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Determination of Sildenafil Citrate Adulterated in a Dietary Supplement Capsule by LC/MS/MS

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ABSTRACT

A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was used to identify and quantify an adulterated substance, in a dietary supplement capsule. The capsule is claimed to be an extract of animal organs and traditional Chinese herbs and indicated for enhancing sexual activity. At first, the sample was extracted with 50% v/v methanol in water and the extract was injected directly into the LC/MS/MS with no separation and a full scan of positive ion eletrospray (ESI^+) analysis was performed. The full scan mass spectrum was compared with the NLFD3/LM library created by Division 3rd of National Laboratories of Foods and Drugs for LC/MS/MS analysis. A sildenafil specific mass spectrum was matched indicating that the capsule may have been adulterated with sildenafil, the active ingredient of VIAGRA®. Furthermore, sildenafil was confirmed by carrying out a daughter ion scan and a parent ion scan. The daughter ion spectrum was compared with the NLFD3/LM library. A negative ion eletrospray (ESI^-) analysis was performed for confirming the citrate salt in the capsule. The results indicated that the adulterated ingredient was sildenafil citrate.

For quantitative analysis of the sildenafil citrate, multiple reaction monitoring (MRM) was performed. Pirenzepine 2HCl was used as the internal standard. A series of standards solution and sample solution with internal standard were analyzed by LC/ MS/MS. The analytical condition is as following: column: Cosmosil 5C18-AR, 4.6 x 150 mm; mobile phase:methanol:acetonitrile:1% acetic acid (25:17:58); capillary voltage: 3 kV;cone voltage: 80 V; collision energy: 25 eV; source temperature: 120˚C; desolvation temperature: 350˚C. The detection limit was 40 ng/mL.

An excellent calibration curve of sildenafil citrate was obtained with the γ^2 correlation coefficient of 0.9977. The RSDs of interday and intraday were between 7.56~7.75% and 9.09~11.25%, respectively. The analytical strategy and method is suitable for identification and measurement of sildenafil citrate adulterated in capsule. Amount of sildenafil citrate in the capsule was determined as 42.8 mg/capsule.

Key words: LC/MS/MS, sildenafil citrate, ESI., MRM, pirenzepine, daughter ion scan, parent ion scan

INTRODUCTION

There are numerous synthetic medicines in the world and it is very difficult to perform systematic analyses to reveal those compounds if sample mixtures contain unknown ingredients without any information. It is even more difficult to screen out unknown compounds adulterated in natural products such as traditional Chinese medicine (TCM), due to the interference caused by the contents of natural compounds during analysis. Thin Layer Chromatography(TLC) is normally used for separating the target compounds in cases such as the above and to detect the UV spectrum[1,2] of each TLC spots. However, ingredients with analogous molecular structure are of similar UV spectrum and may cause confusion for inspection purpose. For many compounds with weak UV absorption, it is difficult to identify them using TLC/UV methods. In recent years, the GC/MS analysis method has gradually become popular. By library searching in mass spectrum and comparing the peak of retention time with library data provided by GC/MS, a more precise inspection result can be acquired[3]. However, there are limitations to GC/MS analysis. For example, GC/MS cannot be adopted in analyzing compounds of high molecule weight, heat instability and difficulty to ionize. Although HPLC has a good separation effect, the peaks presented could not provide any direct information about the unknown ingredients adulterated in the sample. By using LC/MS to compensate the insufficiency of GC/MS and HPLC, and applying Tandem mass(LC/MS/MS) to quantify and qualify the unknown ingredients, the process is faster and the result is more precise. The present study examined samples of food supplement capsule made from animal organ extracts, indicated for enhancing sexuality. Since the claimed effect was quite extraordinary, a consumer submitted the sample to the NLFD for inspection. We used LC/MS/MS and by comparing the MS1 mass spectrum with a library search, found traces of possible adulteration. After performing the daughter ion scan, we were able to identify the adulterants. No complicated separation and purification were required during the analysis processes.

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MATERIALS AND METHODS

I. Materials

(I) Reference Standard: sildenafil citrate provided for registration and inspection on ViagraR by Pfizer Pharmaceutical (Taiwan); pirenzepine 2HCl from Sigma (St. Louis, USA).

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Table 1. Parameters of LC/MS screening scan methods by direct sampling for identification of unknown sample

Mode / Parameters	E S I$^+$	E S I$^-$
Carrier Flow (mL/min)	50%MeOH ，0.1	60%MeOH ，0.1
Capillary (kV)	3.0	3.0
Cone (V)	80	60
Collision (eV) ：Argon	5	5
Source Temp (˚C)	120	120
Desolvation Temp (˚C)	300	350

(II) Reagents: LC grade methanol from Labscan (Ireland), acetonitrile from BDH laboratory (England) and acetic acid from E-Merck (Germany).

(III) Model TCM sample: Huan Shao Tan from Shen Chung Pharmaceutical, Taiwan.

(IV) Sample: sample is pink and cream color capsule, containing dark brown powder with average weight at 375 mg/capsule.

(V) Filter: 0.45 μm Millipore filter film from Gelman Sciences (U.S.A.).

II. *Equipment/Instruments*

(I) Tandem mass: Micromass Quattro Ultima LC/MS/MS (England).

(II) HPLC: Waters 2690 Alliance LC & 996 PDA with Automatic Liquid Sampler and Injector (U.S.A.).

(III) Software: MassLynx NT Quattro Data Acquisition (England).

(IV) Mass spectrum data library: NLFD3/LM Library created by Division 3rd of National Laboratories of Foods and Drugs for LC/MS/MS analysis.

III. *Identification of Unknown Ingredients in the Sample*[4~11]

(I) Preparation of sample solution: Accurately weighed sample powder equivalent to one capsule, put in a 50 mL centrifugal tube and extracted with 30 mL of 50% v/v methanol. Shook in the ultrasonic shaker for 10 minutes and centrifuged under 3000 rpm for 10 minutes. Filtrated the supernatant through filter paper and extracted the residue three times. Collected the filtrate in a 100 mL volumetric flask and added solvent to the mark. Pipette 2 mL of the filtrate and diluted to 50 mL with solvent and filtered through Millipore filter to be used as sample solution.

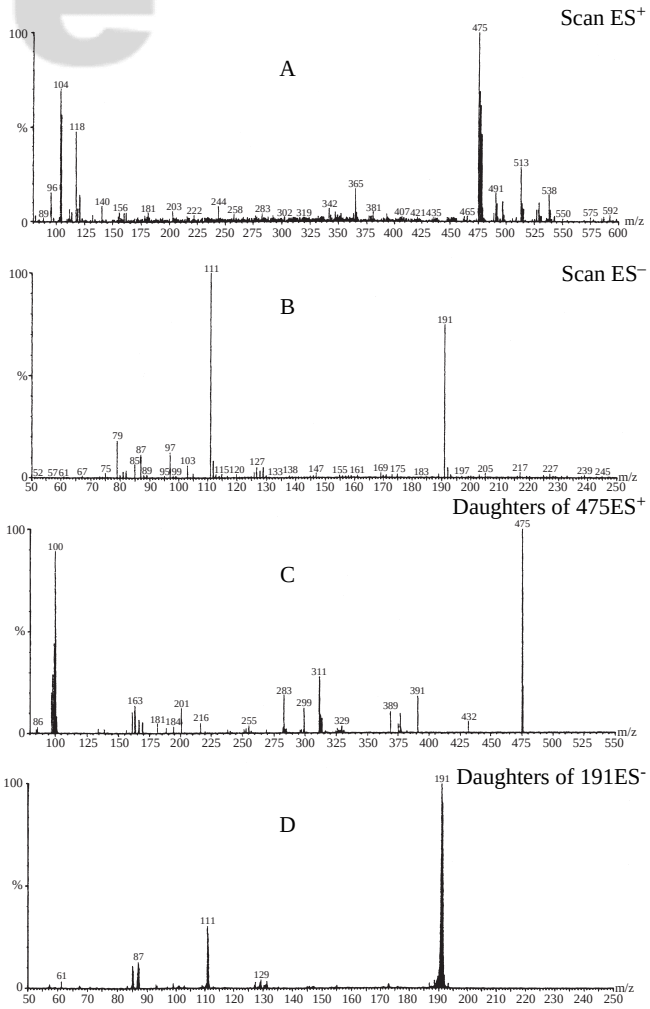

Figure 1. The MS1 mass spectrum (A&B) and daughter ion spectrum of sample solution (C&D).

(II) Screening unknown ingredients with MS1 scan: Directly injected 10 μL sample solution[5] from Divert/Injection valve, performed both positive and negative ion electrospray ionization (ESI$^+$ & ESI$^-$) MS1 scan mode based the analysis conditions on Table 1 and acquired a total ion current (TIC)

Table 2. Parameters of LC/MS/MS daughter ion and parent ion scan by direct sampling for identification of sildenafil citrate

Scan mode	E S I$^+$		E S I$^-$
Selected ion (m/z)	Daughter ion of 475	Parent ion of 100	Daughter ion of 191
Carrier Flow (mL/min)	50%MeOH ，0.1		60%MeOH ，0.1
Capillary (kV)	3.0		3.0
Cone (V)	80		80
Collision (eV): Argon	25		20
Source Temp (˚C)	120		120
Desolvation Temp (˚C)	300		350

chromatogram. After deducting the noise signal process, the mass spectrum (Figure 1A&B) was then compared with the NLFD3/LM library database.

(III) Reconfirming the ingredients by LS/MS/MS analysis: Directly injected 10 µL of the above sample solution to perform ESI^+ daughter ion scan (Table 2). The base peak of fragment (m/z 475) revealed in Figure 1 was selected and a daughter ion TIC chromatogram was acquired. After deducting the noise signal process, a mass spectrum was obtained (Figure 1C) and compared with the NLFD3/LM library database. Took daughter ion fragment m/z 100 to perform ESI^+ parent ion scan and acquired result as the above to confirm whether it was produced from m/z 475 ion fragment (Figure 2C).

(IV) Confirming organic salt: In order to confirm whether sildenafil was combined with citrate, m/z 191 ion fragment was selected to perform ESI^- daughter ion scan following the Table 2 conditions (Figure 1D). Compared the spectrum with the NLFD3/LM library database.

IV. *Quantitating the Content of Sildenafil Citrate in the Sample*[4~10]

(I) Preparation of standard solution and calibration curve: Accurately weighed sildenafil citrate reference standard and dissolved with 50% v/v methanol to make a series standard solution with concentrations at 1.76, 4.40, 8.80, 13.20, 17.60 µg/mL, each containing 1.28 µg/mL pirenzepine 2HCl internal standard solution. Based on the following HPLC/Photodiode array conditions, Multiple Reaction Monitoring (MRM) analysis was performed with the combined analysis condition in Table 2 and 3. Plotted the peak area ratio between sildenafil citrate reference standard and the internal standard as the Y axis, and the concentration of standard solution as the X axis to derive the calibration curve equation of sildenafil citrate Y= m*X + b., the relative coefficients were also calculated.

(II) Preparation of sample solution and quantitating the content of sildenafil citrate in the sample: Accurately weighed five portions of sample powder each equal to one capsule and placed in 50 mL centrifugal tube, respectively. Extracted and filtered as III-(I) procedures. Pipetted 2 mL of the filtrate and 2 mL of 32 µg/mL pirenzepine 2HCl internal standard solution, and added the solvent to make exact 50 mL. Filtered through Millipore filter, used the filtrate as the sample solution. Performed MRM analysis as described previously to determine the content of sildenafil citrate.

(III) *Analysis Condition of HPLC and Photodiode Array* [11]*:*

1. Column: Cosmosil 5C18-AR, 4.6 x 150 mm.
2. Mobile phase: CH_3OH: CH_3CN: 1%HOAc (25: 17: 58).
3. Flow rate: 0.5 mL/min (Split ratio: 2/5).
4. Injection volume: 10 µL.
5. Photodiode array: Scan range: 200~350 nm.
6. Running time: 15 min.

V. *Evaluation of Recovery*

Took Huan Shao Tan model sample powder, adulterated sildenafil citrate reference standard and mixed thoroughly to make 10, 5, 3% w/w sildenafil citrate content in each sample (label as A, B, C adulterated samples). Accurately weighed 100 mg of each group in 50 mL centrifugal tube. Prepared the test solution by following the steps of III-(I), and then quantitated the content of sildenafil citrate by following the above method and calculated the recovery.

VI. *Evaluation of Precision*

Prepared 2.64 µg/mL and 13.20 µg/mL of sildenafil citrate standard solution, performed intra- and inter-day analysis each for five times by following the MRM quantitative methods. Calculated the results by calibration equation derived from previous experiment and calculated the standard deviation (SD) and relative standard deviation(RSD).

VII. *Detection Limit Test*

Took 1.76 µg/mL standard solution, diluted with 50% v/v methanol to 1, 5, 10, 25 and 50 times. Inspected with the MRM quantitative method mentioned previously and acquired individual signal/noise ratio (S/N) at the injection volume of 10 µL. The detection limit was defined when the S/N ratio was 3.

RESULTS AND DISCUSSION

I. *Source of NLFD3/LM Library Database & Establishment*

The LC/MS/MS analysis technology was found to be very useful. For one compound, setting the parameter on the instrument under different ionization mode, capillary voltage, collision energy, cone voltage and source temperature would result in different mass spectra. The analytic parameters of LC/MS/MS are more complex than these of GC/MS. GC/MS has already had library databases Wiley, PMW and

Table 3. Parameters of MRM set for quantitative analysis of sildenafil citrate

Parameters / Ingredients	Parent ion (m/z)	Daughter ion (m/z)	Dwell (sec)	Cone (Volt)	Collision energy (eV)	monitored time interval （min）
pirenzepine 2HCl	352	113	0.08	60	20	1~6
sildenafil citrate	475	100	0.08	80	25	6~15

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NIST ready to use, but not LC/MS/MS. In order to analyze unknown ingredients by LC/MS/MS, the important thing is to establish a mass spectra library database for every ingredient. The mass spectra of sildenafil, citric acid, and pirenzepine were established by NLFD3/LM with the methods described as follows:

(I) Standard Solution: Took the standard of the target ingredients, using appropriate solvents such as water, methanol or 1% acetic acid etc to dissolve into a standard solution of 5 μg/mL concentration.

(II) Selection of ionization mode: Selected ESI^+, ESI^-, $APCI^+$ or $APCI^-$ ionization mode based on the nature of compounds, and tuned the instrument by altering some of the instrument analytic parameters with the standard solutions in order to acquire an obvious $(M+H)^+$ or $(M-H)^-$ ion fragment and some cleaved fragments.

(III) Acquisition of MS1 mass spectrum: Adopted the parameters from the above tune, injected 10 μL standard solution from the divert injection valve, using 50% methanol as mobile phase to perform MSI scan and acquired the TIC chromatogram. MS 1 mass spectra was acquired after deducting the noise signal processes. If the same spectrums were acquired after repeating the processes for three times, the spectra would then be included into NLFD3/LM library with ionization mode, capillary voltage, collision energy, cone voltage, molecular weight and molecular formula.

(IV) Acquisition of MS/MS mass spectrum: Selected target fragment of $(M+H)^+$ or $(M-H)^-$ in general and performed daughter ion scan with different collision energy until the daughter ion spectrum was obvious and stable. If the same mass spectrums were achieved after repeating the processes for three times, the mass spectrum would then be included into the NLFD3/LM library with ionization mode, capillary voltage, collision energy, cone voltage, molecular weight and molecular formula.

II. *Reason for Adopting LC/MS/MS Analysis*

When trying to screen out the unknown adulterants from TCM or food, the complexity of the natural ingredients would interfere with the results due to the enormous categories of the medications. The best method would be to adopt the MS analysis and by compare with the library database. In this study, the sample claimed to be a food capsule with content extracted from animal organs for enhancing sexuality. Since the product label did not specify the content detail, in order to screen out the ingredients, the GC/MS method was adopted but failed. This was because the sildenafil citrate could not be inspected under the routine GC/MS analysis conditions[13] and therefore, LC/MS/MS was adopted instead.

III. *Process of Screening Sildenafil Citrate*

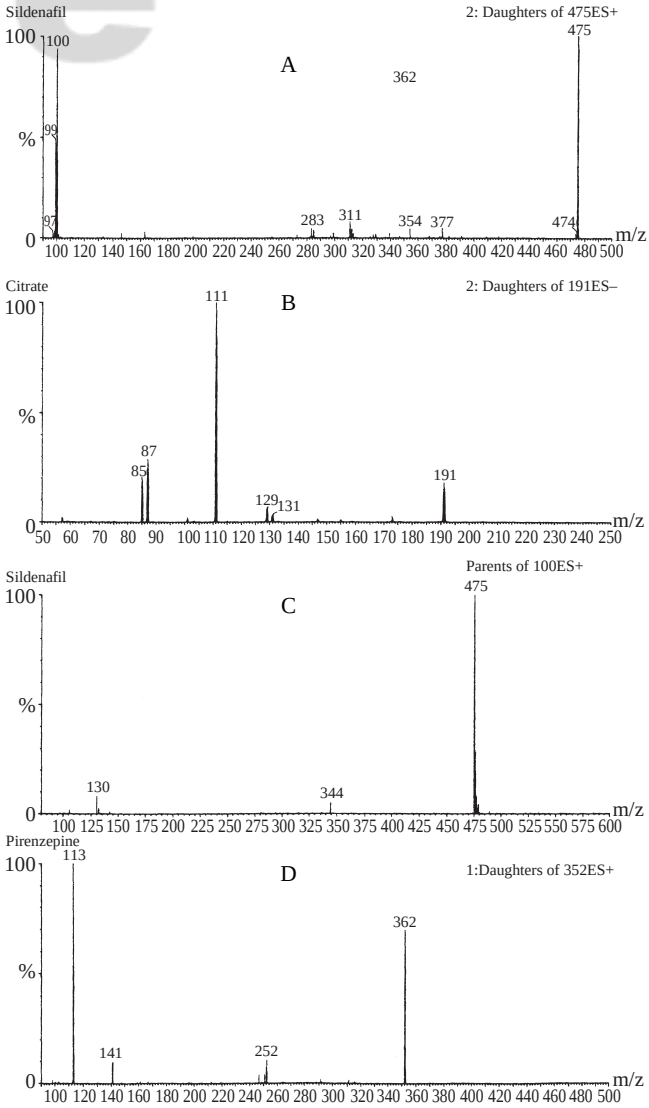

Figure 2. The daughter ion spectrum of sildenafil(A), citrate(B), pirenzepine(D) and parent ion spectrum of sildenafil(C).

After extracting with 50% methanol, the solution went through a MS 1 scan to acquire its spectrum (Figure 1A). The matching quality of this result was 91.8% compared to that of sildenafil as recorded in the NLFD3/LM library database. we therefore suspected the sample was adulterated with sildenafil citrate. We further performed a daughter ion scan on the fragment m/z 475 $(M+H)^+$ of sildenafil to generate a daughter ion mass spectrum (Figure 1C) and compared with the NLFD3/LM library database (Figure 2A). The matching quality was at 92.5%, which certified the adulterant was sildenafil. The result was further confirmed by performing a parent ion scan which proved that the m/z 100 daughter ion was cleaved from the m/z 475 parent ion (Figure 2C) without interference by other ingredients and could be used to set up the MRM quantitative parameter. The accurate result was achieved by using only the 50% methanol, without the complicated extraction and purification process. The analytical time required was shortened as well.

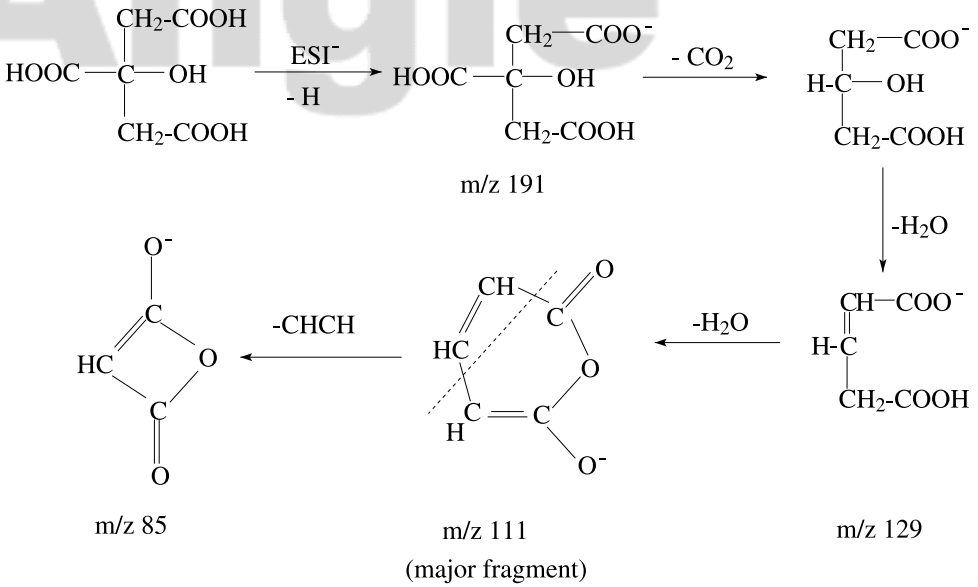

Figure 3. Fragmentation of citric acid by ESI⁻ analysis.

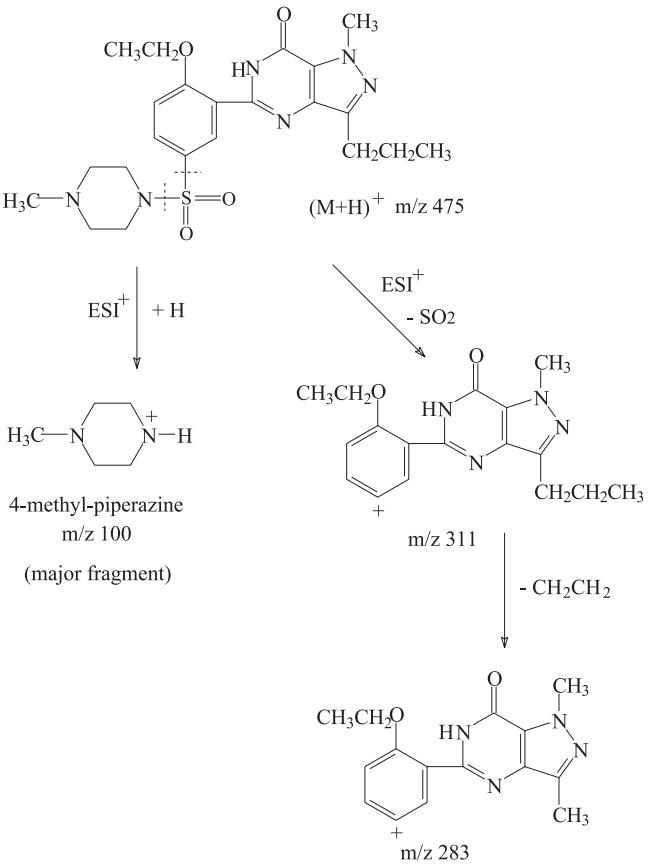

Figure 4. Fragmentation of sildenafil by ESI⁺ analysis.

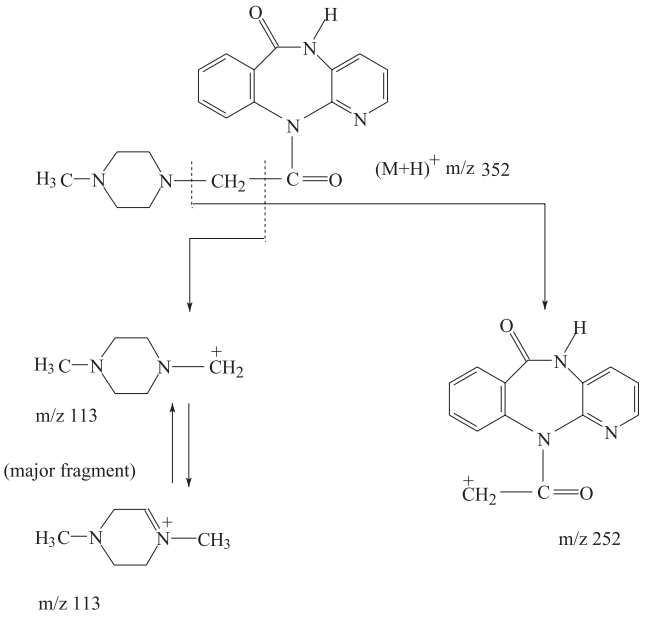

Figure 5. Fragmentation of pirenzepine by ESI⁺ analysis.

IV. *Identification of Citrate*

Through MS1 mass scanning, we suspected that the sample was adulterated with sildenafil citrate. As reported by USP[12], Chp[13], and JP.[14], citrate was generally identified by a chemical coloring reaction. However, this method was easily affected by the complicated natural ingredients or sample solution with color, and it was difficult to identify the citrate salt. Our study used LC/MS/MS to perform ESI⁻ ion analysis and chose $(M-H)^-$ ion fragment m/z 191 ($C_6H_7O_7^-$) of the citrate acid to perform the daughter ion scan (Figures 1D&2B. According to the general rules for predicting prominent fragmentation, one of the three carboxyl groups of citrate acid will be easily eliminated and trigger chemical degradation. It could also easily eliminate water and form a stable structure in the neighboring hydroxyl group after dehydration and cyclization to acquire m/z 111 major fragment (Figure 3).

V. *Choosing Internal Standard*

The best internal standard was isotope isomer of the

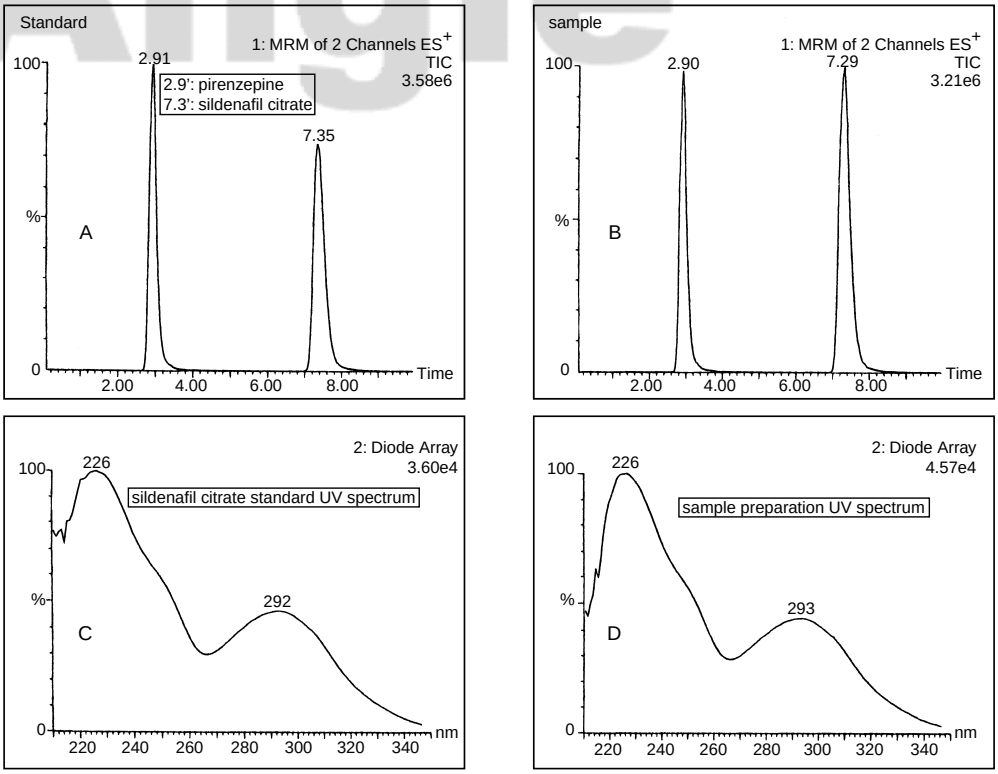

Figure 6. The MRM chromatograms of standard (A), sample solution (B) and the UV spectrums of LC/MS/MS analysis with PDA detector (C&D).

Table 4. Recoveries of sildenafil citrate adulterated in TCM

Group number	Adulterated amount	Mean ± SD (RSD,%)
A	10% w/w	98.8 ± 3.0 (3.0)
B	5% w/w	97.4 ± 4.1 (4.2)
C	3% w/w	98.0 ± 4.8 (5.0)

n = 3.

Table 5. Intraday and interday precision analysis of sildenafil citrate

Concentration of	Intra-day		Inter-day	
standard (μg/mL)	2.64	13.20	2.64	13.20
Mean ± S.D.	2.58 ± 0.20	13.36 ± 1.01	2.49 ± 0.28	12.76 ± 1.16
R.S.D. (%)	7.75	7.56	11.25	9.09

n = 5.

subject when carrying out the quantitative process[13]. Since the compound was difficult to acquire in the market, we chose pirenzepine instead as both sildenafil and pirenzepine could derive 4-methyl-piperazine during the cleavage process (Figures 4 & 5), and could produce intensity fragment under the analytic conditions. In addition, both the analysis subject and the internal standard while being collided by argon in the collision cell of the LC/MS/MS, the m/z 475 of sildenafil $(M+H)^+$ would cleavage into 4-methyl-piperazine ($C_5H_{12}N_2^+$) fragment (m/z 100), when the fragment m/z 352 of pirenzepine $(M+H)^+$ ion would cleavage into 1-methylene-4-methyl-piperazine ($C_6H_{13}N_2^+$, m/z 113) ion fragment (Figure 4). The fragments of the two compounds were distinctive and therefore, during the MRM quantitative process, the fragments would not interfere with each other. Also under the current LC separation conditions, the retention time of sildenafil citrate and pirenzepine were at 7.3 and 2.9 minutes (Figure 6). There was no mutual disturbance problem.

VI. *Plotting the Calibration Curve*

To quantify sildenafil citrate by MRM, the standard solution concentration range was at 1.76 ~ 17.60 μg/mL. The calibration curve was Y = 0.0957X + 0.0231, and the correlation coefficient γ^2 was 0.9977.

VII. *Evaluation of Recovery*

Three groups of model TCM sample were adulterated with sildenafil citrate. The results showed that the average recovery was above 97.4% with relative standard deviation values at 3.0~50% (Table 4). Although the ingredients contained in the sample may not be the same as the model TCM samples, the result indicated that quantitative and qualitative sildenafil citrate by using LC/MS/MS could block out the disturbance caused by natural ingredients and could achieve reliable precision.

VIII. *Precision*

The relative standard variations from intra- and inter-day testing were at 7.53~7.64% and 9.11~11.14%, respectively. These results indicated that the precision was within acceptable range (Table 5).

IX. *Detection Limit Test*

In this study, we found that the S/N ratio was at 2.7 when the standard solution was diluted to 50 times (concentration at 35.2 ng/mL). Should S/N ratio at 3 be the detection limit, 40 ng/mL would then be the relative detection limit concentration.

X. *Quantitative Results*

After quantitation, sildenafil citrate contained in the five sample solutions were at 39.2, 45.0, 43.3, 40.4 and 46.1 mg/capsule, average at 42.8 mg/capsule, with relative deviation at 6.8%. According to the instruction label[16], the suggested dosage of VIAGRA® as suggested by Pfizer Labs., is at one dose of 50 mg per day with maximum dose intake at 100 mg. Sildenafil citrate, as recorded in pharmacology, could induce acute hypotension to cardiovascular patients taking nitrate compounds. A dosage of 3 or more of the testion TCM capsules adulterated with sildenafil citrate could be dangerous.

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應用液相層析串聯式質譜分析法檢出食品膠囊中摻加sildenafil citrate西藥成分之定性定量研究

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摘　要

一種標示由動物器官萃取物製成用於增強性功能之食品膠囊，經以50% 甲醇萃取，取濾液直接注入液相層析串聯式質譜儀（LC/MS/MS ）作正離子電灑法 (ESI^+ ）全質譜掃描（MS 1 scan ）分析，質譜圖與本局自建液相層析電腦質譜資料圖庫比對結果，疑似含sildenafil citrate成分。繼而以子代離子掃描（Daughter ion scan)，獲得質譜圖與對照標準品比對結果均相符，同時以負離子電灑法（ESI^- ）證明檸檬酸鹽之存在。檢品溶液經液相層析及配合附光二極體陣列檢出器（photodiode array ）檢測，獲得之離子峰滯留時間及紫外光光譜圖均與標準品溶液相符，更確切證實檢品摻加sildenafil citrate 西藥成分。為瞭解該食品膠囊中含sildenafil citrate 之量，探討其定量方法而配製系列對照標準品溶液，以pirenzepine 作為內部標準品，分離管柱為Cosmosil 5C18-AR, 4.6 × 150 mm ；移動相溶媒為甲醇：乙腈：1% 醋酸（25:17:58)，流速：0.5 mL/min ，毛細管電壓：3 kV ；進樣圓錐口電壓：80 V ；碰撞能量：25 eV ；離子源溫度：120˚C ；溶媒揮散溫度：350˚C 等分析條件，選定親代及子代離子（m/z 475>100 ）作多重離子裂解監控（MRM, Multiple Reaction Monitoring ）定量分析並製作檢量線，線性關係γ^2 值為0.9977 。

為探討本分析法之精確度及再現性而進行同日內、異日間之測試與添加回收率試驗，結果其相對標準偏差值分別介於7.56~7.75% 及9.09~11.25% 之間，添加回收率之相對標準偏差值分別介於3.0~5.0% 之間，故本分析法之再現性及精確度均可接受。經探討本分析法之最低檢出極限為40 ng/mL 。依上述LC/MS/ MS 分析法定性定量sildenafil citrate 既快速又精確。

本案檢體經定量結果含sildenafil citrate 為42.8 mg/capsule ，對患有心血管疾病正在服用硝酸鹽類心臟藥之患者，在不知情下而服用本檢體，恐有安全之虞。

關鍵詞：液相層析串聯式質譜分析法，電灑法，sildenafil citrate ，pirenzepine ，MRM 定量法