

## Analysis of the Sesquiterpenoids in Processed *Atractylodes Rhizoma*

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In Asia, processed *Atractylodes Rhizoma*, the dried rhizome of *Atractylodes ovata* DE CANDOLLE (Compositae), is widely used as a tonic agent in herbal diets; stir-frying with soil is the most common processing method. In this study, we focused on determining variations in the function and concentrations of sesquiterpenoids in processed *Atractylodes Rhizoma*. Raw *Atractylodes Rhizoma* was processed by stir-frying it with different assistant substrates (*i.e.*, red soil and burnt clay). The results indicated that there was less atractylon in stir-fried materials than in raw materials. However, there were higher levels of atractylenolides II and III in stir-fried materials than in raw materials. We also found that the heavy-metal content in burnt clay exceeded regulations set by the Taiwanese government. Moreover, commercial *Atractylodes Rhizoma* in Taiwan exhibited great differences in concentrations of the active components. In addition, atractylon showed stronger cytotoxicity than atractylenolides II and III in various cell lines. Therefore, we suggest that the toxic effects of atractylon are reduced following atractylon degradation to atractylenolides II and III. In conclusion, the toxicity of *Atractylodes Rhizoma* is reduced through processing.

**Key words** *Atractylodes Rhizoma*; *Atractylodes ovata*; Compositae; processing; sesquiterpenoid; cytotoxicity

In Asia, both Western and Chinese medicines are in regular use. Herb use is an important characteristic of traditional Chinese medicine. *Atractylodes Rhizoma*, the dried rhizome of *Atractylodes ovata* DE CANDOLLE (Compositae), is easily purchased in traditional medicinal markets and is widely used in herbal diets as a tonic agent in Taiwan. In traditional medicine usage, it has several health-promoting functions, including antidiarrheal action, a benefit to the stomach, the arrest of abnormal sweating, a diuretic action, and nourishment. In regard to its chemical constituents, the major ingredients of *Atractylodes Rhizoma* are eudesmane-type sesquiterpenoids, *i.e.*, atractylon, and atractylenolides I, II, and III.<sup>1,2)</sup> Pharmacological functions of *Atractylodes Rhizoma* are closely related to these sesquiterpenoids, including activity against gastrointestinal disease,<sup>3,4)</sup> anti-hepatotoxicity,<sup>5,6)</sup> anti-inflammation,<sup>7)</sup> action against leukemia cells,<sup>8,9)</sup> and inhibition of Na<sup>+</sup>-K<sup>+</sup>-ATPase activity.<sup>10)</sup> A previous study showed that atractylon expressed greater cytotoxicity than atractylenolides I, II, and III in a HL-60 leukemia cell line.<sup>8)</sup> However, atractylon is an excessively unstable sesquiterpenoid. So when exposed to air at room temperature, atractylon will spontaneously oxidize to atractylenolides II and III<sup>11)</sup> (Fig. 1). Therefore, we discuss the effects of variations in the sesquiterpenoid contents of *Atractylodes Rhizoma* after being processed in this study.

According to the traditional Chinese pharmacopoeia, like *Ben Cao Gang Mu* or *Shang Han Lun*, processing (*pao zhi*) plays an important role in preparing Chinese medicines because the herbal properties are changed by different processing methods. Evidence has shown that different drying process conditions alter the volatile oil concentrations in *Laurus nobilis* L. and peppermint.<sup>12,13)</sup> With the same drying process, we were able to change the extraction yield of volatile oil in *Curcuma longa* L. through a steam processing method.<sup>14)</sup> Moreover, the antioxidative abilities of *Cassia tora* L. and *Amygdalus communis* L. were influenced by dif-

ferent roasting processing conditions.<sup>15,16)</sup> We also could change the composition of isoflavones in soybeans by a soaking processing method.<sup>17)</sup> Interestingly, the stir-fry processing method has different functions in the preparation of Chinese herbs. The flavonoid and polysaccharide contents differed in raw or stir-fried *Typha angustifolia* L. because of different stir-fry conditions, including stir-fried temperatures and times.<sup>18)</sup> Moreover, levels of *p*-hydroxybenzoylcholine bisulfate, the active component in the seeds of *Brassica alba* L., were increased after stir-fry processing.<sup>19)</sup> There are still many general processing methods including baking, calcining, carbonizing, and simmering. Besides processing methods, assistant substrates also play a critical role in preparing Chinese prescriptions. All assistant substrates can be divided into liquid assistant substrates, *i.e.*, wine, vinegar, honey, rice-powder solution, and rice-washing solution, and solid assistant substrates, *i.e.*, paddy, wheat bran, red soil, and burnt clay. At the present time, critical problems with the commercial processing of Chinese herbs are the quality and

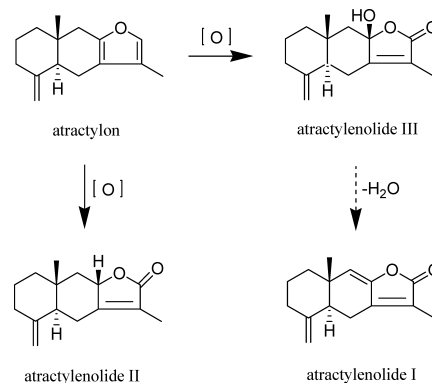


Fig. 1. Structural Variations of Sesquiterpenoids in *Atractylodes Rhizoma* [O], oxidation. The conversion from atractylenolide III to atractylenolide I does not occur spontaneously.

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quantity of the active components. The quality control of *Atractylodis Rhizoma* is an important question, making it necessary to establish standardized analytical methods.

In this study, the sesquiterpenoid concentrations of *Atractylodis Rhizoma* processed by different methods and with different assistant substrates were detected with an HPLC system. We ignored atractylenolide I because of its low concentrations. In addition, the cell cytotoxicity of these three major active components was also further examined. The aim of our study was to investigate the effect of stir-fry of *Atractylodis Rhizoma* to obtain a better understanding of the pharmacological effects of the processing procedures. Moreover, we collected commercially processed *Atractylodis Rhizoma* from different areas of Taiwan and analyzed the sesquiterpenoid contents in this herb to authenticate the quality of this useful Chinese drug in Taiwan.

### Experimental

**Chemical and Reagents** HPLC grade acetonitrile and tetrahydrofuran (THF) were purchased from Merck (Darmstadt, Germany) and Lab-Scan (Dublin, Ireland). Penicillin–Streptomycin, trypsin–EDTA and fetal bovine serum (FBS) were purchased from Gibco (Gibco-BRL, U.K.). Dimethyl sulfoxide (DMSO), adriamycin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Cell culture mediums, Dulbecco's MEM (DMEM) and Nutrient mixture F12 HAM, Kaighn's (Ham's F12K) were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, U.S.A.). Purified deionized water was prepared by Millipore Milli-RO (Milli-QRG, U.S.A.).

**HPLC Analysis of Sesquiterpenoids in *Atractylodis Rhizoma*** The HPLC system was comprised of an LC-10ATvp liquid chromatographic pump system, including an SPD-10A UV–VIS detector, a C-R8A Chromatopac recorder, a SIL-9A auto-injection, and a CTO-10A column oven (Shimadzu Corporation, Tokyo, Japan). Purospher® STAR RP-18e reversed-phase column (5  $\mu$ m, 4 mm i.d.  $\times$  250 mm, Merck, Darmstadt, Germany) was used as the stationary phase, and the mobile phase was CH<sub>3</sub>CN–H<sub>2</sub>O (80:20, v/v) for atractylon and CH<sub>3</sub>CN–H<sub>2</sub>O–THF (37:58:5, v/v) for atractylenolides II and III. All mobile phases were degassed by ultrasonication and filtered through a 0.45- $\mu$ m FP Vericel (PVDF) membrane (Pall Corporation, Ann Arbor, MI, U.S.A.). The flow rate was 1 ml/min, and the oven temperature was maintained at 40 °C. The UV detection wavelengths for atractylon, and atractylenolides II and III were 220 and 236 nm, respectively.

**Calibration Curve Preparation** Atractylon, and atractylenolides II and III were accurately weighed, dissolved, and double-diluted in HPLC-grade methanol to give serial concentrations in the range of 3.91 to 1000, 0.78 to 100, and 3.91 to 500  $\mu$ g/ml, respectively. Calibration curves were plotted after linear regression of the peak areas.

**Method Validation** We evaluated the intra-day and inter-day variations of atractylon, and atractylenolides II and III in methanol-extracted samples. The precision was evaluated in terms of intra-day and inter-day assays. The intra-day assay used triplicate analyses within 24 h, and the inter-day assay used triplicate analyses over 3 d, with each injection separated by at least a 24-h interval. Recovery analysis also used methanol-extracted samples which were spiked with known amounts of sesquiterpenoids to yield final concentrations of 250, 125, and 62.5  $\mu$ g/ml of atractylon, 25, 12.5, and 6.25  $\mu$ g/ml of atractylenolide II, and 125, 62.5, and 31.25  $\mu$ g/ml of atractylenolide III. Each sample was also analyzed in triplicate. The average, standard deviation (S.D.), and relative standard deviation (RSD) were then calculated.

**Atractylodis Rhizoma Processing** Raw *Atractylodis Rhizoma*, red soil and burnt clay were purchased from a traditional Chinese medicinal market in Taipei and identified by Dr. Hsien-Chang Chang. A voucher specimen of *Atractylodis Rhizoma* (AO-1013) was deposited in Graduate Institute of Pharmacognosy Science, Taipei Medical University. The herbs were weighed and subjected to stir-fry processing by our established procedure. The stir-fry method was performed without or with assistant materials, *i.e.*, red soil or burnt clay, for 5, 10, 20, or 30 min respectively. Red soil is the most common assistant material used in modern herb processing and its properties are high content of unhydrated iron oxides, aluminum oxide, and heavy clay. The most region of red soil in China is located in the western Zhejiang province.<sup>20)</sup>

The burnt clay was taken from the lining of a kitchen range, which is a

traditional cooking stove used in China.

**Commercial Atractylodis Rhizoma Collection** Commercial *Atractylodis Rhizoma* was collected from northern (Keelung to Hsinchu), central (Miaoli to Changhua) and southern areas (Yunlin to Kaohsiung) of Taiwan. All materials were identified by Dr. Hsien-Chang Chang. In total, we collected 38 batches of *Atractylodis Rhizoma* samples. Six batches (raw samples, northern Taiwan) were numbered as AO-NR-A to AO-NR-F and these samples were purchased from Taipei County (AO-NR-A to AO-NR-C), Taipei City (AO-NR-D, AO-NR-E) and Hsinchu County (AO-NR-F). Fourteen batches (processed samples, northern Taiwan) were numbered as AO-N1 to AO-N14. They were obtained from Keelung City (AO-N1, AO-N2), Taipei County (AO-N3 to AO-N6), Taipei City (AO-N7 to AO-N11), Taoyuan County (AO-N12, AO-N13) and Hsinchu County (AO-N14). Eight batches (processed samples, central Taiwan) were numbered as AO-C1 to AO-C8. These processed samples were purchased from Miaoli County (AO-C1), Taichung County (AO-C2 to AO-C4), Nantou County (AO-C5), and Changhua County (AO-C6 to AO-C8). Ten batches (processed samples, southern Taiwan) were numbered as AO-S1 to AO-S10, and they were obtained from Yunlin County (AO-S1), Chiayi City (AO-S2 to AO-S6), Tainan County (AO-S7, AO-S8) and Tainan City (AO-S9, AO-S10). The commercial processed samples were processed with red soil. All of these samples were obtained from March to May, 2004.

**Sample Preparation** For the HPLC analysis, three treatments of processed samples (red soil, burnt clay and without substrates) and commercial *Atractylodis Rhizoma* were crumbled and filtered through a 20" mesh. Each 0.5 g of crumbled sample was extracted with 10 ml methanol for 30 min in an ultrasonic water bath. Samples were filtered through a 0.45- $\mu$ m syringe filter (Millipore Corporation, U.S.A.) and a 10- $\mu$ l sample was directly injected into the HPLC system.

**Analysis of Atractylon Oxidation to Atractylenolides II and III** Atractylon, and atractylenolides II and III were previously extracted in our laboratory. We steamed these pure compounds under two different high-pressure conditions: 121 °C for 30 and 60 min, with a steam pressure of 15 psi for both conditions. To monitor the structure variation after steaming method, we used photodiode array HPLC apparatuses for this analysis. Analytical instruments were composed of a SCL-10Avp System Controller, an LC-10ATvp Liquid Chromatograph Pump, an SPD-M10A Diode Array Detector, a SIL-10Avp Auto Injector, a CTO-10A Column Oven, FCV-10Avp Flow-Channel Selection Valves (Shimadzu Corporation, Tokyo, Japan), and an ERC-3415 Degasser (ERC, Altegolfsheim, Regensburg, Germany). The stationary phase consisted of a Purospher® STAR RP-18e reversed-phase column (5  $\mu$ m, 4 mm i.d.  $\times$  250 mm, Merck), and an acetonitrile–water system was used as the mobile phase in the gradient mode as follows: acetonitrile: 0–10 min, 40–45%; 10–20 min, 45–55%; 20–30 min, 55–100%; and 30–40 min, 100–55%. The flow rate was 1 ml/min, and the oven temperature was maintained at 40 °C. We used a UV wavelength of 220 nm to detect structure variation of atractylon, atractylenolides II and III.

**Water and Heavy Metal Content Analysis** The water contents of the raw and processed materials were analyzed using an MX-50 Moisture Analyzer (A&D Company, Tokyo, Japan). We weighed 1 g of each sample, heated it to 105 °C until there was no significant change in the water content of the material, and recorded and calculated the water content. We chose raw and 5 min processed materials to investigate the heavy metal content in *Atractylodis Rhizoma* and assistant substrates. The contents of lead, cadmium, mercury, arsenic, and copper were detected by atomic absorption spectroscopy (AAS) and hydride generation-atomic absorption spectrophotometry (Hg-AAS). The analyses were carried out by Hwayo Tech & Lab, Taipei, Taiwan.

**Cell Culture** The KB (human oral epidermoid carcinoma cells), DU145 (human prostate carcinoma cells), HeLa (human cervical epithelioid carcinoma cells), Hep3B (human hepatocellular carcinoma cells), and WISH (normal human amnion tissue cells) cell lines were maintained in DEME medium, and the AGS (human gastric adenocarcinoma cell) cell line was maintained in Ham's F12K medium. All cultures were supplemented with 10% FBS, 100 mg/l streptomycin, and 100 units/ml penicillin. Cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. When confluent, cells were washed with phosphate-buffered saline (PBS), trypsinized with 0.25% trypsin–EDTA in PBS, washed with fresh culture medium, and transferred to 96-well plates (3  $\times$  10<sup>4</sup> cells/ml) for the cytotoxicity assays.

**Cell Viability Assay** Atractylon, and atractylenolides II and III were initially dissolved in DMSO at a concentration of 10 mM and stored at –20 °C. Adriamycin, a positive control, was initially dissolved in DMSO at a concentration of 4 mM and stored at 4 °C. Serial dilutions of the stock solution were prepared in culture medium in 96-well microtiter plates. The test

samples at appropriate concentrations were added to the carcinoma cell lines (KB, DU145, HeLa, Hep3B, and AGS) and normal cell line (WISH) for 36 h without renewal of the medium. The number of surviving cells was then counted using the MTT assay.<sup>21)</sup> Finally, the products were evaluated by measuring the optical density of each well at 600 nm, using an MRX microplate reader (Dynex Technologies, Guernsey, Channel Islands, U.K.). The IC<sub>50</sub> values are expressed as the cytotoxic effects of these sesquiterpenoids.

**Statistical Analysis** Sesquiterpenoid concentration data were fitted using a Sigma plot. The data are presented as the mean and S.D. Significance was calculated using Student's *t*-test. Differences were considered significant for *p* values <0.05.

## Results

**Linearity** As described above, the chromatographic analysis conditions between atractylon and atractylenolides II and III were different, such as the mobile phase and ultraviolet absorbance. Based on the suitable analysis condition for each compound, the retention times for atractylon, and atractylenolides II and III were 12.9, 34.0, and 18.0 min, respectively. For the preparation of a calibration curve, *y* was regarded as the peak area and *x* was regarded as the spiked concentration (mg/ml). Linear regression equations are shown in Fig. 2. Correlation coefficients (*r*<sup>2</sup>) for atractylon, and atractylenolides II and III were 0.9997, 0.9995, and 0.9996, respectively.

**Precision and Accuracy** Intra- and inter-day assays were chosen to determine the precision of the developed assay, and a recovery test was used to evaluate the accuracy of this method. We used three different spiked concentrations to evaluate the accuracy. The inter- and intra-day variations for atractylon, and atractylenolides II and III are expressed as the relative standard deviation (RSD). The intra-day, inter-day, and recovery data are summarized in Table 1. The RSDs of the inter- and intra-day assays for the three sesquiterpenoids were all below 6.0% and 3.0%, respectively. The average recoveries of spiked samples for atractylon, and atractylenolides II and III were between 90% and 110%.

**Variations in Sesquiterpenoids with Different Processing Methods** According to the different processing methods, we detected variations in sesquiterpenoid concentrations as shown in Fig. 3. Atractylon was significantly lower (*p*<0.05) after the stir-fry process, and this phenomenon had a time-dependent effect. Moreover, unlike the decrease in atractylon, atractylenolides II and III markedly increased (*p*<0.05) (Fig. 3). We found that 5 min of processing (without assistant substrates, red soil) significantly (*p*<0.05) increased the atractylon content, but after 10, 20, and 30 min of processing, this increasing tendency ceased (Fig. 3A). We also compared the contents of atractylenolides II and III with different processing methods. Without any assistant substrates or red soil processing methods, the same tendencies for the atractylenolide II and III contents were expressed, but burnt clay processing displayed different results for which concentrations increased with 5, 10, and 20 min of processing, but with 30 min of processing, the concentrations rapidly decreased (Fig. 3B).

**Water and Heavy Metal Contents in Processed Materials** The water content of the raw materials can affect the results we obtained. The water content of raw material was 12.8%. In the first 5 min of processing, the water content without assistant substrates, with red soil, and with burnt clay decreased by about 5%, 3.2%, and 2.5%, respectively. Moreover, after 10 min of processing, there was still about a

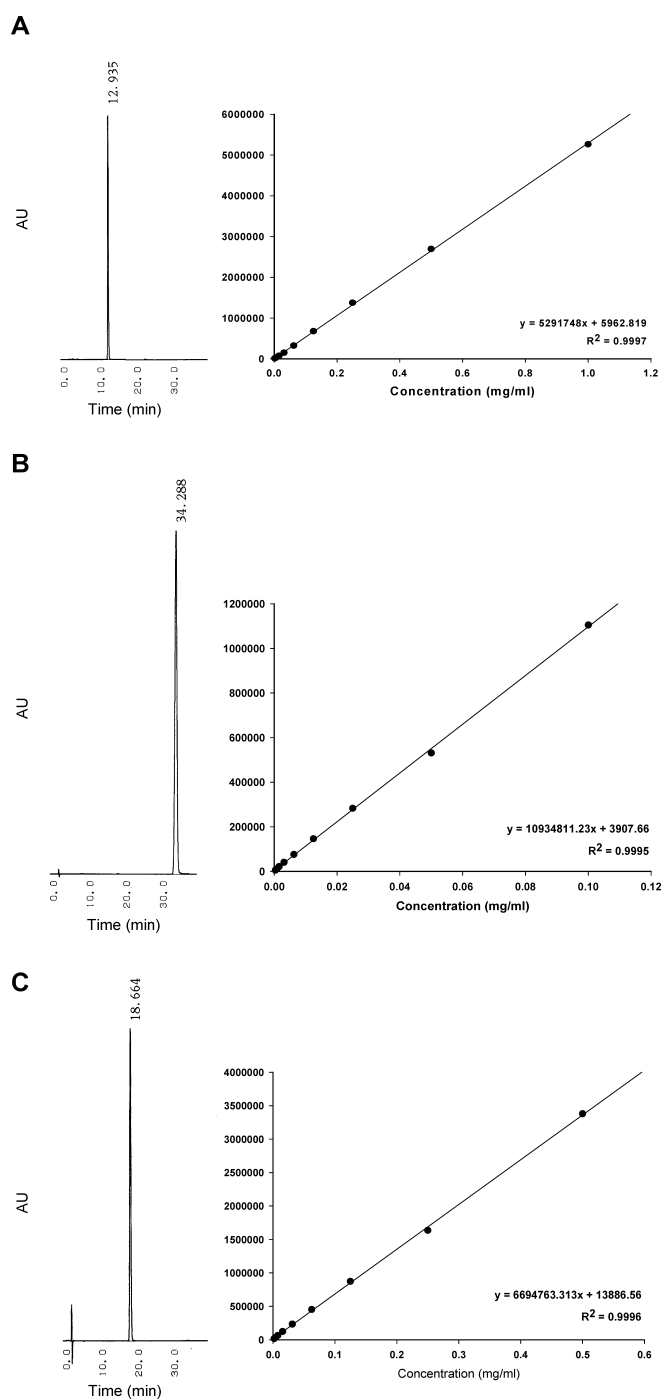


Fig. 2. HPLC Profiles of Atractylon, and Atractylenolides II and III and Their Calibration Curves

(A) Atractylon; (B) atractylenolide II; (C) atractylenolide III.

3% decrease in all groups. The water content loss stabilized after 20 min of processing (Table 2). Concentrations of five heavy metals found in raw and processed samples are also shown in Table 4. The arsenic and copper contents in the raw material and the group without an assistant substrate were almost the same (0.120 ppm arsenic and 14.413 to 14.671 ppm copper). If we processed the *Atractylodis Rhizoma* with different assistant substrates, the heavy metal contents changed. With red soil, arsenic and copper levels were higher than those of the raw materials (0.188 ppm arsenic and 15.650 ppm copper). Furthermore, in the burnt clay group, copper

Table 1. Intra-day and Inter-day Variability and Recovery for the Analysis of Atractylon, and Atractylenolides II and III

Compound	Intra-day variability		Inter-day variability		Recovery (%)		
	Mean±S.D.	RSD (%)	Mean±S.D.	RSD (%)	Amount added <sup>(a)</sup>	Mean±S.D.	RSD (%)
Atractylon	2.959±0.021	0.695	3.034±0.142	4.666	62.5	101.434±0.001	0.001
					125	104.385±0.001	0.001
					250	105.843±0.009	0.008
Atractylenolide II	0.494±0.005	0.957	0.528±0.029	5.418	6.25	99.935±0.034	0.034
					12.5	102.314±0.004	0.004
					25	101.077±0.005	0.005
Atractylenolide III	1.766±0.044	2.487	1.786±0.107	5.989	31.25	107.333±3.990	3.717
					62.5	108.100±0.610	0.560
					125	98.567±0.760	0.770

Each test was run in triplicate. RSD, relative standard deviation. <sup>a)</sup> Unit for amount added in recovery analysis was  $\mu\text{g/ml}$ .

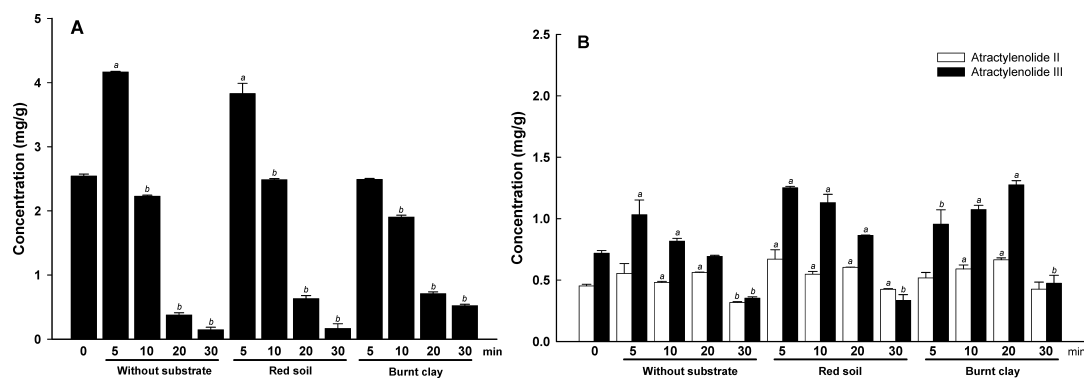


Fig. 3. Variations in the Concentrations of Atractylon, and Atractylenolides II and III after Stir-Fry Processing with Different Assistant Substrates

(A) Atractylon; (B) atractylenolides II and III. All data are expressed as the mean and S.D.  $p$  values were calculated by comparison with raw materials. <sup>a</sup> significant increase ( $p < 0.05$ ); <sup>b</sup> significant decrease ( $p < 0.05$ ).

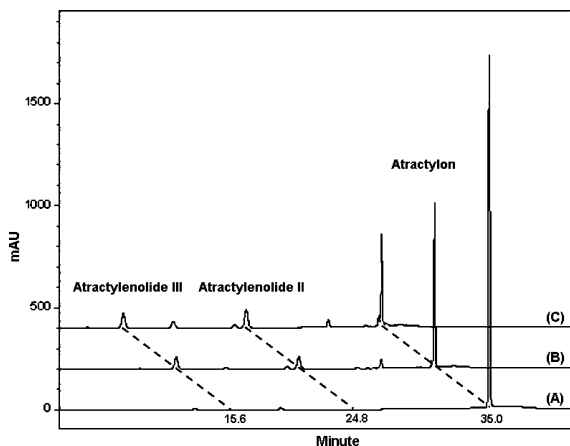
Table 2. Water Contents of Raw and Different Processed Materials

Material	Water content (%)				
	0 min	5 min	10 min	20 min	30 min
Raw	12.8	—	—	—	—
Without substrates	—	7.8	4.6	4.1	3.8
Red soil	—	9.6	7.4	4.7	3.7
Burnt clay	—	10.3	7.6	4.5	4.1

Table 3. The Sesquiterpenoids Content before and after Steam Processing of Atractylon

Stem processing	Content ( $\mu\text{g}$ )		
	Atractylon	Atractylenolide II	Atractylenolide III
Original	426.547±0.731 (1.975±0.003 $\mu\text{mol}$ )	0	0
30 min	166.010±0.208 (0.778±0.001 $\mu\text{mol}$ )	13.787±0.058 (0.059±0.000 $\mu\text{mol}$ )	21.360±0.108 (0.086±0.000 $\mu\text{mol}$ )
60 min	88.585±1.354 (0.410±0.006 $\mu\text{mol}$ )	19.132±0.189 (0.082±0.001 $\mu\text{mol}$ )	24.007±0.273 (0.097±0.001 $\mu\text{mol}$ )

$n=3$ .

Fig. 4. Atractylon Oxidation to Atractylenolides II and III by Autoclaving  
Atractylon steamed for original (A), 30 min (B), and 60 min (C).

showed the highest content (73.204 ppm). We also found a high lead content with burnt clay (10.734 ppm).

**Atractylon Oxidation to Atractylenolides after Steam Processing** The amounts of atractylon, and atractylenolides II and III were detected by a gradient elution-HPLC system. We could monitor and calculate the variations of atractylon oxidation to atractylenolides after steam treatment through this analysis system. From Fig. 4, atractylon, atractylenolides II and III appear at 35.0 min, 24.8 min and 15.6 min, respectively. The atractylon concentration decreased and those of atractylenolides II and III increased after steam processing. After steam-processing for 30 min, atractylon had diminished by about 1.197  $\mu\text{mol}$ , but atractylenolides II and III had respectively increased by 0.059 and 0.086  $\mu\text{mol}$ . In addition, atractylon further decreased to 1.565  $\mu\text{mol}$  with 60 min of

processing time while atractylenolides II and III respectively increased to 0.082 and 0.097  $\mu\text{mol}$  (Table 3). However, the changes of atractylyon to atractylenolides II and III was not

Table 4. Heavy Metal Contents of Raw and Different Processed Materials

Material	Heavy metal content (ppm)				
	Pb	Cd	Hg	As	Cu
Raw	ND	ND	ND	0.120	14.413
Without substrates	ND	ND	ND	0.120	14.671
Red soil	ND	ND	ND	0.188	15.650
Burnt clay	10.734	ND	ND	0.180	73.204

All processed samples were processed for 5 min. ND, not detected (Pb<10 ppm, Cd<2 ppm, and Hg<0.1 ppm).

Table 5. IC<sub>50</sub> Values of Sesquiterpenoids in Various Human Cell Lines

Sesquiterpenoid	IC <sub>50</sub> ( $\mu\text{M}$ )					
	Carcinoma cell line					Normal cells
	KB	DU145	HeLa	Hep3B	AGS	WISH
Atractylyon	96.9	81.2	81.3	110.0	120.6	464.2
Atractylenolide II	205.9	>250	>250	>250	>250	658.9
Atractylenolide III	>250	>250	>250	>250	>250	>800

All cell lines were treated with tested samples for 36 h and calculated from three separate experiments.

Table 6. Sesquiterpenoid Contents of Commercial Raw Atractylodis Rhizoma from Northern Taiwan

No.	Content (mg/g)		
	Atractylyon	Atractylenolide II	Atractylenolide III
A	1.59±0.01	0.71±0.06	1.44±0.02
B	1.80±0.01	0.64±0.06	1.47±0.37
C	2.21±0.03	0.67±0.17	1.19±0.23
D	4.07±0.02	0.74±0.01	1.77±0.01
E	5.87±0.01	0.67±0.04	1.73±0.01
F	1.67±0.02	0.86±0.12	1.15±0.07
Mean	2.87±1.74	0.72±0.08	1.46±0.26

No: A to F indicated AO-NR-A to F. Each test was run in triplicate.

Table 7. Sesquiterpenoid Contents of Commercial Processed Atractylodis Rhizoma from Three Areas of Taiwan

Content (mg/g)											
No.	A <sup>a)</sup>	AII <sup>b)</sup>	AIII <sup>c)</sup>	No.	A	AII	AIII	No.	A	AII	AIII
N1	1.04±0.01	0.44±0.02	1.29±0.07	C1	0.69±0.01	0.86±0.05	1.43±0.04	S1	1.48±0.01	1.09±0.02	2.11±0.01
N2	2.01±0.01	1.16±0.01	2.54±0.01	C2	0.90±0.01	0.73±0.01	1.40±0.05	S2	1.84±0.46	0.89±0.06	1.25±0.02
N3	2.09±0.01	1.34±0.09	3.11±0.05	C3	1.41±0.01	1.04±0.01	2.76±0.19	S3	1.56±0.00	0.65±0.13	0.95±0.00
N4	1.26±0.01	1.31±0.13	2.61±0.02	C4	1.23±0.01	0.84±0.02	1.64±0.01	S4	2.27±0.01	0.79±0.02	1.20±0.02
N5	2.50±0.01	1.13±0.05	2.06±0.14	C5	0.36±0.01	1.02±0.07	2.12±0.01	S5	1.63±0.02	0.54±0.05	1.06±0.01
N6	2.57±0.03	1.01±0.01	2.05±0.08	C6	2.90±0.01	0.57±0.06	1.15±0.00	S6	3.90±0.04	0.69±0.07	1.73±0.15
N7	1.55±0.01	0.74±0.03	1.71±0.63	C7	0.58±0.00	1.58±0.05	3.04±0.04	S7	2.01±0.01	0.54±0.03	1.24±0.25
N8	1.67±0.02	0.55±0.02	1.02±0.08	C8	1.57±0.01	0.89±0.03	2.48±0.02	S8	3.79±0.01	0.68±0.02	1.09±0.01
N9	2.01±0.02	0.86±0.02	1.71±0.07					S9	1.28±0.00	0.98±0.03	1.96±0.09
N10	2.03±0.01	1.04±0.01	1.82±0.02					S10	1.89±0.06	0.98±0.04	1.97±0.20
N11	1.75±0.01	0.93±0.02	1.91±0.02								
N12	2.07±0.01	0.81±0.05	0.44±0.05								
N13	1.58±0.01	0.98±0.05	4.16±0.18								
N14	2.77±0.01	0.96±0.06	1.88±0.03								
Mean	1.92±0.49	0.95±0.26	2.02±0.91	Mean	1.36±0.78	0.94±0.30	2.00±0.70	Mean	2.17±0.93	0.78±0.19	1.46±0.44

No: N1 to N14 indicated AO-N1 to 14; C1 to C8 indicated AO-C1 to 8; S1 to S10 indicated AO-S1 to 10. Each test was run in triplicate. a) A: atractylyon, b) AII: atractylenolide II, c) AIII: atractylenolide III.

stoichiometric, because the other oxidation or degradation compounds (retention time 19.5, 23.5, 30.8 min) were appear in the HPLC chromatogram (Fig. 4).

**Cell Cytotoxicity** The cell cytotoxicity of atractylyon was previously reported by our lab.<sup>8)</sup> To see if the conversion of atractylyon to atractylenolide II and III might change the cytotoxicity, we used carcinoma cell lines and normal cells to examine atractylyon, and atractylenolides II and III. Cell cytotoxicities between atractylyon and atractylenolides II and III had obvious differences. The IC<sub>50</sub> values of atractylyon in various carcinoma cell lines and a normal cell line were 81.2—120.6 and 464.2  $\mu\text{M}$ , respectively. However, the cytotoxicities of atractylenolides II and III were significantly less than those of atractylyon. IC<sub>50</sub> values of atractylenolides II and III in carcinoma cell lines exceeded 200 and in WISH cells were about 600  $\mu\text{M}$ , respectively (Table 5). These data suggest that atractylenolides II and III showed less cytotoxicity than atractylyon. Thus, processing reduced the toxicity of the raw Atractylodis Rhizoma.

**Sesquiterpenoid Contents in Commercial Atractylodis Rhizoma** Raw materials purchased in three different areas of Taiwan displayed greatly different contents of atractylyon, and atractylenolides II and III contents. The average contents of atractylyon in raw and processed materials in the north were 2.87±1.74 and 1.92±0.49 mg/g, respectively (Tables 6, 7). For atractylenolides II and III, the average contents were 0.72±0.08 and 1.46±0.26 mg/g in raw materials, and 0.95±0.26 and 2.02±0.91 mg/g in processed materials. Moreover, the average contents of atractylyon, and atractylenolides II and III in central Taiwan were 1.36±0.78, 0.94±0.30, and 2.00±0.70 mg/g, respectively. In the south, the average contents of atractylyon, and atractylenolides II and III were 2.17±0.93, 0.78±0.19, and 1.46±0.44 mg/g, respectively (Table 7).

## Discussion

In this study, we established two accurate and reproducible HPLC analytical systems to detect the structural and concentration variations of sesquiterpenoids in Atractylodis Rhizoma. Despite the great instability of atractylyon and difficulties with its analysis, the accuracy and reproducibility could

also be well-controlled (Table 1).

Both raw and processed *Atractylodis Rhizoma* are commonly used and easily purchased in Taiwan. To measure differences between raw and processed materials, we processed the raw *Atractylodis Rhizoma* with two common assistant substrates (e.g., red soil and burnt clay). As shown in Fig. 3A, the atractylon content greatly increased in the first 5 min of processing, but eventually it significantly declined after 30 min of processing. Concentrations of atractylenolides II and III were contrary to those of atractylon. Atractylenolides II and III significantly increased in the first 20 min of processing, but declined with a longer time of processing (Fig. 3B). In addition, we also calculated the total amounts of these three sesquiterpenoids in each of the processed samples. Results showed that the total sesquiterpenoid contents with stir-frying without assistant substrates for 5 min, with red soil for 5 and 10 min, and with burnt clay for 5 min were all higher than that of the raw materials. Moreover, the other processed samples (i.e., red soil stir-fried for 30 min) exhibited lower concentrations (data not shown). These data suggest that the actual weight of processed samples was lighter because of water evaporation. So when we prepared HPLC samples, we needed to use greater quantities of the processed samples to achieve the same analysis condition. On the other hand, we suggest that the reason for the decrease in sesquiterpenoids after 20 to 30 min of the stir-fry processing with different assistant substrates was evaporation and dehydration. The evaporable sesquiterpenoids were possibly vaporized with water when we stir-fried the *Atractylodis Rhizoma*. In addition, this dehydration transformed the atractylenolide III into atractylenolide I (Fig. 1). Thus, when we calculated the sum of atractylenolides II and III, we lost the concentrations of atractylenolide I. We also found that the water content of the herb was reduced following a prolonged processing time, especially with 20 min of processing (Table 2). Taken together, we suggest the reason for the large increases in atractylon in the first 10 min of processing was due to the large amount of water lost. This reason could also explain why the changes in atractylon concentrations and water content were not significant after 20 and 30 min of processing.

As previously described, oxidation is the major reason for transforming atractylon to atractylenolides II and III. Consequently, we steamed pure atractylon under two different conditions to monitor the sesquiterpenoid variations after processing. However, according to the physical properties of these sesquiterpenoids, the maximal ultraviolet absorbance of atractylon is 220 nm and of atractylenolides II and III is 236 nm. Thus, we designed a low-pressure-gradient elution HPLC system. Through this analysis condition, we were able to monitor the transformation from atractylon to atractylenolides II and III. We found that atractylon decreased and atractylenolides II and III increased after 30 min of heating; and the longer the heating time was, the greater concentrations of atractylenolides II and III we found (Fig. 4).

In our previous report, atractylon showed greater cytotoxicity than atractylenolides II and III in the HL-60 leukemia cell line.<sup>8)</sup> Furthermore, we examined the cytotoxicity in other cells, including carcinoma and normal cell lines. As summarized in Table 5, the cytotoxicity of atractylon was stronger than those of atractylenolides II and III in all cell lines. Taken together, when we oxidized atractylon into

atractylenolides II and III through heating, the toxicity was significantly reduced. Heavy metal contaminants are extremely dangerous to human health. Chronic copper and lead toxicities may cause severe neurological damage, and disorders of bone formation and calcium metabolism in infants.<sup>22,23)</sup> Therefore, the heavy metal contents in our materials and assistant substrates should be determined during processing. According to Table 4, the lead concentrations in burnt clay-processed *Atractylodis Rhizoma* exceeded the standards for heavy metal contents in Taiwan. In the past, burnt clay was the most common assistant substrates used for *Atractylodis Rhizoma* processing. Because of the heavy metal contamination in burnt clay, people now replace the burnt clay with red soil for the stir-fry processing of *Atractylodis Rhizoma*.

We collected commercial *Atractylodis Rhizoma* from three areas of Taiwan to analyze the sesquiterpenoid concentrations. Our data indicated that there were great differences in sesquiterpenoid contents. The average contents of atractylon, and atractylenolides II and III in the commercially processed *Atractylodis Rhizoma* were  $1.82 \pm 0.80$ ,  $0.89 \pm 0.26$ , and  $1.87 \pm 0.72$  mg/g, respectively (data not shown). Compared with the average contents in Table 7, the differences were all within 50%. In addition, we also found that there were almost no significant differences in sesquiterpenoid contents of commercial *Atractylodis Rhizoma* between northern and central Taiwan, except in the concentrations of atractylon (Table 7). According to the concentration stipulation of the Committee on Chinese Medicine and Pharmacy in Taiwan, these differences are allowable.<sup>24)</sup> The material source of the herbs plays a critical role in determining the quality of Chinese Material Medica. It is difficult to maintain the quality and quantity control of material sources in Taiwan because almost all Chinese herbs are imported from China. The commercial *Atractylodis Rhizoma* sold in Taiwan is mainly imported from the Zhejiang and Anhui Province in Southeast China. But the quality of Chinese herbs in different traditional medicinal markets around Taiwan is quite difficult to control. So, we collected commercially processed *Atractylodis Rhizoma* from three areas in Taiwan to investigate sesquiterpenoid variations in different places of production. Moreover, we also noticed that almost all of the commercially processed *Atractylodis Rhizoma* was stir-fried with red soil (32 of 38 samples had been processed with red soil). As we described above, heavy metal contaminants are found in burnt clay, so perhaps this is the main reason for the popularity of red soil.

In conclusion, we monitored the quality of commercial *Atractylodis Rhizoma* through our established method and made a quality standard enactment. In addition, we characterized the purpose of stir-fry decreasing the concentration of atractylon, the major component exhibiting toxicity. These results provide a better understanding of the reason why *Atractylodis Rhizoma* should be processed.

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