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Untargeted Metabolomics Profiling and Global Semi-quantitation of a Prescription Chinese Herbal Medicine Formula Yinqiaosan Using UPLC-QTOF-MS with a Single Exogenous Reference Internal Standard

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Abstract
Yinqiaosan is a classic Chinese herbal medicine formula that has been used to treat various bacterial and viral infections by Chinese medicine doctors for over two centuries. In this work, we developed a comprehensive qualitative and quantitative method for identification, quantitation, and quality assessment of chemical constituents of Yinqiaosan formula in four different preparation forms (i.e., decoction, granule, pill, and tablet), which employed ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry with a single exogenous reference internal standard for untargeted metabolomics profiling and global semiquantitative analysis. The use of a single exogenous reference internal standard permitted not only qualitative and quantitative analyses of multiple herbal components in a single instrument run, but also cross-comparison of chemical contents in between all four Yinqiaosan preparation forms. The acquired mass chromatograms were analyzed, quantitated, and compared using multivariate data analysis for similarities and differences of chemical constituents in four Yinqiaosan preparation forms. For the first time, we were able to identify over 100 chemical constituents from each preparation form using the available database. Among the 49 commonly identified compounds in the 4 Yinqiaosan preparation forms, 16 have been reported to have pharmacological activities, which may be used in a network pharmacology study of Yinqiaosan for exploring the underlying mechanism of the herbal formula.

ABBREVIATIONS

CHM	Chinese herbal medicine
CV	coefficient of variation
EBM	evidence-based medicine
ESI	electrospray ionization
IS	internal standard
PCA	principal component analysis
PLS-DA	partial least squares discriminant analysis
QC	quality control
Q-marker	quality marker
RCT	randomized controlled trial
SIM	selected ion monitoring
TCM	traditional Chinese medicine
TIC	total ion current
UPLC-QTOF-MS	ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry

Introduction

CHM formulas are the key components of TCM interventions, which have many preparation forms including decoctions, granules, pills, tablets, capsules, powders, medicated teas, medicated wines, etc. [1]. Despite the widespread use of CHM formulas in Asia and their growing use in the West, the fundamental issue hindering CHM integration into the Western mainstream health care system is that rigorous scientific evidence of CHM efficacy and safety obtained through RCTs or systematic reviews of RCTs has been limited from EBM perspectives [2]. This is mainly attributed to two major defects in the RCTs to date: (1) improper differentiation or reporting of a TCM pattern or syndrome since pattern differentiation is crucial in TCM for prescribing CHM therapy, and (2) the lack of a comprehensive quality assessment and control of CHM formulas since the chemical constituents in medicinal plants vary with geographic origin and cultivar, parts in use, time of harvest, ecological environment and potential contamination with heavy metals, pesticides, or mycotoxins, processing and detoxification method as well as various forms of preparations [1].

The methods currently adopted by the international pharmacopeial monographs [3–5] for identification and quality assessment of medicinal plants and CHM formulas emphasize the qualitative and quantitative determination of a group of subjectively selected Q-marker constituents that may or may not have a therapeutic effect. These methods can't distinguish whether a marker constituent is endogenous or falsified because no information on qualitative and quantitative chemical profiles of medicinal plants or CHM formulas is obtained. Therefore, comprehensive qualitative and quantitative chemical analysis methods are much more desirable, not only for phytochemical profiling and quality assessment of medicinal plants and CHM formulas but also for detection of counterfeits. Untargeted metabolomics profiling using LC-QTOF-MS has been successfully applied to fingerprinting herbal medicines [6–8]. If this technique combines with the use of a single exogenous reference IS for semiquantitative analysis and PCA for pattern recognition, it would provide a more adequate method for identification of chemical constituents and quality assessment of medicinal plants and CHM formulas. This work demonstrates such an attempt.

Yinqiaosan is a classic CHM formula developed by the Qing Dynasty famous Chinese doctor Wu Jutong (1758–1836). It is composed of nine herbs including Jinyinhua (*Flos lonicerae*), Lianqiao (*Fructus forsythiae*), Bohe (*Herba menthae*), Jingjie (*Herba schizonepetae*), Niubangzhi (*Fructus arctii*), Jiegeng (*Radix platycodonis*), Dandouchi (*Semen Sojae preparatum*), Gancao (*Radix glycyrrhizae*), and Danzhuye (*Herba lophatheri*) [9]. Based on CHM classification, Yinqiaosan has cool energy, an acrid taste, and properties of releasing exterior, clearing heat and removing toxicity from human bodies [10]. It has been used by TCM doctors for over two centuries as treatment for various bacterial and viral infections such as influenza [11], hand, foot, and mouth disease [12], pharyngitis [13], pneumonia [14], acute tonsillitis [15], measles [16], and mumps [17]. A recent randomized clinical trial indicated that Yinqiaosan plus another CHM formula, Maxingshigan, was as effective as oseltamivir (also known as Tamiflu) for the treatment of H1N1 influenza A virus infections [18].

Yinqiaosan was originally prepared in the form of a decoction. Due to the ease of use, carry, and storage, Yinqiaosan has been prepared in various forms over the course of history, such as Yinqiaosan peifangkeli (granule), Yinqiaojieduwan (pill), and Yinqiaojiedupian (tablet), which are being used interchangeably in clinical interventions. Currently, there are various forms of Yinqiaosan produced by numerous Chinese manufacturers [19]. The lack of comprehensive qualitative and quantitative chemical analysis methods for chemical profiling and quantitation not only impede complete quality assessment of various Yinqiaosan preparation forms, but also confuse the assessment of outcomes of clinical studies [2, 20]. Therefore, we have developed a comprehensive qualitative and quantitative method that combines untargeted metabolomics profiling and global semiquantitative analysis with the use of a single exogenous IS, as well as pattern recognition through PCA for identification, quantitation, and quality assessment of Yinqiaosan in four preparation forms (decoction, granule, pill, and tablet).

Results and Discussion

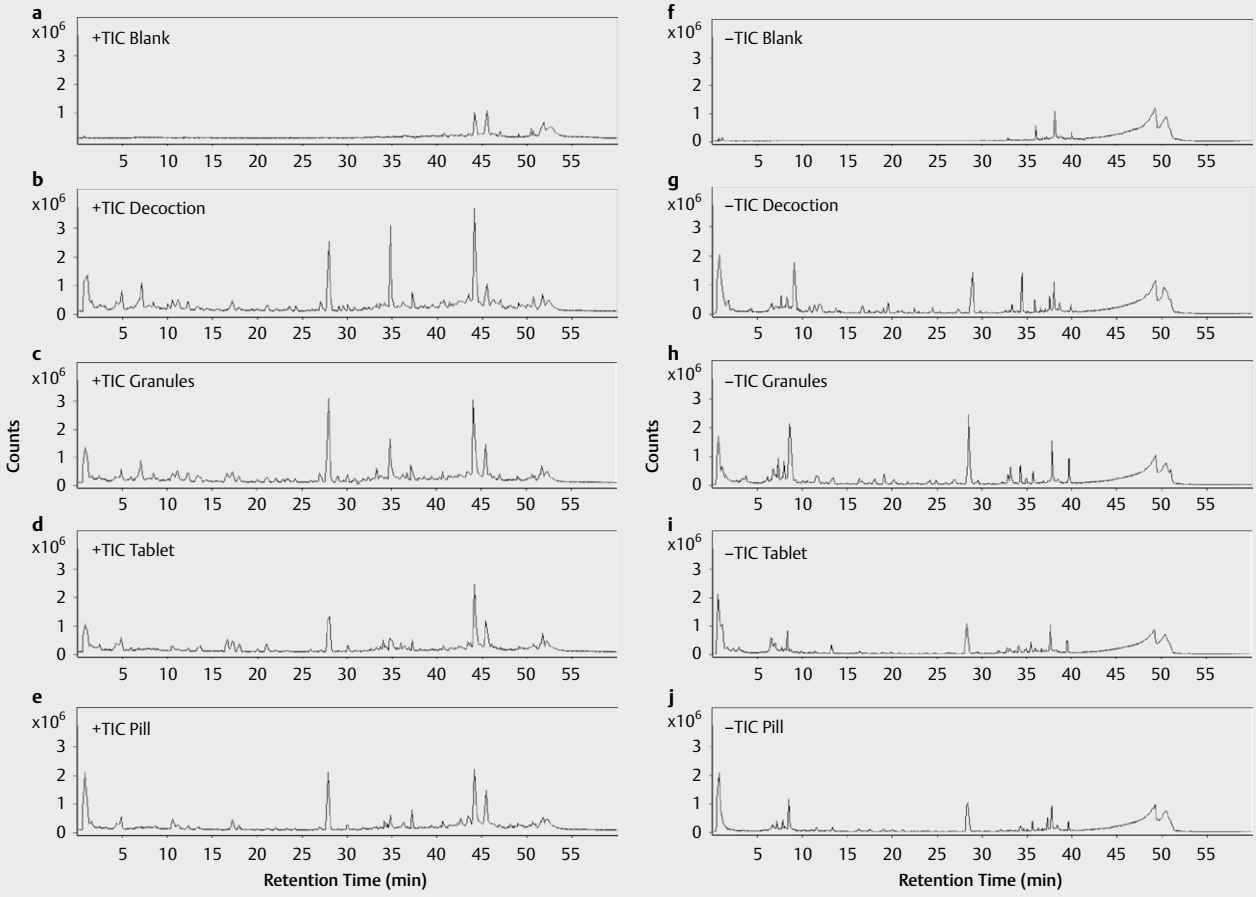
One of the key technical advances of the method developed is the implementation of a single exogenous reference IS (etoposide-d3), which serves multiple roles including selection of retention time and mass shift windows for UPLC-QTOF-MS analysis, assessement and correction of a sample matrix effect, peak normalization for multivariate data analysis, and global semiquantitative analysis of multicomponents in each Yinqiaosan preparation form, as well as cross-comparison of chemical contents in between various Yinqiaosan preparation forms.

Our experimental data (not shown) indicated that there were no chromatographic or mass spectrometric interferences to the IS in the blank solutions and samples of the four Yinqiaosan preparation forms. The matrix effect of each Yinqiaosan preparation form on the mass spectrometric detection of the IS expressed as matrix factor was calculated by the mean peak area of the IS spiked in samples of each Yinqiaosan preparation form over that of the IS spiked

▶ **Table 1** Matrix effects of Yinqiaosan samples on mass spectrometric detection of the IS[a].

Sample matrix (n = 3)	ESI mode	PA_{IS}[b] in extracted sample matrix ± SD[c]	PA_{IS} in solution ± SD	MF_{IS}[d] ± SD
Decoction	+	$(4.75 \pm 0.06) \times 10^5$	$(5.4 \pm 0.2) \times 10^5$	0.88 ± 0.03
	−	$(2.34 \pm 0.02) \times 10^6$	$(2.68 \pm 0.03) \times 10^6$	0.87 ± 0.01
Granule	+	$(5.4 \pm 0.2) \times 10^5$	$(5.4 \pm 0.2) \times 10^5$	1.00 ± 0.05
	−	$(2.52 \pm 0.03) \times 10^6$	$(2.68 \pm 0.03) \times 10^6$	0.94 ± 0.02
Tablet	+	$(5.8 \pm 0.2) \times 10^5$	$(5.4 \pm 0.2) \times 10^5$	1.07 ± 0.05
	−	$(2.37 \pm 0.04) \times 10^6$	$(2.68 \pm 0.03) \times 10^6$	0.88 ± 0.02
Pill	+	$(4.83 \pm 0.04) \times 10^5$	$(5.4 \pm 0.2) \times 10^5$	0.89 ± 0.03
	−	$(2.43 \pm 0.01) \times 10^6$	$(2.68 \pm 0.03) \times 10^6$	0.91 ± 0.01

[a]IS = 1.69 μM, [b]A_{IS} = mean peak area of the spiked IS, [c]SD = standard deviation, [d]MF_{IS} = (PA_{IS} in the extracted sample matrix)/(PA_{IS} in the solution).

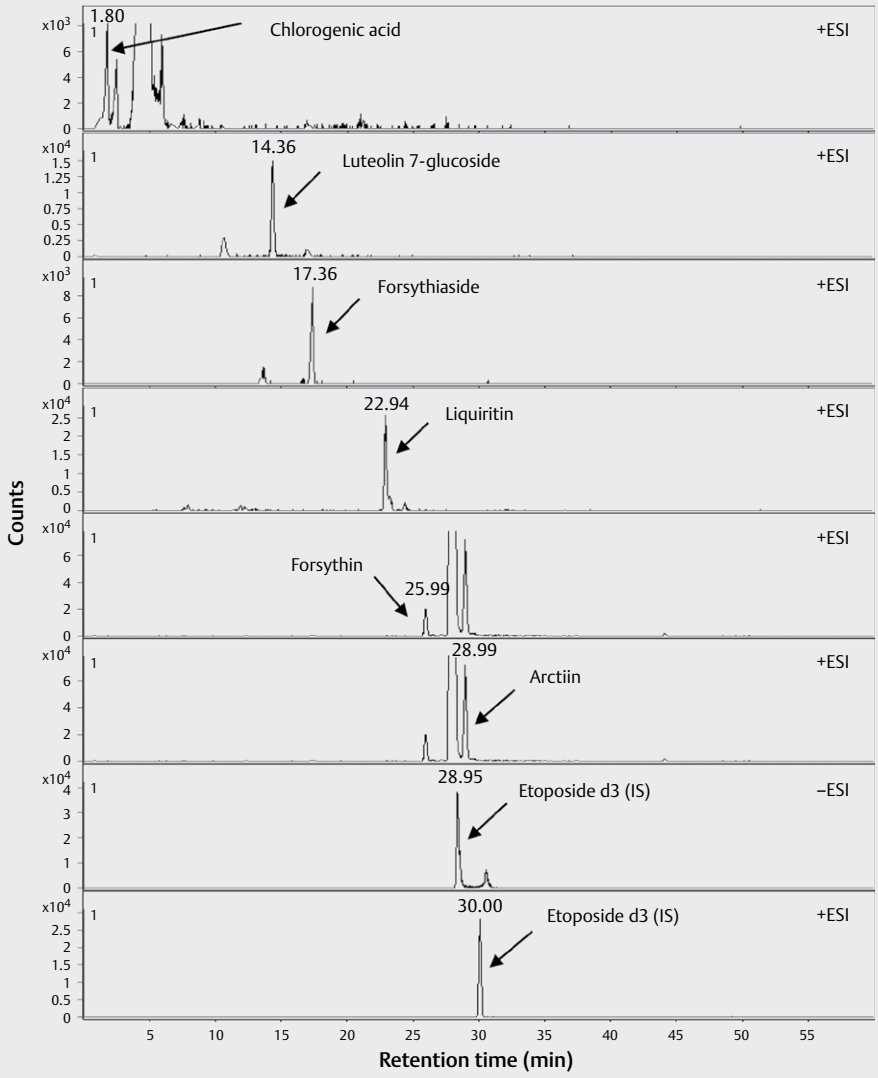


▶ **Fig. 1** Representative TIC chromatograms of the solution blank and samples of various Yinqiaosan preparation forms by both positive ionization mode (**a–e**) and negative ionization mode (**f–j**).

in the blank solutions. The matrix factors of four Yinqiaosan preparation forms ranged from 0.87–1.07 (▶ **Table 1**), indicating there was no significant signal suppression or enhancement of the IS in any of the sample matrices.

For untargeted metabolomics profiling, constituent identification, and global semiquantitation of chemical constituents in Yinqiaosan formula, triplicate samples were prepared for each Yinqiaosan preparation form and the solution blank. A total of 15 Yinqiaosan samples were analyzed using the UPLC-QTOF-MS method developed. The chromatographic and spectra (MS and MS/MS) data were acquired by both positive and negative ESI modes. The representative TIC chromatograms are shown in ▶ **Fig. 1**. Chemical constituents of each Yinqiaosan preparation form were extracted after subtracting TIC chromatograms of the solution blanks from those of Yinqiaosan sample solutions.

There are several nonvolatile, water-soluble Q-markers from the nine herbs of Yinqiaosan [3]. To illustrate this targeted feature of the method developed, these Q-markers were targeted and ex-



▶ **Fig. 2** Representative SIM chromatograms of six marker constituents of Yinqiaosan along with the IS at a concentration of 1.69 µM.

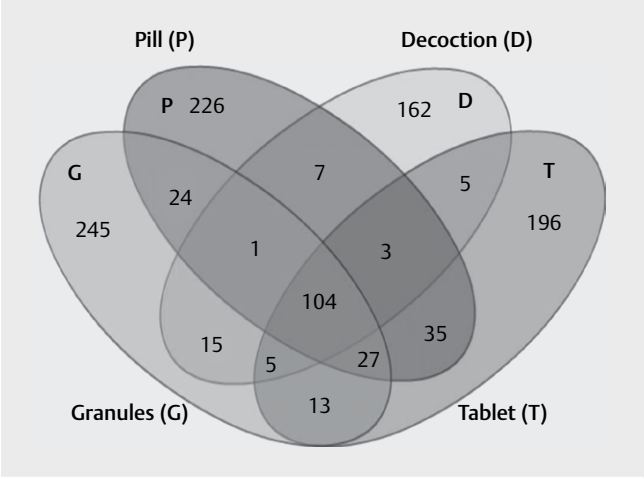
tracted. The representative SIM chromatograms of six Q-markers (i.e., chlorogenic acid, luteolin 7-glucoside, forsythiaside, liquiritin, forsythin, and arctiin) are provided in ▶ **Fig. 2**.

Due to the shortage of standard reference material and the cost concern, determination of multicomponents for quality assessment of CHM formula is currently not required by the Chinese regulatory agency. In the case of Yinqiaosan products, only one of the Q-markers (i.e., arctiin, chlorogenic acid, or forsythin) is regulated by the Chinese Pharmacopoeia [3]. Since Yinqiaosan products contain multiple bioactive components, the current methods for single-component analysis can neither assess the quality of Yinqiaosan products objectively nor detect counterfeits effectively. The method developed in this work provides a much better technical solution for quality assessment of Yinqiaosan products, which not only profiles a product but also quantitates multiple Q-markers simultaneously.

Identification of chemical constituents in each Yinqiaosan preparation form was accomplished by following the procedure detailed in "Data processing, constituent identification, and statistical analysis" in the "Materials and Methods" section. The data obtained were then subjected to molecular feature extractions using possible ion adducts, isotope patterns, and charge states as well as a preset retention time and mass window. It is worth noting that the post-processing filter of the data processing software was set at 3 out of 3 to ensure each molecular feature extracted presented in all three mass chromatograms of the triplicate measurements for each Yinqiaosan preparation form. The total number of constituents identified with chemical names and the number of constituents identified with molecular formulas in each preparation form are summarized in ▶ **Table 2**. The number of chemical constituents found in decoction, granule, pill, and tablet forms of Yinqiaosan were 302, 434, 427, and 388, respectively. Among the 4 preparation forms, there were 104 common constituents (▶ **Fig. 3**), 49 identified with both chemical names and formulas (▶ **Table 3**) and 55 identified only with chemical formulas (▶ **Table 1S**, Supporting Information). The MS/MS spectra of the 49 chemical constituents

▶ **Table 2** Summary of chemical constituents found in four Yinqiaosan preparation forms.

Yinqiaosan	Constituents				Common constituents			
Forms	ESI mode	Identified	Unidentified	Total	ESI mode	Identified	Unidentified	Total
Decoction	+	158	37	302	+	46	21	104
	−	21	86					
Granule	+	79	177	434				
	−	36	142					
Tablet	+	91	163	388	-	3	34	
	−	24	110					
Pill	+	67	186	427				
	−	38	136					



▶ **Fig. 3** Venn diagram of the chemical constituents found in each preparation form of Yinqiaosan.

commonly identified in all four Yinqiaosan preparation forms are provided in (▶ **Fig. 1S**, Supporting Information).

Since each Yinqiaosan preparation form exerts a similar therapeutic effect and is used to treat the same illnesses, it is reasonable to think that pharmacologically active constituents are among the 49 commonly identified chemical constituents in all four preparation forms, whereas the unique chemical constituents in each preparation form (▶ **Tables 2S–9S**, Supporting Information) may come from the herbs used by the manufacturers, where chemical constituents may vary with geographic origin and cultivar, time of harvest and the ecological environment of the herbs, potential agricultural pollutions of pesticides and herbicides or industrial cross-contamination with other pharmaceuticals as well as side reactions and by-products associated with the unique manufacturing conditions (e.g., temperature, pressure, time, solvent, additives, etc.) of each Yinqiaosan preparation form. This study shows that product profiling is an indispensable part of quality assessment, and the untargeted metabolomics approach not only allows us to profile but also fingerprint an herbal product in terms of Q-markers, origin and cultivars, potential contaminations, and signature constituents associated with a particular preparation or manufacturing process.

Multivariate analysis of the acquired MS data (i.e., exact mass to retention time pair and normalized peak area) was first carried out with an unsupervised PCA score plot to assess the similarities of chemical constituents among the four Yinqiaosan preparation forms and the reproducibility of replicate samples of each preparation form by the UPLC-QTOF-MS method, then visualized by a supervised PLS-DA score plot to establish recognition patterns of the four Yinqiaosan preparation forms.

As shown in the PCA score plot (▶ **Fig. 4a**), the differences in chemical constituents among the four Yinqiaosan preparation forms were apparent. The first two principal components encompassed 72.3 % of the total variance, and the tight grouping of triplicate measurements on the samples of each preparation form indicated that the UPLC-QTOF-MS method developed had excellent reproducibility. The PLS-DA score plot (▶ **Fig. 4b**) was in agreement with the PCA score plot, and showed distinctive patterns among the four Yinqiaosan preparation forms. Hence, these patterns may be used for product differentiation and recognition.

A high-throughput UPLC-QTOF-MS method for global semiquantitative analysis of chemical constituents in CHM formula was demonstrated in this work. As illustrated by the contents of 49 commonly identified chemical constituents in four Yinqiaosan preparation forms (▶ **Table 4**), the use of a single exogenous reference IS (etoposide-d3) in the method enabled us to perform not only global semiquantitative analysis of multicomponents in each Yinqiaosan preparation form but also cross-comparison among the four preparation forms. Therefore, the method developed provides an efficient and economical solution for both herbal content analysis and product comparison.

The reproducibility of global semiquantitative analysis by the UPLC-QTOF-MS method was investigated by triplicate measurements of sample of each Yinqiaosan preparation form, and the precision of the semiquantitative measurements was calculated in terms of CV of the chemical contents. It was shown (▶ **Table 4**) that 91.8–98.0 % of the 49 chemical constituents of the 4 preparation forms had CV values that fell within the recommended values (CV ≤ 30 %) [21], which indicated the reliability of the UPLC-QTOF-MS method.

The pharmacological activities of the 49 commonly identified chemical constituents in all 4 Yinqiaosan preparation forms were investigated through text mining and database searching. Sixteen of the forty-nine chemical constituents were found to have various

▶ **Table 3** Common chemical constituents detected and identified in all four Yinqiaosan preparation forms.

No.	Formula	Name	tR (min)	Observed mass	Database mass	Precursor ion, m/z
1	$C_{16}H_{18}O_9$	Chlorogenic acid *	1.80	354.0961	354.0951	355.1026 $[M + H]^+$
2	$C_{11}H_{12}N_2O_2$	D-Tryptophan	3.01	204.0914	204.0891	205.0986 $[M + H]^+$
3	$C_{34}H_{34}O_{18}$	Isorientin 4'-O-glucoside 2''-O-p-hydroxybenzoagte	4.88	730.1764	730.1777	731.1841 $[M + H]^+$
4	$C_{24}H_{24}O_{11}$	Echioidinin 2'-(6''-acetylglucoside)	6.36	488.1332	488.1337	489.1393 $[M + H]^+$
5	$C_{19}H_{25}N_5O_6$	Asn-Trp-Thr	8.11	419.1820	419.1817	420.1895 $[M + H]^+$
6	$C_{20}H_{26}O_9$	Bruceine D	8.40	410.1573	410.1583	411.1644 $[M + H]^+$
7	$C_{17}H_{18}N_4O_5$	202-791	8.49	358.1286	358.1281	381.1177 $[M + Na]^+$; 359.1382 $[M + H]^+$
8	$C_{21}H_{28}O_8$	Vernoflexuoside	8.49	408.1785	408.1793	409.1859 $[M + H]^+$
9	$C_{21}H_{24}O_{12}$	Catechin-4-ol 3-O-BeTa -D-galactopyranoside	9.59	468.1274	468.1279	469.1345 $[M + H]^+$
10	$C_{28}H_{34}O_{12}$	Caohuoside D	9.79	562.2056	562.2066	563.2130 $[M + H]^+$
11	$C_{11}H_{14}O_5$	Genipin	11.14	226.0853	226.0855	227.0925 $[M + H]^+$
12	$C_{20}H_{18}N_4O_6$	Phe-His-OH	11.14	410.1214	410.1226	411.1287 $[M + H]^+$
13	$C_{13}H_{22}N_4O_8S_2$	Cysteineglutathione disulfide	11.19	426.0885	426.0871	214.0506 $[M + 2H]^{2+}$
14	$C_{20}H_{24}N_4O_6$	Asp-Pro-Trp	11.69	416.1681	416.1677	417.1754 $[M + H]^+$
15	$C_{18}H_{26}O_7$	Propofol glucuronide	12.04	354.1676	354.1672	355.1744 $[M + H]^+$
16	$C_{23}H_{20}O_9$	Pongamoside A	12.25	440.1111	440.1112	441.1185 $[M + H]^+$
17	$C_{15}H_{12}O_4$	Liquiritigenin	13.37	256.0748	256.0752	257.0820 $[M + H]^+$
18	$C_{21}H_{21}ClN_2O_8$	Demeclocycline	13.43	464.0977	464.0990	487.0872 $[M + Na]^+$; 465.1052 $[M + H]^+$
19	$C_{32}H_{31}N_3O_5$	KT 5720	13.78	537.2236	537.2250	538.2308 $[M + H]^+$
20	$C_{22}H_{22}O_7$	Artoindonesianin R	14.13	398.1371	398.1369	399.1437 $[M + H]^+$
21	$C_{21}H_{20}O_{11}$	Luteolin 7-glucoside *	14.42	448.1087	448.1038	449.1098 $[M + H]^+$
22	$C_{22}H_{24}O_7$	Dihydroanhydropodorhizol	15.60	400.1520	400.1531	401.1594 $[M + H]^+$
23	$C_{25}H_{23}ClN_2O_7$	TyrMe-Phe4Cl-OH	17.02	498.1188	498.1201	499.1258 $[M + H]^+$
24	$C_{29}H_{36}O_{15}$	Forsythiaside *	17.32	624.2035	624.2054	647.1994 $[M + Na]^+$
25	$C_{28}H_{30}O_{11}$	Ikarisoside D	17.98	542.1811	542.1809	543.1875 $[M + H]^+$
26	$C_{29}H_{32}O_{12}$	Amorphigenin O-glucoside	19.41	572.1902	572.1901	573.1977 $[M + H]^+$
27	$C_{23}H_{28}O_{11}$	Albiflorin	20.08	480.1634	480.1640	481.1706 $[M + H]^+$
28	$C_{21}H_{19}ClN_2O_8$	Oxazepam glucuronide	20.73	462.0831	462.0825	463.0903 $[M + H]^+$
29	$C_{34}H_{30}N_2O_9$	Atalanine	21.18	610.1924	610.1931	611.1997 $[M + H]^+$
30	$C_{21}H_{22}O_9$	Liquiritin *	22.87	418.1293	418.1282	419.1366 $[M + H]^+$
31	$C_{28}H_{50}N_2O_{19}P_2$	N,N'-Diacetylchitobiosyldiphosphodolichol	23.52	780.2486	780.2502	781.2577 $[M + H]^+$
32	$C_{32}H_{42}O_{15}$	3,4,7-Trihydroxy-5-methoxy-8-prenylflavan 4-O-(beta-D-xylopyranosyl-(1-6)-beta-D-glucopyranoside)	24.24	666.2508	666.2508	667.2581 $[M + H]^+$
33	$C_{27}H_{34}O_{11}$	Forsythin *	25.88	534.2086	534.2101	557.2015 $[M + Na]^+$
34	$C_{23}H_{36}O_8$	3''-Hydroxypravastatin	26.01	440.2405	440.2418	441.2477 $[M + H]^+$
35	$C_{21}H_{25}ClN_2O_3$	Cetirizine	27.06	388.1548	388.1547	411.1441 $[M + Na]^+$
36	$C_{27}H_{34}O_{11}$	Arctiin *	29.02	534.2107	534.2101	557.2017 $[M + Na]^+$

▲ **Table 3** Continued

37	$C_{31}H_{42}O_{15}$	Eruberin C	33.29	654.2506	654.2510	655.2577 $[M + H]^+$
38	$C_{20}H_{30}O_5$	19(R)-Hydroxy-PGA2	34.33	350.2093	350.2091	351.2167 $[M + H]^+$
39	$C_{20}H_{32}O_5$	Tuberonic acid	35.72	352.2247	352.2245	353.2322 $[M + H]^+$
40	$C_{15}H_{19}NO$	Pronetalol	38.36	229.1483	229.1475	230.1557 $[M + H]^+$
41	$C_{18}H_{14}O_3$	Ovalitenin A	39.93	278.0935	278.0932	279.1006 $[M + H]^+$
42	$C_{19}H_{26}O_6S$	2-Methoxyestradiol-17β 3-sulfate	40.02	382.1443	382.1441	405.1336 $[M + Na]^+$; 383.1512 $[M + H]^+$
43	$C_{23}H_{28}O_4$	17-Phenyl trinor prostaglandin A2	47.86	368.2011	368.2005	369.2085 $[M + H]^+$
44	$C_{24}H_{40}O_6$	3α,6α,7β,12α-Tetrahydroxy-5α-cholan-24-oic acid	48.05	424.2824	424.2836	425.2898 $[M + H]^+$
45	$C_{24}H_{41}NO_4$	Cassaidine	48.75	407.3044	407.3034	408.3116 $[M + H]^+$
46	$C_{24}H_{38}O_4$	3α,12α-Dihydroxy-5β-chol-6-en-24-oic acid	51.34	390.2787	390.2789	413.2676 $[M + Na]^+$; 391.2861 $[M + H]^+$
47	$C_{36}H_{64}N_8O_{17}$	Glycopeptide	7.86	880.4417	880.4416	879.4333 $[M - H]^-$
48	$C_{31}H_{39}NO_{10}S$	Labriformin	16.28	617.2332	617.2278	616.2259 $[M - H]^-$
49	$C_{49}H_{76}O_{20}$	Lanatoside C	21.15	984.4906	984.4935	491.2383 $[M - 2H]^{2-}$; 983.4851 $[M - H]^-$

*Marker constituent.

▶ **Fig. 4** Visualization of multivariate data analysis. **a** The PCA score plot, and **b** the PLS-DA score plot of four Yinqiaosan preparation forms.

pharmacological activities (▶ **Table 5**), ranging from antibacterial, antiviral, anti-inflammatory, and antifungal to antioxidant, anticancer, and treatment of allergic rhinitis [22–52]. These pharmacological active compounds may serve not only as lead compounds in new drug development, but also as bait for the retrieval of protein targets in a network pharmacology study [53, 54]. The latter may help us to understand the underlying action mechanism of Yinqiaosan formula.

In conclusion, a UPLC-QTOF-MS method with the use of a single exogenous reference IS has been developed for untargeted metabolomics profiling and global semiquantitation of the prescription Chinese herbal medicine formula Yinqiaosan. The chemical profiles of 4 Yinqiaosan preparation forms (i.e., decoction, granule, pill, and

▶ **Table 4** Semiquantitative analysis of 49 commonly identified compounds in 4 Yinqiaosan preparation forms (n = 3).

No.	Formula	Name	D ± SD (μg/g)	CV (%)	G ± SD (μg/g)	CV (%)	T ± SD (μg/g)	CV (%)	P ± SD (μg/g)	CV (%)
1	$C_{16}H_{18}O_9$	Chlorogenic acid *	2.6 ± 0.4	13	14.5 ± 0.3	2	3.9 ± 0.2	4	1.4 ± 0.1	9
2	$C_{11}H_{12}N_2O_2$	D-Tryptophan	18 ± 2	12	61 ± 2	4	3.4 ± 0.1	4	1.8 ± 0.2	13
3	$C_{34}H_{34}O_{18}$	Isorientin 4'-O-glucoside 2''-O-p-hydroxybenzoagte	22 ± 5	22	43 ± 10	24	85 ± 17	20	42 ± 2	5
4	$C_{24}H_{24}O_{11}$	Echioidinin 2'-(6''-acetylglucoside)	13 ± 5	39	16 ± 1	4	5.0 ± 0.2	3	2.7 ± 0.1	5
5	$C_{19}H_{25}N_5O_6$	Asn-Trp-Thr	3.9 ± 0.8	20	22 ± 3	14	15 ± 3	20	5 ± 2	43
6	$C_{20}H_{26}O_9$	Bruceine D	7 ± 2	27	42 ± 16	38	24 ± 3	14	7.5 ± 0.5	6
7	$C_{17}H_{18}N_4O_5$	202-791	13 ± 2	16	113 ± 16	14	14 ± 2	13	7.5 ± 0.5	7
8	$C_{21}H_{28}O_8$	Vernoflexuoside	6 ± 2	30	49 ± 7	13	21 ± 1	6	10 ± 1	9
9	$C_{21}H_{24}O_{12}$	Catechin-4-ol 3-O-BeTa -D-galactopyranoside	4.1 ± 0.5	12	30.6 ± 0.7	2	30 ± 1	5	3.3 ± 0.2	5
10	$C_{28}H_{34}O_{12}$	Caohuoside D	2.8 ± 0.7	24	16 ± 3	20	5 ± 1	28	4 ± 1	35
11	$C_{11}H_{14}O_5$	Genipin	12.5 ± 0.5	4	122 ± 1	1	11 ± 3	28	18 ± 3	15
12	$C_{20}H_{18}N_4O_6$	Phe-His-OH	72 ± 2	3	601 ± 10	2	105 ± 24	23	138 ± 50	38
13	$C_{13}H_{22}N_4O_8S_2$	Cysteineglutathione disulfide	18.3 ± 0.2	1	109 ± 12	11	19 ± 4	21	15 ± 2	12
14	$C_{20}H_{24}N_4O_6$	Asp-Pro-Trp	3.2 ± 0.6	17	27.5 ± 0.7	2	6.8 ± 0.7	11	3.3 ± 0.4	13
15	$C_{18}H_{26}O_7$	Propofol glucuronide	13 ± 2	13	105 ± 44	42	53 ± 10	19	17 ± 2	9
16	$C_{23}H_{20}O_9$	Pongamoside A	34 ± 3	10	292 ± 20	7	119 ± 3	2	69 ± 8	12
17	$C_{15}H_{12}O_4$	Liquiritigenin	11 ± 1	11	88 ± 3	4	17 ± 2	12	24 ± 2	6
18	$C_{21}H_{21}ClN_2O_8$	Demeclocycline	4.1 ± 0.5	13	30.2 ± 0.8	3	22 ± 2	11	3.5 ± 0.4	12
19	$C_{32}H_{31}N_3O_5$	KT 5720	26 ± 3	12	132 ± 5	4	17.0 ± 0.8	5	23 ± 3	11
20	$C_{22}H_{22}O_7$	Artoindonesianin R	4.7 ± 0.9	19	14 ± 3	18	4.1 ± 0.4	9	4.7 ± 0.4	9
21	$C_{21}H_{20}O_{11}$	Luteolin 7-glucoside *	9 ± 1	12	65 ± 3	5	12.2 ± 0.2	2	2.1 ± 0.2	8
22	$C_{22}H_{24}O_7$	Dihydroanhydropodorhizol	4.0 ± 0.4	9	22.2 ± 0.2	1	8.4 ± 0.2	3	4.6 ± 0.4	10
23	$C_{25}H_{23}ClN_2O_7$	TyrMe-Phe4Cl-OH	48 ± 11	23	238 ± 61	26	275 ± 13	5	121 ± 19	16
24	$C_{29}H_{36}O_{15}$	Forsythiaside *	7 ± 2	23	196 ± 38	20	336 ± 23	7	93 ± 7	7
25	$C_{28}H_{30}O_{11}$	Ikarisoside D	27 ± 3	11	454 ± 15	3	666 ± 26	4	145 ± 14	10
26	$C_{29}H_{32}O_{12}$	Amorphigenin O-glucoside	12 ± 2	14	88 ± 2	2	38 ± 3	7	14.0 ± 0.7	5
27	$C_{23}H_{28}O_{11}$	Albiflorin	4 ± 1	29	40 ± 6	14	4 ± 1	24	2.7 ± 0.7	26
28	$C_{21}H_{19}ClN_2O_8$	Oxazepam glucuronide	5 ± 1	25	19 ± 6	32	7.5 ± 0.4	6	3.8 ± 0.5	12
29	$C_{34}H_{30}N_2O_9$	Atalanine	5.9 ± 0.9	15	39.0 ± 0.6	2	16.5 ± 0.7	4	4.9 ± 0.2	4
30	$C_{21}H_{22}O_9$	Liquiritin *	12 ± 3	26	197 ± 10	5	30 ± 3	11	7.6 ± 0.4	5
31	$C_{28}H_{50}N_2O_{19}P_2$	N,N'-Diacetylchitobiosyldiphosphodolichol	22 ± 1	6	72 ± 2	2	9.2 ± 0.3	4	3.2 ± 0.4	12
32	$C_{32}H_{42}O_{15}$	3,4,7-Trihydroxy-5-methoxy-8-prenylflavan 4-O-(beta-D-xylopyranosyl-(1-6)-beta-D-glucopyranoside)	3.7 ± 0.6	15	15.9 ± 0.5	3	1.81 ± 0.08	4	4.3 ± 0.4	9
33	$C_{27}H_{34}O_{11}$	Forsythin *	1.4 ± 0.1	10	95 ± 3	3	138 ± 7	5	13 ± 1	11
34	$C_{23}H_{36}O_8$	3''-Hydroxypravastatin	3.7 ± 0.6	17	5.9 ± 0.7	12	4.9 ± 0.3	6	1.4 ± 0.2	12
35	$C_{21}H_{25}ClN_2O_3$	Cetirizine	2.03 ± 0.09	4	7.0 ± 0.9	12	28 ± 3	11	10.0 ± 0.3	3
36	$C_{27}H_{34}O_{11}$	Arctiin *	2.1 ± 0.3	14	318 ± 9	3	51 ± 5	11	6.8 ± 0.6	8
37	$C_{31}H_{42}O_{15}$	Eruberin C	2.9 ± 0.3	10	91 ± 10	11	12.6 ± 0.3	2	3.9 ± 0.3	7

▲ **Table 4** Continued

38	$C_{20}H_{30}O_5$	19(R)-Hydroxy-PGA2	8 ± 1	12	17 ± 1	6	16.1 ± 0.8	5	6.2 ± 0.8	13
39	$C_{20}H_{32}O_5$	Tuberonic acid	5.6 ± 0.4	7	21 ± 3	16	18 ± 3	14	6 ± 1	17
40	$C_{15}H_{19}NO$	Pronetalol	3.9 ± 0.6	15	23 ± 2	8	10 ± 1	12	9.4 ± 0.7	7
41	$C_{18}H_{14}O_3$	Ovalitenin A	1.3 ± 0.3	21	7 ± 2	28	2.0 ± 0.5	24	2.3 ± 0.2	9
42	$C_{19}H_{26}O_6S$	2-Methoxyestradiol-17β 3-sulfate	4.3 ± 0.6	14	26.4 ± 0.4	2	2.98 ± 0.02	1	8 ± 1	19
43	$C_{23}H_{28}O_4$	17-Phenyl trinor prostaglandin A2	1.4 ± 0.2	12	7.4 ± 0.7	10	6.3 ± 0.3	5	3.5 ± 0.4	11
44	$C_{24}H_{40}O_6$	3α,6α,7β,12α-Tetrahydroxy-5α-cholan-24-oic acid	1.46 ± 0.01	1	11 ± 1	10	7.7 ± 0.6	8	4 ± 2	41
45	$C_{24}H_{41}NO_4$	Cassaidine	0.54 ± 0.06	12	5.0 ± 0.2	4	8.0 ± 0.6	7	2.4 ± 0.4	17
46	$C_{24}H_{38}O_4$	3α,12α-Dihydroxy-5β-chol-6-en-24-oic acid	4 ± 1	31	12 ± 5	43	7 ± 2	22	3.7 ± 0.8	21
47	$C_{36}H_{64}N_8O_{17}$	Glycopeptide	12 ± 1	10	69 ± 7	10	9.4 ± 0.2	3	8.6 ± 0.1	2
48	$C_{31}H_{39}NO_{10}S$	Labriformin	28.7 ± 0.8	3	182 ± 13	7	12 ± 4	33	13 ± 1	11
49	$C_{49}H_{76}O_{20}$	Lanatoside C	8 ± 2	24	284 ± 57	20	17 ± 5	29	83 ± 10	13

*Marker constituents. D = decoction, G = granule, T = tablet; P = pill.

tablet) were obtained, and the 49 common chemical constituents and 16 pharmacological active compounds were identified. Simultaneous semiquantitative analysis of the multicomponents in each Yinqiaosan preparation form was carried out along with untargeted metabolomics profiling, and cross-comparison of the chemical contents in between four preparation forms was accomplished. PCA and PLS-DA analyses showed that the UPLC-QTOF-MS method developed was reproducible and the chemical constituents found in the four Yinqiaosan preparation forms displayed unique patterns for product differentiation and recognition. The method developed is useful for the identification, quantitation, and cross-comparison of chemical constituents not only in Yinqiaosan products, but also in other CHM formulas.

Materials and Methods

Chemicals and Chinese herbal medicine formulas

Ammonium hydroxide and formic acid were purchased from Sigma-Aldrich. Optima LC/MS grade acetonitrile and methanol, and HPLC grade water were from Fisher Scientific. Deionized water was obtained from an in-house Barnstead Nanopuree water purification system (Thermo Scientific) with a resistivity meter reading of 18.2 MΩ-cm. Etoposide-d3 (purity, 97.8 %) was purchased from Toronto Research Chemicals and used as the single exogenous reference internal standard in this work.

The Yinqiaosan decoction was prepared by Jiangsu Provincial Hospital of Traditional Chinese Medicine (Nanjing, Jiangsu, China) with nine kinds of processed CHMs (Jinyinhua, Batch No. 150320; Lianqiao, Batch No. 150718; Bohe, Batch No. 150701; Jingjie, Batch No. 150313; Niubangzhi, Batch No. 150601; Jiegeng, Batch No. 150601; Dandouchi, Batch No. 150401; Gancao, Batch No. 150701; and Danzhuye, Batch No. 150302). *Yinqiaosan peifangkeli* (granules) were manufactured by Jiangyin Tianjiang Pharmaceutical Co. (Jiangyin, Jiangsu, PRC) as individual packages for each CHM [i.e., Jinyinhua (Batch No. 1503112), Lianqiao (Batch No. 1501092), Bohe (Batch No. 1412140), Jingjie (Batch No. 1501094), Niubangzhi (Batch No. 1411156), Jiegeng (Batch No. 1503133), Dandouchi (Batch No. 1410023), Gancao (Batch No. 1502100), and Danzhuye (Batch No. 1501021)]. *Yinqiaojieduwan* (pills) (Batch No. 14033352) was produced by Beijing Tongrentang (Fengtai District, Beijing, PRC). *Yinqiaojiedupian* (tablets) (Batch No. 140695) was made by the Yunnan Tengyao Pharmaceutical Co. (Tengyue, Yunnan, PRC).

Preparation of the internal standard solution

The stock solution of IS was prepared by dissolving 1.00 mg etoposide-d3 powder in 1.00 mL of methanol to a concentration of 1.00 mg/mL. The working solution of the IS was prepared by a 1/10 dilution of the stock solution in methanol to a concentration of 100 µg/mL (or 169 µM).

Preparation of various forms of Yinqiaosan sample solutions

Yinqiaosan decoction sample solution

The Yinqiaosan decoction was prepared using the following procedure: First, seven of the nine processed CHMs, Jinyinhua (Flos Lonicerae, 9.00 g), Lianqiao (Fructus Forsythiae, 9.00 g), Niubangzhi

▶ **Table 5** Pharmacological active constituents found in all four Yinqiaosan preparation forms.

No.	Name	PubChem CID	CAS	Reported pharmacological activity
1	Chlorogenic acid *	1794427	327-97-9	antibacterial, antiviral [26–27]
2	Luteolin 7-glucoside *	5280637	5373-11-5	antibacterial, antifungal, antioxidant, anti-inflammatory [28, 29], antidiabetic [30]
3	Forsythiaside *	5281773	79916-77-1	antibacterial, antiviral, anti-inflammatory [31–32]
4	Forsythin *	101712	487-41-2	antioxidant, anti-inflammatory [33]
5	Liquiritin *	503737	551-15-5	antioxidant [34], antioxidative, anticancer, and neuroprotective [35]
6	Arctiin *	100528	20362-31-6	antiviral, anti-inflammatory [36–39]
7	D-Tryptophan	9060	153-94-6	antibacterial [40]
8	Bruceine D	441788	21499-66-1	anticancer: inhibits hepatocellular carcinoma growth [41]
9	202-791	122114	101342-80-7	a calcium channel agonist [42]
10	Genipin	442424	6902-77-8	anticancer: suppresses colorectal cancer cells [43–45]
11	Liquiritigenin	114829	578-86-9	restores osteoblast damage [46], attenuates cardiac injury [47], prevents palmitate-induced beta-cell apoptosis [48]
12	Demeclocycline	54680690	127-33-3	for the treatment of hyponatremia secondary to SIADH [49]
13	KT 5720	3844	108068-98-0	reverses multidrug resistance, a chemosensitizer [50–51]
14	Albiflorin	51346141	39011-90-0	ameliorates obesity [52]
15	Cetirizine	2678	83881-51-0	for the treatment of allergic rhinitis [53–54]
16	Lanatoside C	656630	17575-22-3	anticancer: induces apoptosis in human gastric cancer cells [55] and human hepatocellular carcinoma cells [56]

* Marker constituents.

(Fructus Arctii, 9.00 g), Jiegeng (Radix Platycodonis, 6.00 g), Dandouchi (Semen Sojae Preparatum, 5.00 g), Gancao (Radix Glycyrrhizae, 5.00 g), and Danzhuye (Herba Lophatheri, 4.00 g) were first soaked in 696 mL of deionized water inside a clay pot for 30 min; next, the pot was heated over a gas range with a high flame. Once the water inside the pot started to boil, the gas flame was adjusted to low and the pot continued to be heated for another 10 min. At this point, the remaining two processed CHMs, Bohe (Herba Menthae, 6.00 g) and Jingjie (Herba Schizonepetae, 5.00 g), were added, and boiling was continued for another 5 min. At the end of heating, the hot decoction (< 500 mL) was poured into a 500-mL glass beaker and cooled to room temperature. The cooled decoction was transferred into a 500-mL volumetric flask, and the beaker was rinsed three times with deionized water. The rinse solution was then combined with the decoction in the flask, and additional deionized water was added to the 500-mL mark of the flask. The solution was vortexed and allowed to settle on a benchtop for 30 min; then, 3.00 mL of the supernatant were pipetted and transferred into a borosilicate glass tube (16 × 100 mm; Fisher Scientific) followed by the addition of 6.90 mL of methanol and 0.100 mL of the IS working solution (169 µM). The mixture was vortexed for 30 s using a MaxiMix II Vortex Mixer (Thermo Scientific). Next, 1.00 mL of the solution was transferred into a 1.5-mL microcentrifuge tube (VWR), and centrifuged at 18 000 × g for 10 min using a Sorvall ST 40R centrifuge (ThermoFisher Scientific). After centrifugation, 500 µL of the supernatant was transferred into an 8-mm clear glass screw thread autosampler vial from ThermoFisher Scientific and subjected to UPLC-QTOF-MS analysis.

Yinqiaosan granule, pill, and tablet sample solutions

First, 1/12.5 of the daily maximum dose of each preparation form of Yinqiaosan was weighed out precisely, and each sample was transferred into a 50.0-mL centrifuge tube (Corning). Then, deionized water was added to the 40.0-mL mark. Each sample was soaked in deionized water for 90 min, followed by vortex mixing for 3 min, then placed in a ThermoFisher Scientific FS-28 ultrasonic bath for 30 min. After settling on a benchtop for 30 min, 3.00 mL of the supernatant were pipetted and transferred into a borosilicate glass tube (16 × 100 mm), and then 6.90 mL of methanol and 0.100 mL of the IS working solution (169 µM) were added. The mixture was vortexed for 30 s. Next, 1.00 mL of the solution was transferred into a 1.5-mL microcentrifuge tube and centrifuged at 18 000 × g for 10 min. After centrifugation, 500 µL of the supernatant were transferred into an 8-mm clear glass screw thread autosampler vial and subjected to UPLC-QTOF-MS analysis.

Preparation of QC sample

The QC sample (800 µL) could be prepared by mixing 200 µL of each of the four Yinqiaosan sample solutions (see " Preparation of various forms of Yinqiaosan sample solutions"), and used with each batch analysis by monitoring the selectivity and reproducibility of the method over the course of analysis on the 49 commonly identified compounds.

UPLC-QTOF-MS system

The UPLC-QTOF-MS system used in this work consisted of an Agilent 1290 Infinity UPLC system coupled with an Agilent 6540 QTOF mass spectrometer. The UPLC system included a solvent reservoir, a degasser, a G4220A binary pump, a G1330B thermostat, a G4226A autosampler, a G1316C thermostatted column compartment, and a G4212A diode array detector. The mass spectrometer was equipped with an Agilent jet stream ESI probe.

Liquid chromatographic separation was achieved using gradient elution on a Waters ACQUITY UPLC BEH C18 (2.1 mm i.d. × 100 mm,

1.7 µm, 130 Å) column with an inline VHP filter (0.5 µm, stainless steel) from Upchurch Scientific. The mobile phase for the positive ESI-QTOF-MS consisted of A) 0.1 % formic acid aqueous solution (v/v) and B) acetonitrile. The mobile phase for the negative ESI-QTOF-MS consisted of A) 0.05 % ammonium hydroxide aqueous solution (v/v) and B) acetonitrile. The gradient elution profile was as follows: 0–4 min, 5 % B; 4–7 min, 5–10 % B; 7–20 min, 10–15 % B; 20–30 min, 15–22 % B; 30–35 min, 22–35 % B; 35–40 min, 35–50 % B; 40–45 min, 50–70 % B; 45–50 min, 70–90 % B; 50–52 min, 5 % B; 52–60 min, 5 % B. The flow rate was 0.4 mL/min. The column temperature was at 60 °C. The sample injection volume was 5.00 µL. Prior to sample analyses, the column was equilibrated with the mobile phase for at least 30 min at a flow rate of 0.4 mL/min.

The QTOF-MS was operated in both positive and negative ESI modes. The chromatographic and spectra data (.d) were acquired for each ESI mode using Agilent MassHunter Data Acquisition software (Version B.05.01). The operation parameters of the mass spectrometer were as follows: drying gas (N_2) temperature, 350 °C; drying gas flow rate, 10.0 L/min; nebulizer gas (N_2) pressure, 35 psi; sheath gas flow rate, 11.0 L/min; sheath gas (N_2) temperature, 325 °C; capillary voltage, 4000 V; nozzle voltage, 500 V; fragmentor voltage, 100 V; skimmer voltage, 65 V; octopole radio-frequency voltage, 750 V; collision energy, 10, 20, and 40 eV. The data were acquired by auto MS/MS mode with an extended dynamic range (2 GHz). The MS scan range was 50–1000 m/z at a scan rate 5 spectra/s. The MS/MS scan range was 50–1000 m/z at a scan rate of 3 spectra/s with an isolation width at narrow (~1.3 m/z). To maintain the mass accuracy, the mass spectrometer was calibrated and tuned before analysis, and internal reference masses from the reference mass solution were used for real-time mass correction at m/z 121.0508 and 922.0098 for the positive ion mode, and m/z 112.9885 and 1033.9881 for the negative ion mode throughout the acquisition process. The reference mass solution was prepared using an Agilent API-TOF reference mass solution kit (Part No: G1969-85001).

Method validation

The specificity and reproducibility of the UPLC-QTOF-MS method were assessed by replicate measurements of samples of four Yinqiaosan preparation forms. PCA score plots were constructed using the acquired MS data, and the CVs were calculated on the concentrations of replicate measurements of 49 commonly identified compounds through global semiquantitative analysis.

Data processing, constituent identification, and statistical analysis

The data files (.d) of replicate measurements for four Yinqiaosan preparation forms acquired by Agilent MassHunter Data Acquisition software at the same ion polarity (i.e., either positive or negative ESI) were first assessed using Agilent MassHunter Qualitative Analysis software (Version: B.06.00) for the signal/noise level, and retention time and mass shifts with respect to the spiked IS, which were then processed using Agilent Profinder software (Version B.06.00) for batch recursive analysis. The data files were grouped into four preparation forms and subjected to molecular feature extraction by selecting a peak height threshold of 1000 counts, possible ion adducts, isotope model of common organic molecules, charge states up to two, a retention time window of 0.10 % + 0.60 min, a mass window of 20.00 ppm + 2.00 mDa (for alignment of the IS in all runs with the same polarity), and a post-processing filter at 3 out of 3 replicate measurements for each Yinqiaosan preparation form.

Each molecular feature extracted data file (.cef) by Profinder software along with the corresponding data file (.d) by MassHunter Data Acquisition software was imported into Agilent MassHunter Qualitative Analysis software. Using "Find by Formula", MS/MS data along with its MS and retention time data were extracted. The new data files (.cef) in the same ion polarity of replicate measurements of each Yinqiaosan preparation form were then imported into Agilent Mass Profiler Professional software (Version: B.13.1.1) for an METLIN AM database search and molecular formula generation. The selection of elements and limits for molecular formula generation were as follows: C (3–156), H (0–180), O (0–40), N (0–20), S (0–14), Cl (0–12), F (0–12), Br (0–10), P (0–9), and Si (0–15) [55]. The top five constituents with the highest scores were annotated, and cross-checked with the Traditional Chinese Medicine Integrated Database [56] and Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform [57] for final name assignments. The combined data file (.cef) of replicate measurements of each Yinqiaosan preparation form with the names of chemical constituents was saved for global semiquantitative analysis.

For multivariate data analysis, the molecular feature extracted data files (.csv) by the Profinder software were imported into MetaboAnalyst 4.0 [58] in terms of mass, retention time, and peak area. The mass tolerance and the retention time tolerance were set at 0.025 and 30 s, respectively. Sample normalization was performed by the IS reference features (i.e., mass, retention time, and peak area), and the data were log transformed and autoscaled. PCA and PLS-DA were performed using the IS for normalization. PCA and PLS-DA score plots were constructed for similarity comparison of the chemical constituents and reproducibility assessment of the UPLC-QTOF-MS method as well as pattern recognition among the four Yinqiaosan preparation forms.

Global semiquantitative analysis

The data files (.d) with the same ion polarity of replicate measurements of each Yinqiaosan preparation form by the MassHunter Data Acquisition software and their corresponding combined data file (.cef) with the names of chemical constituents by the Mass Profiler Professional software were imported into Agilent MassHunter Quantitative Analysis software (Version: B.06.00). In the method setup task, the retention time window was set at 0.6 min, etoposide-d3 ammonium adduct was chosen as the IS and flagged, other identified chemical constituents were chosen as targets relative to the IS, and proper ion polarity was chosen. After validating the method setup, global semiquantitative analysis was performed based on the peak area ratio of each individual target to the IS, and the quantitation data were exported as excel file for reporting.

Supporting Information

The MS/MS spectra of the 49 chemical constituents commonly identified in all four Yinqiaosan preparation forms (▶ **Fig. 1S**), the common chemical constituents detected but unidentified in all four Yinqiaosan preparation forms (▶ **Table 1S**), and the unique constituents found in decoction, granule, pill, and tablet forms of Yinqiaosan (▶ **Tables 2S–9S)** are available as Supporting Information.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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