A Bio-activity Guided in Vitro Pharmacokinetic Method to Improve the Quality Control of Chinese Medicines

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Background

- The current method of quality control for Chinese medicines (CM) involve stability testing using any designated marker(s) present in the product.
- The shortcoming of the current approach is that these marker(s) are chosen entirely arbitrarily which may have no relevance to the product activity inherent in the herbs.
- Without good QC, the efficacy and safety of TCM products are difficult to establish and world acceptance of TCM is unlikely.

There is a need for an improved method that can provide quality advancement of CM.

Objectives

- To demonstrate the feasibility of **a bio-activity guided in-vitro pharmacokinetic method (BAPK)** for quality control of both single herb or complex formulae products
  - **Si-Wu-Tang (SWT) product**

Part I: Bioavailability

Part II: Bioactivity

Part I:
Bioactivity Guided Pharmacokinetics (BAPK) Approach

Sample to be investigated (e.g. SWT product)

↓

Dissolution

↓

Gastrointestinal (GI) metabolism

↓

GI absorption

↓

Identification of relevant bioactive marker(s) → Stability analysis of the identified markers

Part I:
Study Design and Procedures

I. Literature search (data mining) to identify active components in SWT.

II. HPLC-DAD and HPLC-MS/MS assay method development for simultaneous determination of these markers.

III. Preliminary test of raw herbs in accordance with Chinese Pharmacopeia (CP) 2005.

IV. Manufacture of CU-SWT and CU-Si Wu He Ji in accordance with CP 2005 to serve as a “reference” product.

V. Determination of contents of CU-SWT and marketed products.

VI. Identification and comparison of relevant markers via bio-activity guided pharmacokinetics approach.

VII. Stability evaluation of the identified relevant markers in CU-SWT and CU-Si Wu He Ji.

VIII. Establish the most stable marker(s) for SWT product.

Part I Results: Data Mining

Herb	Major active components identified from data mining		
Angelicae	Ferulic acid	Ligustilide	
Chuanxiong	Ligustrazine	Ligustilide	Ferulic acid
	Butylphthalide	Senkyunolide A	
Paeoniae	Paeoniflorin	Paeonol	Gallic acid
Rehmanniae	Catalpol		

Part I Results
Development of HPLC-DAD Assay and Method Validation

HPLC-DAD method development

HPLC-DAD system: Waters 2695 Separation Module

Waters 996 Photodiode Array Detector and Autosampler

LC column: Thermo ODS Hypersil, 4.6 x 250 mm, 5 um, with Waters Delta-Pak C18 guard column, 4.6 x 0.5 mm, 5 um

Mobile phase: 0.04% v/v phosphoric acid and 0.04% v/v diethylamine in water : ACN, gradient elution

Flow rate: 1 mL/min

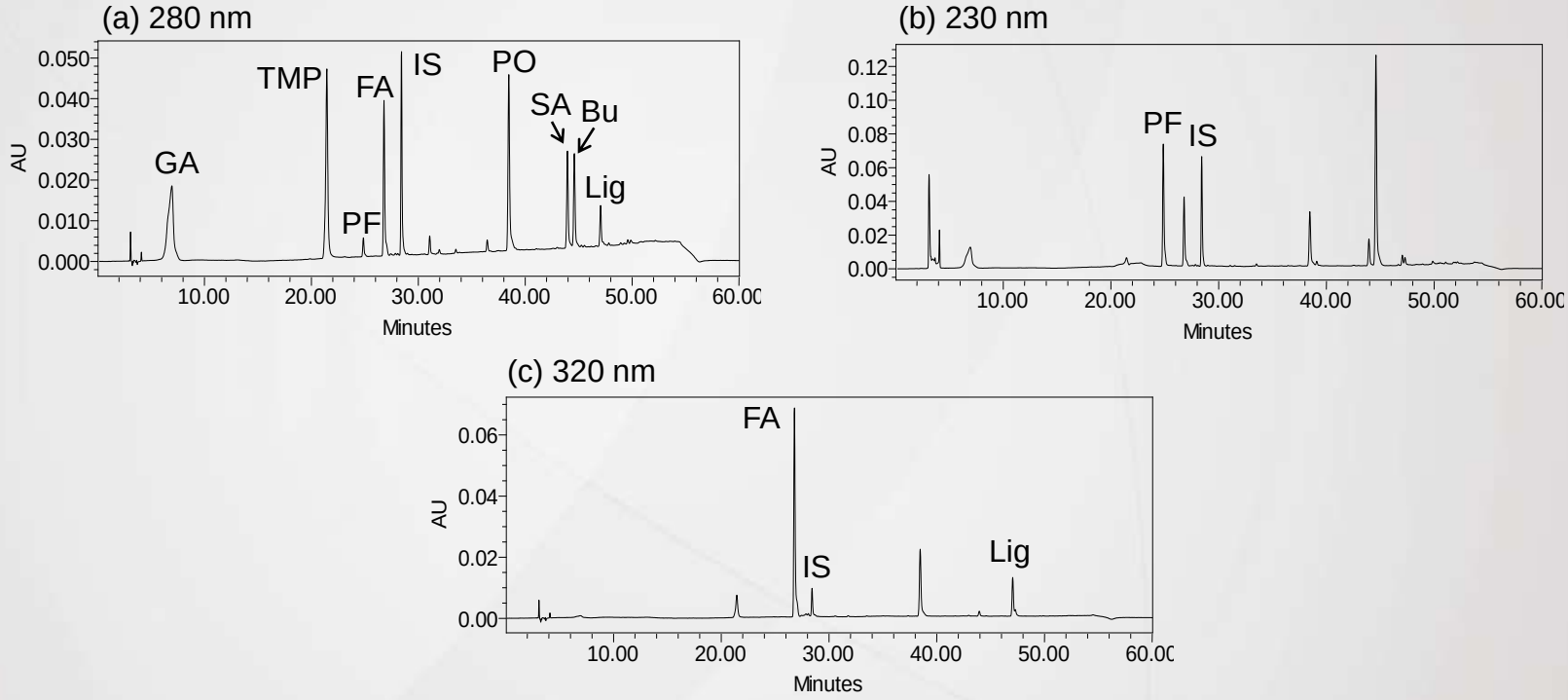
Temperature of LC column: ambient

Detection: 210 to 400 nm

Quantification: internal standard method

Part I Results

HPLC-DAD chromatograms of the nine analytes of interests

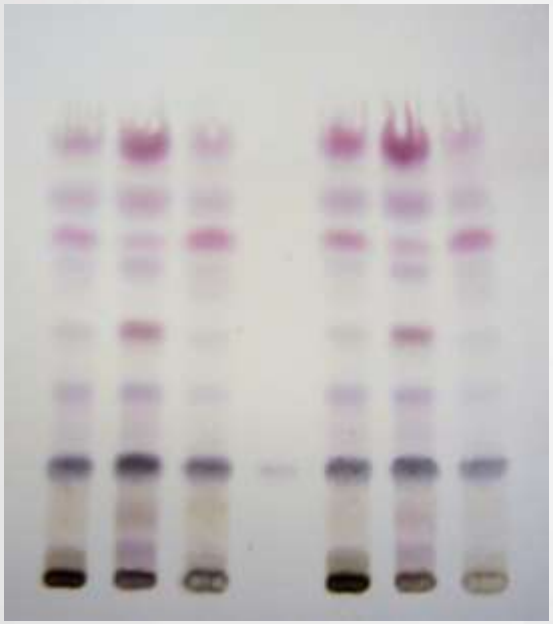


Remark: Gallic acid (GA), senkyunolide A (SA), paeoniflorin (PF), ferulic acid (FA), Z-ligustilide (Lig), butylphthalide (Bu), ligustrazine (TMP) and paeonol (PO)

Part I Results: CP Tests of Raw Herbs

(a) **Radix Paeoniae Alba**

visible light

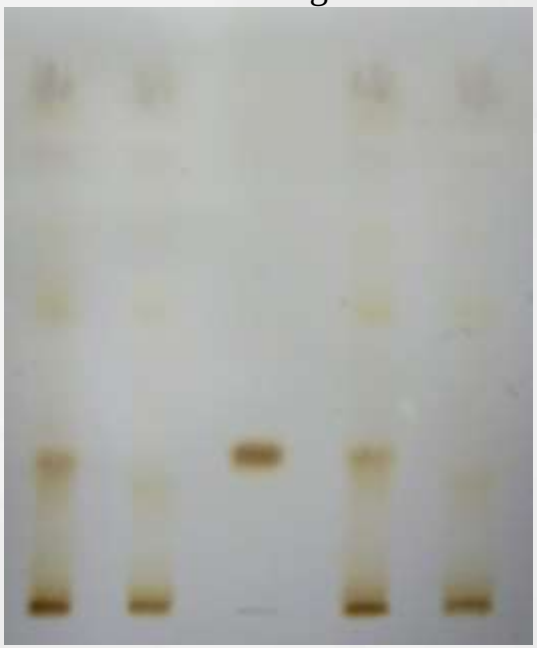


1 2 3 4 1 2 3

1. Test herb (source 1)
2. Test herb (source 2)
3. Reference herb
4. Paeoniflorin standard

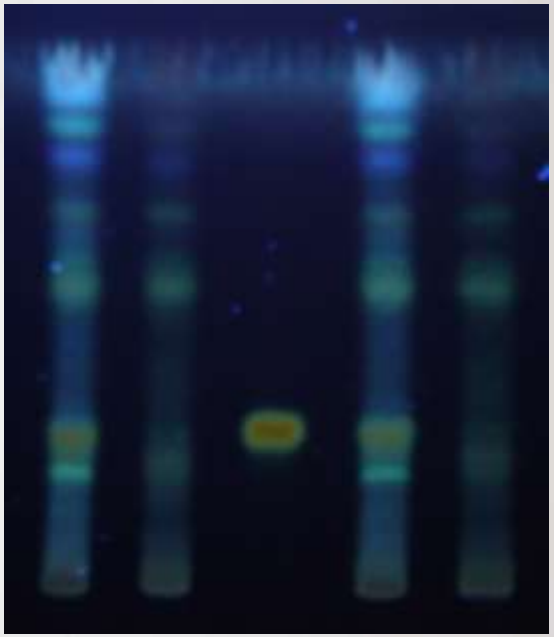
(b) **Radix Rehmanniae Preparata**

visible light



1 2 3 1 2

365 nm

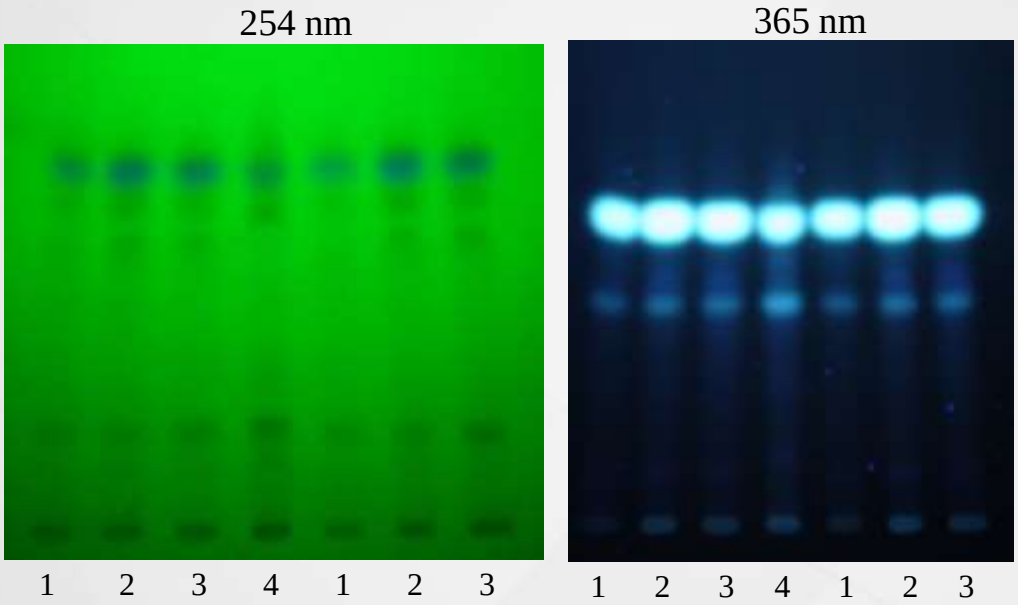


1 2 3 1 2

1. Test herb
2. Reference herb
3. Catalpol standard

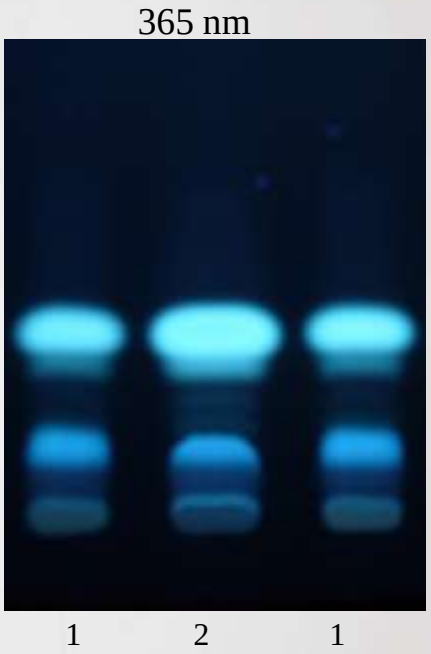
Part I Results: CP Tests of Raw Herbs

(c) **Radix Angelicae Sinensis**



1. Test herb (root)
2. Test herb (body)
3. Test herb (head)
4. Reference herb

(d) **Rhizoma Chuanxiong**



1. Reference herb
2. Test herb

Part I Results: CP Tests of Raw Herbs

Content of raw herbs:

Herbs	Batch	Content (%w/w)				
		FA	Lig	SA	Bu	TMP
Angelicae	#1	0.058	1.26	0.031	N.D.	N.A.
	#2	0.069	1.82	0.027	N.D.	N.A.
	#3	0.051	0.16	0.021	N.D.	N.A.
	#4	0.046	0.69	0.035	N.D.	N.A.
	#5	0.036	0.70	0.023	N.D.	N.A.
Chuanxiong	#1	0.130	2.41	0.350	0.063	N.D.
	#2	0.025	1.68	0.270	0.049	N.D.
	#3	0.081	2.83	0.270	0.028	N.D.
	#4	0.110	2.60	0.350	0.048	N.D.
	#5	0.110	1.08	0.300	0.033	N.D.

Remark: N.A. = not applicable, N.D. = not detectable

- variation among batches of raw herbs was observed
- batch #4 and #5 of Angelicae do not comply with 0.05% ferulic acid (FA) content as stated in CP (2005)
- Ligustrazine (TMP) was not detectable in Chuanxiong

Part I Results: CP Tests of Raw Herbs

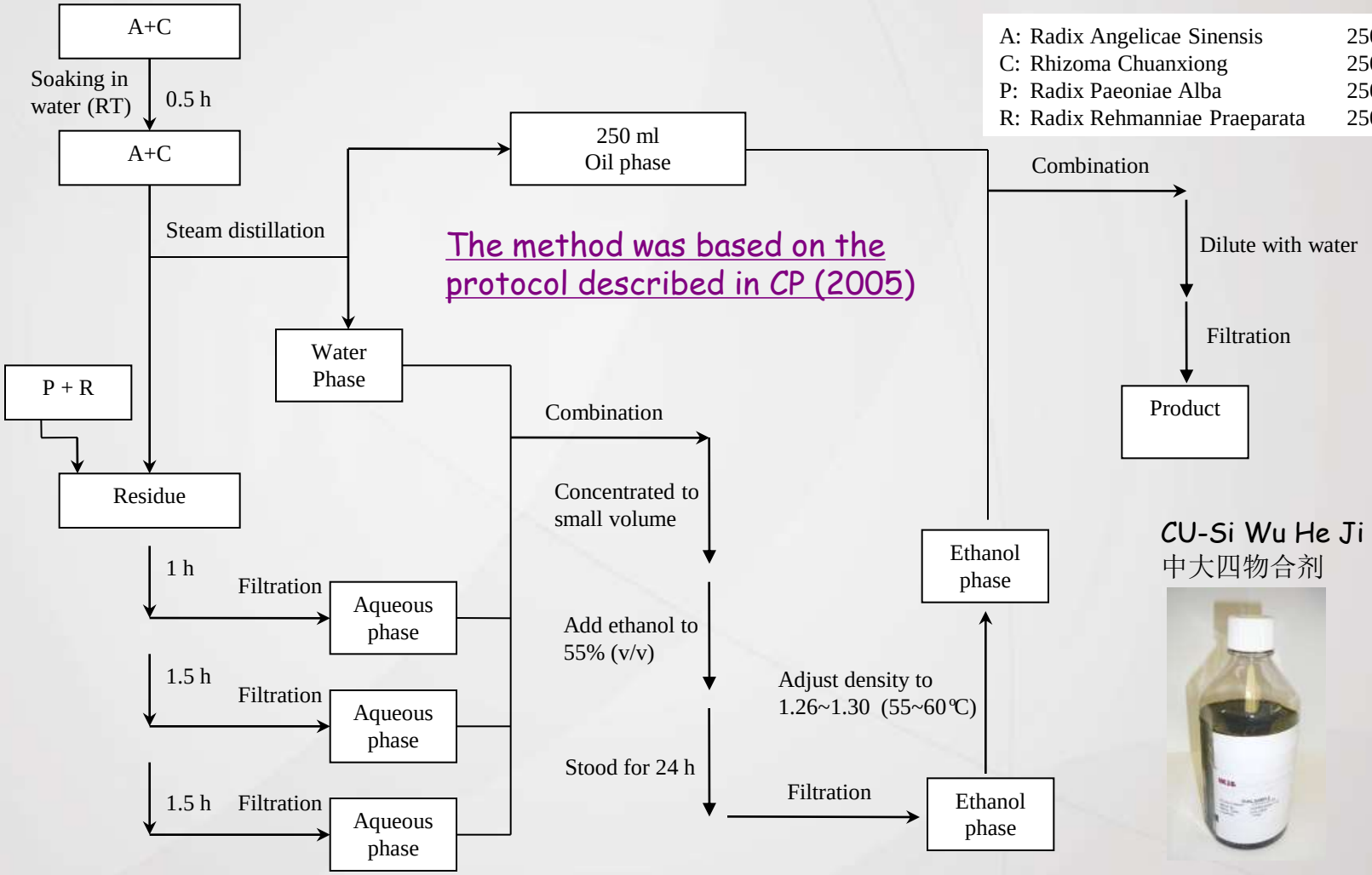
Content of raw herbs: continued

Herbs	Batch	Content (% w/w)			
		GA	PF	Cat	PO
Paeoniae	#1	0.120	2.06	N.A.	N.D.
	#2	0.076	2.11	N.A.	N.D.
	#3	0.065	0.53	N.A.	N.D.
	#4	0.088	2.36	N.A.	N.D.
	#5	0.034	1.25	N.A.	N.D.
Rehmanniae	#1	N.A.	N.A.	0.004	N.A.
	#2	N.A.	N.A.	N.D.	N.A.
	#3	N.A.	N.A.	0.005	N.A.
	#4	N.A.	N.A.	0.004	N.A.
	#5	N.A.	N.A.	0.001	N.A.

Remark: N.A. = not applicable, N.D. = not detectable

- batch #3 and #5 of Paeoniae do not comply with 1.6% paeoniflorin (PF) content as stated in CP (2005)
- very low content of catalpol (Cat) in Rehmanniae; paeonol (PO) cannot be detectable in Paeoniae

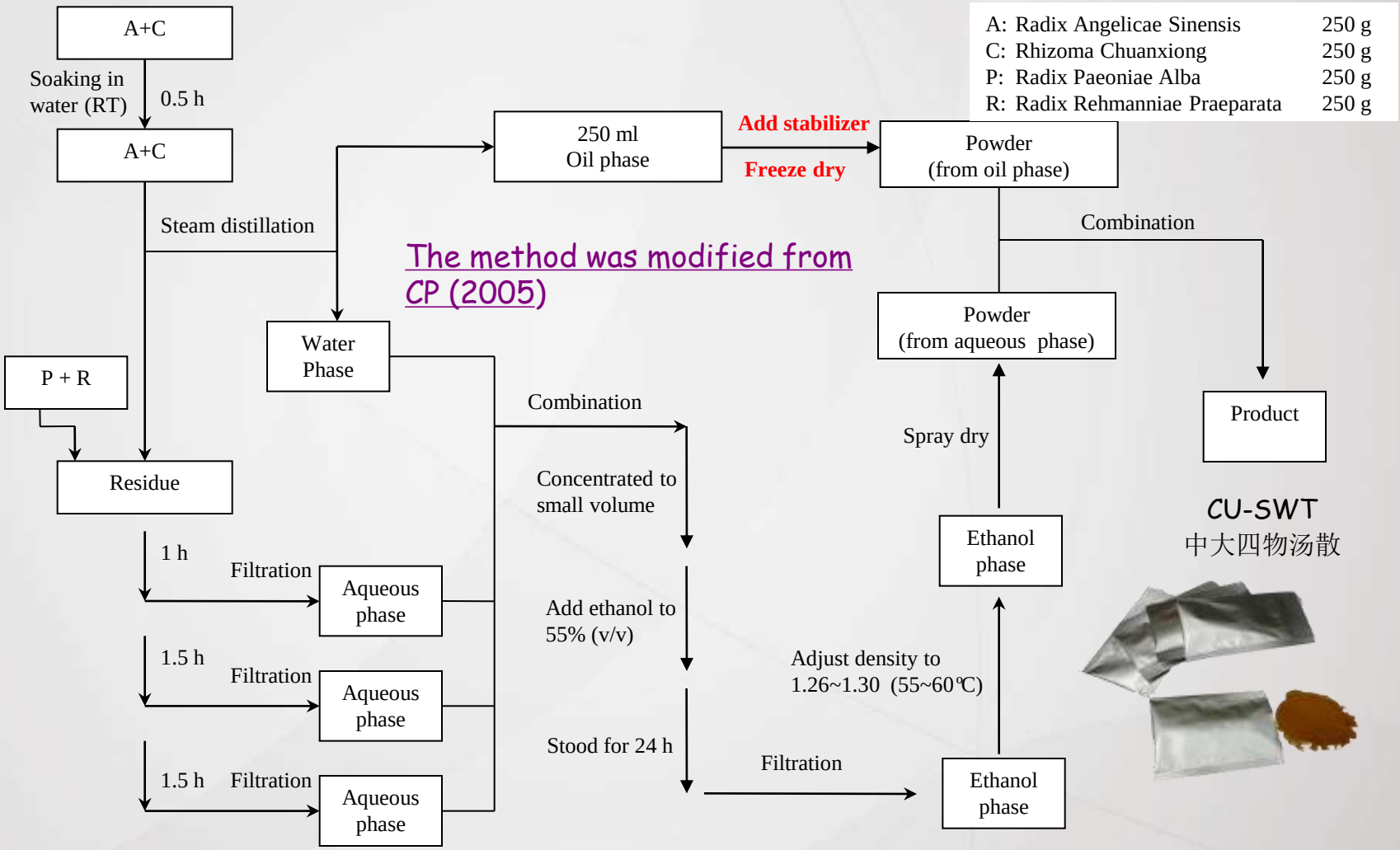
Part I Results – Manufacture CU-Si Wu He Ji



A:	Radix Angelicae Sinensis	250 g
C:	Rhizoma Chuanxiong	250 g
P:	Radix Paeoniae Alba	250 g
R:	Radix Rehmanniae Praeparata	250 g

CU-Si Wu He Ji
中大四物合剂

Part I Results – Manufacture CU-SWT



Products Manufactured

CU-SWT (batch 1 to 3)

CU-Si Wu He Ji (batch 1)

CU-Paeoniae (batch 1 to 3)

CU-Chuanxiong (batch 1 to 3)

CU-Angelicae (batch 1 to 3)

CU-Rehmanniae (batch 1 to 3)

Part I Results – Contents of SWT

(a) SWT product (solid dosage form)

Product	Concentration (mg/g) ± SD (n = 3)				
	GA	SA	PF	FA	Lig
CU-SWT batch 1	0.987 ± 0.089	0.075 ± 0.002	8.651 ± 0.240	0.525 ± 0.015	1.127 ± 0.224
CU-SWT batch 2	0.774 ± 0.030	0.049 ± 0.001	6.353 ± 0.057	0.440 ± 0.044	0.473 ± 0.012
CU-SWT batch 3	0.781 ± 0.101	0.053 ± 0.001	6.362 ± 0.198	0.367 ± 0.015	0.342 ± 0.024
market-1	0.131 ± 0.009	0.044 ± 0.002	0.054 ± 0.004	0.020 ± 0.002	0.037 ± 0.002
market-2	0.506 ± 0.070	0.059 ± 0.001	1.930 ± 0.143	0.154 ± 0.041	0.034 ± 0.003

Remark:
1. Gallic acid (GA), senkyunolide A (SA), paeoniflorin (PF), ferulic acid (FA) and Z-ligustilide (Lig).
2. Butylphthalide (Bu), ligustrazine (TMP), paeonol (PO) and catalpol (Cat) are not detectable.

Part I Results – Contents of SWT

(b) Si Wu He Ji (liquid dosage form)

Product	Concentration (μg/mL) ± SD (n = 3)					
	GA	SA	PF	FA	Lig	Bu
CU-SWHJ	307.9 ± 13.9	26.5 ± 0.9	2794 ± 225	173.3 ± 10.5	33.2 ± 1.0	N.D.
market-3	32.7 ± 1.5	2.07 ± 0.04	5795 ± 366	1.62 ± 0.47	1.02 ± 0.04	0.42 ± 0.03

Remark:
1. Gallic acid (GA), senkyunolide A (SA), paeoniflorin (PF), ferulic acid (FA) and Z-ligustilide (Lig) and butylphthalide (Bu).
2. Ligustrazine (TMP), paeonol (PO) and catalpol (Cat) are not detectable (N.D.).

CU SWT products vs. Commercial SWT

- Among 9 compounds investigated, ferulic acid, gallic acid, paeoniflorin, senkyunolide A and Z-ligustilide are present in all marketed products and CU-SWT & CU-Si Wu He Ji
- The contents of these components are generally higher in CU SWT products than commercial products (in particular the volatile component Z-ligustilide)
- **Large variations** in contents of each component in marketed products

Part I:
Bioactivity Guided Pharmacokinetics (BAPK) Approach

Sample to be investigated (e.g. SWT product)

↓

Dissolution

↓

Gastrointestinal (GI) metabolism

↓

GI absorption

↓

Identification of relevant bioactive marker(s) → Stability analysis of the identified markers

Part I Results – Dissolution Study

SWT in capsule for dissolution test

Good dissolution of ferulic acid, paeoniflorin, senkyunolide A, gallic acid and Z-ligustilide in SWT products in the dissolution medium (pH 2) within 30 min.

Part I:
Bioactivity Guided Pharmacokinetics (BAPK) Approach

Sample to be investigated (e.g. SWT product)

↓

Dissolution

↓

Gastrointestinal (GI) metabolism

↓

GI absorption

↓

Identification of relevant bioactive marker(s) → Stability analysis of the identified markers

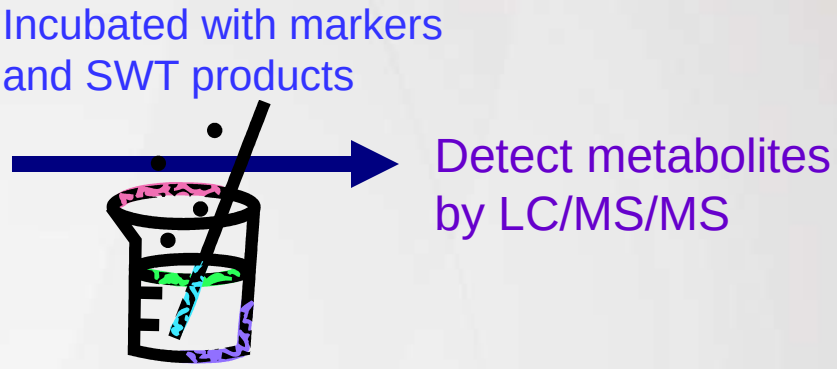
Part I Results – Metabolism Study

GI metabolism models:

✓Rat intestine homogenate

✓ Human intestine homogenate

✓Caco-2 cell lysate



Compound	Possible metabolism
Paeoniflorin	Hydrolysis
Ferulic acid	Sulfation, Glucuronation, Methylation
Gallic acid	Sulfation, Glucuronation, Methylation

Part I:
Bioactivity Guided Pharmacokinetics (BAPK) Approach

Sample to be investigated (e.g. SWT product)

↓

Dissolution

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Gastrointestinal (GI) metabolism

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GI absorption

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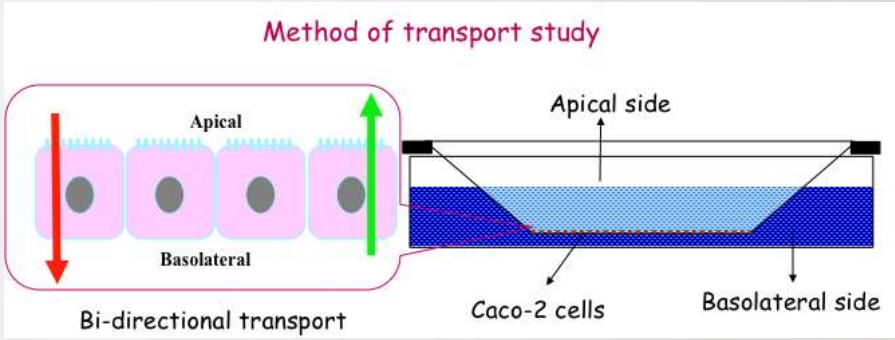
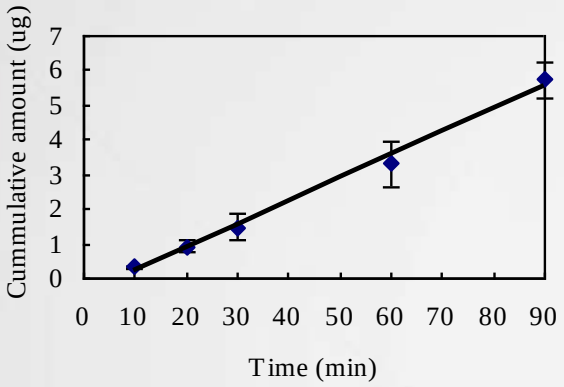
Identification of relevant bioactive marker(s) → Stability analysis of the identified markers



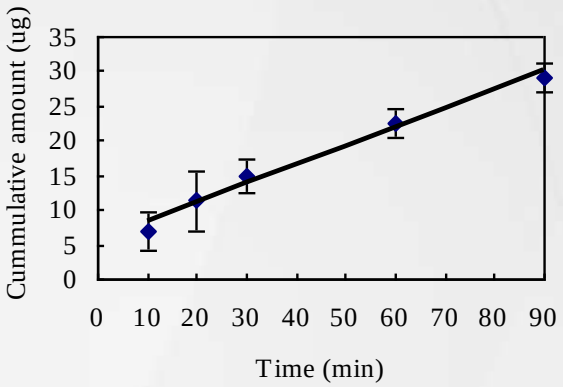
Part I Results – Permeability (GI Absorption)

Ferulic acid



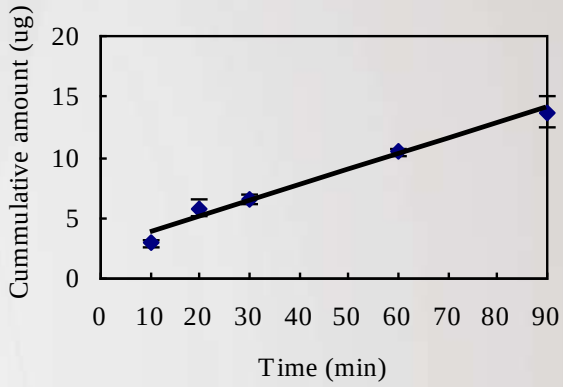
Loading Conc: 19.71 µg/mL
Papp: 1.22 $\times 10^{-5}$ cm/s

Z-Ligustilide



Loading Conc: 62.38 µg/mL
Papp: 1.54 $\times 10^{-5}$ cm/s

Senkyunolide A



Loading Conc: 16.11 µg/mL
Papp: 2.81 $\times 10^{-5}$ cm/s

- **Good permeability for ferulic acid, Z-ligustilide, senkyunolide A**
- **Poor permeability for paeoniflorin and gallic acid**

Part I:
Bioactivity Guided Pharmacokinetics (BAPK) Approach

Sample to be investigated (e.g. SWT product)

↓

Dissolution

↓

Gastrointestinal (GI) metabolism

↓

GI absorption

↓

Identification of relevant bioactive marker(s) → **Stability analysis of the identified markers**

Part I Results: – Stability Study

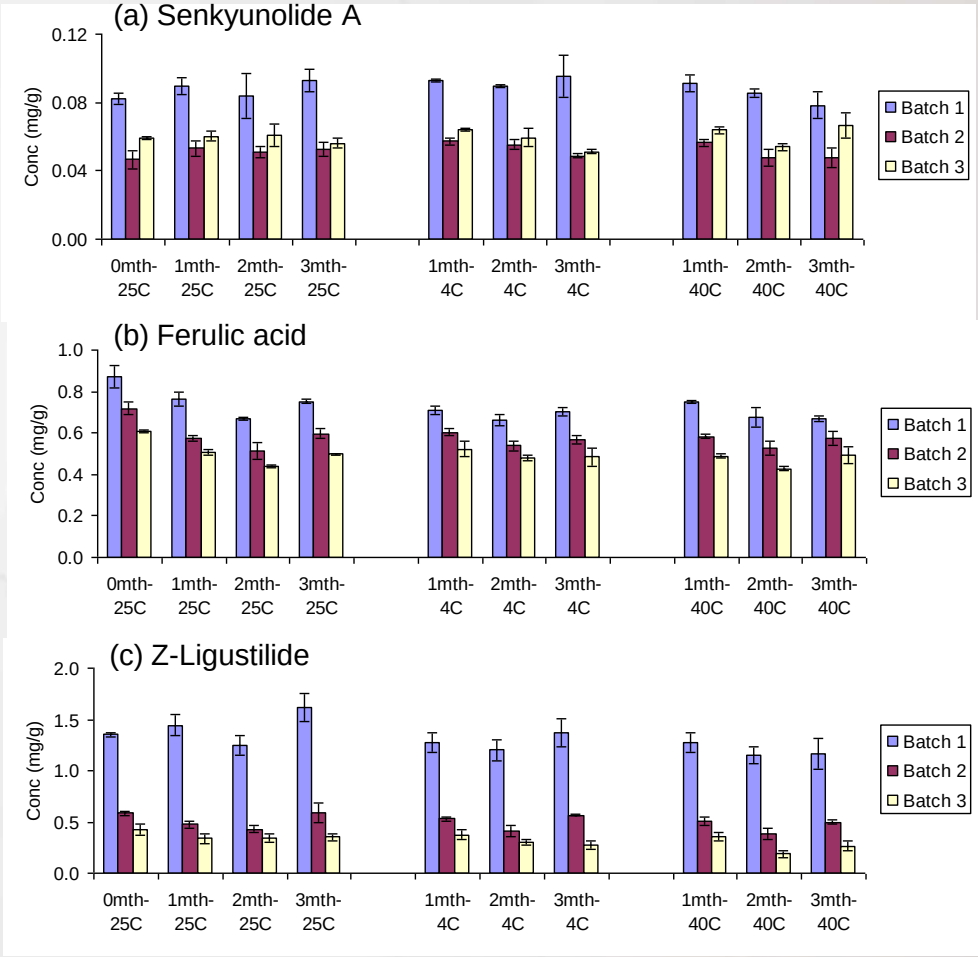
Stability of CU-SWT in various storage conditions

- Temperature at 4, 25 and 40 ^{o}C
- Storage period from 0, 1, 2 and 3 months
- Product tested: CU-SWT powder and CU-Si Wu He Ji, both were manufactured by us according to CP 2005

Part I Results: – Stability Study

Stability results of CU-SWT (solid dosage form)

Senkyunolide A, ferulic acid and Z-ligustilide in CU-SWT (solid dosage form) are relatively stable over 3-month period at 4, 25 and 40 °C storage temperatures.



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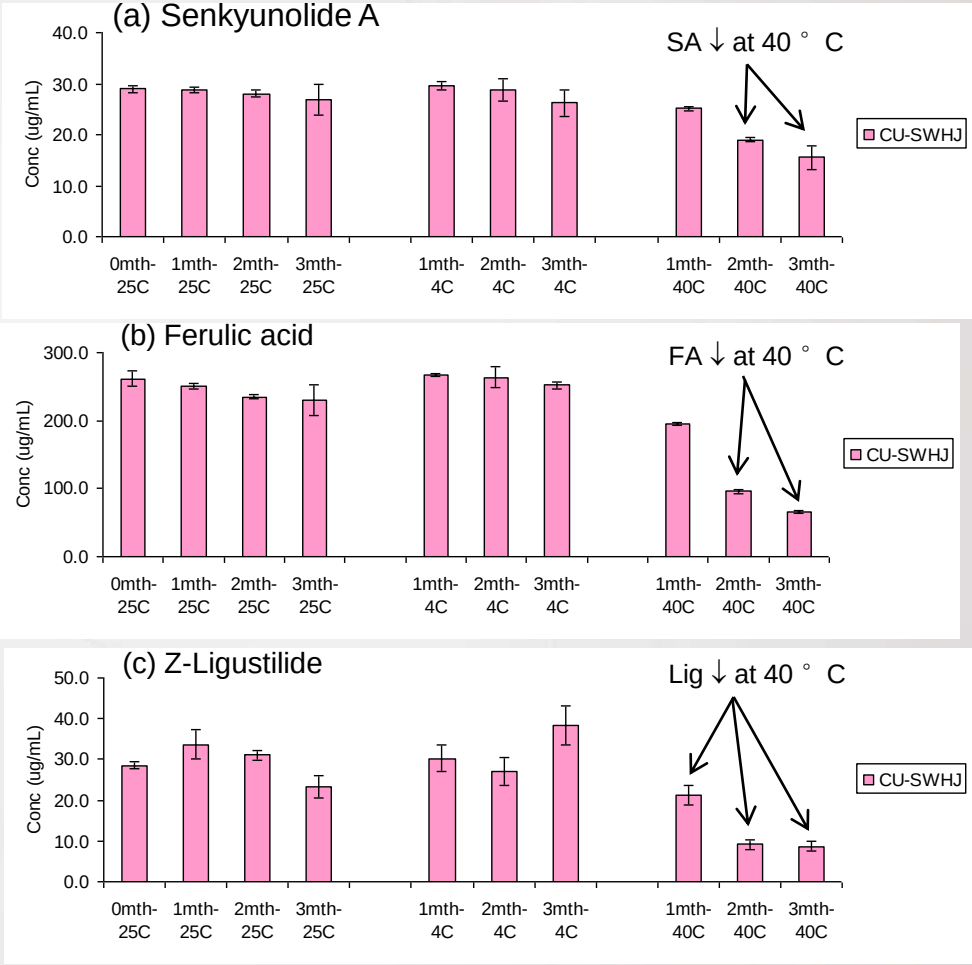
Part I Results: – Stability Study

Stability results of CU-Si Wu He Ji (liquid dosage form)

Degradation of senkyunolide A, ferulic acid and Z-ligustilide was observed in CU-SWHJ stored at high temperature.



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Part I:
Overall Summary in Selection of Relevant Bioactive Markers

- Three compounds, i.e. **ferulic acid**, **senkyunolide A** and **Z-ligustilide**, could serve as a more relevant bioactive markers for QC of SWT due to their good dissolution, permeability and stability characteristics.

- **Paeoniflorin**, a marker compound designated by the Chinese Pharmacopeia, is poorly permeable and thus not suitable to be considered as a “relevant bioactive” marker.

Are we done yet ?

- **Limitation with these markers**: lack of ability to represent the overall activity of a given SWT product.
- Recently, a landmark study was published in Science showing that differentially expressed genes (DEGs) identified via microarray processing and analysis can serve as signature for wide various of chemical components [1].
- We hypothesize that such DEGs can serve as unique composite fingerprint for a given CM product which could be useful for quality control and identity of such product.

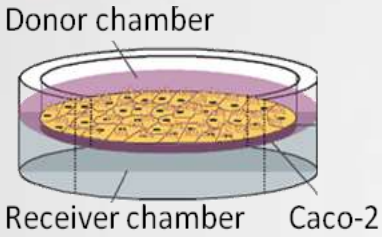
1. Lamb J et al, Science 2006, 313(29): 1929-1935

Part II: Objective

•To investigate the applicability of the different expression genes (DEGs) derived from special microarray processing and analysis in serving as unique fingerprint for a given SWT product and its four single herbs.

Part II: Project scheme

SWT product/herbs samples
LC/MS/MS & MTT

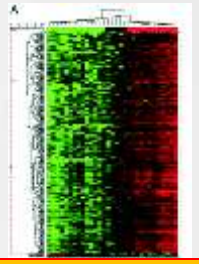


Absorbed fraction

Desalting and concentrate

MCF7

Extract RNA



Microarray processing

DEGs

Multi-gene RT-PCR panel

Verification

Real-time PCR

Specificity

Consistency

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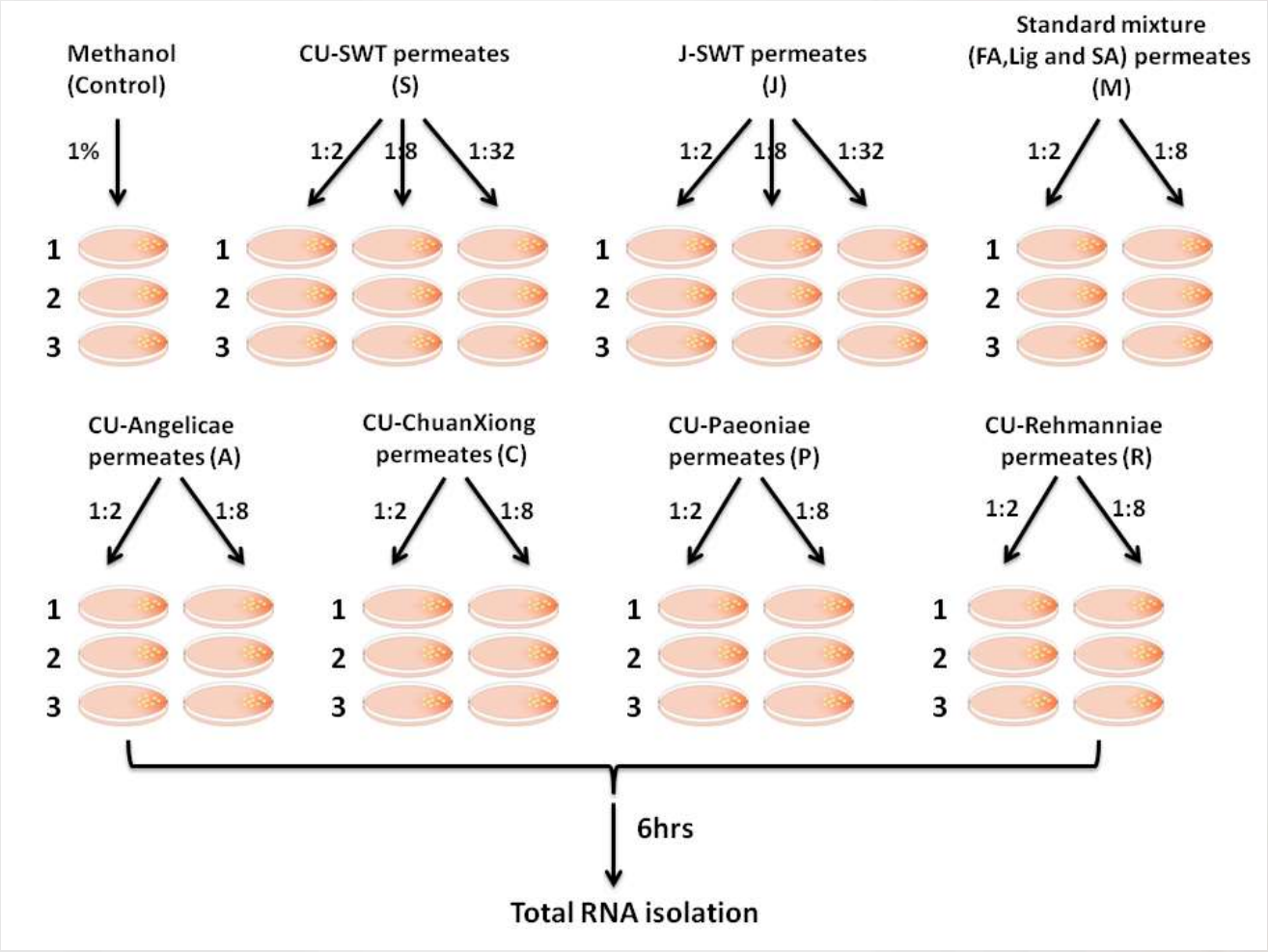
Experimental design of sample treatment for microarray processing / real-time PCR

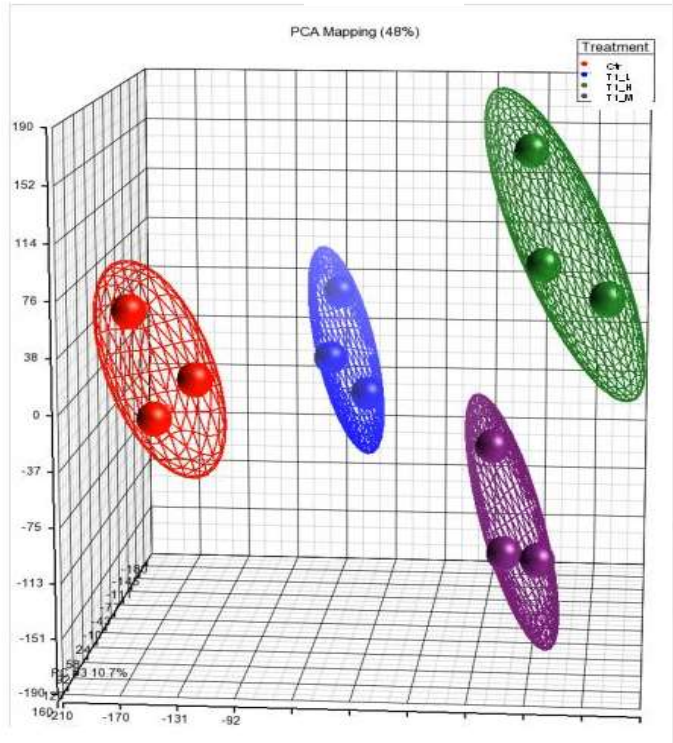


Microarray/Real time PCR

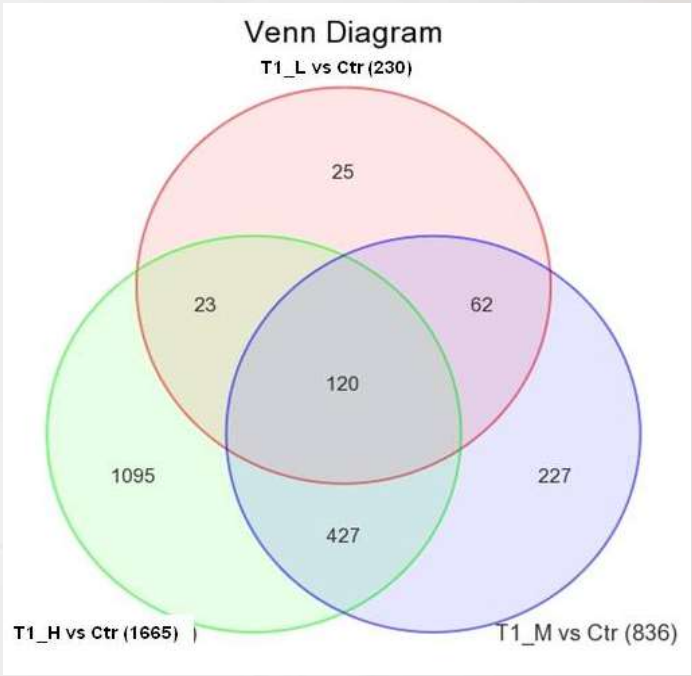
The Principal Components Analysis (PCA) and Venn Diagram of the differentially expressed genes for microarray data of CU-SWT permeates treatment

CU-SWT permeates

a) PCA



b)



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Identification of differentially expressed genes (DEGs), construction of multi-gene RT-PCR panel and method validation.

Step I: DEGs selection criteria:

(1) Fold change (FC) greater than a pre-defined threshold (e.g. FC > 1.5 or FC < -1.5);
(2) The $p < 0.01$ based on unpaired sample t-test;
(3) The expression fold change in a dose-dependent manner;
(4) The gene selected was significantly consistent with the drug's therapeutic use;
(5) The expression of genes selected was consistent in three different batches of microarray work.

Step II: Constructed the multi-gene RT-PCR panel for each treatment group using the DEGs identified.

Step III: Verified the expression fold changes of DEGs using real-time RT-PCR.

Part II Results

The differentially expressed genes (DEGs) for treatment groups

CU-SWT	J-SWT	Standard mixture	CU-Chuanxiong	CU-Angelica	CU-Paeoniae	CU-Rehmanniae
SLC7A11	SLC7A11	SLC7A11	SLC7A11	SLC7A11	TRIM65	SLC7A11
PDK4	CYP1A1	CYP1A1	ALDH1A3	CYP1A1	CYP2B6	CYP1A1
ST3GAL1	AKR1C1/AKR1C2	HMOX1	INPP4B	ALDH1A3	OR2H1	HMOX1
TNFRSF21	SLC7A5	PDK4	MSMB	INPP4B	NAP1L5	CXCR7
THBS1	GCLM	ALDH1A3	NEDD9	LRP8	TMCO6	ALDH1A3
PIGW	TXNRD1	PHLDB2	CCNK	TNFSF10	LCE1E	GCLM
GPER	CDH18	PDE4DIP	HECTD1	SAMD9	OSBPL8	S100A7
PCDH10	CCL28	MAP3K1	OSBPL8	MBNL2	RBAK	ATRX
TNFSF10	METTL7A	ZC3H11A	LARS	SAMHD1	ESCO1	CP

Note: Red color: up-regulated genes; Black color: down-regulated genes

Part II Results

Specificity test

- Test Products (total 21 different products):
  - **Two batches of CU-SWTs** (CU-SWT batch1 and CU-SWT batch2)
  - **Three marketed SWTs** (J-SWT, SWT_NBF, SWTHJ)
  - **Four single herbal extracts** (CU-Ang, CU-ChuanX, CU-Pae,CU-Reh)
  - **Single chemical standards** (ferulic acid, ligustilide, senkyunolide A) and their mixture
  - **Eight independent products** either marketed products (Named M1~M8).
- Treat MCF-7 cells and extract RNA
- Two sets of multi-gene RT-PCR panels for CU-SWT and J-SWT were tested against 21 different products by real-time PCR

Consistency evaluation

- Test Products : Eight -replicate extracts of CU-SWT on 3 separate days
- Treat MCF-7 cells and extract RNA
- The expression fold changes of the CU-SWT DEGs in these products were test using real-time PCR.

Part II Results:

Data analysis of intra-day and inter-day consistency by CU-SWT DEGs panel for CU-SWT

CU-SWT DEGs	Coefficient of Variations (CV)	
	Intra-day (8 batches)	Inter-day (3 days)
SLC7A11	3.3~5.6%	6.9%
PDK4	18.6~26.7%	27.0%
ST3GAL1	25.0~29.6%	13.0%
TNFRSF21	16.3~31.7%	14.3%
THBS1	14.9~17.8%	7.9%
PIGW	19.8~30.6%	21.1%
GPER	14.1~27.3%	20.5%
PCDH10	9.6~22.5%	19.5%
TNFSF10	12.6~25.9%	23.0%

Summary of the findings

- Identify DEGs of two SWTs (CU-SWT, J-SWT), its four single herbal extracts (CU-Angelica, CU-Chuanxiong, CU-Paeoniae and CU-Rehmanniae) and standard mixture (ferulic acid, ligustilide and senkyunolide A) using microarray processing and analysis.
- A panel of specific genes can be constructed using real-time RT-PCR to represent unique "fingerprint" of SWT and single herb tested.
- Convenient and cost-effective manner for "identity test" of the product during its manufacturing and testing of counterfeit products.

Acknowledgements

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