Isolation and Characterisation of Toxins from *Fibrocapsa japonica* (Raphidophyceae)

Dem Fachbereich Chemie der Universität Oldenburg zur Erlangung des Grades

einer Doktorin der Naturwissenschaften

angenommene Dissertation

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geb. am 12. 02. 1975 in China

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Tag der Disputation:	18.08.2003

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PUBLICATIONS

Publications derived from this dissertation:

(1) Fu Meng, Koulman Albert, van Rijssel Marion, Luetzen Arne, de Boer M. Karin, Tyl Monica R., Liebezeit Gerd, 2003, Chemical Characterisation of Three Haemolytic Compounds from *Fibrocapsa japonica* (Raphidophyceae), Toxicon (submitted)

(2) Fu Meng, van Rijssel Marion, Liebezeit Gerd, 2002, Haemolytic compounds from *Fibrocapsa japonica* (Raphidophyceae) In: Harmful Algal Blooms 2002, Proceedings of the Tenth International Conference on Harmful Algal Blooms, Florida, USA (submitted)

Publications derived from previous works in China:

(3) Fu Meng, Yan Tian, Zhou Mingjiang, 2000, Research progress in effects of paralytic shellfish toxin on shellfish and purification of toxin, Journal of Aquaculture, 24(4): 382-387 (in Chinese)

(4) Fu Meng, Yan Tian, Zhou Mingjiang, Li Jun, Yu Rencheng, 2000, Effects of *Alexandrium tamarense* on the early development of scallop, Marine Sciences, 24(3): 8-11 (in Chinese)

(5) Fu Meng, 1999, Effects of *Alexandrium tamarense* on marine bivalves, Thesis for Msc. Degree (in Chinese)

(6) Yan Tian, Fu Meng, Li Jun, Yu Rencheng, Zhou Mingjiang, 2001, Primary study on accumulation, transformation and elimination of PSP in *Mytilus edulis*, Oceanologia et limnology sinica, 32(4): 420-427 (in Chinese)

(7) Yan Tian, Zhou Mingjiang, Fu Meng, Wang Yunfeng, Yu Rencheng, Li Jun, 2001, Inhibition of egg hatching success and larvae survival of the scallop, *Chlamys*

farreri, associated with exposure to cells and fragments of the dinoflagellate *Alexandrium tamarense*, Toxicon, 39(8): 1239-1244

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SUMMARY

Harmful Algal Blooms (HAB) is a natural disaster which has attracted global attention in recent decades since it threatens greatly public health, causes economic damage to fisheries and tourism. *Fibrocapsa japonica* is one of HAB causative organisims which caused significant loss to coastal fisheries in Japan. From the 1990's it was also reported frequently in European coastal waters. The toxicity mechanism behind such fish killing events is so far uncear. Although some views of fibrocapsins (toxins produced by this alga) were ever reported e.g. brevetoxins, but not acceptable for lack of conclusiveness.

In this work, the toxicity of *Fibrocapsa japonica* algal cells was first established by *Artemia salina* biotest. Fibrocapsins was screened step by step through *Artemia salina* biotest, bioluminescence inhibion biotest and erythrocyte lysis assay methods, isolated then in HPLC. The chemical natures of fibrocapsins 1, 2 and 3 were determined finally as 6,9,12,15-octadecatetraenoic acid, all-*cis*-5,8,11,14,17-eicosapentaenoic acid and all-*cis*-5,8,11,14-eicosatetraenoic acid by HPLC-MS, IR, GC-(HR)MS, NMR experiments and biotests.

ZUSAMMENFASSUNG

Schädigende Algenblüten sind natürliche Katastrophe, welche in den letzten Jahrzehnten weltweite Aufmerksamkeit auf sich gezogen haben, da sie die öffentliche Gesundheit gefährden und wirtschaftlichen Schaden für die Fischerei und den Tourismus verursachen. *Fibrocapsa japonica* gehört zu den Organismen, welche toxische Algenblüten verursachen und dabei bedeutenden Schaden für die japanische Küstenfischerei verursacht hat. Seit 1990 wird dieser Dinoflagellat auch häufig in europäischen Küstengewässern gefunden. Der Toxizitätsmechanismus dahinter ist bisher noch nicht geklärt.

In dieser Arbeit wurde die Toxizität von *Fibrocapsa japonica* Algenzellen erstmals über den *Artemia salina*-Biotest ermittelt. Fibrocapsine, die von dieser Algenart produzierten Toxine, wurden Schritt für Schritt mittels *Artemia salina*-Biotest, Biolumineszenzhemmenden Biotest und Erythrozyten-Lysin-Untersuchung gescreent und dann über HPLC isoliert. Die chemischen Eigenschaften der Fibrocapsine wurden letztlich mit Hilfe der HPLC-MS, IR, GC-(HR)MS, NMR und Biotest Methode als mehrfach ungesättigten Fettsäure bestimmt.

CONTENTS

Abbreviations

AA	5,8,11,14-eicosatetraenoic acid; arachidonic acid
amu	atomic mass unit
ASP	Amnestic Shellfish Poisoning
DAD	Diode Array Detector
DHA	docosahexaenoic acid
DIN	Deutsches Institut für Normung (German Institute for Standards)
DSP	Diarrhoeic Shellfish Poisoning
ELA	Erythrocyte Lysis Assay
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	5,8,11,14,17-eicosapentaenoic acid
ESI	Electrospray Ionization
EUROHAB	European Initiative on Harmful Algal Blooms
eV	electron volt
FJJp	*Fibrocapsa japonica* - Japanese strain
FJNZ	*Fibrocapsa japonica* - New Zealand's strain
GC	Gas Chromatography
HAB	Harmful Algal Blooms
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
ICES	International Council for the Exploration of the Sea
IOC	Intergovernmental Oceanographic Commission
IR	Infrared Spectroscopy
LD_{50}	Lethal Dose (the amount of a material, given all at once, which causes the death of 50% of a group of test animals)
m/z	mass/charge
MS	Mass Spectrometry
MSTFA	*N*-methyl-*N*-trimethylsilyltrifluoroacetamide
MUFA	Monounsaturated Fatty Acid

NMR	Nuclear Magnetic Resonance
NSP	Neurotoxic Shellfish Poisoning
NTU	Nephelometric Turbidity Unit
OR	orifice voltage
OTA	octadecatetraenoic acid
PAD	Photodiode Array Detector
PSP	Paralytic Shellfish Poisoning
PSU	Practical Salinity Unit
PUFA	Polyunsaturated Fatty Acid
RNG	ring voltage
ROS	reactive oxygen species
SFA	Saturated Fatty Acid
SPE	Solid Phase Extraction
TLC	Thin Layer Chromatography
TMS	trimethylsilyl
UV	ultraviolet
WG	Working Group

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I. INTRODUCTION

1. Harmful Algal Blooms

There is a very long history of Harmful Algal Blooms (HAB) which can be traced back to the record in the Bible "... all the waters that were in the river were turned to blood. And the fish that was in the river died; and the water stank, and the Egyptians could not drink of the water of the river, ... " (Exodus 7, 20 to 21). However, this phenomenon did not attract worldwide attention until several decades ago when HAB incidences started to increase dramatically (e.g. Smayda 1990, Hallegraef et al. 1993, ICES/IOC WG 2000; 2001).

What are Harmful Algal Blooms? EUROHAB (1999) defined this term as "Microalgae in marine and brackish waters regularly cause harmful effects, considered from the human perspective, in that they threaten public health and cause economic damage to fisheries and tourism. These episodes encompass a broad range of phenomena collectively referred to as Harmful Algal Blooms." As to harmful effects we can classify HAB causing organisms into three groups: 1) toxin producers, which even at low biomass levels can contaminate seafood, causing sickness and potentially death of humans eating the seafood, or causing sickness and death in the shellfish and finfish themselves; 2) high-biomass toxin producers (cyanobacteria), which can have similar harmful effects as toxin producers; 3) high-biomass bloom species, which can cause either anoxia that indiscriminately kills marine life or produce unpleasant foam, gelatinous masses that are a nuisance for e.g. tourists who may a.o. develop allergic skin reactions after bathing.

Table 1. Different harmful algal species, their toxins and their effects (EUROHAB 1999)

Syndrome	Type of toxin	Species	Symptoms
Paralytic Shellfish Poisoning (PSP)	Saxitoxin, Neosaxitoxin and derivatives	*Alexandrium tamarense* *Alexandrium catenella* *Alexandrium minutum*	Headache, dizziness, nausea, tingling paralysis
Diarrhoeic Shellfish Poisoning (DSP)	Okadaic acid, Dinophysis toxins	*Gymnodinium catenatum* *Dinophysis acuminata* *Dinophysis acuta* *Dinophysis caudata* *Dinophysis mitra* *Dinophysis norvegica* *Dinophysis rotundata* *Dinophysis tripos* *Dinophysis sacculum* *Prorocentrum delicatissima* *Prorocentrum lima* *Prorocentrum tepsium sp. indet* *Prorocentrum seriata*	Diarrhoea, nausea, vomiting, abdominal pain; hepatotoxic cardiotoxic; chronic exposure may promote tumour formation in the digestive system
Amnesic Shellfish Poisoning (ASP)	Domoic acid	*Pseudonitzschia multiseries* *Pseudonitzschia pseudodelicatissima* *Pseudonitzschia australis* *Pseudonitzschia seriata*	Nausea, vomiting, diarrhoea, abdominal cramps, dizziness, hallucinations, confusion, short term memory loss seizures
Neurotoxic Shellfish Poisoning (NSP)	Brevetoxins	*Gymnodinium breve*	Chills, headache, diarrhoea, vomiting and abdominal pain, muscular, aches, dizziness, anxiety, sweating and peripheral tingling
Cyanobact-erial toxins	Nodularins, Microcystin, Saxitoxin, Neosaxitoxin	*Nodularia spumigena* *Microcystis spp.* *Anabaena spp.* *Aphanizomenon flos-aquae* New species	Weakness, recumbence, pallor, vomiting, vomiting, diarrhoea. Death occurs due to pooling of blood in the liver and respiratory arrest

2. Diversity of Marine Algal Toxins

So far there are five major classes of marine algal toxins which not only cause intoxication or mortality of marine organisms but also are accepted extensively as human food poisons (Table 1). The toxin producers are mainly microalgae including flagellate, diatom and cyanobacteria species. Among them flagellates are the most important causative organisms being responsible for Paralytic Shellfish Poisoning (PSP), Diarrhoeic Shellfish Poisoning (DSP) and Neurotoxic Shellfish Poisoning (NSP). Marine algal toxins are generally considered to be secondary metabolites, i.e. compounds which are not indispensable to the basic metabolism of the organism, but may act as chemical defences or signals or are evolutionary relics.

3. Fish-Killing HAB Species

Fish-killing HAB species can cause significant damages to fisheries. Fig. 1. shows the food web interactions including fish-killing phytoplankton species. *Gymnodinium mikimotoi* was first recorded in European waters in 1966 and has since then formed recurrent blooms from the Spanish Atlantic border in the south to western Norway in the north, causing death to about 3000 tons of farmed fish and about 900 tons of mussels (Moestrup 1994). The first recorded toxic bloom of a *Chrysochromulina* species occurred in 1988 (Dahl et al. 1988), when *C. ploylepis* formed an extensive bloom in the Skagerrak and Kattegat. The bloom caused death of a wide range of marine organisms including 900 tons of farmed fish. Until now little is known about mechanisms of these toxic effects, but research results showed that fish-killing species produce noxious substances related to their intoxication symptoms (Table 2). *Chatonella* sp. is one of the most frequently appearing noxious red tide phytoplankton

species and highly toxic to fish. Studies of Oda et al. (1997) demonstrated that

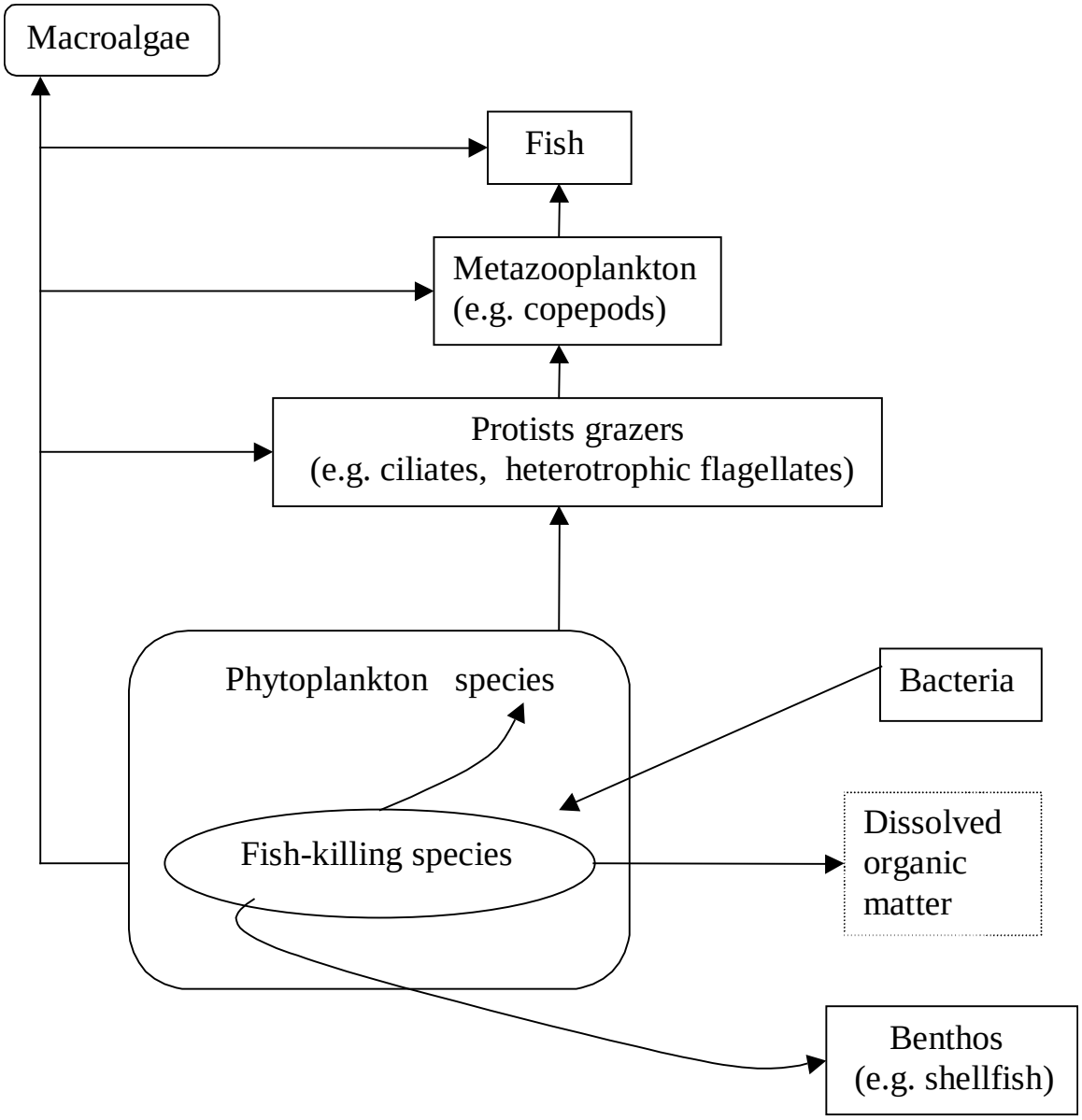


Fig. 1. Food web interactions including fish killing phytoplankton species (EUROHAB 1999)

Chatonella sp. generates reactive oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) which may be responsible for the toxicity. What's more, the other raphidophycean flagellates such as *Heterosigma akashiwo*, *Olisthodiscus luteus* and *Fibrocapsa japonica* also produce H_2O_2 and O_2^- under normal growth conditions. It is suggested that the generation of reactive oxygen species is a common feature of raphidophycean flagellates. In addition, many other works

demonstrated the raphidophycean flagellates to produce neurotoxic, haemolytic and haemo-agglutinating compounds, e.g. *Chattonella marina* (Subrahmanyan) Hara and Chihara (Onoue and Nozawa 1989; Onoue et al. 1990; Ahmed et al. 1995a, 1995b; Khan et al. 1995), *Chattonella antiqua* (Hada) Ono (Khan et al. 1996b) and *Fibrocapsa japonica* (Khan et al. 1996c). Oda et al. (1997) thought that reactive oxygen species are responsible for the gill tissue injury, which eventually causes fish mortality. The common symptoms caused by unknown ichthyotoxins are summarised in Table 2. There is so far no common knowledge of what these unknown ichthyotoxins are, how they function, or whether they work together or individually. The intoxication mechanism seems very complicated in the fish-killing events, depending on different causing organisms, different natural conditions etc.

Table 2. Toxins involved in fish kills and their symptoms (EUROHAB 1999)

Type of toxin	Effects	Species	Symptoms
Irritating substances (unknown)	Physical damage of the gills, Inhibition of oxygen uptake	*Chaetoceros concavicornis* *Ceratium fusus* *Gymnodinium mikitomoi* (=*G. nagakiense*) *Gyrodinium aureolum* *Noctiluca scintillans* *Chrysochromulina leadbeateri* *C. polylepis* *Phaeocystis pouchetii* *Chatonella antiqua* *C. marina*	Abrasion of the gill epidermis, clogging of the gills by excess mucus produced in response to irritating substances, stripping of the protective mucus layers
Harmful chemicals, neurotoxins, haemolytic or blood agglutinating substances (unknown)	Physiological damage to gills, major organs, intestine, respiratory or circulatory systems, interfere with osmoregulation	*Chysochromulina leadbeateri* *C. polylepis* *Prymnesium parvum* *Prymnesium patelliferum* *Chatonella antiqua* *C. marina*	Malfunction of the brain and heart due to blood hypoxia, tissue and blood cell decay, fish death
Unknown	Acute toxicity	*Heterosigma carterae (=H. akashiwo)*	Fish death
Unknown	Acute toxicity	*C. polylepis*	Fish, phytoplankton, copepods, see-stars death

4. *Fibrocapsa japonica*, Fibrocapsins and Brevetoxins

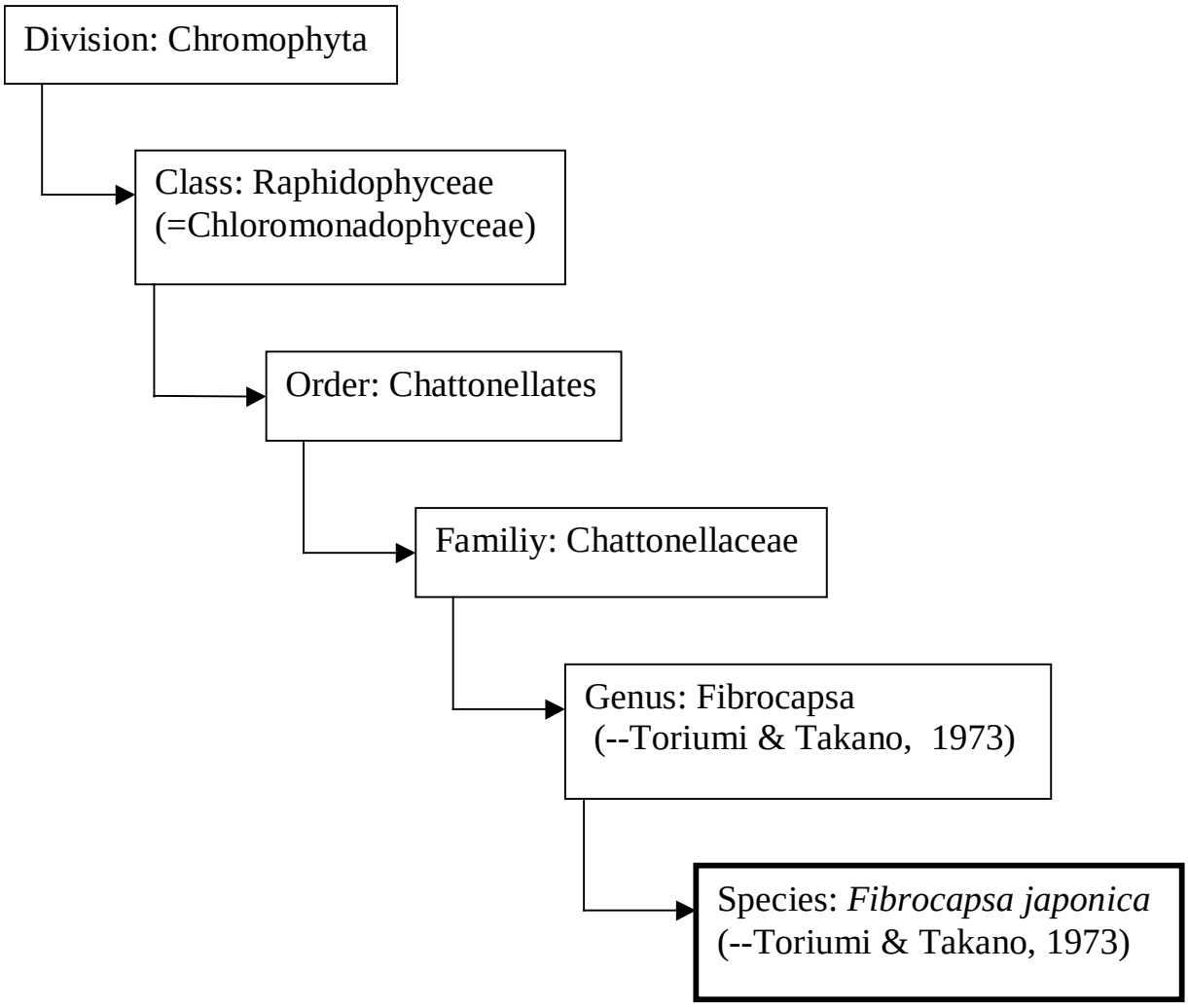


Fig. 2. Position of *Fibrocapsa japonica* in taxonomy (Throndsen 1993)

Fibrocapsa japonica (Raphidophyceae, Fig. 2) is a typical fish-killing algal species. It has caused significant damage to coastal fisheries in Japan. From the 1990´s, it was reported in European coastal waters as well and was isolated and identified from the German Wadden Sea in 1995 (Table 3). In the summer of 1997, this species was found in almost all samples analysed by the Dutch Algal Bloom Programme and listed also in the messages of the German "Algen-Frühwarnsystem (algae-early warning system)" for the German Wadden Sea as well. Under favourable conditions, i.e. warm, sunny weather and a stable water column, it could well form harmful blooms in the coastal

North Sea, as reported for Japanese waters (Rademaker et al 1998). Therefore, although this phytoflagellate has so far not caused mass mortalities in marine fisheries in Germany, its potential hazards to marine mammals, public health and fishery necessitate research especially as far as the nature of the toxins and their mode of action is concerned.

Table 3. History of *Fibrocapsa japonica*

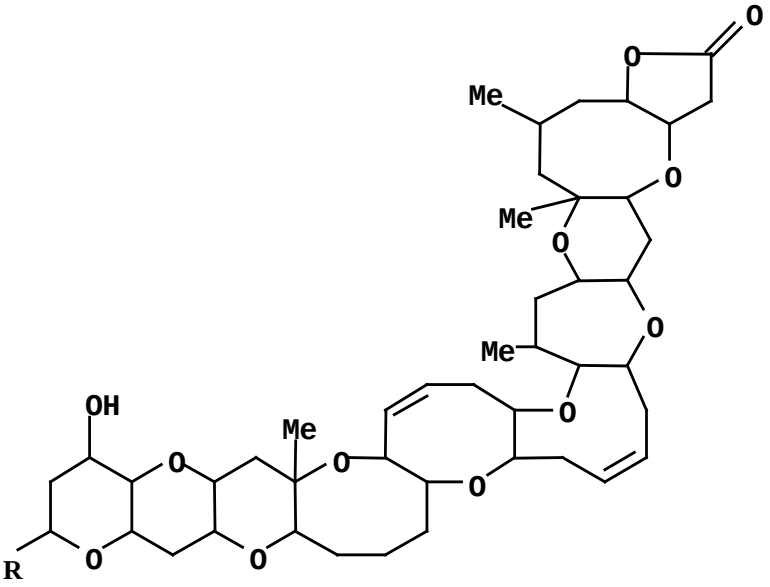
Fibrocapsa japonica Toriumi & Takano		
Time	Events	References
1971	First described as *Botryococcus* sp.	Khan et al. 1996c
1972	A heavy red-tide of this species killed a great number of caged young yellowtail *Seriola quinqueradiata* in Ehime Prefecture, Japan.	Okaichi 1972
1973	Toriumi and Takano described the morphology in detail and renamed it as *Fibrocapsa japonica*.	Toriumi & Takano 1973
Since 1973	Occurrence and blooms of this species have been reported in different coastal areas of Japan.	Toriumi & Takano 1973
		Yoshimatsu 1987
		Iwasaki 1989
		Montani et al. 1995
1991	Reported for the first time on the channel coasts of Normandy, France	Billard 1992
1991	*Fibrocapsa japonica* was observed in 1991 for the first time in Dutch coastal waters.	Vrieling et al. 1995
1993	In May, a minor bloom was observed at a station in the southern central North Sea.	
1992	In spring 1992, a raphidophyte-dominated (*F. japonica* is one of them) phytoplankton bloom occurred along the north-eastern coastline of New Zealand.	Rhodes et al. 1993
From 09/1992 to 02/1993	*Geophyrocapsa oceanica* co-dominated with *F. japonica* along the north-east coast New Zealand in a bloom which immediately proceeded a toxic dinoflagellate event.	Rhodes et al. 1995
1995	Isolated and identified from German Wadden Sea near Büsum.	Nielson 1997

Khan et al. (1996c) tried to investigate the fibrocapsins, toxins produced by *Fibrocapsa japonica*, and found this algal species highly toxic to juvenile red sea

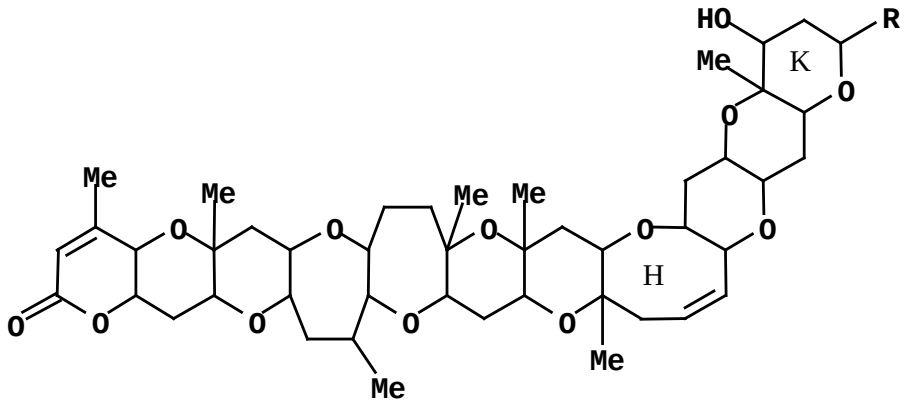
breams. A direct toxicity proof of five isolated neurotoxic components was, however, not supplied.

These five toxic components (FjTx-I, FjTx-II, FjTx-IIIa, FjTx-IIIb, FjTx-IV) were tentatively identified as brevetoxins (PbTx-1, PbTx-2, PbTx-9, PbTx-3, oxidized PbTx-2) as they displayed similar chromatographic (TLC and HPLC) behaviour. Nevertheless, further results of structural investigations are still needed to confirm the identity between fibrocapsins and brevetoxins.

Brevetoxins (Fig. 3) have been originally identified in the unarmored marine dinoflagellate *Gymnodinium breve* (= *Ptychodiscus brevis*), an organism linked to red tide outbreaks along the west coast of Florida, in the Gulf of Mexico, New Zealand, and Japan. This group of toxins is responsible for intoxication in marine organisms and neurotoxic shellfish poisoning in humans. Toxicity of this organism is due to the synthesis and intracellular maintenance of a multiplicity of polyether brevetoxins (PbTxs). A number of toxic compounds were purified both from field blooms and from laboratory cultures: nine toxins are currently known (Fig. 3) (Baden et al. 2000).



Type-A Brevetoxins: PbTx-1, R = $CH_2C(=CH_2)CHO$
PbTx-7, R = $CH_2C(=CH_2)CH_2OH$
PbTx-10, R = $CH_2CH(CH_3)$ CH_2OH

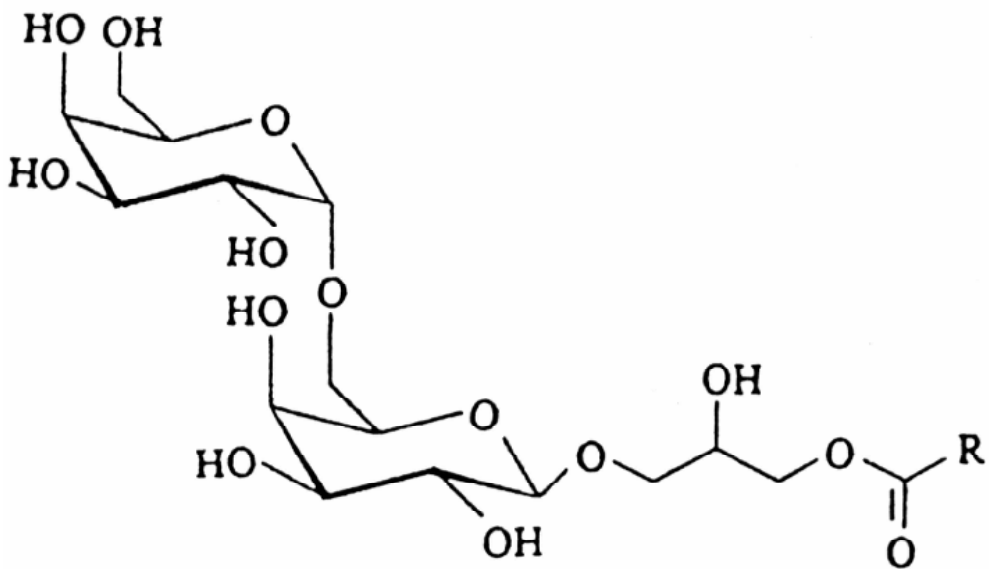


Type-B Brevetoxins: PbTx-2, R = $CH_2C(=CH_2)CHO$
PbTx-3, R = $CH_2C(=CH_2)CH_2OH$
PbTx-5, the K-ring acetate of PbTx-2
PbTx-6, the H-ring epoxide of PbTx-2
PbTx-8, R = CH_2COCH_2Cl
PbTx-9, R = $CH_2CH(CH_3)CH_2OH$

Fig. 3. Structures of the two main types of brevetoxins (Hua 2000)

5. Haemolytic Toxins

Many harmful algal species were reported to show haemolytic activity, for example: *Chrysochromulina*, *Prymnesium*, *Alexandrium* and cyanobacteria species. In many cases the chemical nature of the haemolytic substances produced and consequently the mode of action causing haemolysis are either different among species or unknown (Eschbach et al. 2001). One suggestion in recent works (He 1999; Mitsui et al. 1989; Yaumoto et al. 1987) was that haemolytic toxins are glycolipides or glycosides, an example is given in Fig. 4. So far, only one raphidophycean flagellate - *Chattonella marina* - has been reported to produce haemolytic compound, its molecular structure was, however, not presented (Onoue & Nozawa 1989).



A: R = $CH_3(CH_2)_{11}CH_2$; B: R = $CH_3(CH_2)_{13}CH_2$
C: R = $CH_3(CH_2)_4CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}(CH_2)_5CH_2$

Fig. 4. Structures of haemolytic glycolipides in *Phaeocystis pouchetii* (He et al. 1999)

6. Aim of This Work

Among the many unsolved problems in the research field of *Fibrocapsa japonica*, isolation and characterisation of the toxins still remains a major task. Therefore, in the present work the toxicity of *F. japonica* cells and cell extracts in different biotests will be established first, then these biotests will be applied to individual toxin fractions and toxins isolated after HPLC separation, and finally individual toxic compounds will be characterised with modern structure investigation tools.

II. MATERIAL and METHODS

1. Cultivation and Growth Law for *Fibrocapsa japonica*

1.1 Source of algal strain

The algal strain of *Fibrocapsa japonica* was obtained from M. Karin de Boer (Biological Centre, University of Groningen, The Netherlands). The original strain was isolated from the German Wadden Sea off Büsum and taken into culture at FTZ (Forschungs- und Technologiezentrum, University of Kiel) in 1995 (Rademaker 1998).

1.2 Cultivation and observation under the light microscope

Algal cells were cultivated in 2 L Erlenmeyer flasks in a Gallenkamp Orbital Incubator INR-401 (Fig. 5). Shaking was avoided because any stimulus may cause the mucocyst in the cell to discharge in all directions numerous mucous fibres accompanying breakdown of the cell. F/2-Si culture medium (Guillard & Ryther 1962, Guillard 1975) was used at 20 ± 1°C, an irridiance of ca. 67 µmol quanta$\cdot m^{-2} \cdot s^{-1}$ and a 12 h light/12 h dark photo period. The seawater for the medium came from the Jade Bay (~29-30 PSU) and was filtered through cotton and then autoclaved for 20 minutes at 121°C. Stock solutions of all additions to the medium were either autoclaved or filter-sterilised before they were added to the autoclaved seawater. Normally the medium would stay overnight and over-caps made of aluminium foil were used which prevent fungi from establishing in a damp plug.

The algal samples fixed by Lugol's solution (10 g KI, 5 g I_2 and 5 g CH_3COONa in 100 mL H_2O) were observed with a light microscope (Zeiss Axiophot), and micrographs were taken by a digital camera (Kappa CF 15/4 MCC). The mean cell

size was obtained too by measuring 50 cells with the aid of image analysis software (Soft Imaging System).

Fig. 5. Cultivation of *Fibrocapsa japonica* in the incubator

1.3 Growth Law for Fibrocapsa japonica

Two 500 mL flasks for growth studies were used. The same amount of algal cells from the stationary phase culture were inoculated separately into flasks under a clean bench. Turbidimetry was used here as an easier counting method to monitor the growth law. This is supported by the fact that after fixation with Lugol some algal cells will clump together, so the traditional counting method is not very reliable here. As the culture is a single-species one, the obtained turbidity value therefore reflects only the density of algal cells. Two monitoring methods were used firstly together for the calibration. Every other morning duplicate samples from each flask were taken for

altogether 27 days, and before that flasks were well shaken very slowly and carefully otherwise mucous fibres will be given off in all directions with bursting of cells. The turbidity was read twice for each sample with a DRT-15CE Portable Turbidimeter, data are expressed as Nephelometric Turbidity Units (NTU). For the counting method with Utermöhl chambers, algal samples were fixed with Lugol's solution (1 mL sub-sample + 5 µL Lugol) and counted twice by scanning several transects at regular intervals.

2. Toxicity Biotests with Algal Cells and Culture Filtrate of *Fibrocapsa japonica*

2.1 Artemia salina *test*

A 500 mL flask containing 500 mL cotton-filtered seawater (from Jade Bay) and 0.5 g *Artemia salina* eggs (bought from the pet shop) was maintained in a 28 °C water bath. Since light has a triggering effect on the onset of hatching (Sorgelloss 1973) the flask was illuminated by a 60 W fluorescence lamp for 1 hour. Air was bubbled into the suspension through a bubble stone extending to the bottom of the flask to keep all eggs in continuous motion. Only freshly hatched nauplii were used in the tests within 24 hours. The nauplii were added under light to the experiments as they exhibit phototropic movement. All tests were carried out at room temperature. Observations under a dissecting microscope were done after exposition. As nauplii in this developmental stage are very actively swimming, the sinking percentage was regarded as an appropriate indicator for the physiological state of *A. salina* nauplii. The sinking percentage was calculated as:

$$\frac{\text{number of organisms sinking to the bottom} \times 100}{\text{total number of organisms}}$$

2.2 12 h effect of stationary phase Fibrocapsa japonica *cells and culture filtrate on freshly hatched* Artemia salina *nauplii*

Stationary phase cells were filtered through a 60 µm sieve to remove all dead cell colonies, mucous fibres and so on. The remaining cells were counted twice, giving a mean cell density of 25,900 cells/mL. One part of the cell suspension was filtered through a 47 mm glassfibre filter (Whatman GF/C) to obtain the filtrate. This was proven to contain no algal cells by microscopic examination. The second part of the cell suspension passed through an 11 µm sieve very slowly. Algal cells were carefully rinsed from the sieve into a beaker with filtered seawater. After volume adjustment cell densities were determined in duplicate to be 72,200 cells/mL by microscopic counting method. Two tests were carried out at the same time. One was designed to test toxicity of algal cells, the other to test toxicity of the filtrate. The test of algal cells was composed of one control (filtered seawater) and 6 dilution series (algal cells suspended in filtered seawater): 430, 860, 1700, 3600, 7200, 14000 cells/mL. The filtrate test was composed of one control (f/2 medium) and 5 dilution series (filtrate plus f/2 medium): 1/5, 2/5, 3/5, 4/5, 5/5 filtrate. 5 mL beakers were used, all beakers contained 5 mL test solution and 10 freshly hatched *Artemia salina* nauplii. All tests were run in duplicate at room temperature (20 ± 1°C). Observations under a dissecting microscope were done after 12 h exposure.

3. Toxicity Biotests with Crude Extract of *Fibrocapsa japonica*

3.1 Bioluminescence inhibition test

The luminescent bacteria test was carried out in conformity with DIN 38412 L34, L341. All equipment, luminescent bacteria (*Vibrio fischeri* NRLL-B-11177) and chemicals were purchased from Dr. Lange GmbH & Co. KG, Berlin. The test was carried out at 15°C by mixing 0.5 mL sample dissolved in 2 % NaCl solution and 0.5 mL bacterial solution. Thus, the actual highest concentration of samples in the test can only be 50 %. The pH value was adjusted to 6~8 with 0.1N HCl or NaOH. After 30 minutes incubation the decrease of bioluminescence was measured in comparison to that of the control (2 % NaCl). Samples with bioluminescence inhibition < 20 % are regarded as non-toxic (Lange 1994).

3.2 Artemia salina *and bioluminescence inhibition tests with crude extract*

Algae were inoculated in four 2000 mL flasks. After 14 days, i.e. in the stationary phase, the total volume of 8350 mL (ca. 91,500,000 cells) was filtered gently through 26 pieces of 47 mm GF/C filters (Whatman) which were then stored at –85 °C. Before the experiments, all filters were thawed and extracted at room temperature with 70 mL ice-cold methanol (100 %) aided by ultrasonic treatment for 20 minutes with ice cooling. After shaking thoroughly for 1 min the extract (ca. 1,300,000 cells/mL) was evaporated to dryness at 40°C with a rotatory evaporator and then dissolved in 70 mL 2 % NaCl solution. The solution was filtered through a 0.45 µm single-use syringe filter. This resulting solution (pH = 7.09, S = 2~2.5 %) was used for both *Artemia salina* and bioluminescence inhibition tests. *Artemia salina* tests were performed in two concentration series (0 %, 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, 100 % and 0 %, 4 %, 8 %, 12 %, 16 %, 20 %) in duplicate in 10 mL beakers. All beakers contained 5 mL test solution and 15 *Artemia salina* nauplii. Observations

under a dissecting microscope were done after 24 h exposition. Bioluminescence inhibition tests were also performed in two concentration series (0 %, 3 %, 4 %, 6 %, 8 %, 13 %, 17 %, 25 %, 33 %, 50 % and 0 %, 0.20 %, 0.26 %, 0.39 %, 0.52 %, 0.78 %, 1.04 %, 1.56 %, 2.08 %, 3.13 %).

4. Toxicity Biotests with SPE Fractions of *Fibrocapsa japonica* crude extract

4.1 Separation of toxic fractions with SPE cartridges (C-18) guided by Artemia salina *and bioluminescence inhibition tests*

Superclean™ disposable SPE (solid phase extraction, Supelco, Inc.) 3 mL cartridge (C-18) and a vacuum manifold were employed and connected together. Crude extract (3 mL, stored at –18 °C) from stationary phase algae (ca. 3,800,000 cells/mL extract) was injected into the cartridge which had been conditioned beforehand with 2 mL methanol. The elution scheme is outlined in Table 4. The flow rate was adjusted to ca. 0.5 drop/sec. The different fractions obtained were evaporated to dryness at 40 °C and re-dissolved in 10 mL 2 % NaCl solution (ca. 1,100,000 cells/mL) for the biotests (1 mL for bioluminescence inhibition test in duplicate; 8 mL for *Artemia salina* test in duplicate). All test solutions were adjusted to pH 6~8 and salinity 2~2.5%.

4.2 Separation of toxic fractions with SPE cartridges (C-18) combined with erythrocyte lysis assay

4.2.1 Principle of erythrocyte lysis assay

Erythrocyte lysis assay (ELA) is based on photometrical determination of the released haemoglobin from the lysis of erythrocytes caused by haemolytic compounds (Yariv and Hestrin 1961).

Table 4. Elution scheme of toxic fractions with SPE cartridge (C-18)

Eluent composition/volume	Fraction
3 mL crude extract 2 mL distilled water	1
2 mL 90 % distilled water/10 % methanol 2 mL 80 % distilled water/20 % methanol	2
2 mL 70 % distilled water/30 % methanol 2 mL 60 % distilled water/40 % methanol	3
2 mL 50 % distilled water/50 % methanol	4
2 mL 40 % distilled water/60 % methanol	5
2 mL 30 % distilled water/70 % methanol	6
2 mL 20 % distilled water/80 % methanol	7
2 mL 10 % distilled water/90 % methanol	8
4 mL 100 % methanol	9

4.2.2 Blood source

Human erythrocyte (Group A), obtained from Blutspenderdienst (Blood Donor Service) Oldenburg, was transferred with cooling condition to the laboratory in SAG-mannitol solution and stored at 4°C. ELA assay buffer (Eschbach, 2001) was composed of 150 mM NaCl, 3.2 mM KCl, 1.25 mM $MgSO_4 \cdot 7H_2O$, 3.75 mM $CaCl_2$, and 12.2 mM TRIS base, the pH was adjusted to 7.4 with HCl. The erythrocytes were washed with this assay buffer twice at 4500 rpm for 5 min at 15°C, and re-suspended. Cell numbers were determined by erythrocytometry.

4.2.3 Scanning experiment

A defined amount of human erythrocytes (7×10^6 cells mL^{-1}) was disintegrated completely with an ultrasonic disintegrator (Soniprep 150) with ice-cooling. 1.5 mL

lysed erythrocytes solution (10.5×10^6 cells) was scanned from 350 to 700 nm with a UVIKON 930 photometer to confirm the maximal absorption wavelength of haemoglobin being 414 nm (Fig. 6).

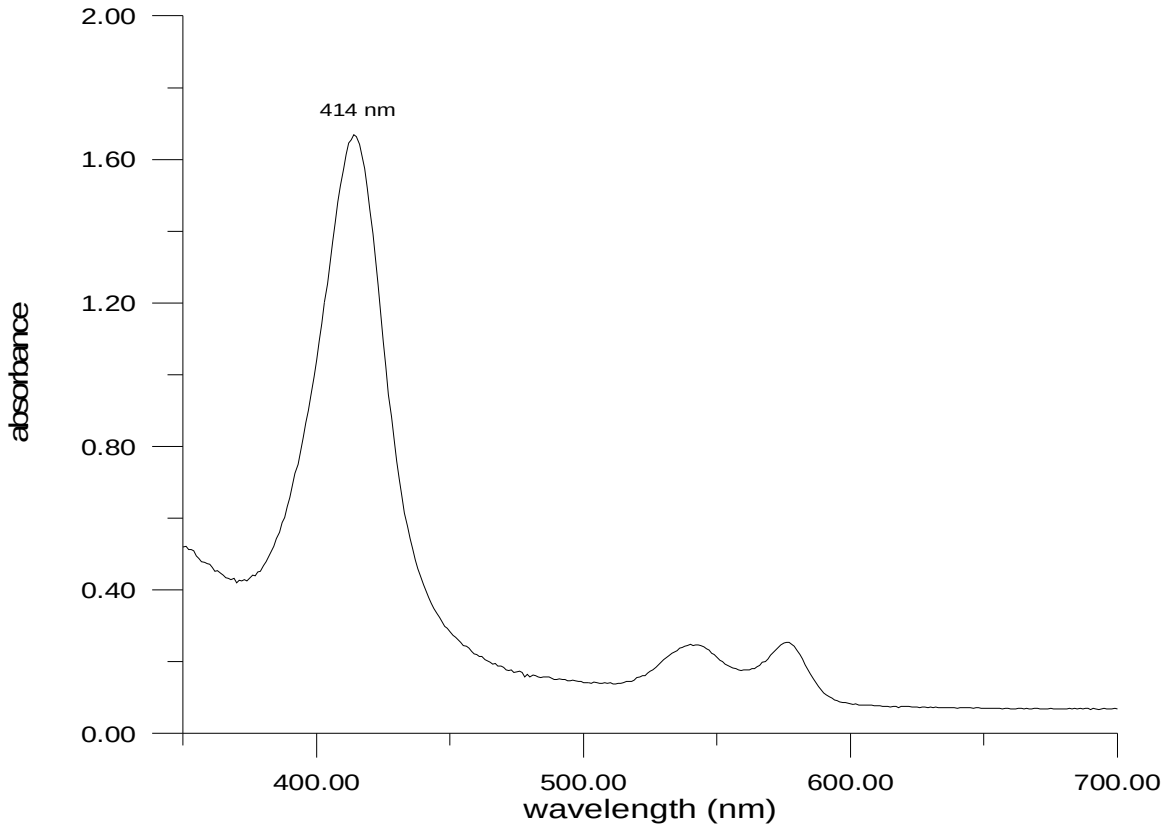


Fig. 6. Photometric scan (350-700 nm) of completely lysed human erythrocytes (7×10^6 cells mL^{-1})

4.2.4 Standard curve experiment

To determine the linear range, the lysed erythrocyte solution ($X = 7\times10^6$ cells mL^{-1}) was diluted with the assay buffer to yield 6 concentrations: X, 0.8X, 0.6X, 0.4X, 0.2X, 0. They were then measured at the maximal absorption wavelength of haemoglobin.

4.2.5 Separation of haemolytic fractions with SPE cartridges (C-18)

Superclean^{TM} disposable SPE (solid phase extraction, Supelco, Inc.) 3 mL cartridge (C-18) and a vacuum manifold were used. Crude extract (3 mL, stored at –18 °C) from stationary phase algae (ca. 2,800,000 cells/mL extract) was injected into the cartridge which had been conditioned before with 2 mL methanol. The elution scheme is outlined in Table 5. The flow rate was adjusted to about 0.5 drops/s. The different fractions obtained were evaporated to dryness at 40°C and re-dissolved in 2 mL assay buffer solution for the erythrocyte lysis assay in duplicate. The background absorption of every fraction was also measured at the maximal absorption wavelength of haemoglobin to correct for possible interferences.

4.2.6 Erythrocyte lysis assay of SPE fractions

12 mL plastic centrifuge tubes were used as the reaction vessels. 1 mL SPE fractions (0,1.....11) were incubated separately with 1.5 mL erythrocyte solutions (7×10^6 cells mL $^{-1}$) at 15°C for 24 h. 1mL ELA buffer together with 1.5 mL erythrocyte solution was taken as the negative control, the positive control was composed of 1 mL ELA buffer and 1.5 mL completely lysed erythrocytes. After 24 h incubation, reaction vessels were centrifuged at 4500 rpm for 5 min at 15°C, and 1.5 mL of each supernant were then transferred and measured at the maximal absorption wavelength of haemoglobin in the photometer (UVIKON 930).

Table 5. Elution scheme of haemolytic fractions with SPE cartridge (C-18)

Eluent composition/volume	Fraction
3 mL crude extract	0
2 mL distilled water	1
2 mL 90 % distilled water/10 % methanol	2
2 mL 80 % distilled water/20 % methanol	3
2 mL 70 % distilled water/30 % methanol	4
2 mL 60 % distilled water/40 % methanol	5
2 mL 50 % distilled water/50 % methanol	6
2 mL 40 % distilled water/60 % methanol	7
2 mL 30 % distilled water/70 % methanol	8
2 mL 20 % distilled water/80 % methanol	9
2 mL 10 % distilled water/90 % methanol	
1.5 mL 100 % methanol	10
3.5 mL 100 % methanol	11

5. Development of a HPLC Separation Method for Fibrocapsins

5.1 HPLC separation method for fibrocapsins

The HPLC system consisted of the following parts: a degassing unit, a Constametric 4100 pump and an AS 100 auto-sampler (all Thermo Separation Products). Detection of separated components was accomplished by a photodiode array detector (Merck PDA L7450) which measured absorption between 200 and 500 nm. A Phenomenex column was used (Aqua, 5 µm, C18, 250 × 4.6 mm). The chromatograms were evaluated by D-7000 HPLC-System Manager software (Merck). All analyses were carried out at room temperature. Isocratic elution was employed using 21 % water containing 0.05 % (v/v) trifluoroacetic acid and 79 % acetonitrile. The flow rate was 1.0 mL/min, and the injected sample was 20 µL concentrated SPE toxic fractions mixture.

5.2 HPLC experiments with fibrocapsins and brevetoxins

Brevetoxin PbTx-2 and PbTx-3 were kindly provided by Alexander Rühl (Department of Food Chemistry, University of Jena). 20 µL crude extract of *F. japonica* with added PbTx-2 and PbTx-3 were injected to the HPLC system, following the same HPLC method as described above (5.1).

6. Haemolytic Activity of Fibrocapins

The mixture of concentrated SPE toxic fractions was subjected to HPLC separation. The elution was repeated many times to collect the three main fibrocapsins according to their retention times. The collected individual fibrocapsins were pooled, evaporated to dryness at 40°C and then weighed. Erythrocyte lysis assay was performed to test the toxicity of the obtained fibrocapsins. Each compound was also re-analysed by HPLC to verify its purity.

40 µg each of fibrocapsins 1, 2 and 3 were re-dissolved separately in 2 mL ELA buffer (concentration: 20 µg/mL) for erythrocyte lysis assay in duplicate. 12 mL plastic centrifuge tubes were used as the reaction vessels. 1 mL fibrocapsin solutions (Fj1, 2 and 3) were incubated separately with 1 mL erythrocyte solution (4.5×10^6 cells mL^{-1}) at 15°C for 24 h, the final concentration of each fibrocapsin in the test solution being 10 µg/mL. 1 mL ELA buffer together with 1 mL erythrocyte solution was taken as the negative control, the positive control was of 1mL ELA buffer and 1 mL completely lysed erythrocyte solution. After 24 h incubation, reaction vessels were centrifuged at 4500 rpm for 5 min at 15°C, and 1.2 mL of each supernant were transferred and measured at the maximal absorption wavelength of haemoglobin (UVIKON 930).

7. HPLC-ESI-MS and ESI-MS-MS Experiments with Fibrocapsins

The three pure fibrocapsins (Fj1, Fj2 and Fj3) isolated by HPLC were measured mass spectrometrically. All samples were diluted in 80 % acetonitrile, and several drops of ammonium hydroxide solution (0.25 % NH_4OH in acetonitrile) were added. All organic chemical reagents used here were from Merck: gradient grade acetonitrile, 25 % ammonia solution (GR for analysis), 98-100 % formic acid (GR for analysis). De-ionised water was UV-treated to remove organic impurities.

7.1 HPLC-ESI-MS experiments with fibrocapsins

The HPLC-system consisted of two Perkin Elmer series 200 micro LC pumps, a Perkin Elmer series 200 autosampler, a Separations 785A programmable absorbance detector (Alltech), a pre-column and an analytical column (Alltima, 5 µm, C18, 150 x 2.1 mm). All analyses were carried out at room temperature. A gradient was employed, starting with 12 min 15 % eluent A (water with 0.1 % formic acid) and 85 % eluent B (acetonitrile with 0.1 % formic acid), followed by 5 % A and 95 % B, held at it for 16 min. The chromatograms were recorded by a chart recorder (Kipp & Zonen). The flow rate was 0.2 mL/min, with an injection volume of 50 µL.

Preliminary experiments showed fibrocapsins to easily lose proton. Mass spectrometry was therefore performed in the negative ion detection mode for which a post-column base condition (0.25 % NH_4OH in acetonitrile) was supplied at 100 µL/min with a syringe pump (Harvard Apparatus 22) which was also connected to the MS system. The MS system used was a PE SCIEX API 3000 triple quadrupole LC/MS/MS mass spectrometer equipped with a pneumatically assisted electrospray ionisation interface. The ionization voltage was set to –4.5 kV. Nitrogen was employed as both nebulising and drying gas (heated to 450°C, 2 bar, 2L/min). Ion

detection was performed in the negative mode under the energy conditions OR = –15 V and RNG = -150 V. The scan data were acquired with a 0.1 u step width and 0.1 ms dwell time.

7.2 HPLC-ESI-MS experiments with crude extract

The crude extract of *F. japonica* was monitored under both positive and negative ion mode to detect whether the size class of brevetoxins, i.e. ions with *m/z* of around 860 or 900, can be found.

7.3 ESI-MS-MS experiments with fibrocapsins

Three pure compounds, fibrocapsin 1, 2 and 3, were injected directly into an API 3000 triple quadrupole MS/MS mass spectrometer with a syringe infusion pump (Harvard Apparatus 22) at 10 µL/min. Ion detection was performed in the negative ion mode under the energy conditions OR = –41V and RNG = -130V. The $[M-H]^-$ precursor masses 275.1 (Fj1) , 301.3 (Fj2) and 303.4 (Fj3) were selected separately. ESI-MS/MS spectra were acquired with nitrogen as the collision gas. All scan data were acquired with 0.1 u step width and 2 ms dwell time.

8. IR Experiment with Fibrocapsins

Three pure fibrocapsins (Fj1, Fj2 and Fj3) isolated by HPLC were measured by infrared spectrometry. All samples were dissolved in 100 % methanol (HPLC grade). Several drops of this solution were taken and dripped on a sodium chloride plate which was then air dried. The plate was then measured in the IR spectrometer (ATI Mattson Genesis Series FTIR™).

9. GC-MS and GC-HRMS Experiments with Fibrocapsins

The isolated compounds Fj1 (170 µg), Fj2 (250 µg) and Fj3 (140 µg) weredissolved separately in 200 µL CH_2Cl_2, then treated ultrasonically for 15 min. 100 µL Fj1, 70 µL Fj2 and 100 µl Fj3 solution were mixed with 50 µL *N*-methyl-*N*-trimethylsilyltrifluoroacetamide solution (MSTFA, CS-Chromatographie Service GmbH) and kept at 80°C for 1 h.

9.1 GC-MS experiments with fibrocapsins

GC-MS analyses of the fibrocapsin-TMS derivatives were performed using an HP 5890 Series II GC (Avondale, PA) coupled with a Finnigan MAT SSQ 710B mass spectrometer (San Jose, CA). The GC was equipped with a KAS 3 injector (Gerstel) and a J&W Scientific DB-5HAT fused silica capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness). Helium was used as carrier gas. The samples were injected at an oven temperature of 60°C. After 1 min, the temperature was raised to 300°C at 3°C/min and kept then at this temperature for 50 minutes.

9.2 GC-HRMS experiments with fibrocapsins

GC-HRMS analyses applied a Finnigan MAT 95 Q mass spectrometer with ICIS 8.2.1 and ICL 10.0 software. The GC equipment was similar to that described above, helium was also used here as carrier gas, but with an HP-1MS column (60 m × 0.25 mm ID, 0.25 µm film thickness). The samples were injected also at an oven temperature of 60°C. After 2 min the temperature was raised to 150°C at 10°C/min , then to 300°C at a 4°C/min and kept there for 10 min.

10. NMR Experiments with Fibrocapsins

The dried haemolytic compounds Fj1 (1.6 mg), Fj2 (2.0 mg) and Fj3 (1.2 mg), obtained by erythrocyte lysis assay guided HPLC purification (see 5.1), were dissolved in methanol-d_4. NMR spectra were recorded from these solutions on an AVANCE 500 NMR spectrometer (Bruker; ^{1}H 500.1 MHz; ^{13}C 125.8 MHz). The measurements were carried out at 300 K. The chemical shifts are reported on the δ-scale in ppm and were referred to the internal standard, signals of non-deuterated methanol (^{1}H: 3.30 ppm) or signals of deuterated methanol (^{13}C: 49.00 ppm). Coupling constants *J* are given in Hz.

11. HPLC and NMR Experiments with Standards

Two standards, all-*cis*-5,8,11,14,17-eicosapentaenoic acid (EPA, C20:5ω3) and all-*cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid AA, C20:4ω6), obtained from Sigma-Aldrich were dissolved in 100 % methanol and stored at -18°C before measurements.

11.1 HPLC experiments with standards

One crude extract sample was measured by HPLC (see 5.1) twice with and without addition of these two standards.

11.2 NMR experiments with standards

After drying in a nitrogen stream, 3.2 mg EPA and 7.8 mg AA were used for the NMR measurements (see 10). The spectra were then compared to those of fibrocapsin 2 and 3.

12. Toxicity Biotests with Standard Compounds

Erythrocyte lysis assay (ELA), bioluminescence inhibition and *Artemia salina* biotests were carried out to test the toxicity of the two standards EPA and AA (at 10 µg/mL each). The biotest methods followed the techniques outlined above (see 6, 3.1 and 2.1).

III. RESULTS

5. Cultivation and Growth Law for *Fibrocapsa japonica*

1.1 Fibrocapsa japonica *algal cells*

The culture displays a yellowish-brown colour. The algal cells are ovoid (Fig. 7) and should have two flagella, one pointing forward, the other pointing backward, although flagella are not so clearly recognizable from Fig. 7. The mean length is 25.9 ± 3.13 µm, the mean width is 23.6 ± 2.33 µm.

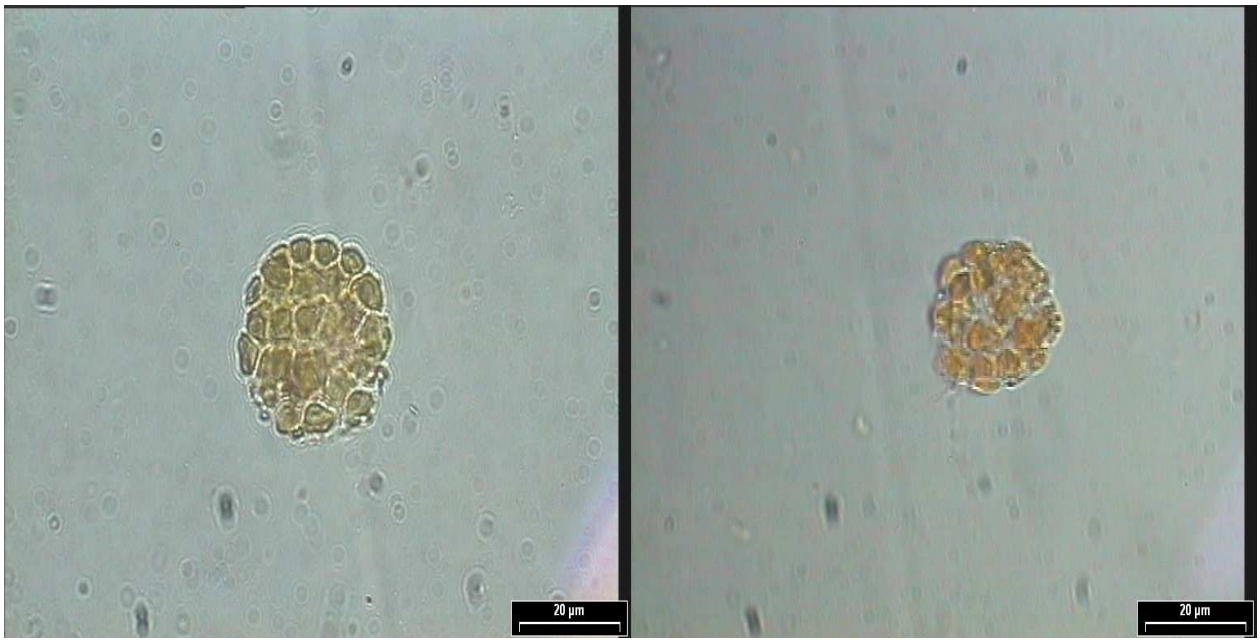


Fig. 7. Light micrographs of *Fibrocapsa japonica* alga cells (× 1,000)

1.2 Growth law for Fibrocapsa japonica

Two growth curves of *Fibrocapsa japonica* culture were obtained with the two monitoring methods (Fig. 8). They clearly show four growth phases: a very short lag phase, a logarithmic phase with a dramatically increasing growth rate, a stationary phase with a very slow growth rate and a senescence phase in which more and more

cells die. A good correlation was demonstrated (Fig. 9) between cell density and turbidity during the growth phase with the following correlation equation:

$$Y = 1268.8\ X + 875.1$$

$$R^2 = 0.9065$$

with X: turbidity [NTU] and Y: cell density [n/mL]

NTU data can thus be easily transferred to cell density values since turbidimetry was used as the major counting method in most experiments of this work.

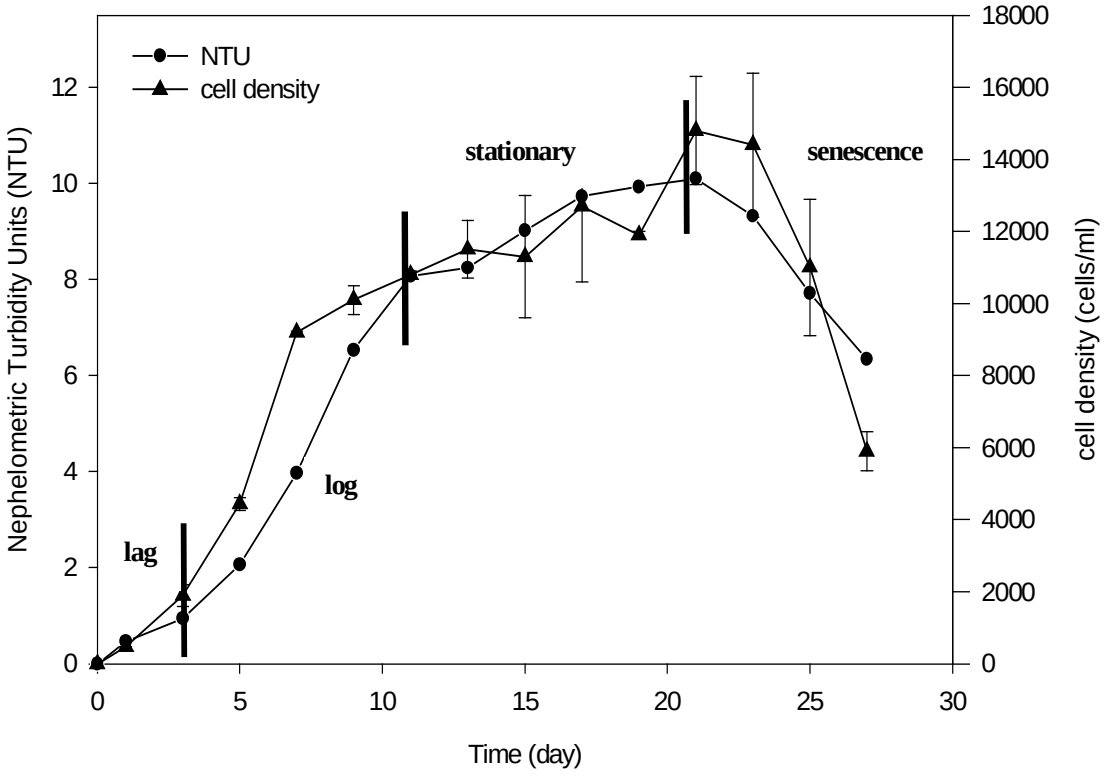


Fig. 8. Growth curves of *Fibrocapsa japonica* culture obtained by different monitoring methods

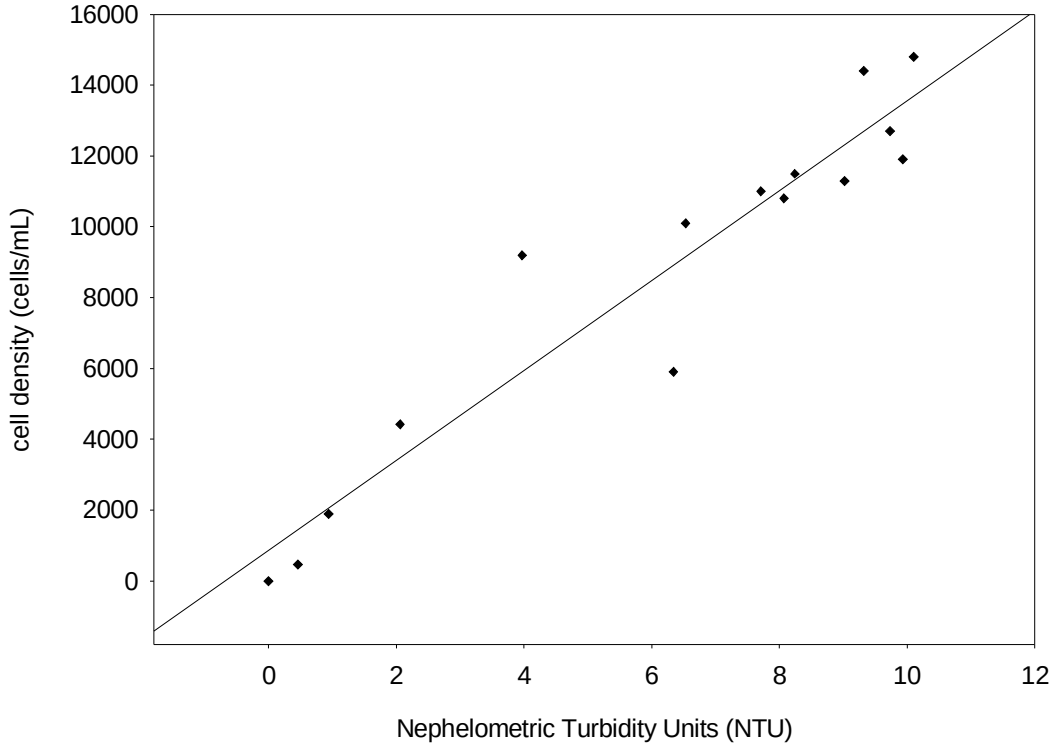


Fig. 9. Correlation between NTU and cell densities of *Fibrocapsa japonica*

2. Toxicity Biotests with Algal Cells and Culture Filtrate of *Fibrocapsa japonica*

Algal cells in the stationary phase have toxic effects on freshly hatched *Artemia salina* nauplii (Fig. 10). As nauplii in this developmental stage are actively swimming, the sinking percentage was regarded as an appropriate indicator for the physiological state of *A. salina* nauplii. During the test abnormal movements were observed for organisms at the bottom of test beakers including swimming inharmoniously, vibrating violently to and fro or rotating around one fixed point extremely fast. For the filtrate no death or abnormal phenomena were observed, indicating that the toxic compounds are not released to the medium.

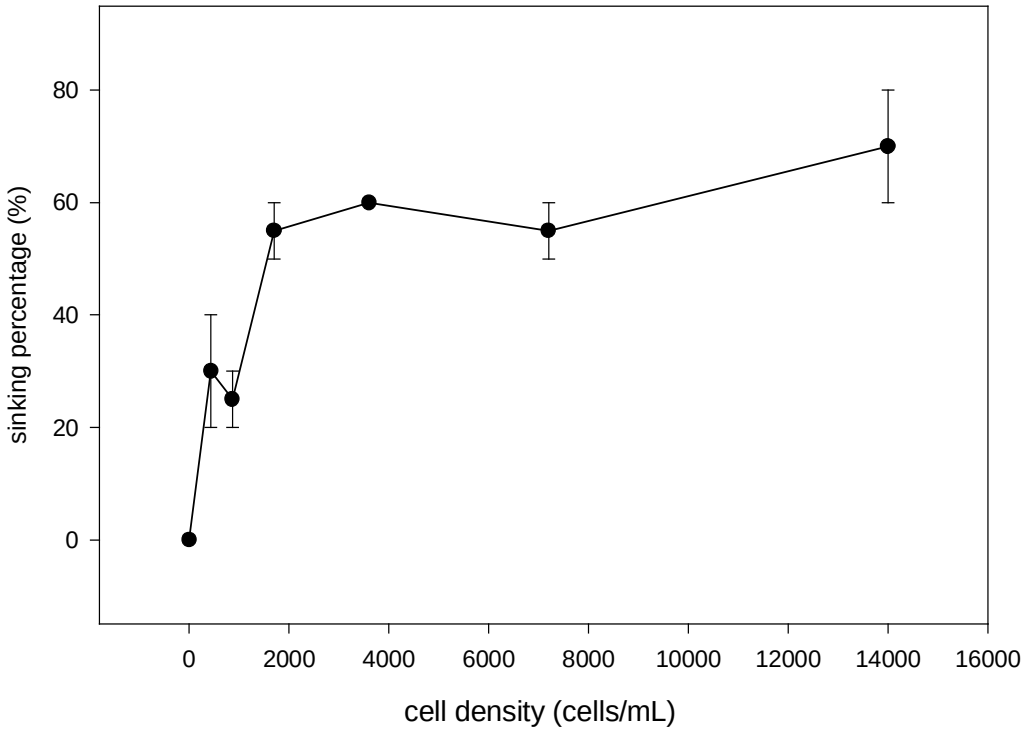


Fig. 10. 12 h effect of *Fibrocapsa japonica* algal cells (stationary phase) on *Artemia salina* nauplii

3. Toxicity Biotests with Crude Extract of *Fibrocapsa japonica*

The crude extract of *Fibrocapsa japonica* also had pronounced toxic effects on *A. salina* not only in the high concentration series but the low concentration series as well (Fig. 11). The organisms that sank to the bottom of the test beaker were differentiated into two parts, those that sank and stayed dead on the bottom (dead), and those which were still alive, but inactive or exhibiting abnormal behaviours (inactive). In the low concentration test series the inactive *A. salina* were predominant, whereas the dead organisms took the dominant position in the high concentration series. The total sinking percentage increased with the increasing percentage of crude extract in both tests.

Similar results were obtained in the bioluminescence inhibition test too (Fig. 12). The bioluminescence test is a sensitive method for monitoring the effects of toxins which directly affect the vitality of luminescent bacteria. Toxic substances inhibit the luminescence intensity through disturbing the normal conditions of cell wall, cell membrane, electron transport system, enzyme and cell plasma components (Lange 1994).

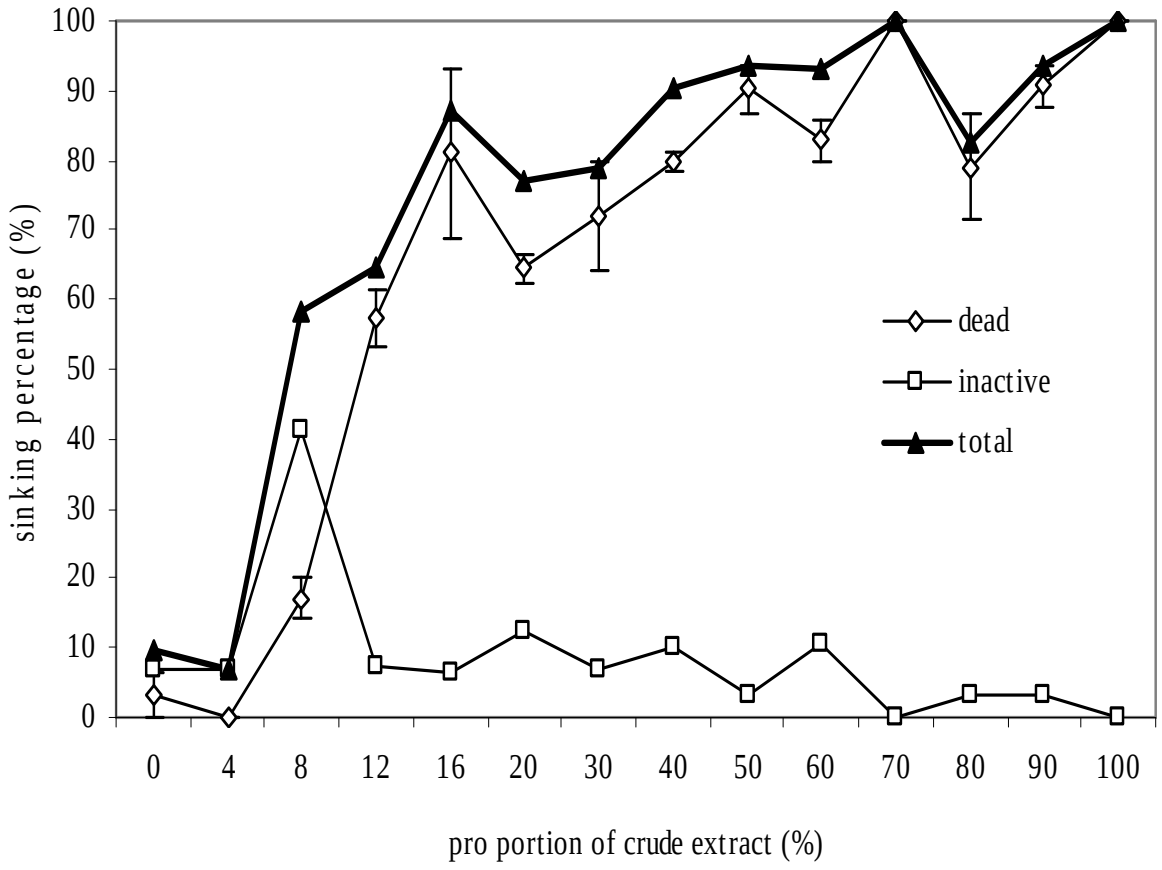


Fig. 11. 24 h effect of *Fibrocapsa japonica* crude extract on *Artemia salina* nauplii

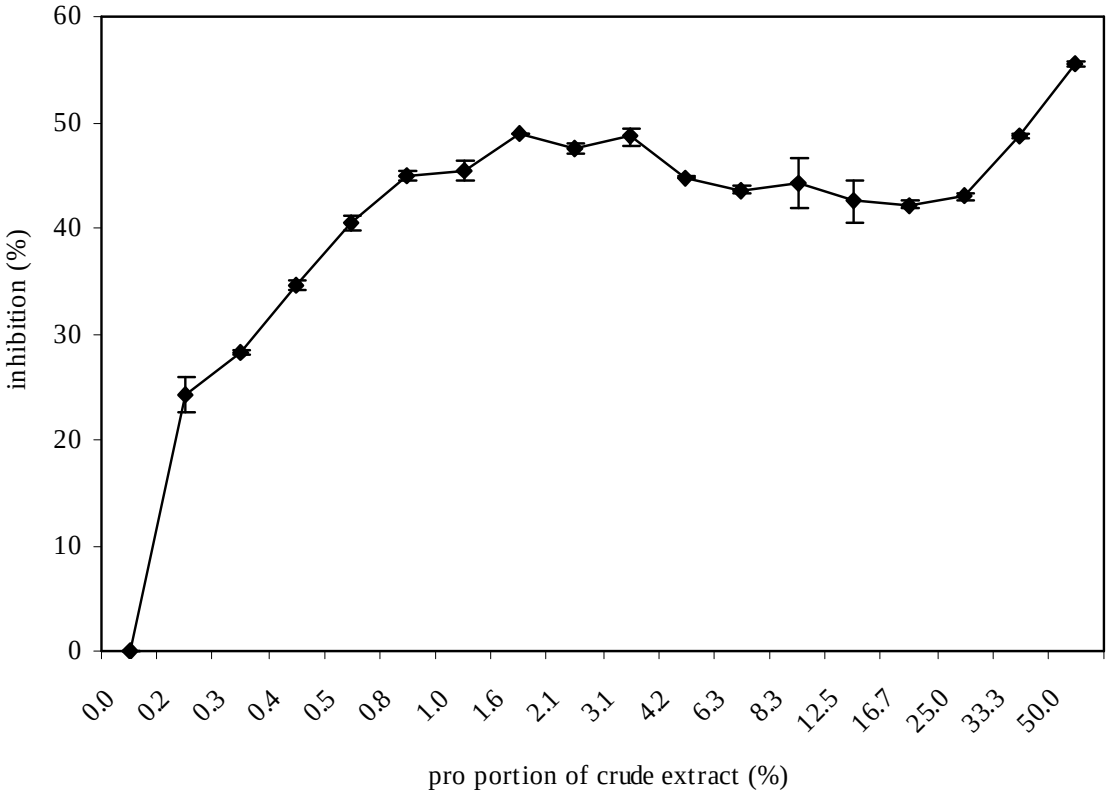


Fig. 12. Bioluminescence inhibition test of *Fibrocapsa japonica* crude extract

4. Toxicity Biotests with SPE Fractions of *Fibrocapsa japonica* Crude Extract

4.1 Separation of toxic fractions with SPE cartridges (C-18) guided by Artemia salina *and bioluminescence inhibition tests*

Fractionation of the crude extracts through C_{18} cartridge resulted in three main toxic fractions identified by both *Artemia salina* (Fig. 13) and bioluminescence inhibition tests (Fig. 14). In the bioluminescence inhibition test, it has to be borne in mind that samples with a bioluminescence inhibition of < 20 % are normally considered non-toxic (Lange 1994). Three toxic fractions (fractions 5, 6 and 7) were eluted, respectively, by 60 % , 70 % and 80 % aqueous methanol.

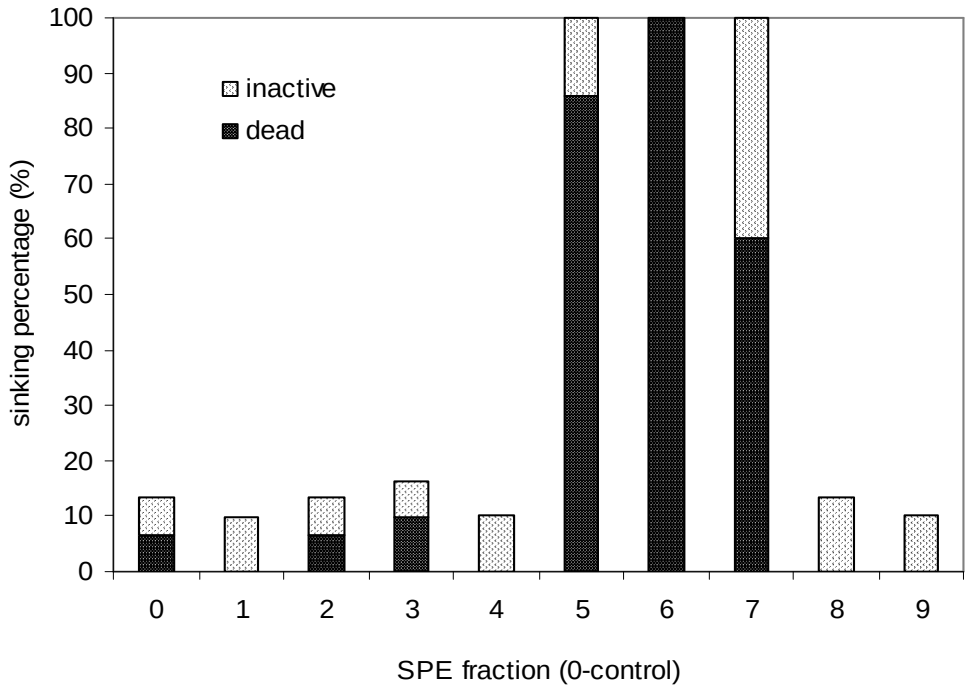


Fig. 13. 24 h effect of SPE fractions of *Fibrocapsa japonica* crude extract on *Artemia salina* nauplii

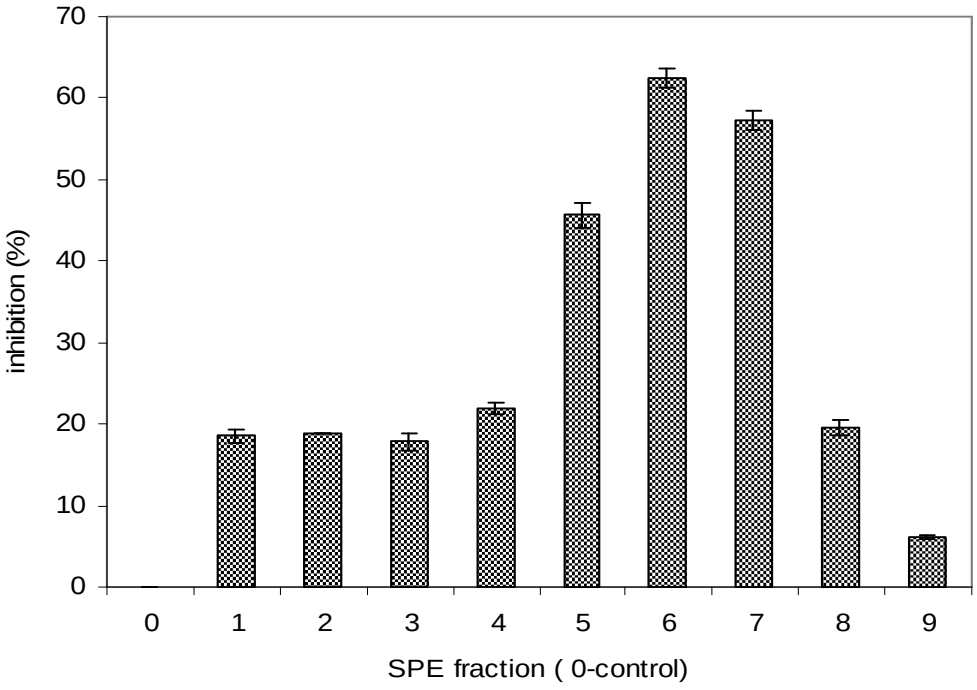


Fig. 14. Bioluminescence inhibition test of SPE fractions of *Fibrocapsa japonica* crude extract

4.2 Separation of toxic fractions with SPE cartridges (C-18) combined with erythrocyte lysis assay

4.2.1 Standard curve experiment

The amount of haemoglobin released by completely lysed human erythrocytes up to a concentration of 7×10^6 cells mL $^{-1}$ showed a good correlation with the corresponding absorbance at 414 nm, i.e. linearity over this range was investigated (Fig. 15).

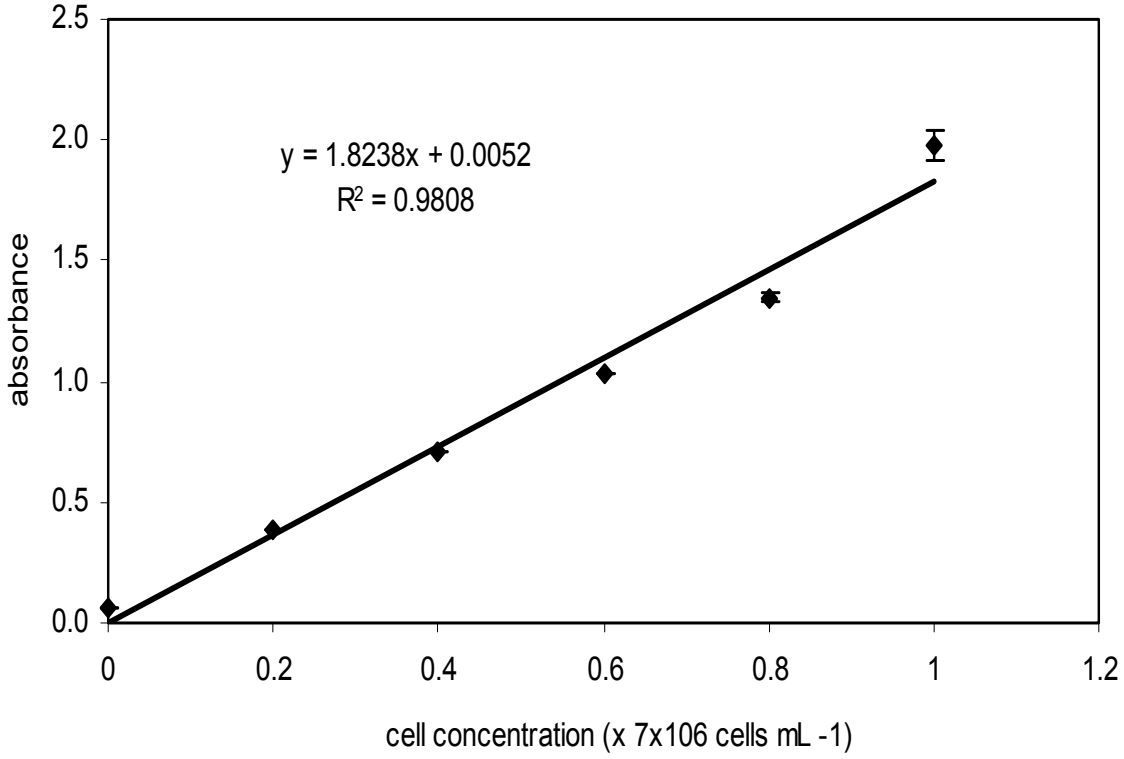


Fig. 15. Standard absorption curve of lysed erythrocytes

4.2.2 Erythrocyte lysis assay of SPE fractions

The absorption of the ELA buffer (blank, neg), the 11 SPE fractions and completely lysed erythrocytes (pos) was measured at 414 nm before incubation with human blood to obtain background absorbance values. Blank (neg) and SPE fractions had only low

absorption values compared to completely lysed erythrocytes (pos). Background values were < 20 % (Fig. 16) and actual data have therefore not been interfered.

After 24 h incubation with human erythrocytes, fractions 7, 8 and 9 showed very strong haemolytic effects (100 %) whereas fractions 5 and 6 exhibited weaker effects (Fig. 16). Fractions 5 to 9 were eluted by 40 %, 50%, 60%, 70 % 80 % and 90 % aqueous methanol, respectively. The photometric results were also consistent with the observations by human eyes. A very clear red precipitate (accumulation of erythrocytes) was seen at the bottom of the tubes without any haemolytic effect after centrifugation (e.g. negative control and all fractions except fractions 5 to 9), whereas tubes with haemolytic effects presented either clear red solutions (e.g. fractions 7 to-9 and positive control) or lighter red solutions with smaller red precipitates (e.g. fractions 5 and 6).

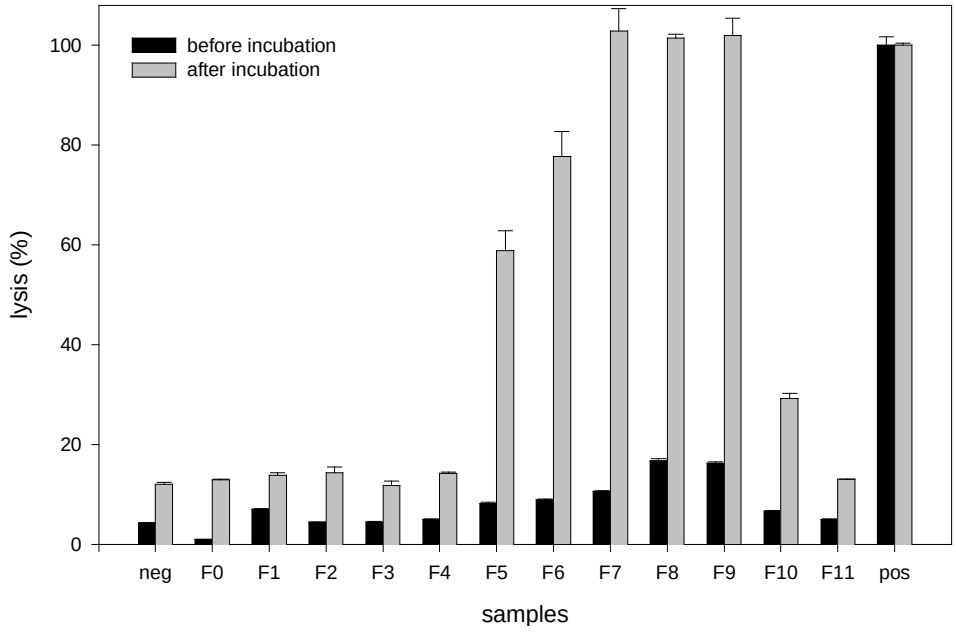


Fig. 16. Background absorption of samples at 414 nm before incubation with human erythrocytes and haemolytic effects of samples after 24 h incubation with human erythrocytes

5. Development of a HPLC Separation Method for Fibrocapsins

5.1 HPLC separation method for fibrocapsins

Concentrated toxic fractions F5 to F9 were mixed and then subjected to high performance liquid chromatography. These three main peaks obtained were tentatively named fibrocapsins 1, 2 and 3 (Fig. 17). Their corresponding retention times were 13.25, 16.80 and 24.32 min.

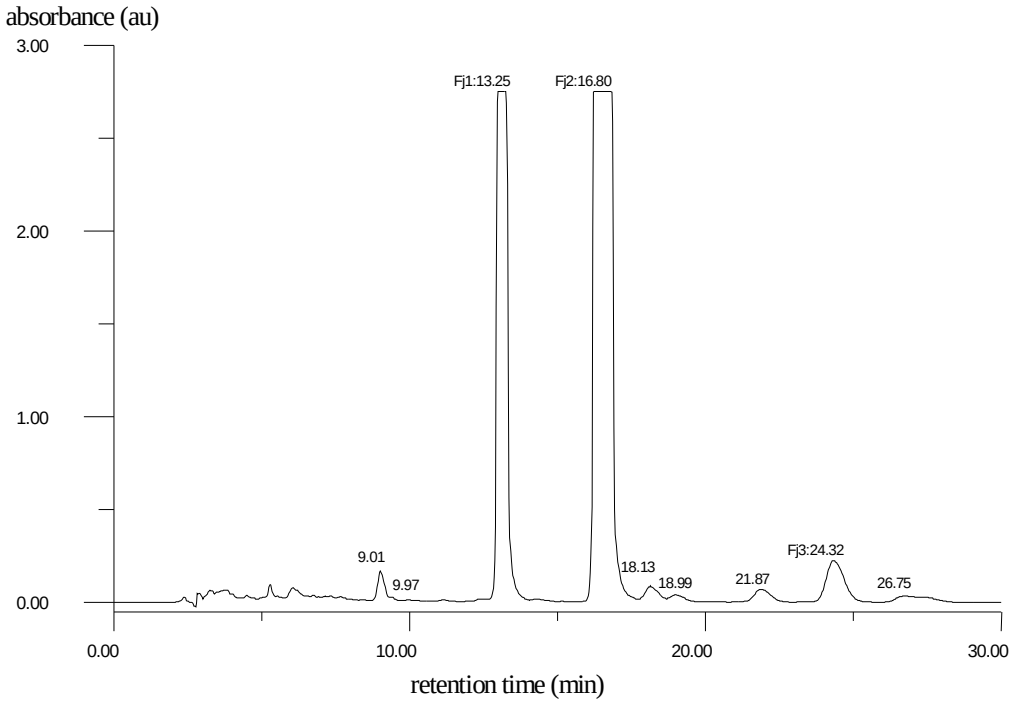


Fig. 17. HPLC chromatogram of fibrocapsins (detection wavelength: 210 nm)

5.2 HPLC experiments with fibrocapsins and brevetoxins

The brevetoxins PbTx-2 and PbTx-3 have the same retention time as Fj1 and an unidentified compound Fj? (Fig. 18), the retention time of Fj? is 9.01 in Fig. 17. Nevertheless, from the DAD contour plot it is evident that fibrocapsins absorb only below 220 nm, whereas brevetoxins have still absorption at 230 nm. The chromatograms also confirm that some brevetoxins do have same retention times with

some fibrocapins which led Khan et al. (1996c) to propose the presence of brevetoxins in *F. japonica*.

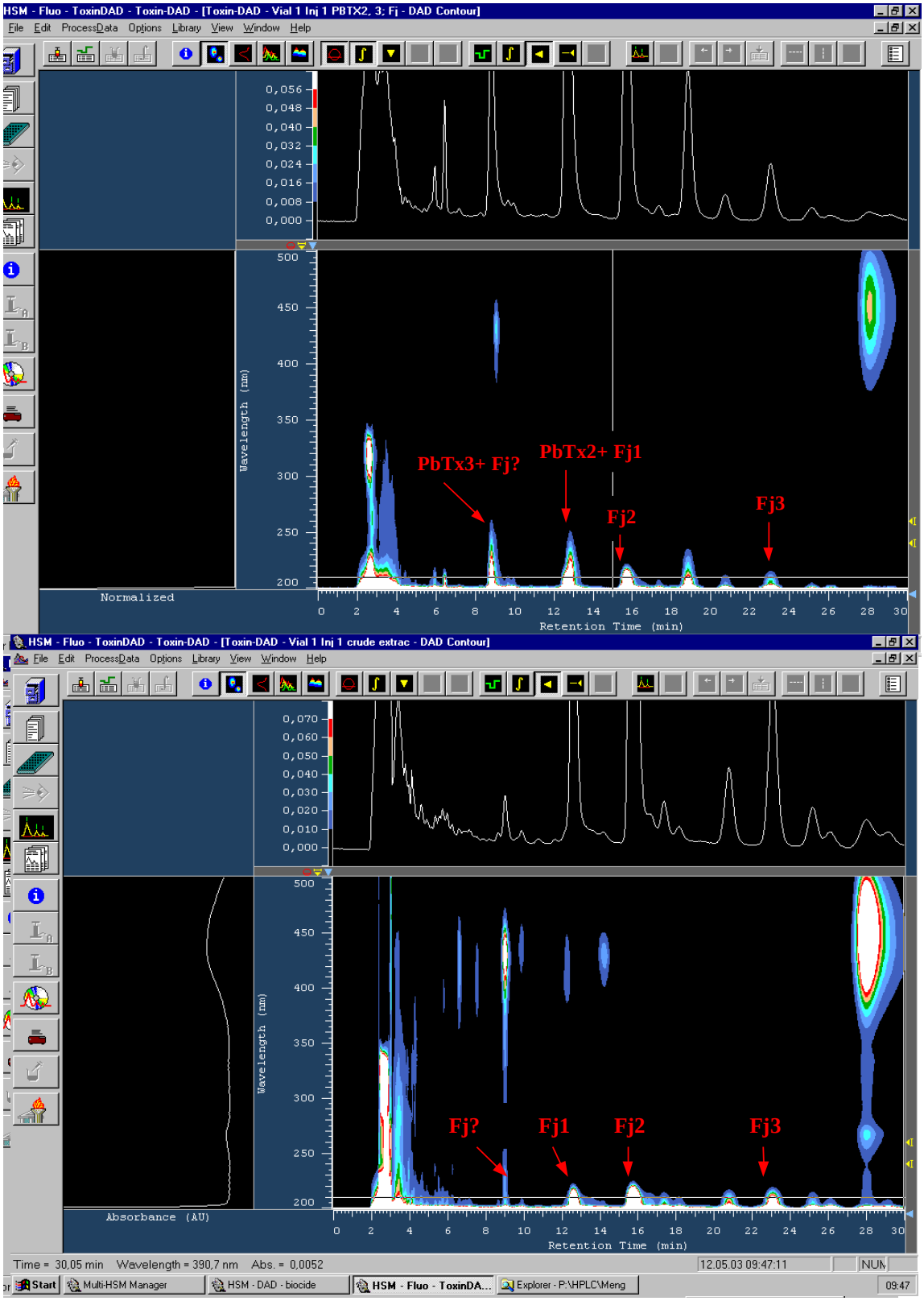


Fig. 18. HPLC-DAD contour plots for fibrocapsins and brevetoxins

6. Haemolytic Activity of Fibrocapsins

Rechromatography proved each fibrocapsin to be relatively pure. In the erythrocyte lysis assay (Fig. 19), fibrocapsins 1 to 3 (10 µg/mL each) showed very strong haemolytic effects on human erythrocytes reaching almost complete lysis.

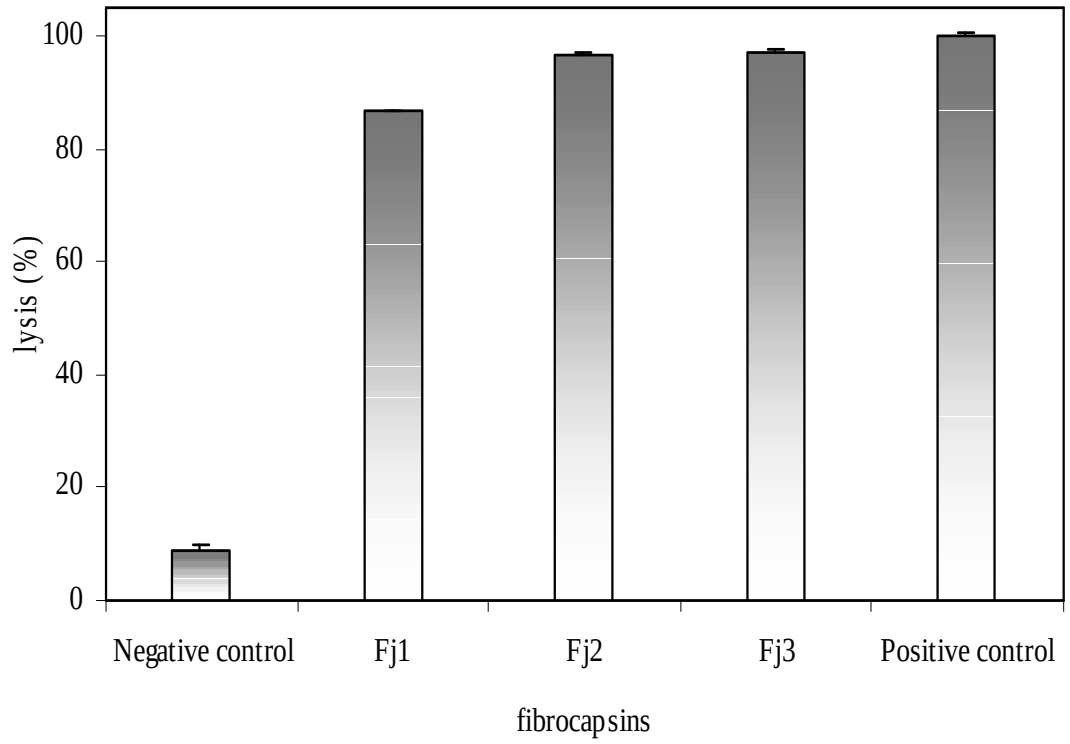


Fig. 19. Haemolytic effects of fibrocapsins on human erythrocytes after 24 h incubation (each fibrocapsin: 10 µg mL^{-1})

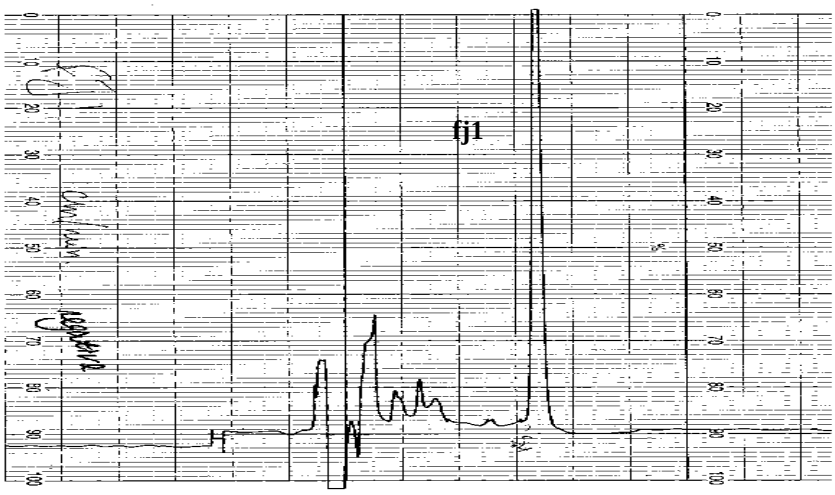
7. HPLC-ESI-MS and ESI-MS-MS Experiments with Fibrocapsins

7.1 HPLC-ESI-MS data for fibrocapsins

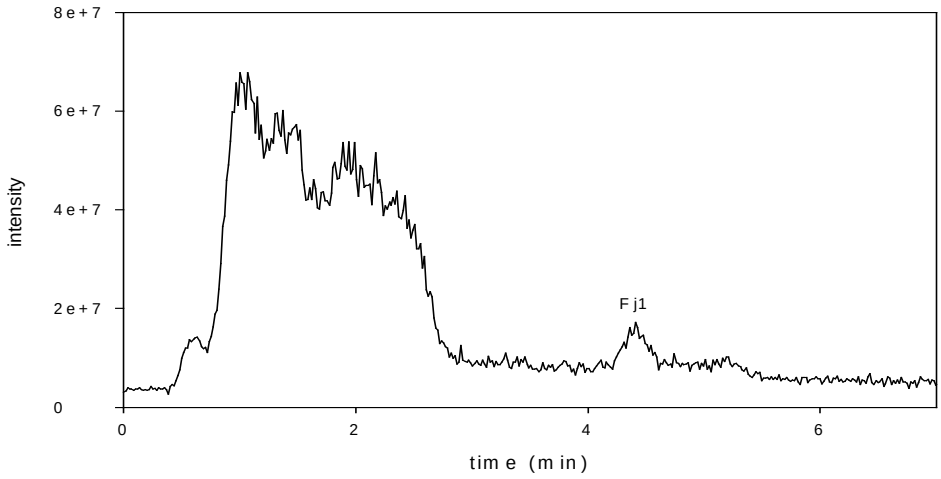
Fj1 (negative mode): *m/z* $[M-H]^-$ 275, $[M+HCOO]^-$ 321, $[2M-H]^-$ 551 (Fig. 20).

Fj2 (negative mode): *m/z* $[M-H]^-$ 301, $[M+HCOO]^-$ 347, $[2M-H]^-$ 604 (Fig. 21).

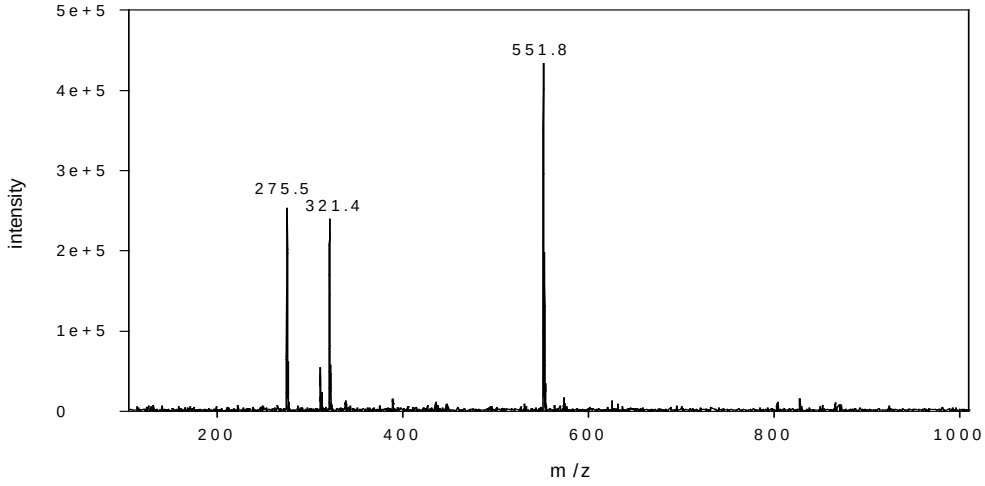
Fj3 (negative mode): *m/z* $[M-H]^-$ 303, $[M+HCOO]^-$ 349, $[2M-H]^-$ 607 (Fig. 22).



HPLC chromatogram of fibropcasin 1 (detection 210 nm)

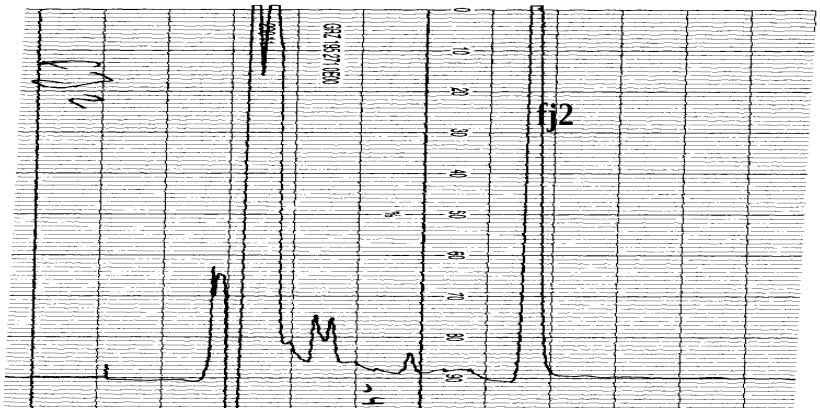


Chromatogram of total ion current of fibrocapsin1 in negative mode

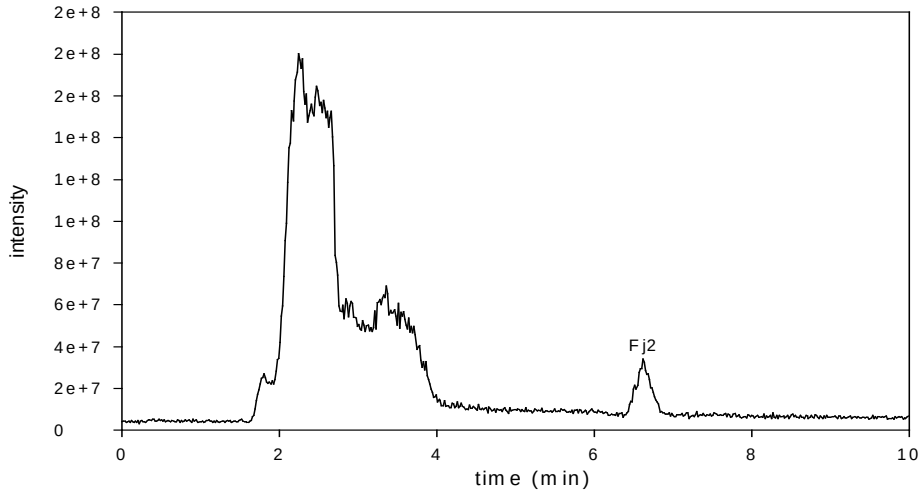


ESI - MS spectrum of fibrocapsin1 in negative ion mode

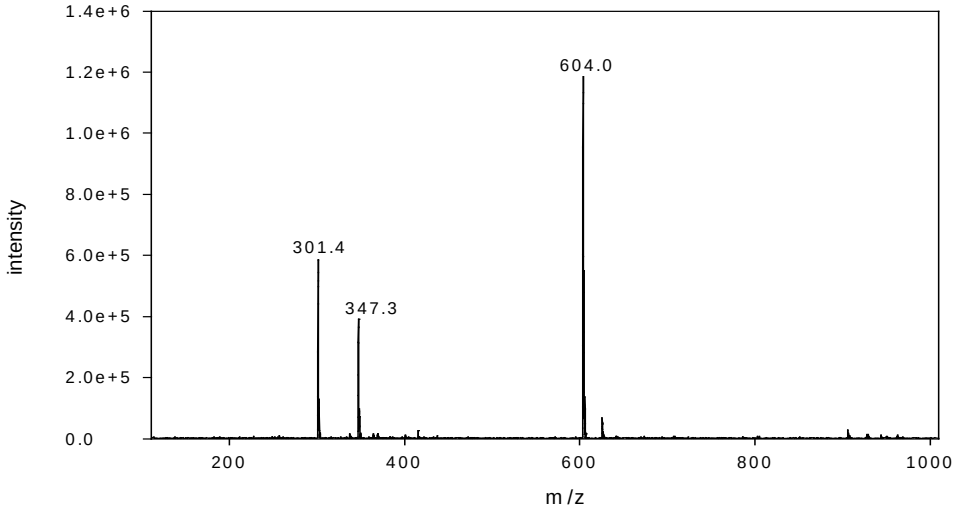
Fig. 20. HPLC-ESI-MS experiment results for fibrocapsin 1



HPLC chromatogram of fibropcasin 2 (detection 210 nm)

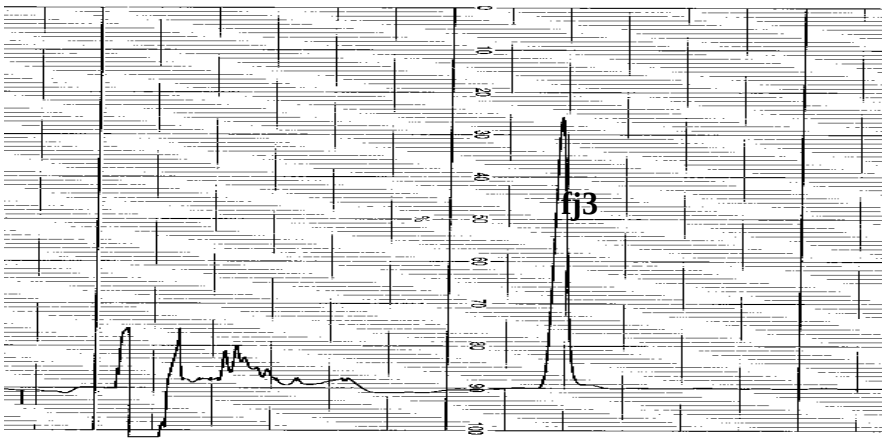


Chromatogram of total ion current of fibrocapsin2 in negative mode

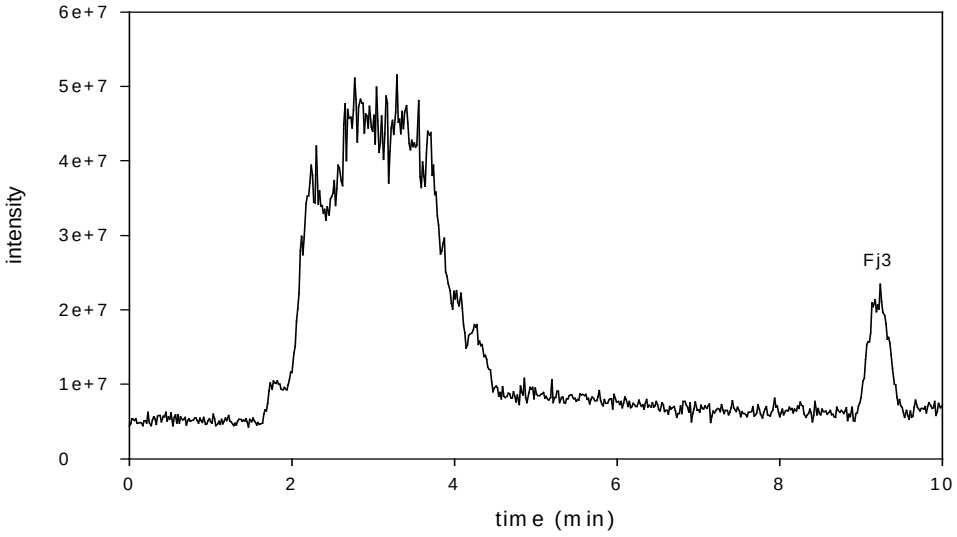


ESI - MS spectrum of fbrocapsin2 in negative ion mode

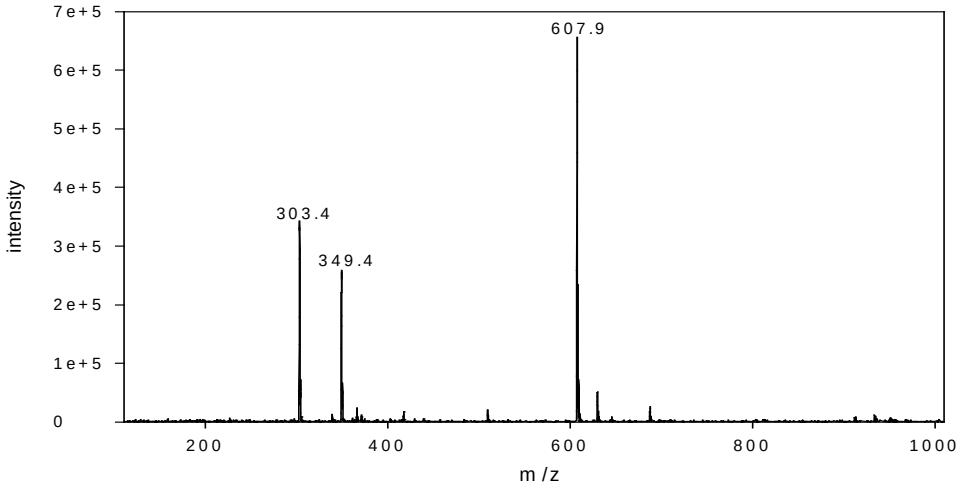
Fig. 21. HPLC-ESI-MS experiment results for fibrocapsin 2



HPLC chromatogram of fibrocapsin 3 (detection 210 nm)



Chromatogram of total ion current of fibrocapsin3 in negative mode



ESI (HPLC-MS) spectrum of Fibrocapsin1 in negative ion mode

Fig. 22. HPLC-ESI-MS experiment results for fibrocapsin 3

7.2 HPLC-ESI-MS experiments with crude extract

The crude extract of *F. japonica* did not contain components in the size class of brevetoxins, i.e. no ions with *m/z* of around 860 or 900 were found.

7.3 ESI-MS-MS experiments of fibrocapsins

For all three compounds similar fragment ions $[M-H]^-$, $[M-H-H_2O]^-$ and $[M-H-CO_2]^-$ were observed (Figs. 23 - 25). The loss of H_2O and CO_2 prove presence of one carboxyl group in each fibrocapsin.

ESI-MS-MS of Fj1: *m* / *z* [M-H, 100 (relative intensity)]$^-$ 275.1, [M-H-H_2O, 0.9]$^-$ 257.3, [M-H-CO_2, 7]$^-$ 231.2.

ESI-MS-MS of Fj2: *m* / *z* [M-H, 100]$^-$ 301.3, [M-H-H_2O, 0.4]$^-$ 282.9, [M-H-CO_2, 16]$^-$ 257.3.

ESI-MS-MS of Fj3: *m* / *z* [M-H, 100]$^-$ 303.4, [M-H-H_2O, 0.2]$^-$ 285.1, [M-H-CO_2, 7]$^-$ 259.4 .

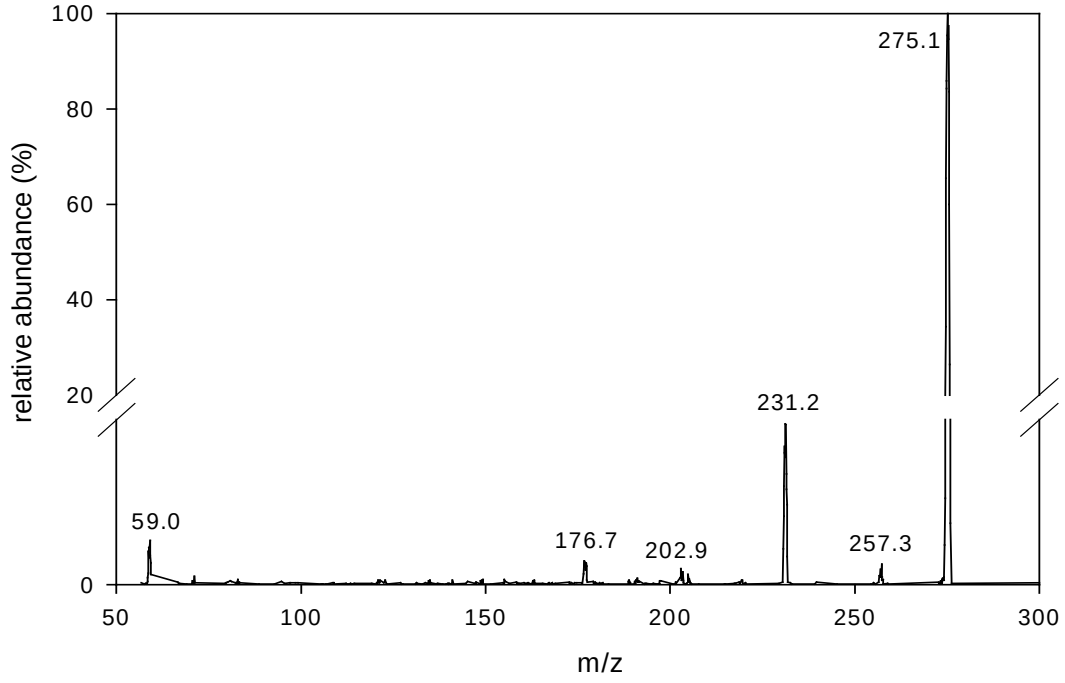


Fig. 23. ESI-MS-MS spectrum of $[M-H]^-$ precursor ion of fibrocapsin 1 in negative ion mode

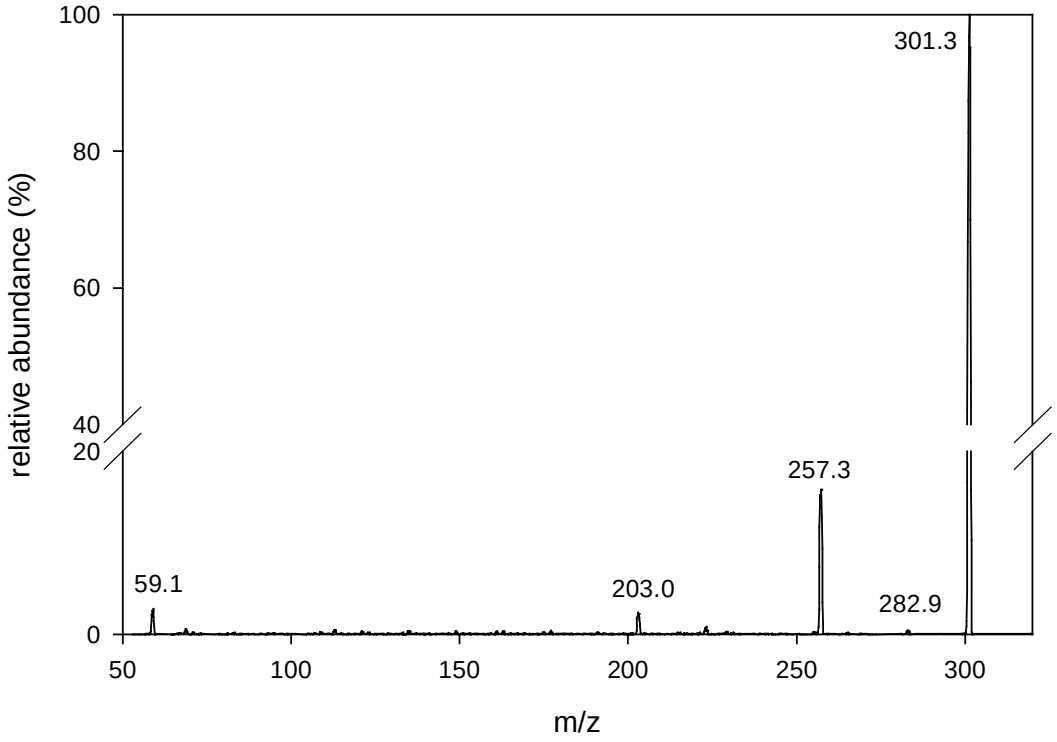


Fig. 24. ESI-MS-MS spectrum of $[M-H]^-$ precursor ion of fibrocapsin 2 in negative ion mode

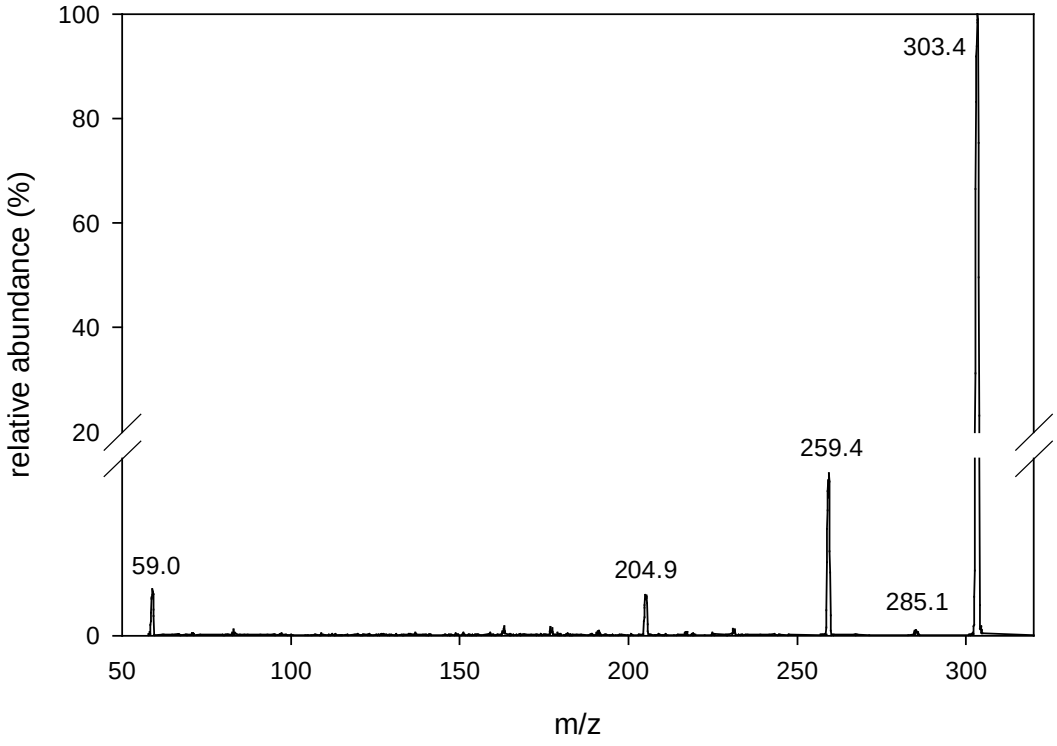


Fig. 25. ESI-MS-MS spectrum of $[M-H]^-$ precursor ion of fibrocapsin 3 in negative ion mode

8. IR Experiments with Fibrocapsins

IR data are presented in Figs. 26 to 28. They show that these three compounds Fj1, Fj2 and Fj3 have similar structural units.

Fj1: IR (dry film) 3000-2500 cm^{-1} (OH), 1685 cm^{-1} (C=O).

Fj2: IR (dry film) 3000-2500 cm^{-1} (OH), 1704 cm^{-1} (C=O).

Fj3: IR (dry film) 3000-2500 cm^{-1} (OH), 1681 cm^{-1} (C=O).

The presence of carboxyl groups in fibrocapsins proven by ESI-MS-MS data were confirmed again by IR experiments. 3000-2500 cm^{-1} represents (O-H) stretching vibration for all types of carboxylic acids. A sharp band around 1710±10 cm^{-1} usually demonstrates C=O group in unsaturated carboxylic acids, the data 1685 cm^{-1} in Fj1 and 1681 cm^{-1} in Fj3 are a bit low, they could be band shift of C=O group to the lower wavenumber.

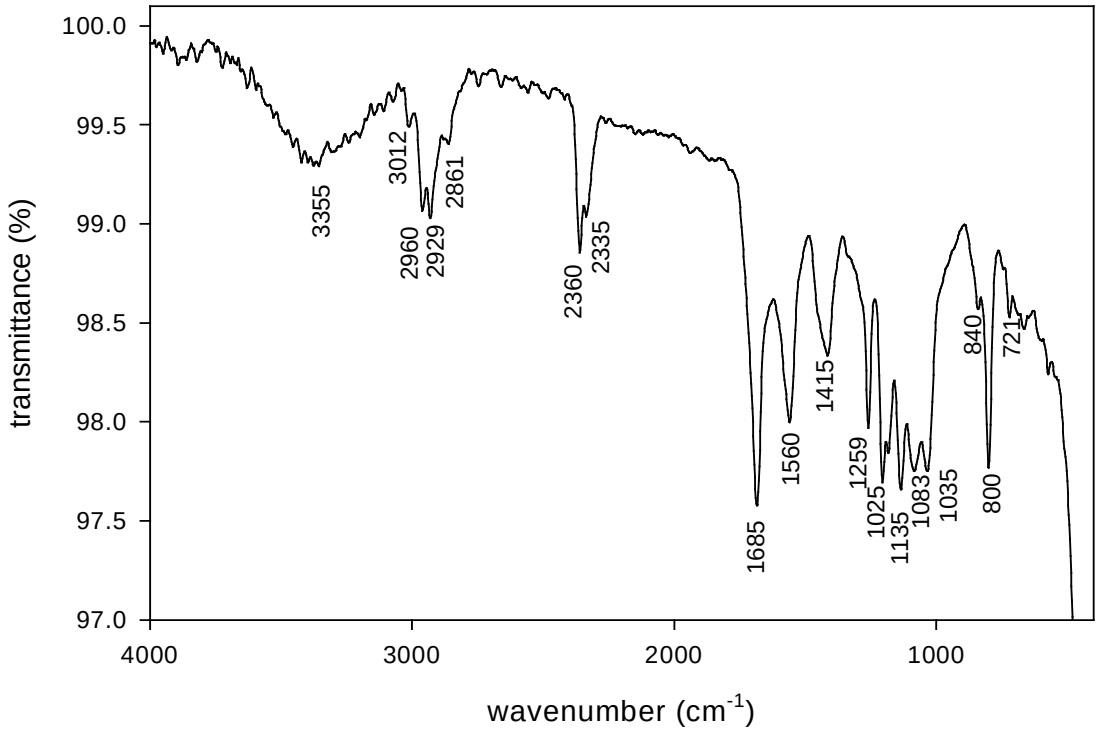


Fig. 26. IR spectrum of fibrocapsin 1

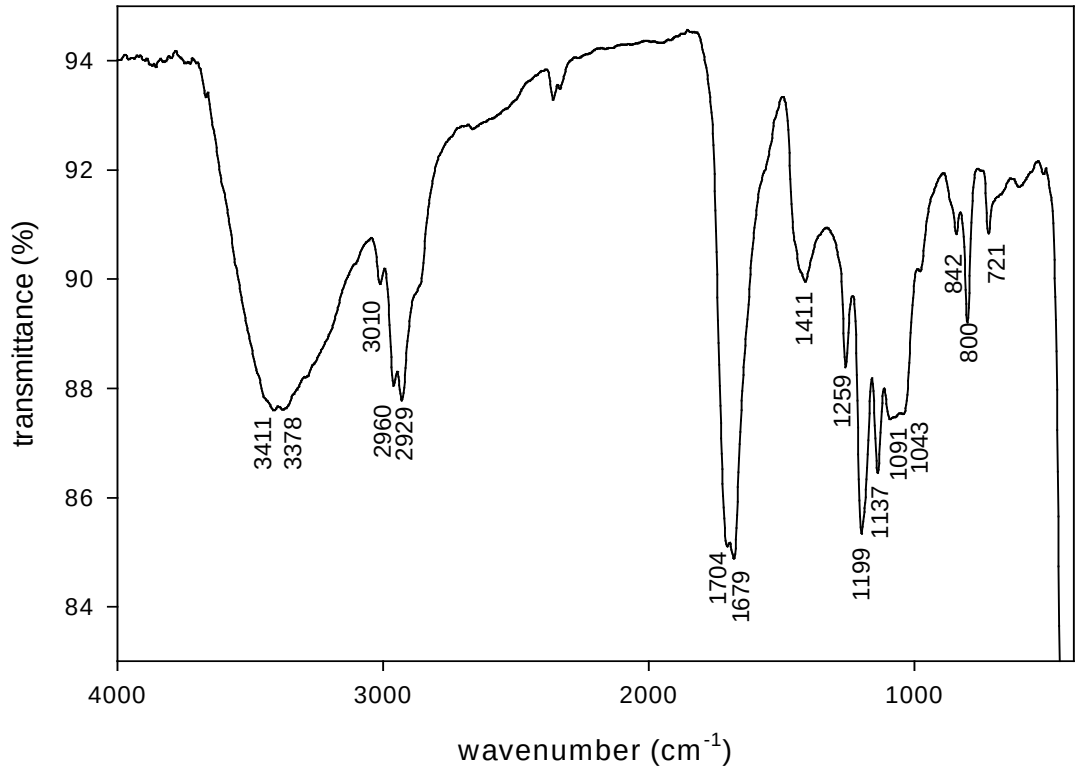


Fig. 27. IR spectrum of fibrocapsin 2

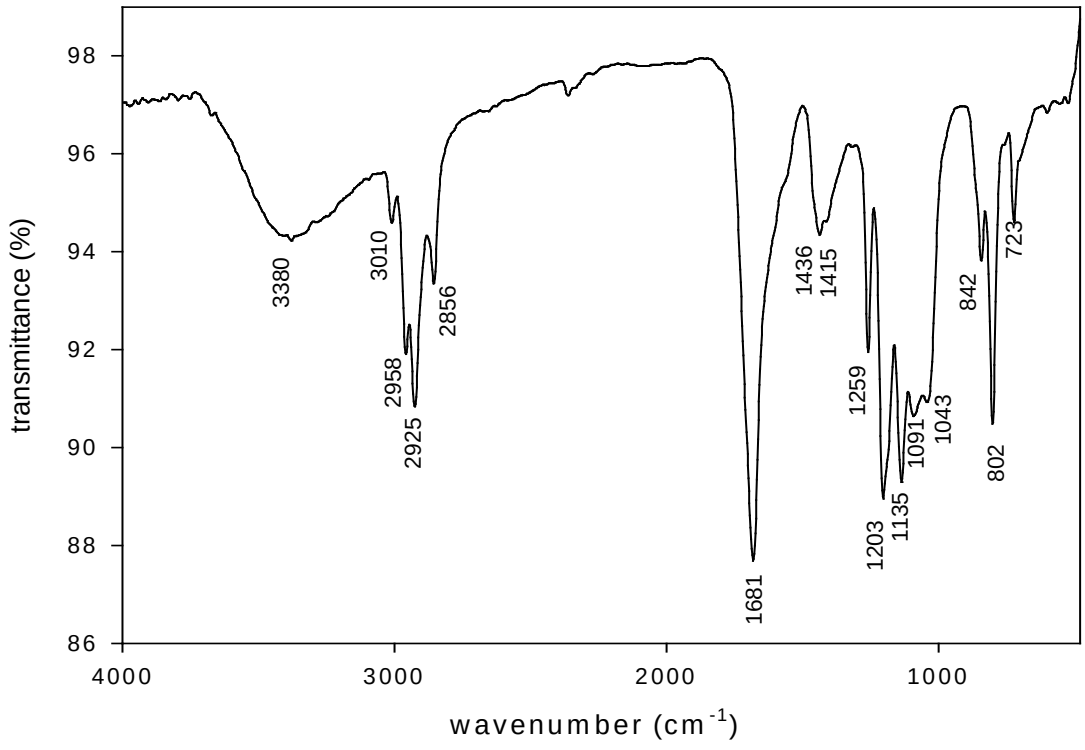


Fig. 28. IR spectrum of fibrocapsin 3

9. GC-MS and GC-HRMS Experiments with Fibrocapsins

9.1 GC-MS and GC-HRMS experiments with fibrocapsin 1

The molecular formula of Fj1-TMS (Fj1-Si(CH_3)$_3$) was calculated for $C_{21}H_{36}O_2Si$ (*m/z* 348.2485) from the experiment data of GC-HRMS (*m/z* 348.2460). Since the molecular weight of Fj1 is 276 proven by HPLC-ESI-MS experiment, it contains one carboxyl group proven by ESI-MS-MS and IR experiments, the formula of Fj1 should be $C_{17}H_{27}$-COOH. The bulk composition information was provided by GC-MS experiment of Fj1 (Fig. 29). The structure of Fj1 was tried to be interpreted (see below), namely, to sum up all the available data until now it could be a polyunsaturated fatty acid (PUFA) (C18:4ω3, Structure. 1) with 18 C atoms, containing a carboxyl group and four double bonds. Fig. 32 and Table 6 provide some literature information for interpretation of GC-MS spectra of PUFA and FA-TMS. A peak at *m/z* = 108, which can be also found in Fig. 29, is characteristic for fatty acids with an omega-3 terminal moiety (Fellenberg et al. 1987).

GC-MS (70 eV) of Fj1-TMS (Fig. 29): *m/z* $[M]^+$ 348 [$C_{21}H_{36}O_2Si$, 7], 333 [$C_{20}H_{33}O_2Si$, 6], 305 [$C_{18}H_{29}O_2Si$, 0.07], 279 [$C_{16}H_{27}O_2Si$, 4], 264 [$C_{15}H_{25}O_2Si$, 1], 258 [M-OH-TMS, 0.01], 252 [$(CH_3)_2$-Si=O-CO-$(CH_2)_4$-$((CH)_2$-$CH_2)_2$-CH_2, 6], 229 [CH_3-$(CH_2$-$(CH)_2)_4$-$(CH_2)_2$-CH=CH, 1], 216 [CH_3-$(CH_2$-$(CH)_2)_4$-CH_2-CH=CH_2, 1], 201 [TMSO-CO-$(CH_2)_5$-CH_2, 3], 189 [CH_3-$(CH_2$-$(CH)_2)_4$-CH_2**,** 13], 175 [CH_3-$(CH_2$-$(CH)_2)_3$-CH_2-CH=CH, 16], 161 [CH_3-$(CH_2$-$(CH)_2)_3$-CH=CH, 27], 149 [CH_3-$(CH_2$-$(CH)_2)_3$-CH_2, 9], 147 [CH_3-$(CH_2$-$(CH)_2)_2$-CH_2-CH=C=CH, 35], 145 [TMSO-CO-CH_2-CH_2, 30], 132 [TMSO-C(OH)=CH_2, 9], 129 [$(CH_3)_2$-SiO-CO-CH=CH_2, 45], 120 [CH_2=$(CH)_2$=CH-CH_2-CH=$(CH)_2$=CH_2, 45], 117 [$(CH_3)_2$-SiO-C(OH)=CH_2, 61], 108 [CH_2=C=CH-CH_2-CH=CH-CH_2-CH_3, 85], 91 [CH≡C-CH_2-$(CH)_2$-CH=CH, 86], 79

[CH_2=C=CH-CH_2-C=CH, 100], 75 [$(CH_3)_2$SiOH, 78], 73 [$(CH_3)_3$Si, 75], 67 [CH_3-CH_2-CH=C=CH, 27], 55 [CH_3-CH_2-CH=CH, 15]; GC-HRMS *m/z* 348.2460 (calculated for $C_{21}H_{36}O_2Si$, 348.2485).

9.2 GC-MS and GC-HRMS experiments of fibrocapsin 2

The molecular formula of Fj2-TMS (Fj2-Si$(CH_3)_3$) was calculated for $C_{23}H_{38}O_2Si$ (*m/z* 374.2641) from the experiment data of GC-HRMS (*m/z* 374.2624). Since the molecular weight of Fj2 is 302 proven by HPLC-ESI-MS experiment, it contains one carboxyl group proven by ESI-MS-MS and IR experiments, the formula of Fj2 should be $C_{19}H_{31}$-COOH. The bulk composition information was provided by GC-MS experiment of Fj2 (Fig. 30). It contains also the characteristic ion (108) of omega-3 fatty acid, in addition, the fragment ions mentioned in Fig. 32 can be found in GC-MS spectrum of Fj2 (Fig. 30) as well. Therefore, the structure of Fj2 was initially supposed as a polyunsaturated fatty acid (C20:5ω3, Structure. 2) with 20 C atoms and five double bonds, see the interpretation below.

GC-MS (70 eV) of Fj2-TMS (Fig. 30): *m/z* $[M]^+$ 374 [$C_{23}H_{38}O_2Si$, 0.51], 359 [$C_{22}H_{35}O_2Si$, 1], 345 [$C_{21}H_{33}O_2Si$, 0.74], 331 [$C_{20}H_{31}O_2Si$, 0.51], 305 [$C_{18}H_{29}O_2Si$, 3], 284 [M-OH-TMS, 0.18], 278 [$(CH_3)_2$-Si=O-CO-$(CH_2)_3$-$((CH)_2$-$CH_2)_3$-CH_2, 2], 242 [CH_3-$(CH_2$-$(CH)_2)_5$-CH=CH_2, 2], 201 [TMSO-CO-$(CH_2)_5$-CH_2, 14], 189 [CH_3-$(CH_2$-$(CH)_2)_4$-CH_2, 3], 175 [CH_3-$(CH_2$-$(CH)_2)_3$-CH_2-CH=CH, 22], 161 [CH_3-$(CH_2$-$(CH)_2)_3$-CH=CH, 15], 149 [CH_3-$(CH_2$-$(CH)_2)_3$-CH_2, 7], 147 [CH_3-$(CH_2$-$(CH)_2)_2$-CH_2-CH=C=CH, 22], 145 [TMSO-CO-CH_2-CH_2, 33], 132 [TMSO-C(OH)=CH_2, 18], 129 [$(CH_3)_2$-SiO-CO-CH=CH_2, 43], 120 [CH_2=$(CH)_2$=CH-CH_2-CH=$(CH)_2$=CH_2, 37], 117 [$(CH_3)_2$-SiO-C(OH)=CH_2, 100], 108 [CH_2=C=CH-CH_2-CH=CH-CH_2-CH_3, 53], 91 [CH≡C-CH_2-$(CH)_2$-CH=CH, 95], 79 [CH_2=C=CH-CH_2-C=CH, 78], 75 [$(CH_3)_2$SiOH,

56], 73 [$(CH_3)_3Si$, 61], 67 [CH_3-CH_2-CH=C=CH, 23], 55 [CH_3-CH_2-CH=CH, 13]; GC-HRMS *m/z* 374.2624 (calculated for $C_{23}H_{38}O_2Si$, 374.2641).

9.3 GC-MS and GC-HRMS experiments of fibrocapsin 3

The molecular formula of Fj3-TMS (Fj3-$Si(CH_3)_3$) was calculated for $C_{23}H_{38}O_2Si$ (*m/z* 376.2798) from the experiment data of GC-HRMS (*m/z* 376.2851). Since the molecular weight of Fj3 is 304 proven by HPLC-ESI-MS experiment, it contains one carboxyl group proven by ESI-MS-MS and IR experiments, the formula of Fj3 should be $C_{19}H_{29}$-COOH. The bulk composition information was provided by GC-MS experiment of Fj3 (Fig. 31). The structure of Fj3 was interpreted initially (see below) as a 20-C polyunsaturated fatty acid (C20:4ω6, Structure. 3) with four double bonds. The data of fragment ions of Fj3 are also consistent with the literature proof in Fig. 32 and Table 6. A peak at *m/z* =150 which defines an omega-6 terminal group (Fellenberg et al. 1987) can be found unequivocally in Fig. 31.

GC-MS (70 eV) of Fj1-TMS (Fig. 31): *m/z* $[M]^+$ 376 [$C_{23}H_{40}O_2Si$, 5], 361 [$C_{22}H_{37}O_2Si$, 6], 305 [TMSO-CO-$(CH_2)_3$-$((CH)_2$-$CH_2)_3$-CH=CH, 1], 278 [$(CH_3)_2$-Si=O-CO-$(CH_2)_3$-$((CH)_2$-$CH_2)_3$-CH_2, 4], 265 [TMSO-CO-$(CH_2)_3$-$((CH)_2$-$CH_2)_2$-CH=CH, 4], 244 [CH_3-$(CH_2)_3$-$(CH_2$-$(CH)_2)_4$-CH=CH_2, 4], 238 [$(CH_3)_2$-Si=O-CO-$(CH_2)_3$-$((CH)_2$-$CH_2)_2$-CH_2, 8], 231 [CH_3-$(CH_2)_3$-$(CH_2$-$(CH)_2)_4$-CH_2, 2], 217 [CH_3-$(CH_2)_3$-$(CH_2$-$(CH)_2)_3$-CH_2-CH=CH, 8], 203 [CH_3-$(CH_2)_3$-$(CH_2$-$(CH)_2)_3$-CH=CH, 12], 187 [TMSO-CO-$(CH_2)_4$-CH_2, 9], 175 [CH_3-$(CH_2$-$(CH)_2)_3$-CH_2-CH=CH, 24], 161 [CH_3-$(CH_2$-$(CH)_2)_3$-CH=CH, 15], 150 [CH_3-$(CH_2)_4$-$(CH)_2$-CH_2-CH=C=CH_2, 48], 145 [TMSO-CO-CH_2-CH_2, 28], 132 [TMSO-C(OH)=CH_2, 19], 129 [$(CH_3)_2$-SiO-CO-CH=CH_2, 49], 120 [CH_2=$(CH)_2$=CH-CH_2-CH=$(CH)_2$=CH_2, 40], 117 [$(CH_3)_2$-SiO-C(OH)=CH_2, 100], 108 [CH_2=C=CH-CH_2-CH=CH-CH_2-CH_3, 9], 91 [CH≡C-CH_2-

$(CH)_2$-CH=CH, 83], 79 [CH_2=C=CH-CH_2-C=CH, 73], 75 [$(CH_3)_2$SiOH, 57], 73 [$(CH_3)_3$Si, 64], 67 [CH_3-CH_2-CH=C=CH, 24], 55 [CH_3-CH_2-CH=CH, 15]; GC-HRMS *m* / *z* 376.2851 (calculated for $C_{23}H_{38}O_2Si$, 376.2798).

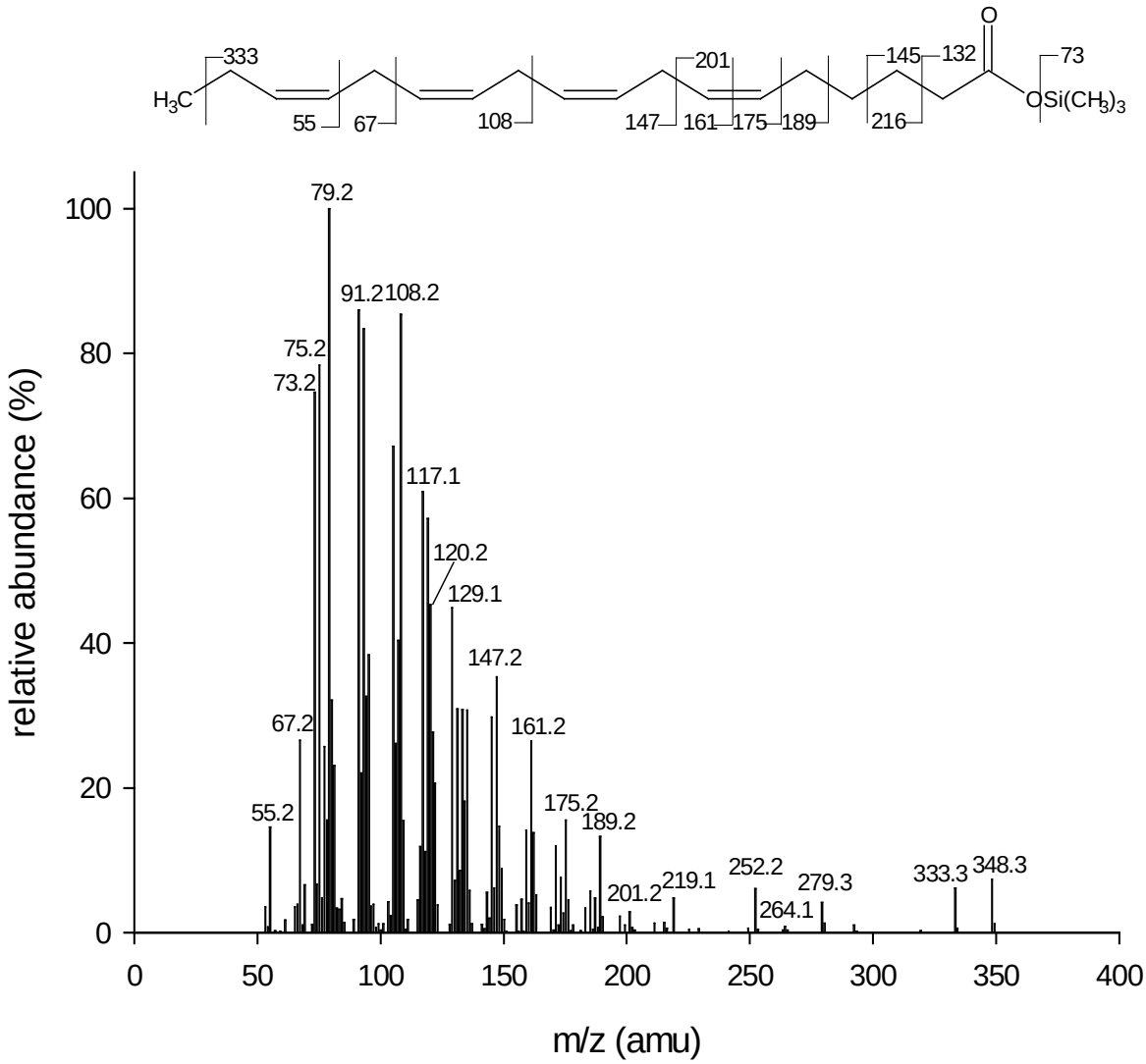
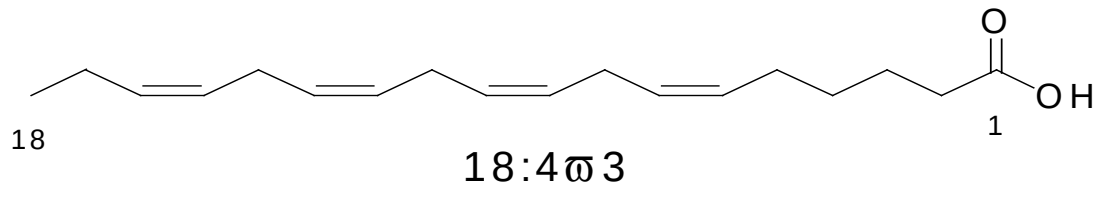


Fig. 29. GC-MS spectrum of fibrocapsin 1-TMS



Structure. 1. Supposition of Fj1's structure

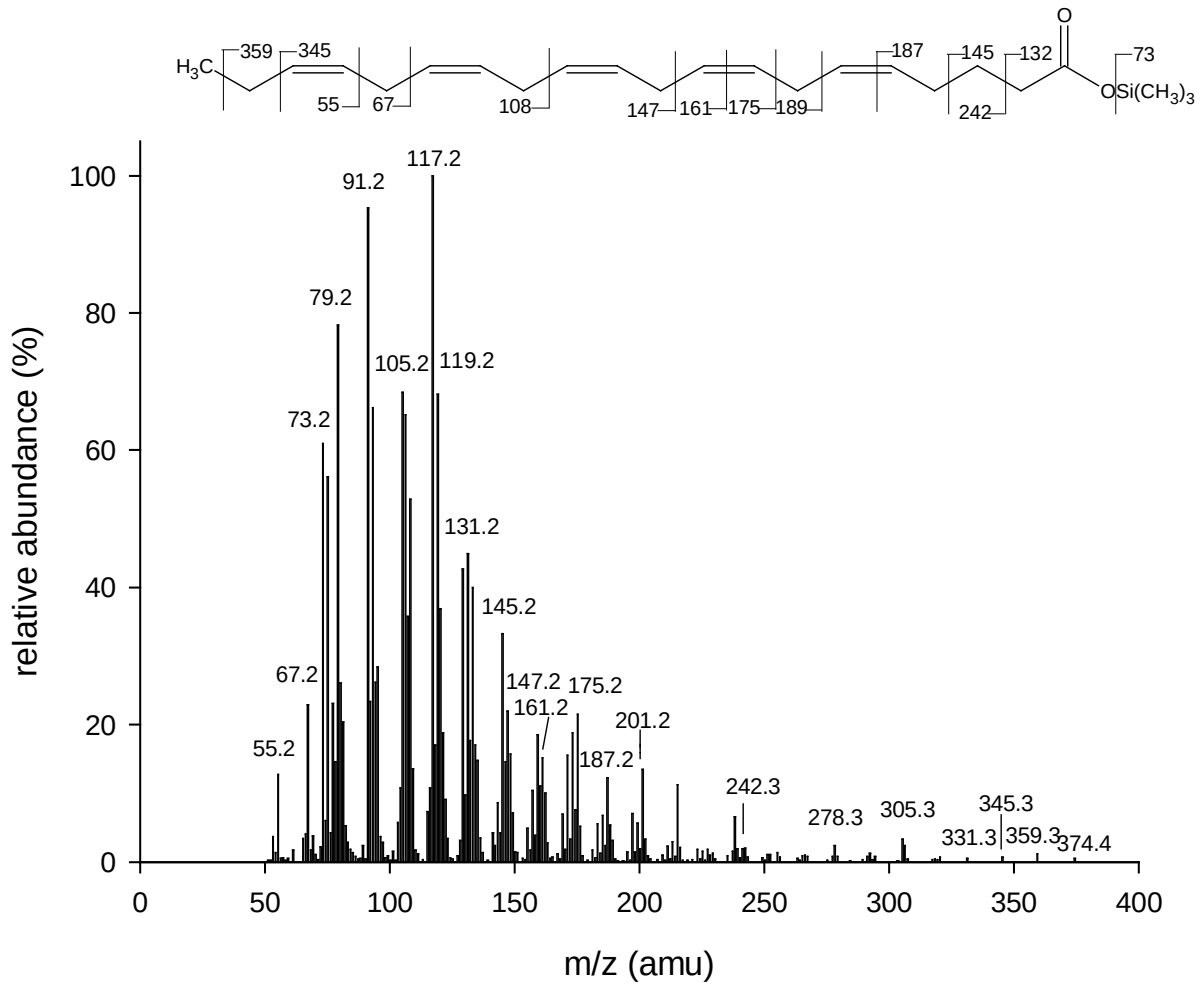
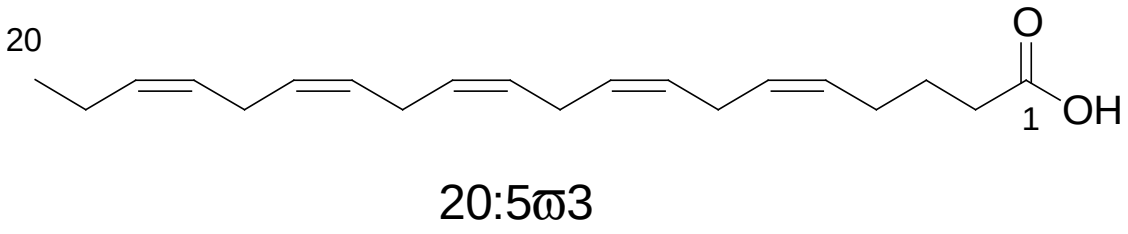


Fig. 30. GC-MS spectrum of fibrocapsin 2-TMS



20:5ϖ3

Structure. 2. Supposition of Fj2's structure

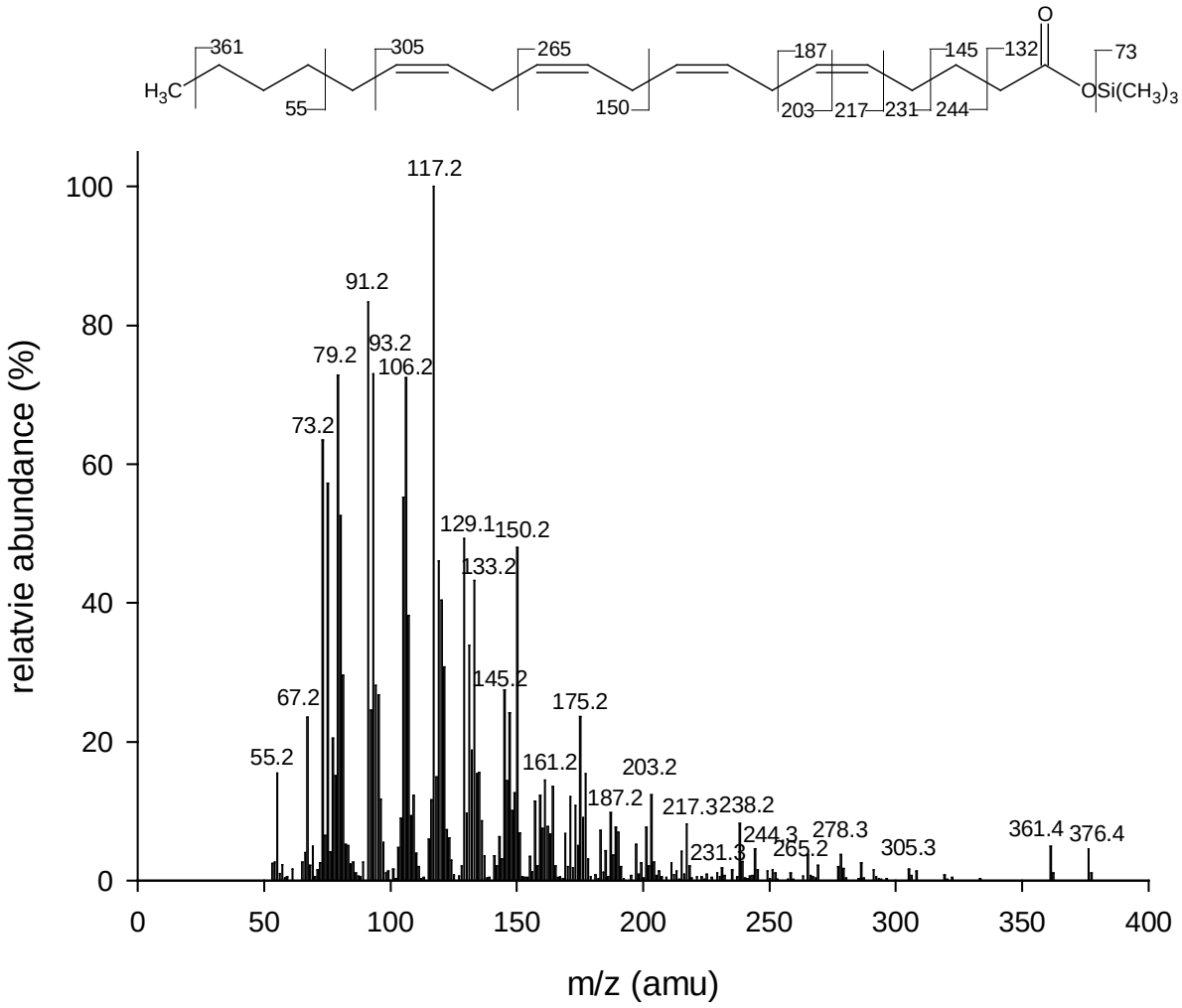
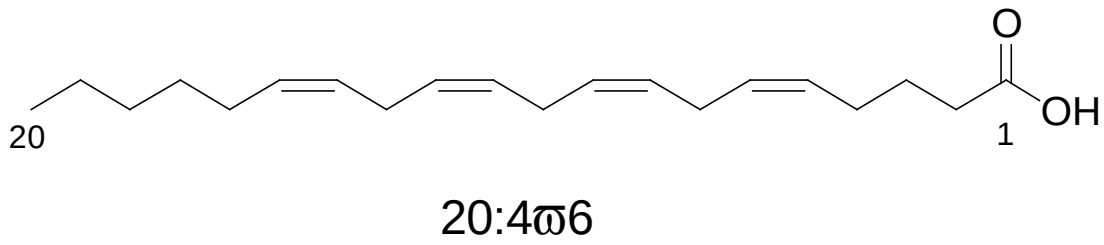


Fig. 31. GC-MS spectrum of fibrocapsin 3-TMS



20:4ϖ6

Structure. 3. Supposition of Fj1's structure

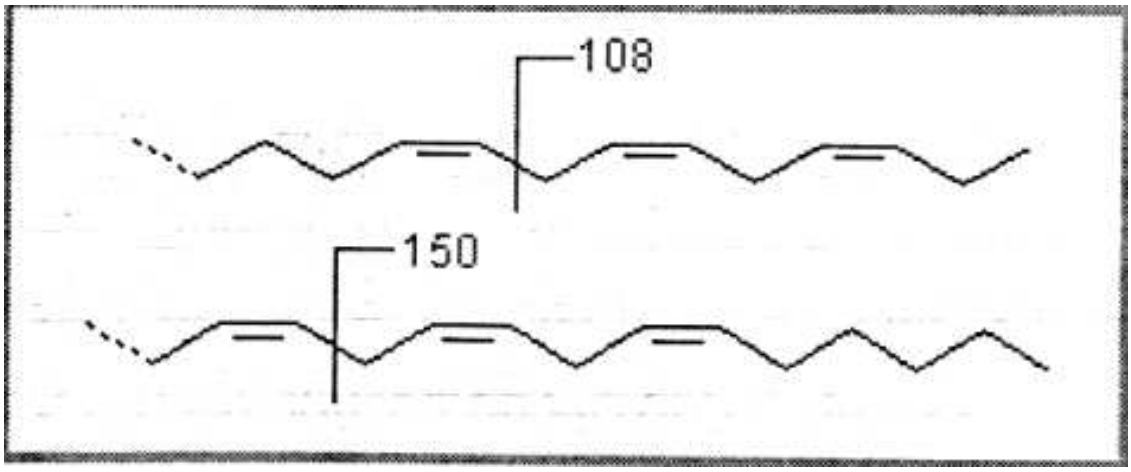


Fig. 32. Diagnostic ions of omega-3 and -6 fatty acids (http://www.lipid.co.uk)

Table 6. Fragment ions of FA-TMS (fatty acid - TMS) (http://www.lipid.co.uk)

m/z	fragment ion
73	$(CH_3)_3Si$
75	$(CH_3)_2SiOH$
117	$(CH_3)_2SiO\text{-}C(OH)=CH_2$
129	$(CH_3)_2SiO\text{-}CO\text{-}CH=CH_2$
132	$TMSO\text{-}C(OH)=CH_2$
145	$TMSO\text{-}CO\text{-}CH_2\text{-}CH_2$
201	$TMSO\text{-}CO\text{-}(CH_2)_5\text{-}CH_2$
M-90	loss of H-OTMS

Efforts in searching for related standard spectra from literatures which can be used to confirm the suppositions above was not successful because there is very little available standard spectra for TMS derivatives of PUFAs. Therefore, the following NMR experiment results appear to be very important for structural confirmation.

10. NMR Experiments with Fibrocapsins

The data of NMR experiments with fibrocapins and interpretation of spectra are as follows:

10.1 NMR experiments with fibrocapsin1

^{1}H-NMR (CD_3OD, 500.1MHz) δ 0.96 (3H, t, *J*=7.7, H-18), 1.40 (2H, m, H-4), 1.61 (2H, m, *J*=7.3, 7.3, H-3), 2.09 (2H, m, H-17), 2.10 (2H, m, H-5), 2.28 (2H, t, *J*=7.3, H-2), 2.85-2.80 (6H, m, H-8, H-11, H-14), 5.38-5.34 (8H, m, H-6, H-7, H-9, H-10, H-12, H-13, H-15, H-16), the proton of carboxyl-group is exchanged against deuterium from the solvent (Fig. 33); ^{13}C-NMR (CD_3OD, 125.8 MHz) δ 14.6 (C-18), 21.5 (C-17), 25.7, 26.4, 26.5, 27.9 (C-3, C-4, C-5,C-8, C-11, C-14), 31.6 (C-2), 128.2, 128.9, 129.0, 129.2, 129.3, 129.8, 130.0, 132.8 (C-6, C-7, C-9, C-10, C-12, C-13, C-15, C-16), the low intensity signal for C-1 could not be clearly distinguished from the noise (Fig. 34).

10.2 NMR experiments with fibrocapsin 2

^{1}H-NMR (CD_3OD, 500.1MHz) δ 0.97 (3H, t, *J*=7.7, H-20), 1.66 (2H, m, *J*=7.3, 7.3, H-3), 2.08 (2H, m, H-19), 2.13 (2H, m, H-4), 2.29 (2H, t, *J*=7.3, H-2), 2.86-2.81 (8H, m, H-7, H-10, H-13, H-16), 5.40-5.30 (10H, m, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15, H-17, H-18), the proton of carboxyl-group is exchanged against deuterium from the solvent (Fig. 35); ^{13}C-NMR (CD_3OD, 125.8 MHz) δ 14.6 (C-20), 21.5 (C-19), 26.0 (C-3), 26.4 (C-16), 26.5 (C-7, C-10, C-13), 27.6 (C-4), 34.4 (C-2), 128.2 (C-17), 128.9, 129.1, 129.1, 129.2, 129.2, 129.5 (C-8, C-9, C-11, C-12, C-14,C-15), 129.8, 130.1 (C-5, C-6), 132.8 (C-18), 177.5 (C-1) (Fig. 36).

10.3 NMR experiments with fibrocapsin 3

^{1}H-NMR (CD_3OD, 500.1MHz) δ 0.90 (3H, t, *J*=7.0, H-20), 1.31 (4H, m, H-18, H-19), 1.36 (2H, m, H-17), 1.66 (2H, m, *J*=7.0, 7.3, H-3), 2.07 (2H, m, H-16), 2.13 (2H, m, H-4), 2.29 (2H, t, *J*=7.3, H-2), 2.80-2.85 (6H, m, H-7, H-10, H-13), 5.31-5.39 (8H, m, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15) (Fig. 37). The available amount of fibrocapsin 3 was too small to record a sufficient ^{13}C NMR.

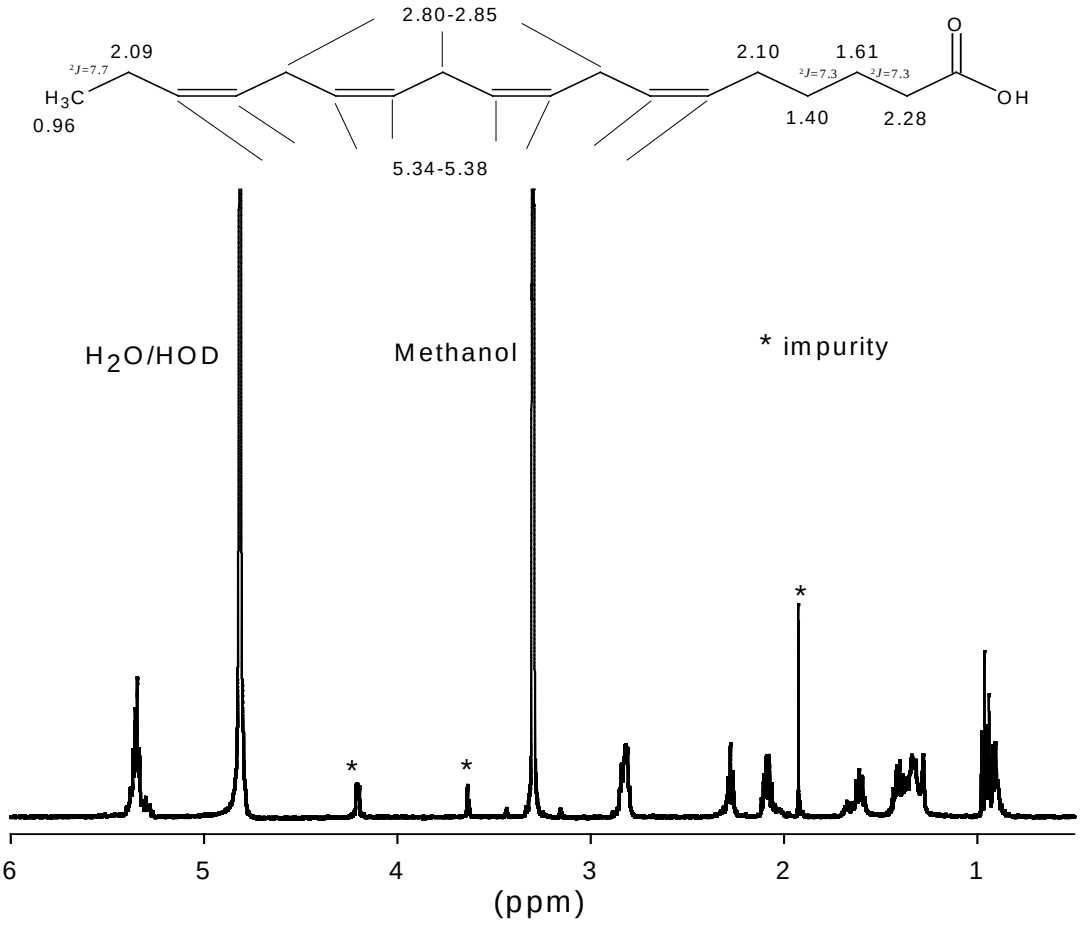


Fig. 33. ^{1}H-NMR spectrum of fibrocapsin 1

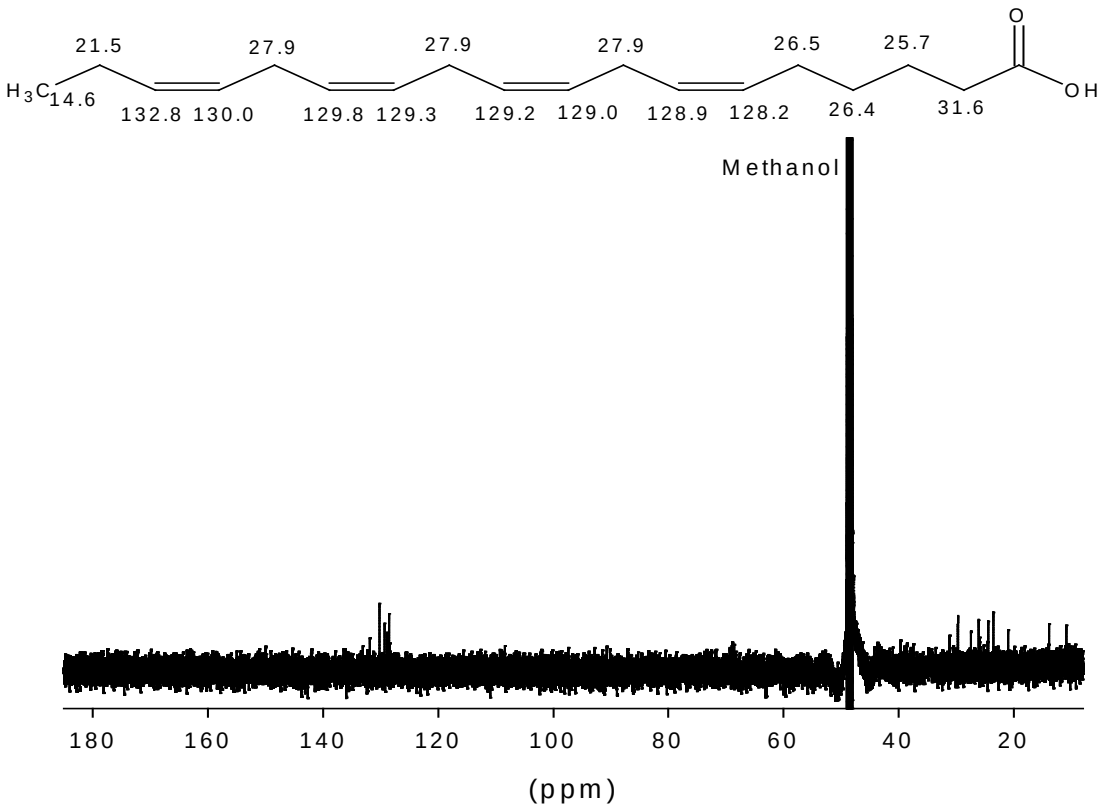


Fig. 34. ^{13}C-NMR spectrum of fibrocapsin 1

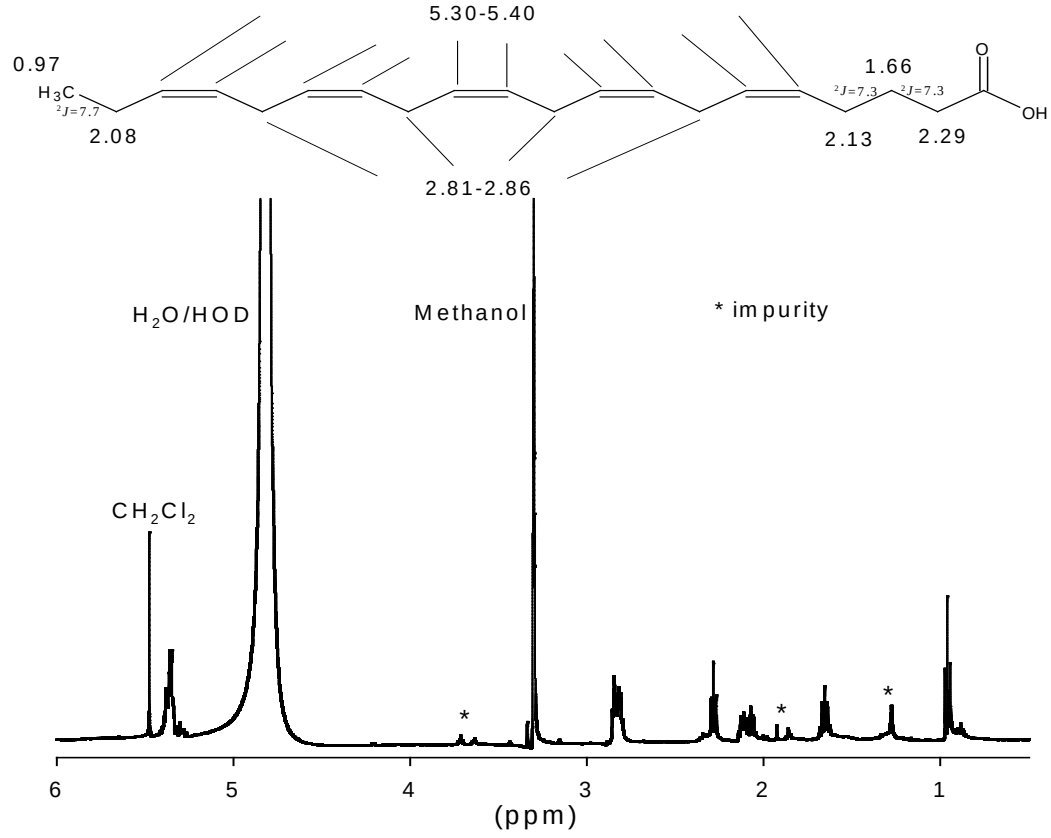


Fig. 35. ^{1}H-NMR spectrum of fibrocapsin 2

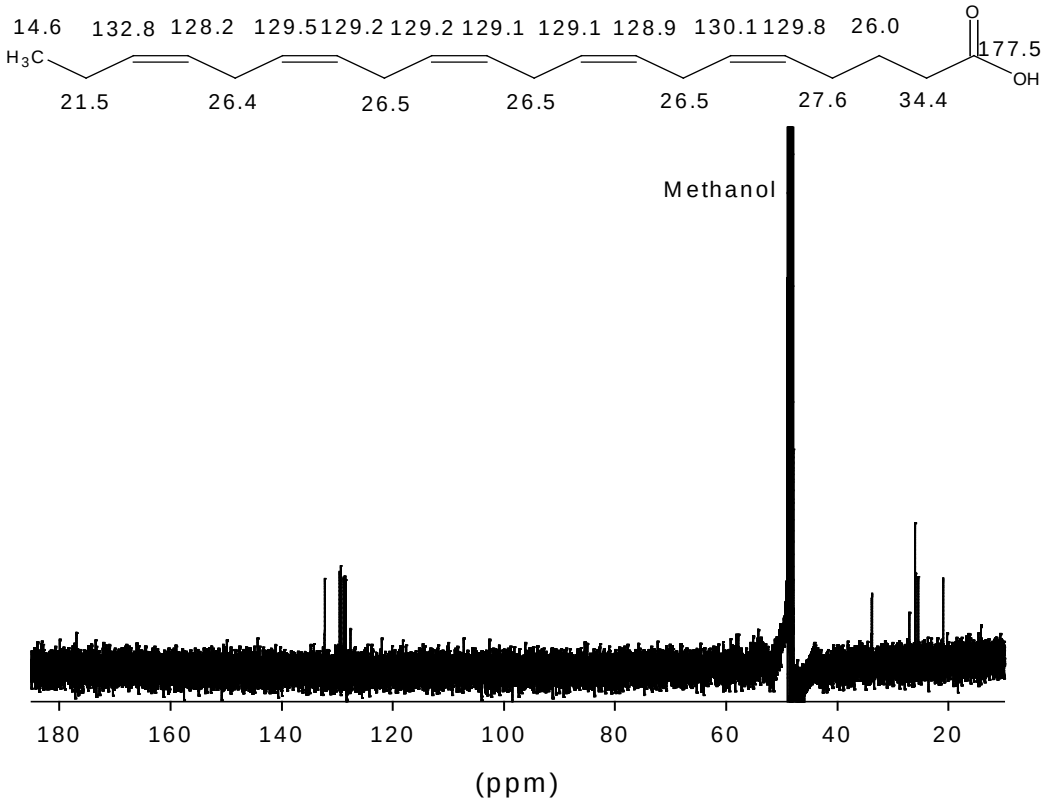


Fig. 36. ^{13}C-NMR spectrum of fibrocapsin 2

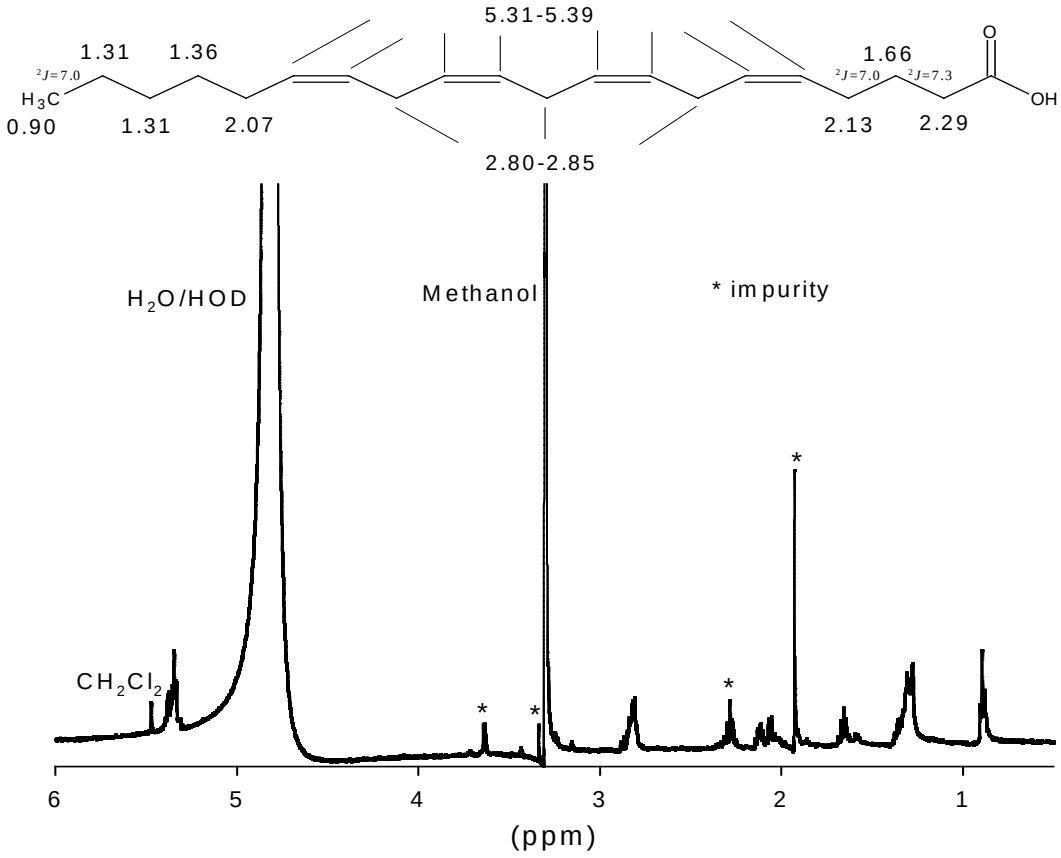


Fig. 37. ^{1}H-NMR spectrum of fibrocapsin 3

The interpretations of NMR spectra above confirm structure suppositions of fibrocapsins derived from data of HPLC-ESI-MS, ESI-MS-MS, IR and GC-(HR)MS experiments, namely, Fj1 as 6,9,12,15-octadecatetraenoic acid (C18:4ω3), Fj2 as 5,8,11,14,17-eicosapentaenoic acid (C20:4ω6) and Fj3 as 5,8,11,14-eicosatetraenoic acid (C20:5ω3).

In ^{1}H-NMR spectra of fibrocapsins (Figs. 33, 35, 37), the chemical shifts (δ) for protons in olefinic CH group are between 5.30-5.40. The cis/trans configuration is actually able to be determined by coupling constants (*J*), i.e. for cis: *J*=*ca.* 10 Hz; for trans: *J*=*ca.* 17-18 Hz (Dr. Arne Luetzen pers. comm. 2003). But it is a pity that in these cases the corresponding coupling constants are unrecognisable since the bad resolution, therefore directly from these NMR experiment results it is difficult to determine cis/trans configurations of fibrocapsins.

11. HPLC and NMR Experiments with Standards

As we have known that naturally occurring unsaturated fatty acids are of the cis configuration, two commercially available standards all-*cis*-5,8,11,14,17-eicosapentaenoic acid (EPA, C20:5ω3) and all-*cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid AA, C20:4ω6) were used here for further confirmation experiments.

11.1 HPLC experiments with standards

The HPLC chromatograms with (Fig. 38) and without (Fig. 39) addition of EPA (C20:5ω3) and AA (C20:4ω6) standards showed Fj2 and Fj3 to have identical retention times with EPA and AA.

11.2 NMR experiments with standards

The ^{1}H-NMR and ^{13}C-NMR spectra of EPA and Fj2 showed no difference (Fig. 40, 41), as far as the ^{1}H-NMR spectra for AA and Fj3 are concerned (Fig. 42).

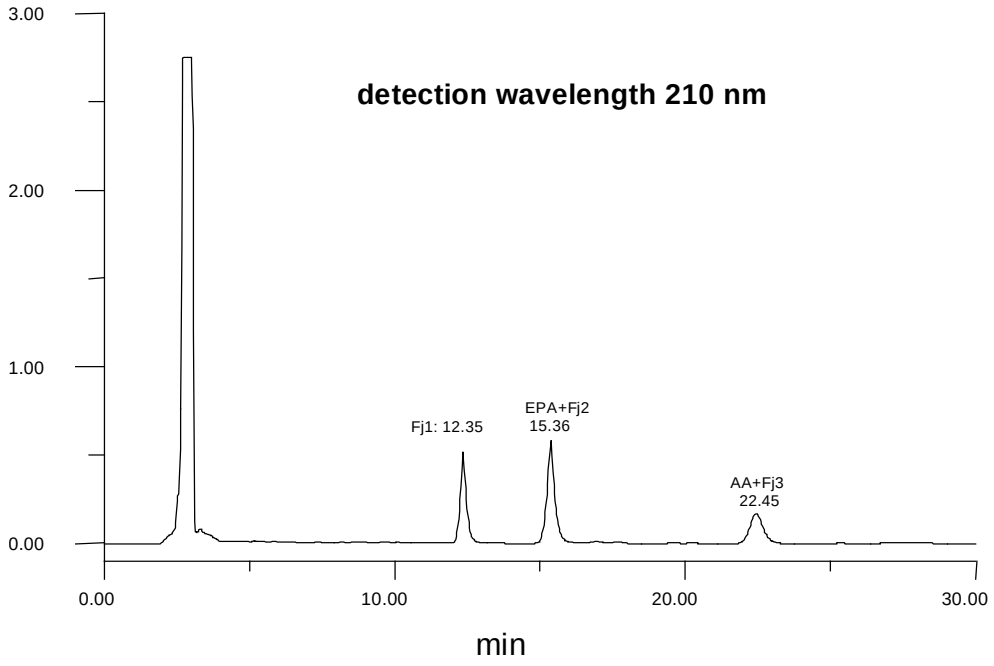


Fig. 38. HPLC chromatogram of crude extract of *Fibrocapsa japonica* (with internal standards)

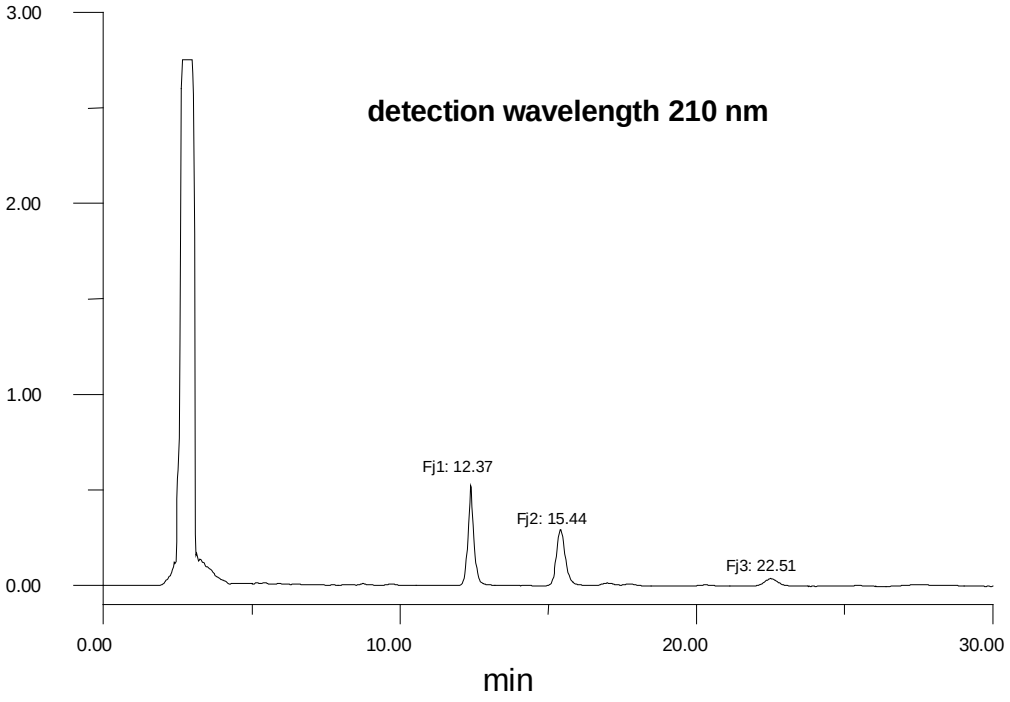


Fig. 39. HPLC chromatogram of crude extract of *Fibrocapsa japonica* (without internal standards)

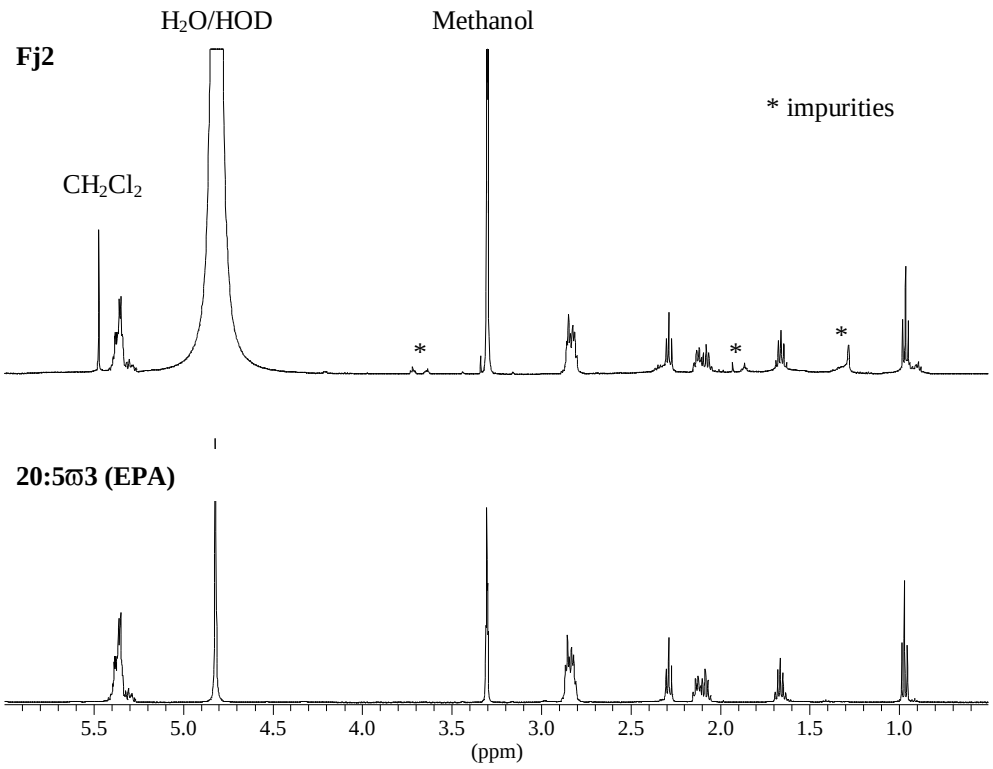


Fig. 40. ^{1}H-NMR spectra of EPA (C20:5ω3) and Fj2

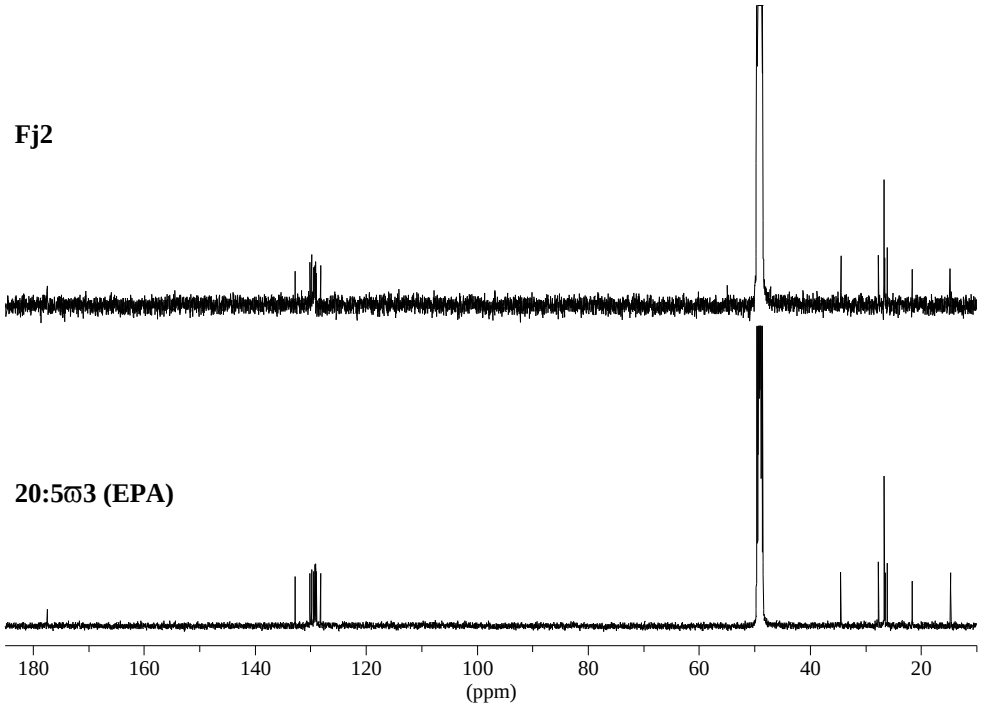


Fig. 41. ^{13}C-NMR spectra of EPA (C20:5ω3) and Fj2

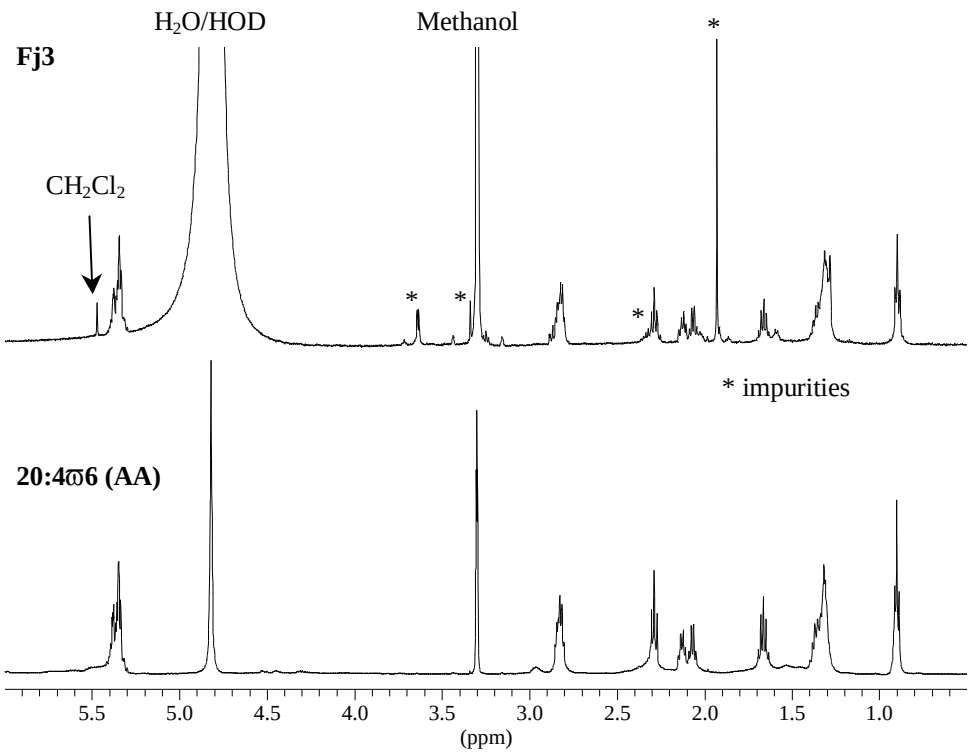


Fig. 42. ^{1}H-NMR spectra of AA (C20:4ω6) and Fj3

There is no doubt any more that Fj2 is all-*cis*-5,8,11,14,17-eicosapentaenoic acid and Fj3 is all-*cis*-5,8,11,14-eicosatetraenoic acid since the NMR spectra of *cis*-standards are identical with those of Fj2 and Fj3. Although the configuration of Fj1's structure is not proven with the help of standard, the similar biological and chemical behaviours which fibrocapins (1, 2 and 3) demonstrated in this whole work, together with the fact of PUFA in nature being dominantly *cis* configuration, Fj1's structure is also supposed as all-*cis*-6,9,12,15-octadecatetraenoic acid.

12. Toxicity Biotests with Standards

Results of all following biotests fully support the above conclusions of structure elucidation.

12.1 Erythrocyte lysis assay with standards

The ELA results (Fig. 43) of the two standards EPA and AA demonstrated the same strong haemolytic effects as those of Fj2 and Fj3 (Fig. 19).

12.2 Bioluminescence inhibition biotest with standards

The two standards EPA and AA showed very strong toxic effects (Fig. 44) in the bioluminescence inhibition biotest, just as SPE toxic fractions and crude extracts of *Fibrocapsa japonica*.

12.3 Artemia salina *biotest with standards*

The two standards also showed similar toxic effects (Fig. 45) on *Artemia salina* as SPE toxic fractions, crude extract and algal cells of *Fibrocapsa japonica*.

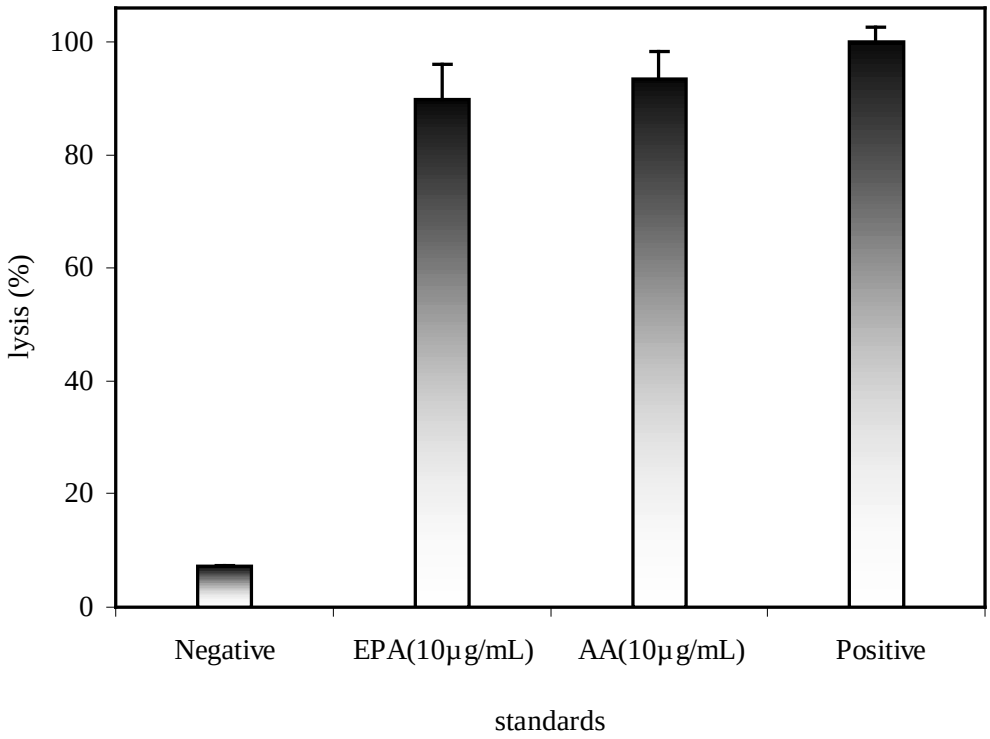


Fig. 43. Haemolytic effects of EPA and AA (10 µg / mL each) after 24 hrs incubation

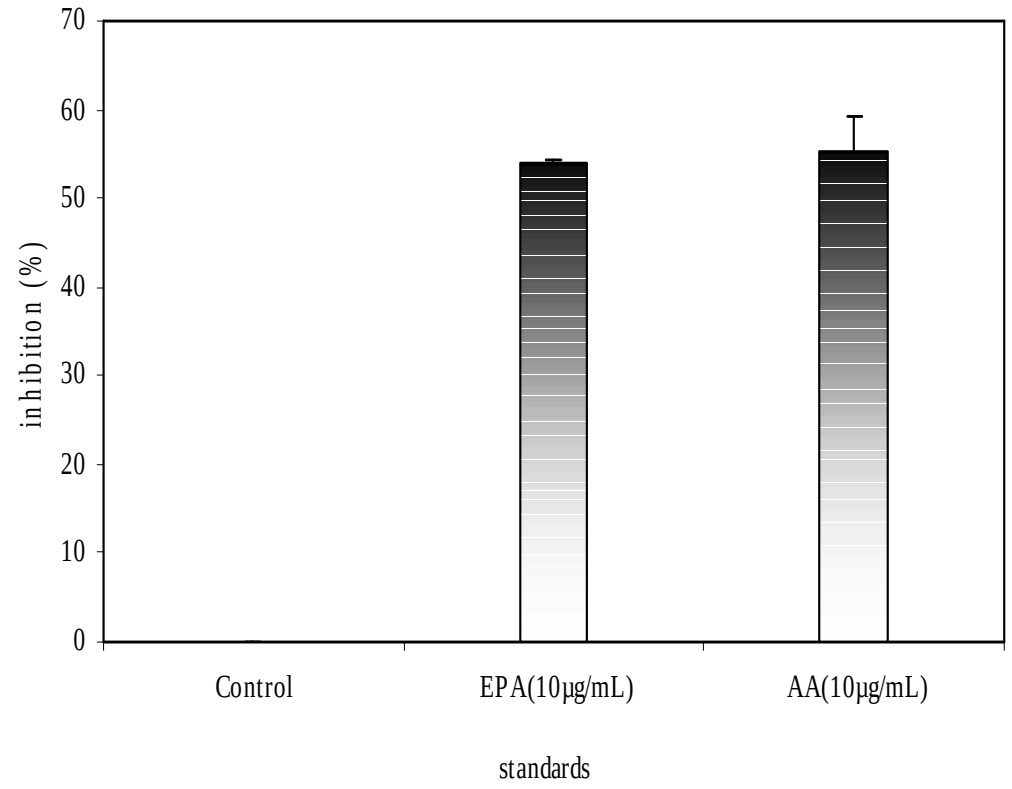


Fig. 44. Bioluminescence inhibition biotest with two standards (EPA and AA)

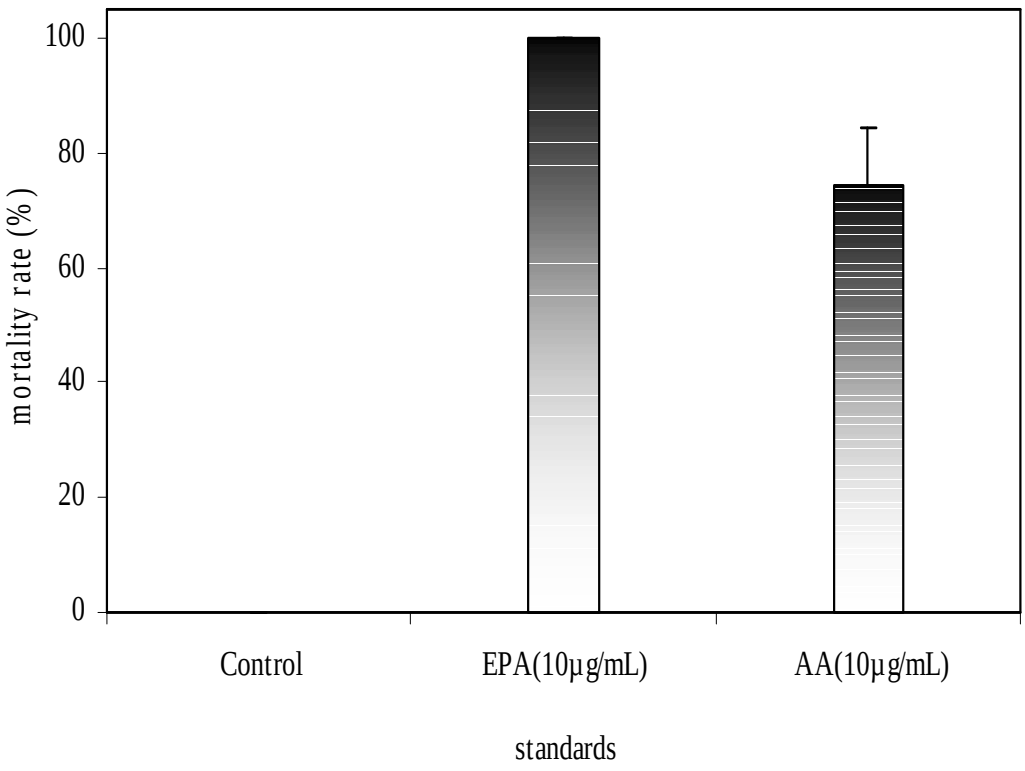


Fig. 45. Toxic effects of two standards (EPA and AA) on *Artemia salina* nauplii

IV. DISCUSSION

1. Cultivation Conditions for *Fibrocapsa japonica*

Fibrocapsa japonica seems to be very sensitive under laboratory conditions. Any mechanical stimulus may cause the mucocyst in the cell to discharge accompanied by cell breakdown. Therefore, after some initial experimentation a stationary cultivation technique was used in which mechanical agitation of the culture flasks was avoided.

However, as one of causative organisms in HAB events, in nature the species can apparently tolerate water movement and the associated shear stress. Although this was not investigated any further in the present work two questions arise from these observations:

- Are there necessary factors missing in the culture? After all, the man-made cultivation condition can not replace the natural environment.
- May this necessary factor be a crucial ingredient missing in the f/2 medium or possibly another co-existing species? Although it is already well known that cyanobateria live tightly together with other chemoorganotrophic bacteria in nature, there are no reports in the literature so far relevant to microalgae.

2. Fibrocapsins and Brevetoxins

All experiments in this work were led closely by biotests. Although a fish biotest was unfortunately not allowed to be used as one of the tools, the three biotest methods applied, i.e. *Artemia salina* and bioluminescence inhibition tests as well as erythrocyte lysis assay, have offered sufficient proof of the toxicity of fibrocapsins.

As discussed above the fibrocapsins were finally identified to be polyunsaturated fatty acids. Brevetoxins could, despite intense search efforts, not be detected. Repeated

attempts to confirm the data provided by Khan et al. (1996b; Fig. 46) failed. Fig. 18 shows that some brevetoxins do indeed have the same retention times as fibrocapsins. However, their different absorption spectra do not lend support to Khan's assignments. Furthermore, there were no peaks at molecular masses around 860 and 900 dalton in HPLC-ESI-MS investigations of bulk extracts which would be expected for brevetoxins.

There could be a number of explanations why Khan's and the present observations are apparently contradictory.

(1) Different strains could actually produce different toxins.

(2) The production of certain toxins could be controlled by culture conditions that probably were different in the different laboratories.

(3) The peaks observed in Khan's study (1996c) were actually PUFAs with a similar retention behaviour as brevetoxins. She used 215 nm as detection wavelength, thus differences in the absorption characteristic would have gone unnoticed.

Marshall et al. (2002a) reported that the investigations on Australian *Chattonella marina* also demonstrated an absence or only low concentrations (0.006-0.03 pg/cell) of brevetoxin-like compounds, but very high concentrations of the polyunsaturated fatty acids. However, recent related research using an ELISA assay for brevetoxins (Bridgers et al. 2002) found that clonal isolates of the raphidophyte species, *Chattonella* (*C. subsalsa, C. marina, C. spp.*) and *Fibrocapsa japonica* isolated from Texas, Florida, South Carolina, North Carolina and Delaware, did produce positive assay results which occurred in late log to stationary phase from supernatants as well as cell pellets. A highly concentrated crude extract of the German strain used here was recently sent to the USA for this ELISA assay. Preliminary results indicate the

existence of brevetoxins at the detection limit amount (Dr. Marion van Rijssel pers. comm. 2003). Therefore, for the German strain of *Fibrocapsa japonica* investigated, it can be concluded that polyunsaturated fatty acids instead of brevetoxins play the key role in the toxic effects of harmful algal blooms.

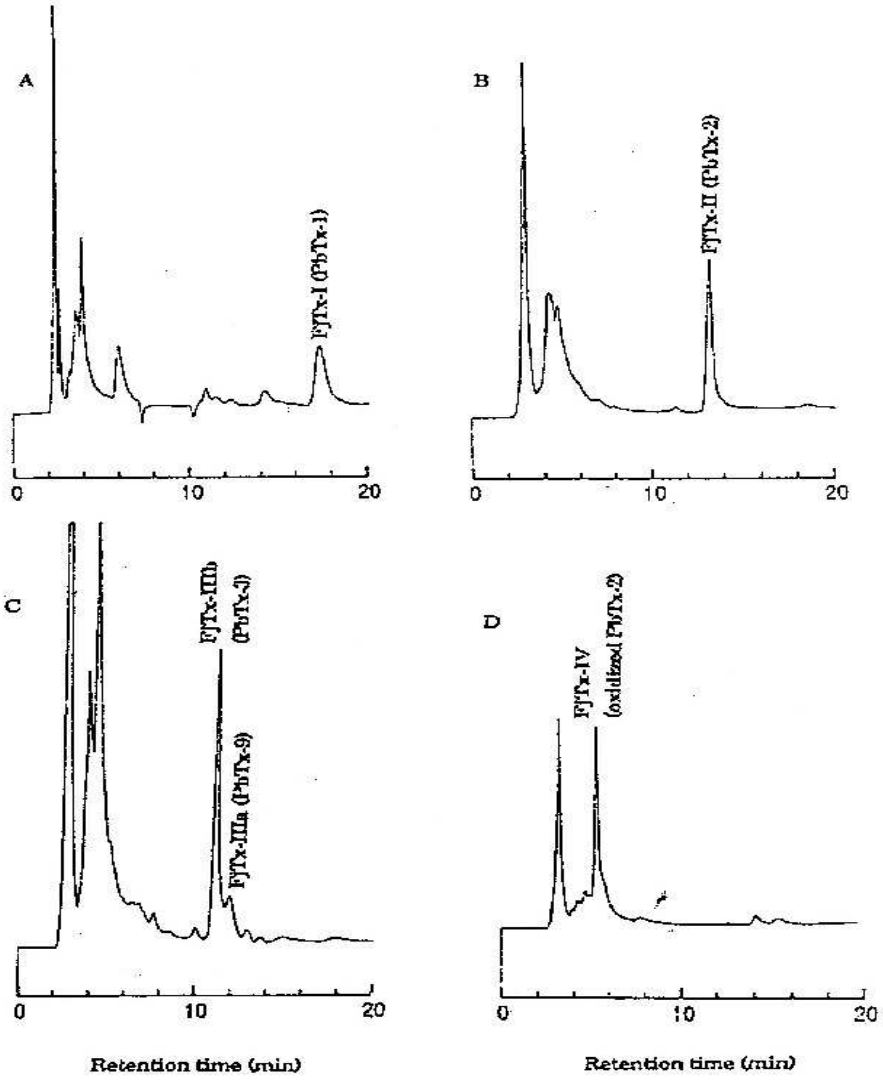


FIGURE 5. *HPLC patterns of* Fibrocapsa japonica. *(A) FjTx-I; (B) FjTx-II; (C) FjTx-IIIa and FjTx-IIIb; and (D) FjTx-IV. Names of matching standard toxins are shown in parentheses.*

Fig. 46. HPLC patterns of fibrocapsin and matched brevetoxins (Khan et al. 1996c)

3. Polyunsaturated Fatty Acids (PUFAs)

Fatty acids are building blocks of lipids which provide energy and support growth throughout cellular life cycles. Fatty acids are taken up by cells, where they may serve

as precursors in the synthesis of other compounds, as fuel for energy production, and as substrates for ketone body synthesis (Fig. 47; internet source: http://medlib.med.utah.edu/NetBiochem/FattyAcids/1_1.html). Fatty acids include polyunsaturated, monounsaturated as well as saturated fatty acids.

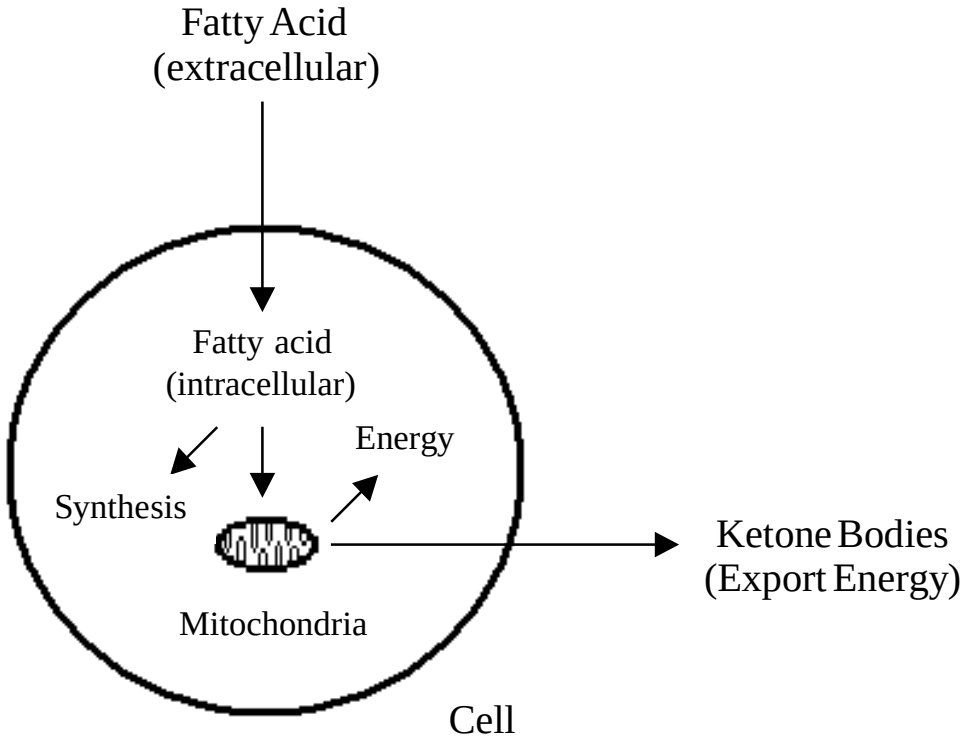


Fig. 47. Significance of fatty acid metabolism

3.1 Are PUFAs beneficial?

Most plant fats are high rich in either polyunsaturated (two and more double bonds) or monounsaturated fats, whereas saturated fatty acids are usually found in animal fats. Saturated and monounsaturated fats are not necessary in the diet as they can be synthesised by the human body. Two PUFAs which can not be made in the body are linoleic acid and α-linolenic acid. They must be obtained from the diet and are therefore known as essential fatty acids. Within the body both can be converted to other PUFAs such as arachidonic acid, eicosapentaenoic acid (EPA) or

docosahexaenoic acid (DHA) (internet source: http://www.vegansociety.com/html/info/info10.html).

Nowadays more and more attention is paid to the nutritional and other functions of PUFAs (e.g. ω-3 fatty acids). More efforts have been given to investigate PUFAs' use as food additives in human nutrition. Some PUFAs are derived from higher plants, whereas others, e.g. EPA, are principally obtained from fish oils. Phytoplankton algae are thought to be a major source of the PUFA in fish oils. PUFAs, either present as free acids, phospholipids or glycolipids, are usually encountered in algal extracts and considered to be membrane components. Their composition can be used for taxonomic identification (species or genus specific PUFAs) and also as an indicator of food quality to herbivores according to the presence or absence of essential fatty acids. At present there is a particular interest in PUFAs for supplementing 'formulas´ used in infant feeding (e.g. Wood et al. 1999; Koletzko & Rodriguez-Palmero 1999).

Recent evidence indicates that long-chain PUFAs (EPA and DHA) can prevent cardiac arrhythmia by a reduction of cardiomyocyte excitability. This was shown to be due to a modulation of the voltage-dependent inactivation of both sodium (I_{Na}) and calcium (I_{Ca}) currents (Vreugdenhil et al. 1996). Furthermore, a number of clinical trials assessed the benefits of dietary supplementation with ω-3 fatty acids in several inflammatory and autoimmune diseases in humans (Borlak & Welch 1994; Simopoulos 2002).

3.2 Are PUFAs harmful?

Contrary to the benefits described above, PUFAs have also been reported to be harmful or toxic. A high proportion of foods rich in ω-6 fatty acids increases the body's production of pro-inflammatory messenger molecules such as cytokines. An

increased ω-6/ω-3 ratio in the diet most likely contributes to a higher incidence of cardiovascular diseases and inflammatory disorders (Simopoulos 2002). Mass use of ω-6-rich vegetable oils is usually the culprit of imbalance ω-6/ω-3 ratio. In addition, excessive heat shapes PUFA from cis to trans configuration which can disrupt normal cellular functions and hinder metabolic activities. Many deep-fried foods and hydrogenated oils contain this kind of fatty acid (internet source: http://www.manitobaharvest.com).

PUFAs seem to be also harmful to lower marine organisms and are known for their haemolytic activities. Yasumoto et al. (1990) identified two haemolytic fractions during a massive fish kill in 1998, associated with a *Gyrodinium aureolum* bloom along the Norwegian coast. The fraction with the highest haemolytic activity against mouse blood cells corresponded to the free fatty acid octadecapentaenoic acid (18:5ω3). Okaichi (1989) discussed the highly unsaturated fatty acids 16:4 and 18:4 as the primary causative substances in finfish mortalities. Among the PUFAs, EPA (20:5ω3) is one of the most harmful ones (Arzul et al. 1995; Jüttner 2001; Takagi et al. 1984). It exhibits lethal toxicity to mice when injected intraperitoneally, causes diarrhea (Sajiki et al. 1993), is haemolytic and also highly repressive for the bioluminescence of *Vibrio fischeri* (reported as *Photobacterium phosphoreum*; Arzul et al. 1998). Although *Heterosigma akashiwo* contains the haemolytic PUFA 18:5ω3 too, 18:4ω3 and 20:5ω3 represent the dominant fatty acids (Bell et al. 1997). *Chattonella* spp. contained 18:5ω3 in a trace amount whereas this component was absent in the two strains of *F. japonica* that Marshall et al. (2002b) studied. EPA was present in high amounts in all Raphidophytes (17-27 %) and produced a similar LD_{50} and fish response to that of *Chattonella marina* cells (Marshall et al. 2002a, b).

The fatty acid profiles showed systematic differences according to taxonomic group, e. g. the distinctly different proportion of DHA EPA and AA in microalgae showed in Fig. 48 (Brown 2002). In addition, although the major fatty acids found in all Raphidophytes are 16:0, 18:4ω3 and 20:5ω3 (Table 7; Marshall et al. 2002b; Mostaert et al. 1998), differences appeared obviously among different strains (e.g. strain FJJp and strain FJNZ in Table 7). That means the geographical and culture conditions do play an important role in toxin production (see points (1) and (2) in page 65). One fact of being worth noting is that EPA, as the predominant component in many microalgae (Fig. 48; Table 7), has different functions, in some non-toxic algal species, it acts as membrane fluidity controlling component, but as the defence agent in some toxic algae (Jüttner 2001), e.g. in *Fibrocapsa japonica*.

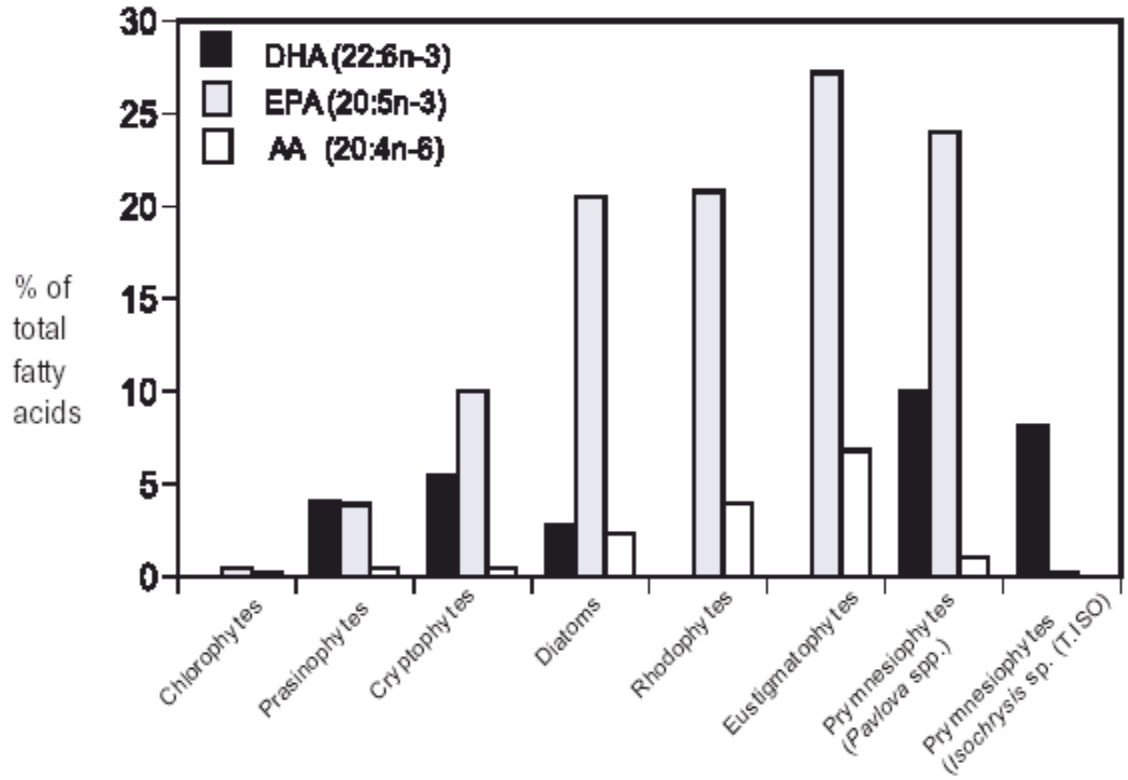


Fig. 48. A summary of the proportion of important PUFAs in 46 strains of microalgae (Brown 2002)

In the HPLC analysis of the *Fibrocapsa japonica* strain studied here, some small peaks near these three main fibrocapsins were found (Figs. 17 and 18). Their retention times are 18.13, 18.99, 21.87 and 26.75 minutes. No efforts were made to identify their structures as too little material was available. However, from Table 7 it appears that these small peaks could be other PUFAs.

Fibrocapsins (OTA, EPA and AA) have already been proven in this work to exhibit very strong haemolytic effects not only on human blood cells but also on rat's (co-operation data will be published elsewhere by Dr. Marion van Rijssel). However, a still unresolved question is why PUFAs, despite being important nutritional supplements in human diets, exhibit haemolytic activity to human erythrocytes. Most probably this is related to the amounts applied although data over a concentration range from beneficial to toxic are not available in the literature. It was reported that dietary supplementation with large doses of PUFAs is potentially harmful (Buffington 1987). High doses of ω-3 fatty acids may alter platelet function to the extent that haemostasis is impaired. Significant increases in bleeding times have been recorded in some studies on fish oil supplements in dogs and cats (Landhmore et al. 1986). Other side effects reported include lethargy, pruritis, diarrhoea and urticaria (Scott and Buerger 1988). It is therefore suggested that PUFAs supplements should be used with caution until new data have been published in this area.

Table 7. Fatty acid composition (%) of raphidophyte species (n = number of replicate samples of the same strain. # = duplicate strains from the same location.) (Marshall et al. 2002b)

	C. marina				*C. antiqua*	*C. subsalsa*	*H. akashiwo*		*F. japonica*		*O. lufeus*
Strain	CMAu#1	CMAu #2	CMJp	CMNZ	CAJp	CSMx n=2	HAAu#2	HANZ	FJJp	FJNZ n=2	OLJp n=2
Saturates											
14:0	11.9	11.8	5.4	7.4	6.8	7.7	5.9	5.8	18.5	20.1	4.6
16:0	22.3	17.1	21.2	19.8	25.8	21.0	25.3	22.8	7.6	10.7	19.4
18:0	0.8	0.8	1.6	1.6	2.2	1.9	1.2	0.6	1.6	0.7	4.4
Sum SFA	35.0	29.8	28.2	28.8	34.8	30.6	32.3	29.2	27.6	31.4	28.4
16:1 ω7c	9.4	7.5	10.1	8.1	9.9	4.4	6.9	7.2	8.1	2.6	1.7	8.4
16:1 ω13t	0.2	1.9	2.6	1.2	2.4	1.7	2.6	3.3	2.7	1.2	1.0	0.2
18:1 ω9c	4.5	5.2	9.9	9.3	6.5	13.5	5.5	5.0	4.4	7.4	6.3	15.6
18:1 ω7c	1.7	1.0	1.7	2.4	1.4	1.6	2.4	1.0	1.6	0.5	5.7	2.6
Sum MUFA	15.8	15.5	24.3	21.0	20.3	21.2	17.5	16.5	16.8	11.6	14.7	26.8
GLA 18:3 ω6	0.2	0.3	0.3	0.1	0.0	0.4	0.3	0.1	0.3	1.3	0.8	0.3
18:5 ω3	0.4	0.7	0.7	0.4	0.4	1.5	7.1	6.7	5.7	0.0	0.0	0.0
18:4 ω3	13.7	19.0	12.7	14.9	12.5	13.2	14.9	19.5	16.5	26.6	12.0	0.3
18:2 ω6	1.9	2.5	1.5	2.0	2.0	3.1	3.2	1.7	2.8	2.9	3.1	2.2
AA 20:4 ω6	1.7	0.0	5.5	4.1	3.7	2.9	1.8	0.8	1.3	4.4	7.2	7.7
EPA 20:5 ω3	22.9	23.4	18.5	19.8	17.7	19.5	16.9	18.7	17.1	17.4	24.5	14.8
20:4 ω3	0.7	0.9	0.6	0.6	0.4	0.9	0.4	0.7	0.4	0.7	1.1	0.3
DPA 22:5 ω6	2.4	2.8	1.4	1.6	2.0	0.4	0.3	0.1	0.1	0.6	0.1	3.6
DHA 22:6 ω3	3.4	3.1	3.3	3.5	2.9	3.2	3.2	3.1	2.8	0.4	0.2	3.4
22:4 ω6	0.2	0.3	1.0	0.8	0.4	0.6	0.3	0.0	0.1	0.3	1.7	2.6
22:5 ω3	0.2	0.4	1.0	0.8	0.4	0.6	0.8	0.3	0.5	0.0	0.0	1.7
Sum PUFA	47.8	53.3	46.4	48.7	42.3	46.3	49.2	51.7	47.4	54.5	50.7	36.6
Other* (%)	1.5	1.5	1.1	1.5	2.6	1.9	1.1	3.9	1.0	6.2	3.2	8.2
Total pg/cell	159	156	419	237	ND	165	286	84	127	2.5	97	ND

* other includes br 15:0, 15:0, 17:0, 20:0, 17:1, 18:1 ω7t. 18:1 ω5t, 20:1, 20:2, 20:3 ω6, 22:1 and an unknown at less than 0.5 pg per cell. ND, not determined

4. Intoxication Mechanisms in *Fibrocapsa japonica* involved HAB events

Both molecular and physiological data on *F. japonica* strains suggest a recent range expansion of this species (Kooistra et al. 2001; De Boer et al. 2002). To my knowledge this is the first report of haemolytic compounds produced by *F. japonica* and the second for the Raphidophyceae. For *Chattonella marina*, three toxic fractions being neurotoxic, haemolytic and hemagglutinative have been described before, but without chemical characterisation of these toxic fractions. All of them were ichthyotoxic to juvenile red seabream (Onoue & Nozawa, 1989).

The mechanism behind the toxicity of *F. japonica* is still under debate. Oda et al (1997) showed the production of reactive oxygen species (ROS) by a Japanese *F. japonica* strain that could damage fish gills. Brevetoxins were recently detected using a competitive ELISA technique in American strains (Bridgers et al. 2002). Jüttner (2001) proposed that the PUFAs observed in algal extracts are in fact the first products of the lipoxygenase cascade that starts upon cell disruption. Lipids are rapidly hydrolysed to yield unsaturated fatty acids, which readily oxidise to hydroperoxyl fatty acids that subsequently cleave into unsaturated aldehydes and ω-oxo-fatty acids. The PUFAs could be part of a defense strategy that rapidly converts an essential cell constituent into a highly toxic grazer toxin. Perhaps the production of PUFAs and ROS (Oda et al. 1992) in *F. japonica* are an indication of such a defense system (Marshall et al. 2002a). Although fish biotests can not be carried out, it is assumed that fishes exposed to a *F. japonica* bloom accumulate algal cells in their gills thereby experiencing strong toxic effects that could be lethal even without neurotoxins being present.

V. CONCLUSIONS

Stationary cultivation technique was used to cultivate *Fibrocapsa japonica* algal cells in which mechanical agitation of the culture flasks was avoided.

The toxicity of algal cells of *Fibrocapsa japonica* was established in *Artemia salina* biotest, whereas the filtrate of it has no toxic effect on *Artemia salina*.

The crude extract of *Fibrocapsa japonica* algal cells was proven to be toxic by both *Artemia salina* and bioluminescence inhibition biotests.

The toxic SPE fractions were screened by three available biotest methods, namely, *Artemia salina* biotest, bioluminescence inhibition biotest and erythrocyte lysis assay.

Three toxic compounds (Fj1 Fj2 and Fj3) were isolated from *Fibrocapsa japonica* SPE toxic fraction mixture in HPLC guided by erythrocyte lysis assay. Their molecular weights were determined as 276, 302 and 304 separately by HPLC-ESI-MS experiments

To sum up the data of ESI-MS-MS, IR, GC-MS, GC-HRMS, ^{1}H-NMR and ^{13}C-NMR experiments, The chemical natures were interpreted initially as polyunsaturated fatty acids i.e. Fj1= 6,9,12,15-octadecatetraenoic acid (OTA, C18:4ω3); Fj2 = 5,8,11,14,17-eicosapentaenoic acid (EPA, C20:5ω3); Fj3 = 5,8,11,14-eicosatetraenoic acid (arachidonic acid AA, C20:4ω6).

The HPLC and NMR experiment results of two commercially available standards all-*cis*-5,8,11,14,17-eicosapentaenoic acid and all-*cis*-5,8,11,14-eicosatetraenoic acid confirm that they are identical with Fj2 and Fj3. The cis/trans configuration of Fj1 is not determined.

The additional three biotest results (*Artemia salina* biotest, bioluminescence inhibition biotest and erythrocyte lysis assay) of these two standards fully support conclusions of structural elucidation.

Neither HPLC nor HPLC-ESI-MS experiment proof can support the existence of brevetoxins in concentrated crude extract of *Fibrocapsa japonica* algal cells, in another word, no brevetoxins were found in this strain of *Fibrocapsa japonica.*

Haemolytic activity is reported for the first time in *Fibrocapsa japonica.*

In addition, a number of other compounds were found at low concentrations in the HPLC separation experiment. They could not be characterised structurally as too little material was available for analysis. The similar absorption characteristics to that of fibrocapsins, however, suggest that they might be polyunsaturated fatty acids as well.

The presence of similarly high amounts of eicosapenatanenoic acid in *F. japonica* as that in other non-toxic algal taxa suggests double roles for the PUFAs in these species, namely acting as membrane fluidity controlling or as defence agent.

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VII. ACKNOWLEDGMENT

Firstly, I would like to give my special appreciation to my supervisor Prof. Dr. Gerd Liebezeit, thanks a lot for your non stop encouragement, help and support not only in my doctor work but also in the daily life, with which I turn to be very courageous to meet strange environment, frustration and overcome different difficulties during my stay in Germany.

I gratefully acknowledge Prof. Dr. Jürgen Rullkötter taking time to be my second referee and your valuable suggestions.

My many thanks also go to Dr. Arne Luetzen for the strong support during the NMR experiments. I have benefited very much from your abundant expertise and even more from your conscientious working attitude.

To my Dutch colleagues Dr. Marion van Rijssel, Dr. Albert Koulman, M. Karin de Boer, Monika R. Tyl, Annie V. Dam, Dr. Andries P. Bruins, to the colleagues from University of Oldenburg Dr. Barbara Scholz-Boettcher, Jochen Maurer, Ralf Woestmann, Bernd Kopke, to Alexander Rühl from University of Jena, I would like to say it was a great co-operative work. All of your efforts and help will be always cherished in my heart.

There are three years in my life that I have spent together with you, all my colleagues in Terramare, "Muschel"-Uwe, Inge, Elke, "Meister"-Uwe, Gisela, Sonja, Angela, Ursula, Brigitte and many others. So many smiling faces, so many warm hearts and so many jokes..... I will never forget them and will miss all of you after I go back to China.

I also would like to thank my best friends, family Horng; Fanny and Hans Karl who let me always feel the warmth of family in Germany. In my hardest time, it were you who gave me the hands, accompanied and comforted me!

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Erklärung:

Hiermit erkläre ich, dass ich mich erstmals um einen Doktorgrad bewerbe und die vorliegende Arbeit selbstaendig verfasst habe. Ich habe keine anderen als die angegebenen Quellen und Hilfsmittel verwendet.

Meng Fu

Den 26. Juni, 2003