the membrane, it can result in the formation of air bubbles in the solvent in hollow fibre [19]. Thus, all experiments were performed at a constant temperature of 22°C and at a fixed solution pH of 6.5. After the extraction by the LPME/DGM-HF method, the extraction yield of OCPs was determined by the peak area of total ion chromatogram (TIC). Fig. 3 shows a TIC of five OCPs extracted from a spiked aqueous solution by the LPME/DGM-HF method. The most abundant ions detected by SIM in the EI/MS spectra were chosen as the quantitative ions. The other two ions were used for confirmation of individual OCPs (see Table 1). Since the dual gauge microsyringe possesses 0.64 and 0.47 mm O.D.s, the commercial hollow fiber

membranes can be easily interfaced to it as long as the I. D. of the fiber is in the range between 0.64 and 0.47 mm. Thus, the commercial hollow fiber membrane we used in this experiment with 0.6 mm I. D. can be easily used to fit tightly with the dual gauge needle.

Probing the effect of solvent selection

The most important step in the development of LPME/DGM-HF method is the selection of a suitable solvent for extraction. The solvent for LPME must possess low volatility [23], low solubility in water, should not leak from the membrane and the analytes must be soluble in it [19]. Four different solvents include toluene, hexane, iso-octane and n-octanol were investigated for the extraction efficiencies of the OCPs from aqueous solution. Table 2 shows the performance of these solvents for the selected OCPs under identical conditions. The results have demonstrated that toluene is the most effective solvent for the extraction of OCPs. The reasons maybe due to it is easily immobilized on the hollow fiber, its solubility in water is low and it also possesses a large OCP enrichment factor [19]. Thus, toluene has been selected as a suitable solvent for the hollow fiber membrane microextraction of OCPs in this study.

Probing the extraction time

The effect of extraction time on the OCP extraction efficiency was also examined by carrying out the extraction at different extraction times. In Table 2, we can observe that Aldrin, Deldrin, 4,4´-DDD has reached equilibrium in 15 minutes. Longer times were needed for 2,4´-DDD and 4,4´-DDT. In the present study, an extraction time of 15 min was selected for the extraction of sample. However, a longer extraction time could be adopted if a larger enrichment factor is desired.

Probing the effect of sample stirring rate

The influence of sample stirring rate on the LPME/DGM-HF extraction was also investigated using different stirring speeds in the range of 0 to 1200 rpm. The increase in the stirring rate improves the OCP extraction efficiency as shown in Table 2, as is evident from the increase in relative chromatographic peak areas with the stirring rate. As can be seen from Table 2 that the extraction efficiency was better for some analytes above 480 rpm. But, it was found that the air bubbles were formed at the stirring rates of 840 and 1200rpm. This leads to errors in quantification of the analytes in the extract. Thus, the stirring rate of 480 rpm was chosen as the optimum value for extraction of OCPs.

Probing the effect of salt concentration of sample

The addition of salt often increases the LLE efficiency due to salting out effect [24]. Thus, the salt concentration effect on extraction of OCPs was also evaluated by adding different amount of sodium chloride to the sample solution and the results are shown in Table 2. It is clear from Table 2 that the peak area decreases with the increase in sodium chloride concentration in the studied range of 0%-20%. Jiang et al. [25] and Wang et al [26] also found that the extraction efficiency in LPME decreased with the increase in salt concentration. The reason for the decrease in extraction efficiency with the increase in salt concentration may be due to the decrease in diffusion rate of OCPs from bulk solution to the organic phase. Because, the addition of salt increases the viscosity of bulk solution leading to decrease in the diffusion rate of analytes. Thus, the best conditions for the LPME/DGM-HF extraction of OCPs were performed without adding any salts.

Method evaluation

The viability of the proposed LPME/DGM-HF combining GC/MS method was evaluated by determining the linearity under the above optimized conditions. Linearity was investigated over a concentration range of 1 μg/l – 500 μg/l for all the studied pesticides. Results shown in Table 3 indicate that, linearity with correlation coefficients > 0.930 can be obtained for all the OCPs at the experimented concentration range.

Conclusion

The determination of OCPs in pure water using the combination of LPME/DGM-HF with the GC/MS has been successfully performed. The roles of some important parameters that influence the extraction efficiency, and the optimal conditions have been established. The method has a high level of linearity over a wide range of analyte concentrations. The novel LPME/DGM-HF technique has proven to be an efficient, rapid, low cost, solvent minimizing and simple method for the qualitative and quantitative analysis of OCPs in aqueous sample.

Acknowledgement

We thank the financial support from the National Science Council of Taiwan, Department of Chemistry and Graduate Institute of Life Sciences, Tamkang University.

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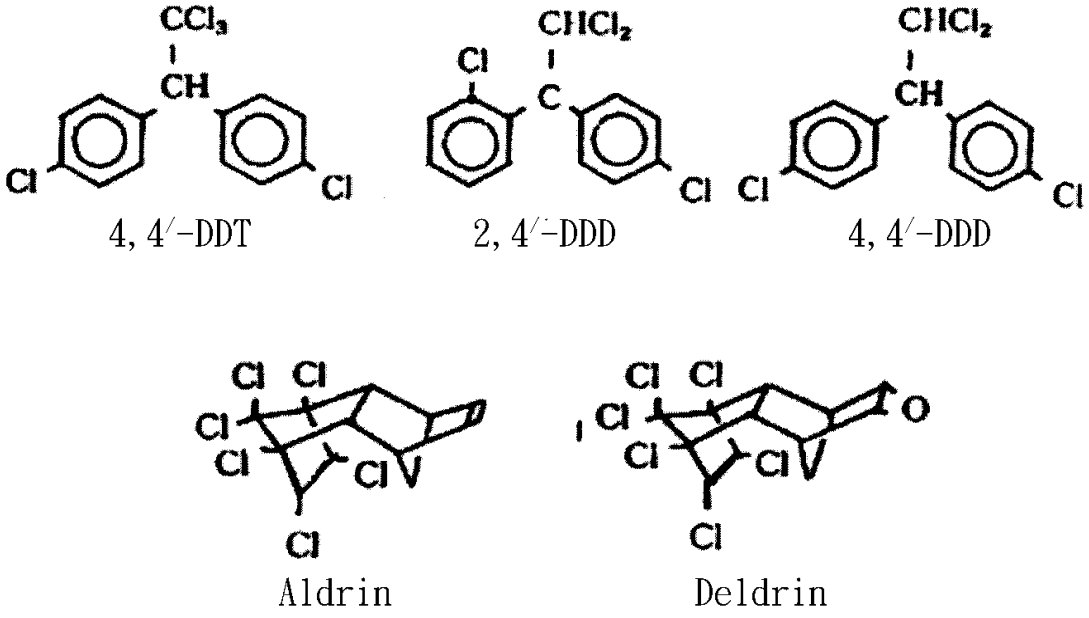


Fig. 1: Structures of Aldrin, Deldrin, 4,4´-DDT 2,4´-DDD and 4,4´-DDD.

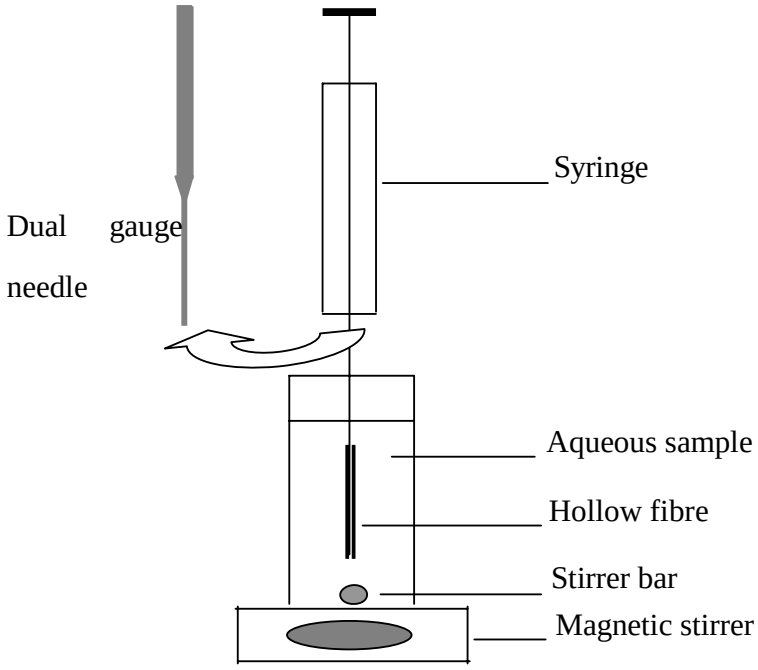


Fig. 2: Schematic of experimental set-up of LPME using hollow fibre membrane and dual gauge micro syringe.

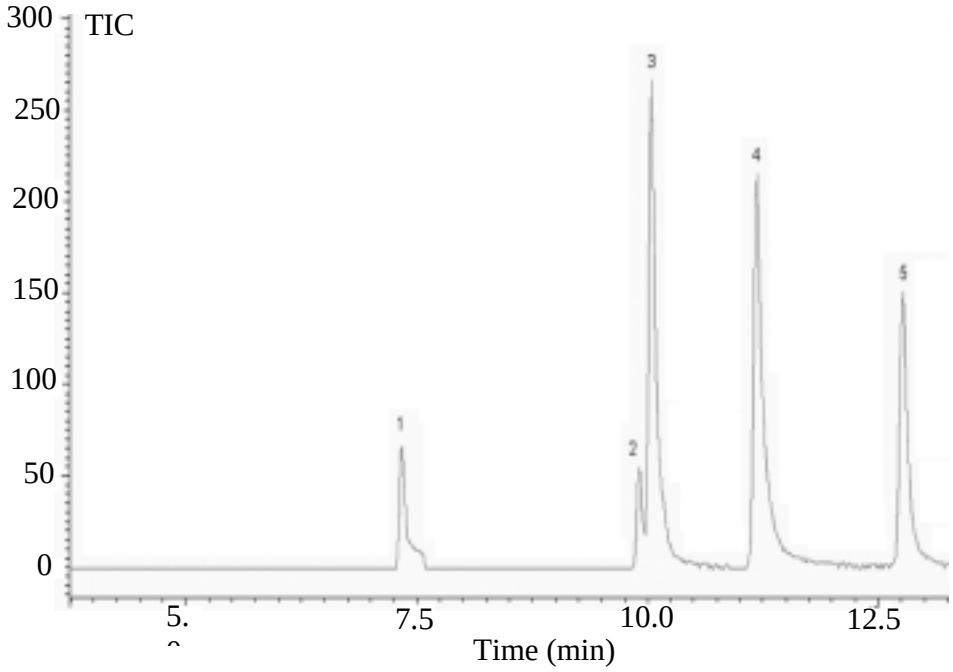


Fig. 3: Chromatogram of five OCPs extracted from an OCP spiked aqueous solution by LPME using hollow fibre membrane. Peaks: 1= Aldrin; 2= Deldrin; 3= 2,4´-DDD; 4= 4,4´-DDD; 5= 4,4´-DDT.

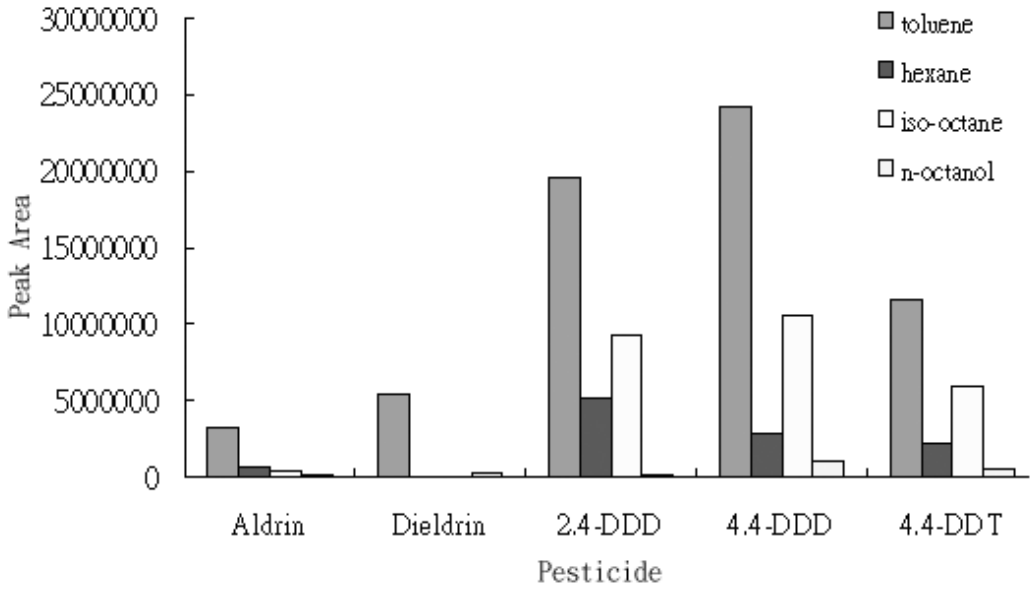


Fig. 4: OCP extraction efficiency from spiked aqueous solution using different solvents.

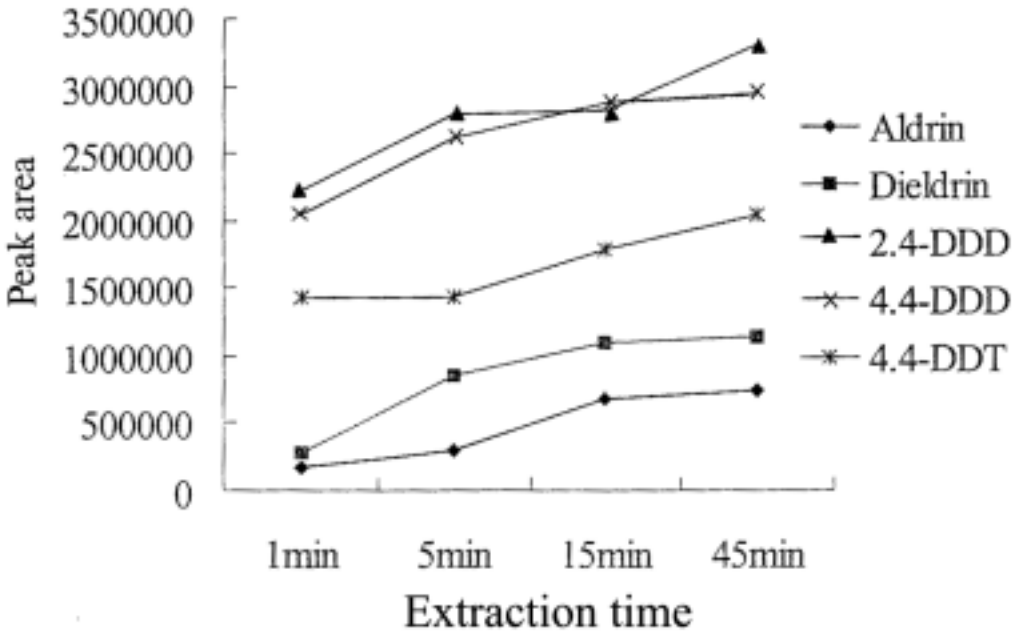
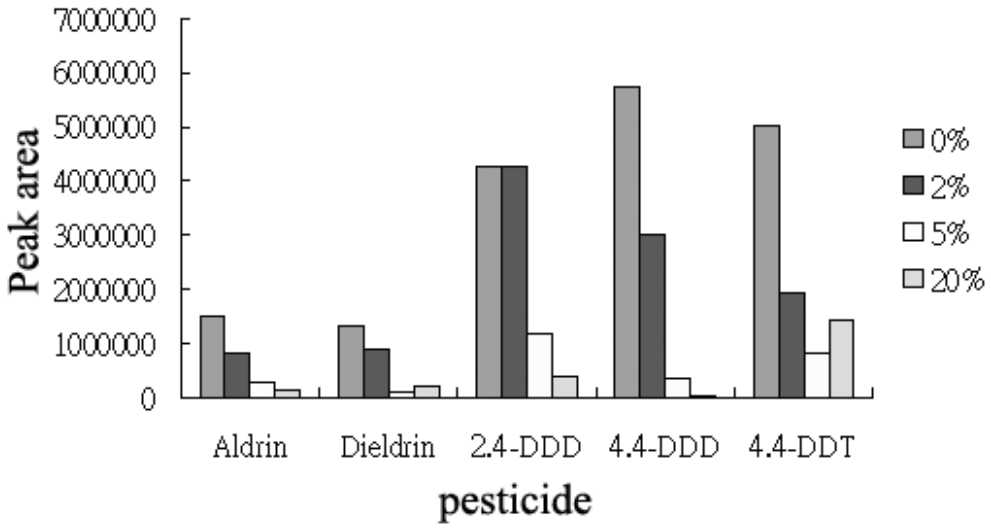


Fig. 5: Equilibrium profile of OCPs during LPME using hollow fibre membrane.


7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
Peak area
0%
2%
5%
20%
Aldrin
Dieldrin
2.4-DDD
4.4-DDD
4.4-DDT
pesticide

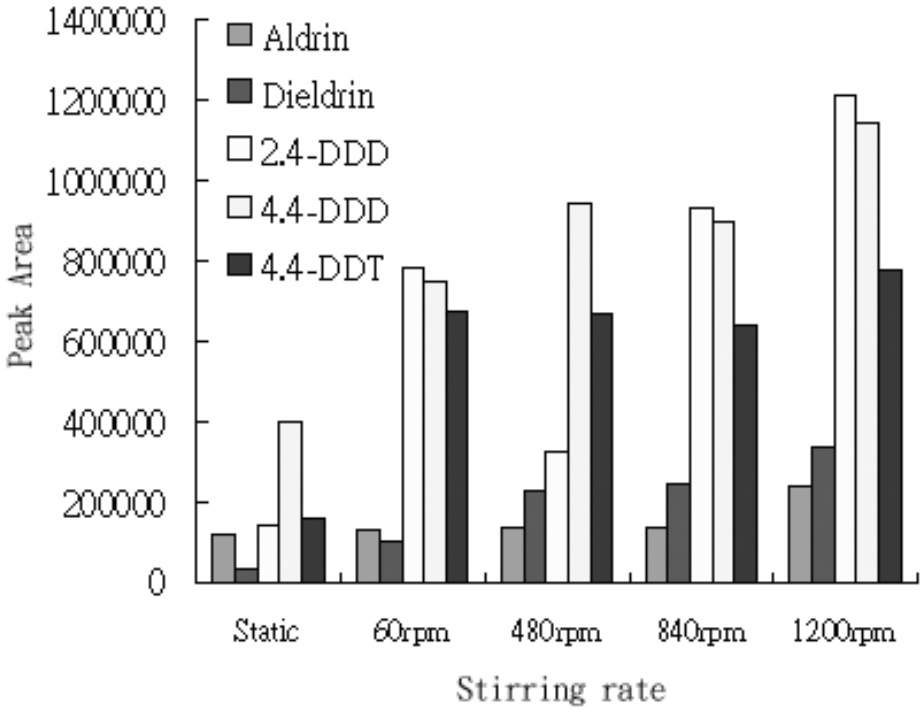


Fig. 7: Effect of sample agitation rate on the extraction efficiency of OCPs

Table 1. GC-MS-SIM conditions for extraction of OCPs by the LPME/DGM-HF method.

Pesticide	Retention time (min.)	Quantitation ion (m/z)	Confirmation ion (m/z)
Aldrin	7.32	66	263, 293
Deldrin	9.91	79	263, 277
2,4´-DDD	10.07	235	165, 200
4,4´-DDD	11.23	235	165, 200
4,4´-DDT	12.76	235	165, 200

Table 2. Effect of selection of solvents, extraction time, stirring rate and salt concentration on the extraction efficiency of OCPs by LPME/DGM-HF. In the table, the peak area for all the measured parameters has been reduced to "X 10^5". In the effect of salt concentration, the values inside the parentheses represent the percentage of the salt concentration (NaCl) added into the sample solution.

Pesticide	selection of solvents	extraction time	stirring rate	salt concentration
Aldrin	32.94 (toluene) 6.44 (hexane) 4.59 (iso-octane) 2.11 (n-octanol)	1.63 (1min) 2.86 (5min) 6.74 (15min) 1.73 (45min)	1.17 (static) 1.32 (60rpm) 1.34 (480rpm) 1.37 (840rpm) 2.39 (1200rpm)	14.90 (0%) 8.12 (2%) 2.88 (5%) 1.22 (20%)
Dieldrin	54.77 (toluene) 0.89 (hexane) 0.73 (iso-octane) 3.08 (n-octanol)	2.69 (1min) 8.51 (5min) 10.82 (15min) 6.78 (45min)	0.31 (static) 1.03 (60rpm) 2.27 (480rpm) 2.46 (840rpm) 3.37 (1200rpm)	13.06 (0%) 9.06 (2%) 1.16 (5%) 2.01 (20%)
2,4´-DDD	195.12 (toluene) 51.78 (hexane) 92.95 (iso-octane) 2.36 (n-octanol)	21.83 (1min) 27.97 (5min) 28.02 (15min) 15.17 (45min)	1.41 (static) 7.83 (60rpm) 3.21 (480rpm) 9.31 (840rpm) 12.12 (1200rpm)	42.80 (0%) 42.82 (2%) 11.64 (5%) 4.04 (20%)
4,4´-DDD	242.43 (toluene) 28.36 (hexane) 105.42 (iso-octane) 10.38 (n-octanol)	20.47 (1min) 26.03 (5min) 28.77 (15min) 15.68 (45min)	4.02 (static) 7.51 (60rpm) 9.43 (480rpm) 8.95 (840rpm) 11.46 (1200rpm)	57.31 (0%) 30.22 (2%) 3.45 (5%) 0.21 (20%)
4,4´-DDT	116.63 (toluene) 22.26 (hexane) 59.21 (iso-octane) 5.01 (n-octanol)	14.21 (1min) 14.34 (5min) 17.83 (15min) 8.92 (45min)	1.58 (static) 6.77 (60rpm) 6.71 (480rpm) 6.39 (840rpm) 7.79 (1200rpm)	50.36 (0%) 19.14 (2%) 8.24 (5%) 14.11 (20%)

Table 3. Linearity for the analysis of OCPs by the LPME/DGM-HF method.

Pesticide	Correlation coefficient	Linearity (µg/l)
Aldrin	0.938	1.0-500
Deldrin	0.932	1.0-500
2,4´-DDD	0.946	1.0-500
4,4´-DDD	0.944	1.0-500
4,4´-DDT	0.935	1.0-500

An application of electrospray ionization tandem mass spectrometry to probe the ion pair interaction of Ca^{+2}/Mg^{+2}/Zn^{+2} and Cl^{-} with gramicidin A

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Abstract

Gramicidin (Gr) has been demonstrated to interact with divalent salts ($CaCl_2$, $MgCl_2$, and $ZnCl_2$) by using electrospray ionization mass spectrometry (ESI/MS). The interaction of Val-GrA and Ile-GrA with divalent salts exhibited two kinds of complexes in ESI-mass spectrum, which are identified as $(Val\ or\ Ile\text{-}GrA+Ca)^+$ and $(Val\ or\ Ile\text{-}GrA+XCl)^+$ where X is Ca or Mg or Zn. All these ions were subjected to collisionally activated dissociation (CAD). MS/MS of $(Val\ or\ Ile\text{-}GrA+Ca)^+$ resulted in the loss of Ca from gramicidin. MS/MS and MS^3 of $(Val\ or\ Ile\text{-}GrA+XCl)^+$ resulted in the elimination of chloride and cation respectively. The results suggest that divalent cation along with chloride as ion pair could interact with gramicidin and chloride interaction is stronger in the presence of Ca relative to Mg and Zn. Further-more, the interaction of Ca with gramicidin is direct evidence for the proposal of a physical basis for the messenger role of Ca (Urry et al., *J. Biol. Chem.*1982, 257:6659-6661).

Introduction

Gramicidin is a small peptide acts as antibiotics[1,2] by disrupting the ionic balance inside the microbial cells. It consists of 15 alternating D- and L-amino acid residues and blocked at both ends with formyl and ethanolamine groups, thus can not form a zwitterion and shows no charge at any pH. The primary structure[3] of gramicidin is: formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Xxx-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine, where Xxx is Trp in GrA, Phe in GrB, Tyr in GrC. GrA referred as Ile-GrA, in-case Val at position one is replaced by Ile in the peptide[4]. Most of the amino acids present in gramicidin are hydrophobic and alternative arrangement of L and D amino acids help to form a helical dimer[5-7] (channel) with

hydrophobic exterior and hydrophilic interior in lipid bilayers. Divalent and monovalent cations bind with aromatic amino acids of the peptide and this cationization is due to the non-covalent interactions between cation and neutral peptide[8].

ESI/MS is a soft ionization method and therefore has been used widely to study the non-covalent interactions of metal ions with oligonucleotides and DNA[9], peptides and proteins[10], and other biomolecules[11,12]. These studies include different metal ions interaction with myoglobin[13], zinc interaction with adenylate kinase[14] and copper interaction with amyloid precursor protein fragment[15] in gas phase. Significantly the comparison of metallothionein protein interactions with zinc and cadmium ions in the solution and gas phase suggested that protein conformation retained in gas phase[16]. In-addition, Hu and coworkers[17] were demonstrated the application of ESI/MS to study the stoichiometry of non-covalent complexes of calcium and four calcium-binding proteins: α-lactalbumin, bovine calmodulin, rabbit parvalbumin and human stromelysin catalytic domain. Information regarding non-covalent complexes was easily obtained from mass spectra and also the solution and gas phase interactions were comparable, made ESI/MS a powerful tool for the evaluation of peptide-metal complexes. Previously, the conformational changes of gramicidin A in different organic solvents were demonstrated by means of ESI/MS[18,19]. Also the ESI/MS study[19] on interaction of gramicidin with monovalent cations revealed their binding strengths: $Na^+ > K^+ > Rb^+ > Cs^+$. The interaction of divalent cations has yet to be studied using mass spectrometry. Among the divalent metallic ions, Ca is well known for being play messenger role in biological system and regulates several intracellular events. This regulation of intracellular events involves the binding of Ca with peptide and proteins. Extensive studies have been made on the

interactions of various divalent and monovalent cations with gramicidin using NMR and CD spectroscopic techniques[6,7,20-23]. These studies demonstrated that Ca has the ability to bind with gramicidin and inhibit the transport of monovalent cations[24-26]. The same studies also suggested that chloride may interact with gramicidin in the presence of divalent cation where as the nature of the physical interaction of calcium along with chloride is remained unclear.

It is well known that ESI-mass spectrometry is a rapidly emerging method and complementary to other biophysical techniques like NMR and CD techniques for the study of peptide and proteins interaction with metal ions. Therefore it is of interest to study the interaction of selected divalent metal chlorides ($CaCl_2$, $MgCl_2$, and $ZnCl_2$) with gramicidin and compare their binding strengths using ESI/MS. These divalent chloride salts were selected based on their metal-ionic radius and biological significance.

Experimental

Materials. Gramicidin D was purchased from Sigma-Aldrich (St.Louis, MO, USA). Commercial preparation of Gramicidin D is a mixture of three components known as gramicidin A, B, and C, consisting of about 80, 5, 15% respectively. Salts ($CaCl_2$, $MgCl_2$ and $ZnCl_2$) were purchased from Aldrich Chem. Co. (Milw., WI, USA) where as solvent *n*-propanol (HPLC grade) was purchased from Sigma-Aldrich (Steinheim, Germany). All these chemicals utilized directly with-out any further purification.

Instrumentation. ESI/MS experiments were carried-out with a Finnigan MAT ion trap mass spectrometer (Finnigan LCQ-Advantage, San Jose, CA, USA). Propanol was used as a solvent in this study and similar results were also observed with ethanol but the spectra observed were little bit noiser. The solution mixture of 20 μM gramicidin and

metal ions with a concentration of 0.1 mM – 1.2 mM was introduced into the ion source. $CaCl_2$ and $MgCl_2$ were used up to 1.2 mM whereas $ZnCl_2$ with < 0.4 mM was used. Using salts above these concentrations made the signals corresponding to the divalent salt and gramicidin adducts lesser intensity with noisy. Gramicidin was incubated with salts for 10 min at 25±2 ^{o}C prior to the experimental measurements. The spectral analysis was carried-out in a positive ion mode with a solvent flow rate of 20 μL/min. The tip was held at 5000V and the temperature of the spray chamber was 250 ^{o}C. The Tube Lens Offset Voltage of 80V and Capillary Voltage of 10V were used in order to obtain good spectra. Data was obtained across the mass to charge range (m/z) 1750 – 2000. Each spectrum is average of 15 individual scans. All these CAD studies were performed using 0.3 mM of corresponding salt with 20 μM gramicidin.CAD was performed on selected precursor ions as described previously[27]. The collision energy used for dissociation of complexes is mentioned as percentage of collision voltage, 5V.

Results and Discussion

In ESI-mass spectrum (Fig. 1), both Val-GrA and Ile-GrA were observed as protonated and sodiated complexes. The m/z of 1883 and 1897 were identified as protonated complexes of gramicidin A (here-after referred as gramicidin), $[Val\text{-}GrA+H]^+$ and $[Ile\text{-}GrA+H]^+$ where as m/z of 1905 and 1919 were sodiated complexes, $[Val\text{-}GrA+Na]^+$ and $[Ile\text{-}GrA+Na]^+$. In-addition to these complexes, sodiated form of Val-GrB was also observed at m/z, 1866 and was identified as $[Val\text{-}GrB+Na]^+$, but the protonated form of this complex was not observed due to its less quantity in gramicidin D. The above-mentioned sodiated complexes were observed with-out any addition of sodium salts. These complexes arise due to the ubiquitous nature of sodium as reported

previously in the ESI/MS study of calcium binding proteins[17]. The addition of $CaCl_2$ to gramicidin D resulted in the formation of (Val or Ile-GrA+Ca)$^+$ and (Val or Ile-GrA+CaCl)$^+$. The singly charged (Val or Ile-GrA+Ca)$^+$ complexes were confirmed from their isotopic peaks. The isotopic peaks were clearly separated by m/z of 1 unit (Fig. 2B), suggest that these complexes are singly charged[18,19]. Similarly, complexes like (Val-GrA+2Na)$^+$ were observed in the ESI/MS study of the conformational changes of gramicidin[18].

The CAD results of the complexes of gramicidin and $CaCl_2$ interaction were showed in Table 1. The MS/MS of (Val or Ile-GrA+Ca)$^+$ leads to the elimination of Ca and thus the protonated complexes of Val-GrA and Ile-GrA were observed in the product mass spectrum. The MS/MS of (Val or Ile-GrA+CaCl)$^+$ leads to the elimination of chloride and the complex of (Val or Ile-GrA+Ca)$^+$ was observed in the product mass spectrum. The MS^3 of (Val or Ile-GrA+Ca)$^+$ is similar to the MS/MS study of this complex observed in the ESI-mass spectrum. The ion pair interaction of chloride and metal ion with gramicidin was also observed in the presence of Mg and Zn salts (Fig. 3 and 4). But the peaks corresponding to Mg and gramicidin interaction were not observed where as Zn and gramicidin complexes were observed with relatively low abundance. This suggests that the interaction of Zn alone (not as ion pair) with gramicidin is weaker relative to Ca interaction where as Mg is unable to interact with gramicidin. Previous study performed with CD spectroscopy also revealed that Mg and Zn ions interact weakly with gramicidin[22]. The CAD of the complexes of gramicidin and $MgCl_2$ or $ZnCl_2$ interaction exhibited similar product ions (Table 1) as observed with the complexes of gramicidin and $CaCl_2$.

The effect of $CaCl_2$ concentration on its interaction with gramicidin has also been studied. At low concentrations (up to 0.4 mM) Ca is able to interact with gramicidin as ion pair along with chloride as we observed the peaks corresponding to gramicidin and CaCl complex with higher abundance than Ca bound gramicidin complex (Fig. 2A). At 0.5 mM and above concentrations, Ca bound gramicidin complex was observed with higher abundance relative to CaCl bound gramicidin complex (Fig. 2B). This could be due to the partial alterations in gramicidin structure, which might be resulted in the dissociation of chloride. Similarly, the increase in the concentration of $MgCl_2$ and $ZnCl_2$ were decreased the abundance of their interaction with gramicidin and also not observed any changes in the abundance of complexes as observed the changes in presence of various concentrations of $CaCl_2$. From the data it is clear that anion could bind to gramicidin along with divalent cation at various concentrations, as we did not observe any interaction of gramicidin with chloride only. The experiments performed in the presence of 1 mM NaCl and 1 mM KCl did not exhibited any chloride bound gramicidin complexes (data not showed), indicates that divalent cations (Ca^{2+}, Mg^{2+}, and Zn^{2+}) facilitate the environment for chloride interaction with gramicidin as ion pair.

Further-more, chloride ion binding strength with gramicidin was studied by means of CAD. The collision energy required to dissociate the chloride bound metal-gramicidin in the presence of $CaCl_2$, $ZnCl_2$, and $MgCl_2$ were 25±0.4, 22.5±0.4, and 22±0.6 % respectively. These results indicate that chloride could bind to gramicidin stronger in presence of Ca relative to Zn and Mg. Similarly it was demonstrated that in solution phase, chloride could bind to gramicidin weakly in presence of Mg relative to Ca[20]. The CAD was also performed on metal bound gramicidin complexes. The collision energy

required for dissociation of Ca and Zn from gramicidin was 36 and 30 % respectively. This revealed that Ca could bind stronger with gramicidin relative to Zn and also suggested that Ca and Zn could bind with gramicidin relatively stronger than chloride. The binding strengths depend on ionic radius of the divalent cation: ionic radius of the divalent cations close to Ca^{2+} (Pauling ionic radius, r = 0.99 A^{o}) is suitable to interact with gramicidin as suggested previously[22]. These results are inconsistent with those results observed during the CD spectroscopic study of gramicidin interaction with multivalent cations[22]. From our CAD studies, we propose that the binding strengths of ion pairs of salts with gramicidin is as follows: $CaCl_2$>$ZnCl_2$>$MgCl_2$.

Conclusion

ESI/MS has been extensively used to determine the molecular weight and primary structure of peptides and proteins. The present study demonstrated that ESI/MS is useful technique to study the ion pair interactions in neutral peptides like gramicidin. The results permit us to assign the binding of divalent cation along with chloride as ion pair with gramicidin for the first time to the best of our knowledge. This study also demonstrated that Ca could bind with gramicidin in a relatively stable form when compared with Mg and Zn. Further-more, these results might serve as baseline for future studies on the role of anion effect besides the cation ability to inhibit the transport of monovalent cations through gramicidin helix and to study the effects of chloride binding on the messenger role of calcium ions.

Acknowledgements

Financial support from National Science Council of Taiwan and Department of Chemistry are greatly acknowledged.

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Figure captions

Fig.1

ESI-mass spectrum of protonated and sodiated complexes of Val and Ile-GrA. The sodiated complex of Val-GrB was also showed.

Fig. 2A and 2B

ESI-mass spectra of gramicidin and $CaCl_2$ interaction. Spectrum showed in Fig. 2A was obtained with 0.3 mM of $CaCl_2$ where as the spectrum showed in Fig. 2B was obtained with 1 mM $CaCl_2$. Inset shows the isotopic peaks of the complexes separated by m/z of 1 unit. Other details were given in the experimental section.

Fig.3

ESI-mass spectrum of gramicidin and $MgCl_2$ interaction. The spectrum was obtained with 0.3 mM concentration of $MgCl_2$. Other details were given in experimental section.

Fig.4

ESI-mass spectrum of gramicidin and $ZnCl_2$ interaction. The spectrum was obtained with 0.3 mM concentration of $ZnCl_2$. Other details were given in experimental section.

Table 1. Shows the ions observed in the ESI-mass spectrum and their product ions generated due to the collision activated dissociation.

Ions observed in the ESI-mass spectrum	**Corresponding product ions (MS^n)**
$[Val\ or\ Ile\text{-}GrA+Ca]^+$	$[Val\ or\ Ile\text{-}GrA+H]^+$ (MS^2)
$[Val\ or\ Ile\text{-}GrA+CaCl]^+$	$[Val\ or\ Ile\text{-}GrA+Ca]^+$ (MS^2), $[Val\ or\ Ile\text{-}GrA+H]^+$ (MS^3)
$[Val\ or\ Ile\text{-}GrA+MgCl]^+$	$[Val\ or\ Ile\text{-}GrA+H]^+$ (MS^2)
$[Val\ or\ Ile\text{-}GrA+Zn]^+$	$[Val\ or\ Ile\text{-}GrA+H]^+$ (MS^2)
$[Val\ or\ Ile\text{-}GrA+ZnCl]^+$	$[Val\ or\ Ile\text{-}GrA+Zn]^+$ (MS^2), $[Val\ or\ Ile\text{-}GrA+H]^+$ (MS^3)

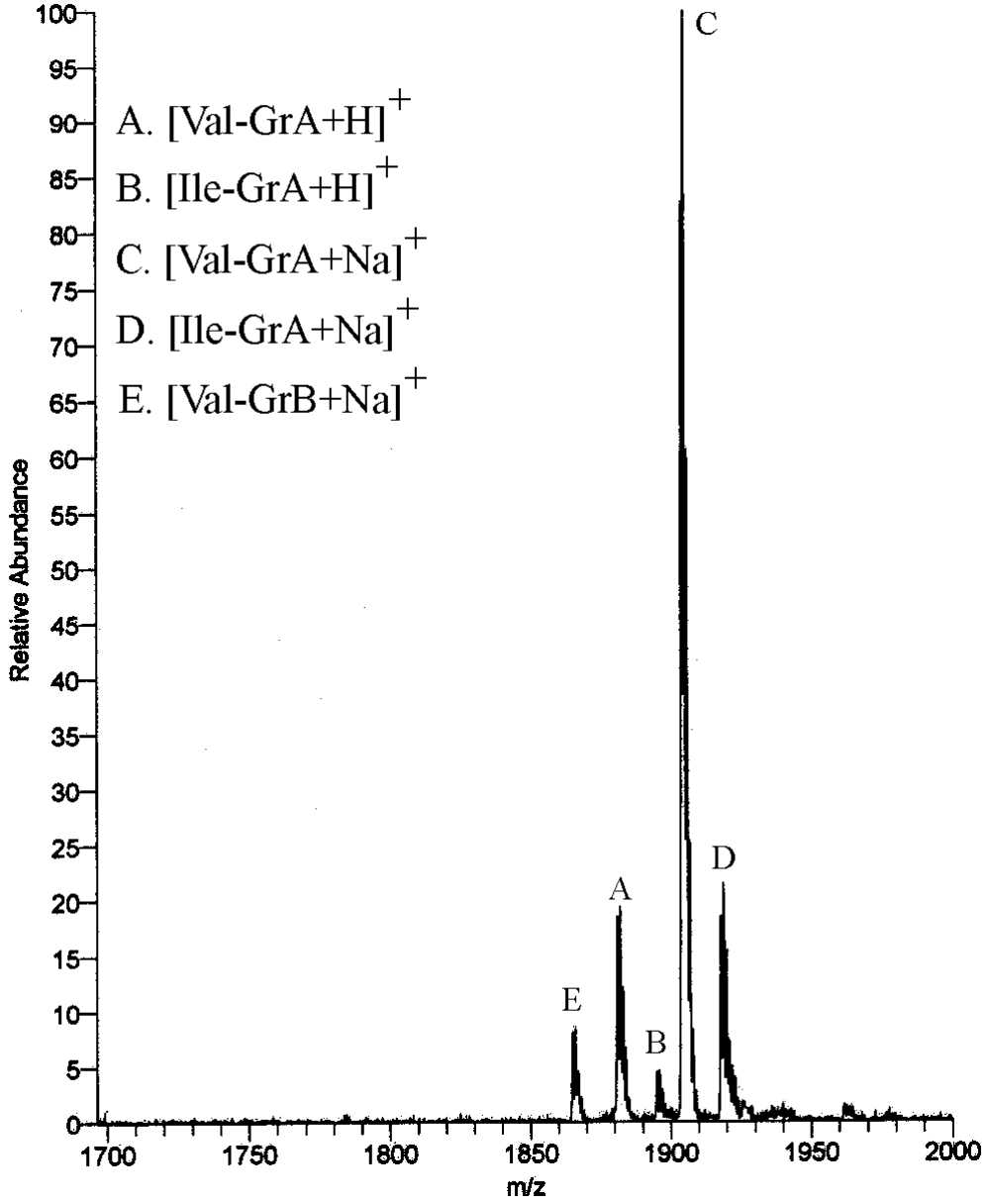
A. [Val-GrA+H]+
B. [Ile-GrA+H]+
C. [Val-GrA+Na]+
D. [Ile-GrA+Na]+
E. [Val-GrB+Na]+
Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1700
1750
1800
1850
1900
1950
2000
A
B
C
D
E

Fig 1

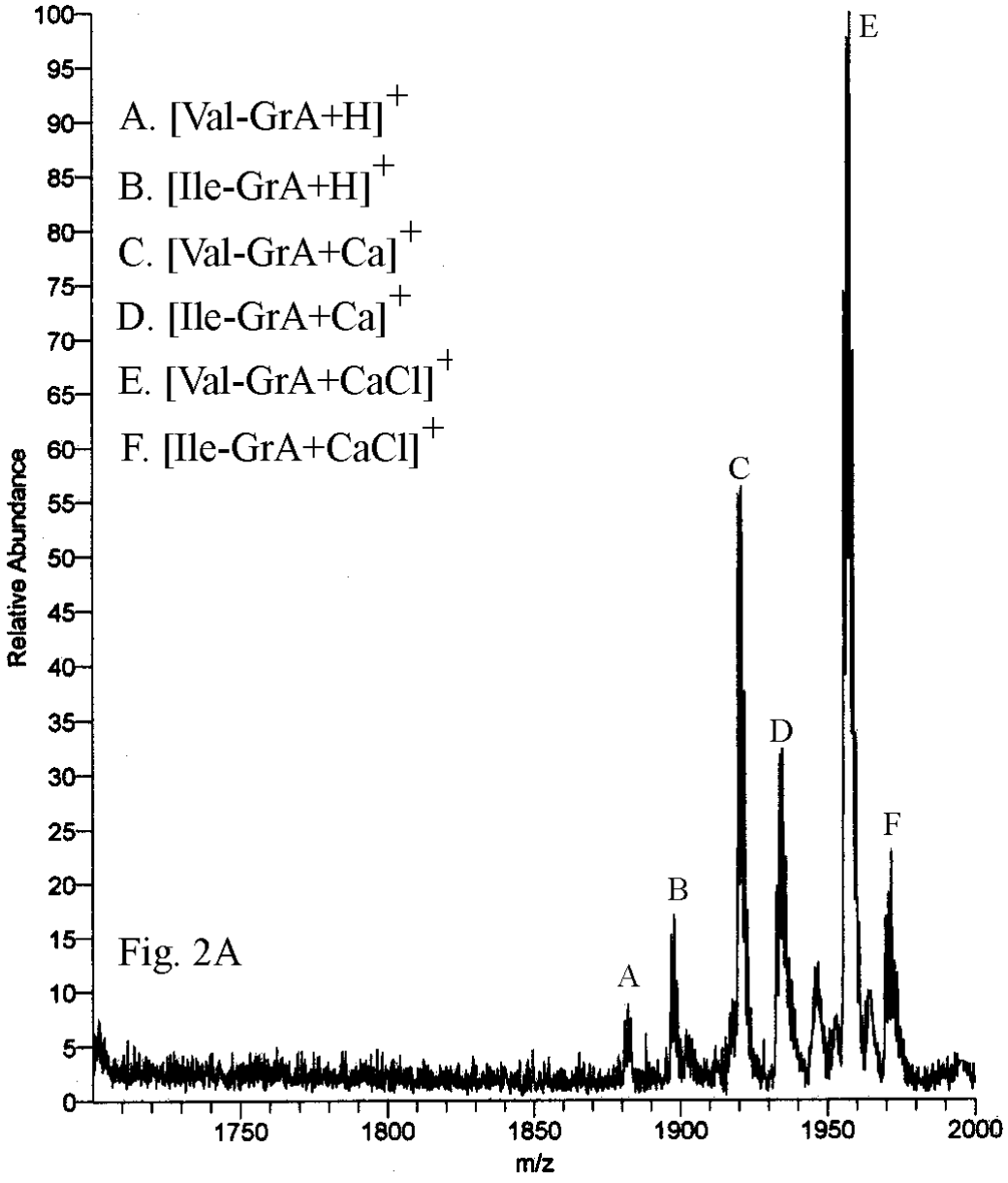
A. [Val-GrA+H]+
B. [Ile-GrA+H]+
C. [Val-GrA+Ca]+
D. [Ile-GrA+Ca]+
E. [Val-GrA+CaCl]+
F. [Ile-GrA+CaCl]+
Relative Abundance
m/z
A
B
C
D
E
F
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1750
1800
1850
1900
1950
2000

Fig. 2A

Fig. 2A

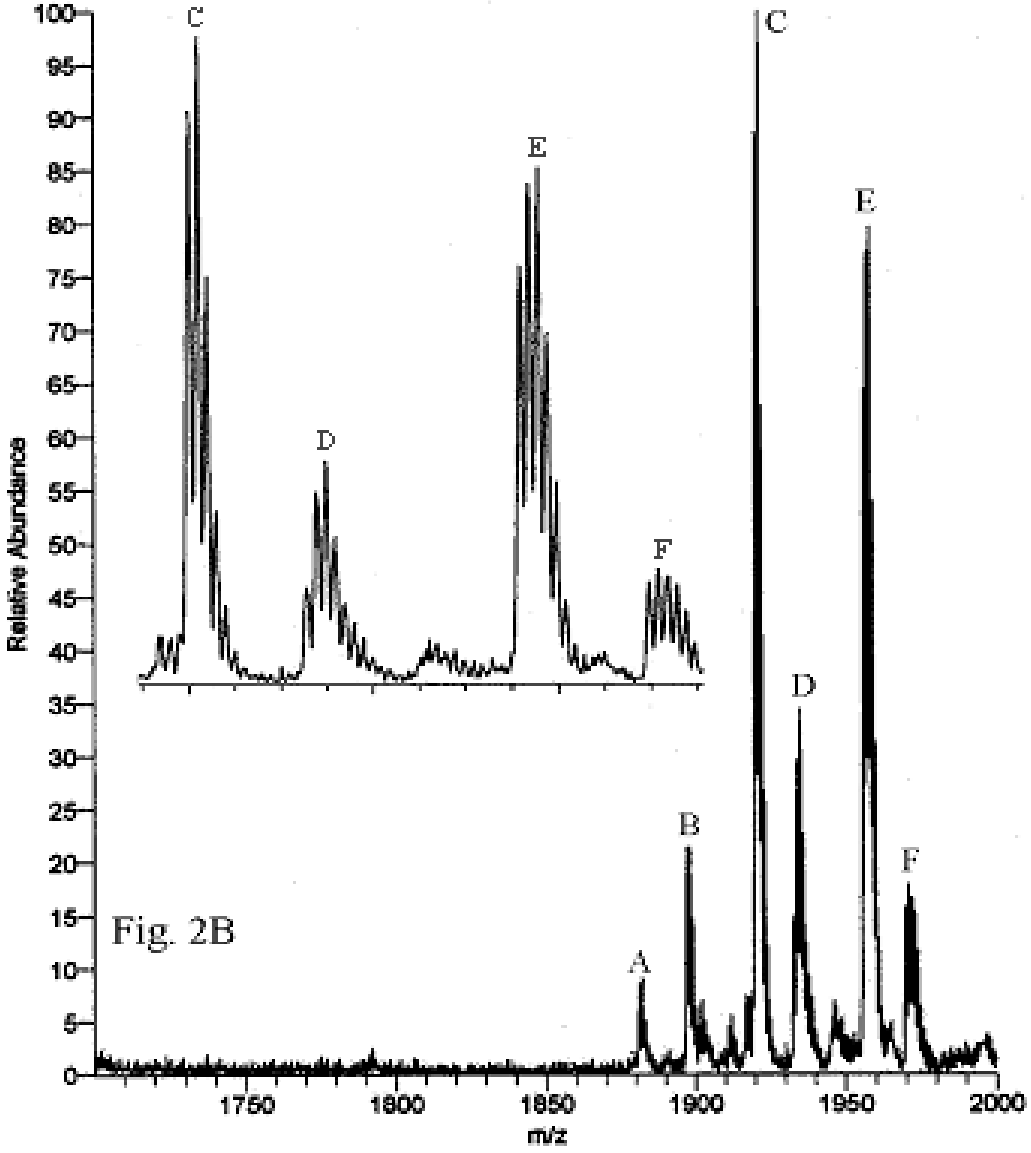

Fig. 2B

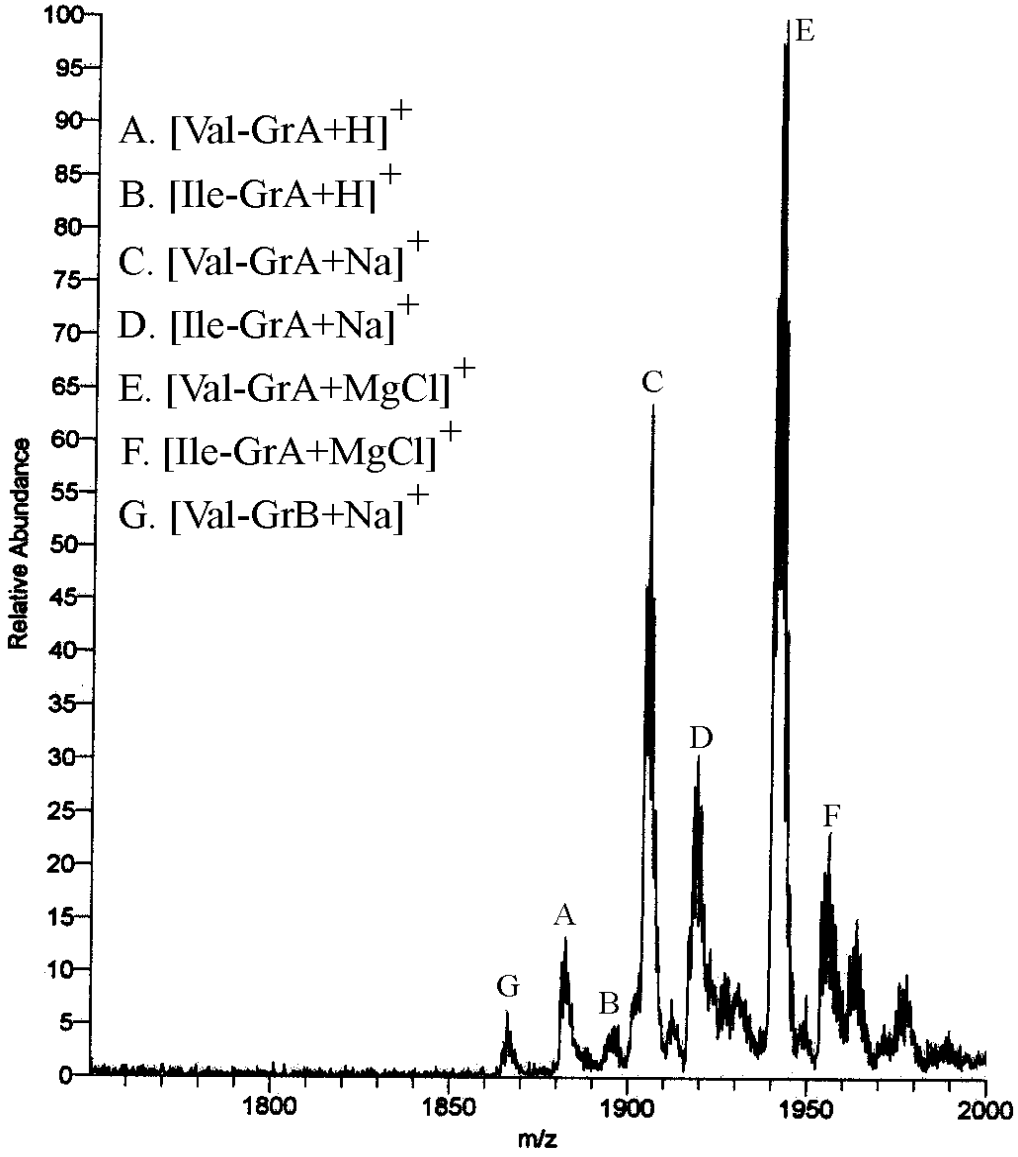
A. [Val-GrA+H]+
B. [Ile-GrA+H]+
C. [Val-GrA+Na]+
D. [Ile-GrA+Na]+
E. [Val-GrA+MgCl]+
F. [Ile-GrA+MgCl]+
G. [Val-GrB+Na]+
Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1800
1850
1900
1950
2000
A
B
C
D
E
F
G

Fig. 3

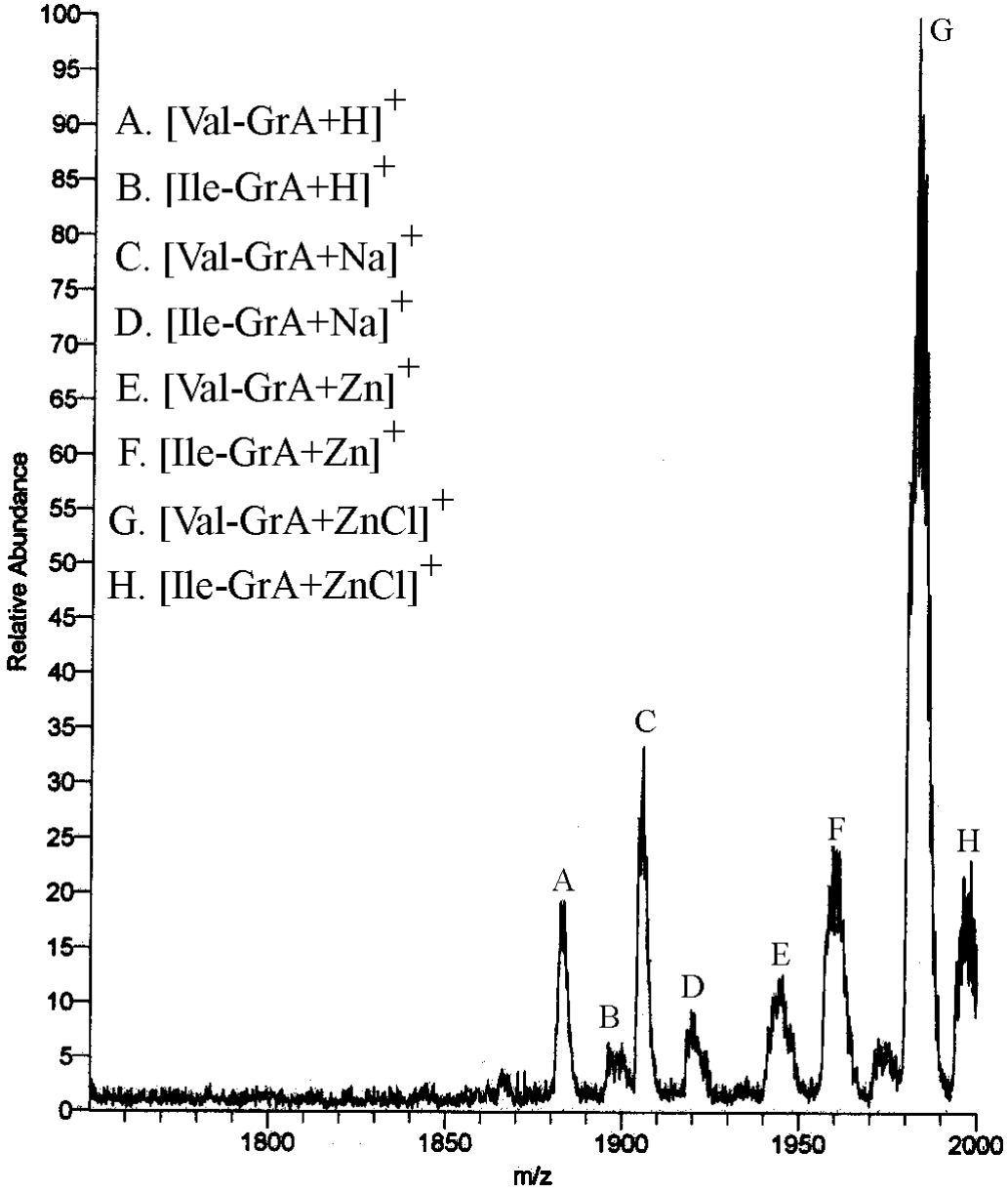
A. [Val-GrA+H]+
B. [Ile-GrA+H]+
C. [Val-GrA+Na]+
D. [Ile-GrA+Na]+
E. [Val-GrA+Zn]+
F. [Ile-GrA+Zn]+
G. [Val-GrA+ZnCl]+
H. [Ile-GrA+ZnCl]+
Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1800
1850
1900
1950
2000
A
B
C
D
E
F
G
H

Fig. 4