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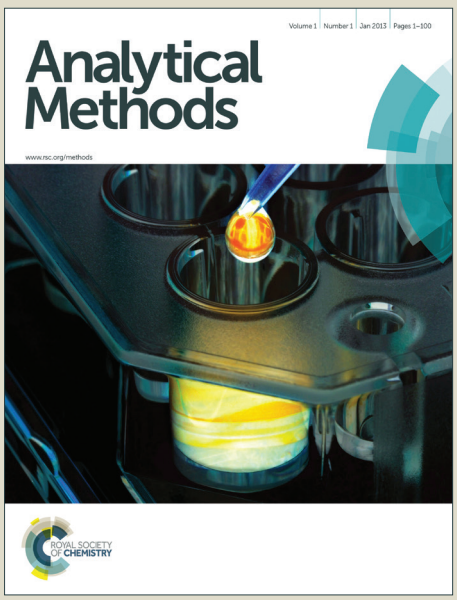

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Comparative analysis of trace elements contained in Rhizoma Curcumae from different origins and their vinegar products by ICP-MS

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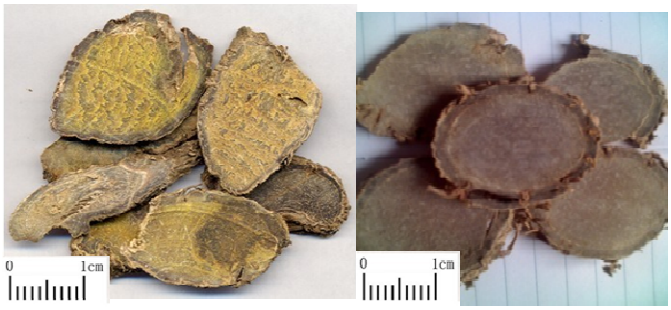

A comparison of trace elements contained in Rhizoma Curcumae and their corresponding vinegar products was undertaken by using ICP-MS, indicating the possible mechanism of the processing technique and sources identification of Rhizoma Curcumae.

Abstract: A comparison of trace elements contained in Rhizoma Curcumae and their corresponding vinegar products, coming from three different origins, namely *Curcuma phaeocaulis* Val., *Curcuma kwangsiensis* S. G. Lee et C. F. Liang and *Curcuma wenyujin* Y. H. Chen et C. Ling, was undertaken using inductively coupled plasma Mass Spectrometry (ICP-MS). SPSS 19 was adopted to do cluster analysis and discriminant analysis. Rhizoma Curcumae is rich in Cu, Zn, Se, Cr, Co, V, Ni and other essential trace elements, and among them Zn is in the first place, followed by Ti, Cu, etc. After processing with vinegar, the contents of some essential trace elements may increase more likely, while the contents of some heavy metal elements tend to decrease. The Rhizoma Curcumae from the same species and origin can be well clustered into one group. The results of the cluster analysis confirm the correlation between genuineness and the material base of Rhizoma Curcumae. In addition, the discriminant function of Rhizoma Curcumae was obtained, with the accuracy of 90.5%. Thus, a new concept was provided that Rhizoma Curcumae, even the other TCMs, from different sources may be identified by the discriminant function. One of the basic mechanisms of the processing technique of TCMs, the “Effect-enhancing and Toxicity-reducing”, may be associated with the increase or decrease of some trace elements, which provides a new conception for the quality control of TCMs and the processed products.

Keywords: ICP-MS; Rhizoma Curcumae; vinegar products; trace elements

1. Introduction

The use of herbal medicines, also called Traditional Chinese Medicine (TCM), is usually recognized as a form of complementary and alternative medicine, which has been used in China for more than 3000 years for the healthcare of human beings [1-2]. In the Chinese clinical practice, many medicinal materials and their processed products were applied. Rhizoma Curcumae, for example, is one of the commonly used TCMs, originating from three species of *Curcuma phaeocaulis* Val., *Curcuma kwangsiensis* S. G. Lee et C. F. Liang and *Curcuma wenyujin* Y. H. Chen et C. Ling. This kind of TCMs are recognised as the efficacy of *qi*-moving, blood-breaking and pain-alleviating and serve as one of the vital medicine for blood-breaking and disorder-eliminating in the clinical practice of TCM [3]. This herb has been widely recorded in many ancient books of TCM such as *Yaoxinglun, Tangbencao, Bencaotujing* and *Xingxubencao* etc, and the modern research has shown that it also has the pharmacologic action of antineoplastic, antiviral, immunoregulation etc [4-5]. In clinical practice, the mostly used form of Rhizoma Curcumae is its products processed with vinegar, which can increase efficacy and decrease toxicity, especially the efficacy of apparent influence on Hemorheology [6]. The trace elements contained in TCMs provide not only their own efficacies, but also some new bio-activeness by facilitating the efficacy of other active components [7]. For instance, the element Zinc (Zn) is one of the most important trace elements in the body, and it is essential as a catalytic, structural and regulatory ion. It has been researched that Zn can enhance the immunity of the body, accelerate the wound-healing [8] and adjust various relative enzymes and receptors [9] , which influence the bio-function of brain [10]. For the long term, however, the research reports are mainly focused on the organic components, and less on the essential trace elements. With regard to the levels of toxic trace elements, such as As, Cd, Hg and Pb in the plant samples, they are detrimental for human beings to a certain degree. For example, the inhalation of cadmium (Cd) fumes or particles can be life-threatening [11] and, although acute pulmonary effects [12] and deaths are uncommon, sporadic cases still occur [13]. Moreover, because the levels of these trace elements are usually at the μg/ml level, the use of ICP-MS is necessary for their determination[14-15]. In recent years, the application of ICP-MS for the analysis of the trace elements has been increasing [16] , with the characteristics of uniqueness, simplicity, accuracy, sensitiveness and rapidness, and it can also detect many trace elements at the same time [17-19]. According to Chinese Pharmacopoeia 2010, this method has been listed as one of the standard detecting methods of detrimental heavy-metal elements in TCMs. Adopting ICP-MS to detect the contents of trace elements in *Curcumae Rhizoma* and *Curcumae Rhizoma* stir- baked with vinegar, to a certain degree, may more roundly reflect the properties of Rhizoma Curcumae. Thus, this paper tends to provide a new method for the research of *Curcumae Rhizoma* and

its processing product and, meanwhile, is intended to offer some scientific proof for reasonable clinical application.

2. Experimental

2.1 Reagent and materials

The 21 batches of decocted pieces of Rhizoma Curcumae were purchased from its main producing areas, that is, Sichuan, Guangxi, Zhejiang and Yunnan etc, which have been identified by Professor Xianming Lu, working in the Traditional Chinese Medicine Center of Chengdu University of TCM (Table 1). The processed products were prepared by using vinegar based on the processing technique optimized from the former accomplishment of our research group (the amount of the 9 °- rice vinegar used was the 20 % of the total weight of the decocted pieces of Rhizoma Curcumae, and a 3 times-amount of water was added, infiltrating for 3 hours, boiling for 1hour over low heat, and then drying in air until the solution was absorbed)[20-22] . The powders of *Curcumae Rhizoma* and *Curcumae Rhizoma* Stir- baked with vinegar after crushing and sieving were stored in drying vessels until analysis. Indium (concentration=1000μg/mL), used as internal standard, were purchased from the National Research Center for Certified Reference Materials (GBW (E) 07160). Multielement standard solutions were purchased from the National Testing Center of Nonferrous Metals and Electronic Materials Analysis (GBW (E) 04-1767-2004). Tuning solutions were purchased from Thermo Electron Corporation, San Jose, CA, USA. Nitric acid was purchased from Merk & Co Inc., Rahway, NJ, USA. The others entire reagents are analytically pure. The standards were certified reference material of tea, provided by Geophysical geochemical survey and research institute, Hebei Province, China.

Table 1 Source of the samples

NO.	Species	Areas of production
1	*Curcuma phaeocaulis* Val.	Shuangliu Sichuan
2	*Curcuma phaeocaulis* Val.	Wenjiang Sichua
3	*Curcuma phaeocaulis* Val.	Shuangliu Sichuan
4	*Curcuma phaeocaulis* Val.	Chongzhou Sichuan
5	*Curcuma phaeocaulis* Val.	Sanjiang Sichuan
6	*Curcuma phaeocaulis* Val.	Shuangliu Sichuan
7	*Curcuma phaeocaulis* Val.	Shuangliu Sichuan
8	*Curcuma phaeocaulis* Val.	Leshan Sichuan
9	*Curcuma phaeocaulis* Val.	Yuxi Yunnan
10	*Curcuma phaeocaulis* Val.	Wenjiang Sichua
11	*Curcuma phaeocaulis* Val.	Shuangliu Sichuan
12	*Curcuma a kwangsiensis* S. G. Lee et C. F. Liang	Lingshan Guangxi
13	*Curcuma a kwangsiensis* S. G. Lee et C. F. Liang	Yuling Guangxi
14	*Curcuma a kwangsiensis* S. G. Lee et C. F. Liang	Guigang Guangxi
15	*Curcuma a kwangsiensis* S. G. Lee et C. F. Liang	Yuling Guangxi
16	*Curcuma a kwangsiensis* S. G. Lee et C. F. Liang	Lingshan Guangxi
17	*Curcuma a kwangsiensis* S. G. Lee et C. F. Liang	Chuxiong Yunnan

18	*Curcuma a kwangsiensis* S. G. Lee et C. F. Liang	Yuling Guangxi
19	*Curcuma wenyujin* Y. H. Chen et C. Ling	Ruian Zhejiang
20	*Curcuma wenyujin* Y. H. Chen et C. Ling	Leqing Zhejiang
21	*Curcuma wenyujin* Y. H. Chen et C. Ling	Yongjia Zhejiang

2.1 Instruments

The analysis was made on Thermo X-2 ICP-MS (Thermo Electron Corporation, Waltham, MA, USA). The microwave digestion instrument was from Milestone Company , Sorisole (BG), Italy. The mili Q-water purification system was from American Millipore Company, Bedford, MA, USA . The Mettler AE240 electronic balance with a precision of ±0.01 mg was from Mettler Instrument AG, Greifensee, Switzerland.

2.2.1 Digestion procedure of microwave

The process of microwave shown in Table 2. Though this process of microwave, finally the sample solutions were obtained.

Table 2 Digestion procedure of microwave

Steps	Microwave digeston furnace power（W）	Beginning time （min）	Stop time （min）	Temperature （℃）
1	500	0	10	120℃
2	700	10	15	180℃
3	700	15	25	180℃
4	500	25	35	120℃

2.2.2 Operating conditions of ICP-MS

Sample solution was measured by ICP-MS. The parameters for ICP-MS were plasma RF-power 1200W, nebulizer gas flow rate 0.89L/min and sampling depth 100 step. The other parameters are shown in Table 3.

Table 3 Operating conditions for ICP-MS

Parameters	Operating conditions
Plasma RF-Power	1.2kW
Sampling depth	100 step
Nebulizer gas flow	0.89L·min^{-1}
Auxiliary gas flow	0.7 L·min^{-1}
Cooling gas flow	13.0 L·min^{-1}
Peristaltic pump speed	30r·min^{-1}
Atomizing chamber temperature	3℃
Dwell time per point	0.6ms
Points/quality	3

Replicates	2
Measurement time/times	0.13s

2.3 Preparation of the solutions

2.3.1 Preparation of the standard solutions

Some reserve solutions of Pb, As, Cd and Cu were weighed accurately, then they were diluted, with 2% HNO_3, to prepare the mixed standard solutions, which respectively contained 0ng, 1ng, 5ng, 10ng, 20ng Pb and As, 0ng 0.5ng, 2.5ng, 5ng, 10ng Cd and 0ng, 10ng, 50ng, 100ng, 200ng Cu per milliliter; and also some reserve solutions of Hg were weighed significantly, then diluted with 10% HNO_3 to obtain the standard solutions, which respectively contained 0ng, 0.2ng, 0.5ng, 1.2ng Hg per milliliter.

2.3.2 Preparation of the internal standard solutions

^{115}In standard solution was diluted by water to prepare the internal standard solution containing 10ng ^{115}In per milliliter.

2.3.3 Preparation of the test solutions

The sample was dried for 2h at 60□ before use. Then, the dried sample weighted 0.25g was digested with 6ml HNO_3 added in the microwave digesting tube. After completing the reaction, 2ml hydrogen peroxide were added, airtight until the digestion finished in the procedure of item 2.2.1, then, it was taken out after cooling down, and poured into 50ml volumetric flask. It was washed 3 times by water, the solutions were collected into the flask, and diluted with water to 50ml, mixing and filtering, ready for measurement.

3. Results and Discussion

3.1 Method validation of quantitative analysis

3.1.1 Linearity, accuracy and precision

The results of regression analysis on calibration curves are presented in Table 4. The regression equation was obtained, Y=aE+ bX + c, and the correlation coefficient of the calibration curves was no less than 0,9990. The present quantitative method had quit satisfactory accuracy with the overall recoveries of these elements ranging from 94% to 108% (Table 4). Then, the same standard solutions of each element were respectively tested 6 times with the RSD less than 3.9% (Table 4). The limit of detection (LOD) were determined by injecting the blank solution for 20 times.

Table 4 The method validation results

Element	Regression equation	Correlation coefficient/ *r*	RSD/%	Recovery%
^{65}Zn	*Y*=5.12E+03*X*+2998	0.9990	2.0	95
^{48}Ti	*Y*=2.47E+04*X*+249	0.9999	3.7	101
^{65}Cu	*Y*=4.40E+03*X*+541	0.9998	2.7	97
^{11}B	*Y*=3.16E+03*X*+121	0.9999	3.5	101
^{51}V	*Y*=1.65E+04*X*+584	0.9997	1.8	102
^{60}Ni	*Y*=3.63E+03*X*+16	0.9998	2.8	99
^{59}Co	Y=2.07E+04X+292	0.9999	3.9	98
^{7}Li	Y=7.04E+03X+23	0.9996	2.4	103
^{75}As	Y=1.39E+03X+18	0.9992	2.3	102
^{111}Cd	Y=2.36E+04X+10	0.9998	1.7	99
^{121}Sb	Y=4.50E+04X+22	0.9995	2.0	100
^{208}Pb	Y=2.89E+04X+11	0.9998	2.2	102
^{202}Hg	Y=2.74E+04X+10	0.9997	2.4	108

3.1.2 Accuracy

The standards were spiked approximately 0.3 g, and then analysed as described for normal samples (as item 2.3.3). Recovery test was used to evaluate the accuracy of this method, and the results are shown in Table 4.

The sample were weighed 0.1g, added with Multielement standard solutions and then analysed as described for normal samples (as item 2.3.3). The present quantitative method had quit satisfactory accuracy with the overall recoveries of these elements ranging from 94% to 108% (Table 5).

Table 5 Analytical results of certified reference material of tea

Element	Standard Amount	Amount found	Recovery%
Zn(10^{-6})	51±2	52	107
Ti(10^{-6})	14	14	100
Cu(10^{-6})	18.6±0.7	19.1	99
B(10^{-6})	14±1	15	106
V(10^{-6})	0.17±0.03	0.19	107
Ni(10^{-6})	3.4±0.3	3.6	97

Co(10^{-6})	0.22±0..02	0.24	98
Li(10^{-6})	0.14±0.02	0.16	102
As(10^{-6})	0.09±0.01	0.08	106
Cd(10^{-9})	62±4	63	108
Sb(10^{-6})	0.022±0.06	0.027	94
Pb(10^{-6})	1.5±0.2	1.6	108
Hg(10^{-9})	3.8±0.8	3.4	104

3.3 Content determination of samples

The trace elements contained in different sources of Rhizoma Curcumae and Rhizoma Curcumae stir-baked with vinegar (the processed products) were determined by ICP-MS for 3 times parallelly. The contents of essential trace elements and potential toxic elements are shown in Fig. 1.

From the table above, it's easy to find out that Rhizoma Curcumae contain essential trace elements such as Zn, Ti, Cu, B, V, Ni, Co, etc. The content of element Zn is on the top, followed by Ti, Cu. After the process, the content of essential trace elements, such as Zn, Ti, B and Ni, was increased more or little. On the other hand, the content of potential toxic elements was decreased, such as Li, As, Cd, Pb.Chinese pharmacopoeia 2010 limited the content of heavy metal Cd to less than 0.3 ug/g, Pb 5 ug/g, As 2 ug/g, and Hg 0.1 ug/g. The result of determination shows that the contents of Cd and Pb in some samples are out of limits, while the contents of As and Hg are within prescribed limits.

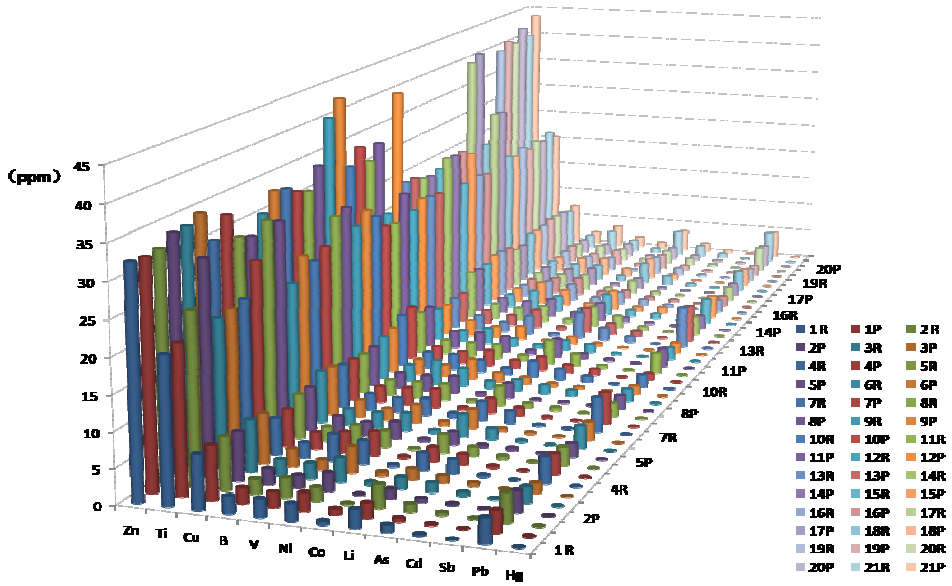

Fig. 1 Determination of trace essential elements（ppm, n=3； R:Raw Products P:Processed Products）

It is generally accepted that Be, Sb, Ti, Ge, Cd, Pb, Hg and other heavy metals, commonly defined as those having a specific density of more than 5 g/cm3, are detrimental to living creatures and human beings [23] .Through the results of the experiment on 21 sample batches, we can see that the contents of Ge and Pb are out of the limit. Heavy metal elements can be gathered into stem, directly impacting the growth and development of plants and reducing the yield. Thus, the heavy metals gathered into the plants can also threaten the health of human being through the food chain [24] . If the solid is polluted by Ge, not only the environment of the yield [25] , but also the quality and safety of Rhizoma Curcumae cultivated in this solid will be influenced tremendously. Clearly, the environmental protection of a planting area is really crucial, because it can directly influence the quality of the herbal medicines. Not to mention the genuine producing area of Curcuma phaeocaulis Val., such as Pengzhou, in Sichuan, which is facing the double challenge of being rebuilt after the earthquake by limiting pollution and adopting a bio-comprehensive governance to heal the polluted solid.

Compared with Rhizoma Curcumae (the raw product), the contents of trace elements, such as Zn, contained in Rhizoma Curcumae stir- baked with vinegar (the processed product) have changed, as well as the efficacy. We can speculate that the reason why the efficacy of blood-breaking and disorder-eliminating enhanced after being processed with vinegar could be closely related to the change of the contents of trace elements. Besides, the contents of some heavy metals, such as Cd, were reduced after being processed with vinegar, quite reducing the toxicity of the heavy metals contained in Rhizoma Curcumae. Thus, both the increase of some essential trace elements and the decrease of some toxic trace elements after processing could serve as an evidence for the “Effect-enhancing and Toxicity-reducing”mechanism, one of the basic mechanisms of the processing technique of TCMs.

3.2 Cluster analysis

The SPSS 19.0 statistical softwarewas used to do the cluster analysis with the determined results of the trace elements content of 21 batches of *Rhizoma Curcumae.* Euclidean was adopted to measure the distance and link two samples by the method of Average linkage, using 13 elements as the cluster variables of 21 *Curcumae Rhizoma;* finally, the tree diagrams of the systemic cluster analysis were obtained (Fig. 2).

As shown in figure 2, it is shown that Rhizoma Curcumae from different areas of production can be mostly distinguished during the hierarchical cluster analysis of the samples gathered into 3 groups. Compared with *Curcuma wenyujin* Y. H. Chen et C. Ling, *Curcuma phaeocaulis* Val. is more similar to *Curcuma kwangsiensis* S. G. Lee et C. F. Liang. The no.9 *Curcuma phaeocaulis* Val. from Yuxi Yunlan and no.17

Curcuma kwangsiensis S. G. Lee et C. F. Liang from Chunxiong Yunlan were not gathered into groups according to their corresponding origins, but they were both gathered into *Curcuma wenyujin* Y. H. Chen et C. Ling, indicating that the environment of the areas of production can certainly influence the capability of gathering of the elements, thus, both of them are different from *Curcuma phaeocaulis* Val. and *Curcuma kwangsiensis* S. G. Lee et C. F. Liang but tend to be similar to *Curcuma wenyujin* Y. H. Chen et C. Ling. From the results of a further classification , the same sources of Rhizoma Curcumae could well get together into one class; it can be demonstrated that Rhizoma Curcumae from different origin has different capability of selective enrichment. In other words, the plant might absorb given elements in a proportional way [26].

This consequence of "Same Quality and Diverse Efficacy"somehow confirms the genuineness of the Chinese medicine theory.

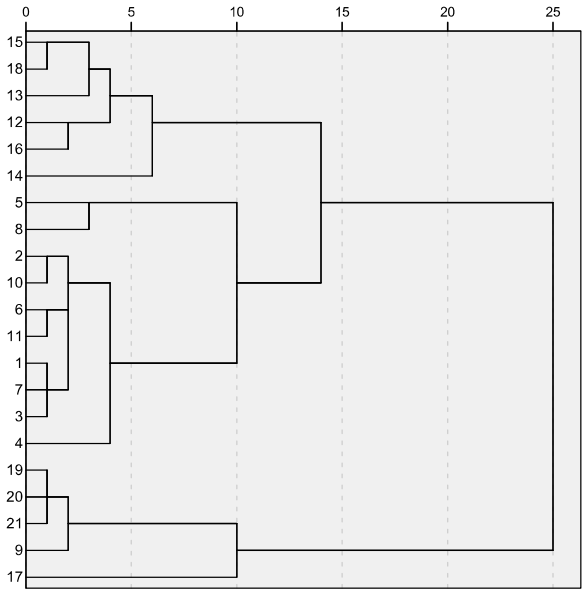

Fig. 2 Results of cluster analysis

3.3 Discriminant Analysis

Based on the results of cluster analysis, the samples were classified into three species according to their origins. Thus, the statistical software, SPSS 19.0, was used to do the discriminant analysis for the categories of the areas of production of Rhizoma Curcumae through the contents of trace elements .

In order to simplify the discriminant function, we only chose the element Zn as index. Through the equivalence test between group statistics and group average, the element Zn showed to contain 0.415 of Wilks'Lambda, 15.811 of chi-square and the P value equal to 0, so the difference among the groups is obvious. This is to say the

contribution of Zn to the discrimination function is large, while the other elements were excluded for their minor influence on the discrimination function. Therefore, the element Zn was chosen as the content of the variable with the highest discriminant efficiency. Classification Function Coefficients were obtained through the Bayes discriminant method. Eventually, the Fisher linear discriminant function result as shown below:

$$\begin{cases} y_1=1.624x-26.636 \\ y_2=1.265x-16.88 \\ y_3=2.019x-42.127 \end{cases}$$

(y1 , y2, y3 separately correspond to Curcuma phaeocaulis Val., Curcuma wangsiensis S.G. Lee et C.F.Liang and Curcuma wenyujin Y.H.Chenet C.Ling; X correspond the content of Zn (ppm))

By bringing the discriminant objects, that is the content of the element Zn, into y1, y2, y3 type, the discriminant function values were calculated. The highest value is the corresponding origin of it. Through verified classification results, the accuracy of the origin discrimination can reach 90.5% in the initial case, consistent with the results of the cluster analysis. Thus, it has been proved that this method can identify the different origins of Rhizoma Curcumae scientifically and effectively.

The reason why contents of Zn are obviously different if the origin is different may be that: in the one hand, Rhizoma Curcumae from different origin show a different capability of gathering the element Zn; on the other hand, it may be related to the distributing difference. However, the mechanism of this hypothesis need further research to be proved.

4. Conclusion

The material base for the efficacy of Rhizoma Curcumae can be generally divided into two categories: organic components and inorganic elements. Both of them can mutually coordinate and restrict each other, participate in various biochemical reactions within the body and facilitate the self-adjustment of the body, attaining the purpose of the therapy [27] .The research of inorganic elements in Rhizoma Curcumae and its processed products are also vital to reflect the properties of Rhizoma Curcumae, provide a guidance for the further research of its active components and offer more scientific evidence for the quality control, quality determination and reasonable clinical application of Rhizoma Curcumae and its processed products. By doing a further discriminant analysis, we innovatively present a concept that Rhizoma Curcumae, even the other TCMs, from different sources can be identified by the discriminant function of trace element, which can identify the different origins of

Rhizoma Curcumae scientifically and effectively. It is expected that the established method can provide a new train of thought for the quality control of TCMs.

Furthermore, the trace elements contained in TCMs have profound significance for the quality control of TCMs [28]. Some of them are closely related with the efficacy of the medicine, while others (heavy metal elements) may directly affect the human heath if out of the limits. The U.S. Pharmacopoeia limits total heavy metals in most oral dietary supplements to 20 ppm, sometimes with lower limits for lead, arsenic, and mercury, and each monograph has a specific requirement for the content determination of heavy metals [29]. In the Chinese Pharmacopoeia 2010 did not recorded the content determination of the heavy metals for each Chinese medicinal materia. However, the decocted pieces are required to be used in the formulation of Chinese Patent Medicines and, as since a long time, in clinical practice [30]. Thus, the accomplishment of the content determination of the trace elements in the decocted pieces (including the processed products) is necessary for the quality control and a further development of TCMs.

Acknowledgements

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