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Journal Name

ARTICLE

Simultaneous determination of 41 components in Gualou Guizhi granule by UPLC coupled with triple quadrupole mass spectrometry

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Gualou Guizhi granule (GLGZG) is a classical formula of traditional Chinese medicine, which has been commonly used to treat dysfunction after stroke, epilepsy and spinal cord injury. In this study, an efficient method using ultra-performance liquid chromatography (UPLC) coupled with triple quadrupole mass spectrometry (QqQ MS) was developed for the rapid determination of forty-one major components in GLGZG. Chromatographic separation was achieved on a Waters ACQUITY UPLC Cortest C18 column (100 mm × 2.1 mm, 1.6 μm) with a gradient mobile phase (A: 0.1% aqueous formic acid and B: acetonitrile with 0.1% formic acid) at a flow rate of 0.25 mL/min. Multiple-reaction monitoring (MRM) was used to quantitative analyze. Ten batches of GLGZG were analyzed with good linear regression relationship (R², 0.9841-0.9998). The present study offered highly sensitive, specific and speedy determination of forty-one components, which promoted the quality control investigation of GLGZG via employing the developed method.

Keywords: Gualou Guizhi granule; ultra-performance liquid chromatography; QqQ mass spectrometry; quantification

Introduction

Traditional Chinese medicine (TCM) has several thousands of years history, and recently, it receive great interest in the treatment of stroke. Historically, these treatments have been described as effective with few side effects. However, these clinical studies were not conducted as Clinical Trials typically approved by the FDA or EMA to determine efficacy and safety (multi-center, double-blinded, placebo controlled, properly powered with proper morbidity/mortality endpoint(s) and statistical analysis) and such studies are planned. Gualou Guizhi granule (GLGZG, Min drug system approval no. S20130001) was originated from Gualou Guizhi decoction, which is consisted of six herbs including *Trichosanthis Radix*, *Cinnamomi Ramulus*,

Paeoniae Radix Alba, *Glycyrrhizae Radix*, *Zingiberis Rhizoma Recens* and *Jujubae Fructus*, which was first recorded in 'Essentials from the Golden Cabinet' in the Eastern Han Dynasty (around 210 AD). GLGZG has been applied clinically to treat muscular spasticity following stroke, epilepsy or spinal cord injury (1-3). Preclinical studies have supported the rational for conducting large clinical trials and suggested mechanisms of action. Recently, we evaluated GLGZG on lipopolysaccharides (LPS)-induced BV-2 murine microglial cells and middle cerebral artery occlusion (MCAO) rat, which indicated that GLGZG had an effect upon toll-like receptor (TLR) 4/nuclear factor (NF)-κB pathway and mitogen-acti-vated protein kinase (MAPK) signaling pathway (4,5) and GLGZG exerted neuroprotective effects via the modulation of excitatory amino acids levels and N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor expression and inhibition neuron apoptosis (6-8). Quality Control (QC) data and methods are lacking. The continued interest in GLGZG and other TCM in Clinical Trials necessitates

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that quantitative methods to standardize preparations be developed and described.

Although many analytical strategies have been developed to evaluate the quality of GLGZG or other TCM (9-12), GLGZG is a complex combination of six natural products, each of which contains numerous chemical compounds, the efficacy of GLGZG should be associated with the synergistic or interactive work of numerous chemical, including monoterpene glycosides from *Paeoniae Radix Alba*, phenolic acids from *Trichosanthis Radix* and *Cinnamomi Ramulus*, flavonoids from *Glycyrrhizae Radix*, gingerols from *Zingiberis Rhizoma Recens* and triterpene saponins from *Jujubae Fructus* and *Glycyrrhizae Radix*, single or several marker compounds quantification do not afford sufficient quantitative information for the other active constituents in GLGZG. Thus, to develop an efficient method to evaluate and control the quality of GLGZG is significant.

Along with the development of analytical technology, ultra-performance liquid chromatography (UPLC) coupled with triple quadrupole mass spectrometry (QqQ MS) can effectively avoid false-positive results with high sensitive and provide a reliable quantification of different compounds (13-15). UPLC-QqQ MS has been proved to be an ultimate solution to the aforementioned problems, which showed faster analytical speed, narrower chromatographic peaks and effectively avoided false-positive results with high sensitive (16). UPLC-QqQ MS method is suitable for the determination of active constituents in GLGZG samples.

Herein an UPLC-QqQ MS method was developed for the rapid simultaneous determination of forty-one major active components in GLGZG. Ten batches of GLGZG were collected for the analysis. The general content ranges of these active compounds were given in this paper, which was benefit for the quality control and clinical usage of GLGZG.

2 Materials and methods

2.1 Samples

Ten batches of GLGZG were provided by Fujian University of TCM Affiliated Second People's Hospital (Fuzhou, China). Voucher specimens were deposited in the College of Pharmacy, Fujian University of TCM.

2.2 Reagents and standards

Acetonitrile, methanol and formic acid (HPLC grade) were bought from Merck (Darmstadt, Germany). Deionized water was prepared by a Millipore Milli-Q purification system (Millipore, Bedford, MA, USA). Standards of gallic acid, protocatechuic acid, chlorogenic acid, catechin, protocatechuic aldehyde, p-hydroxybenzoic acid, paeoniflorin, rutin, liquiritin, luteoloside, ferulic acid, liquiritigenin, cinnamic acid, glycyrrhizic acid and 6-gingerol and glycyrrhetic acid were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Neochlorogenic acid, oxypaeoniflorin, methyl gallate, vanillic acid, schaftoside, 4-hydroxycinnamic acid, ethyl gallate, liquiritin apioside, pentagalloylglucose, astragalgin, 3-

hydroxycinnamic acid, isoliquiritin apioside, isoliquiritin, 2-hydroxycinnamic acid, ononin, 2-methoxycinnamic acid, isoliquiritigenin, licochalcone A and 8-gingerol and 6-shogaol were bought from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). Albiflorin, benzoylpaeoniflorin, jujuboside A, formononetin, jujuboside B, swertiamarin (internal standard, IS 1), nicotiflorin (internal standard, IS 2) and methylparaben (internal standard, IS 3), ginsenoside Rb1 (internal standard, IS 4) were purchased from Manstie Bio-Technology Co., Ltd. (Chengdu, China). The purity of these standard compounds was more than 98% and they structures were shown in Fig.1.

2.3 Preparation of standard and sample solutions

Forty-one standard stock solutions were prepared individually at concentrations ranging from 0.33 to 2.31 mg/mL by dissolving the substance in methanol. Internal standards stock solution were also prepared in a concentration of 4.01 µg/mL for swertiamarin, 2.00 µg/mL for nicotiflorin, 0.5 µg/mL for methylparaben and 1.00 µg/mL for ginsenoside Rb1. Then a mixed solution containing all the forty-one standards were prepared and serially diluted with 50% methanol-water (v/v) to obtain seven reference solutions with different concentrations. All prepared solutions were stored at 4 °C until use.

GLGZG were ground to fine powder and well mixed. 0.050 g powder of GLGZG was precisely weighed, then the powder was ultrasonicated for 30 min with 25 mL 50 % methanol-water (v/v) solution. Then, the extraction were centrifuged at 12000 rpm for 10 min after additional methanol adding to make up the lost weight. 500 µL supernatant was taken out and mixed with 500 µL internal standards working solutions, and then filtered through 0.22 µm PTFE membrane prior to injection. All the samples were stored at 4 °C until use (previous studies have demonstrated stability under these conditions).

2.4 Liquid chromatography

A Waters UPLC system (Waters, USA) equipped with an online vacuum degasser, a binary pump, an autosampler and a thermostated column compartment was employed to do chromatographic analysis. Chromatographic separation was carried out using an Waters ACQUITY UPLC Cortest C18 column (100 mm × 2.1 mm, 1.6 µm) and the column temperature was maintained at 45 °C. The mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile with 0.1% formic acid (B) were used to elute the target components with a gradient program (0-0.5 min, 8-10% B; 0.5-2.5 min, 10-15% B; 0.5-2.5 min, 10-15% B; 2.5-4.0 min, 15-60% B; 4.0-5.0 min, 60-95% B; 5.0-6.3 min, 95-95% B, 6.3-7.5 min, 8-8% B). The flow rate and the sample injection volume was kept at 0.25 mL/min and 2 µL.

2.5 Mass spectrometry

Tandem mass spectrometry was performed on a Waters (Milford, MA) Xevo TQMS with an electrospray ion source (ESI). The MS spectra were acquired in the negative ion MRM mode, which was carried out by optimization of the product ion obtained from the fragmentation of the isolated precursor ion for each standard. Once the product ions were chosen, the MRM conditions for each standard were further optimized to achieve maximum sensitivity. Nitrogen was used as curtain gas

(CUR), nebulizer gas (GS1) and heater gas (GS2). Argon was chosen as the collision gas. The optimized MS conditions were 150 °C of source temperature, 20 ms of dwell time. The most appropriate precursor ion, daughter ion, cone voltage and collision energy (CE) of each analyte were displayed in table 1.

2.6 Analytical method validation

2.6.1 Linearity, LOQs and LODs

At least seven concentrations of calibration standard solution were made and analyzed in triplicate, and then the calibration curves were constructed by plotting the ratios of the peak areas of each standard to IS versus the concentration of each analyte. Linear regression analysis was used to calculate the slope, intercept and the correlation coefficient of each calibration line. Typically, LOD and LOQ are three times and ten times the noise level, respectively. For each target constituent, the LODs and LOQs were determined by serial dilution of standard solution under the described UPLC-QqQ MS conditions.

2.6.2 Precision, repeatability and stability

Intra- and inter-day variations were utilized to evaluate the precision of the developed method. The intra-day precision was investigated for the standards solutions using six replicates within one day, while for inter-day precision test, the standards solutions were determined in duplicates for consecutive three days. Relative standard deviation (RSD) was used to represent the variations.

Repeatability of the developed method was determined by six samples of GLGZG (S101) on three separate days. The RSD was used to evaluate the method repeatability. Meanwhile, the stability of the samples was also investigated at 25 °C. Each sample solution was analyzed every 4 h within 12 h in triplicate.

2.6.3 Accuracy

A recovery test was employed to evaluate the accuracy of the developed method. Standard solutions with three different concentration levels (120%, 100% and 80%) were added to the known amounts of GLGZG sample. The mixture was extracted and analyzed as described above. Three replicates of each level were performed. The recoveries were calculated by the formula: $\text{recovery}\% = (\text{detected amount} - \text{original amount}) / \text{added amount} \times 100\%$, and $\text{RSD}\% = (\text{S.D.} / \text{mean}) \times 100\%$.

3. Results and discussion

3.1 Optimization of UPLC-MS/MS conditions

The selection of UPLC-MS/MS conditions was guided by the requirement for obtaining chromatograms with good peak shapes and high sensitivity within a short time especially when large amounts of samples were analyzed.

In this study, different mobile phases consisting of water-methanol and water-acetonitrile were examined. As a result, the good separation for 41 standards was achieved by using water-acetonitrile. Meanwhile, it was found that formic acid not only improve the chromatographic separation, but also enhance the abundance of [M-H+HCOOH]⁻ in the negative model. Moreover, swertiamarin (IS 1), nicotiflorin (IS 2), and methylparaben (IS 3), ginsenoside Rb1 (IS 4) were chosen as

internal standards due to the similar structures, retention time and ionization response in ESI-MS.

And for the MS conditions, different parameters including declustering potential, collision energy and cone voltage were studied to achieve the abundance of precursor ions and product ions, and at last the most sensitive transition in MRM was selected. As we known, different type compounds present different ionization intensity in different ion modes. In our study, 41 quantitative target compounds including 15 phenolic acids, 14 flavonoids, 4 monoterpene glycosides, 4 triterpenes, 3 gingerols and 1 galloyl glucose. Most phenolic acids, monoterpene glycosides and galloyl glucose, produced stronger ionization intensity in the negative ion mode than positive ion mode, so the negative ion mode was used for the identification and quantitative of this type of compounds; both of flavonoids and triterpenes produced almost the same ionization intensity in the both positive ion mode and negative ion mode, so the negative ion mode were used for the analysis of these two types of compounds. However, gingerols produced stronger ionization intensity in the positive ion mode, but they can not produce stable MS/MS ions (daughter ion), So we choose the negative ion mode([M-H]⁻) to analysis the 41 target compounds. In addition, above these observations in our study were consistent with previous reports (17-19). The optimum conditions were shown in Table 1 and MRM chromatogram of 41 standards and four IS were shown in Fig.2. And we also provide some representative MS/MS figure of some compounds in this version (Fig 3) and all the 41 MS/MS figure in supporting information (Fig S1).

3.2 Validation of method

3.2.1 Linearity, LOD and LOQ

The linear calibration curves with the R^2 , linear range and regression equation, LOD and LOQ of 41 standards were listed in Table 2. It was indicated good linear correlation at these conditions with determination coefficients (R^2) from 0.9841-0.9998. The LODs (S/N=3) and LOQs (S/N=10) for all standards were in the range of 0.03–30.6 and 0.12–70.9 ng/mL, respectively, which indicated that this method was sensitive for the quantitative determination of major components in GLGZG samples under these conditions.

3.2.2 Precision, repeatability, stability and recovery

As shown in Table 3, the precision, repeatability and stability of 41 standards were listed. The RSDs of intra-day precision were in the range of 2.77-4.9%, 3.24-4.78%, 1.51-4.45% and 1.33-4.13%, respectively. The RSDs of inter-day precision were in the range of 3.28-4.97%, 3.32-4.97%, 3.85-4.99% and 3.35-4.96%, respectively. It was suggested that the developed method was precise enough for the quantitative evaluation of the analytes in GLGZG.

Repeatability with RSD<5% of GLGZG suggested that the developed method was reproducible enough for the quantitative evaluation of the analytes in GLGZG. And the The stability of samples was 1.48–4.12% within 12 h at 25 °C, which suggested that the developed method was stable enough for the quantitative evaluation of the analytes in GLGZG.

As shown in Table 4, average recoveries of 41 standards varied from 95.63 to 104.80% and RSDs were all $\leq 5.11\%$. It was revealed that the acceptable recovery and accuracy of this method.

In brief, the developed method had good precision, repeatability and stability, it was demonstrated that the developed method was sufficiently reliable and accurate for the quantitative evaluation of the analytes in GLGZG.

3.2.3 Sample analysis

This developed method was successfully applied for the identification and quantification of the 41 major target components in ten batches of GLGZG. The contents of the investigated 41 components (mg/g) in GLGZG were summarized in Table 5. As the results turns out, albiflorin (2.5668-5.3533 mg/g), paeoniflorin (1.0185-5.8907 mg/g) and glycyrrhizic acid (1.9177-6.7504 mg/g) were found to be the most abundant constituents. In addition, the others were not more than 1 mg/g in all GLGZG. It was reported that the contents of these compounds could be affected mainly by different sources of plant material and processing time or temperature in the manufacturing procedure (20,21). Real sample data of different batches of GLGZG illustrated that the contents of the investigated compounds were not consistent in different batches of GLGZG, despite being prepared by the same protocol and manufacturing process. So, it would be a valuable tool to improve quality control of GLGZG to simultaneous quantitative analysis 41 major components in GLGZG. Also, it may therefore be employed as a useful tool to evaluate the quality of these TCM.

3.3 Comparisons with reported methods

Comparison with the methods reported so far reveal some advantage of this method. First, it greatly reduced the sample analysis time (from 25-105 min to 6.3 min) and promotes analysis efficiency. Secondly, the analysis compounds increased from a few to 6 types of compounds (41 compounds including 15 phenolic acids, 14 flavonoids, 4 monoterpene glycosides, 4 triterpenes, 3 gingerols and 1 galloylglucose). Thirdly, sensitivity was better than the methods reported, LOQ was changed from $\mu\text{g/mL}$ to ng/mL . Fourthly, it required less solvent than the methods reported, its flow rate was only 0.25 ml/min, solvent of one analysis only need 1.575 mL (less than 2 ml), however the methods reported need 50-105 ml solvent. Related data from some references have been added (in support information, Table S1).

Conclusions

More and more use of GLGZG or other TCMs, hence efficient methods to evaluate and control the quality of herbal products are urgently needed. In this paper, a UPLC-QqQ/MS method to simultaneous determinate 41 compounds in GLGZG has been developed and validated for the first time. The proposed method could enable quantitatively analyze of target

compounds with high selectivity even at low concentration by comparison with standards, and rapid analysis performed within 7.5 min, which was useful in controlling the quality of GLGZG and other related pharmaceutical preparations containing *Trichosanthis Radix*, *Cinnamomi Ramulus*, *Paeoniae Radix Alba*, *Glycyrrhizae Radix*, *Zingiberis Rhizoma Recens* and *Jujubae Fructus*.

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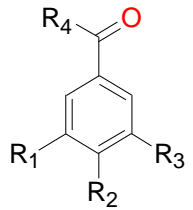
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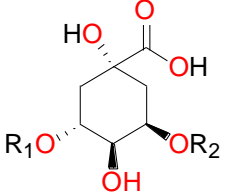
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(a) Phenolic acids



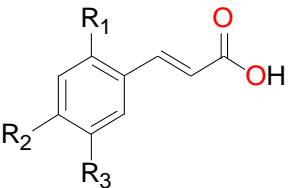
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- (1) R₁=OH, R₂=OH, R₃=OH, R₄=OH
- (2) R₁=OH, R₂=OH, R₃=H, R₄=OH
- (7) R₁=H, R₂=OH, R₃=OH, R₄=H
- (8) R₁=H, R₂=OH, R₃=H, R₄=OH
- (9) R₁=OH, R₂=OH, R₃=OH, R₄=OCH₃
- (10) R₁=OCH₃, R₂=OH, R₃=H, R₄=OH
- (16) R₁=OH, R₂=OH, R₃=OH, R₄=OC₂H₅
- (IS3) R₁=H, R₂=OH, R₃=H, R₄=OCH₃



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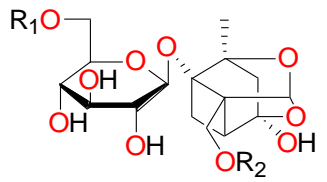
- (3) R₁=Caffeoyl, R₂=OH
- (5) R₁=OH, R₂=Caffeoyl



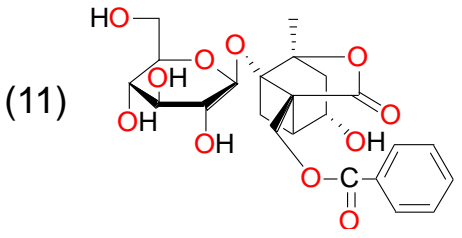
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- (14) R₁=H, R₂=OH, R₃=H
- (21) R₁=H, R₂=OH, R₃=OCH₃
- (23) R₁=H, R₂=H, R₃=OH
- (26) R₁=OH, R₂=H, R₃=H
- (31) R₁=H, R₂=H, R₃=H
- (34) R₁=H, R₂=OCH₃, R₃=H

(b) Monoterpene glycosides

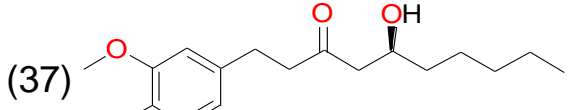


- (4) R₁=H, R₂=*p*-Hydroxy benzoyl
- (13) R₁=H, R₂=Benzoyl
- (29) R₁=Benzoyl, R₂=Benzoyl

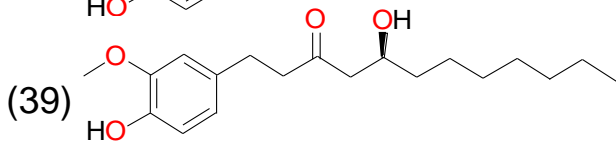


(11)

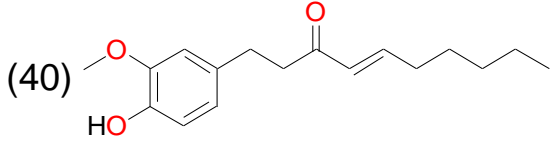
(e) Gingerols



(37)



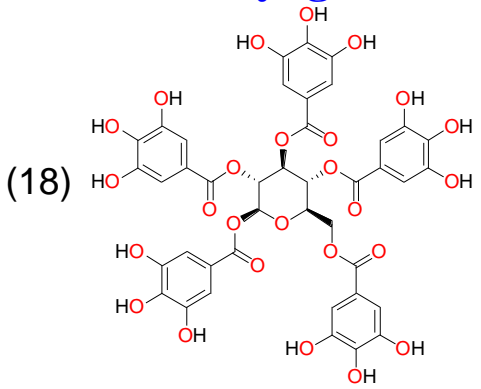
(39)



(40)

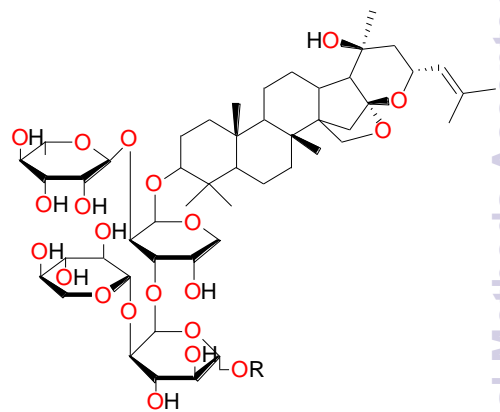
Fig.1

(c) Galloyl glucose



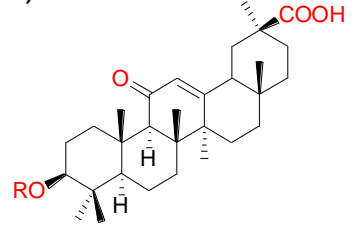
(18)

(f) Triterpenes



(30) R=Glc

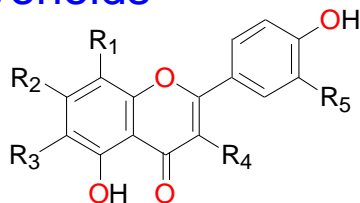
(33) R=H



(36) R=GlcA(2,1)-GlcA

(41) R=H

(d) Flavonoids

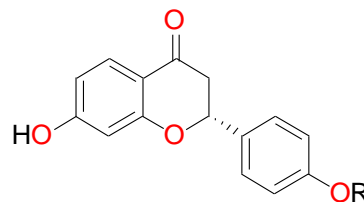
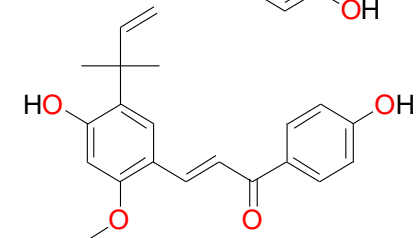
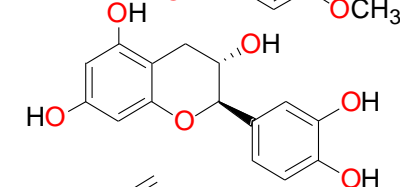
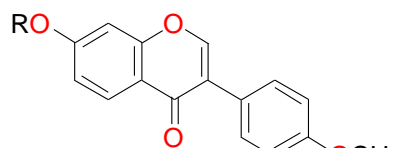
(12) R₁=C-Ara, R₂=OH, R₃=C-Glc, R₄=H, R₅=H(15) R₁=H, R₂=OH, R₃=H, R₄=O-Glc(6,1)-Rha, R₅=OH(20) R₁=H, R₂=O-Glc, R₃=H, R₄=H, R₅=OH(22) R₁=H, R₂=OH, R₃=H, R₄=O-Glc, R₅=H(IS2) R₁=H, R₂=OH, R₃=H, R₄=O-Glc(6,1)-Rha, R₅=H

(27) R=Glc

(32) R=H

(6)

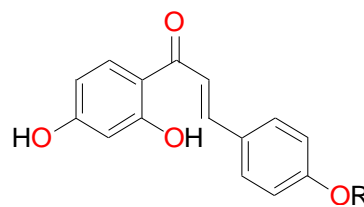
(38)



(17) R=Glc(2,1)-Api

(19) R=Glc

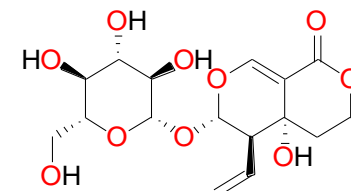
(28) R=H



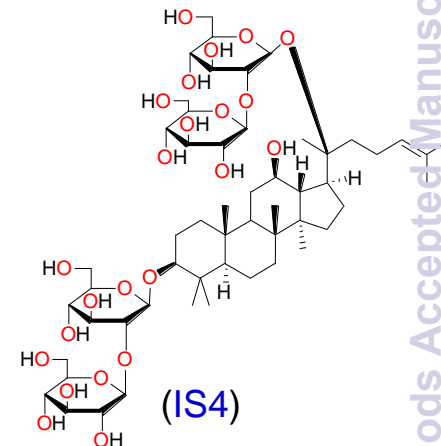
(24) R=Glc(2,1)-Api

(25) R=Glc

(35) R=H

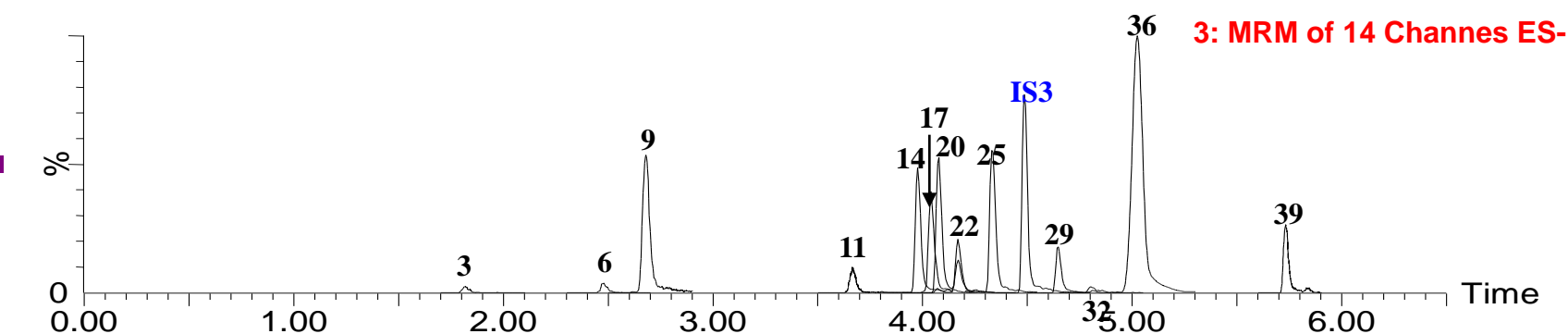
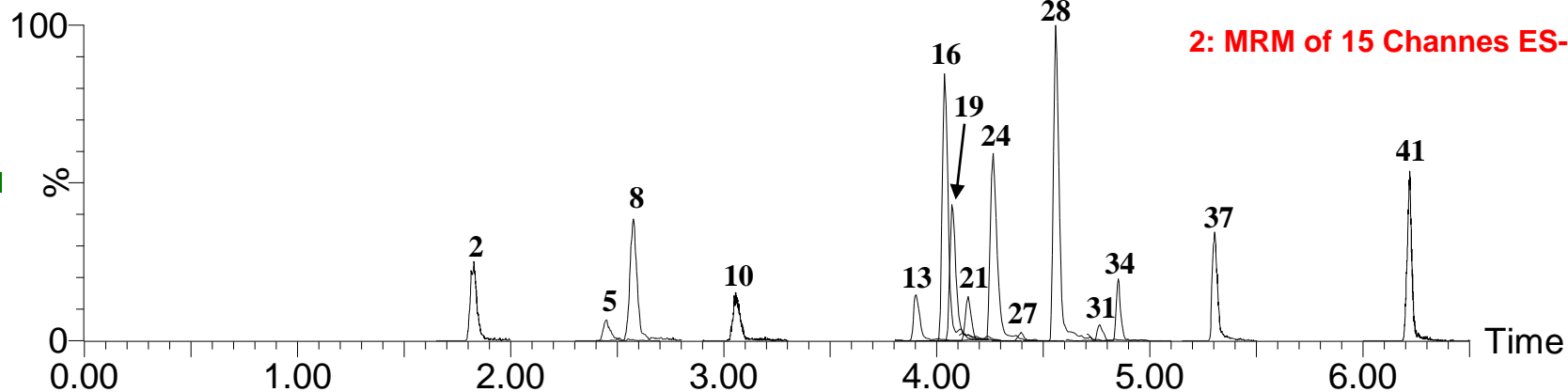
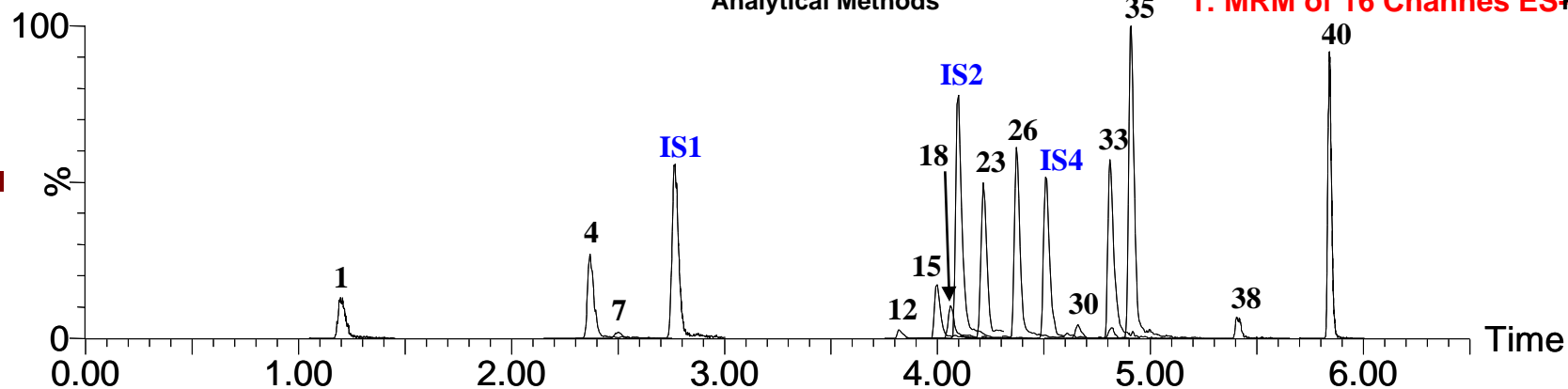


(IS1)



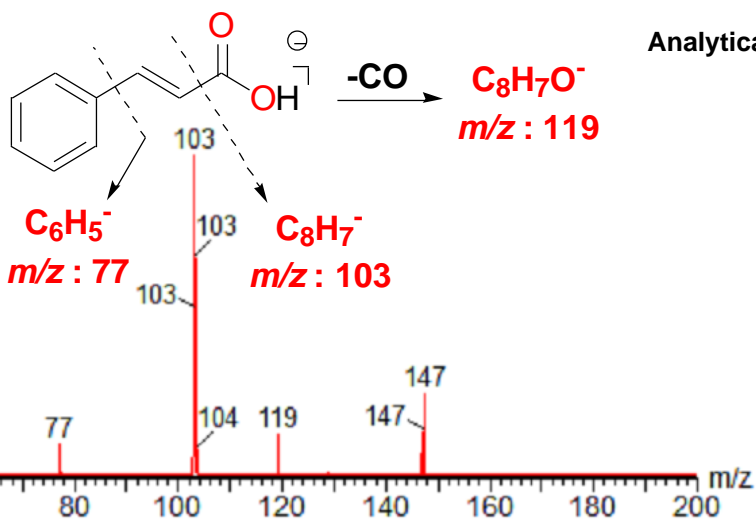
(IS4)

Fig.1 Chemical structures of the 41 Standards

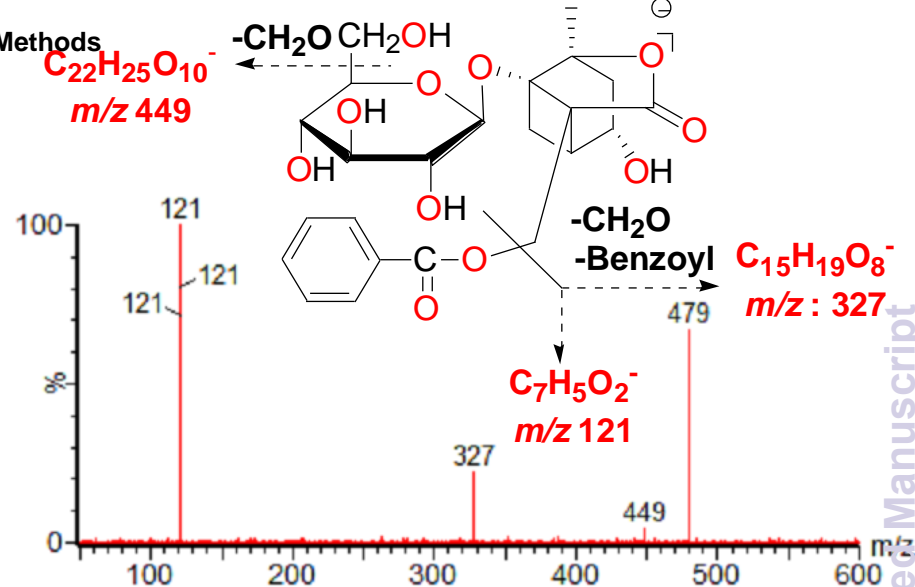


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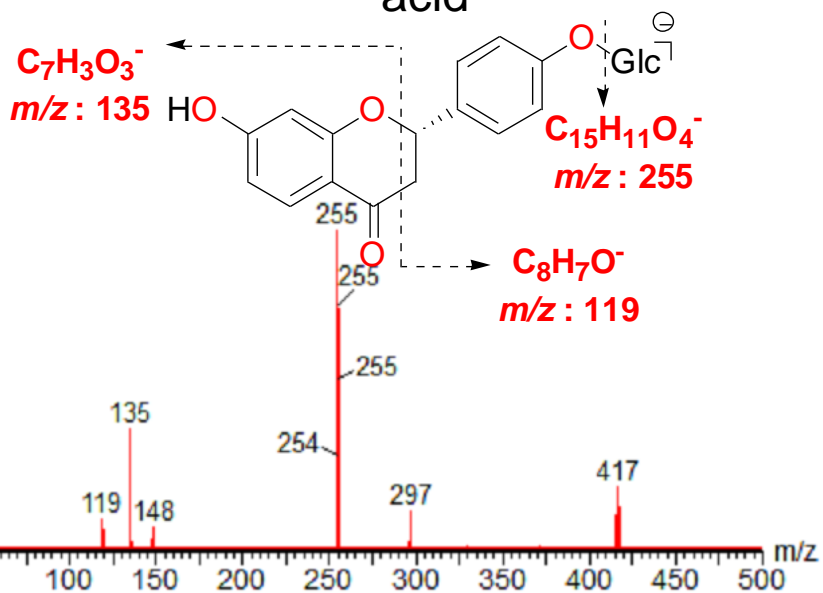
Fig.2 The MRM chromatograms of 41 Standards and 3 internal standards of mixed standards



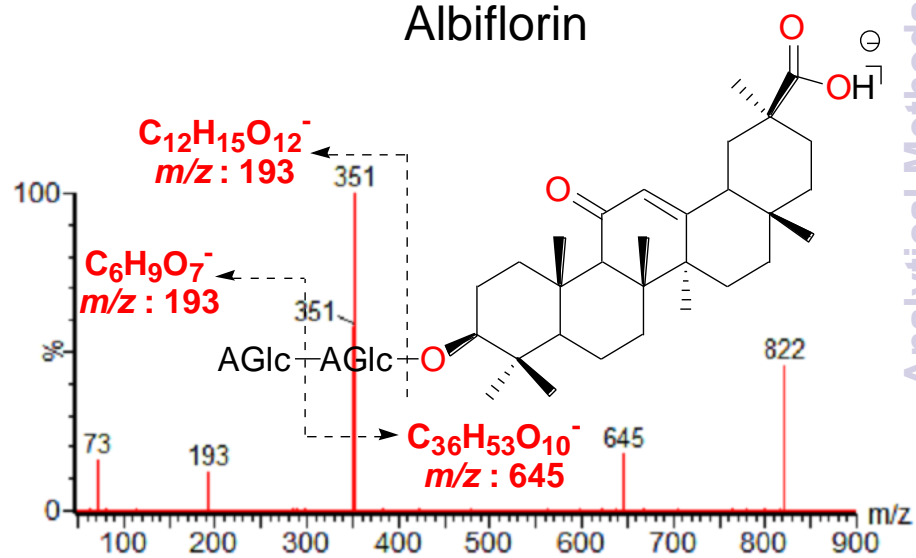
The MS/MS spectra and the proposed fragmentation pathway of Cinnamic acid



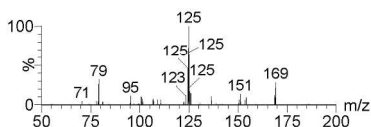
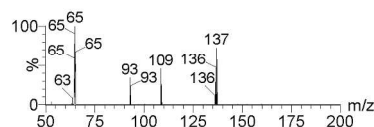
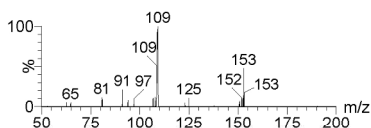
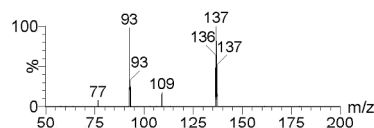
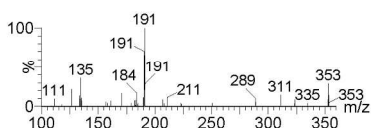
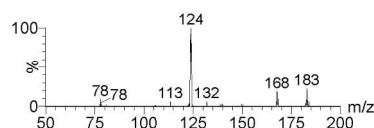
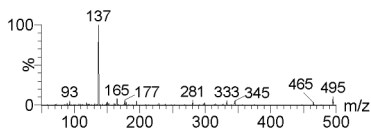
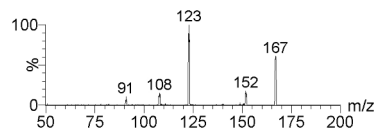
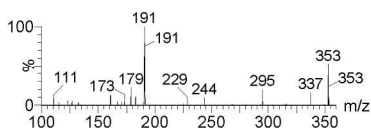
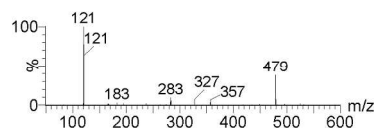
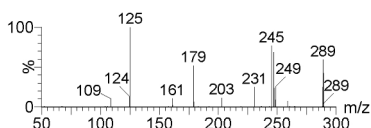
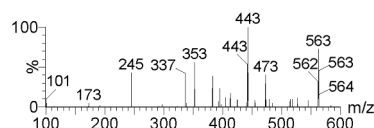
The MS/MS spectra and the proposed fragmentation pathway of Albiflorin



The MS/MS spectra and the proposed fragmentation pathway of Liquiritin

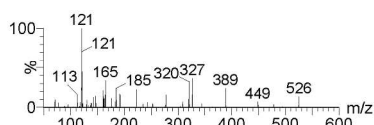
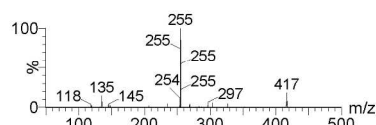
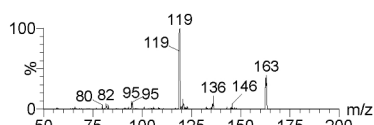
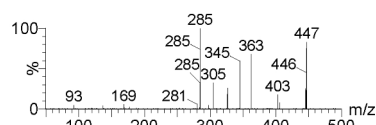
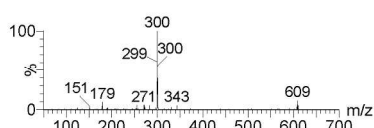
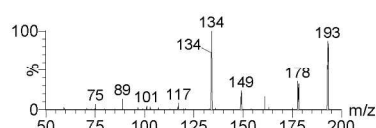
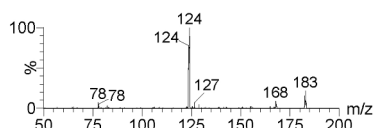
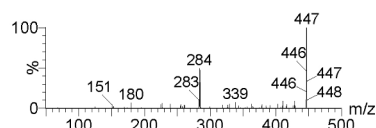
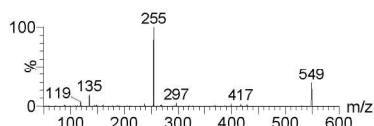
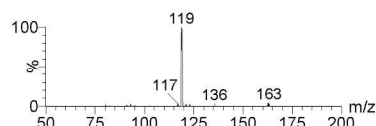
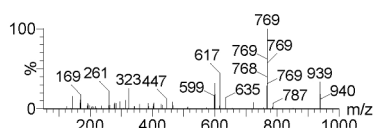
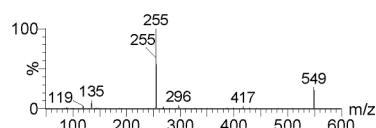


The MS/MS spectra and the proposed fragmentation pathway of Glycyrrhizin

1 Gallic acid**7 Protocatechuic aldehyde****2 protocatechuic acid****8 p-hydroxybenzoic acid****3 Neochlorogenic acid****9 Methyl gallate****4 Oxypaeoniflorin****10 Vanillic acid****5 Chlorogenic acid****11 Albiflorin****6 Catechin****12 Schaftoside**

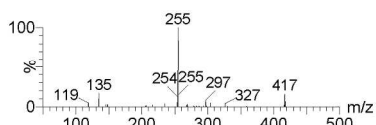
155x234mm (300 x 300 DPI)

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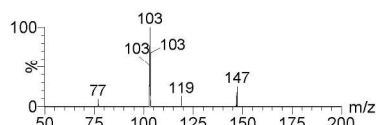
13 Paeoniflorin**19 Liquiritin****14 4-Hydroxycinnamic acid****20 Luteoloside****15 Rutin****21 Ferulic Acid****16 Ethyl gallate****22 Astragalin****17 Liquiritin apioside****23 3-Hydroxycinnamic acid****18 Pentagalloylglucose****24 Isoliquiritin apioside**

154x233mm (300 x 300 DPI)

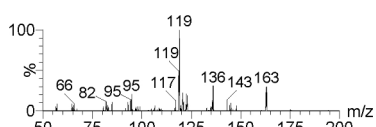
25 Isoliquiritin



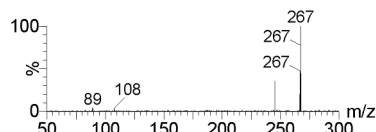
31 Cinnamic acid



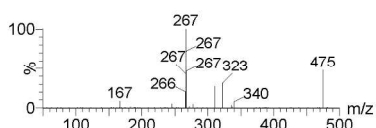
26 2-Hydroxycinnamic acid



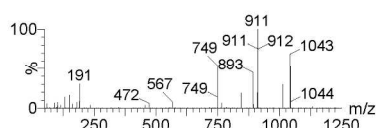
32 Formononetin



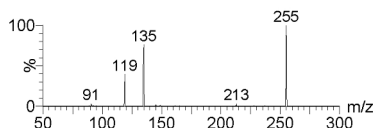
27 Ononin



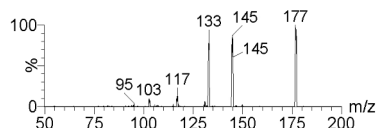
33 Jujuboside B



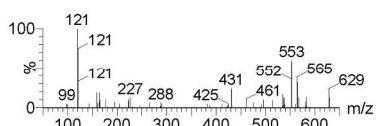
28 Liquiritigenin



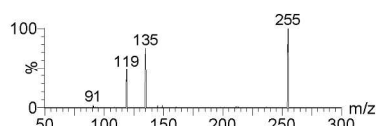
34 2-Methoxycinnamic acid



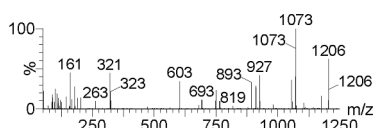
29 Benzoylpaconiflorin



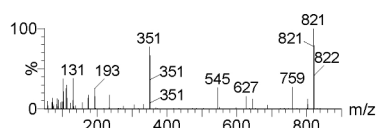
35 Isoliquiritigenin



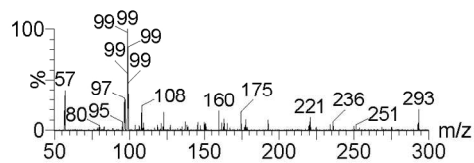
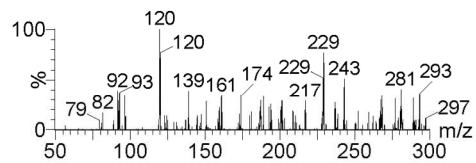
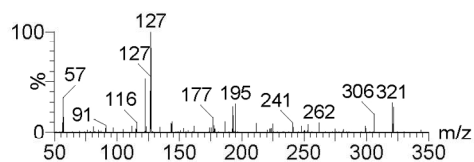
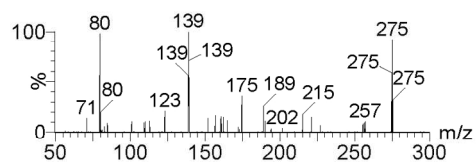
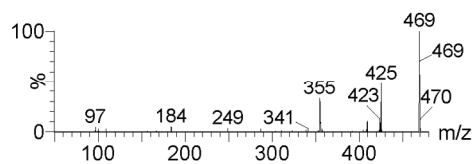
30 Jujuboside A



36 Glycyrrhizic acid



153x233mm (300 x 300 DPI)

37 6-Gingerol**38 licochalcone A****39 8-Gingerol****40 6-Shogaol****41 Glycyrrhetic acid**

154x119mm (300 x 300 DPI)

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Gualou Guizhi granule

quantification

Chemical Profiling

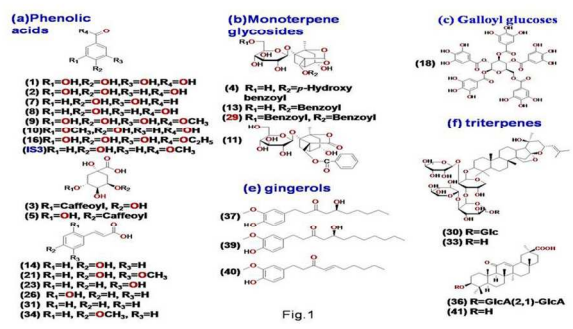
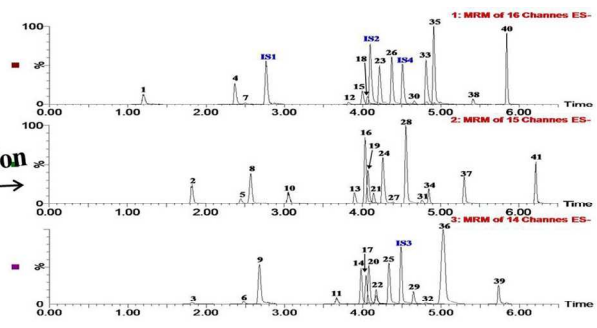


Fig. 1

255x174mm (150 x 150 DPI)

Analytical Methods Accepted Manuscript