Title	Study on a quantitative method for determination of multiple representative components of Zhishi--Xiebai-Guizhi decoction by UHPLC-TQ-MS

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本科生毕业论文

论文题目　　中药经方枳实薤白桂枝汤多指标成分的含量测定方法研究

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2023 年 **4** 月 **12** 日

Statement of Originality

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Study on a quantitative method for determination of multiple representative components of Zhishi-Xiebai-Guizhi decoction by UHPLC-TQ-MS

Abstract: Zhishi-Xiebai-Guizhi decoction (ZXG), a well-known traditional Chinese medicine (TCM) formula, is extensively used for the treatment of coronary heart disease (CHD) in China. The chemical composition of ZXG is complex and contains a variety of structural components. At present, there is no quantitative method to systematically describe the content distribution of each structure type and effective component. In this study，the UHPLC-TQ-MS technology was used to determine the content of 17 representative components in ZXG, including representative structural components of each herbal medicine, components absorbed into the blood, and cardiovascular-related active components. The quantitative method was validated by evaluation of the specificity, linearity, limits of quantification (LOQs), precision, repeatability, stability and recovery, and it was successfully applied for the quality evaluation of 24 batches of ZXG samples. The results showed that flavonoids were the most abundant compounds in ZXG, followed by alkaloids, phenylethanoid glycosides, triterpenoids, lignans, organic acids, steroidal saponins, phenolic glycosides, and coumarins. This study qualitatively described the chemical profile of ZXG, providing a solid chemical basis for revealing the pharmacological components of ZXG.

Key Words: Zhishi-Xiebai-Guizhi decoction, Quantitative method, Multiple representative components, UHPLC-TQ-MS

中药经方枳实薤白桂枝汤多指标成分的含量测定方法研究

摘　要: 枳实薤白桂枝汤(ZXG)是中国著名的中药配方，在中国被广泛用于治疗冠心病。ZXG 的化学成分复杂，含有多种结构成分。目前尚无定量方法可以系统描述其各结构类型和有效成分的含量分布。本研究采用超高效液相色谱-三重四极杆-质谱（UHPLC-TQ-MS）技术对 ZXG 中 17 种代表性成分进行含量测定，包括各中药代表性结构成分、入血成分和心血管相关活性成分。通过评估专属性、线性、定量限、精密度、重复性、稳定性和回收率对该定量方法进行验证，且该方法成功应用于 24 批 ZXG 样品的质量评价。结果表明:黄酮类化合物含量最高，其次为生物碱类、苯乙醇苷类、三萜类、木脂素类、有机酸类、甾体皂苷类、酚苷类和香豆素类。本研究定性描述了 ZXG 的化学特征，为揭示 ZXG 的药理成分提供了坚实的化学基础。

关键词: 枳实薤白桂枝汤；定量方法；多代表成分；UHPLC-TQ-MS

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1. Introduction

1.1 Overview and hazards of coronary heart disease

Coronary atherosclerotic heart disease, referred to as coronary heart disease (CHD). It is a heart disease caused by myocardial ischemia and hypoxia due to the stenosis of the lumen caused by coronary atherosclerosis and the relative insufficient blood supply of the coronary artery (He & Liu, 2015).

CHD, known as the "world coronary disaster",“ is the number one killer of heart disease, its mortality rate ranks second in the world, and the mortality rate of coronary heart disease in China is the highest in the world (He et al., 2015). At present, the number of patients with CHD in our country has reached 11 million, and the annual growth rate is 20% (高润霖, 2017).

Modern medicine shows that CHD is related to hemorheology abnormalities, the role of inflammatory factors, vascular endothelial function or structure abnormalities, coagulation dysfunction and other aspects (卢李娜 & 郑娴，2023). It cannot be truly cured, and it may be recurrent and progressively worse because the cause of CHD cannot be eradicated. Drug therapy is mainly used to delay the progression of the disease and reduce the attack of angina and myocardial infarction.

Western medicine in the treatment of CHD is mainly based on "open", mostly using antihypertensive drugs, lipid-lowering drugs, dilation of coronary arteries and other drugs, and the use of "tonic" drugs, such as myocardial nutrition drugs, less than "open" drugs, so the treatment effect is often not satisfactory. Under the guidance of the holistic concept of traditional Chinese medicine and the thought of syndrome differentiation and treatment, medication pays attention to "tonic" and make up for its needs. When “Qi” is insufficient, “Qi” should be replenished. Only when “Qi” is sufficient can the blood be unblocked. When supplementing “Qi” with blood-activating drugs and applying both “open” and “tonic”, satisfactory curative effect can be achieved (卢李娜 et,al., 2023)

1.2 Interpretation of "chest stuffiness and heartache" and the representative traditional Chinese medicine Zhishi-Xiebai-Guizhi decoction

In traditional Chinese medicine(TCM), CHD is classified into the categories of "chest stuffiness", "chest stuffiness and heartache" and "true heartache".《National Standard Application • Traditional Chinese Medicine Internal Medicine Disease Diagnosis and Treatment Routine》 also clearly equated "chest stuffiness and pain" with CHD.(Kinjo et al., 1985) Zhongjing Zhang pointed out in 《Synopsis of Golden Chamber (Jin Kui Yao Lue)》:Chest stuffiness is a disease of "deficiency in foundation and obvious in symptom" (Wang et al., 2015).

In the past 30 years, the understanding of etiology and pathogenesis of CHD gradually tends to be unified in TCM, that the basic pathogenesis of CHD is “Qi” deficiency and blood stasis. Coronary atherosclerosis and plaque formation can be classified into the category of “blood stasis” in CHD, and the changes of hemorheology are closely related to “Qi” deficiency. “Qi” deficiency as the foundation, blood stasis as a sign, disease location in the heart, involving in the kidney, also related to the spleen, the treatment should pay attention to the important role of “Qi”.

As the research on the syndrome of “Qi” deficiency and blood stasis of CHD has been gradually deepened, the method of treating CHD has been changed to "activate blood circulation to remove blood stasis", and the treatment methods such as invigorating “Qi” and activating blood circulation have been developed on this basis, which has significantly improved the clinical efficacy (Fu, Gao & Wang, 2012).

The treatment of CHD by western medicine focuses on symptomatic treatment such as intervention, anti-platelet aggregation, and anti-angina. Long-term medication often has adverse reactions. The proposed pathogenesis of “Qi deficiency and blood stasis” provides a better direction for the use of TCM in the treatment of CHD. It is also a new exploration for the treatment of CHD (卢李娜 et al., 2023).

In TCM, Zhishi-Xiebai-Guizhi decoction (ZXG), a well-known ancient TCM formulae originally documented in < Synopsis of Golden Chamber (Jin Kui Yao Lue) > written by physician Zhongjing Zhang in the Eastern Han Dynasty, has been

widely used for thousands of years in the treatment of “chest stuffiness and pains”, which corresponds to CHD in western medicine.

It is composed of five commonly used herbal medicines (**Figure 1-1**), including Trichosanthis Fructus (TF) (Gua lou), Allii Macrostemonis Bulbus (AMB) (Xie bai), Aurantii Fructus Immaturus (AFI) (Zhi Shi), Magnoliae Officinalis Cortex (MO) (Hou po) and Cinnamomi Ramulus (CR) (Gui Zhi) (谈晓东, 2019). Modern clinical studies have shown that ZXG can be widely used to improve and treat unstable angina of coronary heart disease (Tang et al., 2018), mid-stage myocardial infarction (Gao et al., 2015), or heart failure (郑萍红 et al., 2019).

ZXG has significant clinical efficacy, a long history of drug use, and high safety, which has become the basic prescription of "Xuanbi Tongyang(宣痹通阳)" treatment of chest stuffiness and heartache. Moreover, the formula was adopted into the <Ancient Classical Chinese Medicine Formula Catalogue (First Edition)> in April 2018, indicating that its clinical benefits have been publicly recognized in China.

Figure 1-1 Five Herbal Medicines in ZXG

1.3 Research progress on pharmacological action of ZXG

Pharmacological studies have shown that ZXG has the effects of anti-myocardial ischemia, anti-myocardial hypoxia, anti-hyperlipidemia, anti-myocardial ischemia-reperfusion injury, stabilizing plaque and anti-inflammation (何湛湛 et al., 2023).

1.3.1 Anti-myocardial ischemia

Myocardial ischemia is the basic pathophysiological process of CHD. ZXG can improve the cardiac contractility during myocardial ischemia, specifically can improve left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP) and other indicators. At the same time, it can reduce the S-T segment of ECG (王灵哲, 2015). Zhao Nan et al (赵楠 et al., 2021). found that ZXG could improve the intravascular environment of rabbit models of myocardial ischemia.

1.3.2 Anti-myocardial hypoxia

Myocardial hypoxia is caused by the decrease of coronary artery blood flow. Experiments have shown that ZXG can effectively improve the heart pumping function of rabbit models of myocardial ischemia (赵楠 et al., 2019).

1.3.3 Anti-hyperlipidemia

Xia Hanxing et al (夏寒星 & 张业, 2012a). confirmed that ZXG could improve hemorheology and reduce blood lipid levels in a rat model of hyperlipidemia. Therefore, it is speculated that ZXG may also play a role in anti-myocardial hypoxia by regulating the hemorheology of coronary artery (夏寒星 & 张业, 2012b).

1.3.4 Anti-myocardial ischemia-reperfusion injury

In the process of restoring the perfused ischemic myocardium, myocardial tissue may be damaged, that is, myocardial ischemia-reperfusion injury (MIRI). Zhao Weili (赵伟丽, 2015) found through experiments that ZXG could reduce the percentage of myocardial water content, increase coronary flow, and improve LVSP, LVEDP indexes and S-T segment of ECG in MIRI model.

1.3.5 Stabilize plaque

Relevant clinical trials have shown that the combination of ZXG and other drugs can effectively reduce the levels of inflammatory factors, mainly reducing high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6) and other indicators (Yan et al., 2020).

1.3.6 Anti-inflammation

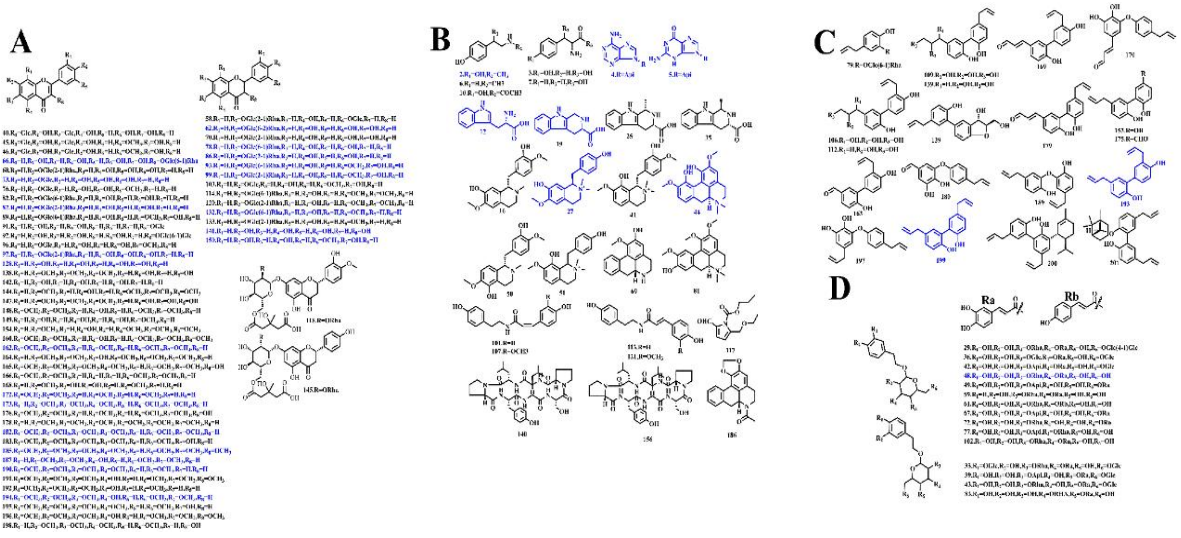
The study of Dai Fei et al (戴飞 et al., 2013) showed that ZXG has the effect of stabilizing plaque by reducing the level of matrix metalloproteinase-9 (MMP-9) to inhibit the degradation or even rupture of plaque, and at the same time, it can increase the level of tissue inhibitor of metalloproteinase-1 (TIMP-1) .

1.4 Research progress on chemical component of ZXG

At present, the overall chemical research of ZGX is very insufficient, and it is impossible to systematically describe the overall chemical profile of ZXG.

In the team's previous research, we identified 201 compounds in ZXG based on the UHPLC-Q/TOF-MS technology, covering 5 herbal medicines and 10 structural types: flavonoids(59), steroidal saponins (20) , alkaloids (27) , lignans (19) ,Phenylethanoid glycosides (16) , phenolic glycosides (14) , triterpenoids (9) , organic acids (11) , coumarin (9) , and others (17) . Among them, 60 compounds were accurately identified by the reference substance.

The overall chemical profile, structural characteristics and the source of the herbal medicines of ZXG were described, which provided a solid chemical basis for revealing the effective components of the compound.



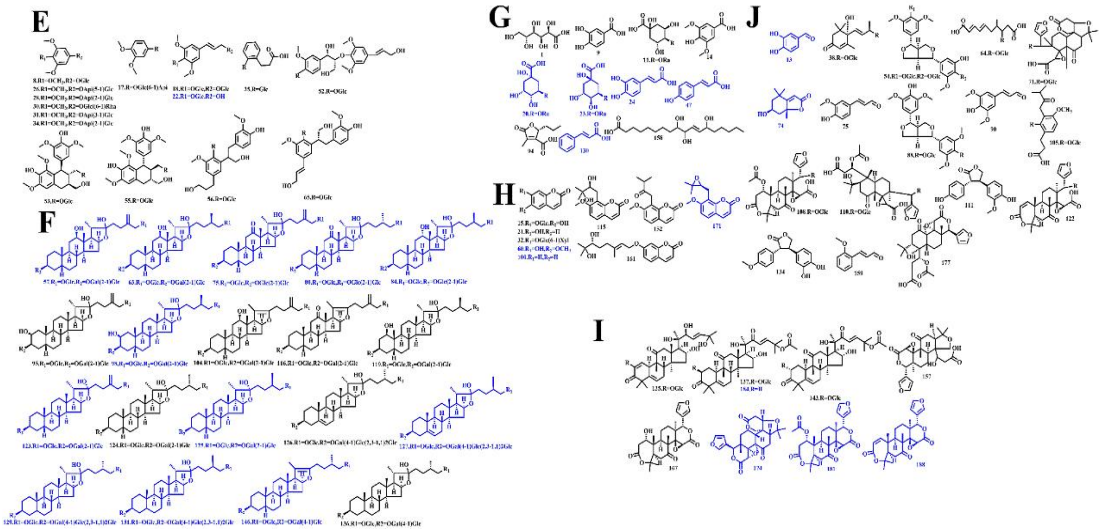


Figure 1-2 Structural Information of Chemical Constituents in ZXG.

Note: Flavonoids (A) , Alkaloids (B) , Lignans (C) , Phenylethanoid glycosides (D) , Phenolic glycosides (E) , Steroidal saponins (F) , Organic acids (G) , Coumarin (H) , Triterpenoids (I) , and others (J). The blue structural formulas are the accurately identified compounds.

1.5 Research progress on metabolism of ZXG in vivo

TCM is mainly administered orally, and its effects in the body may be through the following two forms: one is that the prototype components of TCM are directly absorbed into the body through the gastrointestinal tract to exert their effects; the other is the metabolites that are degraded by gastric acid, metabolized by liver, intestinal tract and other metabolic enzymes, or transformed by intestinal flora. Therefore, characterizing the profile of xenobiotics in vivo after holistic administration of TCM is the primary and indispensable link to reveal the active substances of TCM in vivo.

In the team's previous research, based on the clear outline of ZXG overall chemical profile, we completed in vivo xenobiotics analysis after overall administration of ZXG at the “drug effect dose”. A total of 229 exogenous components (106 prototypes, 123 metabolites) were detected by UHPLC-Q/TOF-MS in blood, urine, bile, feces and intestinal contents of rats after gavage of ZXG. Among them, 60 exogenous components are in plasma (37 prototypes, 23 metabolites). Flavonoids, alkaloids, lignans and steroidal saponins are the main structural types that

enter the blood.

The study depicted the xenobiotic profile of ZXG in rats (**Figure 1-3**), and the metabolic characteristics of various components in rats were elucidated, which provided a solid material basis for revealing the active components of ZXG in vivo.

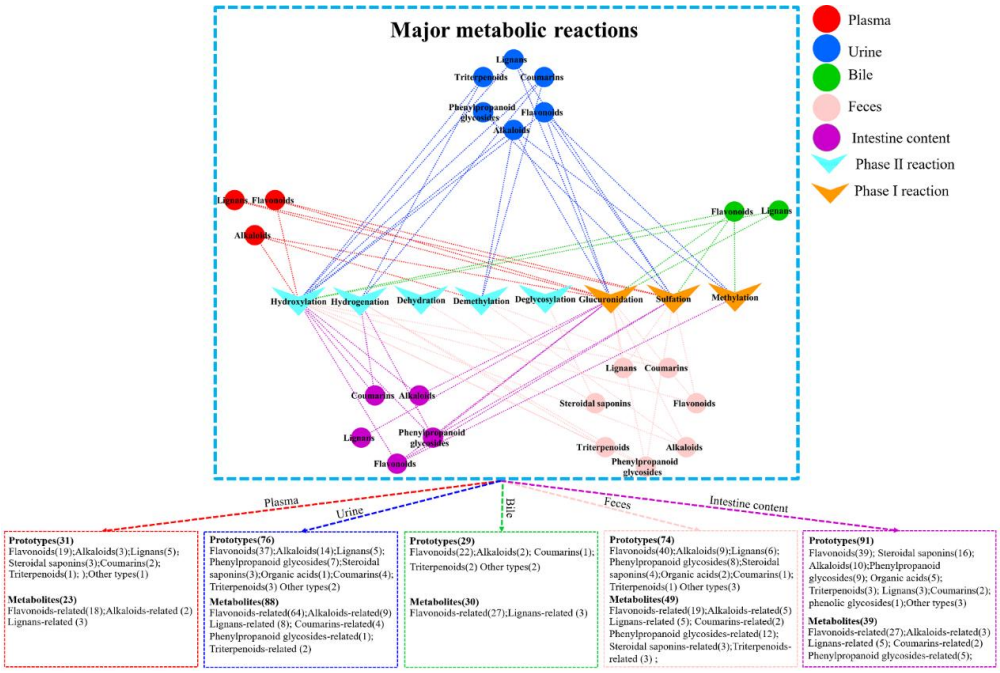


Figure 1-3 Metabolic Profile of ZXG in Rats

1.6 Research progress on content determination of multiple component of ZXG

Yuan Haijian et al.(Yuan et al., 2020) selected representative components with pharmacological activity in ZXG (Synephrine, Quercetin, Cinnamic acid, Magnolol, Naringin, Adenosine, Coumarin, Hesperidin, Honokiol, Neohesperidin) as the investigation indexes and established ZXG standard fingerprint by HPLC method. The established HPLC method can be used for the simultaneous determination of 10 chemical components in ZXG.

Li Mingchan et al.(李明潺 et al., 2022) used LC-MS technology to identify Q-marker (quality marker of TCM) in ZXG and carried out fingerprint methodology investigation. Finally, Hordenine, Acteoside, Neoeriocitrin, Narirutin, Naringin, Hesperidin, Neohesperidin, Isomerancin, Tricin, Magnolol, and Honokiol were identified as Q-markers of ZXG. The quality markers of ZXG were determined, which provided a basis for the quality evaluation of ZXG combined with the fingerprint and the mass transfer study of key components.

Xu Ruijie et al.(Xu et al., 2022) calibrated a total of 11 characteristic peaks through the characteristic map of ZXG reference samples and identified 6 characteristic peaks by comparison with chromatographic peaks of reference materials, namely Synephrine, Hesperidin, Cinnamic acid, Cinnamaldehyde, Honokiol and Magnolol. These six substances were used as index for the multi-component content determination of ZXG reference samples. But feature maps and content determination of chromatographic peaks only from 3 herbs, don't contain AMB and TF.

The indexes in the above content determination methods of ZXG are not comprehensive. These methods could not systematically describe the content distribution of each structure type. It is difficult to reflect the integrity of the formulae and cannot comprehensively and accurately evaluate the quality of ZXG from the aspects of structure and efficacy, so it could not ensure the safety， effectiveness and controllability of the drug.

1.7 Foundation of topic choice and contents of research

The chemical composition of ZXG is complex and contains a variety of structural components. At present, there is no quantitative method to systematically describe the content distribution of each structure type and effective component. The distribution characteristics of multi-component content are unclear, which will undoubtedly hinder the disclosure of pharmacodynamic substances, the disposal characteristics in vivo, and prevent the development of standardization，modernization and globalization.

The experiments in this study will be carried out using ultra - high performance liquid chromatography-triple quadrupole-mass spectrometry (UHPLC-TQ-MS), which will be used based on the research team's earlier investigations of the chemical profile and metabolic profile of ZXG. And as the representative components, which are the analytes, we select representative structural components of each herbal medicine, components absorbed into the blood, and cardiovascular-related active components.

A quantification approach was established to fully explain the content distribution properties of various ZXG components, which might serve as the foundation for clinical drug use, pharmacokinetic investigations, and

pharmacodynamic substance disclosure.

1.8 Technology route of research

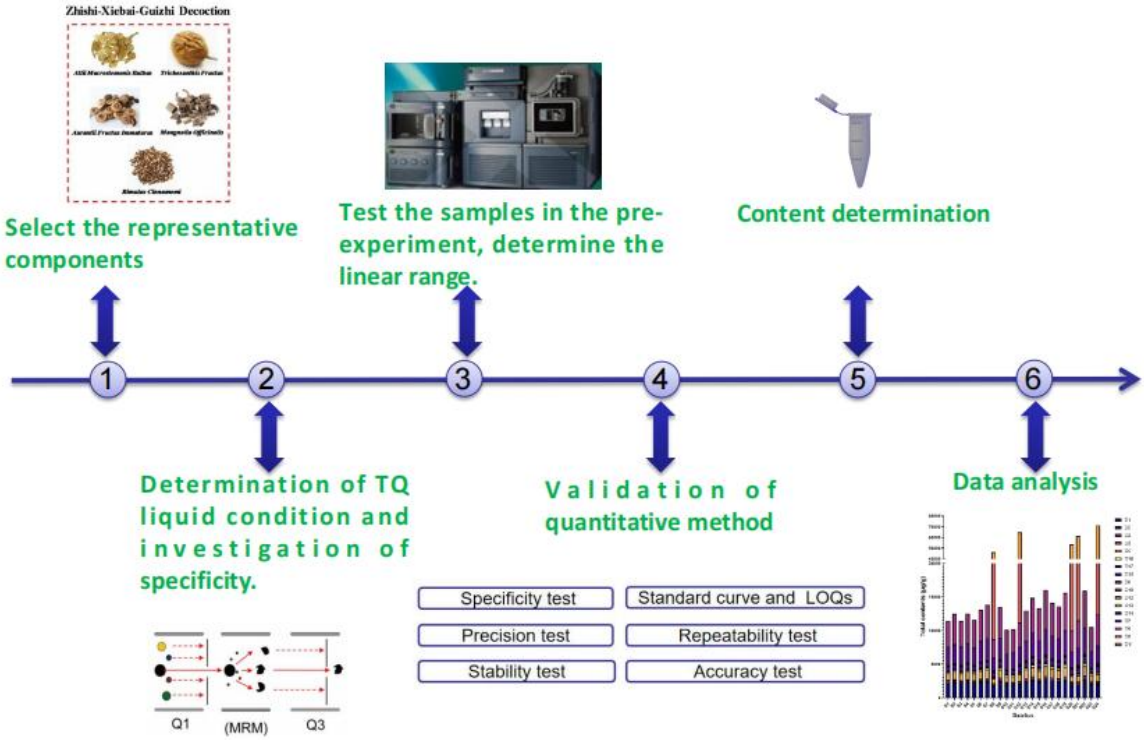


Figure 1-4 Technology Route of Research

2. Materials and methods

2.1 Materials

The 24 batches of AMB, TF, AFI, MO and CR were provided by Shenzhen Tsumura Medicine Co., Ltd. (Shenzhen, China). Synephrine, chlorogenic acid, syringin, magnoflorine, magnoloside A, narirutin, naringin, hesperidin, neohesperidin, coumarin, naringenin, hesperetin, limonin, honokiol, and magnolol were supplied by Chengdu Chroma-Biotechnology Co., Ltd. (25R)-timosaponin B II was purchased from Chengdu Push Biotechnology Co., Ltd. (-)-Loliolide was obtained from Shanghai Chengshao Biotechnology Co., Ltd. All the above standards possess a purity of 98 % by HPLC.

Water, LC–MS grade acetonitrile and methanol were supplied by Fisher Scientific (Fair Lawn, New Jersey, USA). LC–MS grade formic acid was purchased from Sigma Aldrich (St. Louis, USA). Other reagents were analytical grade and

commercially available.

2.2 Methods

2.2.1 Select the representative components

The chemical components of ZXG are diverse, and the quantitative components should cover all 5 herbal medicines and 10 structural types of the whole formula. At the same time, attention should be paid to the potential active components of ZXG, the main components into the blood, and the components related to coronary heart disease activity reported in the literature.

According to these requirements, some appropriate representative components were selected and subjected to the following experiment.

2.2.2 Preparation of sample solutions and standard

12 g of AFI and 12 g of MO were soaked in 1 L of water for 1 h and then decocted for 1.5 h. Following filtration, 30 g of TF, 20 g of AMB and 3 g of CR were added into the filtrate for another 1h of decocting. The solution was filtered and freeze-dried to yield lyophilized powders. The dried extract powders were stored at 4 ℃.

The lyophilized ZXG powder (25mg) was weighed and placed in a 50 mL volumetric flask, add about 48 mL of 60% methanol-water. Then sonicate it (power 250 W, frequency 100 kHz) for 30 min. The solution was removed and cooled to room temperature. Volume the solution with 60% methanol-water solution, shake well, centrifuge at 14000rpm/min for 10 min. The supernatant was taken and diluted 10-fold with 60% methanol-water to obtain the test solution.

Appropriate amounts of 17 reference standards were accurately weighed and dissolved with 60 % methanol-water (v/v) in a 10 mL volumetric flask. Then, a mixture of stock solutions of all 17 reference standards was serially diluted to a series of appropriate concentration ranges for the construction of calibration curves. All solutions were stored at 4 °C before analysis.

2.2.3 UHPLC-TQ-MS analysis

The chromatographic separation was carried on an AcquityTM UHPLC I-Class system (Waters Corp., Milford, MA, USA) coupled a Waters HSS T3 Column (2.1×100 mm, 1.8 μm,) at 40 °C. A binary gradient consisted of water (A) and

acetonitrile (B) (both containing 0.1% aqueous formic acid, v/v). The solvent was delivered at a flow rate of 0.3 mL/min by the following gradient elution program: 0 – 2.0 min, 2 – 20% B; 2.0 – 6.5 min, 20 – 30% B; 6.5 – 8.0 min, 30 – 50% B; 8.0 – 12 min, 50–100% B; 12 – 13 min, 100% B; 13 – 14 min, 100 – 2% B; 14 – 15 min, 2% B. Mass spectrometry detection was performed on Waters Xevo TQ-XS mass spectrometer equipped with an ESI source and operated under multiple reaction monitoring (MRM) mode. The optimal conditions of analysis were designed as follows: capillary voltage was set at 2 kV under positive ion, and -2 kV under negative mode; source temperature 150 °C; desolvation temperature 600 °C; desolvation gas 1000 L/h; and cone gas 150 L/h. In addition, the most appropriate cone voltage, collision energy, precursor ion, and daughter ion were optimized and displayed in **Table S1**. All data were collected and processed by QuanlynxTM program in Masslynx 4.1 software. So the Optimized Multiple Reactions Monitoring (MRM) Parameters of 17 Analytes are in **Table S1**.

2.2.4 Validation of quantitative method

2.2.4.1 Specificity

Analyzing the blank solvent, mixed control solution and a certain concentration of the test product is to obtain the specificity of 17 analytes.

2.2.4.2 Standard curve and lower limit of quantification

A series of seven standard solutions were prepared and analyzed by UHPLC-TQ-MS. Secondly, standard curves for analytes were constructed by plotting relationships between peak areas (y) and corresponding concentrations (x). The limits of quantification (LOQs) were estimated by analyzing a series of known concentration solutions which were acquired via diluting the mixed standard solution at the lowest concentration. The LOQs of analytes were evaluated at signal to noise ratios (S/N) of 10.

2.2.4.3 Instrument precision test

The intra-day precision was examined by six repetitive injections within one day, and the inter-day variability test was carried out for three consecutive days with six repetitive injections each day. The content, average content and RSD of the analytes

were calculated respectively as the result of intra-day precision and inter-day precision.

2.2.4.4 Method repeatability test and Sample stability test

To measure the repeatability， the same ZXG samples were determined for six replicates. And to investigate the stability， the ZXG sample solution was stored in sample manager and performed at 0, 2, 4, 8, 12, and 24 h, respectively. The content, average content and RSD of analytes were calculated as the results.

2.2.4.5 Method accuracy test

The accuracy of method was expressed by the recovery. The known mixed standard solutions at 3 different concentration levels (80 %/100 %/120 %) were put into the same ZXG sample, and three copies of each concentration were prepared. Recoveries were calculated by the following equation: recovery (%) = 100% × (observed amount –original amount) / spiked amount. The results were evaluated by the relative standard deviation (RSD) values.

2.2.5 24 Batches determination of content of ZXG.

The powder of 24 batches of ZXG was used to prepare the test solution (as shown in **2.2.2**).Then,the content of the analytes in 24 batches of ZXG was determined by standard curve method.

3. Results

3.1 Result 1 Select the representative components

According to the requirements of 2.2.3, 17 representative components (**Table 3-1** and **Figure 3-1**) were finally selected to establish the ZXG multi-components simultaneous quantitative method.

Table 3-1 Selection principle of representative components in ZXG

NO	Analyte	Type	Absorbed into the blood	Activity	Source
D1	Synephrine	A		√	AFI
D2	Syringin	PG		√	MO

D3	Chlorogenic acid	OA		√	MO,AFI ,CR
D4	Magnoloside A	P		√	MO,AFI
D5	Magnoflorine	A	√	√	MO
D6	(25R)-12α-OH-Timosaponin B II	S		√	AMB
D7	Narirutin	F	√	√	AFI
D8	Naringin	F	√	√	AFI
D9	Hesperidin	F	√	√	AFI
D10	Loliolide	O	√	√	MO,TK
D11	Neohesperidin	F	√	√	AFI
D12	Coumarin	C		√	CR
D13	Naringenin	F	√	√	AFI
D14	Hesperetin	F	√	√	AFI
D15	Limonin	T		√	AFI
D16	Honokiol	L	√	√	MO
D17	Magnolol	L	√	√	MO

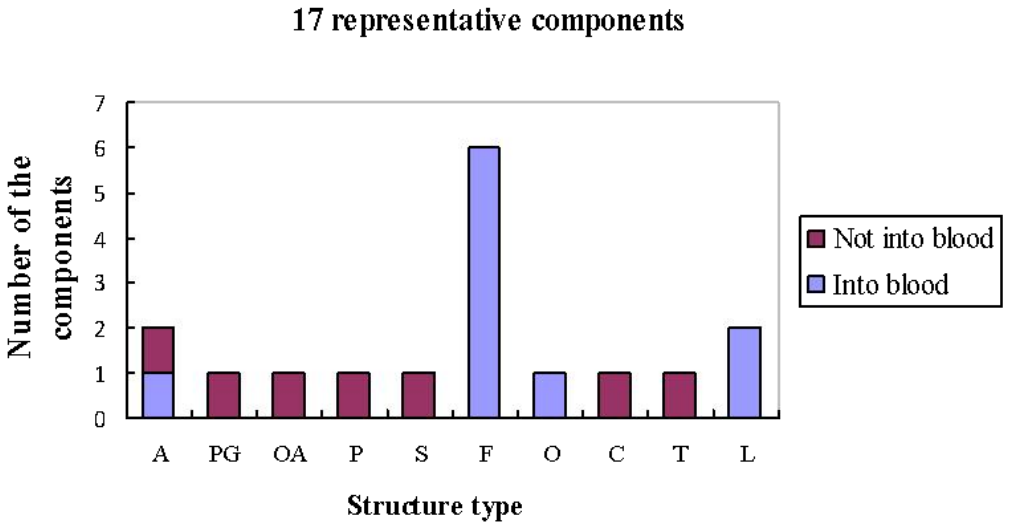


Figure 3-1 Properties of 17 Representative Components in ZXG

Note: F: flavonoids; A: alkaloids; L: lignans; P: phenylethanoid glycosides; PG: phenolic glycosides; S: steroidal saponins; OA: organic acid; C: coumarins; T: triterpenoids; O: other type.

3.2 Result 2 Method Validation

The quantification method of 17 analytes were validated. The calibration curves of 17 analytes displayed good linear correlation over a wide concentration range (**Table 3-2**), and the correlation coefficients (r^2) were higher than 0.999 in the test ranges. The LOQs for the 17 analytes ranged from 0.01 to 0.36 ng/mL (**Table 3-2**). The RSDs of intra-day and inter-day precisions were within the ranges of 0.7 – 3.1% and 0.8 – 3.0% (**Table 3-3**), respectively. The results of repeatability were no more than 2.0% (**Table 3-3**). According to the stability test results (RSDs < 4.0%), it could be found that ZXG sample solution was stable within 24 h (**Table 3-3**). The mean recoveries varied from 98.33% to 102.22% with RSDs less than 2.6% (**Table 3-4**).

All these values were acceptable, indicating that the newly developed quantification method of multiple (17) representative components of ZXG decoction by UHPLC-TQ-MS was accurate, repeatable, reliable, and considered satisfactory for the following content determination of 17 analytes in 24 batches ZXG.

Table 3-2 Calibration Curves, Linear Ranges, LOQs of 17 Analytes in ZXG.

Analyte	Calibration Curves	Linear Range (ng/mL)	r^2	LOQ (ng/mL)
Synephrine	$y = 43044.10\,x + 38580.40$	6.57 -365.00	0.9999	0.03
Syringin	$y = 26.06\,x + 6.99$	2.18 – 121.00	0.9995	0.36
Chlorogenic acid	$y = 12661.10\,x + 736.06$	0.19 – 42.00	0.9999	0.01
Magnoloside A	$y = 930.60\,x - 425.62$	3.31 – 736.00	0.9997	0.07
Magnoflorine	$y = 11408.00\,x + 1962.22$	0.77 – 42.88	0.9995	0.18
(25R)-12α-OH-Timosaponin B II	$y = 221.57\,x – 42.56$	0.28 – 15.38	0.9992	0.10
Narirutin	$y = 498.22\,x - 157.11$	3.06 – 680.00	0.9993	0.07
Naringin	$y = 211.55\,x + 194.60$	23.29 – 5175.00	0.9999	0.23
Hesperidin	$y = 1009.59\,x - 30.95$	4.50 – 1001.00	0.9997	0.20

Loliolide	y = 100570.00 x+ 2701.11	0.13 – 7.42	0.9996	0.02
Neohesperidin	y = 303.53 x+ 208.73	30.24 – 6720.00	0.9992	0.16
Coumarin	y = 68952.30 x+ 7433.61	0.15 – 32.80	0.9995	0.01
Naringenin	y = 65974.10 x+ 6260.99	0.28 – 61.20	0.9995	0.01
Hesperetin	y = 53535.90 x+ 3421.70	0.17 – 36.77	0.9998	0.01
Limonin	y = 4086.64 x+ 557.489	0.64 -148.00	0.9998	0.15
Honokiol	y = 591.12 x+ 30.43	0.40 – 88.00	0.9992	0.01
Magnolol	y = 3584.73 x+ 1619.47	0.33 – 74.56	0.9998	0.01

Table 3-3 Repeatability, Precision, Stability Results of 17 Analytes in ZXG.

Analyte	**Repeatability**		**Precision RSD %**		**Stability**
	Content (μg/g) ± SD	**RSD% (n=6)**	**Intra-Day (n=6)**	**Inter-Day (n=18)**	**RSD% (n=6)**
Synephrine	1710.60 ± 72.28	0.4	0.7	0.8	0.7
Syringin	211.38 ± 3.10	1.5	2.1	2.0	1.8
Chlorogenic acid	150.81 ± 2.82	1.9	1.5	1.6	1.8
Magnoloside A	692.67 ± 4.44	0.6	2.8	2.5	1.7
Magnoflorine	120.32 ± 1.41	1.2	1.3	1.3	1.3
(25R)-12α-OH-Timosaponin B II	51.45 ± 0.63	1.2	1.5	1.8	1.5
Narirutin	1951.92 ± 29.50	1.5	1.6	1.8	1.4
Naringin	16889.99 ±	1.5	2.2	1.9	1.6

	246.46				
Hesperidin	2775.594 ± 30.52	1.1	1.8	1.8	1.3
Loliolide	29.50 ± 0.54	1.8	3.0	2.4	3.2
Neohesperidin	20454.49 ± 136.85	0.7	1.7	3.0	1.4
Coumarin	64.33 ± 0.82	1.3	3.1	2.1	4.0
Naringenin	84.85 ± 1.03	1.2	1.6	1.1	1.6
Hesperetin	88.96 ± 1.76	2.0	1.2	0.9	1.3
Limonin	630.35 ± 11.96	1.9	2.0	1.6	2.0
Honokiol	141.79 ± 2.68	1.9	2.1	2.6	2.7
Magnolol	127.84 ± 1.24	1.0	1.20	1.1	1.2

Table 3-4 Recoveries of 17 Analytes in ZXG

Analyte	**Original (μg)**	**Added (μg)**	**Detected (μg) (n=3)**	**Recovery (%) (n=9)**	**RSD (%)**
Synephrine	21.09	25.20	25.59 ± 0.19	100.76 ± 0.0.91	0.9
		21.00	21.06 ± 0.18		
		16.80	16.87 ± 0.14		
Syringin	2.60	3.12	3.05 ± 0.027	100.21 ± 2.53	2.5
		2.60	2.67 ± 0.015		
		2.08	2.09 ± 0.050		
Chlorogenic acid	1.90	2.28	2.28 ± 0.035	100.32 ± 2.21	2.2
		1.90	1.92 ± 0.057		
		1.52	1.52 ± 0.032		
Magnoloside A	8.70	10.44	10.67 ± 0.14	101.10 ± 1.76	1.7
		8.70	8.73 ± 0.15		

		6.96	7.01 ± 0.15		
		1.80	1.79 ± 0.032		
Magnoflorine	1.52	1.50	1.52 ± 0.032	100.87 ± 2.00	2.0
		1.20	1.21 ± 0. 025		
		0.77	0.77 ± 0.023		
(25R)-12α-OH-Timosaponin B II	0.63	0.64	0.64 ± 0.021	100.45 ± 2.43	2.4
		0.51	0.52 ± 0.0080		
		29.40	29.31 ± 0.20		
Narirutin	24.54	24.50	24.58 ± 0.12	100.64 ± 1.48	1.5
		19.60	19.97 ± 0.40		
		255.60	257.06 ± 5.62		
Naringin	231.01	213.00	210.45 ± 2.59	99.75 ± 2.29	2.3
		170.40	170.17 ± 5.98		
		41.19	41.00 ± 0.21		
Hesperidin	34.33	34.33	33.58 ± 0.63	98.33 ± 1.7	1.7
		27.46	26.81 ± 0.59		
		0.42	0.42 ± 0.0045		
Loliolide	0.34	0.35	0.35 ± 0.0066	99.50 ± 1.31	1.3
		0.28	0.28 ± 0.0034		
		309.00	308.59 ± 6.73		
Neohesperidin	257.92	257.50	260.04 ± 5.80	100.95 ± 2.38	2.4
		206.00	210.11 ± 6.33		
		0.91	0.93 ± 0.010		
Coumarin	0.73	0.76	0.77 ± 0.0081	102.22 ± 2.09	2.0
		0.60	0.62 ± 0.022		
		1.20	1.22 ± 0.015		
Naringenin	1.02	1.10	1.03 ± 0.019	101.52 ± 2.45	2.4
		0.80	0.80 ± 0.027		
Hesperetin	1.09	1.32	1.31 ± 0.033	101.24 ± 2.67	2.6

		1.10	1.11 ± 0.0031		
		0.88	0.92 ± 0.0092		
		9.00	9.12 ± 0.15		
Limonin	7.54	7.50	7.58 ± 0.14	100.60 ± 1.61	1.6
		6.00	5.96 ± 0.044		
		1.98	2.00 ± 0.067		
Honokiol	1.63	1.65	1.62 ± 0.031	99.63 ± 2.38	2.4
		1.32	1.32 ± 0.0063		
		1.92	1.93 ± 0.034		
Magnolol	1.61	1.60	1.64 ± 0.20	101.83 ± 1.62	1.6
		1.28	1.31 ± 0.0088		

3.3 Result 3 Sample analysis

A total of 17 analytes in 24 batches of ZXG were quantified through the UHPLC-TQ-MS methods. The quantitative results are all in **Table 3-5**, and **Figure 3-2** is made by the data from **Table 3-5**.

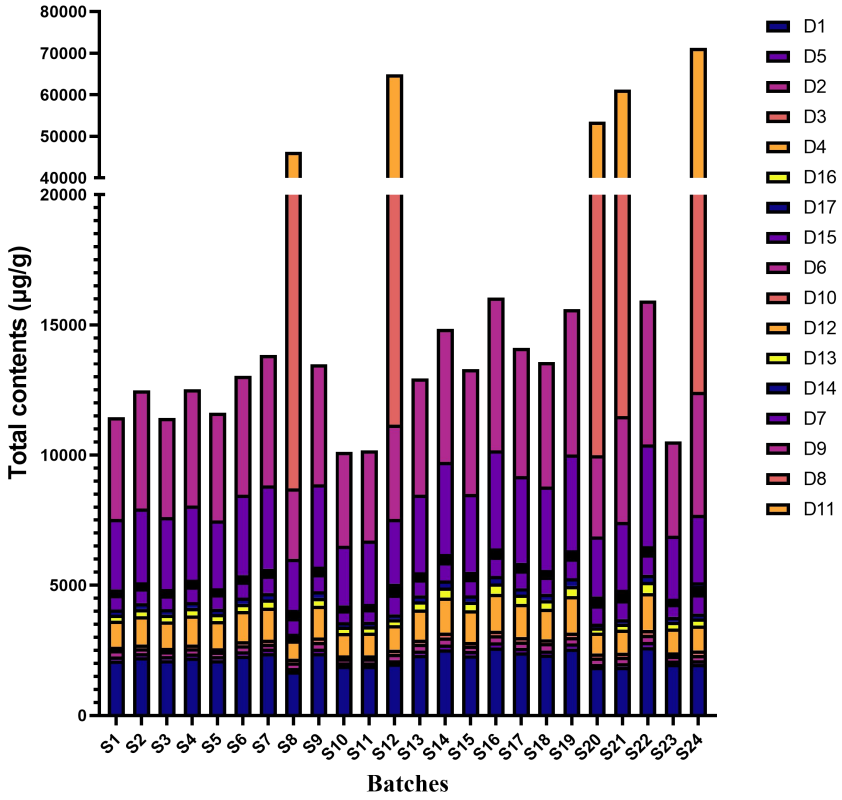


Figure 3-2 Total Contents of 17 Analytes in 24 Batches of ZXG

Table 3-5 Contents of 17 Analytes in 24 Batches of ZXG Samples (μg/g)

Analyte	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
D1	2116.60	2237.89	2125.41	2218.94	2130.80	2302.95	2389.16	1699.51	2389.23	1910.23	1911.28	1989.98	2327.23	2534.93	2315.52	2608.19	2424.50	2335.31	2571.71	1866.55	1870.47	2625.57	1975.58	1975.67
D2	241.02	187.74	186.55	202.70	159.15	230.89	190.08	211.27	265.72	168.29	158.01	238.76	257.39	277.02	192.51	268.19	245.76	276.49	246.35	232.88	244.80	271.28	191.01	204.52
D3	130.21	142.95	130.11	145.68	131.20	151.19	154.24	152.32	163.11	108.71	112.35	156.90	152.29	177.14	153.74	182.22	167.38	152.60	178.03	166.07	162.99	195.37	125.32	172.54
D4	1002.00	1082.96	1021.83	1108.21	1053.92	1157.32	1232.76	696.01	1224.42	857.10	871.10	942.20	1157.19	1349.13	1205.99	1411.50	1274.19	1176.13	1389.24	791.28	875.78	1416.91	915.87	955.47
D5	148.81	160.41	151.21	159.42	157.75	166.51	174.48	121.56	174.40	121.63	129.36	139.76	169.68	187.49	170.11	202.58	175.38	165.11	193.73	119.33	137.28	197.25	127.62	148.93
D6	35.82	31.41	48.15	51.10	41.17	32.33	34.88	50.36	51.08	35.00	38.53	52.55	39.45	55.99	52.65	46.95	54.88	28.60	57.55	40.20	42.62	50.24	32.33	62.98
D7	2697.67	2829.80	2760.74	2856.00	2613.03	3080.26	3197.13	1964.34	3159.59	2283.00	2436.46	2488.13	2982.42	3533.64	2992.85	3772.04	3330.43	3208.41	3675.61	2307.30	2608.43	3895.39	2427.98	2596.44
D8	-	-	-	-	-	-	-	17011.46	-	-	-	22808.57	-	-	-	-	-	-	-	19736.05	21853.50	-	-	24110.13
D9	3913.95	4541.20	3811.80	4457.88	4132.62	4574.21	5019.62	2750.42	4608.34	3599.00	3456.99	3611.98	4457.67	5114.64	4792.00	5868.05	4921.68	4779.82	5589.39	3110.97	4062.52	5532.06	3610.72	4712.64
D10	28.34	30.77	28.84	30.51	28.85	31.74	33.87	29.11	33.58	24.01	24.45	31.34	33.49	37.20	33.45	39.45	36.17	34.05	39.96	29.72	33.60	40.37	25.76	31.69
D11	-	-	-	-	-	-	-	20570.30	-	-	-	30895.38	-	-	-	-	-	-	-	23818.28	27841.86	-	-	34771.40
D12	81.57	84.02	79.35	85.94	81.08	89.28	94.92	63.68	96.04	68.79	67.11	83.98	94.93	103.90	92.66	111.40	102.96	96.37	109.43	72.43	83.38	111.84	70.76	90.74
D13	41.70	44.33	41.86	46.38	41.21	47.34	50.73	84.48	51.97	34.70	35.75	117.13	50.27	56.01	50.34	58.67	52.70	50.69	59.41	102.47	118.27	61.44	37.92	130.62
D14	35.49	40.10	37.12	40.38	35.40	40.97	45.18	87.09	45.70	31.10	31.60	129.35	43.90	52.04	44.93	57.36	47.62	45.60	55.31	107.19	125.69	54.44	34.10	142.26
D15	556.12	569.69	538.73	604.80	552.08	627.20	672.45	626.87	666.58	464.16	481.00	759.84	614.59	712.48	629.00	748.20	681.47	655.93	760.25	695.14	719.43	782.00	494.53	736.80
D16	225.72	278.18	261.51	296.54	262.52	280.70	312.71	140.33	303.78	241.38	242.07	236.15	317.38	386.01	338.89	392.27	349.69	325.29	399.26	204.20	241.59	415.82	268.02	269.29
D17	200.45	223.46	205.03	218.83	207.00	231.71	248.95	127.46	247.17	169.41	176.24	180.94	235.80	269.90	236.12	286.28	249.95	242.38	286.93	145.88	170.13	285.15	180.87	190.22

4. Discussion

In the content determination of 24 batches of ZXG, it could be found that the contents of flavonoids are the most abundant in ZXG. Also, flavonoids are previously reported to have abundant anti-CHD activities. Clearly, naringin, neohesperidin, narirutin and hesperidin are the dominant components in the flavonoids. The compounds with the second-highest content levels are alkaloids, followed by phenylethanoid glycosides, triterpenoids, lignans and organic acids, while steroidal saponins, phenolic glycosides and coumarins were found to be lower in all samples.

And the contents of neohesperidin and naringin were the highest in ZXG, but they were only detected in the ZXG samples of some batches. This result was related to the plant origin of AFI, and neohesperidin and naringin both only existed in Citrus aurantium L. (Suancheng), but not in Citrus sinensis Osbeck (Tiancheng).

5. Conclusion

In this research, we established a quantification approach to fully explain the content distribution properties of various ZXG components (17 representative components) and comprehensive evaluate the quality of ZXG, which might serve as the foundation for clinical drug use, pharmacokinetic investigations, and pharmacodynamic substance disclosure.

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Appendix

Table S-1 The Optimized Multiple Reactions Monitoring (MRM) Parameters of 17 Analytes

NO	Analyte	T_R	Ion mode	Parent ion (*m/z*)	Daughter ion (*m/z*)	Cone (V)	Collision (eV)
D1	Synephrine	0.92	+	168.10	150.09	4	4
D2	Syringin	2.82	-	417.14	209.08	13	12
D3	Chlorogenic acid	2.89	+	355.10	163.04	23	12
D4	Magnoloside A	3.30	-	623.20	161.02	37	39
D5	Magnoflorine	3.39	+	342.17	265.09	30	13
D6	(25R)-12α-OH-Timosaponin B II	4.33	+	919.49	397.31	31	29
D7	Narirutin	4.50	+	581.19	273.08	29	9
D8	Naringin	4.78	+	581.19	273.08	22	11
D9	Hesperidin	5.04	+	611.20	303.09	24	10
D10	Loliolide	5.13	+	197.12	179.11	31	13
D11	Neohesperidin	5.34	+	611.20	303.09	27	10
D12	Coumarin	6.56	+	147.04	103.05	14	15
D13	Naringenin	8.37	+	273.08	153.02	28	22
D14	Hesperetin	8.65	+	303.09	153.02	32	24
D15	Limonin	9.44	+	471.20	425.20	22	18
D16	Honokiol	10.53	-	265.12	224.08	18	23
D17	Magnolol	10.95	-	265.12	247.11	30	23

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