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Research Article

Analysis of the Influence of Different Processing Methods on the Main Effective Components in *Oviductus Ranae* by High-Performance Liquid Chromatography with Diode Array Detector

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Oviductus Ranae (OR) is one of the "treasures of Changbai Mountain" in China. It is used clinically for the treatment of infirmity after illness, fatigued spirit, cough, hemoptysis, and so forth. In this study, a simple and sensitive high-performance liquid chromatography-diode array detector (HPLC-DAD) method was developed for the simultaneous determination of five main effective components (1-methyl hydantoin, estradiol, cholesterol, 7-keto-cholesterol, and 4-cholesten-3-one) in OR and its different processed products. The results indicated that these five components showed good linear relationships within their own concentration ranges along with coefficients of determination ≥0.9996. Average recoveries ranged from 97.67% to 100.06%, with RSDs of 1.45%–1.99%. The proposed method was found to be simple, accurate, and stable, which provided an effective analytical method for quality control and evaluation of OR and its different processed products.

1. Introduction

Oviductus Ranae (OR) is one of the "Treasures of Changbai Mountain" in China, which is also known as "the new three treasures of northeast China" together with ginseng and velvet antler. It was first recorded in the "Compendium of Materia Medica." It is not only used as a nourishing and beautifying food in the folk [1] but also used as a medicine with the functions of tonifying the kidney, benefiting essence, nourishing yin, and moistening the lungs. In addition, OR can be used for treatment of infirmity after illness, fatigued spirit, lack of strength, palpitation and insomnia, perspire during sleep, cough, and hemoptysis [2–4]. Therefore, the OR industry has gradually become one of the pillar industries in northeast China. However, OR is derived from the oviduct of *Rana temporaria* chensinensis David. It is common knowledge that OR needs to be processed, whether used as food or medicine. Through years of research studies on this medicine [5, 6], our research group found that the processing and cooking methods of OR are often determined by the appearances and the tastes of finished products. The processing methods are relatively rough and have no scientific guidance basis.

In this experiment, several processing and edible methods of OR were selected, including drying at 100°C, 60°C, and 40°C, boiling, stewing, wine frying, frying, and charcoal frying [7–10]. In addition, an antitussive-related component (1-methyl hydantoin), a component (estradiol) for the treatment of osteoporosis and the regulation of hormones in vivo, and three antifatigue-related components

(steroidal components, including cholesterol, 7-keto-cholesterol, and 4-cholesten-3-one) were chosen [11–14].

High-performance liquid chromatography is an important method to analyze the content of active components in traditional Chinese medicine or Chinese patent medicine [15–17]. The optimum processing technology was confirmed by the analysis of the influence of different processing and cooking methods on the main effective components in OR by high-performance liquid chromatography with diode array detector (HPLC-DAD). The study in this paper has guiding significance for the edible and processing of OR.

2. Experimental Setup

2.1. Instrumentations. Chromatographic separation was achieved on an Agilent 1260 high-performance liquid chromatography (HPLC) system (including a quaternary low-pressure mixing pump, autosampler, column oven, diode array detector, and ChemStation workstation). AB135-S electronic balance was purchased from Mettler Toledo International Co., Ltd. KQ-250 ultrasonic cleaner was obtained from Kunshan Ultrasonic Instrument Co., Ltd. R series rotatory evaporator was acquired from Shanghai ShenSheng Technology Co., Ltd. DHG-912A heating and drying oven was purchased from Shanghai Precision Experimental Equipment Co., Ltd.

2.2. Materials and Reagents. *Oviductus Ranae* (batch number: 20180301, 20180302, and 20180303) was gathered from Jingyu County of Baishan City in Jilin Province and identified by professor Jiang Dacheng of Changchun University of Chinese Medicine. The samples were in accordance with the relevant requirements of "Pharmacopoeia of People's Republic of China (2015 version, volume I)."

1-methyl hydantoin (batch number: 111836-201102), estradiol (batch number: 100182-201205), and cholesterol (batch number: 111618-200301) were all purchased from the China Pharmaceutical Biological Products Verification Institute (Beijing, China). 7-keto-cholesterol was prepared by the laboratory (purity >95%). 4-cholesten-3-one (batch number: S45539-479) was purchased from Sigma Company (USA). Methanol (Fisher, America) was of chromatographic grade. Phosphoric acid and other reagents (Beijing Chemical Industry Factory) were all of analytical grade. Ultrapure water was purchased from Hangzhou Wahaha Co., Ltd.

2.3. Preparation of Standard Solutions. Appropriate amounts of 1-methyl hydantoin and estradiol were weighted precisely and dissolved with methanol via ultrasound to obtain stock standard solution (I) with the concentration of 1-methyl hydantoin 0.341 $mg{\cdot}mL^{-1}$ and estradiol 0.354 $mg{\cdot}mL^{-1}$, respectively. Appropriate amount of 4-cholesten-3-one was weighted precisely and dissolved with methanol via ultrasound to obtain stock standard solution (II) with the concentration of 4-cholesten-3-one 1.71 $mg{\cdot}mL^{-1}$. Cholesterol (42.00 mg) and 7-keto-cholesterol (4.58 mg) were weighted and placed in a 10 mL volumetric flask. Subsequently, 1 mL stock standard solution (I) and 1 mL stock standard solution (II) were added into the volumetric flask above and dissolved with methanol (final adjusted volume: 10 mL) to obtain the stock standard solution (III) with the concentration of 1-methyl hydantoin 34.1 $\mu g{\cdot}mL^{-1}$, estradiol 35.4 $\mu g{\cdot}mL^{-1}$, cholesterol 4200 $\mu g{\cdot}mL^{-1}$, 7-keto-cholesterol 458 $\mu g{\cdot}mL^{-1}$, and 4-cholesten-3-one 171 $\mu g{\cdot}mL^{-1}$. Finally, 1 mL of stock standard solution (III) was dissolved with methanol (final adjusted volume: 10 mL) and shaken well to obtain mix standard solutions.

2.4. Preparation of Test Solutions. About 5.0 g of OR power was weighed accurately and placed in a round-bottom flask. Then, 100 mL of methanol was added into the round-bottom flask and was reflux extracted for 30 min. The sample was extracted twice in total. Then, the methanol extracts were combined and evaporated to dryness via roller evaporator. Finally, the residue was redissolved with methanol (final adjusted volume: 5 mL) to obtain test solution. The processed products of OR were prepared as per the method above.

2.5. Chromatographic Conditions. Analysis was achieved on Alltima™ C_{18} column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol (A) and 0.1% phosphoric acid solution (B). The concrete gradient elution conditions are displayed in Table 1. The injection volume was 5 μL. The detective wavelengths were monitored at 215 nm (1-methyl hydantoin, estradiol, and cholesterol) and 240 nm (7-keto-cholesterol and 4-cholesten-3-one).

2.6. Processing Methods of OR. Ten processing methods were applied in this study:

A. Boiling: Take an appropriate amount of OR and crush it, add 8 times water, boil for 30 min, and dry by airing

B. Stewing: Take an appropriate amount of OR and crush it, add 20 times water, stew until water is absorbed completely, and cool and dry by airing

C. Wine frying: Take an appropriate amount of OR and crush it, add 1/10 times yellow wine, mix thoroughly, heat gently until ustulate, and cool by airing

D. Charcoal frying: Take an appropriate amount of OR and crush it, place in a hot pot, heat with high heat until burnt black, and cool by airing

E. Frying: Take an appropriate amount of OR and crush it, heat gently until brown, and cool by airing

F. Drying at 100°C: Take an appropriate amount of OR and crush it, heat at 100°C for 4 h, and cool and dry by airing

G. Drying at 60°C: Take an appropriate amount of OR and crush it, heat at 60°C for 4 h, and cool and dry by airing

H. Drying at 40°C: Take an appropriate amount of OR and crush it, heat at 40°C for 4 h, and cool and dry by airing

Table 1: The gradient elution conditions.

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Column temperature (°C)
0	2.0	0	100	30
10	2.0	0	100	30
20	1.0	12	88	30
25	1.0	12	88	40
40	1.0	65	35	40
50	1.0	75	25	40
55	1.0	85	15	30
60	1.0	93	7	30
65	1.0	93	7	30
66	1.0	95	5	30
71	1.0	95	5	30
75	1.0	100	0	30
105	1.0	100	0	30

2.7. Calibration Curves, Limits of Detection, and Quantification. The stock standard solution (precise 0.2 mL, 0.4 mL, 1.0 mL, 2.0 mL, and 4.0 mL) was placed in a 10 mL volumetric flask and was adjusted to scale with methanol, respectively, to obtain the standard serial working solutions. Then, the above standard working solutions were injected into HPLC for analysis, respectively. In addition, the standard solution in Section 3.3 was diluted gradually with methanol. The limits of detection (LOD) and the limits of quantification (LOQ) were determined by three times and ten times of the signal-noise ratio, respectively.

2.8. Precision and Stability

2.8.1. Reproducibility Test. 5 μL of standard solution were taken and injected into HPLC for six times continuously, respectively. Besides, chromatographic peak areas of each analyte were recorded, and RSDs of peak area were calculated, respectively.

2.8.2. Repeatability Test. Six copies of OR with the same batch number (20180301) were taken, prepared into six parallel test solutions, and injected into HPLC for analysis, respectively. Besides, the RSDs of the contents of these five components were calculated, respectively.

2.8.3. Stability. 5 μL of the same test solution (stored at room temperature) was injected into HPLC at 0, 2, 4, 8, 10, and 12 hours for analysis, respectively. Besides, the chromatographic peak areas of each peak were recorded, and the RSDs of each peak were calculated, respectively.

2.9. Recovery Test. Six copies of OR samples (2.5 g) with known contents were accurately weighed and placed in stoppered conical flasks, respectively. Then, 1 mL of 1-methyl hydantoin standard solution (3.51 μg/mL), 1 mL of estradiol standard solution (3.55 μg/mL), 1 mL of cholesterol standard solution (9.57 mg/mL), 1 mL of 7-keto-cholesterol standard solution (45.1 μg/mL), and 1 mL of 4-cholesten-3-one standard solution (17.3 μg/mL) were added into the six conical flasks above and prepared, respectively. The sample solutions were prepared according to the method in Section 3.4 and injected into HPLC for analysis following chromatographic conditions in Section 2.5, respectively.

2.10. Sample Content Determination. Three batches of OR and its processed products were prepared into test solutions based on the method in Section 2.4 and injected into HPLC for analysis according to the chromatographic conditions in Section 2.5, followed by the calculation of the contents of five various analytes via the external standard method.

3. Results and Discussion

3.1. Calibration Curves, Limits of Detection, and Quantification. The standard curve was drawn by using the chromatographic peak area (Y) as the vertical axis and the concentration of the standard solution (X) as abscissa. Besides, the limit of detection (LOD) and limit of quantitation (LOQ) were calculated, respectively. The results are displayed in Table 2, which indicated that five components presented good linear relationships within their determination ranges as well as good sensitivity under the proposed chromatographic conditions.

3.2. Method Validation. The RSD results of average contents of 1-methyl hydantoin, estradiol, cholesterol, 7-keto-cholesterol, and 4-cholesten-3-one were 1.72%, 1.63%, 1.48%, 1.80%, and 1.97%, respectively. The results are displayed in Table 3, which illustrated that this method was of good repeatability.

The reproducibility RSD results of 1-methyl hydantoin, estradiol, cholesterol, 7-keto-cholesterol, and 4-cholesten-3-one were 1.94%, 1.53%, 1.50%, 1.83%, and 1.88%, respectively, indicating that this method had good reproducibility. In addition, the stability RSD results of 1-methyl hydantoin, estradiol, cholesterol, 7-keto-cholesterol, and 4-cholesten-3-one were 1.59%, 1.23%, 1.14%, 1.38%, and 1.74%, respectively. The results are displayed in Table 3, which suggested that test solution was stable within 12 hour.

The contents of five components were measured and the recovery rates were calculated, respectively. The average recoveries of those five components were in the range of

Table 2: Linear relationships of various constituents.

Constituent	Standard curve	Linearity range ($\mu g \cdot mL^{-1}$)	r	LOD	LOQ
1-Methyl hydantoin	$y = 7.6511x - 0.1312$	0.68~34.10	0.9995	0.09	0.32
Estradiol	$y = 14.068x - 1.5682$	0.71~35.40	0.9997	0.11	0.34
Cholesterol	$y = 0.4251x + 1.1244$	84~4200	0.9998	0.13	0.42
7-Keto-cholesterol	$y = 33.722x - 27.624$	9.16~458	0.9999	0.11	0.39
4-Cholesten-3-one	$y = 63.096x + 85.739$	3.42~171	0.9998	0.15	0.50

Table 3: Methodology validation results.

Constituent	Reproducibility	Repeatability	Stability	Recovery	
	RSD (%)	RSD (%)	RSD (%)	Average recoveries (%)	RSD (%)
1-Methyl hydantoin	1.72	1.94	1.59	97.92	1.99
Estradiol	1.63	1.53	1.23	97.67	1.55
Cholesterol	1.48	1.50	1.14	100.06	1.97
7-Keto-cholesterol	1.80	1.83	1.38	99.41	1.54
4-Cholesten-3-one	1.97	1.88	1.74	98.89	1.45

97.67% to 100.06% with RSDs of 1.45%~1.99%. The results are displayed in Table 3, which suggested that the developed method was of good accuracy.

3.3. Sample Content Determination. The proposed HPLC-DAD method was applied to the simultaneous determination of five main effective components in OR and its processing products. The determination results are displayed and summarized in Figures 1 and 2 and in Table 4. Contents of measured components were in the range of 0.0003 to 3.9907 mg/g. The concentration changes of five chemical constituents in OR and OR processing products are shown in Figure 3.

3.4. Discussion

3.4.1. Contents Determination Results. OR not only has good efficacy but also tastes great after proper cooking. It has the reputation of "the head of Eight Treasures" as early as the Qing Dynasty [18, 19]. In the process of its cooking and processing, methods (including stewing, boiling, wine frying, frying, drying, and so forth) were usually applied. Moreover, changes of contents of five components (1-methyl hydantoin, estradiol, cholesterol, 7-keto-cholesterol, and 4-cholesten-3-one) in OR processed by different methods were compared. The results indicated that the contents of each analyte were different in OR processed via different methods.

The contents of five components in OR processed by drying, wine frying, frying, and charcoal frying all decreased in some degree. Contents of 4-cholesten-3-one in OR processed by stewing and frying increased slightly, while the contents of 1-methyl hydantoin, estradiol, cholesterol, and 7-keto-cholesterol were all decreased. The results showed that OR unprocessed or processed by drying at a low temperature was recommended for use of eating and treating.

Drying is an indispensable processing method for food and traditional Chinese medicine, which is also suitable for the processing of OR [20]. It was found that the contents of 1-methyl hydantoin, 4-cholesten-3-one, estradiol, cholesterol, and 7-keto-cholesterol in OR all decreased when dried at the temperature of 100°C, 60°C, and 40°C. The content of estradiol decreased significantly when OR was dried at the temperature of 100°C, while less contents reduction were discovered when OR was dried at other temperatures. Estradiol has good pharmacodynamic effects. It can not only supplement estrogen and regulate hormone balance in vivo but also prevent osteoporosis. Estradiol can be used to treat leukopenia, breast cancer, and prostate cancer [21]. However, estradiol is a double-edged sword for some people. It may lead to hormone abnormality, breast pain, weight gain, hypertension, gallstone, and liver function abnormalities [22]. Therefore, OR should be dried according to treatment and tonic needs.

Frying method is usually applied when OR is processed. The fried OR tastes delicious. And sometimes some yellow rice wine can be added properly in the process of frying, which can reduce the fishy smell and make OR tastes more delicious. In the pursuit of delicious food, people often ignore the content changes of effective ingredients in OR. The local temperature of the medicinal materials is often too high in the process of frying, which may make some heat-sensitive effective ingredients deteriorate. The results displayed that contents of five components in OR processed by wine frying, frying, and charcoal frying decreased significantly. Thus, OR should not be fried at high temperature so as not to affect its effects.

Stewed OR, fried OR, and original medicine materials are the most common way when people eat OR used as food or medicine. The results also showed that contents of 1-methyl hydantoin, estradiol, cholesterol, and 7-keto-cholesterol in OR processed by stewing and frying decreased compared to those of original medicine materials, while content of 4-cholesten-3-one increased slightly. It was found that stewing has a little influence on the content of each analyte, while frying has a great influence on the content of 1-methyl hydantoin and estradiol. OR contains bacteria and microorganisms since it is a kind of animal product.

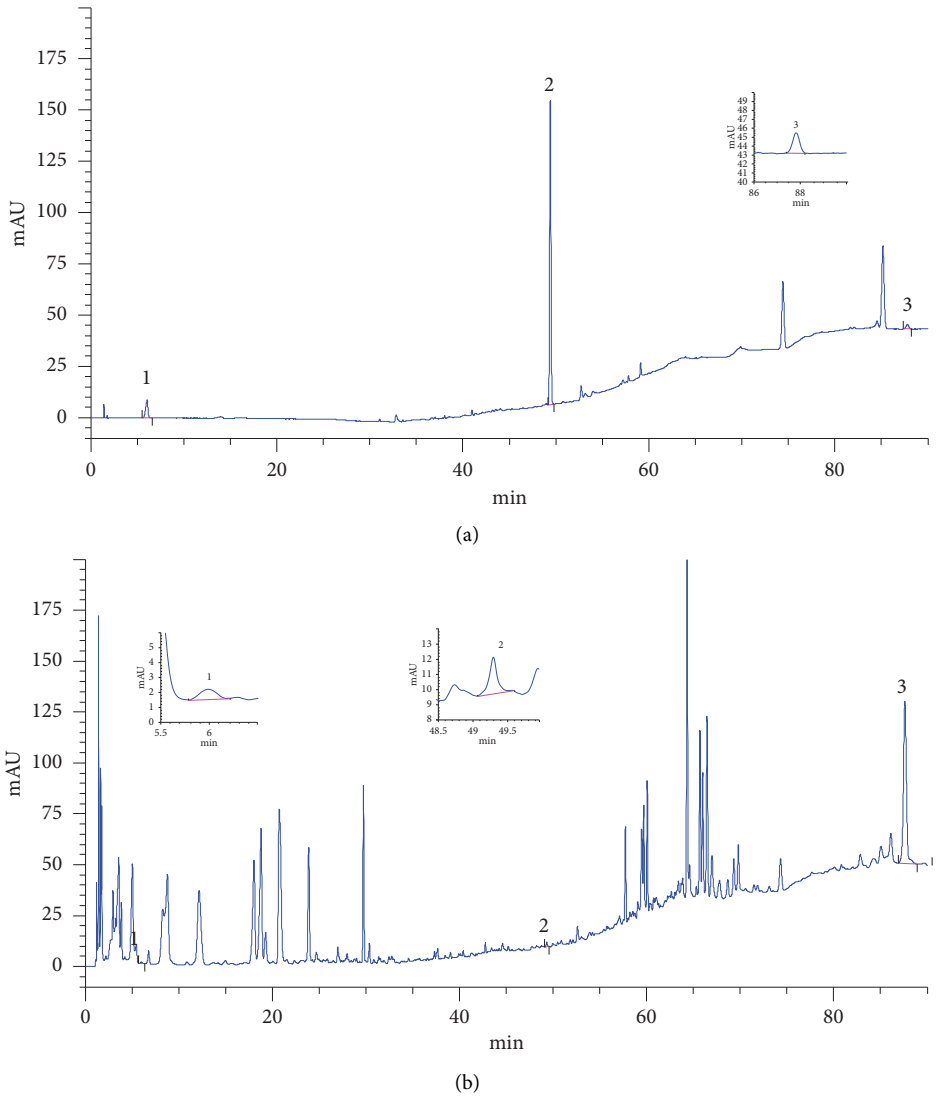


Figure 1: Chromatograms of mixed standard solution (a) and test solution (b) in 215 nm. (1) 1-methyl hydantoin, (2) estradiol, and (3) cholesterol.

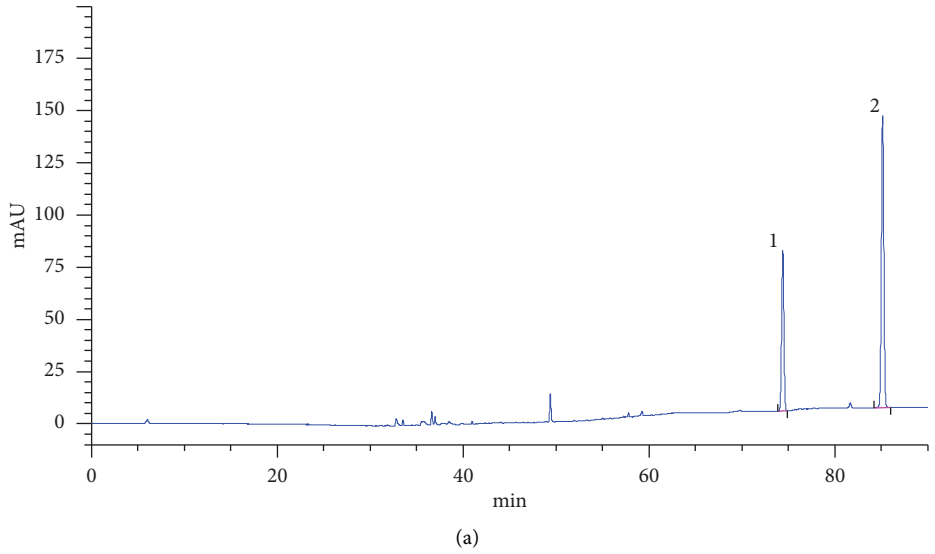
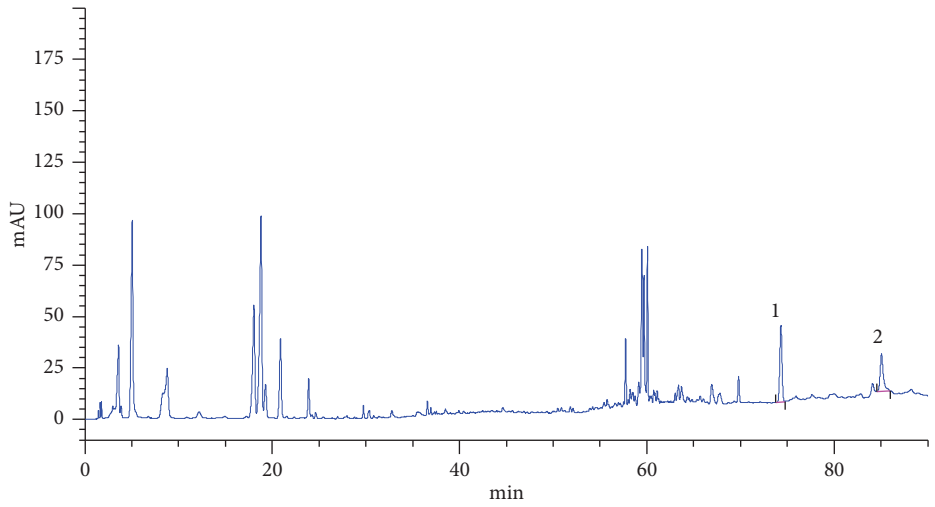


Figure 2: Continued.



(b)

Figure 2: Chromatograms of mixed standard solution (a) and test solution (b) in 240 nm. (1) 7-keto-cholesterol and (2) 4-cholesten-3-one.

Table 4: Results of content determination of various components (mg/g, $n = 3$).

Components	Batch No.	OR	A	B	C	D	E	F	G	H
1-Methyl hydantoin	20180301	0.0014	0.0003	0.0012	0.0008	—	—	0.0011	0.0013	0.0013
	20180302	0.0083	—	0.0078	0.0031	—	—	0.0068	0.0080	0.0082
	20180303	0.0077	—	0.0075	0.0031	—	—	0.0056	0.0072	0.0075
	Average	0.0058	0.0001	0.0055	0.0023	—	—	0.0045	0.0055	0.0057
Estradiol	20180301	0.0014	0.0006	0.0012	0.0010	—	—	0.0010	0.0014	0.0014
	20180302	0.0017	0.0005	0.0013	0.0009	—	—	0.0008	0.0015	0.0016
	20180303	0.0017	—	0.0013	0.0008	—	—	0.0008	0.0016	0.0016
	Average	0.0016	0.0004	0.0013	0.0009	—	—	0.0009	0.0015	0.0015
Cholesterol	20180301	3.7035	3.2729	3.5969	3.2909	3.0834	3.4892	3.5104	3.7784	3.7685
	20180302	3.9379	3.8581	3.9614	2.7178	2.2194	2.7551	3.9066	3.9184	3.9417
	20180303	3.9907	3.6046	3.9445	2.7143	2.3673	3.0290	3.7936	3.8932	3.8625
	Average	3.8774	3.5785	3.8343	2.9077	2.5567	3.0911	3.7369	3.8633	3.8576
7-Keto-cholesterol	20180301	0.0916	0.0780	0.0906	0.0779	0.0584	0.0618	0.0750	0.0754	0.0758
	20180302	0.0260	0.0212	0.0233	0.0161	0.0105	0.0190	0.0208	0.0214	0.0212
	20180303	0.0263	0.0187	0.0234	0.0159	0.0101	0.0171	0.0216	0.0218	0.0218
	Average	0.0480	0.0393	0.0458	0.0366	0.0263	0.0326	0.0391	0.0395	0.0396
4-Cholesten-3-one	20180301	0.0343	0.0379	0.0410	0.0141	0.0146	0.0342	0.0333	0.0310	0.0304
	20180302	0.0101	0.0113	0.0135	0.0084	0.0072	0.0118	0.0098	0.0091	0.0092
	20180303	0.0111	0.0115	0.0143	0.0083	0.0076	0.0100	0.0092	0.0090	0.0089
	Average	0.0185	0.0202	0.0229	0.0103	0.0098	0.0187	0.0174	0.0164	0.0162

A: boiling, B: stewing, C: wine frying, D: charcoal frying, E: frying, F: drying at 100°C, G: drying at 60°C, and H: drying at 40°C.

Therefore, the stewing method can kill bacteria and microorganisms under the premise of ensuring the efficacy of OR, which can guarantee the safety of OR as well.

3.4.2. Experimental Methods. First of all, prepared methods of test samples were investigated in this paper. The influence of different extraction solvents (water, methanol, ethanol, and anhydrous ethanol) on the transfer rates of the test sample and the separation effects of each analyte were studied. The results showed that water had a very poor dissolution effect on estradiol, cholesterol, 7-keto-cholesterol, and 4-cholesten-3-one, making it impossible to extract these three steroidal components. The extraction efficiency of ethanol, anhydrous ethanol, and methanol is similar, and the extraction rate of methanol is about 1.132 – 1.035 times higher than that of ethanol and anhydrous ethanol for each component. The results indicated that the extracted effects of different solvents were as follows: methanol > ethanol > anhydrous ethanol > -water. Therefore, methanol was confirmed as the best extraction solvent. In addition, the influence of different extraction methods (ultrasonic extraction, reflux extraction, and impregnation method) on the contents of 1-methyl hydantoin in test samples was inspected. It was found that ultrasonic extraction was relatively simple and was of high extraction rate, which was determined as the optimum extraction method.

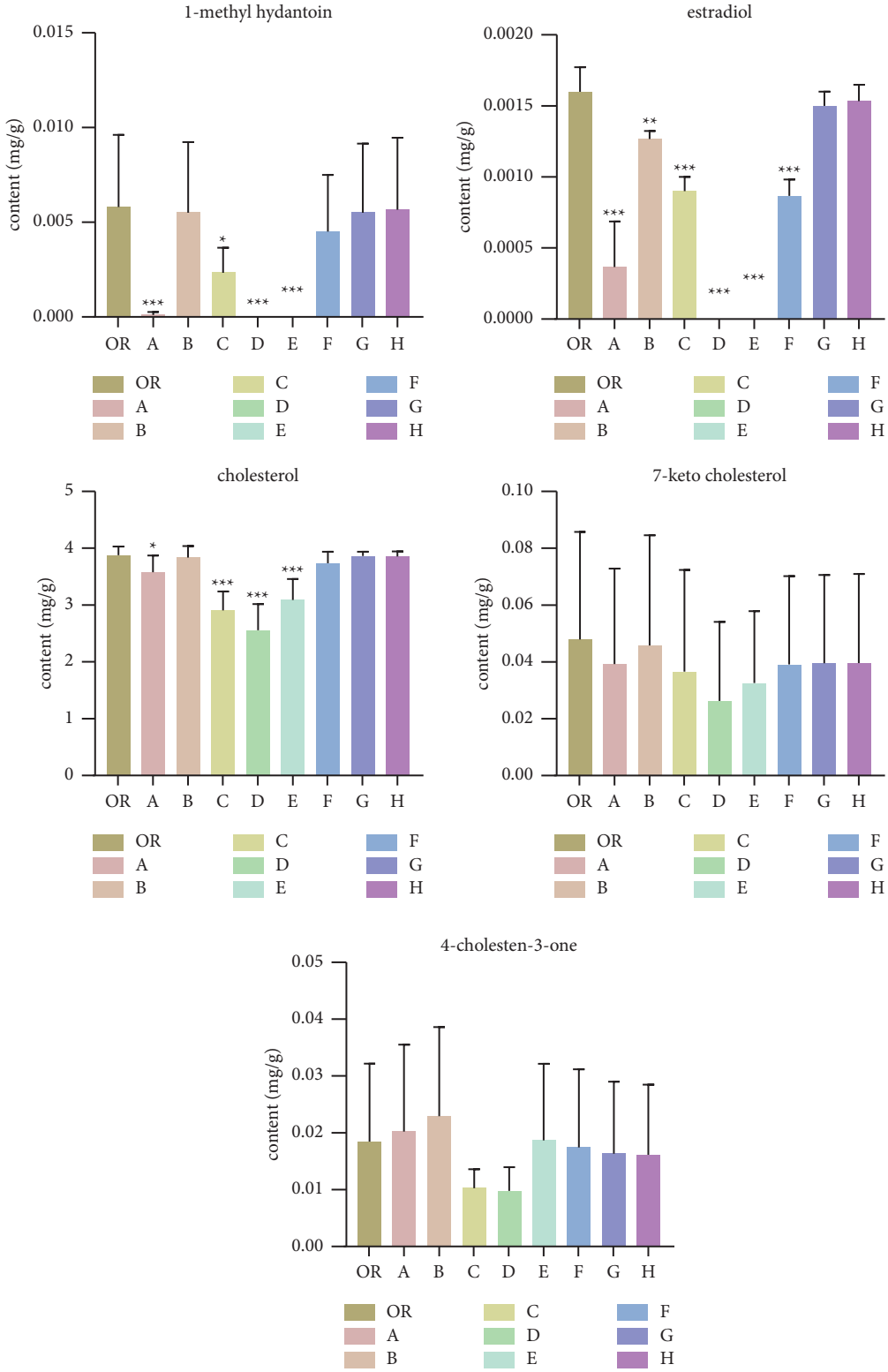


FIGURE 3: The contents change of five chemical constituents in OR and OR processing products, $n = 9$, $\overline{x} \pm s$. (A: boiling, B: stewing, C: wine frying, D: charcoal frying, E: frying, F: drying at 100°C, G: drying at 60°C, and H: drying at 40°C; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, vs. OR group).

Secondly, chromatographic conditions were optimized in this paper. Different chromatographic columns (Alltima™ C_{18}, Angilent TC-C_{18} and Kromasil 100-5 C_{18}) and different mobile phase systems (methanol-water, acetonitrile-water, methanol-0.1% phosphoric acid solution, and methanol-0.2% phosphoric acid solution) were compared, respectively. The results exhibited that the baseline was basically separated and the peak shapes of the chromatographic peak of each analyte

were better using Alltima™ C_{18} column and methanol-0.1% phosphoric acid solution system. In addition, based on the previous study of our research group, the detective wavelengths were confirmed as 215 nm (1-methyl hydantoin, estradiol, and cholesterol) and 240 nm (7-keto-cholesterol and 4-cholesten-3-one), at which each component could be determined at the maximum absorption.

4. Conclusion

In this paper, a HPLC-DAD method was developed and verified for the simultaneous determination of five main effective components (1-methyl hydantoin, estradiol, cholesterol, 7-keto-cholesterol, and 4-cholesten-3-one) in OR. In addition, the proposed method was applied to investigate the changes of five constituents in OR prepared by different processing methods. The results indicated that OR unprocessed or processed by drying at a low temperature was recommended for use of eating and treating. This study provides guidance for the edible and processing technology of OR, which also lays a research foundation for product development and quality research.

Abbreviations

HPLC: High-performance liquid chromatography
DAD: Diode array detector
LOQ: Limit of quantification
LOD: Limit of detection
RSD: Relative standard deviation
OR: *Oviductus Ranae*.

Data Availability

The main table and figure data used to support the findings of this study are included within the article.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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