0014-4754/95/030278-06$1.50 + 0.20

The compositional analysis of recent cephalopod shell carbohydrates by Fourier transform infrared spectrometry and high performance anion exchange - pulsed amperometric detection

Y. Dauphin and F. Marin

Laboratoire de Paléontologie, Bât. 504, URA 723 du CNRS, Université Paris XI-Orsay, F-91405 Orsay Cedex, (France)
Received 26 May 1994; accepted 3 October 1994

Abstract. The organic matrix of four living cephalopod skeletons was extracted, purified, separated in a soluble and an insoluble fraction. These fractions were submitted to infrared analysis. After a light hydrolysis, their monosaccharide content was determined by HPAE-PAD. The results were treated by principal component analysis.

Infrared analysis has been used to estimate the protein/sugar ratios and showed a high amount of chitin within the insoluble fractions. This was corroborated by their high content of glucosamine. The composition of soluble matrices appeared more heterogeneous: the general tendency is an increase of glucosamine from the most mineralized shell (*Nautilus*) to the non-mineralized one (*Loligo*), and a decrease in glucose and galactose. These data would be in agreement with the evolutionary tendency towards shell reduction and disappearance among cephalopods.

Key words. Cephalopods; infrared; chitin; monosaccharides; principal component analysis.

Biologically-formed crystals have sizes and shapes quite different from those found in their nonbiological counterparts. These properties indicate that the crystals form under well-controlled conditions. The regulation of crystal growth is partly by means of proteins. The proteins found in the mollusc shells make up the major part of the organic matrix or conchiolin. Chemical analyses and microstructural observations have shown that the protein part of the conchiolin consists mainly of glyco- or scleroproteins. In vitro experiments have shown that the serine-rich protein-polysaccharide complexes had no noticeable effect on the growing crystals, 'but presumably fulfill an important function in vivo'[1].

All living cephalopods, with the exception of the genus *Nautilus*, belong to the Coleoidea (or Dibranchiata) and have internal shells. Among them, *Spirula* and *Sepia* have mineralized shells. The small number of mineralized shells on the one hand, and the taxonomic differences on the other hand, provide a good sample group with which to test the role of sugars in calcification processes.

The organic matrix of *Nautilus* is composed of a water soluble part (soluble nacrine), insoluble nacrine, and nacroine. Nacroine is a glycoprotein composed of chitin and a polypeptide with high glycin and alanin contents[2,3]. The organic matrix of the nacreous layer is composed of 3.4% chitin in *N. pompilius*, and 2.9 to 6.3% in *N. macromphalus*. The reduced shell of Coleoid Cephalopoda has been shown to contain chitin[4]. The septal organic matrix of *Sepia* contains 25.8% of chitin, and *Loligo* 17.9%[2,3]. However the chitin content of cephalopod shells appears to be variable according to the literature: 17.9 to 41.8% in *Loligo*, 3.0 to 35.1% in *Nautilus*. Such a variability may be due to different analytical methods. The chitin/protein ratios for *Sepia* and *Spirula* shells are similar to those of *Loligo*, but their protein contents are different. *Nautilus* has less chitin than the three other genera[5]. The insoluble matrix of shell and septa of *Nautilus belauensis* is composed of 75% protein and β-chitin[6]. The organic gladius of *Loligo* is not pure chitin (β-chitin[7]), but a protein-chitin complex. Polysaccharides of recent cephalopod shells are composed of a dilute acetic acid-soluble matrix. Galactosamine or mannosamine are present, and glucosamine and another element are present in chitin. Chitin has been characterized by infrared spectra[8,9]. The glucidic content in *Sepia* is 12.1% for horny layers, and 7.4% for the ventral part of the shell. The glucosamine content is 16% in horny layers, 6.4% in the ventral part[10].

Characterization of the polysaccharide content of the shell of squid (*Sepia* or *Loligo*?) was performed by Okutani and Morikawa[11,12]. Polysaccharides of different molecular weights have been isolated. Monosaccharides are: L-arabinose, L-rhamnose, D-xylose, D-mannose, D-galactose, D-glucose, D-glucosamine and D-galactosamine. Using another fractionation mode, these authors have identified 3 major polysaccharides. The first fraction gave two peaks: polysaccharide A (low molecular weight) and B (higher molecular

weight). Infrared spectra indicate the presence of β-linkages (890 cm^{-1}); a band at 1610 cm^{-1} suggests the presence of carboxylate in both components, while a band at 1625 cm^{-1} is present in polysaccharide A. Purified polysaccarides A and B have no amino sugars; they contain only glucose residues.

Material and methods

Material. Specimens of *Nautilus macromphalus* and *Sepia* sp. were collected on the beaches of New Caledonia. Internal shells of *Spirula spirula* were collected in the vicinity of Tulear (Malagasy), those of *Loligo* came from an unknown place.

Aragonitic shells of *Nautilus*, *Sepia* and *Spirula* are composed of three different structural layers: an outer prismatic layer, a nacreous layer and an inner prismatic layer[13–15]. The thickness of the layers varies in the genus: the nacreous layer is thick in *Nautilus*, but thin and weakly mineralized in *Spirula*. Because of the thinness of some layers, the whole shells (outer walls and septa) have been used in this study. The *Loligo* gladius is organic.

γ-Chitin extracted from crustacean shields (Calbiochem, La Jolla, USA, grade B) is not of extra-pure quality. Neutral and amino monosaccharides of the equimolar standard mixture are Sigma chemicals (St. Louis, USA).

Methods. The washed and air-dried shells were ground and decalcified in dilute acetic acid at 20 °C with the pH maintained at 4. After the calcium carbonate was dissolved, the resulting solution was centrifuged at 2000 *g* for 45 min to sediment the insoluble matrix. The insoluble residues remaining after decalcification were rinsed free of acid and lyophilized. After the supernatant containing the soluble matrix was filtered and concentrated by ultrafiltration (Amicon cell, 3 kD membrane), the filtrate was lyophilized.

FT-IR. All spectra were recorded at 4 cm^{-1} resolution with 64 scans on a Perkin-Elmer (Norwalk, USA) Model 1600 Fourier transform infrared spectrometer (FT-IR), from 4000 to 450 cm^{-1}. The spectrometer was equipped with a diffuse reflectance accessory which permits DRIFT measurements with high sensitivity on powders. All spectra were corrected by the Kubelka-Munk function. The system was purged and permanently maintained under nitrogen to reduce the atmospheric CO_2 and H_2O absorptions.

Lyophilized organic matrices and KBr were mixed. Thus, the organic matrices are not denatured by inclusion in KBr pellets[16]. Sample spectra were automatically ratioed against background to minimize CO_2 and H_2O absorptions. Correlation coefficients between two spectra of the same samples are about 99%.

Sugar determination. While monosaccharides such as glucose are usually considered as non-ionic, they act as weak acids at strongly basic pH (11.5 to 13.5) (for pKa values, see Dionex TN20[17,18]). Existing under an anionic form in this pH range, they can be separated by an ion exchange mechanism. The chromatographic separation was performed with HPAE-PAD system (High Performance Anion Exchange-Pulsed Amperometric Detection) developed by Dionex Corporation (Sunnyvale, USA). The whole apparatus consists in an Advanced Gradient Pump (DX-300 serie), a Pulsed Amperometric Detector (PAD), and an Eluent Degas Module (EDM2) allowing the solutions to be degassed and maintained under pressure. Peaks were integrated with a Shimadzu CR-3A integrator. Unknown peaks were not included in the quantification of the molar percentages. Neutral and amino sugars were separated on a 4 × 250 mm anion exchange CarboPac PA1 column, with a NaOH gradient (10 to 100 mM). The column was thermostated (19 °C) with a constant flush of water. The complete separation of monosaccharides occurs within 20 min. The elution order is the following: fucose, deoxyribose, rhamnose, arabinose, galactosamine, glucosamine, galactose, glucose, mannose and xylose (coelution) and ribose.

Lyophilized soluble matrices were rehydrated with deionized water and aliquotes were dried under vacuum. Hydrolysis of polysaccharides was performed with trifluoroacetric acid (TFA), 2 N, 105 °C, for 4 hours. Neutral sugars are easily released within a few hours (4–8 h), but amino-sugars (mainly glucosamine and galactosamine) need longer hydrolysis[19].

Owing to the chitin, lyophilized insoluble matrices require stronger hydrolytic conditions: HCl 2 N, 100 °C. Hydrolysis time is 4 h for the matrices of *Sepia*, *Spirula* and *Nautilus*; it was 16 h for *Loligo*. Soluble and insoluble matrices were dried under vacuum (Speedvac), then neutralized by adding 400 µl of a 20 mM NaOH solution, and filtered through a 0.45 µm filter before injection.

Results

Infrared data. (See fig. 1). Infrared studies have been made on crustacean chitin[20–22]. The crustacean chitin analysed by DRIFT shows the usual main features of KBr pellet method. 3265 cm^{-1} (amide A), 3100 cm^{-1} (amide B), 1656 cm^{-1} and 1636 cm^{-1} (amide I), 1552 cm^{-1} (amide II), 1200 to 1320 cm^{-1} (amide III) bands are present, and there is a general agreement that they indicate a proteic composition. Symmetric (1420 cm^{-1}) and asymmetric (1580–1600 cm^{-1}) carboxylate bands are absent in crustacean chitin. Three bands may be due to sugar groups, at 1070 cm^{-1} and 1155 cm^{-1} (fig. 1).

The soluble matrix of the phragmocone of *Sepia esculenta*[23] and the porous ventral layers of *Sepia pharaonis* have been analysed[10], but the data are scarce.

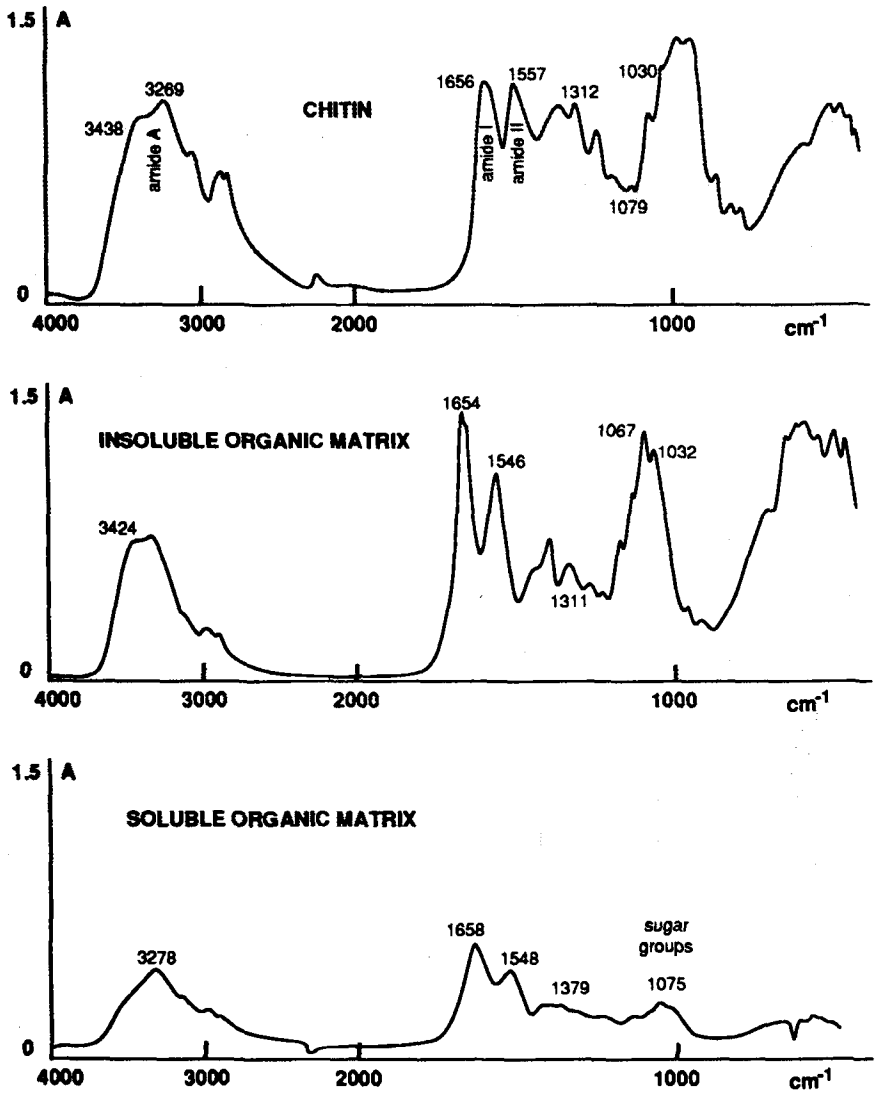

Figure 1. Infrared spectra (absorbance) of γ-chitin and of the organic matrices of *Spirula*. Sugars groups are present on the three spectra.

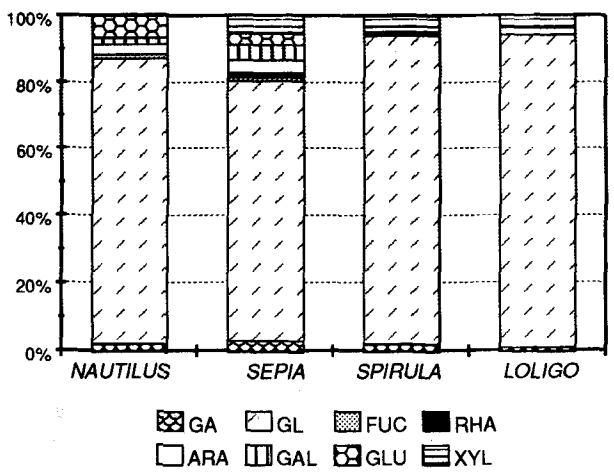

Figure 2. Cumulated bars of the sugar content of the insoluble matrices of cephalopod shells. GA, galactose; GL, glucose; FUC, fucose; RHA, rhamnose; ARA, arabinose; GAL, galactosamine; GLU, glucosamine; XYL, xylose.

The glyco-proteic composition of the insoluble matrix is clearly shown by infrared data. Amide A, amide I and amide II bands are present in all the specimens. Amide B band is present in *Loligo* and *Sepia*. Symmetric and asymmetric carboxylate bands are present in all insoluble matrices. The main sugar groups bands are present in *Loligo*, *Sepia* and *Spirula*, but one of them ($1110\ cm^{-1}$) is absent in *Nautilus*.

Cephalopod soluble matrices show the main features of proteic material: $3295\ cm^{-1}$ (amide A), several bands between $1656\ cm^{-1}$ and $1636\ cm^{-1}$ (amide I), $1567\ cm^{-1} - 1552\ cm^{-1}$ (amide II), 1200 to $1320\ cm^{-1}$ (amide III) bands are present. Symmetric ($1420\ cm^{-1}$) carboxylate bands are also present in the four studied genera. One of the sugar group band is visible: $1075\ cm^{-1}$ (fig. 1). Bands are weaker than those of insoluble matrix. They are also broader; this fact may be indicative of a less 'crystalline' state.

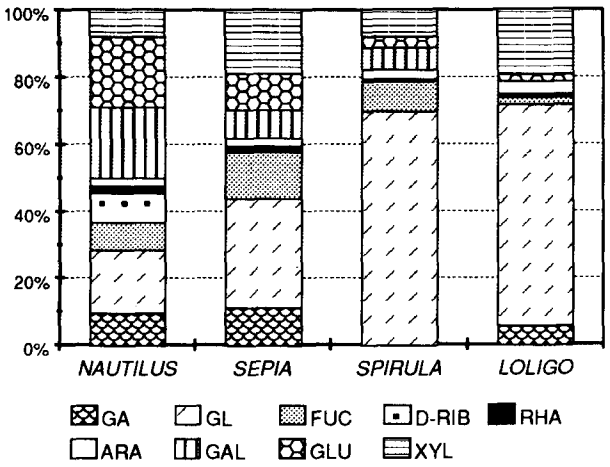

Figure 3. Cumulated bars of the sugar content of the soluble matrices of cephalopod shells. GA, galactose; GL, glucose; FUC, fucose; RHA, rhamnose; ARA, arabinose; GAL, galactosamine; GLU, glucosamine; XYL, xylose.

Sugar determination. The salient features of analysis are summarized in figures 2 and 3.

Monosaccharide contents of the insoluble organic matrices are similar (fig. 2). Glucosamine is higher than 75%, the highest content being 93% in *Loligo* despite being subject to a longer hydrolysis time. *Sepia* and *Loligo* contain small amounts of mannose + xylose, while *Nautilus* and *Sepia* do not. Moreover, *Loligo* does not contain glucose, galactose, rhamnose or fucose.

Mannose, xylose, fructose and ribose are absent in the four soluble organic matrices (fig. 3). Deoxy-ribose is present only in the *Nautilus* shell. All the studied genera are characterized by high glucosamine contents. *Nautilus* is also characterized by the highest content of galactose and glucose, and the lowest glucosamine content. *Loligo* and *Spirula* have the highest glucosamine content, while *Loligo* has the lowest fucose content. It must be noted that the galactosamine content of *Spirula* is zero with this hydrolysis time.

For all the samples, unidentified peaks were detected on the chromatograms. Most of them were eluted after ribose. Unknown sugars were previously reported among the soluble matrix of squid internal shell[11,12]. The proportions of unknown peaks vary greatly between the four genera: low for the soluble matrix of *Nautilus* or *Sepia*, but higher for the soluble matrix of *Loligo*.

Discussion

Sugar determination. The main difference between soluble and insoluble matrices is the high glucosamine content of the insoluble matrix: the highest glucosamine content in the soluble matrix is lower than the lowest content in the insoluble matrix. However, the similarity between the taxa studied on the one hand, and soluble

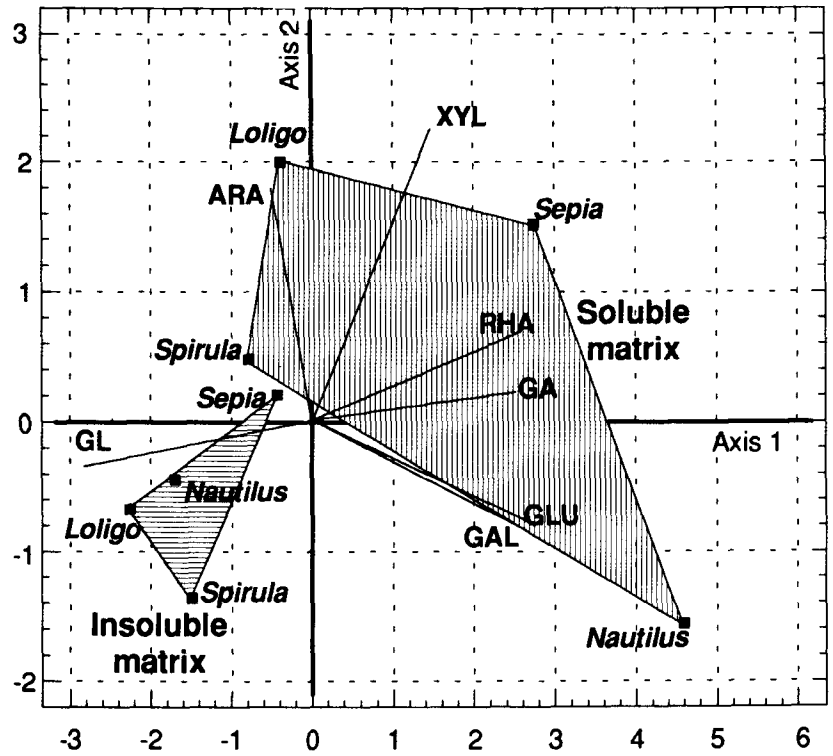

Figure 4. PCA diagram based on sugar amounts of cephalopod shells. The specimens are sorted along axis 1 according to glucose content (GL), opposed to galactose (GA), glucosamine (GLU), rhamnose (RHA) and galactosamine (GAL) contents. They are sorted along axis 2 according to their contents of xylose (XYL) and arabinose (ARA).

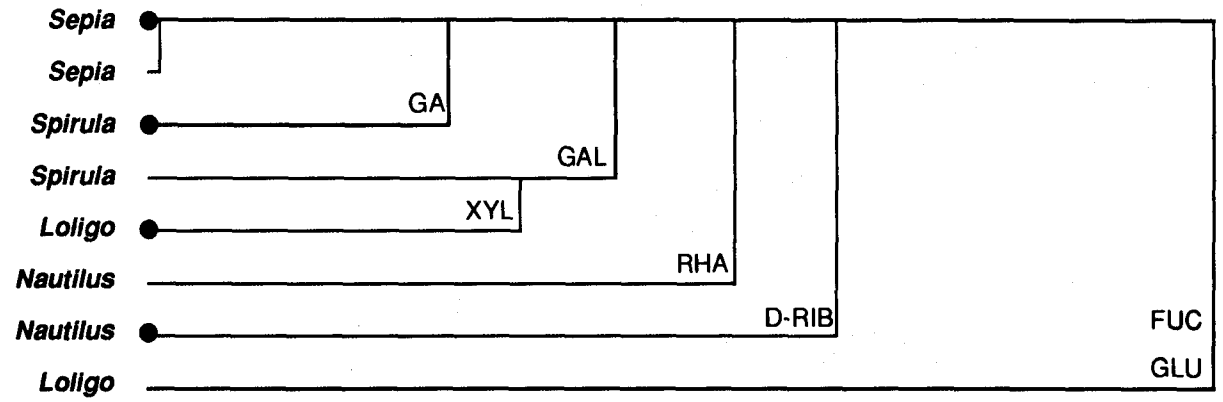

Figure 5. Hierarchic classification diagram based on presence-absence of sugars. Black dots indicate the soluble matrices.

and insoluble matrices on the other, is not easy to estimate. Despite the small number of specimens (this number is correlative from the three recent calcified shells), a multivariate analysis (principal components analysis: PCA) has been performed based on sugar amounts. An ascendant hierarchic classification, using the χ^2 distance, clustered by weighted averages method can be used to detect similarities between organic matrices.

The PCA is based on variance-covariance matrices of sugar amounts (fig. 4). The first axis represent 63.1% of the total variance, the second axis 18.3%. The specimens are sorted along axis 1 according to glucose, opposed to galactose, glucosamine, rhamnose and galactosamine contents. They are sorted along axis 2 according to their contents of xylose (or mannose) and arabinose. In an axis 1–2 graph (fig. 4), soluble and insoluble matrices appear to be different. The insoluble matrices have a high content of glucosamine, and low content of xylose (or mannose). The insoluble matrices are more homogeneous than the soluble matrices.

The hierarchic diagram (fig. 5), based on presence or absence of sugars, shows the similarities between the soluble and insoluble matrices of *Sepia*. The insoluble matrix of *Loligo* is characterized by the absence of fucose and glucose. The soluble matrix of *Loligo* and the insoluble matrix of *Spirula* are differenciated by the xylose (or mannose). It must be noted that the insoluble matrix of *Loligo* is unique if two classes are chosen for hierarchic diagram. *Loligo* is the only non-mineralized shell.

Table. Correlation coefficients between FT-IR data on insoluble matrix and chitin.

	Nautilus	*Sepia*	*Spirula*	*Loligo*
Nautilus	1			
Sepia	0 87	1		
Spirula	0.81	0.95	1	
Loligo	0.88	0.96	0.95	1
chitin	0.58	0.73	0.82	0.78

Infrared data. Correlation coefficients based on infrared spectra of chitin and insoluble matrices show that *Spirula* and *Loligo* are more similar to chitin than is *Sepia*. The lowest coefficient is between *Nautilus* and chitin (table). According to literature[5,24,25], *Sepia*, *Spirula* and *Loligo* have the same chitin/proteins ratios, but are composed of different proteins. *Nautilus* contains less chitin than the other taxa. *Loligo* and *Sepia* are the most similar taxa, while *Nautilus* and *Spirula* differ the most. Two ratios have been calculated from infrared data to estimate the composition of the insoluble organic matrices. The first ratio is based on the sum of the absorbance intensities of 3300 cm^{-1}, 1656 cm^{-1} and 1636 cm^{-1} bands for 'proteic' contents, divided by the sum of intensities of 1070 cm^{-1}, 1110 cm^{-1} and 1155 cm^{-1} bands for sugars (fig. 6A). The second ratio is based on the sum of the intensities of 3300 cm^{-1}, 1656 cm^{-1}, 1636 cm^{-1}, 1750 cm^{-1} and 1720 cm^{-1} bands

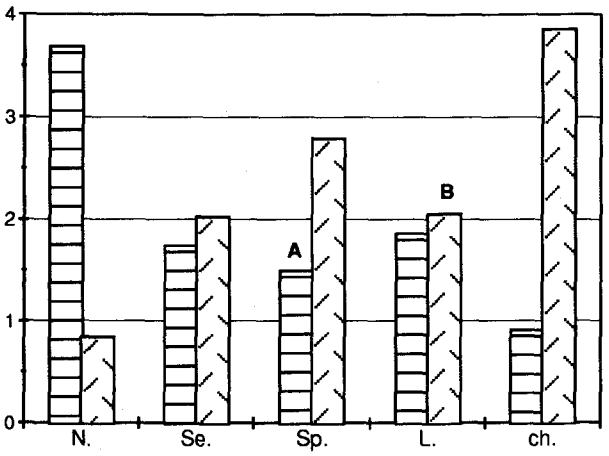

Figure 6. Ratio A is based on the sum of the intensities of 3300 cm^{-1}, 1656 cm^{-1} and 1636 cm^{-1} bands for 'proteic' contents, divided by the sum of intensities of 1070 cm^{-1}, 1110 cm^{-1} and 1155 cm^{-1} bands for sugars. Ratio B is based on the sum of the intensities of 3300 cm^{-1}, 1656 cm^{-1}, 1636 cm^{-1}, 1750 cm^{-1} and 1720 cm^{-1} bands for 'proteic' contents divided by the sum of intensities of 1070 cm^{-1}, 1110 cm^{-1} and 1155 cm^{-1} bands. N, *Nautilus*; Se, *Sepia*; SP, *Spirula*; L, *Loligo*; ch, γ-chitin.

for 'proteic' contents divided by the sum of intensities of 1070 cm^{-1}, 1110 cm^{-1} and 1155 cm^{-1} bands (fig. 6B). Carboxylic bands being absent, these two ratios differ in chitin. *Sepia* and *Loligo* are similar, while *Spirula* is slightly different. In these three specimens, the two ratios are lower than that of chitin, but ratio B is always higher than ratio A. In *Nautilus*, ratio A is higher than ratio B (fig. 6). Ratio A is not a true protein/sugar ratio. However, several authors have noticed that the chitin content in *Nautilus* is lower than in other recent cephalopods. This is in agreement with previously mentioned authors.

Conclusions

DRIFT is a non-time consuming and non-destructive analysis of the organic matrices of shells. The presence of sugar groups can be confirmed, and the protein/sugar ratios can be estimated and compared in various specimens. This method also shows the differences between soluble and insoluble matrices. The range of monosaccharides obtained by hydrolysis of the soluble matrices of recent cephalopod shells is greater than that obtained from the insoluble matrices of the same shells. Despite different methods, the results from the soluble matrix appear to be similar to those previously mentioned[11,12]. The combination of DRIFT and HPAE-PAD analyses shows that the non-mineralized shell of *Loligo* is different from shells of *Sepia* and *Spirula*, all being Coleoid Cephalopoda. This result is in accordance with the hypothesis that polysaccharide complexes are involved in calcification processes[1]. Moreover, the mineralized shell of *Nautilus* is also different. A major difference between the shells of Tetrabranchiata and Coleoida is the structure of the nacreous layer. The nacreous layer of *Nautilus* shell is composed of numerous lamellae, each composed of a single layer of tablets. Organic sheets alternate with mineral lamellae. Each tablet is composed of acicular aragonitic crystals. The main part of the outer shell wall and the septa are nacreous[26]. The nacreous layer of *Sepia* or *Spirula* is devoid of tablets, but shows the acicular crystals[27]. In both cases, acicular crystals are surrounded by organic matrix. It should be noted that the soluble and the insoluble matrices of *Nautilus* are different from those of other shells, reflecting its unique position as the last living externally shelled cepholopod genus. One of the evolutionary trends within the Cephalopoda was toward the loss of mineralized shell, then the loss of shell. The above results suggest that the loss of mineralized shell correlates with the increase of the 'chitinous' nature of the insoluble organic matrix.

1 Addadi. L., and Weiner, S., Proc. natl Acad. Sci. USA *82* (1985) 4110.
2 Jeuniaux, C., Bull. Soc. Chim. biol. *47* (1965) 2267.
3 Goffinet, G., and Jeuniaux, C., Cah. Biol. mar. *XX* (1979) 341.
4 Peters, W., Comp. Biochem. Physiol. *41B* (1972) 541.
5 Hunt, S., and Nixon, M., Comp. Biochem. Physiol. *68B* (1981) 535.
6 Lowenstam, H. A., Traub, N., and Weiner, S., Paleobiology *10* (1984) 268.
7 Rudall, K. M., and Kenchington, W., Biol. Rev. *49* (1973) 597.
8 Stegemann, H., Naturwissenschaften *14* (1961) 501.
9 Stegemann, H., Hoppe-Seyler's Z. physiol. Chem. *331* (1963) 269.
10 Drozdova, T. V., Karyakin, A. V., and Krasnova, V. A., J. evol. Biochem. Physiol. *7* (1971) 350.
11 Okutani, K., and Morikawa, N., Bull. Jap. Soc. Scient. Fish. *44* (1978) 369.
12 Okutani, K., and Morikawa, N., Bull. Jap. Soc. Scient. Fish. *44* (1978) 749.
13 Grégoire, C., Archs int. Physiol. Biochim. *LXIX* (1961) 374.
14 Dauphin, Y., Palaeontographica *A176* (1981) 35.
15 Dauphin, Y., Annls Paléont. (Vert. Invert.) *70* (1984) 87.
16 Okada, K., Ozaki, Y., Kawauchi, K., and Muraishi, S., Appl. Spectrosc. *44* (1990) 1412.
17 Clarke, A. J., Sarabia, V., Keenleyside, W., MacLachlan, P. R., and Whittfield, C., Analyt. Biochem. *199* (1991) 68.
18 Hardy, M. R., LC/GC 7, 3 (1989) 242.
19 Honda, S., Analyt. Biochem. *140* (1984) 1.
20 Carlström, D., Biochim. biophys. Acta *59* (1962) 361.
21 Dweltz, N. E. Biochim. biophys. Acta *51* (1961) 283.
22 Muzarelli, R. A. A., Natural Chelating Polymers-alginic Acid, Chitin and Chitosan. Pergamon Press, London 1973.
23 Hotta, S., and Kamiya, H., Earth Sci. *29* (1975) 111.
24 Degens, E. T., Spencer, D. W., and Parker, R. H., Comp. Biochem. Physiol. *20* (1967) 553.
25 Hunt, S., Polysaccharid-protein Complexes in Invertebrates. Academic Press, London-New York 1970.
26 Grégoire, C., Bull. Inst. r. Sci. nat. Belg. *XXXVIII* (1962) 1.
27 Dauphin, Y., and Keller, J. P., C. r. Acad. Sci. Paris II *294* (1982) 409.