

Natural Deep Eutectic Solvents as extractants and the potential of macroalgae beach wrack as a source of biostimulants for agricultural use

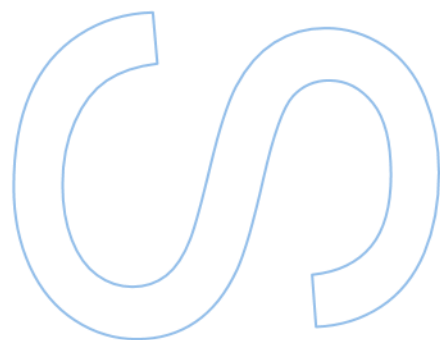
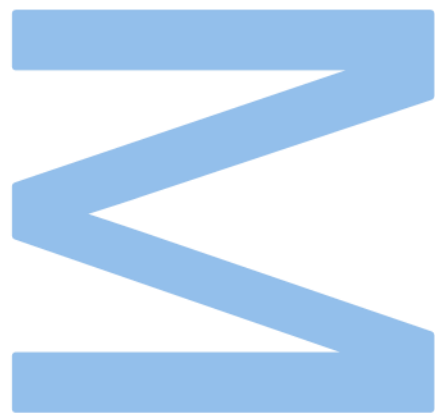
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Dedicated to 10-year-old me who thought we wouldn't make it this far in life

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Resumo

Devido às alterações climáticas, os florescimentos de algas descontrolados tornaram-se mais frequentes, levando a uma série de problemas ambientais e socioeconómicos. As macroalgas arrojadas na praia (popularmente conhecido como “Sargaço”) exigem uma estratégia de gestão melhorada, pois atualmente, esta biomassa é removida e destinada a aterro. Os solventes eutéticos naturais profundos (NADES) surgiram como alternativas “verdes” aos solventes orgânicos tradicionais, mas as suas características físico-químicas, comportamento e toxicidade permanecem pouco compreendidos. Este estudo visou preencher estas lacunas de conhecimento ao investigar as propriedades dos NADES e a sua eficácia como extratores, enquanto se explorou também sargaço como uma potencial fonte de fitohormonas e reguladores do crescimento de plantas (PGRs). Os resultados revelam que os NADES apresentam viscosidade e densidade elevadas, o que exige um ajuste cuidadoso para melhorar a sua viabilidade e eficiência de extração. O ajuste da polaridade dos NADES (e das outras características físicas-“tuning”) é também crucial para otimizar o seu desempenho como solventes. Apesar da caracterização efetuada destes parâmetros, a falta de dados introduz incertezas e desafios significativos na comparação dos NADES com outros solventes. Além disso, as interferências destes extratores em vários métodos utilizados (e.g. Folin-Ciocalteu’s, DMBA), destacam a necessidade de protocolos analíticos adaptados especializados e métodos de calibração para os NADES. Apesar disto, NADES apresentaram toxicidade baixa e potencial estimulante quando aplicados diretamente em sementes em germinação, reforçando a sua classificação enquanto “verdes” e as vantagens de aplicação comparativamente aos solventes convencionais. Esta ausência de padrões estabelecidos para NADES complica a sua avaliação e potencial aplicação. Apesar dos desafios com a qualidade dos extratos e a precisão dos dados, o nosso estudo sublinha o potencial das macroalgas arrojadas na praia como uma valiosa fonte de biomassa e dos NADES como extratores. A análise da biomassa estudada com recurso a LC-MS/MS, revelou a presença de ácido indole-3-acético (IAA), mostrando o potencial bioestimulante deste material orgânico para possível aplicação na agricultura, assim como um guia para o estudo da presença e efeito de fitohormonas em macroalgas. Este projeto representa um passo fundamental na integração de solventes inovadores com materiais tradicionais, visando melhorar aspetos ambientais e práticos dos processos.

Palavras-chave: Sargaço; Reguladores de crescimento; Solventes Eutéticos Profundos Naturais, Ácido Indole-3-acético

Abstract

Due to climate changes, uncontrolled algal blooms have become more frequent, and from there a range of environmental and socio-economic problems have arisen. Stranded macroalgae on the beach (macroalgae beach wrack) urge an improved management strategy, as of current standard, this biomass is removed and landfilled. Natural deep eutectic solvents (NADES) have emerged as promising green alternatives to traditional organic solvents, yet their physical-chemical characteristics, behaviour, and toxicity remain inadequately understood. This study aimed to address these knowledge gaps by investigating the properties of NADES and their efficacy as extractors, while also exploring macroalgae beach wrack as a potential source of phytohormones and plant growth regulators (PGRs). Our findings reveal that NADES exhibit high viscosities and densities, which necessitate careful tuning to enhance their viability and extraction efficiency. The modification of NADES polarity through this tuning process is crucial for optimizing their performance as solvents. Despite successful characterization of these parameters, the lack of comprehensive data introduces significant uncertainties and challenges in comparing NADES with other solvents. Additionally, our analyses were hampered by solvent interferences (e.g. Folin-Ciocalteu's, DMBA), highlighting the need for specialized analytical protocols and calibration methods for NADES. However, NADES presented a low toxicity and stimulant capacity when directly applied to germinating seeds, reinforcing their "green" name and advantages over conventional solvents. Nonetheless, the absence of established standards for NADES in general further complicates their evaluation and potential application. Despite challenges with extract quality and data accuracy, our study underscores the potential of macroalgae beach wrack as a valuable biomass resource, and NADES as extractants. LC-MS/MS analysis of the studied biomass showed us that it contains indole-3-acetic acid (IAA), indicating potential applications in agriculture and guiding further research into phytohormone presence and effect on macroalgae. This project represents a foundational step towards integrating novel solvents with traditional materials, aiming to enhance their environmental and practical benefits.

Keywords: Macroalgae beach wrack, Plant growth regulators, Natural Deep Eutectic Solvents, Indole Acetic Acid

Table of contents

Table of contents	4
List of Tables	7
List of Figures	9
List of Abbreviations	12
Introduction.....	14
Materials and Methods.....	23
1. Reagents.....	23
1.1 NADES reagents	23
1.2 Phytohormone standards.....	23
1.3 MES buffer	23
1.4 Folin & Ciocalteu's method reagents	23
1.5 DMBA method reagents	24
1.6 DPPH method reagents.....	24
1.7 Polarity determination (Nile-Red)	24
1.8 Solid-phase extraction	24
1.9 Biodegradability (Biological/Biochemical Oxygen Demand - BOD)	24
2. Methodology.....	25
2.1. Macroalgae beach wrack sample collection and processing	25
2.2. NADES synthesis & tuning	25
2.3. Viscosity and density measurement.....	26
2.4. Polarity measurements	26
2.4 Biodegradability	27
2.4 Extraction procedure and optimization.....	29
2.4 Total Phenolic Content (TPC) protocol optimization and analysis	30
2.4.1. Reagent order	30
2.4.2. Base and concentration used	30
2.5. Phlorotannins quantification.....	30

2.6.	Scavenging potential measurement.....	31
2.7.	Germination assays.....	31
2.7.1.	MES buffer preparation.....	31
2.7.2.	NADES toxicity assay	32
2.7.3.	NADES vs constituents' toxicity synergy.....	32
2.7.4.	Buffered assays.....	32
2.7.5.	Germination speed	32
2.8.	LC-MS/MS analysis.....	32
2.8.1.	Phytohormones standards for LC-MS/MS analysis.....	32
2.8.2.	Extraction	32
2.8.3.	Phytohormone detection.....	33
	Results and Discussion.....	35
1.	Algae/Sample collection, characterization and processing	35
2.	Natural Deep Eutectic Solvents - NADES.....	37
2.1.	Characterization	37
2.1.1.	Viscosity and density	37
2.1.2.	Polarity	40
2.2.	Degradability	44
3.	Germination.....	46
3.1.	Water volume optimization	46
3.2.	Toxicity.....	47
3.2.1.	NADES.....	47
3.2.2.	NADES vs Components	53
3.2.3.	Buffered vs Unbuffered.....	54
4.	Sample-to-Solvent ratio.....	56
5.	Extract characterization.....	58
5.1.	DMBA – Phlorotannin content.....	58
5.2.	Folin-Ciocalteu's total phenolic content	59

5.3.	Folin-Ciocalteu's protocol optimization	59
5.4.	Folin-Ciocalteu's method results after optimization	60
5.5.	DPPH (2,2'-Diphenyl-1-picrylhydazyl) antioxidant potential assay	61
5.6.	Extracts' effect on germination	63
5.6.1.	Germination rate through time	63
5.6.2.	Seedlings' root growth	66
6.	Phytohormone identification and quantification.....	68
6.1.	Detection optimization	68
6.1.2.	Standards mixture	71
6.2.	Phytohormones in the sample	72
	Conclusion.....	75
	Supplementary Material	78
	References	92

List of Tables

Table 1 - Families/groups of common phytohormones, origin and function	17
Table 2 - DES types based on the chemical characteristics of their components, according to Abbott et al. (2007).	21
Table 3 - Phytohormone standards list and respective molecular weight and precursor ion (m/z).....	23
Table 4- NADES composition, molar ratio and short handle code	26
Table 5 - Mineral medium solutions' constitution.....	27
Table 6- Values of density (ρ / g cm ⁻³) and viscosity (μ / mPa s ⁻¹) of NADES stock and respective tuned solutions with 10, 20, 30 and 40% of water, measured at 20 and 45°C.	37
Table 7 - Viscosity and density values of ChCl:CA stock solution measured at 50°C and 60°C.....	38
Table 8 - $\lambda_{\max}$ and corresponding E_{NR} of some NADES (stock and tunings) and standard solvents (Hexane, DMSO, and H ₂ O)	40
Table 9 - NADES tuning's $\lambda_{\max}$, respective ENR and relative polarity.....	43
Table 10 - Highest tolerable concentrations of NADES on germination, obtained from preliminary toxicity assay, corresponding to the NOEC determined for each NADES .	51
Table 11 - pH of NADES' diluted solutions used in germination assays, buffered and unbuffered	54
Table 12 - Chlorophyll a and carotenoid values determined from different sample/-to-solvent ratios for different NADES and NADES tunings solutions, tuned with 10 to 40% water. Ratios: T1 – 100 mg/mL, T2 – 150 mg/mL, T3 – 250 mg/mL, SD – standard deviation. The quantities of pigments are presented in μ g of pigment/mL of extract ..	56
Table 13 - Chlorophyll a and carotenoid values determined, nNormalized (to μ g of pigment per mg of dry biomass mean pigment content , determinedobtained infrom different sample/-to-solvent ratios for different NADES and NADES tuningssolutions, tuned with 10 to 40% water. Ratios: T1 – 100 mg/mL, T2 – 150 mg/mL, T3 – 250 mg/mL, SD – standard deviation. The quantities of pigments are presented in μ g of pigment per mg of dry biomass extracted.	57
Table 14 - NADES extracts' relative germination speed organized by NADES used to perform the extraction (A) and ranked by relative speed in ascending order (B), against the negative control (H ₂ O). A relative speed of 1 means the extract has the same germination speed as the control, if <1 the germination rate is slower, if >1 it is faster than the control.	65

Table 15 - Phytohormone detection mode selected and retention time (in min). The results were obtained through individual analysis.	71
Table 16 - Phytohormone retention time (in min) in standard mixture matrix.....	72

List of Figures

Figure 1 - Phase diagram of a two-component mixture representing the eutectic point. $Tf(real)$ is the measured freezing point of a mixture at the eutectic composition and $Tf(ideal)$ is the theoretically predicted freezing point for an ideal mixture. Taken from Kalhor and Ghandi (2019).....	20
Figure 2- Pictures showing the representative species collected in trays. A) Brown algae - <i>Sacchoriza polyschides</i> ; B) Green and Red algae - B1) <i>Codium sp.</i> , B2) <i>Ulva rigida</i> , B3) <i>Gigartina pistillata</i> , B4) <i>Condrus crispus</i> , B5) Red algae consortium.	35
Figure 3 - Sample content, in percentage of dry weight, of the 3 <i>phila</i> and the various species identified.	36
Figure 4 - A) Normalized ChCl:Prop tuned solution fluorescence spectrum; B) Zoomed maximum fluorescence peaks of ChCl:Prop tuned solutions and standard solvents ...	41
Figure 5 - A) Normalized ChCl:MA tuned fluorescence spectra; B) Zoomed maximum fluorescence peaks of ChCl:MA tuned solutions and standard solvents.....	41
Figure 6 - A) Normalized ChCl:Urea tuned solutions fluorescence spectra; B) Zoomed maximum fluorescence peaks of ChCl:Urea tuned solutions and standard solvents ...	42
Figure 7 - A) Normalized ChCl:LA tuned solutions fluorescence spectrum; B) Zoomed maximum fluorescence peaks of ChCl:LA tuned solutions and standard solvents	42
Figure 8 – Amount of O ₂ consumed (in mg) to degrade NADES (biodegradability) over a 28-day period (1st assay)	44
Figure 9 - Amount of O ₂ consumed (in mg) to degrade NADES (biodegradability) over a 28-day period (2nd assay)	44
Figure 10 - Water volume optimization for tomato seeds germination assay results – average root length of seedling with 5 different amounts of water (1, 2, 3, 4 and 5 mL). Statistical analysis performed using Kruskal-Wallis non-parametric test: ns $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	46
Figure 11 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:LA. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	47
Figure 12 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:MA. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	48
Figure 13 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:Prop toxicity assay. Statistical analysis performed using Kruskal-Wallis non-	

parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	49
Figure 14 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:Urea. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	50
Figure 15 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:CA toxicity assay. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	51
Figure 16 - Root length observed after exposure to NADES and their respective components separately, at 100 mg/L. A) ChCl:MA; B) ChCl:Urea; C) ChCl:LA; D) ChCl:Prop; E) ChCl:CA. Statistical analysis was performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	53
Figure 17 – Root length (in cm) from toxicity assay with ("Buffered") and without ("Unbuffered") the use of MES buffer in diluted NADES solutions. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	55
Figure 18 - Scavenging potential of various NADES extracts through time, A) ChCl:Prop; B) ChCl:Urea; C) ChCl:MA; D) ChCl:LA.....	61
Figure 19 – Germination rate/speed of NADES extracts over a 7-day period: A) ChCl:Prop; B) ChCl:LA; C) ChCl:MA; D) ChCl:Urea; the bars represent standard deviation. The CNTRL is a NADES solution diluted according to toxicity results obtained previously	64
Figure 20 - NADES extracts effect on root length (in cm), A) ChCl:LA; B) ChCl:Prop; C) ChCl:Urea; D) ChCl:MA. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$	66
Figure 21 - LC/MS-MS chromatograms of JA in A) Negative mode; B) Positive mode; JA LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode	69
Figure 22 - A) JA LC-MS/MS window run ($210.50 \leq m/z \leq 211.50$); B) JA LC-MS/MS CID chromatogram in positive mode; C) JA LC-MS/MS CID spectrum in positive mode	70
Figure 23 - LC-MS/MS chromatograms (A) of standard mixture and corresponding CID spectra (B); top to bottom: IAA, IBA, TZ, BAP, JA, SA, ABA, GA	72
Figure 24 - LC-MS/MS chromatograms (A) and corresponding CID spectra (B), from top to bottom: Phytohormone standard mix (IAA in specific); seaweed extract with biomass	

>250 µm; seaweed extract of biomass <250 µm; doped seaweed extract with biomass	
>250 µm; doped seaweed extract of biomass <250 µm.	73

List of Abbreviations

AA – Absolute Area

ABA – Absciscic acid

BAP – 6-Benzylaminopurine

BOD – Biological Oxygen Demand

BP – Base Peak

CA – Citric Acid

ChCl – Choline Chloride

DES – Deep Eutectic Solvent(s)

GA – Gibberelic acid

HBA – Hydrogen Bond Acceptor

HBD – Hydrogen Bond Donor

IAA – Indole-3-acetic acid

IBA – Indole-3-butyric acid

IL – Ionic Liquid(s)

JA – Jasmonic acid

LA – Lactic Acid

LOEC – Lowest Observed Effect Concentration

MA – Malonic Acid

NADES – Natural Deep Eutectic Solvent(s)

NOEC – No Observed Effect Concentration

PGR – Plant Growth Regulator(s)

Prop – 1,2 – propanediol

RT – Retention Time

SA – Salicylic acid

ThOD – Theoretical Oxygen Demand

TZ – Trans-Zeatin

Introduction

Over the last couple of years, the human impact on the planet has led to an ever-increasing and borderline irreversible global damage. Being through activities such as the mass exploitation and depletion of natural resources (Defeo et al., 2009), or the uncontrolled emission of greenhouse gases, the world's climate has changed in ways that impacted nature and human health. The damages have surpassed the biological part of the environment, and have now been connected to infrastructure, social and economic changes, with impacts on a variety of sectors namely agriculture, fishery, energy and tourism (IPCC, 2023).

One of the impacted ecosystems affected more directly by both direct and indirect human intervention are sandy beaches. Their touristic allure led to their urbanization, impacting biodiversity straightforwardly by the increased presence of human leisure activities (nautical and fishing sports practitioners, beach goers, etc.) or, in a more roundabout way, with the construction of structures like breakwaters that lead to changes in tide movement - causing reduction in species abundance, by changing the sea floor landscape (Aguilera et al., 2019), or light blocking by sediment displacement and accumulation (Masucci et al., 2020). These beaches are an important marine-terrestrial transition ecosystem, serving as sink of organic and inorganic matter and therefore as sources of nutrients and other compounds, making them able to sustain and serve as habitats (Schlacher et al., 2008).

These matter deposits, which more and more contain man-derived subproducts, waste and trash (Burlat and Thorsteinsson, 2022), are what is commonly called "beach wrack". This wrack is variable and dependent on tidal movement, seasons, climate events and location (Orr et al., 2005; Martins et al., 2024; Rodil et al., 2024), but is generally composed of multiple taxa of macroalgae and seagrasses (Orr et al., 2005; Guerrero-Meseguer et al., 2023). Although a common and evidently necessary phenomenon, the occurrence of bigger and more frequent piles of this macroalgae beach wrack, has been rising over the past decades (Smetacek and Zingone, 2013). This can lead to a myriad of effects both biological, by the alteration of habitats and ecosystems' interactions (Rodil et al., 2024), and socio-economical in places where fishing, navigation and beach tourism are big influences in a city or country's revenue, as these biomass clumps can block engines, fishing nets and are usually considered visually unpleasant (Martins et al., 2024). The economic impact extends further since the removal of beach wrack tends to be expensive and with no return as it is mostly wasted and sent to be landfilled, which

itself entails other complications (health concerns related to compounds released on decomposition or disease vectors propagation) (Mainardis et al., 2021; Pardilhó et al., 2022; Martins et al., 2024).

Macroalgae is a broad term for macroscopic eukaryotic pluricellular marine algae, photosynthetic non-vascular organisms, with an important role as primary producers in the oceans. These beings are usually divided into 3 categories based on the predominant pigment they present (and therefore their colour) which are: brown algae (phylum *Ochrophyta*) – chlorophylls a and c, and higher content of carotenoids such as β -carotene; green algae (phylum *Chlorophyta*) – higher chlorophyll a and b content; red algae (phylum *Rhodophyta*) – higher zeaxanthin and lutein content as well as the presence of phycobiliprotein R-phycoerythrin (Pereira, 2021). One other important taxonomical classification to keep in mind is that both red and green algae belong to the *Plantae* kingdom, and brown algae to *Chromista* (Almeida, 2007; Santos, 2016). Besides this main way of division, there are many morphological characteristics that separate macroalgae like the thalli aspect, the ramification (type and number) and means of reproduction (Gaspar et al., 2020), as well as biochemical ones. The presence of cellulose is visible in the cell walls of all these algae reinforcing the similarities to their terrestrial counterparts. The usage of starch as energy storage in green and red algae further emphasizes this proximity, while brown algae use a compound named laminarin, not found in plants (Almeida, 2007).

Apart from their role as primary producers, these “sea trees” impact the environment in other ways such as, their photosynthetic ability allied to radiation attenuation provide refuge to UV-sensitive biota – reinforcing their ecosystem importance - and their canopies can help reduce shore erosion by slowing down local currents (Duarte et al., 2022). Not only that, macroalgae growth (rate) is faster than terrestrial plants, does not take up arable land or fresh water and product pre-treatment is not as intensive (Palatnik and Zilberman, 2017). In addition to that, macroalgae produce secondary metabolites and compounds of interest for a variety of fields (both directly by the usage of the raw biomass – food, feed, biofuel - or indirectly by extraction and purification of compounds – pharmaceutical, cosmetics, nutraceutical), they are regarded as an economical asset (Ghadiryannar et al., 2016; Palatnik and Zilberman, 2017; Thiyagarasaiyar et al., 2020; Duarte et al., 2022).

Taking all this potential of macroalgae into account, it is possible to speculate that macroalgae beach wrack may present/keep at least some of these capabilities when it washes ashore. It is believed that for as long that humans lived by the sea, this beach

wrack has been implemented in agriculture and cattle raising, not only by the seaside, but wherever it could be taken and used, both fresh and dried, depending on what was needed (Ribeiro et al., 2021). In Portugal, while much of actual evidence was lost and left only to word-of-mouth and folklore, there are records of its usage as far back as the 18th century, where it was mentioned to be used as a fertilizer and soil conditioner (e.g. usage of “sargaço” in masseiras of Apúlia and of “moliço” in Ria de Aveiro) (Nabti et al., 2017; Pereira and Cotas, 2019). May it be by changing the soil water retention capacity, the increase of organic content in soils, pH stability/correction, carbon and nitrogen ratio (C/N) variation (benefiting productivity especially if this ratio is lowered by the increase of protein from algal addition), being a source of macro and micronutrients and/or heavy metal remediation, there is a clear benefit for macroalgae beach wrack use in agriculture or forestry overall (Ammar et al., 2022).

One additional explanation that has been raised for the results observed in agricultural applications, is the possibility of algae, just like their terrestrial relatives, producing plant growth regulators (PGR's)/phytohormones which could also explain their similarities in morphology and topology organization (i.e. apical dominance) (Evans and Trewavas, 1991), especially if taken their taxonomical proximity to plants into account (mostly of green and red algae).

Phytohormones are natural signalling molecules responsible for regulating a range of physiological events in higher plants, from growth, morphological changes, and metabolism coordination to the planning of life-cycle events (senescence, maturation, flowering, etc.)(Agudelo-Morales et al., 2021). Being fairly universal among these higher organisms (meaning they are and act very similarly in all of them), these molecules also allow for communication between different individuals of the same or different species (sometimes even different kingdoms) (Pichersky and Gershenzon, 2002; Bari and Jones, 2009; Kamiya, 2009). The main groups and families which these hormones fall under, some of their compounds and described roles, are presented in the table (Table 1) below.

Table 1 - Families/groups of common phytohormones, origin and function

Family	Compound	Origin	Role	Reference
Auxins	Indole-3-acetic acid (IAA)	Natural	Cell division; Shoot cell elongation; Foliar expansion	Mashiguchi et al. (2011)
	Indole-3-butyric acid (IBA)	Natural	Adventitious root induction	Ludwig-Müller (2000)
	2,4-Dichlorophenoxyacetic acid (2,4-D)	Synthetic	Herbicide	NPIC (2009)
Absciscic acid	Absciscic acid	Natural	Seed maturation; drought stress response (stomatal closure)	Xiong and Zhu (2003)
Cytokinins	Trans-Zeatin	Natural	Plant immunity modulation	Großkinsky et al. (2013)
	6-Benzylaminopurine (BAP)	Natural	Stress protection (antioxidant activity increase)	Zhiponova et al. (2024)
	Isopentenyladenine (IP)	Natural	Shoot proliferation	Jaakola et al. (2001)
	<i>N</i> -(2-Chloro-4-pyridyl)- <i>N</i> -phenylurea (CPPU)	Synthetic	Fruit nutritional value increase (starch, vitamins, etc.)	Jiang et al. (2024)
Salicylic acid	Salicylic acid	Natural	Biotic and abiotic stress response; Flowering induction	Hayat et al. (2007)
Gibberellins	Gibberellin A1 (GA1)	Natural	Germination induction and fruit ripening	Li et al. (2019)
	Gibberellin A3 (GA3)	Natural	Flower maturation and fertility	Gupta and Chakrabarty (2013)
Jasmonates	Jasmonic acid	Natural	Pathogen defence response and prevention	Creelman and Mullet (1997)
	Methyl jasmonate	Natural	Seed germination delay/suppression (regulation)	Babenko et al. (2015)
Brassinosteroids	Brassinolide	Natural	Seedling growth promotion; Biotic and abiotic stress response	Clouse (2011); (Kaur Kohli et al., 2020)
Plant peptides hormones	Phytosulphokines	Natural	Enhanced cell division; somatic cell embryogenesis	Kobayashi et al. (1999)
Strigolactones	Strigol	Natural	Mycorrhizome stimulation	Akiyama et al. (2005)
Gases	Ethylene	Natural	Abscission and ripening of fruits	Chen et al. (2018)
	Nitric Oxide	Natural (inorganic)	Plant defence	Baudouin and Hancock (2014)

It is worth noting that, like any other hormone/regulator, some of the effects presented are dose, time of application/action and species dependent (e.g. auxins are generally related to shoot elongation, however, different tissues react differently and have different responses to different auxins – IBA is more associated with adventitious root induction

while IAA present effects more related to shoot elongation and foliar expansion). Not only that, there are varying interactions with other regulators and molecules in the organisms also altering their effect when produced (endogenous) or when externally applied (exogenous effect) (Agudelo-Morales et al., 2021).

Apart from these found compounds, new plant growth regulators have been found/developed. These can be synthetic molecules that are physically and chemically similar to other phytohormones (as mentioned previously), new synthetic molecules or even more complex systems and matrixes like bacterial inoculates (Lerma et al., 2018). Nowadays, PGR is the most global and inclusive term that encompasses the previously mentioned phytohormones and all other new things that may have an impact on plant function.

Both algae and plants, unlike their animal counterparts, do not possess specialized organs/glands or cells for hormone production, raising challenges related to the understanding and study of their metabolic pathways, especially their synthesis and regulation, as these photosynthetic organisms also lack a central nervous system. Together with the lack of a vascular system in algae, and the hardships related to studying these organisms in the wild, the presence of phytohormones in them is still up for debate (Mori et al., 2017). However, some genetic, metabolic and experimental evidence have been found, proving the existence of at least some of these PGR's, mainly in green and red algae, probably explained by the closer taxonomical proximity of these algae to plants, whereas brown ones are not as close (Kalasariya et al., 2021; Saebmehar et al., 2022; Chen et al., 2024; Galal et al., 2024).

With everything presented so far, we should not consider macroalgae beach wrack a dispensable resource but invest in the upcycling of this material. This will have an impact on multiple fronts especially biologically and economically, keeping algal blooms and deposition in check (controlling amounts and time it is left exposed to the elements) and implementing what is now a lost sink of investment in other profitable sectors (namely agriculture).

Since this wrack is quite variable, it is difficult to replicate the composition and therefore effects, which may complicate their industrial applications. While it has been used directly in the past with great effects, this variability may come as a hindrance in product development. A workaround is the creation of extracts and/or formulations, that are easier to characterize and adjust in a lab, making them more uniform (and therefore with higher quality control) and easier to apply onto the fields (irrigation, pulverization, etc.).

Not only that but the effects are easier to replicate and therefore study, meaning more specificity can be achieved with research leading to a wide market coverage with products appropriate for every situation.

For this, extracting solvents with known properties and toxicity are required. While the more conventional (organic) solvents are better studied and effective in their extracting potential, due to years of usage and study, they are also known for their toxicity to both the user and the environment in general.

Over the past couple of years, the scientific community has been trying to implement “green chemistry” principals, focused on making analytical procedures more environmentally friendly and safer to humans. The amounts and toxicity of reagents, generated waste, energy requirements, the number of procedural steps, miniaturization, and automation are just a few of the multitude of criteria considered when assessing an analytical methodology’s greenness (Pena-Pereira et al., 2020). Green chemistry is defined by US EPA as “the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substances” (United States Environmental Protection Agency, 2023).

One of the approaches has been the development and synthesis of “green” solvents, where Ionic Liquids (IL), Deep Eutectic Solvents (DES) and Natural Deep Eutectic Solvents (NADES) fall under. These green solvents present a variety of advantages namely their ease of preparation, low cost, low (cyto)toxicity and ecotoxicity, biodegradability and biocompatibility, all due to the use of environmentally friendly components and procedures (Gorgani et al., 2017). Even though these solvents present similar properties under the “green” umbrella, it is important to distinguish them composition-wise, while IL are pure compounds (Lei et al., 2017), DES are mixtures. First introduced onto the scene by Abbott et al. (2003), still under IL classification, he showcased their functionality in simple terms, the mixing of some compounds (in this case salts) to generate eutectic mixtures, decreasing the temperature for molten salt applications.

To understand this work, a few terms and definitions need to be clarified, eutectic mixtures (or systems), eutectic point, eutectic temperature, eutectic composition and deep eutectic solvents. An eutectic mixture is a mixture of compounds whose melting point is lower than the one of its constituents. The eutectic composition is the proportion of the components where the temperature is the lowest (eutectic temperature), and the meeting point is called the eutectic point. All mixtures of compounds that are at least partly immiscible while in the solid phase do present an eutectic point (Martins et al., 2019). What differentiates a regular eutectic mixture from a deep eutectic one is still a controversial topic, and up for debate.

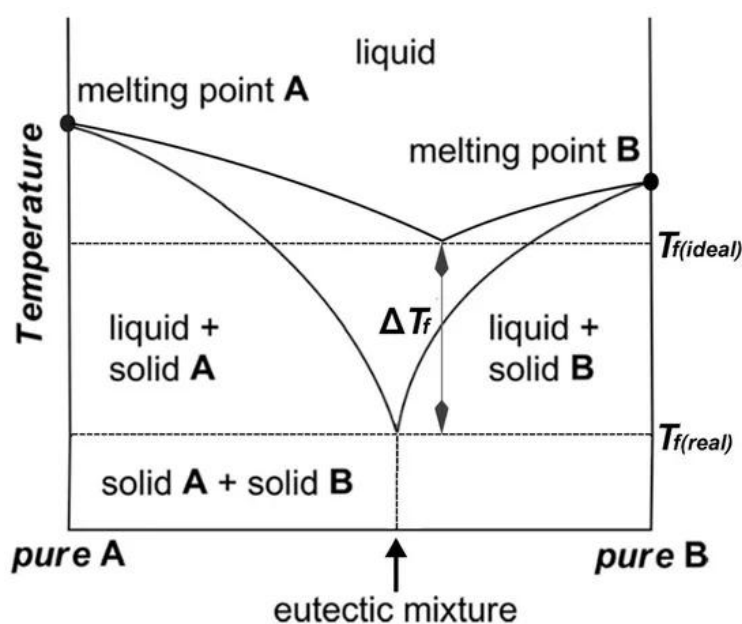


Figure 1 - Phase diagram of a two-component mixture representing the eutectic point. $T_{f(real)}$ is the measured freezing point of a mixture at the eutectic composition and $T_{f(ideal)}$ is the theoretically predicted freezing point for an ideal mixture. Taken from Kalhor and Ghandi (2019)

While the initial definition of DES being a mixture of Lewis or Brønsted acids and bases, that also need to act as Hydrogen Bond Acceptor (HBA) and Hydrogen Bond Donor (HBD) (necessity of an acidity difference in the environment but no proton transference occurs) (Abbott et al., 2007; Smith et al., 2014) is still pretty agreed on, some papers (Martins et al., 2019; Abranches and Coutinho, 2023) raised some inconsistencies and proposed more restrictive parameters to classify DES more accurately and separately from the average eutectic mixture.

As stated in Abranches and Coutinho (2023): “An eutectic solvent is a eutectic-type system that is a liquid at a given desired temperature where at least one of its components would, otherwise, be a solid unfit to be applied as a solvent. A deep eutectic solvent is a eutectic solvent whose components present enthalpic-driven negative

deviations from thermodynamic ideality.” - to simplify, a deep eutectic solvent is a solvent in which the interactions between HBA and HBD are more favourable than the interactions of each component with itself (HBA-HBA and HBD-HBD), deviating negatively from an ideal liquid where the interactions HBA-HBD, HBA-HBA and HBD-HBD are thermodynamically similar – DES are more than the sum of its parts.

One last point of contention amongst researchers is whether all molar ratios/compositions of DES components presenting eutectic properties (liquid at temperatures where the components would not be) should technically be considered DES. It is common practice to test multiple fixed stoichiometric ratios (1:1, 1:2, 1:3, 2:1, etc.) and see which ones form stable eutectic mixtures, however as mentioned above, one will form likely either way, so the dependence and driving force should not be a made-up fixed ratio, as no correct information can be derived from it. Eutectic points should be obtained with information from the solid phase(s) and not the liquid one (Alizadeh et al., 2020). On the other hand, maybe this variability is the leeway necessary to bring out DES’ full potential, as the less rigid restrictions, counterintuitively, allow for a higher specificity of the properties needed for a particular application (Martins et al., 2019; Abranches and Coutinho, 2023). One final worthy mention about DES capabilities is the “tuning” these solvents can undergo. This is a process in which the addition of varying amounts of water (% v/v) to an already prepared and stable DES can drastically change their physical properties, be it viscosity, density, polarity or conductivity, allowing for the creation of solvents/working solutions with very specific objectives in mind (Dai et al., 2013).

There are five types of DES based on the chemical nature of their components (Abbott et al., 2007) (Table 2):

Table 2 - DES types based on the chemical characteristics of their components, according to Abbott et al. (2007).

Type I	Quaternary ammonium salt + metal chloride
Type II	Quaternary ammonium salt + metal chloride hydrate
Type III	Quaternary ammonium salt + hydrogen bond donor
Type IV	Metal chloride hydrate + hydrogen bond donor
Type V	Non-ionic molecular compound + non-ionic molecular compound

When the origin of the compounds that are used in DES synthesis is considered, rather than their chemical nature, we find a new group of these solvents, Natural Deep Eutectic Solvents (NADES). First proposed by Choi et al. (2011), NADES are DES (meaning all former definitions and classifications discussed still apply) but composed of primary metabolites such as sugars, alcohols, organic acids, amino acids, etc. (Hikmawanti et al., 2021). Over the last couple of years there have been theories that these solvents can occur naturally, to a certain degree, inside cells, explaining some previously not understood processes. From enzyme activity being higher *in vivo* than what was expected, to the solubility of certain compounds being different than what was previously seen in water or fat, these led to the speculation of the existence of a third phase within cells (other than aqueous and lipophilic/organic), reinforced by the presence of certain molecules in high amounts without apparent purpose (Choi et al., 2011). In addition, some NADES have shown interesting properties by themselves, namely antifungal (Juneidi et al., 2016) and biostimulant to some algal, diatom and marine bacterial species (Vieira Sanches et al., 2023).

If NADES have a function of compound transportation and solubility in organisms, it is not unreasonable to think they can have a great extracting potential and stabilization capability of the said extracted compound as well as being able to increase their availability and viability even outside cell environment – characteristics already used in pharmaceutical and cosmetical industries (Villa et al., 2024) – coming as a desired effect in biostimulant/biofertilizer making. However, this larger bioavailability comes as a double-edged sword. While the beneficial effects can be felt with smaller amounts of the responsible compounds, so do the negative ones, possibly making normally innocuous amounts of a substance have much harsher effects, reinforcing the necessity of understanding how these solvents work, and of characterizing the macroalgae wrack composition down to a chemical level.

In this project, we aim to unlock the potential of macroalgae beach wrack as a source of PGR's/phytohormones and bioactive compounds, by implementing NADES as extracting solvents. The characterization of the physic-chemical properties of these solvents and tuned solutions should allow a better understanding of the correlation between to the properties and the extracting efficiency they present. The effect of extracts on crop plants, by direct application (possible with NADES but not with conventional solvents) to seeds will also be analysed.

Materials and Methods

1. Reagents

1.1 NADES reagents

L-(+)-Lactic acid, ca 85-90% aqueous solution was acquired from Alfa Aesar; Choline Chloride (2-Hydroxy-N,N,N-trimethylethanaminium chloride) >98.0% purity was acquired from TCI Europe; 1,2-Propanediol, ACS reagent was acquired from Acros Organics; Urea for electrophoresis was acquired from Sigma-Aldrich; Malonic acid for synthesis was acquired from Merck Millipore; Citric acid, Reagent grade was acquired from Sigma-Aldrich.

1.2 Phytohormone standards

3-Indoleacetic acid analytical standard, 4-(3-Indolyl)-butyric acid, 6-Benzylaminopurine and (±)-Jasmonic acid analytical standard were acquired from Supelco; Gibberellic acid, gibberellin A3 basis (90% purity, HPLC) and Salicylic acid, ≥99.0% purity were acquired from Sigma-Aldrich; (+)-Absciscic acid, 98% purity was acquired from Acros Organics; Trans-Zeatin was acquired from TCI Europe. The phytohormone standards list, molecular weight and precursor ion (m/z) are presented in Table 3.

Table 3 - Phytohormone standards list and respective molecular weight and precursor ion (m/z)

Phytohormone	Molecular weight (g/mol)	Precursor ion (m/z)
3-Indoleacetic acid	175.184	176.10
4-(3-Indolyl)-butyric acid	203.24	204.20
6-Benzylaminopurine	225.2492	226.01
(±)-Jasmonic acid	210.27	211.13
Gibberellic acid (A3)	346.4	345.13
Salicylic acid	138.12	139.04
(+)-Absciscic acid	264.32	263.13
Trans-Zeatin	219.248	220.12

1.3 MES buffer

2-(N-morpholino)ethanesulfonic acid (MES acid form) was obtained from Sigma-Aldrich.

1.4 Folin & Ciocalteu's method reagents

Folin & Ciocalteu's phenol reagent and Gallic acid (3,4,5-Trihydroxybenzoic acid) 97.5-102.5% purity, were acquired from Sigma-Aldrich; Sodium hydroxide ACS Reagent,

pellets, ≥97.0% purity was obtained from Honeywell Research Chemicals; Sodium carbonate anhydrous for analysis, EMSURE® ISO was obtained from Merck Millipore.

1.5 DMBA method reagents

2,4-Dimethoxybenzaldehyde, 98% purity was acquired from Thermo Scientific; Phloroglucinol Anhydrous, ≥99.0% purity was acquired from TCI Europe; Acetic acid (glacial) 100% anhydrous for analysis, EMSURE® ACS ISO Reagent was acquired from Merck Millipore; Hydrochloric acid, ACS Reagent, fuming, ≥ 37.5% purity was acquired from Honeywell Research Chemicals.

1.6 DPPH method reagents

2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazine-1-yl was acquired from Sigma-Aldrich.

1.7 Polarity determination (Nile-Red)

Nile red lipophilic dye was acquired from Carl Roth.

1.8 Solid-phase extraction

Oasis® PRIME HLB 3 cc/60 mg cartridges were acquired from Waters Corporation, Milford Massachusetts USA.

1.9 Biodegradability (Biological/Biochemical Oxygen Demand - BOD)

Potassium dihydrogen phosphate, purity ≥ 99.5%, Potassium hydrogen phosphate P.A. grade reagent, Calcium chloride P.A. grade reagent, Magnesium sulphate P.A. grade reagent, and Iron chloride (III) hexahydrate P.A. grade reagent were acquired from Merck Millipore; Ammonium chloride was acquired from José M. Vaz Pereira, S.A.; Sodium hydrogen phosphate dodecahydrate, purity > 99% was acquired from Honeywell Fluka™.

The bacterial inoculum used was obtained from activated muds supplied by Gaia Litoral's wastewater treatment plant (SIMDOURO).

Twenty-eight days (20°C) Biological Oxygen Demand (BOD) analyses were performed according to the OECD guideline for testing of chemicals 301F – Manometric Respirometry (OECD, 1992) with an AL6060 AQUALYTIC® Sensomat System.

2. Methodology

2.1. Macroalgae beach wrack sample collection and processing

2.1.1 Sample collection

Macroalgae beach wrack was collected from Praia da Memoria, Porto, Portugal, 41.23170°N, -8.72231°W, in early September 2023 (18/09/23 – late/end summer). A random collection pattern was used, disregarding the age and size of the algae. The fresh unwashed sample was brought to the laboratory, and separated from epiphytes, sand, foreign organisms and other beach wrack waste. Afterwards, this biomass was thoroughly washed with fresh water and weighted and a portion was separated for characterization and identification. Macroalgae were sorted into brown, green or red algae, and identified - “Sorted” portion. The classification of the macroalgae was up to genus or species, when possible, with the help of CIIMAR’s researcher Francisco Arenas, phycologist and expert in local coastal ecosystems and marine forests.

2.1.1 Sample processing

The washed macroalgae beach wrack was dried and milled to be used in extractions and other assays. The drying process was as follows: the biomass was spread onto trays and set to dry in a convection oven (MMM Venticell oven), at 60°C for 40h-120h. Afterwards, it was roughly chopped and crushed, and then milled using a Retsch Ultra Centrifugal Mill ZM 200, at 10,000 rpm, and sieved using a Retsch AS 200 sieve shaker, set out with a 250 µm sieve. Both fractions, bigger and smaller than 250 µm, were stored in the dark at 4°C until needed.

2.2. NADES synthesis & tuning

NADES were prepared through the heating and stirring method. Components were mixed in the molar ratios presented in Table 4, heated to 60°C under continuous stirring until a clear liquid formed. For ChCl:MA components were mixed and stirred occasionally, without heating (to avoid degradation/conversion of malonic acid to acetic acid), until a clear liquid formed. Adding low but variable amounts of water was needed to stabilize some of the NADES at room temperature (20°C-22°C). NADES were stored at 37°C.

NADES were tuned by addition of 10%, 20%, 30% and 40% (v/v) of distilled water and mixed thoroughly until there was no visible separation of phases.

Table 4- NADES composition, molar ratio and short handle code

NADES composition	Molar Ratio	Shortening/code
Choline Chloride: Citric Acid	1:1	ChCl:CA
Choline Chloride: Lactic Acid	1:1	ChCl:LA
Choline Chloride: Malonic Acid	1:1	ChCl:MA
Choline Chloride: 1,2-Propanediol	1:1	ChCl:Prop
Choline Chloride: Urea	1:1	ChCl:Urea

2.3. Viscosity and density measurement

Viscosity and density of NADES were measured using a Lovis 2000 M/ME micro viscometer and a DMA™ 5000 M densimeter, respectively.

2.4. Polarity measurements

The usage of Nile-Red for polarity was adapted from Dai et al. (2013) and Lacerda et al. (2013).

2.2.1 Nile-Red UV/Vis analysis in microplate

For polarity analysis of NADES and standard solvents (ultrapure water, DMSO and hexane), a Nile-Red UV/Vis protocol was proportionally microscaled to fit in 96-well plates. Briefly, to 157 µL of sample, 5 µL of Nile-Red dye (1 mg/mL in absolute ethanol) were added and mixed. Ethanol was evaporated with a N₂ stream, and an absorbance spectrum was traced to find λ_{max} (wavelength where the absorbance hits its peak), using BioTek® Synergy HT microplate reader.

2.2.2 Nile-Red fluorescence spectroscopy

To 1.5 mL of solvent (NADES, or standards – ultrapure water, DMSO and hexane), 6 µL of Nile-Red dye (1 mg/mL in absolute ethanol) were added and mixed. Using a PerkinElmer LS 55 Fluorescence spectrometer an emission spectrum was traced (from 370 nm-800 nm), with excitation wavelength fixed at 370 nm, excitation and emission slit set to 5 nm, speed 1200 nm/min and a gain of 775 V.

For both methods, the determination of ENR was obtained using the following equation (Equation 1) was used, as determined by Ogiyara et al. (2004).

Equation 1 - E_{NR} calculation (kcal/mol):

$$E_{NR} \text{ (kcal mol}^{-1}\text{)} = \frac{28.591}{\lambda_{max}}$$

2.4 Biodegradability

2.3.1 Theoretical Oxygen Demand (ThOD)

Biodegradability was determined according to OECD's 301F protocol (OECD, 1992). For the selection of the suitable settings for the AQUALYTIC® sensor system, theoretical oxygen demand (ThOD) was calculated for the compound or mixture of compounds to be determined, according to the following equation:

Equation 2 - Theoretical Oxygen Demand equation

$$ThOD = \frac{16[2C + \frac{1}{2}(H - Cl) + \frac{5}{2}N + 3S + \frac{5}{2}P + \frac{1}{2}Na - O]}{M} \text{ mg O}_2/\text{mg test substance}$$

Where ThOD can be used as an estimate of the ultimate BOD (theoretical biological degradation of a compound) expressed in mg of O₂ consumed for oxidation per mg of compound, *C* is the number of carbon atoms in the compound studied, *H* the atoms of hydrogen, *Cl* the atoms of chlorine, *N* the atoms of nitrogen, *S* the atoms of sulphur, *P* the atoms of phosphorus, *Na* the atoms of sodium, *O* the atoms of oxygen and *M* the molar weight of the compound. For NADES, it was calculated taking into account the molar ratio of their components.

2.3.2 Mineral medium preparation

The mineral medium was prepared according to OECD's 301F protocol (OECD, 1992). Four solutions (A, B, C and D) were prepared in deionized water as shown below (Table 5le 5).

Table 5 - Mineral medium solutions' constitution

Solution	Reagent	Concentration/ g L ⁻¹
A	KH ₂ PO ₄	8.5
	K ₂ HPO ₄	21.75
	Na ₂ HPO ₄ .12H ₂ O	44.64
	NH ₄ Cl	0.5
B	CaCl ₂	27.5
C	MgSO ₄	12.632
D	FeCl ₃ .6H ₂ O	0.25

To avoid metal/ion precipitation, 0.1 g of EDTA was added to solutions B, C and D. The pH of solution A was adjusted to 7.5.

2.3.3 Inoculum preparation

For the preparation of the inoculum, 50 mL sterile conical centrifuge tubes were filled with activated muds, centrifuged at 2000 rpm, 5 min, and the supernatant discarded. The tubes were refilled to 50 mL with mineral medium and centrifuged at 2000 rpm, 10 min, and the supernatant discarded. Finally, the mud pellets of 2 tubes (without supernatant) were transferred volumetric flasks. These were filled with mineral medium and left to rest for 5 days in the AQUALYTIC® AL606 BOD measuring system, at 20 °C with continuous airflow and automatic magnetic agitation. After the acclimatisation time of the inoculum in the mineral medium, it was used as the seeded mineral medium either as blanks or for dilution of the NADES.

2.3.4 Assay set-up

BOD flasks were prepared by diluting 1 mL of NADES stock solution in the proper size overflow measurement flask (244 mL) with the seeded mineral medium. This mixture is transferred to 1 L brown BOD flasks, the seal gasket is filled with 3-4 drops of KOH 45% and inserted in the neck of the bottle. The flask was hermetically sealed with the BOD pressure sensor and placed on the AQUALYTIC® AL606 thermostatically controlled cabinet, while stirring the sample continuously with the inductive stirring system and the experiment was started. Every day, automatic readings were taken and stored for a total of 28 days.

2.3.5 Biodegradability determination

According to OECD's 301F protocol, a compound is determined as (readily) biodegradable only if the result of Equation 4 is $\geq 60\%$.

Equation 3 - Determination of BOD (Biological Oxygen Demand)

$$BOD \text{ (mg O}_2\text{/mg test substance)} = \frac{\text{mg O}_2\text{/l uptake by test substance} - \text{O}_2\text{/l uptake by blank}}{\text{mg test substance/l in vessel}}$$

Equation 4 - Determination of % of degradation

$$\% \text{ degradation} = \frac{BOD}{ThOD} \times 100$$

2.4 Extraction procedure and optimization

Ultrasound-heat-assisted extraction was used for biomass extraction. Five sample to solvent ratios were tested: R1 – 100 mg/mL; R2 – 150 mg/mL; R3 – 250 mg/mL; R4 – 400 mg/mL; R5 – 600 mg/mL. The biomass was measured according to the ratios, into 1.5 mL Eppendorfs, the solvent added and vortexed until all the biomass was soaked and suspended. The samples were put into an Elmasonic® S40H ultrasound bath (220-240 V; 340 W; 50/60 Hz) at 45°C, for 40 min, in a continuous mode. Afterwards, the samples were let to cool to room temperature, and centrifuged at 11,000 g, for 20 min at 22°C. The supernatant was collected and stored at 4°C, in the dark, until further use/analysis.

2.4.1 Extraction efficiency analysis

To evaluate extraction efficiency, chlorophyll a and carotenoid content were used as proxies. The quantification of these compounds was done spectrophotometrically using a BioTek® Synergy HT plate reader, as follows: in a 96-well plate, 200 µL of sample or blanks are loaded in triplicate and the absorbance read at 470 nm (Abs470), 648 nm (Abs648) and 664 nm (Abs664). After correction of the path length (0,314 cm – height of the well with 200 µL of sample), the formulas below were used to calculate the content of the pigments. The formulas used for spectrophotometric determination of pigments are solvent dependent as they take the solvents' ϵ (molar absorption coefficient) into account (Parsons and Strickland, 1963; Jeffrey and Humphrey, 1975). However, such formulas are yet to be developed for NADES. The formulas below refer to absolute methanol as the extracting solvent, as it is one of the most commonly used for pigment extraction, with high efficiency.

Equation 5 -Chlorophyll a content (µg/mL)

$$13,36 \times Abs664 - 5,19 \times Abs648$$

Equation 6 -Carotenoids content (µg/mL)

$$\frac{1000 \times Abs470 - 2,13 \times Chlorophyll\ a\ content(\mu g/mL)}{209}$$

2.4 Total Phenolic Content (TPC) protocol optimization and analysis

TPC original protocol taken from Barroso et al. (2016) was adapted for microplate application. Briefly, in a 96-well plate, 7.5 μL of sample (distilled water for blanks), 7.5 μL of Folin & Ciocalteu's reagent, 60 μL of Na_2CO_3 (75 g/L) and 150 μL of distilled water are mixed and incubated in the dark for 1h. Afterwards, the absorbance was measured at 725 nm, using BioTek® Synergy HT plate reader. For the calibration curve, different concentrations of gallic acid were used as a standard.

Due to the interference of the NADES with this methodology, an optimization of this protocol was required.

Based on the literature, 2 variables were tested simultaneously to try and deal with/remove the interferences, 1) the order of the reagents addition and 2) the base/alkali used.

2.4.1. Reagent order

The order of mixing/application of the reagents was tested as follows: For situation 1, the sample, Folin reagent and base are mixed and left to rest for 5 min. Afterwards, the distilled water is added, and the mixture is left to incubate for 1 h in the dark. For situation 2, the sample, distilled water and base are mixed and left to rest for 5 min. Afterwards, Folin reagent is added, and the mixture left to incubate for 1 h in the dark.

2.4.2. Base and concentration used

For this variable, Na_2CO_3 and NaOH were used as the base of the TPC reaction, at the following concentrations 0%, 0.75%, 3.75% and 7.5%. The protocol was performed as mentioned above.

2.5. Phlorotannins quantification

To quantify phlorotannins, the protocol used was taken from Stern et al. (1996). A HCl 6 % in glacial acetic acid solution – blank solution - and a DMBA 2%, in glacial acetic acid, solution – reaction mixture - were prepared in advance. Briefly, to 50 μL of the sample (50 μL of HCl 6%: Glacial acetic acid (1:1 – v/v) for the blanks), 250 μL of the reaction mixture (DMBA 2%: HCl 6% (1:1 – v/v)) were added. The samples were left to incubate in the dark for 1 h and afterwards, the absorbance was measured at 515 nm, using a BioTek® Synergy HT plate reader. For the calibration curve, different concentrations of phloroglucinol were used as a standard.

2.6. Scavenging potential measurement

For the evaluation of the scavenging potential of the extracts, the protocol was adapted from Fukumoto and Mazza (2000) and goes as follows: in a 96-well plate, 25 μ L of sample (methanol for the blanks) and 200 μ L of DPPH 150 μ M solution (prepared in pure methanol and with the absorbance adjusted to 0.8-0.9) were mixed and the absorbance read during a 15 min period (once every minute – 16 total reads), at 515 nm, using BioTek® Synergy HT plate reader.

2.7. Germination assays

Tomato seeds (*Solanum lycopersicum* L. – “Rei dos Temporões” variety) were obtained from Luso Sementes, HORTOSEMENTES, Lda.

Seeds were sterilized by submersion in a 4 % bleach solution supplemented with Tween-20 (0.2 %) for 10 min, followed by rinsing in ethanol 70 %, and washing 6 times in distilled water, shaking the seeds' tube every so often in every step (Lindsey III et al., 2017).

To evaluate effects on seed germination and growth 2 mL of testing solution (extracts, controls, etc.) were placed in petri plates and disinfected under UV light for 15 min. A Whatman™ Grade 1 filter paper (85 mm in diameter) was placed in each dish (assuring the filter paper is fully soaked in the testing solution), 10 seeds placed on top, and the plates sealed with Parafilm.

Seed in plates were grown in the dark, at 25°C for 7 days. Every day, the germination was evaluated by counting the germinated seeds. On the 7th day, the seedlings are removed from the plates, laid flat on a flat surface and a picture was taken for further processing.

Root and shoot lengths were measured using the pictures taken of the seedlings on the 7th day of incubation, with the help of ImageJ Software (v.1.8.0_345), National Institutes of Health, USA.

2.7.1. MES buffer preparation

MES (acid form) buffer was prepared according to Gold Biotechnology® protocol (GoldBio, 2024). Briefly, 97.62 g of MES were dissolved in 750 mL of distilled water and the pH adjusted with NaOH 10N to pH=6.5. This solution was filled/completed to 1 L and autoclaved. MES buffer was stored at 4°C until necessary.

2.7.2. NADES toxicity assay

NADES preliminary toxicity assay was performed by diluting NADES stock solutions to the following concentrations: 10,000 mg/L, 3,333 mg/L, 1,111 mg/L, 370 mg/L and 123 mg/L. The assay was performed as mentioned in 2.7, in duplicate. After extraction with NADES, the resulting extracts were diluted to the concentrations determined in this assay.

2.7.3. NADES vs constituents' toxicity synergy

To evaluate the toxicity of NADES compared to their corresponding constituents, solutions of 100 mg/L of NADES and of each of their components were prepared and an assay was performed as mentioned in 2.7, in duplicate.

2.7.4. Buffered assays

To evaluate the effect of pH of NADES solutions in germination and root growth, two sets of solutions of known concentration were prepared (123 mg/L for ChCl:MA, 100 mg/L for ChCl:Urea, 370 mg/L for ChCl:LA, 123 mg/L for ChCl:Prop, 123 mg/L for ChCl:CA), one unbuffered and the other buffered to a pH of 6.40-6.45, using MES buffer 0.5 M.

2.7.5. Germination speed

The relative germination speed of NADES extracts was calculated using the following equation (Equation 7).

Equation 7 - Relative germination speed calculation

$$\frac{\text{Earliest day where the average number of germinated seeds is max (H}_2\text{O control)}}{\text{Earliest day where the average number of germinated seeds is max (Extract)}}$$

2.8. LC-MS/MS analysis

2.8.1. Phytohormones standards for LC-MS/MS analysis

Phytohormone standards were dissolved in absolute ethanol to obtain 1 mg/mL standard solutions. A mixture of these standard solutions was made at 1 ppm final concentration of each hormone.

2.8.2. Extraction

To prepare the beach wrack macroalgae extracts, 10 mL of 70% methanol, acidified with 0.1% formic acid, were added to 1.1 g of biomass and then sonicated in an ice bath (95%

amplitude, 5 min, 30 s pulse at 20 Hz). The resulting mixture was centrifuged (5,000 g, 4°C, 5 min), the supernatant collected, and the extraction process was repeated two times to ensure maximum recovery.

The three supernatants were pooled together, methanol evaporated in a rotary evaporator (Buchi R-210, Switzerland) at room temperature and the remaining water submitted to solid phase extraction – SPE. Using a negative pressure/vacuum pump and a manifold, the sample were run through Oasis® PRIME HLB 3 cc/60 mg cartridges and the cartridges washed with 2 mL of distilled water. The washing fractions were discarded, the cartridges were fully dried using negative pressure and eluted with 2 mL of absolute methanol. Eluates were collected in collection/test tubes, dried using a N₂ stream and stored at -20°C. Samples were resuspended in 1 mL of methanol LC-MS grade acidified with 0.1% formic acid for analysis.

2.8.3. Phytohormone detection

The detection of the phytohormones through Electrospray Ionization-Liquid Chromatography-Tandem Mass Spectrometry (ESI-LC-MS/MS) followed the method described by Yalçın et al. (2019). First, a standard evaluation was performed with the phytohormones standards, mentioned in 1.2.

Samples were injected in a Liquid Chromatograph Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA), coupled with a Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA). The optimization of the Mass Spectrometer tune method required direct injection of each phytohormone at a concentration of 1 ppm in LC-MS grade methanol. The mass spectrometer was operated in Single Ion Monitoring (SIM) or in Collision-Induced Dissociation (CID) modes, depending on the quantitative or qualitative objectives. Positive and/or negative mode was chosen to obtain the best signal-to-noise values.

The spray voltage was maintained at 5 kV, with the capillary temperature set at 350°C. The capillary voltage and tube lens voltage were held at 20 kV and 120 kV, respectively. Nitrogen was used as the sheath gas at a flow rate of 80 and as the auxiliary gas at 20 (arbitrary units). Helium served as the buffer gas.

Separation was conducted on an ACE Excel C18 column (50 × 2.1 mm I.D., 1.7 µm, Batch: V17-1253, Avantor® ACE®, VWR, PT) maintained at 40°C, with a flow rate of 0.3 mL/min, and an injection volume of 10 µL, in a no-waste mode. The eluents used were methanol (MeOH) and water, both acidified with 0.1 % (v/v) LC-MS grade formic acid (FA) (>99%). The gradient program commenced at 10 % MeOH, increased to 50%

MeOH over 10 minutes, and then to 80% MeOH over 5 minutes before returning to 10% MeOH in 15 minutes and re-equilibrating to the initial conditions over 10 minutes. The quantification of the hormones was done using the standard addition method.

Results and Discussion

1. Algae/Sample collection, characterization and processing

After collection, 2 portions of macroalgae were obtained, one used for the assays of this project (unsorted portion) and another for characterization and species identification (sorted portion). The sorted portion is representative of the sample used for this project and of the average environmental sample of the selected collection spot at the designated time of the year (Figure 2).



Figure 2- Pictures showing the representative species collected in trays. A) Brown algae - *Sacchoriza polyschides*; B) Green and Red algae - B1) *Codium sp.*, B2) *Ulva rigida*, B3) *Gigartina pistillata*, B4) *Condrus crispus*, B5) Red algae consortium.

After drying, both portions were weighed and a loss of 90% of the weight was found. Each of the species in the sorted portion was weighted and the proportion of each phylum and each species relative to the total dry weight of the sample is shown in Figure 3.

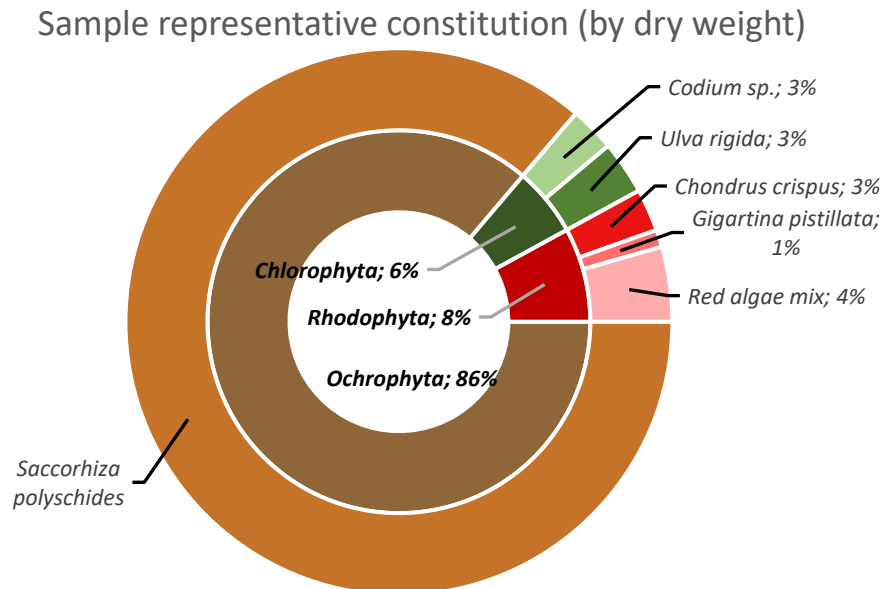


Figure 3 - Sample content, in percentage of dry weight, of the 3 *phyla* and the various species identified.

Brown algae (*Ochrophyta* phylum) make up most of the sample with 86% of the total dry weight, followed by red algae (*Rhodophyta*) with 8% and finally green algae (*Chlorophyta*) the remaining 6%. Only one species of brown algae was identified, this being *Saccorhiza polyschides*. When it comes to red algae, two species were identified, *Chondrus crispus* and *Gigartina pistillata*, constituting 3% and 1% of the total weight respectively, and a consortium of unidentified species was separated making up 4% of the total sample weight. For green algae, two species were identified, *Codium sp.* and *Ulva rigida*, both composing 3% of the total sample dry weight, each.

This does match with the overall literature where, despite not being the most abundant species-wise, brown algae are the biggest and heaviest and end up representing a higher weight portion of the sample, followed by red algae (with higher number of species) and green algae (Pereira, 2021). The species found and identified are described as existing on the northern coasts of Portugal by the “Observatório Marinho de Esposende” (OMARE, 2024), and their classification was confirmed by the macroalgae expert Francisco Arenas (CIIMAR), a collaborator of that Observatory.

After grinding the unsorted sample, two particle sizes sub-samples were obtained after sifting, and stored into zip-lock bags as shown in the figure below, one with particles smaller than 250 μm and the other containing particles bigger than 250 μm .

2. Natural Deep Eutectic Solvents - NADES

2.1. Characterization

2.1.1. Viscosity and density

In the table below (Table 6) are presented the values of viscosity and density measured for the various NADES studied, stock solutions and upon being tuned with different amounts of water, from 10 to 40%, at two temperatures, 20°C and 45°C.

Table 6- Values of density ($\rho/\text{g cm}^{-3}$) and viscosity ($\mu/\text{mPa s}^{-1}$) of NADES stock and respective tuned solutions with 10, 20, 30 and 40% of water, measured at 20 and 45°C.

NADES	Tuning	$\rho/\text{g cm}^{-3}$		$\mu/\text{mPa s}^{-1}$	
		20°C	45°C	20°C	45°C
ChCl:LA	Stock	1.15792	1.14331	498.7	112.3
	10%	1.14245	1.12826	79.43	26.02
	20%	1.12869	1.11455	26.42	10.80
	30%	1.11317	1.09927	11.76	5.245
	40%	1.09679	1.08327	5.904	2.990
ChCl:MA	Stock	Error*	1.21450	Error*	212.6
	10%	1.21068	1.19556	144.5	43.80
	20%	1.09014	1.07644	17.38	7.564
	30%	1.08199	1.06855	9.693	4.149
	40%	1.06897	1.05602	5.370	2.696
ChCl:Prop	Stock	1.09500	1.08137	83.28	28.36
	10%	1.08905	1.07535	33.32	13.13
	20%	1.08138	1.06773	17.00	7.368
	30%	1.07200	1.05861	9.029	4.164
	40%	1.06212	1.04920	5.612	2.770
ChCl:Urea	Stock	1.16032	1.14783	196.7	53.25
	10%	1.14478	1.13234	32.29	13.58
	20%	1.12947	1.11707	12.76	6.149
	30%	1.11243	1.10013	6.051	3.269
	40%	1.09672	1.08459	3.807	2.173
ChCl:CA	10%	1.28777	1.27194	1658	269.1
	30%	1.21839	1.20305	27.95	11.28

"Error*" - For ChCl:MA stock solution, the equipment could not achieve consistent readings leading to unmeasurable values.

Due to higher density and viscosity, measuring ChCl:CA stock solution was only possible at higher temperatures. The values obtained at 50 and 60°C are presented in the table below (Table 7).

Table 7 - Viscosity and density values of ChCl:CA stock solution measured at 50°C and 60°C

NADES	Tuning	$\rho / \text{g cm}^{-3}$		$\mu / \text{mPa s}^{-1}$	
		50°C	60°C	50°C	60°C
ChCl:CA	Stock	1.30264	1.29647	6889	2965

Due to these properties, ChCl:CA was not considered for extraction and no other properties aside from toxicity will be studied for this solvent (and respective tuned solutions).

Based on these results there are some conclusions we can take:

- The viscosity and density values vary as expected when it comes to temperature variation, being higher the lower the temperature is.
- These properties decrease their values the more water is added to a solution, likely due to the undoing/relaxation of the NADES matrix

The order of viscosity and density, from highest to lowest is as follows: ChCl:CA, ChCl:MA, ChCl:LA*, ChCl:Urea* and finally ChCl:Prop. (*ChCl:LA and ChCl:Urea present similar density but the viscosity is higher for ChCl:LA). These results can be related to the chemical structure and hydrogen bond saturation capacity of the corresponding HBD of each NADES.

- CA is a small tri-protic acid, meaning a lot of electronegativity displaced, allowing the 3 carboxyl groups of each molecule to form hydrogen bonds with ChCl, giving rise to a stronger matrix, lower fluidity and higher viscosity. As discussed previously, the interactions between HBA and HBD in NADES are favourable, leading to a compression of volume but keeping the same mass therefore attributing a higher density to the solvent.
- MA is a small di-protic acid. The same principles discussed for CA before also apply but since only 2 groups can form hydrogen bonds, the density and the viscosity are lower.
- LA is mono-protic, presenting one hydroxyl which can also form hydrogen bonds, despite on a smaller scale than a di-protic acid like MA does.

- Urea has 2 amine groups and a carbonyl, which can interact in two ways, both as HBD (from the amines) and as HBA (from the carbonyl). The 3 possible interactions may interfere with each other, reducing the strength of the overall bonds, making it weaker than the di-protic acid, but still strong enough to be comparable to a mono-protic hydroxyl acid like LA.
- Prop is a small diol (2 hydroxyl groups) that can form two bonds, but hydroxyl groups are not as electronegative as those previously mentioned, making the interactions in this solvent the weakest of the bunch.

The varying amounts of hydrogen bonds formed per molecule of HBA give each NADES a different saturation degree, which has been correlated with the difference in density and viscosity (Stefanovic et al., 2017). Similarly, the length of the bonds and the electronegativity of the molecules also impact these properties (Fan et al., 2021).

2.1.2. Polarity

For UV-Vis analysis, the protocol was microscale adapted to 96-well plates to increase the throughput of the polarity analysis and save the amount of sample and reagents used, relative to a regular spectrophotometric cuvette. Multiple samples can be read simultaneously, using less than 1/3 of solvent (1 mL cuvette vs 300 μ L 96-well plate's wells).

In the table below (Table 8) are the results of the UV-Vis analysis for some of the NADES and standard solvent tested.

Table 8 - λ_{max} and corresponding E_{NR} of some NADES (stock and tunings) and standard solvents (Hexane, DMSO, and H_2O)

Solvent	$\lambda_{\text{max}}/\text{nm}$	E_{NR} (kcal/mol)
ChCl:Urea Stock	565	50.604
ChCl:Urea 10%	565	50.604
ChCl:Urea 30%	570	50.160
ChCl:Prop Stock	570	50.160
ChCl:Prop 10%	575	49.724
ChCl:Prop 30%	585	48.874
ChCl:LA Stock	590	48.459
ChCl:LA 10%	590	48.459
ChCl:LA 20%	590	48.459
ChCl:LA 30%	590	48.459
ChCl:LA 40%	590	48.459
ChCl:MA 10%	640	44.673
ChCl:CA 10%	600	47.652
Hexane	490	58.349
DMSO	550	51.984
H₂O	520	54.983

Knowing that polarity and E_{NR} are inversely co-related, lower E_{NR} corresponding to higher polarity, it was expected that H_2O would present the lowest E_{NR} of our standard solvents while hexane should present the highest polarity index (Frontier, 2024), since water is the most polar solvent among the standard solvents tested, and hexane the least. Since the expected values for the standard solvents were not obtained with this method, it was

decided to not follow this adapted UV/vis protocol and opted for the traditional fluorometric analysis in cuvettes, with the setback of only being able to read 1 sample at the time and requiring higher volumes of sample.

The inconsistent values found can be traced to the lower sensitivity of the micro plate reader, trouble mixing Nile-Red in NADES due to their viscosity, as well as the dimerization and subsequent precipitation of Nile-Red in water (Jose and Burgess, 2006). This precipitation has a big impact on this method of reading, as the light beam crosses the bottom of the well, where these dimers have likely deposited and gathered, blocking the light sensor fully or partially, leading to the inaccurate results obtained.

The figures presented below (Figures 4 – 7) show the wavelength shift of NADES tuned with different amounts of water, and their positioning relative to the used standards, as well as a table (**Erro! A origem da referência não foi encontrada.** Table 9) presenting each solvent E_{NR} and respective relative polarity. The lack of data for NADES stock solutions and ChCl:CA is again due to the difficulties mixing Nile-Red onto these solutions, and so no analyses were performed for these solvents.

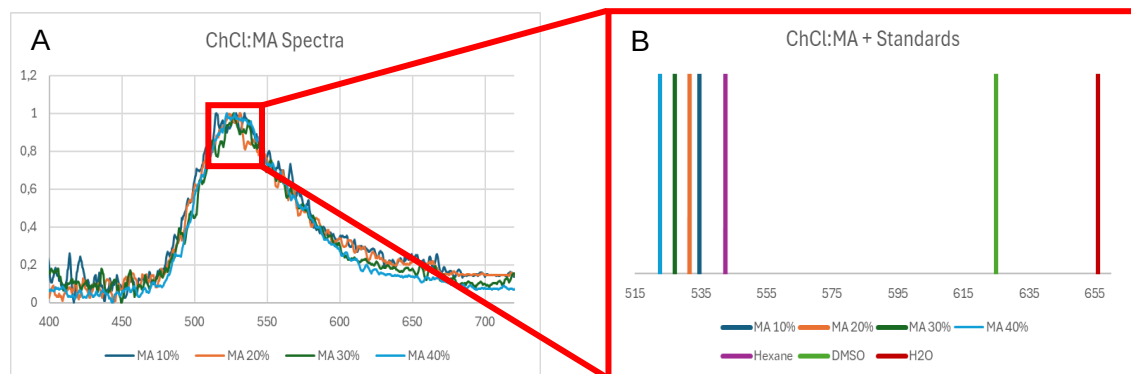


Figure 5 - A) Normalized ChCl:MA tuned fluorescence spectra; B) Zoomed maximum fluorescence peaks of ChCl:MA tuned solutions and standard solvents

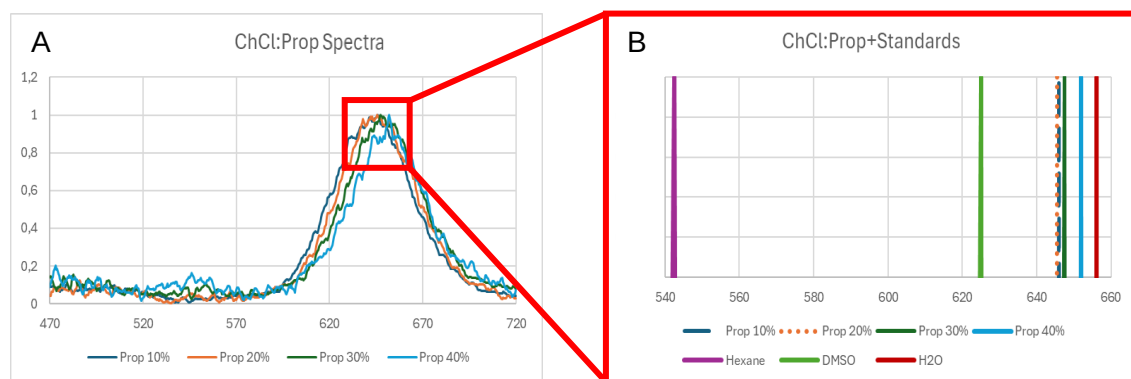


Figure 4 - A) Normalized ChCl:Prop tuned solution fluorescence spectrum; B) Zoomed maximum fluorescence peaks of ChCl:Prop tuned solutions and standard solvents

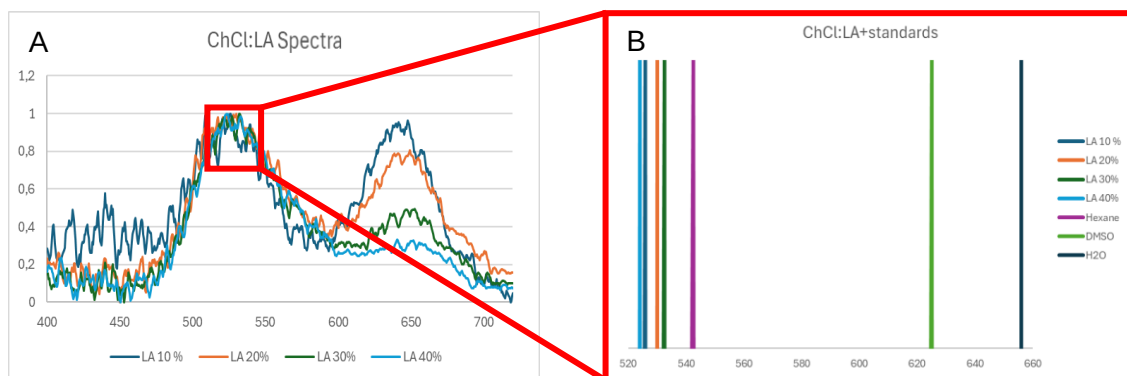


Figure 7 - A) Normalized ChCl:LA tuned solutions fluorescence spectrum; B) Zoomed maximum fluorescence peaks of ChCl:LA tuned solutions and standard solvents

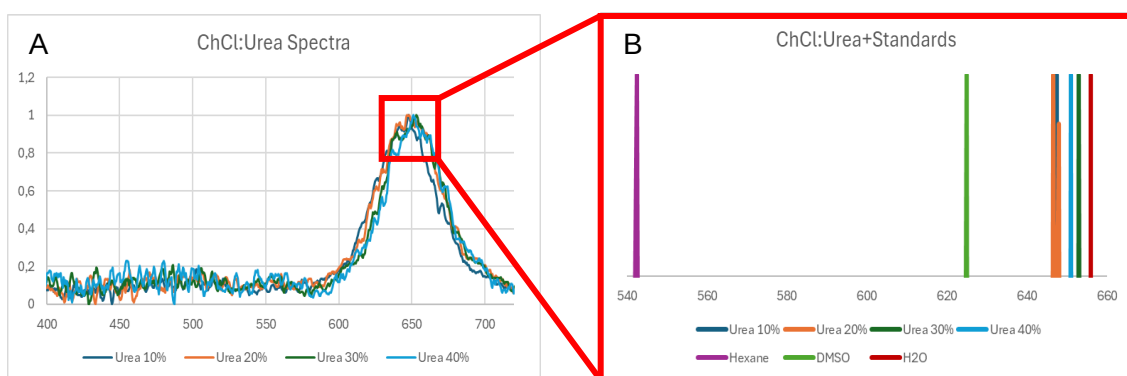


Figure 6 - A) Normalized ChCl:Urea tuned solutions fluorescence spectra; B) Zoomed maximum fluorescence peaks of ChCl:Urea tuned solutions and standard solvents

There is a clear shift in $\lambda_{\max}$ for the different tuned solutions of each NADES, an expected result since, as previously discussed, the addition of water alters the properties of these solvents. The undoing of the matrixes leads to the tuned NADES becoming closer to a solution of the compounds rather than an organized web of bonds, meaning that their characteristics should resemble the ones of water. In theory, it would be expected that, with the increase in water content, the polarity of the tuned solutions should become closer to the polarity of water.

The results allow us to draw some conclusions about the relative polarity of each NADES group. The acid-based NADES (ChCl:LA and ChCl:MA) present low relative polarity (supposedly lower than hexane), while ChCl:Urea and ChCl:Prop present a relative polarity between the ones of DMSO and water, but closer to that of water.

The calculated E_{NR} value of each solvent tested and its relative polarity, are presented in the table below (Table 9) in increasing order of relative polarity. **Erro! A origem da referência não foi encontrada.**

Table 9 - NADES tuning's $\lambda_{\max}$, respective ENR and relative polarity

	$\lambda_{\max}$ (nm)	E_{NR} (kcal mol ⁻¹)	Relative polarity
MA 40%	522.5	54.720	-0.339
LA 40%	524	54.564	-0.321
LA 10 %	526	54.356	-0.298
MA 30%	527	54.253	-0.287
LA 20%	530	53.946	-0.252
MA 20%	531.5	53.794	-0.235
LA 30%	532.5	53.693	-0.224
MA 10%	534.5	53.492	-0.202
Hexane	542.5	52.703	0.009
DMSO	625	45.746	0.444
Prop 20%	645.5	44.293	0.825
Prop 10%	646	44.259	0.829
Urea 20%	646.5	44.225	0.832
Prop 30%	647.5	44.157	0.840
Urea 10%	647.5	44.157	0.840
Urea 40%	651	43.919	0.867
Prop 40%	652	43.852	0.874
Urea 30%	653	43.785	0.882
H ₂ O	656	43.585	1.000

However, when we take a closer look at the data, while it was expected that the 40% tuned solutions of each NADES would present the highest polarity within each group, that was not the case. This can occur for a few reasons. We see from the graphs that this method gives out results with a lot of noise (probably due to low intensity of fluorescence), which can lead to inaccurate readings if they are too close together. The concentration of Nile-Red dye used can explain this noise (lower concentration/amounts, lower intensity), being one possible optimization point. The homogenous mixing of Nile-Red in the tuned solutions is also a problem due to the viscosity of these solutions.

Unlike the usage of the microplate reader, the Nile-Red dimers interfering with the light sensor is not as impactful in this method, since the reading is done halfway through the cuvette height, where fewer or no deposits are present.

2.2. Degradability

Below are graphs (Figure 8 and Figure 9) depicting the biodegradability of NADES.

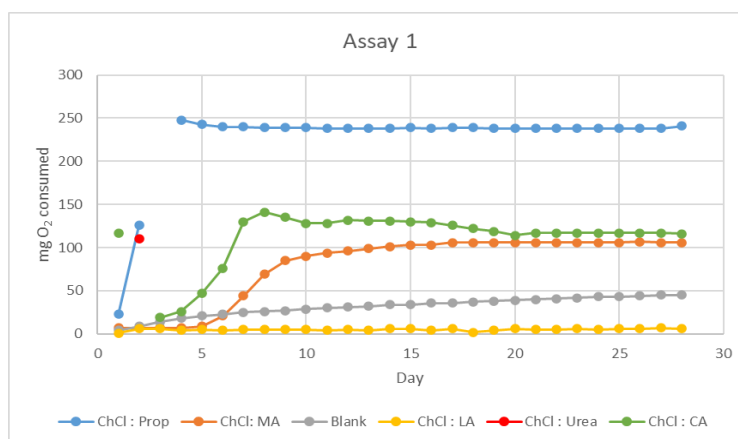


Figure 8 – Amount of O₂ consumed (in mg) to degrade NADES (biodegradability) over a 28-day period (1st assay)

As we can see there are inconsistent/missing measurements for ChCl:Urea, ChCl:Prop and ChCl:LA. For ChCl:Urea, the missing values were due to an overflow of the assay (the oxygen consumed was higher than what could be measured for the chosen flask). ChCl:Prop and ChCl:LA present opposite problems: ChCl:Prop values were very high throughout the assay, while ChCl:LA results were very low. To try and rectify and confirm the results, a second assay was done, repeating the previous. Below are the results (Figure 9).

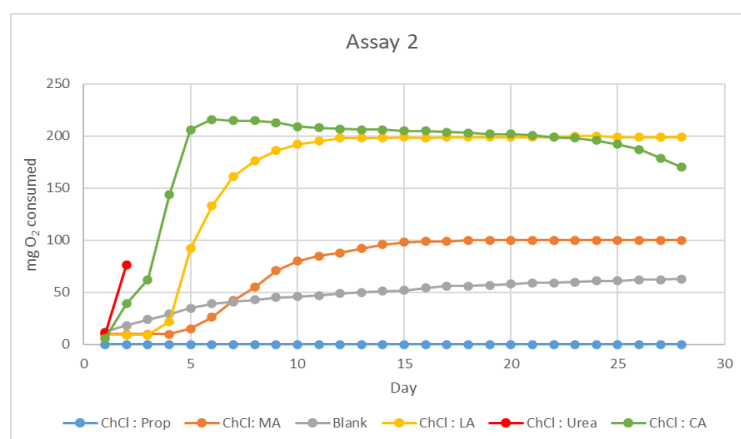


Figure 9 - Amount of O₂ consumed (in mg) to degrade NADES (biodegradability) over a 28-day period (2nd assay)

Unfortunately, despite the new assay no new concrete information was obtained and we ran into the same problems as before. While we can speculate that there might be a certain degree of biodegradability for these NADES, according to OECD's 301F protocol,

for a compound to be declared as (readily) biodegradable, the ratio of experimental biodegradability at day 28 and ThOD must be $\geq 60\%$. Due to difficulties on the preparation of the assay, and the inconsistency of our results we can't take any conclusions about the specific biodegradability of NADES. We can, however, analyse their biodegradability profile and make some speculations.

Overall, NADES present a regular degradability profile, where it starts with low values, has an exponential growth (between 3rd and 5th day) and then plateaus. One of the difficulties that also seems to be messing with the results is a possible toxicity of NADES. When looking at ChCl:CA in assay 1 we see the 1st day presenting a high value, followed by a lack of record and when new values arise, they are very low. This could be evidence of an adaptation to the toxic environment (some bacteria cannot handle the presence of this material in the environment on the first couple of days but end up adapting to it leading to a regular profile afterwards). This is evidence of the necessity of further study of these compounds, that although claimed as "green" and biodegradable, there is still a lack of concrete evidence to classify them as such, probably due to their novelty.

3. Germination

3.1. Water volume optimization

On the next figure (Figure 10) are presented the results of the optimization assay of water volume used in petri dishes for tomato seed germination.

Water volume optimization assay

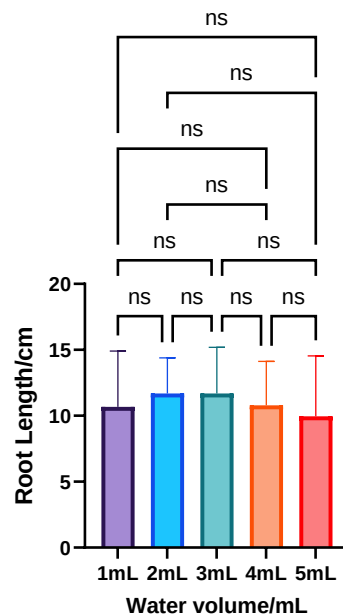


Figure 10 - Water volume optimization for tomato seeds germination assay results – average root length of seedling with 5 different amounts of water (1, 2, 3, 4 and 5 mL). Statistical analysis performed using Kruskal-Wallis non-parametric test: ns $p > 0.05$; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$

Despite there being no significant differences between the amounts of water, when it came to lab work and the visual aspect of the seeds there were some noteworthy mentions. 1 mL led to very weak and dried out seedlings, while 4 mL and 5 mL would have the seeds floating around and gathering in one spot, which lead to the roots tangling and breaking very easy when needed to be analysed. From the 2 mL and 3 mL, these show the greatest performances and although not significantly different from each other, 2 mL led to slightly higher germination averages (9.5 and 9 seeds per plate respectively). With all this information, we opted to do all the assays using 2 mL.

3.2. Toxicity

3.2.1. NADES

A preliminary toxicity assay was performed by diluting weighted amounts of NADES stock solution to obtain solutions of the following concentrations: 10000 mg/L, 3333 mg/L, 1111 mg/L, 370 mg/L, 123 mg/L and 100 mg/L. These solutions were applied in a germination assay of *Solanum lycopersicum* seeds, to determine their phytotoxicity. The graphs presented depict the effects of exposure to these solutions, for 7 days at 25°C, on tomato seedlings' root and shoot growth. These results will allow us to determine the concentrations of NADES seaweed extracts that will be used in future assays, based on the concentrations at which NADES present toxicity or not (either No Observed Effect Concentration – NOEC - or Lowest Observed Effect Concentration – LOEC). For the selection of the concentrations both measurements were used and can be seen in detail below (Figure 11 to Figure 15).

For ChCl:LA exposure (Figure 11), the assay present similar results for root and shoot length, where up to 370 mg/L, there were no significant differences in either measurement compared to the control. So, we opted to use this concentration for future assays.

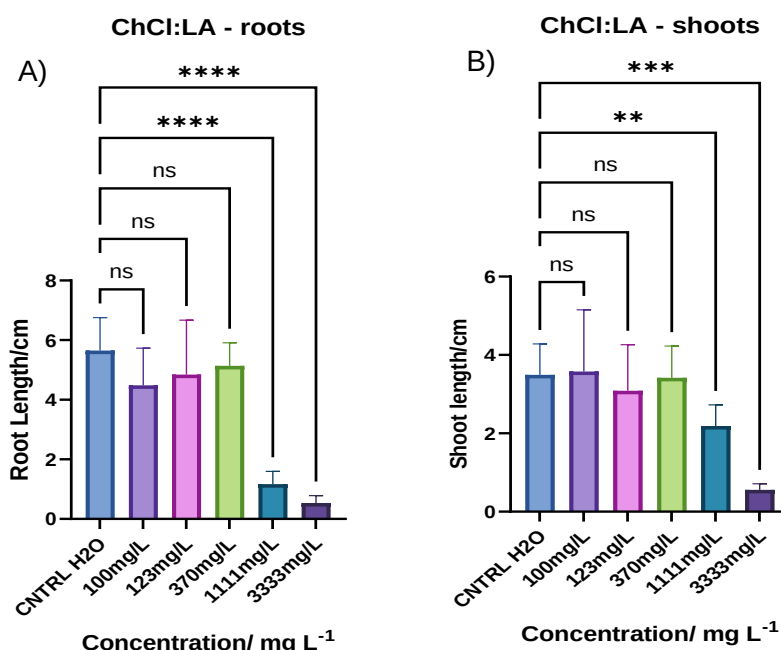


Figure 11 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:LA. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$

For ChCl:MA (Figure 12), while there were no significant differences when comparing shoot lengths, we can see a clear impact on the root length. This was the most toxic NADES. To minimize the impact of toxicity, we chose to use a concentration of 100 mg/L for the following ChCl:MA assays.

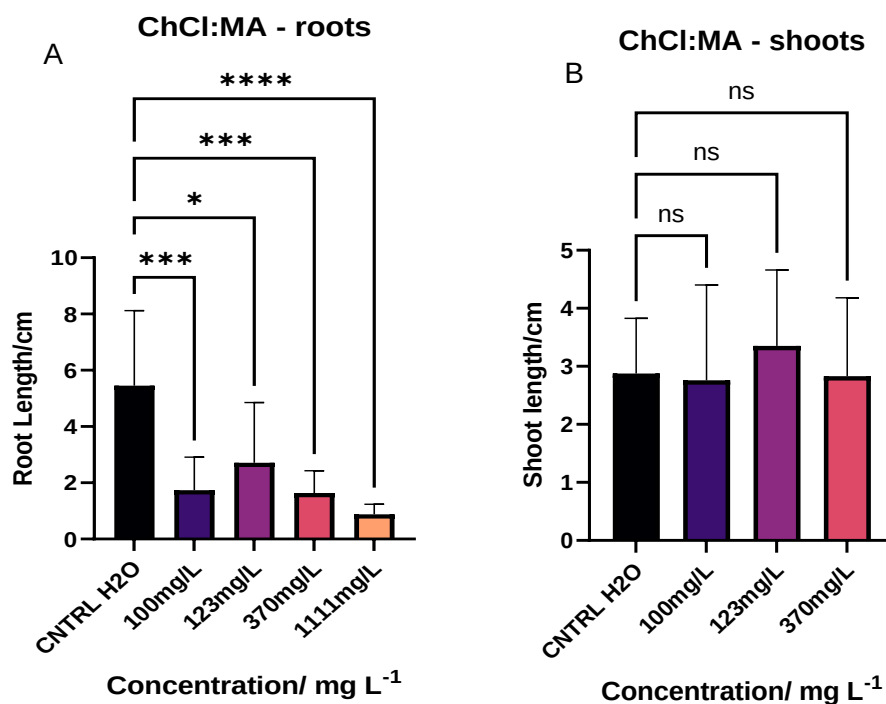


Figure 12 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:MA. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p>0.05$; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$; **** $p<0.0001$

There are discrepancies for ChCl:Prop results (Figure 13), likely due to the small number of replicas analysed, as it was a preliminary assay. To account for that we went for the concentration of 123 mg/L, where there was a bigger consistency of NOEC values for both root and shoot length.

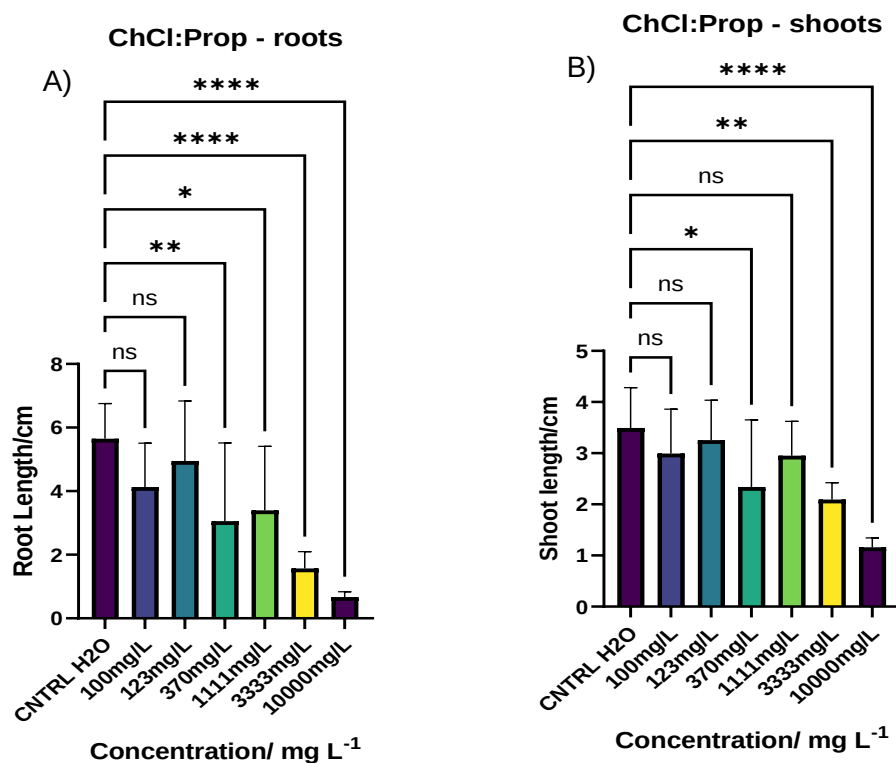


Figure 13 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:Prop toxicity assay. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$.

For ChCl:Urea (Figure 14), roots suffer less impact of the toxicity when compared to shoots, but since there were significant differences for the shoots, the concentration of 123 mg/L was chosen, aiming for a healthy seedling.

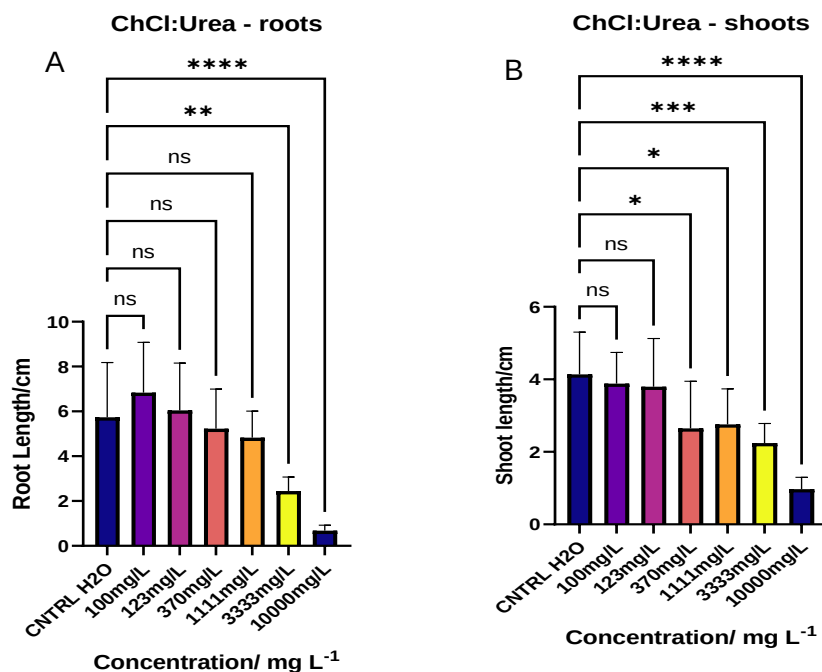


Figure 14 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:Urea. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$

For ChCl:CA (Figure 15) the concentration chosen was of 123 mg/L.

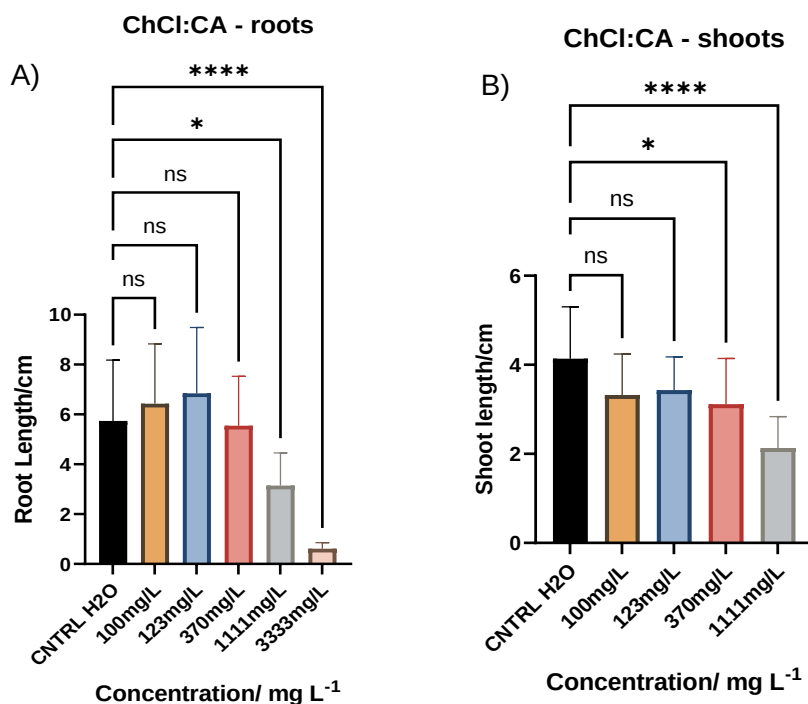


Figure 15 - Root (A) and shoot (B) length of seedlings exposed to different concentrations of ChCl:CA toxicity assay. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$.

To sum up, the table below (Table 10) shows the NOEC and LOEC values observed for this assay, based upon which the concentrations that will be used in future NADES' extracts assays.

Table 10 - Highest tolerable concentrations of NADES on germination, obtained from preliminary toxicity assay, corresponding to the NOEC determined for each NADES

EFFECT	Root Length		Shoot Length	
	NOEC (mg/L)	LOEC (mg/L)	NOEC (mg/L)	LOEC (mg/L)
ChCl:LA	370	1111	370	1111
ChCl:MA	-	100	370	1111
ChCl:Prop	123	370	123	370
ChCl:Urea	1111	3333	123	370
ChCl:CA	370	1111	123	370

The parameter "shoot length" is more sensible than "root length" for the majority of NADES. As so, the NOEC observed on shoot growth was selected to determine the

working concentrations for the posterior assays. ChCl:LA and ChCl:Prop presented similar values of NOEC and LOEC for both root and shoot growth.

The toxicity ranking of the NADES used, considering the NOEC of both parameters, root growth and shoot length, was the follow: MA > Prop; Urea; CA > LA. According to the hazard ranking presented by Passino and Smith (1987) based on LC50's of fish, these NADES follow in the category "practically harmless" (effect observed between 100-1000 mg/L) for tomato seed. Considering that NOEC is a parameter more conservative than LC50, these NADES may be very close to the category below, which is "relatively harmless" (effects above > 1000 mg/L), the one where the lowest toxic compounds fall.

3.2.2. NADES vs Components

To evaluate the toxicity of diluted NADES compared to that of a solution of each of its components, 100 mg/L solutions were tested on a germination assay where root length was examined. The graphs (Figure 16) below present the results obtained.

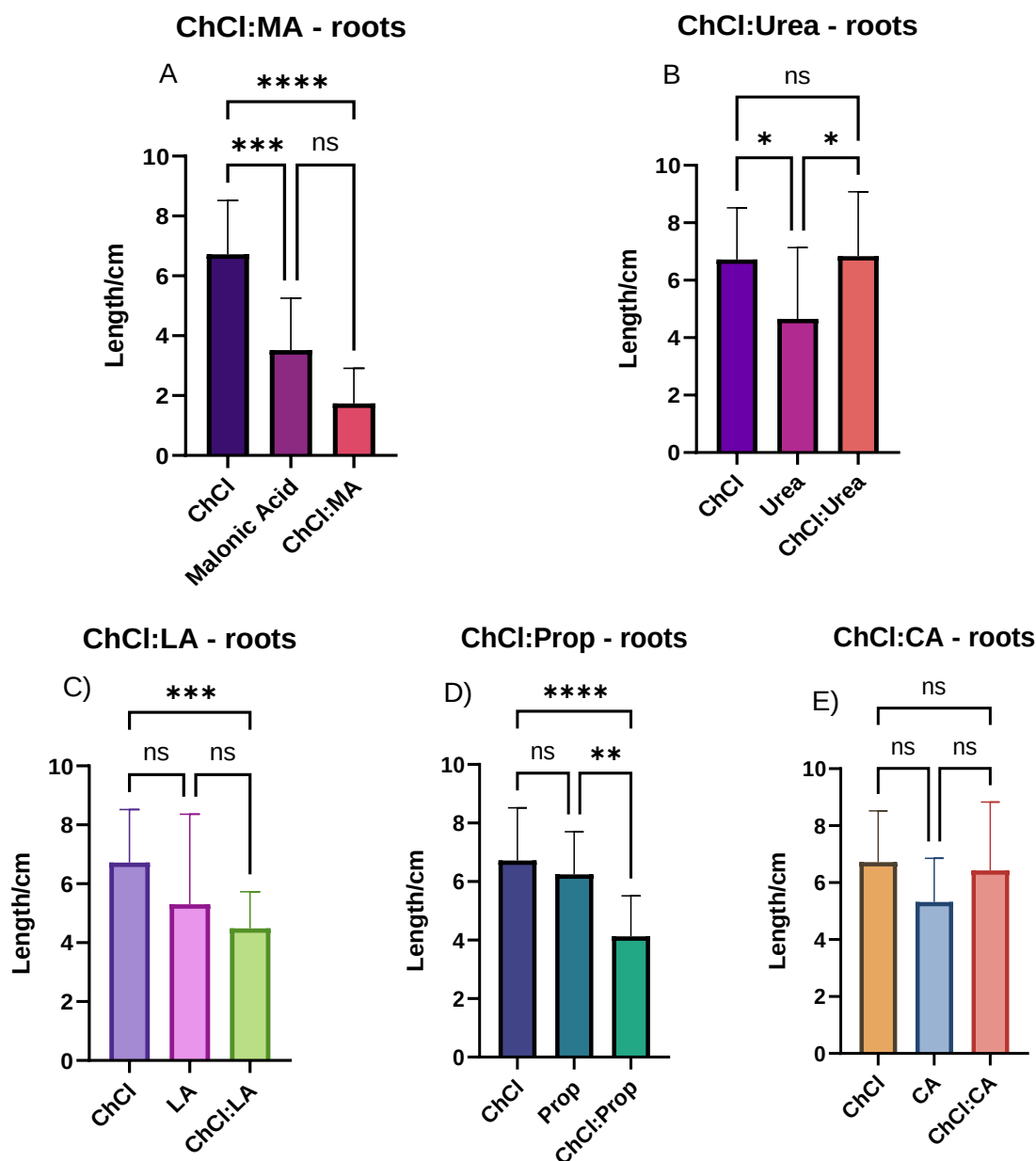


Figure 16 - Root length observed after exposure to NADES and their respective components separately, at 100 mg/L. A) ChCl:MA; B) ChCl:Urea; C) ChCl:LA; D) ChCl:Prop; E) ChCl:CA. Statistical analysis was performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$.

From these results we can see that there was no overall correlation between NADES toxicity and the corresponding components, and this varies on a case-to-case basis. For ChCl:MA, ChCl:LA and ChCl:Prop, the root growth is significantly smaller than the one of ChCl, indicating higher toxicity of the NADES, while for ChCl:Urea and ChCl:CA show

no differences to this compound. When comparing to the HBD of each NADES, ChCl:Urea and ChCl:Prop present higher toxicity, but for ChCl:MA, ChCl:LA and ChCl:CA no significant differences were found.

ChCl:Prop was a unique case, where the NADES was significantly more toxic than any of the components. In the case of ChCl:MA and ChCl:LA, their toxicity was similar to their HBD, while in the case of ChCl:Urea its toxicity was similar to that of the HBA.

What we can take from these results is that, although not independent, there doesn't seem to exist a direct correlation between the toxicity of NADES and their respective compounds. However, in general it was observed that ChCl tends to be less toxic than the various HBDs and NADES. These results are in accordance with those observed by Morais et al. (2015) for the bacteria *Vibrium fischeri* (Microtox test), whom concluded that the HBA is the less toxic component and that the toxicity observed is mainly due to the HBD. In general, the toxicity of the NADES is intermediate when compared to starting materials (ChCl and acids).

3.2.3. Buffered vs Unbuffered

Since the NADES' diluted solutions used on the toxicity assays presented a variable pH, lower than the ideal for tomato seed germination and seedling growth (Table 11) a new assay using MES buffer was performed to evaluate the impact of the pH on the previously obtained results.

Table 11 - pH of NADES' diluted solutions used in germination assays, buffered and unbuffered

NADES	Unbuffered pH	Buffered pH
ChCl:LA (370 mg/L)	3.29	6.40
ChCl:MA (123 mg/L)	3.54	6.41
ChCl:Prop (123 mg/L)	6.15	6.43
ChCl:Urea (100 mg/L)	3.58	6.45
ChCl:CA (123 mg/L)	5.45	6.40

The graph (Figure 17) below shows the effect of MES buffering of NADES solutions on root growth.

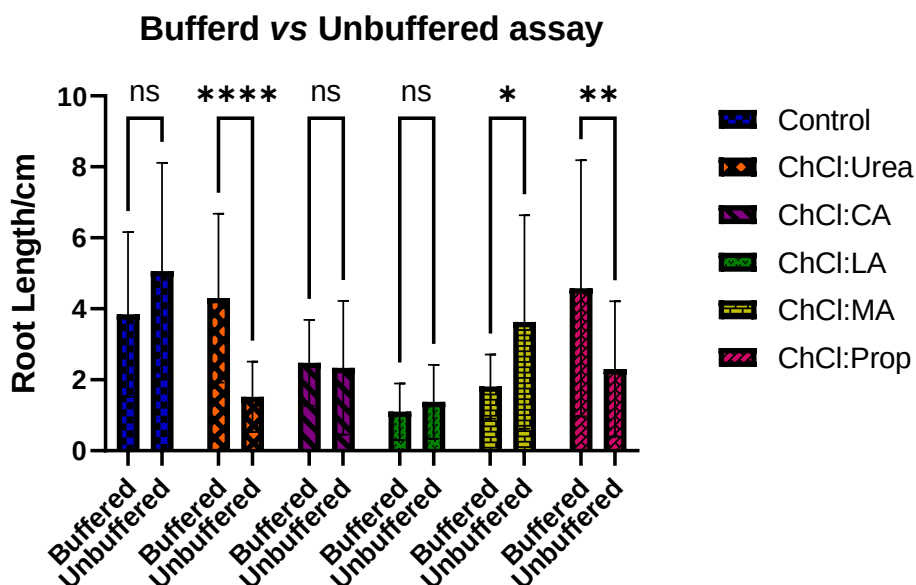


Figure 17 – Root length (in cm) from toxicity assay with ("Buffered") and without ("Unbuffered") the use of MES buffer in diluted NADES solutions. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$

While there is an increase in root length when ChCl:Urea and ChCl:Prop NADES are buffered, this is not a consistent result. We can see that the application/use of MES buffer can also have no (Control, ChCl:CA, and ChCl:LA) or even an inhibiting effect (ChCl:MA). Since the beneficial effect does not apply to the majority of NADES, and for the sake of uniformity, we decided not to use MES buffer in future assays.

4. Sample-to-Solvent ratio

For NADES extraction, multiple sample-to-solvent ratios were found throughout the literature review. Taking that into account as well as the toxicity of NADES and differences in the tunings' properties, multiple ratios were tested to try and obtain the maximum extraction efficiency for each solvent. Below is a table (Table 12) showing the chlorophyll a and carotenoids content of each solvent for ratios T1 – 100 mg/mL, T2 – 150 mg/mL and T3 – 250 mg/mL. Ratios T4-400 mg/mL and T5- 600mg/mL were discarded as they were too high for the full coating and suspension of the biomass to occur and therefore unworkable extraction-wise.

Table 12 - Chlorophyll a and carotenoid values determined from different sample/-to-solvent ratios for different NADES and NADES tunings solutions, tuned with 10 to 40% water. Ratios: T1 – 100 mg/mL, T2 – 150 mg/mL, T3 – 250 mg/mL, SD – standard deviation. The quantities of pigments are presented in µg of pigment/mL of extract

NADES	Working solution	Compound	T1	SD	T2	SD	T3	SD
ChCl:L.A.	10%	Chll a	1,835	1,106	1,498	0,231	2,697	0,079
		Carotenoids	1,143	0,652	1,022	0,177	1,927	0,054
	20%	Chll a	0,708	0,077	1,057	0,171	0,707	0,045
		Carotenoids	0,595	0,068	0,897	0,133	0,633	0,040
	30%	Chll a	0,457	0,113	0,865	0,114	0,520	0,175
		Carotenoids	0,463	0,072	0,805	0,091	0,627	0,050
	40%	Chll a	0,509	0,042	0,709	0,027	0,710	0,191
		Carotenoids	0,493	0,016	0,687	0,016	0,805	0,087
ChCl:M.A.	10%	Chll a	0,978	0,266	1,066	0,338	2,815	0,936
		Carotenoids	0,665	0,206	0,738	0,254	2,026	0,794
	20%	Chll a	0,803	0,168	0,811	0,334	0,783	0,453
		Carotenoids	0,637	0,135	0,696	0,254	0,752	0,331
	30%	Chll a	0,504	0,030	0,515	0,138	0,579	0,287
		Carotenoids	0,446	0,022	0,554	0,077	0,690	0,164
	40%	Chll a	0,409	0,220	0,515	0,093	0,590	0,181
		Carotenoids	0,367	0,141	0,499	0,060	0,719	0,107
ChCl:Prop	10%	Chll a	0,247	0,155	0,481	0,241	0,583	0,348
		Carotenoids	0,261	0,140	0,490	0,227	0,621	0,326
	20%	Chll a	0,402	0,040	0,332	0,179	0,116	0,028
		Carotenoids	0,478	0,054	0,431	0,213	0,218	0,032
	30%	Chll a	0,244	0,104	0,315	0,060	0,107	0,030
		Carotenoids	0,337	0,122	0,448	0,079	0,236	0,054
	40%	Chll a	0,169	0,090	0,287	0,032	0,183	0,122
		Carotenoids	0,264	0,103	0,431	0,039	0,361	0,160
ChCl:Urea	10%	Chll a	0,252	0,064	0,251	0,097	0,544	0,173
		Carotenoids	0,287	0,078	0,301	0,104	0,582	0,178
	20%	Chll a	0,232	0,049	0,231	0,046	0,587	0,115
		Carotenoids	0,318	0,051	0,349	0,041	0,783	0,142
	30%	Chll a	0,253	0,035	0,362	0,097	0,351	0,147
		Carotenoids	0,376	0,048	0,516	0,113	0,679	0,153
	40%	Chll a	0,332	0,005	0,334	0,044	0,466	0,008
		Carotenoids	0,476	0,012	0,539	0,072	0,809	0,059

The results are presented in Table 13, after normalization to the dry biomass extracted.

Table 13 - Chlorophyll a and carotenoid values determined, nNormalized (to μg of pigment per mg of dry biomass mean pigment content), determined obtained from different sample/solvent ratios for different NADES and NADES tuning solutions, tuned with 10 to 40% water. Ratios: T1 – 100 mg/mL, T2 – 150 mg/mL, T3 – 250 mg/mL, SD – standard deviation. The quantities of pigments are presented in μg of pigment per mg of dry biomass extracted.

NADES	Working solution	Compound	T1	SD	T2	SD	T3	SD
ChCl:L.A.	10%	Chll a	1,84E-02	1,11E-02	9,98E-03	1,54E-03	1,08E-02	3,17E-04
		Carotenoids	1,14E-02	6,52E-03	6,82E-03	1,18E-03	7,71E-03	2,16E-04
	20%	Chll a	7,08E-03	7,73E-04	7,04E-03	1,14E-03	2,83E-03	1,80E-04
		Carotenoids	5,95E-03	6,79E-04	5,98E-03	8,84E-04	2,53E-03	1,62E-04
	30%	Chll a	4,57E-03	1,13E-03	5,76E-03	7,60E-04	2,08E-03	6,99E-04
		Carotenoids	4,63E-03	7,25E-04	5,37E-03	6,07E-04	2,51E-03	2,00E-04
	40%	Chll a	5,09E-03	4,18E-04	4,73E-03	1,79E-04	2,84E-03	7,66E-04
		Carotenoids	4,93E-03	1,65E-04	4,58E-03	1,06E-04	3,22E-03	3,47E-04
ChCl:M.A.	10%	Chll a	9,78E-03	2,66E-03	7,11E-03	2,25E-03	1,13E-02	3,75E-03
		Carotenoids	6,65E-03	2,06E-03	4,92E-03	1,70E-03	8,10E-03	3,18E-03
	20%	Chll a	8,03E-03	1,68E-03	5,41E-03	2,23E-03	3,13E-03	1,81E-03
		Carotenoids	6,37E-03	1,35E-03	4,64E-03	1,69E-03	3,01E-03	1,32E-03
	30%	Chll a	5,04E-03	2,96E-04	3,43E-03	9,22E-04	2,32E-03	1,15E-03
		Carotenoids	4,46E-03	2,15E-04	3,69E-03	5,15E-04	2,76E-03	6,56E-04
	40%	Chll a	4,09E-03	2,20E-03	3,43E-03	6,21E-04	2,36E-03	7,22E-04
		Carotenoids	3,67E-03	1,41E-03	3,33E-03	4,03E-04	2,87E-03	4,28E-04
ChCl:Prop	10%	Chll a	2,47E-03	1,55E-03	3,21E-03	1,61E-03	2,33E-03	1,39E-03
		Carotenoids	2,61E-03	1,40E-03	3,27E-03	1,52E-03	2,48E-03	1,30E-03
	20%	Chll a	4,02E-03	3,99E-04	2,22E-03	1,19E-03	4,66E-04	1,14E-04
		Carotenoids	4,78E-03	5,41E-04	2,87E-03	1,42E-03	8,71E-04	1,29E-04
	30%	Chll a	2,44E-03	1,04E-03	2,10E-03	4,02E-04	4,27E-04	1,18E-04
		Carotenoids	3,37E-03	1,22E-03	2,99E-03	5,27E-04	9,43E-04	2,17E-04
	40%	Chll a	1,69E-03	9,03E-04	1,91E-03	2,12E-04	7,34E-04	4,88E-04
		Carotenoids	2,64E-03	1,03E-03	2,88E-03	2,61E-04	1,44E-03	6,38E-04
ChCl:Urea	10%	Chll a	2,52E-03	6,37E-04	1,67E-03	6,44E-04	2,18E-03	6,92E-04
		Carotenoids	2,87E-03	7,80E-04	2,01E-03	6,93E-04	2,33E-03	7,11E-04
	20%	Chll a	2,32E-03	4,95E-04	1,54E-03	3,03E-04	2,35E-03	4,61E-04
		Carotenoids	3,18E-03	5,10E-04	2,33E-03	2,73E-04	3,13E-03	5,70E-04
	30%	Chll a	2,53E-03	3,50E-04	2,41E-03	6,47E-04	1,40E-03	5,86E-04
		Carotenoids	3,76E-03	4,84E-04	3,44E-03	7,55E-04	2,72E-03	6,11E-04
	40%	Chll a	3,32E-03	4,57E-05	2,23E-03	2,96E-04	1,86E-03	3,36E-05
		Carotenoids	4,76E-03	1,24E-04	3,60E-03	4,80E-04	3,24E-03	2,36E-04

The highest pigment (chlorophyll a and carotenoids) content pair measured for each NADES solution is highlighted in blue. The corresponding ratios were the ones used for extraction. It is to be noted that for ChCl:LA 20%, T1 and T2 gave us similar amounts, but to try and obtain other possible compounds in higher amounts we decided to go for the higher ratio T2.

It is important to mention that there are no formulas for spectrophotometric quantification of these pigments specific for NADES yet, unlike other commonly used solvents, meaning these values are likely not the real/accurate ones, but should allow us to take some conclusions of the extraction efficiency of our synthesized solvents.

5. Extract characterization

Macroalgae are known for being a source of a variety of compounds applicable in a wide range of industries. Brown algae specifically are associated with high content of phlorotannins, a class of polyphenols, and other antioxidant products. The extracts we obtained are very crude, presenting an array of metabolites from various classes and effects (proteins, sugars, secondary metabolites, etc.).

5.1. DMBA – Phlorotannin content

The quantitative measurement of phlorotannins in the macroalgae extracts, obtained with NADES, was not possible to attain due to the interferences of some of the NADES, or their components in the reaction of DMBA method. The interferences present themselves as both a powdery precipitate and a high-intensity coloration of the solution, which lead to inaccurate and inconsistent readings among the successive dilutions of the calibration curve.

Because no literature was found concerning possible interferences or optimizations for this protocol, for the sake of uniformity, and since only two of the NADES did not present interferences (ChCl:MA and ChCl:Prop), we opted for the Folin-Ciocalteu's total phenolic content analysis protocol.

This was a disappointing outcome, since DMBA is a colorimetric method specific for 1,3- and 1,3,5-substituted phenols, making it very specific when working with brown macroalgae, as the high content in phenolic compounds is normally associated to phlorotannins, polymers of phloroglucinol (a 1,3,5- substituted phenol, 1,3,5-trihydroxybenzene), very abundant in this type of algae.

When discussing interferences, while it is known that phlorotannins can bind to proteins and form complexes that precipitate under certain pH conditions (Vissers et al., 2017), not much else is known, especially when talking about NADES. However, due to the nature of our NADES being composed of an ammonium salt (ChCl) and in some cases carboxylic acids (LA, CA, and MA), we could speculate the precipitation is due to similar interactions, as these mixtures could present an amino-acid-like behaviour based on their constituents.

5.2. Folin-Ciocalteu's total phenolic content

When the traditional Folin protocol was performed, a yellow precipitate was visible after the addition of the Folin reagent to our synthesized NADES, as well as a false positive in the case of ChCl:MA.

As this assay is based on redox reaction of the Folin reagent composed of phosphotungsten and phosphomolybdenum ions, by phenolate ions, turning the solution yellow to blue, the precipitate is likely due to an interference in the solubility of these ions. Phosphomolybdate has a known affinity for ammonium salts, forming ammonium phosphomolybdate which is typically insoluble in water, leading to the precipitation of a yellow powder. Despite other researchers, who used the same protocol for TPC measurement for analysis of NADES extracted samples, also reporting similar experiences, the reviewed bibliography does not report this phenomenon thoroughly.

However, there has been some suggestions concerning the delay or prevention of formations of these solids depending on the initial base and its concentration (Cicco and Lattanzio, 2011). The change of base can also help prevent or minimize the appearance of false positives derived from the reaction with reducing sugars and/or other compounds with free hydroxyl groups (Magalhães et al., 2010; Lawag et al., 2023).

Due to the nature of NADES, both the presence of both ammonium salts and reducing hydroxyl groups is a common motif be it from the use of some alcohols, carboxylic acids, amino acids and/or choline chloride (common HBA), leading to higher rate of interferences and false positives.

With this in mind, despite being a commonly used method for TPC, if we want to make NADES (and other synthetic solvents) a viable alternative, a deeper understanding of their interferences in these day-to-day reactions is necessary, as well as the development of solutions, either for the removal of interferences in well-known protocols, or the development of new methods.

5.3. Folin-Ciocalteu's protocol optimization

As mentioned previously, the traditional Folin-Ciocalteu's protocol may be optimized, by changing the reagent addition order, and the kind and concentration of base/alkali used.

The only alteration to the original protocol that prevented precipitation was changing the base from the traditional Na_2CO_3 7.5% to NaOH 7.5%. For uniformization's sake, all TPC analysis going forward followed the microscale standard Folin-Ciocalteu's protocol with this alteration.

5.4. Folin-Ciocalteu's method results after optimization

When the new optimized protocol was implemented to develop the calibration curves necessary for this protocol, new problems arose. When gallic acid was added, the previously seen precipitation formed again, even after the increased concentration of NaOH (despite being smaller and allowing for a small colour shift), interfering with the absorbance reading. The precipitation likely occurs by the formation of molybdenum complexes with ChCl, making them insoluble (Akyildiz, 2021). While adding a stronger base (NaOH) likely prevents complexation, the addition of gallic acid is probably causing a neutralization reaction, taking away the "power" of NaOH to make Folin's metallic ions soluble again.

In conclusion, further optimization of this protocol is needed to quantify TPC in NADES-extracted samples using Folin's reagent.

5.5. DPPH (2,2'-Diphenyl-1-picrylhydrazyl) antioxidant potential assay

The results of the antioxidant potential of beach wrack extracts obtained with different NADES and determined by the DPPH method are presented below (Figure 18).

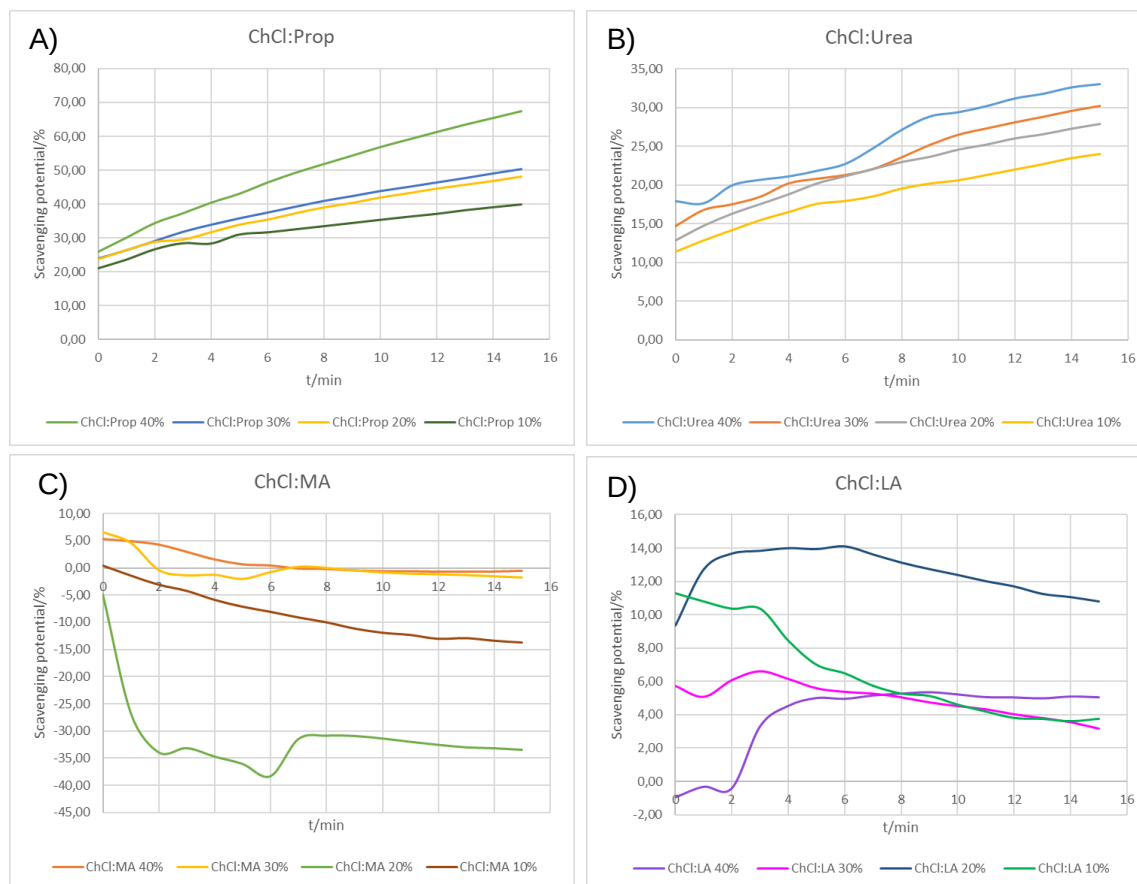


Figure 18 - Scavenging potential of various NADES extracts through time, A) ChCl:Prop; B) ChCl:Urea; C) ChCl:MA; D) ChCl:LA

From the analysis of the results, it was observed that the extracts that presented the highest antioxidant potential are those extracted with the ChCl:Prop NADES, mainly the 40% tuned solution, with a maximum scavenging potential of 67.45%, and a 41.47% increase with time. It was followed by the remaining ChCl:Prop NADES tuned solutions, ChCl:Urea, ChCl:LA and ChCl:MA by this order.

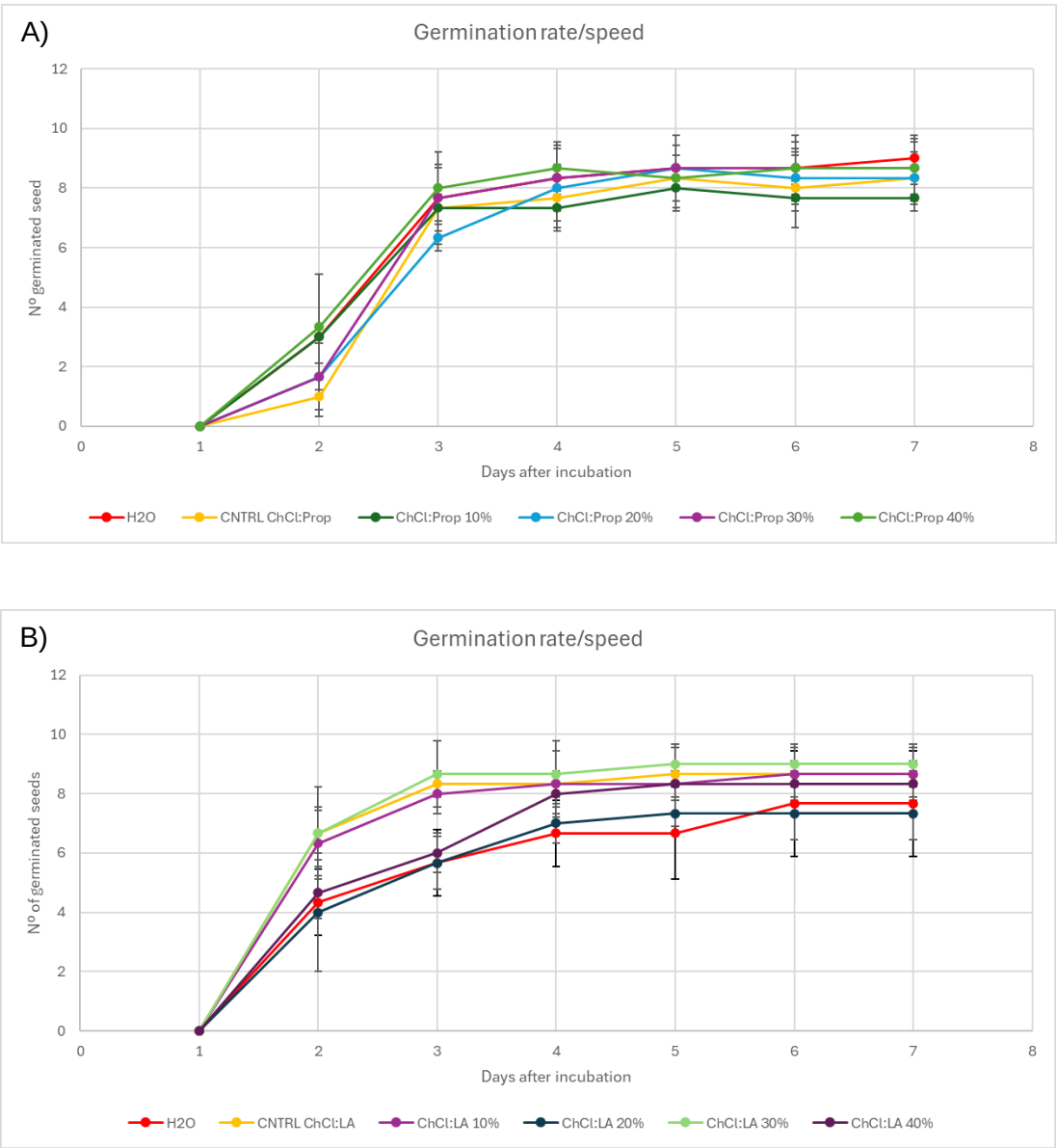
ChCl:MA and ChCl:LA presented antioxidant capacity with an inconsistent pattern and negative potential relative to the blank. However, “negative potential” is not an accurate description, as the values presented took into account the inherent scavenging potential of the corresponding NADES, whose values were high, masking the extracts’ effect. Based on the extracts obtained with ChCl:Urea and ChCl:Prop, it is unlikely that the

potential of these extracts is smaller, but their antioxidant potential may be obfuscated by the higher values obtained from the analysis of their respective solvents by this method. This high potential derives from the way DPPH interacts with acids or hydrogen donors in general, where the unpaired electron of this free radical pairs with the hydrogen ions available from the acids, stabilizing itself (Fukumoto and Mazza, 2000; Nithya and Madhavi, 2017). From this reaction arises the problem of analysing the extracts obtained with these acidic solvents in a precise manner, as interferences are bound to appear.

5.6. Extracts’ effect on germination

5.6.1. Germination rate through time

The following figure (Figure 19) show the germination rate of seeds after extract application.



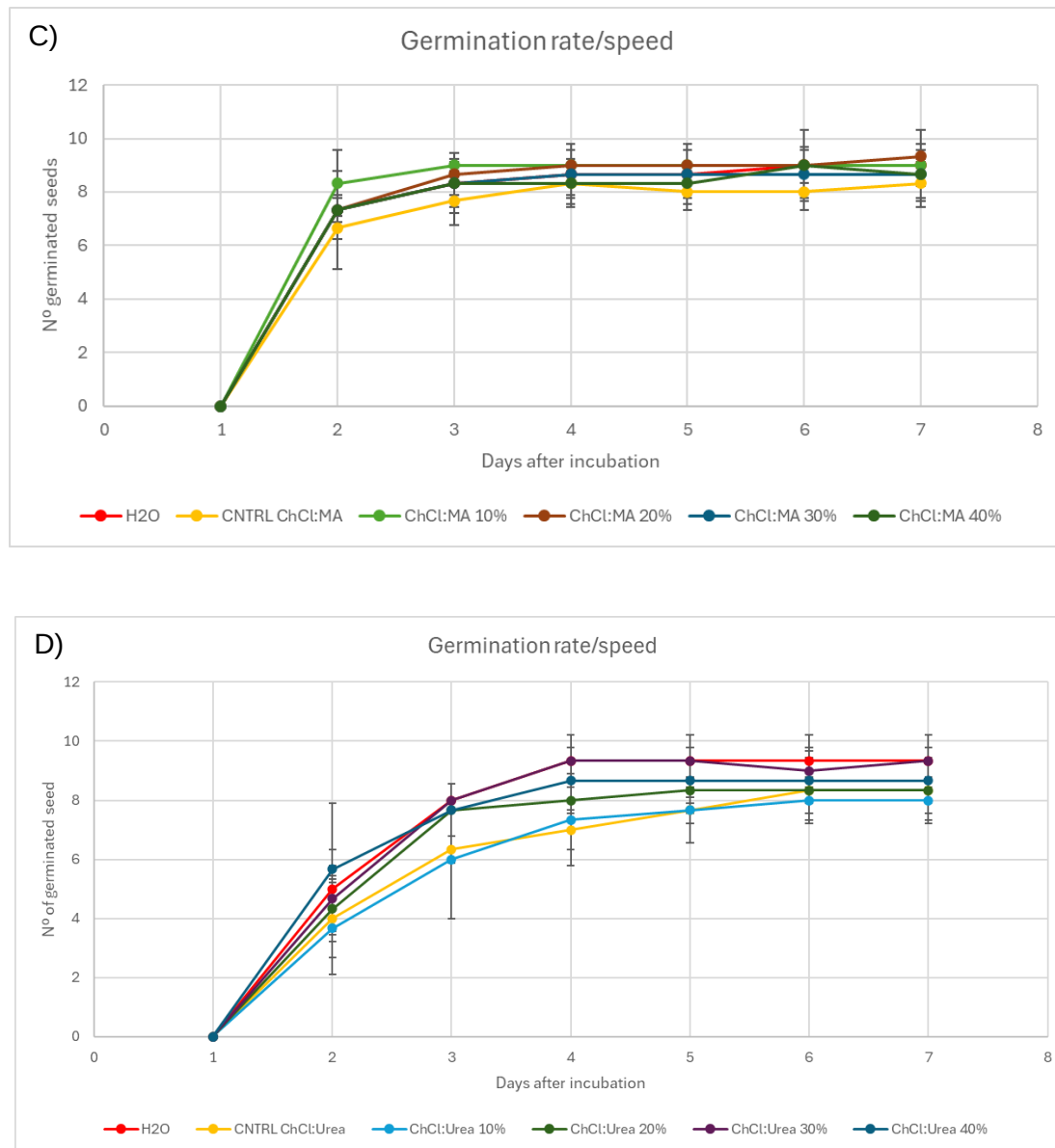


Figure 19 – Germination rate/speed of NADES extracts over a 7-day period: A) ChCl:Prop; B) ChCl:LA; C) ChCl:MA; D) ChCl:Urea; the bars represent standard deviation. The CNTRL is a NADES solution diluted according to toxicity results obtained previously

From the results, we can take away that since the germination speed and number of the NADES controls are overall similar to the corresponding water controls, the concentrations of NADES used are indeed below (or identical) to the toxicity limits determined earlier.

The variation within consecutive days, where some later days present smaller germination average than the previous, is due to the difficulty of counting and managing seed germination without opening the Petri plates. This sealed environment causes the

fogging of the lids due to respiration and transpiration, causing the inconsistent values for some days.

In the table below (Table 14) we can see the relative speed of each extract compared to their respective negative control (H₂O), calculated using Equation 7 – a relative speed of 1 means the extract has the same germination speed as this control, if <1 it's slower, if >1 it's faster.

Table 14 - NADES extracts' relative germination speed organized by NADES used to perform the extraction (A) and ranked by relative speed in ascending order (B), against the negative control (H₂O). A relative speed of 1 means the extract has the same germination speed as the control, if <1 the germination rate is slower, if >1 it is faster than the control.

NADES	Relative speed	NADES	Relative speed
CNTRL ChCl:Prop	1.40	CNTRL ChCl:Urea	0.67
ChCl:Prop 10%	1.40	ChCl:Urea 10%	0.67
ChCl:Prop 20%	1.40	ChCl:Urea 20%	0.80
ChCl:Prop 30%	1.40	ChCl:MA 20%	0.86
ChCl:Prop 40%	1.75	ChCl:MA 40%	0.86
CNTRL ChCl:LA	1.2	ChCl:LA 10%	1.00
ChCl:LA 10%	1.00	ChCl:Urea 30%	1.00
ChCl:LA 20%	1.20	ChCl:Urea 40%	1.00
ChCl:LA 30%	1.20	CNTRL ChCl:LA	1.20
ChCl:LA 40%	1.20	ChCl:LA 20%	1.20
CNTRL ChCl:MA	1.50	ChCl:LA 30%	1.20
ChCl:MA 10%	3.00	ChCl:LA 40%	1.20
ChCl:MA 20%	0.86	CNTRL ChCl:Prop	1.40
ChCl:MA 30%	1.50	ChCl:Prop 10%	1.40
ChCl:MA 40%	0.86	ChCl:Prop 20%	1.40
CNTRL ChCl:Urea	0.67	ChCl:Prop 30%	1.40
ChCl:Urea 10%	0.67	CNTRL ChCl:MA	1.50
ChCl:Urea 20%	0.80	ChCl:MA 30%	1.50
ChCl:Urea 30%	1.00	ChCl:Prop 40%	1.75
ChCl:Urea 40%	1.00	ChCl:MA 10%	3.00

Overall, all extracts cause an increase in germination speed, except for ChCl:MA 20% and 40%, and ChCl:Urea 10% and 20%, relative to the water control. When it comes to the controls (NADES diluted solutions with concentrations determined in **3.2.1**), most of the NADES do improve germination speed, with the exception of ChCl:Urea.

The most noteworthy extracts are ChCl:Prop 40% and ChCl:MA 10%, as not only do they surpass the effect of their respective controls but also manage to reduce the germination timing by almost half (1.75 for ChCl:Prop 40%) and by a third (3.00 for ChCl:MA 10%).

5.6.2. Seedlings' root growth

The following graphs (Figure 20) show the effect of the extracts of beach-casted macroalgae obtained with different NADES tuned solutions on tomato seedling's root growth.

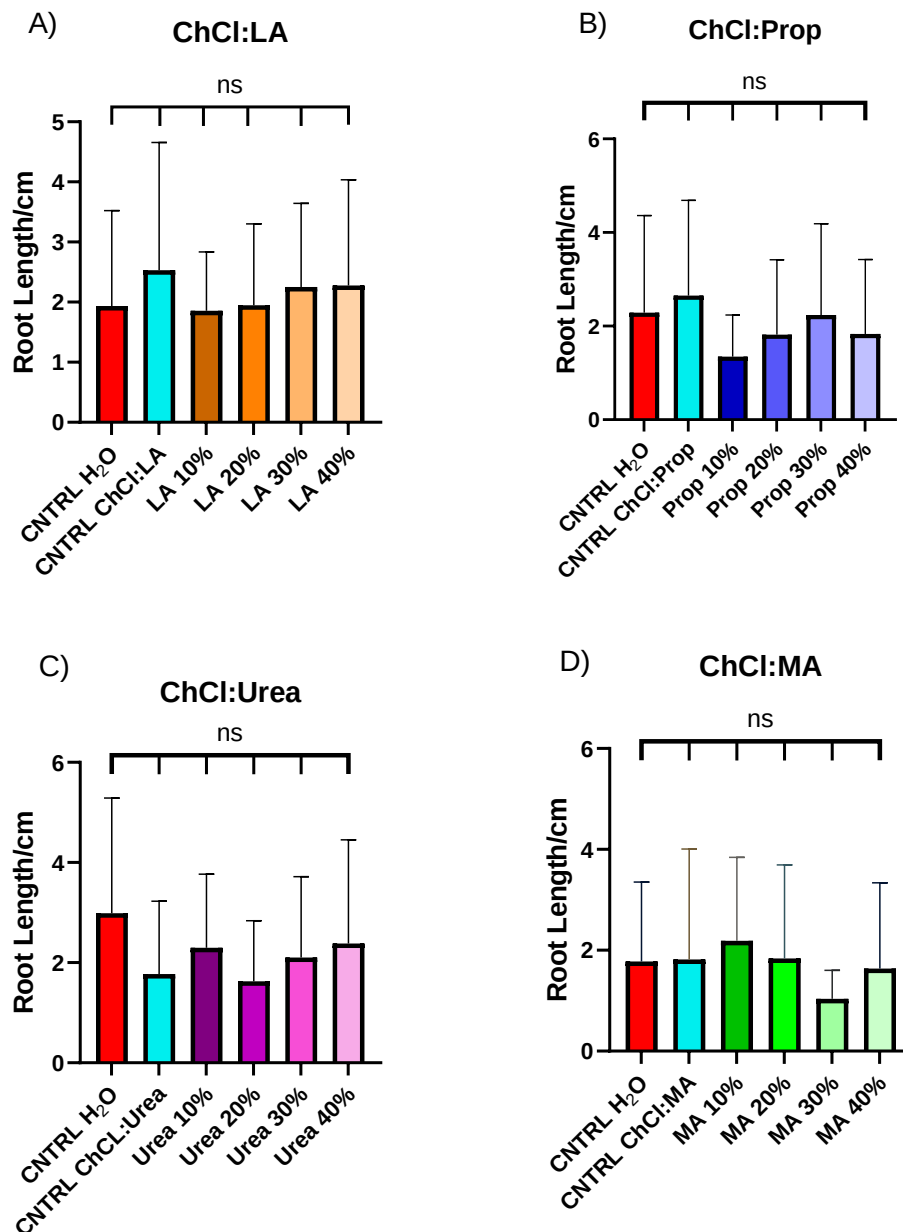


Figure 20 - NADES extracts effect on root length (in cm), A) ChCl:LA; B) ChCl:Prop; C) ChCl:Urea; D) ChCl:MA. Statistical analysis performed using Kruskal-Wallis non-parametric test: ns (non-significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$

No significant effect on root growth was verified with the application of NADES extracts. Even in the cases when seeds germinated earlier and the roots had more time to grow, ChCl:Prop 40% and ChCl:MA 10%, no significant effects of those seaweed extracts were

observed. Due to, the mean root length values presenting high variations, and non-parametric tests having been used to analyse the results, might have contributed to this situation. In a future experimental design, more replicates than the 3 petri dishes used for each condition, may have to be considered to define more clearly the effect of the extracts on the root growth. A comparison with extracts obtained with conventional solvents should also be included to comprehend the efficiency of extracts obtained with NADES against traditional solvents. However, it may also be the case that the effect of the extracts applied is indeed not significant, and various hypotheses may be postulated to explain the fact: 1) The extracts don't possess stimulant activity; 2) The compounds that would affect the root growth are not present in the extract, or not present in insufficient concentrations; 3) Possible degradation of the biostimulant compounds that might exist in the extracts, during the extraction, storing procedures or interference/interaction with the NADES (possibly lowering the bioavailability, or absorption by the seedlings). However, regarding this last hypothesis, NADES have been described to prolong the life and increase the stability of extracted compounds (Hikmawanti et al., 2021; Gómez-Urios et al., 2022). Despite that, and since NADES can't be frozen in temperatures that would ensure the longevity of certain bioactive compounds ($<-20^{\circ}\text{C}$), as it would lead to their crystallization, the storage and longevity of extracts are also in need of optimization.

6. Phytohormone identification and quantification

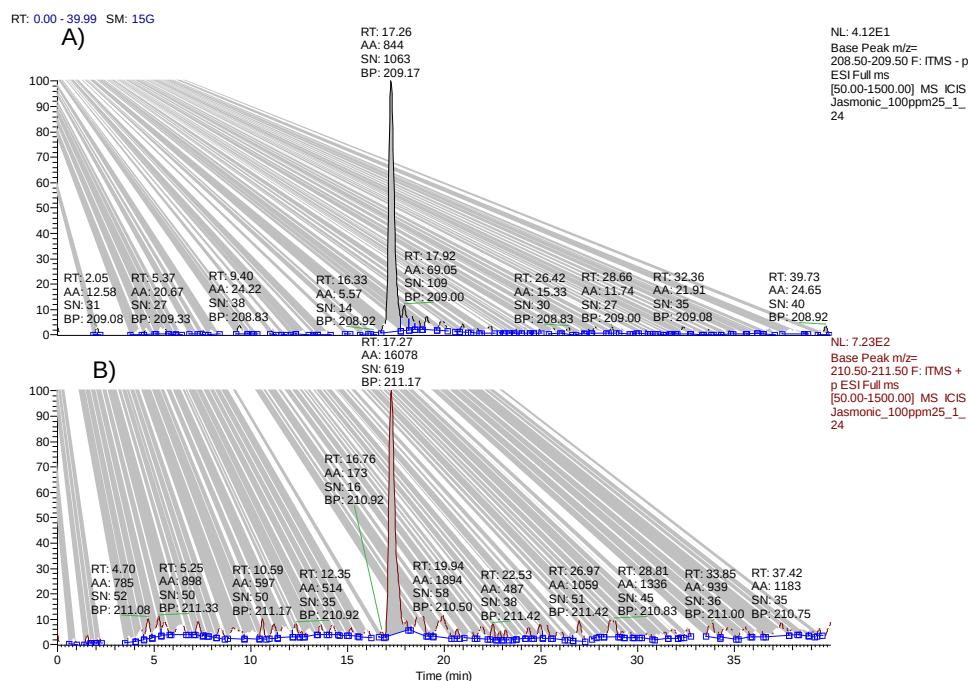
6.1. Detection optimization

6.1.1. Single phytohormone analysis

For analysis and detection optimization the chromatograms and spectrums for all studied phytohormones in positive, negative and collision-induced dissociation (CID) modes were analysed.

The full-run chromatograms, at both positive and negative modes, are used to optimize the detection settings of the equipment for the compounds that will be analysed. The most adequate is the mode that presents the chromatogram with lower noise/interference and with the biggest base peak (BP) area (AA) to the corresponding expected m/z. To identify the expected m/z, the full run spectrum is analysed, where for positive mode $m/z \approx M+1$, and negative mode $m/z \approx M-1$, with M being the target compound's molecular weight. This also provides a window of BP m/z's to be searched in the chosen mode, which allows for a more specified analysis in future runs.

The following figure (Figure 21) presents the full scan chromatograms and respective spectrums for both positive and negative modes, of JA, as an example.



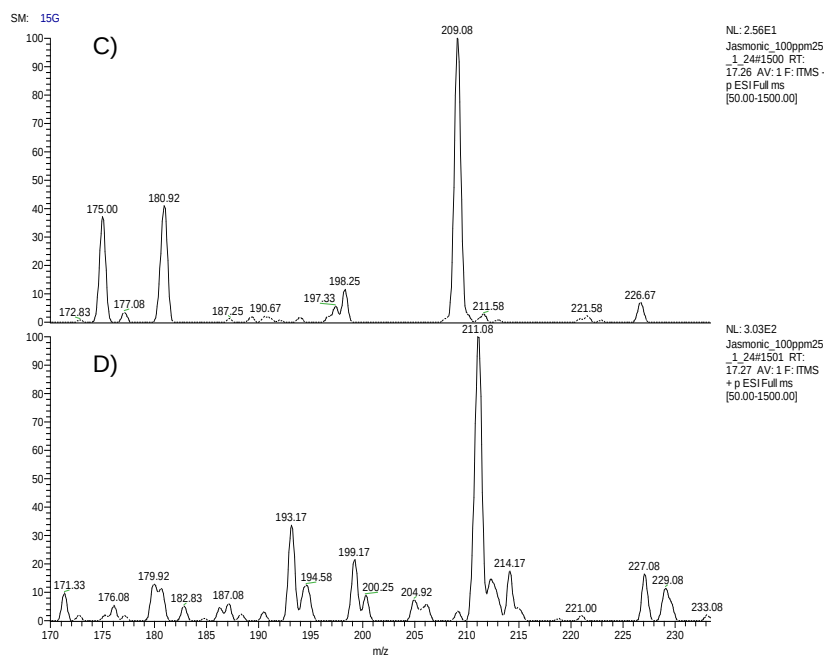


Figure 21 - LC/MS-MS chromatograms of JA in A) Negative mode; B) Positive mode; JA LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode

Looking at the results, the retention time for JA is 17.26min, and the best mode was the positive mode.

These chromatograms and spectra are not enough to identify compounds in more complex matrixes, so collision-induced dissociation (CID) is used. This mode breaks down the compound into smaller characteristic fragments. These present recognizable ratios and m/z's, with the main fragment presenting similar a RT to the one of the whole compound (± 0.3 min, according to lab standards), within the window analysed. Below, specific window run, CID chromatogram and spectrum of JA are presented (Figure 22).

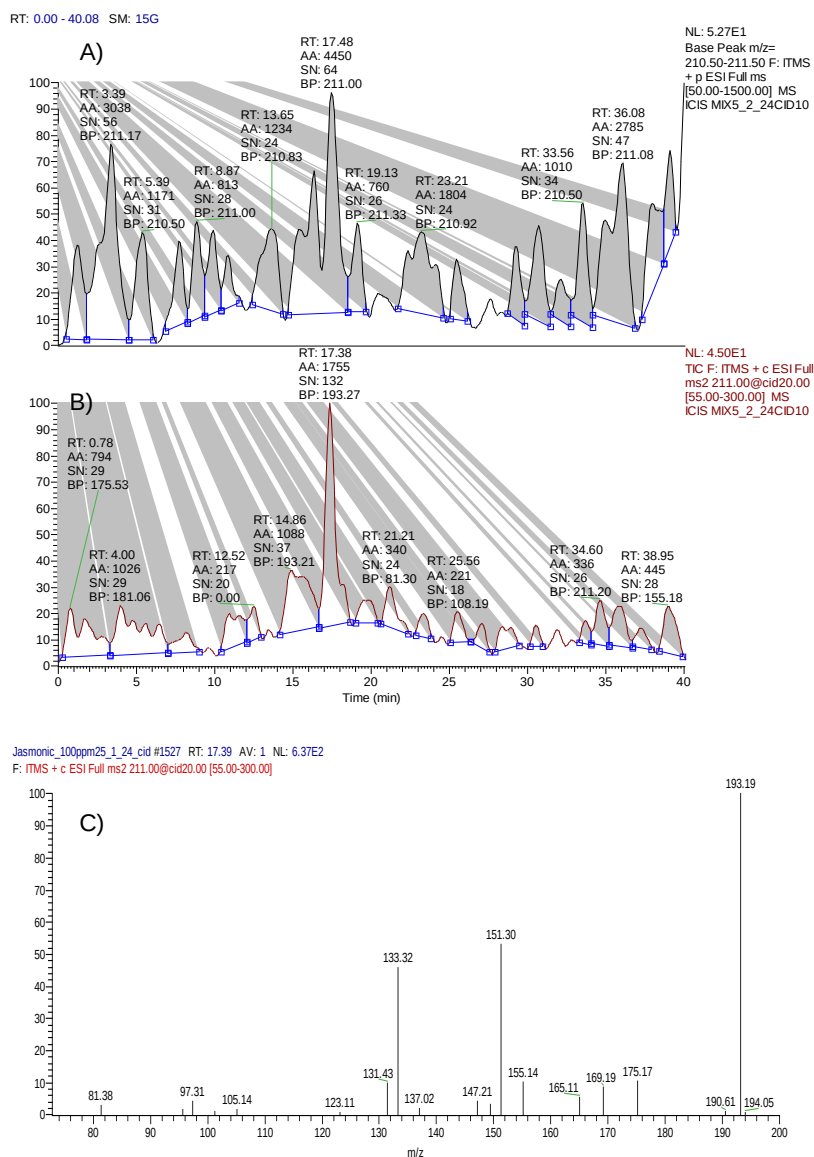


Figure 22 - A) JA LC-MS/MS window run ($210.50 \leq m/z \leq 211.50$); B) JA LC-MS/MS CID chromatogram in positive mode; C) JA LC-MS/MS CID spectrum in positive mode

The results for the other phytohormones are similar in nature and interpretation, are presented in the supplementary material section (Figures S1 to S7) and will be summed up to the chosen mode and RT in Table 15.

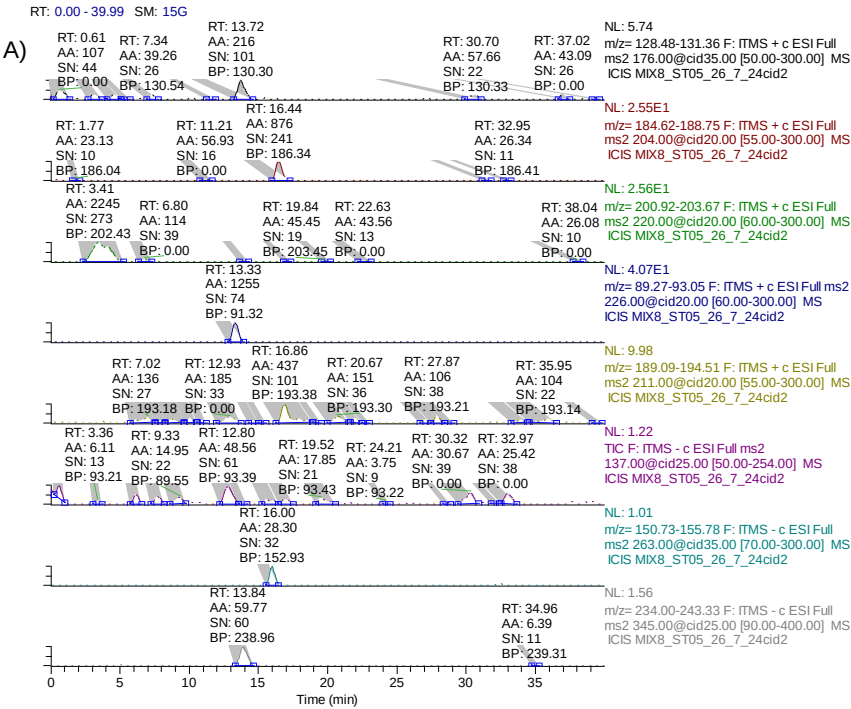
The following table (Table 15) sums up the detection modes that provided better results for each phytohormone, as well as their retention time.

Table 15 - Phytohormone detection mode selected and retention time (in min). The results were obtained through individual analysis.

Hormone	Detection mode	Retention time/min
JA	Positive	17.48
SA	Negative	14.05
IAA	Positive	14.26
GA	Negative	14.53
IBA	Positive	17.04
TZ	Positive	4.23
BAP	Positive	13.91
ABA	Negative	16.50

6.1.2. Standards mixture

The mixture of all standards at equal concentrations was analysed according to the most adequate modes, previously determined on single phytohormone analysis. This was to evaluate the effect of the interaction among the various phytohormones, their behaviour within a matrix and the characteristic retention time of each phytohormone for said matrix. The results are presented below (Figure 23 and Table 16).



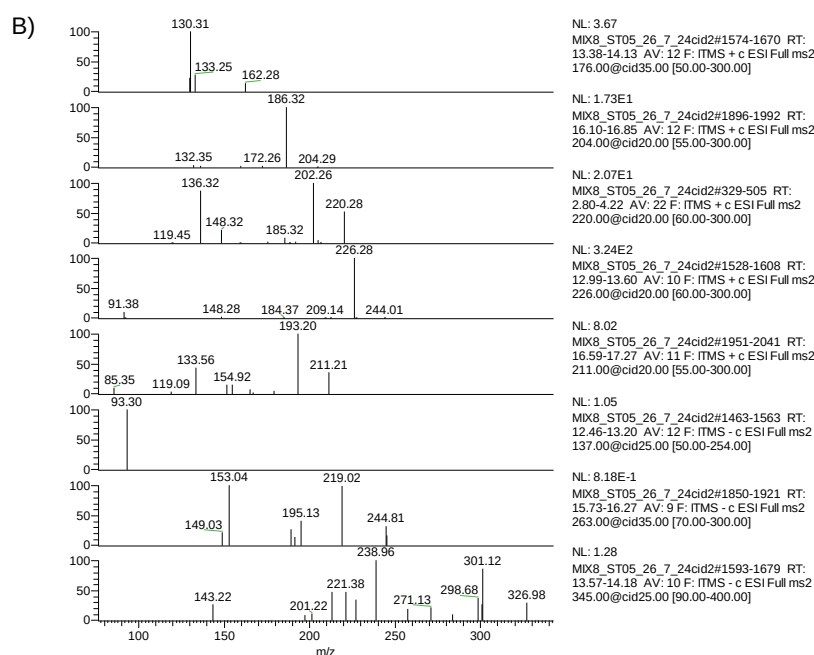


Figure 23 - LC-MS/MS chromatograms (A) of standard mixture and corresponding CID spectra (B); top to bottom: IAA, IBA, TZ, BAP, JA, SA, ABA, GA

Table 16 - Phytohormone retention time (in min) in standard mixture matrix

Phytohormone	Retention time/min
IAA	13.72
IBA	16.44
TZ	3.41
BAP	13.33
JA	16.86
SA	12.80
ABA	16.00
GA	13.84

6.2. Phytohormones in the sample

After the extraction of the seaweed biomass described in **2.9.2** using 70% methanol as a solvent, the LC-MS/MS CID chromatograms presented a peak with characteristics similar to the one of IAA (RT $\approx$ 13.72 and BP $\approx$ 130.30). It was decided to focus the analysis on this phytohormone, with the CID chromatograms and spectra below (Figure 24).

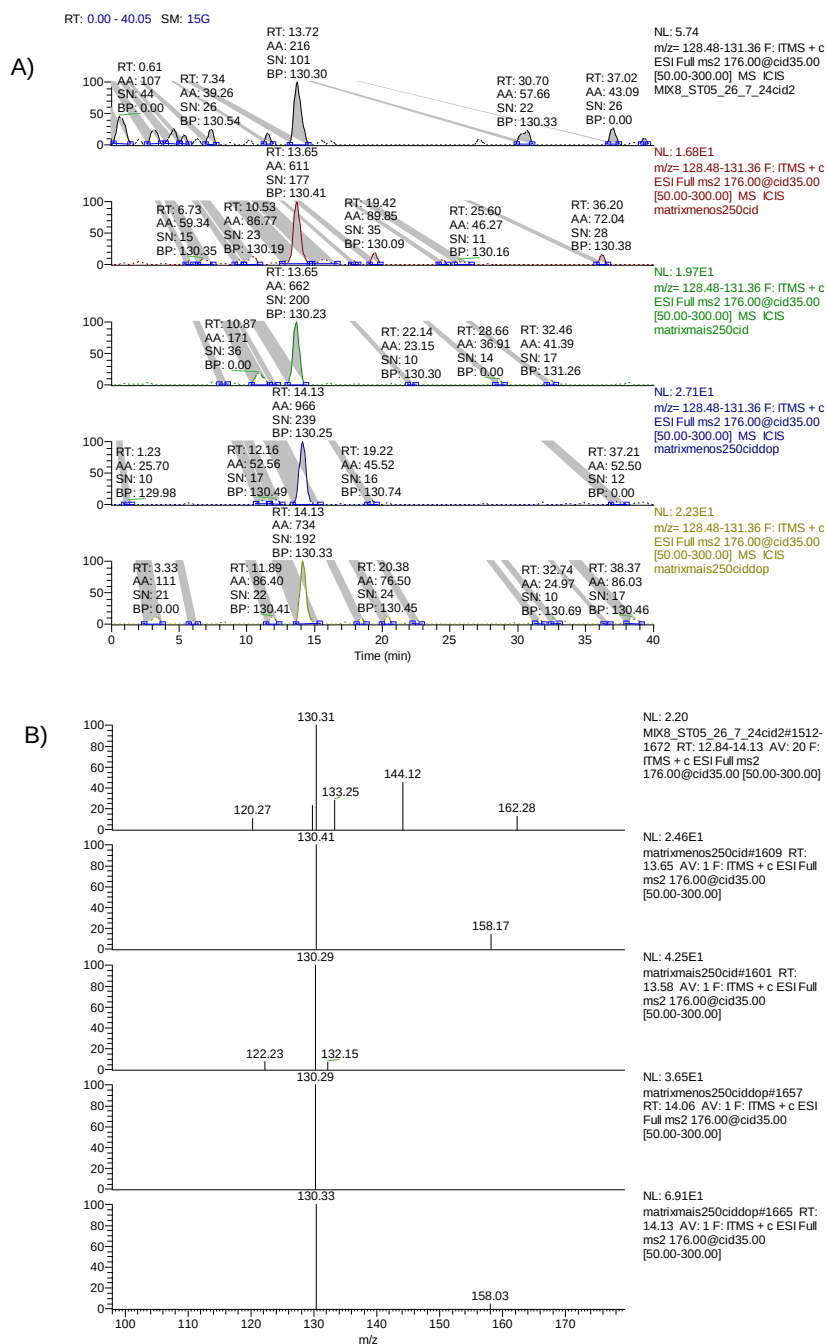


Figure 24 - LC-MS/MS chromatograms (A) and corresponding CID spectra (B), from top to bottom: Phytohormone standard mix (IAA in specific); seaweed extract with biomass >250 µm; seaweed extract of biomass <250 µm; doped seaweed extract with biomass >250 µm; doped seaweed extract of biomass <250 µm.

From the analysis of the chromatograms of the two non-doped extracts, there is the repeated appearance of a BP and associated RT that correspond to IAA (RT= 13.72 ± 0.5). The CID fragment profile of these samples also match that of the compound on the standard mixture.

To better assure the presence of IAA, the sample was doped with the standard mixture of phytohormones, which led to an increase in peak area for both extracts, keeping the BP and CID profile. The increase in peak area indicates greater quantity of the compound of interest (IAA in this case), and since there was no shift in the CID profile, this was evidence for the presence of IAA in the seaweed extract. The variation of RT in the doped solutions, was due to the change of the matrix, caused by adding the mixture of phytohormone standards solution, which diluted the sample. Since the change is still very close to the lab standard of $RT = 13.72 \pm 0.5$, together with all other mentioned pieces of evidence, the seaweed extract (and therefore the powder blend) appears to contain some amount of IAA. No other phytohormone was detected in the extracts.

Since the analysis done was of extracts obtained from a blend of macroalgae, it was not possible to pinpoint its source. However, there has been evidence of IAA and other phytohormones in the three main groups of macroalgae. Mostly in red and green algae (Chen et al., 2024; Galal et al., 2024), probably due to their already mentioned evolutionary proximity to terrestrial plants, but in rarer cases brown macroalgae as well, where IAA seems to have a more prevalent presence when compared to other concurrent phytohormones (Sunarpi et al., 2021; Saebmehr et al., 2022).

The lack of other phytohormones in the extracts obtained may have multiple explanations, such as a low amount of said compounds, making it hard for them to be detected; the degradation of these stimulants during the processing of the biomass or during the extraction process; the inability to extract the phytohormones using the chosen protocol; or simply the non-existence of such compounds in the macroalgae studied, being it absolute (no production of phytohormones occur ever in these macroalgae in their life cycle), or at the time of life they were collected and studied.

Future studies of the species individually, both at the same and at different times of collection, may reveal more information about the purpose and production of these phytohormones, and possibly even show the presence of other phytohormones not studied in this project.

Conclusion

While presenting much potential and having become a green alternative to common organic solvents in the last couple of years, little is known about NADES physical-chemical characteristics, extracting behaviour and toxicity. Our work, aimed to fill in gaps about said properties of these solvents, and evaluate both their potential as extractors of bioactive compounds, namely from macroalgae beach wrack and evaluate their potential as a source of phytohormones and other PGR's.

NADES physical properties elucidated the problems found in the lab, as after analysis we found that these solvents present high viscosities and densities, making their tunability an essential step for making NADES workable/viable as solvents. This tuning process also allows for an increase in specificity as it changes the solvents' polarity, a crucial factor for extraction efficiency. While we managed to characterize the aforementioned parameters, it was not without some trouble. The current lack of information on NADES leads to an uncertainty of results as there is very possible comparisons that can be made, which ends up downplaying their potential.

While very efficient solvents in theory, many analyses used to evaluate their extraction efficiency are hindered by interferences of these solvents with the methodologies commonly used, as seen in the work presented. A focus on NADES is needed, but from a foundational perspective, it is necessary to start by characterizing them and develop analytical protocols that are compatible with such solvents before using them in laboratorial or industrial settings.

From simple direct spectrophotometric analysis (photosynthetic pigments) that require calibrating equations for compound measurements (which take into account the molar extinction coefficient – ϵ - of the solvents - there isn't a record of such for any NADES so far), to more indirect approach methods that require chemical reactions to occur (colorimetric reactions such as Folin's, DMBA and DPPH) without interferences occurring with the solvents (leading to unusable or unreliable assays results), there are no specific protocols set-up for these solvents. Our best bet so far for any conclusions about their effectiveness, is the inaccurate comparisons to other solvents, which leads to unreliable conclusions, that can downplay NADES (and extracts) potential.

Not only that, the lack of consensus on what defines a DES (and subsequently NADES) may cause problems down the line, as many roughly similar solvents end up in the same

categories, where they may be susceptible to a certain prejudice if a portion of them do not prove the potential attributed to the group.

The potential of this project also came from our macroalgae beach wrack blend. Macroalgae biomass has been used for centuries, and over the last couple of years more and more industries have found interesting in it as a source of compounds applicable in cosmetics, nutraceuticals, food and feed, among many others.

Despite our results seeming less-than-ideal when it comes to the extracts (and a bit contradicting to the previously mentioned potential), there is a variety of parameters that could not be measured, due to the usage of NADES as extractants. However, the antioxidant capacity and the presence of IAA do seem to indicate potential and a future where the extracts of this biomass obtained with NADES, can be implemented in agriculture, as the raw biomass was many years ago. Not only that, the presence of IAA can also help reinforce the knowledge and guide the study of phytohormone presence, production and effect on macroalgae, a still very open-ended debate whose elucidation can make possible the usage of this raw material in more specific ways.

It was concluded that the NADES tested are not toxic to plants at concentrations below 123 mg/L, or even 370mg/mL in some cases, making their use safe to be used in agriculture, despite, other toxicity tests need to be done relative to soil organisms. From their constituents, choline chloride, the HBA, was in general the least toxic of the components. Only in the case of ChCl:Prop, the NADES was more toxic than both NADES components.

Despite the incongruencies observed in biodegradability assays, the patterns seen indicate potential and good perspectives in future assays, which may help cement the potential of extracts obtained with some NADES in agricultural applications.

Regarding the efficiency of extraction, the highest extraction efficiencies occurred at 100 mg/mL of sample-to-solvent ratio, and at 10% tuning for the most polar NADES (ChCl:LA and ChCl:MA), as compared to 20% ChCl:Prop and 40% ChCl:Urea, the least polar NADES.

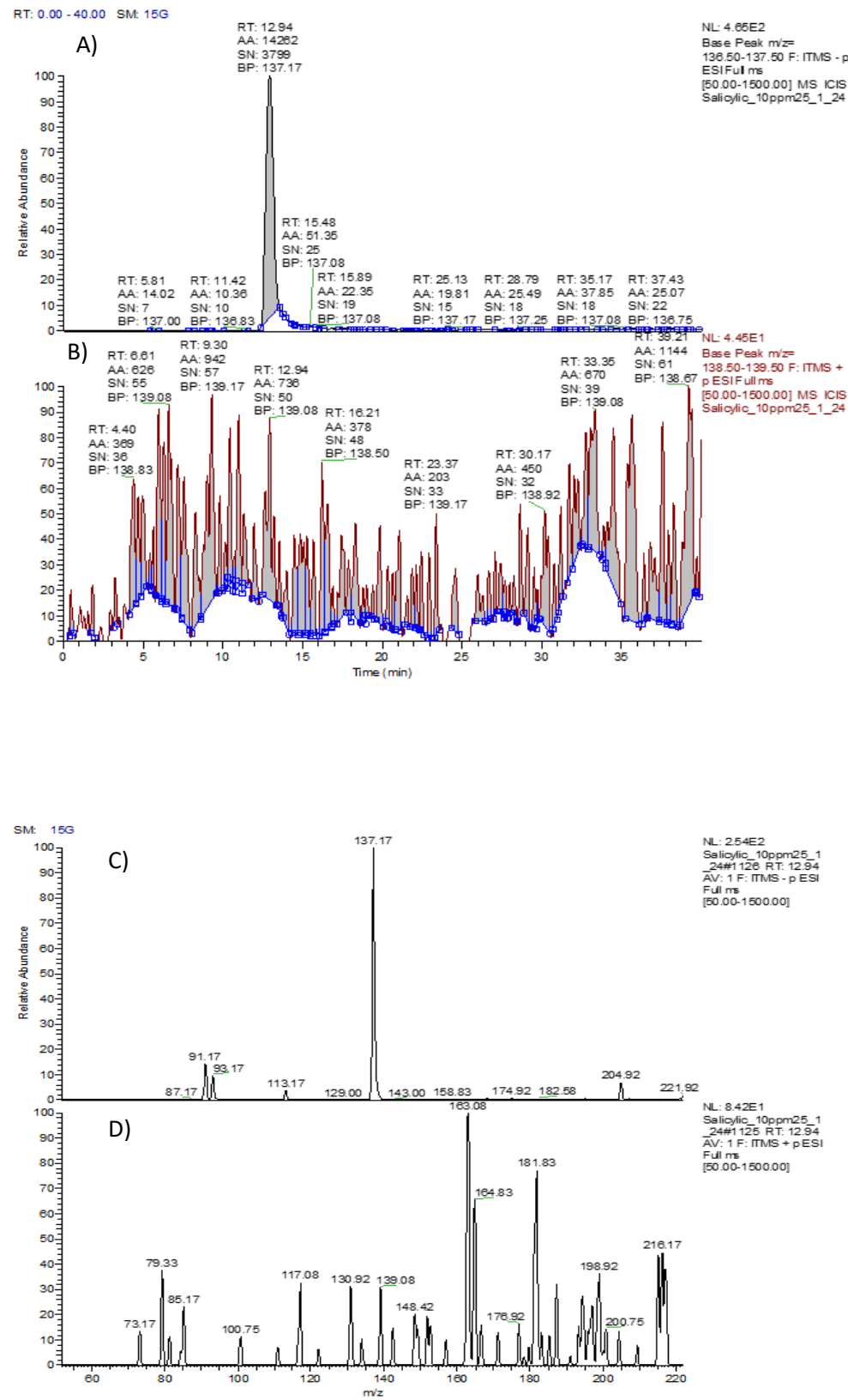
Relatively to the Folin-Ciocalteu's method, although no total optimization was achieved, it could be concluded that the least precipitate was observed at higher alkali concentrations, particularly with NaOH.

The extracts presenting the highest antioxidant potential were those extracted with the ChCl:Prop 40% water-tuned solution, with a maximum scavenging potential of 67.45%,

and a 41.47% increase with time. It was followed by the remaining NADES tuned at 40% water as well: ChCl:Prop > ChCl:Urea > ChCl:LA > ChCl:MA. The higher radical-scavenging potential was achieved with the least polar NADES.

Overall, despite the lack of results concerning extract effects, we believe the work done in the project was a crucial 1st step in paving the road of knowledge that will allow the scientific community to join both new solvents and old traditional experiences, improving the application of both, with strong evidence and with the well-being of the environment in mind.

Supplementary Material



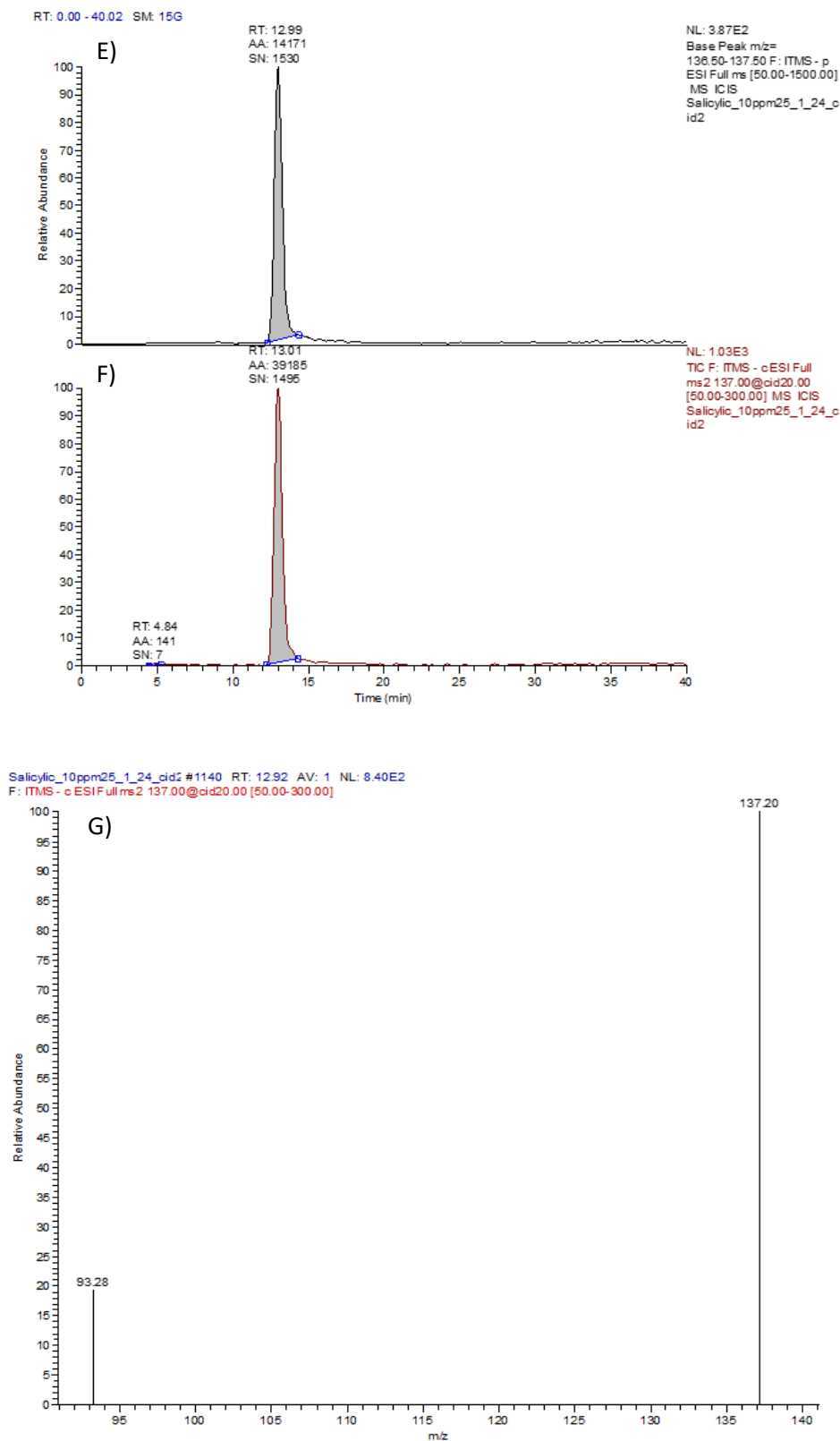
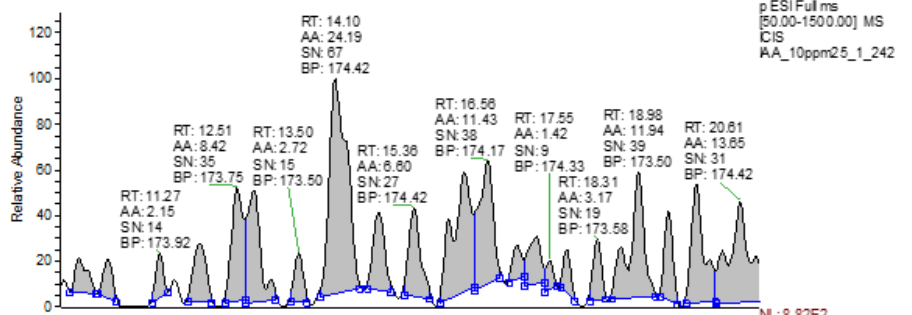


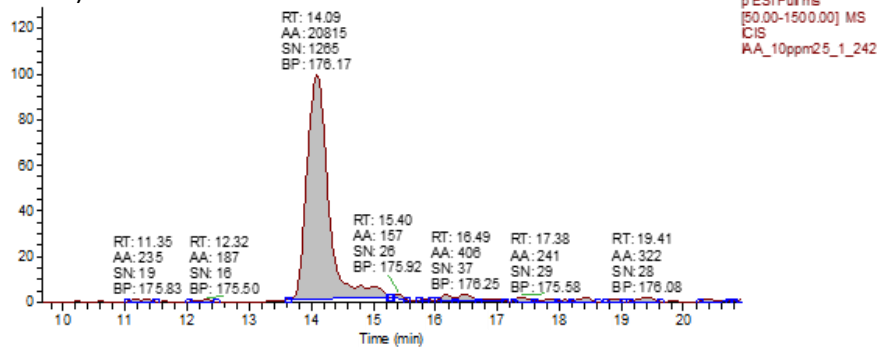
Figure S 1 - LC/MS-MS chromatograms of SA in A) Negative mode; B) Positive mode; SA LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode; E) SA LC-MS/MS window run ($136.50 \leq m/z \leq 137.50$); F) SA LC-MS/MS CID chromatogram in negative mode; G) SA LC-MS/MS CID spectrum

RT: 9.67 - 20.92 SM: 15G

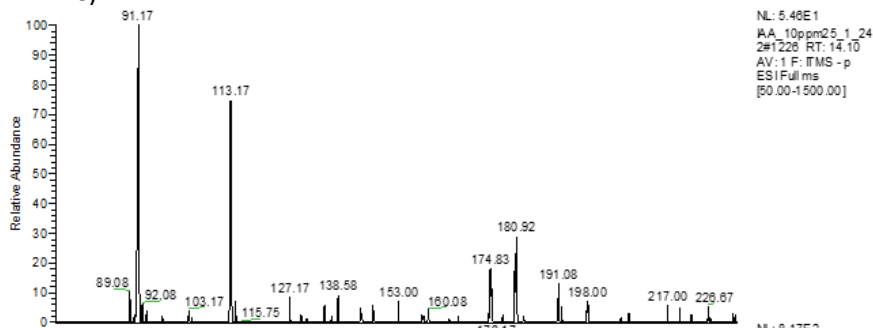
A)



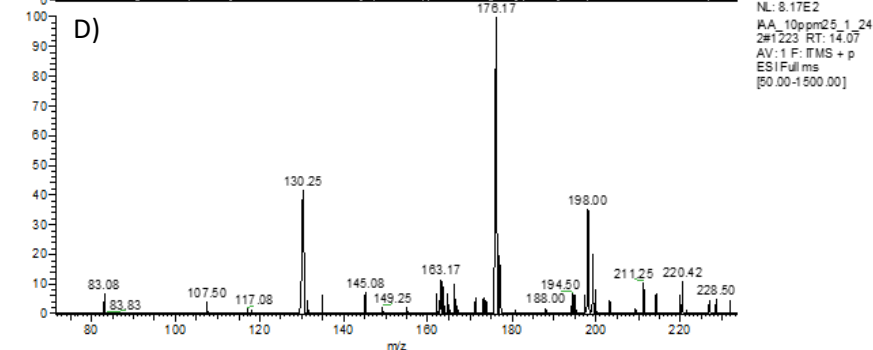
B)



C)



D)



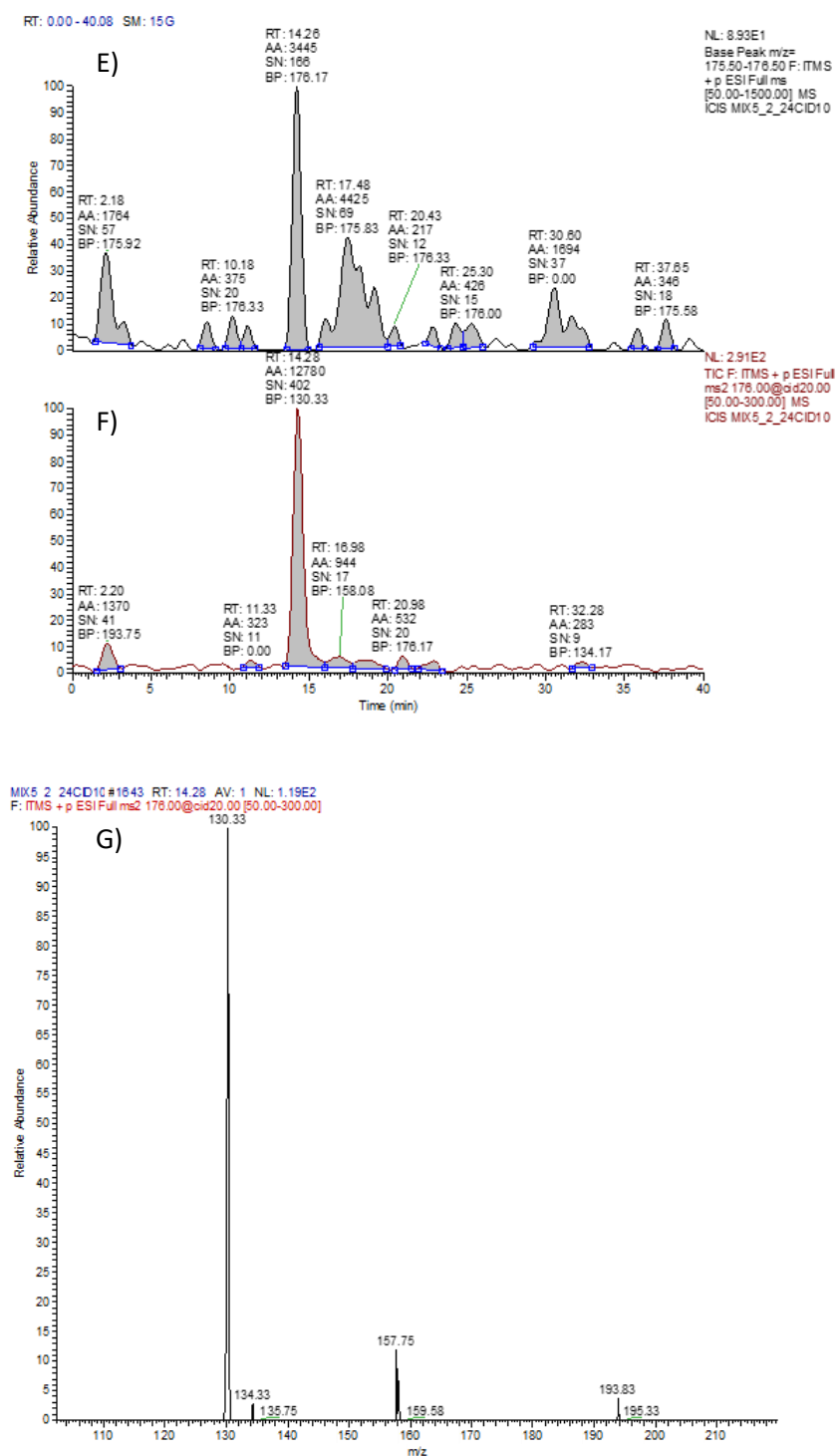
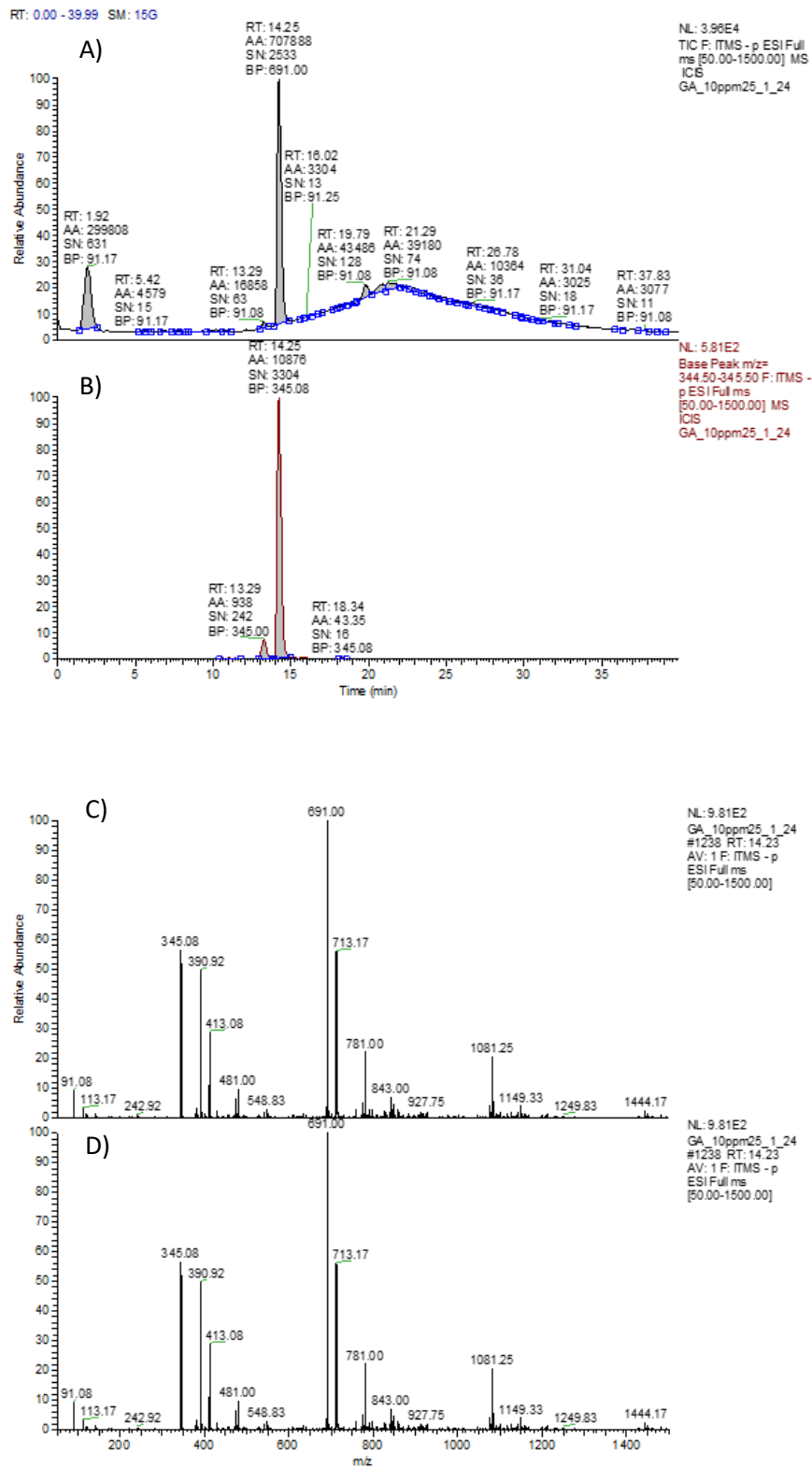


Figure S 2 - LC/MS-MS chromatograms of IAA in A) Negative mode; B) Positive mode; IAA LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode; E) IAA LC-MS/MS window run (175.50 ≤ m/z ≤ 176.50); F) IAA LC-MS/MS CID chromatogram in positive mode; G) IAA LC-MS/MS CID spectrum



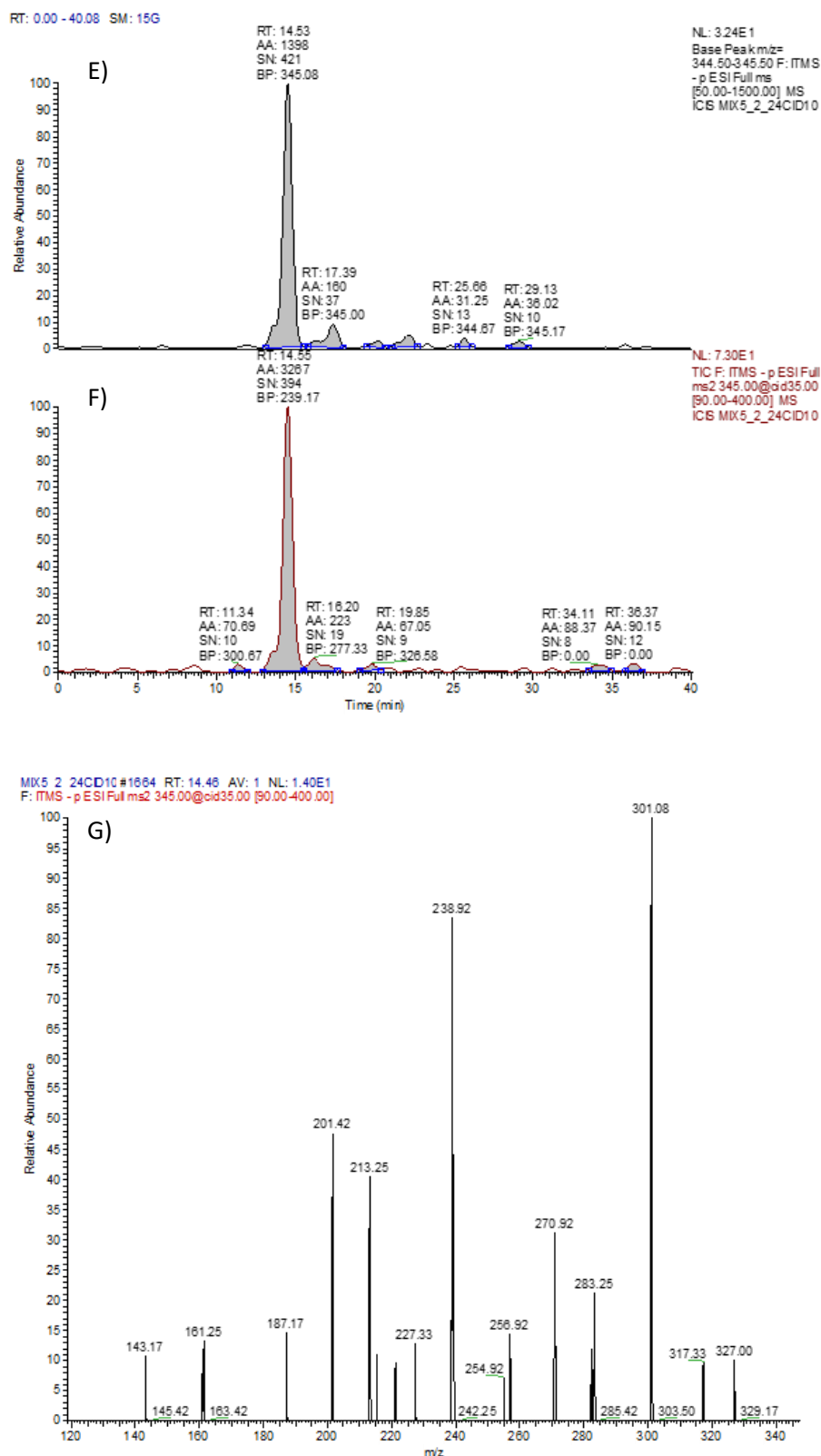
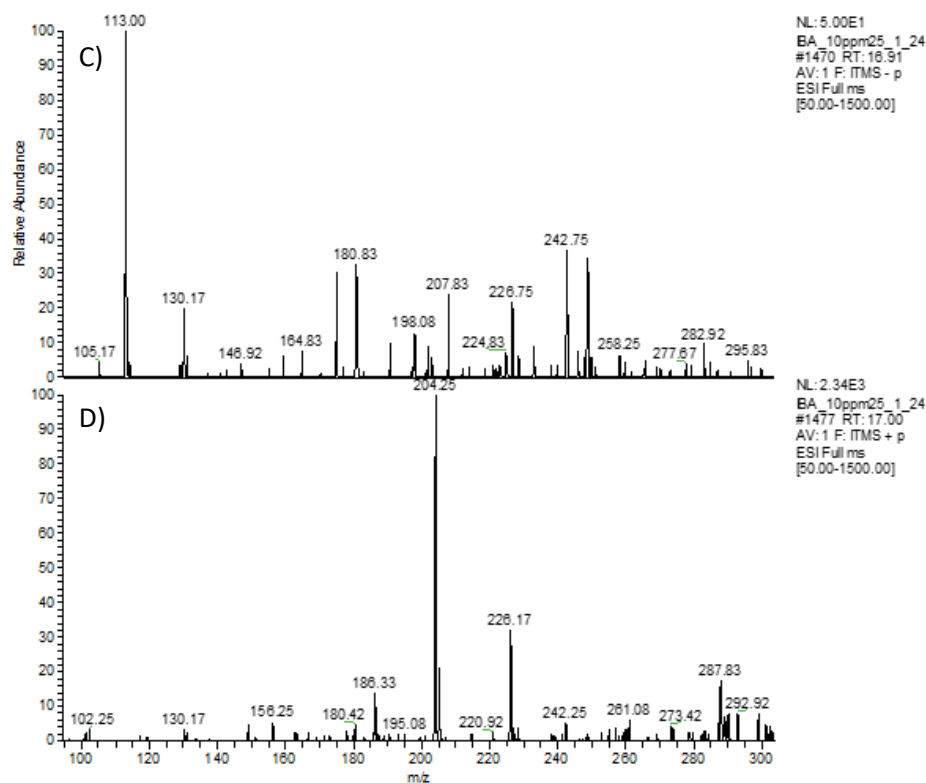
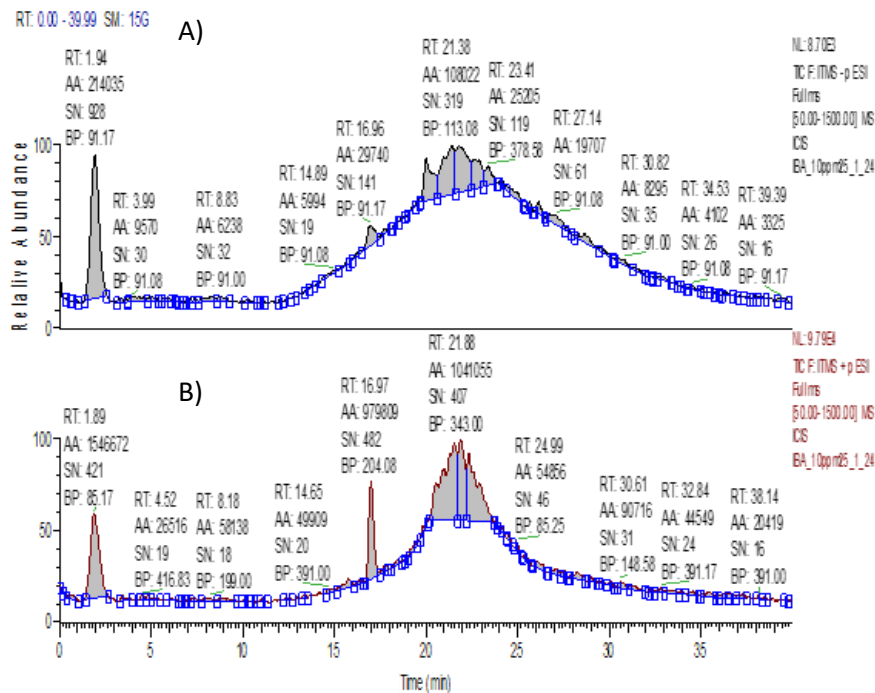


Figure S 3 - LC/MS-MS chromatograms of GA in A) Negative mode; B) Positive mode; GA LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode; E) GA LC-MS/MS window run ($344.50 \leq m/z \leq 345.50$); F) GA LC-MS/MS CID chromatogram in negative mode; G) GA LC-MS/MS CID spectrum



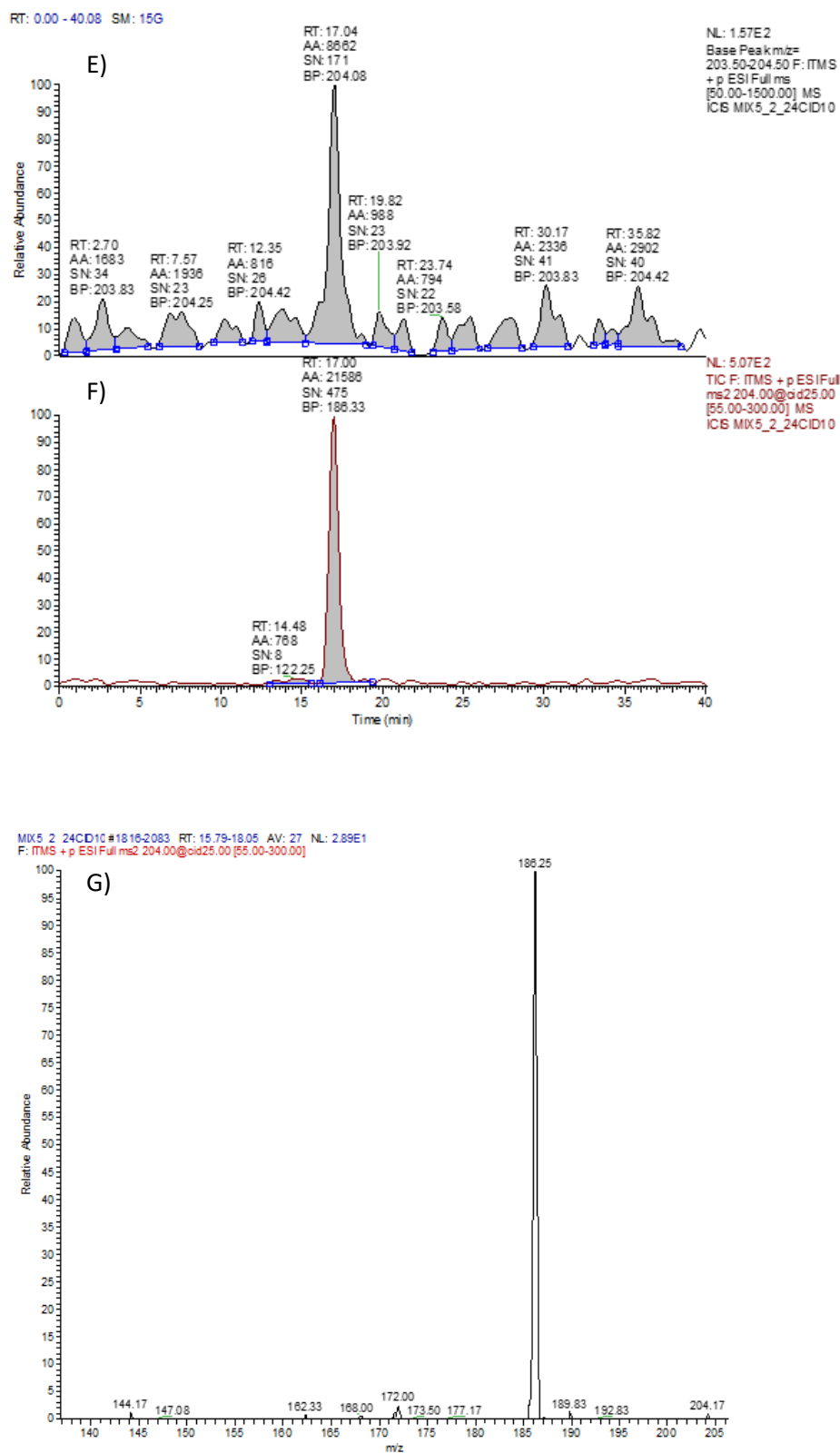
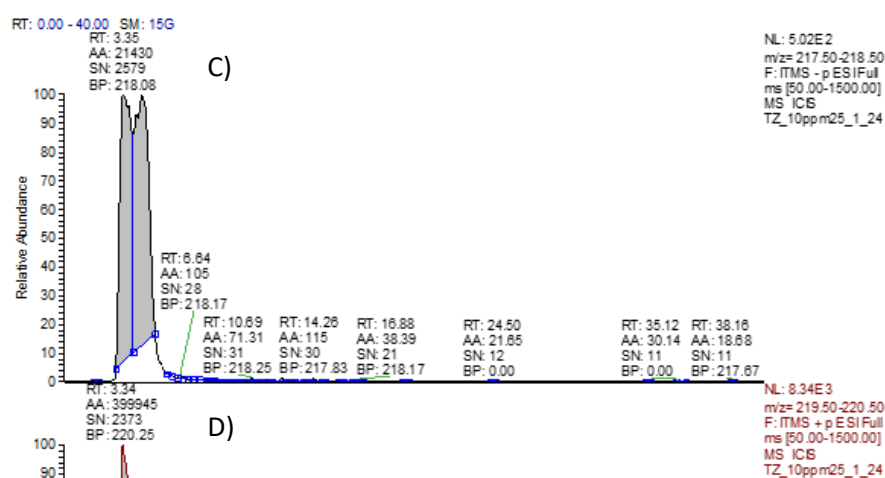
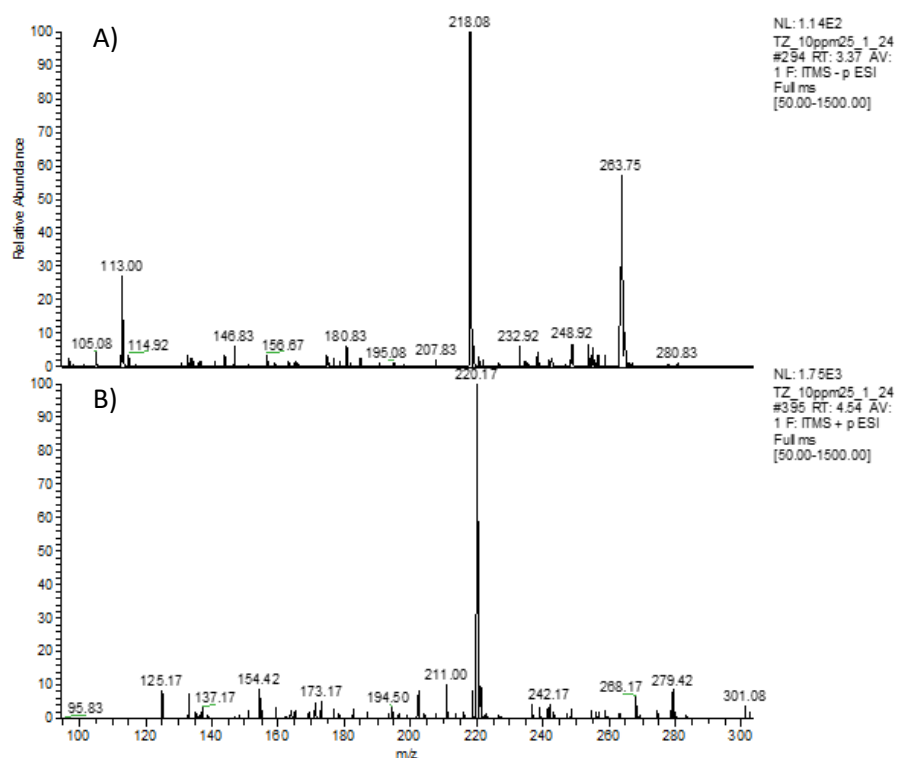


Figure S 4 - LC/MS-MS chromatograms of IBA in A) Negative mode; B) Positive mode; IBA LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode; E) IBA LC-MS/MS window run (203.50 ≤ m/z ≤ 204.50); F) IBA LC-MS/MS CID chromatogram in positive mode; G) IBA LC-MS/MS CID spectrum



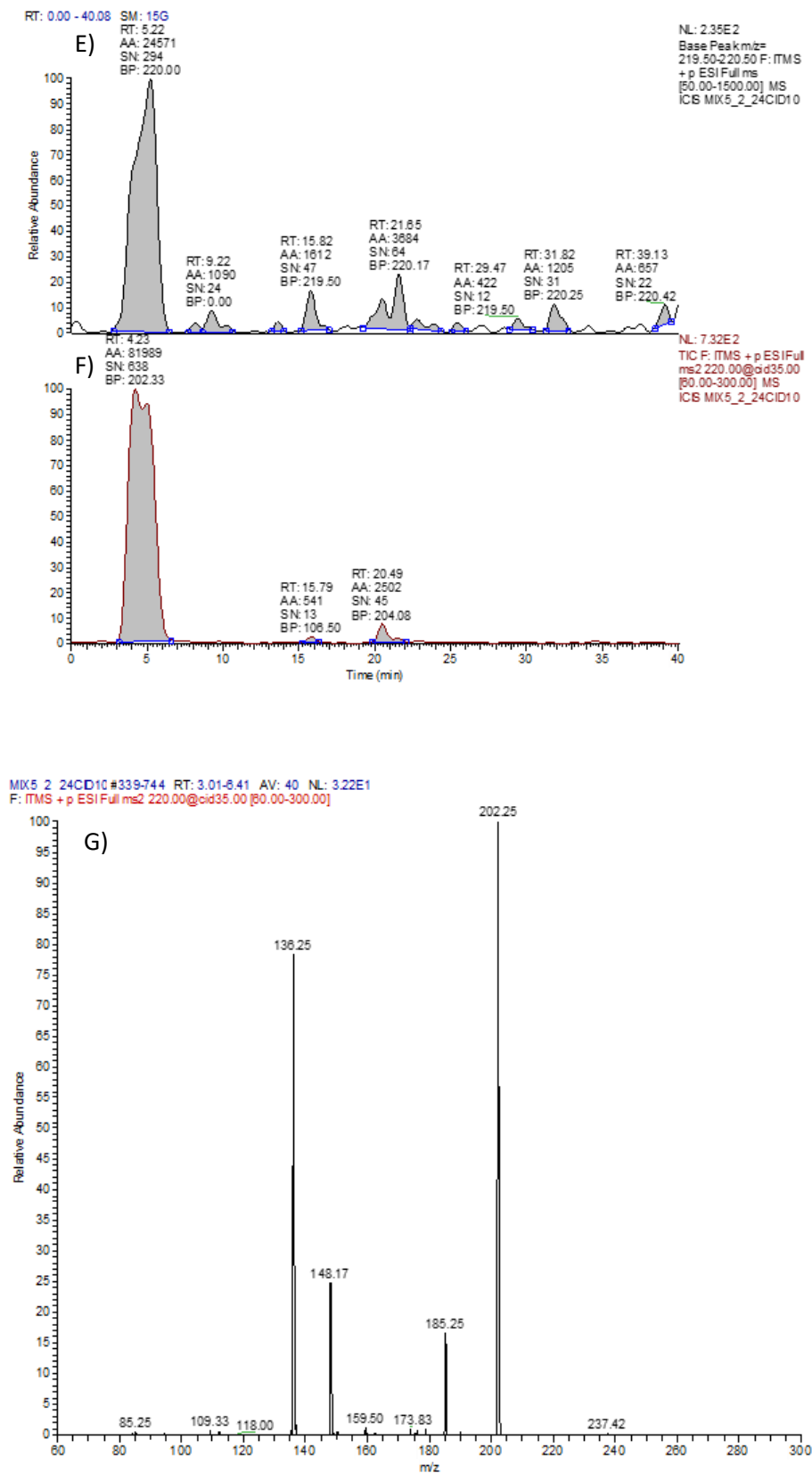
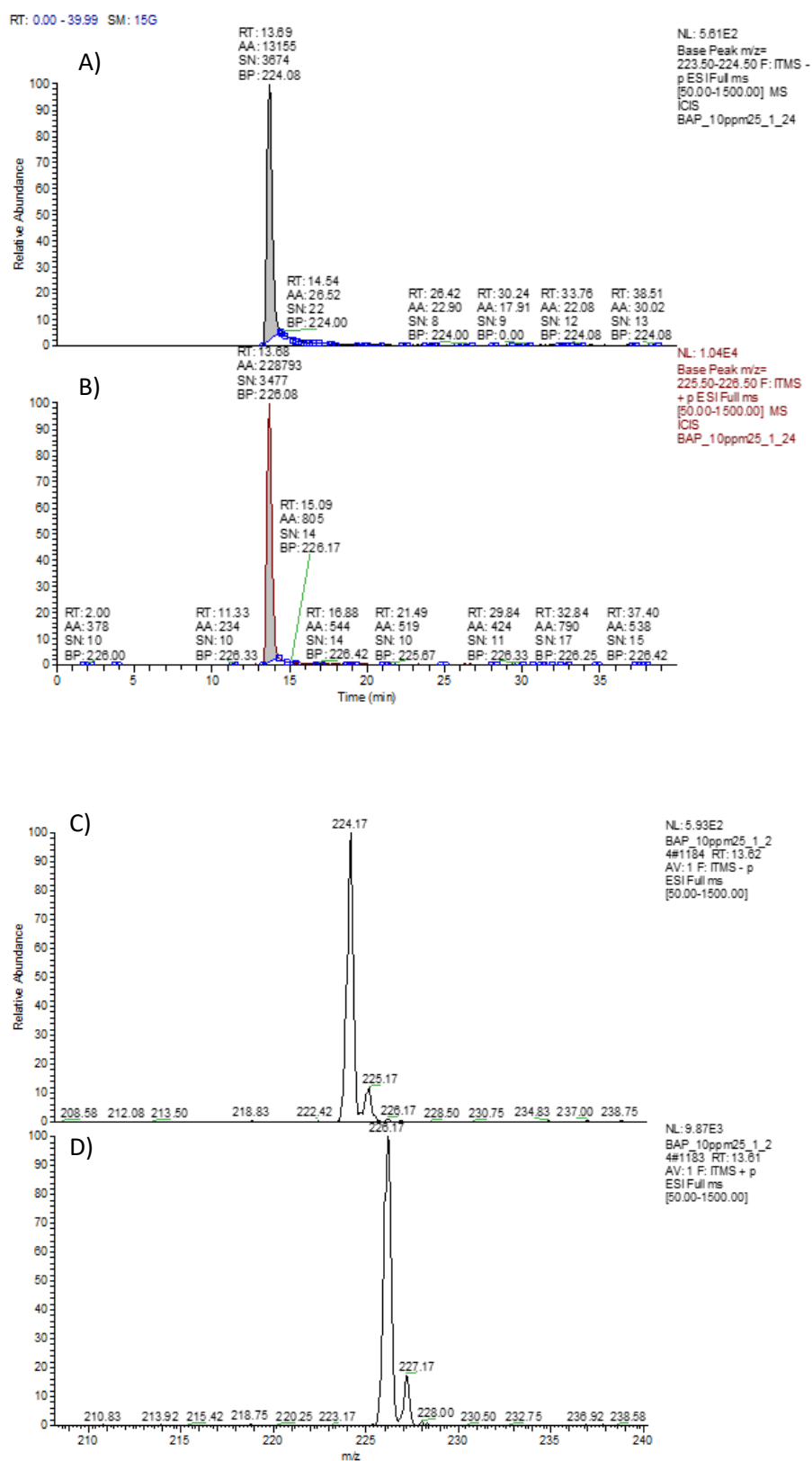


Figure S 5 - LC/MS-MS chromatograms of TZ in A) Negative mode; B) Positive mode; TZ LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode; E) TZ LC-MS/MS window run ($219.50 \leq m/z \leq 220.50$); F) TZ LC-MS/MS CID chromatogram in positive mode; G) TZ LC-MS/MS CID spectrum



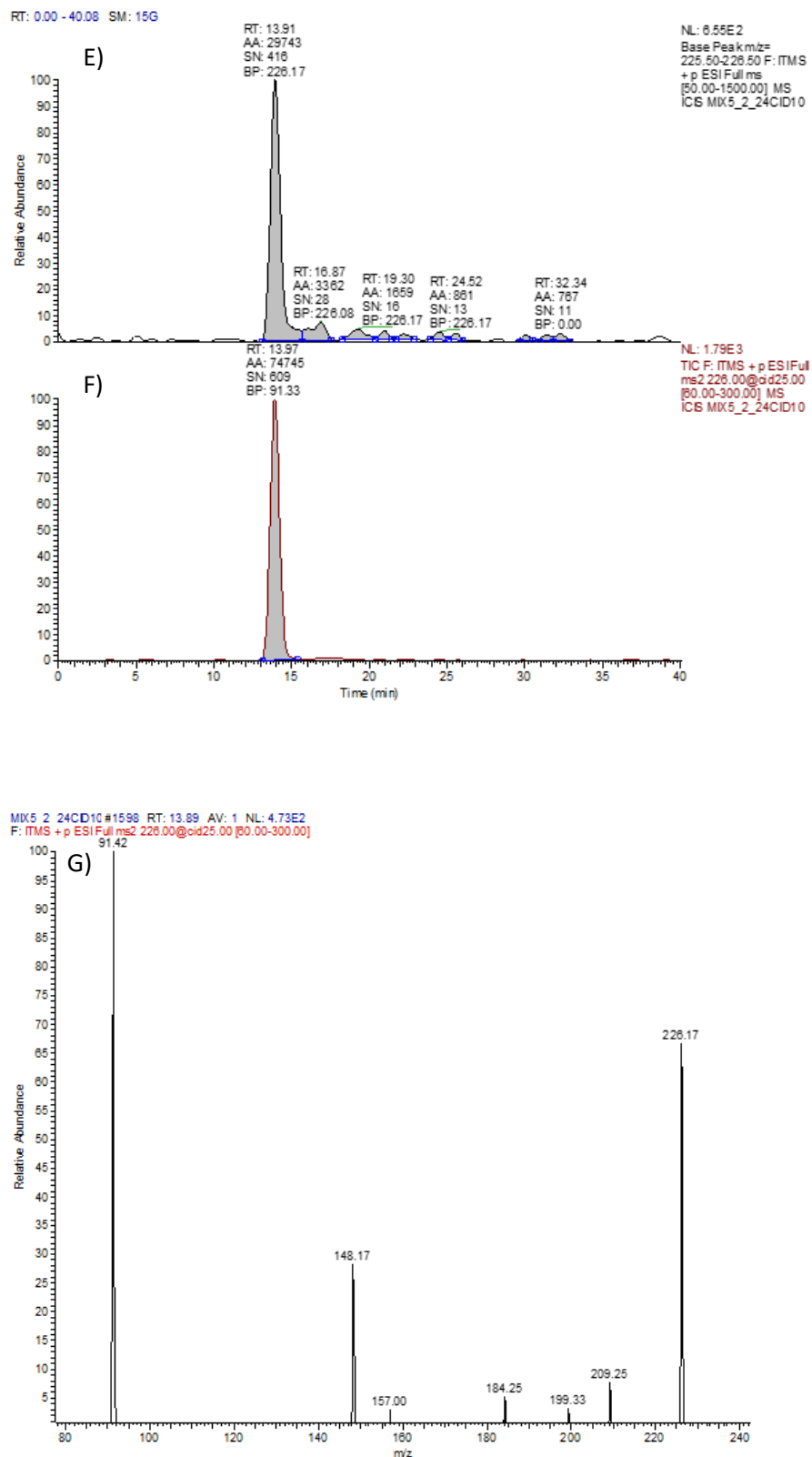
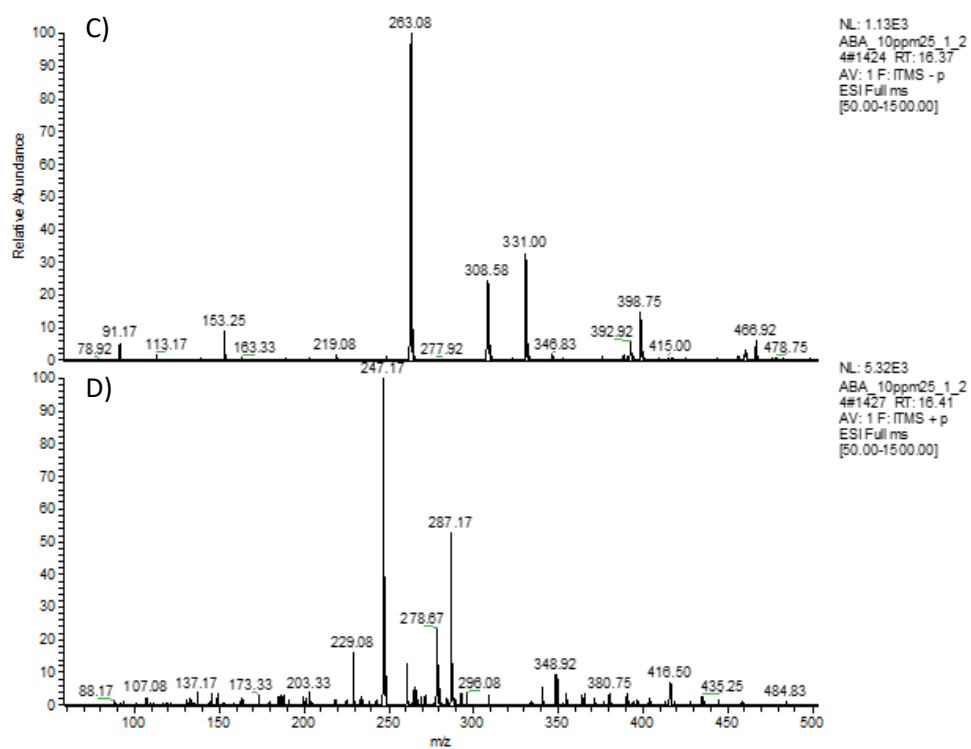
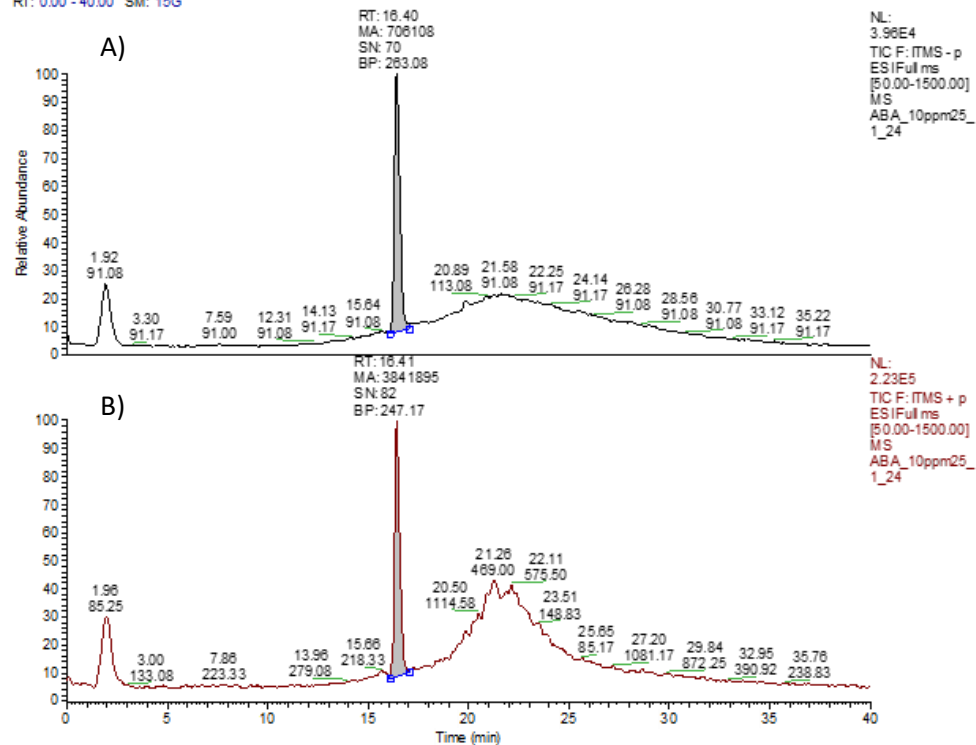


Figure S 6 - LC/MS-MS chromatograms of BAP in A) Negative mode; B) Positive mode; BAP LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode; E) BAP LC-MS/MS window run ($225.50 \leq m/z \leq 226.50$); F) BAP LC-MS/MS CID chromatogram in positive mode; G) BAP LC-MS/MS CID spectrum

RT: 0.00 - 40.00 SM: 15G



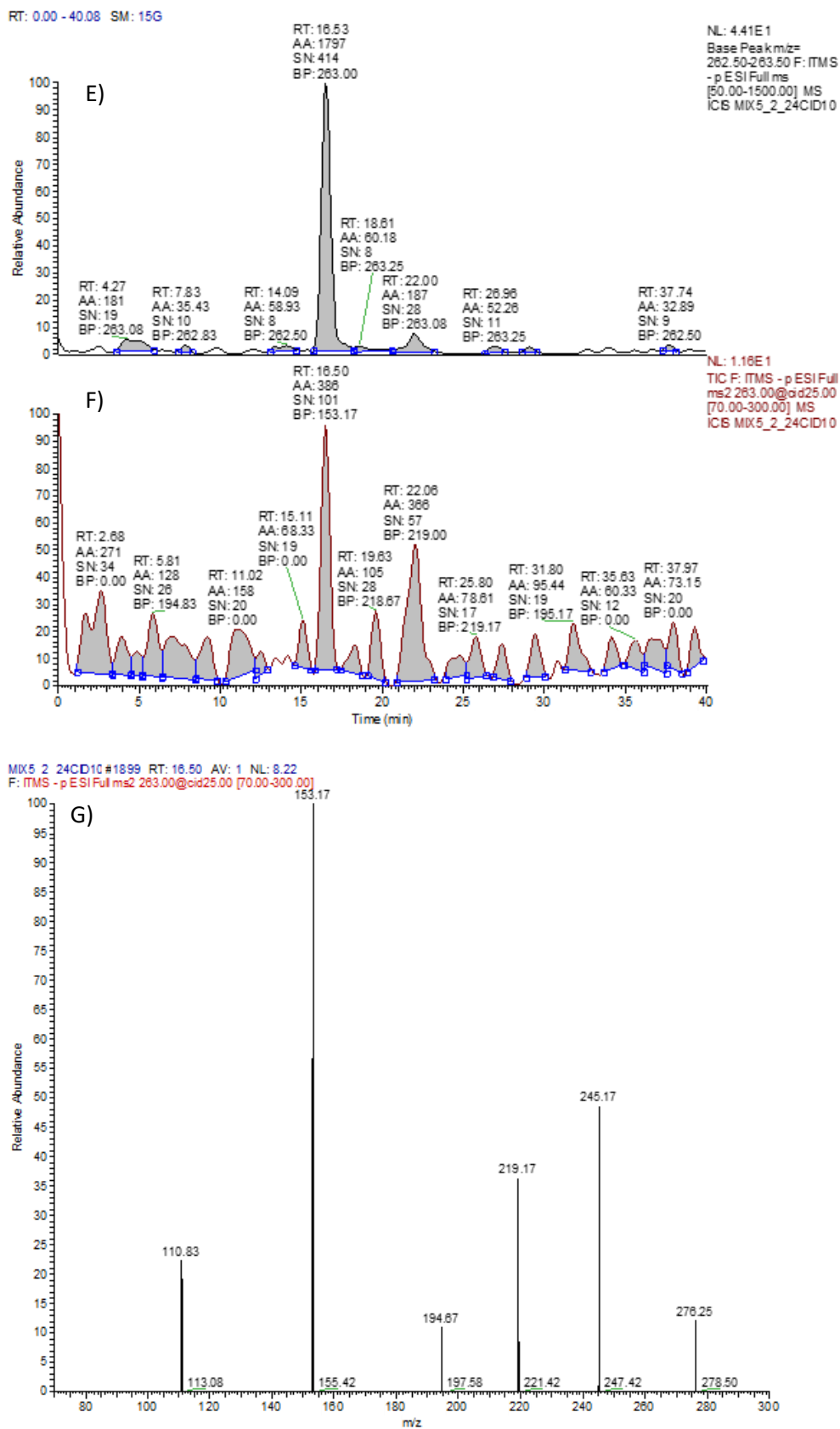


Figure S 7 - LC/MS-MS chromatograms of ABA in A) Negative mode; B) Positive mode; ABA LC-MS/MS spectra C) Peak m/z in negative mode; D) Peak m/z in positive mode; E) ABA LC-MS/MS window run (262.50 ≤ m/z ≤ 263.50); F) ABA LC-MS/MS CID chromatogram in negative mode; G) ABA LC-MS/MS CID spectrum

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