



Fungi isolated from Madagascar shrimps - investigation of the *Aspergillus niger* metabolism by combined LC-MS and NMR metabolomics studies

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1. Introduction

In the last few years, Madagascar shrimp cultures especially giant tiger prawns *Penaeus monodon*, have been affected by diseases, impacting one of the main economic resources of Madagascar (Food and Agriculture Organization of the United Nations, 2017). The giant tiger prawn remains the most widely farmed species in the world because of its rapid growth rate, low cost food requirements and ability to reach a larger size when compared with other commonly farmed species (Kenway et al., 2006). In the natural environment, it has been shown that different microorganisms are associated to tiger shrimps such as bacteria (Flegel, 1997; Rungrassamee et al., 2013; Kusumaningrum and Zainuri, 2015) and viruses (Mohr et al., 2015), many of them being detrimental to their growth. One of the affections reaching the livestock is the white spot syndrome (WSS), for which the pathogenic agent has not clearly been identified and is currently assumed to be of viral origin (Corsin et al., 2002; Sparagano et al., 2003; Jeswin et al., 2015). Indeed, viral pathogens such as the White Spot Syndrome Virus (WSSV), are believed to be the major cause responsible for the dramatic worldwide decline in *P. monodon* production since 1997 (Spann and Lester, 1997; Tanticharoen et al., 2008; Flegel, 2012). While mortality usually occurs within 5–10 days, the appearance of white spots over the exoskeleton, pinkish-red body color, lethargy and anorexia are the common clinical signs associated with the WSSV and usually appear 48 h post infection (p.i.). Marked histomorphological changes characteristic of WSSV have been observed in infected tissues (Lightner, 1996; Rajendran et al., 1999; Sahoo et al., 2014), allowing early diagnosis from 18 h p.i. by the observation of the subcuticular ectoderm of eyestalk and pleopod (Rajendran et al., 2005).

While a global effort has been established to understand the pathogen WSSV, the present work investigated the presence of fungi in

infected WSS-tiger shrimps collected at different fisheries along the Madagascar coast. While various fungi have been reported to be associated to *P. monodon* and closely related species (Wu and Salunkhe, 1978; Lightner, 1985; Lightner, 1993; Lavilla-Pitogo et al., 2000; Khoa et al., 2004; Leão et al., 2005; Zaini et al., 2009; Silva et al., 2011; Karthikeyan and Gopalakrishnan, 2014), no study investigated the metabolite production by these strains. This could be of interest as fungal metabolites include toxic compounds, which may impair growth (Karthikeyan et al., 2015) of the tiger shrimp as previously shown in feeding experiments (Wiseman et al., 1982; Boonyaratpalin et al., 2001; Suppamataya et al., 2005; Santos et al., 2010; Gonçalves et al., 2016). On the opposite, specific compounds produced by fungi associated to tiger shrimps may also have a direct immunostimulatory effect as described by Subramanian et al. (2014).

The present work describes the isolation of fungi in tiger shrimps and focuses on one of the isolated strains *Aspergillus niger*, which is a widely distributed filamentous fungus and has been extensively studied (Martins et al., 2005; Nielsen et al., 2009; Frisvad et al., 2011; Gautam et al., 2011). Because fungal metabolite production can be highly variable depending on culture conditions, due to differential biosynthetic pathway regulation (Rédou et al., 2016), the metabolome of this specific strain was investigated on different media. Special attention was given to the metabolites which could be enhanced or repressed when cultured in the shrimp matrix. Indeed, the compounds produced by the fungus could either be toxic to the shrimp, and ultimately pose a threat to human, but they could also be beneficial for the shrimp in order to fight against diseases such as the WSS.

To tackle this question, metabolomics studies were then performed in order to compare metabolic profiles of *Aspergillus niger* isolated from tiger shrimps, when cultivated on different media using the OSMAC approach (Bode et al., 2002), including a shrimp-based medium. The

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latter medium was designed to mimic part of the nutrimental conditions the fungus may experience inside the shrimp.

2. Material and methods

2.1. Fungal strains isolation

Fungi were isolated from shrimps affected by the WSS from the West coast of Madagascar. The samples were collected at two different sites, Morondava and Majunga. The first location is on the southwest coast of Madagascar and the second on the northwest coast with their respective geographical coordinates being 19° 50' 03.74" S, 44° 30' 41.98" E and 18° 46' 1" S, 46° 52' 9" E. These collection sites were chosen based on their resources of both wild and cultured shrimps together with the appearance of the WSS in livestock. For this study, WSSV-positive shrimps were chosen to isolate fungi as they enter into the daily consumption of the local population but also because those collected in the wild environment did not present any symptoms of mortality after one week. Moreover WSSV-negative shrimps could not be found during the sampling campaign, showing how much the disease has impacted Madagascar coast. The shrimp samples were successively washed with Dakin's solution to avoid development of other microorganisms in the isolation medium and prevent contamination of the samples. Fungal strains were isolated from the wild shrimps (carapace and flesh, from head, abdomen, tail and whole body) after incubation for 13 days on a malt agar medium composed of 30 g of malt extract, 15 g of agar, 1 L of sterile water, oxytetracyclin and streptomycin at a concentration of 400 mg/L at 25 °C under natural light.

2.2. Identification of fungi

Genomic DNA was extracted from fresh mycelium grown for 5–10 days depending on the strains. The Qiagen DNeasy Plant Mini Kit (Qiagen, Ltd. Crawley, UK) was used for extraction. PCR was performed in a 25 µL reaction mix, containing 12.5 µL genomic DNA (dilution: 10^{-2} after extraction), 0.125 µL Taq DNA Polymerase (Q-Biogen, 5 units/µL), 5 µL of PCR Direct loading buffer with MgCl₂ (Q-Biogen), 0.5 µL of dNTPs (6.25 mM, dNTP Mix, Q-Biogen), 1 µL of each 10 µM primer (Eurogentec) and 4.875 µL sterile water.

To identify fungi, different DNA barcode markers were used depending on the group of fungi (Schoch et al., 2012): the nuclear ribosomal DNA large subunit for yeasts, the internal transcribed spacer region of the ribosomal DNA for filamentous fungi combined with a secondary marker in taxa showing low ITS interspecific variability such as *Aspergillus* and *Byssoschlamys*, the β -tubulin-coding marker. The oligonucleotide primer sets used in this study were: LROR/LR6 (Vilgalys and Hester, 1990; Vilgalys and Sun, 1994) to amplify a 600 bp portion at the 5' end of the nuclear ribosomal DNA large subunit, ITS4/ITS5 (White et al., 1990) to amplify the internal transcribed spacer region of the ribosomal RNA gene; Bt2a/Bt2b (Glass and Donaldson, 1995) to amplify a part of the 5' end of the β -tubulin.

Amplifications were performed using a BioRad DNA Engine Peltier Thermal cycler with 30 cycles of 30 s at 94 °C, 30 s at either 55 °C (for ITS4/ITS5 or Bt2a/Bt2b) or 50 °C (for LROR/LR6) and 40 s at 72 °C. All PCR programs were followed by a final 5 min extension step at 72 °C. PCR products were purified and sequenced by GenoScreen (Lille, France) in both directions to confirm the accuracy of each sequence. Sequences were assembled with CodonCode Aligner v.3.7.1 (Codon Code Corporation), checked by visual inspection of chromatograms and edited if necessary. Sequences were identified using BLAST option at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Best hits were carefully examined to attribute species names ($\geq 97\%$ of sequence similarity). Sequences have been deposited in the GenBank database under accession numbers from MF084287 to MF084291.

2.3. Culture media for metabolomics analysis

Aspergillus niger strain was grown on seven different culture media prepared with 15 g/L of agar in sterilized natural seawater (salinity of 32.8 g/L) containing additionally: 5 g/L Czapek extract, 5 g/L yeast extract and 30 g/L saccharose for Czapek Yeast extract Agar medium (CYA); 40 g/L glucose and 10 g/L casein enzymatic digest for Dextrose Casein Agar medium (DCA); 5 mg/L CuSO₄, 10 mg/L ZnSO₄, 1 g/L peptone, 20 g/L malt extract and 20 g/L glucose for Malt Extract Agar medium (MEA); 5 mg/L CuSO₄, 10 mg/L ZnSO₄, 4 g/L potato extract and 20 g/L glucose for Potato Dextrose Agar medium (PDA); 5 mg/L CuSO₄, 10 mg/L ZnSO₄, 0.5 g/L MgSO₄, 20 g/L yeast extract and 150 g/L saccharose for Yeast Extract Sucrose medium (YES); 2,4 g/L MgSO₄, 2,4 g/L NH₄NO₃, 1,21 g/L Tris buffer and 5 g/L saccharose for Kohlmeyer Solid Medium (KMS). Shrimp Extract Saccharose (SES) medium was replacing the 20 g/L of yeast extract of the YES medium by 20 g/L of a freshly prepared shrimp extract. The shrimp extract was obtained by crushing fresh *Penaeus monodon* with 900 mL sterile water, which was and centrifugated at 3500g, at 4 °C for 10 min. The supernatant was filtered (50 µm; Sartorius, Göttingen, Germany) and lyophilized. The dried extract obtained was ground in order to obtain a homogeneous powder.

2.4. Culture of fungi and extract preparation

Culture of fungi was carried out in triplicates for each medium in Erlenmeyer flasks containing 50 mL of solid medium; cultures were incubated at 27 °C for 12 days (until complete covering of the medium) under natural light. Fungal mycelium was extracted together with the agar layer, in order to obtain both intra- and extracellular compounds. The cultures were extracted twice with 100 mL of dichloromethane/ethyl acetate 1:1 (v/v). Mixtures were then sonicated for 30 min and macerated overnight. After filtration on Büchner funnel, the organic phases were combined and dehydrated on anhydrous sodium sulfate. Spores were removed by filtration on regenerated cellulose 0.45 µm filters (Sartorius, Göttingen, Germany). Organic phases were evaporated to dryness leading to crude extracts. Media without inoculated fungi were also extracted following the same protocol, to be used as controls (blank samples).

2.5. HPLC-HRMS analyses

The crude extracts were analyzed at 1 mg/mL in UPLC grade MeOH, following a 2 µL injection on a UPLC-HRESIMS (IT-TOFMS) Shimadzu instrument (Prominence Ultra Fast Liquid Chromatography coupled to High Resolution Electrospray Ionization Mass Spectrometry combining Ion trap and Time of Flight analyzers). Blank samples with UPLC grade MeOH were also injected randomly during the analysis sequences. Elution was performed on a Kinetex™ C18 column (100 × 2.1 mm, 2.6 µm) maintained at 40 °C using the following gradient: 15% ACN (+ 0.1% FA) for 2 min; from 15% ACN (+ 0.1% FA) to 100% ACN (+ 0.1% FA) in 23 min; hold at 100% ACN (+ 0.1% FA) for 5 min; and back to the initial conditions and equilibration for the last 5 min. The detection using ESI was performed both in positive and negative modes with a coupled ion trap and TOFMS analyzer in the mass range m/z 100–1000 with a mass accuracy of 7 ppm and a resolution of 10,000 at m/z 500. MS² acquisition was added in the protocol in the positive mode for a selection of samples to improve compound identification.

2.6. ¹H NMR spectroscopic analyses

The extracts (20 mg) were dissolved in 600 µL of dimethyl sulfoxide-*d*₆ (Eurisotop, France) and the solution was then transferred into a 5-mm diameter NMR tube. High resolution NMR experiments were performed on a Bruker Avance III 500 spectrometer (Bruker, Karlsruhe, Germany), operating at 500.13 MHz and equipped with a dual ¹H/¹³C

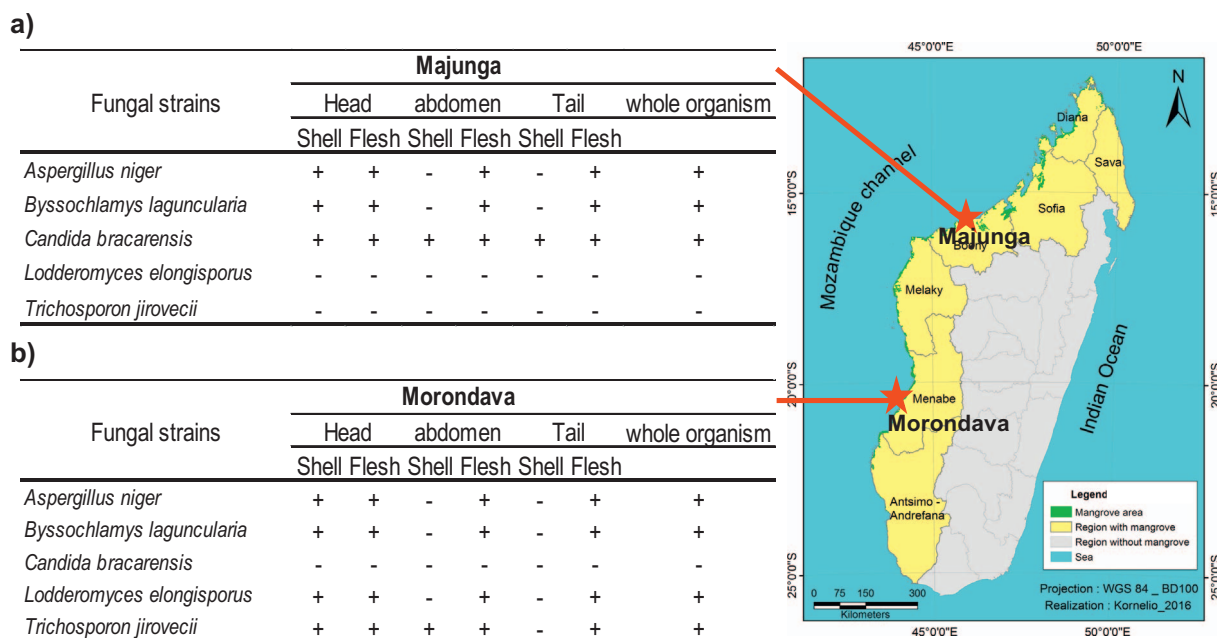


Fig. 1. Identified fungal species in *Penaeus monodon* (in the different body compartments) in relation to their collection sites: a) Majunga and b) Morondava.

cryogenic probe. ^1H NMR experiments were acquired at 298 K using a conventional 1D experiment with water presaturation in F2 (zgpg30). For the acquisition, 64 K data points over a spectral width of 18 ppm (9000 Hz) and 128 scans were collected. Data were processed using the Bruker TopSpin software 2.1 with one level of zero-filling and Fourier transformation after multiplying the FIDs by an exponential line-broadening function of 0.3 Hz.

2.7. Data treatment

For global analysis, raw LC-MS files were converted to netCDF (Kessner et al., 2008). Automatic peak detection and integration were performed using software R (version 3.0.2) (R Core Team, 2015) with XCMS package (version 1.38.0) (Smith et al., 2006). The script R was developed in MMS laboratory and generates a matrix with two entries (variables/samples). The process is based on several steps: peak picking for each sample, peak grouping (with m/z and R_t), retention time alignment and missing data completion with the “fillPeaks” function. The parameters for XCMS were as follows: find peak method using centWave, $mzdiff$ set to 0.05, signal-to-noise ratio threshold to 3, $mzwid$ to 0.05 and ppm to 50. The profmethod used was binlinbase as it was demonstrated to be best suited for data generated by this LC-MS method (Roullier et al., 2016). The pcgroups were generated by CAMERA (version 1.18.0) R package (Kuhl et al., 2012) and the extracted ion chromatograms were manually inspected and filtered (isotopes removal). The resulting data matrix was then used for multivariate statistics. Principal Component Analysis (PCA) was applied to describe patterns in the data with Pareto scaling using the muma package. A total of 21 extracts (3 replicates per medium) were then analyzed. The PCA was performed on the data acquired in ESI^+ mode considering the totality of the extracted MS signals with all features detected in blank samples removed leading to a total of 966 features.

^1H NMR spectra were transferred to the AMIX software (Bruker). The bin area method was used to segment the spectra between 0 and 15.6 ppm using a fixed bucketing size of 0.01 ppm. 1560 variables were generated with one signal (or noise) per bucket. The bins corresponding to the signals of HOD (3.54–3.55 ppm) and DMSO (2.44–2.60 ppm) resonances were excluded. A manual filtering procedure was applied to the whole spectrum to exclude buckets which contained only noise. A total of 1327 variables were thus obtained. Bin areas were integrated to

generate a matrix consisting of rows representing the 28 samples and columns representing the 1327 variables. The integrated regions were normalized by dividing each area by the total area of the spectrum. In order to get rid of the signals arising from the culture medium, the statistical analyses were carried out on the 8.4–15.6 ppm region only. A total of 504 variables was thus retained for the subsequent statistical analyses. First, an in-house package developed in the R environment (Balayssac et al., 2013) was used to locate potential outlier samples or metabolites with atypical variations through a univariate approach based on the analysis of variance for each variable. Discriminating variables were identified through a supervised univariate approach with t -tests and boxplots. After mean-centering and auto-scaling, the data matrix was subjected to several multivariate statistical analyses using SIMCA-P + 12.0 software (Umetrics, Umeå, Sweden).

2.8. Compounds identification

Major compounds on LC-MS and NMR profiles were identified using accurate mass, isotopic pattern, adducts prediction, UV spectra and ^1H NMR characteristic signals in $\text{DMSO}-d_6$. Comparison to databases such as the online dictionary of natural products (DNP 25.2 Online Copyright © 2016, <http://dnp.chemnetbase.com>) and published data allowed high level of confidence in the annotations performed (see Supplementary information).

Reported mycotoxins from *A. niger* were searched in the crude extracts obtained from the shrimp-derived *A. niger* strain, by systematically tracing LC-MS extracted ion chromatograms from twelve possible adducts/fragments either in positive and negative modes: $[\text{M} - \text{H}_2\text{O} + \text{H}]^+$, $[\text{M} + \text{H}]^+$, $[\text{M} + \text{NH}_4]^+$, $[\text{M} + \text{Na}]^+$, $[\text{M} + \text{K}]^+$, $[\text{M} + \text{CH}_3\text{CN} + \text{H}]^+$, $[2\text{M} + \text{H}]^+$, $[\text{M} - \text{H}_2\text{O} - \text{H}]^-$, $[\text{M} - \text{H}]^-$, $[\text{M} + \text{Na} - 2\text{H}]^-$, $[\text{M} + \text{FA} - \text{H}]^-$, $[\text{M} + \text{TFA} - \text{H}]^-$. When a peak occurred for one or several searched adducts, it was carefully compared with expected accurate mass and isotopic profile. Co-eluting ions were also inspected and only when all these data matched with one regulated toxin, it was considered as “potentially present” in the extracts. When signal intensities of searched ions were lower than 5000 units and/or no typical Gaussian-type profile peak was observed, the corresponding toxin was considered “not detected”.

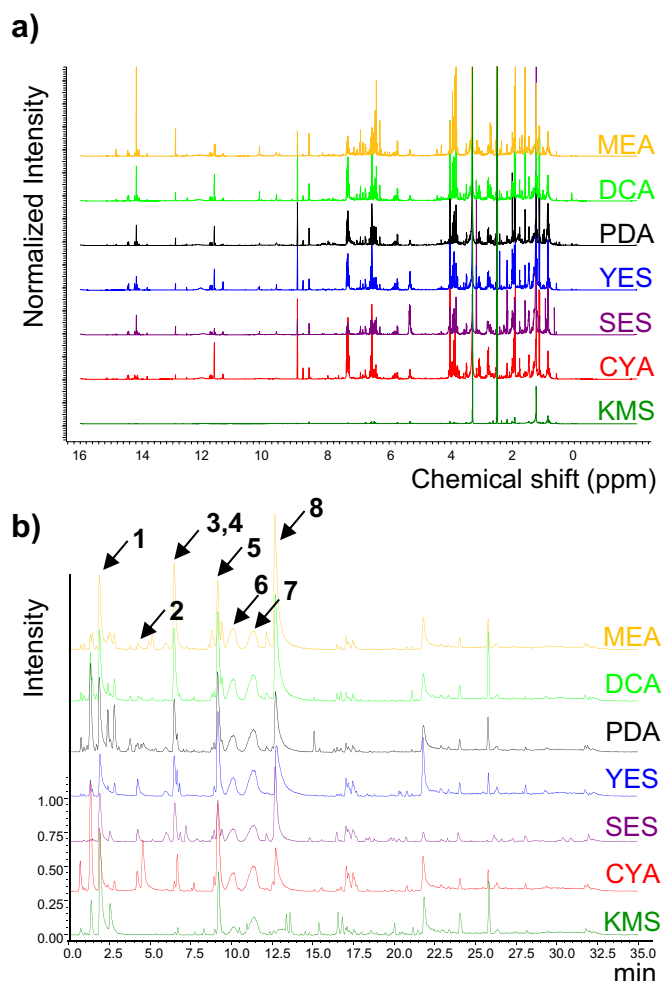


Fig. 2. Metabolic profiles acquired by a) ^1H NMR and b) LC-MS of samples from the *Aspergillus niger* fungal strain isolated from *Penaeus monodon* in relation to culture media. The samples corresponded to the crude extracts obtained from the different culture media: MEA (Malt Extract Agar), DCA (Dextrose Casein agar), PDA (Potato Dextrose Agar), YES (Yeast Extract Sucrose), SES (Shrimp Extract Sucrose), CYA (Czapek Yeast extract Agar) and KMS (Kohlmeyer Solid Medium). Peaks highlighted with an arrow correspond to the compounds (1–8) identified.

3. Results and discussion

3.1. Identification of fungal strains associated to *Penaeus monodon*

Fungi were isolated from tiger shrimps displaying the WSS on their shell typical of an infection by the virus *Nimaviridae* (Tang et al., 2007). No necrotic symptoms indicative of fungal infection could be observed. Microscopic observation and molecular sequencing were used to identify the associated fungal strains isolated from these shrimps.

Five species were identified: *Aspergillus niger*, *Byssoschlamys lagunculariae*, *Candida bracarensis*, *Lodderomyces elongisporus* and *Trichosporon jirovecii* (Fig. 1.) Among these strains, none had been previously isolated from crustaceans. Only close species of *Aspergillus* had been identified, namely *Aspergillus awamori* from *Litopenaeus vannamei* (Karthikeyan et al., 2015) and *Aspergillus flavus* from *Penaeus vannamei* (Sun et al., 2014).

Overall, fungi were more frequently observed from the flesh than the shell. *Aspergillus niger* and *B. lagunculariae* were common to both studied sites. *Lodderomyces elongisporus* and *T. jirovecii* appeared to be specific to Morondava as they were not found in Majunga. On the contrary, *C. bracarensis* appeared to be specific to Majunga, which is more populated than Morondava. Many hypotheses could be raised to explain these site-specific observations. As Majunga receives more

pollution, this could have an impact on fungal survival but also shrimps in this location could be less healthy than in Morondava and then be more sensitive to fungal colonization by *Candida* species.

The role of the fungi isolated from the flesh of the tiger shrimps has long been debated. As the studied shrimps presented the WSS on their shell but were still alive with no necrotic symptom, could these fungi help for shrimp survival or represent a risk for further infection? Moreover, most of these fungi had already been reported to produce different types of metabolites, some of which presenting toxicity.

Among the different fungal strains identified in *P. monodon*, *A. niger* represents one of the most important food and feed contaminant, with the production of mycotoxins (Nielsen et al., 2009). These mycotoxins are generally toxic to animals and humans (Gautam et al., 2011). The most problematic secondary metabolites known to be produced by this species are aflatoxins, ochratoxins and fumonisins (Abarca et al., 1994; Noonim et al., 2009; Palencia et al., 2010). Concerning *B. lagunculariae*, a heat-resistant strain has also been reported to produce toxic secondary metabolites (Samson et al., 2009) such as byssoschlamic acid (Rice et al., 1977), which was also described from the closely related species *B. fulva* (Raistrick and Smith, 1933), and byssotoxin A (Kramer et al., 1976). Furthermore, other secondary metabolites produced by the genus *Byssoschlamys* include mycophenolic acid (Puel et al., 2005), the carcinogen patulin (Escoula, 1975), and byssoschlamysol, a cytotoxic steroid active on IGF-1 dependent cancer cells (Mori et al., 2003). *Candida bracarensis* is a recently described species which is phenotypically and biochemically similar to *C. glabrata* (Warren et al., 2010). The genus *Candida* includes some of the most common human pathogens (Ahearn, 1998). The other yeasts isolated included *Lodderomyces elongisporus* (Van Der Walt, 1966) a species closely related to *Candida parapsilosis* (Lockhart et al., 2008), and *Trichosporon jirovecii*, which was first described based on a strain isolated from human nails by Fragner in 1969. The three latter species found in *P. monodon*, namely *C. bracarensis*, *L. elongisporus* and *T. jirovecii* have not been reported to cause infections in crustaceans nor to produce toxic compounds so far.

3.2. Metabolism investigation of the *Aspergillus niger* strain isolated from *Penaeus monodon*

As *A. niger* was here isolated from the tiger shrimp for the first time and as it is known to produce a vast array of compounds including toxins, a further study on the metabolism of this specific species isolated from *P. monodon* was performed. Many studies clearly had demonstrated that microbial metabolism is highly variable and that biosynthetic gene clusters implied in the production of compounds sometimes needed specific nutritional conditions to be expressed (Reen et al., 2015; Rédou et al., 2016). In order to assess the spectrum of metabolites the *A. niger* strain isolated from *P. monodon* is able to produce, it was cultured on different media following the OSMAC approach (One Strain Many Compounds) (Bode et al., 2002). This represents a powerful tool to elucidate the secondary metabolome of microbes (the overall number of all secondary metabolites from one organism). Moreover, the shrimp extract medium tried to mimic the environment in which the organism lives. The effect of the media on metabolite production was then investigated by a metabolomics study involving both LC-MS and NMR data obtained from culture extracts (Fig. 2). Metabolomics allows a better understanding of systems biology by highlighting important variations between samples based on their global metabolite production. To the best of our knowledge, no studies had investigated OSMAC approaches on *A. niger* in combination with metabolomics by LC-MS and NMR.

On principal component analysis (PCA) score plots obtained from ^1H NMR (Fig. 3a) and LC-MS (Fig. 3b) data, replicates of cultures were very close indicating that the fungus behaved similarly on each medium. This homogeneity of the replicates allowed to further interpret the results. Both types of analyses were consistent with differential metabolite production depending on culture media. In all cases, on the

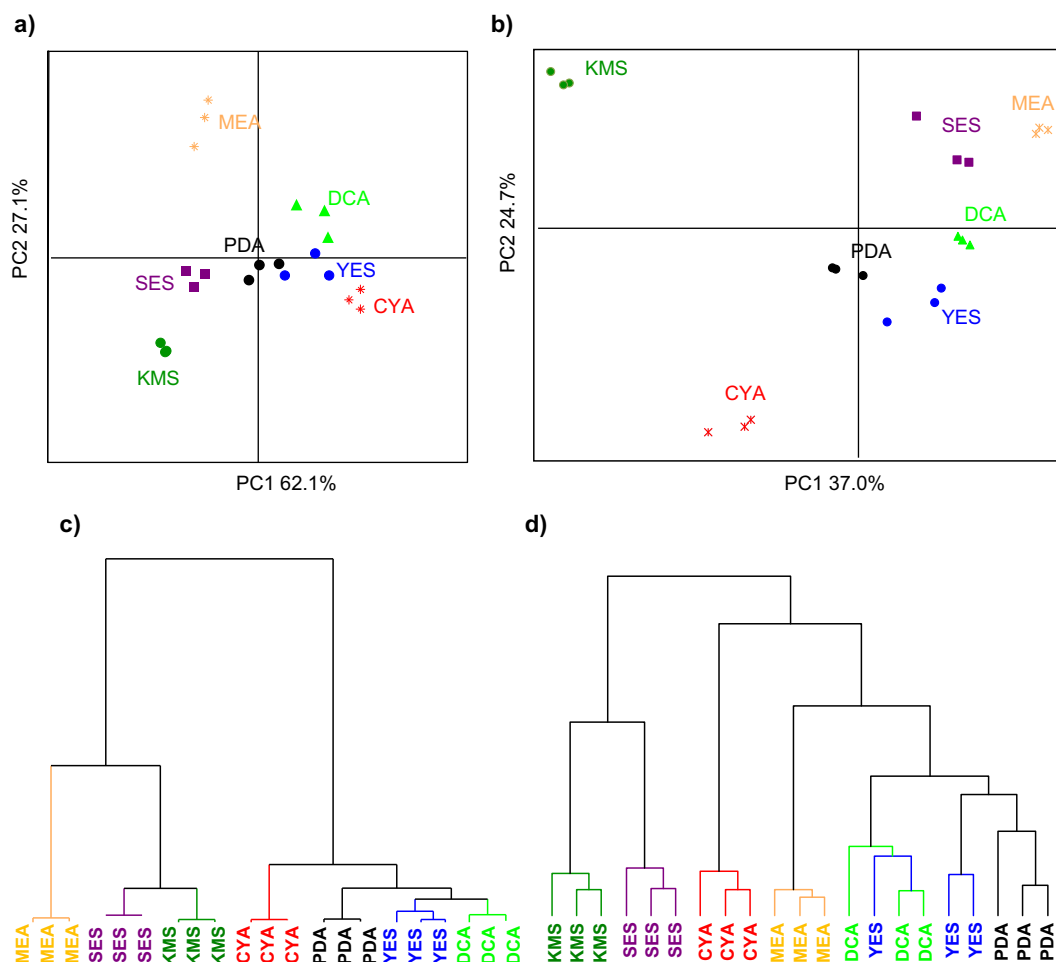


Fig. 3. Metabolomics study of the *Aspergillus niger* fungal strain isolated from *Penaeus monodon* in relation to culture media, following the OSMAC approach. Score plots on Principal Component Analyses (PCA) of the different samples based on a) ^1H NMR and b) LC-MS data together with corresponding hierarchical clustering c) and d) are here represented. The data used corresponded to the profiles obtained by these two methods on the crude extracts obtained from the different culture media: MEA, DCA, PDA, YES, SES, CYA and KMS.

graphs, four media (DCA, PDA, SES, YES) are grouped near the center (with a rather low dispersion) and three media (CYA, KMS, MEA) are somewhat further away. This means that the KMS, MEA and CYA media induce metabolite productions that are significantly more contrasted compared to other culture media. It can also be noted that MEA and CYA are diametrically opposed, meaning a negative correlation between the two samples. Some variables (and therefore metabolites) strongly expressed in one are less expressed in the other and vice versa. The KMS medium appeared to induce significantly different metabolic profiles in *A. niger* samples, especially on LC-MS data. This could be explained by the low nutrient content of this medium leading to slow growth of the fungus when compared with other media. Hierarchical clustering of the samples obtained from ^1H NMR (Fig. 3c) and LC-MS (Fig. 3d) data confirmed this separation between the media, with SES being even more excluded. It mainly distinguished metabolic profiles arising from three media (DCA, PDA, YES) to each of the others (SES, KMS, CYA, MEA). Data from both LC-MS and NMR led to similar conclusions, the main difference being a different clustering for extracts from CYA and MEA media. By NMR, CYA extract resembled to PDA, YES and DCA extracts, while LC-MS revealed differences in profiles. On the contrary, MEA extract better clustered with PDA, YES and DCA extracts by LC-MS, while by NMR, they were distant. However, this approach highlighted the fact that the medium enriched with shrimp extract (SES) induced the production of different metabolites in the investigated strain.

In-depth analysis of the PCA loadings plot, representing the contribution of the different variables in the construction of the PCA model,

highlighted some important features (see Supplementary information S-1), most of them corresponding to the major compounds of the extracts. For this reason, to better understand the metabolic production of this shrimp-sourced *Aspergillus* strain, a focus was given on the major compounds produced on the different media investigated. The analysis of the 21 crude extracts led to the identification of eight major compounds (Fig. 4). High resolution mass spectral data allowed molecular formula prediction based on mass accuracy, isotopic profiles and adducts or fragments annotations. After a search in the natural products database, the different proposed compounds were discriminated based on corresponding ^1H NMR signals and UV spectra (see Supplementary information S-2).

Pyranonigrins A-C (1–2) were first reported from an EtOAc extract of a static culture of *A. niger* isolated from the Mediterranean sponge *Axinella damicornis* (Hiort et al., 2004). Pyranonigrin A, later isolated in 2007 from another marine strain of *A. niger* (LL-LV3020) originating from mangrove wood, had its structure reassigned (Schlingmann et al., 2007). Pyranonigrins B and C are close analogues, with the opposite arrangement of the respective substituents at C-2 and C-3, making them very difficult to differentiate based on mass spectrometry or UV/visible absorption. An antioxidant activity has been described for pyranonigrin A (Miyake et al., 2007) and for all three compounds no toxicity against brine shrimp nor human leukemia and carcinoma cells was detected by Hiort et al. (2004). Fonsecin (3) is an isomer of rubrofusarin (Ashley et al., 1937), a naphtho- γ -pyrone compound. It is a yellow pigment which was produced by an ultraviolet mutant of the fungus *A. fonscaeus* (Galmarini et al., 1962). It displayed interesting inhibition of IL-4

a)

Rt	m/z	Adducts /Fragments	UV ($\lambda_{\max}$)	Formula	Name	NMR signals
2.0	224.0542	$[M+H]^+$	208 249 314 434 442	C ₁₀ H ₁₉ NO ₅	Pyranonigrin A (1)	yes
4.6	254.0654	$[M+H]^+$	244 330 459	C ₁₁ H ₁₁ NO ₆	Pyranonigrin B/C (2) too low	
	276.0479	$[M+Na]^+$				
6.6	291.0866	$[M+H]^+$	231 275 322 334 404	C ₁₆ H ₁₅ O ₅	Fonsecin (3)	yes
	313.0677	$[M+Na]^+$				
6.7	230.0811	$[M+H]^+$	246 313 476	C ₁₃ H ₁₁ NO ₃	Carbonarone A (4)	yes
	252.0609	$[M+Na]^+$				
9.2	334.1137	$[M+H]^+$	208 445	C ₁₈ H ₁₇ NO ₆	Pestalamide A (5)	yes
	366.0938	$[M+Na]^+$				
10.1	593.1648	$[M+H]^+$	234 283 326 409	C ₃₁ H ₂₈ O ₁₂	Aurasperone C (6)	yes
11.4	607.1908	$[M+H]^+$	235 283 325 410	C ₃₂ H ₃₀ O ₁₂	Aurasperone B (7)	too low
12.8	287.0915	$[M+H]^+$	240 280 373 487	C ₁₆ H ₁₄ O ₅	Flavasperone (8)	yes
	272.0690	$[M-CH_3+H]^+$				

b)

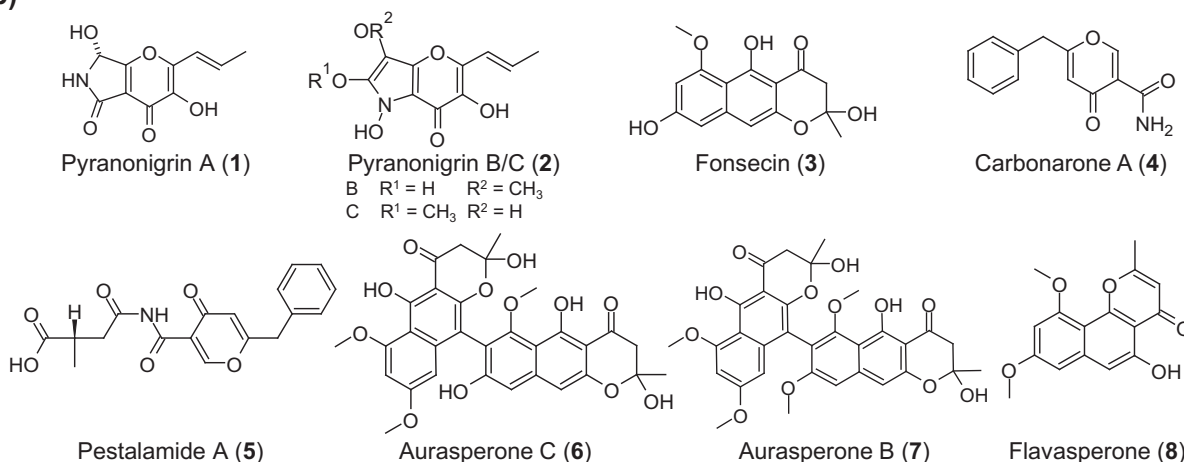


Fig. 4. a) Compounds identified in the shrimp-sourced *Aspergillus niger* crude extracts based on MS, NMR and UV data together with their corresponding structures b).

signal transduction with an IC₅₀ of 30 μ M, and has thus been claimed as a potential useful compound in the treatment of allergic disease (Sakurai et al., 2002). Carbonarone A (4) corresponding to the revised structure of tensidol A (Fukuda et al., 2006; Henrikson et al., 2011) is a secondary metabolite isolated from both *A. niger* and *A. carbonarius* (Zhang et al., 2007). This compound has been reported to potentiate miconazole activity against *Candida albicans* in a dose-dependent manner (Fukuda et al., 2006) and to display very moderate cytotoxic activity against K562 cell line (IC₅₀ 56 μ g/mL) (Zhang et al., 2007) but it had no activity on HIV-1, *Candida albicans*, *Geotrichum candidum* or *Aspergillus fumigatus* (Ding et al., 2008). Pestalamide A (5) corresponding to the revised structure of tensidol B (Fukuda et al., 2006; Henrikson et al., 2011), was first isolated from cultures of *A. niger* and named after its isolation from the phytopathogenic fungus *Pestalotiopsis theae* (Ding et al., 2008). This compound displayed potentiation of miconazole antifungal activity on *C. albicans* (Fukuda et al., 2006), inhibitory effects on HIV-1 replication in C8166 cells (EC₅₀ 64.2 μ M) and a very potent antifungal activity against *A. fumigatus* (IC₅₀/MIC values of 1.50/57.8 μ M) (Ding et al., 2008). Aurasperone B (7) and C (6) are asperpyrone-type bis-naphtho- γ -pyrones. They are yellow pigments initially isolated from the mycelia of *A. niger* and *A. awamori* (Tanaka et al., 1966; Tanaka et al., 1972). Aurasperone C corresponds to the monodemethylated aurasperone B. Aurasperone B has recently been shown to display potent radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), with IC₅₀ values of 0.01 μ M, which

was more potent than the positive control, ascorbic acid (IC₅₀, 20.0 μ M) (Leutou et al., 2016). Flavasperone (8) (“Asperxanthone”) is a naphtho- γ -pyrone monomer, and corresponds to a bright-yellow metabolite produced in small yield by various strains of *A. niger*. It was first isolated by Lund et al. (1953) from the dried mycelium of *A. niger* or *A. fonsecaeus* with its structure assigned later by Bycroft et al. (1962). This compound was reported as a selective acyl-CoA cholesterol acyltransferase isoenzyme 2 (ACAT2) inhibitor (Sakai et al., 2008). It was shown to have no obvious antiproliferative activity (IC₅₀ > 100 μ M) against A549, MGC-803 and HL-60 (Li et al., 2016), MCF-7, MDA-MB-435, Huh7, SNB 19 cell lines and very weak cytotoxicity (50 μ M < IC₅₀ > 100 μ M) against Hep3B and U87MG cell lines (Huang et al., 2011).

While naphtho- γ -pyrones have been claimed to be mycotoxins with marked central nervous system depressant effects (Ghosal et al., 1979), this group of compounds, often encountered in filamentous fungi (*Aspergillus* and *Fusarium* spp.) but also in plants and echinoderms, is now considered of an industrial interest with its broad range of biological activities (Choque et al., 2015) including potent antibiotic effects against drug-resistant pathogens (He et al., 2016).

These eight compounds were detected in all used media (Fig. 5), except pyranonigrin B/C (2), which displayed very low intensities in SES and DCA media. Other differences were observed in terms of intensities on different culture media. The majority of compounds were present in low amounts in the crude extracts obtained from KMS, which

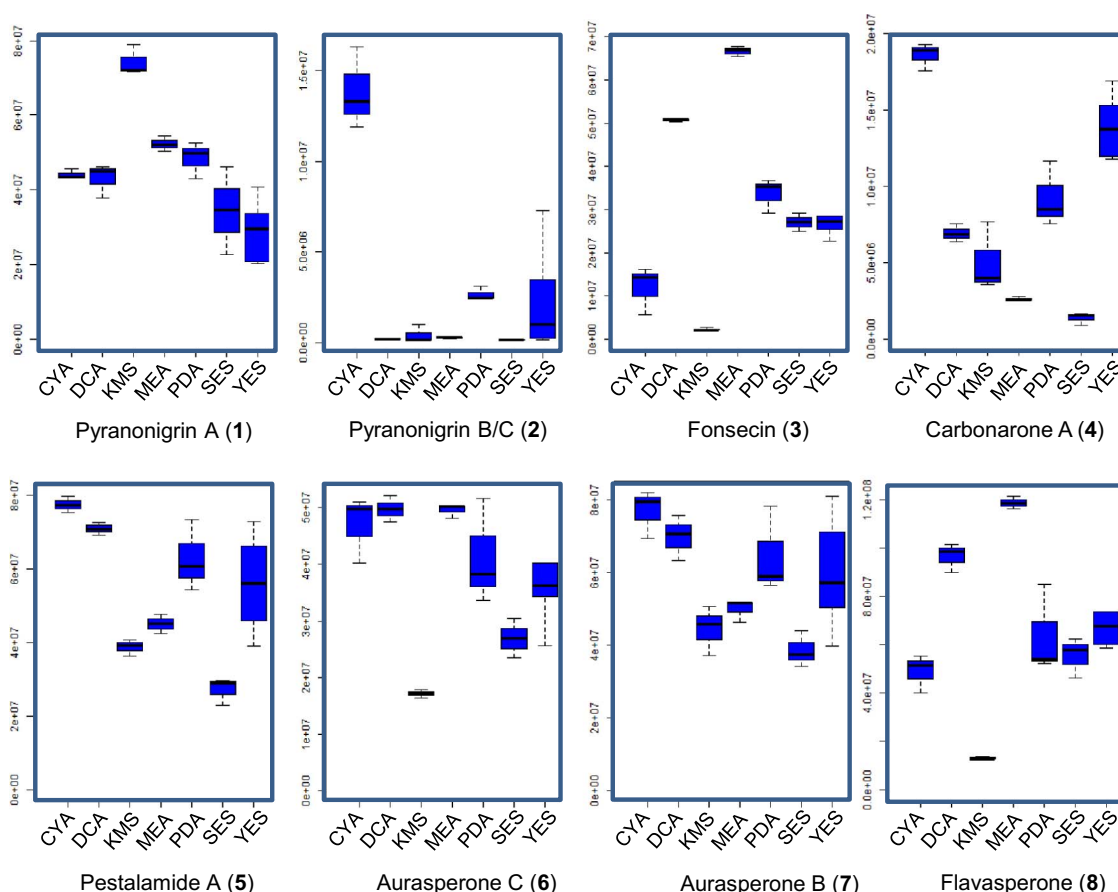


Fig. 5. Boxplots representing the intensity of the eight identified compounds depending on culture media: MEA, DCA, PDA, YES, SES, CYA and KMS.

confirmed the low secondary metabolite production observed on this medium. However, interestingly, pyranonigrin A (1) was overexpressed in this medium compared to the others, suggesting its biosynthetic pathway is favored on this medium. CYA was an interesting medium for chemodiversity as it allowed the production of most compounds (5 out of 8). SES did not much induce the expression of any of the 8 major compounds observed.

As mentioned earlier, *A. niger* is a very productive strain of mycotoxins, which can be toxic to animals and humans. In contrast, aspergilli from marine sources are also interesting producers of new compounds with potential bioactivity (Rateb and Ebel, 2011; Bugni and Ireland, 2004), in addition to toxic secondary metabolites (Abarca et al., 1994; Samson et al., 2001; Schuster et al., 2002; Al-Abdalall, 2009; Noonim et al., 2009). During this study, all acquired chromatograms were systematically explored to find ions, which could correspond to mycotoxins reported from *A. niger* (see Supplementary information S-3). Among the searched mycotoxins only sterigmatocystin was annotated as potentially present. While this was not the primary purpose of this study, further investigations specifically quantifying toxins in *A. niger* infected shrimps together with biological assays would be necessary to further confirm these shrimps are safe for consumers. A comparison with normal shrimps would also be interesting to carry out.

Last but not least, although many *A. niger* metabolites have been investigated in the past, some of the peaks displaying important variability between the different media could not be identified because no hit was found in natural product databases. Putatively unknown metabolites were then produced by *A. niger* and these will need further investigation.

4. Conclusion

In this report, fungal strains were isolated from *P. monodon* for the first time. The study of the chemical behavior of the isolated *A. niger* strain using the OSMAC approach revealed global differential expression of metabolites. Eight compounds were identified with none reported as toxin so far. Additionally, the medium enriched with *P. monodon* extract did not induce the expression of any of these 8 molecules. However, sterigmatocystin was annotated in some samples, notably in the SES medium. While medium SES was designed to mimic the shrimp matrix in laboratory conditions, transposition to the conditions in cultured or wild shrimps remains difficult. Moreover some features could not be assigned and would need further investigation to identify their structure. Further studies would then be necessary to determine if these metabolites are effectively produced in situ and to investigate their potential roles on WSS agents, whether they are beneficial or detrimental to the shrimp. Finally, as some metabolites could not be identified, this study also suggests that *A. niger* may produce interesting secondary metabolites, which could be of interest for drug discovery.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.aquaculture.2017.07.015>.

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Supplementary information for:

Fungi isolated from Madagascar shrimps - investigation of the *Aspergillus niger* metabolism by combined LC-MS and NMR metabolomics studies

Maherizo Gedice Fernand ^a, Catherine Roullier ^{b,c,*}, Yann Guitton ^d, Julie Lalande ^e, Sandrine Lacoste ^f, Joëlle Dupont ^f, Nicolas Ruiz ^b, Yves François Pouchus ^b, Christian Raheriniaina ^a, Ranaivoson Eulalie ^a

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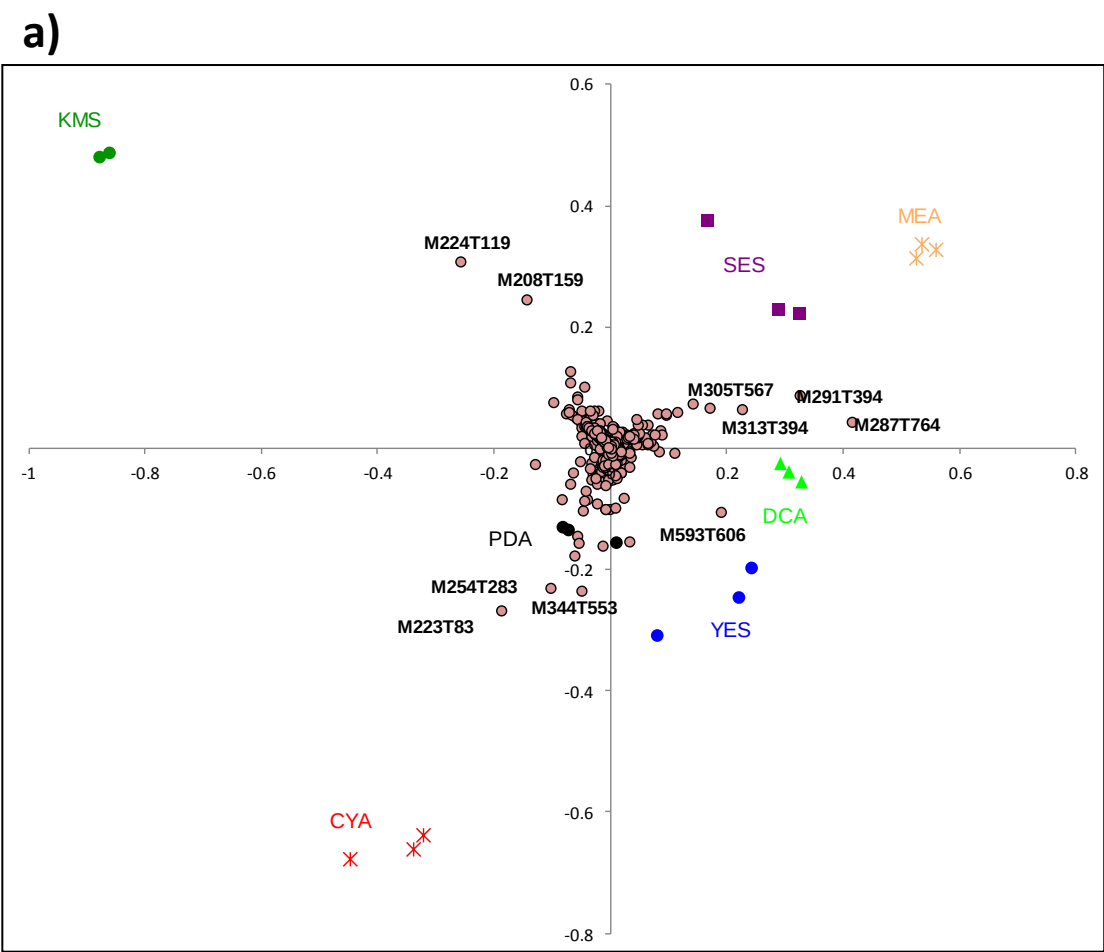
^c ThalassOMICS, Plateforme CORSAIRE- Biogenouest, 44000 Nantes, France

^d Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), Oniris, 44000 Nantes, France

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S-1: a) Biplot of scores and loadings according to the first two components of the PCA performed on LC-MS data of the crude extracts of *Aspergillus niger* isolated from *P. monodon* obtained on different culture media. Features deviating from the center and thus strongly contributing to the construction of components 1 and 2, thus explaining the dispersion of the samples in this space, are annotated on the graph as *m/z* (M) - retention time (T in s) ; b) their intensity values in the various crude extracts depending on culture media (CYA, DCA, KMS, MEA, PDA, SES, YES) are given and colour-coded from green (less intense) to red (most intense) :



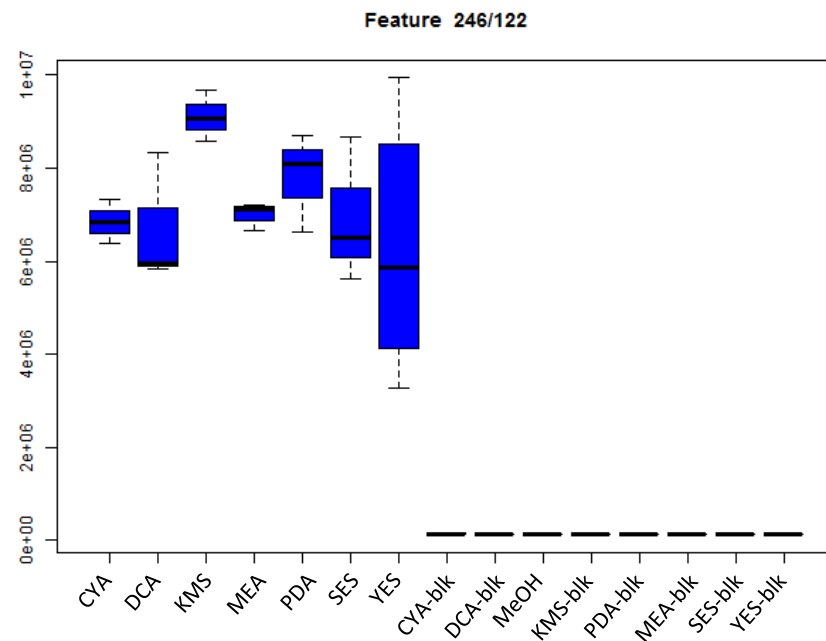
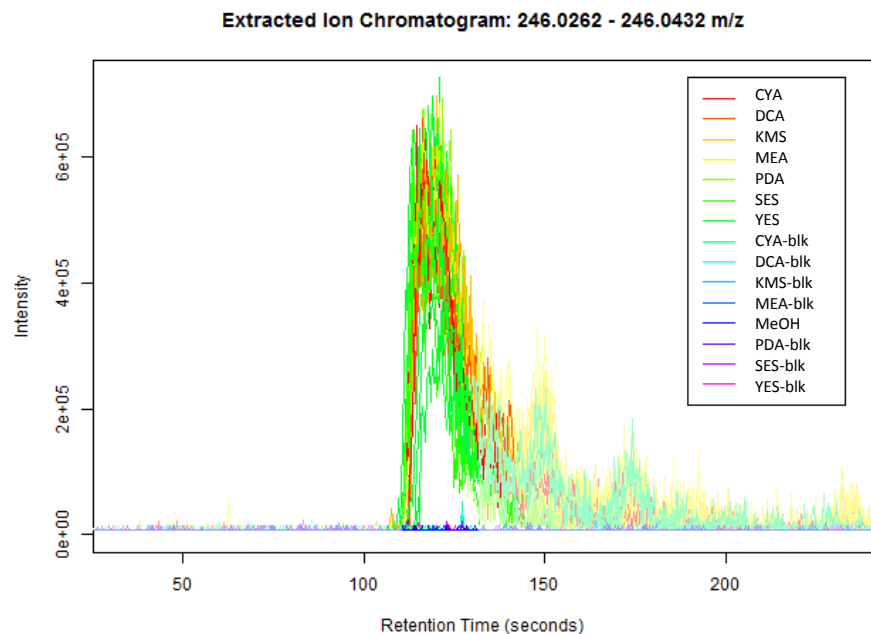
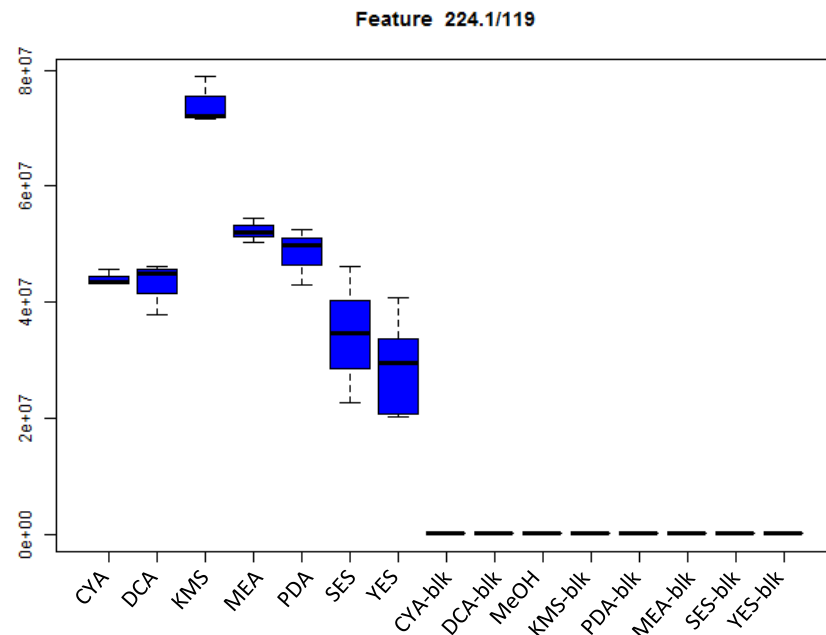
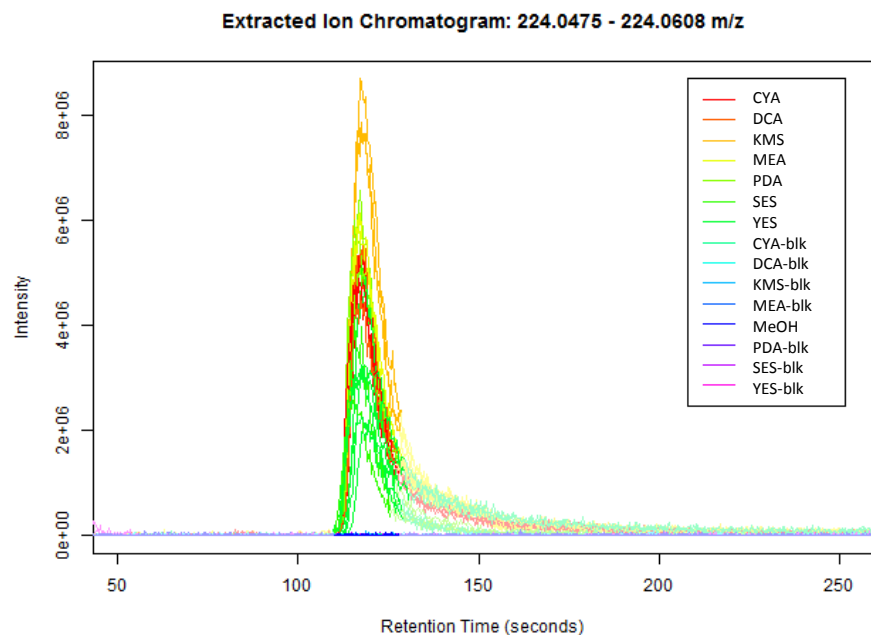
b)

	CYA	DCA	KMS	MEA	PDA	SES	YES
M224T119	4.41E+07	4.30E+07	7.41E+07	5.23E+07	4.84E+07	3.44E+07	2.82E+07
M208T159	2.26E+06	8.45E+06	2.63E+07	1.61E+07	1.00E+07	7.46E+06	4.45E+06
M223T83	7.21E+07	2.70E+07	2.05E+07	9.76E+06	5.15E+07	1.31E+06	7.96E+06
M254T283	3.89E+07	8.32E+05	2.85E+06	2.70E+06	8.42E+06	2.61E+05	5.57E+06
M344T553	7.75E+07	7.09E+07	3.87E+07	4.51E+07	6.28E+07	2.71E+07	5.95E+07
M593T606	4.70E+07	4.97E+07	1.72E+07	4.95E+07	4.12E+07	2.69E+07	3.73E+07
M313T394	3.95E+06	2.42E+07	2.90E+05	2.90E+07	1.48E+07	1.39E+07	1.28E+07
M305T567	2.34E+06	1.54E+07	9.78E+05	1.97E+07	7.82E+06	7.17E+06	6.58E+06
M291T394	1.20E+07	5.08E+07	2.18E+06	6.67E+07	3.37E+07	2.70E+07	2.68E+07
M287T764	4.89E+07	9.66E+07	1.29E+07	1.19E+08	6.38E+07	5.53E+07	6.57E+07

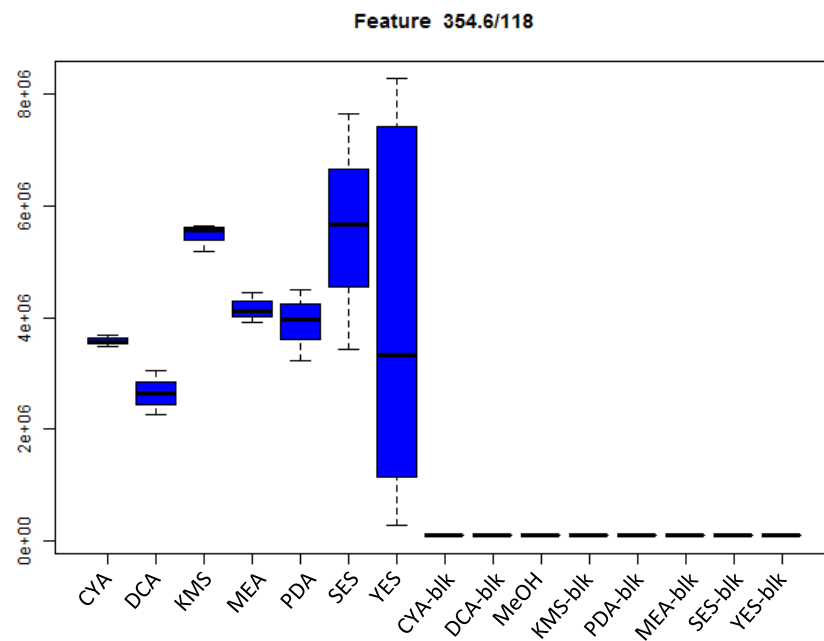
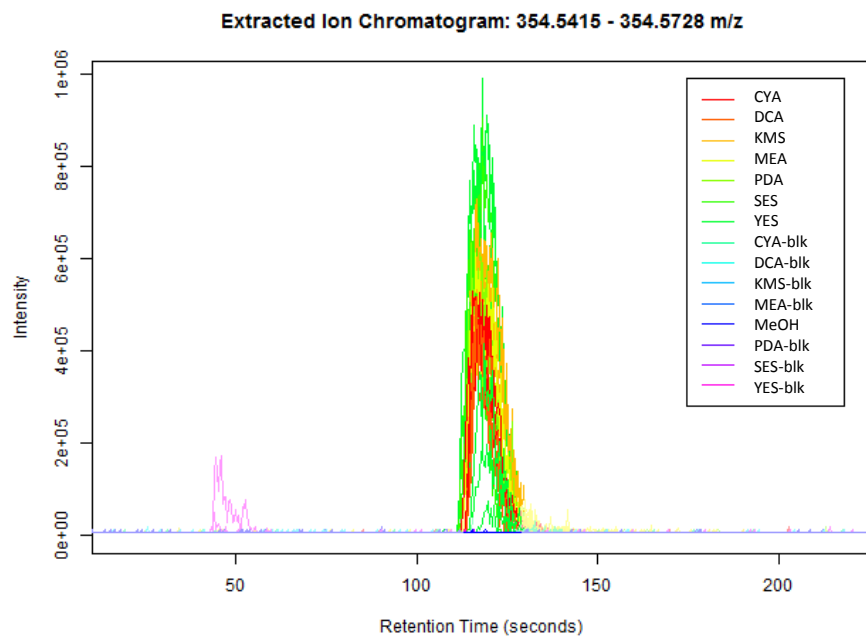
S-2: Dereplication of the eight compounds from the different crude extracts (from peak 1 to peak 8) with, for each of them:

- a) the major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed
- b) the corresponding HRMS spectra obtained by ESI in positive and negative modes together with UV spectrum
- c) the proposed structure
- d) the corresponding ^1H NMR signals observed on spectra obtained for the different extracts.

a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed.

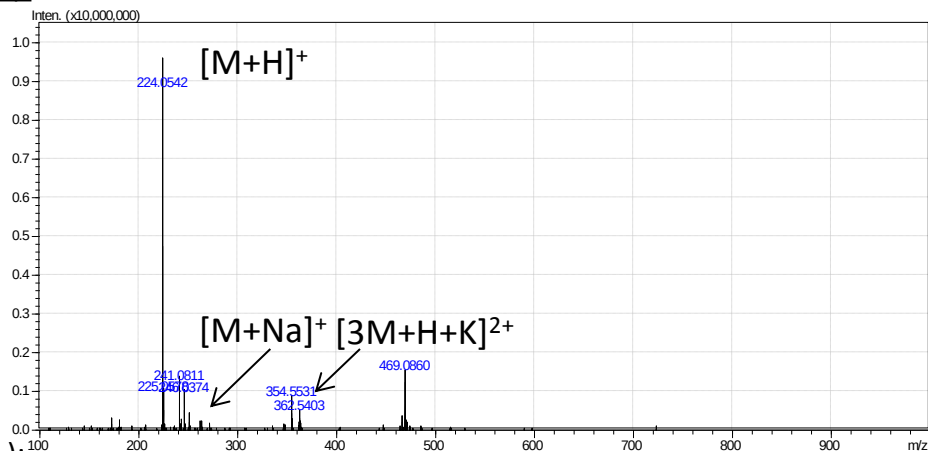


a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed. (*continued*)

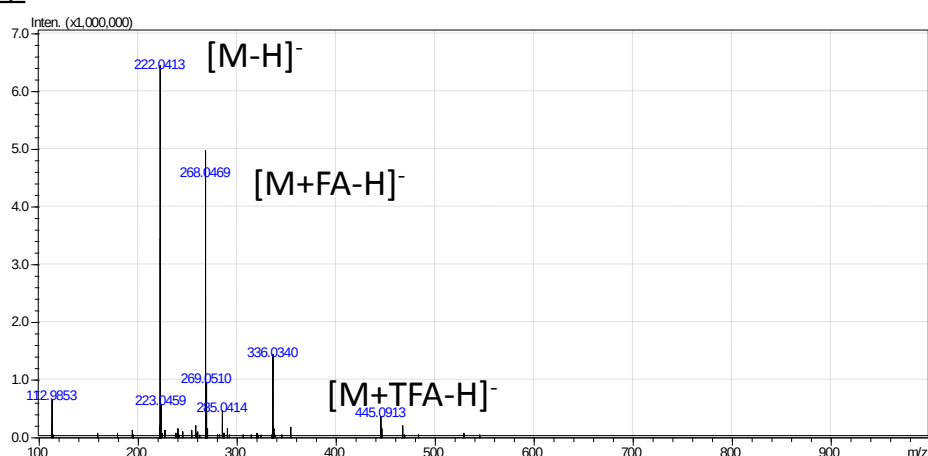


b) Corresponding HRMS spectra obtained by ESI in positive and negative modes (left side) together with UV spectrum (right side).

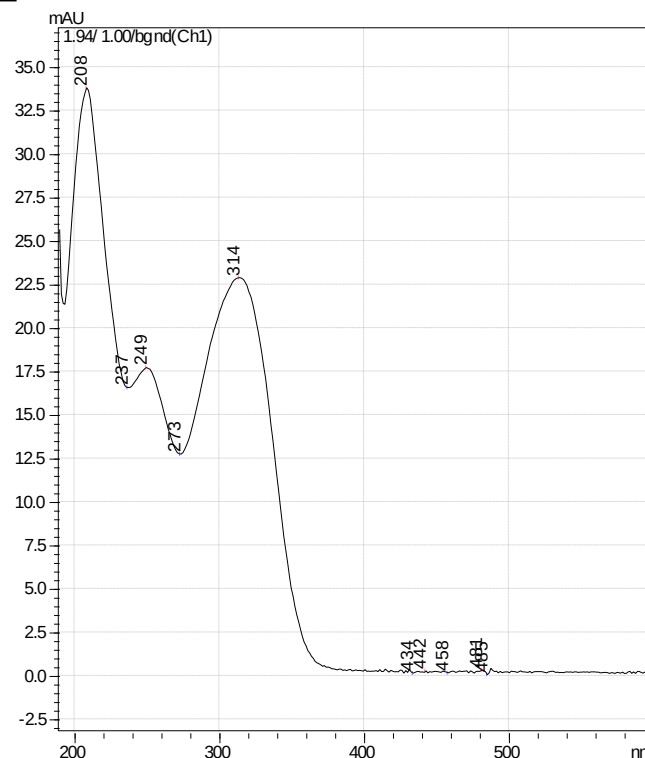
ESI(+):



ESI(-):

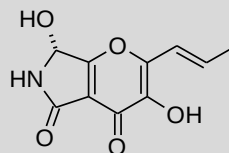


UV:



c) Proposed structure

Proposed MF: C₁₀H₉NO₅
ppm error: -4.91



Pyranonigrin A

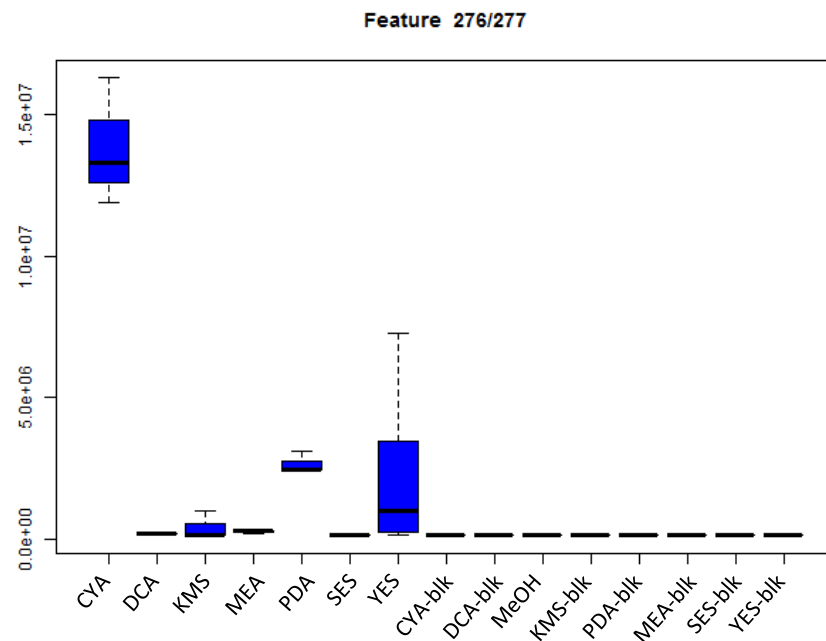
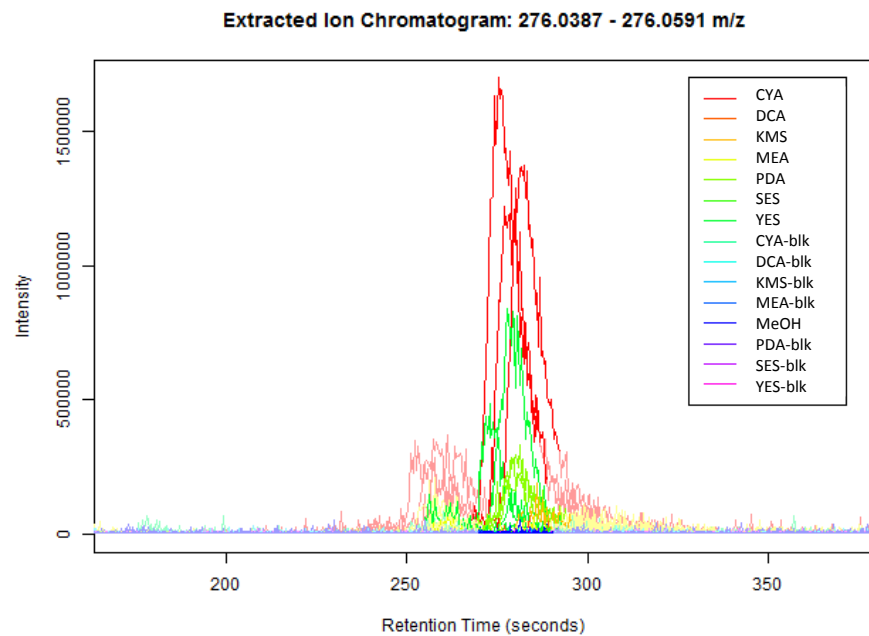
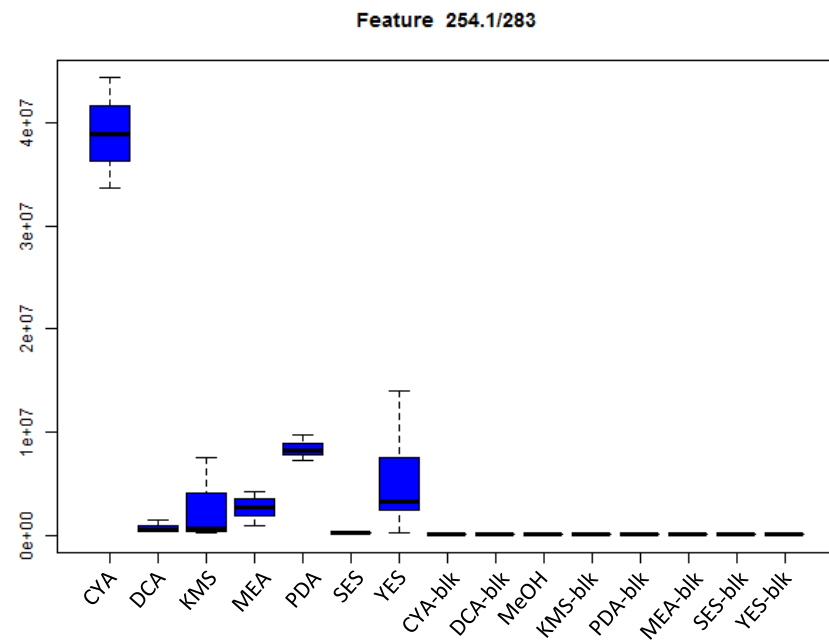
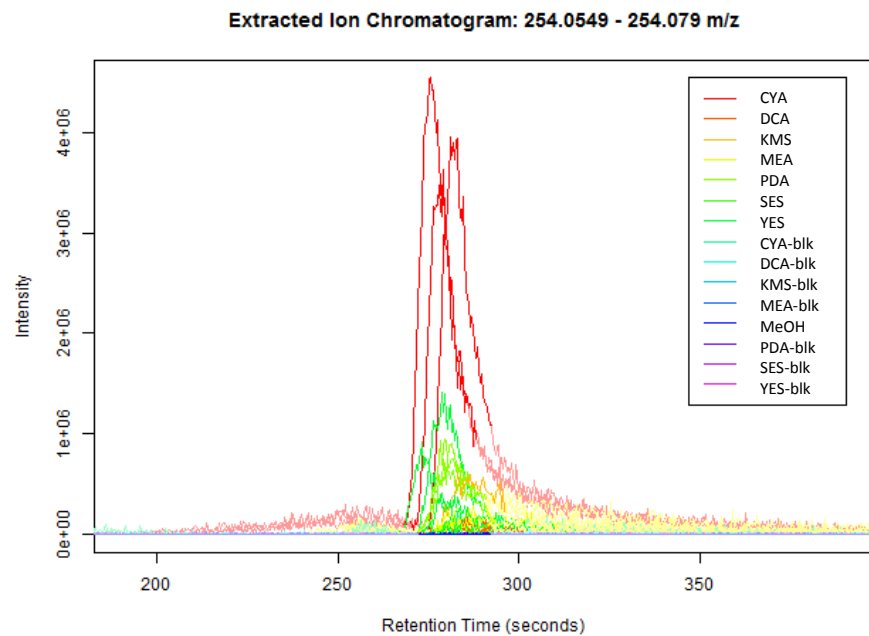
d) Corresponding ¹H NMR signals observed on spectra obtained for the different extracts.

¹H NMR signals, δ (DMSO-d₆):

- | | |
|-----------------------------|-----------------------------|
| - 9.67 (s) | - 6.45 (dq, J=15.9, 6.9 Hz) |
| - 8.60 (d, J=1.0 Hz) | - 5.72 (dd, J=8.8, 1.0 Hz) |
| - 6.77 (d, J=8.8 Hz) | - 1.92 (dd, J=6.9, 1.3 Hz) |
| - 6.57 (dq, J=15.9, 1.6 Hz) | |

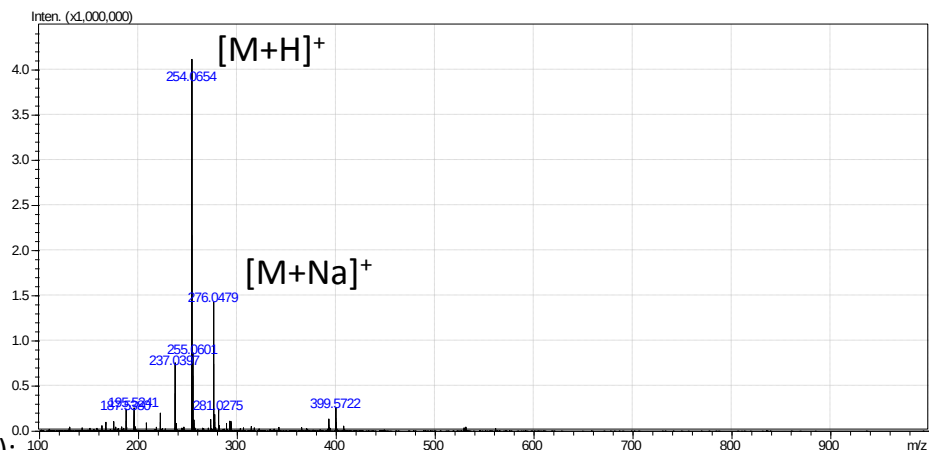
References: Hiort et al., 2004; Schlingmann et al., 2007; Nielsen et al., 2009

a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed.

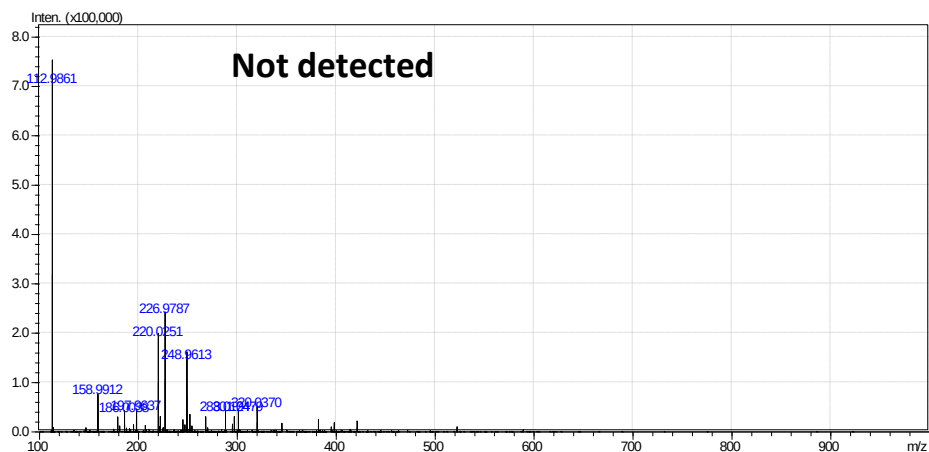


b) Corresponding HRMS spectra obtained by ESI in positive and negative modes (left side) together with UV spectrum (right side).

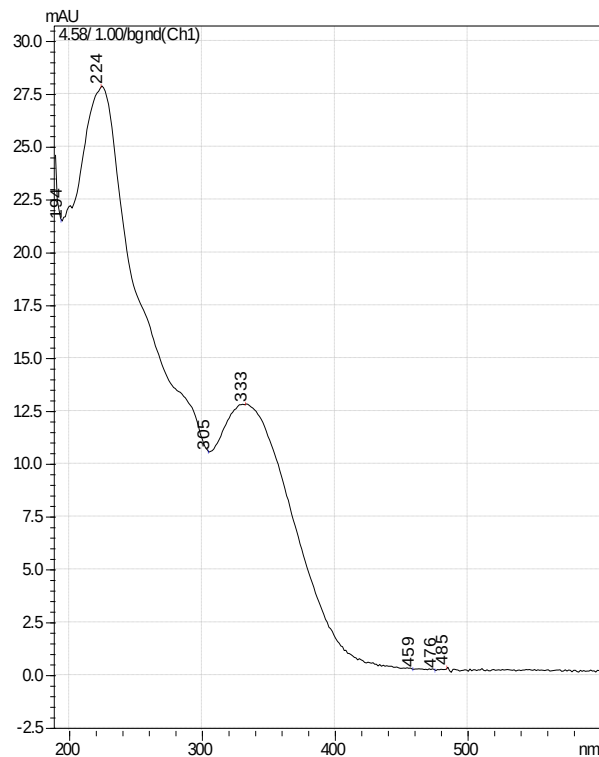
ESI(+):



ESI(-):

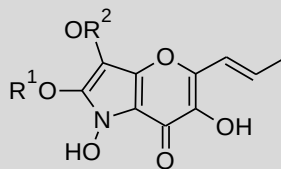


UV:



c) Proposed structure

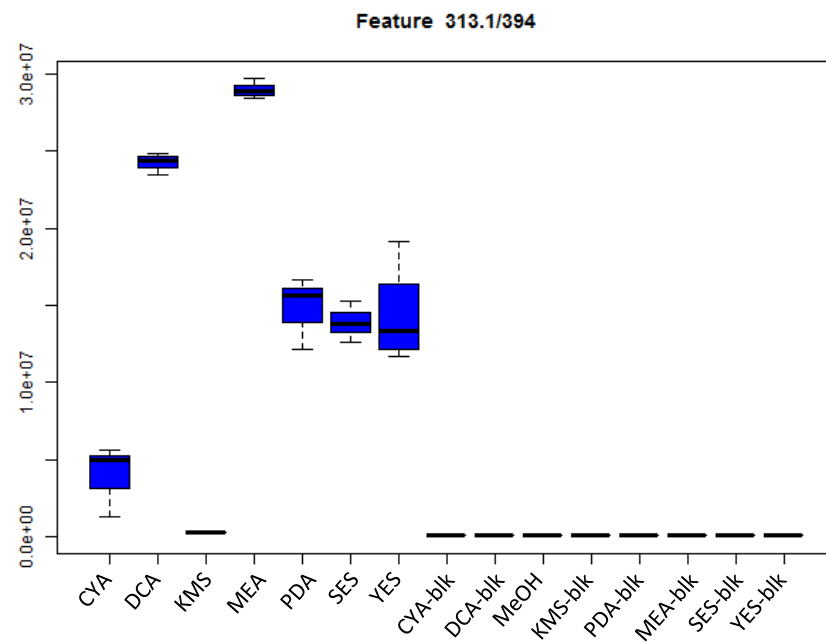
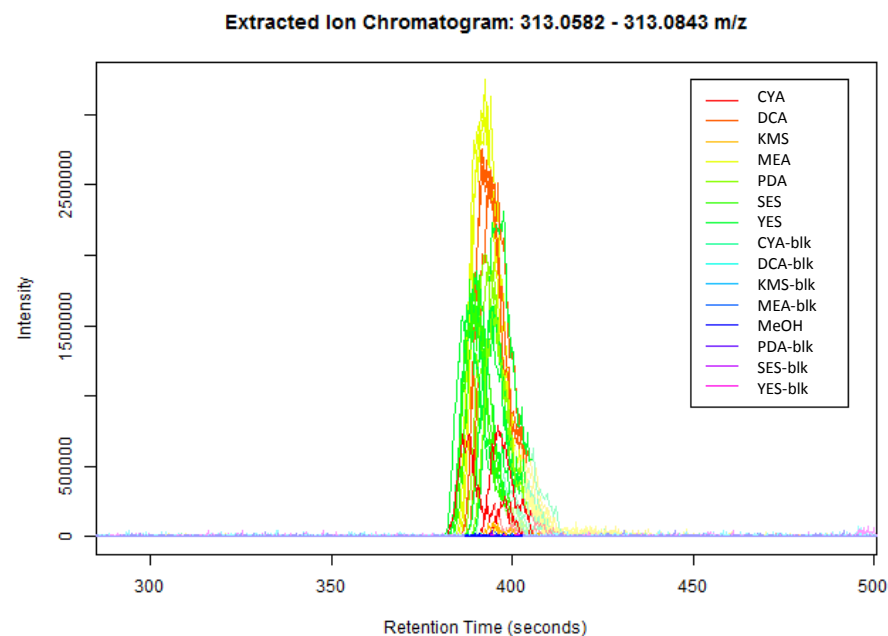
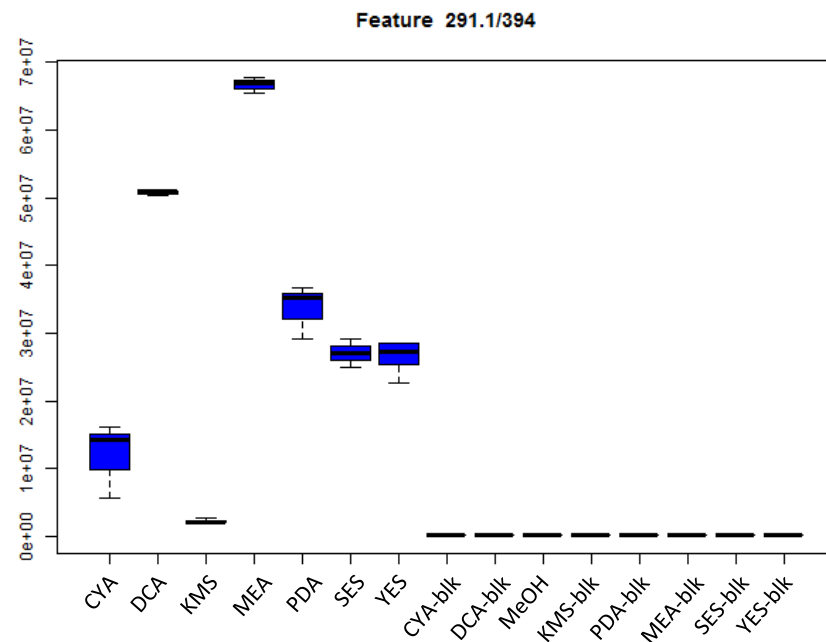
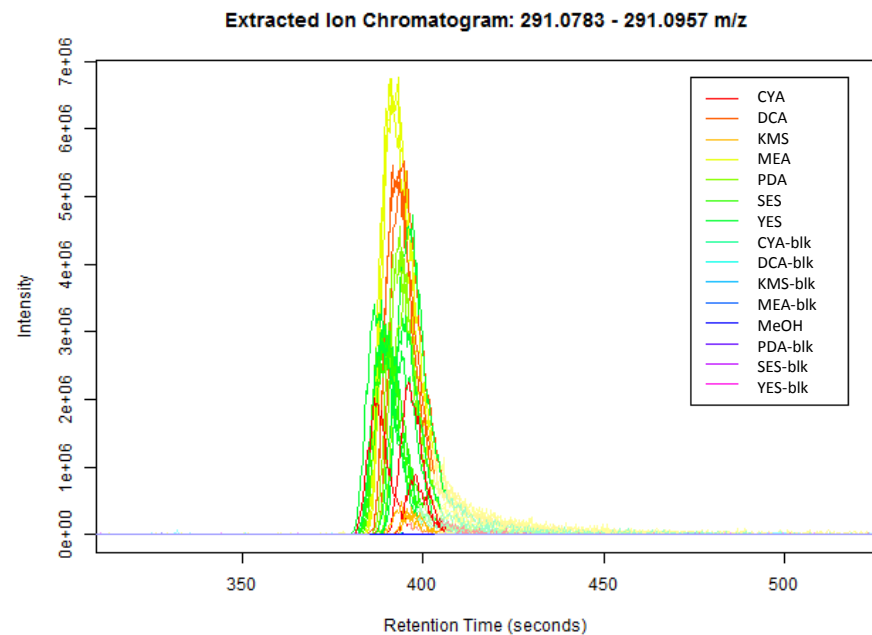
Proposed MF: $C_{11}H_{11}NO_6$
ppm error: -1,97



$R^1 = H$ $R^2 = CH_3$ **Pyranonigrin B**
 $R^1 = CH_3$ $R^2 = H$ **Pyranonigrin C**

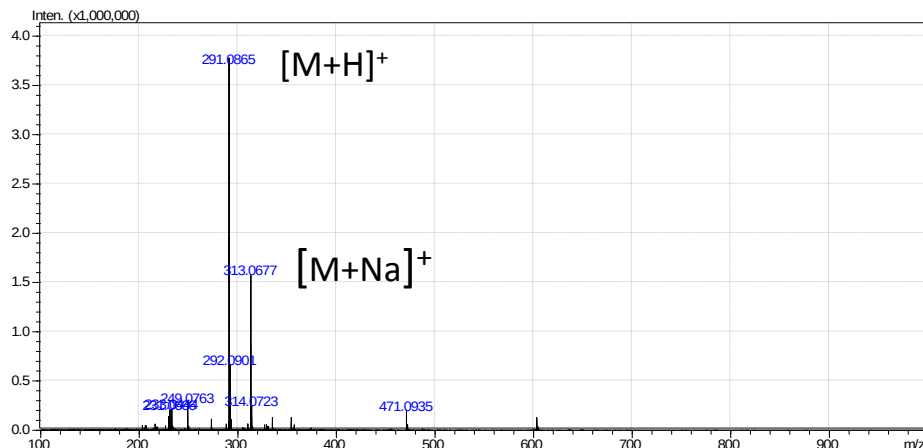
References: Hiort et al. 2004 ; Ióca et al., 2016

a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed.

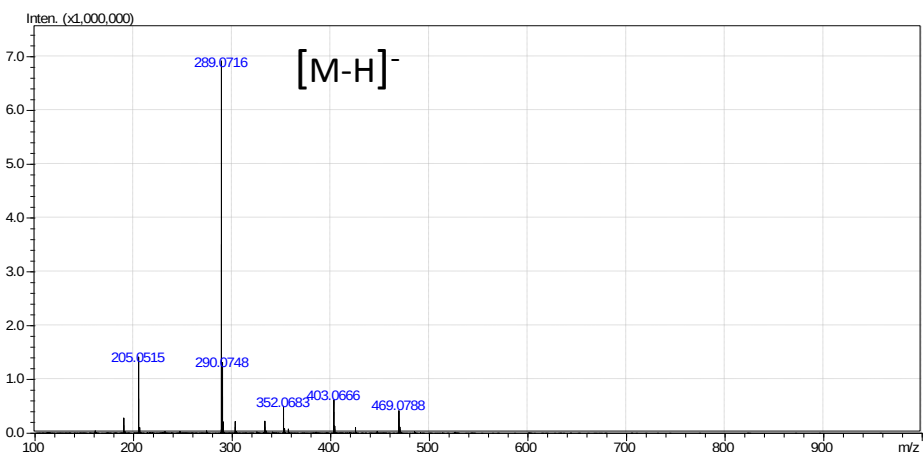


b) Corresponding HRMS spectra obtained by ESI in positive and negative modes (left side) together with UV spectrum (right side).

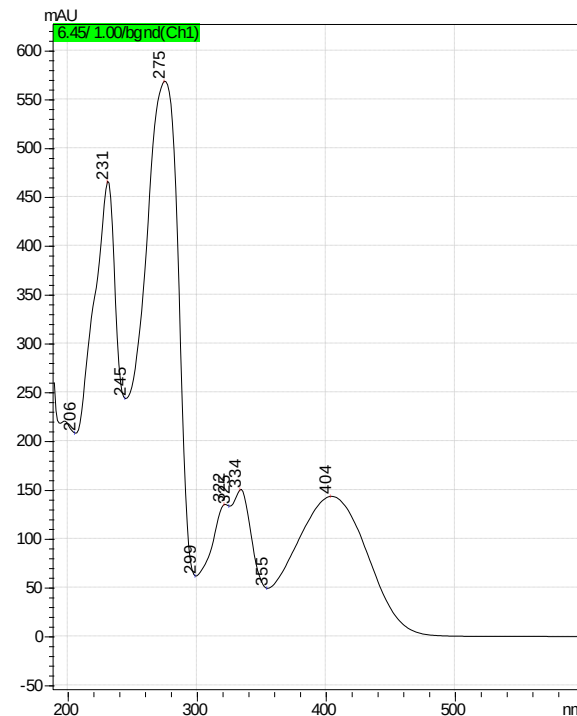
ESI(+):



ESI(-):

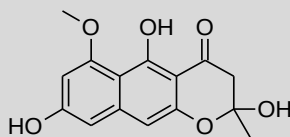


UV:



c) Proposed structure

Proposed MF: C₁₅H₁₄O₆
ppm error: 0.34



Fonsecin

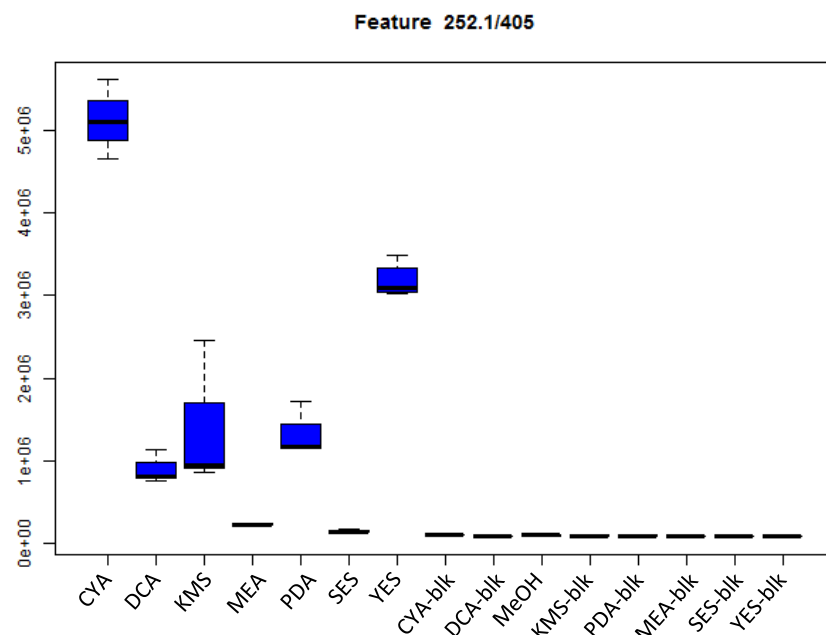
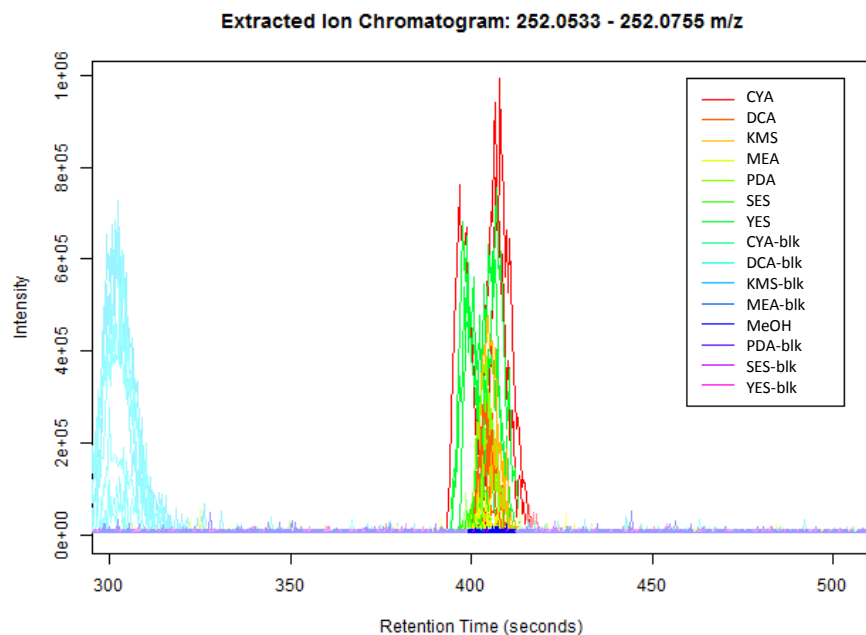
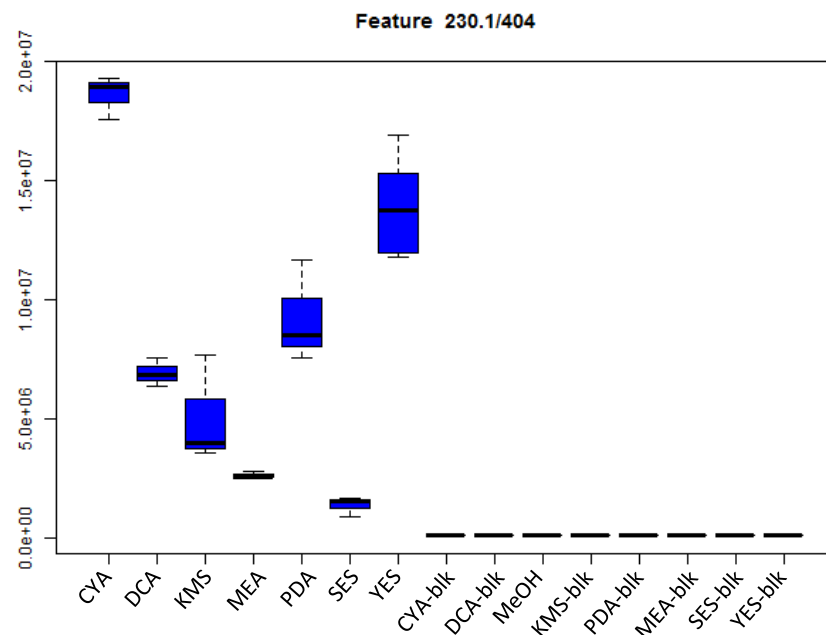
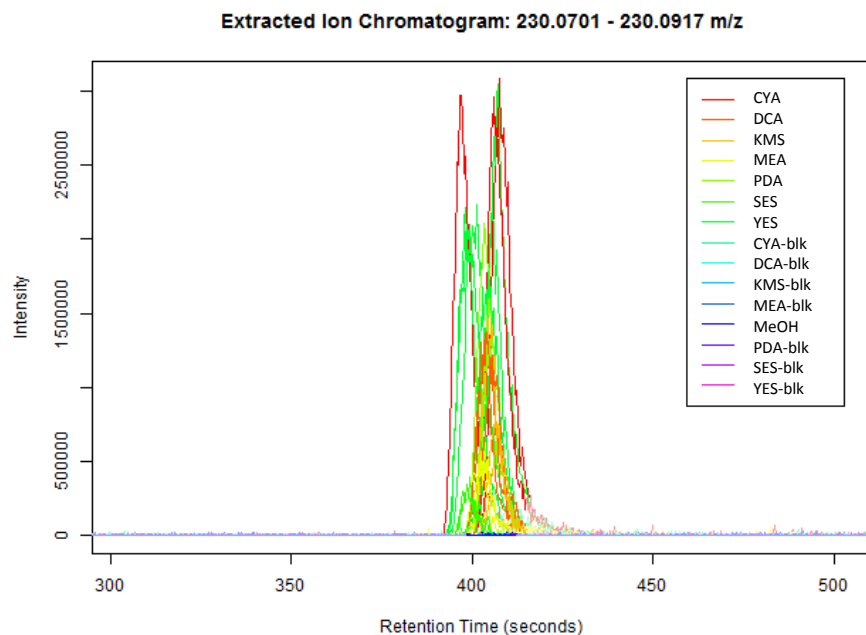
d) Corresponding ¹H NMR signals observed on spectra obtained for the different extracts.

¹H NMR signals, δ (DMSO, d₆):

- | | |
|----------------------|-----------------------|
| - 14.18 (s) | - 3.83 (s) |
| - 10.20 (s) | - 2.71 (d, J=16.8 Hz) |
| - 6.41 (s) | - 1.60 (s) |
| - 6.30 (d, J=2.2 Hz) | |

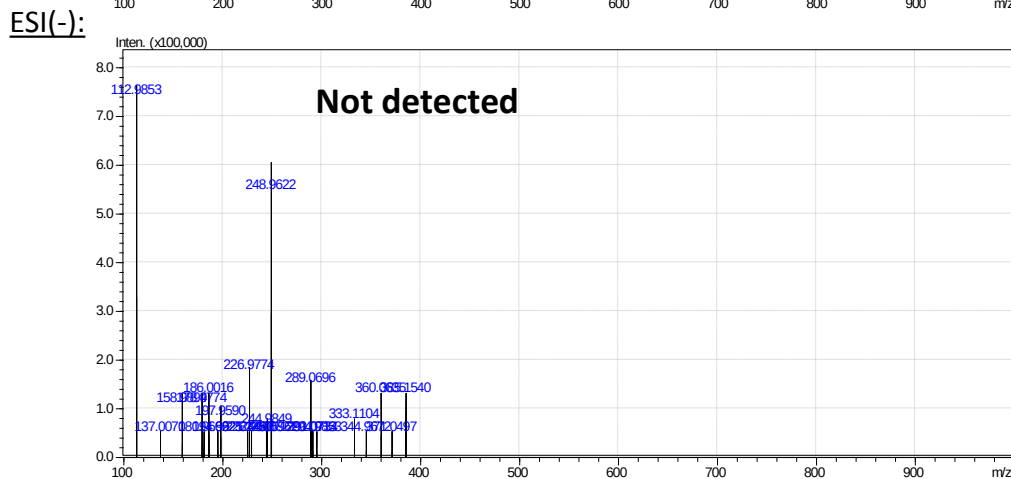
References: Galmarini et al., 1962; Tanaka et al., 1966; Priestap et al., 1984

a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed.



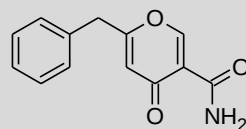
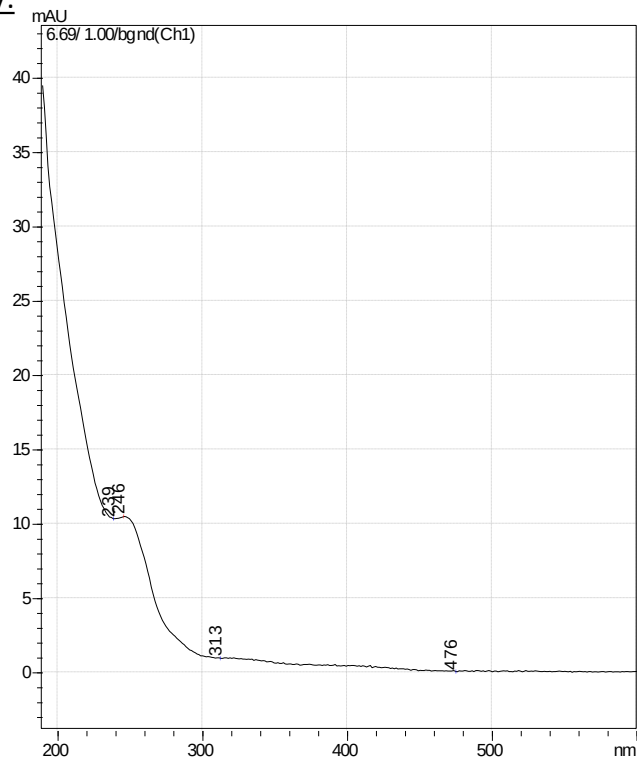
Peak #4 at 6.7 min

c) Proposed structure



d) Corresponding ^1H NMR signals observed on spectra obtained for the different extracts.

- 8.78 (s)
- 3.99 (s)

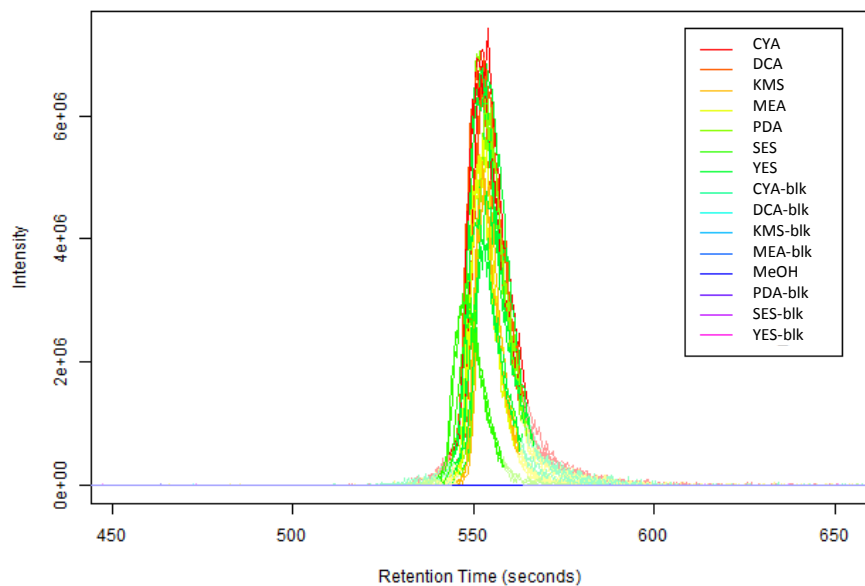


Carbonarone A

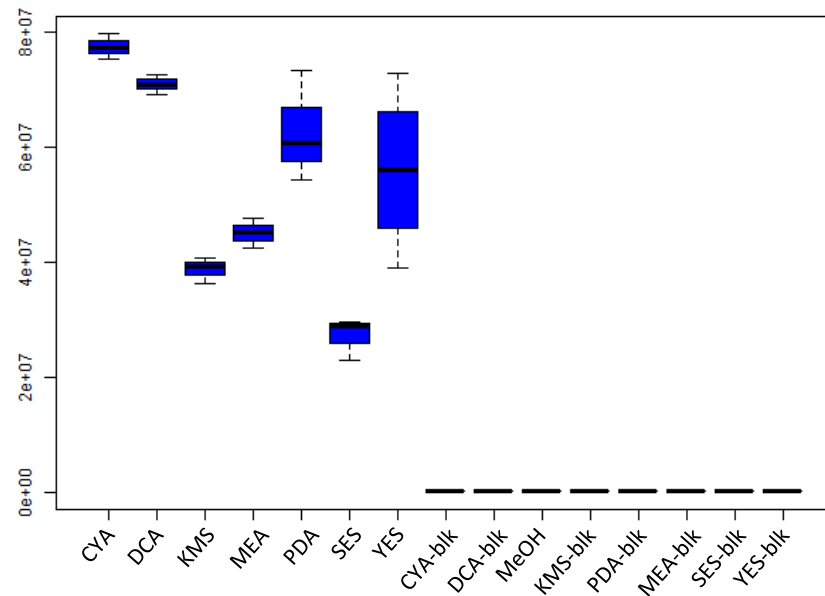
References: Henrikson et al. 2011 (NMR in CDCl_3); Fukuda et al., 2006; Zhang et al., 2007 (NMR in CDCl_3)

a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed.

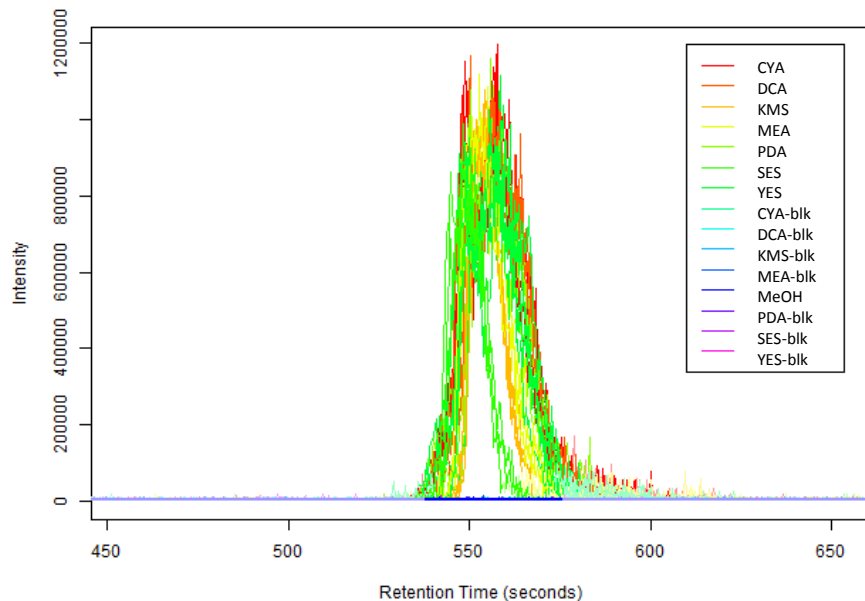
Extracted Ion Chromatogram: 344.107 - 344.1258 m/z



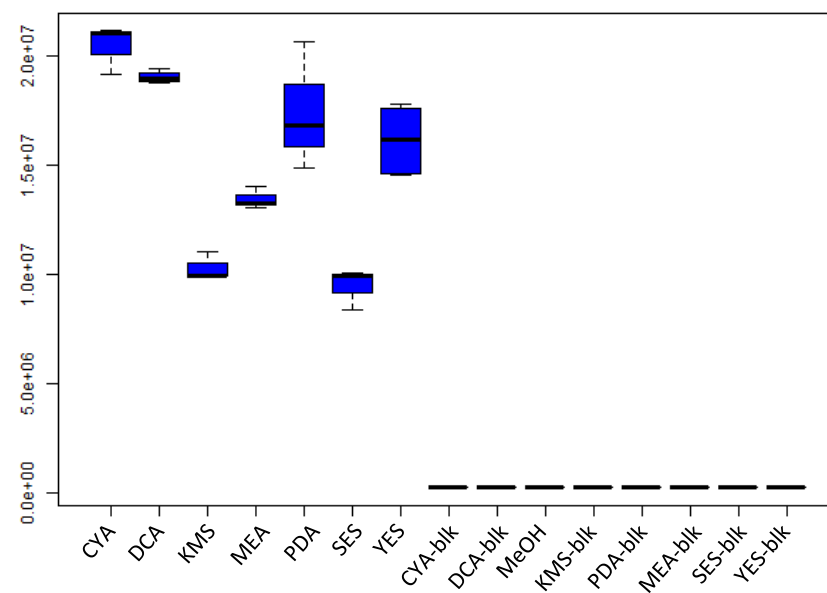
Feature 344.1/553



Extracted Ion Chromatogram: 366.0734 - 366.1097 m/z

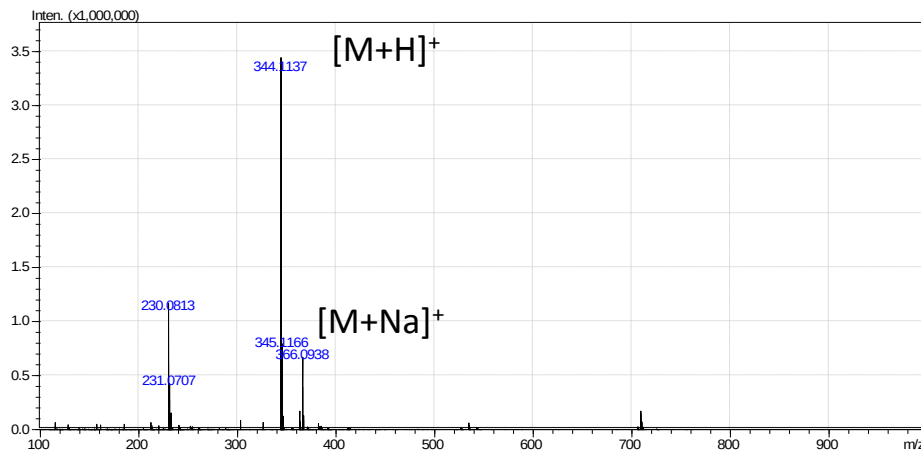


Feature 366.1/554

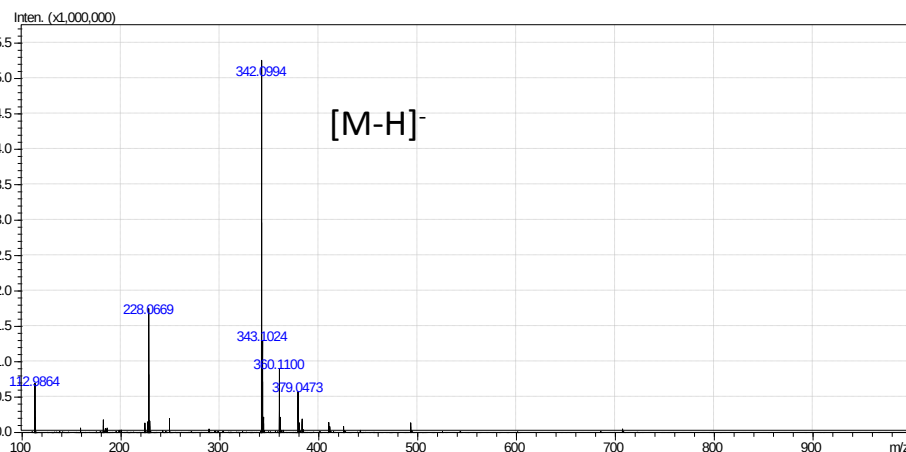


b) Corresponding HRMS spectra obtained by ESI in positive and negative modes (left side) together with UV spectrum (right side).

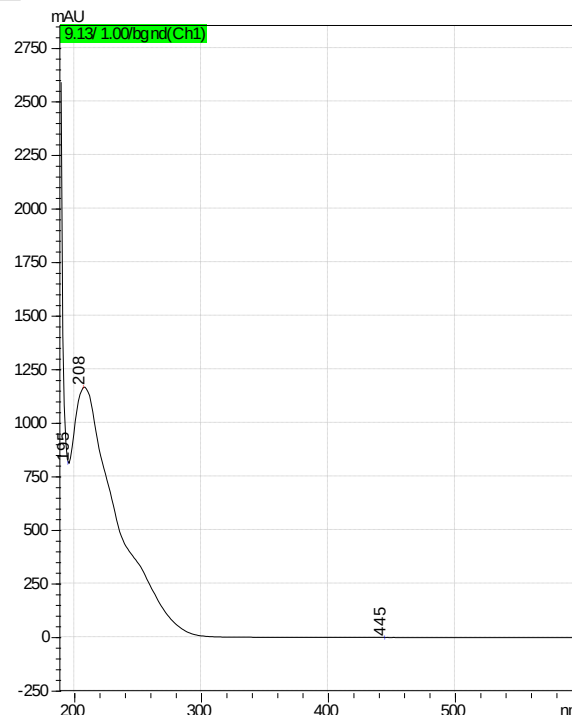
ESI(+):



ESI(-):

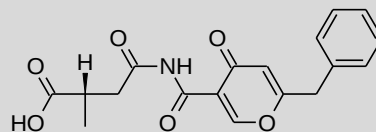


UV:



c) Proposed structure

Proposed MF: C₁₈H₁₇NO₆
ppm error: -2.03



Pestalamide A

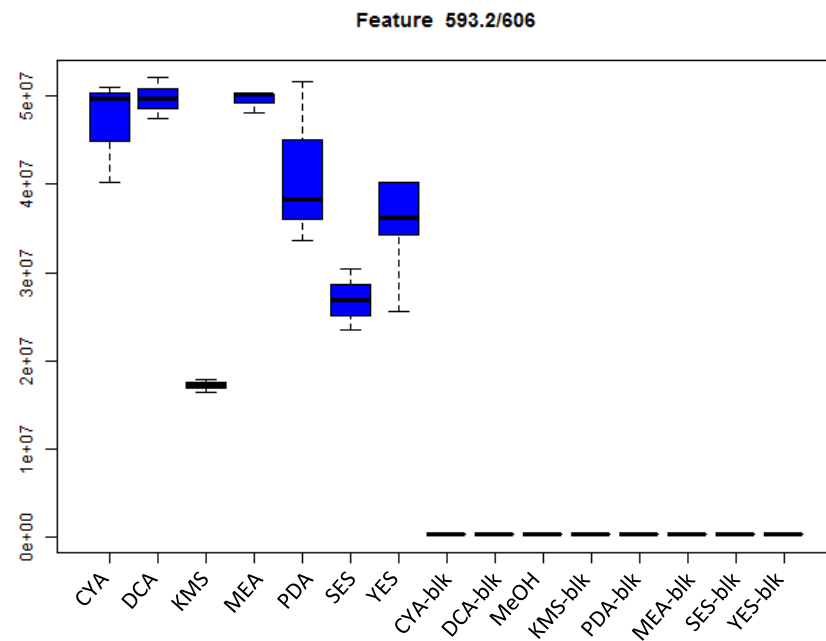
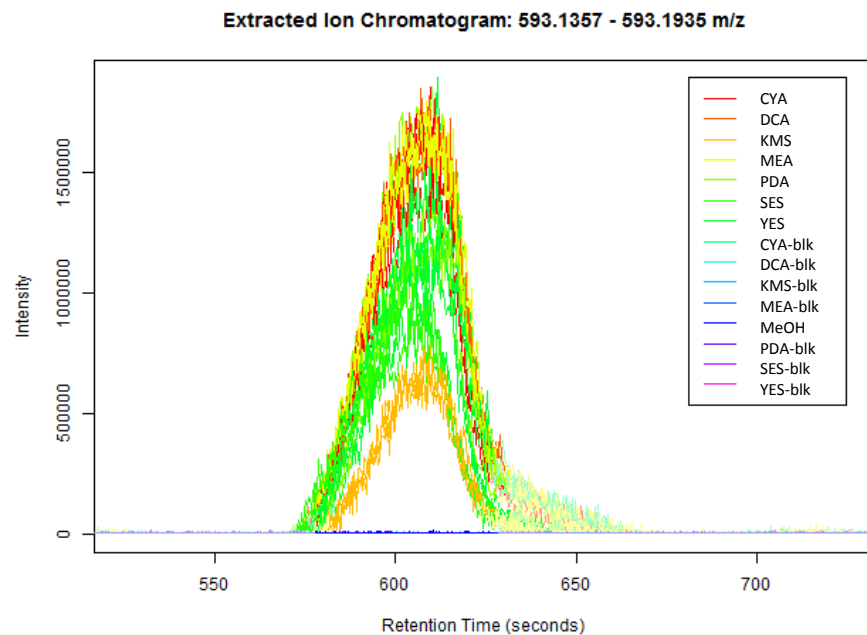
d) Corresponding ¹H NMR signals observed on spectra obtained for the different extracts.

¹H NMR signals, δ (DMSO, d₆):

- | | |
|-------------|-----------------------------|
| - 11.66 (s) | - 4.03 (s) |
| - 8.96 (s) | - 3.09 (dd, J=18.9, 9.6 Hz) |
| - 7.33 (m) | - 2.79 (m) |
| - 6.55 (s) | - 1.24 (d, J=7.2 Hz) |

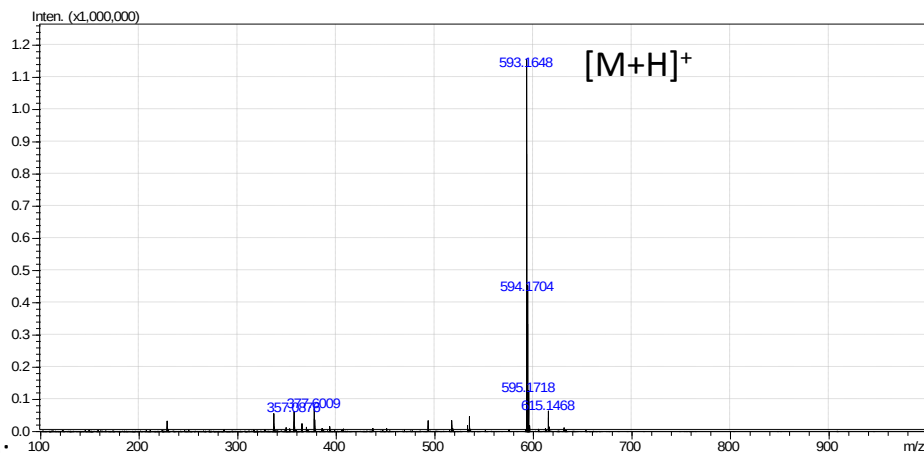
References: Henrikson et al. 2011 (NMR in CD₃OD); Takagi et al. 2010 (NMR in CDCl₃); Ding et al., 2008 (NMR in Acetone, d₆); Fukuda et al., 2006 (NMR in CDCl₃)

a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed.

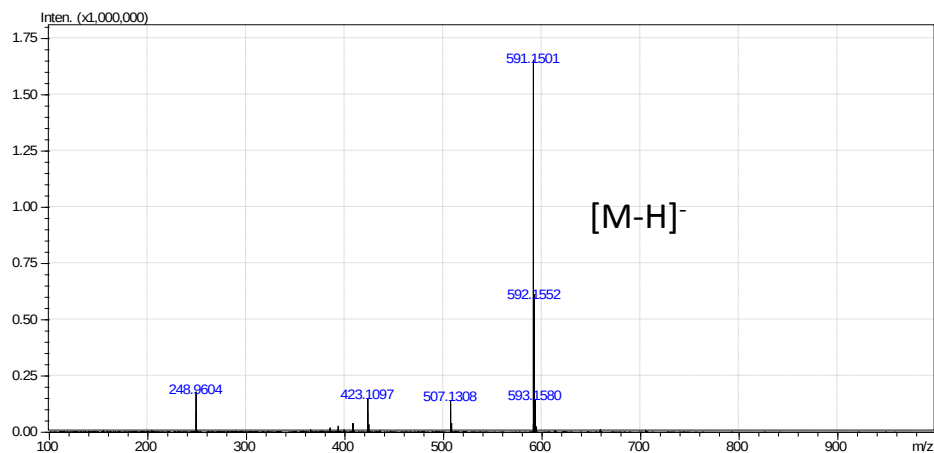


b) Corresponding HRMS spectra obtained by ESI in positive and negative modes (left side) together with UV spectrum (right side).

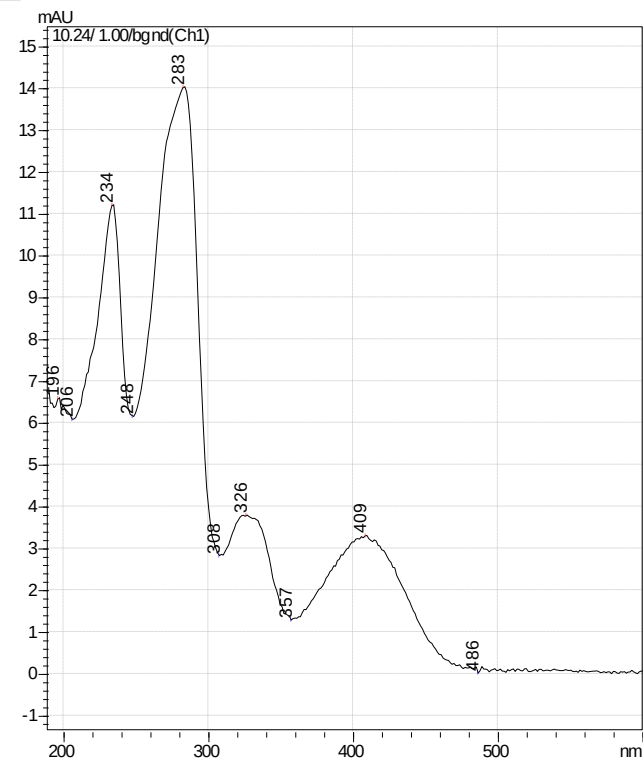
ESI(+):



ESI(-):

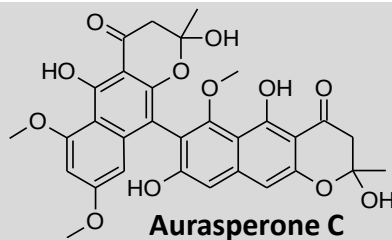


UV:



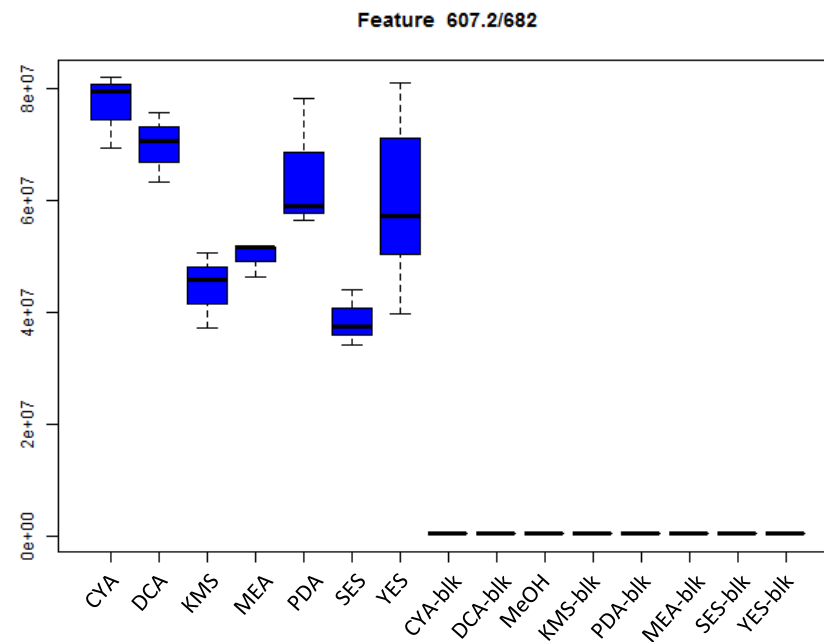
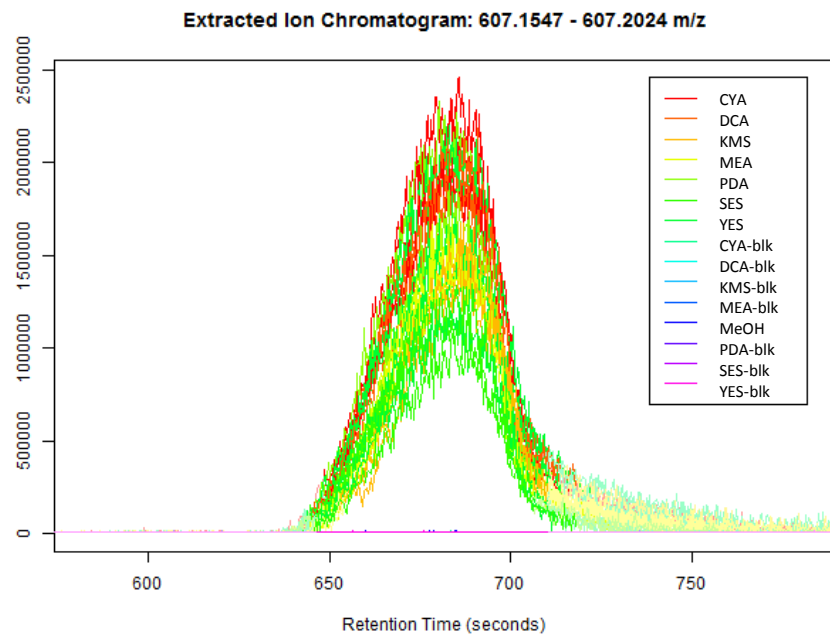
c) Proposed structure

Proposed MF: C₃₁H₂₈O₁₂
ppm error: -0.34



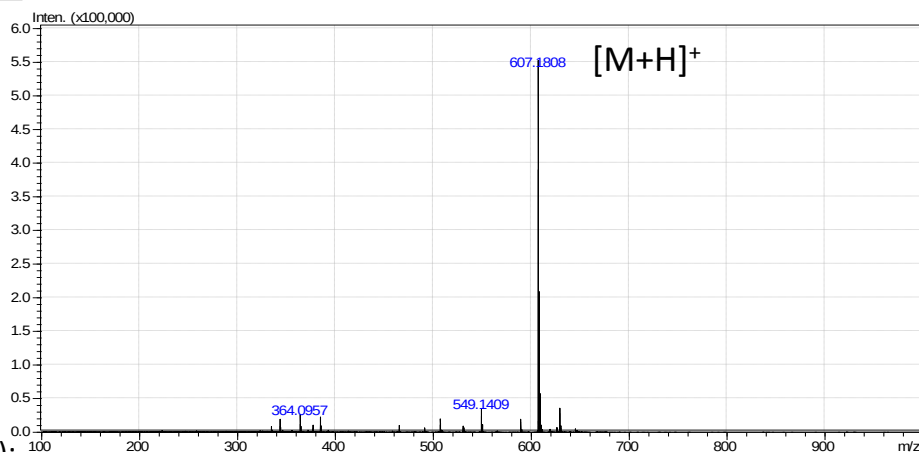
References: Tanaka et al., 1966; Tanaka et al., 1972; Shaaban et al., 2012

a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed.

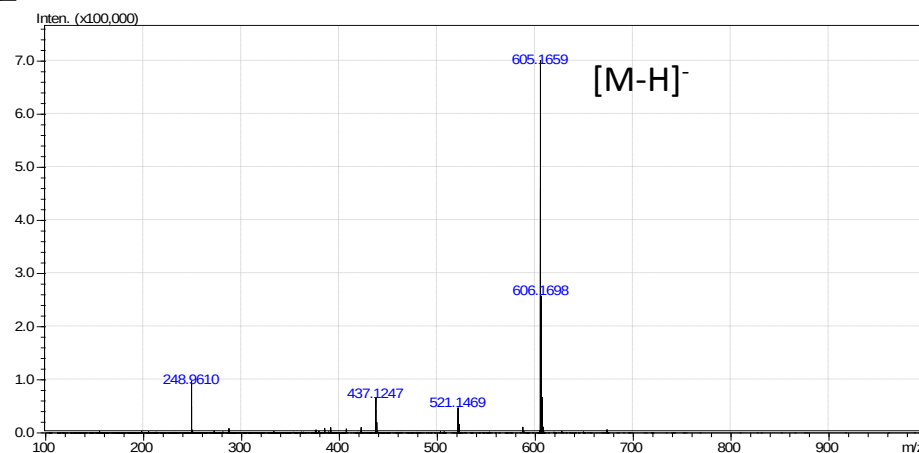


b) Corresponding HRMS spectra obtained by ESI in positive and negative modes (left side) together with UV spectrum (right side).

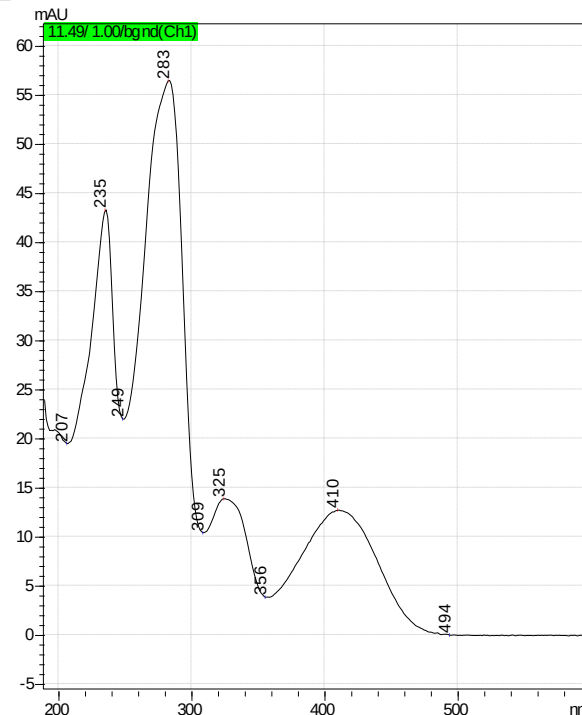
ESI(+):



ESI(-):

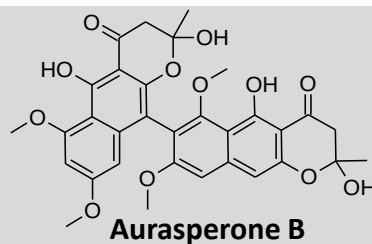


UV:



c) Proposed structure

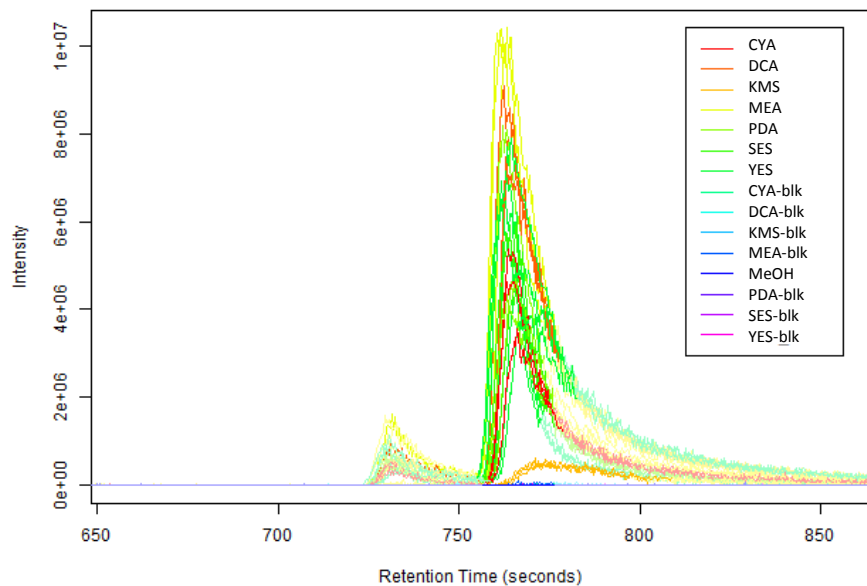
Proposed MF: C₃₂H₃₀O₁₂
ppm error: -0.33



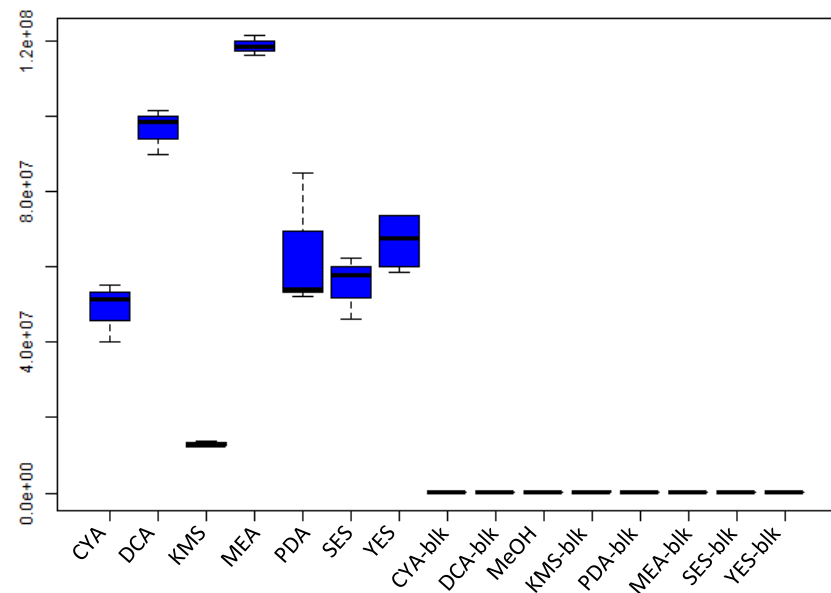
References: Tanaka et al. 1966 ; Shaaban et al., 2012 (NMR in CDCl₃), Wu et al, 2015 (NMR in MeOH-*d*₄)

a) Major ions observed and picked by the XCMS package together with box plots graphs representing their intensities in the different samples analyzed.

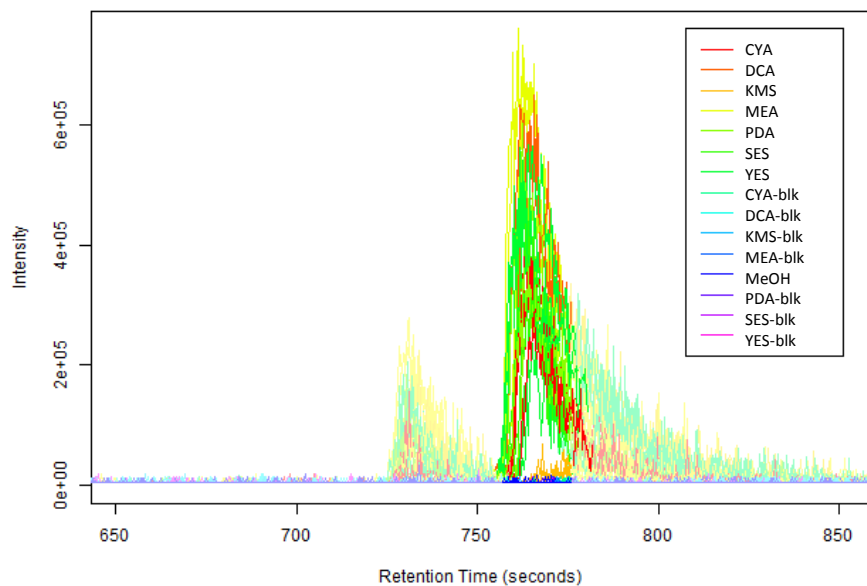
Extracted Ion Chromatogram: 287.081 - 287.1051 m/z



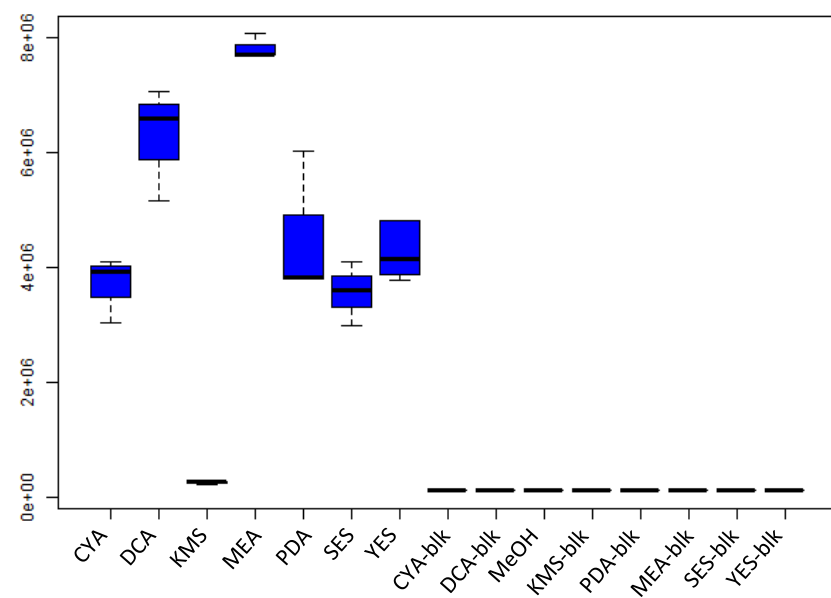
Feature 287.1/764



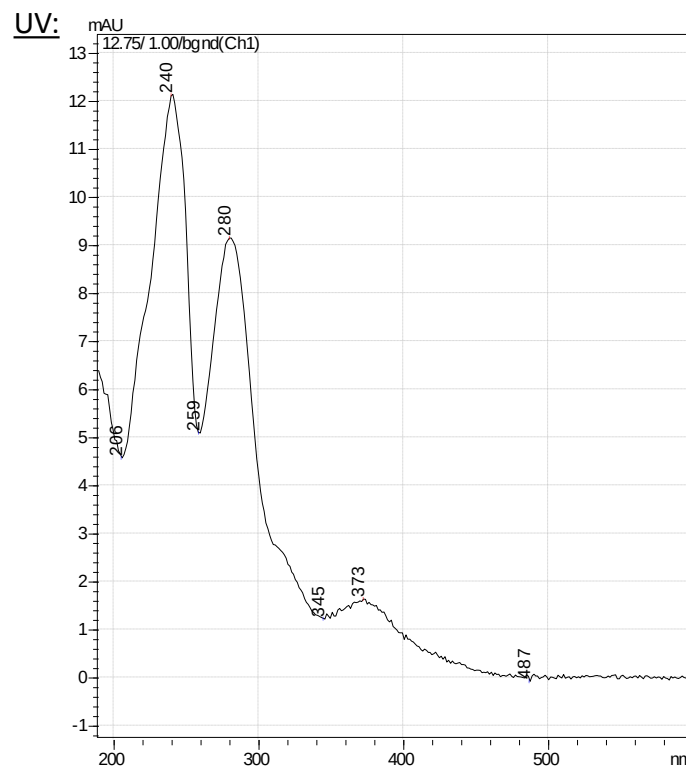
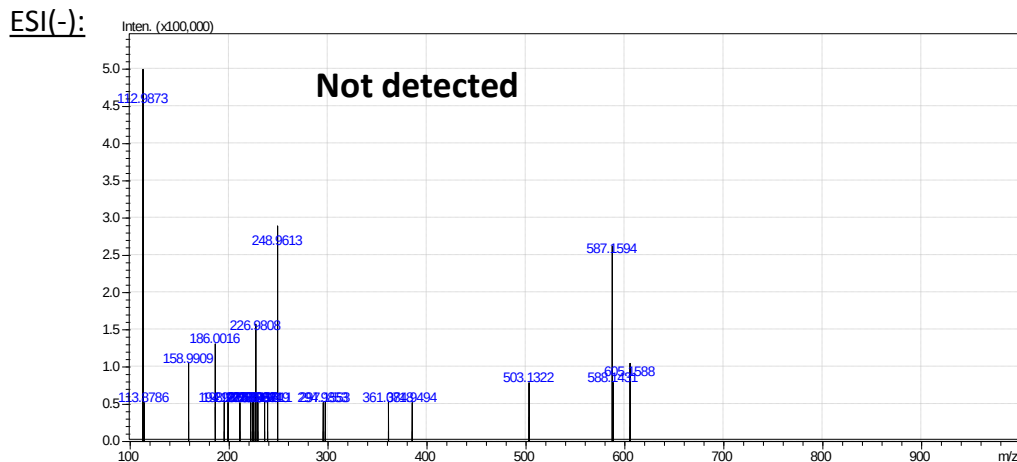
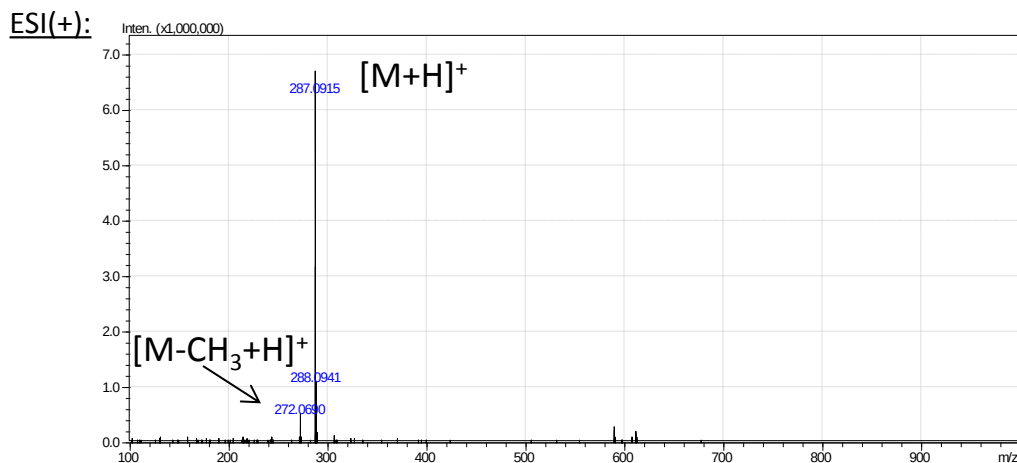
Extracted Ion Chromatogram: 272.0611 - 272.0779 m/z



Feature 272.1/766

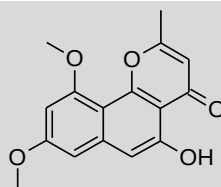


b) Corresponding HRMS spectra obtained by ESI in positive and negative modes (left side) together with UV spectrum (right side).



c) Proposed structure

Proposed MF: $C_{16}H_{14}O_5$
ppm error: 0.35



Flavasperone

d) Corresponding ^1H NMR signals observed on spectra obtained for the different extracts.

^1H NMR signals, δ (DMSO, d_6):

- 12.81 (s) - 3.95 (s)
- 6.91 (s) - 3.88 (s)
- 6.84 (d, $J=2.2$ Hz)

References: Fukushima et al., 1964; Tanaka et al., 1966; Sakurai et al., 2002

S-3: Detection of mycotoxins from the shrimp-derived *A. niger*

Mycotoxins	Molecular Formula	Production by the shrimp-sourced <i>Aspergillus niger</i> strain studied							Maximal intake in µg/Kg bw	Described toxicity
		CYA	DCA	KMS	MEA	PDA	SES	YES		
3-Nitropropionic acid	C ₃ H ₅ NO ₄	ND	ND	ND	ND	ND	ND	ND	NE	neurotoxic (Blumenthal, 2004)
aflatoxin B1	C ₁₇ H ₁₂ O ₆	ND	ND	ND	ND	ND	ND	ND	10 µg/Kg (Creppy 2002)	hepatocarcinogen (Creppy et al., 2002)
aflatoxin G1	C ₁₇ H ₁₂ O ₇	ND	ND	ND	ND	ND	ND	ND	10 µg/Kg (Creppy 2002)	carcinogenic (Frisvad et al., 2007)
aflatoxin G2	C ₁₇ H ₁₄ O ₇	ND	ND	ND	ND	ND	ND	ND	10 µg/Kg (Creppy 2002)	carcinogenic (Frisvad et al., 2007)
aflatoxin M1	C ₁₇ H ₁₂ O ₇	ND	ND	ND	ND	ND	ND	ND	0,05 µg/Kg (Creppy 2002)	carcinogenic (Frisvad et al., 2007)
aflatoxin B2	C ₁₇ H ₁₄ O ₆	ND	ND	ND	ND	ND	ND	ND	10 µg/Kg (Creppy 2002)	carcinogenic (Frisvad et al., 2007)
Citrinin	C ₁₃ H ₁₄ O ₅	ND	ND	ND	ND	ND	ND	ND	NE	genotoxic (Wügler et al., 1991)
Fumonisin B1	C ₃₄ H ₅₉ NO ₁₅	ND	ND	ND	ND	ND	ND	ND	2 µg/Kg TDI (EC, 2006)	carcinogenic (Gelderblom et al., 1993 ; Bartók et al., 2006)
Fumonisin B2	C ₃₄ H ₅₉ NO ₁₄	ND	ND	ND	ND	ND	ND	ND	2 µg/Kg TDI (EC, 2006)	carcinogenic (Gelderblom et al., 1993 ; Bartók et al., 2006)
Fumonisin B3	C ₃₄ H ₅₉ NO ₁₄	ND	ND	ND	ND	ND	ND	ND	2 µg/Kg TDI (EC, 2006)	carcinogenic (Gelderblom et al., 1993 ; Bartók et al., 2006)
Ochratoxin A	C ₂₀ H ₁₈ ClNO ₆	ND	ND	ND	ND	ND	ND	ND	1,2 µg/Kg TWI (EC, 2006)	genotoxic (Creppy et al., 1985)
Ochratoxin B	C ₂₀ H ₁₉ NO ₆	ND	ND	ND	ND	ND	ND	ND	NE	nephrotoxic and renal carcinogenic effects (Mally et al. 2005)
Patulin	C ₇ H ₆ O ₄	ND	ND	ND	ND	ND	ND	ND	0.4 µg/Kg PMTDI (EC, 2006)	genotoxic (Wügler et al., 1991), carcinogenic (Tournas, 1994)
Sterigmatocystin	C ₁₈ H ₁₂ O ₆	ND	+	ND	+	+	++	+	NE	carcinogenic (Schroeder and Kelton, 1975)

ND: non detected

NE: non evaluated

EC, 2006: European Commission 2006. Commission Regulation No 1881/2006 of December 19th setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union No. L 364/5 20th December, 2006.

PMTDI: provisional maximum tolerable daily intake

bw: body weight

TWI: tolerable weekly intake

TDI: tolerable daily intake