UiT The Arctic University of Norway

The Faculty of Bioscience, Fisheries and Economics

Naphthoquinone pigments from the green sea urchin, *Strongylocentrotus droebachiensis*

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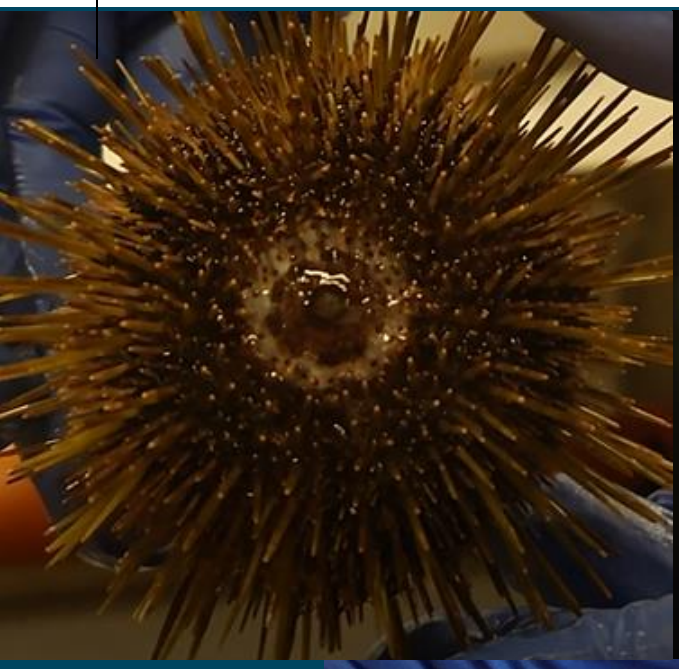

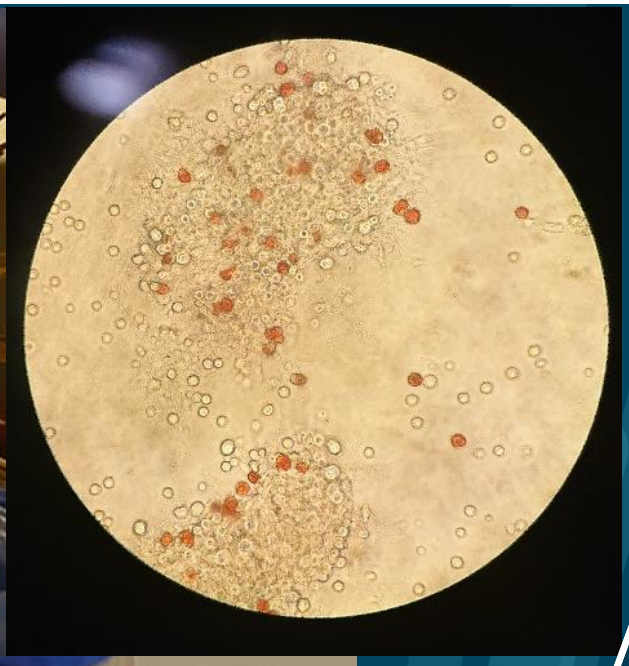

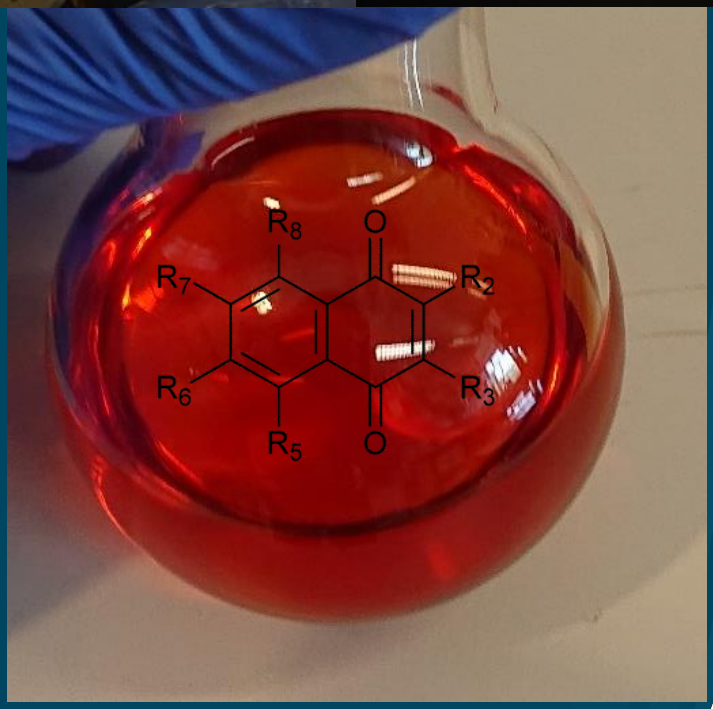

Abstract

Spinochromes are part of a subgroups of quinones called naphthoquinones. These molecules have a series of bioactivity making them a promising target for marine researchers all over the world. Sulphated spinochromes was recently identified, but their structure or bioactivity is yet to be elucidated. In this project, an attempt to extract these sulphated spinochromes from red spherule cells are made. This was done with two different solvents, Milli-Q water, using the principle of hypotonic condition to extract cell content, and Methanol + Trifluoracetic acid which will lyse the cell membrane so that the content leaks out. The extracts were then separated and analysed with UHPLC-DAD-MS. It was determined that none of the extracts contained any of the sulphated spinochromes at the time of the analysis. Due to time restrictions and limited access to an LC-MS system, the data was limited. Therefore, the extracted compounds were identified by available mass but mainly based on their UV/Vis spectra. Spinochrome E, Spinamine E, Spinochrome 502 dimer and spinochrome 536 dimer were identified, as well as the finding of a possible novel PHNQ pigment based on its UV/Vis spectra. Due to limited amount of sample material, the bioactivity tests were limited to antibacterial testing against *Bacillus subtilis* and ORAC antioxidative assay. The bioactivity assays showed limited antibacterial activity, but antioxidative activity was detected in fractions or close to fractions containing PHNQ pigment according to the UV/Vis spectra. An optimized protocol suggests an improved focus on stability and solubility for further studies of the compounds.

Foreword

The practical work connected to this master thesis was conducted at the Faculty of Biosciences, Fisheries, and Economics in lab facilities of the Marine bioprospecting group. I am grateful to Klara Stensvåg and Jonathan Hira for their help finding a project for my thesis and to the supervisor team with Tor Haug.

I would like to extend my gratitude toward all the supervisors, but also those who have helped me in the lab. Thanks to Hege Devold for growing my bacteria and showing me around in the lab. Thanks to Christoffer Sivertsen for teaching me how to operate the HPLC. I would also like to thank Celine Edvardsen, from the Seafood research group, that instructed me in how to perform ORAC. I am grateful for Tor Haug and his input and experience. A special thank you to Ida Kristine Østnes Hansen who have helped me with her analytical knowledge. Her willingness to answer my question, but also tried to keep me on track and steer me in the right direction, has been valuable. This have been a challenging time, but I always felt welcome in the lab facilities (except for when the lockdown happened, and I wasn't allowed in the lab). Thanks to everyone in the Marine bioprospecting group who welcomed me with open arms.

A special thank you to Klara Stensvåg who never gave up on me, always was available to answer questions, helping improve my writing and always showing understanding for whatever I had to say. It made me not give up on this thesis.

Lastly, I would like to thank my mom and my brothers. This would literally not be possible without you guys. Thank you.

Haakon Knutsen

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Abbreviations

Abbreviation	Full text
amu	atomic mass units
AUC	Area under curve
CFP	Coelomic fluid pellet
CMFSW	Ca^{2+} +Mg^{2+}-Free sea water
CSC	Colourless spherule cells
Da	Dalton
DAD	Diode-array detector
FACS	Fluorescence-activated cell sorting
HPLC	High-performance liquid chromatography
LC	Liquid Chromatography
m/z	Mass-to-charge
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
OD600/595	Optical density at 600/595 nm
ORAC	Oxygen radical absorbance capacity
P	Peak
PDA	photodiode array detector
PHNQ	Polyhydroxylated 1,4-naphthoquinones
Q-TOF	Quadropol-time of flight
RSC	Red spherule cells
TIC	Total ion current
UV/Vis spectra/maxima	Ultraviolet/visual
WERM	Water extract of rest material

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1 Introduction

Information about cells in invertebrates and particularly, sea urchins, is interesting. These invertebrates are ancient animals and have evolved for millions of years. Still, we do not have much information of some of the cell types, called coelomocytes, that they have in their coelomic fluid and is the equivalent to a blood system.

Of particular interest, particularly in a marine bioprospecting perspective, is what these different cell types produce of metabolic compounds. Recently, in the research group of marine bioprospecting at The Norwegian College of Fishery Science, UiT, they have focused on research on sea urchins for some years. In one of the recent works, Hira and co-workers (ref) succeeded in making a pure cell populations of red spherule cells and isolate pigments form them. These pigments were extracted from both pure red spherule cell (RSC) population and mixed population of coelomocytes (MPC), collected from live green sea urchins, *Strongylocentrotus droebachiensis*, collected off the coast of Tromsø, Norway. The RSC were separated from the coelomic fluid using fluorescence-activated cell sorting (FACS), with the use of flow cytometry, due to the RSC having a distinct autofluorescence. Chromatographic separation of the pigment solutions from the green sea urchin, both a mix of population of coelomocytes (MPC) and red spherule cells (RSC), were done on a pentafluorphenyl (PFP) column [1]. One of the results of the PHNQ profiling from the RSC and the MPC population showed a significant higher concentration of PHNQ in the sample from the pure population of RSC [1]. However, the bioactivities of these compounds are unclear. Thus, more material is needed to do such studies, and to identify a more convenient method for isolations of more material is essential for a further characterization for these pigments.

1.1 Sea urchins

Sea urchins belong to a large group of exclusively marine invertebrates called Echinodermata, a phylum where it's accompanied by the sand dollar, sea stars, sea cucumbers and about 7000 other living species [2]. There are hundreds of species of sea urchins, varying in colours from purple, brown, red, and green. They are evolutionary ancient creatures that lack specialized respiratory or circulatory systems found in other animals. This means they have no blood vessels, pump or oxygen binding molecules in their body fluid (coelomic fluid) [3].

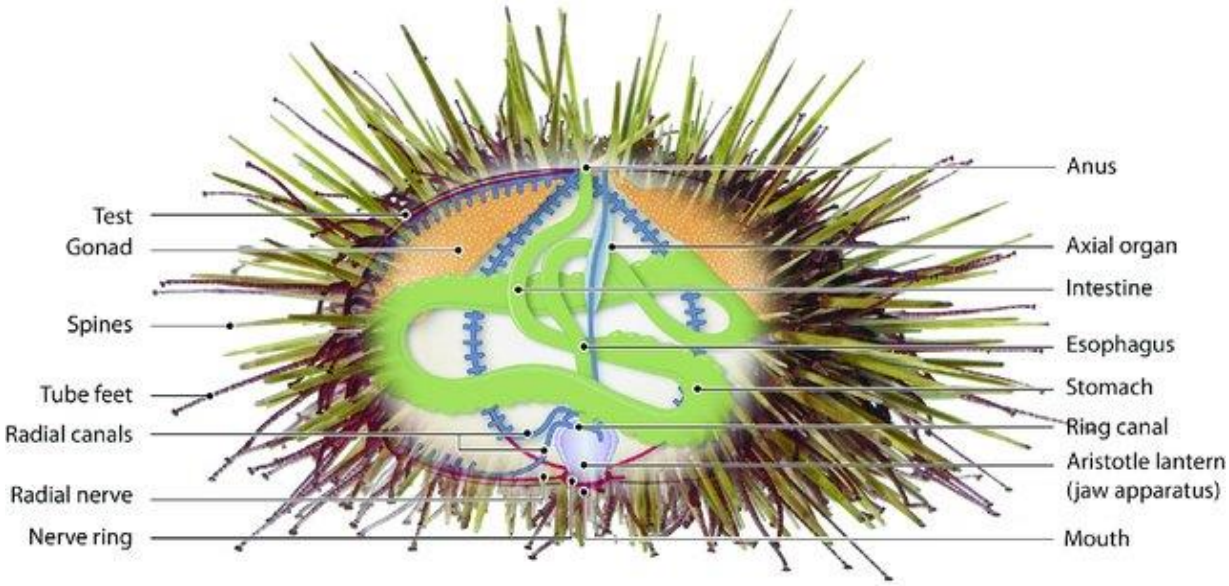

Figure 1 A schematic overview of exterior and interior organs and general anatomy of sea urchins of the Strongylocentrotus spp. [3]

Strongylocentrotus spp. is a genus of sea urchins. Even though the sea urchins of the *Strongylocentrotus spp.* have a simple anatomy, compared to other species on this planet, but the same can't be said about their physiology. The outer shell of the sea urchin forms the coelomic cavity which protects the intestine, gonads, canal system and other organs, which are described in **Feil! Fant ikke referansekilden.** [4].

Table 1. Overview of organs and their known function

Name of organ structure	Functions
Spines	Movements and protects the main body from predators. Will differ in size between species [5].
Tube feet	A part of sea urchins hydraulic system acting as sensory, anchoring and locomotive appendages that can extend and contract [6].
Teeth	They possess a set of five self-sharpening teeth [7]. A part of the digestive system.
Aristotle's lantern	The jaw apparatus whit a five-fold symmetry which are five jaw sections each containing a single tooth. Chews and ingests food [7]. A part of the digestive system. The major driving force of circulation of the perivisceral fluid [8].
Gonads	The gonads for some species are edible and are more commonly known as uni. Produces gametes.
Intestines/gut	Contains digestive enzymes to digest ingested food/nutrients.
Axial organ	It is believed to provide a propulsive force for the movement of coelomic fluid, though its function is not fully known [9]
Anus	Secretion of waste and bi-products. Placed at the top of the sea urchin.
Perivisceral cavity	Located between Aristotle's lantern and test [8].
Radial canal	Part of the water vascular system. A canal between ampullae and the ring canal. Allows for flow of water [10].
Ring canal	Part of the water vascular system
Ampullae	They are flattened thin sacks which are connected to the radial canal. When contracted, it lets water flow into the tube-feets allowing them to move [10].
Madreporite	It connects the internal cavity of the water-vascular system, from the upper end of the stone canal, to the external seawater. Part of fluid volume regulation [11].

Strongylocentrotus droebachiensis, also known as the green sea urchin, is one of the most widely distributed members in the Strongylocentrotidae family (Figure 2**Feil! Fant ikke referansekilden.**). Its gonads are edible and are popular in sushi where it is called uni (Figure 2). The market for sea urchins is very traditional with Japan consuming 80-90% of the total global supply per 2017 [12].

Figure 2. An open S. droebachiensis showing the eatable roe. (Photo: Sten I. Siikavuopio) [13].

1.2 Innate immunity and coelomocytes

The immune response in echinoderms is divided into humoral and cellular responses. Humoral immune responses is mediated by molecules present in coelomic fluid and cellular is, like the name suggests, cell mediated [14]. The compiling name for the cells responsible for the innate immunity is called coelomocytes [15].

The urchins ability to defend themselves from invasion and infection from a foreign organisms are dependent on the coelomocytes [16]. Coelomocytes are moving freely in all coelomic spaces, including the perivisceral coelomic cavities and rest of the water vascular system. There are six types of coelomocytes identified in the coelomic fluid of sea urchins, which is more closely described in the next section (1.2.1). The coelomic fluid (from *S. droebachiensis)* have shown antibacterial activity both against Gram-positive and Gram-negative bacteria [17].

1.2.1 Cell types of coelomic fluid

The cells involved in the coelomic fluid are divided into categories depending on their morphology [16]. Though there are six cell types, not all six are identified in all classes or species of sea urchins. The four main cell types of the coelomic fluid of the sea urchins are therefore phagocytes, vibratile cells and the colourless and red spherule cells [4, 18, 19].

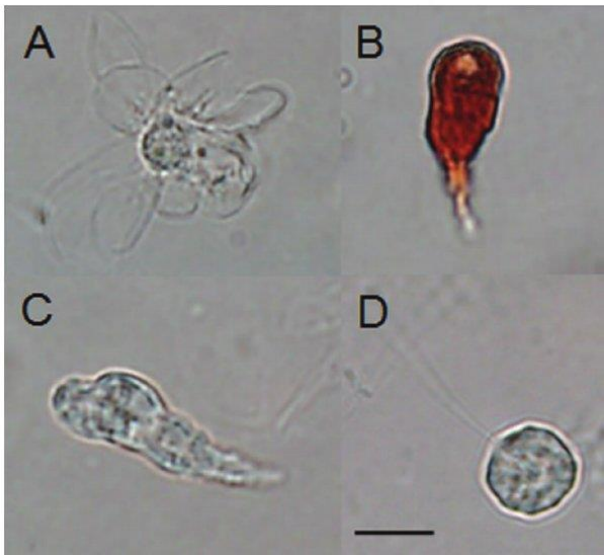

Figure 3 Coelomocyte types of the sea urchin, Paracentrotus lividus. A) petaloid phagocyte. B) red spherule cell. C) colourless spherule cell. D) vibratile cell. Scale bar = 5 microns. Images taken by R. Bonaventuea. Smith et al [20].

The most abundant out of these four are the phagocytes where they, depending on the species, make up 40-80% of the coelomocytes and range in sizes from 20 to 50 µm, in diameter [16, 20]. They are associated with phagocytosis and the capture/encapsulation of foreign invaders and cytotoxic responses in addition to expression and secretion of anti-microbial peptides (AMP), though AMPs have been associated with the other three cell types as well[4]. They are also able to self-aggregate and are involved in the clotting mechanisms of the coelomic fluid [16].

The vibratile cells which are spherical in shape, have large cytoplasmic granules and are 5-10x smaller than the phagocytes, with a diameter of 5-10 µm. [16]. Though they are a lot smaller than the phagocytes, they still make up for 8-20% of the coelomic fluid. They have a high motility and are more distinctive due to their single long flagellum, and they are believed to be

involved in the circulation of the coelomic fluid [20, 21]. They are also involved in the clotting of coelomic fluids. They degranulate during clotting processes and are believed to be a source of some clotting proteins [21].

The spherule cells are divided into two groups, colourless spherule cells (CSC) and red spherule cells (RSC). They have a diameter of 8-10 µm and have a nucleus of condensed chromatin. They also contain large cytoplasmic granules. These granules are filled with proteins and mucopolysaccharides. The CSC make up 3.7-25% and the RSC 7-40% of all the cells in the coelomic fluid as the homeostasis will depend on the health of the sea urchin [15, 20].

What distinct the RSC from the other coelomocytes is its distinct red colour, hence its name. It is believed that the RSC get their colouration from a PHNQ double-ring structure in the cytoplasmic granules, called Echinochrome A [16], more on these structures in 1.3.2. They also have a weak, but distinct autofluorescence when exited at 633 nm, making it possible to extract pure populations of RSC [1]. The RSC tend to move towards tissue damage/are under stress (like infection sites), where they self-granulate and release bactericidal substances [1]. The released compounds also have other types of bioactivity, such as RSC respond to lipopolysaccharides (pathogen-associated molecular pattern and damage-associated molecular pattern) by undergoing exocytosis involving Ca^{2+} influx [22]. The released substances are believed to stunt the growth of bacteria in the urchins by chelating iron in the area [22].

1.3 Bioactive compounds from Strongylocentrotus spp.

Sea urchin research have gained more attention the last couple of years due to their edibility (gonads of some species), and their pharmaceutical capabilities. The diversity of compounds that they produce are a potential as a promising reservoir of new bioactive compounds antimicrobial peptides and PHNQ pigments.

1.3.1 Antimicrobial peptides (AMPs)

Antimicrobial peptides and proteins are a class of natural occurring molecules that are a line of defence for multicellular organisms [23]. The function to these peptides and proteins are to kill bacteria, yeasts, fungi, viruses and sometimes cancer cells [23], and many show a high specificity for prokaryotes and low toxicity for eukaryotic cells [24]. In addition they are described as host defence molecules that harbour other important defence functions such as chemotactic effect, antitoxic effect of lipopolysaccharides and intercellular up-regulatory substances like chemokines [25]. AMPs in sea urchins have been linked to the four main cell types in the coelomocytes of some species, as seen in Table 2, since the first reported AMP isolation from sea urchins, Li. C et al 2008 [26].

Table 2 Antimicrobial peptides isolated from the sea urchin species S. droebachiensis and S. purpuratus

Species	AMP	Mw (Da)	Transcripts from	References
S. droebachiensis	Strongylocins 1	4488.30	Phagocytes, vibratile cells and/or CSP	[4, 27]
	Strongylocins 2	4396.27	Phagocytes, RSC	[4, 27]
	Centrocin 1	4409.1	Phagocytes	[4]
	Centrocin 2	4317.0		[27]
S. purpuratus	Strongylocins 1			[28]
	Strongylocins 2			[28]

1.3.2 Polyhydroxylated 1,4-naphthoquinones (PHNQ)

PHNQ pigments, a subgroup of quinones, are secondary metabolites found in echinoderms. Their existence have been known for over a hundred years as the first record of PHNQ pigments is echinochrome recorded in 1883 by MacMunn, found in Echinus, but a pure sample was not obtained until 1934 [29]. Spinochromes get their name as they were first found in the spines. They are polyhydroxylated derivatives of either juglone (5-hydroxy-1,4,naphthoquinone) and naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) that's substituted with functional groups such as methoxyl, acetyl, ethyl, and amino groups[30]. They are found within and isolated from shells and spines from various sea urchins, as well from the intestines, tube feet, larvae and coelomic fluid [30].

The overall structure for spinochromes are similar, as shown in Figure 4, but the different types have different functional groups and placement which is shown in Table 3, and known quinone pigments and their mass and UV/Vis maxima is shown in **Feil! Fant ikke referansekilden.**Table 3.

Polyhydroxylated 1,4-naphthoquinones

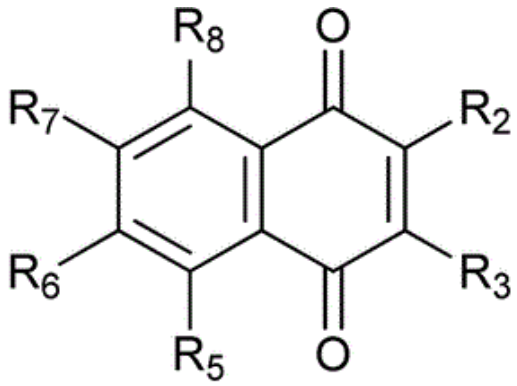

Figure 4 General structure of a non-dimer spinochrome. R_n are functional groups, which differ between the different spinochromes. Modified picture from Hou et al, 2018 [30].

Table 3 Spinochromes/quinonoid pigments of sea urchins and their different functional groups at different positions (Rn)

R_n Spinochromes↓	R_2	R_3	R_5	R_6	R_7	R_8
Echinochrome A	OH	OH	OH	C_2H_5	OH	OH
Spinochrome A	$COCH_3$	OH	OH	OH	H	OH
Spinochrome B	OH	OH	OH	H	OH	H
Spinochrome C	$COCH_3$	OH	OH	OH	OH	OH
Spinochrome D	OH	OH	OH	H	OH	OH
Spinochrome E	OH	OH	OH	OH	OH	OH
Echinamine B	NH_2	OH	OH	OH	C_2H_5	OH
7-Ethyl-3,5,6,8-tetrahydroxy-2-methoxy-1,4-naphthoquinone	OCH_3	OH	OH	OH	C_2H_5	OH

In general, spinochromes' UV-vis spectra are characteristic, and consist of absorption maxima at 254-290 nm, 310-480 nm and near 500 nm. The absorbance at 254-290 nm consists of a combined benzenoid (a six sided ring structure) and quinonoid structure [1, 30]. The

absorbance at 310-480 are due to a small quinonoid band [1, 30], whereas The absorbance at 500 nm [1, 29-31] is a benzenoid structure. A methylated 1,4-napthazarin will however have a bathochromic shift from the 500 nm to 520-530 nm [32]. Based on the data from Hira et al [1], there seems to be a hypsochromic shift in the absorbance at 254-290 and at the absorbance at 310-480 nm, and a bathochromic shift near the 500 nm absorbance [1].

Table 4 ***Quinonoid pigments with established structure found in sea urchins****. This list is largely based on supplementary table S2 from Vasileva, et al 2021 [32]. Listed in order of monoisotopic mass*

Pigment name	Molecular formula	Calculated monoisotopic mass, Dalton (Da)	UV/Vis maxima
Spinochrome B	$C_{10}H_6O_6$	222.0164	265, 320, 390, 471
Mompain	$C_{10}H_6O_6$	222.0164	270, 315, 515, 559
Spinochrome D	$C_{10}H_6O_7$	238.0114	251, 327, 463
3-Acetyl-2-hydroxynaphthazarin	$C_{12}H_8O_6$	248.0321	250(sh), 296, 490, 525(sh), 568(sh)
Spinamine E	$C_{10}H_7NO_7$	253.0150	275, 370, 473
Spinochrome E	$C_{10}H_6O_8$	254.0063	264, 350,478
Spinochrome A	$C_{12}H_8O_7$	264.0270	266, 312, 508
Echinamine A	$C_{12}H_{11}NO_6$	265.0586	278, 352, 477
Echinamine B	$C_{12}H_{11}NO_6$	265.0586	274, 352, 477
Echinochrome A	$C_{12}H_{10}O_7$	266.0427	254, 338, 471
Namakochrome	$C_{11}H_8O_8$	268.1769	262, 340, 480, 524
Spinochrome C	$C_{12}H_8O_8$	280.1870	290, 456
7-Ethyl-3,5,6,8-tetrahydroxy-2-methoxy-1,4-naphthoquinone	$C_{13}H_{12}O_7$	280.2302	252, 330, 491, 525
7-Ethyl-2,5,6,8-tetrahydroxy-3-methoxy-1,4-naphthoquinone	$C_{13}H_{12}O_7$	280.2302	256, 332, 474, 497, 538
Dehydroechinochrome	$C_{12}H_{12}O_9$	300.0481	256, 321, 391
7,7'-Anhydroethylidene-6,6'-bis(2,3,7-trihydroxynaphthazarin)	$C_{22}H_{12}O_{13}$	484.0203	265, 316, 470
Mirabiquinone	$C_{22}H_{12}O_{13}$	484.0205	264, 325, 452

Ethylidene-3,3'-bis(2,6,7-trihydroxynaphthazarin)	$C_{22}H_{14}O_{14}$	502.0384	254, 339, 471

Bioactive properties of PHNQ

Anti-oxidative: Echinochrome contains several isomers with different location of methyl group with varied proportions, and it's considered that the anti-oxidative effect are from the structures multiple hydroxyl groups [33]. The mechanism of antioxidative action for Echinochrome A includes the capture of free radicals [33], chelation of metal ions [34], regulating cell redox potential and inhibiting lipid peroxidation [35].

Anti-inflammatory: By reducing Reactive Oxygen Species (ROS) it decreases the expression of NFκB and TNF-α [33], which both are involved in acute inflammation response.

Anti-diabetic: The suggested mechanism of Echinochrome A activity in reduction of diabetic complications in liver, is through the hypoglycaemic and the anti-oxidative role of Echinochrome. A [36].

Anti-microbial: In a study where echinochrome A and spinochrome A-E were tested, only echinochrome A had some bioactivity. This towards the bacteria *Staphylococcus aureus* and the fungi *Candida utilis. Trichophyton* and *Saccharomyces [30].*

Anti-viral: More recent, there have been results indicating that spinochrome A and echinochrome A have potential as therapeutic and antiviral agent against SARS-CoV-2 due to their affinity for the viral main protease (M^{pro}) based on *in silico* experiment [37]. M^{pro} is a key enzyme of coronaviruses and has an important role in mediating viral replication and transcription, thus making it an important drug target for SARS-CoV-2 [38].

In the Russian Federation, echinochrome is approved as an experimental treatment for acute myocardial infarction and ischemic heart disease in humans, under the name *Histochrome* [33].

Despite spinochromes have been studied for quite some time, their function in sea urchins are still not fully known.

1.4 Spinochrome extraction and purification/separation methods

Due to quinones mostly being extracted from the shell and spines of the sea urchin, the preferred extraction methods have been to use a fairly high amount of strong acids like HCl [39-41] H_2SO_4 [42], to solubilise the sample both with and without EtOH. Then extracting the pigment from the acidic solution by using one or more organic solvents. The crude extracts are fractionated with some form of chromatography [30]. Due to quinones mostly being extracted from the shell and spines of the sea urchin, the preferred extraction methods have been to use a fairly high amount of strong acids like HCl [39-41] H_2SO_4 [42], to solubilise the sample both with and without EtOH. Then extracting the pigment from the acidic solution by using one or more organic solvents. The crude extracts are fractionated with some form of chromatography [30].

A typical extraction method with HCl could be crushing the material and adding up to 5 g dried sample to 10 ml HCl with a final concentration of 6 M for 1 h before filtration under vacuum. The sample will then be partitioned with diethyl three times and the diethyl phases pooled and partitioned again. This time its partitioned against 30 ml aqueous NaCl 5% solution. The ethereal phase is then collected and evaporated either at 30°C nitrogen stream or 60°C low pressure rotary evaporator. The dried sample is the re-dissolved in 50% MeOH centrifuged at 10^4 RCF for 10 min [41].

1.5 Sulphated derivatives

In 2014, Powel et al extracted and identified PHNQ pigments from the sea urchin, *Psammechinus miliaris* [43].[43]. Their conclusion was that the extract contained PHNQ pigments identified in other sea urchins species, but that there was additional results (based on their liquid-chromatography mass spectrometry data) indicating the presence of sulphated or phosphorylated PHNQ structures, due to a neutral loss of 80 atomic mass units (amu) from the MS peak m/z 300.8 (giving m/z 221.0 $[M-H]^{-1}$ spinochrome B) and m/z 332.8 (giving m/z 253.0 $[M-H]^{-1}$ spinochrome E) [43, 44].

The suspected sulphated spinochromes were recently observed by Hira et al [1] by chromatographic separation of the pigment solutions from the green sea urchin *S. droebachiensis*, with a mix of population of coelomocytes (MPC) and red spherule cells (RSC). The pure population of RSC showed a significant higher concentration of PHNQ pigments, as well as spinochrome dimers and suspected spinochrome E, spinochrome C, and spinochrome D [1].

1.6 Principles for methods used in this project

1.6.1 Anti-clotting and gradient centrifugation

The use of EDTA or EGTA will chelate away Ca^{2+}, thus removing one of the factors needed for CF clotting which would make separation of coelomic cell impossible. Where EDTA is a general chelator of divalent cation (Ca^{2+}, Mg^{2+}, Zn^{2+}), EGTA is more specific, where it has a higher affinity to chelate Ca^{2+}. The CF will be directly added to Anti-clot buffer and centrifuged at 4°C and 150 g for 2 min, which will allow the colourless- and red spherule cells to sediment and the other cell types can be removed by removing the supernatant [45].

1.6.2 Principles of cell content extraction

A hypotonic solution is a solution with a lower solute concentration compared with another solution. Due to a difference of osmolarity, water will migrate from the side of the lower concentration to the side of the higher concentration of solutes. This migration will end when the concentration of osmotic active molecules is equal on the two sides of the membrane. By suspending cells in Milli-Q water (reverse osmosis and UV-lights purified), the osmotic conditions will be in favor of water influx into the cell, eventually lysing them.

Methanol is known to denature cell membrane proteins and thereby facilitate the extraction of contents inside a cell [46]. Quite recently, it was published results from a study comparing different methods to monitor the extraction efficiency of metabolites of different polarity from adherent growing mammalian cells [47]. The best extraction method was based on a two-phase

solvent system with both methyl *tert*-butyl ether (MTBE) and 75% 9 : 1 methanol : chloroform. [46].

1.6.3 Isolation, quantification, and characterization of compounds using LC-MS

This is a combinational separation and analytical technique of high-performance liquid chromatography (HPLC, separation) and mass spectrometry (MS, analytical).

Crude extracts and mixtures may consist of many different compounds with different chemical properties. With HPLC, an advanced form of column chromatology, different compounds from the same extract can be separated by polarity, charge or size by [48, 49]. Reverse phase has a non-polar stationary phase and a polar mobile phase, resulting in that more polar compounds will eluate faster than non-polar compounds. The gradient of the mobile phase can be regulated, resulting in better separation and customization of the method. In terms of HPLC, the term preparative refers to a more large scale process which would imply a larger column and larger sample pool and flow rate [49].

The analytical part of LC-MS, mass spectrometry, can detect the mass of components in a sample. MS consists of three primary components which are an ion source that converts compounds into charged particles, one or more mass analysers that separate the ions and produces the basis of their mass-to- charge ratio (m/z) and an ion detector [48].

1.6.4 Solid-phase extraction

A low-resolution chromatographic process which can be used to extract compounds based on polarity and size. The column is firstly primed with water. components in the sample will adsorb to the material of the column and unwanted, polar compounds (such as salts) are washed out. By flushing with different gradients of the chosen mobile phase (such as methanol or acetonitrile), the components of the sample will gradually eluate through the column and will be separated based on its hydrophobicity [50].

1.6.5 Bioactivity testing

1.6.5.1 Antibacterial activity assay

Minimum inhibiting concentration (MIC) is the lowest concentration of an extract that will inhibit the growth of an organism (bacteria). The growth of the bacteria is determined by measurements of the optical density of the bacteria solution starting at a predetermined concentration.

1.6.5.2 Antioxidative assay

Oxygen radicel absorbance capacity (ORAC) is a method that measures a compound, biological fluids, or extracts ability to scavenge free radicals. AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) will produce free radicals (peroxyl radicals) which will react with fluorescein (a fluorescent probe). This will result in a gradual loss of fluorescence. If test material/compound/fraction contains/is an antioxidant it will neutralize the peroxyl radical and the fluorescent signal won't dissipate at the same rate or at all [51].

The activity of the tested material is determined by the area under the kinetic curve (AUC) normalized by subtracting the are under the for the blank sample (AUC_0). AUC_0 contain fluorescein and AAPH, but not sample or standard. The activity is defined as Trolox equivalent. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which is an analogue to vitamin E, is used to make a standard curve and as such a measurement for unknown antioxidative compounds/solutions [51].

2 Objective

The main objective of the project it to evaluate different isolation procedures of spinochromes from the sea urchin *S. droebachiensis,* to get enough yield of the suspected sulphated spinochromes to reveal information about the activity.

Hypothesis

Extraction and isolation of compounds from echinoderms using acidic conditions will dissociate a potential sulphate group from some compounds in red spherule cells whereas a water-based extraction procedure will keep the sulphated compounds intact.

Sub goals:

From this hypothesis, the following sub goals are:

- Isolation and characterization by:
    - identify spinochromes to obtain a high yield of the different spinochromes by using a new method
    - identify possible sulphated spinochromes
- Evaluate different bioactivities
    - Elucidate activity of the isolated compounds (antibacterial, antioxidative.)
- Document the effect of a putative optimized isolation methods of both the RSC and the sulphated spinochromes

3 Materials and Methods

3.1 Ethical statement

Animals (sea urchin) used on this study do not belong to any group of endangered species and any ethical restrictions to use for research purpose.

3.2 Biological material

3.2.1 Sea urchins

As many as 162 animals from the species *S. droebachiensis*, was captured by divers of FF Hyas, the research vessel of UiT the Artic university of Tromsø, February 2020 in the sub-Artic areas near the coast outside Tromsø. The diameter of the animals ranged between 4-7 cm.

3.2.2 Bacterial strain

In this project, a bacterial strain of *Bacillus subtilis* (B.s, ATCC 23857) was used as the target bacteria in activity testing against fractions from two different extraction methods.

The bacterial strain was stored in 15% glycerol stock Mueller Hinton media at -80°C until use. Then material of the frozen bacteria was added to a Müller Hilton agar plate and grown at 30-37°C overnight and kept in refrigerator for a maximum of 14 days.

3.3 Chemicals, buffers, and media

All the chemicals used in this study are taken from the Norwegian College of Fishery Science chemical store and are listed in Table 5.

Table 5 Overview for chemicals used through this masters' thesis

Chemicals	Abbreviations	Manufacturer
2,2´-Azobis(2-methylpropionamidine) dihydrochloride	AAPH	Aldrich chemicals
Acetonitril, ≥99.9% for LC/MS	ACN	Honeywell
Acetonitril, ≥99,8% for HPLC	ACN	Honeywell
Ethylene glycol-bis(2-aminoethylether)-N,N,N´,N´-tetraacetic acid	EGTA	Sigma-Aldrich
Formic Acid, For LC/MS	FA	VWR
Methanol	MetOH/MeOH	VWR
Milli-Q H_2O	MQ	Millipore
Mueller Hinton Agar-05040	Agar	Sigma-Aldrich
Mueller Hinton broth-275730	Broth	BD DIFCO
Sodiumhydrogencarbonate		MERCK
Potassium chloride		Sigma-Aldrich
Sodium bicarbonate		Merck
Sodium chloride		VWR
Sodium hydroxide		Sigma-Aldrich
Sodium phosphate dibasic		Sigma
Sodium sulfate		MERCK
(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxyl acid	Trolox	Aldrich chemicals
Trifluoroacetic acid, for HPLC	TFA	VWR

The protocol of making the different buffers and media are listed in the appendix Buffers and agar.

3.4 Research design

The design that was used for the experiments in this project and the overview of the methods used in the different processes, are highlighted in Figure 5.

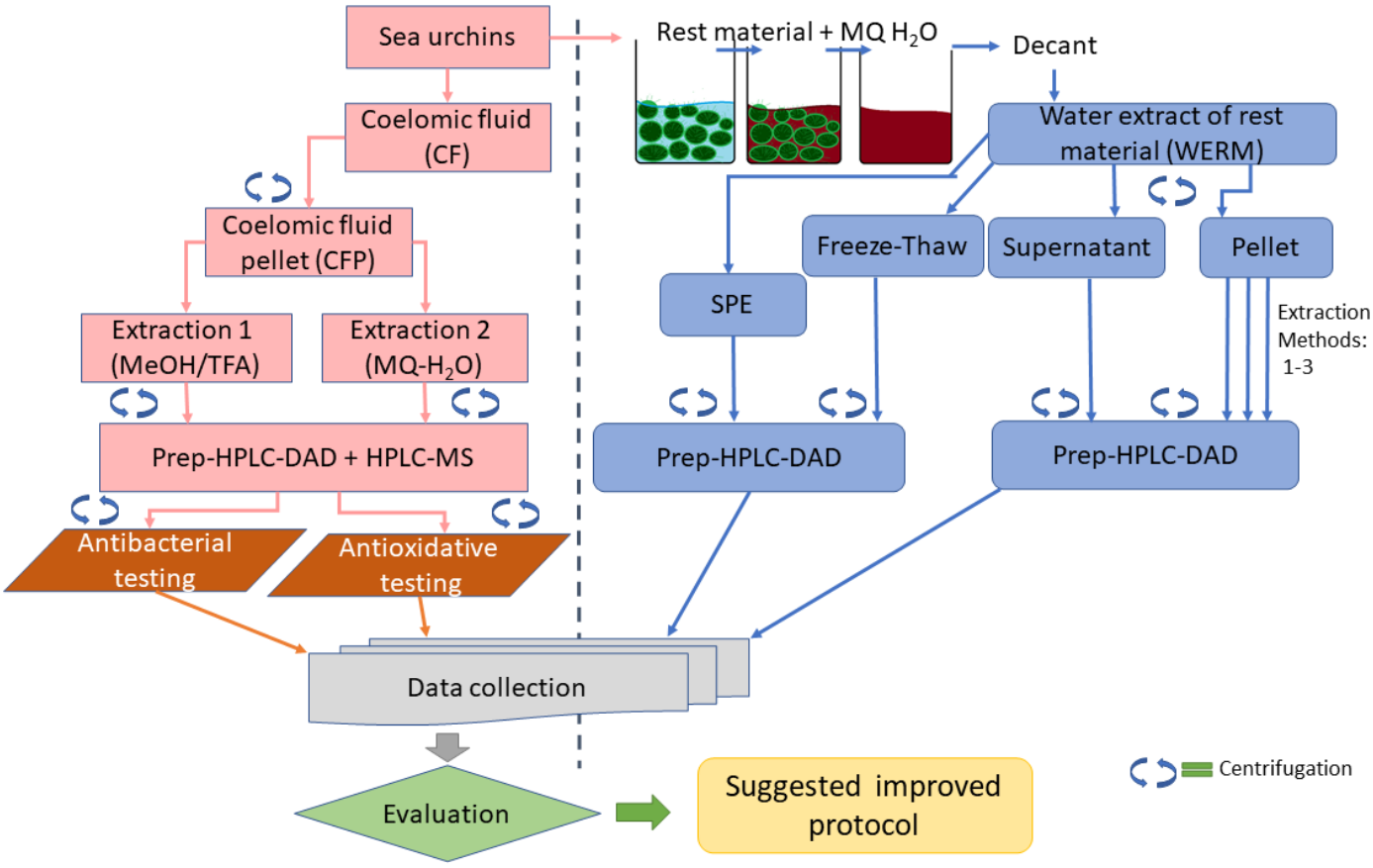

Figure 5 ***Flowchart illustrating the overall procedures of this thesis.*** *The more detailed explanation is found under the materials and methods section. Steps with centrifugation are marked with a double rotating arrow.*

3.5 Cell separation and extraction

The cell separation method is largely based on an article in *Journal of Cell Biology* by Brian J. Hiller and Victor D. Vacquier, 2003 [45], expertise in the bioprospecting research group, work published by Hira et al [1] and unpublished ongoing work.

The amount of CF from each sea urchin depended on its size. Also, the amount of red spherule cells (RSC) in the CF differed, which was evaluated based on the colour of the CF. More colour (darker red) was expected to contain more RSC.

The CF was extracted by cutting through the soft tissue around the mouth of the sea urchin. The jaw apparatus was then promptly removed before pouring the CF into a flask, where it was immediately mixed 1:1 with an anti-clotting buffer (AB) containing 20 mM EGTA (end concentration 10 mM) or 100 mM (end concentration 50 mM) EDTA in Ca^{2+} and Mg^{2+} free sea water (AB). The CF was then centrifuged at 150 g for 2 min, on a Heraeus Multifuge 1 S-R, which allowed the colourless- and red spherule cells to sediment which should make a spherule cell population when the supernatant was removed [1, 45]. The CFP was frozen and kept at -80°C.

3.5.1 CFP content extraction

Two different solvents were used to extract compounds from the CFP. Extraction with solvent 1, MeOH+TFA (described in 3.5.2). Extraction with solvent 2, Milli-Q described in 3.5.3, was based on an article from Hira et al (2020) [1].

3.5.2 Extraction with MeOH/TFA

About 7.5 ml of the CFP were subjected to extraction with this solvent. A volume of six ml extraction Solvent (ES) were added to the ≈7.5 ml pellet, changing the total concentration of the ES to 20% MeOH+0.004%TFA. This was vortexed for 30 sec before centrifuging for 10 min at 3500 min^{-1} for 10 min at 10 °C. five ml of supernatant was then moved to another 50 ml Falcon tube. Five ml of the ES was added to the CFP, vortexing, centrifugation and sampling

of five ml supernatant. This was repeated a total of six times. The pooled supernatant of 35 ml was partially dried on a rotavapor, (VWR IKA HB 10, CVC 3000 vacuum controller and RV 10 rotary evaporator) bath at 20 rpm and 40 °C, due to the supernatant containing a high concentration of MeOH before complete drying was performed using a Scanspeed 40 vacuum centrifuge (LabogeneApS, Denmark) overnight at 2000 min^{-1} at 20 °C. The dried material was rehydrated in Milli-Q water before HPLC-fractionation.

3.5.3 Extraction with Milli-Q

About 10 ml of the CFP were subjected to extraction with Milli-Q water. A volume of six ml Milli-Q were added to the ≈10 ml pellet. This was vortexed for 30 sec before centrifuging for 10 min at 3500 min^{-1} for 10 min at 10 °C. five ml of supernatant was then moved to another 50 ml Falcon tube. Five ml of Milli-Q water was added to the CFP, vortexing, centrifugation and sampling of five ml supernatant. This was repeated a total of six times. The pooled supernatant of 35 ml was dried using a Scanspeed 40 vacuum centrifuge (LabogeneApS, Denmark) overnight at 2000 min^{-1} at 20 °C. The dried material was rehydrated in Milli-Q water before HPLC-fractionation.

3.6 Extraction of rest material

The remains of the sea urchins were put in a container with Milli-Q-H_2O after removal of the CF. When the water had turned dark red/purple, the remaining sea urchins were removed, and the dark coloured liquid (water extract of rest material, WERM) were collected. There were therefore two types of material to work with after the extraction from the sea urchin, CFP and WERM.

3.6.1 WERM Content extraction

The supernatant still had a strong red colour after centrifugation, and after it was removed from the sediment/pellet. Therefore, it was collected by centrifuging 30 ml WERM in 50 ml FALCON tubes for 10 min at 4500 min^{-1}. The supernatant was then again centrifuged for 5 min at 14400 min^{-1} before directly injecting it into the HPLC at a volume of 400 µl.

Falcon tubes, 50 ml, containing 40 ml of sample was frozen at -80°C overnight. The WERM was vortexed after thawing. 1 ml of the freeze-thawed WERM was then transferred to a 1,5 ml Eppendorf tube before centrifuging for 5 min at 14000 min^{-1} in an Eppendorf table centrifuge. 400 µl of supernatant was injected into the HPLC system.

Pellet samples from WERM were prepared by centrifuging 30 ml the WERM in 50 ml Falcon tubes for 10 min at 4700 min^{-1} in a Heraeus Multifuge 1 S-R. The supernatant was removed. From the sample, yielding approximately 300 µl of pellet in each tube.

A volume of 500 µl Milli-Q water was added to the pellet in one Falcon tube, and the solution was vortexed for 30 sec and subsequently transferred to a 1.5 ml Eppendorf tube. The sample was then centrifuged at 5 min at 14400 min^{-1} on an Eppendorf table centrifuge before injecting 400 µl of the supernatant into the preparative HPLC system.

A volume of 1 ml 5% ACN+0.01 TFA was added to the pellet, then vortexed for 30 sec and transferred to a 1.5 ml Eppendorf tube. The solution was then centrifuged for 5 min at 14400 min^{-1} on an Eppendorf table centrifuge before injecting 400 µl of the supernatant into the preparative HPLC system.

A volume of 1 ml MeOH + 0,005% TFA was added to a WERM pellet. The sample was vortexed for 30 sec and centrifuged for 3 min at 10000 min^{-1} in an Eppendorf table centrifuge. The supernatant was removed and another ml of the 50% MeOH+0,005% TFA solution was added to the pellet. The sample was vortexed and centrifuged before removing the supernatant. This procedure was repeated three times and the collected supernatants were pooled and dried in a vacuum centrifuge overnight at 2000 min^{-1} at 20 °C.

3.7 Separation and characterization

3.7.1 Solid-phase extraction (SPE), WERM

Salt was removed from the aqueous phase by SPE using a C18 35cc Sep-Pak Vac cartridge (Waters, MA, USA) and a vacuum manifold (Supelco). The SPE cartridge was washed with 45 ml ACN, then equilibrated with 45 ml Milli-Q water. A volume of 15 ml of sample was thereafter run through the cartridge before washing with 15 ml water. The compounds in the sample were eluted sequentially from the cartridge with 30 ml of 20% ACN, 30 ml of 100% ACN, and finally 70% EtOH 15 ml. The eluates were dried overnight in a vacuum centrifuge at 2000 min^{-1} at 20°C.

3.7.2 Preparative High-Performance Liquid Chromatography

Preparative High-Performance Liquid Chromatography (Prep-HPLC) was performed on an Agilent Technologies 1260 Infinity, Prep star pump system, Waters In-Line Degasser AF, Agilent 440-LC Fraction collector system.A Phenomenex Kinetex F5 UHPLC 5 µm 100 Å, 10 x 250 mm column was used for all Prep-HPLC run during this project. **Pump A;** Milli-Q + 0.05% trifluoracetic acid (TFA). Pump B; ≥99,8% HPLC grade Acetonitril + 0.05% TFA.

Gradient pump B, **MeOH/TFA** and **Milli-Q** extract: 0%, 0-5 min. 0-20%, 5-25 min. 20-100%, 25-35 min. 100%, 35-40 min. Flowrate 4 ml/min. 0.8 ml injected volume.

Gradient pump B, decanted rest material water: 0%, 0-10 min. 0-20%, 10-30 min. 20-100%, 30-40 min. 100%, 40-50 min. Flowrate 4 ml/min. 0.4 ml injected volume.

Fraction collector were set to collect with 1 min intervals. The total fraction volume was therefore 4 ml. Fractions were based on the ending minute. Example, fraction 7, which would be eluent collected from the start of minute 6 to the start of minute 7. The collected eluent was then dried in a Vacuum centrifuge after collection to remove ACN and TFA which would interfere with the results of bioactivity testing.

3.7.3 Mass spectrometry

LC-MS was performed on an Agilent Technologies 1290 Infinity II LC, 6540 UHD Accurate-Mass Q-TOF LC/MS system. A Phenomex Kinetex F5 UHPLC, 1.7 100 Å, 150 x 2.1 mm column was used. Pump A; Milli-Q + 0.1% formic acid (FA). Pump B; ≥99.9% LC/MS Acetonitrile + 0.1% FA. Gradient pump B; 5-100%, 15 minutes with a flow of 0.3 ml/min. Total run-time 18 minutes (15 min analysis + 4 minutes flush/wash).

3.8 Bioactivity assays

3.1.1 Antibacterial assay

The Minimal Inhibitory Concentration (MIC) Assay setup were based on a method from CLSI [52] adapted by Paulsen et al (2013) [53] for use of activity testing in the labs of NFH and the group for marine bioprospecting. The Fractions were tested against one species of bacteria (*Bacillus subtilis)* due to limited material for some of the fractions. The Fractions were diluted in Milli-Q water to 400 µl/ml.

The bacteria were grown on Mueller Hinton (MH) Agar at room temperature until colonies were visible. One colony where then transferred to 5 ml MH-broth where it was incubated and shaken overnight at 37°C. The next day, 20 µl of the culture was transferred to another 5 ml MH-broth, and incubated while shaken for two hours at 37°C. The culture was diluted 1/10 in uncultivated MH-broth (37°C) before determining the density of the bacterial growth. This was done by reading the Optical Density at 600 nm (OD_{600}) and transferring the needed amount (**Feil! Fant ikke referansekilden.**) to 10 ml uncultivated MH-broth (room temperature).

The fractions and positive control (*Oxytetracycline)* were two-fold diluted. Milli-Q water was used as negative control. Each fraction had two parallels. The MIC assay was performed in 96-well microtiter plates. 50 µl Milli-Q were added to each well, except to row A, where the undiluted fractions/positive control were to be added. 100 µl of the fractions at 2x wanted start concentration were added to row A. Then 50 µl were transferred to row B and mixed with the pipette. 50 µl was then transferred from row B to C. This step was repeated until the last row

(H) was reached. 50 µl were then removed from row H after mixing up and down with the pipette. This gave a fraction concentration from 100 µg/ml to 0,76 µg/ml.

The plate was then placed in a Perkin Elmer EnVision 2105 Multimode plate reader, where it was incubated at 35 °C for 48 hours. The well density was read with 1-hour intervals at OD 595. Growth curves were made in excel with data for optical density at 595 nm provided.

3.1.2 Antioxidative assay

The antioxidative properties of the fractions from the two extractions methods were determined using the Oxygen Radical Absorbance Capacity assay, ORAC for short. All fractions were diluted in Milli-Q water equal to the amount of available material so that the start concentration was 400 µg/ml. All samples were centrifuged for 15 min at 10000 g and at 4 °C. The method used is based on Dávalos, W., Gómez-Cordovés, C. and Bartolomé, B, 2004[51].

The ORAC assay was performed in black non-transparent 96-well from Corning Costar. 25 µl fraction and Trolox-standard (pos/neg-control) were pipetted to assigned wells. 125 µl Fluorescein was then added the sample/Trolox/blank wells (not in the plate blank well). This was then incubated for 15-30 min at 37 °C to get the temperature homogenous inn all wells. 50 µl was then immediately added and placed in a plate reader (SpectraMax i3, Molecular Devices) which recorded 90 measurements at 1-minute intervals. The final volume for the assay mix was 200 µl (Table 6).

Table 6 ***ORAC assay set up****. Volume of solutions are added.*

	Sample volume (µl)	PB (µl)	Fluorescein (µl)	AAPH (µl)	Total Volume (µl)
Fraction	25	-	125	50	200
Trolox	25	-	125	50	
Blank/Trolox 0 µl	0	25	125	50	
Plate blank	0	200	0	0	

The fractions and the Trolox-standard had two parallels each. Fractions and Trolox-standard were two-fold diluted with PB, giving a final sample concentration of 100-6.25 μg/ml and Trolox standard concentration 12.5-0.76 mM.

The fluorescence data for the standard and sample were normalized by subtracting the blanks' AUC measurements (Trolox 0 μl). The Sample value was expressed as Trolox equivalents (TE). The Trolox standard (**Feil! Fant ikke referansekilden.**) were used for the standard curve and the calculations for TE were based on the linear function formula for the standard curve, one for each plate in the setup.

4 Results

The focus of this project is extracting spinochromes from sea urchins without using high amounts of strong acids, based on the hypothesis that the acids will alter the structure of the spinochromes. This was done with two different extraction methods which was compared based on reverse phase HPLC chromatograms, UV/Vis-spectre of peaks and available LC/MS data. The bioactive properties of HPLC-fractions for both methods were also evaluated by antibacterial and antioxidative assays. The rest material which was put in Milli-Q water yielded about 7 L of liquid after the sea urchins were removed and the liquid was decantated into 1 L bottles.

4.1 Yield from CFP extraction, extract 1 and 2

The coelomic fluid was extracted from 162 sea urchins of the species *S. droebachiensis.* A total of ≈20 ml pellet remained after centrifugal coelomocyte separation which was then extracted with two methods. The volume of CFP extracted with 50% MeOH+0.001 TFA was 7.5 ml. A total of 35 ml supernatant was pooled and dried yielding 295.02 mg extract. The combined weight of the fractions after fractionation was 250.85 mg. that's a loss of 44.17 mg. The volume of CFP extracted with **Milli-Q** was ≈10 ml. A total of 35 ml supernatant was pooled which gave 766.63 mg dry extract. Combined weight of the fractions 235.75 mg, meaning a loss of 530.88 mg. The dried weight was not able fully dissolve in Milli-Q water when rehydrating before reverse phase preparative HPLC hence the loss of material.

Figure 6 MeOH/TFA extract before drying on rota vapor to remove MeOH and TFA.

4.2 Prep-HPLC for CFP extracts

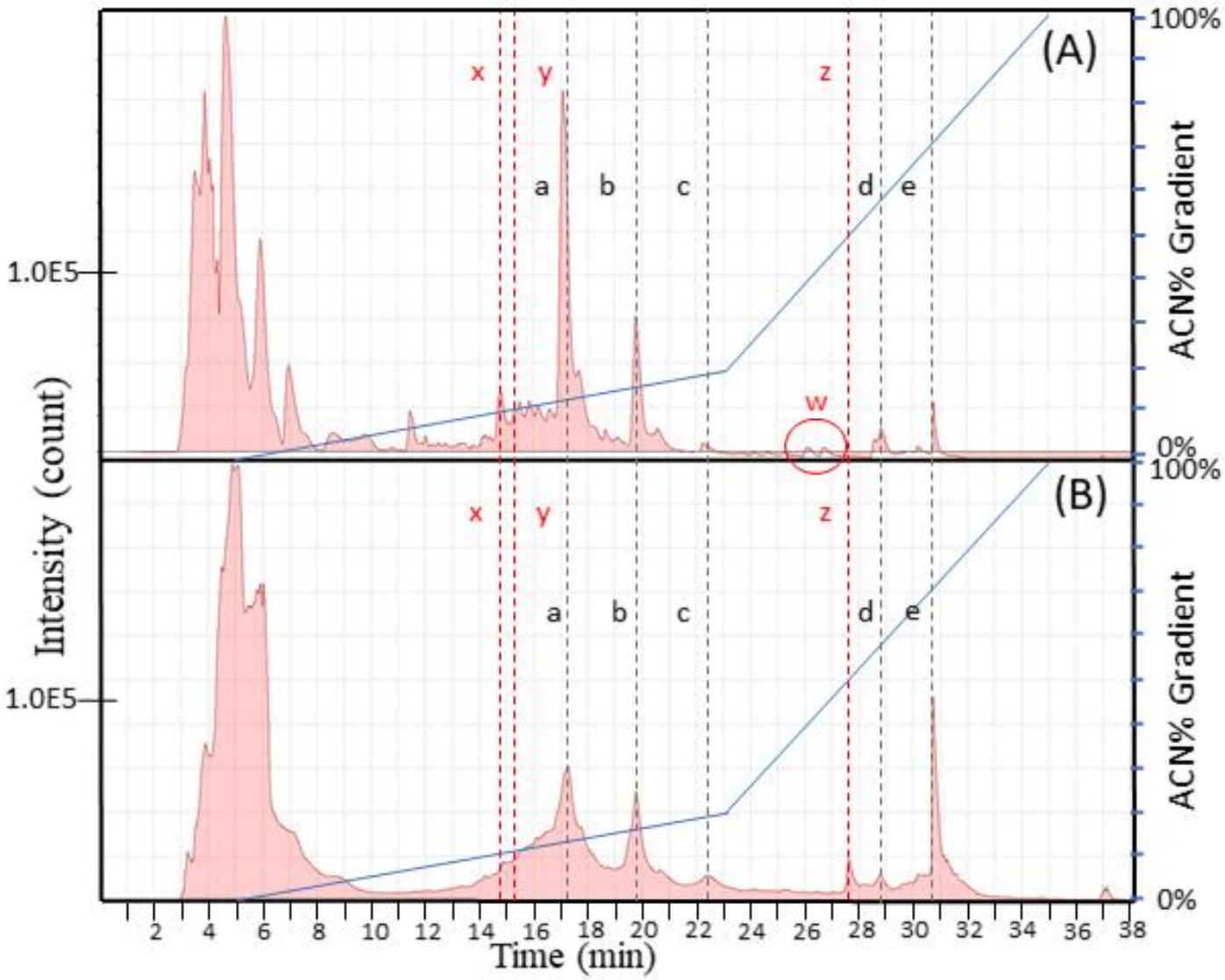

Figure 7 ***HPLC chromatogram of sea urchin extracts at 280 nm from reverse phase.*** *(A); MeOH/TFA extract and (B); Milli-Q extract. An amount of 0.8 ml was injected for HPLC and the material was separated with an ACN (with 0.005% TFA) gradient of 0% from 0-5 min, 0. Peaks found which correlate with both extract are marked with a, b, c, d and e. Peaks which was only visible in one of the extracts are marked with x, y and z.*

Both extracts have some similarities such as peaks at, but there are some clear differences in the chromatograms for **MeOH/TFA** and **Milli-Q** extract, as seen in Figure 7**.** Fraction 18, 20, 29, 31 and 35 have peaks at about the same retention time (RT). By comparing the UV/Vis spectre for the peaks, similarities are observed. After the HPLC fractionation, the total yield for the **MeOH/TFA** extract was 250.85 mg and 235.75 mg for the **Milli-Q** extract for their collected fractions. Most of the material was fraction 4-7. In the **MeOH/TFA** extract 239.9 mg

was in fraction 4-6 leaving a total of 10.95 mg for the other fractions. In the milli-Q extract 232.31 out of 235.75 was in fraction 4-7, leaving a total of 3.44 mg for the other fractions.

Fraction 18 (Figure 8) for both the **MeOH/TFA** (A) extract and **Milli-Q** (B) extract have similar absorption with a slight difference which could be due to difference in intensity quantity. UV/Vis-maxima for **(A)a** at 266 nm, 352 nm, 477 nm. UV/Vis-maxima for **(B)a** at 264 nm, 350 nm, 474 nm.

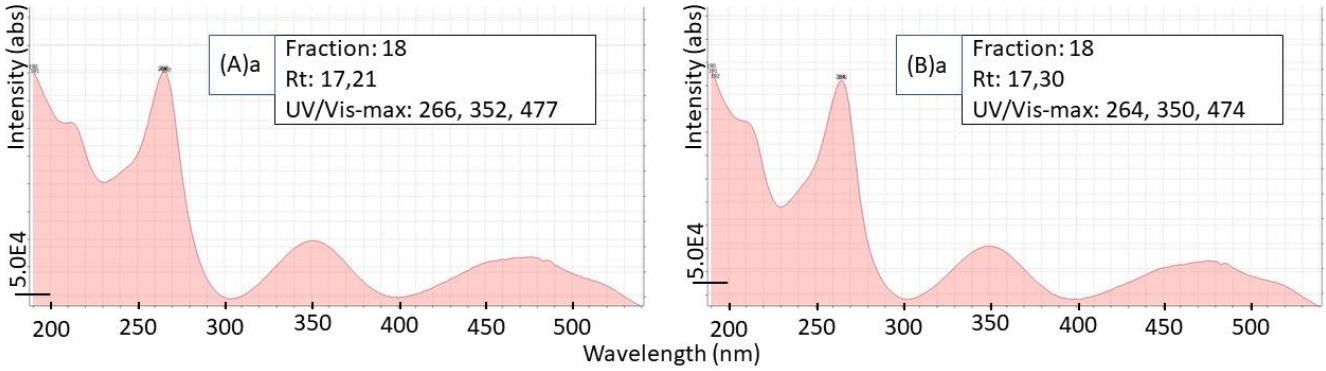

*Figure 8 (**AB)a, UV/Vis spectre at RT 17.21(MeOH) and RT 17.30 (Milli-Q) compound in fraction 18 compared.** Shown as vertical line a in Figure 7. Intensity (abs) is marked at 5.0E4 in both fraction for intensity comparison.*

Fraction 20 (Figure 9) show little differences between the two extract. The most noticeable difference is in the peak at 477 for **(A)b** and 468 for **(B)b**. This is due to a "dent" around the area for the peak which can be seen in most of the fraction around the same wavelength. UV/Vis-maxima for **(A)b** at 215(sh) nm, 273 nm, 368 nm, 477 nm. UV/Vis-maxima for **(B)b** at 214 (sh) nm, 272 nm, 368 nm, 468 nm.

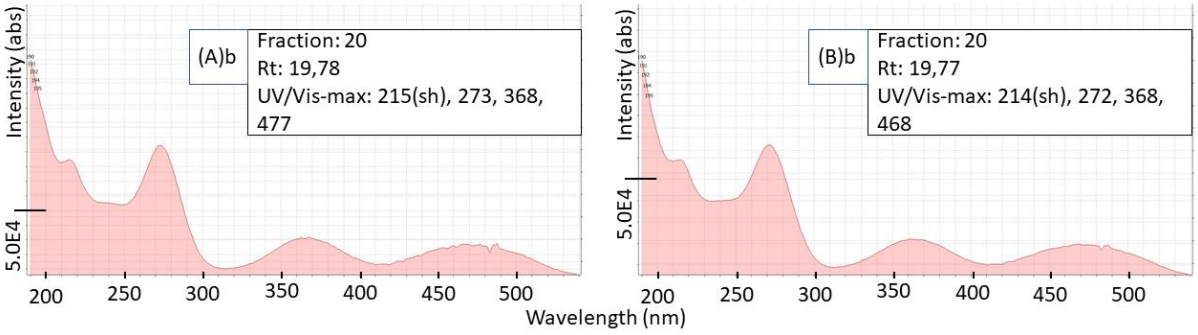

Figure 9 (AB)b UV/Vis- spectra at RT 19.78 (MeOH) and RT 19.77 (Milli-Q) compound in fraction 20 compared. Shown as vertical line b in Figure 7. Shoulder is abbreviated as sh. Intensity (abs) is marked at 5.0E4 in both fraction for intensity comparison.

Fraction 23 (Figure 10) shows higher intensity in the **Milli-Q** extract for this peak as seen at the vertical line *c* (**Feil! Fant ikke referansekilden.**). UV/Vis-maxima for **(A)c** at 240 nm, 270 nm, 363 nm. UV/Vis-maxima for **(B)c** at 274 nm, 356 nm, 467 nm.

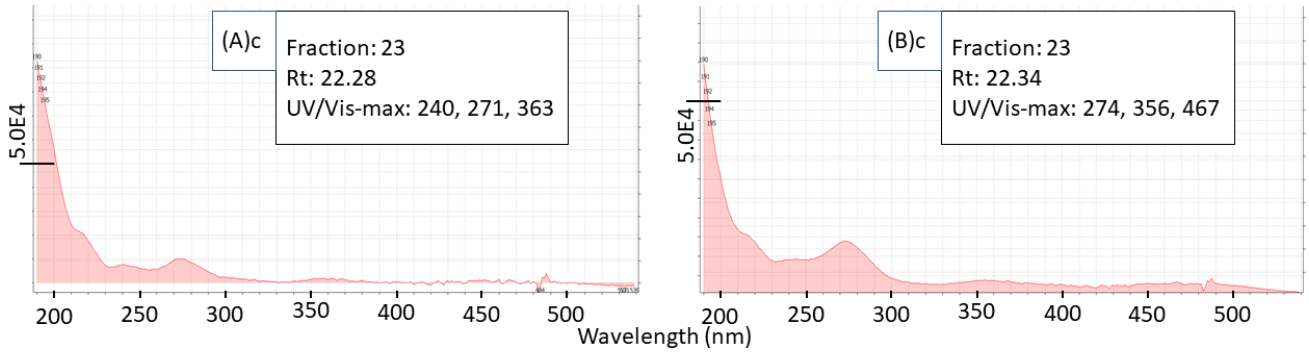

Figure 10 ***(AB)c UV/Vis-spectra at RT 22..28 (MeOH) and RT 22.34 (Milli-Q) compound in fraction 23 compared.*** *Shown as vertical line c in Figure 7. Intensity (abs) is marked at 5.0E4 in both fraction for intensity comparison.*

Fraction 29 (Figure 11) have low intensity (abs) making it hard to determine peaks. UV/Vis-maxima for **(A)d** at 220(sh) nm, 240 nm, 275 nm, 460 nm. UV/Vis-maxima for **(B)d** at 216(sh) nm, 244 nm, 270 nm, 467 nm.

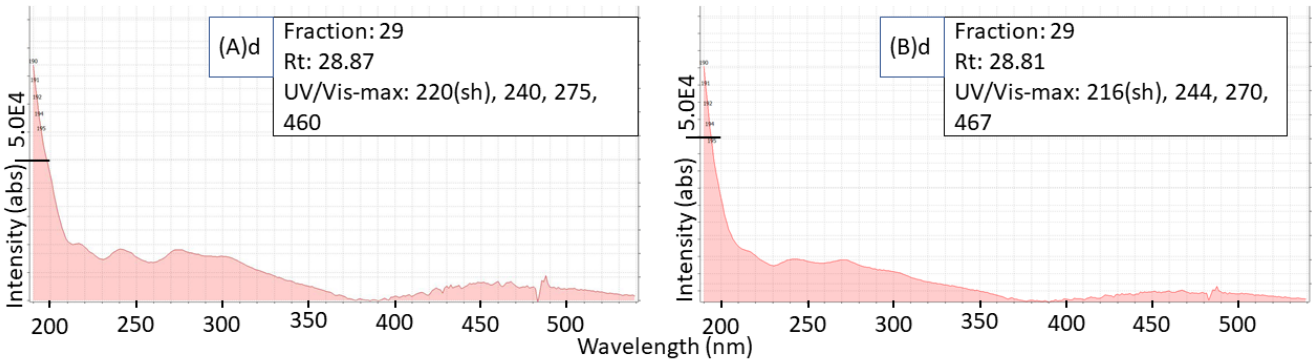

Figure 11 ***(AB)d UV/Vis-spectra at RT 28.87 (MeOH) and RT 28.81 (Milli-Q) compound in fraction 29 compared.*** *Shown as vertical line d in Figure 7. Shoulder is abbreviated as sh. Intensity (abs) is marked at 5.0E4 in both fraction for intensity comparison.*

Fraction 31 (Figure 12) shows higher intensity in the **Milli-Q** extract for this peak as seen at the vertical line *d* (**Feil! Fant ikke referansekilden.**). UV/Vis-maxima for **(A)e** at 214(sh) nm, 264 nm, 340 nm, 477 nm. UV/Vis-maxima for **(B)e** at 214(sh) nm, 264 nm, 339 nm, 485nm. Due to the «dent» at around 480 for **(A)e**, UV/Vis-min is listed to help compare **A(d)** and **(B)e**.

UV/Vis-min for **(A)e** at 230 nm, 297 nm, 390 nm. UV/Vis-min for **(B)e** at 228 nm, 297 nm, 390 nm.

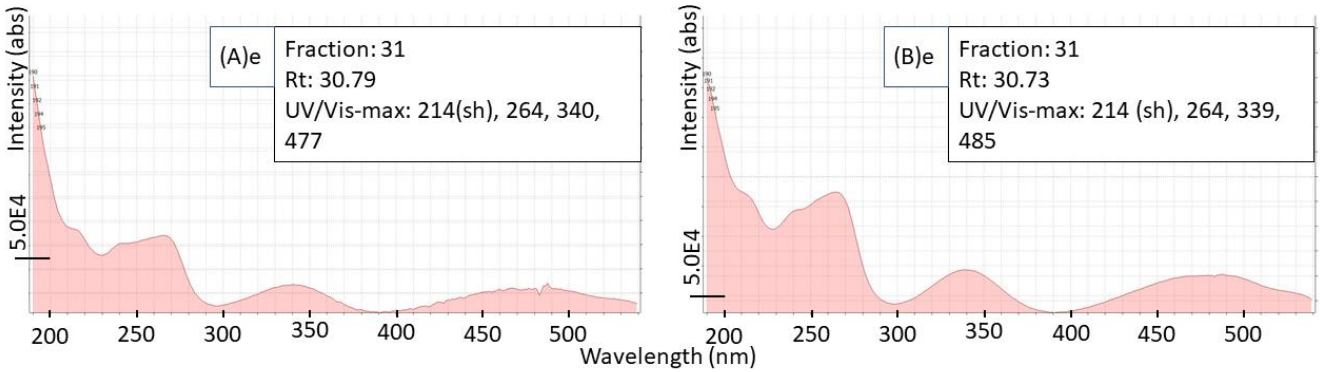

Figure 12 ***(AB)e UV/Vis-spectra at RT 30.79 (MeOH) and RT 30.73 (Milli-Q) compound in fraction 31 compared****. Shown as vertical line e in Figure 7. Shoulder is abbreviated as sh. Intensity (abs) is marked at 5.0E4 in both fraction for intensity comparison.*

Although there are similarities between the two extracts, mainly at the main peaks which all have PHNQ-like UV/Vis spectra, there are some peaks that are only in the **MeOH/TFA** and vice versa. These are those with PHNQ-like UV/Vis spectra.

Fraction 15 (**Feil! Fant ikke referansekilden.**). UV/Vis-maxima for **(A)x** at 269 nm, 358 nm, 460 nm. UV/Vis-maxima for **(B)x** at 267 nm, 350 nm, 468 nm. Peak **(A)x** is visible at Rt 14.77. There is no clear peak **(B)x**. Nevertheless, each extract seems to have a similar UV/Vis maxima at Rt 14.77 suggesting that the same, or a closely related compound. The difference in intensity makes the UV/Vis spectra harder to compare.

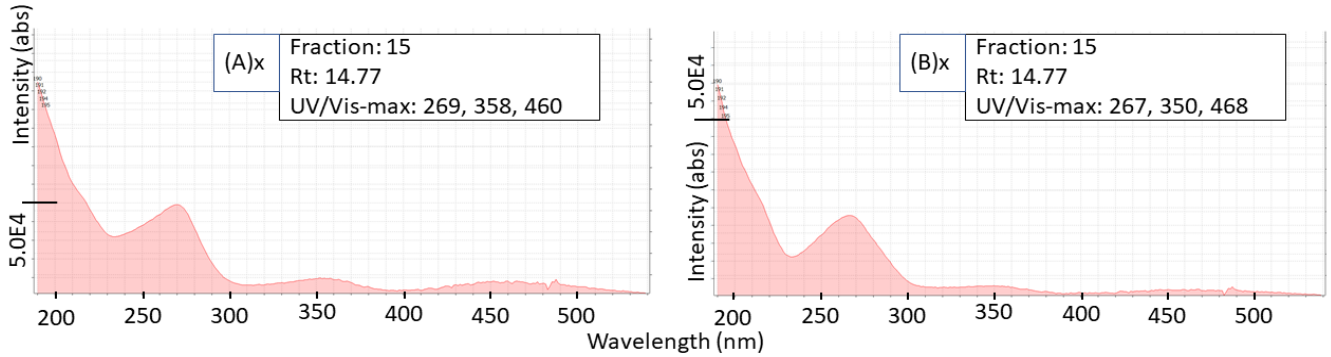

Figure 13 (AB)x UV/Vis spectre at Rt 14.77 (MeOH) and Rt 14.77 (Milli-Q) compound in fraction 15 compared. Shown as vertical line x in Figure 7. Intensity (abs) is marked at 5.0E4 in both fraction for intensity comparison.

Fraction 16 (Figure 14). UV/Vis-maxima for **(A)y** at 267 nm, 327 nm, 460 nm. UV/Vis-maxima for **(B)y** at 264 nm, 350 nm, 467 nm. Peak **(A)y** is visible at Rt 15.30. Here there is a clear difference between the two extracts despite the intensity difference, as seen with the 327 nm absorbance in the **(A)y** contra the 350 nm absorbance in **(B)y**.

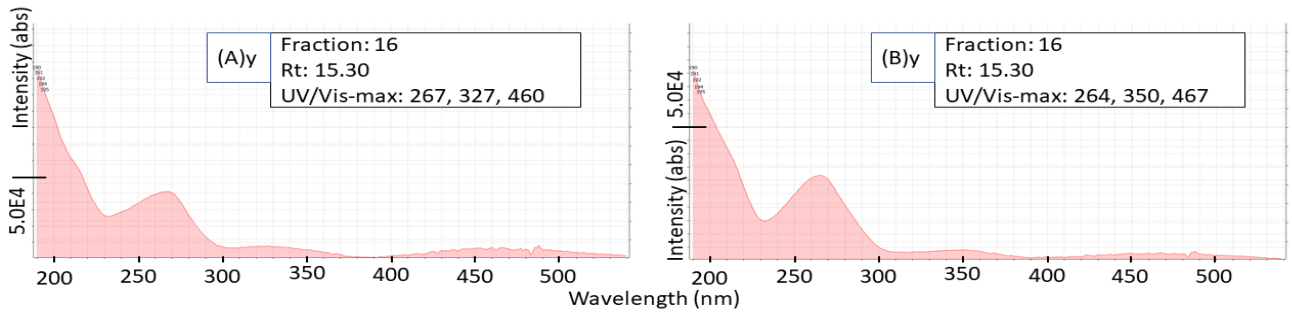

Figure 14 ***(AB)y UV/Vis spectre at Rt 15.30 (MeOH) and Rt 15,30 (Milli-Q) compound in fraction 16 compared****. Shown as vertical line y in Figure 7. Intensity (abs) is marked at 5.0E4 in both fraction for intensity comparison.*

Fraction 16 (Figure 15). UV/Vis-maxima for **(A)z** at 218 nm, 340 nm, 472 nm. UV/Vis-maxima for **(B)z** at 258 nm, 330 nm, 400 nm, 468 nm. Peak **(A)x** is visible at Rt 15.30. No peak at **(A)z** at 280 nm.

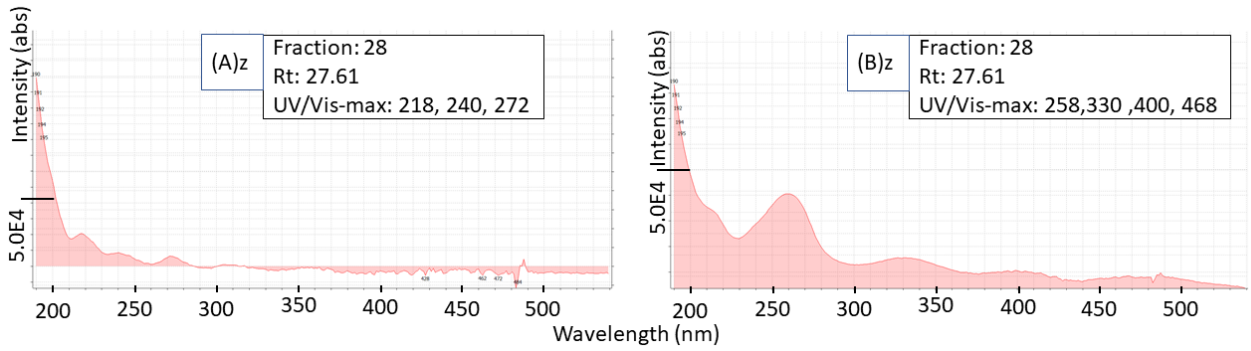

Figure 15 ***(AB)z UV/Vis spectre at Rt 27.61 (MeOH) and Rt 27.61 (Milli-Q) compound in fraction 28 compared.*** *Shown as vertical line z in Figure 7. Intensity (abs) is marked at 5.0E4 in both fraction for intensity comparison.*

There were two small peaks in fraction 27. They have slightly different retention time, but the same UV/Vis-maxima for **(A)w** at 225 nm and 276 at both peaks.

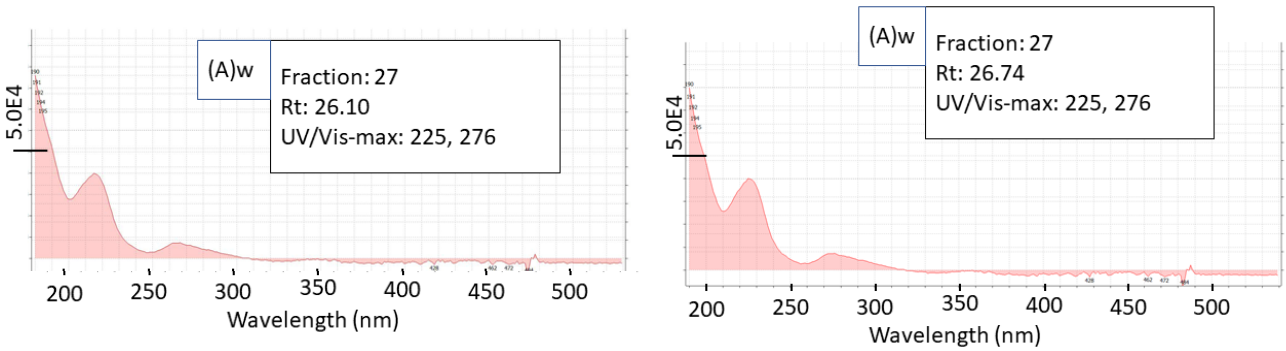

Figure 16 ***(A)w with*** *two peaks at Rt 26.10 and 26.74 in Fraction 27 (Figure 7). UV/Vis maxima 225, 276*

4.3 HPLC-diode array detector (DAD)-MS

An aliquot of 20 µl of the MeOH/TFA extract were injected for LC/MS. Based on m/z, photodiode array detector (PDA)-data (UV/Vis absorption), and Retention time (Rt) compared to the already known literature some compound could be identified. The focus was on Spinochrome/quinoids-like compounds. In Figure 17, **(A)** which Shows the Total Ion Current (TIC) of the **MeOH/TFA** extract, all compounds are shown, regardless of the absorption ability of the compound. **(B)** shows all absorption at 280 nm. This seems to have a cut-off after approximately 5 min, except for at Rt 10.86 that can also be observed in the blank sample run.

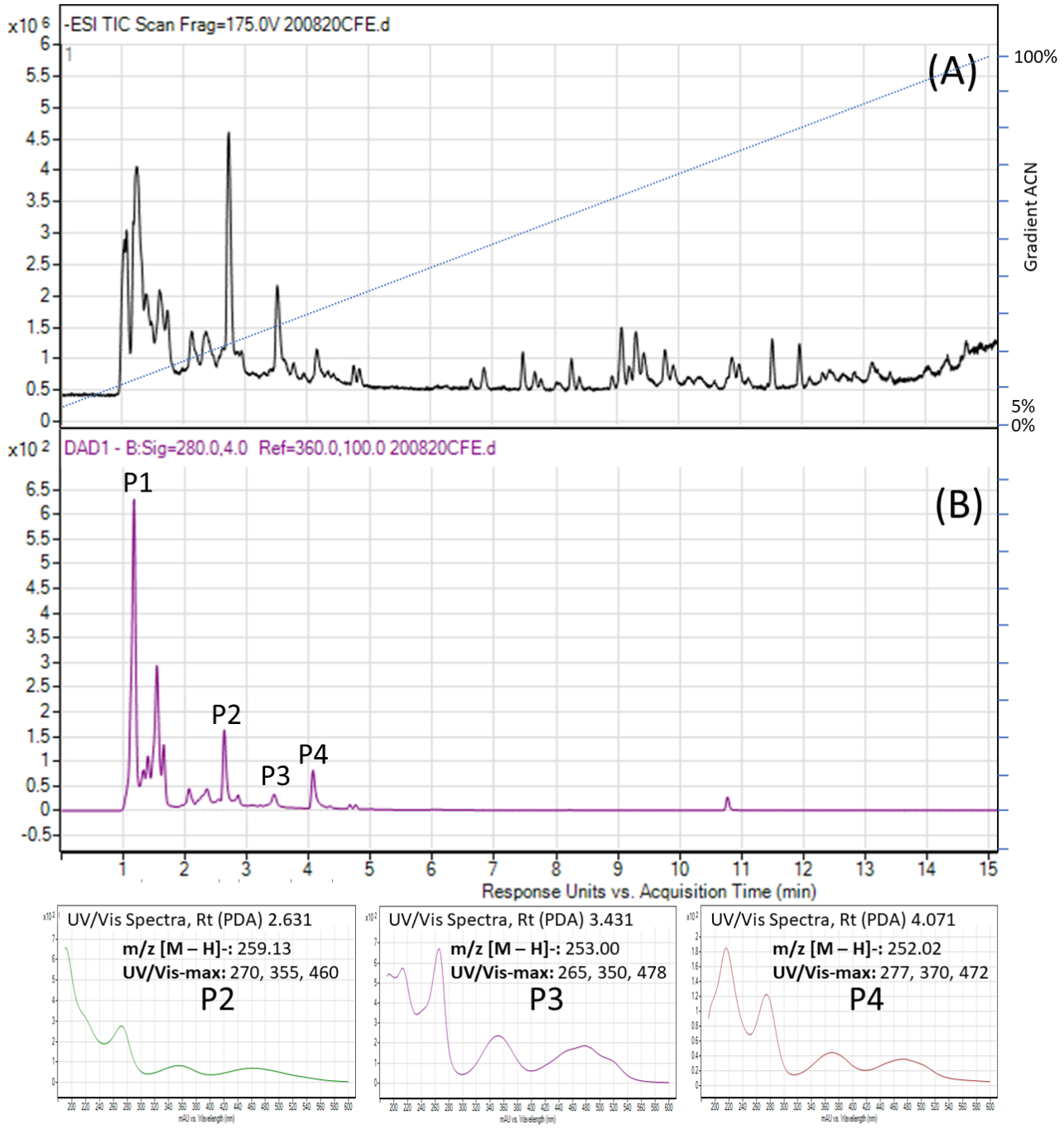

Figure 17 ***(A); TIC LC/MS for MeOH/TFA CFP extract. (B); DAD signal at 280 nm for MeOH/TFA CFP extract. b;*** *a spinochrome-like spectra at PDA Rt 2.631.* ***c;*** *a spinochrome-like spectra at PDA Rt 3.431.* ***d;*** *a spinochrome-like spectra at PDA Rt 4.071. Detector in negative mode with 1µl injected. The material was separated by a gradient of 5-100% ACN (with 0,1% FA) over 15 min.*

The m/z [M – H]- for peak (P) P2, P3 and P4 are shown in Figure 18. The next mass is 1 amu apart which idicates that the compounds have only one charge.

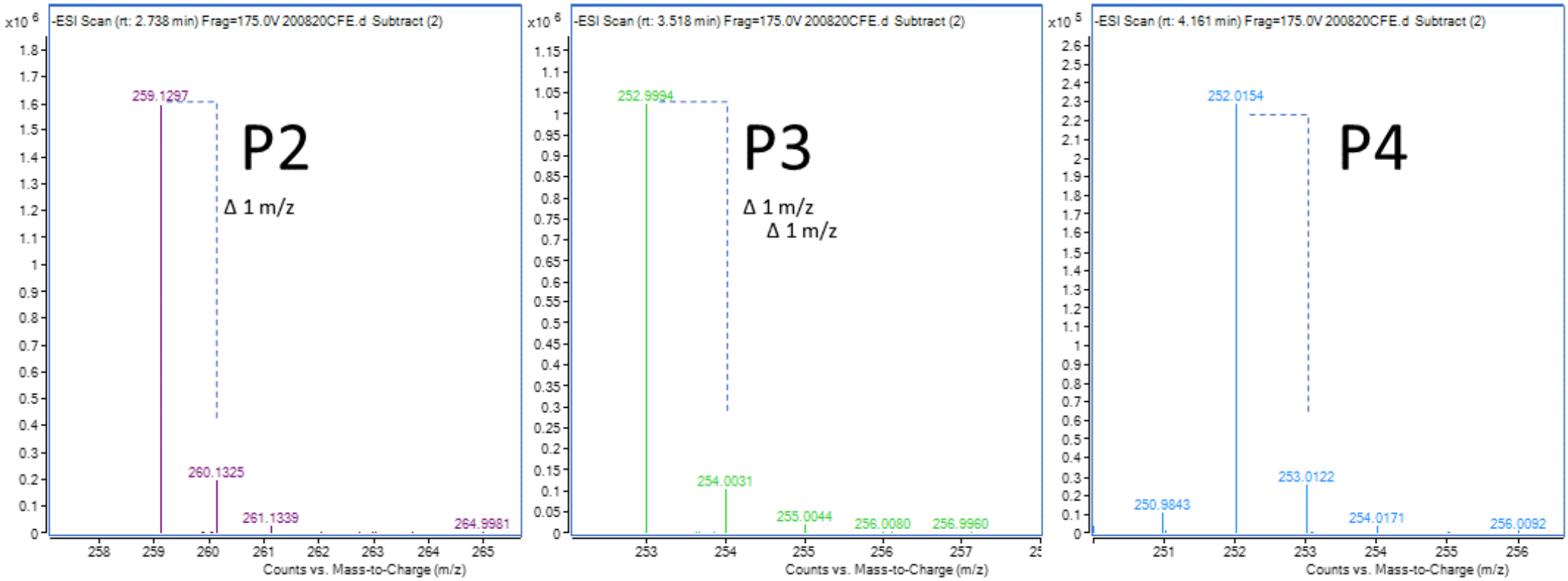

Figure 18 MS data for P2, P3 and P4. Negative mode [M – H]-. P2; 259.1297 m/z, one charge. P3; 252.9994 m/z, one charge. P4; 252.0154 m/z, Δ1 M/Z.

Peak 2, 3 and 4 have spinochrome-like UV/Vis specter (Figure 17). Their m/z ([M – H]-), Rt and Molecular formula are described in Table 7. Peaks after 5 min did not have quinone-like UV/Vis-spectra.

Table 7 ***PHNQ-pigments identified in MeOH/TFA extract based on PDA and LC/MS data.*** *Suggested compounds based on retention time, m/z and UV/Vis maxima compared to literature*

Peak ID	**Retention time, min (TIC)**	**Measured m/z [M – H]-**	**Calculated Monoisotopic mass, Da [M – H]-**	**Molecular Formula**	**Suggested Compound**	**ACN%**
1	1,25	379,14	379.1352	$C_{14}N_2O_{10}H_{24}$	EGTA	11
2	2,74	259,13			Unknown	21
3	3,53	253,00	252.9984	$C_{10}H_6O_8$	Spinochrome E	26
4	4,17	252,02	252.0144	$C_{10}H_7NO_7$	spinamine E	31

By combining the data from LC/MS and prep-HPLC and comparing against the literature for already known compounds, the following PHNQ-pigments were identified (Table 8).

Table 8 ***Combined HPLC*** *(Figure 8-Figure 15****) and LC/MS data*** *(Figure 17 and Table 7) . Identifying compounds based on available LC/MS, HPLC and literature data. Possible compounds marked with * based on UV/Vis data and Rt for already known compounds. UV/Vis spectra compared against data from Vasileva et al, 2021[54] and Hira et al, 2020[1].*

Peak ID HPLC	**F**	**Rt, HPLC**	**UV/Vis maxima**	**$[M - H]^-$**	**Possible compound**	**ref**
(A)a	18	17.21	266, 352, 477	252.9984	Spinochrome E	[1, 54]
(B)a	18	17.30	264, 350, 474			
(A)b	20	19.78	215(sh), 273, 368, 477	252.0144	Spinamine E	[1, 54]
(B)b	20	19.77	214(sh), 272, 368, 468			
(A)c	23	22.28	240, 271, 363	N/A	Unknown	
(B)c	23	22.34	274, 356, 467	N/A		
(A)d	29	28.87	220(sh), 240, 275, 460	N/A	Unknown	
(B)d	29	28.81	216(sh), 244, 270, 467	N/A		
(A)e	31	30.79	214(sh), 264, 340, 477	N/A	Spinochrome 502 dimer*	[1]
(B)e	31	30.73	214(sh), 264, 339, 485			
(A)x	15	14.77	269, 358, 460	259.1294	Unknown	
(B)x	15		267, 350, 468			
(A)y	16	15.30	267, 327, 460	N/A	Unknown	
(B)y	16		264, 350, 467	N/A	Unknown	
(A)z	28	27.61				
(B)z	28		258, 330, 400, 468	N/A	Spinochrome 536 dimer*	[1]
(A)w	27	26.10	225, 276	N/A	Unknown	
		26.74				

4.4 HPLC for decanted Sea urchin rest material water

This was a side project, and the focus was on finding spinochromes, and preferably sulphated. Thus, the only kind of assessment done on the decanted rest material water was looking for additional peaks after extraction and look for PHNQ-like UV/Vis spectra. There were varying

results based on method of "extraction", with new peaks showing up (Figure 19). None of the peaks had PHNQ-like UV/Vis spectra.

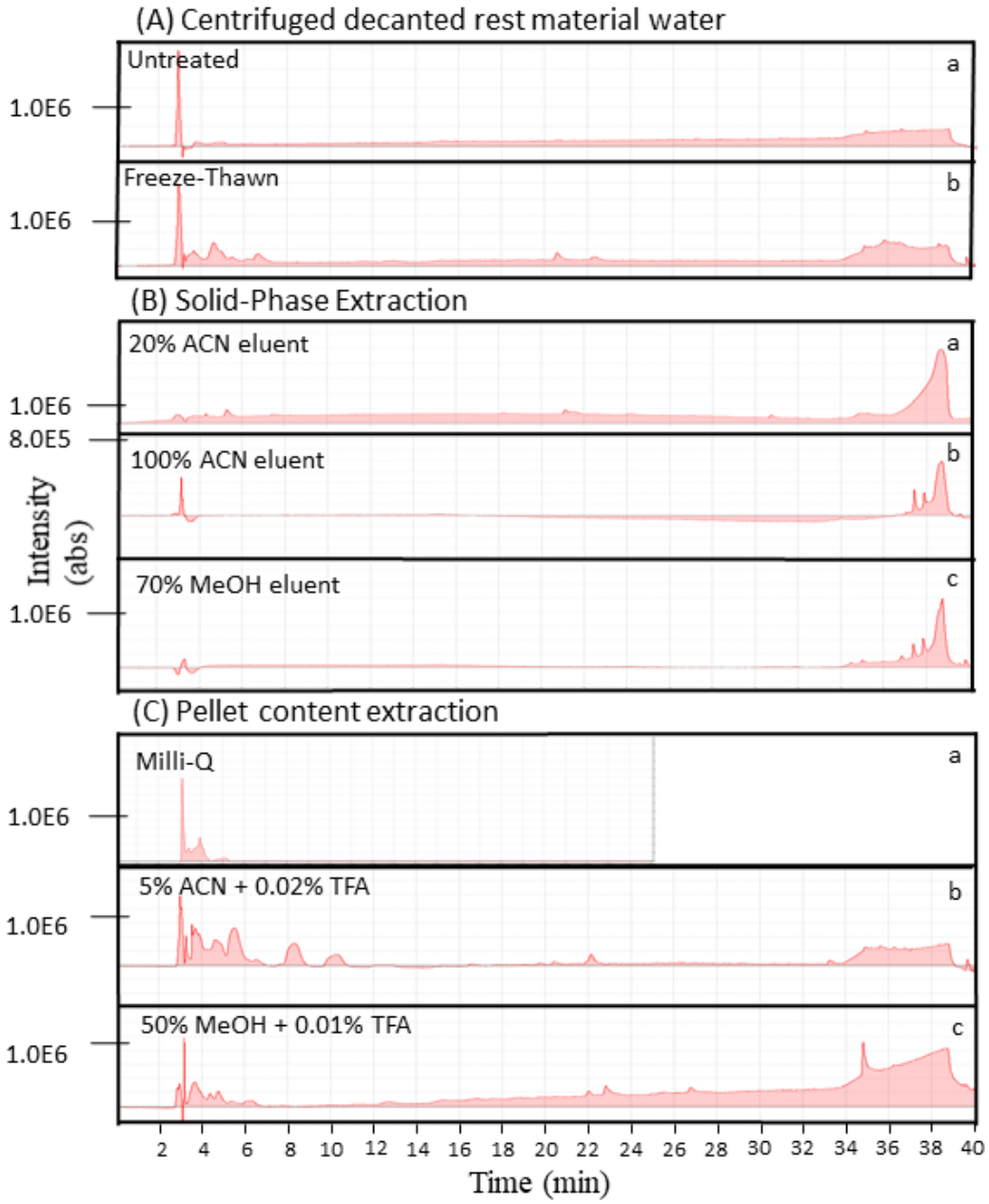

Figure 19 ***Chromatogram showing all absorption between 190-550 nm from prep-HPLC separation for decantated sea urchin rest material****. (A)a; Centrifuged decanted Sea urchin rest material water where the supernatant was injected for HPLC. (A)b; same material as (A)a which was Freeze-thaw before centrifugation. (B); Solid-phase extraction on decanted Sea urchin rest material water, a=20% ACN, b=100% ACN, c=70% MeOH eluent. (C); Centrifuged decanted Sea urchin rest material water where the supernatant was removed. a; Milli-Q was added to create hypertonic conditions, b; 5% ACN+ 0,02% TFA was added, c; 50% MeOH+0,005% TFA was added. 0,4 ml was injected for HPLC and the material was separated with an ACN (with 0,005% TFA) gradient of 0% from 0-10 min, 0-20% from 10-30 min, 20-100% from 30-40 min, 100% from 40-50 min to flush the column*

4.5 Bioactivity

3.1.1 Antibacterial activity

None of the fractions can completely inhibit growth of *B. subtilis*. There is however a prolonged lag-phase in fraction 4, 5 and 6 from the **Milli-Q** extract shown in Figure 20. Fractions from the **MeOH/TFA** extract shows no antibacterial activity against *B. subtilis.*

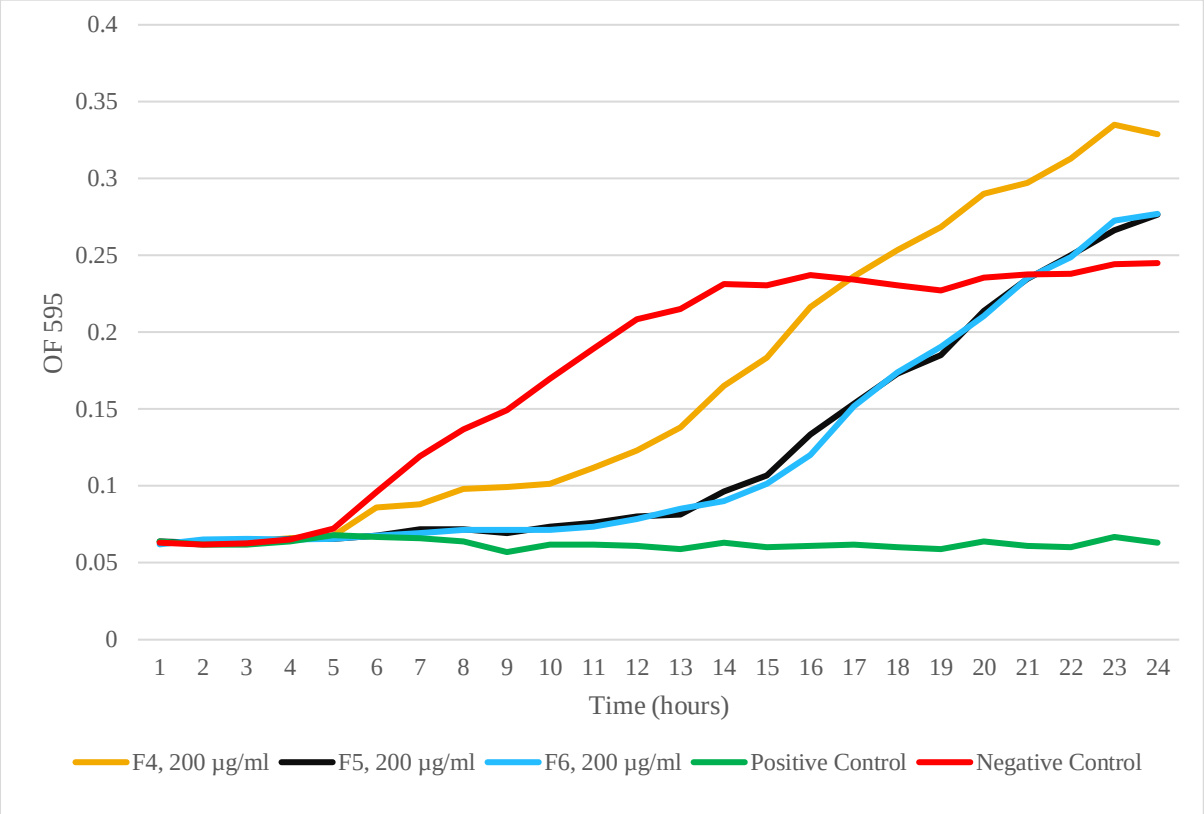

Figure 20 ***Antibacterial activity Assay results in different fractions of separations of material from coelomocytes of S. droebachiensis.*** *Fraction activity against B. subtilis shown as growth OD over time. Only fractions with some activity are shown. Negative control is B. subtilis against Milli-Q water. Positive control is B. subtilis against Oxytetracycline.*

The targeted fraction concentration was 200 µg/ml, with two parallels. There wasn't enough material for all the fractions to test at this concentration, some fractions were therefore tested at a lower concentration, none of them positive.

3.1.2 Antioxidative activity

The fraction was tested at several different concentrations ranging from 100-6.25 µg/ml. Most of the tested fractions show some antioxidative activity (Figure 21). The most active fractions

from the **MeOH/TFA** extract are 15, 17, 22 and 27 where all have an activity higher than 6.25 µM Trolox equivalent. The most active fraction for the **Milli-Q** extract is fraction 33 with a higher activity than 6.25 µM Trolox equivalents. Fraction 8-14, 24-26 from the **Milli-Q** extract were not kept after prep-HPLC and therefore not tested. Fraction 23 and 29 from the **Milli-Q** extract were tested at half the concentration of the other fractions due to a lower amount of available material. To compensate for this, their values have been doubled. Fraction 15, 19, 22 and 35 have uncertain concentrations because inaccurate amount detected during weighing. **Milli-Q** fraction 33 showed the highest activity of all tested fractions regardless of extract.

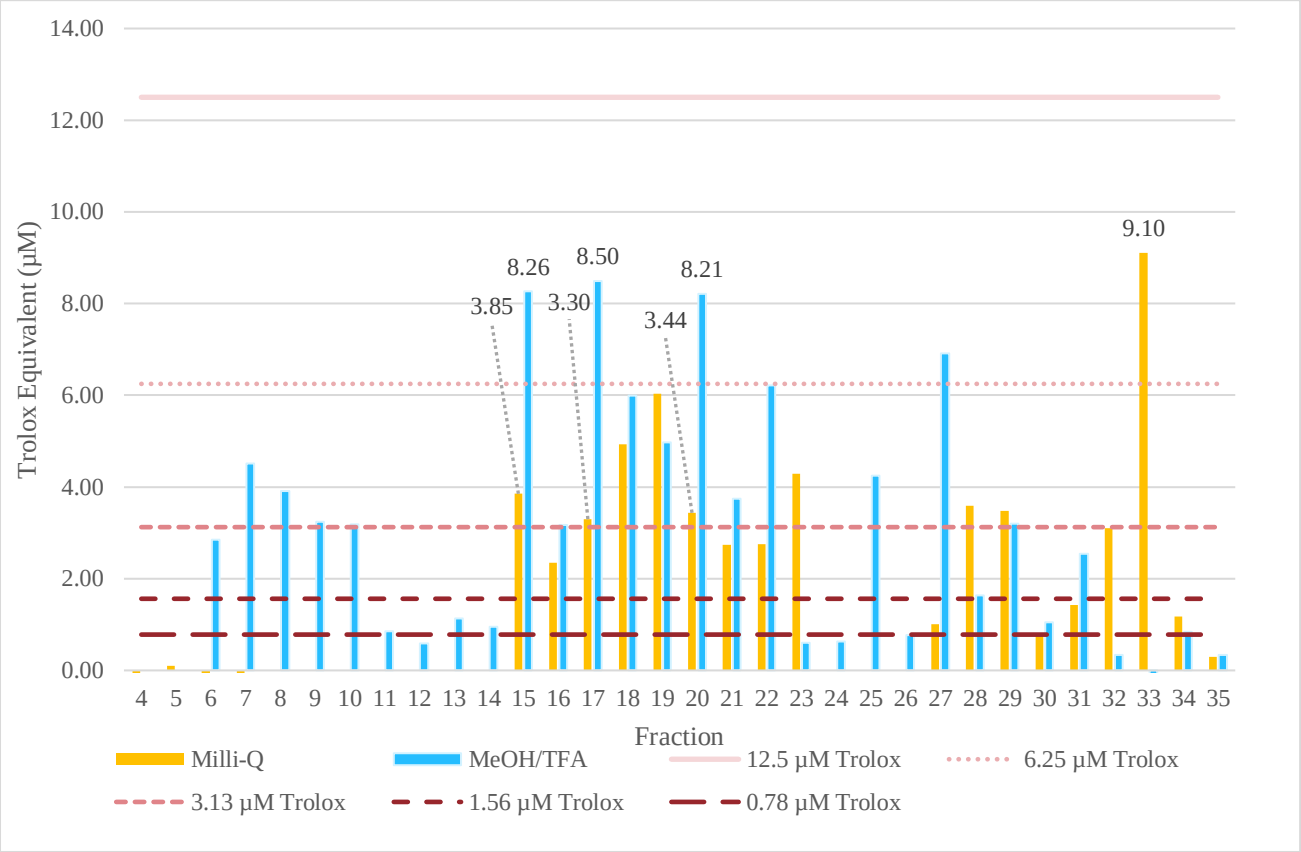

Figure 21 ***Oxygen radical absorbance capacity of fractions from MeOH/TFA and Milli-Q extract from* S. droebachiensis.** *Results are expressed as 12.5 µg/ml sample (columns) vs Trolox (horizontal lines) at concentrations ranging from 12.5 µM (top) to 0.78 µM (bottom). All fractions were set up with two parallels. Mean are parallels used as values in the calculations.*

4.6 Suggested improved method

A suggested flow of isolation of PHNQ pigments from, mainly RSC is shown in Figure 22, based on the results of this project. The improvements are discussed further in 5.8.

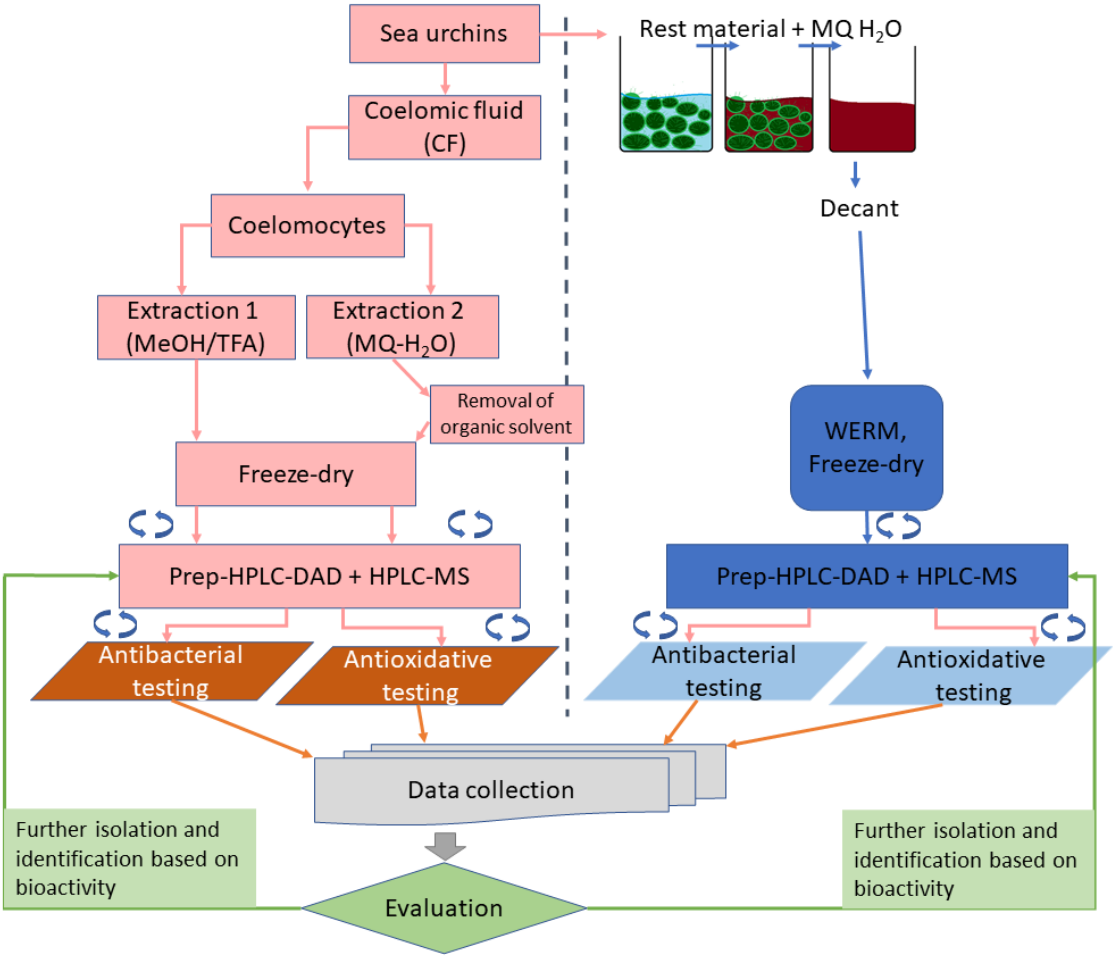

Figure 22 Suggested improved method for isolation of PHNQ pigments from the green sea urchin, S. droebachiensis

5 Discussion

The aim for this project was primarily to evaluate different isolation methods for spinochromes made by the sea urchin, *S. droebachiensis*. This idea of the project was based on findings published by Hira and co-workers [20] where suspected sulphated spinochromes were identified in the data. They based their results mainly on relatively pure cell populations of red spherule cells obtained by flow cytometry. That is a time-consuming method that cannot be used to get enough material to characterize the features and test the bioactivities of the spinochromes and PHNQ compounds of the sea urchin. Hence, in this master project two different extractions procedures were used and compared. It turned out to be possible to extract and identify naphthoquinone pigments. However, it was not possible to identify any of them as sulphated. Moreover, it was not identified a high antibacterial activity against *B. subtilis* in the HPLC fractions containing PHNQ compounds, except for a delayed growth effect of the bacteria at high concentrations of fraction materials. Interestingly, the fractions containing identified PHNQs based on UV/vis and MS data, showed antioxidant activity. These findings and aspects of the work will be discussed in more details below.

5.1 Red spherule cell extraction

From 162 animals, the total amount of pellet [1, 45] spherule cells were 17-20 ml by centrifugal separation [45]. The overall concentration of RSC is unknown as the gradient centrifugation will sediment both CSC and RSC. Based on the status of the animals, the percentage of RSC will also vary. An improvement to the separation for RSC could have been by fluorescence-activated cell sorting (FACS) which is a form of flow cytometry if one has a flow cytometry available. This would be possible due to the autofluorescence of the RSC, distinguishing them from other coelomocytes[1]. This was done in Hira et al, 2020 [1], where used FACS to get a >99% RSC population. Unfortunately, this is very time consuming and would end up occupying a flow cytometer for days. get enough material to characterize the features and test the bioactivities of the spinochromes and PHNQ compounds of the sea urchin.

The amount of RSC collected during the extraction of coelomic fluid was, based on the pellet size, less than expected (J. Hira, personal communication, 2020). Hira collected 40 ml of RSC from a lower number of animals when performing a similar study, Hira et al, 2020 [1]. This is could be due to seasonal variations, life stages of the sea urchin, influence of environmental factors, stress, or a combination of factors. A possible explanation is given in the following paragraph.

There are evidence showing that the abundance of red spherule cells in the coelomic fluid could be linked to environmental stress, such as pollution or injuries[55, 56], but coelomocytes have been shown to activate their response machinery when exposed to physical and chemical stress such as temperature shock and pH drop, expressed with an elevated level of heat shock protein 70 (Hsp70) [57]. The same can be seen when exposed to UV-B radiation [58]. It is suggest that red spherule cells arise from a maturation/differentiation process occurring to white amoebocytes in response to stress, which is a hypothesis stemming from the notion that sea urchins from polluted sea waters or exposed to cold stress or injuries show an increased number of RSC [58], although this lacks sufficient evidence. Therefore, this could indicate a significant difference in stress levels when extracting the coelomic fluid was extracted during this project contra when it was extracted in Hira et al [1].

5.2 Evaluation of PHNQ extraction with MeOH/TFA and Milli-Q water

The choice of the two extraction solvents (methanol + low amounts of trifluoracetic acid, and Milli-Q water) were based on the results of, and personal communication with Hira and co-workers. Both solvents are simple to execute when the coelomic fluid is extracted from the sea urchins. The actual size of the RSC pellet is not fully known due to that there still being some coelomic fluid which was difficult to remove. Therefore, not that much care was taken into the amount of solvent used when extracting content under hypotonic conditions with Milli-Q water. One also runs into the risk of degrading wanted material due to spinochromes not being stable when in water.

The **MeOH/TFA** extract was exposed to light and heat at 40°C over longer amounts of time when removing the MeOH and TFA on a rotavapor before vacuum centrifuging as vacuum drying with high amounts of MeOH could damage the centrifuge.

They based their results mainly on relatively pure cell populations of red spherule cells obtained by flow cytometry. That is a time-consuming method that cannot be used to get enough material to characterize the features and test the bioactivities of the spinochromes and PHNQ compounds of the sea urchin. Thus, this is the reason for the focus and the aim of this master project. Here we tested out two different extraction procedures, it was possible to extract and identify PHNQ pigments. However, it was not possible to identify some of them as sulphated.

5.3 Identification of compounds and bioactivity

The LC/MS was not calibrated for negative mode prior to the analysis. Therefore, some leniencies were given when interpreting the data from the mass spectrometer. A considerable amount of EGTA still remains in **MeOH/TFA** extract as shown in Figure 17 and Table 7, and due to the similarities between the extracts one can assume that the **Milli-Q** extract also contains EGTA. Fraction 4 and 5 from both extracts could, most likely, contain EGTA. When looking at Table A 3 and Table A 4, there is a difference in the clear fractions weight. Where The **MeOH/TFA** extract fraction 4 weighs 219.18 mg, fraction 4, 5 and 6 weighs 77.95 mg, 85,63 mg and 55.98 mg, which could mean that content of fraction 4 in the **MeOH/TFA** extract (most likely EGTA based on LC-MS data, Figure 17 andTable 7) is in fraction 4-6 of the **Milli-Q** extract.

Three PHNQ-pigments have been identified by m/z, UV/Vis-maxima and Rt (Table 7) from pre-fractionated **MeOH/TFA** extract. Because of time restrictions and unavailable LC-MS system, no other LC-MS data are available, therefore there is a high emphasis on the UV/Vis spectra data from the Prep-HPLC for both CFP extraction solvents.

Peak **(AB)a (**Figure 8**)**, **(AB)b (**Figure 9**)** and **(AB)x (**Figure 13**)** shown in Figure 7 are most likely, based on the similar pattern in UV/Vis-maxima and retention time, the peaks in LC-MS (Figure 17) marked as **P2**, **P3** and **P4**. The UV/Vis spectra for **(AB)x** indicate its relation to PHNQs, but unlike **(AB)a** and **(AB)b**, which had matching UV/Vis-maxima compared to established compounds Spinochrome E and spinamine E, there was none for **(AB)x**. If one would go by the assumption that the compound is of the spinochrome class, solely based on the UV/Vis spectra its proximity to other spinochromes in retention time then one could predict a theoretical structure based on the scaffolding structure provided in Vasileva et al (Figure 23) and the knowledge of functional groups such as methoxyl, acetyl, ethyl, and amino groups already established by the literature [30].

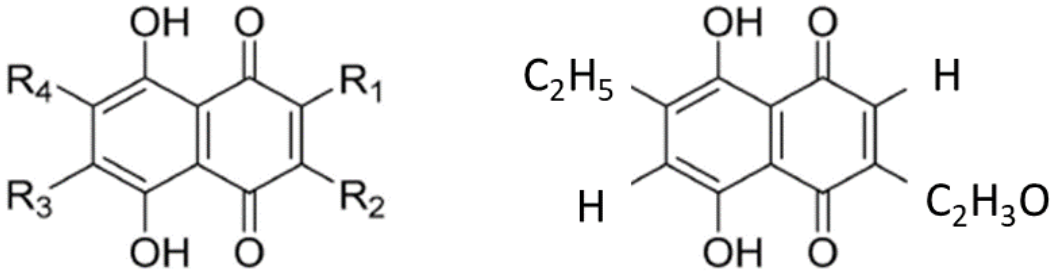

Figure 23 Scaffolding structure of spinochromes (left), taken from Vasileva et al, 2021[54], and suggested structure functional groups (right) Rn are functional groups.

The basic structure as provided in Figure 23, have a molecular mass of 185.9953 ($C_{10}H_2O_4$). That leaves 74.1419 for the undetermined functional groups. Suggested functional groups are:

R_1 = H (1.0078 Da) R_2 = C_2H_3O (43.0184 Da)

R_3 = H (1.0078 Da) R_4 = C_2H_5 (29.0391 Da)

This giving a molecular formula of $C_{14}H_{12}O_5$ and a Monoisotopic mass of 260.0685 Da. As for the order of the functional group, more data is required.

The LC-MS data is only available for the **MeOH/TFA** extract and the **Milli-Q** extract is closer to the method of extraction in Hira et al [1], where they found different possible sulphated spinochromes by extracting a population of 100% RBC with H_2O (ratio ~1.5:40 of H_2O to

original coelomic fluid volume) [1]. In Hira et al, 2020 [1] the most abundant suspected sulphated spinochrome D, which were not identified in its established or sulphated form in none of the two extract. This could be due to the stability of the compounds, more on this in 5.6, or the fact that there was no Spinochrome D as *S. droebachiensis* have been found to vary in pigment composition depending on which depth they are collected from [59].

The hypothesis for this thesis was as follows:

- *Extraction and isolation of compounds from echinoderms using acidic conditions will dissociate a potential sulphate group from some compounds in red spherule cells whereas a water-based extraction procedure will keep the sulphated compounds intact*.

No sulphated compounds of PHNQ characteristics have been found when identifying compounds with the available data. These data is not enough to conclude on a falsification of the hypothesis because other variables, like stability (see below), could be the reason for the hypothetical sulphate group to dissociate.

5.4 Antibacterial activity

The fractions of both extracts were tested against *B. subtilis* by following the growth curves for 24 hours. The reasoning behind using one bacteria strain was to limit the use of material because of shortage of fraction material. There is data available regarding antibacterial activity against *B. subtilis* using crude extract and isolated spinochromes from sea urchins which could help with the identification of the compounds. *B. subtilis* also have more options for follow up test available in the group for marine bioprospecting, such as biosensor assay. Oxytetracycline was used as a positive control.

None of the fractions tested against *B. subtilis* were able to stop the growth of the bacteria. However, **Milli-Q** extract, fraction 4, 5 and 6, had a longer lag phase than the negative control sample (Figure 20), which was *B. subtilis* tested against Milli-Q water. None of these fractions have PHNQ-like UV/Vis spectra.

EGTA could be the reason for the activity in these fractions as Ca^{2+} depletion can slow down bacterial growth. Growing *B. subtilis* in LB-media in the presence of EGTA have shown to slow down or even stop exponential growth curves [60].

5.5 Antioxidative activity

The fractions from both **MeOH/TFA** and **Milli-Q** extract have positive results regarding antioxidative activity. With that said, there were only two parallels for each fraction tested so a standard deviation could not be determined. This should be taken into consideration when evaluating the reliability of the results. Both parallels for all active fractions have values high enough values to which one can say there are antioxidative activity.

When talking antioxidative activity of spinochromes several articles reporting that they are strong antioxidants, though most is on Echinochrome A or crude extracts. They can block free radical reactions [39, 61, 62], chelate and reduce metal ions [34, 39] and inhibit lipid peroxidation [39, 61].

The cluster of active fractions from 6-10 have no PHNQ-like spectra. As such they have not been the focus of this thesis and the active compound have not been identified. In theory all the fractions were diluted to the same concentration, said for a few which had less material available. In practice, this was not the case due to the difference in solvability when rehydrating and diluting in Milli-Q water before testing. There is also a difference in activity between the two extracts at some comparable fractions even though they have the same compound identified. This could be due to purity of the fraction and solely based on the HPLC chromatogram the **MeOH/TFA** extract seems to be purer due to the more prominent peaks at **(A)a**, **(A)b** and **(A)x** (Figure 7).

Fraction 15, which is the second most antioxidative active in the **MeOH/TFA** extract at 8.26 TE, is **(AB)x**, further reenforcing the assumption that this is an unidentified PHNQ-like compound. The **MeOH/TFA** extract have TE value which is x2.15 higher than that of the **Milli-Q** extract for this fraction. The **Milli-Q** extract was less soluble for this fraction and

EtOH/TFA extract fraction seemed to be fully dissolved however the concentration is unknown due to difficulties during weighing which resulted in a negative number.

Fraction 17, the most antioxidative peak for the **MeOH/TFA** extract with a value of 8.50 TE, is identified with the available data to most likely be spinochrome E **(AB)a**. The **MeOH/TFA** extract have TE value which is x2.58 times higher than that of the **Milli-Q** extract for this fraction. The difference could be due to the purity of the tested fraction as **(A)a** have a more distinct peak than **(B)a**. There is some overlapping from fraction 17 to 18 and 19 for both peaks, which would explain the activity. Fraction 19 have more activity in the **Milli-Q** fraction than the **MeOH** fraction, which could be due to an overlapping from both peak 18 and 20. Both extracts were not able to fully dissolved when diluting.

Fraction 20, the third most active fraction in the **MeOH/TFA** extract at 8.21 TE, is identified to be Spinamine E. This fraction also shows a significantly higher activity in the **MeOH/TFA**, with it having x2.39 higher activity than its counterpart from the **Milli-Q** extract. The difference could be due to the purity of the tested fraction as **(A)b** have a more distinct peak than **(B)b (**Figure 7**)**. Both extracts were not able to fully dissolved when diluting. Fraction 21 activity seems to be due to overlapping peaks between fraction 20 and 21.

Fraction 22-27 have unknown components. Fraction 23 have a PHNQ-like UV/Vis spectra in both extracts. The abundance seems to be higher in the **Milli-Q** extract due to the intensity (Figure 10). No distinct peak in fraction 25. Parallels have good corelation, and there is a clear dose response. Fraction 27 have two small peaks **(A)w** which both have the same UV/Vis spectra (Figure 16), compound unknown.Fraction 28, possible Spinochrome 536 dimer in the **Milli-Q** extract. **Milli-Q** extract fraction 33, which is the most active, have no distinct peak in Figure 7. This could be a compound which does not absorb light in the UV/Vis range (190-550).

5.6 Stability of PHNQ pigments

There were no measures taken to protect the extracts and fractions from exposure to light. The stability in water, or rather lack thereof, were also not taken into consideration since the project was to try and extract sulphated spinochromes with a water-based extraction and a methanol-based extraction. There was some care to not keep the fractions in room temperature longer than needed, but they were exposed to temperatures at 20°C for hours when vacuum drying the extracts. The **Milli-Q** extract was the first to be fractionated with prep-HPLC. The fractions were dried overnight in a vacuum centrifuge. Greater care was taken when drying the MeOH/TFA fractions. They were dried during the day with approximately bi-hourly checkups. This could have had an impact on the difference in some of the fractions where the **MeOH/TFA** seemed to be more potent than the **Milli-Q** counterpart. The effects of storing in water overnight can be seen in the difference of peak signal between DAD 280 in the LC/MS run and Prep-HPLC, where the identified spinochrome E clearly is the peak with most absorbance during the fractionation of the MeOH/TFA extract (peak **(AB)a** in Figure 7) contra the next day during the LC/MS run where it is barely visible (Peak **(B)c** in Figure 17). The same can be observed for spinamine E (peak **(AB)b** in Figure 7 and **P4** in Figure 17). This also indicate that the unknown compound at mass 260 could be more stable than spinochrome E and spinamine E, though available in lower amounts.

In Hira et al [1], the supernatant was collected and immediately submitted for UHPLC-DAD-MS/MS analysis, and where therefore not exposed to the same challenges as in this project. PHNQ pigments from *Strongylocentrotus nudus* have been found prone to degradation when exposed to light, and even at room temperature and when solved in water [63] and its therefore recommended to store them in dark and low- temperature conditions. When storing over longer amount of time, storing with another antioxidant such as vitamin C can protect and enhance the colour of the pigment [63]. Their stability in solvents vary. As for their stability in alkaline conditions, it seems to vary depending on the PHNQ pigment. Echinamines B have shown to be resistant to degradation under weakly alkaline conditions (HEPES buffer at pH 7.5) and Spinamine E and spinochrome E were prone to degradation [64].

5.7 Solubility of material

Most spinochromes seems to be poorly soluble in water [30]. Each extract was dissolved in Milli-Q water after vacuum drying to prepare for prep-HPLC. Both extracts were not fully dissolved when rehydrating the extracts in Milli-Q water, and when rehydrating/diluting the fractions before bioactivity assays. Hence, the practical concentration was lower than the theoretical concentration. This was the case for both **EtOH/TFA** and **Milli-Q** extract fractions alike with it visually being a little more prominent in the **Milli-Q** extract fractions. The fractions that would not completely dissolve in milli-Q water is listed in appendix A 2 **Feil! Fant ikke referansekilden.** (**MeOH/TFA**) and **Feil! Fant ikke referansekilden.** (**Milli-Q**).

5.8 Improvements in methods to obtain more material

In this project, several steps were not performed optimally. This is mainly based on three factors, the stability of the compounds, the solubility, and the compounds availability in the animal. A proposed optimized protocol is shown by a glimpse in the flow presented in Figure 22.

For question about availability, additional experiments can be considered to get a better understanding of PHNQ pigments in sea urchins under certain conditions. Although time consuming, a study on the difference in PHNQ pigments, or RSC, based on seasons could be interesting to optimize a potential harvest. Also, the potential to stimulate the sea urchin under certain conditions to upregulate a stress response can be considered.

As for direct improvements, the focus should be on optimizing the yield of the extraction and taking heights for the instability of the compounds under certain conditions. In Luo et al [47] they tested four extraction methods on metabolite recovery from mammalian cells. They found that a novel Two-phase solvent system extraction could be used to somewhat specialize depending on what was going to be extracted [47]. The novel two-phase solvent system extraction method with both methyl tert-butyl ether (MTBE) and 75% 9 : 1 methanol : chloroform showed a clear advantage with high extraction efficiency for global

metabolomics [47] among four extraction methods, which should be taken into consideration if this were to be attempted again.

As for the stability. The extract should be protected from light exposure and temperatures over 20°C. During drying, the extracts were exposed to temperatures at 20°C for as long as overnight. Instead of vacuum centrifuging, it might be better to freeze dry the sample due to lower temperatures and easier to managed light exposure as the sample can be covered said from some few holes. This does however mean that possible organic solvents need to be removed prior to freeze-drying. Always working with aliquots of the sample and suspending in water as late as possible would be preferable, but this will be a challenge when doing assays with fractions.

For the solubility, the use of dimethyl sulfoxide (DMSO) could be a solution should be considered based on the problems with solubility. DMSO have an excellent capacity to solvate both organic and inorganic compounds with a relatively low toxicity. It is also commonly used with small molecules. It could however interfere with bioactivity test, so it needs to be removed or diluted to <1% concentrations before tests [65].

6 Conclusion

Red spherule cells extracted from the green sea urchin *S. droebachiensis*, exposed to two solvents, gave extracts containing PHNQ pigments. However, none of them were identified as sulphated.

Spinochrome E and spinamine E were identified based on mass, UV/Vis spectra and Rt. Although there were no LC-MS data, spinochrome 502 dimer and spinochrome 536 dimer were identified based on UV/Vis spectra and Rt. There is also the suspected novel compound which is suspected to be of PHNQ nature based on UV/Vis spectra, Rt and antioxidative activity. All showed antioxidative activity, reinforcing the notion of them being PHNQ pigments. Unfortunately, the amount extracted were not enough to do extensive isolation, identification, and characterization.

The data show a huge loss of material due to water insoluble material, based on weight, and problems with stability, as seen in the difference between prep-HPLC and LC-MS PDA data. This led to a low amount of available material for testing. Low antibacterial activity was detected against *B. subtilis*. The antioxidative assay ORAC showed that there was activity in and around the fractions containing PHNQ pigments, as well as in some fractions with no absorbance at 280 nm.

There is not enough available information of sulphated spinochromes.

The hypothesis: *Extraction and isolation of compounds from echinoderms using acidic conditions will dissociate a potential sulphate group from some compounds in red spherule cells whereas a water-based extraction procedure will keep the sulphated compounds intact*, can therefore not be verified.

More studies are needed to reveal the effect of seasonal variations, physical status, or effect of different chemicals and their influence on the PHNQ content. Greater care should be taken to protect the extracts from light, temperature and solvents that can degrade spinochromes.

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8 Appendix

8.1 Buffers and agar

Ca^{2+} +Mg^{2+}-Free sea water (CMFSW)

The beaker was filled halfway up (500 ml) before adding the reagents (Table 5). The beaker was then filled up to 1 L and mixed after adding the chemicals.

Table A 1 Ca2++ Mg2+ Free Sea Water chemicals used for anti-clotting buffer. Contains name, formula, molar concentration, amount needed for 1 L and manufacturer

Formula	MW(g/mol)	mM	g/L
NaCl	58.44	462	27
KCl	74.55	10.7	0.8
Na_2SO_4	142.04	7	1
$NaHCO_3$	84	2.1	0.18

Anti-clot buffer (AB)

For a volume of 1 L at concentration 100 mM, an amount of 38 g EGTA is needed. To solve the EGTA in Milli-Q water a pH of 7.5 or higher is needed. Sodium hydroxide was used to regulate the pH up. EGTA is added while mixing and the solution was done when the mix was clear. The 100 mM EGTA was Mixed 1+4 with CMFSW, and the pH was adjusted down to 8 with Potassium chloride if needed.

Mueller-Hinton (MH) broth and agar

To make MH-agar, weigh in the dry stuff (**Feil! Fant ikke referansekilden.**) and add Milli-Q water. Dissolve the media while stirring at 70-100 °C. For the broth, don't add the agar.

Table A 2 Ingredients for Mueller-Hinton agar and broth

Mueller-Hinton Growth media	
Ingredients	Amount
Mueller-Hinton broth	21 g
Milli-Q water	1000 ml
Mueller-Hinton agar	20 g

Phosphate buffer (PB)

The phosphate buffer is made with 10.649 g of Na_2HPO_4 (Mw = 141.988 g/mol) was solved in 1 L Milli-Q water. The pH was adjusted to 7.4 with HCl.

8.2 Bioactivity, solvability, and dry weight

Table A 3 ***Summery from bioactivity testing, MeOH/TFA extract.*** *Shows activity, targeted test concentration and solvability for MeOH/TFA extract*

MeOH/TFA fractions						
Fraction	Antibacterial activity (*Bacillus subtilis)*	Antibacterial test dilution (µg/ml)	Antioxidative activity, 12.5 µg/ml TE	Antioxidative test dilution (µg/ml)	Difficulties dissolving in Milli-Q water (+ yes)	Available material for testing (mg)
4	-	200-1.56	0	100-6.25		219.18
5	-	200-1.56	0	100-6.25		19.04
6	-	200-1.56	2.86	100-6.25		1.68
7	-	200-1.56	4.51	100-6.25		0.43
8	-	200-1.56	3.91	100-6.25		0.43
9	-	200-1.56	3.24	100-6.25		0.27
10	-	200-1.56	3.19	100-6.25		0.28
11	-	200-1.56	0.85	100-6.25		0.41
12	-	200-1.56	0.59	100-6.25		0.72
13	-	200-1.56	1.13	100-6.25		0.81
14	-	200-1.56	0.95	100-6.25		0.58
15	-	N/A	8.26	N/A		N/A
16	-	200-1.56	3.17	100-6.25		0.1
17	-	200-1.56	8.50	100-6.25	+	0.44
18	-	200-1.56	6.0	100-6.25	+	0.6
19	-	200-1.56	4.98	100-6.25		1.06
20	-	200-1.56	8.21	100-6.25	+	0.6
21	-	200-1.56	3.74	100-6.25	+	0.12

22	-	N/A	6.22	N/A	+	N/A
23	-	200-1.56	0.60	100-6.25	+	0.59
24	-	200-1.56	0.63	100-6.25	+	0.44
25	-	200-1.56	4.24	100-6.25	+	0.35
26	-	200-1.56	0.77	100-6.25		0.53
27	-	200-1.56	6.92	100-6.25	+	0.39
28	-	200-1.56	1.64	100-6.25	+	0.43
29	-	200-1.56	3.21	100-6.25		0.27
30	-	N/A	1.05	N/A	+	N/A
31	-	200-1.56	2.55	100-6.25	+	0.21
32	-	200-1.56	0.33	100-6.25	+	0.26
33	-	200-1.56	-0.74	100-6.25	+	0.29
34	-	90	0.84	100-6.25		0.12
35	-	200-1.56	0.33	100-6.25		0.22
				Combined fraction weight		250.85
				Combined fraction weight – not collected **Milli-Q** fractions		246.03

Table A 4 ***Summery from bioactivity testing, Milli-Q extract.*** *Shows activity, targeted test concentration and solvability for Milli-Q extract.*

Milli-Q fractions						
Fraction	Antibacterial activity (*Bacillus subtilis)*	Antibacterial Test Concentration Highest-lowest (µg/ml)	Antioxidative Activity, 12.5 µg/ml TE	Antioxidative test concentration Highest-Lowest (µg/ml)	Difficulties dissolving in Milli-Q water (+ yes)	Available material for testing (Mg)
4	(+)	200-1.56	-0.87	50-6.25		77.95
5	(+)	200-1.56	0.10	50-6.25		85.63
6	(+)	200-1.56	-0.62	50-6.25		55.98
7	-	200-1.56	-0.20	50-6.25		12.75

15	-	N/A	3.85	N/A	+	N/A
16	-	200-1.56	2.35	50-6.25	+	0.38
17	-	200-1.56	3.30	50-6.25	+	0.45
18	-	200-1.56	4.93	50-6.25	+	0.38
19	-	N/A	6.03	N/A	+	N/A
20	-	200-1.56	3.44	50-6.25	+	0.32
21	-	200-1.56	2.74	50-6.25	+	0.23
22	-	N/A	2.76	N/A	+	0.01
23	-	100-0.78	4.30	25-3.13	+	0.07
27	-	200-1.56	1.01	50-6.25	+	0.14
28	-	200-1.56	3.59	50-6.25	+	0.24
29	-	100-0.78	3.48	25-3.13	+	0.14
30	-	200-1.56	0.77	50-6.25	+	0.21
31	-	200-1.56	1.43	50-6.25	+	0.47
32	-	50-0.39	3.10	50-6.25	+	0.11
33	-	76.50-0.59	9.10	50-6.25	+	0.09
34	-	200-1.56	1.17	50-6.25	+	0.18
35	-	N/A	0.29	N/A		N/A
				Combined fraction weight		235.75

8.2.1 MIC bacteria setup concentration

Table A 5 ***Bacterial dilution based on OD600.***

OD_{600}	Amount transferred to 10 ml MH-broth (µl)
0,003-0,010	20
0,010-0,030	10
0,030-0,075	5
0,075-0,100	4
0,100-0,150	3

8.3 Calculations

ORAC standard curves MeOH/TFA Extract

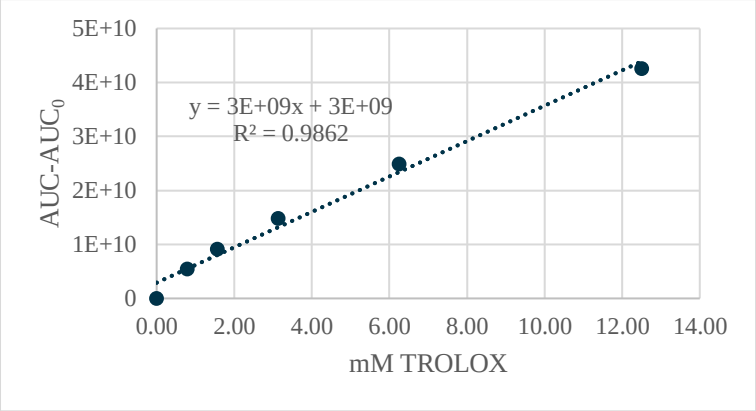

Figure A 1 Standard curve, plate 1 MeOH/TFA fractions.

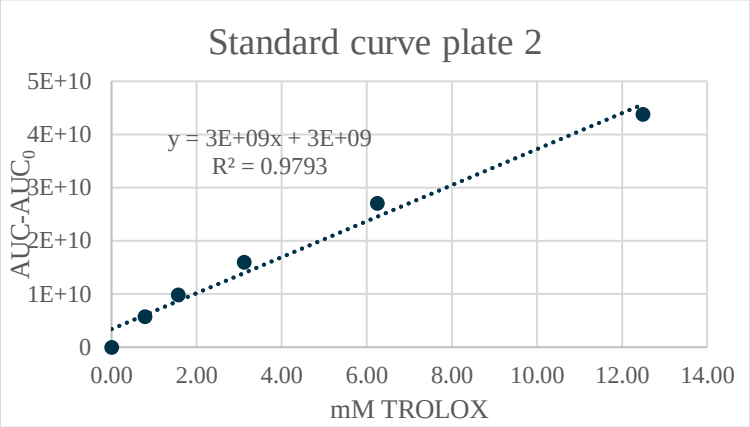

Figure A 2 Standard curve, plate 2 MeOH/TFA fractions.

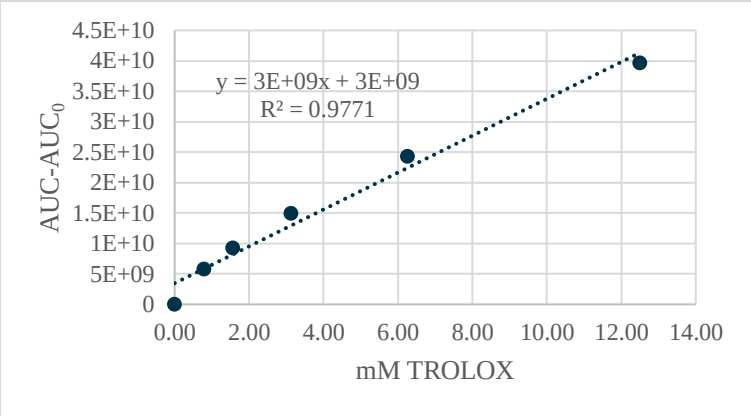

Figure A 3 Standard curve, plate 3 MeOH/TFA fractions

ORAC standard curves Milli-Q extract

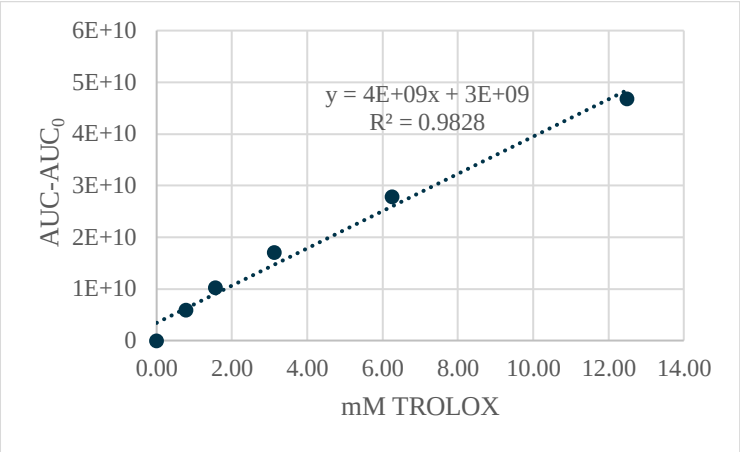

Figure A 4 Standard curve, Plate 1 MilliQ fractions.

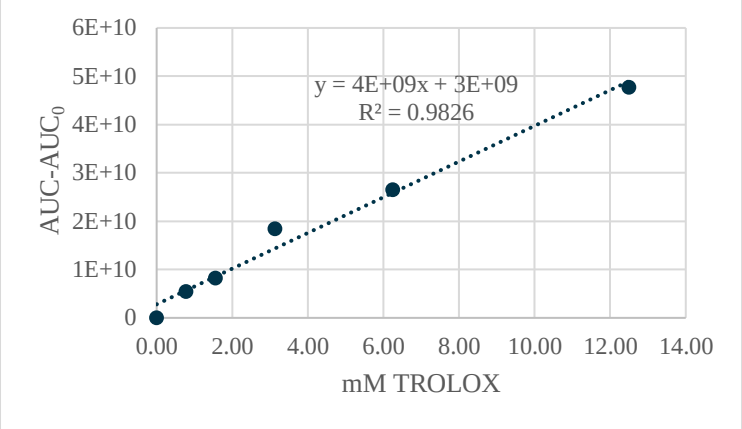

Figure A 5 Standard curve, Plate 2 Milli-Q fractions.

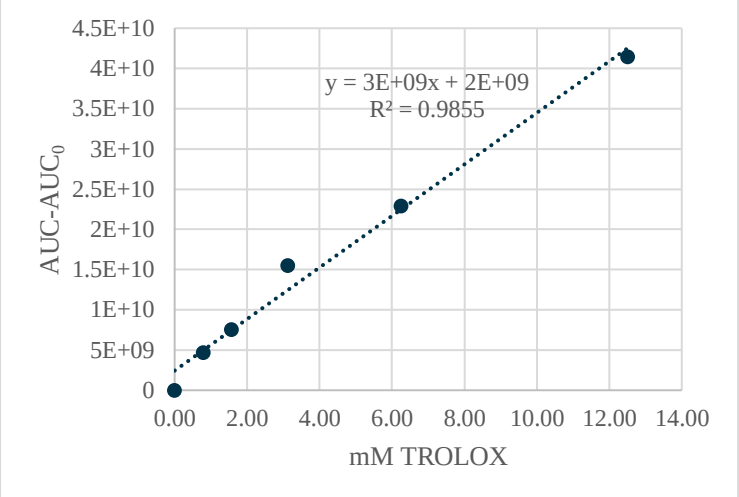

Figure A 6 Standard curve, plate 3 Milli-Q fractions

ORAC calculations

Use normalized data for calculations.

AUC=Area Under Curve

AUC_0=Area Under Curve blank

$$Area\ under\ curve\ netto = sample\ AUC - AUC\ blank$$

Trolox equivalent were calculated by using the linear formula from the standard curves. The formula used was

$$y = ax + b$$

By rearranging the formula, we get

$$x = \frac{y - b}{a}$$

Plot normalized sample values for y.

Here the values for the fractions was set in for y. b and a can be found in the corresponding plate standard curve. Some fractions had a lower amount of material available. They were tested at half the concertation. To compare them to the other fractions, their value was doubled.