Analysis and Comparison of Cuttlebone Nutrient Composition in Sepia pharaonis at Different Growth Stages: Postprint

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Abstract

This study investigated the changes in nutritional components and mineral element contents in the internal shell of Sepia pharaonis at different growth stages, aiming to provide a theoretical basis for its development and utilization. National standard methods were employed to determine the contents (on a dry matter basis) of nutritional components (moisture, crude ash, crude protein, crude fat, polysaccharides, amino acids, fatty acids) and mineral elements [5 macroelements—sodium (Na), potassium (K), magnesium (Mg), aluminum (Al), calcium (Ca) and 8 trace elements—copper (Cu), iron (Fe), zinc (Zn), selenium (Se), manganese (Mn), cadmium (Cd), arsenic (As), strontium (Sr)] in the internal shell of Sepia pharaonis at different growth stages (60, 90, 120, and 150 days of age). The results showed that at different growth stages, crude ash was the main component of the internal shell of Sepia pharaonis, with contents ranging from 89.98% to 92.06%, and its content increased continuously with growth, being significantly higher at 150 days of age than at other ages ($P<0.05$); crude protein content ranged from 2.82% to 3.11%, and its content increased continuously with growth, being significantly higher at 150 days of age than at 60 days of age ($P<0.05$); moisture content ranged from 3.93% to 6.21%, and its content decreased continuously with growth, being significantly higher at 60 days of age than at other ages ($P<0.05$); compared with other nutritional components, the contents of crude fat and polysaccharides in the internal shell of Sepia pharaonis were relatively low, being 0.41%–0.47% and 0.34%–0.38%, respectively. Seventeen amino acids, including 7 essential amino acids (EAA), were detected in the internal shell of Sepia pharaonis at all growth stages. The contents of total amino acids (TAA), EAA, and acidic amino acids (AAA) in the internal shell of Sepia pharaonis at different growth stages were 2.79%–2.93%, 0.87%–0.98%, and 0.69%–0.87%, respectively, with AAA content increasing continuously with growth and being significantly higher at 150 days of age than at other ages

(P<0.05). At all growth stages, glutamic acid (Glu) content (0.31%-0.43%) was the highest and histidine (His) content (0.03%-0.07%) was the lowest in the internal shell of Sepia pharaonis. Seven fatty acids were detected in the internal shell of Sepia pharaonis at all growth stages, including 3 saturated fatty acids (SFA), 1 monounsaturated fatty acid (MUFA), and 3 polyunsaturated fatty acids (PUFA); the fatty acid composition was dominated by C22:6n-3, C16:0, and C18:0, with C22:6n-3 content (35.68%-37.62%) being the highest. Growth stage had a significant effect on the contents of macroelements Na, Mg, Al, and Ca and trace elements Cu, Zn, and Sr (P<0.05), particularly Ca, Mg, and Zn contents, which increased continuously with growth; the heavy metal elements Cd and As were present at very low levels, being 0.001-0.003 mg/g and 0.012-0.030 mg/g, respectively. In summary, the internal shell of Sepia pharaonis is rich in nutrients, with high contents of Ca, Mg, and Zn that increase continuously with growth, indicating good prospects for development and application.

Full Text

Comparative Analysis of Nutritional Component Contents in Cuttlebone of *Sepia pharaonis* at Different Growth Stages

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Abstract

This study investigated changes in nutritional components and mineral element contents in cuttlebone of *Sepia pharaonis* at different growth stages to provide a theoretical basis for its development and utilization. National standard methods were employed to determine the contents of nutritional components (moisture, crude ash, crude protein, crude fat, polysaccharide, amino acids, fatty acids) and mineral elements [five major elements—sodium (Na), potassium (K), magnesium (Mg), aluminum (Al), calcium (Ca) and eight trace elements—copper (Cu), iron (Fe), zinc (Zn), selenium (Se), manganese (Mn), cadmium (Cd), arsenic (As), strontium (Sr)] (dry weight basis) in cuttlebone of *Sepia pharaonis* at different growth stages (60, 90, 120, and 150 days of age).

The results showed that crude ash was the predominant component in cuttlebone across all growth stages, with contents ranging from 89.98% to 92.06%. Ash content increased significantly with age, reaching its highest value at 150 days (P<0.05). Crude protein content ranged from 2.82% to 3.11% and also increased with growth, with the 150-day group showing significantly higher values than the 60-day group (P<0.05). Moisture content decreased progressively from 6.21% to 3.93% during development, with the 60-day group exhibiting significantly higher values than other age groups (P<0.05). In contrast, crude fat (0.41%-

0.47%) and polysaccharide (0.34%–0.38%) contents remained relatively low and showed no significant variation among growth stages.

A total of 17 amino acids were detected at all growth stages, including seven essential amino acids (EAA). Total amino acid (TAA), EAA, and acidic amino acid (AAA) contents ranged from 2.79% to 2.93%, 0.87% to 0.98%, and 0.69% to 0.87%, respectively. Notably, AAA content increased progressively with age, reaching significantly higher levels at 150 days compared to other stages ($P<0.05$). Glutamic acid (Glu) was the most abundant amino acid (0.31%–0.43%), while histidine (His) was the least abundant (0.03%–0.07%) across all growth stages. Seven fatty acids were identified, comprising three saturated fatty acids (SFA), one monounsaturated fatty acid (MUFA), and three polyunsaturated fatty acids (PUFA). The fatty acid profile was dominated by C22:6n-3, C16:0, and C18:0, with C22:6n-3 showing the highest content (35.68%–37.62%).

Growth stage significantly affected the contents of major elements (Na, Mg, Al, Ca) and trace elements (Cu, Zn, Sr) ($P<0.05$), particularly Ca, Mg, and Zn, which all increased with age. Heavy metal elements Cd and As were present at very low concentrations of 0.001–0.003 mg/g and 0.012–0.030 mg/g, respectively. In conclusion, cuttlebone of *Sepia pharaonis* is nutritionally rich, with high contents of Ca, Mg, and Zn that increase progressively during growth, indicating promising prospects for development and application.

Keywords: *Sepia pharaonis*; cuttlebone; different growth stages; nutritional components; mineral elements

Introduction

Cuttlebone, commonly known as "hai piao xiao" or "cuttlefish bone," is a frequently used marine animal medicine in traditional Chinese medicine with salty, astringent, and warm properties. It functions to astringe and stop bleeding, restrain seminal emission and leukorrhea, neutralize acid, and promote wound healing [1]. The medicinal use of cuttlebone dates back to ancient times, with the *Compendium of Materia Medica* documenting its efficacy in treating various internal and external hemorrhages, gastric ulcers, duodenal ulcers, and chronic gastritis, as well as its clinical application for skin ulcers and other conditions. The *Shennong Bencao Jing* also recorded its ability to "treat women' s red and white leukorrhea." Recent research on cuttlebone has primarily focused on clinical applications [2-5], physiological studies [6], and extraction and application of active components [7-11]. Currently, aside from limited use in traditional Chinese medicine, most cuttlebone is discarded as waste from food processing, representing a serious resource waste.

Sepia pharaonis belongs to the phylum Mollusca, order Sepioidea, family Sepiidae, and genus *Sepia*. This marine cephalopod species is economically important, characterized by large body size, heavy cuttlebone (exceeding 1 kg), and

rapid growth. Previous research on *S. pharaonis* has primarily concentrated on biological characteristics [12-13], reproductive biology [14-15], nutritional composition [16-17], and aquaculture technology [18], with no reports on the nutritional composition of its cuttlebone. Studies on mineral element contents in cuttlebone are also scarce, limited to *Sepia esculenta* [19-20] and *Sepiella maindroni* [21]. This paper analyzes the nutritional components and mineral element contents in cuttlebone of *S. pharaonis* at different growth stages to provide a theoretical basis for the utilization and development of cuttlebone resources.

1.1 Experimental Materials

The experiment was conducted from June to October 2014 at the Zhujiajian Base of the Zhoushan Fisheries Research Institute in Zhejiang Province. Juvenile *S. pharaonis* were obtained through artificial breeding by our research group. Healthy juveniles with uniform size and good vitality were selected for the experiment, with initial mantle lengths of 2.2–2.6 cm and body weights of 1.8–2.3 g. Experimental animals were cultured in concrete tanks (4.5 m × 4.0 m × 1.4 m) under the following conditions: water temperature 24.2–28.6 °C, salinity 23.7–25.4, pH 7.81–8.04, water depth 0.6–1.2 m, and continuous aeration (1 air stone/m^2). The cuttlefish were fed frozen small fish twice daily at 10%–15% of body weight. Water exchange (50%–60% of tank volume) was performed 1–2 times daily using sand-filtered natural seawater, with siphoning to remove dead individuals and waste. The culture period lasted 150 days. Sampling was conducted every 30 days, with 8–10 individuals randomly collected from the tanks each time. Specimens were transported on ice to the laboratory for measurement of body weight and mantle length, followed by dissection to obtain cuttlebone for measurement of shell weight and shell length (Table 1). Cuttlebone samples were then dried to constant weight in a forced-air oven (DGG-9620A) at 105 °C, ground into powder, and stored for subsequent analysis of nutritional components and mineral element contents.

1.2.1 Determination of Basic Nutritional Components

National standard methods were employed for determining basic nutritional components: moisture content by oven drying at 105 °C to constant weight [22]; crude ash content by ignition at 550 °C to constant weight [23]; crude protein content by the Kjeldahl method [24]; and crude fat content by the Soxhlet extraction method [25].

1.2.2 Determination of Polysaccharide Content

Preparation of glucose standard curve [26]: Standard glucose solutions of 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mL were accurately pipetted into 25 mL colorimetric tubes, diluted to 2 mL with ultrapure water, then mixed with 1.0 mL of 5% phenol solution and 5.0 mL of sulfuric acid. After mixing and standing at room

temperature for 30 min, absorbance was measured at 490 nm. The standard curve regression equation was:

$C = 0.1231A + 0.0012$ ($R^2 = 0.9956$)

where C represents absorbance and A represents glucose content (mg/mL).

Polysaccharide extraction [27]: A precise amount of dried cuttlebone powder was extracted twice with 95% ethanol under reflux. After evaporating the solvent, the residue was extracted twice with water under reflux for 2 h each time. The aqueous extracts were combined and concentrated, then 95% ethanol was added to achieve a final ethanol concentration of 80%. The solution was stored overnight at 4 °C, then centrifuged. The precipitate was de-alcoholized and lyophilized to obtain cuttlebone polysaccharide. Polysaccharide content was determined by the phenol-sulfuric acid method [28].

1.2.3 Determination of Amino Acid Content

Amino acid content was determined according to national standard methods [29]. Cuttlebone samples were microwave-digested and derivatized with o-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) reagents for chromatographic analysis. Detection conditions: Zorbax Eclipse-AAA amino acid analysis column (4.6 mm × 150 mm, 5 m); mobile phase A: 40 mmol/L Na_2HPO_4 (pH adjusted to 7.8 with 8 mol/L NaOH); mobile phase B: acetonitrile-methanol-water (45:45:10); gradient elution at 2 mL/min; detection wavelengths 338 nm and 262 nm; column temperature 40 °C.

1.2.4 Determination of Fatty Acid Content

Following crude fat determination by Soxhlet extraction, the crude fat extract was saponified and methylated for analysis using an Agilent 7890A gas chromatograph. Chromatographic conditions: DB-WAX polyethylene glycol capillary column (30 m × 0.25 mm × 0.25 m); 10 L automatic liquid sampler (ALS); injection volume 1 L; injector temperature 250 °C; splitless injection; constant pressure temperature control mode; column head pressure 5.3 MPa. Temperature program: initial temperature 50 °C held for 2 min, then increased to 250 °C at 10 °C/min and held for 23 min. Detector: flame ionization detector (FID) at 300 °C; carrier gas: nitrogen (N_2). Gas flow rates: hydrogen (H_2) 40 mL/min, air 450 mL/min.

1.2.5 Determination of Mineral Element Content

Cuttlebone samples from different growth stages were analyzed for five major elements [sodium (Na), potassium (K), magnesium (Mg), aluminum (Al), calcium (Ca)] and eight trace elements [copper (Cu), iron (Fe), zinc (Zn), selenium (Se), manganese (Mn), cadmium (Cd), arsenic (As), strontium (Sr)].

Sample pretreatment: Accurately weighed 0.1000 g of dried cuttlebone sample was placed in a digestion tube and digested with 20–30 mL of mixed acid (per-

chloric acid:nitric acid = 1:4) until the solution became clear. After cooling to room temperature, the solution was diluted to 100 mL with ultrapure water. Analysis was performed using inductively coupled plasma optical emission spectrometry (Optima 5300DV) with imported mixed standard stock solution (1000 mg/L) provided by PerkinElmer.

Preparation of mixed standard series: The 1000 mg/L mixed standard stock solution was diluted with 10% HNO_3 to prepare mixed standard solutions containing 0.10, 1.0, 5.0, and 10 mg/L of Na, K, Mg, Al, Cu, Fe, Zn, Se, Mn, Cd, As, Sr, and Ca.

ICP-AES operating parameters: plasma gas flow 15 L/min, carrier gas flow 0.8 L/min, auxiliary gas flow 0.2 L/min, nebulizer flow 0.8 L/min, pump flow 1.5 mL/min, axial viewing distance 15 mm, instrument stabilization delay 30 s, power 1300 W.

1.3 Data Analysis

Experimental data were analyzed using Excel 2003 and SPSS 18.0 statistical software. Descriptive statistics were expressed as mean ± standard deviation, with $P < 0.05$ as the criterion for significant difference.

2.1 Basic Nutritional Components

As shown in Table 2 , crude ash was the predominant component in cuttlebone of *S. pharaonis* across all growth stages, with contents ranging from 89.98% to 92.06%. Ash content increased progressively with age, reaching its maximum at 150 days (92.06%), which was significantly higher than other age groups ($P<0.05$). Moisture content ranged from 6.21% to 3.93%, with the highest value observed at 60 days (6.21%), significantly exceeding values at 120 and 150 days ($P<0.05$). Crude protein content varied from 2.82% to 3.11%, peaking at 150 days (3.11%), which was significantly higher than at 60 days ($P<0.05$). Other nutritional components were present in relatively small amounts, with crude fat content of 0.41%–0.47% and polysaccharide content of 0.34%–0.38%.

2.2 Amino Acid Composition and Content

A total of 17 amino acids were detected in cuttlebone of *S. pharaonis* at all growth stages (Table 3 ; tryptophan not detected), including seven essential amino acids. Total amino acid (TAA) content showed no significant differences among growth stages ($P>0.05$), with values of 2.79%, 2.84%, 2.85%, and 2.93% at 60, 90, 120, and 150 days, respectively. Essential amino acid (EAA) content also showed no significant differences ($P>0.05$), with values of 0.87%, 0.97%, 0.82%, and 0.98% at the respective ages. Glutamic acid (Glu) was the most abundant amino acid (0.31%–0.43%) across all stages, while histidine (His) was the least abundant (0.03%–0.07%). Although serine (Ser), lysine (Lys), and

glycine (Gly) showed no significant differences among growth stages (P>0.05), they were present at relatively high concentrations of 0.25%-0.27%, 0.23%-0.24%, and 0.19%-0.20%, respectively. Acidic amino acid (AAA) [aspartic acid (Asp) + Glu] content increased progressively with age, reaching 0.56%, 0.62%, 0.69%, and 0.78% at 60, 90, 120, and 150 days, respectively, corresponding to 20.07%, 21.83%, 24.2%, and 26.62% of TAA content. The AAA content at 150 days was significantly higher than at other ages (P<0.05).

2.3 Fatty Acid Composition and Content

Seven fatty acids were detected in cuttlebone of *S. pharaonis* at all growth stages (Table 4), including three saturated fatty acids (SFA), one monounsaturated fatty acid (MUFA) (C18:1), and three polyunsaturated fatty acids (PUFA) [C20:4, C20:5 (EPA), and C22:6 (DHA)]. Unsaturated fatty acid content showed no significant differences among growth stages (P>0.05), with values of 57.38%, 57.71%, 58.50%, and 57.00% at 60, 90, 120, and 150 days, respectively. The fatty acid profile was dominated by C22:6n-3, C16:0, and C18:0, with C22:6n-3 showing the highest content (35.68%-37.62%) and C16:0 content ranging from 25.36% to 26.61%.

2.4 Mineral Element Content

As shown in Table 5 , growth stage significantly affected the contents of major elements Na, Mg, Al, and Ca, as well as trace elements Cu, Zn, and Sr (P<0.05), particularly Ca, Mg, and Zn, which all increased progressively with age. Among major elements, Ca was the most abundant, with contents of 334.23, 393.30, 452.39, and 474.28 mg/g at 60, 90, 120, and 150 days, respectively. Mg was the second most abundant, with values of 29.85, 36.11, 39.89, and 54.82 mg/g at the respective ages. Among trace elements, Zn showed the highest content (14.05, 16.36, 19.21, and 23.02 mg/g), followed by Cu (2.34, 2.85, 4.33, and 5.47 mg/g). Heavy metal elements Cd and As were present at very low concentrations of 0.001–0.003 mg/g and 0.012–0.030 mg/g, respectively.

3.1 Changes in Nutritional Component Contents of Cuttlebone at Different Growth Stages

The chemical composition of cuttlebone reflects the accumulation of nutrients from dietary intake through digestion, metabolism, and absorption during growth and development. Variations in chemical composition among growth stages are influenced by both internal factors (such as ontogenetic regulation and metabolic patterns at different developmental phases) and external factors (including diet and aquatic environment), which collectively affect material and energy metabolism as well as nutrient and mineral accumulation [30].

In this study, crude ash and crude protein contents in cuttlebone of *S. pharaonis*

increased significantly with individual growth and development, whereas crude fat and polysaccharide contents showed no significant changes. Crude ash was the predominant component in cuttlebone across all growth stages, reaching 89.98%–92.06% and increasing progressively with age to a maximum of 92.06% at 150 days, significantly higher than at other stages. The high ash content is closely related to cuttlebone structure formation. During early development, cuttlebone consists primarily of organic matter, but as growth proceeds, calcium ion adsorption increases gradually, forming calcified lamellar structures with progressively thicker calcified layers that eventually develop into densely arranged mineralized crystals [31].

Total amino acid (TAA) content showed no significant differences among growth stages (2.79%–2.93%), which was higher than that reported for *Sepiella maindroni* (2.32%) [21] and similar to *Sepia esculenta* (2.86%) [20]. Essential amino acid (EAA) content also showed no significant differences (0.87%–0.98%), comparable to values reported for *S. maindroni* (0.966%) [21] and *S. esculenta* (0.994%) [20]. Acidic amino acid (AAA) content increased progressively with age, peaking at 150 days (26.62% of TAA), significantly higher than at other stages ($P<0.05$). The AAA content accounted for 20.07%–26.62% of TAA, similar to the range of 22.178%–24.107% reported for different cuttlefish species [32] but higher than the 19.96%–21.29% reported for *S. maindroni* and *S. esculenta* [33]. Although serine (Ser), lysine (Lys), and glycine (Gly) showed no significant differences among growth stages, they were present at relatively high concentrations of 0.25%–0.27%, 0.23%–0.24%, and 0.19%–0.20%, respectively. Mollusk shell proteins are rich in Asp+asparagine (Asn), Glu+glutamine (Gln), Gly, and Ser. Keith et al. [34] compared amino acid compositions in shells of nautilus, abalone, and mussel, finding substantial amounts of Asp, Gly, and Ser in soluble proteins from all three species, with particularly high levels of Asp+Asn and Gly in abalone shell. Weiner and Hood [35] conducted in-depth studies on abalone and oyster shell formation, concluding that shell formation is regulated by organic matter, with proteins, polysaccharides, and chitosan forming network scaffolds during calcification. These scaffolds facilitate mineralization, particularly through the strong calcium-binding capacity of AAA, enabling crystal formation. The organic content in cuttlebone is approximately 4%, and these organic macromolecules regulate crystal structure orientation and spatial morphology at the nanoscale, conferring unique strength and order [36-37].

3.2 Changes in Mineral Element Contents of Cuttlebone at Different Growth Stages

Cuttlebone exhibits strong binding capacity for environmental mineral elements. Determination of trace element species and contents in mollusk shells can serve not only for water quality monitoring but also as an important indicator of aquaculture environment health and the wellbeing of cultured shellfish themselves.

Crude ash is the main component of cuttlebone and contains abundant mineral

elements. Comparative analysis revealed that Ca was the most abundant element in cuttlebone of *S. pharaonis* at all growth stages, followed by Mg. Both Ca and Mg contents increased progressively with age, reaching maximum values at 150 days (474.28 and 54.82 mg/g, respectively), significantly higher than at other stages. Calcium is the most abundant element in the human body, concentrated primarily in bone, teeth, and hard tissues, where it exists as calcium phosphate in the cytoplasm to form rigid structures. Beyond its role as the main component of bone, calcium enhances capillary wall density to prevent tissue fluid exudation, providing anti-inflammatory, anti-edema, and anti-histamine effects. Calcium also plays crucial roles in blood coagulation, muscle contraction, nerve structure composition and signal transduction, and activation of various enzymes [38]. Therefore, cuttlebone of *S. pharaonis* can serve as a dietary or medicinal source of calcium and magnesium supplementation. Calcium is the most abundant element in mollusk shells (accounting for 89%–95% of ash content) [39-40], and in *S. pharaonis* cuttlebone, Ca comprises 89.98%–92.06% of ash content. The calcified structure of cuttlebone provides porosity exceeding 90% and exhibits excellent biocompatibility, making it valuable for novel biomedical material applications including hydroxyapatite preparation, tissue engineering scaffolds, and composite protein artificial bone formation and revascularization [2-4].

Among trace elements, Zn showed the highest content in cuttlebone across all growth stages (14.05, 16.36, 19.21, and 23.02 mg/g). Zinc serves as a cofactor and activator for numerous enzymes, influencing the biological activity of over 160 enzymes and participating in nucleic acid and protein synthesis. Zinc deficiency delays gonadal maturation in animals and causes gonadal atrophy and fibrosis in mature animals. Zinc is abundant in male testes and participates in the entire process of spermatogenesis, maturation, and capacitation. Zinc deficiency also affects skin system growth and development, leading to dermatitis. Appropriate zinc supplementation in infants and children can effectively prevent respiratory infections and diarrhea. Therefore, cuttlebone of *S. pharaonis* represents a promising dietary or medicinal source of zinc supplementation. Additionally, heavy metal elements Cd and As were detected at very low concentrations of 0.001–0.003 mg/g and 0.012–0.030 mg/g, respectively, far below the EU food safety limits (1.0–1.5 mg/kg) [41].

In summary, crude ash, crude protein, and AAA contents in cuttlebone of *S. pharaonis* increased progressively with growth. The most distinctive feature across growth stages was the high content of major elements Ca and Mg and trace element Zn, all of which increased with age, while heavy metal elements Cd and As remained at low levels.

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Note: Figure translations are in progress. See original paper for figures.