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Qualitative and Quantitative Studies on Marine Shell TCM Concha Arcae Based on Multi-Analysis Methods

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ABSTRACT

Concha Arcae is a widely used marine shell traditional Chinese medicine (TCM), which is commonly called 'Walengzi' in China. Concha Arcae is often used to treat stomachache. Its main and active composition is calcium carbonate ($CaCO_3$) which content is more than 90%. The quality control standard in China is not sufficient and limited, and the quality co ntrol studies become difficult, due to the high $CaCO_3$ content. Here, we have developed some studies on Concha Arcae to reveal the natures of it, in order to create a foundation to support the quality control study and development of Concha Arcae. Powder microscopic identification was performed using powder slide-making method. HPLC characteristic spectrum was developed with an Agilent 1260 Series HPLC-DAD system. The IR, Raman and X-ray diffraction (XRD) spectra were detected. The heavy metals were determined and $CaCO_3$ content were measured by EDTA titration. These techniques are the first comprehensive applications to study the natures of Concha Arcae, and these are helpful to develop the quality standard of Concha Arcae.

Keywords: Marine shell TCM Concha Arcae, Multi-analysis method, Qualitative and Quantitative studies, Walengzi

INTRODUCTION

Marine traditional Chinese medicine is an important composition of TCM, where Concha Arcae as a commonly used marine TCM is one of 12 kinds of marine shell TCM which was recorded in 2010 edition of Chinese Pharmacopoeia [1]. It is the shell of Cockle family animals Arca subcrenata Lisehke, Arca granosa Linnaeus and Arca inflata Reeve which is mainly distributed in coastal regions of China. It is divided into crude products and calcined products for use in clinic. The crude products will expel at eliminating phlegm and stasis, softening the hardness and dissolving nodules. The calcined products will also expel at relieving gastric pain by reducing acid.

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Previous studies have shown that $CaCO_3$ is the main composition of Concha Arcae,the content of which is more than 90% [2], meanwhile it is the active composition so as to neutralizing stomach acid. That leads the quality control study of Concha Arcae becomes rather difficult, therefore, we are trying to reveal the natures of Concha Arcae by some exploratory researches. We are looking forward to better understand Concha Arcae which has a long use history of these researches and make it better use in clinic. The 2010 edition of Chinese Pharmacopoeia just simply has described the resource and characters of Concha Arcae. There are no any qualitative and quantitative indexes to control its quality in clinical application. Additionally, it is applied in clinic in powder form so that the resources and characters description in 2010 edition of Chinese Pharmacopoeia can not be effectively controlled for the commercial quality. In addition modern pharmacology researches have demonstrated that Arca subcrenata Lisehke showed anti-tumor activity in vivo and vitro models [3-4].

In the present report, qualitative and quantitative studies including the powder microscopic identification, HPLC characteristic spectrum, IR, Raman, XRD spectra, heavy metal determination and EDTA complexicretric titration were done on 16 batches of Concha Arcae to develop better understanding of Concha Arcae, and so that these developed researches create the foundation of quality control studies on Concha Arcae.

MATERIALS AND METHODS

Plant materials

A summary of 16 batches of crude Concha Arcae is given in Table 1, collected from different habitats in coastal area of China, and identified by Prof. Dr. Wei Wang. The crude products samples were burned to get 16 batches of calcined products. The voucher specimens have been deposited in TCM and Ethnomedicine Innovation & Development Laboratory, School of Pharmacy, Hunan University of Chinese Medicine.

Instruments and reagents

Nikon Eclipse 50i microscope was used for microscopic identification. Mettler-Toledo analytical balance (AE100S) was used for weighing samples. All HPLC analyses were performed with an Agilent 1260 Series HPLC-DAD (Santa Clara, CA, USA) system consisting of a vacuum degasser, quaternary pump, automatic sampler, thermostated column compartment and DAD detector. An ultrasonic cleaner with a frequency of 40 kHz (KQ-2200DB, Kunshan Ultrasonic Instrument Co. Ltd., China) was used for extraction. Agilent 7700e ICP-MS (Santa Clara, CA, USA) was used to test the heavy metals of Walengzi. Nicolet FTIR 6700 (American); Thermo Fisher DXR Raman microscope (American); Bruker D8 advance powder XRD instrument (Germany) were used for the IR, Raman and XRD spectra researches. All solvents for heavy metal detection were electronic grade. All solvent for microscopic identification and EDTA complexicretric titration are analytical grade, and those for HPLC analysis were chromatographic grade.

Microscopic identification

Powder slide-making method was utilized to do the microscopic identification [8]. The crude products and calcined products were shattered and passed through an 80-mesh sieve. A little powder was taken and placed in a clean glass slide and to it are added 1 to 2 drops chloral hydrate and diluted glycerol, then covered with the coverslip. Thereafter, observed under a microscope.

HPLC characteristic spectrum [9]

Ten gms of crude Concha Arcae powder was exactly weighed into the conical flask and to it 10 mL MeOH was added into the conical flask and ultrasonic extracted for 60 min. The extraction was filtered, and the filtrate solution was diluted to 10 mL. Lastly, the solution was filtered through a 0.45 μm nylon filter membrane before HPLC analysis. The ZORBAX Eclipse XDB-C18 HPLC column (4.6×250mm, 5μm) was applied for chromatographic separation. Acetonitrile and water containing 0.01% phosphoric acid (21:79) was used as the mobile phase of HPLC with isocratic elution. Analysis was performed at 20 °C and at the flow rate of 0.8 mL/min with 210 nm DAD detection.

$CaCO_3$ content determination

EDTA complexocretric titration method was adopted to determine the content of $CaCO_3$ [5-7]. Weighed exactly 0.15 gram of Concha Arcae powder, transfer it into the conical flask, and add 10 mL diluted HCl, then heat till it is dissolved completely, add 20 mL H_2O and one drop of methyl red indicator solution. Titrate by 10% KOH making the solution turn yellow and continue to add 10 mL 10% KOH to adjust the pH to about 13. Then titrate by EDTA (0.05 mol/L) until the color of the solution changes from the yellow-green fluorescence to orange. Each 1mL EDTA solution (0.05 M) is equivalent to 5.004 mg $CaCO_3$.

Heavy metal determination

ICP-MS method was adopted to determine the heavy metal [1, 10]. After digestion by nitric acid and the perchloric acid, the samples were induced in the ICP-MS to test the content of Pb, Cu, As, Cd, Hg.
2.7 IR, Raman and X-ray diffraction spectra researches [11-13]

KBr disc technique was adopted for IR spectroscopic analysis. Weigh sample 1-2 mg and 200-mesh KBr powder 200 mg, put together then grind to uniform under the IR lamp. Put the mixture into pressure mold and press 2 min under 27 MPa pressure. Then scan from 4000-400 cm^{-1} by Nicolet FTIR 6700 IR instrument.

DXR Raman microscope was used to test the Raman spectra. The laser power level is 10.0 mW, the filter is 532 nm, and the exposure time is 1 s for exposing over 10 times.

X-ray was detected under Bruker D8 advance XRD. The experimental conditions are as follows: X ray tube potential and current: 40 kV, 40 mA. Scan speed: 0.1 s / step. Scan range: 3°- 40°.

RESULTS AND DISCUSSION

$CaCO_3$ is the main and active composition of Concha Arcae, it is essential to determine the content of $CaCO_3$. But the only $CaCO_3$ content determination can not be enough to reveal the properties and control the quality of Concha Arcae. Microscopic identification is one of the most popular and effective methods to identify TCM. Microscopic identification of TCM can be intuitive expression of the particular characteristics by the microscopic pictures or figures so as to effectively identify the TCM. HPLC characteristic spectrum is another widely used method to identify the TCM. With the help of chromatographic characteristic spectrum, the authentication and identification of herbal medicine can be accurately conducted. IR, Raman and XRD spectra are widely used methods to do the qualitative analysis of unknown materials. They have the advantages of widely suitability, conveniences, high sensitivity, good repeatability and rapidly determinations. Where as exceeding heavy metals in TCM is a common problem which lead to the toxicity and influence the market development of TCM. So in this communication, microscopic identification and HPLC characteristic spectrum, IR, Raman, XRD spectra, heavy metal determination and $CaCO_3$ content determination were utilized to reveal the properties of Concha Arcae, so as to provide strong basis to quality control study of Concha Arcae.

The research on microscopic identification revealed that 16 batches of crude products and 16 batches of calcined products have the same powder microscopic characteristics. The powder is off-white, where in long strip, quadrilateral, triangle, quasi-circular or irregular fragments can be seen. The powder is obviously granularity and can be seen straight or slightly curved stripes on the surface of some fragments (Fig.1). Briefly, 3 kinds of resource of Concha Arcae and 2 types of decoction have the same powder microscopic characteristics. The microscopic identification has provided a strong qualitative evidence for identification of Concha

Arcae.

HPLC chromatograms of 16 batches of crude Concha Arcae can be seen in Fig. 2. The HPLC characteristic spectrum was established (Fig.3). Three common characteristic peaks were separated in the crude products within 20 minutes. The similarities of 16 batches of crude products were over 0.90 (Table 2). The RSDs of precision, repeatability are smaller than 3%. The stability of the method indicated that the sample was stable within 48 h. In short, a rapid, simple and stable HPLC characteristic spectrum method of raw products was established to identify the crude products.

The $CaCO_3$ content of 16 batches of crude Concha Arcae and 16 batches of calcined Concha Arcae indicated that the $CaCO_3$ content of crude products is more than 94% (Table 3), that of the calcined products is more than 95% (Table 4). That indicated that $CaCO_3$ is the main composition of Concha Arcae which is more than 94%. The calcined products have higher $CaCO_3$ content than the crude products indicated that the $CaCO_3$ content became higher after calcining.

The heavy metals (Pb, Cu, As, Cd, Hg) content of crude products and calcined products can be seen in Table 5 and 6. The data indicated that Pb, Cd, As, Cd, As, Hg were less than 0.7, 0.2, 0.7, 0.2, 4 mg/kg respectively. These contents of heavy metals are much less than the safety level of them so it is safe when be used in clinic.

The IR spectra of crude products are similar to that of calcined products (Fig. 4 and 5). The IR characteristic peaks of $CaCO_3$ are as follows: 3419.17~3434.60 cm-1 belongs to the water symmetric H-O stretching mode, asymmetric H-O stretching mode peak; 2512.79~2522.43 cm-1 belongs to the stretching vibration (?1+ ?3) peak of calcite; 1787.69~1797.33 cm-1 belongs to the stretching vibration (?1+ ?4) combination bands of calcite; 858.17~875.32 cm-1 belongs to the [CO3]2- out-of-plane deformation mode peak of calcite; 711.60~713.53 cm-1 belongs to O-C-O bending (in-plane deformation) mode band of calcite. All of these IR peaks are authentic to $CaCO_3$.

The calcite Raman characteristic peaks can be found in the Raman spectra of crude and calcined products (Fig. 6-9). The assignments of characteristic peaks are as follows: 1084.62 cm-1 belongs to the symmetric C-O stretching mode peak; 704.70 cm-1 belongs to the O-C-O bending (in-plane deformation) mode of calcite, 280.78 and 151.99 cm-1 belongs to the Ca2+ and $[CO_3]$2- combination lattice vibration bands.

The XRD spectra indicated that the crude products only consist of aragonite apart from R-7 and R-9 can found the characteristic peaks of calcite and aragonite (Fig. 10-11). And all the calcined products can find the characteristic peaks of calcite and aragonite (Fig. 12-13). The differences of XRD spectra between crude products and calcined products showed that the crystal form of $CaCO_3$ changed after calcining.

CONCLUSION

Powder microscopic identification, HPLC characteristic spectrum, IR, Raman, XRD spectra, heavy metal and CaCO3 content determination were conducted to research Concha Arcae. All these works can provide useful guidelines for quality control study of Concha Arcae. Through these studies, Concha Arcae has shown better understanding and better applications.

The microscopic identification, HPLC characteristic spectrum, IR, Raman, XRD spectra provide the qualitative methods to distinguish the true from the false of Concha Arcae where $CaCO_3$ is over 94%. Heavy metal and $CaCO_3$ content determination provide the quantitative indexes to assess the quality of Concha Arcae.

It is rather a need than desire to develop the quality control standard of Concha Arcae under the situation of current poor quality standard. The microscopic identification, HPLC characteristic spectrum and $CaCO_3$ content determination will give the quantitative and qualitative control indexes of Concha Arcae.

ACKNOWLEDGEMENTS

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Table 1: 16 Batches of Crude Concha Arcae

Code	Producing Area	Resources	Code	Producing Area	Resources
R-1	Shandong	Arca subcrenata Lisehke	R-9	Liaoning	Arca subcrenata Lisehke
R-2	Shandong	Arca subcrenata Lisehke	R-10	Guangdong	Arca subcrenata Lisehke
R-3	Shandong	Arca subcrenata Lisehke	R-11	Fujian	Arca subcrenata Lisehke
R-4	Guangxi	Arca subcrenata Lisehke	R-12	Shandong	Arca subcrenata Lisehke
R-5	Guangxi	Arca subcrenata Lisehke	R-13	Shandong	Arca subcrenata Lisehke
R-6	Guangxi	Arca subcrenata Lisehke	R-14	Anhui	Arca subcrenata Lisehke
R-7	Fujian	Arca subcrenata Lisehke	R-15	Guangdong	Arca granosa Linnaeus
R-8	Fujian	Arca subcrenata Lisehke	R-16	Ningbo	Arca inflata Reeve.

Table 2: The Similarities of 16 Batches of Crude Concha Arcae

Code	Similarity	Code	Similarity
R-1	0.993	R-9	0.935
R-2	0.889	R-10	0.889
R-3	0.980	R-11	0.960
R-4	0.952	R-12	0.903
R-5	0.964	R-13	0.961
R-6	0.923	R-14	0.901
R-7	0.993	R-15	0.865
R-8	0.938	R-16	0.925

Table 3: $CaCO_3$ Content of Crude Concha Arcae

Code	$CaCO_3$ content (%) (n=3)			Average content (%) ((%))
R-1	95.59	96.30	95.94	95.94±0.36
R-2	95.25	95.79	96.60	95.88±0.68
R-3	95.48	95.75	95.11	95.45±0.32
R-4	95.78	95.40	96.08	95.75±0.34
R-5	95.79	95.14	95.58	95.50±0.33
R-6	95.87	95.64	95.42	95.64±0.23
R-7	95.79	95.13	95.57	95.50±0.34
R-8	96.19	95.66	96.13	95.99±0.29
R-9	95.80	95.78	95.65	95.74±0.08
R-10	95.36	94.89	95.48	95.24±0.31
R-11	95.12	94.55	94.62	94.76±0.31
R-12	94.62	94.47	94.25	94.45±0.19
R-13	95.26	95.09	94.99	95.11±0.14
R-14	95.54	95.25	94.34	95.04±0.63
R-15	94.32	95.04	94.03	94.46±0.52
R-16	94.58	94.96	94.30	94.61±0.33

Table 4: $CaCO_3$ Content of Calcined Concha Arcae

Code	CaCO3 content (%) (n=3)			Average content (%) ((%))
P-1	97.84	96.65	96.05	96.84±0.91
P-2	96.33	96.16	96.96	96.48±0.42
P-3	96.62	96.80	97.12	96.85±0.25
P-4	96.64	96.84	96.34	96.61±0.25
P-5	97.11	97.65	98.17	97.64±0.53
P-6	96.17	95.54	95.92	95.88±0.32
P-7	97.30	96.70	96.57	96.86±0.39
P-8	96.64	96.84	96.34	96.61±0.25
P-9	97.30	96.70	96.57	96.86±0.39
P-10	97.04	96.65	97.66	95.81±0.51
P-11	95.86	96.66	96.39	96.30±0.41
P-12	96.16	95.66	95.61	95.81±0.30
P-13	95.30	95.59	95.45	95.45±0.15
P-14	96.19	96.17	96.18	96.18±0.01
P-15	96.23	96.88	96.73	96.61±0.34
P-16	95.43	95.77	95.48	95.56±0.18

Table 5: The Heavy Metal Content of Crude Products

Code	*Heavy metal Content (mg/kg)*				
	Cu	*As*	*Cd*	*Hg*	*Pb*
R-1	1.069	0.375	0.017	0.111	0.404
R-2	0.423	0.397	0.017	0.074	0.533
R-3	0.406	0.316	0.017	0.171	0.343
R-4	0.195	0.271	0.013	0.051	0.270
R-5	3.421	0.318	0.043	0.174	0.468
R-6	0.620	0.245	0.034	0.099	0.352
R-7	0.271	0.219	0.111	0.087	0.690
R-8	0.384	0.222	0.019	0.079	0.386
R-9	0.499	0.304	0.027	0.073	0.266
R-10	0.880	0.570	0.029	0.092	0.405

Table 6: The Heavy Metal Content of Calcined Products

Code	*Heavy metal Content (mg/kg)*				
	Cu	*As*	*Cd*	*Hg*	*Pb*
P-1	0.2925	0.3215	0.0152	0.0752	0.3478
P-2	0.3135	0.3176	0.0147	0.0768	0.3624
P-4	1.0433	0.2856	0.0113	0.0875	0.3105
P-5	1.5250	0.2638	0.0102	0.1035	0.2620
P-6	1.2712	0.2231	0.0432	0.0869	0.4362
P-7	1.7994	0.2174	0.0832	0.0873	0.5251
P-8	1.8364	0.1937	0.0569	0.0694	0.5328
P-10	0.8922	0.1963	0.0251	0.0631	0.3581
P-13	0.9832	0.2746	0.0224	0.0863	0.2498
P-14	0.9212	0.4986	0.0271	0.0875	0.3682

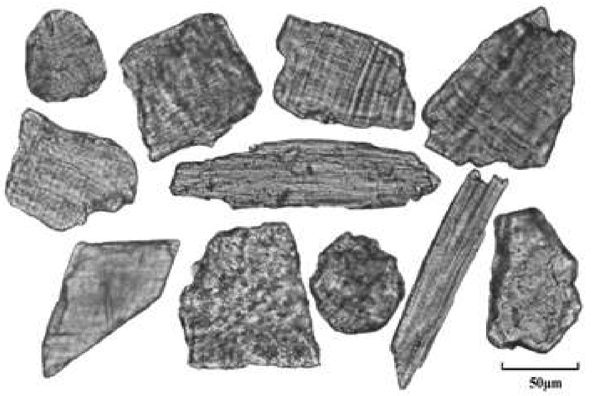

Figure 1: The Microscopic Characteristics of Concha Arcae

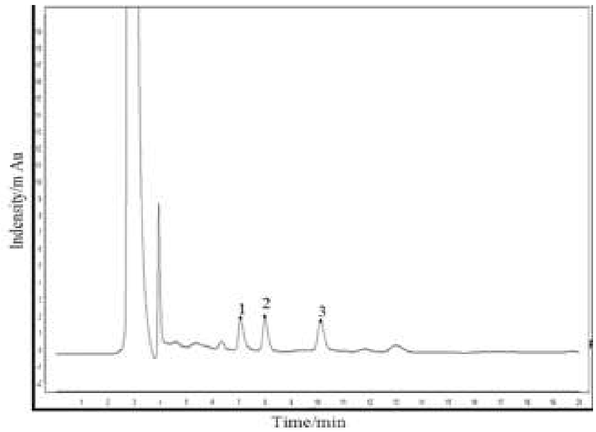

Figure 2: The HPLC Characteristic Spectrum of Crude Concha Arcae

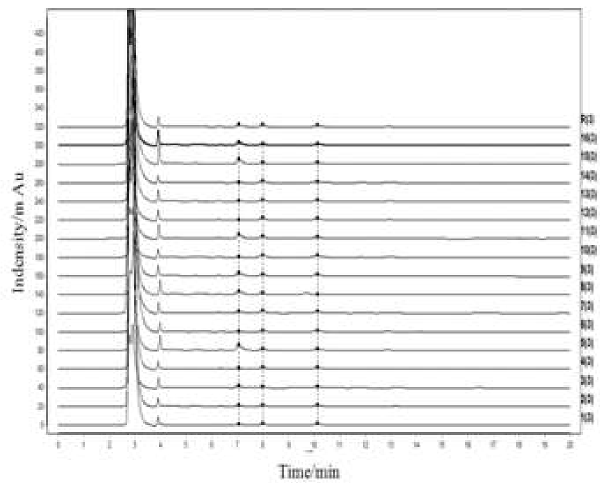

Figure 3: Hplc Chromatograms of 16 Crude Concha Arcae and Simulative Median Chromatogram

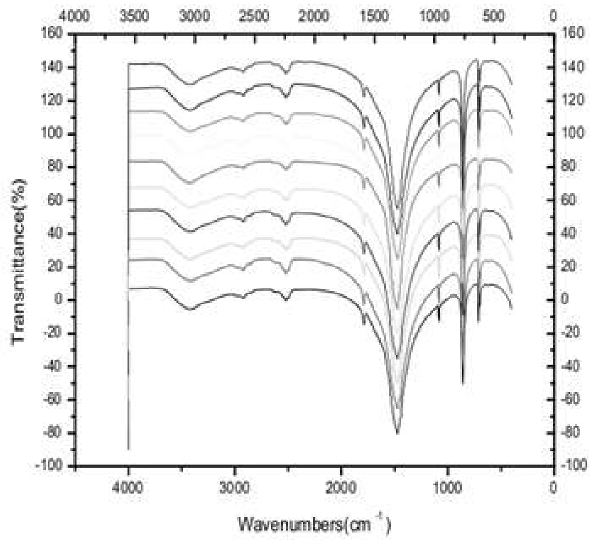

Figure 4: IR Spectra of 10 Batches of Crudeproducts

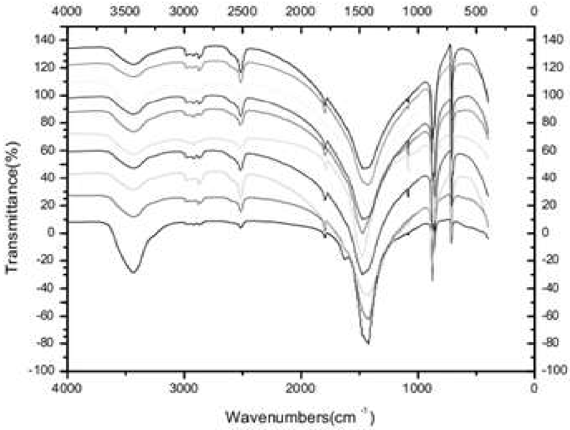

Figure 5: IR Spectra of 10 batches of calcined products

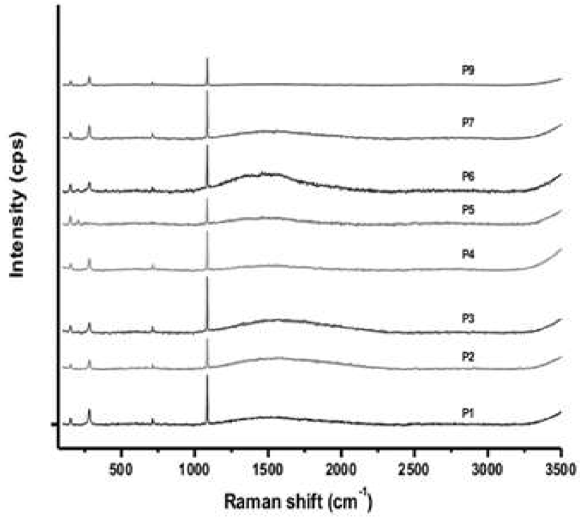

Figure 8: Raman spectra of calcined products (p-1~p-9)

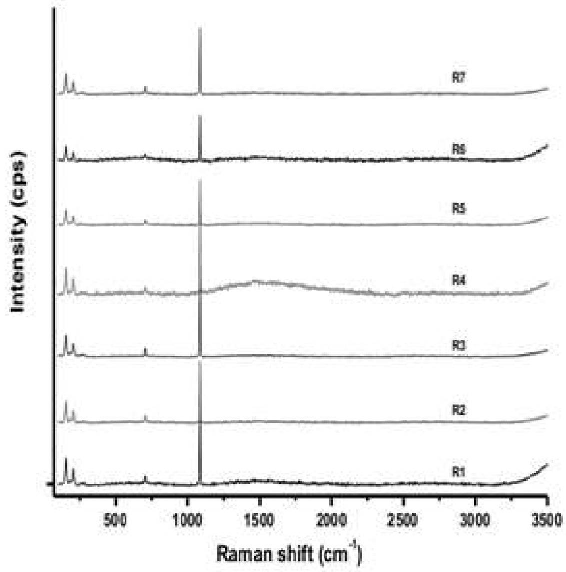

Figure 6: Raman spectra of crude products (r-1~r-7)

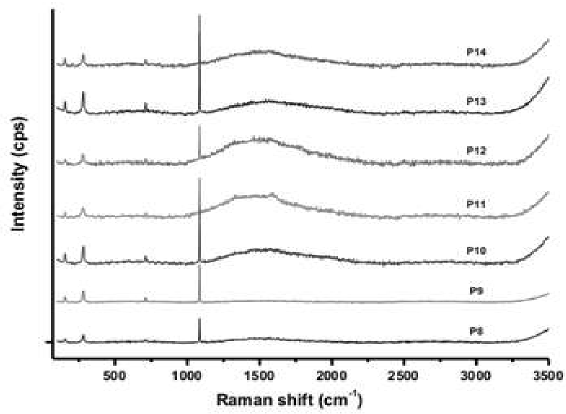

Figure 9: Raman spectra of calcined products (p-8~p-14)

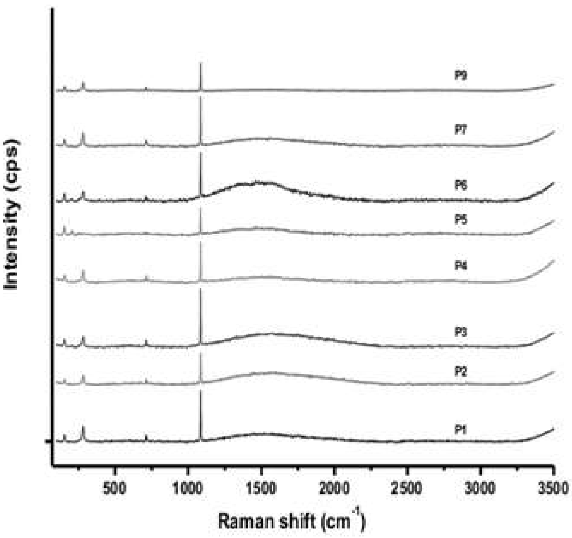

Figure 7: Raman spectra of crude products (r-8~r-14)

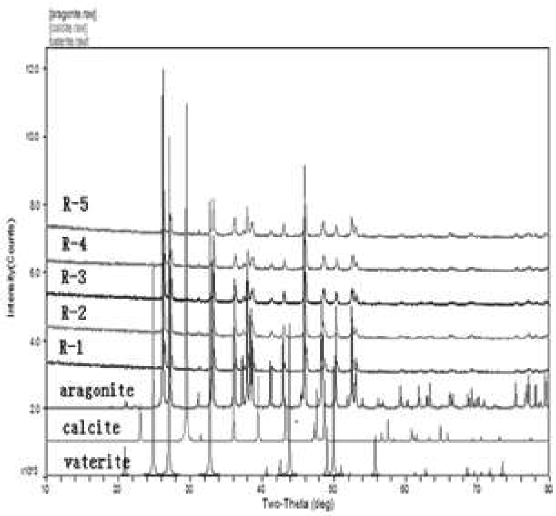

Figure 10: Xrd of Crude Products (R-1~R-5) And 3 Kinds of $CaCO_3$ Crystals

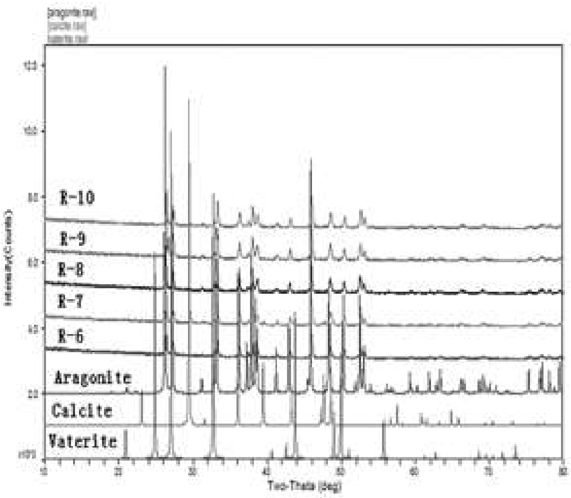

Figure 11: Xrd of crude products (r-6~r-10) and 3 kinds of $CaCO_3$ crystals

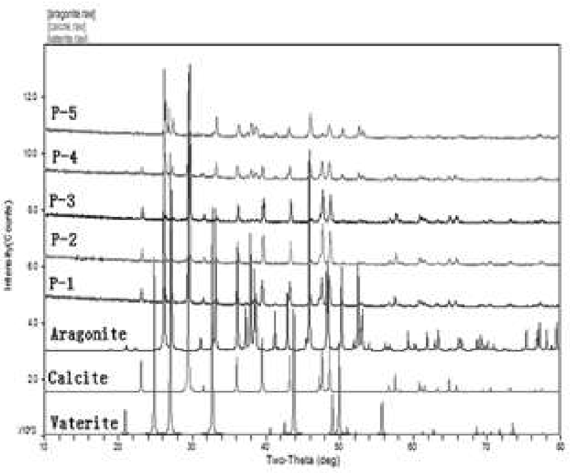

Figure 12: Xrd of calcined products (p-1~p-5) and 3 kinds of $CaCO_3$ crystals

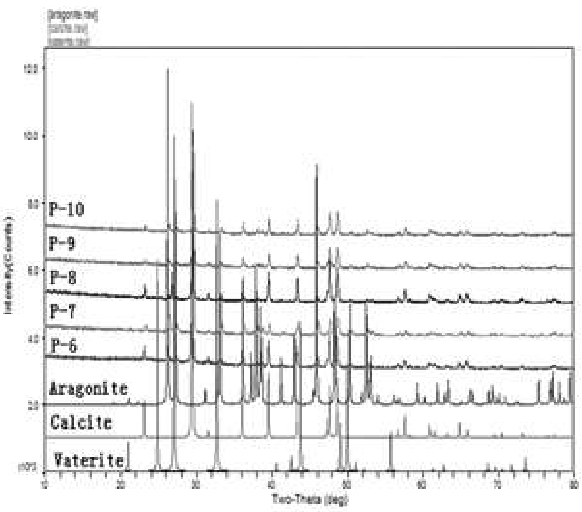

Figure 13: Xrd of calcined products (p-6~p-10) and 3 kinds of $CaCO_3$ crystals

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