Isolation and characterization of bioactive compounds from *Scenedesmus* sp. by Spiral Coil Countercurrent Chromatography (*spiral*-CCC)

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Introduction:

This BMELV/ BLE funded project 'FENA – Fish meal and oil alternatives for a durable, sustainable aquaculture' has the aim to develop a high quality fish feed based on yeast and algae to reduce the demand for fish meal and oil, which are currently the main components of many fish feed [1].

To date there is little evidence of the metabolic profile of *Scenedesmus* sp. (Chlorophyceae, Chlorococcales, Scenedesmaceae), a micro green algae [2,3]. Antioxidant components have been described in *Scenedesmus* species [4] and detected by HPLC-onlineTEAC, but not yet sufficiently characterized. The focus is the characterization of compounds that are responsible for the high antioxidant activity (Figure 3).

Sample preparation:

Lyophilized cells of *Scenedesmus* sp. were exhaustively macerated in MeOH (over 5 days). The metabolites of the extract were partitioned by Liquid-Liquid-Extraction with solvents of different polarities (*n*-hexane, CH_2Cl_2, EtOAc).

Spiral-CCC:

Then 5 g of the CH_2Cl_2 extract were fractionated in a 5.5 liter preparative *spiral*-CCC unit [5,6]. The two-phase system *n*-hexane/ACN [1:1 *(v/v)*] was operated in the 'head - to-tail' mode (Rotation: 280, Flow 15 mL/min]. By using elution and extrusion over 10 hours the spiral-CCC separation was obtained 28 fractions.
The collected fractions were analyzed by TLC and APCI-MS/MS offline injection.
The further chromatographic separations were performed using column chromatography, SPE and/or preparative HPLC.

HPLC-DAD-onlineTEAC & LC-ESI-MS/MS:

Each isolated fraction was analyzed by HPLC-DAD-onlineTEAC (Knauer, Berlin, Germany) and LC-ESI-ion trap-MS/MS (Dionex, Sunnyvale, USA; Bruker Daltonics, Billerica, USA) using a Kinetex column (C18; 150 mm × 2.1 mm i.d., 2.6 µm, Phenomenex Inc., Torrance ,CA, USA). The separation was achieved by a gradient using a composition of deonized water, ACN and formic acid. The column temperature was kept at 25 °C with a flow rate of 0.25 mL/min was used. By using the LC-ESI-MS/MS, the positive and negative ion traces were analyzed by detecting the 2 most abundant precursor ions.

A final structure elucidation of isolated compounds was performed by 1D/2D-NMR-spectroscopy and biological- chemical evaluation.

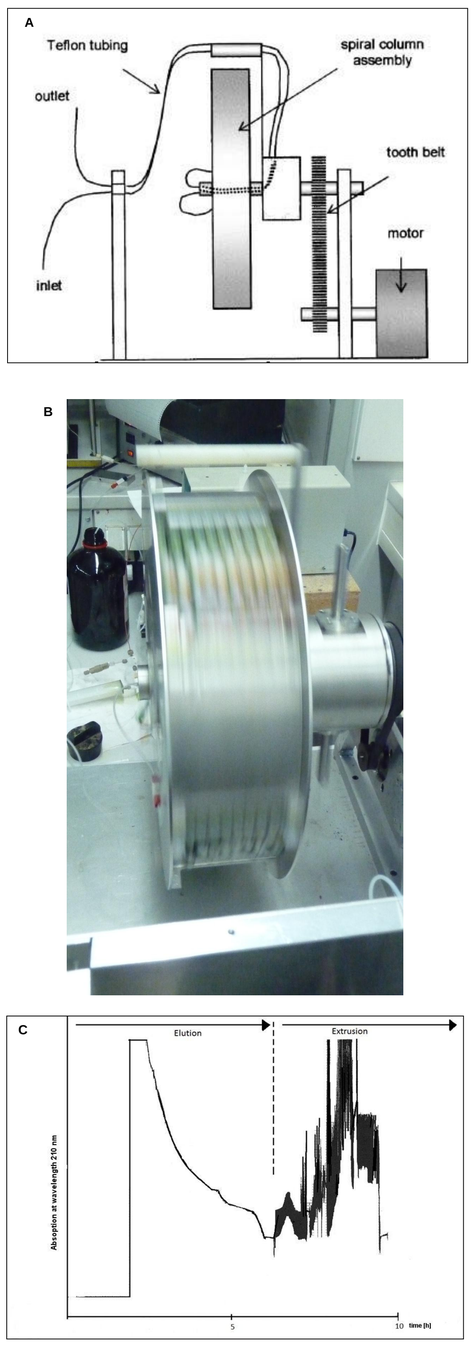

Figure 1
A: Side view, schematic construction of the *spiral*-CCC [5]

B: *spiral*-CCC in operation; visual separation of chlorophyll pigments and carotenoids

C: Chromatogram of the *spiral*-CCC-separation at a wavelength of 210 nm

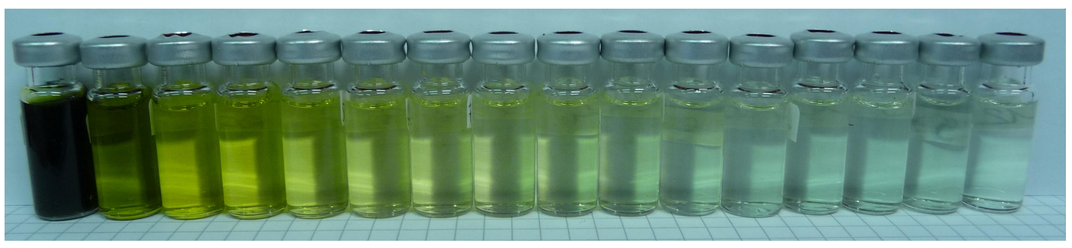

Figure 2 Color change of the *spiral-CCC* separation by taking every 10th test tube

APCI-MS/MS profile:

To check the purity of the *spiral*-CCC fractions, the samples were diluted by a factor of 4 and analyzed by offline continuous injection APCI-MS/MS in positive and negative ionization mode. For generating this profile, every 8th test tube was injected. The APCI-MS/MS system was set to monitor the most abundant 7 precursor ions with its respective MS/MS fragment ions. This APCI-MS/MS profiling of preparative *spiral*-CCC fractions enabled a targeted and precise fractionation of the metabolites. Figure 3 shows the offline-profile of the *spiral*-CCC over a 2.5 h duration in the positive detection mode. By profiling it can be shown, that there is a clear separation of carotenoid (**5**) and chlorophyll (**3,4**) derivates and their degradation products (**1,2,5**) [4,7].

Through the enrichment of the preparative *spiral*-CCC is a clearly separation from chlorophyll *a* (**4**) and pheophobide *a* (**1**) visible. In stead of this, there is no separation of chlorophyll *b* (**3**) and its degradation products like pheophobide *b*. Accordingly to carotenoid derivates (**2,5**), astaxanthine, adonixanthine, adonirubine and canthaxanthine were detected.

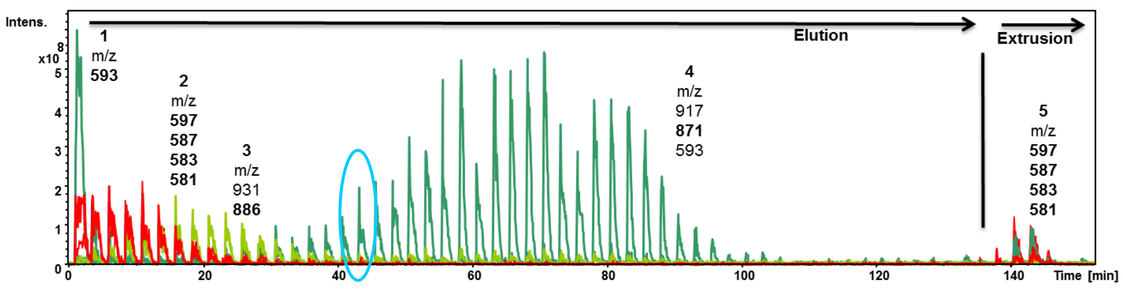

Figure 3 Ion-traces of the *spiral*-CCC by offline APCI-MS/MS (pos. Mode) infusion: *m/z* $[m+H^+]^+$; Fractions 16/17 are blue encircled

Structure elucidation of selected *spiral*-CCC fractions

At first, to start to identify components in Scenedesmus, the *spiral*-CCC fractions 16 and 17 [Figure 3, highlighted in blue] were taken and cleaned by column chromatography (Silica gel). At first the fractions were eluted with an eluent composition 1:1 *(v/v)* (Dichloromethane/ Methanol) and later with 100% Methanol. This separation provided 13 fractions.

In the LC-ESI-MS/MS spectra of the components of the *spiral*-CCC fractions 16/17 [Figure 4] are signals at the *m/z* values which correspond to the molecular weights of chlorophyll *a* and it's breakdown catabolites: chlorophyll *a* sodium adduct (916.9), pheophytin *a* (871.7), pheophytin *a* hydroperoxyde (917.7) and the typical fragmentation ions of pheophorbide *a* (593.3) and 533.3 for chlorophyllone $[M\text{-}CH_3COOC_{20}H_{39}]^+$.
Further components such as carotenoid derivates cannot be observed in this fractions or were under the limit of detection.

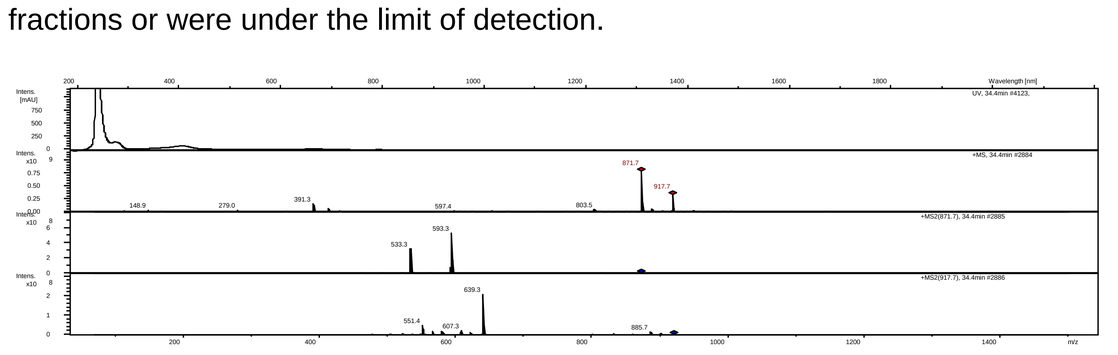

Figure 4 LC-ESI-MS/MS of the main component of spiral-CCC fractions 16/17

Literatur
[1] Verbundprojekt: FENA - Fischmehl- und -öl Ersatzstoffe für eine nachhaltige Aquakultur - Teilprojekt 5, http://www.fisaonline.de/ [2] M.D. Guiry In: Guiry, M.D. & Guiry, G.M. (2013) *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org [3] The National Center for Biotechnology Information, www.straininfo.net/taxa/373792/browser/ncbi_approx [4] C. Grewe, Untersuchungen zur Astaxanthin-Biosynthese in den Grünalgen Scenedesmus sp. und Haematococcus pluvialis, 2009, http://www.dnb.de [5] Köhler et al. (2004), J. Liq. Chromatogr. Relat. Technol., 27:16, 2547 [6] Ito (2005), *J. Chromatogr. A* 1065 (2), 2005, 145. [7] Jerz et al., Recent Advances in the Analysis of Food and Flavors, pp 145-165. *ACS Symposium Series*; American Chemical Society: Washington, DC, 2012.

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