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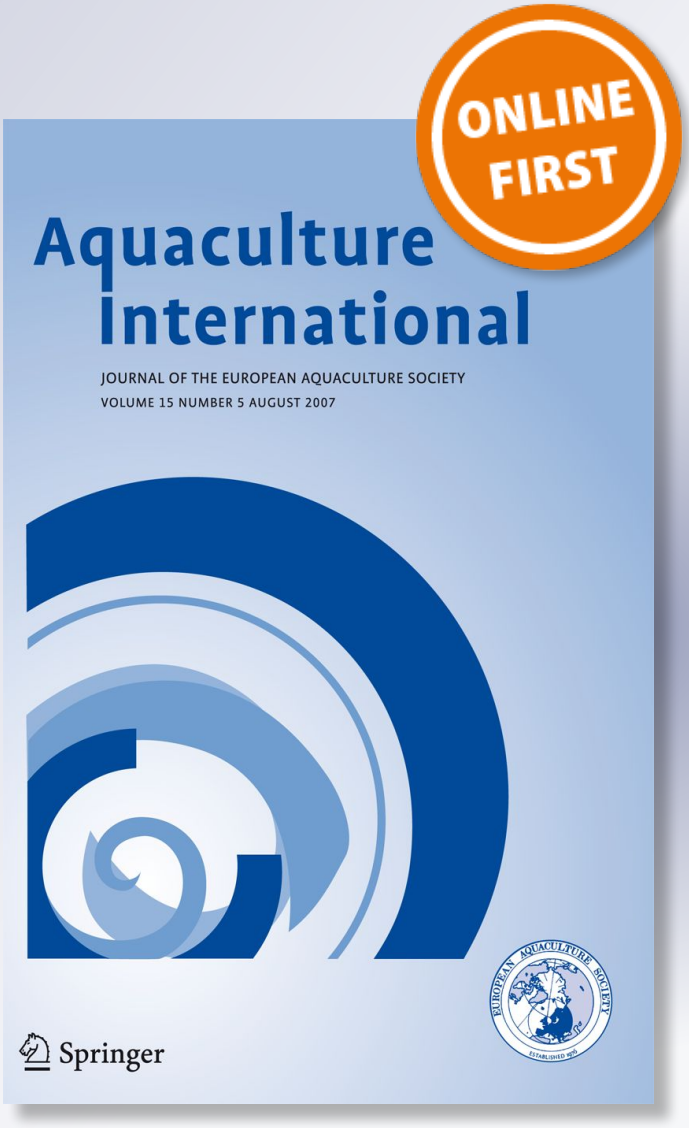
Aquaculture International
Journal of the European Aquaculture Society

ISSN 0967-6120

Aquacult Int
DOI 10.1007/s10499-012-9569-6



Springer

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Amino acids composition and protein quality evaluation of marine species and meals for feed formulations in cephalopods

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Received: 14 December 2011 / Accepted: 21 July 2012

Abstract The amino acid composition and protein levels of three species of cephalopods (*Octopus vulgaris, Loligo gahi* and *Todarodes sagittatus),* the natural diets of common octopus (*O. vulgaris*) and different kinds of meals were determined in order to optimise the content of these nutrients in artificial feeds. Arginine, leucine and lysine were the most abundant essential amino acids in cephalopods, while glutamate and aspartate represented the main non-essential amino acids. Arginine and leucine were the limiting amino acid in most samples, with maximum Chemical Score values for mussel (79–98 %), squid (84 %)

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Published online: 08 August 2012

and crustaceans (65–91 %); medium for fish (41–70 %); and minimum for meals (29–64 %). Mussel, squid, crustaceans and fish showed a high essential amino acid index according to Oser (OI: 88–99 %) suggesting a suitable amino acid balance. The protein from animal meals (fish and krill) covered all the essential amino acids except arginine and lysine in fish meal. The vegetable meal presented the worst amino acid balance (OI: 74–89 %) with several deficiencies in essential amino acids, including arginine, threonine, lysine and methionine. Supplementation with arginine or leucine and protein complementation of crustaceans and bivalves with fish or animal meal are proposed as alternatives for improving the performance of protein in feed for cephalopods.

Keywords Amino acid · Cephalopod · Feed composition · Feed formulation · Meal · Nutrition · Octopus

Abbreviations

AA	Amino acid
AAR	Amino acid ratio
CS	Chemical Score
OI	Oser's Index

Introduction

One of the main reasons for the lack of development in cephalopod aquaculture is that there are no feeds available, which are palatable with a balanced nutritional composition for all stages of their growth (Vaz-Pires et al. 2004; Cerezo Valverde et al. 2008). The culture of the early life stages of cephalopods (including octopus, squid and cuttlefish) has depended on the supply of live prey to achieve acceptable growth and survival (Boletzky and Hanlon 1983; Baeza-Rojano et al. 2010). However, the culture of some species of octopus, such as *Octopus vulgaris*, is problematic and shows high mortality rates during their planktonic life stage when live prey (Navarro and Villanueva 2003; Iglesias et al. 2007) or formulated diets (Villanueva et al. 2002) are used, emphasising our poor knowledge of their nutritional requirements. Nevertheless, in both juvenile and adult stages, the best results have been obtained with natural diets (Aguila et al. 2007; Domingues et al. 2008).

Whatever the case, the successful commercial ongrowing of any species needs a formulated diet, given the advantages of such compared with natural diets, and this is the case with sea bream, sea bass and turbot (Cho and Bureau 2001; Davies et al. 2009). It is also necessary to know the correct nutritional composition of the feed to obtain good growth and feed efficiency. One starting point for optimising the nutritional composition of artificial octopus feeds could be to analyse the macro and micronutrients of major ingredients such as crabs, mussels and fish, which are commonly used in the natural diets (Chapela et al. 2006; García García and Cerezo Valverde 2006; García García et al. 2009). In addition, the ratios of the macro and micronutrients in the tissues of several cephalopods species and in the raw materials may be used to develop an artificial diet.

In this respect, protein is the most expensive nutritional component for feed formulation in aquaculture. This is especially relevant considering that cephalopods are exclusively

carnivorous species (Guerra and Nixon 1987) and the high protein/energy ratio needed to achieve maximum growth (up to 50 g protein/MJ in *Sepia officinalis* according to Lee 1994). Such a high value can be explained by the predominance of amino acid metabolism and its use for energy purposes. Therefore, unlike in the case of fish, the substitution of proteins by fats or carbohydrates does not seem a good way of formulating feeds for cephalopods, although a minimum level of carbohydrates dry matter is necessary for producing extruded dry feeds with suitable physical properties (Thomas et al. 1998). For this reason, the quality of the protein or the amino acid balance may be the best measure of nutritional value for cephalopod diets. With such a procedure, it will be possible to obtain maximum growth and protein retention with the lowest possible percentage of proteins in the diet.

In recent years, researchers have developed feed formulations that have been found acceptable by octopus and have resulted in significant growth (Cerezo Valverde et al. 2008; Quintana et al. 2008; Rosas et al. 2008; Estefanell et al. 2011). However, the choice of major ingredients is extremely important in this respect. With this in mind, the coordinated project, JACUMAR (2007–2009), which is directed at optimising octopus ongrowing in Spain, has among its objectives the detailed biochemical analysis of different cephalopod species, their natural diets, waste products from the canning industry, and several different plant and animal meals as well. In this study, we evaluate the results obtained for the amino acids composition found in molluscs, crustaceans, fish and meals, selecting the most appropriate to elaborate cephalopod diets by reference to an index of nutritional quality.

Materials and methods

Collection and keeping of samples

Forty-two samples were gathered, including fish, crustaceans, molluscs and meals, from different participants in the National Plan entitled 'Optimising the ongrowing of the octopus *Octopus vulgaris* 2007–2009'. Some samples were collected both during summer and winter (Tables 1, 2 and 3). For molluscs, only the edible portion was selected, except in the case of *O. vulgaris* when, besides the whole animals, the gonad, digestive gland and muscle tissue were analysed. For fish, the filets, gonads and viscera were included, rejecting bony structures and fins. As for crustaceans, all the animals were emptied, including the meat from the claws, inside the shell, gills and gonads, rejecting only the skeletal structure. As an exception, the whole heads of *Penaeus* sp. from the canning industry were analysed, obtaining between 500 and 1,000 g of sample per species, ensuring that it came from at least six different specimens. All the animals were triturated to obtain a homogeneous mixture, which was vacuum-packed in 100 g portions and frozen -20° C until use (a maximum of 3 months until amino acid analysis).

Analytical methods and determination of amino acids

Prior to chemical analyses, all samples were freeze-dried and then analysed according to AOAC (1997). Briefly, dry matter was obtained by drying (105 ± 1 °C) to constant weight, and crude protein ($N \times 6.25$) was determined by the Kjeldahl method after acid digestion (Kjeltec 2300 Auto Analyser, Tecator Höganas, Sweden). All analyses were performed in triplicate. Following the method previously described by Bosch et al. (2006), amino acids of the samples were determined using a Waters HPLC system (Waters 474,

Table 1 Samples used to determine amino acids in molluscs and crustaceans

Id	Group species	Common name	Sample	Sampling period	Location (Spain)	*n**	Fresh weight ± SD (g)
Molluscs							
1	*Loligo gahi*	Squid	Edible portion	Feb-08	Andalucía (S)	28	40.6 ± 6.4
2	*Mytilus galloprovincialis*	Mussel	Edible portion (boiled)[a]	Jul-07	Galicia (NW)	90	4–9 (without shell)
3	*Mytilus galloprovincialis*	Mussel	Edible portion (boiled)[a]	Feb-08	Galicia (NW)	90	4–9 (without shell)
4	*Mytilus galloprovincialis*	Mussel	Edible portion	Feb-08	Asturias (N)	40	48.6 ± 0.4
5	*Mytilus galloprovincialis*	Mussel	Edible portion	Jun-08	Asturias (N)	40	40–50
6	*Octopus vulgaris*	Common octopus	Whole animal	Jul-07	Murcia (SE)	6	1,005.0 ± 291.9
7	*Octopus vulgaris*	Common octopus	Whole animal	Mar-08	Murcia (SE)	6	868.0 ± 46.6
8	*Octopus vulgaris*	Common octopus	Muscle	Jul-07	Murcia (SE)	10	1,448.9 ± 337.6
9	*Octopus vulgaris*	Common octopus	Muscle	Mar-08	Murcia (SE)	10	771.7 ± 138.2
10	*Octopus vulgaris*	Common octopus	Digestive gland	Jul-07	Murcia (SE)	24	1,369.0 ± 316.9
11	*Octopus vulgaris*	Common octopus	Digestive gland	Mar-08	Murcia (SE)	36	967.2 ± 394.4
12	*Octopus vulgaris*	Common octopus	Gonad	Mar-08	Murcia (SE)	36	967.2 ± 394.4
13	*Todarodes sagittatus*	Sea arrow	Mantle, arms and fins[a]	Jul-07	Galicia (NW)	100	100–150
Crustaceans							
14	*Carcinus maenas*	Common shore crab	Edible portion	Feb-08	Asturias (N)	24	50–60
15	*Carcinus maenas*	Common shore crab	Edible portion	Jun-08	Asturias (N)	30	50–60
16	*Carcinus maenas*	Common shore crab	Edible portion	Jul-07	Murcia (SE)	86	41.6 ± 10.4
17	*Carcinus maenas*	Common shore crab	Edible portion	Mar-08	Murcia (SE)	178	39.0 ± 14.0
18	*Penaeus* sp.	Prawn	Heads[a]	Jul-07	Galicia (NW)	150	9.0 ± 2.1
19	*Procambarus clarkii*	Red crayfish	Edible portion	Feb-08	Andalucía (S)	268	11.4 ± 1.8

[a] From the canning industry. * Number of animals used to obtain a homogeneous mixture

Table 2 Samples used to determine amino acids in fish

Id	Group species	Common name	Sample	Sampling period	Location (Spain)	*n**	Fresh weight ± SD (g)
Fish							
20	*Boops boops*	Bogue[a]	Edible portion	Feb-08	Murcia (SE)	118	20.6 ± 11.3
21	*Boops boops*	Bogue[b]	Edible portion	Feb-08	Murcia (SE)	11	177.4 ± 67.6
22	*Boops boops*	Bogue[a]	Edible portion	Jul-07	Canary Islands	20	92.0 ± 23.0
23	*Boops boops*	Bogue[b]	Edible portion	Jul-07	Canary Islands	6	333.0 ± 34.0
24	*Gadus poutassou*	Blue whiting[a]	Edible portion	Jul-07	Galicia (NW)	25	36.8 ± 7.5
25	*Gadus poutassou*	Blue whiting[a]	Edible portion	Feb-08	Galicia (NW)	25	30–50
26	*Gadus poutassou*	Blue whiting[a]	Edible portion	Feb-08	Asturias (N)	10	103.2 ± 4.2
27	*Gadus poutassou*	Blue whiting[a]	Edible portion	Jun-08	Asturias (N)	10	90–110
28	*Mugil* sp.	Mullet[a]	Edible portion	Jun-07	Cataluña (NE)	6	300–2,000
29	*Mugil* sp.	Mullet[a]	Edible portion	Ene-08	Cataluña (NE)	6	300–2,000
30	*Sardina pilchardus*	Sardine[a]	Edible portion	Jul-07	Murcia (SE)	32	49.9 ± 11.3
31	*Sardina pilchardus*	Sardine[a]	Edible portion	Feb-08	Murcia (SE)	135	20.4 ± 5.7
32	*Sparus aurata*	Sea bream[b]	Edible portion	Feb-08	Canary Islands	6	421.0 ± 76.0
33	*Trachurus trachurus*	Scad[a]	Edible portion	Feb-08	Asturias (N)	10	112.9 ± 1.7
34	*Trachurus trachurus*	Scad[a]	Edible portion	Jun-08	Asturias (N)	10	100–120
35	*Gadus minutus*	Poor cod[a]	Edible portion	Jun-07	Cataluña (NE)	75	15–30
36	*Gadus minutus*	Poor cod[a]	Edible portion	Ene-08	Cataluña (NE)	75	15–30

[a] From artisanal fisheries; [b] From the bycatch of fish farms. * Number of animals used to obtain a homogeneous mixture

Table 3 Samples used to determine amino acids in meals

Id	Group	Company
Plant meals		
37	Sunflower	Piensos y Cereales Desco S.L., Valencia, Spain.
38	Pea (75 % protein)	Dibaq-Diproteg, S.A., Segovia, Spain.
39	Soybean	COCERVA, Náquera, Valencia, Spain.
40	Wheat	Piensos y Cereales Desco S.L., Valencia, Spain.
Animal meals		
41	Krill	Sopropeche, Barcelona, Spain.
42	Fish	COCERVA, Náquera, Valencia, Spain.

Waters, Milford, MA, USA) consisting of two pumps—(Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters)—and a temperature control module. Aminobutyric acid (Sigma-Aldrich Co.) was added as an internal standard patron before hydrolysation. The amino acids were derivatized with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and cysteic acid after oxidation with performic acid. Amino acids were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm) and then transformed to methionine and cystine. It was not possible to differentiate the amino acid arginine of taurine (a non-protein nitrogen substance) because both compounds had the same retention time by the analytical technique used. In this regard, the nomenclature 'Arg^{T}' (arginine plus taurine) has been indicated in tables. In any case, taurine was determined by an automatic amino acid analyser (Biochrom 20®, Pharmacia Biotech, Cambridge, UK) in several samples (*O. vulgaris*: Id. 6, 7; *Carcinus maenas*: Id. 17; *Boops boops*: Id. 20) using a cation exchange high performance column (200 × 4.6 mm column size; Pharmacia Biotech) and ninhydrin as derivative agent (Ultra Ninhydrin Reagent, Pharmacia Biotech). When the values of arginine include taurine has been clearly specified throughout the manuscript.

Data analysis

Crude protein is expressed as g kg^{-1} dry weight, with the mean and standard deviation shown for three replicates. Each replica came from the same homogeneous pool of triturated animals. The amino acid (AA) content is expressed as grams of AA kg^{-1} of protein and was obtained in a single sample from the pool. With the data obtained, the values of the following indices were calculated:

- Amino acid ratio (AAR, %) = $(AA_{sample})/(AA_{reference})*100$, where AA_{sample} and $AA_{reference}$ are the amino acid contents in the test sample and whole *O. vulgaris*, which was taken as reference (mean values taking into account summer and winter samples). Amino acid ratios for arginine were calculated by subtracting the values of taurine in samples from octopus to avoid underestimation of the ratios.
- Chemical Score (CS, %): Minimum value from AARs calculated for essentials amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val).
- Limiting amino acid: This is the amino acid corresponding to CS in the test sample.

- Oser's Index (OI, %) was used as index of nutritional quality and obtained as the geometric mean ratio of amino acids in the samples to those detected in *O. vulgaris*, which were taken as reference, according to the formula:

$$\mathrm{OI}(\%) = \left(10^{(1/n*(\log(\mathrm{AAR1})+\log(\mathrm{AAR2})\ldots+\log(\mathrm{AAR}n))}\right),$$

where AAR1, AAR2,…AARn are the ratios of essential amino acid and '*n*' the number of essential amino acids detected. When the ratio is above 100, this was taken as reference (Oser 1951).

All the differences were analysed by one-factor ANOVA and Tukey's test to establish homogenous groups, with the level of significance of $P < 0.05$. A Neperian logarithmic transformation was made before the ANOVA to achieve homogeneity of variances. Principal component analysis (PCA) was performed to obtain a more integrated interpretation of the protein and amino acid composition in the different groups of samples (cephalopods, bivalves, crustaceans, fish, plant meals and animal meals).

Results

Protein content

In molluscs, the highest protein values were detected in *O. vulgaris* (801.9–810.3 g kg^{-1}) and *L. gahi* (797.9 g kg^{-1}) and the lowest in *M. galloprovincialis* (634.5–651.2 g kg^{-1}), the difference being significant ($P < 0.05$; Table 4). These values remained constant regardless of the season ($P > 0.05$). In crustaceans, the protein content was significantly higher in *P. clarkii* (695.2 g kg^{-1}) compared to *Penaeus* sp. (570.1 g kg^{-1}) or *C. maenas* (543.0–607.1 g kg^{-1}; $P < 0.05$). No differences were found in *C. maenas* between summer and winter samples from the same geographical area ($P > 0.05$; Table 4). Fish from artisanal fisheries such as *B. boops* and *G. poutassou* had higher protein levels than the rest of the samples analysed (922.1 and 919.0 g kg^{-1}, respectively; $P < 0.05$; Table 5). In contrast, fish from bycatch of fish farms like *B. boops* (356.3–501.4 g kg^{-1}) and *S. aurata* (520.2 g kg^{-1}) showed the lowest values of all the fish analysed ($P < 0.05$). *Mugil* sp., *S. pilchardus*, *T. trachurus* and *G. minutus* had intermediate values (567.2–884.6 g kg^{-1}). Significant seasonal variations were observed in protein levels. For example, protein levels were higher in *S. pilchardus* in winter than in summer (877.9 vs. 567.2 g kg^{-1}, respectively; $P < 0.05$), but higher levels were observed in summer in *G. poutassou* and *T. trachurus* ($P < 0.05$). In the meals, the highest protein content was found in the pea meal (785.1 g kg^{-1}) and fish meal (748.9 g kg^{-1}) and the lowest in sunflower (344.5 g kg^{-1}) and wheat (124.7 g kg^{-1}; $P < 0.05$; Table 5). The soy (533.5 g kg^{-1}) and krill (559.3 g kg^{-1}) meals had intermediate values but with significant differences from the rest of the meals analysed ($P < 0.05$).

Amino acid content

Arginine, lysine and leucine were the main essential amino acid in the molluscs, with contents that reached 156.7, 72.5 and 64.3 g AA kg^{-1} protein in whole *O. vulgaris*, respectively, with glutamate the main non-essential amino acid (from 104.6 in *M. galloprovincialis* to 145.0 g AA kg^{-1} protein in *T. sagittatus*; Table 6). The same amino acids predominated in all crustacean samples (91.4–128.2, 59.6–81.6, 58.0–75.0 and

Table 4 Moisture (g kg^{-1} fresh weight) and crude protein in molluscs and crustaceans (g kg^{-1} dry weight)

Id	Group/Species	Moisture	Crude protein
Molluscs			
1	*L. gahi*	802.7 ± 0.9^{c}	797.9 ± 5.1^{ab}
2	*M. galloprovincialis*	759.1 ± 0.6^{e}	651.2 ± 21.9^{efg}
3	*M. galloprovincialis*	762.0 ± 2.6^{e}	648.6 ± 3.0^{efg}
4	*M. galloprovincialis*	798.0 ± 1.9^{c}	652.9 ± 5.2^{efg}
5	*M. galloprovincialis*	799.7 ± 1.4^{c}	634.5 ± 3.0^{fg}
6	*O. vulgaris*	798.5 ± 2.3^{c}	810.3 ± 17.6^{a}
7	*O. vulgaris*	780.4 ± 6.2^{d}	801.9 ± 23.6^{ab}
8	*O. vulgaris* (muscle)	832.8 ± 6.8^{b}	778.6 ± 57.2^{abc}
9	*O. vulgaris* (muscle)	798.2 ± 2.7^{c}	780.7 ± 36.8^{ab}
10	*O. vulgaris* (digestive gland)	682.0 ± 1.6^{g}	$738.5 \pm 4.3b^{cd}$
11	*O. vulgaris* (digestive gland)	694.1 ± 1.8^{f}	619.5 ± 3.5^{g}
12	*O. vulgaris* (gonad)	720.2 ± 4.2^{d}	712.6 ± 21.9^{cde}
13	*T. sagittatus*	880.2 ± 2.2^{a}	693.6 ± 13.7^{def}
Crustaceans			
14	*C. maenas*	737.0 ± 10.4^{d}	543.0 ± 19.3^{c}
15	*C. maenas*	788.4 ± 1.7^{c}	584.5 ± 3.0^{bc}
16	*C. maenas*	801.5 ± 2.3^{bc}	571.6 ± 2.1^{bc}
17	*C. maenas*	819.8 ± 1.9^{a}	607.1 ± 2.7^{b}
18	*Penaeus* sp.	805.4 ± 6.7^{ab}	570.1 ± 29.8^{bc}
19	*P. clarkii*	813.5 ± 4.6^{ab}	695.2 ± 21.6^{a}

Different superscripts indicate significant differences ($P < 0.05$) in the moisture or protein content between samples of the same group

115.3–165.8 g AA kg^{-1} protein for arginine including taurine, lysine, leucine and glutamate, respectively). The protein content of all the fish species was characterised by high lysine levels (88.0–109.4 g AA kg^{-1} protein in *G. poutassou* and *S. pilchardus* in winter, respectively; Table 7). The principal non-essential amino acid in all the fish species was glutamate (124.2–166.4 g AA kg^{-1} protein). In the sunflower, pea and fish meals, the main essential amino acid was arginine including taurine, (78.6, 92.2 and 78.0 g AA kg^{-1} protein, respectively) and in the soy, wheat and krill meals leucine (72.91, 64.2 and 83.2 g AA kg^{-1} protein, respectively; Table 8). The main non-essential amino acid in meals was glutamate (113.6–285.3 g AA kg^{-1} protein).

Protein quality evaluation

L. gahi was deficient in histidine, threonine and phenylalanine (AAR: 84, 93 and 95 %, respectively). *T. sagittatus* and one sample of *M. galloprovincialis* were deficient in arginine compared with the *O. vulgaris* protein (AAR between 63 and 84 %, respectively; Fig. 1a). Furthermore, most of the samples of *M. galloprovincialis* had low levels of isoleucine (AAR: 84–102 %) and leucine (ARR: 78–99 %). None of the crustacean samples reached the arginine levels observed in *O. vulgaris* (AAR of between 65 and 91 % in *P. clarkii* and *C. maenas*, respectively; Fig. 1b). In general, the rest of the essential

Table 5 Moisture (g kg^{-1} fresh weight) and crude protein in fish and meals (g kg^{-1} dry weight)

Id.	Group/Species	Moisture	Crude protein
Fish			
20	*B. boops*	802.6 ± 5.4^{a}	922.1 ± 33.6^{a}
21	*B. boops*	627.9 ± 2.7^{j}	501.4 ± 5.4 ^{h}
22	*B. boops*	759.0 ± 2.0^{de}	854.7 ± 2.0^{bc}
23	*B. boops*	526.0 ± 3.0 ^{k}	356.3 ± 2.2i
24	*G. poutassou*	775.4 ± 1.6^{bcd}	919.0 ± 17.3^{a}
25	*G. poutassou*	736.0 ± 5.3^{f}	727.8 ± 4.0^{ef}
26	*G. poutassou*	720.5 ± 2.4 ^{g}	804.4 ± 13.7 ^{cd}
27	*G. poutassou*	763.4 ± 2.3 ^{cde}	811.3 ± 1.3^{cd}
28	*Mugil* sp.	761.7 ± 4.3^{cde}	759.1 ± 2.0^{de}
29	*Mugil* sp.	755.6 ± 4.0^{e}	735.3 ± 3.1^{ef}
30	*S. pilchardus*	657.7 ± 7.9^{i}	567.2 ± 18.5 ^{g}
31	*S. pilchardus*	777.3 ± 6.9^{bc}	877.9 ± 30.0^{ab}
32	*S. aurata*	681.3 ± 3.0 ^{h}	520.6 ± 1.6^{hg}
33	*T. trachurus*	709.6 ± 8.4 ^{g}	677.8 ± 28.1^{f}
34	*T. trachurus*	762.4 ± 4.1^{de}	732.4 ± 2.4^{e}
35	*G. minutus*	775.0 ± 5.7^{bcde}	871.0 ± 4.0^{ab}
36	*G. minutus*	787.0 ± 7.2^{ab}	884.6 ± 2.5^{ab}
Meals			
37	Sunflower	93.0 ± 1.5^{b}	344.5 ± 10.0^{c}
38	Pea	89.0 ± 2.2^{b}	785.1 ± 4.0^{a}
39	Soybean	100.0 ± 2.3^{a}	533.5 ± 9.1^{b}
40	Wheat	103.0 ± 0.9^{a}	124.7 ± 4.4^{d}
41	Krill	63.3 ± 4.3^{c}	559.3 ± 2.9^{b}
42	Fish	89.1 ± 2.2^{b}	748.9 ± 33.1^{a}

Different superscripts indicate significant differences ($P < 0.05$) in the moisture and protein content between samples of the same group

amino acid levels was covered, with the exception of lysine in *Penaeus* sp. (AAR 87 %) and slight deficiency in leucine, isoleucine and methionine (AAR > 90 %) in some samples of *C. maenas*. In fish, there was a good amino acid profile with the exception of arginine (AAR from 41 % in *B. boops* to 69 % in *G. minutus*; Fig. 2). Similarly, all the meals were deficient in arginine (AAR from 29 % in wheat meal to 63 % in pea meal) but exceeded histidine, phenylalanine and valine level (Fig. 3). The animal meals covered the rest of the amino acids, except lysine in the fish meal (AAR 90 %). The vegetal meals showed an even worse balance, with a lysine deficiency in sunflower, soy and wheat meals, and methionine deficiency in all meals except sunflower, and threonine deficiency in all of them without exception.

Therefore, the limiting amino acid in most of the samples was arginine, except in *L. gahi* where it was histidine (Fig. 4a), two samples of *M. galloprovincialis* where it was leucine (Fig. 4a), one sample of *C. maenas* (methionine; Fig. 4b) and sunflower meal (lysine; Fig. 4d). The Chemical Score pointed to a gradient with the lowest values in fish (41–70 %) and meals (29–64 %), intermediate levels in crustaceans (65–91 %) and the highest levels in molluscs (84–98 %; Fig. 4). According to the Oser's Index, the best balanced protein as regards essential amino acids would be *M. galloprovincialis* (88–99 %; Fig. 4a), squid *L. gahi* (96 %; Fig. 4a) and all the crustacean samples (95–97 %; Fig. 4b).

Table 6 Amino acid content expressed as grams of AA $kg^{-1}$of protein in molluscs and crustaceans

*Id.	Molluscs													Crustaceans					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Essential amino acids (g AA kg^{-1} protein)																			
Arg^{T}	148.6	138.9	118.1	184.0	156.5	231.0	189.0	230.9	201.6	145.0	147.6	223.6	89.0	128.2	108.1	112.0	111.7	113.8	91.4
His	16.0	21.0	17.5	19.4	15.8	21.3	17.1	18.0	17.7	24.5	21.9	21.0	19.5	23.8	27.6	26.8	24.7	30.9	21.8
Ile	36.0	37.8	35.6	34.9	31.2	36.5	37.5	33.2	36.7	44.0	42.2	37.3	43.0	34.7	39.2	34.7	36.9	40.2	42.1
Leu	65.4	63.5	57.6	55.3	50.5	63.8	64.3	60.0	64.1	69.2	64.1	61.3	76.6	59.9	64.9	58.0	60.6	66.1	75.0
Lys	81.0	74.9	87.6	64.9	73.0	51.7	72.5	54.6	66.9	65.2	72.7	74.3	73.5	59.6	78.4	63.3	67.9	54.4	81.6
Met	24.7	19.5	16.3	15.1	17.3	15.3	18.2	15.4	16.9	18.7	18.0	16.5	20.4	15.3	25.9	16.6	51.0	19.2	20.0
Phe	30.0	33.8	29.3	51.3	26.5	36.4	26.5	30.2	27.4	41.0	36.8	31.5	30.9	36.4	35.8	37.0	34.1	46.3	37.4
Thr	38.4	53.3	45.2	53.0	33.9	44.9	37.6	41.3	40.2	52.2	44.2	42.0	44.8	44.7	42.3	43.5	44.2	42.6	42.6
Val	35.9	43.5	39.3	38.8	31.0	35.2	36.4	32.8	35.9	43.2	42.9	39.5	41.0	38.9	42.0	40.2	42.8	47.5	45.2
Non-essential amino acids (g AA kg^{-1} protein)																			
Ala	50.1	50.1	51.6	47.6	44.0	43.6	51.0	44.0	49.2	44.1	45.3	42.1	63.5	48.3	41.6	54.9	53.7	53.9	68.2
Asp	106.1	81.6	103.5	87.5	89.9	75.8	102.4	76.6	101.0	84.3	102.9	92.1	102.4	82.5	99.4	86.7	97.9	90.4	113.4
Cys	22.2	42.0	21.3	28.2	23.2	18.5	21.4	17.6	20.2	39.5	44.8	30.8	19.0	21.5	23.5	23.3	24.0	26.2	20.5
Gly	48.2	78.7	76.8	93.3	79.2	69.6	62.7	57.6	70.1	52.0	45.3	43.6	86.5	71.5	55.1	54.2	62.1	97.3	56.4
Glu	143.4	104.6	125.4	109.2	118.3	110.0	142.4	110.5	140.0	112.0	120.0	117.5	145.0	115.3	146.0	114.9	125.2	115.7	165.8
Pro	51.5	36.9	37.9	32.6	35.9	37.7	35.8	35.4	37.1	32.5	32.5	33.8	50.7	38.6	55.9	47.9	51.8	43.3	35.6
Ser	41.2	55.5	59.5	47.0	44.4	44.7	46.2	40.7	47.7	48.1	49.0	49.9	44.3	40.7	45.3	38.3	40.9	45.4	43.2
Tyr	24.1	35.0	26.6	25.3	25.9	34.2	23.9	32.1	24.8	32.7	31.1	29.4	25.9	34.3	32.6	35.9	29.6	34.9	29.5
Tau	n.a.	n.a.	n.a.	n.a.	n.a.	74.3	64.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	64.0	n.a.	n.a.

* Id.: 1-*L. gahi*; 2, 3, 4, 5-*M. galloprovincialis*; 6, 7-*O. vulgaris* (whole); 8, 9-*O. vulgaris* (muscle); 10, 11-*O. vulgaris* (digestive gland); 12-*O. vulgaris* (gonad); 13-*T. sagittatus*; 14, 15, 16, 17-*C. maenas*; 18-*Penaeus* sp.; 19-*P. clarkii*; [T] Including taurine; n.a. = not analysed

Table 7 Amino acid content expressed as grams of AA kg^{-1} of protein in fishes

*Id.	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Essential amino acids (g AA kg^{-1} protein)																	
Arg^{T}	83.6	57.7	69.2	66.2	70.3	71.4	73.6	65.8	74.8	79.3	79.6	76.8	78.8	79.4	73.5	81.0	97.7
His	31.5	51.4	28.6	53.6	19.2	17.5	22.8	18.3	23.6	31.5	52.4	36.8	26.1	32.4	28.6	19.6	22.9
Ile	44.1	40.8	42.3	42.0	42.9	38.4	43.4	42.5	44.8	44.1	41.4	44.7	41.8	40.7	39.5	47.8	41.5
Leu	80.3	75.0	77.3	76.9	81.9	71.5	80.0	76.6	79.5	78.2	74.9	79.3	75.7	73.9	69.4	83.4	75.9
Lys	100.1	100.1	103.7	103.3	95.7	93.5	88.0	100.6	94.9	91.3	78.0	109.4	104.3	90.5	89.2	94.5	85.5
Met	28.0	25.6	28.6	27.1	27.9	18.2	26.8	28.5	26.5	28.8	28.3	30.5	29.9	26.8	25.6	27.4	28.7
Phe	33.9	31.6	34.3	35.4	37.8	33.0	42.9	35.6	32.9	38.9	45.7	35.4	34.6	35.8	35.4	37.2	46.7
Thr	47.7	41.1	46.5	44.2	45.0	41.9	47.9	37.9	45.2	48.9	47.6	45.5	45.6	44.7	38.0	45.9	47.5
Val	50.2	48.1	47.7	47.9	49.4	44.3	47.9	42.7	52.5	49.2	48.8	51.8	48.7	46.3	40.4	53.8	46.7
Non-essential amino acids (g AA $kg^{1}-$ protein)																	
Ala	62.7	60.6	63.6	61.7	65.5	70.4	60.1	60.7	66.8	59.4	56.6	65.6	63.8	60.3	55.5	67.9	60.1
Asp	115.0	112.0	112.7	110.8	107.4	112.4	97.4	110.2	108.3	93.2	85.8	119.9	112.9	99.9	91.9	101.8	93.0
Cys	28.2	26.7	21.3	17.6	22.7	12.7	20.7	20.8	21.4	24.0	21.4	20.0	23.7	18.6	16.7	20.5	23.3
Gly	51.3	45.7	48.0	47.6	50.4	71.9	44.0	56.3	51.3	55.3	56.2	51.8	57.4	56.5	61.4	45.2	50.0
Glu	166.2	153.9	161.9	159.0	152.0	161.8	141.7	166.4	150.0	134.0	124.2	166.1	159.6	142.7	138.1	147.1	142.3
Pro	34.2	29.6	30.5	29.1	30.2	38.6	33.8	38.8	32.9	33.0	33.3	35.1	33.4	33.6	32.3	30.5	25.9
Ser	48.0	40.4	42.5	43.8	42.9	48.0	42.6	42.4	41.0	43.8	42.3	45.5	44.8	39.5	39.6	40.4	43.5
Tyr	29.3	26.8	30.4	30.2	30.3	21.1	42.6	26.1	25.3	35.2	35.9	28.7	29.8	26.5	23.7	29.5	38.6
Tau	23.8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

* Id.: 20, 21, 22, 23-*B.boops*; 24, 25, 26, 27-*G. poutassou*; 28, 29-*Mugil* sp.; 30, 31-*S. pilchardus*; 32-*S. aurata*; 33, 34-*T. trachurus*; 35, 36-*G. minutus*. [T] Including taurine; n.a. = not analysed

Table 8 Amino acid content expressed as grams of AA kg^{-1} of protein in plant and animal meals

Id.	Plant meals				Animal meals	
	37 (Sunflower)	38 (Pea)	39 (Soybean)	40 (Wheat)	41 (Krill)	42 (Fish)
Essential amino acids (g AA kg^{-1} protein)						
Arg[T]	78.6	92.2	68.5	42.3	73.9	78.0
His	27.9	29.5	26.0	23.8	22.4	33.6
Ile	43.3	47.8	46.2	35.4	56.9	44.7
Leu	62.0	87.4	72.9	64.2	83.2	71.5
Lys	27.5	68.2	58.1	30.5	67.3	56.0
Met	18.4	10.7	9.6	11.9	29.6	25.8
Phe	59.3	59.3	54.8	49.3	53.0	62.2
Thr	37.2	34.2	37.2	30.2	48.9	45.4
Val	49.2	50.3	45.3	43.6	55.7	48.8
Non-essential amino acids (g AA kg^{-1} protein)						
Ala	40.5	40.8	40.6	37.5	58.0	56.2
Asp	81.8	117.7	115.3	52.7	105.5	79.9
Cys	41.7	24.7	20.2	37.0	6.7	17.7
Gly	65.2	27.4	42.5	44.5	48.7	67.7
Glu	177.9	163.3	181.8	285.3	131.7	113.6
Pro	84.6	42.5	71.1	107.3	40.3	89.1
Ser	44.2	51.8	50.5	46.8	43.3	37.7
Tyr	24.9	20.5	30.1	13.1	47.8	45.5

[T] Including taurine

The values in fish ranged from a minimum of 90 % in *B. boops* from bycatch of fish farms and a maximum of 96 % in *G. minutus* (Fig. 4c). The animal meals showed similar values to fish (92 %), the worst results being observed in vegetable meals (74–89 %; Fig. 4d).

Relationship between amino acids composition and group of samples based on PCA

The three selected principal components (PC) explained 64.8 % of the total variance. The first PC explained 31.5 % of the variance and was related mainly to the leucine, lysine and alanine content with high positive factor loadings (Table 9). The second PC explained 20.5 % of the variance and was positively related to the glutamate content and negatively related to the arginine content. The third PC explained 12.7 % of the variance and was positively related to the tyrosine content. Other PCs were not included and explained percentage of variance less than 8.3 % with eigenvalues less than 1.5. Figure 5 shows a clear separation between the different groups of samples according to the first two principal components. Bivalves and cephalopods are separated from fish and crustaceans based mainly on the first PC, with factor scores negatively related in the first two groups of samples and positively in the others. Furthermore, cephalopods and bivalves showed the lowest scores for the second PC. Between crustacean samples, Penaeus heads are separated based on their low scores for the first PC. Plant meals are separated from the remaining samples based on their low value for the first PC and high values for the second PC, and especially in the case of wheat flour. Similarly, fish and krill meals are separated based on their high scores for the second PC and also the krill meal based on the first PC.

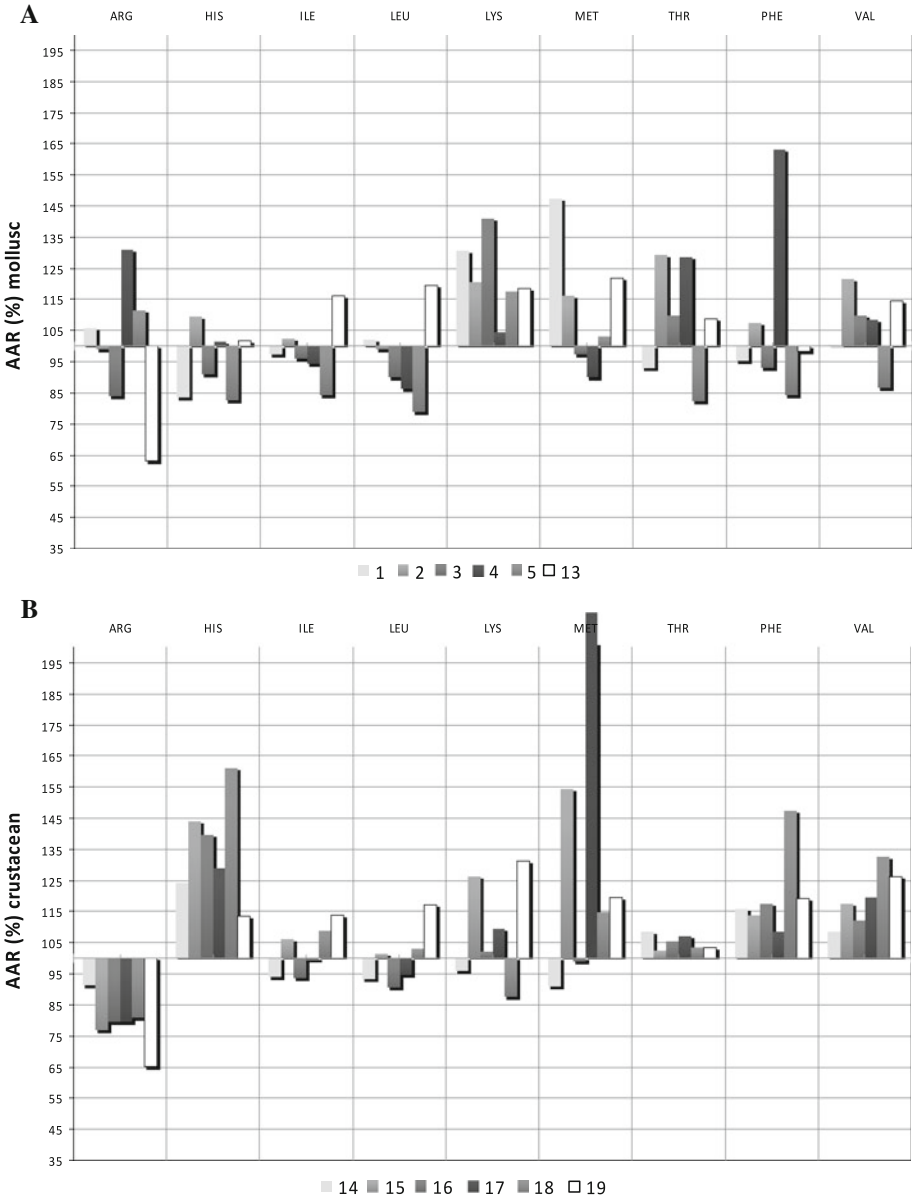

Fig. 1 Amino acid ratios (%) for essential amino acids in molluscs (**a** (*1*)—*L. gahi*; (*2*), (*3*), (*4*), (*5*)—*M. galloprovincialis*; (*13*)—*T. sagittatus*) and crustaceans (**b** (*14*), (*15*), (*16*), (*17*)—*C. maenas*; (*18*)—*Penaeus* sp.; (*19*)—*P. clarkii*)

Discussion

The most obvious difference between the proximal composition of cephalopods and other marine organisms is the high protein content and low lipid and mineral content of the former (Lee 1994; Rosa et al. 2005; Ozogul et al. 2008; Cerezo Valverde et al. 2012a).

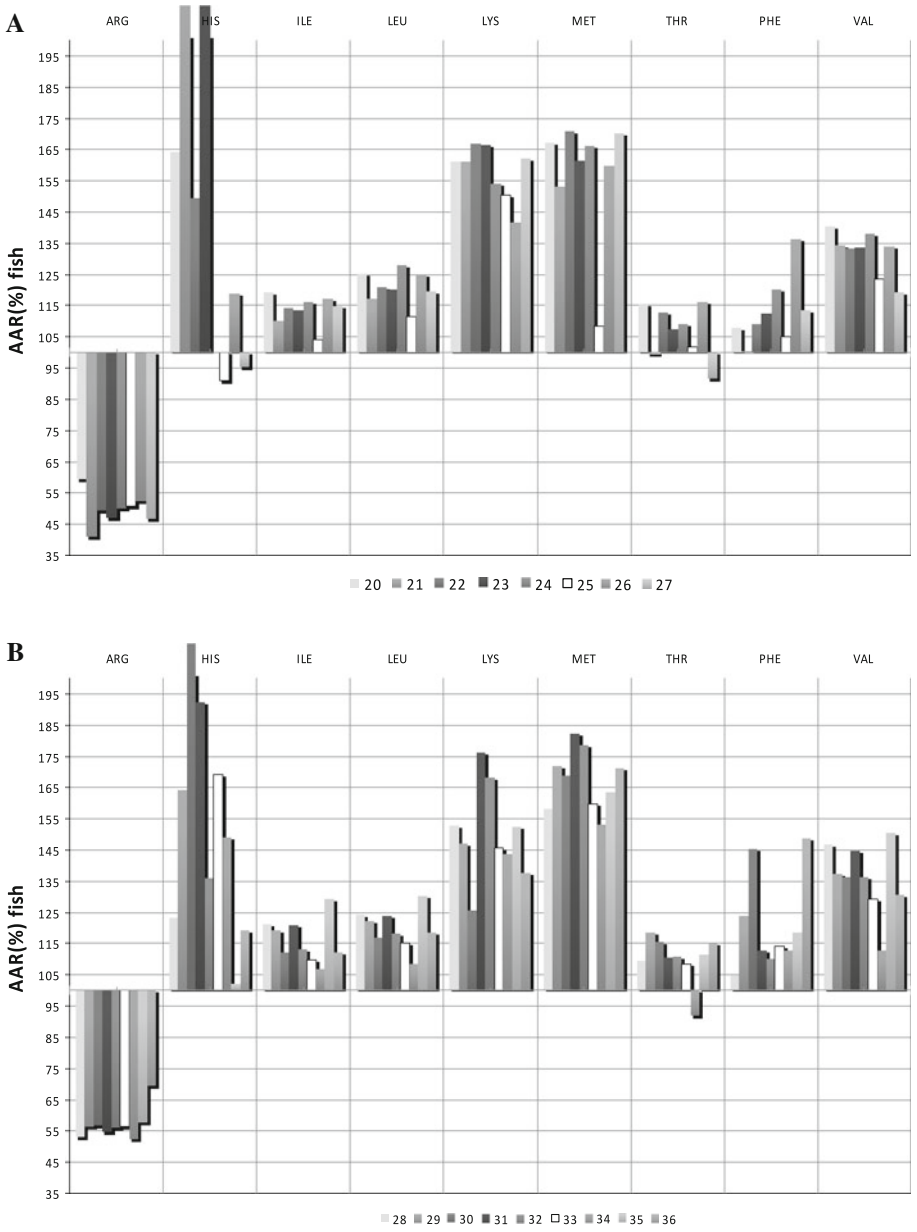

Fig. 2 Amino acid ratios (%) for essential amino acids in fish (**a** (*20*), (*21*), (*22*), (*23*)—*B.boops*; (*24*), (*25*), (*26*), (*27*)—*G. poutassou*. **b** (*28*), (*29*)—*Mugil* sp.; (*30*), (*31*)—*S. pilchardus*; (*32*)—*S. aurata*; (*33*), (*34*)—*T. trachurus*; (*35*), (*36*)—*G. minutus*)

In the present study, protein levels reached between 800 and 810 g kg^{-1} dry weight in *O. vulgaris* and *L. gahi*. In other species, such as *Sepia officinalis* and *Loligo vulgaris*, these levels exceeded 820 g kg^{-1} (Zlatanos et al. 2006). Generally speaking, all the samples of bivalve molluscs (648–653 g kg^{-1}), crustaceans (543–695 g kg^{-1}), fish or krill meals

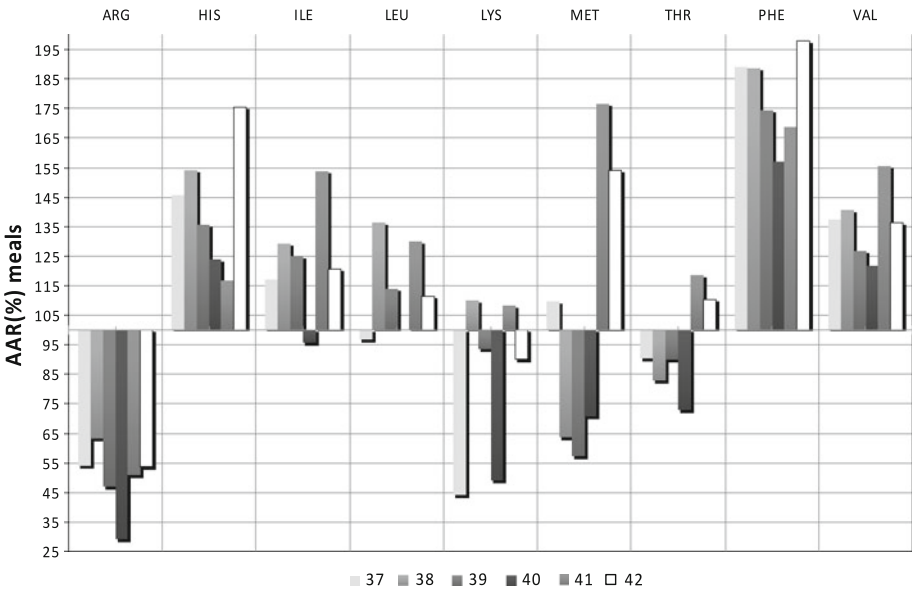

Fig. 3 Amino acid ratios (%) for essential amino acids in vegetable and animal meals ((*37*)—Sunflower; (*38*)—Pea; (*39*)—Soybean; (*40*)—Wheat; (*41*)—Krill; (*42*)—Fish)

(559–749 g kg^{-1}) and plant meals (124–785 g kg^{-1}) had lower protein content, with a few notable seasonal exceptions for fish samples. *B. boops*, *G. poutassou*, *S. pilchardus* and *G. minutus* had protein contents ranging between 850 and 930 g kg^{-1}. These extremely high levels can be explained by their low fat content or the assay coinciding with the season of the year when such deposits were at their lowest. This phenomenon has been described in many species and is explained by the mobilisation of energy reserves during the time of least food availability and their accumulation if present in high amounts (Bandarra et al. 1997; Luzia et al. 2003; Pazos et al. 2003). Similarly, the high lipid content in species from the bycatch of fish farms (e.g. *B. boops* or *S. aurata*; Cerezo Valverde et al. 2012a) would have led to the very low protein content (350–520 g kg^{-1}), even in winter.

The results of this study also underline the marked genetic character of the amino acid composition of the samples, great similarity being observed within the same taxonomic group. Based on the results from the PCA, there was a great similarity between amino acid composition of cephalopods and bivalves, both separated from the remaining samples from the first and second PCs. The first PC would suggest low contents of leucine, lysine and alanine, and in the second PC high content of arginine but low of glutamate in relation to other samples. As in the results obtained by Villanueva et al. (2004) and Rosa et al. (2004), the predominant amino acids in cephalopods were, in order, arginine, lysine and leucine (essential) and glutamic and aspartic acids (non-essential). A similar pattern is qualitatively preserved in crustaceans and may be one reason for the excellent growth recorded in the cephalopods when they are fed solely a crustacean-based diet (Cagnetta and Sublimi 2000; Aguado Giménez and García García 2002) or mixed diets containing crustaceans and fish or bivalves (García García and Cerezo Valverde 2006; Rodríguez et al. 2006; Biandolino et al. 2010; Prato et al. 2010). Fish and crustaceans would be characterised by higher content of leucine, lysine and alanine according to PCA. Furthermore, crustaceans showed

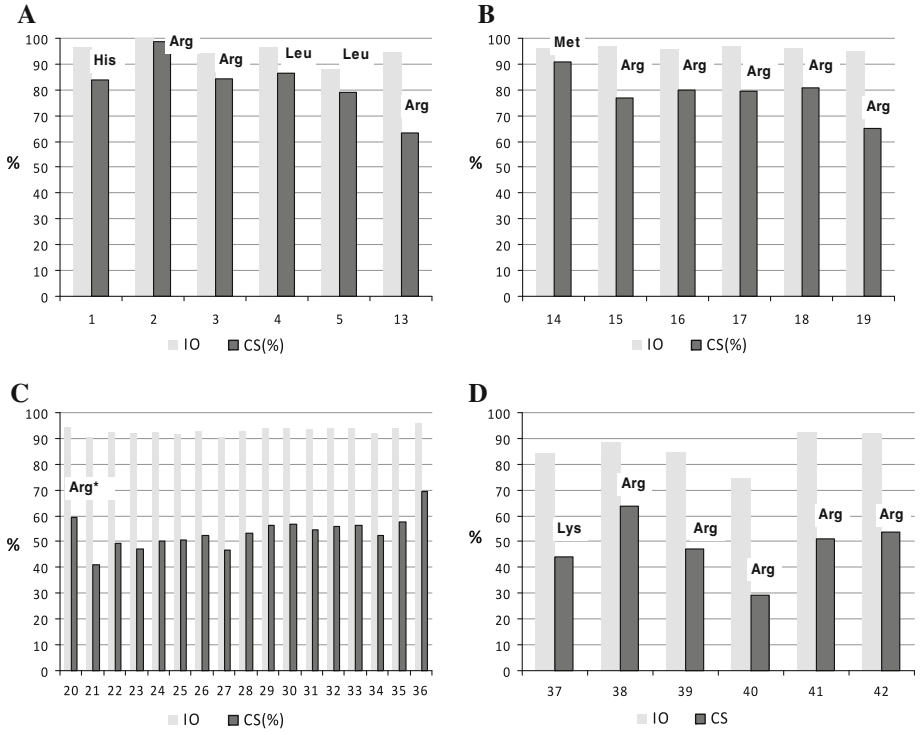

Fig. 4 Oser's Index (OI), Chemical Score (CS) and limiting amino acid in mollusc (**a**: (*1*)—*L. gahi*; (*2*), (*3*), (*4*), (*5*)—*M. galloprovincialis*; (*13*)—*T. sagittatus*), crustacean (**b**: (*14*), (*15*), (*16*), (*17*)—*C. maenas*; (*18*)—*Penaeus* sp.; (*19*)—*P. clarkii*), fish (**c** (*20*), (*21*), (*22*), (*23*)—*B.boops*; (*24*), (*25*), (*26*), (*27*)—*G. poutassou*. B: (*28*), (*29*)—*Mugil* sp.; (*30*), (*31*)—*S. pilchardus*; (*32*)—*S. aurata*; (*33*), (*34*)—*T. trachurus*; (*35*), (*36*)—*G. minutus*) and meal (**d** (*37*)—Sunflower; (*38*)—Pea; (*39*)—Soybean; (*40*)—Wheat; (*41*)—Krill; (*42*)—Fish) samples. * Arginine is the limiting amino acid for all fish samples

higher arginine content and better Chemical Score than fish. However, the high growth rates obtained with crustaceans are accompanied by high rates of ingestion and low feed efficiency and protein retention compared with the mixed or monodiets that include fish (García García and Cerezo Valverde 2006; Prato et al. 2010). In most of the fish species, the predominant essential amino acids were lysine and leucine, with low levels of CS (41–70 %) compared with crustaceans (65–91 %), suggesting that the amino acid profile would not explain the greater protein retention observed in fish-based diets. Since the Chemical Score is indicative of the maximum percentage of protein that may be retained for growth, these results coincide with the hypothesis proposed by García García and Cerezo Valverde (2006) concerning the existence of a nutritional factor present in fish but absent from crustaceans that would lead to better protein use. In this sense, cephalopods are exclusively carnivorous species (Guerra and Nixon 1987) and rarely use carbohydrates or lipids as energy source (O'Dor et al. 1984; Lee 1994). The latter, in particular, are poorly assimilated by cephalopods in general (Sánchez et al. 2009; Seiça Neves et al. 2010). However, several recent studies have demonstrated efficient lipid dietary utilisation (Estefanell et al. 2011) and a significant contribution of lipids and carbohydrates to the energy metabolism in octopus (García-Garrido et al. 2010; Morillo-Velarde et al. 2011). The low fat content detected in crustaceans compared with fish would explain the greater

Table 9 Results obtained for principal component analysis taking into account the biochemical variables analysed

	PC1	PC2	PC3
Eigenvalues	5.68	3.69	2.29
Per cent total variance	31.55	20.5	12.75
Crude protein	0.50	−0.51	−0.10
Arg	−0.54	−0.67	0.11
His	0.33	0.33	0.21
Ile	0.70	0.46	0.26
Leu	0.85	0.36	−0.05
Lys	0.78	−0.34	−0.40
Met	0.60	−0.15	0.14
Phe	−0.11	0.65	0.57
Thr	0.43	−0.47	0.55
Val	0.72	0.53	0.26
Glu	−0.04	0.77	−0.52
Ala	0.82	−0.25	−0.11
Asp	0.70	−0.10	−0.44
Cys	−0.51	0.25	−0.02
Gly	−0.48	−0.55	0.25
Pro	−0.55	0.66	0.15
Ser	−0.29	−0.02	−0.23
Tyr	0.34	−0.18	0.82

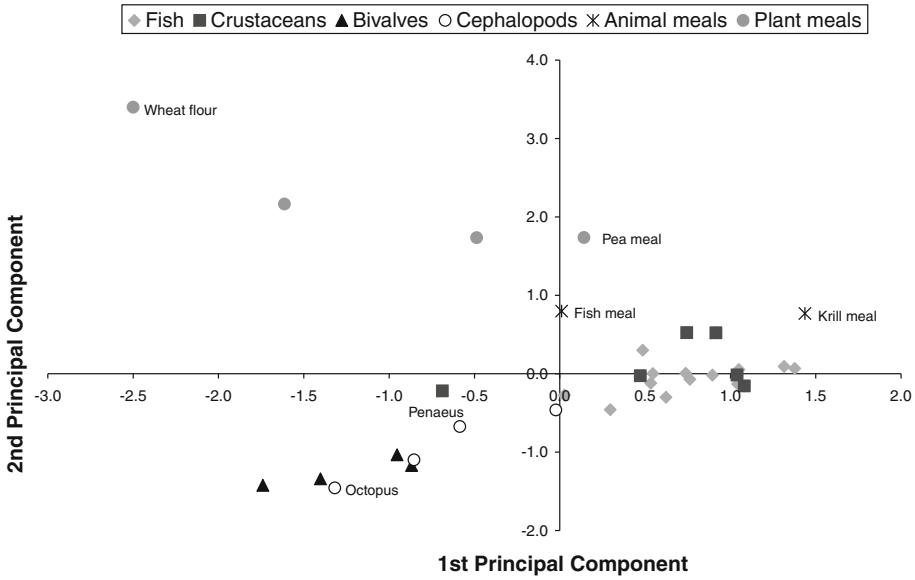

Fig. 5 Separation of the different group of samples on the basis of the first two principal components according to their biochemical composition

use of protein for energetic ends and lower retention of the same. Moreover, the amino acid profile of fish characterised by the marked deficiency in arginine, together with the lower ingestion rates compared with crustaceans may explain the lower growth obtained with a fish-based monodiets.

Therefore, the results of the present study suggest that the incorporation of fish protein in feeds destined for cephalopods should be supplemented with arginine to improve yields. When using primary materials from crustaceans, two strategies might be followed: (a) a moderate increase in lipids accompanied by supplementation with low levels of leucine, isoleucine and methionine, and a higher level of arginine; or (b) complement proteins from crustaceans with proteins derived from fish—high leucine, isoleucine, lysine and methionine levels, but with moderate levels of fat—incorporating both sources in the same feed and supplemented with arginine. In our case, the use of *B. boops* from artisanal fisheries may be the best approach because of its high arginine levels, better overall amino acid balance, moderate fat content (Cerezo Valverde et al. 2012a) and low market price (García García and García García 2011). Besides, bogue has a lower commercial value with a minimum demand for consumption.

Several researchers have attempted to add hydrolysed proteins or amino acids in crystalline form in artificial diets for cephalopods or added crystalline amino acids in culture water during the early stages of larval development. In the first case, the feeds resulted in low or negative growth rates in *S. officinalis* (Castro and Lee 1994; Domingues et al. 2005) and *O. maya* (Aguila et al. 2007) and moderate rates in *O. vulgaris* (Cerezo Valverde et al. 2012b), although this was largely due to the low degree of acceptability of the diets. Cerezo Valverde et al. (2012b) observed that if the feed is cohesive and stable in water and it is accepted by the octopus, the beneficial effects of supplementation with pure amino acids are evident and could be an effective tool to slow feeding animals. Domingues et al. (2005) also obtained best results with a diet representing the highest degree of amino acid supplementation in *Sepia officinalis*. Villanueva et al. (2004) observed the beneficial effect of adding a water solution of amino acids on octopus paralarval survival, although this did not translate into higher growth. Therefore, the role of dissolved amino acids in culture water remains uncertain.

In fish, the best growth and nitrogen retention results were obtained by complementing a deficient protein source by adding the limiting amino acid in the form of another protein source that contains it (Ketola 1982). The worst results obtained with diets supplemented with crystalline amino acids were attributed to their rapid absorption since optimal protein synthesis requires availability of all amino acids in the tissues simultaneously and in sufficient quantities (Schuhmacher et al. 1997). In the case of fish diet formulation, a combination of different raw materials is a good solution to alleviate the amino acid deficiencies of several protein sources (Kaushik et al. 2004; Sánchez-Lozano et al. 2009). However, all analysed fish samples in the present study were deficient in arginine, meaning that dietary mixtures do not fully meet requirements for *O. vulgaris*. Furthermore, the problem of samples deficient in arginine is more pronounced than what is stated in this paper. Arginine ratios remained extremely low in fish, crustacean and meal samples despite the inclusion of taurine in the value of the arginine and taurine deduction in the whole octopus samples. According to our results and other authors, taurine is particularly high in the tissues of molluscs and crustaceans (Robertson et al. 1992; D'Aniello et al. 1995; Babarro and Fernández Reiriz 2006). Taurine values for *O. vulgaris* in the present study (64–75 AA kg^{-1} protein) were similar to those detected for *O. maya* (65–80 g AA kg^{-1} protein according to George-Zamora et al. 2011).

According to our results, *L. gahi* and *M. galloprovincialis* samples would better cover arginine requirements of octopus and displayed a balanced essential amino acids

composition. However, in this respect, it should be noted that the ideal nutritional composition of a diet does not necessarily imply greater acceptability. Indeed, ongrowing experiments with diets containing *M. galloprovincialis* were associated with low ingestion and growth rates (López et al. 2009; Biandolino et al. 2010; Prato et al. 2010). There is a clear need for preliminary experiments with materials that improve acceptability before any new material is incorporated in diet formulation.

All meals, both animal and plant, are separated from the rest of the samples according to the PCA analysis. Vegetable meals would be characterised by low contents of leucine, lysine and alanine based on the first PC and high values of glutamate and low of arginine based on the second PC. Krill and fish meal noted for their separation in the second component (low content of arginine and high of glutamate) and also the krill meal by the high levels of leucine, lysine and alanine according to the first PC. By far, the worst balanced proteins were those contained in the vegetal meals. Both the wheat and soy meals should be supplemented with proteins rich in lysine, threonine, methionine and arginine. Similarly, while the pea meal should be supplemented with threonine, methionine and arginine, the sunflower meal should be supplemented with arginine and lysine. Of the plant meals analysed, pea is the most suitable given its high protein content and best CS (63 %) and OI (88 %). Both the fish and krill meals offer similar benefits as the different species of fish taking into account the amino acid composition, CS or OI values. The fish meal has the advantage of a higher protein content and CS than krill, although it has the disadvantage of needing lysine supplementation.

In conclusion, the suitability of molluscs and crustaceans for developing cephalopod meals is evident, although their protein quality indices could be improved with arginine or leucine supplementation and the joint use of protein from fish or krill meals. None of the vegetable meals assayed could on their own offers a good nutritional balance and would need supplementation or would have to be used alongside other raw materials. In the present study, the amino acid quality of feed for octopus was tested by amino acid ratios alone; however, in future studies, the digested essential amino acid estimation for each raw material should be taken into account for octopus diets formulation.

Acknowledgments Project financed by the National Marine Culture Plans of JACUMAR.

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