Article

SYNTHESIS, PURITY CHECK, HYDROLYSIS AND REMOVAL OF O-CHLOROBENZYLIDEN MALONONITRILE (CBM) BY BIOLOGICAL SELECTIVE MEDIA

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Abstract: The military taxonomy of class indices for chemical and biological warfare agents considers halogenated tear agents (CS) to be incapacitating causing local pain and discomfort with associated reflexe. The removal yield of the organic substances present in the water depends on the environmental conditions, on the chemical composition of the water and on the chemical substance dissolved in the water, which constitutes the substrate of the metabolic activities of the microorganisms that use these substances in the biochemical reactions of the cellular enzyme complexes. The two -CN groups in the malonic nitrile molecule are strongly electron-attracting and activate the CH2 group to which they are linked, and because of this, malononitrile has the role of a methylene component in condensation reactions with aldehydes or ketones. The results obtained after analyzing the degree of hydrolysis in the samples that contained the biological suspension indicated that no CBM metabolites were detected in any biological sample, regardless of the test concentration, analyzed 24 h after incubation. In the parallel samples, the aqueous solutions of CBM without biological treatment had metabolites in the samples., at 30 minutes the degree of hydrolysis is 0.21%, at 60 minutes, the degree of hydrolysis is 5.42%, after 90 minutes, the degree of hydrolysis is 6.19%, and after 24 hours. the degree of hydrolysis is 23.41%. Chemical substances in contact with microorganisms are used by them in the biochemical processes in which they are involved, the retention time and biodegradation capacity have led to an increased effect of the metabolism of toxic substances, in metabolic reactions, organic substances are the source of carbon and energy for biochemical processes. The tests performed indicate that the suspension of *Chlorella* sp. consumed the entire amount of CBM and metabolites from the analyzed samples The tests prove that the biological material can be used for the decontamination of the affected areas.

Keywords: CS gas; O-Chlorobenzylidene malononitrile; ecotoxicity; Chlorella sp., FTIR spectroscopy; GC-MS

1. Introduction

From the riot control agent (RCA) category, halogenated tear agents cause acute physiological effects due to the physical and chemical properties of the substances they contain and due to their decomposition products. The military taxonomy of class indices for chemical and biological warfare agents considers halogenated tear agents (CS) to be incapacitating causing by inhalation local pain, discomfort with associated reflexes. Among the adverse reactions generated by exposure to halogenated lacrimal agents is the "Kratschmer Reflex" which causes tension of the aortic artery, apnea, due to the excitation of the sensory receptors of the nervous system .https://doi.org/10.1016/B978-0-12-386454-3.00922-2 [1]

Grenades containing o-chlorobenzylidene malononitrile (CS: tear gas) have been used since the First World War. [2-4]. Halogenated organic compounds are widely used

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for mass dispersion and for crowd control in incendiary devices. There are concerns about the use of these substances due to its toxicity on violent criminals but also on law enforcement agents, or other type of the personnel who are involved in such operations. Also, the use of these substances as a means of self-defense is available and could be used for civil protection. The toxicity of the substance can affect anyone who is incidentally exposed, such as health care personnel, bystanders, noncriminal, or nonviolent offenders, with concerns over long-term or repeated exposure to halogenated toxicity .https://doi.org/10.1016/B0-12-369399-3/00077-X

The medical problems generated by exposure to halogenated tear agents require the search for prophylactic antagonists against them because they are very persistent chemicals, remaining present in the environment for extended periods of time. http://dx.doi.org/10.1016/j.taap.2008.04.005 The evaluation of the chemical risk induced by the persistence of chemical substances used in military operations requires the development of economically feasible methods of remediation of contaminated areas. The integration of effective depollution methods that decontaminate and at the same time reduce the risks of chemical pollution requires the use of biological methods. Natural environments, based on the bioavailability of microbiological suspensions capable of developing on selective media, with the aim of streamlining the performance of the process of eliminating toxic substances from the environment, and preventing the risk of new contamination due to military activities.http://dx.doi.org/10.1016/j.scitotenv.2022.157007

Chlorobenzylidene Malononitrile (CBM – our notation in the experimental part) is a substance used in military operations that is used for crowd control, in explosives where it is found as a component of military projectiles, it is the chemical component of dispersive devices such as aerosolization. https://doi.org/10.1016/B0-12-369399-3/00077-X,https://doi.org/10.1016/0305-4179(95)00063-h,The toxicological effects generated by this substance have serious consequences for the population and biofauna.

Dissemination of CS can be carried out on the targeted population in the case of a military operation by explosive dispersion of powder or a solution containing CS, or by release in the form of smoke from a pyrotechnic mixture. The method of disseminating the toxic can generate a serious situation due to the severity of the injuries to the eyes or the respiratory system of the affected subjects. CS tends to agglomerate when used in powder form, which is why hydrophobic formulations of CS have been developed that contain siliconized powder with hydrophobic silicon airgel, thus making the active substance have a long-lasting effect, its persistence being several weeks.[5]

The removal yield of the organic substances present in the water depends on the environmental conditions, the chemical composition of the water and the chemical substance dissolved in the water that constitutes the substrate for the metabolic activities of the microorganisms that use these substances in the biochemical reactions of cellular enzyme complexes. As a result of the metabolic reactions that take place in the cells of microorganisms, simple or complex organic compounds (carbohydrates, amino acids, fatty acid esters) are formed in the reaction medium. Some organic substances present in water are easily degraded by some microorganisms selected and adapted to chemical stress conditions. [6]

In the presence of pollutants from the irritating substances category, it is necessary to adapt the microbial cultures and test the biodegradation capacity of these toxins, taking into account the speed and detoxification capacity, as well as the cellular toxicity exerted by the harmful substance in a biological environment.[7]

The biological suspension can influence the biodegradation of chemical compounds through its genetic ability to metabolize organic substances in close correlation with the rate of assimilation of the toxicant. To accelerate these processes, certain optimal conditions for cell development are needed (pH, temperature, nutrient substrate, agitation, incubation). [8],[9]

The toxic substances present in the waters influence the enzymatic system of microorganisms. A very important aspect in the biodegradation of toxic compounds is the chemical nature of the substance that determines the persistence of the compound in the

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reaction medium. The contact time of microorganisms from a biocenosis with a toxic substance is important, the retention time and biodegradation capacity lead to an increased effect of the metabolism of toxic substances, which increases the reaction speed in the tested environment. [10]

According to the MICHAELIS-MENTEN relationship in enzyme kinetics, it was established that the speed of biochemical processes in the cell increases with the increase in the concentration of the substrate until a lag phase where the cell growth no longer changes in relation to the concentration of the substrate. Following this process, percentages of the undegraded substance remain in the reaction medium, and metabolites resulting from biochemical processes also accumulate.[11]

Chemical substances are degraded through different metabolic pathways, through the oxidation of carbon and hydrogen from organic substances, through the oxidation of nitrogen from nitrites, or from chemical substances that contain nitrogen in the molecule, hydrolysis or the removal of water at C=C atoms by addition to the double bond, splitting and forming C-C bonds by decarboxylating or carboxylating ketones, adding or removing the N atom in the form of NH_3. In addition to the metabolic reactions of the cells, there are also reactions by which the toxic organic substances are inactivated and eliminated from the reaction medium through methylation, acetylation.[11]

The biochemical processes that take place in the cells of microorganisms are mechanisms that can vary at different speeds because chemical substances can be attacked sequentially, which generate a sequential increase due to some metabolism products. The metabolic reactions involved in the cells of microorganisms are catabolic in which the oxidation of substances takes place in the purpose of obtaining energy and anabolism or assimilation where microorganisms use the energy resulting from degradation reactions.[6][14]

CS toxicity was highlighted by testing CBM solutions of different concentrations put in contact with suspensions of microorganisms of *Chlorella* sp., *Saccharomyces sp, Lactobacillus* sp and the ciliate *Paramecium* sp. It was observed that the most effective biological strain for CBM degradation it was *Chlorella* sp because it had the best cell development and regeneration under increasing concentration conditions, from 10 ppm to 150 ppm. https://doi.org/10.3390/toxics11030285. After the tests, it was observed that the cellular inhibition of *Chlorella* was due to the oxidative stress to which they were subjected, which generated a change in the growth of biomass compared to the control sample that was treated under identical conditions but without toxic. In order to deepen the scientific research, it was necessary to study the chemical reaction of CS in the aqueous environment by analyzing the degree of hydrolysis of CS over a period of time. At the same time, it is necessary to study the concentration of CBM metabolites that break down through conversion into 2-chlorobenzyl malononitrile (CSH_2), 2-chlorobenzaldehyde (o-CB), 2-chlorohippuric acid ($C_9H_8ClNO_3$) and thiocyanate.[12]

The toxic effects of CBM through studies on BarbusCapoetaTetrazone fish, highlighted the fact that the toxicity limits were established by LC50 estimated at 24 h 2.9 mg /L CBM and LC50 estimated at 72 h 1.2 mg /L CBM.[13] https://doi.org/10.37358/Rev

2. Experimental design, Materials and Methods

2.1. Aspects on the synthesis reaction of the o-chlorobenzylidene malononitrile

The following reagents were used in the synthesis reaction of *o*-chlorobenzylidene malononitrile (CBM): *o*-chlorobenzaldehide (C_7H_5ClO CAS 89-98-5, MW 140 g/mol), malononitrile ($C_3H_2N_2$ CAS 109-77-3, MW 66g/mol, and diethylamine ($C_4H_{11}N$, MW 73 g/mol, CAS Registry Number 109-89-7) as a catalyst. *o*-chlorobenzylidenmalononitrile was synthesized in-house by a condensing reaction between *o*-chlorobenzaldehide and malononitrile (Figure 1), in the presence of diethylamine. [15] : https://www.wuerth.ro/articole/fise-tehnice-securitate.html

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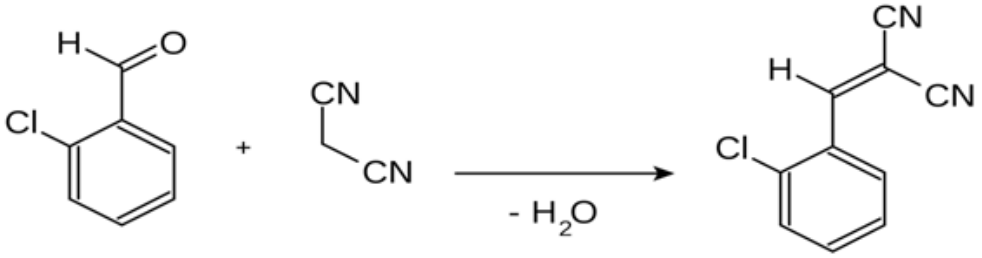

o-chlorobenzaldehide	malononitrile	*o*-chlorobenzylidenemalononitrile
C_7H_5ClO	$C_3H_2N_2$	$C_{10}H_5ClN_2$
CAS 89-98-5	CAS 109-77-3	CAS 2698-41-1
MW 140 g/mol	MW 66 g/mol	MW 188 g/mol

Figure 1. The synthesis reaction of the o-chlorobenzylidene malononitrile.

The two -CN groups in the malonic nitrile molecule are strongly electron-attracting and activate the CH_2 group to which they are linked, and because of this, malononitrile has the role of a methylene component in condensation reactions with aldehydes or ketones. The reaction takes place in the presence of catalysts such as secondary or tertiary amines (piperidine, diethylamine, etc.). In the synthesis reaction of CBM, we used diethylamine. The working installation for the synthesis of CBM is presented in Figure 2. Purity of CBM was checked by gas chromatography mass-spectrometry (GC-MS) in methylene chloride (DCM, Supelco) as organic solvent.

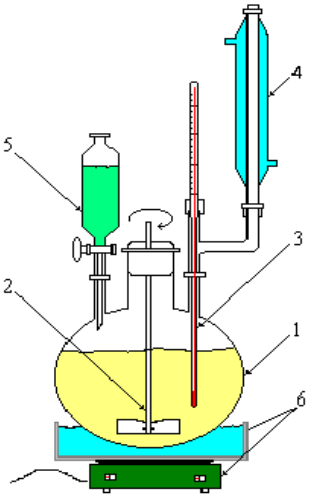

Figure 2. The working installation for the synthesis of CBM (1 - round bottom balloon, 2 - mechanical stirrer, 3 - thermometer, 4 - refrigerant, 5 - drip funnel).

2.2. Preparation of biological material

The biological material used in the experiments was Microalgae *Chlorella* sp. was obtained from the Culture Collection of Algae of Petroleum-Gas University of Ploiesti and it was adopted as model organism for experiments. The biological material was prepared in a specific culture medium and incubated in an ORBITAL SHAKER.[16] https://doi.org/10.3390/toxics11030285

An analytical balance OHAUS model AX224M was used to prepare the CBM solutions and the culture medium for the development of the algal suspension, the monitoring of cell viability during the experiment was done by using CELESTRON Microscope,

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model 4434, the biomass growth was evaluated by determining the optical density OD at 600 nm by using an UV-Vis spectrophotometer, model T85+, PG Instruments,

To be able to evaluate the toxicity of CBM on the culture of *Chlorella* sp. solutions of different concentrations of the substance (o-chlorobenzylidene malononitrile) were prepared by dissolution in water. CBM concentrations were prepared by ultrasonic dispersion by using an Ultrasonic SONICA S3 equipment - Soltec model.

In the tests, 2 replicate series were used (series A and series B), each series contains 7 test tubes (bioreactors) coded PA1-PA7, PA10 respectively PB1-PB7, PB10. In all test tubes 2 ml (104 cells /ml) from the algal suspension *Chlorella* sp. in the phase of exponential growth. PA10 and PB10 were diluted to 10 ml with distilled water and marked as blank. The rest of the test tubes were loaded up to 10 ml with the established concentrations of CBM, obtaining the following solutions in the bioreactors: PA1 (20 μg/ml), PA2 (40 μg/ml), PA3 (60 μg/ml), PA4 (80 μg /ml), PA5 (100 μg/ml), PA6 (120 μg/ml), PA7 (140 μg/ml), respectively PB1 (20 μg/ml), PB2 (40 μg/ml), PB3 (60 μg/ml ml), PB4 (80 μg/ml), PB5 (100 μg/ml), PB6 (120 μg/ml), PB7 (140 μg/ml). The tubes were incubated for 96h, by mechanical stirring in an ORBITAL SHAKER and were kept at a temperature of 35 °C with a photoperiodism of 12h day/night.

After 24 H from the incubation of the biological samples, from series A (PA1-PA7) and PA10 blank, FTIR characterizations were performed using the TRACER IR spectrophotometer. After the FTIR analysis, the samples were incubated for up to 96h.

From series A (PA1-PA7) and PA10 blank after 96 h of incubation and from series B (PB1-PB7), PB10 blank after 24 h of incubation, the metabolites resulting from the solubilization of the substance were analyzed by GC-MS as well as the degree of hydrolysis of CS in aqueous solution.

2.3. Chemical characterization and confirmation of the synthesized compound by GC-MS

A Thermo Electron chromatograph GC Trace 1310 equipped with a mass detector TSQ 9000 was used for the study. The GC was fitted with a 15 m x 0.25 mm i.d. TR-5MS bonded phase column (5% Phenyl Methyl Siloxan), 0.25 μmfilm thickness (Thermo Electron Corporation, USA). Helium (99,9999 purity) was used as the carrier gas with a flow rate of 1 ml/min. (36.2 cm/s) constant pressure. The oven temperature was held initially at 40°C for 1 minute, programmed from 40°C to 250°C at 10 $^{\circ}$C min^{-1}, and held at 250°C for 1 minute. Splitless injections of 1μL volume were made using a Thermo Electron Corporation AI800 autosampler. [17]

The compound was identified based on the reference chemical from the NIST Database.[15]

The spectrometric technique used was mass spectrometry, in electron impact ionization (EI) mode, for a mass range between 40 and 650 amu. The electron ionization MS operating conditions were as follows: ion source pressure approximately 1.5 x 10^{-5}torr; source temperature, 250°C; electron energy, 70 eV; and electron multiplier voltage +400 V relative to the autotune setting.

3. Results obtained

The CBM substance code CBM04 of 99% purity was synthesized in the laboratory according to the description in point 2.1. Following the GC-MS analysis, 3 other substances were identified in a percentage of 1%, according to table 1, coded as follows: o-Chlorobenzaldehydes (C1), o-Chlorobenzylmalononitrile (C2), 2-Chlorobenzalmalononitrile (CBM-C3), 2-(3-Chlorobenzylidene)malononitrile (C4).[18]

Table 1. The sample components, sample code CBM04, detected and identified by GC-MS.

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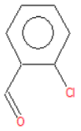
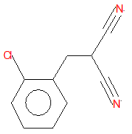
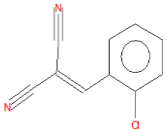
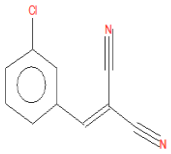
Chemical name	Retention time (minutes)	Molecular formula	Chemical structure	Molecular weight (g/mole)	CAS Registry Number
o-chlorobenzaldehide (C1)	6.10	C_7H_5ClO		140	89-98-5
o-chlorobenzylmalononitrile (C2)	11.31	$C_{10}H_7ClN_2$		190	40915-55-7
2-chlorobenzalmalononitrile (CBM-C3)	11.93	$C_{10}H_5ClN_2$		188	2698-41-1
2-(3-chlorobenzylidene)malononitrile (C4)	12.82	$C_{10}H_5ClN_2$		188	2972-73-8

The following figures show the total ion chromatogram (Figure 3), the EI mass spectra(Figure 4) of the synthesized chemical (CBM - retention time of 11.93 min.) and the reference chemical from the NIST Database.[15]

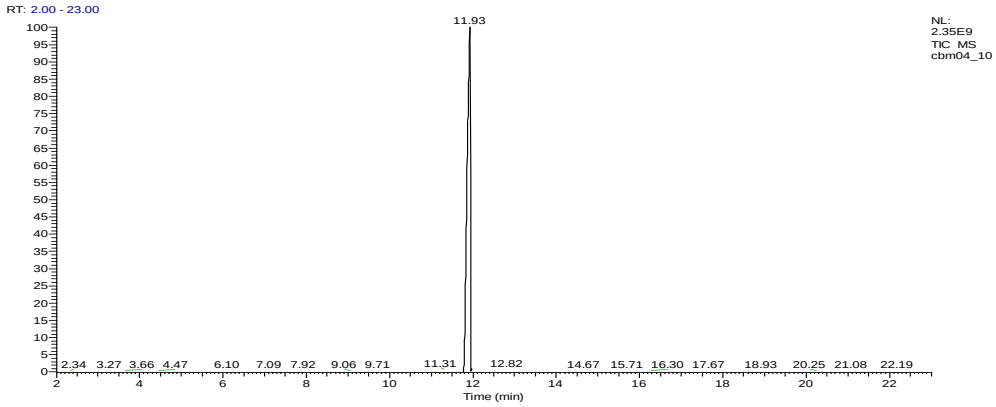


Figure 3. The total ion chromatogram for the sample containing CBM (retention time of 11.93 min.), sample code CBM04.

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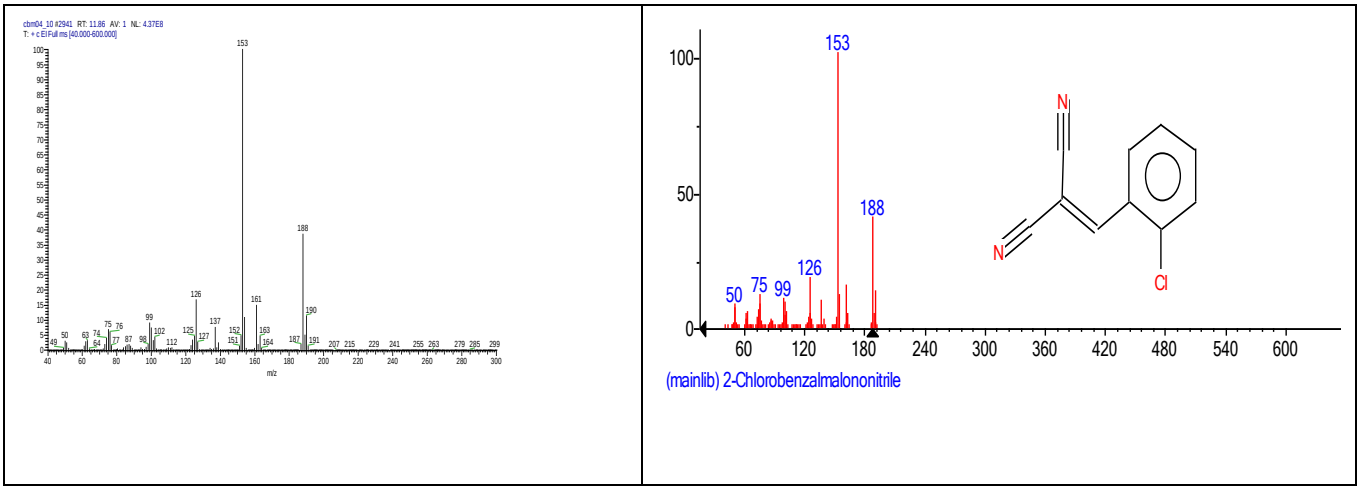
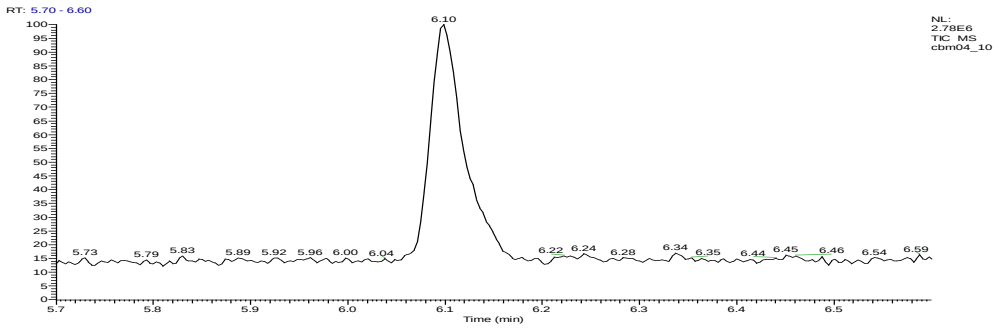
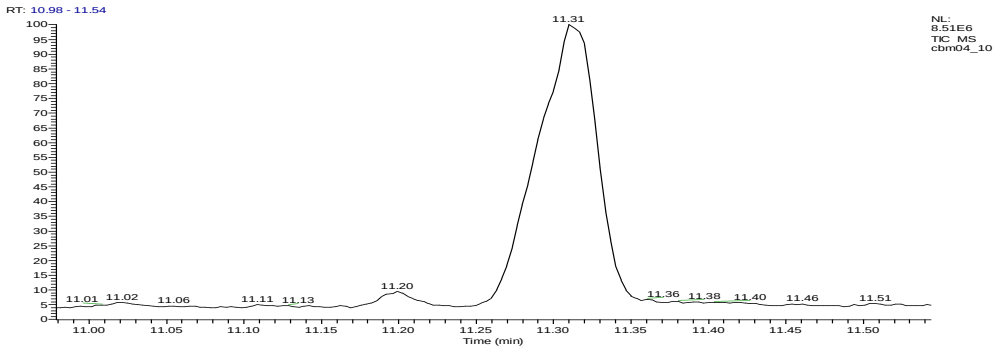


Figure 4. EI mass spectra of o-chlorobenzylidenemalononitrile (CBM) - own synthesis - (left), compared with the reference chemical from the NIST database (right).

EI chromatogram supporting identification of o-CB, retention time of 6.10 minutes, o-chlorobenzylmalononitrile, retention time of 11.31 minutes, and 2-(3-chlorobenzylidene)malononitrile, retention time of 12.82 minutes, the other component of the reference chemical, CBM, are presented in Figure 5, and the mass spectra of the chemicals, in comparison with the references from the NIST database are presented in Figure 6.



a)



b)

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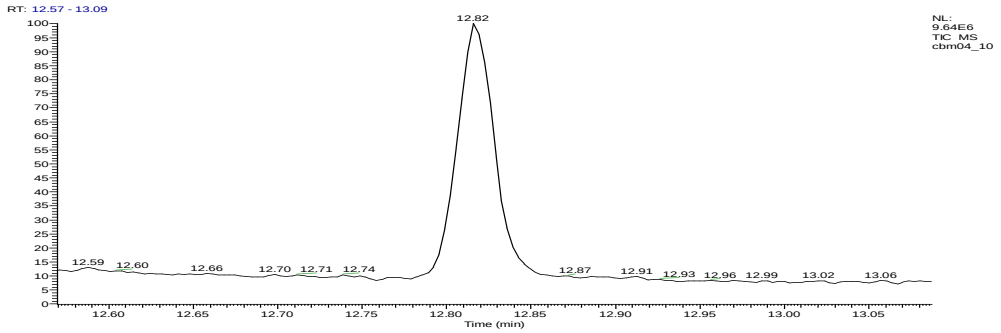
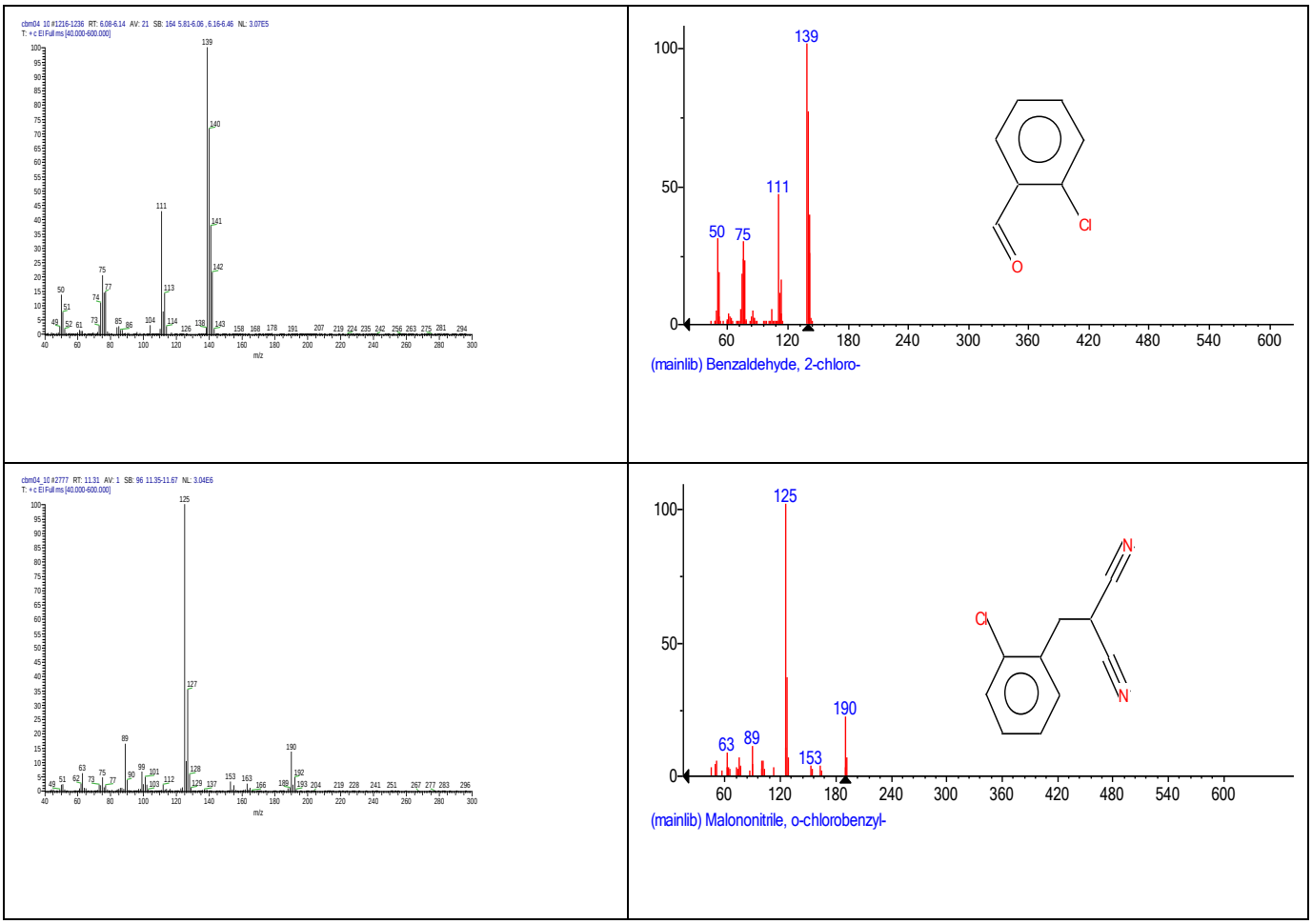


c)

Figure 5. EI chromatograms supporting identification of o-CB, retention time of 6.10 minutes (a), o-chlorobenzylmalononitrile, retention time of 11.31 minutes (b), and 2-(3-chlorobenzylidene)malononitrile, retention time of 12.82 minutes (c).



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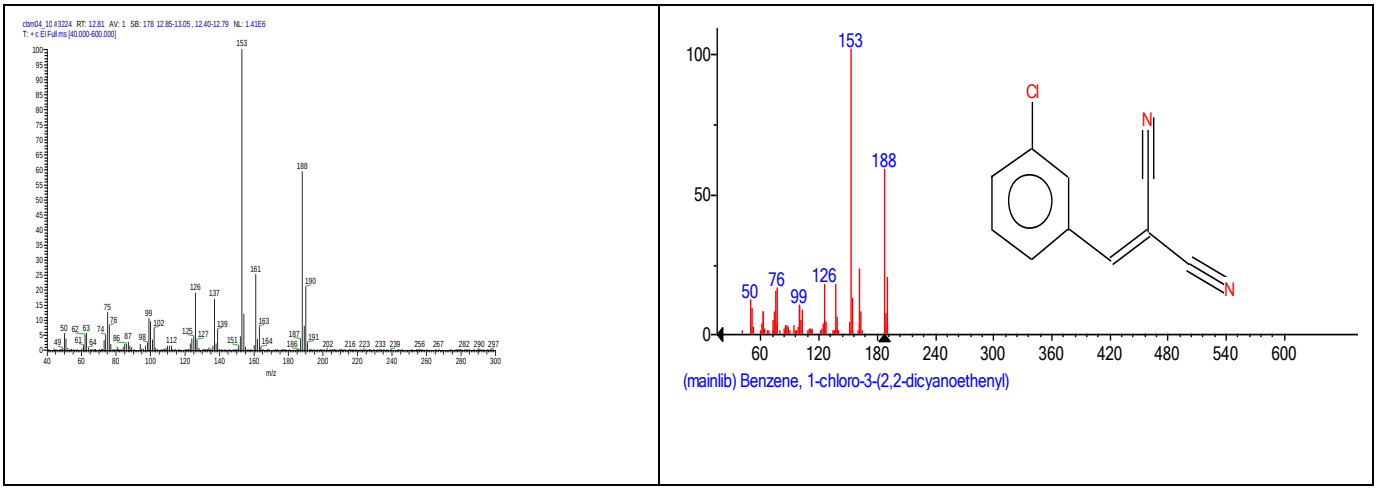

Figure 6. EI mass spectra of o-CB, o-chlorobenzylmalononitrile, and 2-(3-Chlorobenzylidene)malononitrile - (left), compared with the reference chemical from the NIST database (right).

3.1. Calibration curve of CBM and 2-CB

A Thermo Electron chromatograph GC Trace 1310 equipped with a mass detector TSQ 9000 was used for the study. Additional equipments are as follows: KERN ABJ electronic balance.The dilution series of CBM in DCM (µg/ml), sample code: S01CBM 2000 (µg/ml), S02CBM 300 (µg/ml), S03CBM 200 (µg/ml), S04CBM 150(µg/ml), S05 CBM 100 (µg/ml), S06 CBM 50(µg/ml), S07CBM 20(µg/ml), S08 CBM 10(µg/ml), S09CBM 4(µg/ml), S10CBM 2(µg/ml).

The dilution series of 2-CB in DCM (µg/ml), sample code: S01CB 2700 (µg/ml), S02CB 189 (µg/ml), S03CB 135 (µg/ml), S04CB 81(µg/ml), S05CB 54 (µg/ml), S06 CB 27 (µg/ml), S07CB 13b(µg/ml), S08 CB 5.4 (µg/ml), S09CB 2.7 (µg/ml),

3.1.1. Method quantification

The quantification of the interest chemicals (CBM and 2-CB) was based on nine-point calibration curve (for CBM) and 8-point calibration curve (for 2-CB) obtained by plotting the area ratio of the target compounds and concentration of the calibration standards.

Excellent linearity of response is observed over the concentration range of 2 to 300 ppm for CBM and 2,7 to 189 ppm for 2-CB. Typical results of multi-level concentrations are shown in Figure 7 where the correlation coefficient is 0.999 for CBM and also for 2-CB.

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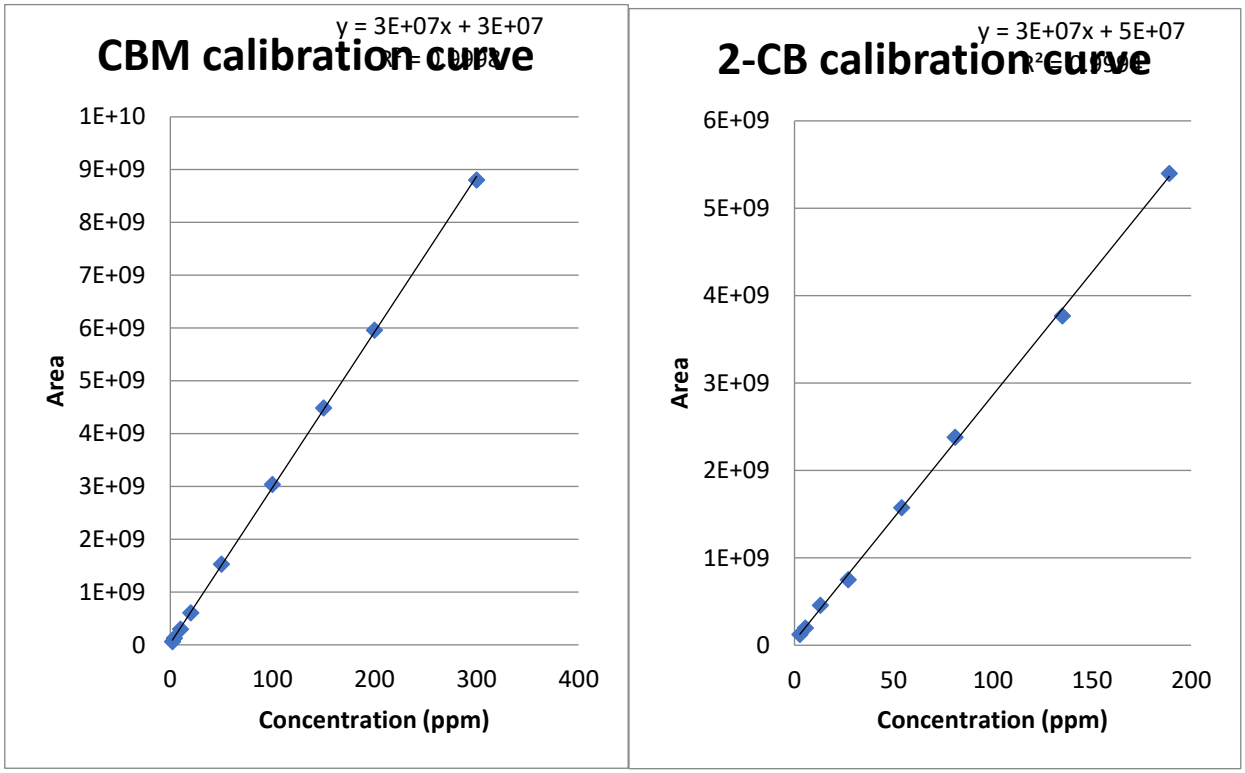

Figure 7. Calibration curves of CBM and 2-CB.

3.1.2. Extraction of the spiked samples with CBM and biological suspension

Biological samples were prepared by extraction of 10mL of the incubated water sample with 3 mL of DCM by manual agitation for 10 minutes. The organic extract was dried on sodium sulphate anhydrous for 1 hour, filtered through a HPLC filter unit (0.45 µm, Millipore Millex-HV), transferred to silanized glass autosampler vials and analyzed by GC-MS. A water sample which wasn't spiked with CBM, just contain the biological suspension, was used as blank (sample code P10blank).

In Figure 8 are compared the blank sample, coded PA10BLK, that means the biological suspension without CBM, the CBM dilution of 20 ppm, sample code S07CBM, and the sample with the microorganism and CBM at 20 ppm, with 24 hours incubation, coded PB124EI and with 96 hours incubation, coded PA196EI. In Figure 9 are expanded region of 2-7 minutes, with the evidence of 6.10 minutes of o-chlorobenzaldehide in the reference sample, sample code S07CBM. No peak of this chemical in the samples PB124EI and PA196EI.

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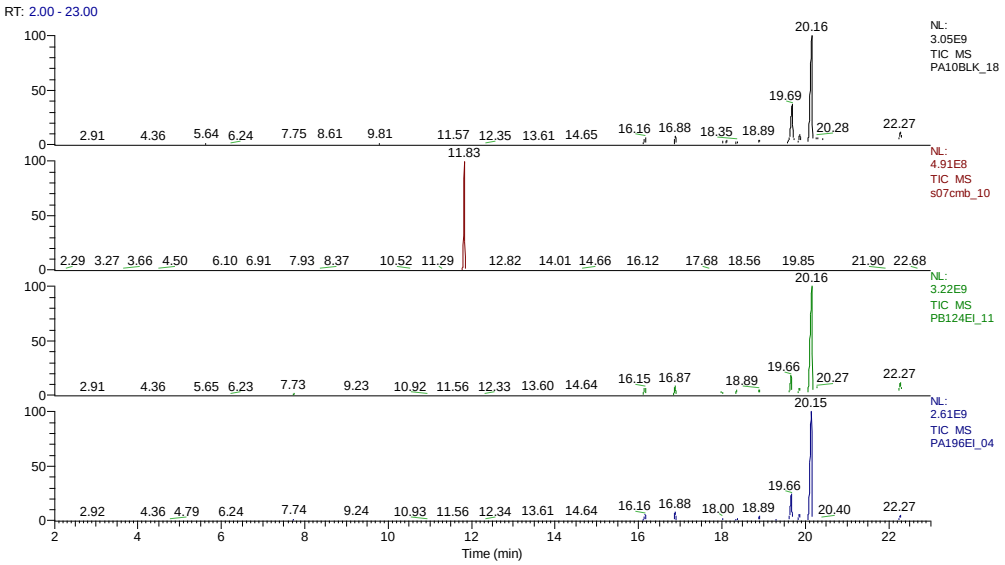

Figure 8. EI total ion chromatogram of a blank sample, coded PA10BLK, CMB solution of 20 ppm, sample code S07CBM, and the 2 solutions of 24 and 96 hours incubation, sample code PB124EI and PA196EI. CBM has the retention time of 11.83 minutes.

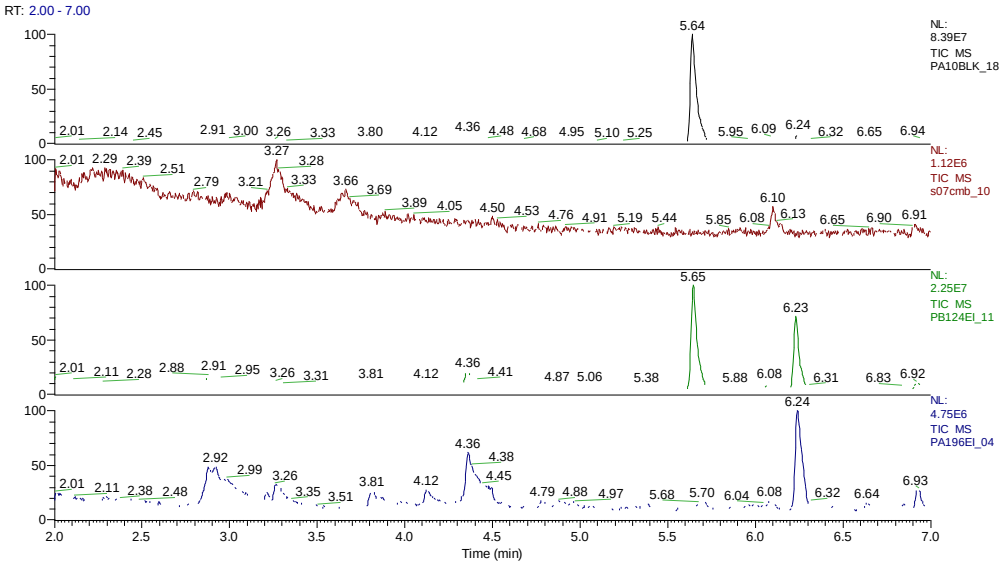

Figure 9. Expanded region of 2-7 minutes, with the evidence of 6.10 minutes of o-Chlorobenzaldehide in the reference sample, sample code S07CBM. No peak of this chemical in the samples PB124EI and PA196EI.

In the sample reference of CBM, sample code S07CBM at the retention time of 6.10 minutes, is found a minor chromatographic peak that support the identification of o-chlorobenzaldehide, from the synthesis reaction. The absence of this chemical from the sample codes PB124EI and PA196EI is proved by the mass spectra of the chromatographic peaks at 6.10 minutes (Figure 10). Figure 11 highlights the fact that there is no peak signal for substances C2 and C4.

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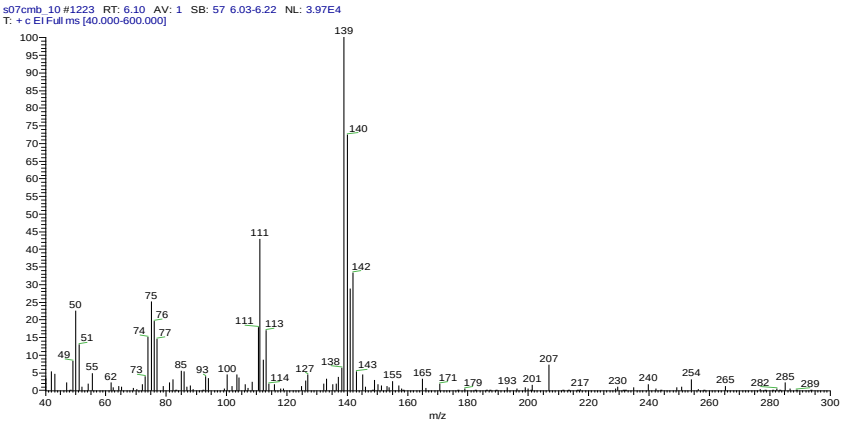
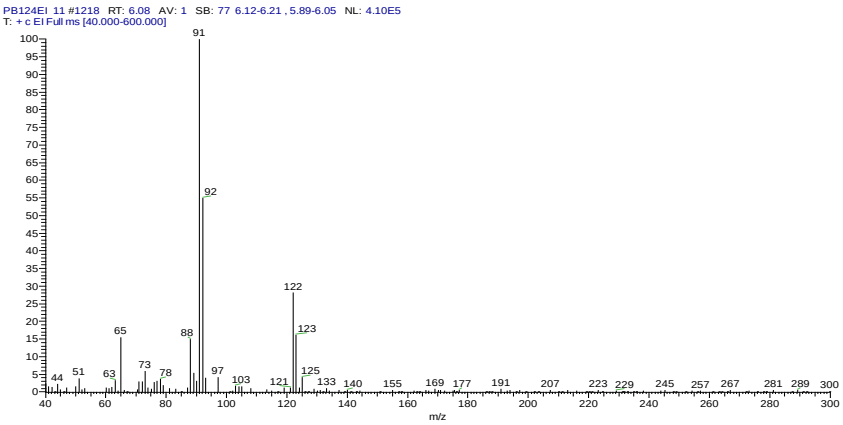
Expanded region of 7-11 minutes and 11-14 minutes, with the evidence of 11.83 minutes of *o*-chlorobenzylidene malononitrile (CBM) in the reference sample, sample code S07CBM. No peak of this chemical in the samples PB124EI and a trace level in sample PA196EI.





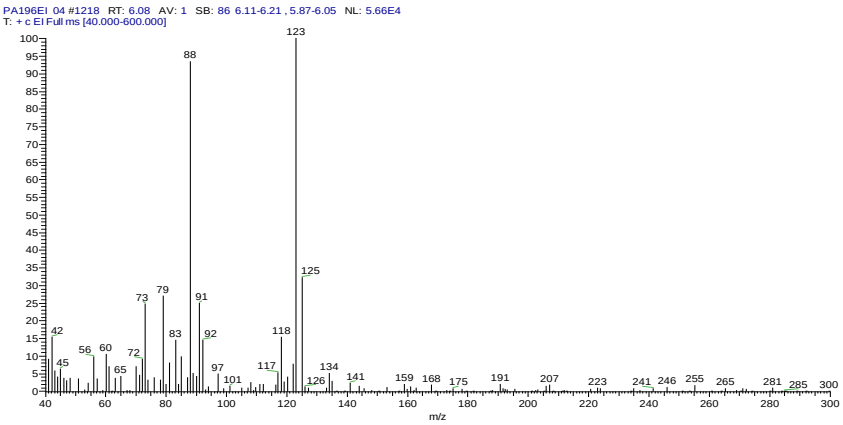


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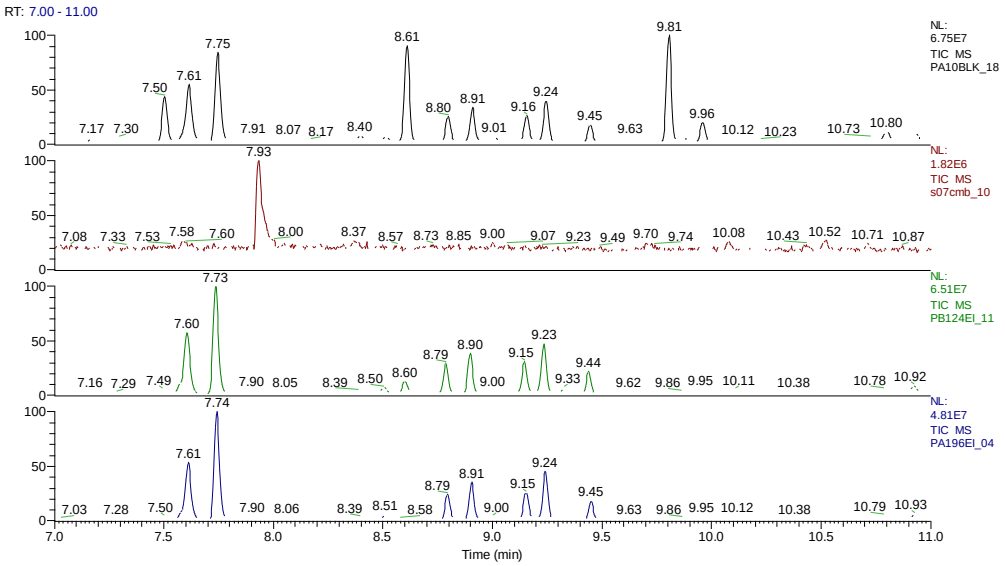
Figure 10. The absence of the o-chlorobenzaldehide, retention time of 6.10 minutes,.from the samples PB124EI and PA196EI



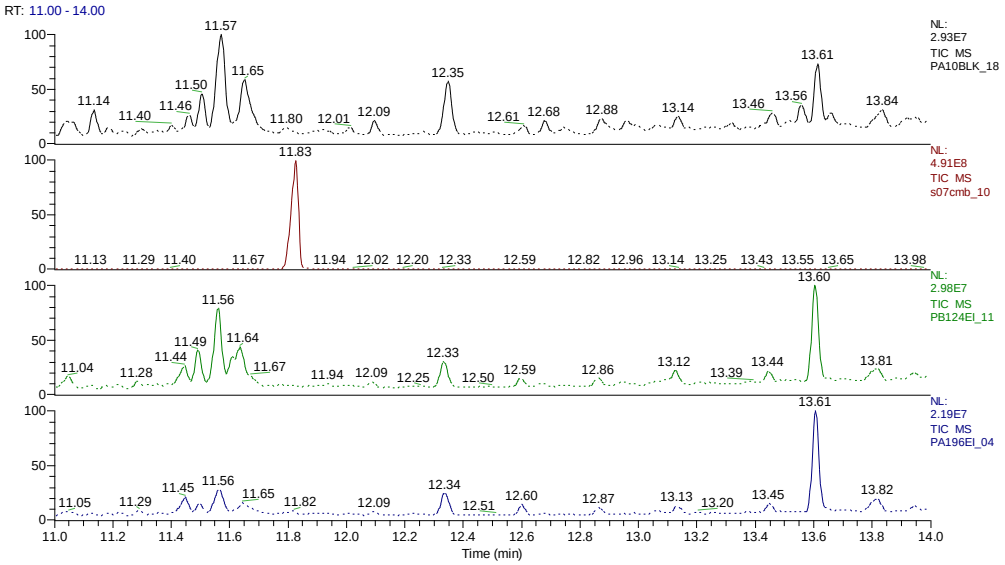


Figure 11. Expanded region of 7-11 minutes and 11-14 minutes, with the evidence of 11.83 minutes of *o*-chlorobenzylidene malononitrile (CBM) in the reference sample, sample code S07CBM. No peak of this chemical in the samples PB124EI and a trace level in sample PA196EI. No peak for chemicals C2 and C4.

In Figure 12, the absence of the o-chlorobenzylidene malononitrile (CBM) is observed, retention time of 11.83 minutes, from the samples PB124EI. In Figure 13 is presented expanded region of 14-19 minutes and 19-23 minutes, with no chemicals of interest. In Figure 14 is presented the series PA196EI- PA796EI, in comparison with the CBM solution from the calibration curve, of 100 ppm, sample code S05CBM. In Figure 15 is presented expanded regions of 2-7 minutes and 5.9-6.5 minutes, with the evidence of 6.10

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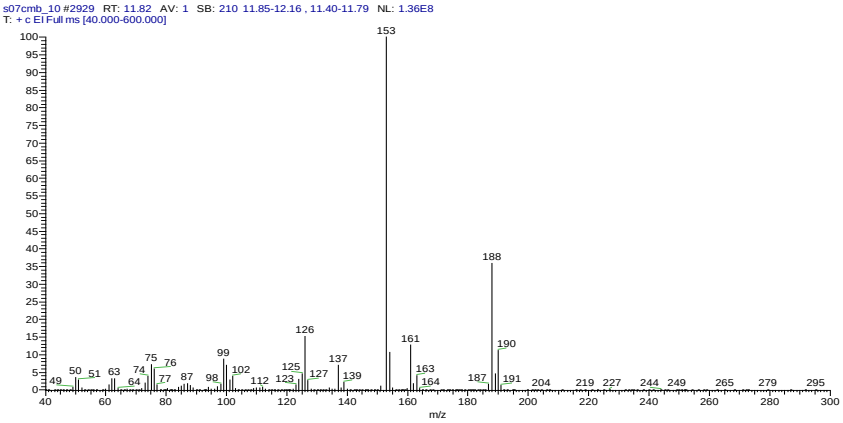
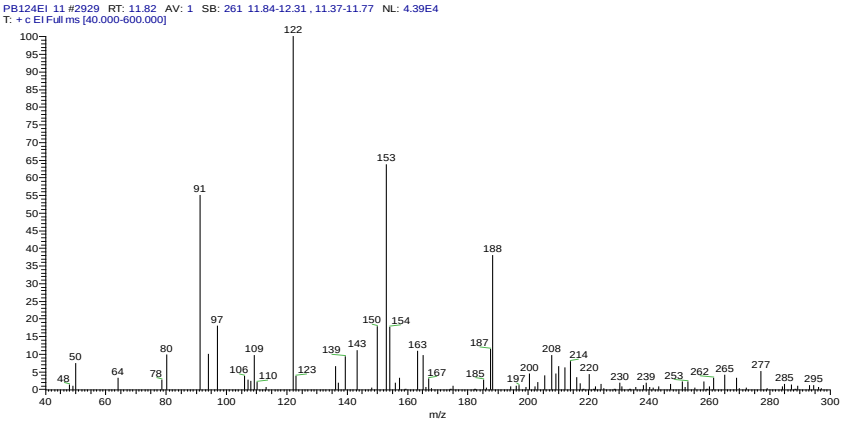
minutes of o-Chlorobenzaldehide in the reference sample, sample code S05CBM. No peak of this chemical in the samples PA196EI- PA796EI. Figure 16 presented expanded region of 7-11 minutes and 11-14 minutes, with the evidence of 11.86 minutes of *o*-chlorobenzylidene malononitrile (CBM) in the reference sample, sample code S05CBM. A trace level was observed in the sample PA196EI and no peak in the other samples; no peak for chemicals C2 and C4. Figure 17 shows expanded region of 14-19 minutes and 19-23 minutes, with no chemicals of interest.





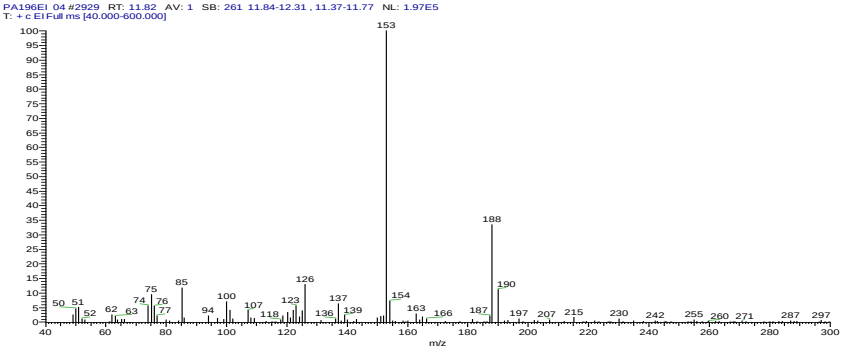


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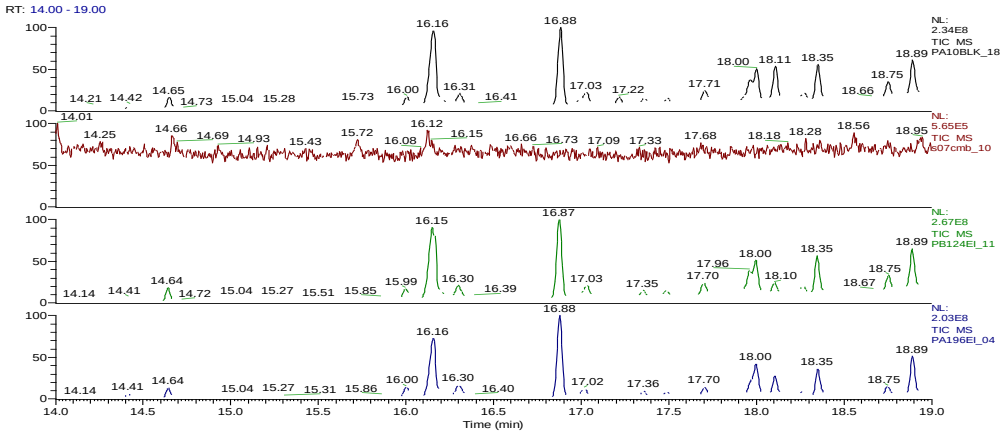
Figure 12. The absence of the *o*-chlorobenzylidene malononitrile (CBM),.retention time of 11.83 minutes, from the saples PB124EI.



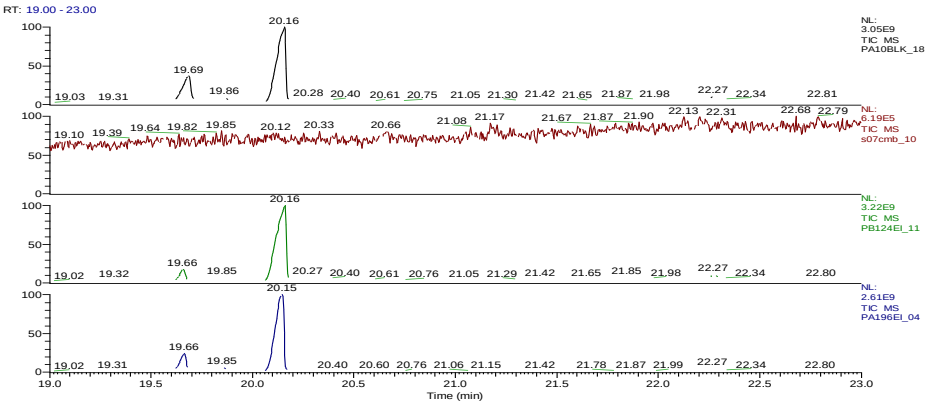


Figure 13. Expanded region of 14-19 minutes and 19-23 minutes. No chemicals of interest.

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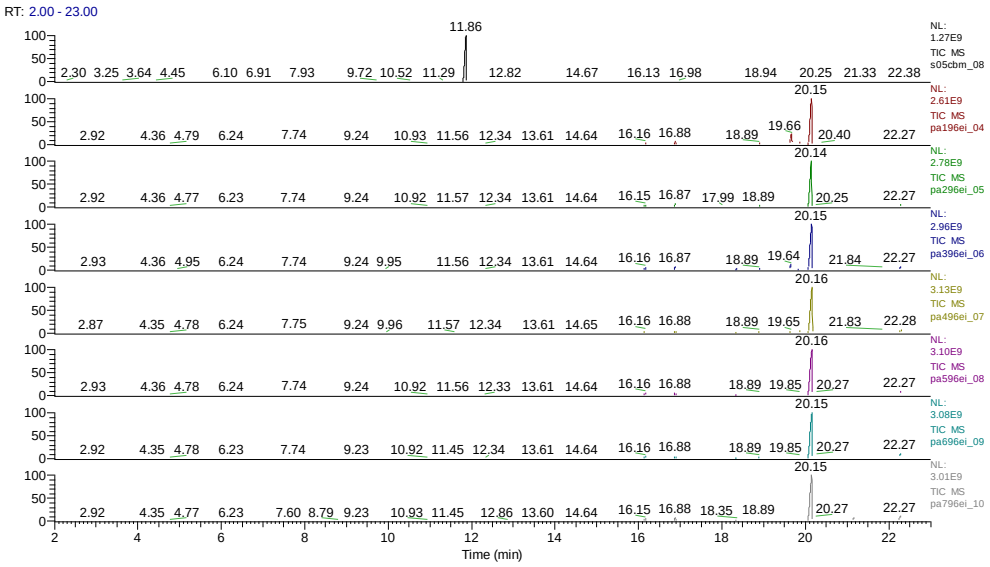
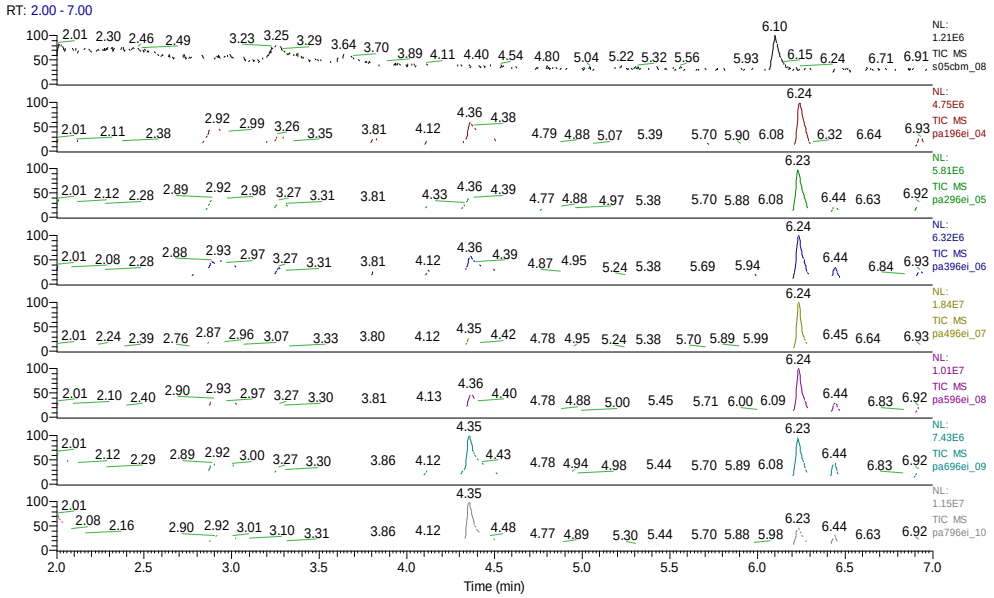


Figure 14. The series PA196EI- PA796EI, in comparison with the CBM solution from the calibration curve, of 100 ppm, sample code S05CBM.



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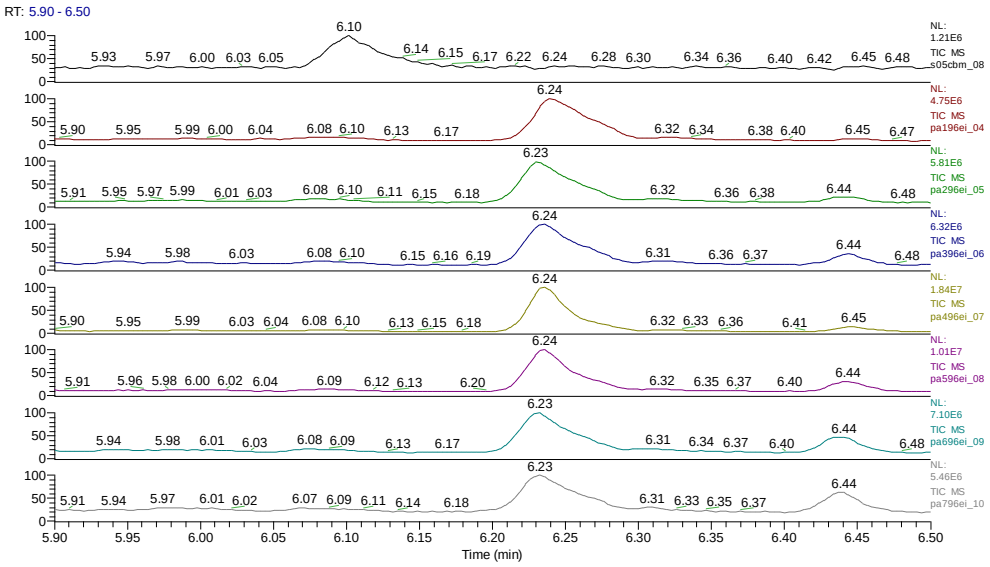
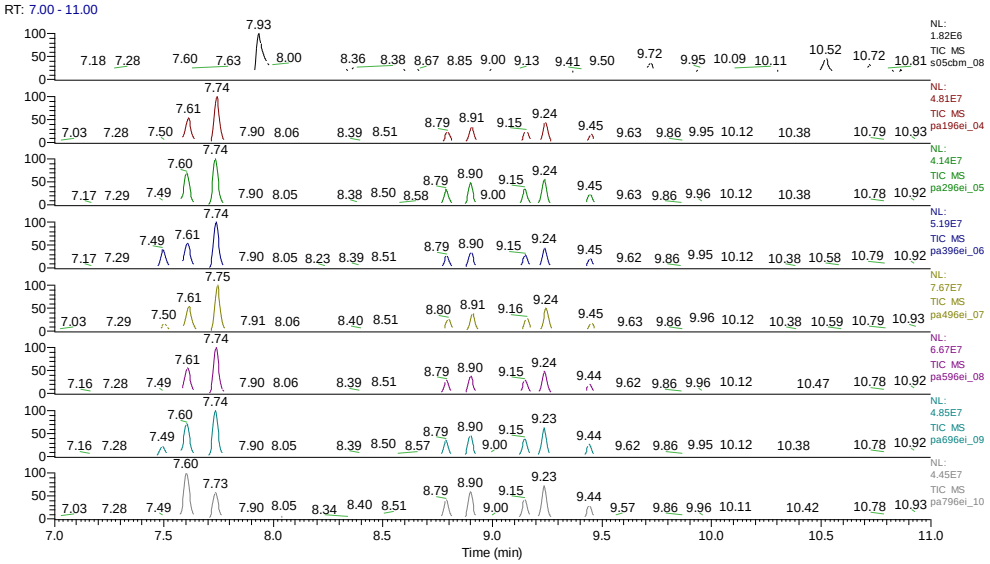


Figure 15. Expanded regions of 2-7 minutes and 5.9-6.5 minutes, with the evidence of 6.10 minutes of o-Chlorobenzaldehide in the reference sample, sample code S05CBM. No peak of this chemical in the samples PA196EI- PA796EI.



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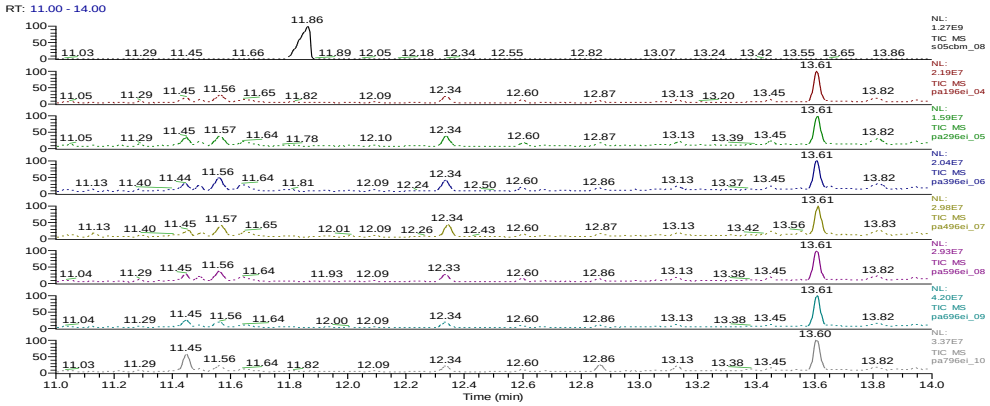
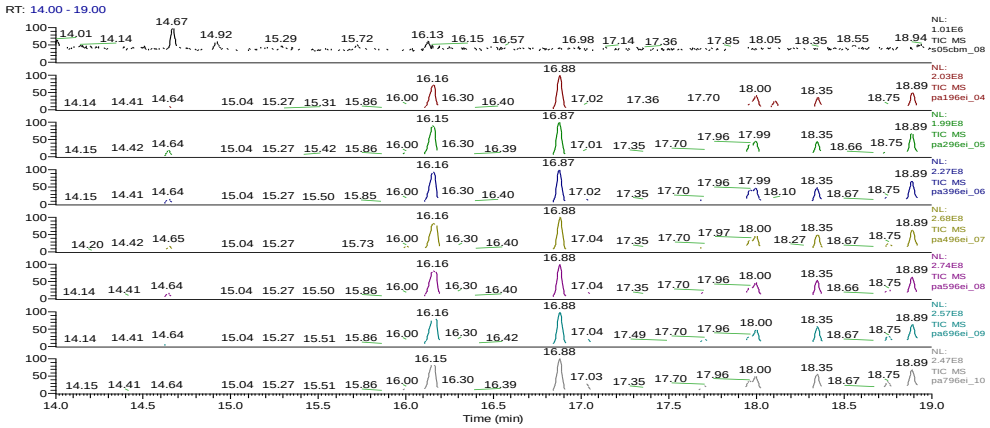


Figure 16. Expanded region of 7-11 minutes and 11-14 minutes, with the evidence of 11.86 minutes of *o*-chlorobenzylidene malononitrile (CBM) in the reference sample, sample code S05CBM. A trace level was observed in the sample PA196EI and no peak in the other samples. No peak for chemicals C2 and C4.



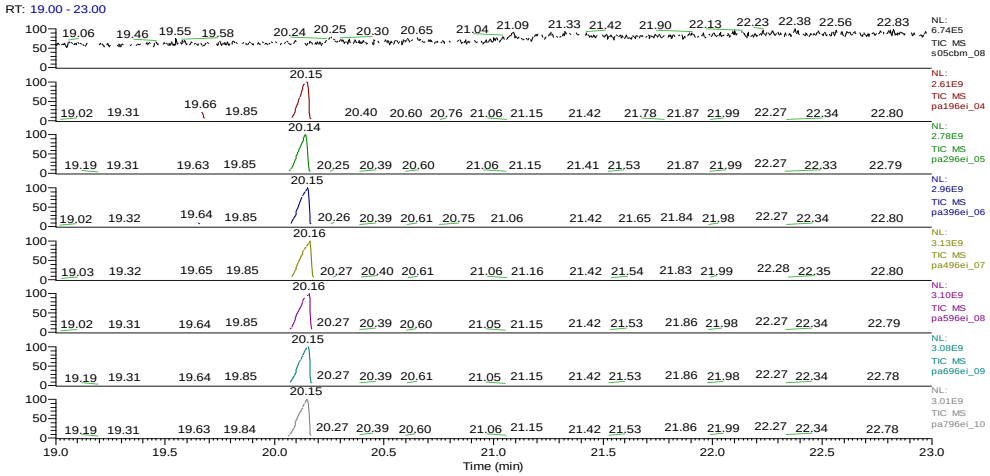


Figure 17. Expanded region of 14-19 minutes and 19-23 minutes. No chemicals of interest.

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Figure 18 presents the series PB124EI- PB724EI, in comparison with the CBM solution from the calibration curve, of 100 ppm, sample code S05CBM. In Figure 19 are presented expanded regions of 2-7 minutes and 5.9-6.5 minutes, with the evidence of 6.10 minutes of o-chlorobenzaldehide in the reference sample, sample code S05CBM; no peak of this chemical in the samples PB124EI- PB724EI. In Figure 20 are presented expanded regions of 7-11 minutes and 11-14 minutes, with the evidence of 11.86 minutes of *o*-chlorobenzyl-idene malononitrile (CBM) in the reference sample, sample code S05CBM. No peak of this chemical in the samples PB124EI- PB724EI. No peak for chemicals C2 and C4. In Figure 21 is presented expanded region of 14-19 minutes and 19-23 minutes, with no chemicals of interest.

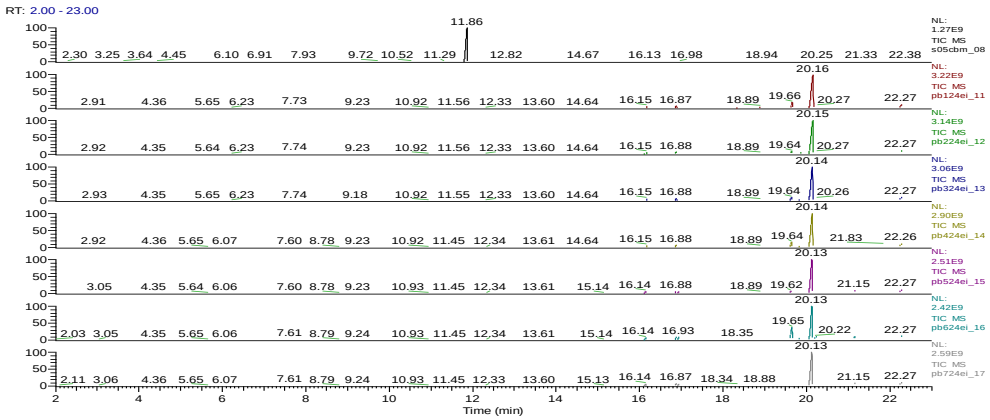
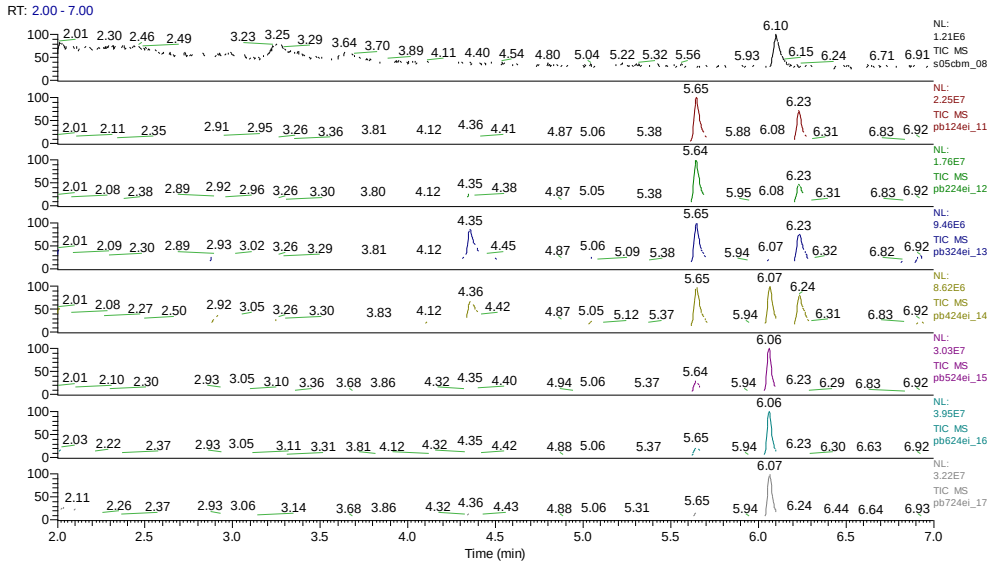


Figure 18. The series PB124EI- PB724EI, in comparison with the CBM solution from the calibration curve, of 100 ppm, sample code S05CBM.



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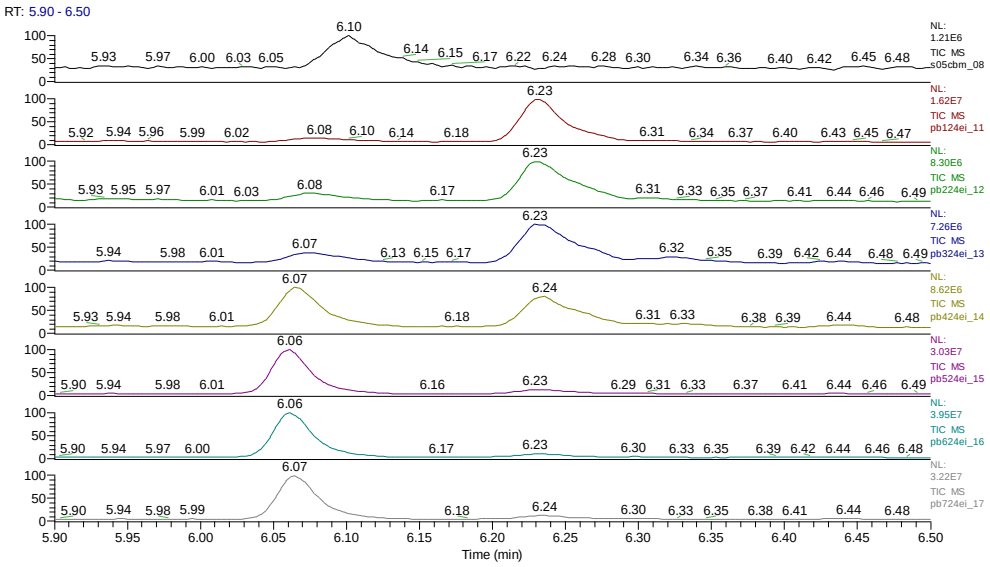
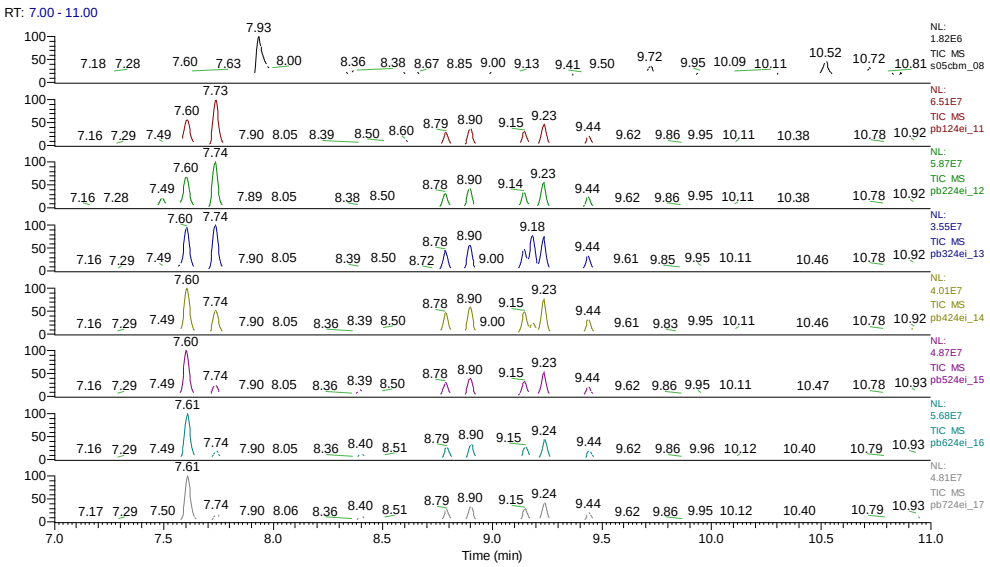


Figure 19. Expanded regions of 2-7 minutes and 5.9-6.5 minutes, with the evidence of 6.10 minutes of o-Chlorobenzaldehide in the reference sample, sample code S05CBM. No peak of this chemical in the samples PB124EI- PB724EI.



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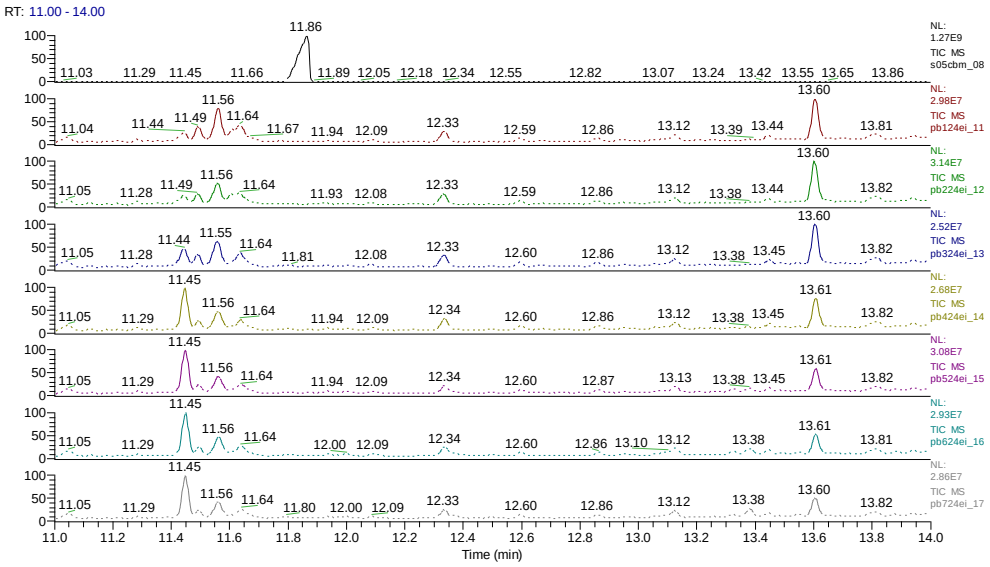
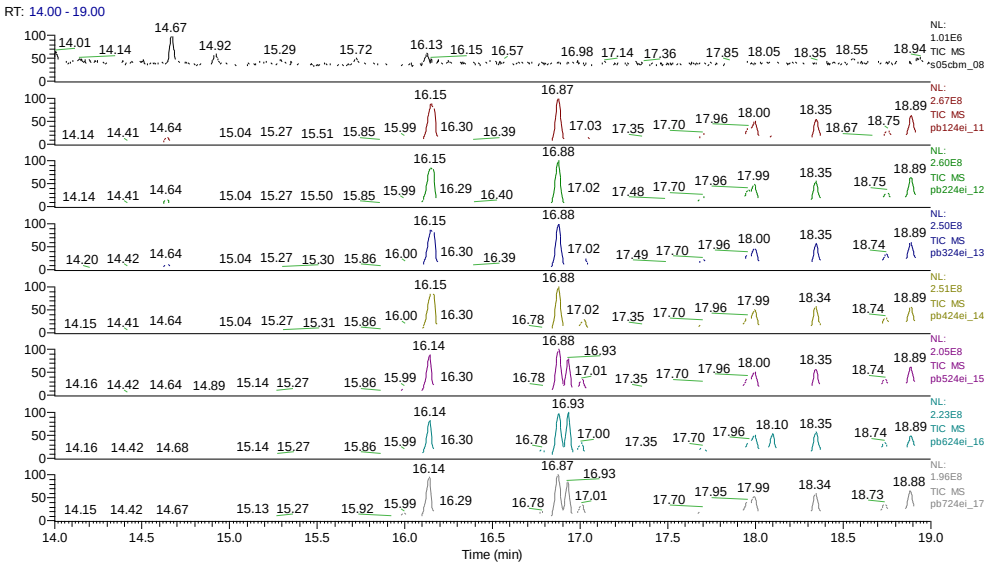


Figure 20. Expanded region of 7-11 minutes and 11-14 minutes, with the evidence of 11.86 minutes of *o*-chlorobenzylidene malononitrile (CBM) in the reference sample, sample code S05CBM. No peak of this chemical in the samples PB124EI-PB724EI. No peak for chemicals C2 and C4.



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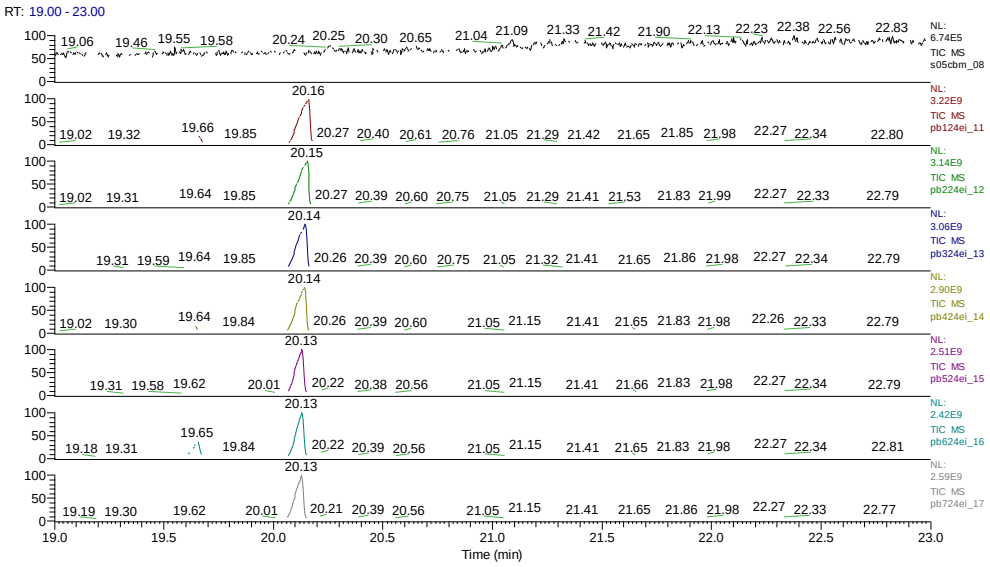

Figure 21. Expanded region of 14-19 minutes and 19-23 minutes. No chemicals of interest.

Vibrational spectroscopy including Fourier Transform Infrared (FT-IR), has been used to study the chemical composition of biological suspension for samples coded PA1-PA7, PA10 blank.

All test tubes were inoculated with 2 ml (104 cells/ml) of the algal suspension *Chlorella* sp. in the phase of exponential growth. PA10 and PB10 were diluted to 10 ml with distilled water. The rest of the test tubes were loaded up to 10 ml with the established concentrations of CBM, obtaining the following solutions in the bioreactors: PA1 (20 µg/ml), PA2 (40 µg/ml), PA3 (60 µg/ml), PA4 (80 µg /ml), PA5 (100 µg/ml), PA6 (120 µg/ml), PA7 (140 µg/ml), respectively PB1 (20 µg/ml), PB2 (40 µg/ml), PB3 (60 µg/ml ml), PB4 (80 µg/ml), PB5 (100 µg/ml), PB6 (120 µg/ml), PB7 (140 µg/ml), The tubes were incubated by mechanical stirring in an ORBITAL SHAKER and were kept at a temperature of 35 °C with a photoperiodism of 12h day/night. After 24 H from the incubation of the biological samples, FTIR characterizations were performed using the Fourier Transform Infrared Spectrometer. Each peak was assigned a functional group. FTIR spectra distinct bands and were assigned a range of vibrationally active chemical groups.

The surface chemistry of the samples was studied using an FTIR Spectrophotometer (Shimadzu IR TRACER-100, Kyoto, Japan) in the region of 4000–400 cm^{-1}. The spectra were examined and compared in order to identify the functional groups (Figure 22). In Figure 23, we can see differences obtained by FTIR scanning of control sample PA10 compared to sample PA7 which contained 140 ppm CBM. The differences obtained indicate FTIR spectra distinct bands and were assigned a range of vibrationally active chemical groups: lipids bands at 2930-2850 cm^{-1}, protein amide I band mainly (C=O) stretching 1583-1700 cm^{-1}, Protein as (- CH_2) and as (- CH_3) bending of methyl, Lipid as (CH_2) bending of methyl 1425-1477 cm^{-1} and pectin (bands at 1610 cm^{-1}, 1424 cm^{-1}); Carboxylic group of esters (bands 1720-1700 cm^{-1}).[19]http://dx.doi.org/10.1080/05704920902907440 , https://doi.org/10.1002/jat.767

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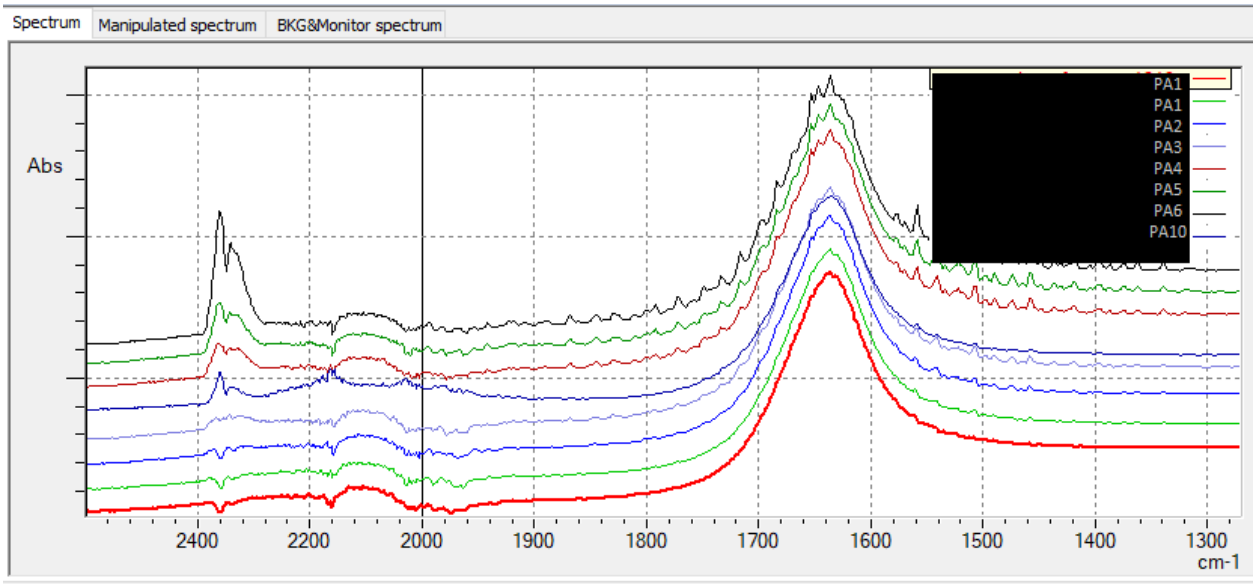

Figure 22. FTIR spectra of PA10 blank, PA1-PA7 after 24h of incubation.

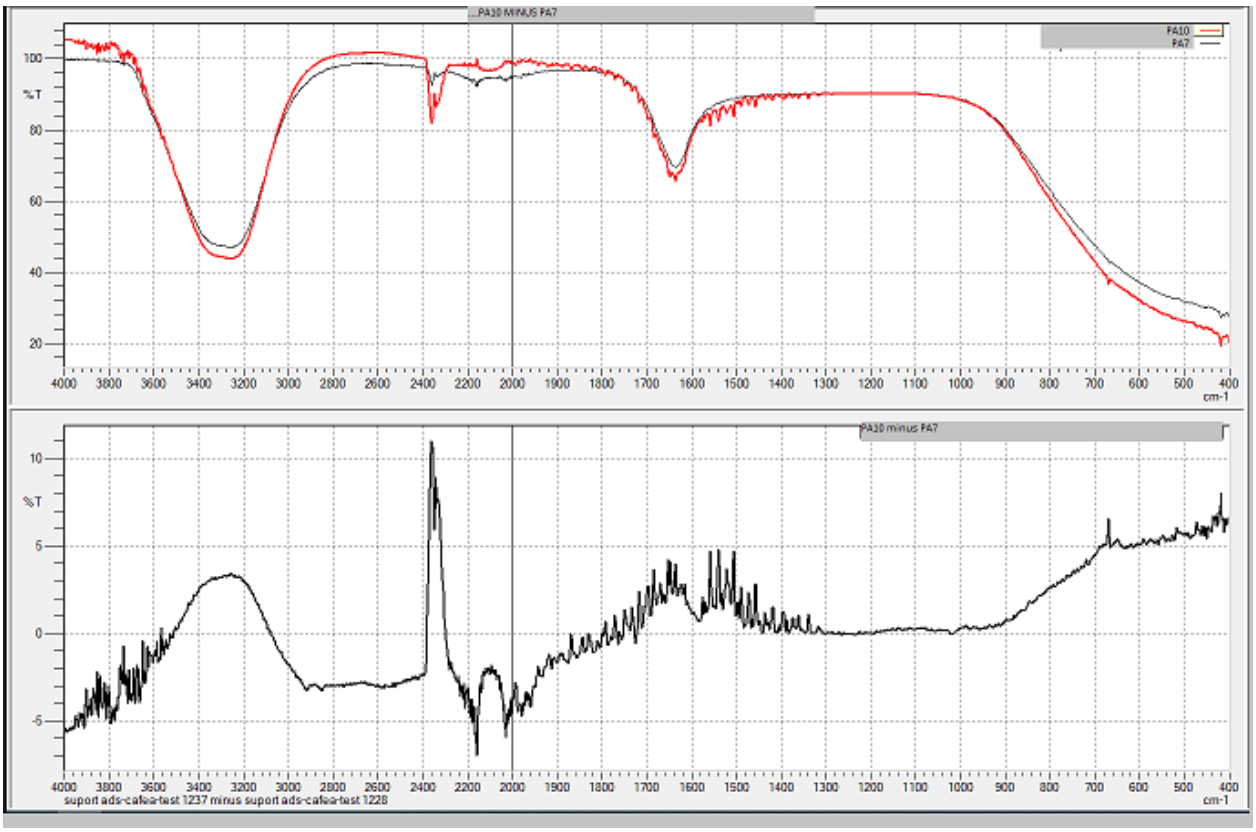

Figure 23. FTIR spectra with the difference between PA10blank and PA7 after 24h of incubation.

The degree of hydrolysis of CBM in water was analyzed by 5 samples of 20 mg of CBM each dissolved in 40 ml of tap water and extracted with 10 ml of DCM, at different time periods, as follows: CBMHYT0 – reference sample, CBMHYT30 – 30 minutes, CBMHYT60 – 60 minutes, CBMHYT90 – 90 minutes, CBMHYT24 – 24 hours. The toxic did not completely solubilize CBM in tap water, but it was the same volume of extraction solvent for each sample. The chromatographic peak at 2.52 minutes corresponds to malononitrile and at the retention time of 6.10 minutes we found 2-CB, as hydrolysis products.

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The sample preparation procedure included extraction of the sample with DCM, manual stirring for 10 min, separation of aqueous and organic phases, drying over anhydrous sodium sulfate for 1h, filtered through an HPLC filter unit (0.45 µm, Millipore Millex-HV), transferred to silanized glass autosampler vials and analyzed by GC-MS.

The chromatographic peak areas of interest and degree of hydrolysis of CBM in tap water are shown in Table 2.

Table 2. The hydrolysis grade of CBM in the tap water.

Sample code/Chemical	Malononitrile (Tr 2.52 min.)	2-CB (Tr 6.10 min.)	CBM (Tr 12.14 min.)	Mass of CBM (mg)	Hydrolysis grade (%)*
			GC area		
CBMHYT0	0	64347004	52890605168	17.62	-
CBMHYT30	8974787	814678251	52777364000	17.58	0.21
CBMHYT60	16137050	1185660077	50024457883	16.66	5.42
CBMHYT90	17355009	1257452113	49620527787	16.53	6.19
CBMHYT24h	264863119	10837442146	40513601453	13.49	23.41

*Hydrolysis grade (%) = (initial mass – final mass)/ initial mass x 100./ is calculated according with the calibration curve of CBM.

The content of 2-CB is increased from the reference sample, CBMHYT0, of 0.005 mg to 0.402 mg (according to the calibration curve of 2-CB) in the sample extracted after 90 minutes from the contact of CBM with tap water, and 3.59 mg in the sample extracted after 24 hours from the contact of CBM with tap water (sample code CBMHYT24h). The obtained results were used to study CBM hydrolysis in the samples that were in contact with the biological suspension. Comparison between the hydrolysis of CBM in tap water, at room temperature, 24 hours, sample code CBMHYT24h and the sample with biological suspension and CBM, a period time of incubation of 24 hours, sample code PB724EI (with a concentration of CBM of 140 ppm). Total ion chromatograms for the organic extracts (2-23 minutes), sample code of CBMHYT0 – CBMHYT24h are presented in Figure 24,

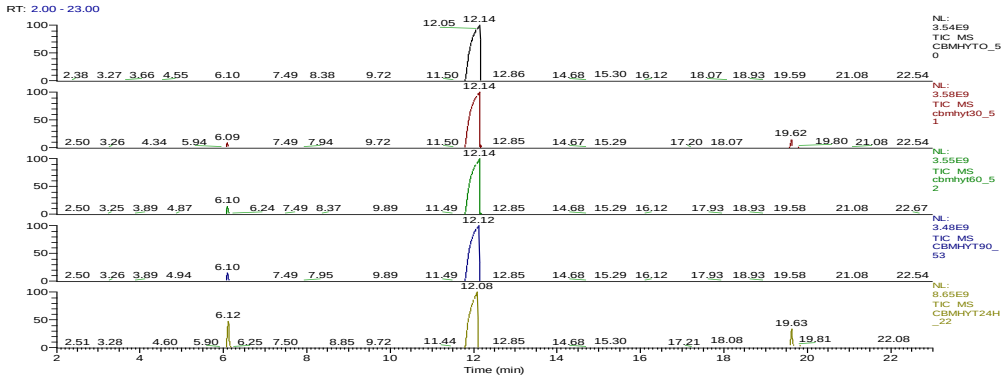

Figure 24. Total ion chromatograms for the organic extracts, sample code CBMHYT0, CBMHYT30, CBMHYT60, CBMHYT90, and CBMHYT24h.

Malononitrile chemical was detected and identified at the retention time of 2.50 minutes in the samples CBMHYT30, CBMHYT60, CBMHYT90 and CBMHYT24h and the total ion chromatograms are identified in Figure 25.

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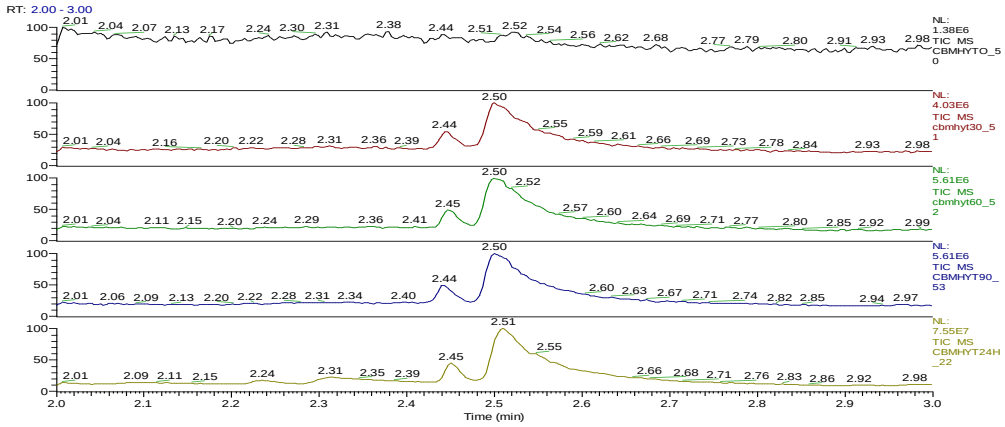

Figure 25. Total ion chromatograms of the organic extracts, coded CBMHYT0, CBMHYT30, CBMHYT60, CBMHYT90, and CBMHYT24h domain of 2-3 minutes; chemical malononitrile with retention time of 2.50 minutes.

2-CB chemical was detected and identified at the retention time of 6.10 minutes in the samples CBMHYT0, CBMHYT30, CBMHYT60, CBMHYT90 and CBMHYT24h, and the total ion chromatograms are identified in figure 26.

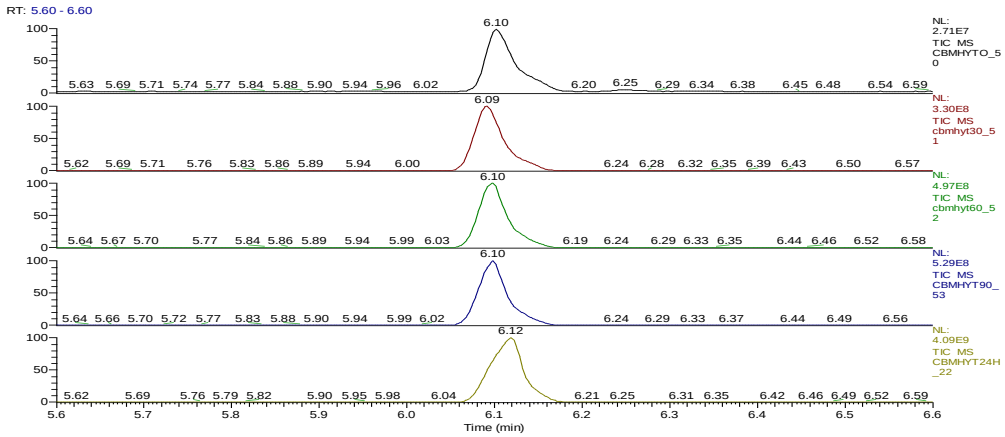

Figure 26. Total ion chromatograms of the organic extracts, coded CBMHYT0, CBMHYT30, CBMHYT60, CBMHYT90, and CBMHYT24h, domain of 5.6-6.6 minutes; chemical 2-CB with retention time of 6.10 minutes.

In figure 27 are presented the mass spectra of malononitrile and 2-CB, the hydrolysis products of CBM, confirmed by the reference chemicals from NIST database.

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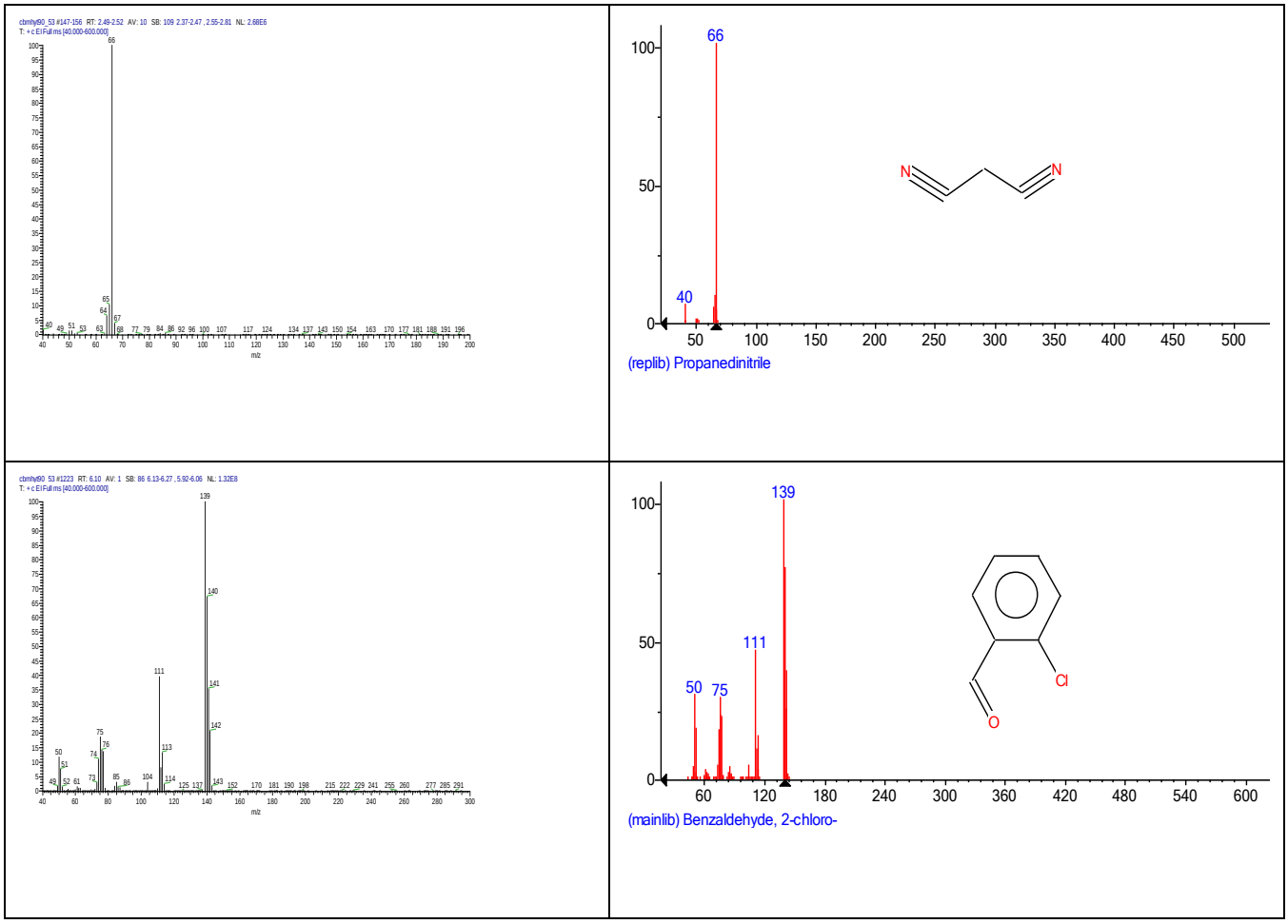

Figure 27. Mass spectra of malononitrile ($C_3H_2N_2$, M 66 g/mol, CAS 109-77-3) and 2-CB, from the sample code CBMHYT90, confirmed by the reference chemicals from the NIST database.

Comparison between the hydrolysis of CBM in tap water, room temperature, 24 hours, sample code CBMHYT24h and the sample with biological suspension and CBM, a period time of incubation of 24 hours, sample code PB724EI is presented in Figure 28, were is compared the hydrolysis of CBM in tap water, room temperature, 24 hours, sample code CBMHYT24h and the sample with biological suspension and CBM, a period time of incubation of 24 hours, sample code PB724EI (with a concentration of CBM of 140 ppm).

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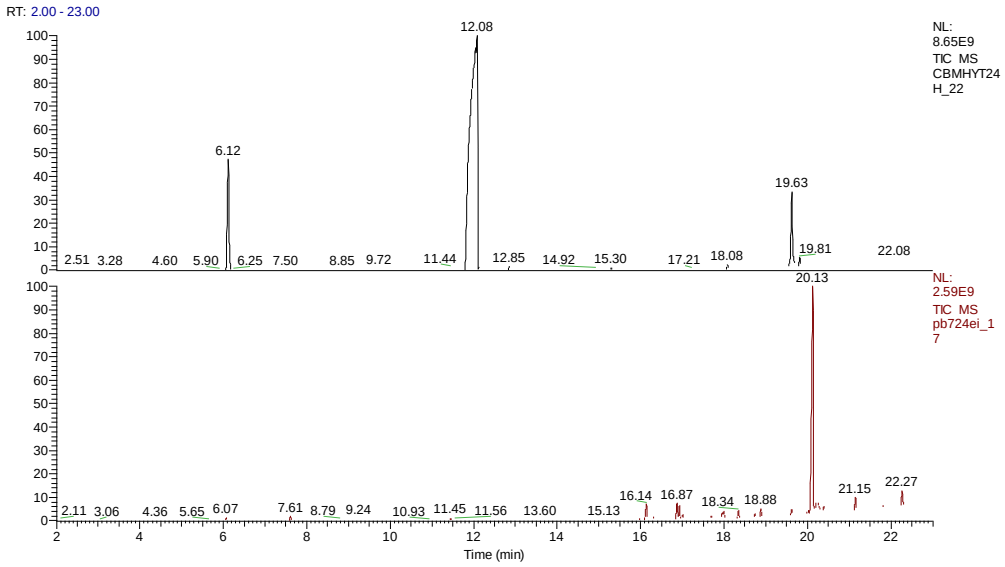

Figure 28. EI total ion chromatogram of the organic extract of a hydrolysis reaction of CBM after 24 hours, sample code CBMHYT24h and the solution of 24 hours incubation, sample code PB724EI.

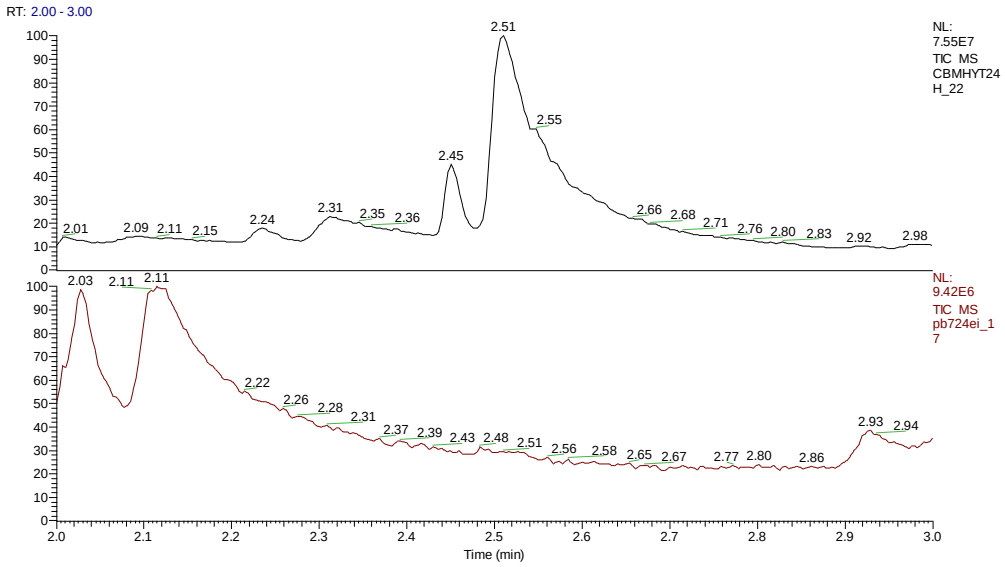

Figure 29. EI total ion chromatogram of the organic extract of a hydrolysis reaction of CBM after 24 hours, sample code CBMHYT24h and the solution of 24 hours incubation, sample code PB724EI, domain of 2-3 minutes. No evidence of malononitrile in PB724EI.

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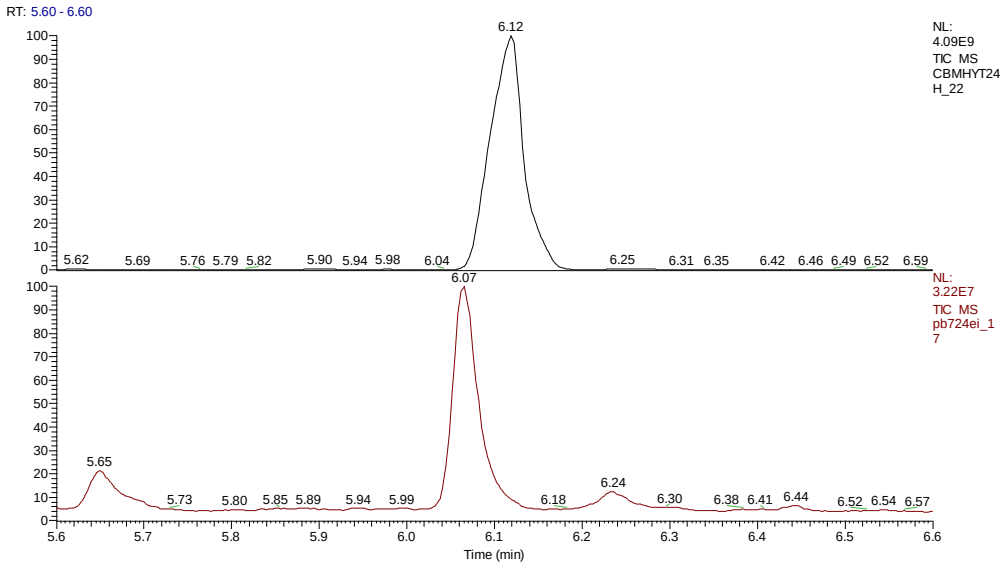
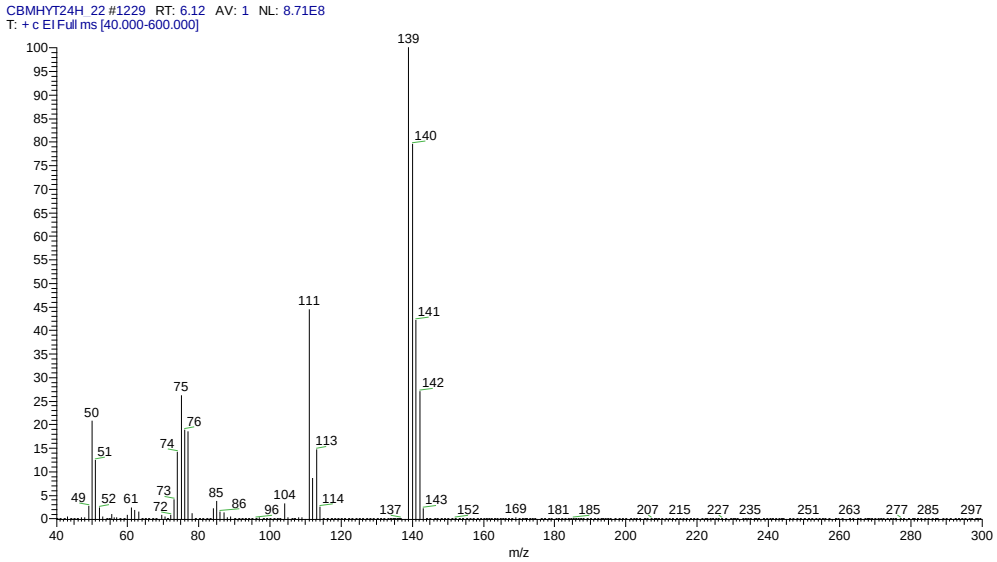


Figure 30. EI total ion chromatogram of the organic extract of a hydrolysis reaction of CBM after 24 hours, sample code CBMHYT24h and the solution of 24 hours incubation, sample code PB724EI, domain of 5.6-6.6 minutes. No evidence of 2-CB in PB724EI.

Mass spectra of 2-CB from CBMHYT24h (retention time of 6.12 minutes) and no evidence of the chemical in PB724EI are presented in Figure 31.



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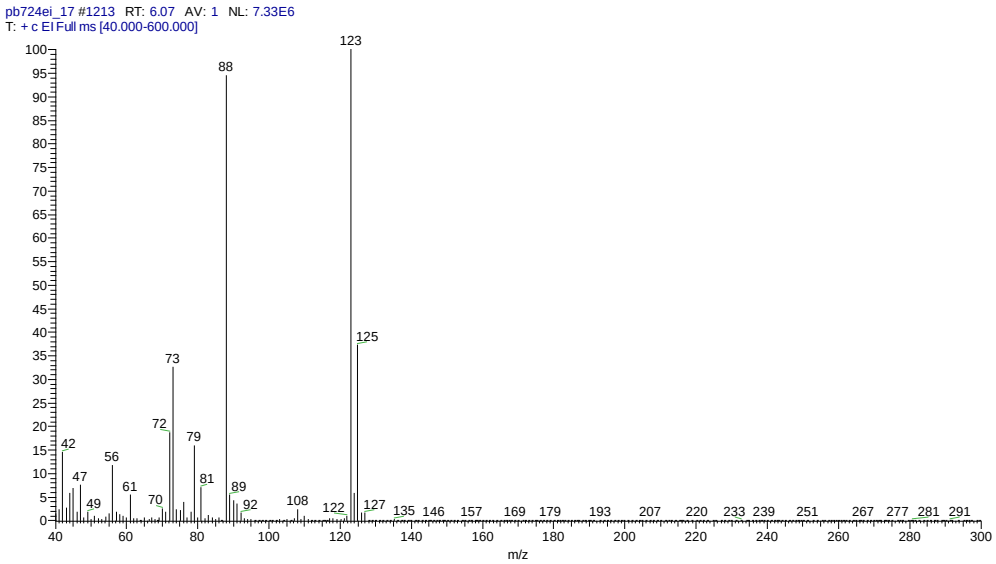

Figure 31. No evidence for chemical 2-CB in sample PB724EI.

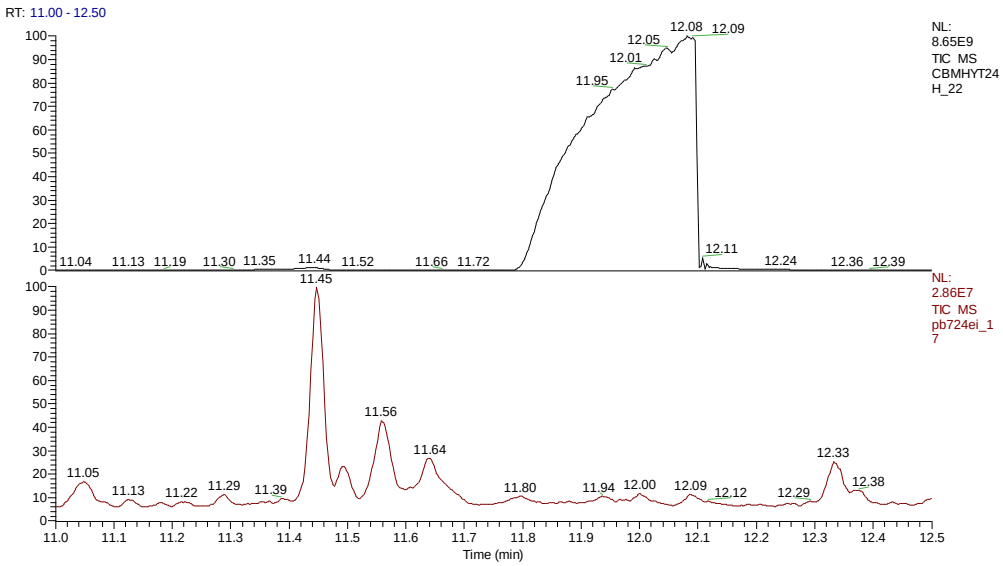

Figure 32. EI total ion chromatogram of the organic extract of a hydrolysis reaction of CBM after 24 hours, sample code CBMHYT24h and the solution of 24 hours incubation, sample code PB724EI, domain of 11-12.5 minutes. No evidence of CBM (retention time of 12.05 minutes in sample CBMHYT24h) in PB724EI.

4. Conclusions

The two -CN groups in the malonic nitrile molecule are strongly electron-attracting and activate the CH2 group to which they are linked, and because of this, malononitrile has the role of a methylene component in condensation reactions with aldehydes or ketones.

After the tests, it was observed that the cellular inhibition of the *Chlorella* microorganism was due to the oxidative stress to which the biological suspension was subjected,

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which generated a change in the composition of the biomass compared to the control sample that was treated under identical conditions but without toxic. The differences observed by FTIR scanning showed that in the sample that contained the concentration of 140 ppm CBM, weaker signals of specific functional groups were identified compared to the control sample that was not under oxidative stress. However, the concentration of 140 ppm does not cause total damage to the culture of *Chlorella* sp.

The degree of hydrolysis of the CBM sample dissolved in water indicates that at 30 minutes the degree of hydrolysis is 0.21%, at 60 minutes, the degree of hydrolysis is 5.42%, after 90 minutes, the degree of hydrolysis is 6.19%, and after 24 hours. the degree of hydrolysis is 23.41%.

The results obtained after analyzing the degree of hydrolysis in the samples that contained the biological suspension indicated that no CBM metabolites were detected in any biological sample, regardless of the test concentration, analyzed 24 h after incubation.

CBM in contact with microorganisms are used by them in the biochemical processes in which they are involved, the retention time and biodegradation capacity led to an increased effect of the metabolism of toxic substances, in metabolic reactions, organic substances are the source of carbon and energy for the biochemical processes. Thus, we conclude that the suspension of *Chlorella* sp. consumed the entire amount of CBM from the samples. The tests prove that the biological material can be used to decontaminate the affected areas.

In future tests, we propose to analyze the effect on *Chlorella* sp. of the toxic CBM, by studying the minimum inhibitory concentration it generates on axenic cultures, looking for information that could be useful in the medical field for the treatment of specific diseases.

The evaluation of the chemical risk induced by the persistence of chemical substances used in military operations requires the development of economically feasible methods of remediation of contaminated areas. The integration of effective depollution methods that decontaminate and at the same time reduce the risks of chemical pollution requires the use of biological methods as a viable soliution.

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Data Availability Statement:

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Conflicts of Interest:

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