



## Chemical characterisation of three haemolytic compounds from the microalgal species *Fibrocapsa japonica* (Raphidophyceae)

Meng Fu<sup>a,\*</sup>, Albert Koulman<sup>b</sup>, Marion van Rijssel<sup>c</sup>, Arne Lützen<sup>d</sup>, M. Karin de Boer<sup>c</sup>,  
Monika R. Tyl<sup>c</sup>, Gerd Liebezeit<sup>a</sup>

<sup>a</sup>Research Centre Terramare, Schleusenstasse 1, 26382 Wilhelmshaven, Germany

<sup>b</sup>Department of Pharmaceutical Biology, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands

<sup>c</sup>Department of Marine Biology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

<sup>d</sup>Department of Chemistry, Carl von Ossietzky University Oldenburg, P.O. Box 2503, 26111 Oldenburg, Germany

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### Abstract

The molecular structures of the three main haemolytic compounds (Fj1, Fj2 and Fj3) isolated from the ichthyotoxic microalgal species *Fibrocapsa japonica* have been investigated by NMR, LC–ESI–MS, ESI–MS–MS, IR, GC–MS and GC–HRMS methods. They are polyunsaturated fatty acids which we identified as: 6,9,12,15-octadecatetraenoic acid (OTA, C18:4 $\omega$ 3), 5,8,11,14,17-eicosapentaenoic acid (EPA, C20:5 $\omega$ 3) and 5,8,11,14-eicosatetraenoic acid (arachidonic acid AA, C20:4 $\omega$ 6). The identity of the latter two was confirmed on the basis of commercial standards (C20:5 $\omega$ 3 and C20:4 $\omega$ 6). Both displayed the same spectroscopic and chromatographic characteristics as Fj2 and Fj3 and had a similar strong haemolytic effect. We propose that when *F. japonica* cells accumulate in fish gills during blooms these compounds could be the cause of ichthyotoxicity.

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**Keywords:** *Fibrocapsa japonica*; Haemolytic compounds; 6,9,12,15-Octadecatetraenoic acid (OTA, C18:4 $\omega$ 3); 5,8,11,14,17-Eicosapentaenoic acid (EPA, C20:5 $\omega$ 3); 5,8,11,14-Eicosatetraenoic acid (arachidonic acid AA, C20:4 $\omega$ 6); High performance liquid chromatography; Nuclear magnetic resonance; Infrared spectroscopy; Liquid chromatography–electrospray ionisation–mass spectrometry; Gas chromatography–mass spectrometry; Gas chromatography–high resolution mass spectrometry; Erythrocyte lysis assay

### 1. Introduction

In the class Raphidophytes, the species *Fibrocapsa japonica*, *Heterosigma akashiwo* and *Chattonella* spp. have been reported to produce red-tide blooms that induce mass mortality of cultured fish in Southeast Asian coastal waters (Toriumi and Takano, 1973; Nakamura and Watanabe, 1983). The species *F. japonica* has caused significant damage to coastal fisheries in Japan (Toriumi and Takano, 1973; Yoshimatsu, 1987; Montani et al., 1995). From the beginning of the 1990s *F. japonica* has been observed in

European coastal waters (Billard, 1992; Vrieling et al., 1995; Rademaker et al., 1998). 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).

The mechanism behind the toxicity of *F. japonica* is still under debate. Oda and co-workers (1997) showed the production of reactive oxygen species by a Japanese *F. japonica* strain that could damage fish gills. Polyether neurotoxins were found in a European strain (W420) and identified as brevetoxins on the basis of their chromatographic behaviour in HPLC (Khan et al., 1996), and brevetoxins were also recently detected using a competitive ELISA technique in American strains (Bridgers et al., 2002).

\* Corresponding author. Fax: +49-4421-944199.

E-mail addresses: meng.fu@terramare.de (M. Fu); gerd.liebezeit@terramare.de (G. Liebezeit).

From the crude extract of *F. japonica* from the German coast (CCRuG, C13, Culture Collection, University of Groningen, The Netherlands) that did not contain components in the size class of brevetoxins (LC–MS), we purified three compounds with pronounced haemolytic activity (Fu et al., 2002). The elucidation of the structures of these three purified compounds is described in the present communication.

## 2. Materials and methods

### 2.1. NMR experiments

The dried haemolytic compounds Fj1 (1.6 mg), Fj2 (2.0 mg) and Fj3 (1.2 mg), obtained by ELA-guided HPLC purification (Fu et al., 2002), were dissolved in methanol- $d_4$  and NMR spectra were recorded from these solutions on an AVANCE 500 NMR spectrometer (Bruker) ( $^1\text{H}$  500.1 MHz;  $^{13}\text{C}$  125.8 MHz). The measurements were carried out at 300 K. The chemical shifts are reported on the  $\delta$ -scale in ppm and were referred to the internal standard, signals of non-deuterated methanol ( $^1\text{H}$ : 3.30 ppm) or signals of deuterated methanol ( $^{13}\text{C}$ : 49.00 ppm). Coupling constants  $J$  are given in Hz. Assignment of the signals was done according to the  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra.

### 2.2. LC–ESI–MS and ESI–MS–MS experiments

Fj1, Fj2 and Fj3 were diluted in 80% acetonitrile (gradient grade from Merck, made with UV-treated deionised water), and several drops of ammonium hydroxide solution (0.25%  $\text{NH}_4\text{OH}$  in acetonitrile) were added. 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  $\mu\text{m}$ , C18, 150  $\times$  2.1  $\text{mm}^2$ ). 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 16 min isocratic 5% A and 95% B. The chromatograms were recorded by a HPLC recorder (Kipp & Zonen). The flow rate was 0.2 ml/min, with an injection volume of 50  $\mu\text{l}$ .

Preliminary experiments showed fibrocapsins to easily lose protons. Mass spectrometry was therefore performed in the negative ion detection mode for which a post-column base condition (0.25%  $\text{NH}_4\text{OH}$  in acetonitrile) was supplied at 100  $\mu\text{l}/\text{min}$  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 ionisation voltage was set to  $-4.5$  kV. Nitrogen was employed as both

nebulizing gas and drying gas (heated to 450  $^\circ\text{C}$ , 2 bar, 2 l/min). Ion detection was performed in negative mode under the energy condition (OR =  $-15$  V, RNG =  $-150$  V). The scan data were acquired with a 0.1 u step width and 0.1 ms dwell time.

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  $\mu\text{l}/\text{min}$ . Ion detection was performed in the negative ion mode under the energy conditions OR =  $-41$  V and RNG =  $-130$  V. 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.

### 2.3. IR experiments

Three haemolytic compounds Fj1, Fj2 and Fj3 were each dissolved in 100% methanol (HPLC grade from Merck). Several drops of solutions were taken and dripped on the sodium chloride plate, which was then air dried. The plate was measured in the IR spectrometer (ATI Mattson Genesis Series FTIR<sup>TM</sup>).

### 2.4. GC–MS and GC–HRMS experiments

The haemolytic compounds Fj1 (170  $\mu\text{g}$ ), Fj2 (250  $\mu\text{g}$ ) and Fj3 (140  $\mu\text{g}$ ) dissolved separately in 200  $\mu\text{l}$   $\text{CH}_2\text{Cl}_2$ , were treated ultrasonically for 15 min. Hundred microlitres Fj1, 70  $\mu\text{l}$  Fj2 and 100  $\mu\text{l}$  Fj3 were then incubated with 50  $\mu\text{l}$  *N*-methyl-*N*-trimethylsilyltrifluoroacetamide solution (MSTFA, CS-Chromatographie Service GmbH) for 1 h at 80  $^\circ\text{C}$ .

GC–MS analyses of the volatised TMS-fibrocapsins were performed on a 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 quartz capillary column (30 m  $\times$  0.25 mm ID, 0.25  $\mu\text{m}$  film thickness) with helium as the carrier gas. The samples were injected at an oven temperature of 60  $^\circ\text{C}$ . After 1 min the temperature was programmed to raise to 300  $^\circ\text{C}$  at 3  $^\circ\text{C}/\text{min}$  and kept for 50 min at this temperature.

GC–HRMS analyses applied a Finnigan MAT 95 Q mass spectrometer with ICIS 8.2.1, 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  $\times$  0.25 mm ID, 0.25  $\mu\text{m}$  film thickness). The samples were injected also at an oven temperature of 60  $^\circ\text{C}$ . After 2 min, the temperature was raised to 150  $^\circ\text{C}$  at a 10  $^\circ\text{C}/\text{min}$  rate and then to 300  $^\circ\text{C}$  at a 4  $^\circ\text{C}/\text{min}$  and kept there for 10 min.

### 2.5. HPLC, ELA and NMR experiments with standards

Two standards, all-*cis*-5,8,11,14,17-eicosapentaenoic acid (EPA, C20:5 $\omega$ 3) and all-*cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid AA, C20:4 $\omega$ 6) obtained from Sigma-Aldrich, were dissolved in 100% methanol and stored at  $-18^{\circ}\text{C}$  before measurements. One crude extract sample of *F. japonica* was measured twice, once with and once without addition of these two standards. The HPLC protocol followed Fu et al. (2002).

ELA was carried out to test the haemolytic effects of these two standards (10  $\mu\text{g}/\text{ml}$  each). The method was derived from Eschbach et al. (2001) using human erythrocytes instead of erythrocytes from fish and has been described in Fu et al. (2002).

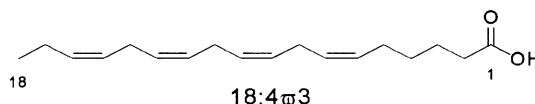
Dried EPA (3.2 mg) and AA (7.8 mg) were used for the NMR measurements as described above (Section 2.1).

## 3. Results

### 3.1. Physical and spectroscopic data of Fj1

Fj1 was obtained as colourless oil: UV  $\lambda_{\text{max}}$  (HPLC-DAD) 210 nm; LC-ESI-MS (negative mode)  $m/z$  [M-H] $^{-}$  275, [M + HCOO] $^{-}$  321, [2M-H] $^{-}$  551; ESI-MS-MS (negative mode)  $m/z$  [M-H, 100] $^{-}$  275.1, [M-H-H<sub>2</sub>O, 0.9] $^{-}$  257.3, [M-H-CO<sub>2</sub>, 7] $^{-}$  231.2; IR (dry film) 3000–2500  $\text{cm}^{-1}$  (OH), 1685  $\text{cm}^{-1}$  (CH=O); <sup>1</sup>H-NMR (CD<sub>3</sub>OD, 500.1 MHz)  $\delta$  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; <sup>13</sup>C-NMR (CD<sub>3</sub>OD, 125.8 MHz)  $\delta$  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 clearly be distinguished from the noise.

GC-MS (70 eV) of Fj1-TMS:  $m/z$  [M] $^{+}$  348 [C<sub>21</sub>H<sub>36</sub>O<sub>2</sub>Si, 7], 333 [C<sub>20</sub>H<sub>33</sub>O<sub>2</sub>Si, 6], 305 [C<sub>18</sub>H<sub>29</sub>O<sub>2</sub>Si, 0.07], 279 [C<sub>16</sub>H<sub>27</sub>O<sub>2</sub>Si, 4], 264 [C<sub>15</sub>H<sub>25</sub>O<sub>2</sub>Si, 1], 258 [M-OH-TMS, 0.01], 252 [(CH<sub>3</sub>)<sub>2</sub>-Si-O-CO-(CH<sub>2</sub>)<sub>4</sub>-(CH)<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 6], 229 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-(CH<sub>2</sub>)<sub>2</sub>-CH=CH, 1], 216 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-CH<sub>2</sub>-CH=CH<sub>2</sub>, 1], 201 [TMSO-CO-(CH<sub>2</sub>)<sub>5</sub>-CH<sub>2</sub>, 3], 189 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-CH<sub>2</sub>, 13], 175 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH<sub>2</sub>-CH=CH, 16], 161 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH=CH, 27], 149 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH<sub>2</sub>, 9], 147 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-CH<sub>2</sub>-CH=C=CH, 35], 145 [TMSO-CO-CH<sub>2</sub>-CH<sub>2</sub>, 30], 132 [TMSO-C(OH)=CH<sub>2</sub>, 9], 129 [(CH<sub>3</sub>)<sub>2</sub>-SiO-CO-CH=CH<sub>2</sub>, 45], 120 [CH<sub>2</sub>=(CH)<sub>2</sub>=CH-CH<sub>2</sub>-CH=(CH)<sub>2</sub>=CH<sub>2</sub>, 45], 117



Structure 1. Fj1: 6,9,12,15-octadecatetraenoic acid (C18:4 $\omega$ 3).

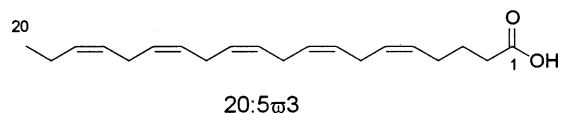
[(CH<sub>3</sub>)<sub>2</sub>-SiO-C(OH)=CH<sub>2</sub>, 61], 108 [CH<sub>2</sub>=C=CH-CH<sub>2</sub>-CH=CH-CH<sub>2</sub>-CH<sub>3</sub>, 85], 91 [CH $\equiv$ C-CH<sub>2</sub>-(CH)<sub>2</sub>-CH=CH, 86], 79 [CH<sub>2</sub>=C=CH-CH<sub>2</sub>-C=CH, 100], 75 [(CH<sub>3</sub>)<sub>2</sub>SiOH, 78], 73 [(CH<sub>3</sub>)<sub>3</sub>Si, 75], 67 [CH<sub>3</sub>-CH<sub>2</sub>-CH=C=CH, 27], 55 [CH<sub>3</sub>-CH<sub>2</sub>-CH=CH, 15]; GC-HRMS  $m/z$  348.2460 (calculated for C<sub>21</sub>H<sub>36</sub>O<sub>2</sub>Si, 348.2485).

The data show that Fj1 is a molecule with a molecular weight of 276, containing 18 C atoms, a carboxyl-group and four double bonds at positions 6, 9, 12 and 15. The first double bond from the terminal methyl group C is at the third position. From these data we propose the structure of Fj1 to be polyunsaturated fatty acid (PUFAs) 6,9,12,15-octadecatetraenoic acid (C18:4 $\omega$ 3, Structure 1).

### 3.2. Physical and spectroscopic data of Fj2

Fj2 was also obtained as colourless oil: UV  $\lambda_{\text{max}}$  (HPLC-DAD) 210 nm; LC-ESI-MS (negative mode)  $m/z$  [M-H] $^{-}$  301, [M + HCOO] $^{-}$  347, [2M-H] $^{-}$  604; ESI-MS-MS (negative mode)  $m/z$  [M-H, 100] $^{-}$  301.3, [M-H-H<sub>2</sub>O, 0.4] $^{-}$  282.9, [M-H-CO<sub>2</sub>, 16] $^{-}$  257.3; IR (dry film) 3000–2500  $\text{cm}^{-1}$  (OH), 1704  $\text{cm}^{-1}$  (C=O); <sup>1</sup>H-NMR (CD<sub>3</sub>OD, 500.1 MHz)  $\delta$  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; <sup>13</sup>C-NMR (CD<sub>3</sub>OD, 125.8 MHz)  $\delta$  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).

GC-MS (70 eV) of Fj2-TMS:  $m/z$  [M] $^{+}$  374 [C<sub>23</sub>H<sub>38</sub>O<sub>2</sub>Si, 0.51], 359 [C<sub>22</sub>H<sub>35</sub>O<sub>2</sub>Si, 1], 345 [C<sub>21</sub>H<sub>33</sub>O<sub>2</sub>Si, 0.74], 331 [C<sub>20</sub>H<sub>31</sub>O<sub>2</sub>Si, 0.51], 305 [C<sub>18</sub>H<sub>29</sub>O<sub>2</sub>Si, 3], 284 [M-OH-TMS, 0.18], 278 [(CH<sub>3</sub>)<sub>2</sub>-Si-O-CO-(CH<sub>2</sub>)<sub>3</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH<sub>2</sub>, 2], 242 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>5</sub>-CH=CH<sub>2</sub>, 2], 201 [TMSO-CO-(CH<sub>2</sub>)<sub>5</sub>-CH<sub>2</sub>, 14], 189 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-CH<sub>2</sub>, 3], 175 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH<sub>2</sub>-CH=CH, 22], 161 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH=CH, 15], 149 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH<sub>2</sub>, 7], 147 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-CH<sub>2</sub>-CH=C=CH, 22], 145 [TMSO-CO-CH<sub>2</sub>-CH<sub>2</sub>, 33], 132 [TMSO-C(OH)=CH<sub>2</sub>, 18], 129 [(CH<sub>3</sub>)<sub>2</sub>-SiO-CO-CH=CH<sub>2</sub>, 43], 120 [CH<sub>2</sub>=(CH)<sub>2</sub>=CH-CH<sub>2</sub>-CH=(CH)<sub>2</sub>=CH<sub>2</sub>, 37], 117 [(CH<sub>3</sub>)<sub>2</sub>-SiO-C(OH)=CH<sub>2</sub>, 100], 108 [CH<sub>2</sub>=C=CH-CH<sub>2</sub>-CH=CH-CH<sub>2</sub>-CH<sub>3</sub>, 53], 91 [CH $\equiv$ C-CH<sub>2</sub>-(CH)<sub>2</sub>-CH=CH, 95], 79 [CH<sub>2</sub>=C=CH-CH<sub>2</sub>-C=CH, 78], 75

Structure 2. Fj2: 5,8,11,14,17-eicosapentaenoic acid (C20:5 $\omega$ 3).

[(CH<sub>3</sub>)<sub>2</sub>SiOH, 56], 73 [(CH<sub>3</sub>)<sub>3</sub>Si, 61], 67 [CH<sub>3</sub>-CH<sub>2</sub>-CH=C=CH, 23], 55 [CH<sub>3</sub>-CH<sub>2</sub>-CH=CH, 13]; GC-HRMS *m/z* 374.2624 (calculated for C<sub>23</sub>H<sub>38</sub>O<sub>2</sub>Si, 374.2641).

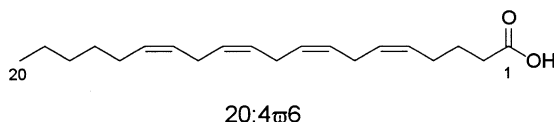
The above data prove the structural identity of Fj2 with the polyunsaturated fatty acid 5,8,11,14,17-eicosapentaenoic acid (C20:5 $\omega$ 3, Structure 2).

### 3.3. Physical and spectroscopic data of Fj3

Fj3 was obtained as colourless oil, too: UV  $\lambda_{\max}$  (HPLC-DAD) 210 nm; LC-ESI-MS (negative mode) *m/z* [M-H]<sup>-</sup> 303, [M + HCOO]<sup>-</sup> 349, [2M-H]<sup>-</sup> 607; ESI-MS-MS (negative mode) *m/z* [M-H, 100]<sup>-</sup> 303.4, [M-H-H<sub>2</sub>O, 0.2]<sup>-</sup> 285.1, [M-H-CO<sub>2</sub>, 7]<sup>-</sup> 259.4; IR (dry film) 3000–2500 cm<sup>-1</sup> (OH), 1681 cm<sup>-1</sup> (CH=O); <sup>1</sup>H-NMR (CD<sub>3</sub>OD, 500.1 MHz)  $\delta$  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). The amount of material was too little to record <sup>13</sup>C-NMR spectrum.

GC-MS (70 eV) of Fj3-TMS: *m/z* [M]<sup>+</sup> 376 [C<sub>23</sub>H<sub>40</sub>O<sub>2</sub>Si, 5], 361 [C<sub>22</sub>H<sub>37</sub>O<sub>2</sub>Si, 6], 305 [TMSO-CO-(CH<sub>2</sub>)<sub>3</sub>-((CH<sub>2</sub>)<sub>2</sub>-CH<sub>2</sub>)<sub>3</sub>-CH=CH, 1], 278 [(CH<sub>3</sub>)<sub>2</sub>-Si=O-CO-(CH<sub>2</sub>)<sub>3</sub>-((CH<sub>2</sub>)<sub>2</sub>-CH<sub>2</sub>)<sub>3</sub>-CH<sub>2</sub>, 4], 265 [TMSO-CO-(CH<sub>2</sub>)<sub>3</sub>-((CH<sub>2</sub>)<sub>2</sub>-CH<sub>2</sub>)<sub>2</sub>-CH=CH, 4], 244 [CH<sub>3</sub>-(CH<sub>2</sub>)<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-CH=CH<sub>2</sub>, 4], 238 [(CH<sub>3</sub>)<sub>2</sub>-Si=O-CO-(CH<sub>2</sub>)<sub>3</sub>-((CH<sub>2</sub>)<sub>2</sub>-CH<sub>2</sub>)<sub>2</sub>-CH<sub>2</sub>, 8], 231 [CH<sub>3</sub>-(CH<sub>2</sub>)<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-CH<sub>2</sub>, 2], 217 [CH<sub>3</sub>-(CH<sub>2</sub>)<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH<sub>2</sub>-CH=CH, 8], 203 [CH<sub>3</sub>-(CH<sub>2</sub>)<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH=CH, 12], 187 [TMSO-CO-(CH<sub>2</sub>)<sub>4</sub>-CH<sub>2</sub>, 9], 175 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH<sub>2</sub>-CH=CH, 24], 161 [CH<sub>3</sub>-(CH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-CH=CH, 15], 150 [CH<sub>3</sub>-(CH<sub>2</sub>)<sub>4</sub>-(CH<sub>2</sub>-CH<sub>2</sub>-CH=C=CH<sub>2</sub>, 48], 145 [TMSO-CO-CH<sub>2</sub>-CH<sub>2</sub>, 28], 132 [TMSO-C(OH)=CH<sub>2</sub>, 19], 129 [(CH<sub>3</sub>)<sub>2</sub>-SiO-CO-CH=CH<sub>2</sub>, 49], 120 [CH<sub>2</sub>=(CH)<sub>2</sub>=CH-CH<sub>2</sub>-CH=(CH)<sub>2</sub>=CH<sub>2</sub>, 40], 117 [(CH<sub>3</sub>)<sub>2</sub>-SiO-C(OH)=CH<sub>2</sub>, 100], 108 [CH<sub>2</sub>=C=CH-CH<sub>2</sub>-CH=CH-CH<sub>2</sub>-CH<sub>3</sub>, 9], 91 [CH=C-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-CH=CH, 83], 79 [CH<sub>2</sub>=C=CH-CH<sub>2</sub>-C=CH, 73], 75 [(CH<sub>3</sub>)<sub>2</sub>SiOH, 57], 73 [(CH<sub>3</sub>)<sub>3</sub>Si, 64], 67 [CH<sub>3</sub>-CH<sub>2</sub>-CH=C=CH, 24], 55 [CH<sub>3</sub>-CH<sub>2</sub>-CH=CH, 15]; GC-HRMS *m/z* 376.2851 (calculated for C<sub>23</sub>H<sub>38</sub>O<sub>2</sub>Si, 376.2798).

Fj3 is therefore proposed to be the third polyunsaturated fatty acid, namely 5,8,11,14-eicosatetraenoic acid (C20:4 $\omega$ 6, Structure 3).

Structure 3. Fj3: 5,8,11,14-eicosatetraenoic acid (C20:4 $\omega$ 6).

### 3.4. HPLC, ELA and NMR experiments with standards

The HPLC chromatograms with and without addition of EPA (C20:5 $\omega$ 3) and AA (C20:4 $\omega$ 6) standards showed Fj2 and Fj3 to have identical retention times with EPA and AA (Fig. 1). The <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of EPA and Fj2 showed no differences (Fig. 2), as did the <sup>1</sup>H-NMR spectra for AA and Fj3 (Fig. 3). The ELA results (Fig. 4) of these two standards demonstrated the same strong haemolytic effects as those of Fj2 and Fj3 (Fu et al., 2002).

## 4. Discussion

The three haemolytic compounds isolated from a methanol extract of *F. japonica* were identified as polyunsaturated fatty acids (PUFAs). PUFAs, either present as free acids, phospholipids or glycolipids, are usually encountered in algal extracts and considered to be membrane components. Fatty acid composition can be used in taxonomic identification (species or genus specific PUFAs) and also serves as an indicator of food quality to herbivores according to the presence or absence of essential fatty acids. These compounds have also been investigated for their use as food additives in human nutrition (Wood et al., 1999) and their effects on various inflammatory and autoimmune diseases (Borlak and Welch, 1994; Simopoulos, 2002). Furthermore, the role of PUFAs in infant nutrition also has received considerable attention (cf. Koletzko and Rodriguez-Palmero, 1999).

Although essential for some herbivores, high concentrations of PUFAs have been reported to be toxic. 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 (18:5 $\omega$ 3). Okaichi (1989) reported that the highly unsaturated fatty acids 16:4 and 18:4 may be the primary causative substances in finfish mortalities. Among the PUFAs, EPA (C20:5 $\omega$ 3) is one of the most harmful (Arzul et al., 1995; Jüttner, 2001; Takagi et al., 1984). It exhibits lethal toxicity to mice when injected intraperitoneally, it can cause diarrhea (Sajiki et al., 1993), it is haemolytic, and it is also highly repressive for the bioluminescence of *Vibrio fischeri* (reported as *Photobacterium phosphoreum*, Arzul et al., 1998).

The major fatty acids found in the Raphidophytes are 16:0, 18:4 $\omega$ 3 and 20:5 $\omega$ 3 (Marshall et al., 2002b; Mostaert

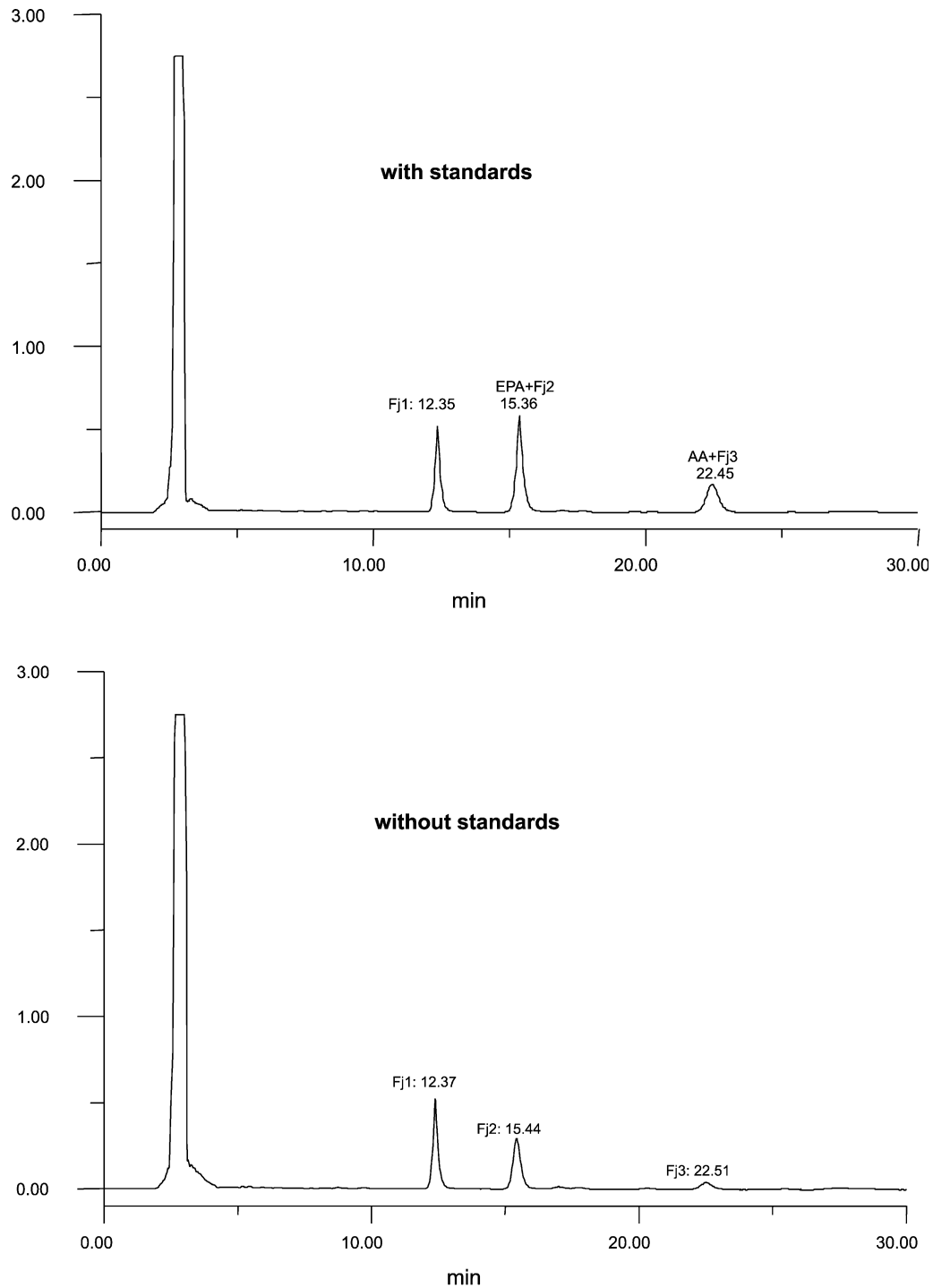
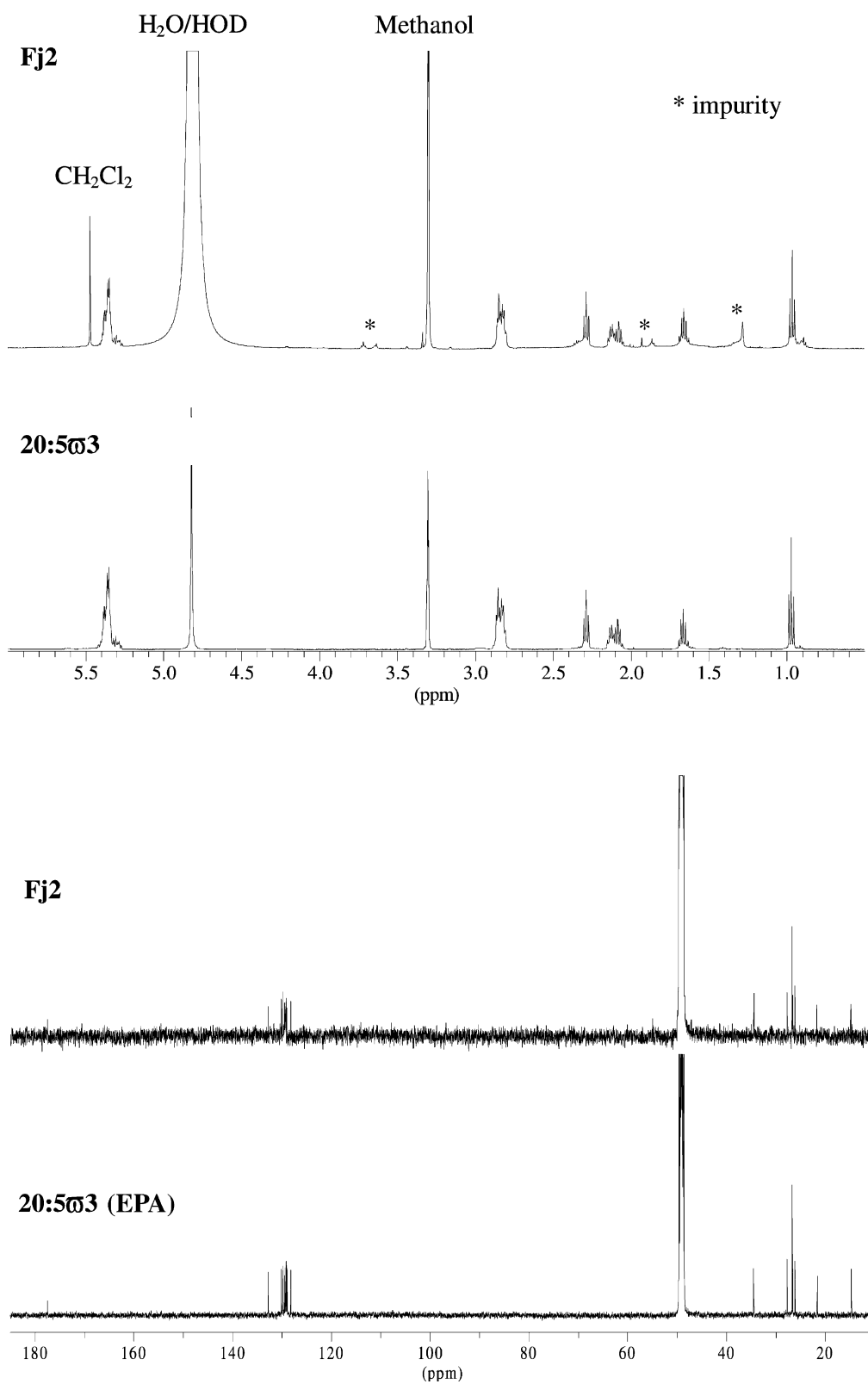


Fig. 1. HPLC chromatogram of crude extract of *Fibrocapsa japonica* (with and without internal standards, detection wavelength 210 nm).

et al., 1998). *Heterosigma akashiwo* was reported to contain the haemolytic PUFA 18:5 $\omega$ 3 too (Bell et al., 1997), but 18:4 $\omega$ 3 and 20:5 $\omega$ 3 were dominant and no C20:4 $\omega$ 6 was detected. *Chattonella* spp. contained 18:5 $\omega$ 3 as trace while

this component was absent in the two strains of *F. japonica* that Marshall and co-workers studied. EPA was present in a high amount in all the raphidophytes species studied (17–27%) (Marshall et al., 2002b).

Fig. 2. <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of EPA (C20:5ω3) and Fj2.

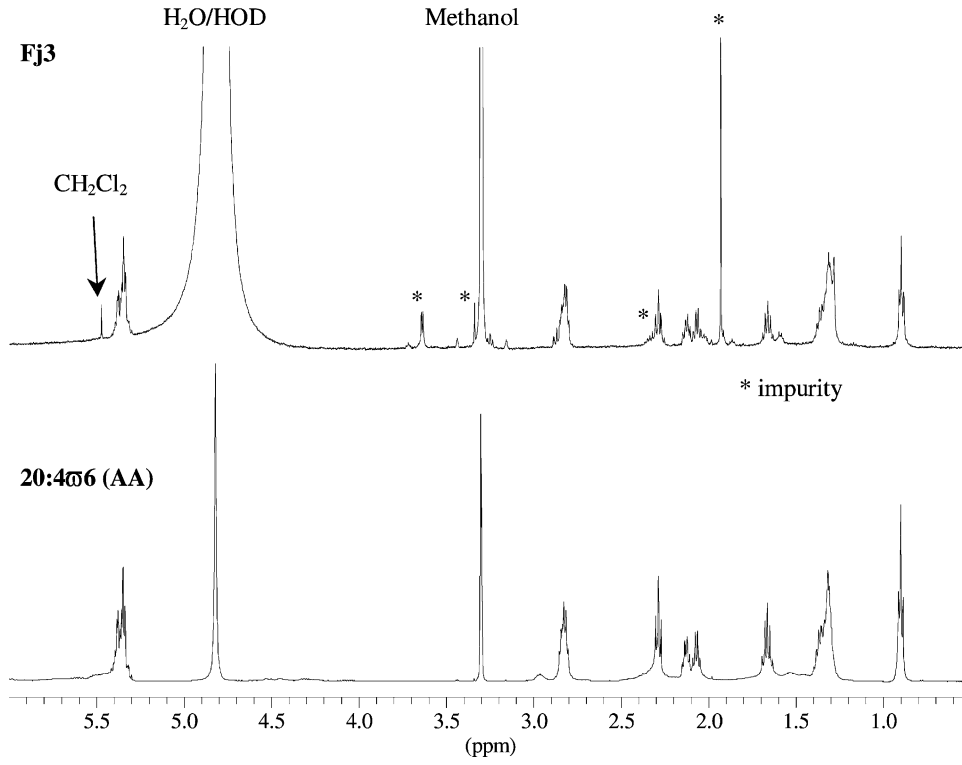


Fig. 3. <sup>1</sup>H-NMR spectra of AA (C20:4ω6) and Fj3.

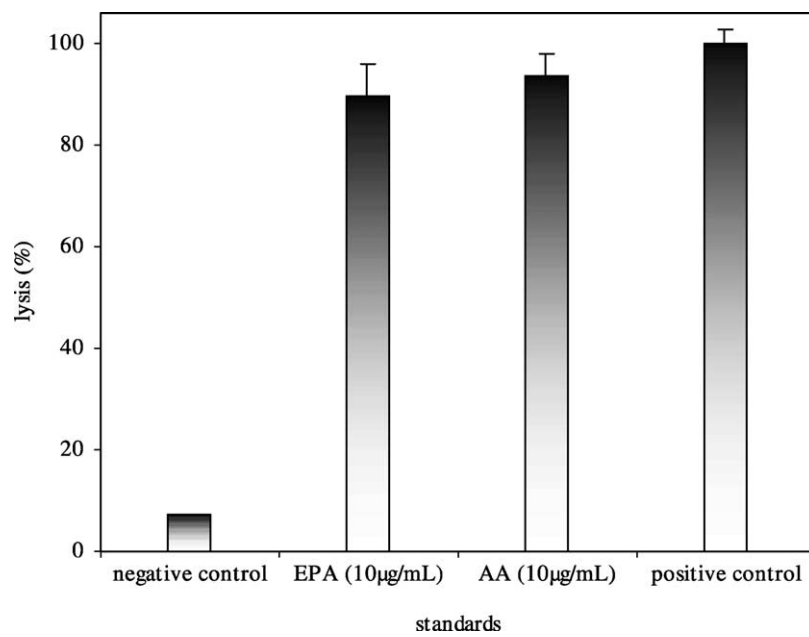


Fig. 4. Haemolytic effects of two standards (10 µg/ml each) after 24 h incubation (negative control: ELA buffer and human erythrocytes; positive control: ELA buffer and completely lysed human erythrocytes).



Jüttner (2001) proposed that the polyunsaturated fatty acids 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 hydroperoxy fatty acids that subsequently cleave into unsaturated aldehydes and  $\omega$ -oxo-fatty acids. The PUFAs could be part of a defence 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 defence system (Marshall et al., 2002a). Although we did not carry out any fish biotest, we assume that fishes involved in a *F. japonica* bloom accumulate cells in their gills and thereby experience strong toxic effects that could be lethal even without neurotoxins being present.

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