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Dedicated to my parents, Miguel and Margarida
and brother, Miguel
“We have enough consciousness and humanity to ensure a sustainable tomorrow.”

Paul Anastas

(Father of Green Chemistry)
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Abstract

Agar is a seaweed polysaccharide (SP), mainly used in the gel-form in food applications, although its interest for the production of new materials towards the development of emerging areas such as, tissue engineering or ‘smart materials’ has recently gained great attention. Due to its unique molecular structure and behavior supported by strong intra- and intermolecular hydrogen bonds, agar has excellent gelling and film-forming properties. It is also easy to process which makes it attractive to be used in other forms such as bioplastic films (aqueous-blended and melt-processed) or fibers.

Agar industry is a well-established sector in the field of hydrocolloids mostly sustained by long-term implemented machineries and processes, often lacking efficiency. In the current global economic scenario, the use of more efficient technologies, processes and products, not only complies with sustainable development but is also vital for companies to keep competitiveness.

Recent incentives given to the bio-energetic sector and the need to move away from food crops have opened new market opportunities for a second generation of feedstocks based in non-food crops such as algae and by-products. In parallel, the expected growth demand for plastics will demand greater production and consumption of bioplastics.

*Gracilaria vermiculophylla* is a red algal species currently dominant in Ria de Aveiro, northwestern Portugal (40°38'N, 8°43'W). As non-native species may have a negative impact in the recipient ecosystems intensive joint efforts have been carried out to explore the potentialities of this national underexploited resource. The use of *G. vermiculophylla* as biofilter in an integrated multitrophic aquaculture (IMTA) system, which is a sustainable farming approach, has shown to be very promising. In wild, cultivated or as waste of emerging industries, seaweeds hold the potential to become a sustainable resource for hydrocolloid and/or polymer transformation industries. Considering the seasonal variation on the abundance and quality of the biomass harvested from wild populations, the production of seaweeds in IMTA systems can offer a continuous supply of raw material with reliable quality to the transformation industries.

In the first stage of the PhD project, microwave-assisted extraction (MAE) of native (NA) and alkali-treated (AA) agars from IMTA *Gracilaria*, was tested and optimized using response surface methodology. The influence of the MAE operational parameters (extraction time, temperature, solvent volume and stirring speed) on the physical and chemical properties of agar (yield, gel strength (GS), gelling and melting temperatures, as well as
sulfate and LA contents) was evaluated in a $2^4$ orthogonal composite design. The found optimal MAE conditions were applied to the same wild biomass to study the influence of the seaweeds’ growth conditions in the quality of the extracted SP. In addition, MAE agars were compared in terms of their properties with SP extracted using a traditional water extraction (TWE) method, previously optimized by our group (2 hours, 200 mL, 85 °C, no agitation).

The quality of the AA obtained by MAE compared favourably with that attained using TWE. The gelling properties were best when the SP were extracted from IMTA *Gracilaria* and the extracts compared favourably with a commercial sample used as reference. Maximum GS of AA were attained at relatively mild MAE conditions (5 minutes of extraction, 90 °C, 20 mL, maximum stirring speed) which could be very important for industrial implementation (avoid safety concerns). Higher AA amounts were recovered when using 20 minutes of extraction, 110 °C, 40 ml of solvent and no agitation.

MAE optimal conditions (15 min of extraction, 120 °C, 40 mL of solvent and medium stirring speed) led to higher NA recoveries than TWE yet, the lower $M_w$ and $M_v$ indicated substantial depolymerization of the SP backbone; this resulted in lower GS, in any case, fulfilling the commercial standards for application in soft-texture food products. The higher tendency for degradation when exposed to microwaves could be attributed to the higher sulfate content of NA (more labile character of L6S units). AA in turn, with higher LA fraction were more stable to microwaves. The MAE approach supports sustainable development, as it requires less energy and solvent than conventional processes, while generating fewer wastes. This work suggests the feasibility of exploitation of *G. vermiculophylla*, wild or produced in IMTA systems, for the production of agar gels with commercial quality.

A case study concerning the influence of the extraction process on the rheological and structural properties of representative AA from wild *Gracilaria* was carried out. While TWE agar showed a more open and irregular 3D network, MAE agar gel imaged by cryoSEM was denser and fairly uniform. The rheological (higher thermal stability and consistency) and mechanical (higher GS) behaviors of MAE agar seemed consistent with a positive effect of molecular mass and LA content. MAE produced non-degraded agar comparable with commercial ones, and if properly monitored, could be a promising alternative to TWE.

In order to assess the influence of MAE conditions on the behavior of NA and AA in dilute and concentrated aqueous media, a second case study was performed also using agars from wild *Gracilaria*. Different MAE routes led to SP with unique properties which in turn, resulted in different molecular assemblies imaged by AFM and cryoSEM, in dilute and concentrated aqueous media. The molecular assemblies of agars can be tuned via MAE which can be of great interest towards the development of new agar-based materials for
emerging applications. The image studies supported well the view of double helix formation followed by intensive double helix association proposed for agar gelation.

The 2nd stage of the PhD project focused on exploring the obtained optimal agar extracts for the production of sustainable materials. The first considered applications were the production of aqueous agar-blended films and hydrogels. For these studies, representative agar extracts were used: NA and AA from IMTA *Gracilaria* extracted using TWE (*i.e.* NA\_IMTA\_TWE\textsuperscript{opt} and AA\_IMTA\_TWE\textsuperscript{opt}). Agar/locust bean gum (LBG) mixtures were prepared at different mass ratios (*i.e.* 100/0, 75/25, 50/50, 25/75, 0/100) using water as solvent and processed in the form of films or hydrogels.

The addition of LBG to agar made easier the films' fabrication by dramatically increasing the viscosity and decreasing the gelling character of the solutions as confirmed by rheology. It also led to a significant improvement of the films' final properties. Best films were found at 50/50 and 25/75 agar/LBG mass ratios for AA\_IMTA\_TWE\textsuperscript{opt} while NA\_IMTA\_TWE\textsuperscript{opt} prepared at 25/75 yielded the best results. For instance, the mechanical properties for the mentioned optimal AA\_IMTA\_TWE\textsuperscript{opt} formulations fell in the range, TS \~80-110 MPa and YM \~69-84 MPa, while for 25/75 NA\_IMTA\_TWE\textsuperscript{opt}/LBG, TS \~80-120 MPa and YM \~67-93 MPa. All agar/LBG films were very brittle (*EB* in the range \~1.6-2.4 %). The obtained data show the potential of NA to fabricate aqueous-blended films with comparable or even improved properties than alkali-modified SP. This can be very cost-attractive since the extraction of native SP doesn’t need the inclusion of an alkaline step prior to the extraction. Moreover, the addition of LBG in percentages as high as 50-75%, will further reduce the cost of the films since this polysaccharide is cheaper than agar.

The addition of LBG to agar hydrogels led to mixed systems with various rheological properties which can be very attractive for the manufacture of new products with interesting textures and mouth feel to the consumers. As seen in the films' case, the physicochemical nature of agar was a relevant factor in the definition of the binding mechanisms between agar and LBG. SAOS and LAOS studies gave different and insightful information on the microstructure of the mixed gels as well as the established interactions between both polysaccharides. The rheological tests seemed to indicate different interactions of NA and AA with LBG, which could be related with the different physicochemical nature of the SP. Yet, further studies are clearly needed to shed more light on this topic. The LBG incorporation to AA\_IMTA\_TWE\textsuperscript{opt} solutions, decreased the GS and increased the deformability of the gels. The interactions established between agar and LBG were also dependent on the material's form.
In order to improve the typical brittle nature of agar films other explored application was the production of non-aqueous agar films. To this end, we tested the suitability of ChCl/urea (DES-U) and ChCl/glycerol (DES-G) eutectic mixtures, at 1:2 molar ratios, used as both solvent and plasticizer. For this application, a commercial agar sample was used. A three-step process was proposed: pre-solubilization of polymer in DES followed by compression-molding and subsequent drying. The mechanical properties, water resistance and microstructure of the films were evaluated at different agar concentrations (i.e. 2 to 6% wt). DES-U showed by far the best film forming ability. Agreeing with the diffusion and SEM data, films with best mechanical properties were found at the lowest and highest concentrations of agar (e.g. TS of 24.2-42 MPa and EB of 15.4-38.9%). Clearly, by replacing water by an alternative solvent such as DES-U, films with remarkably different functional properties can be obtained which might be very attractive for the development of new materials based on agar.

The last form of material to be tested was agar fibers produced by electrospinning technique. Micro- to nano-sized fibers were obtained by processing agar and agar/PVA solutions with different mass ratios (100/0,…, 50/50,…,0/100) at 50 °C. In water, the addition of a co-blending polymer to 1% wt agar solution was needed to attain spinnability. Above 60% PVA content in the final blend (from a 10% wt PVA starting solution), defect-free electrospun nanofibers were obtained. Pure agar nanofibers were only obtained when replacing water by DES-U. The effectiveness of the electrospinning was strongly impacted by the viscoelasticity of the DES-U systems. Solutions with very good spinnability were obtained even at 1% wt agar concentration if working far beyond the gelation transition. In both cases (using water or DES-U), the apparent viscosity increased with the addition of PVA. The morphology of agar composite fibers in DES-U was greatly affected by the composition of the spinning solution and processing conditions. In the quest for highly functional and sustainable materials this can be quite promising.

**Keywords:** Gracilaria vermiculophylla; Integrated multi-trophic aquaculture; Polysaccharides; Native agars; Alkali-treated agars; Microwave-assisted extraction; Deep eutectic solvents; Hydrogels; Films; Fibers; Electrospinning; Rheology; AFM; SEM; Sustainability.
Resumo

O agar é um polissacarídeo que surge como material intracelular em várias espécies de algas marinhas vermelhas, maioritariamente usado como agente gelificante e estabilizante em aplicações alimentares. Em anos recentes, o seu uso no fabrico de novos materiais para áreas emergentes, tais como engenharia de tecidos ou produção de ‘materiais inteligentes’, tem também suscitado amplo interesse. Devido à sua estrutura molecular única, suportada por fortes ligações de hidrogénio intra- e intermoleculares, o agar possui excelentes propriedades gelificantes e formadoras de filme. É também facilmente processável, o que o torna atrativo para ser usado noutras formas para além de gel, tais como filmes bioplásticos (aquosos e termo-processados) ou fibras.

A produção industrial do agar possui uma longa tradição na indústria dos hidrocolóides sendo sustentada por tecnologias e equipamentos antigos e, por isso, muitas vezes pouco eficientes. Na atual conjuntura económica, o uso de tecnologias, processos e produtos mais eficientes não só cumpre com os princípios de desenvolvimento sustentável como também é vital para as empresas se manterem competitivas.

Os incentivos dados, recentemente, ao setor bioenergético e a necessidade de não competirmos com o cultivo de alimentos, possibilitaram uma nova oportunidade de mercado para uma segunda geração de matérias-primas com origem em produtos não alimentares tais como algas e subprodutos. Paralelamente, o esperado aumento da procura de plásticos exigirá uma maior produção e consumo de bioplásticos.

A *Gracilaria vermiculophylla*, é uma espécie de alga vermelha, atualmente dominante na Ria de Aveiro, noroeste Portugal (40°38’N, 8°43’W). Dado os impactos negativos que espécies não nativas podem ter nos ecossistemas recetores, têm sido levados a cabo esforços conjuntos entre diferentes áreas de investigação com o intuito de explorar as potencialidades deste recurso nacional subexplorado. O uso da *G. vermiculophylla* como biofiltro em sistemas de acuicultura multitrófica integrada (AMTI), que é um tipo de cultivo sustentável, mostrou ser bastante promissor.

No seu estado natural, cultivadas ou como subprodutos de indústrias emergentes, as algas têm um grande potencial de se tornarem um recurso sustentável para as indústrias dos hidrocolóides ou outros setores transformadores. Considerando a variabilidade na abundância e qualidade da biomassa resultante das populações naturais, o cultivo de algas em sistemas de AMTI pode permitir um fornecimento contínuo de matérias-primas, com qualidade regular, para as indústrias transformadoras.
Na primeira fase do presente projeto de doutoramento, foi testada e otimizada a extração assistida por microondas (EAM) de agares nativos (AN) e pré-tratados (APT) de *G. vermiculophylla* cultivada em sistemas de AMTI, usando a metodologia de superfície de resposta. Foi estudado o efeito de diferentes parâmetros da EAM – tempo de extração, temperatura, velocidade de agitação e volume de solvente – sobre o rendimento da extração e as características do agar extraído, nomeadamente, teor em sulfatos e em 3,6–anidro–L–galactose (LA), força de gel (FG) e temperaturas de gelificação e de fusão. Um desenho ortogonal $2^4$ foi a abordagem estatística escolhida para a otimização do processo de extração. Os agares da EAM foram comparados, em termos de propriedades, com agares obtidos pelo método de extração convencional (extração aquosa a quente, EAQ), previamente otimizado pelo nosso grupo (2 horas, 200 mL, 85 °C, sem agitação). Adicionalmente, com o intuito de estudar a influência das condições de crescimento das algas nas propriedades finais dos agares extraídos, foram aplicadas as condições ótimas de EAM e EAQ a amostras de populações naturais de *G. vermiculophylla* (*in natura*).

A qualidade dos APT obtidos por EAM foi claramente superior aos obtidos por EAQ. As propriedades gelificantes foram melhores nos agares extraídos da *Gracilaria* cultivada e, independentemente das condições de crescimento das algas, os resultados foram superiores ao da amostra de agar comercial usada como referência. A FG máxima para os APT foi obtida a condições de EAM relativamente suaves (5 minutos de extração, 90 °C, 20 mL, agitação máxima), o que poderá ser muito relevante em termos da implementação industrial do processo (minimização dos riscos de segurança). O rendimento ótimo dos APT foi registado para 20 minutos de extração, 110 °C, 40 ml de volume e sem agitação.

As condições de EAM ótimas: 15 minutos de extração, 120 °C, 40 mL de volume de solvente e agitação média, permitiram obter maiores rendimentos de AN que o método convencional; no entanto, as massas moleculares mais baixas estimadas para estes agares evidenciaram uma maior degradação das cadeias poliméricas quando comparados com os polissacarídeos obtidos por EAQ; isto traduziu-se em valores de FG mais baixos, em todo o caso, de acordo com os valores comerciais de referência para aplicações alimentares com texturas suaves. A maior suscetibilidade à degradação por microondas poderá ser atribuída ao maior teor em sulfatos dos AN (maior caráter lâbil dos monômeros L6S). Por seu turno os APT, com maior teor em LA foram mais estáveis às microondas. A EAM suporta o desenvolvimento sustentável pois requere menores gastos de energia e de solventes que os processos convencionais, simultaneamente gerando menos resíduos. Os resultados obtidos sugerem a viabilidade de exploração da *G. vermiculophylla*, cultivada em AMTI ou *in natura*, para a produção de agares com qualidade comercial.
A influência do processo de extração nas propriedades reológicas e estruturais dos agares foi estudada através de um caso de estudo com extratos representativos de agares de Gracilaria in natura. Os ensaios de microscopia de varrimento eletrónico evidenciaram redes poliméricas 3D mais densas e uniformes para os géis de agar obtidos por EAM, e redes mais abertas e irregulares para os géis de polissacarídeos obtidos por EAQ. Os comportamentos reológicos (maior consistência e estabilidade térmica) e mecânicos (maior FG) do agar obtido por EAM foi consistente com um efeito positivo da massa molecular e do teor em LA. A EAM produziu agares não degradados com propriedades comparáveis a amostras comerciais, e se bem otimizado, pode constituir uma alternativa promissora à EAQ.

Um segundo caso de estudo foi levado a cabo para perceber a influência das condições operacionais da EAM no comportamento de AN e APT, em meio aquoso diluído e concentrado. Para o efeito, também foram considerados agares de Gracilaria in natura. Diferentes condições de EAM produziram agares com propriedades únicas, o que, por sua vez, resultou em aglomerados moleculares únicos observados por microscopias de força atómica e eletrónica de varrimento. As imagens obtidas foram concordantes com o modelo atualmente proposto para o mecanismo de gelificação do agar: formação de hélices duplas seguida de intensa associação entre hélices. A arquitetura molecular do agar pode ser moldada via EAM, o que pode ter bastante interesse no desenvolvimento de novos biomateriais, feitos à base de agar, para aplicações em áreas emergentes.

A segunda fase do projeto de doutoramento focou-se na produção de materiais sustentáveis feitos à base dos agares extraídos. As primeiras formas de materiais exploradas foram filmes aquosos e hidrogéis à base de agar e goma de alfarroba. Para estes estudos, foram usados AN e APT de Gracilaria cultivada, obtidos por EAQ (i.e. AN_AIMT_EAQ\textsuperscript{opt} and APT_AIMT_EAQ\textsuperscript{opt}). As misturas de agar/goma de alfarroba foram preparadas com diferentes composições mássicas (i.e. 100/0, 75/25, 50/50, 25/75, 0/100) e processadas na forma de filmes e/ou hidrogéis.

A adição da goma de alfarroba ao agar facilitou o processamento delle soluções durante o fabrico dos filmes através de um aumento significativo da viscosidade e diminuição do caráter gelificante das soluções. Também possibilitou uma melhoria significativa nas propriedades finais dos filmes. As formulações ótimas para o APT_AIMT_EAQ\textsuperscript{opt} foram as 50/50 e 25/75 agar/goma de alfarroba, enquanto que o AN_AIMT_EAQ\textsuperscript{opt} misturado a 25/75 produziu os melhores resultados. Por exemplo, as propriedades mecânicas para as misturas ótimas de APT_AIMT_EAQ\textsuperscript{opt} situaram-se entre ~80-110 MPa para a tensão-na-rutura (\textit{TR}) e ~69-84 MPa para o modulo de Young (\textit{MY}),
enquanto que para o filme 25/75 de AN_AIMT_EAQ<sup>opt</sup>/goma de alfarroba, TR ~80-120 MPa e MY ~67-93 MPa. Todos os filmes de agar/goma de alfarroba produzidos foram muito quebradiços (deformação-na-rutura, DR entre ~1.6-2.4 %). Os resultados reunidos nesta fase mostraram o potencial dos AN para o fabrico de filmes aquosos com propriedades comparáveis ou mesmo melhores do que os APT. Isto pode ser economicamente bastante atrativo uma vez que os AN não necessitam de pré-tratamento alcalino antes do processo de extração. A adição da goma de alfarroba em percentagens tão elevadas como 50-75% permitirá reduzir ainda mais o custo final do bioplástico, uma vez que é um polissacarídeo mais barato de produzir que o agar.

A adição da goma de alfarroba aos hidrogéis de agar resultou em sistemas com propriedades reológicas variadas, o que pode ser bastante atrativo para o desenvolvimento de produtos com novas e interessantes texturas, mais apelativas ao consumidor. Tal como nos filmes, a natureza físicoquímica do agar foi um fator relevante na definição dos mecanismos de interação entre o galactano e a goma de alfarroba. Os estudos reológicos levados a cabo a pequenas e elevadas deformações deram informação importante e complementar relativamente à microestrutura dos géis obtidos, bem como às interações estabelecidas entre os polissacarídeos. Os resultados reológicos obtidos a elevadas deformações pareceram indicar interações diferentes entre cada agar e a goma de alfarroba. Contudo, mais estudos serão necessários para fundamentar esta sugestão. A adição da goma de alfarroba aos géis de APT_AIMT_EAQ<sup>opt</sup> diminuiu a FG e aumentou a sua deformabilidade. As interações estabelecidas entre o agar e a goma de alfarroba foram diferentes, dependendo do material (filmes aquosos ou hidrogéis).

A fim de diminuir o caráter quebradiço tipicamente associado aos filmes aquosos de agar, foi explorada a produção de filmes de agar não aquosos. Para este efeito, foi testada a aplicabilidade, como solventes e plastificantes, das misturas eutéticas de cloreto de colina/ureia (DES-U) e cloreto de colina/glicerol (DES-G) com uma razão molar de 1:2, na produção de filmes de agar comercial a diferentes concentrações de polímero (i.e. 2 to 6% wt). A metodologia de fabrico dos filmes consistiu na pré-solubilização do agar no DES seguida por termo-compressão do sistema e posterior secagem. A mistura eutéctica de DES-U revelou, de longe, as melhores propriedades formadoras de filme. Concordando com os resultados de permeabilidade e de microscopia, as melhores propriedades mecânicas foram obtidas para as concentrações mais baixa e mais alta de agar (p.ex. TR entre 24.2-42 MPa e DR entre 15.4-38.9%). Claramente, substituindo a água por um solvente alternativo como o DES-U, podem ser produzidos filmes de agar com propriedades funcionais substancialmente diferentes dos materiais aquosos habitualmente produzidos.
O último estudo dos materiais focou a produção de fibras de agar por eletrofiação ('electrospinning'). Micro- e nanofibras com diferentes morfologias foram produzidas através do processamento, a 50 °C, de misturas de agar/PVA preparadas em água e em DES-U, com diferentes proporções mássicas (100/0, ..., 50/50, ..., 0/100). Em meio aquoso, a adição do PVA a uma solução de agar 1% wt foi necessária para obter uma amostra eletrofiável. Acima de 60% de teor de PVA na mistura final (a partir uma solução-mãe de 10% wt PVA), foram produzidas nanofibras sem defeitos.

Nanofibras de agar puras só foram conseguidas quando se substituiu a água pelo DES-U. Neste caso, a eficácia do processo de eletrofiação dependeu fortemente da viscoelasticidade dos sistemas de agar/PVA. Quando operando longe da temperatura de gelificação do sistema, foram obtidas soluções facilmente eletrofiáveis mesmo quando usando uma concentração de agar de 1% wt. Nos dois tipos de sistemas (aquosos ou em DES-U), a viscosidade aparente aumentou com a adição do PVA. A morfologia das fibras compósitas de agar, em DES-U, foi muito afetada pela composição da solução e das condições de processamento. Na busca pelo desenvolvimento de novos materiais altamente funcionais e sustentáveis, os resultados obtidos podem ser bastante promissores.

Palavras-chave: Gracilaria vermiculophylla; Aquacultura multitrófica integrada; Polissacarídeos; Agares nativos; Agares pré-tratados; Extração assistida por micro-ondas; Solventes eutéticos; Hidrogéis; Filmes; Fibras; Eletrofiação; Reologia; Microscopia de Força Atómica; Microscopia Eletrônica de Varrimento; Sustentabilidade
List of Publications

List of publications in peer-reviewed journals included in this PhD thesis:


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\textbf{PART II}

\textit{Chapter II.1}

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fabrication of agar/PVA fibers using water as solvent. The following conditions were fixed in all experiments: temperature of 50 °C, initial sample volume of 3 mL and distance tip-to-collector of 8 cm. A drum collector was used in all cases. For more details concerning the overall process please see the experimental section. **Table II.6.2** – Electrospinning conditions used for the fabrication of agar and agar/PVA fibers in DES-U. In all experiments an ethanol bath was used as collector. For more details concerning the overall process please see the experimental section.
List of Symbols, Abbreviations and Shorthand Notations

**Symbols:**

- $a_w$: Water activity
- $A_{930}/A_{1250}$: Absorbance ratio of peaks attributed to LA (930 cm$^{-1}$) and total sulfate (1250 cm$^{-1}$) obtained from FTIR analysis
- $\Delta A$: Droplet surface area variation
- $B_0$: Magnetic field
- $B_{loc}$: Local magnetic field
- $C$: GAB constant
- $\Delta C$ or $C_1-C_2$: Concentration gradient
- $d$: Thickness
- $D$: Diffusivity constant
- $\Delta M$: Molar mass dispersity
- $E_B$: Elongation-at-break
- $g$: Gap
- $G'$: Elastic or storage modulus
- $G''$: Viscous or loss modulus
- $G_S$: Gel Strength
- $h$: Planck’s constant
- $\Delta H_S$: Enthalpic variation caused by the solubilization of the diffusant in the polymer matrix
- $J$: flux of diffusant that transfers through unit area of polymer film
- $k$: GAB parameter
- $K$: Dynamic consistency index
- $K_H$: Huggins constant
- $K_K$: Kraemer constant
\( m \) \hspace{1cm} \text{Dimensionless constant of the Cross model}

\( M \) \hspace{1cm} \text{Torque}

\( M_n \) \hspace{1cm} \text{Number-average molar mass}

\( M_w \) \hspace{1cm} \text{Mass-average molar mass}

\( \Delta m \) \hspace{1cm} \text{Weight gain}

\( n \) \hspace{1cm} \text{Dynamic power-law factor}

\( N \) \hspace{1cm} \text{Dimensionless constant of the Carreau model}

\( p \) \hspace{1cm} \text{Gas partial pressure}

\( P \) \hspace{1cm} \text{Permeability}

\( \Delta P \text{ or } p_1-p_2 \) \hspace{1cm} \text{Pressure gradient}

\( Q \) \hspace{1cm} \text{Amount of diffusant that transfers through the polymer film}

\( r \) \hspace{1cm} \text{Plate radius}

\( R \) \hspace{1cm} \text{Universal gas constant}

\( R^2 \) \hspace{1cm} \text{Quadratic correlation coefficient}

\( R_h \) \hspace{1cm} \text{Hydrodynamic radius}

\( S \) \hspace{1cm} \text{Solubility parameter in Henry's law}

\( t \) \hspace{1cm} \text{Efflux time of the solution}

\( t_s \) \hspace{1cm} \text{Efflux time of the solvent}

\( T \) \hspace{1cm} \text{Absolute temperature}

\( T_g \) \hspace{1cm} \text{Gelling temperature}

\( T_m \) \hspace{1cm} \text{Melting temperature}

\( TS \) \hspace{1cm} \text{Tensile strength-at-break}

\( \Delta T \) \hspace{1cm} \text{Thermal hysteresis}

\( \tan \delta \) \hspace{1cm} \text{Tangent of the phase angle}

\( V_h \) \hspace{1cm} \text{Hydrodynamic volume}

\( \Delta V \) \hspace{1cm} \text{Droplet volume variation}

\( X_0 \) \hspace{1cm} \text{Monolayer moisture content on dry basis}

\( X_A \) \hspace{1cm} \text{Weight fraction of polysaccharide A}

\( X_B \) \hspace{1cm} \text{Weight fraction of polysaccharide B}

\( YM \) \hspace{1cm} \text{Young's modulus}
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_c$</td>
<td>Time constant of the Cross model</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Strain</td>
</tr>
<tr>
<td>$\gamma_0$</td>
<td>Maximum strain</td>
</tr>
<tr>
<td>$\gamma_c$</td>
<td>Critical strain</td>
</tr>
<tr>
<td>$\dot{\gamma}$</td>
<td>Shear rate</td>
</tr>
<tr>
<td>$\Delta \Theta$</td>
<td>Contact angle variation</td>
</tr>
<tr>
<td>$\varepsilon'$</td>
<td>Real permittivity (real part of the complex permittivity)</td>
</tr>
<tr>
<td>$\varepsilon''$</td>
<td>Loss Factor (imaginary part of the complex permittivity)</td>
</tr>
<tr>
<td>$\varepsilon^*$</td>
<td>Complex permittivity</td>
</tr>
<tr>
<td>$\eta'$</td>
<td>Dynamic viscosity</td>
</tr>
<tr>
<td>$\eta_0$</td>
<td>Zero-shear rate viscosity</td>
</tr>
<tr>
<td>$\eta_\infty$</td>
<td>Infinite-shear rate viscosity</td>
</tr>
<tr>
<td>$\eta_A$</td>
<td>Apparent viscosity of the polysaccharide A</td>
</tr>
<tr>
<td>$\eta_B$</td>
<td>Apparent viscosity of the polysaccharide B</td>
</tr>
<tr>
<td>$\eta_{\text{app}}$</td>
<td>Apparent viscosity</td>
</tr>
<tr>
<td>$[\eta]$</td>
<td>Intrinsic viscosity</td>
</tr>
<tr>
<td>$\eta^*$</td>
<td>Complex viscosity</td>
</tr>
<tr>
<td>$\Theta$</td>
<td>Contact angle</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
</tr>
<tr>
<td>$\lambda_c$</td>
<td>Time constant of the Carreau model</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Stress amplitude</td>
</tr>
<tr>
<td>$\sigma_0$</td>
<td>Maximum stress amplitude</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Density of the solution</td>
</tr>
<tr>
<td>$\rho_s$</td>
<td>Density of the solvent</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Angular frequency</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Angular velocity</td>
</tr>
</tbody>
</table>
### Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Alkali-treated Agar</td>
</tr>
<tr>
<td>ABS</td>
<td>Acrylonitrile Butadiene Styrene</td>
</tr>
<tr>
<td>ADC</td>
<td>Analog to Digital Converter</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>APIP</td>
<td>Portuguese Association of the Plastic Industry</td>
</tr>
<tr>
<td>APT</td>
<td>Attached Proton Test</td>
</tr>
<tr>
<td>bio-PE</td>
<td>Bio-polyethylene</td>
</tr>
<tr>
<td>bio-PET</td>
<td>Bio-polyethylene terephthalate</td>
</tr>
<tr>
<td>bio-PU</td>
<td>Bio-polyurethane</td>
</tr>
<tr>
<td>BOPP</td>
<td>Bi-oriented polypropylene</td>
</tr>
<tr>
<td>CA</td>
<td>Commercial Agar</td>
</tr>
<tr>
<td>cryoSEM</td>
<td>cryoScanning Electron Microscopy</td>
</tr>
<tr>
<td>D1</td>
<td>Relaxation Delay or delay between repetitions</td>
</tr>
<tr>
<td>DES</td>
<td>Deep Eutectic Solvent</td>
</tr>
<tr>
<td>DF</td>
<td>Degree of Freedom</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>FID</td>
<td>Free Induction Decay</td>
</tr>
<tr>
<td>GAB</td>
<td>Guggenheim-Anderson-de Boer</td>
</tr>
<tr>
<td>GG</td>
<td>Guar Gum</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
</tr>
<tr>
<td>GTT</td>
<td>Glass Transition Temperature</td>
</tr>
<tr>
<td>HDPE</td>
<td>High Density Polyethylene</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear Double Quantum Coherence</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>IMTA</td>
<td>Integrated Multi-Trophic Aquaculture</td>
</tr>
<tr>
<td>IPNs</td>
<td>Interpenetrating Networks</td>
</tr>
<tr>
<td>Symbol</td>
<td>Abbreviation/Explanation</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LAOS</td>
<td>Large Amplitude Oscillatory Shear</td>
</tr>
<tr>
<td>LBG</td>
<td>Locust Bean Gum</td>
</tr>
<tr>
<td>LDPE</td>
<td>Low Density Polyethylene</td>
</tr>
<tr>
<td>LLDPE</td>
<td>Linear Low Density Polyethylene</td>
</tr>
<tr>
<td>MAE</td>
<td>Microwave-Assisted Extraction</td>
</tr>
<tr>
<td>MS</td>
<td>Mean Square</td>
</tr>
<tr>
<td>MW</td>
<td>Microwaves</td>
</tr>
<tr>
<td>NA</td>
<td>Native Agar</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Effect Spectroscopy</td>
</tr>
<tr>
<td>NS</td>
<td>Number of Scans</td>
</tr>
<tr>
<td>PAD</td>
<td>Pulsed Amperometric Detector</td>
</tr>
<tr>
<td>PBAT</td>
<td>Poly(butylene adipate-co-terephthalate)</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly-ε-caprolactone</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene oxide</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>PHA</td>
<td>Polyhydroxyalkanoate</td>
</tr>
<tr>
<td>PHB</td>
<td>Polyhydroxybutyrate</td>
</tr>
<tr>
<td>PHBV</td>
<td>Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)</td>
</tr>
<tr>
<td>PHBH</td>
<td>Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PP</td>
<td>Polypropylene</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
</tbody>
</table>
RTIL  Room Temperature Ionic Liquid
SP    Seaweed Polysaccharides
TWE   Traditional hot-Water Extraction
UV    Ultraviolet
Vis   Visible
WVP   Water Vapor Permeability

**Shorthand Notations:**

The chemical nomenclature of residues followed the shorthand notation system defined by Knutsen *et al.* (Knutsen *et al.*, 1994. *Botanica Marina*, 37(2), 163-169),

C1  Carbon at position one (other carbons were also represented by the number of the position in the residue and the respective letter)
H1  Proton at position one (other protons were also represented by the number of the position in the residue and the respective letter)
DA  4-linked 3,6-anhydro-α-D-galactose
G   3-linked β-D-galactose
L   4-linked α-L-galactose
LA  4-linked 3,6-anhydro-α-L-galactose
M   O-methyl
S   ester sulfate

A simple notation was used for the deep eutectic solvents,

ChCl  (2-hydroxyethyl)trimethylammonium chloride (choline chloride)
DES-U ChCl/Urea at 1:2 molar ratio
DES-G ChCl/anhydrous glycerol at 1:2 molar ratio
For clarity purposes, the different sets of extraction parameters used to obtain the polysaccharides were identified by the following shorthand notation (Table i),

<table>
<thead>
<tr>
<th>Id.</th>
<th>Conditions</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAE(^{\text{opt}})</td>
<td>15 min under microwave heat, 120 °C, 40 mL of water and medium stirring speed</td>
<td>Optimal conditions of NA</td>
</tr>
<tr>
<td>MAE(^{1})</td>
<td>15 min under microwave heat, 100 °C, 40 mL of water and no agitation</td>
<td>Additional tested conditions of NA</td>
</tr>
<tr>
<td>MAE(_{Y}^{\text{opt}})</td>
<td>20 min under microwave heat, 110 °C, 40 mL of water and no stirring speed</td>
<td>Optimal yield conditions of AA</td>
</tr>
<tr>
<td>MAE(_{GS}^{\text{opt}})</td>
<td>5 min under microwave heat, 90 °C, 20 mL of water and maximum stirring speed</td>
<td>Optimal GS conditions of AA</td>
</tr>
<tr>
<td>TWE(^{\text{opt}})</td>
<td>2 h under conventional heat, 85 °C, 200 mL of water and no agitation</td>
<td>Optimal TWE conditions of NA and AA obtained in previous a study (Villanueva et al., 2010)</td>
</tr>
</tbody>
</table>

Finally, a shorthand notation was adopted for identifying the different agar extracts (Table ii). Each sample designation will first identify the agar type (i.e. NA or AA), second, the biomass used to extract the polysaccharides (i.e. wild or IMTA Gracilaria) and finally, the extraction method (i.e. MAE or TWE). In the case of AA, where two sets of operational conditions were considered for the MAE method, the abbreviations MAE\(_{Y}^{\text{opt}}\) and MAE\(_{GS}^{\text{opt}}\) will refer to, respectively, maximum yield and optimal GS. For instance, the alkali-treated agar (AA) obtained from farmed Gracilaria (IMTA) using MAE parameters which led to optimal GS (MAE\(_{GS}^{\text{opt}}\)) will be designated as AA\_IMTA\_MAE\(_{GS}^{\text{opt}}\). The table below lists the shorthand notation considered for each optimal extract. Two additional samples were considered throughout this project, a commercial agar (CA) used as reference (from Sigma-Aldrich) and an additional extract used to infer the effect of more draconian MAE conditions in the backbone of the native polysaccharides (NA\_IMTA\_MAE\(^{1}\)).
<table>
<thead>
<tr>
<th>Sample Id.</th>
<th>Description</th>
<th>Case Study / Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA_IMTA_MAE^{opt}</td>
<td>Native Agar from IMTA <em>Gracilaria</em> extracted using MAE^{opt}</td>
<td>Chapters I.3 and I.4</td>
</tr>
<tr>
<td>NA_IMTA_MAE^{t}</td>
<td>Native Agar from IMTA <em>Gracilaria</em> extracted using MAE^{t}</td>
<td>Chapter I.4</td>
</tr>
<tr>
<td>AA_IMTA_MAE_{Y}^{opt}</td>
<td>Alkali-treated Agar from IMTA <em>Gracilaria</em> extracted using MAE_{Y}^{opt}</td>
<td>Chapters I.3 and I.4</td>
</tr>
<tr>
<td>AA_IMTA_MAE_{GS}^{opt}</td>
<td>Alkali-treated Agar from IMTA <em>Gracilaria</em> extracted using MAE_{GS}^{opt}</td>
<td>Chapters I.3 and I.4</td>
</tr>
<tr>
<td>NA_Wild_MAE^{opt}</td>
<td>Native Agar from Wild <em>Gracilaria</em> extracted using MAE^{opt}</td>
<td>Chapters I.4 and I.6</td>
</tr>
<tr>
<td>AA_Wild_MAE_{Y}^{opt}</td>
<td>Alkali-treated Agar from Wild <em>Gracilaria</em> extracted using MAE_{Y}^{opt}</td>
<td>Chapters I.4 and I.6</td>
</tr>
<tr>
<td>AA_Wild_MAE_{GS}^{opt}</td>
<td>Alkali-treated Agar from Wild <em>Gracilaria</em> extracted using MAE_{GS}^{opt}</td>
<td>Chapters I.4, I.5 and I.6</td>
</tr>
<tr>
<td>NA_IMTA_TWE^{opt}</td>
<td>Native Agar from IMTA <em>Gracilaria</em> extracted using TWE^{opt}</td>
<td>Chapters I.4, II.3 and II.4</td>
</tr>
<tr>
<td>AA_IMTA_TWE^{opt}</td>
<td>Alkali-treated Agar from IMTA <em>Gracilaria</em> extracted using TWE^{opt}</td>
<td>Chapters I.4, II.3 and II.4</td>
</tr>
<tr>
<td>NA_Wild_TWE^{opt}</td>
<td>Native Agar from Wild <em>Gracilaria</em> extracted using TWE^{opt}</td>
<td>Chapters I.3 and I.4</td>
</tr>
<tr>
<td>AA_Wild_TWE^{opt}</td>
<td>Alkali-treated Agar from Wild <em>Gracilaria</em> extracted using TWE^{opt}</td>
<td>Chapters I.4 and I.5</td>
</tr>
<tr>
<td>CA</td>
<td>Commercial Agar from Sigma-Aldrich (A-7002)</td>
<td>Reference Sample</td>
</tr>
</tbody>
</table>
Preface

In a world facing great technological and industrial developments, the sustainability concept has caught the attention of researchers, companies, governmental authorities and consumers, worldwide. Four years ago, when I joined the doctoral program on Sustainable Chemistry of REQUIMTE, I could not imagine we would now be living in a time where the word ‘sustainability’ would cut across so many disciplines. Indeed, society in general has become aware of the need to build sustainable platforms of development, in order to ensure the future of mankind.

At R&D level, the search for greener processes as well as sustainable materials that can replace the conventionally used synthetic materials seems now a top priority, as we all recognize the need to use safer and more efficient technologies, processes and products. Most importantly, an optimal use of sustainable resources in efficiently interconnected cascade schemes to produce eco-friendly products, seems the key answer towards ‘true’ sustainability.

European priorities for the coming years are clearly stated in strategic plans; Horizon 2020 lists as main challenges (http://ec.europa.eu/research/horizon2020/index_en.cfm?pg=better-society):

- Health, demographic change and well-being;
- Food security, sustainable agriculture and forestry, marine and maritime and inland water research, and the bioeconomy;
- Secure, clean and efficient energy;
- Smart, green and integrated transport;
- Climate action, environment, resource efficiency and raw materials;
- Europe in a changing world - inclusive, innovative and reflective societies;
- Secure societies - protecting freedom and security of Europe and its citizens.

The present research project seems in perfect agreement with these simple guidelines. The idea was to build a sustainable platform as schemed below:
Goals

In this context, the three main goals set for this PhD project were:

i) The development and optimization of an alternative and more environmentally friendly agar extraction process, namely Microwave-Assisted Extraction (MAE);

ii) To explore the potential of *Gracilaria vermiculophylla*, a red algal species currently dominant in Ria de Aveiro, along the northwestern coast of Portugal, for the production of agars with commercial quality. Wild and cultivated *Gracilaria* in sustainable farming systems (Integrated-Multitrophic Aquaculture, IMTA) were explored with the intent of adding-value to a national underexploited resource. As invasive species may have a negative impact in the recipient community, this action could benefit the coastal ecosystems equilibrium. Moreover, the use of IMTA systems for the production of seaweeds with constant and reliable levels of quality for the transformation industries could help solving the problems commonly associated with wild populations (*i.e.* variability in abundance and quality due to seasonality or other physiological and environmental factors).

iii) To test the feasibility of the extracted agars for the production of sustainable materials in the form of films, hydrogels and fibers.
Overall, the goals set in the beginning of the PhD project were successfully accomplished. Obviously, and because science is not static, over the course of these 4 years, the project guidelines were readjusted and redefined whenever needed, much due to the new and exciting results that were constantly being attained. For that reason, it became impossible to use all the extracted agars in the several forms of tested materials discussed in Part II. Up to the moment of writing this PhD thesis, two of the considered applications, non-aqueous films and fibers (presented in Chapters II.5 and II.6, respectively), were only tested with commercial agar. However, the very promising results obtained in these studies definitely motivated us to continue this line of research and currently, there are ongoing investigations focusing on the use of our sustainably extracted agars for the fabrication of these novel materials.

Finally, it is our hope that this project will provide useful information and sustainable tools to be used by other researchers in the same or related fields.
This thesis is organized in four sections (General Introduction, Part I, Part II and Final Conclusions & Remarks) comprising a total of 14 chapters.

The current chapter, General Introduction, comprises the preface, main goals and motivations of the work. The organization of the thesis is also outlined.

The core of the thesis will comprise Part I and Part II, each one organized in several chapters.

Part I will concern the first stage of the PhD thesis, “Development and Optimization of an Alternative and Environmentally Friendly Agar Extraction Process”. Six chapters will form this section (Chapters I.1 to I.6):

In Chapter I.1, a detailed literature review concerning the background information related to the first stage of the project is given. Agar structure, properties, gelation mechanism and conventional extraction methodologies are some of the addressed topics. An overview of the hydrocolloids industry is tentatively given, with special attention being paid to the agar sector. The most relevant commercial agar applications, as well as the main manufacturers, are also listed. The main seaweed sources of agar are identified, as well as their cultivation and harvesting methods, with particular emphasis being given to the IMTA approach. The motivations behind the use of the red seaweed G. vermiculophylla, a national underexploited resource, as raw-material for this project are detailed. Finally, the principles of microwave heating and MAE technique are discussed in the light of other conventionally used (and less environmentally friendly) extraction methods. The applications of microwaves in the extraction of seaweed and/or seaweed components and the MAE scale-up are other relevant topics focused in this chapter.

Chapter I.2 will concern the experimental procedures and techniques adopted throughout the first stage of the PhD project. Due to the significant number of used techniques, special emphasis will be given to the experimental methods used on a more regular basis. In these cases, besides the description of the experimental procedure, the theoretical background of the technique and the basic operating principles of the instrumentation will also be presented. The seaweed sampling (wild and IMTA G. vermiculophylla) and collection are also explained in detail by clearly stating the differences between the different batches of seaweeds used to carry out the project. It is also included a list of the most relevant chemicals used during this stage.
Chapter I.3 details the optimization approach used to find the optimal MAE conditions. Two process optimizations were carried out for NA and AA using IMTA Gracilaria (1st batch) and the results are discussed throughout the chapter. As many factors can influence the characteristics of the extracted SP, RSM was applied to fit and exploit mathematical models representing the relationship between the responses (extraction yield, GS, $T_g$ and $T_m$, as well as, sulfate and LA contents) and input variables (extraction time, temperature, solvent volume and stirring speed).

In Chapter I.4, an extensive structural and physicochemical characterization of the extracted agars is presented. The optimal MAE conditions, for each type of agar, i.e. NA and AA, obtained in Chapter I.3, were applied to the 2nd batches of wild and IMTA seaweeds. For comparison purposes, agars from the same seaweeds were extracted using the TWE method according to previously optimized conditions. A commercial agar sample was also used as reference.

Throughout the chapter, we intend to study the influence of the i) extraction process and ii) seaweed growth conditions on the final properties of the extracted polysaccharides. Due to the large amount of data to analyze, the chapter was divided in two main sections, the first concerning the data discussion of agars from IMTA Gracilaria, and the second focusing on the SP from the wild biomass. The discussion concerning the IMTA biomass is divided by agar types, i.e. NA and AA, while the data collected for the wild Gracilaria is organized by extraction methods, i.e. MAE vs TWE.

All relevant parameters influencing the quality of the extracts were considered. The backbone structure of the SP, investigated through FTIR and NMR spectroscopy, is described in detail. Sulfate and sugar contents (including LA units), estimated by turbidimetry, colorimetry and/or liquid chromatography, are also presented. Other determined properties include GS (penetration tests), $M_w$ (SEC), intrinsic viscosities ($[\eta]$; viscometry), $T_g$ and $T_m$ (rheology). In order to keep the number of figures/tables to a reasonable level, NMR and FTIR spectra, as well as respective chemical shift assignments, will only be shown for representative samples.

A case study concerning a comparison between agars extracted using conventional (thermal heat; TWE) and non-conventional (microwaves; MAE) energy sources is presented in Chapter I.5. AA from wild Gracilaria were chosen as model samples. The viscoelastic behaviors of the SP were monitored through rheological measurements and compared with a commercial sample. AFM and cryoSEM techniques were used to image the molecular associations of MAE and TWE agars in solutions of, respectively, low and high polymer concentrations. The obtained results were related with relevant physicochemical properties of the SP.
Chapter I.6 encompasses a second case study comparing the behavior in dilute and concentrated aqueous media of agars but, now, focusing on the influence of the MAE conditions. Again, representative SP extracts were used (NA and AA from wild Gracilaria). The assemblies evolution upon concentration increase of NA and AA was investigated by imaging dilute (5, 10, 50 and 100 µg/mL) and concentrated (1.5% wt) aqueous media by means of AFM and cryoSEM, respectively. The obtained data were discussed in the light of the theoretical model proposed for agar gelation, as well as the nature of the polymeric chains (i.e. sulfate/LA ratio and \( M_w \)). Large deformation studies were performed on the equilibrated gels (i.e. concentrated regime) and correlated with the 3D polymeric networks observed by cryoSEM.

Part II, will concern the second stage of the PhD thesis, “Suitability of the extracted agars for the fabrication of sustainable materials”. Six chapters will form this section (Chapters II.1 to II.6):

The first chapter (Chapter II.1) will comprise an extensive literature review covering the most relevant forms of materials from seaweeds and SP, with particular emphasis being given to the forms explored throughout the project (i.e. films, hydrogels and fibers).

First, the concept of ‘bioplastic’ will be introduced and the placement of the bioplastic sector in the current global plastic scenario will be discussed. The main drivers behind the sector’s expected growth for the coming years, as well as current challenges, will also be addressed.

Moving forward in the chapter, the focus will turn on bioplastic from seaweeds and SP. The reasons why seaweeds are now seen as a potential sustainable resource for the sector will be debated. Other forms of materials from seaweeds and SP, production methodologies and strategies to improve the functional properties of the marine materials will also be detailed.

Another highlight of the chapter will be the properties evaluation of the materials where the importance of using standardized rules, norms and methodologies will be focused.

The description of the experimental techniques used to fabricate and characterize each agar-based material will be presented in several sub-sections throughout Chapter II.2. Albeit some techniques are common to the considered applications, part of the studies was developed in Portugal (aqueous-blended films, hydrogels) and part in the USA (non-aqueous films, fibers). For this reason, it becomes easier to adopt the suggested organization, i.e. by material’s form.
The first two materials to be considered were aqueous-blended films (Chapter II.3) and hydrogels (Chapter II.4) of agar and LBG. Mixtures of the gelling and non-gelling polysaccharides were prepared at different mass ratios and taken to assume the intended form of material. ‘Knife-coating’ technique was used to fabricate the films while the gels were formed upon cooling of the hot mixed aqueous systems. The potential synergistic interactions between both polysaccharides were investigated through rheological studies and by evaluating important functional properties of the produced materials. Two different kinds of agar were considered for these studies, one native and one alkali-modified, to check whether the strongest interactions with LBG were established between highly or poorly substituted agars. Due to the limited amounts of agar that could be extracted from the MAE apparatus we chose to use extracts from the TWE to find the optimal agar/LBG formulations.

With the intent of overcoming the brittle nature of agar aqueous films, a different approach was considered for the fabrication of agar materials in the film-form. This study will comprise Chapter II.5. To this end, we tested the suitability of two eutectic mixtures, ChCl/urea (DES-U) and ChCl/glycerol (DES-G) at 1:2 molar ratios, for the production of non-aqueous agar films by compression-molding. For this study, a commercial agar (CA) sample was used. The idea was to find the optimal CA/DES formulation and, then, apply it to the optimal agar extracts obtained in the 1st stage of the PhD project. As previously mentioned, this goal was not fully accomplished at the time of writing this thesis and it is still an ongoing research.

Chapter II.6 will focus on our attempts to produce agar fibers by the electrospinning technique using water and the DES, ChCl/Urea at 1:2 molar ratio (DES-U), as solvents. The addition of a co-blending polymer with known spinnability, such as PVA, was also explored, to improve the electrospinnability of agar pure solutions. The morphology of the electrospun materials was examined by SEM. To support the SEM data, a comprehensive study, concerning the rheological properties of the spinning solutions used to fabricate the fibers, was carried out.

The last chapter will outline the Final Conclusions and Remarks concerning the PhD project. The main conclusions gathered throughout the project and suggestions for future work will be discussed. The follow-up of MAE implementation at an industrial scale, in what agar is concerned, current market trends for marine based materials, as well as future prospects for the sector, close the discussion of the chapter.
PART I

DEVELOPMENT AND OPTIMIZATION OF AN ALTERNATIVE ENVIRONMENTALLY FRIENDLY AGAR EXTRACTION PROCESS.
I.1 LITERATURE REVIEW

CHAPTER I.1
I.1.1. Seaweeds: An Overview

Placed among the first signs of life on Earth, algae are one of the most fascinating living organisms. In the animal kingdom algae have a vital function promoting the equilibrium of marine ecosystems, serving as food, energy sources or even shelter for several marine species (http://www.teara.govt.nz/en/seaweed/page-1).

Algae biomass is also recognized to have fascinating properties to be used in clear benefit of mankind. A fair measure of algae tremendous success comes from a very rich composition, in components such as proteins, polysaccharides, lipids, polyphenols and many others (Rinaudo, 2008). These substances have been reported to have a variety of biological activities such as antitumor, antiviral, anticoagulant and antioxidant, just to name a few (Fitzgerald et al., 2012; Mohamed et al., 2012; Salvador et al., 2007; Toskas et al., 2011; Vishchuk et al., 2013; Wang et al., 2012; Yuan et al., 2005).

Seaweeds, also designated as macroalgae, distinguish from the micron-size blue-green algae not only by its greater dimensions but also for the distinct composition; while microalgae typically possess high lipid content, macroalgae are very rich in carbohydrates.

Based on pigmentation, seaweeds can be classified as red (Rhodophyceae), brown (Phaeophyceae) and green (Chlorophyceae) (Kadam et al., 2013). The size of red and green seaweeds goes from a few centimeters up to 1 meter long while brown seaweed species can reach dimensions as high as ~20 meters (McHugh, 2003).

The majority of the world’s seaweed supplies are consumed directly as food particularly in Pacific-Asian countries, e.g. Japan, China, Korea, Taiwan, Philippines, Vietnam and Indonesia, but also in Peru, the Canadian Maritimes, Scandinavia, Ireland, Wales, Scotland, and others (http://www.oilgae.com/ref/glos/seaweed.html). Most of algae carbohydrates are dietary fibers (i.e. insoluble carbohydrates not digested by the human gastrointestinal tract) and hence, can serve as very important food supplements. By moderating human appetite they can help to fight obesity (Mohamed et al., 2012). Other health benefits include the protection of the intestinal flora, reduction of the glycemic response (when ingested with high glycemic foods) or even the prevention of colon cancer (Dawczynski et al., 2007). Seaweeds are also a rich source of proteins. The content of essential amino-acids in some red seaweed species (e.g. Porphyra sp) can be as high as of soy or egg protein (Fleurence, 1999). Wakame produced from the brown seaweeds Undaria pinnatifida, and nori, obtained from the red seaweeds Porphyra, are very consumed by humans (Taboada et al., 2013). The latter is used to make sushi which has become a very popular food style worldwide.
The second most important use of seaweeds concerns the extraction of seaweed polysaccharides (SP). Agar, carrageenan and alginates are the most relevant SP isolated from seaweeds. These SP are also termed hydrocolloids due to their ability of forming a viscous (or gelled) solution when dissolved in hot water, even at very low polymer concentrations. This exquisite feature makes them suitable to be used as thickening, gelling and stabilizing agents in a wide array of applications such as food, biomedical, pharmaceutical and biotechnological. Alginate and carrageenan are also used as moisturizers in cosmetic products such as creams and lotions (McHugh, 2003).

The hydrocolloid industry has grown to become a well-established sector yet, older producers, with less efficient machineries and processes, struggle to maintain competitiveness in the current global economic scenario (Bixler & Porse, 2011).

SP have a linear backbone composed of sugar units conjugated to form complex structures. The –OH groups, predominant in the polymeric chains can establish strong intra- and intermolecular hydrogen bonds and are responsible for the excellent gelling and film-forming properties of SP. Their easy processability makes them even more attractive; hence, SP are used in several other forms besides gels; they can be processed as bioplastic films (aqueous-blended and melt-processed), beads, capsules and fibers (Rinaudo, 2008). The high fiber content of seaweeds makes them also very attractive as reinforcing and/or filler agents of composite materials (Chiellini et al., 2009; Cinelli et al., 2008).

Other typical carbohydrates of brown seaweeds include fucoidans, laminaran, cellulose and mannitol while compounds such as floridean starch, cellulose, xylan and mannann are commonly found in red seaweeds (Dawczynski et al., 2007). In recent years, some of these less exploited SP became focus of great attention due to their potential benefits for medical purposes. For instance, the anti-tumor activity of fucoidans from brown seaweeds was reported with success (Senthilkumar et al., 2013; Vishchuk et al., 2013). These polysaccharides have also shown anticoagulant, antithrombotic, antioxidant and anti-inflammatory properties (Balboa et al., 2013; Dore et al., 2013). The bioactivity of ulvan, named after the seaweed from which it can be isolated (green seaweed Ulvales), has been demonstrated in several studies (Toskas et al., 2011).

Another recent trend is to use marine algae for the biosynthesis of stable nanoparticles. For instance, the red seaweed Gelidiella acerosa successfully synthetized silver nanoparticles with antifungal properties while fucoidan has been used in a green route to fabricate gold nanoparticles (Asmathunisha & Kathiresan, 2013). These particles have shown great interest in fields such as biological, medical and electronic (Shukla et al., 2012).

SP have also been used as substitute of antibiotics often used to treat diseases in fish from aquaculture. The immunostimulant and prebiotic activity of alginate, carrageenan
and ulvan showed to benefit marine aquaculture when added to fish diets (Peso-Echarri et al., 2012).

Throughout the decades, the use of seaweeds to improve the water retention and plant growth of soils has been a common practice (McHugh, 2003). Seaweed fertilizers promote the growth of plants and roots development, enhance photosynthesis, strengthen stalks, and prevent plant diseases. It can also stimulate the development of fruits, ameliorate the soil texture and preservation of soil moisture (http://yssi.en.ecplaza.net/seaweed-fertilizer--30192-12172.html).

The ability to bind and concentrate heavy metal ions, makes seaweeds very interesting for wastewater treatments (Bhatnagar et al., 2012; Vilar et al., 2008; Vilar et al., 2009). To this end, Laminaria sp have shown to be one of the most promising.

II.1.2. Seaweeds: A Promising Resource for Sustainable Development

With this amazing potential, it is not at all surprising that the seaweed industry has grown to become a multi-billion dollar business (www.seaweedindustry.com). If in the beginning, it mainly relied on naturally grown (i.e. wild) biomass currently, it can also benefit from a tremendous amount of cultivated (i.e. farmed) seaweeds generated by the outstanding growth of aquaculture in the last decades (FAO Fisheries and Aquaculture Department, 2009). While brown seaweeds are too costly to be farmed, red seaweeds have proved to be excellent candidates to this end; cost-effective cultivations of red seaweeds enabled the expansion of Agar and Carrageenan industries (McHugh, 2003).

Another huge breakthrough for the algae industry came recently, with the incentives given to the bioenergetic sector. Currently, the biofuels produced at industrial scale come mainly from food crops (e.g. corn, sugar cane). However, to achieve the targets set out for the biorefinery sector and with the increasing demand for food, non-food crops are getting a lot of attention (Wei et al., 2013). Early this year, the Obama administration has qualified algae as “qualified feedstock” and attributed a tax credit to algae fuels. This measure is expected to have a tremendous impact in other industries build up from the exploitation of the generated algae wastes (Williams & Hillmyer, 2008) (http://www.algaeindustrymagazine.com/the-2013-playing-field-levels-for-algae/). An efficient use of resources supported by economic platforms from governmental authorities can outline a sustainable implementation of seaweeds as resource for biorefineries (Dave et al., 2013). To this end, seaweeds are currently being explored for the biogas production where rich
carbohydrate contents, and low lignocellulose fractions can be particularly advantageous (Dave et al., 2013; Hinks et al., 2013; Jung et al., 2013). Very recently, *Laminaria hyperborea* was efficiently used for the first time as sole feedstock for biogas production through anaerobic fermentation (Hinks et al., 2013) while *Gracilaria vermiculophylla* (also termed *G. verrucosa*) proved its potential for bioethanol production (Kumar et al., 2013).

Whether in wild, cultivated or as an industrial by-product, seaweeds are unquestionably, a sustainable resource with tremendous potential of exploitation.

I.1.3. Seaweed Polysaccharides (SP)

Perhaps more than any other components, agar, carrageenan and alginate have been responsible for the widespread use of seaweeds (Matsuhashi, 1990). These polysaccharides can be easily isolated from various red (agars, carrageenans) and brown (alginates) seaweed species (Table I.1.1). Agar and carrageenan are water-soluble sulfated galactans differing from one another mainly by the sulfate content (higher in carrageenan; ~22 to 35% against ~1-5% in agar) and the configuration of the anhydride galactose units. While in agar this monomer assumes the L- form (3,6-anhydro-α-L-galactose, *i.e.* LA) in carrageenan the D-configuration is observed (3,6-anhydro-α-D-galactose, *i.e.* DA). The three most important commercial carrageenans can be identified according to their composition: κ (kappa-) and ι (iota-), with lower sulfate ester contents, are the gelling forms of the polymer while non-gelling λ (lambda-) carrageenan exhibits more sulfate groups. Biological precursors of main carrageenans can also be found. In its natural form, carrageenan has truly a complex nature; it often exhibits a hybrid structure composed mainly by two types of carrageenan or even by a mixture of carrageenan and agar (van de Velde, 2008).

Alginate comprises a mixture of Ca, Mg and Na salts of alginic acid, alginic acid derivatives and alginic acid itself (1,4-linked β-D-mannuronic acid and α-L-guluronic acid units in GG, MM and MG domains). Only the Na form of the alginic acid is soluble in water. Hence, during the extraction process the other forms of salts are converted into the soluble form and subsequently, recovered from the aqueous media.

Agar is the SP with greatest gelling capability, being able to form firm gels even without the addition of other components (*e.g.* salts). Detailed information concerning this SP will be presented in the following sections.

Agar is mainly extracted from *Gelidium* sp and *Gracilaria* sp while *Euchema* sp and *Gigartina* sp are the most popular sources of carrageenophytes. Good sources of alginate include the genus *Laminaria*, *Ascophyllum* and *Durvillaea*. In wild, SP can be found in the
plant cell-walls where they promote flexibility and mobility of seaweeds for an easy adaptation to different marine environments (Matsuhashi, 1990).

<table>
<thead>
<tr>
<th>COMPOSITION</th>
<th>SEAWEED SOURCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>Gelidium,</td>
</tr>
<tr>
<td></td>
<td>Gracilaria,</td>
</tr>
<tr>
<td></td>
<td>Pterocladia</td>
</tr>
<tr>
<td>Alginate</td>
<td>Laminaria,</td>
</tr>
<tr>
<td></td>
<td>Ascophyllum,</td>
</tr>
<tr>
<td></td>
<td>Durvillaeae</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>Kappaphycus alvareizii (Euchema cottonii),</td>
</tr>
<tr>
<td></td>
<td>Eucheuma denticulatum (Euchema Spinosum),</td>
</tr>
<tr>
<td></td>
<td>Gigartina,</td>
</tr>
<tr>
<td></td>
<td>Chondrus crispus</td>
</tr>
<tr>
<td>Kappa-</td>
<td>4-sulfated on the G units (G4S-DA)</td>
</tr>
<tr>
<td>Iota-</td>
<td>4-sulfated on the G units and 2-sulfated in DA (G4S-DA2S) units</td>
</tr>
<tr>
<td>Lambda-</td>
<td>2-sulfated G and 2,6-disulfated G backbone, G2S-D2S,6S (residual or no D-LA content and highest percentage of sulfate groups)</td>
</tr>
</tbody>
</table>

I.1.3.1. Agar

Agar is the name given to a complex mixture of water-soluble and gel-forming polysaccharides, found in selected marine red seaweeds. It was the first hydrocolloid to be found when in the 17th century, a Japanese inn keeper named Minora Tarazaemon, noticed that some leftovers of a seaweed soup gelled, after being thrown away during a cold winter night (Matsuhashi, 1990).

Probably the most common way to describe agar structure is to consider the polymer divided in two fractions: agarose, the unbranched gelling segment of the polymer, and
Agaropectin, comprising all substituted and non-gelling units (Matsuhashi, 1990). Agarose is considered the ideal structure since it accounts for the polymer gelling ability and is formed by 3-linked β-D-galactose (G) and 4-linked 3,6-anhydro-α-L-galactose (LA) repeating units. Quite often, the anhydride bridge of the LA units is absent and several substituent groups such as sulfate esters, methyl ethers, pyruvate acid ketals and more rarely, other sugar residues, occur throughout the polymer skeleton, Fig. I.1.1. This will globally comprise the non-gelling fraction of agar (i.e. agaropectin). For instance, common methylations occur at C6 of G and at C2 of LA units.

Due to its complex nature, structural studies of agar have been made by fractionation, followed by chemical and enzymatic hydrolysis and subsequently, by spectroscopic methods (Armisen & Galatas, 1987). Several methods have been proposed to separate agarose from agaropectin, although none of them is able to yield agarose totally free of electronegative charged groups. For that reason, successive fractionation methods are often employed and chemical modifications are needed to achieve agarose of commercial grade. The most popular methods used to prepare agarose include the acetylation method of Araki (Araki, 1937), the quaternary ammonium precipitation proposed by Hjerten (Hjerten, 1962; Hjerten, 1971) or the classical method of Polson based on the use of polyethylene glycol (Russell et al., 1964). After hydrolysis, agaropectin has shown sulfate and pyruvate residues as well as small to moderate amounts of LA units (Armisen & Galatas, 1987). The most commonly found sulfate units are L-galactose-6-sulfate (L6S) and D-galactose-4-sulfate (G4S) while the presence of pyruvic acid groups were clearly identified in 4,6-O-(1-carboxyethylidene)-D-galactose residues.

![Agar structure](image)

**Fig. I.1.1 – Agar structure.**
A modified nomenclature proposed by Knutsen et al. (Knutsen et al., 1994), considers agar to be formed by two main polysaccharides, agarose and agaran. When the anhydride bridge is absent, the polysaccharide is termed agaran and is composed by 3-linked β-D-galactose (G) and 4-linked α-L-galactose (L) residues. Here as well, the structural regularity of agar chains may be masked in numerous ways due to the presence of substituent groups (Rodriguez et al., 2009; Sousa et al., 2013; Usov, 1998).

Regardless the choice of nomenclature, different arrangements and relative contents of monomers will result in agars with distinct properties. The ability to form thermo-reversible physical hydrogels with large thermal hysteresis (i.e. difference between melting and gelling points) (Boral et al., 2008; Clark & Ross-Murphy, 1987; Lahaye & Rochas, 1991; Nijenhuis, 1997) makes agar widely used in several industrial applications such as food, pharmaceutical, biotechnological and biomedical.

Gel strength (GS), is often used as a reference parameter for agar quality and can be defined as the maximum stress required to break the gel surface (Matuhashi, 1990). The concept of GS was first presented by Tanii in 1957 and has become a relative standardized test for industries. GS is typically measured for standard gels with 1.5% wt agar concentration according to simple procedures the measure the load (g/cm²) that causes the gel to break.

### I.1.3.1.1. Agar Gelation

Agar hydrogels form upon cooling of a homogeneous solution prepared in boiling water. The complexity underlying agar gelation made it a widely discussed subject among researchers in the past years (Arnott et al., 1974; Clark & Ross-Murphy, 1987; Djabourov et al., 1989; Foord & Atkins, 1989; Manno et al., 1999; Nijenhuis, 1997; Xiong et al., 2005). At high temperatures, agar molecules are believed to exist in a random coil state and move towards an ordered conformation when cooled to temperatures below the polymer’s gelation point (~30-40 °C) (i.e. coil-to-helix transition or 2n(coil) ↔ n(double helix)). Intensive interhelical association then takes place (i.e. helix-to-helix aggregation or n(double helix) ↔ double helix assemblies; Fig. I.1.2) until the system approaches the global minimum of free energy (Aymard et al., 2001; Ayyad et al., 2010; Djabourov et al., 1989; Labropoulos et al., 2002; Normand et al., 2000). Agar’s route to gelation will be markedly influenced by its physicochemical properties (Nijenhuis, 1997). While the anhydride bridge of the LA residues will confer high stability to the polymer and enhance its capability of forming the double helices, the sulfate groups will introduce kinks during the intermolecular associations leading to softer hydrogels (Arnott et al., 1974; Labropoulos et al., 2002). Also, the molecular mass
(Nijenhuis, 1997; Normand et al., 2000; Rodriguez et al., 2009) of agars is reported as an influential parameter in the gelation process.

![Proposed model for agarose network formation considering intensive interhelical association (Source: Clark & Ross-Murphy, 1987).](image)

**I.1.3.1.2. Sources of Agar**

As previously mentioned, *Gracilaria* and *Gelidium* genera constitute the main agar sources. *Gracilaria* genus comprises the greatest number of species in *Gracilariaceae* (Rhodophyta). Because of its favorable cultivation and wide availability, *Gracilaria* sp started to be increasingly explored when *Gelidium* sp became unable to fully supply the hydrocolloid industry. *Gracilaria* sp can grow in cold (e.g. Canada, Chile), temperate (e.g. Spain, Portugal, Morocco) and tropical (e.g. Indonesia, Philippines) environments (McHugh, 1991; McHugh, 2003). The main focus of interest of *Gracilaria* sp concerns agar extraction although part is also consumed directly as food. Although *Gelidium* extracted agar has typically better quality (higher GS), the inclusion of an alkaline treatment prior to the extraction to enhance the gelling properties, allows *Gracilaria* genus to be currently the major agar source worldwide (Freile-Pelegrin & Murano, 2005; Marinho-Soriano, 2001; Marinho-Soriano et al., 2001; Pereira-Pacheco et al., 2007). Nonetheless, both type of seaweeds serve a specific niche of applications and for that reason, are equally important; while *Gelidium* genus is particularly interesting for the production of bacteriological and pharmaceutical agar and agarose, agar from *Gracilaria* sp is most relevant for the food sector. This topic will be discussed in more detail in the following sections.

*Gelidium* is a small and slow-growing plant and for that reason is mostly reported as economically unprofitable for aquaculture (McHugh, 2003). Hence, its use as raw-material for agar industry comes from wild capture mainly from countries such as Spain, Japan, Korea,
Indonesia, Morocco, Mexico and others (McHugh, 2003). Meanwhile, the most affected countries by the recent Fukushima nuclear disaster could have increased their need for external seaweed supplies.

*Gracilaria* genera, in turn, have been successfully cultivated in tanks (Buschmann et al., 1994; Buschmann et al., 1996; Martinez-Aragon et al., 2002), offshore systems (Abreu et al., 2009; Buschmann et al., 2008) and in ponds (Jones et al., 2002; Marinho-Soriano et al., 2002). In the beginning, *Gracilaria* supplies mainly relied in wild capture from countries such as Chile, Argentina and Indonesia, yet the proliferation of farming sites as well as the development of cultivation methods in countries such as China, Korea or Philippines, made possible for *Gracilaria* to be more readily available for industries (McHugh, 2003).

Major *Gracilaria* producers can be found in the Asian-Pacific and American regions. A good example is the Chinese company Hainan Wanzhou Agar Co. Ltd whose cultivation base can supply 400 MT/year of *Gracilaria* seaweeds. However, China producers struggle to provide raw-materials that can deliver high-quality hydrocolloids (Bixler & Porse, 2011). Numerous small-scale *Gracilaria* suppliers can be found by performing a quick on-line search which difficult an accurate estimation of this seaweed production worldwide. Most of them come from countries such as Vietnam, Indonesia or China.

Pond cultivation is less laborious than other methods (e.g. rope/net farming) yet the final agar typically presents lower gelling capability. *Gracilaria* sp grown in tanks have been widely studied but the high costs and laborious nature of the method, hinders its implementation at commercial scale.

Chilean *Gracilaria* is particularly known for providing high-quality agars and for that reason the country has become a top supplier of these wild seaweeds worldwide (McHugh, 2003) (Table I.1.2). Countries such as China, who struggle to produce high-grade hydrocolloids from their own seaweed supplies, are the main interested in Chilean resources. During this year, the merging of the Chilean company Productoras de Algas Marinas Ltda (Prodalmar) and the Chinese algae group Qingdao Mingyue Seaweed Co. Ltd, world leader in alginate production, shows how strategic partnerships established between different regions of the globe can contribute for the development of the hydrocolloid sector (http://www.algaeindustry.com/chinese-chilean-algae-company-launched/).

SeaCop Pacific Earl and Anivien Manufactura & Exportacion y Compania Ltda are two other examples of Chilean *Gracilaria* producers. In Argentina, Soriano SA has currently operating centers for seaweed collection and production. However, smaller production volumes or lack of official data could explain why it’s not accounted for in FAO 2010 data displayed in Table I.1.2.
I.1.4. Integrated Multitrophic Aquaculture (IMTA)

Aquaculture is one of the fastest expanding agricultural industries in the world with a three times faster sector growth compared with terrestrial farm production of animal meat (FAO Fisheries and Aquaculture Department, 2009). As main fishing areas have reached their full potential, and with the expected increase of the global population, the need for more food that can ensure human life quality will become a major issue. In parallel, a rational use of resources such as land and freshwater will limit the agriculture expansion. Hence, the demand for aquatic food seems most likely to occur but with wild living resources provided by capture fisheries becoming clearly insufficient, aquaculture could be the obvious solution (Diana et al., 2013).

The world seaweed production comes almost exclusively from aquaculture reaching roughly 20 million tonnes in 2010 according to FAO statistics (Table I.1.2). This practice has allowed access to some seaweed species that otherwise wouldn’t be readily available for human consumption due to its high demand (McHugh, 1991). Europe however, doesn’t seem to follow the global trend and only a residual amount of seaweed supplies comes from aquaculture (<0.3% of the total production in 2010; Table I.1.3). France, Ireland and Spain were the only European contributors to these digits. Portugal only registered wild capture (~500 metric tons; Table I.1.3). Almost 60% of the global farmed marine plant production came from China with countries like Indonesia and Philippines also presenting high production levels.

Despite the promising scenario for the future of aquaculture, economic and ecological aspects must be taken into consideration. For instance, the economic viability of seaweed farming is always dependent on several factors such as costs of harvesting, drying, transportation, water supply and others (McHugh, 1991).
Table I.1.2 - World Seaweed and other Aquatic Plants Production by Capture (wild) and Aquaculture in 2010 according to FAO (data shown in metric ton (mt), and wet-weight basis; Source: ftp://ftp.fao.org/STAT/summary/a-0a.pdf).

<table>
<thead>
<tr>
<th>Country</th>
<th>Capture (mt)</th>
<th>Aquaculture (mt)</th>
<th>Total (mt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Australia</td>
<td>1923F</td>
<td>-</td>
<td>1923F</td>
</tr>
<tr>
<td>Brazil</td>
<td>-</td>
<td>730</td>
<td>730</td>
</tr>
<tr>
<td>Canada</td>
<td>37 632</td>
<td>-</td>
<td>37 632</td>
</tr>
<tr>
<td>Chile</td>
<td>368 580</td>
<td>12 179</td>
<td>380 759</td>
</tr>
<tr>
<td>China</td>
<td>246 620</td>
<td>11,092 270F</td>
<td>11,338 890F</td>
</tr>
<tr>
<td>China, H. Kong</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>China, Macau</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>China, Taiwan</td>
<td>106</td>
<td>4888</td>
<td>4994</td>
</tr>
<tr>
<td>Indonesia</td>
<td>2697</td>
<td>3,915 017</td>
<td>3,917 714</td>
</tr>
<tr>
<td>Japan</td>
<td>96 600F</td>
<td>432 796</td>
<td>529 396F</td>
</tr>
<tr>
<td>Korea D P Rp</td>
<td>-</td>
<td>444 300</td>
<td>444 300</td>
</tr>
<tr>
<td>Korea Rep</td>
<td>13 043</td>
<td>901 672</td>
<td>914 715</td>
</tr>
<tr>
<td>Malasya</td>
<td>-</td>
<td>207 892F</td>
<td>207 892F</td>
</tr>
<tr>
<td>Morocco</td>
<td>7405</td>
<td>-</td>
<td>7405</td>
</tr>
<tr>
<td>Peru</td>
<td>4368</td>
<td>0</td>
<td>4368</td>
</tr>
<tr>
<td>Philippines</td>
<td>473</td>
<td>1,801 272</td>
<td>1,801 745</td>
</tr>
<tr>
<td>Russian Fed</td>
<td>5917</td>
<td>614</td>
<td>6531</td>
</tr>
<tr>
<td>USA</td>
<td>9027</td>
<td>-</td>
<td>9027</td>
</tr>
<tr>
<td>Vietnam</td>
<td>-</td>
<td>35 000F</td>
<td>35 000F</td>
</tr>
<tr>
<td>Zanzibar</td>
<td>-</td>
<td>125 157</td>
<td>125 157</td>
</tr>
<tr>
<td>World (1)</td>
<td>885 650</td>
<td>19,007 053</td>
<td>19,892 703</td>
</tr>
</tbody>
</table>

1 World data comprises other countries not represented in the Table; F - FAO estimate from available sources of information or calculation base on specific assumptions.
Most importantly, the massive production of aqua feed resources has some negative impacts caused by intensive nutrients (nitrogen, N, and phosphorus, P) released to the environment due to animal excretion. This release may cause environmental and socio-economic problems, compromising aquaculture activity itself (Matos et al., 2006).

In this context, integrated multitrophic aquaculture (IMTA), which promotes economic and environmental sustainability, is rising. In this farming approach, seaweeds and other extractive organisms convert dissolved nutrients produced by fed aquaculture (e.g. finfish and shrimp), into additional crops (Abreu et al., 2009). Autotrophic plants like seaweeds, work as biofilters, using solar energy and the excess nutrients to photosyntethize new biomass, effectively stripping nutrients from aquaculture effluent prior to its release to the environment (Neori et al., 2004). From an economic point of view, the possibility to add value to waste nutrients such as N and P by producing new biomass is seen with great interest (Wei et al., 2013). However, this should be associated with high productivities to ensure profitability and hence, interest from stakeholders. A schematic representation of IMTA approach is given in Fig. I.1.3.
From a sustainable perspective, the use of seaweeds and/or seaweed wastes in integrated production cascade schemes comprising different industrial segments, will demand a regular supply of feedstocks with controlled properties. Indeed, it can be challenging to obtain regular feedstocks from wild seaweeds due to physiological and environmental aspects. In this regard, aquacultured biomass can open a straightforward access to products of controlled quality.

In Portugal, the pioneer company, Algaplus, is exploring the IMTA approach. This encouraging national example will be discussed in the next section. Currently, the top five genera in aquaculture production are *Laminaria*, *Undaria*, *Porphyra*, *Euchema*, and *Gracilaria* (76% of the total macroalgae production) (Wei et al., 2013).

### I.1.5. *Gracilaria vermiculophylla*: a National Underexploited Resource

Located in the Mediterranean-Atlantic region, Portugal presents an extensive coastal area formed by rocks, sandy beaches and surrounded by temperate water, ideal for the development of a diverse marine flora. The early references to Portuguese algal resources concern the agarophytes *Gelidium corneum* (formerly *G. sesquipedale*) and *Pterocladiella capillacea*, initially explored by the agar industry.
Currently, the central and northern shores are dominated by native seaweed species such as *Himanthalia elongata*, *Gelidium cornum*, *Bifurcaria bifurcata*, *Chondrus crispus*, *Mastocarpus stellatus*, *Calliblepharis jubata*, *Gigartina pistillata*, *Chondracanthus acicularis*, *Osmundea pinnatifida*, *Gelidium pulchellum* and *Pterosiphonia complanata* (Pereira & van de Velde, 2011).

*Gracilaria vermiculophylla* (Gracilariales, Rhodophyta) is a red algal species, originally described from Japan and recently established in European waters as an invasive species. One possible strategy to mitigate the impact of these invasive seaweeds, that threatens the ecological balance of coastal ecosystems, is its mechanical removal (harvesting) which would yield a tremendous biomass that can be utilized for various applications.

The great similarities among *Gracilaria* sp often leads to misidentifications. *Gracilaria vermiculophylla* has been termed *Gracilaria verrucosa* and *Gracilaria bursa-pastoris*. New populations of *G. vermiculophylla* are established in Ria de Aveiro, northwestern Portugal (40°38’N, 8°43’W). Initially, *G. vermiculophylla* from Portuguese waters had an important function as soil conditioner but now it is a national unexploited resource (Abreu, 2010).

The IMTA of *G. vermiculophylla* was tested with success by Maria H. Abreu and co-workers from CIIMAR during her PhD thesis (Abreu, 2010), in Coelho & Castro Aquaculture, Rio Alto, Portugal. This seaweed was successfully cultivated in IMTA tanks serving as biofilter for turbot (*Scophthalmus rhombus*), sea bass (*Dicentrarchus labrax* Linnaeus) and Senegalese sole juveniles (*Solea senegalensis* Kaup) fish effluents. CIIMAR was the seaweed supplier of our project.

*G. vermiculophylla* is widespread in marine environments throughout the globe and so, its potential is being explored by researchers from different backgrounds and regions of the globe. For instance, *G. vermiculophylla* grown along Russian coasts have also shown great capacity to effectively remove ammonium and phosphates in effluents from bivalves’ aquaculture (Skriptsova & Miroshnikova, 2011).

This species has also been presented has a potential source of fatty acids, a benefic compound to human health. Over 50% of the fatty acid content of *G. vermiculophylla* is believed to be a prostaglandin precursor, arachidonic acid, 20:4n-6 (Illijas et al., 2008). The efficiency of *G. vermiculophylla* from Japanese waters to byosyntethize prostaglandin enzyme complex was not affected by its growth environment (wild or after grown in mariculture) (Imbs et al., 2012). The potential of *G. vermiculophylla* in obesity prevention has been suggested by Kim and co-workers (Kim et al., 2012).

Very recently, *G. vermiculophylla* was shown to have great potential for bioethanol production and agar extraction (Kumar et al., 2013). This work is quite interesting since it
successfully explores the use of this seaweed in cascade production schemes hence, promoting sustainability.

It is worth pointing out that the excellent study carried out by our CIIMAR co-workers led to the creation of the pioneer Portuguese company, AlgaPlus, dedicated to the production of marine macroalgae and its application as biofilters in IMTA systems. AlgaPlus is located in Aveiro and more information related to this pioneer project can be found in the company’s website, www.algaplus.pt.

I.1.6. Agar Traditional Water Extraction (TWE)

Agar is traditionally hot-extracted with water (TWE) for several hours using thermal heat. The general process could be described as follows: dried seaweeds (previously washed) are heated in boiling water for several hours. After being cooked, the extract (i.e. agar and water) is separated from the seaweed residues through hot-filtration. The filtrate is left to cool forming a gel composed mostly by water (with salts and soluble carbohydrates) and only a small agar fraction (~1% wt). In order to remove color as well as other components the gel is bleached and subsequently, washed. Then, the solvent needs to be removed whether by freeze/thawing or by pressure/syneresis techniques. Finally, the product is dried and milled (Armisen & Galatas, 1987; McHugh, 2003).

*Gelidium* cell walls are harder to break and for that reason the extraction is enhanced if performed under pressure. Another common strategy to overcome the rigid nature of *Gelidium* is to treat the seaweeds with a very weak acidic solution. Typical temperature and extraction times for *Gelidium* sp fall in the ranges, 105-120 °C and 2-4 hours.

When using *Gracilaria* seaweeds and depending on the final application, two types of agars can be extracted (Bulone et al., 1999). If soft-texture products are desired, native agars (NA) with low gelling character (i.e. GS~30-200 g/cm²) are the most appropriate (Pereira-Pacheco et al., 2007). Contrarily, if hard-texture modifiers are needed, alkali-modified agars (AA) should be considered. Superior-grade agars, such as AA (i.e. GS>700 g/cm² in a 1.5% wt solution) (Pereira-Pacheco et al., 2007), are obtained by the inclusion of an alkaline step prior to the extraction process which will promote the conversion of α-L-galactose 6-sulfate (L6S) in LA units, responsible for the polymers' gelling ability, Fig. 1.I.4 (Lahaye & Rochas, 1991; Murano, 1995).

![Conversion of L6S in LA units during the alkaline treatment.](image)
For the alkaline treatment, the seaweeds are heated in an alkaline solution (typically, sodium hydroxide with 2-7% wt concentration at 85-95 °C for 1-3.5 h). Subsequently, the seaweeds are washed to remove the alkaline solution and frequently, a neutralization step is performed to eliminate possible alkaline residues (Arvizu-Higuera et al., 2008; Villanueva et al., 2010). *Gracilaria* sp, with softer talus need less severe extraction conditions (typically, 95-100 °C for 2-4 hours) (McHugh, 2003).

At an industrial scale, the extraction of agar from seaweeds is performed according to well-established methodologies, although some efforts have been made in order to make the process more energy-efficient. Currently, agar production is mostly performed using high-pressure membrane presses to separate agar from the extraction-water in order to reduce the energy costs associated with the refrigeration step of freeze/thawing technique (Bixler & Porse, 2011; McHugh, 2003). Nonetheless, the process is still clearly very time and solvent consuming. Agar extraction from *Gracilaria* seaweeds implies the use of even greater solvent amounts than *Gelidium* sp. Therefore, recycling of water is necessary, which restricts the factory location (McHugh, 2003).

In the case of Iberagar, the only company engaged in agar production located in Portugal, the extraction of agar from *Gelidium corneum* takes 6h plus two consecutive re-extractions of 8 and 12h (Vilar, 2006). The TWE of agar from wild *G. vermiculophylla* has been extensively documented in the literature (Arvizu-Higuera et al., 2008; Mollet et al., 1998; Orduna-Rojas et al., 2008; Villanueva et al., 2010).

### I.1.7. Agar Industry

In a recent review, Bixler and Porse gave an updated overview of the hydrocolloid industry (Bixler & Porse, 2011). The data presented by the authors, describe a reasonable evolution in agar production volumes over the last decade especially considering the global economic crisis. Asian-Pacific emerging economies, were clearly the driving force behind this positive trend with their productivity levels being significantly reinforced in that time frame; from 3,000 metric tons registered in 1999, went up to 5,300 metric tons in 2009. Countries such as Chile, Brazil and Argentina are responsible for the positive result in agar production volumes in the American continent. The Argentinean firm Soriano SA (http://www.soriano-sa.com.ar/), Agargel (http://www.agargel.com.br/empresa.html) from Brazil or the Chilean companies Productoras de Algas Marinas Ltda (Prodalmar) and Proagar S.A. (http://www.proagar.cl/home_en.html) were good contributors for these numbers.

In clear contrast, the European agar production volumes had a 30% decay in 10 years, reaching 700 metric tons by the end of 2009. These data reflected by one hand, the
extinction of several small agar factories with outdated machineries and inefficient processes in countries such as Spain, Portugal or Italy. On the other hand, new companies with optimized operation lines were born in countries such as China, Indonesia and Chile. Also, some European producers chose to open new facilities or move their operating lines to other regions with cheaper cost-labor and advantageous geographical positioning (closer to the seaweed suppliers). For instance, the Italian company B&V, with 40 year-old experience in the production of agar and other hydrocolloids, is currently operating in Africa and Asia (http://www.agar.com/old/history.html) while, Hispanagar, other major player in the sector, has opened new facilities in China (Bixler & Porse, 2011).

Programs engaged on bridging the gap between Asian-Pacific and European countries have also benefited the agar productive sector. Despite the adverse european conjecture, Agarindo Bogatama, a major player in the agar producing sector, saw its sales to European markets significantly increase (8%), after participating in the European Food Ingredients trade fairs in 2011 (http://www.s-ge.com/switzerland/import/en/content/new-markets-agarindo-bogatama). This Indonesian company and Algas Marinas from Chile account for roughly 40% of the total agar production worldwide (Bixler & Porse, 2011). Another leader in agar manufacture is the Chinese firm Hainan Wanzhou Agar Co. Ltd with an annual production of 1,000 tons for both internal and external markets (http://www.21food.com/showroom/164412/aboutus/Hainan-Wanzhou-Agar-Co.,-Ltd..html). Once leader in agar production, Japan is currently preferring to import agar from other countries due to economic and environmental aspects.

In terms of sales volume, agar productive sector experienced a ~35% growth in a 10-year time frame, representing the smallest hydrocolloid sector (Bixler & Porse, 2011). Despite, being a well-established industry, agar is less produced worldwide due to its higher cost when compared with similar hydrocolloids. In terms of price fluctuations, this SP was also the most instable. In 2009, the total volume of sales for the hydrocolloid industry was estimated around 86,100 metric tons reflecting 1,018 million US$ in annual sales (~63% growth in ten years). Agar accounted for 9,600 tons (173 million US$), alginates for 26,500 tons (318 million US$) while the largest contribution came from carrageenan (50,000 tons; 527 million US$; (Bixler & Porse, 2011)).

Currently, agar productive sector is not operating at its full capacity. The 12,500 metric tons produced worldwide in 2009, accounts for roughly 80% of plant utilization which is clearly below the desired by companies. To this end, more efficient processes and technologies would clearly benefit the sector’s sustainability.
I.1.7.1. Agar Industry in Portugal

In the early 1940s, the Asian interest for agar led to the search for agar-producing seaweeds (i.e. agarophytes) worldwide. *Gelidium corneum* started to be collected along Portuguese waters for exportation up to the year 1947, when the first Portuguese agar producing company started to function. The great abundance of *Pterocladia capillacea* (formerly *Pterocladiella capillacea*), another agarophyte similar to *Gelidium* sp from Azores, further foster the development of the Portuguese agar industrial sector. By 1971, there were six operating lines of agar production, two of them in Azores. Currently, only one national company continues active; Iberagar is a Portuguese leading company engaged in the production and distribution of hydrocolloids ([http://www.iberagar.com/](http://www.iberagar.com/)). Iberagar resulted from the merging of two companies, Biomar and AGC, shortly after World War II. After acquiring the Japanese company, Unialgas, in 1970, Iberagar transferred its facilities to Barreiro (close to the Portuguese capital, Lisbon) where is still currently operating. The company’s unique process, particularly during the freezing stage, allows the recovery of agar with very high purity, widely recognized at global scale.

During the 90s, Iberagar started to import *Gelidium corneum* from Morocco as well as *Pterocladiida* from Azores to face the lower availability of *Gelidium* seaweeds in national waters. At this time, the company was readjusting to lower market demands, producing around 160 tons in the end of the decade, in opposition to the 250-300 tons fabricated during the 80s ([Melo, 1998](http://www.iberagar.com/food.html)). Currently, Iberagar exports most of its agar production, mainly to asian countries where the demand for this hydrocolloid is much more significant.

I.1.7.2. Agar Applications

While alginate and carrageenan are almost exclusively sold as powders, agar can be sold in other forms such as strips or squares ([Bixler & Porse, 2011](http://www.iberagar.com/); [McHugh, 2003](http://www.iberagar.com/)). Commercial agar is sold in different grades, meeting the specific needs of different types of applications (Table I.1.4).

The interest of agar as thickening and gelling agent for food products relies on its remarkable gelling power even at very low polymer concentration, as well as odorless and colorless nature. Unlike other food hydrocolloids such as carrageenan and pectin, agar does not require additives (such as salts) to form gels. Hence, it has much less ash content than other gelling polysaccharides ([http://www.iberagar.com/food.html](http://www.iberagar.com/)).

When mixed with other gums, agar can create interesting textures with various mouth feel to the consumer. For instance, agar and the non-gelling plant polysaccharide locust bean
gum (LBG) can be mixed seeking to combine the properties of each individual component to fabricate mixed systems with improved and complex features. The production of agar/LBG hydrogels will be addressed in detail in Chapter II.4.

Food-grade agar is used as ingredient (E-406) in a wide array of products. It can be used in confectionary, bakery and pastry, dairy products and canned foods. Several examples of these applications are listed in Table I.1.4. Within the food segment, agars with distinct properties can be produced in order to meet specific applications. Gold Agar from Hispanagar is produced from Gelidium seaweeds while Agarite™ is obtained from Gracilaria biomass. While the former presents a low gelling temperature, which is particularly suitable for applications where the solution must be kept liquid for a longer time period, the latter has excellent compatibility with high sugar contents and presents higher gelling temperature. Hence, Agarite™ is used in confectionery, bakery, icings and others where a faster gelling time is preferred. Agar’s remarkably high content of dietary fibers makes it very attractive for low-calories food products or as meat substitute in healthier style foods such as vegetarian and vegan.

For biotechnological applications, agars from Gelidium are used primarily because of their higher thermal hysteresis when compared to Gracilaria SP. The inert nature, high GS as well as transparency of the gels are also desired requisites. Bacteriological agar is used as propagation media for microbial growth as well as in vitro plant tissue culture cells. The need for very high purity levels increases the cost productions of this type of agar making it more expensive than food grade SP (McHugh, 2003).

In the pharmaceutical industry, agar is used mainly as an excipient, enabling active compounds to reach target areas, or as an efficient laxative due to its high fiber content. The high purity of Iberagarâ€™ from Iberagar is well recognized by the pharmaceutical sector.

Plantagar from Agarindo Bogatama is commercialized as an effective fertilizer and soil conditioner for ornamental plants, fruits, vegetables and tree crops.

In more sensitive fields, where the charged groups (i.e. sulfate esters) of agar may interact with other macromolecules (e.g. in nucleic acid electrophoresis), agarose gels have to be used. Agarose is also used as stationary phase for certain chromatographic applications.
### Table I.1.4 - Commercial Agar-grades, Main Applications and Properties.

<table>
<thead>
<tr>
<th>AGAR TYPE</th>
<th>APPLICATION</th>
<th>EXAMPLES</th>
<th>PROPERTIES</th>
<th>COMMERCIAL AGARS</th>
</tr>
</thead>
</table>
| **Food Agar** | Confectionery     | water dessert jellies, sweets and candies, fruit jellies, candy fillings, jellies and jams | • High gelling capacity  
• Wide pH working range  
• Resistance to heat treatment  
• Large thermal hysteresis  
• No effect on flavors and colors  
• Thermo-reversible gels  
• Gel stability  
• Very high fiber content | Gold Agar, Agarite, Grand Agar (Hispanagar);  
Agarin (Agarindo Bogatama) |
| Pastry/bakery |                   | icings and glazes for pastries, cakes and donuts                          |                                                                                                  |                                                                                   |
| Dairy products |                   | yoghurts, milk desserts, fermented dairy products                         |                                                                                                  |                                                                                   |
| Canned food  |                   | canned meat, meat in jelly                                                |                                                                                                  |                                                                                   |
| Beverages    |                   | clarification and refining of juices, beers, wines and vinegars           |                                                                                                  |                                                                                   |
| **Bacteriological Agar** | Culture media | clinical diagnosis, quality control and food safety procedures; in vitro plant tissue culture cells | • Large thermal hysteresis  
• Inert nature  
• Transparency  
• High gelling capacity  
• Reproducibility | Agar B (Iberagar):  
Bacteriological Agar, Purified Bacteriological and PTC Agar (Hispanagar);  
Bioagar (Agarindo Bogatama) |
<table>
<thead>
<tr>
<th>AGAR TYPE</th>
<th>APPLICATION</th>
<th>EXAMPLES</th>
<th>PROPERTIES</th>
<th>COMMERCIAL PRODUCTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmaceutical Agar</td>
<td>Drugs</td>
<td>excipient tablets, capsules, emulsions</td>
<td>• High purity</td>
<td>Iberagarâ€™™</td>
</tr>
<tr>
<td></td>
<td></td>
<td>laxative</td>
<td>• Inert nature</td>
<td></td>
</tr>
<tr>
<td>Plant Propagation Agar</td>
<td>Fertilizer or land conditioner</td>
<td>ornamental plants, fruits, vegetables,</td>
<td>• Water binding capacity</td>
<td>Plantagar (Agarindo</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tree crops</td>
<td>• High mineral and fiber content</td>
<td>Bogatama)</td>
</tr>
<tr>
<td>Agarose</td>
<td>Electrophoresis, Chromatography, Immunodiffusion, solid culture media, growth of protein crystals</td>
<td>nucleic acids electrophoresis stationary phase in chromatography</td>
<td>• Neutral structure</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• Large thermal hysteresis</td>
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<td></td>
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<td>• Inert nature</td>
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<td>• Transparency</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• High gelling capacity</td>
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</tbody>
</table>
Naturally motivated by a greater interest from the food industry, agar R&D was initially, mainly focused on food applications. In recent years, however, emerging areas such as tissue engineering (Cao et al., 2009; Jin et al., 2013; Nayar et al., 2012; Verma et al., 2007; Yamada et al., 2012) and “smart” materials (Singh et al., 2010) production, have become promising fields. For instance, agar hydrogels are also being explored for tissue engineering and controlled drug delivery applications (Santoro et al., 2011). In these cases however, the ideal form of agar, i.e. agarose, with unique gelling capability and structure, is preferred.

Extensive studies have been presented describing conventional agar extraction methodologies (i.e. lab-scale procedures of agar industrial process) from a wide array of red seaweed species (Kumar & Fotedar, 2009; Rodriguez et al., 2009; Villanueva & Montano, 1999; Villanueva et al., 2010). Considering, the challenges ahead for Agar Industry, particularly in regions more exposed to the economic crisis, such as Europe and the USA, these new applications could become the life-saver of many companies exclusively devoted to agar production.

I.1.8. Microwave-Assisted Extraction (MAE)

In recent years, the application of non-conventional energy sources in the extraction of natural compounds has gained great interest. Microwave-assisted extraction (MAE), which promotes the simultaneous heating of the whole sample matrix by using microwave irradiation, has been successfully applied in the extraction of a wide variety of natural products (Chan et al., 2011; Chi et al., 2011; Fishman et al., 2008; Perino-Issartier et al., 2011; Santana et al., 2005). The use of specialized microwave systems as opposed to the classical domestic devices many times used in the past by researchers, allowed the proper control of vital operational parameters such as pressure and temperature, thus becoming of great interest for the industries.

MAE technique can be performed under atmospheric pressure (open vessels) or controlled temperature and pressure (closed vessels). By using closed vessels, the extraction can be performed at elevated temperatures accelerating even more the mass transfer of target compounds from the sample matrix. In most cases, reproducibility and extraction yields are improved compared to those reached by traditional methods while using less energy and solvent volume (Castro et al., 2009; Herbert et al., 2006; Portet-Koltalo et al., 2008; Srogi, 2006).
During the MAE process, microwaves which promote a type of volumetric heating (Fig. I.1.5) interact with the extractive system (i.e. matrix-solvent) via dipolar rotation and ionic conduction (Boldor et al., 2010; de la Hoz et al., 2005). If working with liquid media with small enough volume, virtually there is no temperature gradient upon heating, because of the unique way microwaves heat. When the electromagnetic field is applied, the dipoles of the solvent molecules align in the direction of the electric field and rotate at a rate of 4.9×10⁹ times per second (i.e. dipole rotation) (Kou & Mitra, 2003). In ionic conduction, charged molecules will dissipate kinetic energy, generating heat via frictional forces between the ions and the surrounding media (Boldor et al., 2010; Kou & Mitra, 2003). This will lead to an “inverted heat transfer” from the core of the extractive system towards the exterior causing the rapid heat of the hole system (de la Hoz et al., 2005). If the volume is larger, things are very different - microwaves heat just the outer layer of the media and the heating won’t be uniform.

The ability of a solvent to absorb microwaves can be described by the loss tangent, $\tan \delta$ (Eq.I.1.1) (Dariusz & Prociak, 2007):

$$\tan \delta = \frac{\varepsilon''}{\varepsilon'}$$  \hspace{1cm} (I.1.1)

where $\varepsilon'$ is the real permittivity which characterizes the ability of the solvent to be polarized by the applied electric field (i.e. ability of microwaves to pass through the material) and $\varepsilon''$ is the imaginary part of the complex permittivity, $\varepsilon^* (i.e. \varepsilon^* = \varepsilon' - i \varepsilon'')$, known as the loss factor, and indicates the ability of the material to dissipate the energy (i.e. efficiency of conversion of microwaves into heat).
The dielectric constant, which is characteristic of each material, is easily obtained from \( \varepsilon^* \), if entering with the correspondent phase displacement. Hence, the dielectric properties of the extractive system will be determinant for the MAE success. Polar solvents such as water will absorb more microwaves than non-polar solvents (e.g. hexane). In turn, methanol will heat much more rapidly than water (higher \( \varepsilon'' \)) even though having lower \( \varepsilon' \) (i.e. less efficient barrier to the microwaves as they pass through). In summary, the ability to rapidly heat the sample-solvent mixture is inherent to MAE and the main advantage of this technique (Srogi, 2006).

Thermal heat which is a slow superficial heat is used in conventional extractions (Fig. I.1.3). In opposition to the MAE, the success of conventional extraction methods rely on the solubility of the compounds in the solvent and depends on conduction and convection phenomena (Boldor et al., 2010; Kou & Mitra, 2003).

I.1.8.1. MAE of Seaweed Compounds

In the case of hydrocolloids extraction from seaweeds, we can interpret the MAE process as follows: when microwave energy is applied to the seaweed-water system, microwaves directly heat the plant matrix causing a pressure gradient upon temperature increase (water evaporation). As the pressure further increases, the seaweed cell-walls are further swelled and disrupted forcing the seaweed compounds to migrate into the solvent (Hahn et al., 2012; Routray & Orsat, 2012).

At the time of performing the process optimization of the present PhD project only two works were found concerning extraction of biopolymers from seaweeds with some kind of microwave-based technique. Navarro and Stortz (2005) used a domestic microwave oven to study the alkaline modification and desulfation of red seaweed galactans while Uy et al. suggested a microwave procedure as a promising and efficient commercial method for extraction of carrageenan (Navarro et al., 2007; Navarro & Stortz, 2005; Uy et al., 2005). To the best of our knowledge we were the first research group to apply MAE technique to the recovery of SP (Sousa et al., 2010). In the following of our research, the process was successfully applied to the extraction of other SP. Rodriguez-Jasso and co-workers successfully applied MAE to recover fucoidans from the brown seaweeds *Fucus vesiculosus* (Rodriguez-Jasso et al., 2011). Optimum yield conditions were found at 800 kPa, 1 min using 1g of seaweed/25 mL of water. Very recently, Francavilla et al. isolated NA and AA from *Gracilaria gracilis* using MAE (Francavilla et al., 2013). The authors chose the experimental conditions based on our optimization study (Sousa et al., 2010) and preliminary tests.
Other components besides SP have been efficiently recovered from algae biomass by MAE. In recent years, metal speciation has become focus of great interest due to growing food safety and human health concerns. Some forms of metalloids such as arsenic are toxic and commonly found in marine organisms, many of them used for human consumption (Hsieh & Jiang, 2012; Rubio et al., 2010). From four tested methods for the extraction of arsenic from seaweeds, MAE was the most efficient (93% of extracted metal in just 5 minutes of extraction) (Zhang et al., 2013).

A method for the analysis of pesticides in wild and farmed seaweeds was reported by García-Rodríguez et al. using MAE coupled with chromatographic techniques (García-Rodríguez et al., 2010).

The feasibility of MAE to assist the solubilisation of seaweeds in tetramethylammonium hydroxide has also been investigated to extract iodine and bromine (Romaris-Hortas et al., 2009).

MAE compared favorably with other techniques for carotenoid extraction from the microalgae Dunaliella tertiolecta and Cylindrotheca closterium (Pasquet et al., 2011). Antioxidant compounds from the red seaweed Palmaria palmata (Li et al., 2012a) and the green alga Caulerpa racemosa (Li et al., 2012b) were efficiently obtained by MAE. Also, Crespo and co-workers extracted essential oils from microalgae with 10-times lower extraction times and yields 50-500% higher than the conventional extraction methods (Crespo et al., 2006).

**I.1.8.2. MAE Scale-up**

Even though MAE has been extensively explored at lab scale in innumerous research fields when it comes to process scale-up the main challenge is to ensure economic viability. The MAE implementation has to be more profitable than conventional extraction for a company to make the decision towards new processing technologies.

The extraction of natural compounds from plant and/or seaweed biomass through microwave heating at larger scales is already a reality (http://www.atlantachemical.com/), yet several requirements should be fulfilled to ensure the economic feasibility.

The first concern should be guaranteeing the absence of gradient temperatures inside the microwave reactor. When larger volumes are chosen, microwaves will heat just the outer layer of the media due to limited penetration depth of microwaves defined by the material’s dielectric properties. Even with propeller mixing the heating would not be uniform and to scale-up all of these formed turbulent areas in the vessel with uneven microwave energy
distribution it would be almost impossible. By heating fast and continuously, small volume of the liquid (or semi-liquid) media, the heating is still uniform and easy to be scaled up. Since new applications require not only the development of the microwave equipment but also understanding the processing principles associated to the intended industrial use, several companies focus exclusively in the construction, test and validation of microwave equipments as well as microwave process optimizations. Atlanta Chemical Engineering L.L.C. is a successful US company working to bring effectiveness and profit viability to industrial projects where microwave processing and/or microwave extraction can be advantageous. According to the customer’s requirement for the magnitude of the end-product and equipment operating hours, and based on mathematical equations and data from lab experiments, the company will choose the adequate microwave system. Due to the limited maximum power of the magnetrons available on the market (i.e. 100 kW) if it is necessary several microwave extraction systems could be connected in parallel. For large production scales, MAE of plant or seaweed biomass is carried out in mono-mode microwave equipments (Fig. I.1.6). These units allow a homogeneous energy distribution inside the flowing medium through the formation of a single “standing microwave”. More details concerning the scale-up of microwave processing and Atlanta Chemical Engineering L.L.C can be found on the company’s website, http://www.atlantachemical.com/.

![Fig. I.1.6 – Schematic representation of a microwave extractor for large production (image gently provided by Atlanta Chemical Engineering L.L.C.: http://www.atlantachemical.com/).](image)

Besides the typical MAE advantages already extensively listed throughout the current Chapter (i.e. higher recoveries, less time, solvent and energy consumptions, and so forth)
the economic feasibility of microwave processing will be even more favored when the cost of
the material is high, the available plant space is limited, the energy costs are low, electricity
is a better energetic option than other sources, the use of clean energy is of interest and
finally, the minimal product handling is seen as an advantage (Table I.1.5; Dariusz & Prociak,
2007).

Table I.1.5 – Advantages and disadvantages of microwave processing (Dariusz & Prociak, 2007).

<table>
<thead>
<tr>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volumetric heating</td>
<td>Field complexity (particularly, for multi-mode equipments)</td>
</tr>
<tr>
<td>Energy savings</td>
<td>Temperature uniformity</td>
</tr>
<tr>
<td>Instantaneous control of power</td>
<td>Temperature measurement</td>
</tr>
<tr>
<td>Reduced equipment size</td>
<td>Initial capital cost</td>
</tr>
<tr>
<td>Selective heating</td>
<td>Limited power supply (100 kW)</td>
</tr>
<tr>
<td>Clean energy transfer</td>
<td>Need for R&amp;D prior to implementation</td>
</tr>
<tr>
<td>Chemical reactions driven (solvent-free reactions, …)</td>
<td></td>
</tr>
</tbody>
</table>

The spectrum of available microwave reactors at production scale is getting wider and more
efficient as manufacturers seem truly committed to make microwave technology as versatile
as possible to meet the specific needs of industries. Hence, the future seems bright for
microwave processing. In the particular case of natural compounds extraction from
seaweeds microwave technology can be extremely useful to help overcome the low
efficiencies currently associated to this industrial segment, mostly supported by outdated
machineries and processes.
References.


Yuan, Y. V., Carrington, M. F., & Walsh, N. A. (2005). Extracts from dulse (*Palmaria palmata*) are effective antioxidants and inhibitors of cell proliferation in vitro. *Food and Chemical Toxicology, 43*(7), 1073-1081.

I.2 MATERIALS AND METHODS

CHAPTER I.2
I.2.1. Chemicals

Commercial agar from Sigma-Aldrich Co. (A-7002, St. Louis, MO, \((\text{C}_{12}\text{H}_{18}\text{O}_9)\_n\)) was used as reference. The reagents used in these experiments included: hydrochloric acid (Panreac, 37% v/v, HCl), resorcin (Sigma-Aldrich, ≥99.0%, \(\text{C}_6\text{H}_8\text{O}_2\)), acetaldehyde diethyl acetal (Fluka, ≥95.0%, \(\text{C}_3\text{H}_6\text{O}_2\)), trichloroacetic acid (Panreac, \(\text{CCl}_3\text{COOH}\)), ethanol (Aga, 96% v/v), sodium hydroxide (Pralab, ≥98.6%, \(\text{NaOH}\)), acetic acid (Sigma-Aldrich, ≥99%, \(\text{CH}_3\text{COOH}\)), sodium nitrate (Merck, ≥99.5%, \(\text{NaNO}_3\)) all of analytical grade and used as received. The standards, D(+) xylene (95729, \(\text{C}_5\text{H}_{10}\text{O}_5\)), D(+) glucose (G5767, \(\text{C}_6\text{H}_{12}\text{O}_6\)), D(+) galactose (G0750, \(\text{C}_6\text{H}_{12}\text{O}_6\)), D(-) fructose F0127, \(\text{C}_6\text{H}_{12}\text{O}_6\)) and agarose, type II (A6877, \((\text{C}_{24}\text{H}_{38}\text{O}_{19})\_n\)) were purchased from Sigma-Aldrich; pullulans were from Shodex (Showa Denko, Japan; \(\text{C}_6\text{H}_{12}\text{O}_5\)) and sodium sulfate from Fluka (puriss p.a. ACS ≥ 99.0%). 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP, Sigma-Aldrich) and deuterium oxide (Merck, ≥ 99.9%, \(\text{D}_2\text{O}\)) were used in NMR analysis.

I.2.2. Preparation of Seaweed Samples

Two types of \textit{Gracilaria vermiculophylla} were explored throughout this project, wild and farmed. Agars from each biomass were analyzed to check their quality and understand how the growth environment of the seaweeds affected the final properties of the extracted SP. Farmed \textit{Gracilaria} was supplied by Laboratório de Biodiversidade Costeira from CIIMAR and produced in an IMTA system located at Coelho & Castro Aquaculture, Rio Alto, in northwestern Portugal. The samples were obtained after a cultivation period of four weeks. Biomass of twelve different tanks (subjected to different nutrients amounts) was mixed, washed with freshwater and dried in an oven at 60 °C during 72 h. The algal material was then ready for extraction. Details of the used farming approach can be found in the PhD thesis of H. Abreu (Abreu, 2010). Wild \textit{G. vermiculophylla} was collected at Ria de Aveiro, northwestern Portugal (40°38’N, 8°43’W).

Two supplies of both wild and farmed \textit{Gracilaria} were considered in this project differing in the date of collection: January of 2009 (1\textsuperscript{st} batch) and January of 2011 (2\textsuperscript{nd} batch). The month of harvesting as well as the geographical location was chosen based on previous studies (Villanueva \textit{et al.}, 2010). Each time, the seaweeds were washed with freshwater to remove extraneous matter such as other seaweeds, mollusks or crustaceans. Finally, the cleaned seaweeds were dried in an oven at 60°C.
The 1st batch of IMTA *Gracilaria* was used to obtain the optimal MAE conditions of alkali-treated (AA) and native (NA) agars (*Chapter I.3*). In order to study the influence of the seaweed growth conditions in the quality of the extracted polysaccharides wild *Gracilaria vermiculophylla* harvested at the same time (January 2009) was used to extract agars under the determined optimal MAE conditions.

To carry out the remaining part of the project, the optimal MAE conditions were applied to the 2nd batch of wild and farmed seaweeds (collected in January 2011). With these samples the following studies were carried out: i) characterization of the extracted agars (*Chapter I.4*), ii) case studies concerning the SP behavior in solution (dilute aqueous regime) and in gel (concentrated aqueous regime) (*Chapters I.5 and I.6*) and finally, iii) application of the extracted agars for the production of sustainable materials (*Chapters II.3 to II.6*).

Agars obtained by MAE were also compared with SP obtained by the TWE method. To this end, optimal TWE conditions obtained in our early optimization study (Villanueva *et al.*, 2010), were applied to the considered seaweed batches. The experimental details of the conventional extraction process will be addressed in section I.2.2.2. of the current chapter.

A schematic representation of the experimental steps followed to prepare the seaweed samples for the extractions is given in Fig. I.2.1 (steps 1-6 and 1,7 for respectively, AA and NA).

In the case of AA, the alkaline treatment step was performed according to the traditional method using optimal parameters obtained in our previous work (Villanueva *et al.*, 2010). Briefly, dried seaweed samples (1 g for MAE (1) and 4 g for TWE) were soaked in a 6% wt NaOH solution (100 mL for MAE and 200 mL for TWE) at 85 °C for 3.5 h (2, 3). The NaOH solution was then discarded and the algal material was washed with fresh water until the removal of the slimy feel (4). Then, the samples were neutralized with 100 or 200 ml of 0.5% (v/v) acetic acid for 1 h at room temperature after which, the acid was discarded and the seaweed samples were again washed with freshwater. The algal material was then ready for the MAE (5,6). In the case of NA, the dried biomass was directly put in the extraction vessels without previous treatment (7).
I.2.1 Schematic representation of the experimental steps followed during the preparation of *Gracilaria vermiculophylla* samples (1-11) and subsequent MAE of NA (7-11) and AA (2-10, 12). Images identified with numbers are mentioned throughout the text.
I.2.3. Agar Extractions

Agar traditional hot-water extraction (TWE) was performed in a Binder GmbH heating oven (FD 53, Tuttlingen, Germany) according to previous optimization studies (Villanueva et al., 2010). Briefly, 4 g of dried seaweed were hot extracted in 200 mL of distilled water at 85 °C, during 2h, after the pre-treatment step followed by acid neutralization, described above. An extensive description of the followed conventional extraction methodology can be found in the Master thesis of A.M.M. Sousa (Sousa, 2008).

Images of the experimental steps followed during MAEs of NA and AA are also displayed in Fig. I.2.1 (steps 8-12). Illustrative details concerning the TWE procedure can be found in the above mentioned thesis. For the TWE of NA, no pre-treatments were performed on the biomass and the seaweeds were directly heated at 85 °C for 2h. Contrarily to our previous work (Villanueva et al., 2010), no steps prior to TWE were performed (i.e. soaking in hot-water) as the main goal of the current study was to infer the induced differences in NA extracted under different heat sources (microwave irradiation or conventional heating) (Sousa et al., 2012).

MAEs were performed with a MARS-X 1500 W (Microwave Accelerated Reaction System for Extraction and Digestion, CEM, Mathews, NC, USA) configured with a 7 or 14 position carousel (8). In the case of NA, one-gram of dried seaweed was transferred to Teflon (NA) extraction vessels with the tested desionized water volume; then the vessels were closed. Each NA run was performed with 14 vessels. Glass extraction vessels were used in the case of AA and runs with 7 vessels were considered (limited by the maximum number of available vessels). The option for Teflon vessels in the 2nd optimization study was motivated by the possibility of using larger volumes and using more vessels in the same run. Each time, the MAE apparatus operated at 100 % magnetron power and with a time to reach settings of 10 min. During operation, both temperature and pressure were monitored in a single vessel (control vessel).

![Image](image-url)  
*Fig. I.2.2 – Pressure (1) and temperature (2) probes placed in the control vessel and connected to the MAE apparatus.*
Magnetic stirring in each extraction vessel and a sensor registering the solvent leaks in the interior of the microwave oven were also utilized. MAEs were carried out according to the experimental design defined for each agar type by varying the extraction time, solvent volume, temperature and stirring speed (please see Chapter I.3 for details regarding the extraction conditions). After the extraction, the vessels were opened still warm because of agar gelling properties. In each case (TWE and MAE), the hot-mixtures were filtered using filter cloths (8,9 for MAE). The recovered NA solutions (11) were typically darker than the AA’s (12) due to the different methods of seaweed preparation. Agar was recovered through freeze-thawing process after which it was washed and dehydrated with ethanol (96 %, v/v), and finally, oven dried at 60 °C. The agar yield (%) was calculated as percentage of dry matter.

I.2.4. Purification of Agar Extracts

Optimal extracts from both processes were purified through the centrifugation of 0.2% wt agar aqueous solutions using a Beckman Coulter centrifuge (Model Alegra 25R) at 40 °C, 21 000 g, 9 ACC for 1 hour.

I.2.5. Structural Characterization

I.2.5.1. NMR and FTIR Spectroscopies

I.2.5.1.1. Basic Principles

Much progress has been accomplished in the field of compound identification through the use of modern spectroscopic methods (Bross-Walch et al., 2005). Spectroscopy comprises a broad range of techniques that, despite having the same theoretical background, present specific features that distinguish them from one another.

Nuclear Magnetic Resonance (NMR) and Fourier transform Infrared (FTIR) techniques constitute two of the most important spectroscopic methods used by researchers when working with polysaccharides identification. A deeper knowledge on the structure of such complex compounds through the use of advanced and sensitive instrumentation has brought new insightful information at molecular level that could be related with biological and physicochemical properties of the considered polysaccharides. This constituted a major
breakthrough in the commercial acceptance of these compounds by enabling a stricter control of the polymers’ final quality.

NMR fundamentals are based on the absorption of radio frequencies by certain nuclei in a molecule when in the presence of a strong magnetic field ($B_0$ in Fig. I.2.4). To produce a NMR signal the equilibrium populations within the molecule must be perturbed by an energy source (i.e. oscillating electromagnetic radiation) generated by an alternating current (Cabrita, 2010).

Most atomic nuclei intrinsically possess spin and when this magnetic field is applied, spin polarization will move around the field due to the contribution of two forces: one tries to align the spin with $B_0$ and the other tries to keep the spin angular momentum. Quantum mechanics will indicate the possible orientations of the nuclear spin; when $B_0$ is applied, the energy levels will split and a magnetic quantum number will correspond to each level.

To measure a NMR signal two main relaxation processes must take place: longitudinal (or spin-lattice relaxation) and transversal (or spin-spin relaxation). The principles of the former rely on the movement of nuclei within the lattice caused by the magnetic field (i.e. lattice field). Some of the components of the lattice field will interact with nuclei in the high energy state and cause them to lose energy (returning to the lower state). Consequently, this will lead to an increase in vibration and rotation within the lattice. The spin-spin relaxation describes the interaction between neighboring nuclei with the same frequency but differing magnetic quantum states. Here, a nucleus in the lower energy level can be excited while the excited nuclei can relax to lower energy states (Cabrita, 2010).

The nuclei magnetic fields are not equal to the applied magnetic fields. The electrons around each nucleus shield it from the applied magnetic field (Fig. I.2.3). The difference between the field at the nucleus, i.e. local magnetic field, $B_{loc}$, and $B_0$ is called magnetic shielding.
The chemical shift is used to describe the NMR signals. It is affected by the nucleus neighborhood (atom type, functional group, ...) and is measured relatively to a reference compound.

The Free Induction Decay (FID) is generated by a radiofrequency pulse exciting a range of frequencies within a spectral window. The detected signal is a combination of all those frequencies and it is called an interferogram. The NMR spectrum is obtained through the Fourier transformation of the FID.

The general operation of a NMR spectrometer can be schematized as described in Fig. I.2.4. The sample should be placed in the magnet where the magnetic field will be applied. Meanwhile, the acquisition parameters must be defined while the instrument reaches the desired temperature of the experiments. Considering the study in question, the sample is excited according to a pre-defined sequence of radiofrequency pulses and delays. Relevant acquisition NMR parameters include the spectral width (SW), total number of points for the FID sampling (TD), spectrum resolution (SR), carrier frequency offset (O1, O2), number of scans (NS), delay between repetitions or relaxation delay (D1), just to name a few. An analog to digital converter (ADC) will allow the data acquisition after the signal being adjusted to its dynamic range.

![Fig. I.2.4 – General NMR spectrometer scheme (adapted from 'High resolution NMR techniques in organic chemistry', 2nd Ed, Elsevier, 2009).](image-url)

NMR spectroscopy might well be considered the most successfully used technique in the structural elucidation of SP (Bilan et al., 2007; Usov, 2011; Usov et al., 1980; van de Velde et
al., 2002). In reality, SP exhibit hybrid compositions far more complex than the ideal molecular structures attributed to these compounds. For instance, the high signals resolution in the anomeric region of $^{13}$C NMR experiments allows the confirmation of the LA configuration in the backbone of seaweed galactans. It also provides a way to determine the relative content between important monomers of the SP (Usov, 1984, 1998; Usov et al., 1980). It is possible to distinguish the L6S residues typical of NA from the $^1$H spectral data through a minor peak at ~5.28 ppm.

In the last decade or so, the development of modern NMR techniques mainly relied in the development of proton-detected spectroscopies. In an initial stage, 2D homonuclear experiments broadened the possibilities in structure identification. Applications of these experiments include correlation spectroscopy (COSY) and the nuclear Overhauser effect spectroscopy (NOESY). The nuclear Overhauser effect (NOE) is a manifestation of cross-relaxation between two nuclear spins which are close to each other in space. Since NOE depends on the spatial relationship of two nuclei it is a tool for studying intermolecular interactions (Cabrita, 2010). The turning point to multidimensional NMR experiments came with the heteronuclear single quantum coherence (HSQC) and heteronuclear double quantum coherence (HMQC), all other 2D NMR modules developing from these two (Mandal & Majumdar, 2004). In this type of experiments, the origin of the cross peaks is based on a type of polarization transfer detecting high-sensitivity nuclei through an ‘inverse technique’. HMQC and HSQC correlate coupled heteronuclear spins across a single bond identifying directly correlated nuclei. HSQC provides better resolution than HMQC, so it is better suited for crowded spectra but it can be more sensitive to experimental imperfections (Cabrita, 2010).

NMR is a non-destructive approach and despite needing longer acquisition times for some experiments than for instance, other techniques such as FTIR, the information that can be extracted from the spectral data can reach much greater detail.

Infrared (IR) is one of the most widely used techniques in the structural elucidation of polysaccharides. It constitutes a non-invasive, rapid and relatively inexpensive and sensitive method for sample identification. IR radiations cover the wavenumbers from 14285-100 cm$^{-1}$ but most of the IR applications occur within the mid IR region, 4000-400 cm$^{-1}$. The initial IR equipments were of filtering or dispersive types; the frequencies corresponding to the emitted energy were measured individually, at each frequency that passed through the sample which made the scanning process slow. A significant technical improvement came with the incorporation of an interferometer in the spectrometers which allowed the IR frequencies to be measured simultaneously. Basically, this simple optical device splits the incoming IR
beam in two, one beam is reflected from a fixed mirror and the other from a moving mirror. The interference between these two beams along the path length will result in a signal (interferogram). Next, the beam will enter the sample compartment where part of the energy will be absorbed by the sample and part will pass through it. To decode the measured frequencies that arrive to the detector and build up a frequency spectrum Fourier transform algorithms are used. An illustrative scheme of the basic FTIR operating principles is given in Fig.I.2.5.

![FTIR Basic Operating Principles](image)

**Fig. I.2.5** – Schematic representation of FTIR basic operating principles.

FTIR analysis rely on vibrational energy emitted when a molecule absorbs IR radiation causing the atoms to oscillate in two types of movements: stretching and bending. Because each material has a unique atomic combination, the absorption peaks of a given molecule serve as fingerprint of the material. Stretching can be symmetric or antisymmetric and represents the movement along the bond axis. Bending movements occur when the bond angle between two atoms or a group of atoms might change relatively to the rest of the molecule. Several types of bending such as scissoring, wagging, rocking and twisting can be identified.

The developed softwares for FTIR equipments allow extracting valuable information. For instance, the conversion of the acquired data to second derivative spectra allows a better spectral resolution by sharpening the signals of corresponding bands and shoulders in parent spectra. This can be used to distinguish agars from carrageenans (Matsuhiro & Rivas, 1993). Additionally, FTIR spectra have been used to estimate the total sulfate content of these
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SP (Rochas et al., 1986). As for NMR, FTIR employs Fourier transforms to process the spectral data.

I.2.5.1.2. Experimental Procedures

The NMR experiments were carried out non-spinning at 80 °C in a 400 MHz Bruker Avance III spectrometer, operating at 400.15 MHz for protons and 100.62 MHz for carbon, equipped with pulsed gradient units, using a 5 mm QNP probe equipped with a z gradient coil capable of producing magnetic field pulsed gradients in the z-direction of 56.0 G/cm. The studies were carried out at CEMUP (Centro de Materiais da Universidade do Porto), Porto, Portugal and at Universidade Nova de Lisboa, Lisboa, Portugal. The adopted procedures followed a previous work (Villanueva et al., 2010) with some modifications and, in both cases, TSP was used as internal reference (δ_H = -0.017 ppm; δ_C = -0.18 ppm).

For the study concerning the characterization of NAs from IMTA biomass (Sousa et al., 2012), described in Chapter I.4, section I.4.1.1.1, the NMR spectra were acquired as follows:

Agar was dissolved in D_2O to a final concentration of 30 mg mL^{-1} and TSP was added as reference. Standard 1D ^1H NMR experiments were performed with 30° pulses for excitation (typical 90° degree pulse width of 12.5 μs), acquisition time 3.98 s, relaxation delay 1 s, 16 transients of a spectral width of 8000 Hz were collected into 64 K time domain points. For the full range broad-band proton decoupled ^13C NMR spectra the following acquisition parameters were used: experiments with 30° excitation pulses (90° degree pulse width of 9 μs), acquisition time of 0.8 s, relaxation delay of 2 s, spectral with 24000 Hz, 32 K time domain points and 5K transients were collected. Two-dimensional gradient-selected ^1H/^13C heteronuclear single quantum coherence (HSQC) spectra were recorded using the standard Bruker software via double inept transfer, using sensitivity improvement and decoupling during the acquisition. A total of 1024 data points in F2 over a spectral width of ca. 6000 Hz for ^1H and 256 data points in F1 over a spectral width of 16000 Hz for ^13C were collected, with 8 scans per increment and a relaxation delay of 1.5 s between scans. The delay for the inept transfer was set to 1.73 ms corresponding to a C-H coupling constant of 145 Hz. All the NMR spectra were processed in MNova Software version 7.1.1.

Other extracts were also characterized using this procedure and some representative examples will be shown in the Chapter I.4 concerning the samples characterization.
In the case of NAs and AAs from wild Gracilaria obtained by MAE, slight adjustments were made to the procedure described above with the intent of improving the quality of the acquired spectra. The samples were dissolved in D$_2$O to a final concentration of 15 mg/mL. The $^1$H NMR spectra were obtained with a 5.11 s acquisition time, 6400 Hz spectral width and an average number of 16 scans while the $^{13}$C NMR data were acquired with a 1.42 s acquisition time, a spectral width of 23000 Hz and an average number of scans of 15000. The $^1$H-$^{13}$C correlations were recorded through a phase-sensitive HSQC experiment using the following parameters: 0.2 s acquisition time, (5000, 20000) Hz spectral width and 128 average scans. The Wild_MAE_GS$^{opt}$ sample was further investigated through 1D $^{13}$C APT (attached proton test; a relaxation delay of 2.0 s, 1.42 s acquisition time, a spectral width of 23000 Hz and 5000 scans) and 2D HMBC (heteronuclear multiple bond correlation; relaxation delay of 1.5 s, 0.39 s acquisition time, (5000, 23000) Hz spectral width and 72 average scans) experiments.

Thin polysaccharide films of 0.5% wt concentration were analyzed in a Bomem MB-series FTIR spectrometer (ABB Bomem, Inc., Quebec) according to a previous procedure (Villanueva et al., 2010). The samples were prepared by drying in an oven (60ºC) ~3.5 mL of each 0.5% wt agar solution, placed in a plastic container (4 cm diameter). Each spectrum is the average of 8 scans acquired at 2 cm$^{-1}$ resolution. The absorbance ratio of peaks attributed to LA (ca. 935 cm$^{-1}$) and total sulfate (ca. 1250 cm$^{-1}$), hereby denoted as A$_{935}$/A$_{1250}$, was determined for representative extracts, by the estimation proposed by Rochas et al. (Rochas et al., 1986). Peak base lines and heights were determined in transmittance mode and calculated peak heights were converted to absorbance (absorbance = log (1/transmittance).

The chemical nomenclature of residues followed the shorthand notation system defined by Knutsen et al. (Knutsen et al., 1994). For details please see the List of Symbols, Abbreviations and Shorthand Notations provided in the begging of the thesis. The degrees of methylation at C6 of G and at C2 of LA units were estimated from the $^1$H NMR spectral data by the ratio between 1/3 of the area of the peak at ~3.41 and areas of low intensity singlets in the range ~ 3.54-3.44 ppm, respectively, and the peak area of H1 of the 4-linked residues [LA (5.13 ppm) + L6S (5.28 ppm)] (Villanueva et al., 2010). CA was used as reference in both spectroscopic measurements.
I.2.6. Physicochemical Characterization

I.2.6.1. Determination of the Sulfate and Sugar Contents

During the MAE optimization of AA, the sulfate concentrations of each extract were determined based on the method described by Matos et al., using a Dionex ion exchange chromatography system (Dionex Corporation, USA) constituted by an ED 50 electrochemical detector, an Analytical AS9 (4 mm) column and an Anion Suppressor-ULTRA (4mm) (Matos et al., 2008). The mobile phase used was Na\textsubscript{2}CO\textsubscript{3}\textsubscript{9} m mol/L, pH = 13, at a flow rate of 1 mL/min. 10-20 mg of dried agar were hydrolyzed under reflux in 10 mL of HCl 1 mol/L for at least 4 h and afterwards diluted to a final volume of 25 mL. Prior to the analysis, the hydrolysate was diluted by a factor of ten (1:10) and passed through a filter with a porosity of 0.2 μm. Sulfate standards were prepared using Na\textsubscript{2}SO\textsubscript{4} and an HCl solution with the same pH as the diluted hydrolyzed agar solution.

For the optimal agar extracts, the xylose, glucose and sulfate contents were also examined by High-performance Liquid Chromatography (HPLC) but using a different apparatus, a DIONEX equipment, model ICS 3000. The hydrolysis of the samples were carried out as described above. The sulfates determination was carried out in an IonPac AS15 column (Dionex) with an IonPac AG15 pre-column, equipped with a conductivity detector with suppression (suppressor ASRS300 4 mm). The analyses were performed at 30 °C using NaOH (38 mM) as eluent, at a flow rate of 1.2 mL/min.

Additional sulfate (%) determinations were carried out by the turbidimetric method of Jackson & McCandless (Jackson & McCandless, 1978) and each time, confirmed by HPLC. In all cases, the results were concordant.

For the sugars (glucose, xylose) content, a CarboPac column with a pre-column BioLC AminoTrap, equipped with a pulsed amperometric detector (PAD) was used. The experiments were carried out at 30 °C with NaOH (18 mM) as eluent, at a flow rate of 0.9 mL/min. The system was washed with a 200 mM NaOH solution between each injection and an 18 mM NaOH solution was passed through the system prior to each analysis. The LA content was determined by the colorimetric method of Yaphe and Arsenault using the resorcinol-acetal reagent and with fructose as standard (Yaphe & Arsenault, 1965). Experiments were performed, at least, in triplicate.
I.2.6.2. Size Exclusion Chromatography (SEC)

I.2.6.2.1. Basic Principles

Size exclusion chromatography (SEC) also known as gel permeation chromatography (GPC) is a chromatographic technique used to separate compounds based on the particles sizes through the use of specific columns. The basic SEC system must include an injector (manual or automatic), a column or a set of columns, the detector and finally, the chromatograph interface to handle the experimental data. To begin the SEC experiments, the mobile phase should pass through the system in a continuous manner using a pump. As the sample travels along the column particle separation occurs and the eluted sample is screened in a concentration detector (single or in series). The most commonly used detector in SEC is the refractive index (RI) whose signals result from the difference in RI between the eluent in the reference side and the sample + eluent in the sample side. Other detectors include the UV, protodiode array (PDA), viscometer and light scattering. When a triple detector (i.e. light scattering, viscometer and concentration detectors) is used simultaneously, the absolute molecular weight, molecular size and intrinsic viscosities can be extracted as well as information concerning the molecular structure and conformation. In a single detector case, the physical parameters will be determined relative to a standard. Finally, the SEC apparatus may also have an in-line solvent degasser to minimize the influence of gas bubbles in the recorded signal. A schematic representation of a SEC basic operating unit is presented in Fig. I.2.6.

![Schematic representation of a general SEC equipment.](image)

The separation of particles is based on their hydrodynamic radius ($R_h$) or volume ($V_h$), not molecular weight ($M_w$). The columns are packed with porous microparticles (typically, 5 to 20 µm in size) such as styrene/divinylbenzene copolymer gel, silica gel, etc. Basically, the larger
molecules of the sample are excluded from some of the pores in the column and consequently, are eluted faster through the column than the smaller molecules. As result, the detector placed outside the column will read first the eluted volume of the largest molecules while the smallest ones will be detected in last.

In the case of conventional calibration a series of polymer or protein standards of narrow and known $M_w$ must be injected. The measured retention volumes ($RV$) or times ($RT$) will allow us to build a calibration curve of $\log M_w$ vs $RV$ (or $RT$). The use of $RV$ instead of time will allow the acquired data to be independent of the used flow rate. Obviously, the chosen standards must cover the entire range of $M_w$ intended to be measured. The problem with this calibration is that not always there are available standards matching the sample we want to characterize and, even though we assume both compounds (sample and standard) as equal, this is never the case. The presented information concerning the SEC principles and instrumentation can be found in detail in the following website, http://www.waters.com/waters/pt_PT/GPC-Basic-Chemistry/.

When studying SP by SEC, special cautions must be taken in the preparation of the solutions due to the great aggregation propensity of these compounds, even at very low polymer concentrations. Typically, solvents known to prevent gelation are preferred, the solutions are prepared at very low concentrations (the minimum possible that guarantees a good signal) and the experiments are performed at temperatures above the gelation point of the sample.

I.2.6.2.2. Experimental Procedure

The apparent number-average ($M_n$) and mass-average ($M_w$) molar masses of agars were determined by SEC using a low temperature Waters Co. apparatus with a Waters Ultrahydrogel linear column (7.8x300 mm) and a differential refractive index detector (Waters 2410). The molar mass dispersity of the samples was estimated from the ratio between $M_w$ and $M_n$ ($\bar{M}_w = M_w / M_n$). Agar solutions (5 μg/mL) were prepared in 0.1M NaNO$_3$, a non-aggregating salt, in order to minimize as far as possible, the aggregation of the SP molecules. The solutions were filtered through a 0.45 μm filter and injected with a 200 μL sample loop in a Waters 510 Solvent Delivery System. Measurements were performed at 40 °C and a flow rate of 0.8 mL/min using 0.1M NaNO$_3$ as eluent. Pullulan standards with known $M_w$ values (P-5, 5,900; P-20, 22,800; P-50, 47,300; P-100, 122,000; P-200, 212,000; P-400, 404,000; P-800, 788,000) were used in the calibration. Chromatograms of at least two
injections were obtained with the data being recorded and analyzed with the Millenium 32 Software package.

I.2.7. Dilute Aqueous Regime Studies

I.2.7.1. Atomic Force Microscopy (AFM)

I.2.7.1.1. Basic Principles

Atomic force microscopy (AFM) is a type of scanning probe technique widely used to image polysaccharides. A schematic representation of a general AFM setup is given in Fig.I.2.7. The basic idea of AFM relies on mapping the sample surface with a sharp probe which allows building a topographic profile of the surface. The cantilever on which the tip is mounted permits the tip to move up and down with great precision due to a very low force constant. Both tip and cantilever are typically made of very resistant materials generally silicon or silicon nitride. The scanning process is made by a piezoelectric transducer and the detection system is usually of the optical type and serves as a highly sensitive mechanical amplifier, able to detect atomic scale movement of the tip as it maps the sample. Interferometers were used initially as optical AFM detectors, yet their high sensitivity to thermal drift and variation in laser frequency made its installation more difficult. Currently, laser beam deflection is the most employed detection method because it’s cheap, simple and versatile.

Fig. I.2.7 – Schematic representation of a general AFM setup. Illustration of the different type of interactions established between the tip and the surface of the sample is also provided (Adapted from Borges, 2012 and Morris, 2010).
The forces between the tip and sample causing the tip to move can be of short or long-range interactions. In the former case, van der Waals are the most relevant forces established between the atoms of the tip and the atoms of the sample's surface. At long-range the most important forces include electrostatic, capillary and magnetic while at intermolecular level, van der Waals are the most significant interactions.

Two basic modes of operation can be considered when performing AFM studies: static (or contact mode) and dynamic (non-contact mode and tapping mode). In contact mode, DC current is applied and the tip moves along the surface of the sample in close physical contact to it. This can become problematic if attractive forces established between the tip and the sample become too significant leading the tip to ‘dip into’ the sample. Ultimately, this can damage the sample and compromise the acquired data.

To solve these problems AFM studies can be carried out in AC modes: non-contact mode or in tapping mode. Non-contact experiments are particularly useful when imaging softer samples. In this case, AFM images are built by scanning the attractive forces established between the tip and the sample, with the former never touching the surface of the latter. Because the magnitudes of these forces (e.g. van der Waals) are quite low when compared to the contact mode, a slight oscillatory movement is given to the tip so that the detectors can measure reasonable signals. The second AC method is the tapping mode which consists on the tip tapping the surface of the sample intermittently. Tapping mode eliminates the effects of capillary adhesion and reduces the lateral forces on the sample because the tip spends less time in contact with the surface of the sample (Morris et al., 2010).

The samples need to be deposited onto a rigid substrate to be able to be manipulated and imaged by AFM. Mica is the most common substrate due to its low cost and large availability. It is ideal for high resolution measurements (individual molecules) and it is negatively charged in aqueous media at neutral pH. Other substrates include glass, more adequate for larger molecules, and graphite, which is extremely hydrophobic and can be particularly interesting when the material to study interacts with the mica surface (Morris et al., 2010).

Imaging fibrous polysaccharides in water or buffers can be particularly challenging. The weakly attached adsorbed molecules move from the surface of the sample during the tip scanning and consequently, compromising the acquired data. In this regard, the use of non-solvents such as alcohols can prevent the molecular motion. As previously mentioned, the use of contact mode can also damage the observed structures. A way to overcome these issues is to drop the polysaccharide sample onto mica and dry it in air, before imaging the
material in the tapping mode. This procedure also allows to image polysaccharides in water and buffer media.

### I.2.7.1.2. Preparation of Agar Solutions

AFM technique was used to carry out two case studies concerning agar behavior in aqueous media with low polymer concentration.

For the case study presented in *Chapter I.5*, concerning the comparison between the behaviors in solution of TWE and MAE alkali-treated agars (model samples: AA\_Wild\_MAE\_GS\textsuperscript{opt} and AA\_Wild\_TWE\textsuperscript{opt}), dilute aqueous solutions of 0.05% wt (ca. 500 µg/mL) agar concentration were prepared following two different procedures. The first one consisted in dispersing the appropriate amount in distilled water under vigorous stirring. The dispersions were then heated at ~95 °C for one hour and the resulting solutions were left to slowly cool to room temperature (‘slow cooling’ method). The other adopted procedure consisted in diluting with cold distilled water the concentrated solutions (1.5% wt; prepared as described in section 1.2.2.7.1) while hot (i.e. random coil state), to a final concentration of 500 µg/mL (‘fast cooling’ method). The pH of the solutions fell in the range ~6.4-6.7.

For the case study presented in *Chapter I.6*, concerning the extracts from wild *Gracilaria* obtained by MAE at different operational conditions (model samples: NA\_Wild\_MAE\textsuperscript{opt}, AA\_Wild\_MAE\_Y\textsuperscript{opt} and AA\_Wild\_MAE\_GS\textsuperscript{opt}), the solutions of 0.05% wt concentration were prepared as described above. However, after complete solubilization of the polymer, the solutions were left to cool at room temperature for 40 minutes after which they were diluted to the desired concentrations (5, 10, 50 and 100 µg/mL). The pH of the solutions fell in the range ~6.1-6.9.

### I.2.7.1.3. Experimental Procedure

Aliquots (25 µL) of each dilute solution were deposited onto freshly-cleaved discs of mica (Muscovite V-4, 15 mm diameter, SPI Supplies®), allowed to air dry for about 20 min, washed thoroughly with Millipore water and dried under a soft stream of nitrogen. AFM imaging was performed in air at room temperature using a PicoLe Atomic Force Microscope (Molecular Imaging, USA) operating in dynamic tip deflection mode (Acoustic Alternating Current mode, AAC). An intermittent contact regimen (tapping mode) was selected in order
to avoid damage on surfaces and typical tip sticking problems. AFM images of each dilute media (prepared as described in section I.2.2.6.2) were obtained by imaging the force of the oscillating contacts of the tip with the sample surface. silicon cantilever (ACT-50, AppNano, USA) with a tip (pyramidal shape) height in the range of 14-16 μm, radius of curvature (ROC) lower than 10 nm, spring constant of 25-75 N/m, and a typical resonance frequency in the range 200-400 kHz was used for these purposes. Several macroscopically separated areas of the mica surface were scanned in topography, amplitude, and phase modes with a resolution of 512 × 512 pixels and are representative of 5 μm × 5 μm and/or 3 μm × 3 μm regions over different locations on the studied mica surfaces. AFM images were corrected for bow/tilt by a second-order flattening using the PicoViewTM 1.8.2 software (Agilent Technologies). The average heights \( H_{av} \) as well as the height profiles along the lines drawn over the topographic AFM images were obtained using the free Gwyddion 2.22 software.

I.2.7.2. Intrinsic Viscosity Measurements

1.2.7.2.1. Basic Principles

The term ‘rheology’ describes the study of the flow of matter or in other words it concerns the manner how materials, in the liquid or solid states, respond to an external disturbance (i.e. stress or strain). Different kind of rheometers (rotational or tube) can be used to measure the rheological properties of a given material. Viscometers constitute a particular case of tube rheometers, limited to the measurement of viscosity (Steffe, 1996). The general concept of ‘solution viscosity’ translates its resistance to flow due to internal friction.

The determination of the intrinsic viscosity \([\eta]; \text{mL/g}\) of polymeric solutions is a good and simple alternative to estimate the average molecular mass of a given polymer. Viscometry is a common practice in the field of SP due to its simple instrumentation/manipulation and possibility to obtain the results quickly, constituting a great complementary methodology of other techniques such as SEC.

At sufficient low concentration (dilute regime), SP molecules in aqueous media assume a random coil conformation acting as isolated particles. If increasing the polymer concentration, the system will eventually reach a critical point where the molecules start to interact with each other, consequence of a decrease in the total excluded volume of the system (critical concentration, \( C^* \)). At this stage, the solution is moving towards the formation of entangled networks which will gradually occur with increase in polymer concentration. A second critical concentration limit will then mark the transition between this semi-dilute
concentration window and the concentrated regime, where individual molecules are no longer distinguishable and entangled polymeric mass starts to predominate within the system, becoming steeply coarser with the increase of concentration (Morris, 1995).

The concept of \([\eta]\) works in the dilute regime (\textit{i.e.} below \(C^*\)) and is defined as the limiting value of reduced viscosity at infinite dilution (Eq. I.2.1),

\[
[\eta] = \lim_{C \to 0} \eta_{\text{red}}
\]  
(I.2.1)

The \(\eta_{\text{red}}\) can be defined as the ratio between the specific viscosity, \(\eta_{\text{sp}}\), and the concentration of the solution, \(C\):

\[
[\eta] = \lim_{C \to 0} \frac{\eta_{\text{sp}}}{C}
\]  
(I.2.2)

where \(\eta_{\text{sp}} = \left[\frac{(\eta-\eta_s)}{\eta_s}\right] = \eta_{\text{rel}} - 1\) is the dimensionless specific viscosity and \(\eta_{\text{rel}}\) is the dimensionless relative viscosity, representing the ratio between the viscosity of the solution, \(\eta\), and the viscosity of the solvent, \(\eta_s\).

The two relations commonly used to estimate \([\eta]\) came by the hands of Huggins (Eq.I.2.3) and Kraemer (Eq.I.2.4), respectively:

\[
\eta_{\text{red}} = [\eta](1 + K_H[\eta] C)
\]  
(I.2.3)

\[
\frac{\ln(\eta_{\text{rel}})}{C} = [\eta](1 - K_K[\eta] C)
\]  
(I.2.4)

where \(\ln \eta_{\text{rel}} / C\) is the inherent viscosity and \(K_H\) and \(K_K\), the Huggins and Kraemer constants, respectively (Huggins, 1942; Kraemer, 1938).

The \(\eta_{\text{rel}}\) can be determined by the equation of Hagen-Poiseuille (Eq. I.2.5),

\[
\frac{\eta}{\eta_s} = \left(\frac{\rho}{\rho_s}\right)\left(\frac{L}{L_s}\right)
\]  
(I.2.5)
where $\rho$ and $\rho_s$ are the densities of the solution and the solvent, respectively, while $t$ and $t_s$, are the respective efflux times of each component. When working with dilute aqueous solutions, $\eta_{rel} \approx t / t_s$.

### 1.2.7.2.2. Experimental Procedure

The $[\eta]$ were determined by dilute solution viscometry using a Cannon-Fenske viscometer (size 50; Comecta S.A., Barcelona), at 35.0±0.1 °C. Agar dilute solutions were prepared using 0.75 mol/L NaSCN as solvent as it inhibits the aggregation between agar molecules (Rochas & Lahaye, 1989). Average flow times were used for the calculations of $\eta_{rel}$ and $[\eta]$ was obtained by extrapolation to zero concentration of Huggins (Eq.I.2.5) and Kraemer (Eq.I.2.6) relations.

The viscosity-average molecular masses ($M_v$; Da) were determined using the Mark-Houwink equation (Eq.I.2.6) for the described experimental conditions (Rochas & Lahaye, 1989):

$$[\eta] = 0.07 M_v^{0.72} \quad \text{(I.2.6)}$$

### I.2.8. Concentrated Aqueous Regime Studies

#### I.2.8.1. Preparation of Agar Solutions and Gels

Concentrated agar aqueous solutions were prepared by dispersing the appropriate amount of SP in distilled water under vigorous stirring and heating at ~95 °C for one hour. Approximately fifteen grams of the hot 1.5% wt (ca. 1.5% g/mL) solutions were transferred to a cylindrical container with 30 mm diameter and properly covered to avoid water evaporation. Agar gels were left to set at room temperature and to equilibrate for 20 h. The gel depths were approximately 21-22 mm. The remaining hot agar solutions were used in the oscillatory rheological measurements.
I.2.8.2. Rheological Measurements

Contrarily to viscometers, rotational rheometers are highly versatile instruments (several types of tests can be carried out in a continuous manner) able to strictly control temperature and stress/strain. The data acquisition can be followed on the course of the experiments allowing the operator to get ‘on-line’ information about the rheological behavior of the sample. To interpret the rheological data several theoretical models have been developed providing insightful information on the microstructure of the most complex systems in analysis.

The rheological properties of a given material are typically investigated by two ways: linear viscoelastic tests under small amplitude oscillatory shear (SAOS) and non-linear viscoelastic experiments which can be of several kinds (Hyun et al., 2002). Good examples of the late measurements are the steady state shear and the large amplitude oscillatory shear (LAOS) tests. The former consists in measuring the shear viscosity as a function of shear rate and covers a much wider spectrum of deformations than the linear viscoelastic experiments. The later normally refers to strain sweep tests used to measure the viscoelastic response of various strains. Although LAOS is usually only used to check the region of linear viscoelastic responses, the information that can be extracted from the curves in the region of large deformations can be quite relevant. This topic will be properly addressed in Part II of the current thesis, in Chapter II.4, focusing the study of agar/LBG hydrogels.

I.2.8.2.1. Basic Principles

The rheological measurements all along this thesis were performed with a controlled stress rheometer ARG2 (TA Instruments, USA) (Fig. I.2.8). The operating principles of this rheometer can be described as follows: the temperature control of the apparatus is performed by a Peltier system in the bottom plate (1). Water circulates in soft plastic tubes (3), pumped to the Peltier plate with a small aquarium pump (4), allowing the equipment to operate over a broad temperature range, fast and continuously and with an accurate temperature control.
Two different geometries were considered to carry out the rheological measurements: cone-and-plate (2 in Fig. I.2.8 A) and parallel plate (Fig.I.2.8 B).

For liquid homogeneous media containing small particles (< 1µm) the cone-and-plate geometry offers uniform shear, small inertia, small sample volume and easy sample handling. Cone-and-plate geometries are usually distinguished by their diameter and cone angle. The cone is often truncated. The gap definition is automatically performed by the equipment to ensure that the 'virtual' tip of the cone is in contact with the bottom plate when placing the samples, Fig. I.2.9 A (Sittikijyothin, 2006).

For systems with micron-sized particles, the parallel plate geometry is more adequate (Fig. I.2.9 B). The gap in this case, can be set by the operator in the rheometer and typically should be at least 3-5 times the size of the biggest particles of the system. The main disadvantage of this geometry is the non-uniformity of the shear stress applied to the sample. To prevent slippage problems during the measurements, crosshatched parallel plates are normally used.
I.2.8.2.1.1. Dynamic Oscillatory Testing

During this 1st stage of the PhD thesis, only dynamic oscillatory measurements were considered.

The theoretical background of this type of rheological tests assumes that the material is exposed to sinusoidal disturbances of shear strain, $\gamma$ (controlled-rate rheometers) or shear stress, $\sigma$ (controlled-stress rheometers) (Steffe, 1996). This is the most common rheological procedure to examine the viscoelastic properties of a given material. For instance, when small sinusoidal strains are applied to the system, i.e. the viscoelastic components behave linearly to the applied disturbance,

$$\gamma = \gamma_0 \sin(\omega t) \quad (I.2.7)$$

The resulting shear stress will also respond in a sinusoidal manner at the same oscillation frequency, yet with a phase angle displacement, if the material behaves linearly,

$$\sigma = \sigma_0 \sin(\omega t + \delta) \quad (I.2.8)$$

where $\gamma_0$ and $\sigma_0$ are respectively, the maximum strain and stress amplitudes, $\omega$ is the oscillatory frequency in rad.s$^{-1}$, $t$ is the time in seconds and $\delta$, the phase angle between stress and strain.

The phase angle displacement will vary between an ideal solid material (direct proportionality of stress-strain),

$$\sigma = k \gamma = k \gamma_0 \sin(\omega t) \quad (I.2.9)$$

and a Newtonian fluid (direct proportionality of stress-shear rate),

$$\sigma = \eta \dot{\gamma} = \eta \omega \gamma_0 \cos(\omega t) \quad (I.2.10)$$
In terms of sinusoidal responses, this means that the shear stress wave for an ideal solid will be in phase with the γ wave (i.e. δ=0) while for a Newtonian fluid δ=90°. The response of a viscoelastic material will fall between these two limits (Fig. I.2.10).

![Ideal sinusoidal responses](image)

**Fig. I.2.10** – Ideal sinusoidal responses of completely elastic (solid), completely viscous (fluid) or viscoelastic systems to sinusoidal oscillatory shear strain (solid curves) and/or shear stress (dashed curves) (source: (Sittikijyothin, 2006)).

The above equation can be written as follows, if the notion of viscoelastic components is introduced,

\[
\sigma = \gamma_0 G' \sin(\omega t) + \gamma_0 G'' \cos(\omega t)
\]  

(I.2.11)

where \( G' \) is the storage modulus and \( G'' \) the loss modulus, two fundamental rheological parameters. The former refers to the elastic behavior of the system representing the deformation energy stored in the sample for each oscillatory cycle. The latter corresponds to the viscous behavior and indicates the energy dissipated in the system through viscous flow (Steffe, 1996). Both components constitute, respectively, the real and imaginary parts of the complex modulus, \( G^* \):

\[
G^* = \frac{\sigma}{\gamma^*} = G' + i G''
\]  

(I.2.12)

\( G' \) and \( G'' \) are functions of frequency and can be defined as a relation between the amplitude ratio and the phase angle:
The data from dynamic oscillatory measurements can also be expressed in terms of complex viscosity,

\[ \eta^* = \frac{G^*}{\omega} = \eta' - i \eta'' \]  

composed by the dynamic viscosity, \( \eta' \), and the out of phase component of the complex viscosity, \( \eta'' \):

\[ \eta' = \frac{G''}{\omega} \]  

\[ \eta'' = \frac{G'}{\omega} \]

Another common parameter used to describe the viscoelastic behavior of the system is the loss tangent of the phase shift or phase angle, \( tan \delta \), that represents the ratio between \( G'' \) and \( G' \) or alternatively, \( \eta' \) and \( \eta'' \):

\[ tan \delta = \frac{G''}{G'} = \frac{\eta'}{\eta''} \]

In what SP are concerned, dynamic oscillatory testing are particularly useful to investigate the structure of the gels and gelation mechanisms leading to its formation. This is accomplished by monitoring the viscoelastic properties in a continuous manner under small amplitude oscillatory shear which avoids structural damage of the gel. The sol-gel and gel-sol transitions (i.e. gelling and melting points) are two other estimations of interest for researchers in the field that is possible to extract from this type of rheological tests.
I.2.8.2.1.2. Steady Shear Measurements

Flow properties can be quite complex to determine however, if certain conditions are obeyed (small cone angle (< 5°), sufficiently low rotational speeds and no errors due to surface tension effects at the free fluid surface), the flow curves of a given material can be obtained directly through simple mathematical relations (Steffe, 1996). The cone is rotated at a known angular velocity (Ω) and the resulting torque (M) is measured on the fixed plate or through the cone. The shear rate (\( \dot{\gamma} \)) and shear stress (\( \sigma \)) used to obtain the flow curves can be estimated by the following equations,

\[
\dot{\gamma} = \frac{\Omega}{R}
\]

(I.2.19)

\[
\sigma = \frac{3M}{2\pi R^3}
\]

(I.2.22)

where \( R \) is the cone angle and \( r \) is the cone radius as described in Fig. I.2.9 A. Steady shear measurements were performed in Part II of the thesis and for that reason will be addressed in more detail later on. The experimental procedure presented next will only concern the rheological tests carried out over the course of Part I.

I.2.8.2.2. Experimental Procedure

Dynamic rheological measurements were performed on 1.5% wt agar sols and gels in the stress-controlled rheometer. The experimental procedure used for the \( T_g \) and \( T_m \) determinations during the optimization studies (Chapter I.3) is described next (Sousa et al., 2010; Sousa et al., 2012). A parallel plate geometry was used with a crosshatched acrylic geometry (4 cm diameter, 2 mm gap) to avoid slippage. The agar solution (prepared as described in section I.2.8.1) was loaded on the Peltier plate (pre-heated to 80 °C) after being degassed for 5 minutes in a vacuum oven, at 80 °C, to minimize the air influence in the tests (the degasification time chosen guaranteed a negligible water evaporation percentage of the sample solution). Excess sample was removed and its periphery was coated with paraffin oil to minimize evaporation. Hot solutions were cooled down to 25 °C at a rate of 2.33 °C /min,
while small amplitude oscillatory shear strain with 1% amplitude was applied at 1 Hz (6.28 rad/s), in order to probe the temperature evolution of linear viscoelastic properties such as tan δ. The $T_g$ was defined as the crossover point of the $G'$ and $G''$ (*i.e.* when the tan δ, equals 1). However, it is important to have in mind that this criterion is rather simplistic if one considers the kinetic nature of agar gelation process (Mohammed *et al.*, 1998). Nonetheless, this concept works quite well for the purpose of the present work. The time evolution of the storage modulus $G'$ and loss modulus $G''$ was followed at 25 °C (1% strain at 1 Hz) allowing the gel to equilibrate. Gels mechanical spectra were then measured in the linear regime by performing frequency sweeps at 1% strain. Finally, a heating scan (2.33 °C/min) to 95 °C was made with 1% strain at 1 Hz (6.28 rad/s), enabling the determination of the $T_m$ defined as the point at which tan δ=1.

In the case of Chapters I.4 and I.5(Sousa *et al.*, 2013), the rheological studies followed the procedure described above yet with some adjustments. A cone-and-plate geometry (4 cm diameter, 2° angle and a 54 µm gap) was used in all the determinations since preliminar tests showed consistent results with the parallel plate geometry. The hot agar 1.5 % wt solutions were placed in the Peltier at 80 °C. The cooling step for gel formation was performed at a constant rate of 1 °C/min and a fixed frequency of 1 Hz (6.28 rad/s) from 80 to 25 °C (at 1% strain amplitude). Gels were left to equilibrate at 25 °C before recording frequency sweeps (mechanical spectra) over the range 0.05 - 100 rad/s at 1% strain amplitude. Finally, the gel-sol transition was examined by submitting the samples to a heating step from 25 to 95 °C at a constant rate of 0.1 °C/min, at 1 % strain amplitude and a fixed frequency of 6.28 rad/s. Both $T_g$ and $T_m$ were determined according to the same criterion (*i.e.* tan δ=1) and the thermal hysteresis ($\Delta T$) was determined as $T_m$-$T_g$. The first order approximation of the Kronig-Kramers relations (Eq.I.2.21) proposed by Tschoegl (Tschoegl, 1989),

$$ G'' = \frac{\pi d}{2} \left( \frac{\sigma' (\omega)}{\ln(\omega)} \right) $$

(I.2.21)

was used to calculate $G''(\omega)$ from $G'(\omega)$ data and check if the frequency-sweep experiments were performed in the linear viscoelastic region (Sittikijyothin *et al.*, 2007).

The relationship between the magnitude of the complex viscosity, $|\eta^*|$, and the frequency, $\omega$, was determined using the power law equation (Eq.I.2.22),

$$ |\eta^*| = \eta_0 \left( \frac{\omega}{\omega_0} \right)^n $$

(I.2.22)
\[ |\eta^*| = K \omega^n \]  

(I.2.22)

Where

\[ |\eta^*| = \left( \frac{G_r^2 + G_i^2}{\omega} \right)^{1/2} \]  

(I.2.23)

\( K \) is the dynamic consistency index and \( n \) the dynamic power-law factor. For completely elastic or completely viscous systems \( n \) equals -1 and 0, respectively (Noda et al., 2008). All measurements were performed at least three times.

### I.2.8.3. Texture Analysis

#### I.2.8.3.1. Basic Principles

Texture analysis (TA) consists in the mechanical testing of consumer products such as food, pharmaceutical, adhesives and many others, with the intent of measuring some of their physical properties (http://www.stablemicrosystems.com/). It is a simple and cost-attractive methodology that allows the manufacturer to tune the product’s formulation according to the desired end-use application. It operates in the region of large deformations and, depending on the material, different tests can be carried out (e.g. tensile, compression, penetration). From the 'mouth feel' texture in foods or the flow behavior of creams and pastes to the break or stretching of a packaging material, this analysis covers a broad spectrum of crucial properties, in terms of consumer’s acceptance. In the development of new materials, it is also vital for the manufacturer to attest the quality of their product according to standardized tests. This will give the consumer/buyer comparable specifications between competitive products making easier the decision towards a given material.

The basic instrumentation used to carry out TA, i.e.a texture analyzer, is simple and easy to operate (Fig. I.2.11). The equipment measures the response of the material under large deformation and according to a predefined test speed. In the case of penetration tests, the arm (1) of the texture analyzer moves the loadcell (2) down, with the attached probe (3), to penetrate the material (4) while force, distance and time data are acquired and displayed in the interface (6). The test conditions can be set in the control panel (5). The test ends
when the arm returns to its initial position. This type of studies are particularly important to analyze food gels and were used to assess the quality of our agar gels. The quality indicator of reference for industries, GS, can be estimated from these experiments. These tests are standardized by different industries and for that reason cannot be considered universal. An example of a widely known standard universal test carried out by TA is the ‘Bloom test’ performed on gelatin gels (Gregson et al., 1999).

The basic principles underlying the tension tests, usually performed on packaging films, are similar, but instead of moving down, the load cell will move the superior grip up, stretching the material until its rupture. These tensile experiments were performed during the development of the 2nd stage of this PhD thesis, to assess the mechanical properties of the agar-pure and agar-based films, and will be presented in detail in Part II.

Depending on the sample and property we intend to measure, different probes can be considered. Probes can compress, extrude, cut, strecht, bend or fracture the material according to the customer desire. Examples of different probes are given in Fig. I.2.12. The mini otawa/kraemer cell used to compress coated apples (A), cylinder probes used to estimate GS of agar gels (B) and tensile grips (C) used to assess the mechanical resistance of packaging films. Several other devices can be incorporated in the texture analyzer to obtain other important properties such as acoustic energy released during the mechanical test, powder flows, volume and pressure, just to name a few.
I.2 MATERIALS AND METHODS

Fig. I.2.12 – Examples of probes and grips used to carry out texture analysis (A- mini Otawa/Kraemer cell; B- cylinder used for agar gels; C- tensile grips used for packaging films). The choice of the probe depends on the sample and property intended to determine.

Data from TA is considered empirical although attempts have been made to take a leap forward in terms of turning the extracted information more fundamental (Gregson et al., 1999). When used simultaneously with rheology, TA can provide very important and complementary information to the operator. For instance, the shear modulus estimated from the penetration tests data were shown to be correlated with rotational rheological data of gelatin gels, by a converting factor (Gregson et al., 1999). In the case of SP gels however, this relation was harder to infer since the results were highly dependent on the probe radius.

In the next section, the experimental procedure concerning the penetration tests of agar gels will be described while the tensile studies concerning the mechanical characterization of agar films will be detailed in Chapter II.2 concerning the Materials and Methods description of Part II.

I.2.8.3.2. Penetration Tests of Agar Gels

I.2.8.3.2.1. Experimental Procedure

The behavior of the equilibrated hydrogels under large deformations was examined using a texture analyzer (Stable Micro Systems model TA-XT2, Surrey, England). The failure stresses required for breaking the gels' surfaces and respective strains were determined through penetration tests performed with a cylindrical probe of 10 mm diameter at a penetration rate of 0.2 mm/s. At least three measurements were performed for each agar sample. GS, used as an indicator of agar gels' quality, was determined for each agar extract. This parameter is defined as the stress required for breaking the gel surface.
I.2.8.4. cryoScanning Electron Microscopy (cryoSEM)

I.2.8.4.1. Basic Principles

Electron microscopy is a high-resolution and non-destructive microscopic technique developed in the early 1930s to surpass the limited resolution of conventional light microscopy (LM). It is based on the simple idea that the wavelength of electrons is much smaller than the wavelength of visible light (used in LM) and uses electromagnetic coils to focus the high-energy electron beam instead of glass lenses. The first EM equipment to be built was the transmission electron microscopy (TEM). This equipment measures the electrons that pass through a thin and small area of material. It is particularly useful for instance, to investigate cellular components.

Scanning Electron Microscopy (SEM) is used to scan the surface of solid materials and generate 2D or 3D images by measuring electrons that are reflected and knocked off the sample (Figs. I.2.13 and I.2.14). Like in TEM, SEM can provide images at nano-scale, typically ~50-100 nm, scanning just a very small area of the material's surface. (source: http://serc.carleton.edu/research_education/geochemsheets/techniques/SEM.html by Susan Swapp, University of Wyoming). Different types of interactions between the incident electrons and the material will result in different signals, from which it can be extracted various information. The secondary electrons (SE) and the backscattered electrons (BSE) are responsible for the SEM images. While the former focus on the morphology and topography of the material the latter highlights contrasts in composition when imaging multiphasic samples. Information concerning the chemical composition of the samples can be extracted from characteristic X-ray signals using energy dispersive X-ray spectroscopy (EDS). These signals arise from the return of excited electrons to lower energy levels consequence of interactions between incident electrons with electrons in discrete atomic orbitals of the sample. This energetic difference will result in X-rays of fixed wavelengths, specific to each chemical element. The diffracted backscattered electron (EBSD) signals can be measured by simply attaching an adequate detector to the SEM apparatus. This device is particularly useful for determining crystalline structure and orientation of minerals. Other signals include visible light (cathodoluminescence- CL) and heat.
Fig. I.2.13 – Schematic representation of a basic scanning electron microscopy (SEM) equipment. Main components are identified accordingly. Abbreviations used: SE - secondary electrons detector; EDS – energy dispersive X ray spectroscopy; EBSD – electron backscatter diffraction detector; V- scanning control; SC- Signal conditioning.

Fig. I.2.14 – General scanning electron microscopy (SEM) setup (image gently provided by CEMUP, Porto, Portugal). Main components are identified by numbers (1- N₂ gas injector, 2 – EDS detector, 3- SE detector, 4- sample chamber, 5- electron column, 6- BSE detector, 7- computer).

CryoSEM (cryoScanning electron microscopy) is a particular type of SEM ideal for imaging things that contain moisture (e.g. food, hydrogels). In cryoSEM, the samples must be frozen in liquid nitrogen before the analysis. This avoids the need for critical drying needed to image samples in conventional SEM. The main advantage of this EM technique is the preservation of the material in its natural form (minimal artifacts induction). The sample preparation for this
type of analysis is carried out in a cryo chamber specially designed for the effect (inset of Fig. I.2.15).

![Cryo SEM setup](image)

**Fig. I.2.15 –** Cryo scanning electron microscopy (cryoSEM) setup. The inset figure highlights the cryo preparation chamber (image gently provided by CEMUP, Porto, Portugal).

### I.2.8.4.2. Experimental Procedure

The cryoSEM studies of agar hydrogels (1.5% wt) were performed at CEMUP, Porto, Portugal. A small sample volume (approx. 1-3 mm$^3$) was mounted on aluminum stubs and rapidly freeze-dried through quick plunging in a nitrogen slush (-210 °C). After freezing, the sample was transferred to an ALTO 2500 cryo preparation chamber and placed onto a cool stage (-150 °C), where it was fractured. The ice formed on the exposed fractured gel surface was removed by sublimation at -90 °C for 1.5 min. Afterwards, the sample was coated with sputtered Au-Pd thin film at -150 °C for 40 sec, from a sputter head using ultrapure argon gas. The analysis was performed at -150 °C in a JEOL JSM 6301F scanning electron microscope equipped with a Gatan ALTO 2500 cryo preparation chamber using an accelerating voltage of 15 kV and working distances of 15 mm (Fig. I.2.15).
References.


Cabrita, E. J. (2010). NMR from theory to practice. *NMR courses FCT-UNL*.


CHAPTER I.3

I.3 MAE OPTIMIZATION
As explained in the previous chapter, concerning the Materials and Methods, both process optimizations (i.e. NA and AA) were carried out using IMTA biomass. For clarity purposes, in the current chapter it will only be presented the statistical approach used to find the optimal MAE conditions. Everything concerning the comparison between extracts from both processes and types of biomasses will be shown in the following chapter (Chapter I.4, Characterization of the Extracted Agars). Two papers were published from the work developed during this stage of the PhD thesis (Sousa et al., 2010; Sousa et al., 2012).

I.3.1. Optimization Strategy

MAE optimizations of AA and NA were made using response surface methodology, RSM (Montgomery, 1991). It is a combination of mathematical and statistical techniques used to analyze problems where the response of interest is affected by several factors with complex interactions. The main goal of RSM is to optimize this response or determine the region that satisfies the operating specifications. This procedure involves fitting a function to the experimental data and then using optimization techniques to obtain the optimum parameters (Garg et al., 2008).

The first performed optimization concerned MAE of AA. Due to the lack of information related to agar MAE, the experimental domain was defined taking into account preliminary tests as well as the operative limits of the instrument. All significant parameters in a typical MAE process were chosen: extraction time ($X_1$; min), temperature ($X_2$; °C), solvent volume ($X_3$; ml) and stirring speed ($X_4$; 4 positions are available in modern apparatus: turned off, minimum, medium and maximum speed). The response variables studied in the case of AA were, yield ($Y_1$; %), GS ($Y_2$; g/cm$^2$), $T_g$ ($Y_3$; °C) and $T_m$ ($Y_4$; °C), and sulfate ($Y_5$; %) and LA ($Y_6$; %) contents. An orthogonal central composite design with four parameters, $2^4$, was the approach made to the optimization problem. This design included 36 experiments to estimate the models coefficients: 16 points of a factorial design at levels $\alpha=\pm1.000$, 8 axial points at a distance $\alpha=\pm2.000$ from the center, and a center point with 12 replications (Table I.3.3). The respective factors and levels of the AA process optimization are described in Table I.3.1.
Table I.3.1 - Factors (X) and Levels (α) of the $2^4$ Orthogonal Central Composite Design of AA from IMTA G. vermiculophylla.

<table>
<thead>
<tr>
<th>Xi</th>
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</table>

In the case of NA, the optimization approach was slightly readjusted taking into consideration the specificities of this type of agars as well as the knowledge obtained with the MAE optimization of AA (Table I.3.2). Since yield and GS are the most important variables for agar industry and because all other responses considered during the first MAE optimization ($T_g$, $T_m$ as well as LA and sulfate contents) were found to be somehow related with GS, only yield ($Y_1$; %) and GS ($Y_2$; g/cm$^2$) were considered as response variables for the second MAE optimization. The $T_g$ and $T_m$ as well as chemical properties were only determined for the optimal NA extracts. Optimization results of the process optimization of NA are listed in Table I.3.5.

Table I.3.2 - Factors (X) and Levels (α) of the $2^4$ Orthogonal Central Composite Design of NA from IMTA G. vermiculophylla.

<table>
<thead>
<tr>
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</table>

The 12 replicates at center point allowed estimating the experimental error and checking the fit. The results in the initial set of experiments (e.g. runs 1-16 in Table I.3.3 for AA) were fitted to a first order model and its adequacy was checked. If the lack of fit was not significant, steepest ascent method should be applied in order to move rapidly to the optimum region. On the contrary, if the first order model lack of fit reached significance, probably due to a quadratic effect, additional runs were performed to improve the model adjustment. Then, experimental data were fitted to the following second order model, Eq. I.3.1 (Montgomery 1991),
where \( Y_i \) is the experimental response, \( X_i \) are the studied factors, \( b_0 \) is the average response, \( b_i \) are the average effects of the different factors, \( b_s \) are the average effects of second interaction factors, \( b_{ii} \) are the quadratic components and \( \varepsilon \) is the experimental error. The lack of fit in the second order model is desired to be not significant and, if it persists, steepest ascent method should be used.

All statistical analyses were made using the software Statistica version 6.0 (StatSoft, Inc., Tulsa, UK), namely, multifactor variance analysis (ANOVA) and response surface 3D plots. The two factors not represented by the horizontal axes were fixed at their 0 level values.

In order to validate a model, appropriate analysis of variance (ANOVA) must be carried out (Masmoudi et al., 2008). The total sum of squares of the mathematical model is divided into the sum of squares due to the regression (SS model in Tables I.3.4 and I.3.5) and the residual sum of squares (SS residual in Tables I.3.4 and I.3.5). The latter, can be divided in two parts: one part due to pure experimental error and is computed as the sum of squared deviations (SS pure error in Tables I.3.4 and I.3.5) in the center point experiments, and the second part corresponds to the lack of fit (SS lack of fit in Tables I.3.4 and I.3.5). The fitted models are considered adequate if they reach significance (p-value<0.05 for a 95% confidence level) and their lack of fit is not significant (p-value>0.05 for the same confidence level).

Significance of each coefficient present in regression equations was determined by the student’s t-test and p-values. Optimal extraction conditions were obtained by surface 3D plots inspection and based on statistical information. The p-value at a 95% confidence level for AA and 90% for NA was also used to confirm the significance of the studied factors and their interaction effects. The relationship between two agar properties was determined by Pearson’s correlation analysis. All experiments were performed in randomized order to minimize bias effect.

### I.3.2. MAE Optimization of AA

#### I.3.2.1. Yield

Yield results obtained in the first set of runs were adjusted to a first order model (runs 1-16 in Table I.3.3) which revealed a very significant lack of fit (p<0.0001), probably due to a
quadratic effect. Therefore, additional runs were performed in order to achieve optimum conditions (runs 29-36 in Table I.3.3). Due to experimental limitations, runs 33 (10 mL of solvent are not enough for totally immersing the sample) and 36 (the equipment does not have a stirring speed higher than the maximum one) were not performed nor statistically considered by the software. The second order model showed high statistical significance (p<0.0001; Table I.3.4) however, its remarkable lack of fit persisted (p<0.0001; Table I.3.4) suggesting steepest ascent method should be applied. This apparent contradiction may be due to the insufficient number of experimental observations to produce an appropriate analysis of the residues because of the high number of studied parameters (Domingos et al., 2008). The second order model quadratic correlation coefficient, $R^2=0.9024$, can be considered acceptable for data of chemical nature (>0.8; (Lundstedt et al., 1998)), advocating a good correlation between observed and predicted values. Steepest ascent method suggested operational parameters values impossible to apply, consequently, optimum yield was determined by 3D surface plots analysis and statistical information. Additional tests showed that higher temperatures, 100 and 110 °C (with remaining parameters set at center point values) produced an increase on yield, respectively, 10.6±0.4 and 12.4±1.5%, although GS decreased but still fulfilling industrial standards (> 700 g/cm$^2$ in a 1.5% solution (Pereira-Pacheco et al., 2007)). Complete polysaccharide degradation occurred at 120 °C due to heating excess. Low temperatures (<60 °C) resulted in poor polymer recovery (less than 1%).

As reported in other MAE studies concerning polymers (Costley et al., 1997; Marcato & Vianello, 2000), temperature had the most significant influence on yield (p<0.0001) with higher temperatures clearly improving results (Fig.I.3.1 and Table I.3.3). At high temperatures, the rate of extraction increases because the viscosity and the surface tension decrease, while solubility and diffusion rate into the sample increase. However, draconian extraction conditions usually affect negatively the extraction selectivity. Therefore, 110 °C was admitted as an optimum possibility.
Solvent volume showed a negative influence (p<0.05) on yield (except on runs 5-7 where a slight increase in response was verified when increasing solvent volume and runs 2-4, 9-11 which revealed equal yields, Table I.3.3), contrary to its quadratic effect that positively influenced yield results (p<0.05). 3D surface plots analysis revealed that, enhanced yields were achieved with higher temperatures (100 °C or more) coupled with maximum solvent volume (50 ml). In order to ensure enough space in the vessels to promote solvent volatilization to attain the selected temperature (above the solvent’s boiling point at atmospheric pressure), a 40 ml solvent volume was chosen as the most appropriate to assure a reproducible and safe process.

Stirring speed interacted significantly with temperature (p<0.05) with high temperatures needing lower stirring speed rates (minimum or without) in order to achieve better yields (Fig. I.3.1). Stirring speed quadratic effect also reached negative significance (p<0.05). On the contrary, all the runs performed at 70 °C (runs 1-4 and 9-12, in Table I.3.3), produced enhanced yields when increasing stirring speed. This behavior was also observed for runs with 10 minutes extraction time and 90 °C (runs 5-8 in Table I.3.3). This pattern of variation inverted when high temperatures (≥90 °C) and longer extraction times (≥20 minutes) were applied (runs 13 to 16, in Table I.3.3) reaching the best yields. 3D surface plot analysis corroborated this information, where longer extraction times (20-25 minutes) and minimum speed of agitation seemed to produce the best results. Because extraction time was not an influential parameter in yield response (p>0.05), 20 minutes was the extraction time chosen as optimum (Fig.I.3.1). Maximum yields were obtained with runs 6 and 13 operational
condition, respectively, 13.5% and 13.7% (Table I.3.3). Therefore, possible optimum yield conditions were studied at 110 °C, 20 minutes of extraction, 40 ml of solvent, and minimum/without stirring. Five replicates were done for each set of operational parameters. Runs 6 (13.2±0.4%) and 13 (13.4±0.3%) were also investigated and no significant difference (p>0.05) was found among treatments. Regarding 110 °C optimum possibilities, the agar yields reached with the first set (with minimum stirring speed) were 14.8±0.6% and 14.4±0.4% with the second one (with no stirring). A Student’s t-test was applied and no significant difference (p>0.05) between both groups was detected, consequently 20 minutes, 110 °C, 40 ml of solvent and no agitation was chosen as best option. On the contrary, significant differences were observed when comparing runs 6 and 13 with 110 °C optimum, therefore, optimum yield conditions were defined as: 20 minutes extraction, 110 °C, 40 ml of solvent and no agitation.

The extractions with yield values lower than 3% were statistically ignored in terms of the other response variables.

I.3.2.2. Gel Strength (GS)

In the initial set of experiments (runs 1-16 in Table I.3.3), the lack of fit of GS first order model was not significant (p>0.05) suggesting steepest ascent method should be applied, however, it suggested parameters values impossible to put in practice. Therefore, additional experiments were carried out to achieve optimum conditions (runs 29-36 in Table I.3.3). Second order model lack of fit was very significant (p<0.01), as well as its statistical significance (p<0.01) (Table I.3.4). Steepest ascent method could not be applied and GS second order model did not compute a satisfactory solution. GS quadratic correlation coefficient was considered satisfactory, R²=0.8313 (Lundstedt et al., 1998). ANOVA results, revealed that quadratic effect of extraction time reached positive significance in GS (p<0.01) (runs 29 and 30 in Table I.3.3). 3D surface plots analysis showed that temperatures in the range of 90-100 °C produced stronger gels for shorter times (5 minutes) (Fig.1.3.2), as well as maximum stirring speed and the same extraction time. Considering energy savings and that the highest GS value was obtained for 5 minutes extraction time (~1103 g/cm²), this was the optimum time chosen.
Solvent volume had a negative effect in GS (runs 2-4, 5-7, 10-12, 13-15 and 14-16 in Table I.3.3) and 3D surface plots analysis revealed that higher GS values were obtained for 20 ml of solvent with shorter (5 minutes) and/or longer (25 minutes) extraction times. Also, low solvent volumes (20 ml) associated with temperatures in the range 80-90 °C produced stronger gels. Clearly, maximum stirring speed favored GS (runs 1-16 in Table I.3.4 with the exception of runs 5-6). This information was corroborated by 3D surface plots where temperatures in the range 80-90 °C with maximum speed ensured stronger gels, as well as low solvent volume (20 ml) with the same stirring rate. Therefore, MAE conditions for optimum GS were considered to be: 5 minutes of extraction, 90 °C, 20 ml of solvent and maximum stirring speed. Five independent extractions were carried out using the above referred parameters and a GS of 1331±51 g/cm² was obtained.

I.3.2.3. Gelling and Melting Temperatures

For T_g, the first order model lack of fit was significant (p<0.05) probably due to a quadratic effect, and so, remaining experiments were performed (runs 29-36 in Table I.3.3). The second order model lack of fit was significant (p<0.05) and the model reached high statistical significance (p<0.001) (Table I.3.4). Again, steepest ascent method could not be successfully
applied, indicating values for the operational parameters impossible to put in practice. $T_g$ canonical form of the model predicted a saddle point, as so, optimum conditions were found by 3D plots observation and statistical analysis. The quadratic correlation coefficient, $R^2=0.8623$, may be considered acceptable stating good model predictability (>0.8; (Lundstedt et al., 1998)). Globally, stronger gels revealed higher $T_g$, with the highest value, 40.7 °C, being associated to the strongest gel. Temperature and solvent volume had a very significant positive interaction ($p<0.001$) on $T_g$. On the contrary, extraction time and temperature produced a negative interaction, with temperatures in the range of 90-100 °C and 5 minutes extraction time producing the highest $T_g$. Extraction time quadratic effect was very significant ($p<0.01$). 3D surface plot analysis also revealed that shorter extraction times (5 minutes) and lower solvent volumes (20 ml) produced best responses. The same happened with shorter extraction times and maximum stirring speed or longer extractions (25 minutes) with no agitation (Fig.I.3.3).

Fig. I.3.3 - Response surface of gelling temperature, $T_g$ (°C) of AA from G.vermiculophylla obtained by MAE as a function of extraction time ($X_1$) and stirring speed ($X_4$) (temperature ($X_2$) = 80 °C, solvent volume ($X_3$) = 30 ml).

A positive correlation was found between GS and $T_g$ results ($r=0.73$, $p<0.01$). Therefore, the set of operational parameters to attain the optimal $T_g$ is the same of optimal GS: 5 minutes extraction, 90 °C, 20 ml solvent volume and maximum stirring speed. Five independent experiments were also performed to verify the selected conditions. A $T_g$ of 40.7±0.2 °C was measured for AA produced by MAE which was in the range defined by the US Pharmacopoeia (32-43 °C; (Orduna-Rojas et al., 2008)) and is suitable for international market.
For $T_m$, the lack of fit of the first order model was not significant ($p>0.05$) suggesting steepest ascent method should be applied in order to move more rapidly to optimum vicinity. This technique could not be successfully applied and so, the remaining experiments were carried out (runs 29-36; Table I.3.3). As desired, second order model lack of fit was not significant ($p>0.05$) and it revealed high statistical significance ($p<0.01$) (Table I.3.4). The model predicted a saddle point, thus optimum $T_m$ was obtained by statistical information and 3D surface plots analysis. The quadratic correlation coefficient ($R^2=0.7784$) was slightly below the minimum value acceptable for data of chemical nature. In accordance with several authors (Freile-Pelegrin & Murano, 2005; Orduna-Rojas et al., 2008; Villanueva & Montano, 1999; Villanueva et al., 2010), $T_g$ and $T_m$ were positively correlated ($r=0.72$, $p<0.01$), and both temperatures had the same kind of correlation with GS (for $T_m$, $r=0.92$, $p<0.01$), with best results for the three responses occurring in the same run (run 29; Table I.3.3). Temperature and solvent volume had a significant positive interaction in $T_m$ ($p<0.05$). Extraction time quadratic effect reached positive significance ($p<0.01$). 3D surface plots analysis revealed that short extraction times (5 minutes) and temperatures in the range of 90-100 °C, as well as long times (25 minutes) coupled with temperatures in the range of 70-80 °C, produced the best responses. This information was corroborated by runs 29 (5 minutes extraction time; 92.9 °C) and 30 (25 minutes; 92.4 °C). Also, solvent volumes in the range of 20-45 ml and 5 minutes extraction time produced higher melting temperatures, and the same was verified for longer extractions (25 minutes) and 20-40 ml of solvent. Maximum stirring speed was the most favorable parameter for shorter and longer extractions times (Fig. I.3.4). Also, 20 ml solvent volume produced best results when using maximum speed (runs 1-2 and 13-14). Therefore, $T_m$ optimum conditions were considered to be the same as GS and $T_g$ and were verified performing five independent experiments. AA yielded a high $T_m$ (93.1±0.5 °C) clearly meeting the commercial standards, >85 °C (Orduna-Rojas et al., 2008).
I.3.2.4. Sulfate Content

Sulfate content first order model did not show a significant lack of fit (p>0.05) suggesting steepest ascent application in order to move more rapidly to optimum vicinity. This technique could not be operationally applied. Remaining experiments were carried out and second order model reached high statistical significance (p<0.05), yet, revealing significant lack of fit (p<0.05) (Table I.3.5). The model did not compute a satisfactory solution ($R^2 = 0.7258$), therefore, optimum conditions were found by surface graphs observation and statistical information analysis. Temperature quadratic effect reached positive significance (p<0.05), with temperatures in the range of $85\text{-}100 ^\circ C$ and 5 minutes extraction time giving best responses. Experimental data revealed that increasing the temperature (70 to 90 °C) resulted in decreasing sulfate contents (runs 1-5, 2-6, 4-8, 10-14 and 12-16 in Table I.3.3). Low solvent volume (20 ml) and time (5 minutes) originated the best results. Maximum stirring speed applied simultaneously with a 90 °C extraction temperature and 20 ml solvent volume (runs 5-6 and 13-14) presented better experimental responses. Interpretation of 3D plots corroborated this behavior.

Sulfate content was negatively correlated with GS, $T_g$ and $T_m$ (respectively, $r=-0.72$ (p<0.01); $r=-0.76$ (p<0.01); $r=-0.69$ (p<0.01)). Therefore, the optimum conditions selected were the same (5 minutes extraction, 90 °C, 20 ml solvent volume and maximum speed).
Five independent extractions were performed and allowed to verify this assumption. AA from IMTA *Gracilaria* had a sulfate content of 1.73±0.13%. The international food market currently requires sulfate content less than 4%, usually 1.5-2.5% (Armisen, 1995) and all results reached are within the acceptable range.

### 1.3.2.5. LA Content

LA content first order model did not reveal a significant lack of fit (p>0.05) suggesting steepest ascent method should be applied. This technique suggested operational parameter values impossible to apply; so, remaining experiments were performed (Table I.3.3). In the second order model the lack of fit was not significant (p>0.05) however, the model did not reach statistical significance (p>0.05). The quadratic correlation coefficient was low, $R^2=0.6730$, meaning that only 67.30% of the variability in the data was accounted by the model. This poor model predictability may be due to more complex parameters interactions that were not sufficiently explained by the number of runs performed. Therefore, optimum LA content was found based only on the experimental results (Table I.3.3).

Five experiments were carried out at maximum LA content operational conditions: 10 minutes extraction, 90 °C, 40 ml of solvent and minimum stirring speed (run 7 in Table I.3.3) reaching an average value of 39.4±0.3%. Pearson’s correlation analysis revealed that all gel properties were correlated positively with LA content (GS, $r=0.67$ (p<0.01); $T_g$ $r=0.70$ (p<0.01) and $T_m$ $r=0.64$ (p<0.01) temperatures), except the sulfate content which was negatively correlated ($r=-0.81$ (p<0.01)). Marinho-Soriano and Bourret reported for *G. dura* a positive correlation of LA with GS, however no significant correlation between sulfate content and GS was observed (Marinho-Soriano & Bourret, 2005).
Table I.3.3 - Real Values and Coded Levels for the Experimental Design $2^4$ of AA ($X_1$ – Extraction Time; $X_2$ – Temperature; $X_3$ – Solvent Volume; $X_4$ – Stirring Speed) and Results for all the Response Variables Studied, yield ($Y_1$ %), GS ($Y_2$ g/cm$^2$), $T_g$ ($Y_3$ °C), $T_m$ ($Y_4$ °C), sulfate ($Y_5$ %) and LA ($Y_6$ %) contents.

<table>
<thead>
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<th>Run</th>
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<th>$X_3$</th>
<th>$X_4$</th>
<th>$Y_1$</th>
<th>$Y_2$</th>
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$2^4$ factorial design with twelve replicates at the C.P.
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**Additional runs – model expansion**

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n.a. – not available, the equipment does not have a stirring speed higher than the maximum one; n.d. – not determined; C.P. – center point; t.o.- turned off.
### Table I.3.4 - Analysis of Variance (ANOVA) for Regression Models of AA.

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SS=Sum of squares; DF=Degree of freedom; MS=Mean square; R²=quadratic correlation coefficient.
I.3.3. MAE Optimization of NA

The coefficient estimates of the regression models for the two studied response variables (i.e. yield, $Y_1$ and GS, $Y_2$) were determined according to experimental results (data not shown). By eliminating the non-significant parameters (p>0.10 in Table I.3.5), response surface regression gave the following model equations for the recoded factor values ($\pm 1, 0, \pm \alpha$):

\begin{equation}
Y_1 = 19.00 + 1.410X_1 + 2.208X_2 + 0.658X_3 - 1.683X_1^2 - 1.280X_2^2 - 1.205X_3^2 - 1.434X_4^2 + 0.838X_1X_3 + 1.088X_2X_4
\end{equation}

\begin{equation}
Y_2 = 31.36 + 7.524X_1 + 22.56X_2 + 8.634X_3 + 15.46X_4 - 8.519X_2^2 + 12.44X_2X_4 + 8.915X_3X_4
\end{equation}

As desired, yield second order model (Eq. I.3.2) reached high statistical significance (p<0.0001) contrarily to its lack of fit (p>0.05). The high F-value of the model (9.21) was indicative of the factors significant effect in the response (Table I.3.5). The significant (p<0.05) effects assumed by the prediction equation were $X_1$, $X_2$, $X_1^2$, $X_2^2$, $X_3^2$ and $X_4^2$, while $X_3$ and $X_1X_3$ were marginally significant (p<0.10). Clearly, a strong curvature effect of the factors in yield was observed. The value of $R^2=0.87$ and adjusted $R^2=0.77$ stated a good degree of correlation between observed and predicted values. $R^2$ was clearly above the minimum accepted for data of chemical nature (>0.8; (Lundstedt et al., 1998)) and so, the model was selected. The software predicted a maximum agar yield of, $Y_1=20.4\%$, at critical values $X_1=19.5$ min; $X_2=114.9$ °C; $X_3=48.3$ mL; $X_4=2.9$ (maximum speed) which was considered.

GS 2nd order model (Eq. I.3.3) also reached high statistical significance (p<0.0001) and the lack of fit was found to be insignificant (p>0.05) meaning that the fitted model adequately represented the experimental results (Table I.3.5). Again, $R^2$ value (0.85) was above the minimum limit of acceptance for chemical data. All the main effects ($X_1$, $X_2$, $X_3$, $X_4$) were considered significant (p<0.05) by the model as well as, $X_3^2$, $X_2X_4$ and $X_3X_4$. The software predicted a saddle point close to a minimum and so, it was not considered.

An accurate observation of the experimental data, surface 3D plots and ANOVA results was carried out in order to ensure an adequate interpretation of RSM. All parameters were considered in the design with the intent of minimizing the error. $X_2$ was the most influential parameter in the yield of NA MAE (p<0.0001; Table I.3.5) with values in the range 110-120 °C clearly improving results, reaching its maximum at around 115 °C (e.g. Fig. I.3.5). As seen before for AA, solubility of the target compound and diffusion rate to the
sample-matrix are favored by high temperatures due to the decrease in surface tension and viscosity (Sousa et al., 2010).

Additional experiments performed at higher temperatures (130 °C) showed that, after this point, polysaccharide degradation took place, decreasing the recovery (~30% less; results not shown). Therefore, operating with temperatures above 120 °C should be avoided. The negative effect of \( X_2^2 \) (p<0.01) was in line with these findings. High speeds of agitation (medium and maximum) coupled with temperatures in the optimum range (110-120 °C) attained the best recoveries (p<0.05; Fig. I.3.5). A positive relation was found between the solvent amount and the time used in the extraction process (p<0.10; figure not shown). Besides the predicted maximum point, the best sets of experimental conditions (set1: 20 min, 110 °C, 30 mL and minimum speed, \( Y_1=18.3\% \); set2: 20 min, 110 °C, 50 mL and maximum speed, \( Y_1=19.8\% \); set3: 15 min, 120 °C, 40 mL and medium speed, \( Y_1=19.7\% \)) were tested. Replicates of each set of parameters showed that the response obtained with the critical point (19.3±1.1%) was not significantly different (p>0.05) from the remaining conditions (set1: 18.3±4.8%; set2: 19.7±2.2% and set3: 19.6±1.0%). This could be attributed to the variability not accounted for by the regression equation.

\( X_2 \) was also the most influential effect on the GS of NA (p<0.0001; Table I.3.5) with an increase in temperature clearly producing an improvement in agar gelling properties (e.g. Fig. I.3.6). A strong \( X_2X_4 \) interaction was found (p<0.01), with high values of both parameters.
producing the best results (figure not shown). Highest GS was achieved with 15 min, 120 °C, 40 mL of solvent and medium speed of agitation (set3; $Y_2=80.5 \text{ g/cm}^2$). Additional experiments proved that, above this temperature limit, NA were degraded and their gelling properties were compromised. $X_3$ had a positive influence in GS ($p<0.05$) with values in the range 40-60 mL being the best option. Outside these limits, a decrease in response was observed ($X_3^2$ negative effect; $p<0.05$). The $p$-value of $X_3X_4$ (<0.05) stated a positive interaction between volume and speed of agitation in the GS of the polymer. Finally, extraction times in the range of 15-25 min favored GS ($p<0.05$; Table I.3.5 and Fig. I.3.6).

![Fig. I.3.6 - Response surface of gel strength, GS ($Y_2; \text{ g/cm}^2$) of MAE NA from G.vermiculophylla as a function of extraction time ($X_1; \text{ min}$) and temperature ($X_2; ^\circ\text{C}$) (solvent volume ($X_3$) = 40 mL and stirring speed ($X_4$) = medium).](image)

Considering energy and solvent saving concerns, lower range limits of solvent volume (40 mL) and extraction time (15 min) were chosen, as no significant differences were found in the response when considering lower/upper limit values. Remaining parameters were fixed at their maximum levels: 15 min of extraction, 120 °C, 40 mL of solvent and maximum stirring speed (set4 conditions). Experimental run that led to the highest GS (set3) was also investigated. Replicates of the tested set of optimal conditions showed that, clearly, set3 led to the best gelling properties (78.0±2.3 g/cm² against 53.3±1.0 g/cm² of set4 conditions; $p<0.05$). The observed differences could be related to problems with the model predictability (15.17% of unexplained variability in the data by the model); therefore, optimal GS conditions were considered to be set3 conditions: 15 min of extraction, 120 °C, 40 mL of solvent and
medium stirring speed. As no significant differences were found between all tested set of parameters for agar recovery ($Y_1$), set3 conditions were also chosen for $Y_1$.

Table I.3.5 - Analysis of Variance (ANOVA) for the Regression Models of NA MAE. $X_1$ – Extraction Time (min); $X_2$ – Temperature (°C); $X_3$ – Solvent Volume (mL); $X_4$ – Stirring Speed.

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F-value</th>
<th>p</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
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<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8132.43</td>
<td>14</td>
<td>580.89</td>
<td>7.99</td>
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</tr>
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<td>11.93</td>
<td>14.78</td>
<td>0.0010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>339.68</td>
<td>1</td>
<td>339.68</td>
<td>4.67</td>
<td>0.0429&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
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<td>1</td>
<td>447.29</td>
<td>6.15</td>
<td>0.0221&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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<td>24.14</td>
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<td>143.19</td>
<td>1.97</td>
<td>0.1758&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.0013&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.8319&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.3119&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.2057&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.0770&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.25</td>
<td>0.6201&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.02</td>
<td>0.8864&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>211.56</td>
<td>2.91</td>
<td>0.1035&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.08</td>
<td>0.09</td>
<td>0.7627&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.14</td>
<td>1</td>
<td>6.14</td>
<td>0.08</td>
<td>0.7744&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>$X_2X_4$</td>
<td>4.73</td>
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<td>4.73</td>
<td>5.86</td>
<td>0.0251&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>618.52</td>
<td>8.51</td>
<td>0.0085&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.3694&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.0495&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>Resid.</strong></td>
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<td>20</td>
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<td>1454</td>
<td>20</td>
<td>72.70</td>
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<tr>
<td><strong>Lack of fit</strong></td>
<td>5.25</td>
<td>9</td>
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<td>0.59</td>
<td>0.7822&lt;sup&gt;c&lt;/sup&gt;</td>
<td>864.91</td>
<td>9</td>
<td>96.10</td>
<td>1.79</td>
<td>0.178&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><strong>Total</strong></td>
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<td></td>
<td>9586.35</td>
<td>34</td>
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</table>

$R^2$= 0.87, $R^2$ adjusted = 0.77

| SS=Sum of squares; DF=Degree of freedom; MS=Mean square; Resid.= residual; $R^2$= quadratic correlation coefficient; <sup>a</sup>significant ($p<0.05$); <sup>b</sup>marginally significant ($p<0.10$); <sup>c</sup>not significant ($p>0.10$).
I.3.4. Conclusions

The statistical approach outlined for MAE optimization was efficient at obtaining optimal extraction conditions for NAs and AAs. For clarity purposes, further conclusions about the MAE process won't be taken at this point. Everything concerning this topic will be discussed in the following Chapter I.4, Characterization of the Extracted Agars, where differences between MAE and TWE processes, seaweed cultivation conditions as well as NA and AA will be addressed in detail.

References.


I.4 CHARACTERIZATION OF THE EXTRACTED AGARS: INFLUENCE OF EXTRACTION PROCESS AND SEAWEED GROWTH CONDITIONS

CHAPTER I.4
As explained in the introductory section, the structural regularity of agar’s backbone is often modified by the presence of substituent groups. These modifications, induced by physiological (seaweeds’ life cycle) and environmental (growth conditions, season of collection) aspects as well as the extraction conditions, will define the final properties of the SP (Murano, 1995; Rodriguez et al., 2009). For instance, the alkaline treatment of agar causes the conversion of the L6S monomers in LA units (Rodriguez et al., 2009; Romero et al., 2008). Sulfates at C6S are an exception in that they are eliminated during the formation of the anhydride rings. All the other sulfate groups present in agar skeleton are stable to this alkaline modification (Lahaye & Rochas, 1991). Throughout the current chapter all these aspects will be extensively covered.

To this end, the optimal conditions of each process (i.e. MAE and TWE) were applied to wild and farmed Gracilaria to extract NA and AA. Once again, it is worth pointing out that, the data presented in the current chapter might differ from the ones obtained during the optimization step (Chapter I.3) because two different batches of seaweeds were used. Each batch was a mixture of the several IMTA seaweed tanks (thus minimizing differences of cultivation conditions) but originated from different cultivation periods. Since this cultivation system is outdoors, some influence from irradiance and/or temperature seasonal differences may be expected.

Part of the results presented here can be found in three publications (Sousa et al., 2012; Sousa et al., 2013a; Sousa et al., 2013b).

I.4.1. Agars from IMTA Gracilaria

I.4.1.1. Native Agars (NA)

I.4.1.1.1. Structural Analysis

Tables I.4.1 and I.4.2 show respectively, the obtained $^{13}$C and $^1$H chemical assignments of NA from IMTA Gracilaria obtained by MAE and TWE. Resonances found by other authors are also included in the tables for comparison purposes. The observed displacement (~2.2 ppm more downfield) between the obtained chemical shifts when compared with other reports (Murano, 1995; Usov et al., 1980) are attributed to differences in the chemical shift of the reference used by the authors relative to what was recently found when measured in polar solvent (Villanueva et al., 2010). As temperature and stirring speed were the most influential parameters in the studied responses of NA MAE, an additional sample was
considered in the structural analysis in order to better understand the changes induced by microwave heating in the NA structure (MAE: 15 min, 100 °C, 40 mL of solvent and no agitation). HSQC spectra of NA.IMTA.MAEopt (A), NA.IMTA.TWEopt (B) and NA.IMTA.MAE1 (C) are presented in Fig. I.4.1. The 12 characteristic signals of agarose were assigned in the $^{13}$C NMR spectra of the three NA samples. This was positively compared with CA (spectra not shown). L6S residues were detected in both MAE agars (Figs. I.4.1 A and C)) through the presence of a minor cross-peak at (~5.28; 103.5) ppm attributed to H1 and C1 of L6S, respectively (Villanueva et al., 2010). This peak was absent in the CA spectrum. Typical C1 signal of G (~105.6 ppm) in G-L6S diads was barely discernible in $^{13}$C NMR spectra (Table I.4.1)(Rodriguez et al., 2009; Usov et al., 1980). The absence of the respective cross peak of L6S units in NA.IMTA.TWEopt(Fig. I.4.1 B)) was expected since this sample needed to be diluted due to its high viscosity. Nevertheless, it was clear from its $^1$H NMR spectrum the presence of the typical signal of the LA precursor residues (5.28 ppm; horizontal trace in Fig. I.4.1 B)).
### Table I.4.1 - Assignments of Major Chemical Shifts (ppm) in the $^{13}$C NMR Spectra of NA_IMTA_TWE$^{opt}$, NA_IMTA_MAE$^1$ and NA_IMTA_MAE$^{opt}$. Comparison with Assignments of other Reports. The Displacement Observed in the Assignments Results from Differences in the Reference Compound Used (See Discussion for Details).

<table>
<thead>
<tr>
<th>CARBON CHEMICAL SHIFT (PPM)</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>M</th>
<th>Ref.</th>
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<td>70.1</td>
<td>82.2</td>
<td>68.6</td>
<td>75.2</td>
<td>61.3</td>
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<td>69.7</td>
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<td>(Usov et al., 1980)</td>
</tr>
<tr>
<td><strong>LA</strong></td>
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<td>70.0</td>
<td>80.0</td>
<td>77.4</td>
<td>75.6</td>
<td>69.0</td>
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<td>59.1</td>
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<td>68.7</td>
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<tr>
<td><strong>G(L6S)</strong></td>
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<td>70.5</td>
<td>80.9/81.0</td>
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<td>61.6</td>
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<td>(Falshaw et al., 1999)</td>
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<tr>
<td><strong>LA</strong></td>
<td>98.1</td>
<td>69.8</td>
<td>80.0</td>
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<td>78.1</td>
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**CARBON CHEMICAL SHIFT (PPM)**

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<td>MAE&lt;sup&gt;opt&lt;/sup&gt;</td>
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<tr>
<td>G6M</td>
<td>104.6</td>
<td>72.2</td>
<td>84.6</td>
<td>71.2</td>
<td>76.0</td>
<td>72.8</td>
<td>61.5</td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>100.7</td>
<td>72.6</td>
<td>82.5</td>
<td>79.8</td>
<td>78.2</td>
<td>71.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>104.9</td>
<td>72.3</td>
<td>n.d.</td>
<td>71.3</td>
<td>78.2</td>
<td>63.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA2M</td>
<td>100.7</td>
<td>80.5</td>
<td>80.1</td>
<td>79.8</td>
<td>78.1</td>
<td>71.5</td>
<td>61.5</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>105.9&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>71.6</td>
<td>84.4</td>
<td>71.3</td>
<td>77.9</td>
<td>63.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L6S&lt;sup&gt;(1)&lt;/sup&gt;</td>
<td>103.5</td>
<td>71.3</td>
<td>72.8</td>
<td>80.5</td>
<td>72.5</td>
<td>69.9</td>
<td></td>
<td></td>
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<tr>
<td>G</td>
<td>104.8</td>
<td>72.3</td>
<td>84.6</td>
<td>71.2</td>
<td>77.8</td>
<td>63.8</td>
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</tr>
<tr>
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<td>100.7</td>
<td>71.8</td>
<td>82.5</td>
<td>79.8</td>
<td>78.1</td>
<td>71.4</td>
<td></td>
<td>TWE&lt;sup&gt;opt&lt;/sup&gt;</td>
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<td>G6M</td>
<td>104.9</td>
<td>72.6</td>
<td>84.6</td>
<td>71.2</td>
<td>76.0</td>
<td>72.5</td>
<td>61.5</td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>100.8</td>
<td>72.5</td>
<td>82.5</td>
<td>79.9</td>
<td>78.2</td>
<td>71.4</td>
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<tr>
<td>G</td>
<td>104.9</td>
<td>72.3</td>
<td>n.d.</td>
<td>71.3</td>
<td>78.2</td>
<td>63.9</td>
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<td></td>
</tr>
<tr>
<td>LA2M</td>
<td>100.8</td>
<td>81.1</td>
<td>80.0</td>
<td>79.9</td>
<td>77.8</td>
<td>71.5</td>
<td>61.5</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>n.d.</td>
<td>71.6</td>
<td>84.5</td>
<td>71.3</td>
<td>77.8</td>
<td>64.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L6S&lt;sup&gt;(2)&lt;/sup&gt;</td>
<td>103.5</td>
<td>71.4</td>
<td>72.8</td>
<td>81.1</td>
<td>72.5</td>
<td>70.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d. – not discernible.

1 Corroborated by a signal in $^1$H NMR spectra (~5.28 ppm) and FTIR analysis. 2 Other lower intensity peaks were observed in this region; see discussion for details.

3 Two low intensity signals at 105.6 and 105.9 ppm; see discussion for details.
Table I.4.2 - Assignments of Major $^1$H Chemical Shifts (ppm) of NA_IMTA_TWE$^{opt}$, NA_IMTA_MAE$^{opt}$ and NA_IMTA_MAE$^1$. Comparison with Assignments of Villanueva et al. (2010).

<table>
<thead>
<tr>
<th></th>
<th>CARBON CHEMICAL SHIFT (PPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA_IMTA_MAE$^1$</td>
</tr>
<tr>
<td>G1</td>
<td>4.54</td>
</tr>
<tr>
<td>G3</td>
<td>3.75</td>
</tr>
<tr>
<td>G4</td>
<td>4.10</td>
</tr>
<tr>
<td>G5</td>
<td>3.71</td>
</tr>
<tr>
<td>G6</td>
<td>3.67$^{(1)}$/3.75$^{(2)}$</td>
</tr>
<tr>
<td>G6M</td>
<td>3.40</td>
</tr>
<tr>
<td>LA1</td>
<td>5.13</td>
</tr>
<tr>
<td>LA2</td>
<td>4.10</td>
</tr>
<tr>
<td>LA3</td>
<td>4.52</td>
</tr>
<tr>
<td>LA4</td>
<td>4.65</td>
</tr>
<tr>
<td>LA5</td>
<td>4.54</td>
</tr>
<tr>
<td>LA6</td>
<td>4.01$^{(3)}$/4.25$^{(4)}$</td>
</tr>
<tr>
<td>LA2M</td>
<td>3.49</td>
</tr>
<tr>
<td>L6S</td>
<td>5.28</td>
</tr>
</tbody>
</table>

$^1$G6 proton; $^2$G6' proton; $^3$LA6exo proton; $^4$LA6endo proton; n.d. – not discernible.
I.4 CHARACTERIZATION OF THE EXTRACTED AGARS: INFLUENCE OF EXTRACTION PROCESS AND SEAWEED GROWTH CONDITIONS

(A)

(B)
An additional signal in the region of C1 of L6S residues (105.9 ppm) was observed for NA_IMTA_MAE\textsuperscript{opt} and could be attributed to different positions of the adjacent groups of the sulfate substituents. Considerable methylation at C6 of the G residue was detected through the presence of a cross peak at (3.41; 61.5) ppm in all spectra (Fig. I.4.1). The presence of M groups in the polysaccharides results in a peak at about 59 ppm due to an upfield shift of the carbon M signal when compared with remaining carbons of the sugar residues in $^{13}$C NMR spectra (Usov, 1984). The resonances observed at $\sim$75.9 and $\sim$72.8 ppm result of an upfield shift of 2 ppm of C5 and a downfield of almost 10 ppm of C6 in the G units, respectively, and are also associated with this type of substitution (Murano, 1995; Usov, 1984). The signal splitting of the M group ($\sim$3.43 ppm; almost imperceptible in the horizontal 1D spectra of Figs. I.4.1 A) and B)) could be attributed to different configurations (axial and/or equatorial) of the –OH and CH$_2$OH (Usov, 1984; Usov et al., 1980). Despite the different energy sources used in the extraction process, NA_IMTA_MAE\textsuperscript{1} (15 min under microwave heat, 100 °C, 40 mL of solvent, no agitation) and NA_IMTA_TWE\textsuperscript{opt} (2 h under conventional heat, 85 °C, 200 mL of solvent, no agitation) revealed identical methylation degree at C6 of G units ($\sim$30% mol). On the contrary, more severe MAE conditions (MAE\textsuperscript{opt}: 15 min, 120 °C, 40 mL, medium agitation) led to a decrease of the methylation level of NA_IMTA_MAE\textsuperscript{opt} (25.6% mol; Table I.4.4). This could be related to some instability of the M groups that are more likely to suffer
degradation under more extreme heating conditions. Two additional minor cross-peaks at around (3.10; 56.0) and (3.21; 42.0) ppm were detected in both MAE samples (Figs. I.4.1 A and C)). Additional studies would be needed in order to clarify the nature of these signals. Another methylation occurring in lower extent was observed at C2 of the LA units through the appearance of small intensity peaks in the region (3.54-3.45) ppm in the 1H spectra of all extracts. Distinct configurations of the adjacent groups were assumed to cause the presence of several chemical shifts (Usov, 1984; Usov et al., 1980). NA_IMTA_MAE1 evidenced a minor amount of this kind of substitution (6.4 % mol) while NA_IMTA_TWEopt and NA_IMTA_MAEopt showed similar levels (7.7 and 7.9% mol in Table I.4.3). In this case, more extreme MAE conditions (MAEopt) did not decrease the methylation level and this could be attributed to the high stability of the residues. The absence of a cross peak in 2D spectra was attributed to a concentration below the detection limit, as observed before in our previous work using wild Gracilaria (Villanueva et al., 2010). G units with six-membered (4,6)-carboxylethylidene cyclic ketals (Usov, 1984) and more rarely five-membered cyclic ketals with O-3 and O-4 (Bilan et al., 2007), can be found in SP. Typical signals of these pyruvate residues such as methyl, acetal and carbonyl may be detected in the regions 15-60, 103.8-105.3/108.3 and 165-180 ppm, respectively (Bilan et al., 2007). No correlations in the HSQC spectra of the NA from IMTA seaweeds were detected in the referred methyl region. Due to the typical low concentration of this type of residues in agars from Gracilaria (Murano, 1995) further 1D 13C APT (attached proton test) were performed with more scans than the previous 13C spectra in order to check for the existence of typical signals of acetal C. No methyl, acetal or carbonyl carbons could be detected in the APT spectrum (data not shown). Therefore, we concluded that no traceable amounts of pyruvated G segments existed in the extracts and that the signal around (1.30; 30) ppm could be related with non-galactan signals (Gordonmills et al., 1990). No detectable starch contamination was found in NA from IMTA seaweed (absence of typical C NMR signals of (1→4) (1→6)-a-D-glucan and no glucose measured by liquid chromatography, Table I.4.3) (Rodriguez et al., 2009) contrarily to wild G. vermiculophylla from French waters (Mollet et al., 1998).

FTIR analysis showed typical bands of agar-like galactans and were well related with NMR results. All agars (including the commercial) showed similar spectra regardless of the extraction method used (Fig. I.4.2). A broad band at 930 cm⁻¹ attributed to C-O vibration of LA units and diagnostic bands of total sulfate, at 1250 cm⁻¹, related to the S=O antisymmetric stretching vibration (used for calculating total S content) (Andriamanantoaoinina et al., 2007; Mollet et al., 1998; Rochas et al., 1986; Ruperez et al., 2002) and at 1370 cm⁻¹, related to ester sulfates (Mollet et al., 1998), were observed. As expected, the A₉₃₀/A₁₂₅₀ ratio was higher for CA (11.60±0.41) and lower for NA (MAE¹: 4.17±0.47; MAEopt: 4.76±0.53 and TWEopt: 3.86±0.55). An intense absorption region centered at ~1060 cm⁻¹ and a band at ~
1150 cm\(^{-1}\) could be assigned to C-O and C-C stretching vibrations of pyranose ring common to all polysaccharides. Two bands were assigned to the C-O-C bending mode in glycosidic linkages at ~712 and 740 cm\(^{-1}\) (Mollet et al., 1998). The first is specific of agar-like polysaccharides and refers to the L-configurations present throughout the polymer skeleton while the latter is assigned to the skeletal bending of the pyranose rings (Matsuiro, 1996). Typical bands of S substitutions are assigned in the region 800-850 cm\(^{-1}\). The peak at 845 cm\(^{-1}\) indicative of an axial S at C4 of G units was present in all samples but was higher in MAE agars (Fig. I.5.2) while 2-sulfate G moieties usually detected at 830 cm\(^{-1}\) (Rochas et al., 1986; Romero et al., 2008) were absent. The same pattern of absorptions was found previously for the same wild species (Mollet et al., 1998; Villanueva et al., 2010). A low sulfation level at C2 of LA units was detected through the presence of a minor shoulder at 805 cm\(^{-1}\). This result, not detected in TWE agars from our early study concerning wild G. vermiculophylla (Villanueva et al., 2010), could not be confirmed by \(^{13}\)C NMR spectra as no splitting of the C1 resonance of the LA moiety was observed probably due to the detection limit of the analysis. The shoulder at 810-820 cm\(^{-1}\) was indicative of a minor S substitution in L residues (L6S) (Romero et al., 2008; Ruperez et al., 2002). A broad band at 2920 cm\(^{-1}\) attributed to C-H groups was observed in all spectra. Additionally, NA from IMTA biomass presented a shoulder on this band at 2845 cm\(^{-1}\) due to M groups. This pattern, expected in FTIR spectra of highly methylated agars of Gracilaria species (Rochas et al., 1986), was not detected in the CA.

![FTIR spectra](image_url)

**Fig. I.4.2** - FTIR spectra of NA_IMTA_TWE\(^{opt}\) (a), NA_IMTA_MAE\(^{opt}\) (b) and NA_IMTA_MAE\(^{1}\) (c). CA was used as reference (d). Bands assigned to total sugar content (C-H; 2920 cm\(^{-1}\) (1)) and respective shoulder of CH\(_3\) (2845 cm\(^{-1}\) (2)), total sulfate
I.4 CHARACTERIZATION OF THE EXTRACTED AGARS: INFLUENCE OF EXTRACTION PROCESS AND SEAWEED GROWTH CONDITIONS

(1370 cm⁻¹ (3) and 1250 (4)), LA (930 cm⁻¹ (5)), sulfate at 4-position in G units (845 cm⁻¹ (6), weak signal) are marked accordingly.

### I.4.1.1.2. Yield and Physicochemical Properties

The extraction yields as well as the physicochemical properties of NA from IMTA seaweeds extracted using both processes are summarized in Tables I.4.3 and I.4.4. Yields were marginally increased (15.8±1.4%; p<0.05) when using MAE instead of TWE (13.5±0.7%). MAE allowed the recovery of higher agar amounts while reducing drastically the extraction time (eight times less) and the achieved yield was within the range accepted worldwide for agar industry (15-25%) (Armisen, 1995). Recent data from Francavilla et al. agreed well with these findings; higher amounts of NA were recovered from wild *Gracilaria gracilis* when using MAE instead of TWE (~26% vs 21%). The authors found the best results at relatively mild conditions: 100 °C, 10 min, 40 mL of solvent (Francavilla et al., 2013). Moreover, higher yields could be attained if processing of the thaw-water was carried out as some native SP are leached out during the freeze-thawing process. NA typically produce weak gels, which upon the freeze-thawing step do not yield firm polymeric networks hence, becoming difficult to recover even upon partial dehydration with ethanol (Villanueva et al., 2010).

On the contrary, the GS for NA_IMTA_MAEopt was significantly lower (~115 g/cm²) when compared with NA_IMTA_TWEopt (~227 g/cm²). The [n] obtained by extrapolation of Huggins (Eq.I.2.1) and Kraemer (Eq.I.2.2) equations were well related with structural analysis and GS values. NA_IMTA_TWEopt was much more viscous (~300 mL/g) than NA_IMTA_MAEopt (~179 mL/g) proving a higher degree of degradation of NA_IMTA_MAEopt backbone. While in TWE the energy is transferred to the sample matrix through conduction and convection, in MAE the energy is transformed into heat through ionic conduction and dipole rotation. The polarized molecules rotate to align themselves with the electromagnetic field at a rate of 4.9x10⁹ times per second and so, molecules are more likely to suffer degradation (Srogi, 2006). The $K_h$ is used as an approximation of the state of aggregation of the polymer molecules as well as their interactions with the solvent (Harding, 1997). For flexible molecules in a good solvent, $K_h$ ~0.35, but due to aggregative phenomena between the molecules of the polymer, deviations from this ideal value are often observed (Harding, 1997). When significant polymer-polymer aggregation occurs, $K_h$ can be higher than 1 (Bastos et al., 2010). Both samples showed $K_h$ values in the same order of magnitude (0.47 and 0.45 for NA_IMTA_MAEopt and NA_IMTA_TWEopt, respectively) and stated a good polymer-solvent interaction. Also, the constraint $K_h + K_k$ ~0.5 was satisfied regardless the extraction method. [n] values were used in the Mark-Houwink equation (Eq.I.2.2) to determine the $M_v$ of NA extracts from IMTA *Gracilaria*. As expected, NA extracted using
microwave irradiation presented a significantly lower $M_v$ (~54 kDa for NA_IMTA_MAE$^{\text{opt}}$ against ~111 kDa for NA_IMTA_TWE$^{\text{opt}}$).

Table I.4.3 - Chemical Properties of NA_IMTA_MAE$^{\text{opt}}$ and NA_IMTA_TWE$^{\text{opt}}$.

<table>
<thead>
<tr>
<th>CHEMICAL PROPERTIES</th>
<th>NA_IMTA_MAE$^{\text{opt}}$</th>
<th>NA_IMTA_TWE$^{\text{opt}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA (%)$^{(1)}$</td>
<td>31.7±0.8</td>
<td>27.8±1.4</td>
</tr>
<tr>
<td>Sulf (%)$^{(2)}$</td>
<td>2.7±0.3</td>
<td>3.1±0.9</td>
</tr>
<tr>
<td>Sulf (%)$^{(3)}$</td>
<td>2.82</td>
<td>3.39</td>
</tr>
<tr>
<td>Gluc (% wt)$^{(3)}$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Xyl (% wt)$^{(3)}$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>% mol M (G6M)$^{(4)}$</td>
<td>25.6</td>
<td>30.2</td>
</tr>
<tr>
<td>% mol M (LA2M)$^{(4)}$</td>
<td>7.9</td>
<td>7.7</td>
</tr>
<tr>
<td>Water (%)</td>
<td>10.4</td>
<td>11.1</td>
</tr>
</tbody>
</table>

1 determined through the colorimetric method of Yaphe & Arsenault (Yaphe & Arsenault, 1965);
2 Sulfate (Sulf) content determined by the turbidimetric method of Jackson & McCandless (Jackson & McCandless, 1978);
3 Sulfate (Sulf), glucose (Gluc) and xylose (Xyl) contents determined through HPLC; maximum standard deviation of 0.17%;
4 estimated from the $^1$H NMR spectral data (see Chapter I.2, Materials and Methods for details).

Table I.4.4 - Yield and Physical Properties of NA_IMTA_MAE$^{\text{opt}}$ and NA_IMTA_TWE$^{\text{opt}}$.

<table>
<thead>
<tr>
<th>PROPERTIES</th>
<th>NA_IMTA_MAE$^{\text{opt}}$</th>
<th>NA_IMTA_TWE$^{\text{opt}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield(%)</td>
<td>15.8±1.4</td>
<td>13.5±0.7</td>
</tr>
<tr>
<td>GS(g/cm$^2$)$^{(1)}$</td>
<td>115.1±7.3</td>
<td>227.2±31.9</td>
</tr>
<tr>
<td>$[\eta]$ mL/g$^{(2)}$</td>
<td>178.9±0.8</td>
<td>300.3±2.1</td>
</tr>
<tr>
<td>$K_H \times 10^{(2)}$</td>
<td>4.7</td>
<td>4.5</td>
</tr>
<tr>
<td>$K_K \times 10^{(2)}$</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>$M_v$ (kDa)$^{(2)}$</td>
<td>54.0±0.4</td>
<td>110.9±1.1</td>
</tr>
<tr>
<td>$T_g$ (°C)$^{(3)}$</td>
<td>38.4±0.3</td>
<td>36.7±0.7</td>
</tr>
<tr>
<td>$T_m$ (°C)$^{(3)}$</td>
<td>81.9±2.2</td>
<td>84.7±0.5</td>
</tr>
<tr>
<td>$M_v \times 10^{3}$ (g/mol)$^{(4)}$</td>
<td>341±2</td>
<td>865±52</td>
</tr>
<tr>
<td>$M_n \times 10^{3}$ (g/mol)$^{(4)}$</td>
<td>129±0.2</td>
<td>246±51</td>
</tr>
<tr>
<td>$\bar{M}_w$ (g/mol)$^{(4)}$</td>
<td>2.63±0.02</td>
<td>3.63±0.77</td>
</tr>
</tbody>
</table>

1 determined through penetration tests on 1.5% wt agar gels; 2 determined through viscometry; 3 determined through rheological studies on 1.5% wt agar gels; 4 determined through SEC.

The gelation of agar takes place during cooling of the polymer's solution at temperatures below the gelation point ($T_g$), which allows the aggregation between the helical chains
through hydrogen bonding. Contrarily to what has been reported by other authors (Murano, 1995; Rodriguez et al., 2009), NA_IMTA_MAE\textsuperscript{opt}, with lower methylation degree, revealed slightly higher $T_g$ (~38.5 °C) when compared with the conventional extract (~37 °C for NA_IMTA_TWE\textsuperscript{opt}; Table I.4.4). The more draconian conditions of MAE did not lower agar’s gelation point. Both extracts exhibited $T_g$ that were appropriate for commercial purposes (32-43 °C) (Orduna-Rojas et al., 2008). NA_IMTA_MAE\textsuperscript{opt}, with significantly lower $M_v$, could have lower $T_m$ (81.9±2.2 °C) when compared with NA_IMTA_TWE\textsuperscript{opt} (~85 °C). This finding could be related to the lower degradation suffered by NA_IMTA_TWE\textsuperscript{opt} (higher $M_v$), as molecular chains of higher size are expected to promote the formation of more stable interactions with the consequent increase in $T_m$ (Rodriguez et al., 2009). NA_IMTA_TWE\textsuperscript{opt} could attain higher total sulfate (~3.4%) and lower LA (~28 %) contents than NA_IMTA_MAE\textsuperscript{opt} (2.8 and 32%, respectively), yet the differences in GS seemed mainly driven by differences in the $M_v$; NA_IMTA_MAE\textsuperscript{opt} with higher LA fraction showed lower GS. This could be explained by its significantly lower $M_v$ as larger molecules have been associated to the formation of more rigid networks (Ruperez et al., 2002). In spite of being extracted under more severe conditions, the LA units were found in higher extent in NA_IMTA_MAE\textsuperscript{opt} and this could be related to the high stability of these residues which was in line with the results of structural analysis. The opposite was observed for sulfate content (more labile character).

I.4.1.2. Alkali-treated Agars (AA)

I.4.1.2.1. Structural Analysis

Alkali-treated agars (AA) from IMTA Gracilaria obtained with the optimal MAE conditions (MAE\textsubscript{Y\textsuperscript{opt}} and MAE\textsubscript{GS\textsuperscript{opt}}) showed similar $^{13}$C and $^1$H chemical assignments than their native counterparts (not shown). Exception made to the low intensity cross-peak at (~5.28; 103.5) ppm attributed to H1 and C1 of L6S, which was absent due to the alkali-treatment inclusion (e.g. HSQC spectrum of AA_IMTA_MAE\textsubscript{Y\textsuperscript{opt}} in Fig. I.4.3). This agreed well with the chemical analysis; low sulfate/LA ratios were estimated for AA (Table I.4.5) when compared to NA (Table I.4.3). In the particular case of AA_IMTA_MAE\textsubscript{GS\textsuperscript{opt}} the attained values were similar to the reference sample, CA (LA~43% and sulfates~1.5%; Table I.4.5).
I.4.1.2.2. Yield and Physicochemical Properties

Table I.4.6 lists the extraction yields as well as relevant physical properties of AA from IMTA seaweeds obtained by MAE (AA_IMTA_MAE_Y\textsuperscript{opt} and AA_IMTA_MAE_Y\textsuperscript{opt}) and TWE (AA_IMTA_TWE\textsuperscript{opt}).

The optimal conditions of the TWE method (2 h, 85 °C, 200 mL of solvent and no agitation) obtained in our earlier study (Villanueva et al., 2010), were applied to the same alkali-treated biomass. The obtained yield, ~12%, was significantly lower than the value reached using MAE, ~15.6% (AA_IMTA_TWE\textsuperscript{opt} vs AA_IMTA_MAE_Y\textsuperscript{opt} in Table I.4.6) Francavilla et al. reported significantly lower yields of AA from wild Gracilaria gracilis when using MAE instead of TWE (~7% vs ~14%). In any case, upon increasing the temperature to 140 °C higher yields were obtained (16%) (Francavilla et al., 2013). Since the authors chose the experimental conditions based on our optimization work (Sousa et al., 2010) as well as preliminary tests rather than carrying out a complete process optimization, the results could mean that they were not in the optimal region when working at lower temperatures.

As expected, an improvement in agar quality was observed upon inclusion of the alkaline step. Remarkably stronger gels, were obtained using MAE (~1569±59 g/cm\textsuperscript{2}) when
compared with gels produced by the TWE method (834±124 g/cm²). AA_IMTA_MAE_GS\textsuperscript{opt} compared favorably with the commercial sample (~1177 g cm²; CA in Table I.4.10). Even at optimal yield conditions, high-quality agar (GS> 700 g/cm² in a 1.5% wt solution; Armisen, 1995)) was extracted from IMTA Gracilaria by MAE (~1031 g/cm² for AA_IMTA_MAE_Y\textsuperscript{opt} in Table I.4.6). Furthermore, the reproducibility was clearly enhanced by the MAE process.

Contrarily to what was observed for NA, a significant enhancement in agar gelling properties was found when using microwaves in opposition to conventional heating. As extensively debated, the inclusion of an alkali-treatment step prior to the extraction promotes the conversion of L6S in LA (Rodriguez et al., 2009). These anhydride structures are much more stable than their precursor units and for that reason, are more unlikely to suffer degradation under microwave heating. Nevertheless, GS values for NA_IMTA_MAE\textsuperscript{opt} (Table I.45.2) clearly fell in the range considered adequate for soft-texture food products applications (30-200 g/cm²) (Pereira-Pacheco et al., 2007).

The highest GS (~1570 g/cm²) of AA_IMTA_MAE_GS\textsuperscript{opt} agreed well with the measured \(T_m\) (95 °C; Table I.4.6). This was indicative of a more thermally stable polymeric network, harder to disrupt upon heating the system. The largest thermal hysteresis (\(\Delta T\sim50\) °C) of this extract could have interest for commercial applications. Even though its \(T_g\) wasn’t as low as Gelidium agars the attained \(\Delta T\) value wasn’t far from the reference sample, CA (~53 °C).

Table I.4.5 - Chemical Properties of AA_IMTA_MAE_Y\textsuperscript{opt}, AA_IMTA_MAE_GS\textsuperscript{opt} and AA_IMTA_TWE\textsuperscript{opt}. CA from Sigma-Aldrich was used as reference.

<table>
<thead>
<tr>
<th>PROPERTIES</th>
<th>AA_IMTA_MAE_Y\textsuperscript{opt}</th>
<th>AA_IMTA_MAE_GS\textsuperscript{opt}</th>
<th>AA_IMTA_TWE\textsuperscript{opt}</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA (%)</td>
<td>35.9±0.2</td>
<td>43.0±2.5</td>
<td>34.4±0.3</td>
<td>43.5±0.9</td>
</tr>
<tr>
<td>Sulf (%)\textsuperscript{(1)}</td>
<td>1.73</td>
<td>1.54</td>
<td>1.37</td>
<td>1.42</td>
</tr>
<tr>
<td>Gluc (% wt)</td>
<td>tr.</td>
<td>tr.</td>
<td>tr.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Xyl (% wt)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>% mol M (G6M)</td>
<td>19.2</td>
<td>21.7</td>
<td>18.1</td>
<td>5.4</td>
</tr>
<tr>
<td>% mol M (LA2M)</td>
<td>6.7</td>
<td>4.6</td>
<td>5.6</td>
<td>6.3</td>
</tr>
<tr>
<td>Water (%)</td>
<td>13.1</td>
<td>12.1</td>
<td>13.2</td>
<td>12.9</td>
</tr>
</tbody>
</table>

\textsuperscript{(1)} determined through HPLC; maximum standard deviation of 0.17%.  

Table I.4.6 - Yield and Physical Properties of AA_IMTA_MAE_Y\textsuperscript{opt}, AA_IMTA_MAE_GS\textsuperscript{opt} and AA_IMTA_TWE\textsuperscript{opt}.

<table>
<thead>
<tr>
<th>PROPERTIES</th>
<th>AA_IMTA_MAE_Y\textsuperscript{opt}</th>
<th>AA_IMTA_MAE_GS\textsuperscript{opt}</th>
<th>AA_IMTA_TWE\textsuperscript{opt}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield(%)</td>
<td>15.6±0.7</td>
<td>8.4±0.1</td>
<td>11.9±1.0</td>
</tr>
<tr>
<td>GS(g/cm\textsuperscript{2})</td>
<td>1013±51</td>
<td>1569±59</td>
<td>834±124</td>
</tr>
<tr>
<td>T\textsubscript{g} (°C\textsuperscript{(1)})</td>
<td>40.8±0.1</td>
<td>40.4±0.4</td>
<td>40.5±0.35</td>
</tr>
<tr>
<td>T\textsubscript{m} (°C\textsuperscript{(1)})</td>
<td>87.2±0.7</td>
<td>95 (\textsuperscript{(1)})</td>
<td>87.1±0.3</td>
</tr>
<tr>
<td>M\textsubscript{w} ×10\textsuperscript{3} (g/mol)</td>
<td>285±17</td>
<td>665±30</td>
<td>532±7</td>
</tr>
<tr>
<td>M\textsubscript{n} ×10\textsuperscript{3} (g/mol)</td>
<td>103±5</td>
<td>208±21</td>
<td>190±2</td>
</tr>
<tr>
<td>ĐM</td>
<td>2.78±0.29</td>
<td>3.21±0.18</td>
<td>2.80±0.004</td>
</tr>
</tbody>
</table>

\textsuperscript{(1)}Gelation point (\tan δ=1) measured during the time sweep performed at 95 °C (please see Chapter I.2 for details).

Overall, it was found that from the 1\textsuperscript{st} batch of IMTA seaweeds (January 2009; used for the MAE optimization) to the 2\textsuperscript{nd} one (January 2011), the GS improved yet, lower amounts of SP were extracted. Nonetheless, taking into consideration the great variability in agar properties observed when using wild biomass, it is clear that a stricter control of the feedstock properties can be accomplished with the IMTA approach.

**I.4.2. Wild Gracilaria**

**I.4.2.1. MAE Agars**

**I.4.2.1.1. Structural Analysis**

The alkaline modification minimized the proton signal related to the L6S residues (around 5.28 ppm) in the \textsuperscript{1}H NMR spectra of AA (\textit{e.g.} AA\_Wild\_MAE\_GS\textsuperscript{opt} spectrum in Fig. I.4.4 b)). Further proof of the conversion of these sulfated monomers into their anhydride ‘precursor’ units during the alkaline treatment was obtained with the 2D HSQC experiments\textsuperscript{(Sousa et al., 2013b)}. Typical signals of additional monosaccharides such as xylose and glucose were not resolved which fairly agreed with the residual amounts detected through liquid chromatography (Table I.4.7). The chemical analysis showed higher sulfate content for NA\_Wild\_MAE\textsuperscript{opt} (3.22%) while the sulfates detected in AA (1.73% for AA\_Wild\_MAE\_Y\textsuperscript{opt} and 1.54% for AA\_Wild\_MAE\_GS\textsuperscript{opt}) could be attributed to akali-stable ones present in the 3-linked monomers (Murano, 1995; Romero et al., 2008). The amount of anhydride monomers showed an inverse trend; NA\_Wild\_MAE\textsuperscript{opt} showed by far, the lowest value (~23%) in opposition to the higher values for AA\_Wild\_MAE\_Y\textsuperscript{opt} (~34%) and AA\_Wild\_MAE\_GS\textsuperscript{opt} (~41%), the latter falling closer to the reference (~43.5%).
I.4 CHARACTERIZATION OF THE EXTRACTED AGARS: INFLUENCE OF EXTRACTION PROCESS AND SEAWEED GROWTH CONDITIONS

Fig. I.4.4 - $^1$H NMR spectra (400 MHz) of NA_Wild_MAE$^{opt}$ (a) and AA_Wild_MAE_GS$^{opt}$ (b) samples in D$_2$O (data obtained at 80°C with the following conditions: 5.11 s acquisition time, 1.0 s relaxation delay, a spectral width of 6400 Hz and an average of 16 scans). The alkaline modification minimizes the peak of the C1 of the L6S residues. The region of the methylated segments, LA2M and G6M, is identified in the marked inset.

Table I.4.7 - Chemical Properties of NA_Wild_MAE$^{opt}$, AA_Wild_MAE_Y$^{opt}$ and AA_Wild_MAE_GS$^{opt}$.

<table>
<thead>
<tr>
<th>CHEMICAL PROPERTIES</th>
<th>NA_Wild_MAE$^{opt}$</th>
<th>AA_Wild_MAE_Y$^{opt}$</th>
<th>AA_Wild_MAE_GS$^{opt}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA (%)</td>
<td>23.2±0.4</td>
<td>34.2±1.1</td>
<td>40.7±1.1</td>
</tr>
<tr>
<td>Sulfate (%)</td>
<td>3.22</td>
<td>1.73</td>
<td>1.54</td>
</tr>
<tr>
<td>Gluc (% wt)</td>
<td>tr.</td>
<td>tr.</td>
<td>tr.</td>
</tr>
<tr>
<td>Xyl (% wt)</td>
<td>tr.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>% mol M (G6M)</td>
<td>36.3</td>
<td>20.1</td>
<td>24.3</td>
</tr>
<tr>
<td>% mol M (LA2M)</td>
<td>11.4</td>
<td>5.6</td>
<td>8.0</td>
</tr>
<tr>
<td>Water (%)</td>
<td>12.0</td>
<td>14.3</td>
<td>13.2</td>
</tr>
</tbody>
</table>

* determined through HPLC; maximum standard deviation of 0.17%; tr- traces (< 1%); n.d. – not detected.
Higher agar recoveries were observed when using wild biomass to carry out the MAEs. For instance, ~16% yield was attained for AA_IMTA_MAE_Yopt (Table I.4.6) while ~22% agar was recovered from wild Gracilaria (AA_Wild_MAE_Yopt; Table I.4.8). However, agars with increased gelling ability (maximum GS) were recovered from seaweeds with high tissue nitrogen, due to IMTA conditions (Abreu et al., 2011) (e.g. ~1570 g/cm^2 of AA_IMTA_MAE_GSopt in Table I.4.6 and ~1320 g/cm^2 of AA_Wild_MAE_GSopt in Table I.4.8). Certain parameters, such as talus nitrogen content and plant growth can be related to the improvement in gel quality of SP from seaweeds produced in aquaculture systems despite the lower recoveries (Marinho-Soriano & Bourret, 2003). Nonetheless, the GS measured for AA from wild biomass also compared favorably with CA (~1177 g cm^2; CA in Table I.4.10) which confirms that wild G.vermiculophylla from Ria de Aveiro can also be envisaged as a good and cheap raw material for the agar industry, and replace Gelidium species (known for better quality agars) in specific applications (Armisen & Galatas, 1987).

The apparent mass-average molar masses, M_w, determined by SEC (Table I.4.8), seemed to suggest a substantial depolymerization of AA_Wild_MAE_Yopt when compared to AA_Wild_MAE_GSopt ((230±30)×10^3 and (676±110)×10^3 g/mol, respectively). This could be attributed to the more draconian MAE conditions used in the case of AA_Wild_MAE_Yopt. NA_Wild_MAEopt showed an intermediate value of M_w between those of AA_Wild_MAE_Yopt and AA_Wild_MAE_GSopt ((476±21)×10^3 g/mol) and was comparable to NA from other Gracilaria species extracted with thermal heating (Rodriguez et al., 2009). The chemical conversion occurring during the alkaline step is expected to produce some decrease in molar mass (Murano, 1995). Although the intermediate M_w for NA_Wild_MAEopt could again be
assigned to differences in the MAE conditions we could suspect of possible occurrence of aggregative phenomena during the SEC measurements. AA_Wild_MAE\(_{\text{opt}}\) showed higher variability between different injections (i.e. an error range of ±110×10\(^3\) g/mol) and had simultaneously the highest molar mass dispersity, \(D_M\) (~3.1±0.3). Similar broad \(M_w\) distribution was found for the commercial sample (~3.4±0.1) in opposition to the narrowest one observed for NA_Wild_MAE\(_{\text{opt}}\) and AA_Wild_MAE\(_{\text{Y opt}}\) (~2.6). The \(D_M\) of NA_Wild_MAE\(_{\text{opt}}\) fell in the range reported by Rodríguez and co-workers (Rodríguez et al., 2009) for Gracilaria gracilis agars (1.7-2.8). If one considers that only the agarose fraction present in agar’s skeleton is monodisperse (Labropoulos et al., 2002) it should be expected higher \(D_M\) for NA_Wild_MAE\(_{\text{opt}}\) (higher sulfate/LA ratio). In the case of MAE however, the spectacular molecular accelerations induced by the use of microwave heating during extraction (i.e. in order to align with the electromagnetic field the molecules rotate at 4.9×10\(^9\) times per second (Srogi, 2006)) could explain the observed values (AA_Wild_MAE\(_{\text{GS opt}}\) extracted at maximum stirring in opposition to the other SP). Nonetheless, problems related to either particulate material not separated during the purification step or possible aggregation are commonly associated with this type of measurement (Milas et al., 2001; Vold et al., 2006) and for that reason, caution must be taken when interpreting the results.

I.4.2.2. TWE Agars

I.4.2.2.1. Structural Analysis

Spectroscopic data of NA_Wild_TWE\(_{\text{opt}}\) and AA_Wild_TWE\(_{\text{opt}}\) showed similar backbones with the chemical conversion of L6S in LA unis resulting, as previously, in the absence of the low intensity cross-peak at (~5.28; 103.5) ppm (not shown). Also, it could be concluded that the growth conditions of Gracilaria didn’t affect the structural backbone of the extracts; despite in variable amounts, the building blocks were the same in all studied samples.

I.4.2.2.2. Yield and Physicochemical Properties

The extraction yields and physicochemical properties of agars from wild Gracilaria obtained using conventional energy sources (TWE) are given in Tables I.4.9 and I.4.10.

When using the TWE method, closer yields were obtained if treating the seaweeds with alkaline solution (~17-21% for AA_Wild_TWE\(_{\text{opt}}\) and NA_Wild_TWE\(_{\text{opt}}\); Table I.4.10). On the contrary, for the cultivated biomass higher recoveries were attained (roughly, 13.5% and 17% for respectively, NA_IMTA_TWE\(_{\text{opt}}\) and AA_IMTA_TWE\(_{\text{opt}}\); Tables I.4.4 and I.4.6) which could be explained by greater losses of non-treated SP during the freeze-thawing step.
Nitrogen availability is known to affect phycocolloid synthesis and gel quality; generally, seaweeds grown in enriched nitrogen media will synthesize less phycocolloid but with better gel quality. For NAs however, the GS seemed independent of the type of seaweed; close GS were obtained when applying the same extraction conditions to different biomass. For instance, the GS values for NA recovered using microwave heating were in the range ~110-200 g/cm$^2$ (NA_Wild_MAE$^{opt}$ and NA_Wild_MAE$^{opt}$; Tables I.4.4 and I.4.8) while an average GS of ~227 g/cm$^2$ (NA_Wild_TWE$^{opt}$ and NA_Wild_TWE$^{opt}$; Tables I.4.4 and I.4.10) was estimated for the conventional method. As seen for IMTA biomass, NA from wild *Gracilaria* was more degraded after exposure to microwaves than when thermally heated. In fact, the M$_w$ of NA_Wild_MAE$^{opt}$ measured by SEC was half the M$_w$ of NA_Wild_TWE$^{opt}$ (~476×10$^3$ g/mol against ~1060×10$^3$ g/mol; Tables I.5.9 and 1.5.11). This extract presented the highest M$_w$ and M$_v$ (~162 kDa; Table I.5.11) of all samples. Overall, IMTA grown *Gracilaria* resulted in NAs with lower M$_w$ and in the case of the MAE process, with higher LA/sulfate ratios than the NA from wild seaweeds. The conjugation of these properties resulted in close GS values for the non-treated SP.

In our early study focusing wild *G.vermiculophylla*, collected at the same geographical area and season but in different years (Villanueva *et al.*, 2010), NAs extracted with thermal heat showed significantly lower M$_v$ values (44-63 kDa) as well as T$_g$ (~26.5 °C) and T$_m$ (~70 °C). In the present study, NA_Wild_TWE$^{opt}$ exhibited the lowest T$_g$, ~38.5 °C (Table I.4.10) while maintaining a large ΔT (~ 50 °C) which could be attractive for commercial applications. Wild *G.vermiculophylla* from Mexico led to NAs with significantly lower GS (72 g/cm$^2$) (Arvizu-Higuera *et al.*, 2008).

As seen for agars from IMTA biomass, GS was significantly higher for AA obtained by MAE (e.g. AA_Wild_MAE_GS$^{opt}$ ~1319 g cm$^{-2}$ in Table I.4.6 against ~1027 g cm$^{-2}$ for AA_Wild_TWE$^{opt}$ in Table I.4.8). Nonetheless, both GS values fulfilled the market requirements for high-grade agars (GS >700 g cm$^{-2}$ at 1.5% wt) (Armisen, 1995; Sousa *et al.*, 2013a).

Despite the dramatic acceleration of extraction observed when using MAE, the right choice of the operational parameters (MAE_GS$^{opt}$: 5 minutes, 90 °C, 20 mL, with maximum stirring speed) allowed the recovery of AA with high molecular mass (e.g. M$_v$, ~138 kDa for AA_Wild_MAE_GS$^{opt}$; Table I.4.6), determined by viscometry (Sousa *et al.*, 2012; Souza *et al.*, 2012). The longer exposure of the extraction mixture to thermal heating in TWE (2 hours, 85 °C, 200 mL, and without agitation; Villanueva *et al.*, 2010) seemed to promote the degradation of the polymeric chains which explains the low M$_v$, ~74 kDa in Table I.4.8 of AA_Wild_TWE$^{opt}$ (Hurtado-Ponce, 1992; Lai & Lii, 1998). In this context, the extraction of AAs based on non-conventional energy sources such as microwaves can be a good strategy to
obtain non-degraded agars at relatively mild processing conditions while significantly reducing the extraction times. The molecular mass, in turn, decreased in accordance with the polymer GS. This positive relation has been reported previously (Lai & Lii, 1998; Murano, 1995; Romero et al., 2008), although within certain limits beyond which either the parameters become independent of molecular mass (upper limit) or the agar gelation does not take place (lower limit) (Murano, 1995).

Compared with previous reports on agar TWE, the current data are very promising (Arvizu-Higuera et al., 2008; Meena et al., 2008; Orduna-Rojas et al., 2008; Villanueva et al., 1999; Villanueva et al., 2010). Agar MAE was much less time and solvent consuming than other conventional methodologies extensively documented for Gracilaria sp. Moreover, the quality of our extracts compared favorably with the published data found in literature concerning G. vermiculophylla (Orduna-Rojas et al., 2008; Rodriguez-Montesinos et al., 2013). A detailed comparison with previous studies can be found in our paper (Sousa et al., 2010).

Table I.4.9 - Chemical Properties of NA_Wild_TWE\textsuperscript{opt} and AA_Wild_TWE\textsuperscript{opt}.

<table>
<thead>
<tr>
<th>CHEMICAL PROPERTIES</th>
<th>NA_Wild_TWE\textsuperscript{opt}</th>
<th>AA_Wild_TWE\textsuperscript{opt}</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA (%)</td>
<td>29.4±1.2</td>
<td>33.1±1.6</td>
</tr>
<tr>
<td>Sulfate (%)</td>
<td>3.53</td>
<td>1.71</td>
</tr>
<tr>
<td>Gluc (% wt)</td>
<td>tr.</td>
<td>tr.</td>
</tr>
<tr>
<td>Xyl (% wt)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Water (%)</td>
<td>12.4</td>
<td>13.1</td>
</tr>
</tbody>
</table>

Table I.4.10 - Yield and Physical Properties of NA_Wild_TWE\textsuperscript{opt} and AA_Wild_TWE\textsuperscript{opt}.

<table>
<thead>
<tr>
<th>PROPERTIES</th>
<th>NA_Wild_TWE\textsuperscript{opt}</th>
<th>AA_Wild_TWE\textsuperscript{opt}</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (%)</td>
<td>17.6±1.6</td>
<td>19.1±1.4</td>
<td>n.a.</td>
</tr>
<tr>
<td>GS (g cm\textsuperscript{-2})</td>
<td>222±27</td>
<td>1027±46</td>
<td>1177±43</td>
</tr>
<tr>
<td>M\textsubscript{v} (kDa)</td>
<td>162±1</td>
<td>73.7±2</td>
<td>138±1</td>
</tr>
<tr>
<td>M\textsubscript{w} \times 10\textsuperscript{3} (g/mol)</td>
<td>1060±43</td>
<td>85±7</td>
<td>392±40</td>
</tr>
<tr>
<td>M\textsubscript{n} \times 10\textsuperscript{3} (g/mol)</td>
<td>341±3</td>
<td>40±0.4</td>
<td>116±8</td>
</tr>
<tr>
<td>D\textsubscript{M}(b)</td>
<td>3.11±0.16</td>
<td>2.13±0.16</td>
<td>3.38±0.10</td>
</tr>
<tr>
<td>T\textsubscript{g} (°C)</td>
<td>38.5±0.4</td>
<td>40.9±0.5</td>
<td>38.9±0.3</td>
</tr>
<tr>
<td>T\textsubscript{m}(°C)</td>
<td>88.5±0.83</td>
<td>76.9±1.7</td>
<td>92.1±0.7</td>
</tr>
</tbody>
</table>
I.4.3. Conclusions

The present study shows that different types of agars can be obtained by changing the process and process parameters as well as the seaweeds cultivation conditions/origin. Some of the studied properties (e.g. T_9 and T_m) showed by Gracilaria agars could be interesting for agar industry specially if one considers Gracilaria sp ease of cultivation when compared to Gelidium sp.

Both farmed and wild G. vermiculophylla yielded high quality agars. The GS of AA was more favored by the IMTA growth conditions (N-enriched environment), whose values compared favorably with those of the commercial agar sample used as reference. However, lower yields were found for extractions with IMTA Gracilaria when compared to wild biomass. Remarkable gel quality was accomplished at relatively mild MAE conditions (MAE_GS_{opt}: 5 min of extraction, 90 °C, 20 mL of water and maximum stirring speed). This could be very attractive for industries since draconian conditions outcomes safety issues.

MAE allowed extracting higher amounts of NA than TWE yet the SP seemed more prone to degradation when exposed to microwaves (lower GS) which could be attributed to its higher sulfate content (more labile character of L6S units). Nonetheless, the gel quality of NA from MAE still comply with the reference values defined for soft texture products (30-200 g/cm^2; Pereira-Pacheco et al., 2007). Opposite behavior was observed for the extracts from pre-treated seaweeds, richer in alkali-stable LA moieties and hence, more stable under microwaves. Overall, MAE yields fell in the range considered acceptable for industrial applications, 15-25% (Pereira-Pacheco et al., 2007).

Considering the seasonal variation on the abundance and quality of the biomass harvested from wild populations, the production of seaweeds in IMTA systems can offer a continuous supply of raw material with reliable quality to the transformation industries.

The MAE approach supports sustainable development, as it requires less energy and solvent than conventional processes, while generating fewer wastes. This work suggests the feasibility of the exploitation of G. vermiculophylla, wild or produced in IMTA systems, for production of agar gels with superior quality. The exploitation of a national underused resource such as G. vermiculophylla, helping to maintain the equilibrium of coastal ecosystems, is also a good commitment towards sustainability.
References.


I.5 INFLUENCE OF THE EXTRACTION PROCESS ON THE RHEOLOGICAL AND STRUCTURAL PROPERTIES OF ALKALI-TREATED AGARS – CASE STUDY 1

CHAPTER I.5
This chapter, will focus a case study published in our recent paper Sousa et al. (Sousa et al., 2013) concerning the influence of the extraction process on the rheological and structural properties of agars. The alkali-treated extracts from wild biomass, AA_Wild_MAE_GS\textsubscript{opt} and AA_Wild_TWE\textsubscript{opt}, were chosen as model samples and the results were compared with a reference sample, CA.

### I.5.1. Physicochemical Properties of MAE and TWE Agars

As discussed in detail in the previous chapter (section I.4.2), the right choice of MAE conditions led to non-degraded AA with enhanced gelling capability (AA_Wild_MAE_GS\textsubscript{opt}: $M_v \sim 138$ kDa and $\sim 1319$ g cm$^{-2}$ in Table I.4.6 against $M_v \sim 74$ kDa and $\sim 1027$ g cm$^{-2}$ for AA_Wild_TWE\textsubscript{opt}).

AA_Wild_MAE_GS\textsubscript{opt} showed significantly higher LA fraction ($\sim 41 \%$) than AA_Wild_TWE\textsubscript{opt} agar ($\sim 33 \%$) while the sulfate content was comparable ($\sim 1.6 \%$; measured by HPLC). Therefore, in this case, the gelling ability of the SP seemed mainly governed by the molecular mass and the LA content.

### I.5.2. AFM Studies of Low Concentration Agar Solutions

AFM was used to image AA_Wild_MAE_GS\textsubscript{opt} and AA_Wild_TWE\textsubscript{opt} in aqueous solutions with low polymer concentration (500 $\mu$g mL$^{-1}$, ca. 0.05 % wt). The samples were either slowly cooled to room temperature or rapidly quenched by dilution as described in Chapter I.2, section I.2.6.2.2. At room temperature, none of the prepared solutions formed macroscopic gels readily visible to the naked eye. In each case, the agar systems were then deposited onto freshly cleaved mica and air-dried.

Despite the amount and richness of information one can obtain when imaging gel-forming polysaccharides by the AFM technique, attention should be given to a couple of points. The first concerns the structural modifications that will most likely occur during the drying step when preparing the sample for analysis (Ikeda et al., 2001; Kirby et al., 1996; Morris et al., 2010; Noda et al., 2008; Robic et al., 2009). The other relevant aspect is the difficulty to observe ‘true solutions’ (Morris et al., 2010); very often, AFM images of such polysaccharides show large aggregates of intensively associated molecules caused by an insufficient solubilization of the polymer during the solution preparation and/or formed upon
drying of the solution on the mica surface (Ikeda et al., 2004; Morris et al., 2010). To minimize this problem the agar powders were dispersed in the solvent under a vigorous vortex stirring and the dispersions were heated at high temperatures (~96 °C) until a homogeneous solution was obtained.

Fig. I.5.1 shows the topographic and equivalent amplitude AFM images of the AA_Wild_MAE_GS\textsuperscript{opt} (Figs. I.5.1A-B) and AA_Wild_TWE\textsuperscript{opt} (Figs. I.5.1C-D) solutions, when prepared by the ‘slow cooling’ method. The images were acquired at a scanning size of 5 µm × 5 µm.
Fig. I.5.1 - Representative topographic (A, and C) and equivalent amplitude (B, and D) AFM images obtained in tapping mode for 0.05% wt ca. 500 µg mL⁻¹ aqueous solutions of AA_Wild_MAE GS⁹⁶ (A, and B) and AA_Wild_TWE⁹⁵ (C, and D), prepared by the ‘slow cooling’ method, deposited onto freshly cleaved mica after being cooled to room temperature. The image size is 5 µm × 5 µm. The curves below the images represent the height profile along the white line drawn in the images. Arrows indicate local aggregates.
For the considered conditions, both SP formed thin gelled layers on the mica surface (Fig. I.5.1). Since the samples were deposited onto the substrate as liquids, the formation of the gels probably occurred as result of a substantial concentration increase upon drying of the samples (Ikeda et al., 2004). The height profiles illustrated below Figs. I.5.1A (AA_Wild_MAE_GS\textsuperscript{opt}) and I.5.1C (AA_Wild_TWE\textsuperscript{opt}) seemed in line with this view; even if some compression should result in underestimated measured heights (Morris et al., 2010), the obtained values were higher (AA_Wild_MAE_GS\textsuperscript{opt} typically ~5-10 nm and AA_Wild_TWE\textsuperscript{opt} ~1-3.5 nm) than expected for single agar molecules (Arnott et al., 1974). According to Arnott and co-workers each agarose chain with an average molecular weight of 120,000 is expected to form a three-fold helix of 1.9 nm pitch with an axial translation of 0.95 nm. Despite the evident massive aggregation state, it was clear that the structures formed by the two agar extracts were different and seemed considerably larger in the case of the MAE sample. In both cases, spherical and more rarely odd-shape aggregates (indicated with arrows) could be seen occasionally, buried in the polymeric networks, reaching greater dimensions in the case of AA_Wild_MAE_GS\textsuperscript{opt}. Similar gelled structures were reported by Roesch et al. (2004) when imaging κ-carrageenan by AFM.

When diluting the 1.5 % wt agar solutions while hot, different structures were imaged by AFM (Fig. I.5.2). Again, no discrete fibers were recognizable in the acquired images and polymeric gelled layers covered the mica surface. The distribution of heights illustrated below Figs. I.5.2A and I.6.2C seemed to support this interpretation (typically ~5-10 nm for both agars). The aggregates (indicated by arrows in Fig. I.5.2) seemed more frequent than for slowly cooled agar solutions (Fig. I.5.1) and were mainly spherical.
Fig. I.5.2 - Representative topographic (A, and C) and equivalent amplitude (B, and D) AFM images obtained in *tapping mode* for a 0.05% wt ca. 500 µg mL⁻¹ aqueous solutions of AA_Wild_MAE_GSopt (A, and B) and AA_Wild_TWEopt (C, and D). The samples were deposited onto freshly cleaved mica after diluting while hot a 1.5% wt agar solution (*rapid cooling* method). The image size is 5 µm × 5 µm. The curves below the images represent the height profile along the white line drawn in the images. Arrows indicate local aggregates.
Differences between the two agar extracts were more difficult to establish. Values of surface roughness ($R_{ms}$) and average height ($H_{av}$) were derived from Figs. I.5.1A and I.5.2A (AA_Wild_MAE_GS$^{opt}$) and Figs. I.5.1C and I.5.2C (AA_Wild_TWE$^{opt}$). Rapid cooling (Fig. I.5.2) led to surfaces with similar roughness ($R_{ms}$ = 4.51 and 4.60 nm for AA_Wild_MAE_GS$^{opt}$ and AA_Wild_TWE$^{opt}$, respectively), but when the solutions were allowed to cool slowly (Fig. I.5.1) a significantly smoother surface was formed by the AA_Wild_TWE$^{opt}$ ($R_{ms}$ = 1.13 nm, in comparison with 4.26 nm for the AA_Wild_MAE_GS$^{opt}$). An identical trend was observed for $H_{av}$ data (not shown). Although we were tempted to suggest that the structures in Fig. I.5.2 were less organized than when slowly cooled (Fig. I.5.1), the concentration used makes this interpretation purely speculative. However, one should expect that rapid quenching by dilution would decrease the interhelical association (Ikeda et al., 2001; Roesch et al., 2004), thus forming less organized structures than when slowly cooling the agar solutions. Mohammed and co-workers found that the formation of agarose gels was deeply affected by the cooling rate, suggesting that slow cooling promoted more aggregation of double helices than rapid quenching (Mohammed et al., 1998). Nonetheless, based only on the present AFM data, it seems reasonable to say that the systems formed by each SP were different and that AA_Wild_MAE_GS$^{opt}$ showed, apparently, larger structures than AA_Wild_TWE$^{opt}$, particularly when the solutions were cooled slowly.

I.5.3. Rheological Studies of High Concentration Agar Solutions/Gels

In order to further understand the sol-gel transition of AA_Wild_MAE_GS$^{opt}$, AA_Wild_TWE$^{opt}$ and CA, the elastic ($G'$) and viscous ($G''$) moduli dependence on temperature of 1.5% wt agar solutions was recorded at 1 ºC/min from 80 to 25 ºC. The $T_g$ was considered as the crossover point between the two moduli (i.e. $\tan \delta=G''/G'=1$). For the sake of clarity, only $G'$ and $G''$ data of AA_Wild_MAE_GS$^{opt}$ and AA_Wild_TWE$^{opt}$ are shown in Fig. I.5.3 (main graphic) while the $\tan \delta$ data of the three samples are compared in the inset graphic of Fig. I.5.3.
The agar gelation occurred gradually, the sol-gel transition happening in a broad temperature window (~43-30 °C) and the shape of the curves was identical for AA_Wild_MAE_GS\textsuperscript{opt} and AA_Wild_TWE\textsuperscript{opt}. Similar curves (not shown) were obtained for the CA, but the transition started later in the cooling process (~40 °C).

At high temperatures (T>T\textsubscript{g}), agar molecules tended to exist in a random coil state where a predominance of the viscous character was observed (G">G' or tan δ>1). In the early stage of the cooling ramp, the values of G' were extremely small, close to the detection limit of the equipment, and are therefore not shown. The moduli of AA_Wild_MAE_GS\textsuperscript{opt} and AA_Wild_TWE\textsuperscript{opt} remained fairly constant until temperatures close to 43 °C (Note: despite the scattering of the acquired data this was the observed trend). Then, both moduli started to increase, G' increasing faster and crossing G" at ~ 41-42 °C. There was consequently a sharp decrease of tan δ (inset Fig. I.5.3), indicative of the system's elasticity gain, and was caused by the initial formation of double helices. For CA, the helical organization started at lower temperatures (i.e. sharp decrease of tan δ at ~40 °C; inset Fig. I.5.3). After the gelation point (tan δ=1), the elastic behavior became predominant (G">G" or tan δ<1) with the continuous increase in G' being a consequence of the side-by-side association of the helical structures to form larger aggregates which, at some point, would percolate into three-
dimensional networks. $G''$ followed the same trend but kept much lower values. Thus, at the end of the cooling ramp, the $\tan \delta$ of the three systems evolved to an asymptotic value, $\sim 0.025$, indicating comparable elasticity. Also, the $G'$ of AA_Wild_MAE_GS$^{opt}$ ($\sim 15$ kPa) was almost twice that of the AA_Wild_TWE$^{opt}$ ($\sim 8$ kPa) and clearly closer to that of the commercial agar ($\sim 19$ kPa). Nonetheless, all values were around 40-times higher than the respective $G''$ which was in favor of ‘true gels’ formation, $G' >> G''$ (Kavanagh & Ross-Murphy, 1998).

Immediately before the equilibration step, both moduli were still increasing yet at a much slower rate. Similar results were reported by other authors when studying agarose gels (Labropoulos et al., 2001; Lahrech et al., 2005; Mohammed et al., 1998; Nordqvist & Vilgis, 2011). The obtained curves are in accordance with the view that agar gelation is a two stage kinetic process in which the limiting step is the side-by-side association of the helices (Mohammed et al., 1998). The estimated $T_g$ values of AA_Wild_MAE_GS$^{opt}$ and AA_Wild_TWE$^{opt}$ differed slightly (respectively, $\sim 42.3$ °C and $\sim 40.9$ °C; respectively, Tables I.4.8 and I.4.10), and were higher than the CA $T_g$ value ($\sim 39$ °C; Table I.4.10). Differences in the methoxyl contents of the polymers could explain these results (Murano, 1995). Another possible explanation could be the positive influence of the size of the polymer chains on the gelation process. In agreement with previous reports (Lahrech et al., 2005; Marinho-Soriano & Bourret, 2005; Romero et al., 2008), the higher number of potential bonding sites in AA_Wild_MAE_GS$^{opt}$ (higher $M_v$ and LA content) seemed to promote the aggregation of the double helices (Norziah et al., 2006), with gelation occurring sooner in the cooling ramp (i.e. higher $T_g$).

The time evolution of the viscoelastic properties at 25 °C (Fig. I.5.4), with $G' >> G''$ throughout the entire time window, corresponds to a progressive reinforcement of the polymeric networks. It is noticeable that the AA_Wild_MAE_GS$^{opt}$ gel ($G' = \sim 25$ kPa) is more rigid than the AA_Wild_TWE$^{opt}$ gel ($G' = \sim 14$ kPa). At the end of the time sweep, both moduli were still increasing, but slowly enough to allow the frequency sweep to be recorded with some confidence.
The ‘true gel’ behavior of the polymers prevailed in the recorded frequency sweeps (mechanical spectra) for the applied angular frequencies (0.05-100 rad/s in Fig. I.5.5). Here, $G'$ and $G''$ appeared practically frequency-independent which agrees well with what was found by Mohammed et al. (1998) and Labropoulos et al. (2001).
There is, however, a slight monotonic increase in $G'$ with increasing $\omega$ accompanied by a slight decrease in $G''$ which is typical of high molecular weight amorphous polymers (Labropoulos et al., 2001). Overall, $G''(\omega)$ values calculated by the first order approximation to Kraemers-Kronig relation (Eq.I.2.4) were in good agreement with the measured ones (e.g. AA_Wild_MAE_GS$^{opt}$ in Fig. I.5.5b), thus confirming that the data were recorded within the linear viscoelastic region of the gels (Sittikijyothin et al., 2007). Plotting the mechanical spectra in terms of $|\eta^* (\omega)|$ data (Eq.I.2.5; e.g.AA_Wild_MAE_GS$^{opt}$ in Fig. I.5.5b)) confirmed the typical ‘true gel’ behavior of the three agar systems (Kavanagh & Ross-Murphy, 1998) with $n$ assuming close values, respectively, -1.01, -1.02 and -1.01 for AA_Wild_MAE_GS$^{opt}$, AA_Wild_TWE$^{opt}$ and CA. The estimated dynamic consistency index $K$, indicated a less cohesive gel for AA_Wild_TWE$^{opt}$ (20 kPa.s). The MAE polymer in turn, showed values closer to the reference (31 and 37 kPa.s, respectively). Accordingly, the elastic character of the systems throughout the entire frequency window followed the trend: CA > AA_Wild_MAE_GS$^{opt}$ > AA_Wild_TWE$^{opt}$. For instance, taking as reference the elastic storage modulus measured at 6.28 rad/s ($G_0$), AA_Wild_MAE_GS$^{opt}$, AA_Wild_TWE$^{opt}$ and CA showed values of respectively, ~34, ~26 and ~17 kPa. For the considered frequency, the three gel systems showed comparable elasticity, $\tan \delta$ (Table I.5.1).
Agar gels were heated from 25 to 95 °C at a very slow heating rate (0.1 °C/min) to allow the systems to equilibrate at each measured point. Results from the moduli dependence on temperature of AA_Wild_TWE$^{opt}$ and AA_Wild_MAE_GS$^{opt}$ are illustrated in Fig. I.5.6. The evolution with temperature of tan δ of the three agars (AA_Wild_MAE_GS$^{opt}$, AA_Wild_TWE$^{opt}$ and CA) is presented in the inset of Fig. I.5.6.

![Fig. I.5.6](image-url)  
**Fig. I.5.6** - Temperature dependence of elastic ($G'$; filled symbols) and viscous ($G''$; open symbols) moduli of 1.5 % wt agar gels/sols during heating ramp from 25 to 95 °C (AA_Wild_MAE_GS$^{opt}$ (squares) and AA_Wild_TWE$^{opt}$ (triangles)). **Inset Figure:** Temperature dependence of the loss tangent of 1.5 % wt agar gels/sols during heating ramp from 25 to 95 °C (AA_Wild_MAE_GS$^{opt}$ (open squares), AA_Wild_TWE$^{opt}$ (open triangles) and CA (open circles)). The measurements were recorded at 6.28 rad/s and 1% strain amplitude.

### Table 1.5.1 - Rheological properties of AA_Wild_MAE_GS$^{opt}$ and AA_Wild_TWE$^{opt}$. CA was used as reference.

<table>
<thead>
<tr>
<th>PROPERTIES</th>
<th>AA_Wild_MAE_GS$^{opt}$</th>
<th>AA_Wild_TWE$^{opt}$</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_g$ (°C)</td>
<td>42.3±0.2</td>
<td>40.9±0.5</td>
<td>38.9±0.3</td>
</tr>
<tr>
<td>$T_m$ (°C)</td>
<td>89.7±1.7</td>
<td>76.9±1.7</td>
<td>92.1±0.7</td>
</tr>
<tr>
<td>$\Delta T$ (°C)</td>
<td>46.8±1.7</td>
<td>36.0±1.1</td>
<td>53.1±1.0</td>
</tr>
<tr>
<td>$\tan \delta \times 10^2$</td>
<td>1.84±0.13</td>
<td>1.67±0.07</td>
<td>2.09±0.76</td>
</tr>
<tr>
<td>$G_0$ (kPa)$^{(1)}$</td>
<td>25.6±2.6</td>
<td>16.9±2.0</td>
<td>33.9±2.0</td>
</tr>
</tbody>
</table>

1 Average values (three replications) from mechanical spectra at 25 °C for 6.28 rad/s.
Upon heating, the gel networks are expected to undergo a gradual transformation (i.e. helix-to-coil transition) until reaching again the sol state. This conformational change will occur at much higher temperatures than the gelation point due to the high stability of the formed networks (Labropoulos et al., 2001). In the beginning of the heating step, $G'$ was much higher than $G''$ and remained fairly constant over a broad temperature range for the three agars, which seems to indicate that the dissociation of the aggregated helices was an equilibrium process (Mohammed et al., 1998). A sharp decrease in both moduli happened sooner for AA_Wild_TWE\textsuperscript{opt} (~56 °C) with the melting occurring around 76 °C ($T_m$). Subsequently, both moduli started to evolve asymptotically and, at the end of the heating period, the attained values of $G'$ and $G''$ were respectively, 0.019 and 0.072 Pa. At this point, the system was still changing, but at a slower rate. The inverse behavior was seen for $\tan \delta$, which remained fairly constant, rising sharply after $T_m$ (inset of Fig. I.5.6).

Sharp decrease in the viscoelastic moduli of AA_Wild_MAE\textsubscript{GS}opt\textsuperscript{opt} started later in the heating ramp (~70 °C; Fig. I.5.6) and consequently, the estimated $T_m$ was significantly higher (~90 °C). Accordingly, a wider thermal hysteresis (~47 °C) was observed for the AA_Wild_MAE\textsubscript{GS}opt\textsuperscript{opt} when compared to AA_Wild_TWE\textsuperscript{opt} (~36 °C). As seen from the inset of Fig. I.5.6, the sharp increase of $\tan \delta$ of AA_Wild_MAE\textsubscript{GS}opt\textsuperscript{opt} occurred at higher temperatures, with the elasticity loss of the system evolving in a similar way to the commercial sample. This seemed consistent with the higher thermal stability of the AA_Wild_MAE\textsubscript{GS}opt\textsuperscript{opt} and CA networks which, in turn, could be related to a positive effect of both $M_v$ and LA content. After the melting point, the viscous behavior became predominant ($G''>G'$) for the three samples marking the agar transition to the sol state. For the highest temperatures, where $G'$ and $G''$ assumed extremely small values (typically <0.1 Pa), it was difficult to obtain valid data. Indeed, high variability was found in the estimated $T_m$ ($G''=G'$ or $\tan \delta=1$) for the MAE sample. This was not surprising in the sense that, in the temperature window where the dissociation of the junction zones takes place, the sample viscosity decreases drastically and the applied deformation can damage the gel structure to a certain point (Norziah et al., 2006). Thus, it was difficult for the rheometer to acquire valid data. Overall, a closer resemblance was found between the rheological behavior of AA_Wild_MAE\textsubscript{GS}opt\textsuperscript{opt} and CA.
I.5.4. CryoSEM Studies of Agar Gels

The 3D networks of 1.5% wt AA_Wild_MAE_GS\textsuperscript{opt} and AA_Wild_TWE\textsuperscript{opt} gels, whose topology depends on the type of bonding and on the conformational constraints of the polymer chains, were observed by cryoSEM imaging (Fig. I.5.7).

![CryoSEM images of agar gels](image)

**Fig. I.5.7** - Representative cryoSEM pictures obtained for 1.5% wt AA_Wild_MAE_GS\textsuperscript{opt} (A) and AA_Wild_TWE\textsuperscript{opt} (B) gels at 2000 x, and 10 000 x (inset figure). The accelerating voltage was 15 kV and working-distances 15 m.

Rapid freezing of the gel structure reduces damage during sample preparation, particularly drying artifacts (critical in conventional SEM), thus making cryoSEM of great interest (Kaminskyj & Dahms, 2008). Agar obtained by MAE showed a more compact and regular polymeric network with well-defined pores (Fig. I.5.7A). The pores were homogeneously distributed throughout the structure, and presented similar and smaller sizes. The denser structure obtained for AA_Wild_MAE_GS\textsuperscript{opt} resembled that of the CA (not shown) and seemed to confirm the positive contribution of molecular mass and LA content to the assembly formation. Moreover, the attained results were in line with the higher GS value measured for AA_Wild_MAE_GS\textsuperscript{opt} (~1319 g/cm\textsuperscript{2} against ~1027 g/cm\textsuperscript{2} for AA_Wild_TWE\textsuperscript{opt}; Chapter I.4), reported as an indicator of more rigid networks (Labropoulos et al., 2001). Also, it agreed well with the higher reinforcement of the polymeric networks upon gelation recorded during the rheological measurements for AA_Wild_MAE_GS\textsuperscript{opt}. Similar images were found by Tuvikene and co-workers (2008) for agarose gels (Tuvikene et al., 2008). By contrast, agar extracted using conventional heating formed a less rigid network with the internal pores often exhibiting open irregular cavities (Fig. I.5.7B). The larger pores of AA_Wild_TWE\textsuperscript{opt} (2-3 µm, against roughly 1 µm in AA_Wild_MAE_GS\textsuperscript{opt}) with free dangling and thinner walls,
came as no surprise and were mainly attributed to the lower LA and molecular mass of the sample.

Overall, a very good agreement was found between the physicochemical properties, rheological and imaging studies of the SP. The higher aggregation capability of AA_Wild_MAE_GS\textsuperscript{opt} relatively to AA_Wild_TWE\textsuperscript{opt} under comparable conditions, matched its enhanced gelling properties, as determined in texture (higher GS) and rheological (higher thermal stability and consistency of the system) measurements. These findings seemed also in favor of a positive contribution of molecular mass and LA content to the assembly formation, rather than a direct consequence of the negative influence of the sulfate groups (i.e. formation of kinks during association of helices), which were in similar number in both agars.

### I.5.5. Conclusions

AA_Wild_MAE_GS\textsuperscript{opt} showed greater capacity for self-association than AA_Wild_TWE\textsuperscript{opt}, at comparable conditions, which seemed in accordance with its higher GS, consistency and thermal stability. At low polymer concentration, AFM images of both agars showed thin gelled layers. Depending on the cooling rate of the solutions, different structures were formed and seemed larger for AA_Wild_MAE_GS\textsuperscript{opt}. The 3D gel network of AA_Wild_MAE_GS\textsuperscript{opt} was compact and regular contrasting with a more opened structure of TWE polysaccharide. Self-association seemed mostly favored by the molecular mass and LA content. If properly controlled, non-conventional energies like microwaves could extract non-degraded agars with comparable properties to commercial ones, thermal-extracted.
References.


microscopy in relation to the rheological behavior in aqueous systems. 1. Gellan gum with various acyl contents in the presence and absence of potassium. Food Hydrocolloids, 22(6), 1148-1159.


I.6 INFLUENCE OF MAE CONDITIONS ON THE BEHAVIOR OF NATIVE AND ALKALI-MODIFIED AGARS IN DILUTE AND CONCENTRATED AQUEOUS MEDIA – CASE STUDY 2

CHAPTER I.6
This chapter will focus a case study concerning the influence of the MAE conditions on the behavior of NA and AA in dilute and concentrated aqueous media. The extracts from wild biomass, NA_Wild_MAE_{opt}, AA_Wild_MAE_GS_{opt} and AA_Wild_MAE_Y_{opt}, were chosen for this study. The SP behaviors in the sol-gel state will be compared in the light of relevant physicochemical properties previously discussed in section I.4.2 of Chapter I.4. The data presented here can be found in a recent publication (Sousa et al., 2013).

**I.6.1. Dilute Regime Studies**

Details on the molecular assemblies of NA_Wild_MAE_{opt}, AA_Wild_MAE_Y_{opt} and NA_Wild_MAE_GS_{opt} were obtained by imaging dilute aqueous media of increasing concentration (5, 10, 50 and 100 µg/mL) by AFM. Previous AFM studies have provided relevant knowledge on other polysaccharides behavior in solution and gels (Fishman et al., 2007; Ikeda et al., 2001; Morris et al., 2011; Roesch et al., 2004). As stressed out in Chapter I.5, polysaccharides are prone to certain artifacts during the sample preparation procedure for AFM analysis (Ikeda et al., 2001; Kirby et al., 1996; Robic et al., 2009).

**I.6.1.1. Influence of Media Concentration**

5 µm × 5 µm topographic and equivalent amplitude AFM images of NA_Wild_MAE_{opt} for the 5, 10, 50 and 100 µg/mL solutions are illustrated in Fig. I.6.1.
Fig. I.6.1 - Topographical (A,C,E,G) and equivalent amplitude (B,D,F,H) AFM images of NA_Wild_MAE\textsuperscript{opt} structures formed on the 5 (A-B), 10 (C-D), 50 (E-F) and 100 (G-H) \(\mu\)g/mL dilute aqueous solutions when deposited onto mica and air dried. The image size is 5 \(\mu\)m \(\times\) 5 \(\mu\)m. Examples of NA_Wild_MAE\textsuperscript{opt} aggregates (1), local networks (2), cyclic structures (3), individual fibers (4) and branching (5) are identified by numbered arrows.
As the concentration increased, a progressive structural reinforcement could be observed. At 5 μg/mL, short individual fibers were predominant on the NA_Wild_MAEmax background although longer and thicker particles were sporadically seen (Figs. I.6.1 A/B and I.6.5 A/B). Small aggregates were also visible (see arrow 1) which, could be interpreted as highly associated fibers aggregated upon drying of the samples on the substrate (Morris et al., 2010). Local networks (2) were very rarely seen, suggesting that the chosen concentration could be close to the critical value for network formation. At 10 μg/mL (Figs. I.6.1 C/D), NA structures included individual fibers (4) with occasional branching (5), cyclic structures (3), aggregates (7) and local networks (2). The strands were mainly long although shorter particles could still be observable. Further association of agar helices at higher concentrations (50 and 100 μg/mL, respectively, Figs.I.6.1 E/F and I.6.1 G/H) resulted in a polymeric network covering the substrate. Occasionally, brighter dots could be seen in the AFM micrographs matching points of higher heights (1 in Figs.I.6.1 E/F and I.6.1 G/H) which were attributed to fibrous aggregates. NA images seemed to suggest some orientation of the fibers probably due to a less rigid nature of the polysaccharide (Ikeda et al., 2001).

Fig. I.6.2 shows the 5 μm × 5 μm topographical and equivalent amplitude AFM images of AA1 dried solutions on the mica surface.
Fig. I.6.2 - Topographical (A,C,E,G) and equivalent amplitude (B,D,F,H) AFM images of AA1 structures formed on the 5 (A,B), 10 (C,D), 50(E,F) and 100 (G-H) μg/mL dilute aqueous solutions when deposited onto mica and air dried. The image size is 5 μm × 5 μm. Examples of AA_Wild_MAE_Yopt aggregates (*) are identified accordingly.
At the lowest polymer concentration, highly entangled fibers formed networks with no particular ordering (‘random networks’) (Figs. I.6.2 A/B). No clear indication of orientation or branching of the material was obtained from the AFM images examination. Small spherical-or odd shaped aggregates were sporadically seen resulting as previously, in brighter regions in the AFM micrographs (1 in Figs. I.6.2 A/B). Deposition of AA_Wild_MAE_Y_{opt} at 10 μg/mL resulted in reinforced ‘random networks’ which further developed into polymeric layers at higher concentrations (50 and 100 μg/mL). Here as well, occasional aggregates resulted in brighter dots in the AFM images (1 in Figs. I.6.2 E/F and G/H, respectively).

Fig. I.6.3 provides information on the molecular assemblies of AA_Wild_MAE_GS_{opt} in dilute aqueous media, when adsorbed onto mica.
Fig. 1.6.3 - Topographical (A,C,E,G) and equivalent amplitude (B,D,F,H) AFM images of AA_Wild_MAE_GSopt structures formed on the 5 (A-B), 10 (C-D), 50 (E-F) and 100 (G-H) μg/mL dilute aqueous solutions when deposited onto mica and air dried. The image size is 5 μm × 5 μm. Example of AA_Wild_MAE_GSopt aggregates (1), side-by-side aggregation (2) and partner switching with end association (3) of the fibers, cyclic structures (4) are identified accordingly.
At 5 μg/mL, well-defined fibers with different degrees of entanglement could be seen with great detail (Figs. I.6.3 A/B). The elongated shape and variable lengths of the strands suggested polymer chains with non-uniform sizes (Ikeda et al., 2001). This agreed well with the \( D_M \) estimations by SEC (higher \( D_M \sim 3.1 \) for AA_Wild_MAE_GS\textsuperscript{opt}; Table I.4.8). Also, the fibers seemed rigid enough to prevent orientation during the spreading of the sample (Ikeda et al., 2001).

Side-by-side association of the strands was suggested from the images examination (2 in Fig. I.6.3 A/B). At some point, this association ceased, guessing the presence of alkali-stable sulfates in the polymer skeleton, and the ends of the fibers would connect to a different partner (3 in Figs. I.6.3 A/B) (Arnott et al., 1974; Clark & Ross-Murphy, 1987). In our own view, these “primary” structures would lead to the formation of the fibrous rings (4 in Figs. I.6.3 A/B) that would percolate into local networks when sufficiently entangled. The considerable widths and only occasionally seen ends of the fibers further confirmed a low-level of aggregation of AA_Wild_MAE_GS\textsuperscript{opt} helices (Ikeda et al., 2001). The critical value for network formation in this case seemed clearly below the minimum chosen concentration. Similar fibrous particles were reported by Ikeda et al. (Ikeda et al., 2001) and Roesch et al. (Roesch et al., 2004) in studies of the gelation mechanism of κ-carrageenan, a red seaweed polysaccharide resembling agar.

A considerable structural reinforcement was seen when doubling the polymer concentration (Figs. I.6.3 C/D). The intensive association of agar helices formed an extremely well organized network of thick strands covering the entire mica surface. Aggregates were rarely seen in the AFM images (1 in Figs. I.6.3 C/D). The impressive organization of AA_Wild_MAE_GS\textsuperscript{opt} molecules, seen with higher clarity at an enlarged scanning size (3 μm × 3 μm) in Fig. I.6.4, suggested fairly rigid and stable fibers (Ikeda et al., 2004). As previously seen for NA_Wild_MAE\textsuperscript{opt} and AA_Wild_MAE_Y\textsuperscript{opt}, the percolation threshold of AA_Wild_MAE_GS\textsuperscript{opt} occurred between 10-50 μg/mL. At higher concentrations, multilayer polymeric networks, formed by even thicker AA_Wild_MAE_GS\textsuperscript{opt} strands, covered the substrate (Figs. I.6.3 E/F and I.6.3 G/H for respectively, 50 and 100 μg/mL), with intensive aggregation of fibers resulting at some point in large agglomerates (1).
I.6.1.2. Influence of the Nature of Agar Chains

From image observation, one can also confirm the greater lengths and considerable widths of AA_Wild_MAE_GS$^{opt}$ fibers (Figs. I.6.5 E/F) when compared with the other samples (Figs. I.6.5 A/B and I.6.5 C/D).
Fig. I.6.5 - Enlarged scanning size (3 μm × 3 μm) of topographical (A, C, E) and equivalent amplitude (B, D, F) AFM images of NA_Wild_MAE_opt (A-B), AA_Wild_MAE_Y_opt (C-D) and AA_Wild_MAE_GS_opt (E-F) for the 5 μg/mL polymer concentration. The curves below the images represent the height profile along the white line drawn in the images.

Also, the ring-shaped fibrous segments percolated into localized networks could be seen with remarkable detail. The ‘random networks’ of AA_Wild_MAE_Y_opt (Figs. I.6.5 C/D) and the visible lower self-association capability of NA_Wild_MAE_opt helices (Figs. I.6.5 A/B)
contrasted greatly with the AA_Wild_MAE_GS$^{opt}$ ordering (Figs. I.6.5 E/F). The greater aggregation propensity of AAs could be easily attributed to their lower sulfate/LA ratio (Murano, 1995). Assuming as valid the overall trend of the $M_w$ data (AA_Wild_MAE_GS$^{opt}$>AA_Wild_MAE_Y$^{opt}$; Table I.4.8), the reinforcement of the AAs networks should arise from longer chain lengths, which seems to agree with the findings of Normand et al. (Normand et al., 2000). The LA units, with higher expression in the AA skeletons, favored the perfect helices alignment by substantially increasing the potential hydrogen bonding sites between two agar chains (Boral et al., 2008; Labropoulos et al., 2001). Therefore, it seems rather intuitive that longer chain lengths would further increase the network connectivity, thus adding stability to the polymeric structure. Also, methylated segments such as G6M and L2M clearly identified in the studied samples (ins of Fig. I.4.4 in Chapter I.4), are reported to influence the ordering temperature (i.e. gelation point) of agar systems (Lahaye & Rochas, 1991; Nijenhuis, 1997).

**I.6.1.3. Comparison with Previous Studies**

The average heights ($H_{av}$) of the formed agar structures were estimated from the 3 μm × 3 μm topographic AFM images (Figs. I.6.4, I.6.5 and others not shown) and interpreted in the light of the helical model proposed for agar gelation (Arnott et al., 1974; Djabourov et al., 1989). In order to eliminate possible contributions of abnormal size aggregates to the estimated AFM indicators, height profiles of representative cross sections in the above mentioned images were also examined. The estimated widths of NA_Wild_MAE$^{opt}$ and AA_Wild_MAE_GS$^{opt}$ fibers displayed in Figs. I.6.5 A/B and E/F (at least 10-times greater than the measured heights) confirmed some compression of the samples (Ikeda et al., 2001; Morris et al., 2010). Thus, only the structures’ heights were interpreted as advised by other authors (Ikeda et al., 2001; Morris et al., 2010).
### Table I.6.1 - Estimated average heights ($H_{\text{av}}$) of NA$_{\text{Wild\_MAE}}$$^{\text{opt}}$, AA$_{\text{Wild\_MAE\_Y}}$$^{\text{opt}}$ and AA$_{\text{Wild\_MAE\_GS}}$$^{\text{opt}}$ for the 5, 10, 50 and 100 µg/mL dilute solutions. The parameters were obtained from the 3 µm × 3 µm AFM topographic images using the free Gwyddion 2.22 software.

<table>
<thead>
<tr>
<th>DILUTE MEDIA CONC. (µg/mL)</th>
<th>AVERAGE HEIGHT, $H_{\text{av}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA$_{\text{Wild_MAE}}$$^{\text{opt}}$</td>
</tr>
<tr>
<td>5</td>
<td>1.73</td>
</tr>
<tr>
<td>10</td>
<td>2.11</td>
</tr>
<tr>
<td>50</td>
<td>2.53</td>
</tr>
<tr>
<td>100</td>
<td>2.91</td>
</tr>
</tbody>
</table>

As data in Table I.6.1 show, the estimated $H_{\text{av}}$ increased with the polymer concentration, and at each concentration, they were significantly higher for AAs. At 5 µg/mL, the measured $H_{\text{av}}$ for NA$_{\text{Wild\_MAE}}$$^{\text{opt}}$ was 1.73 nm (Figs. I.6.5 A/B) whereas, 2.09 and 5.09 nm was obtained for, respectively, AA$_{\text{Wild\_MAE\_Y}}$$^{\text{opt}}$ (Figs. I.6.5 C/D) and AA$_{\text{Wild\_MAE\_GS}}$$^{\text{opt}}$ (Figs. I.6.5 E/F). X-ray diffraction and optical rotation studies of Arnott and co-workers (Arnott et al., 1974) suggested agarose double helix to be formed by two parallel left-handed helices with a 3-fold symmetry (three G-LA units per turn) of 1.9 nm pitch and an axial translation of 0.95 nm. The idea that the formation of agarose networks should also involve intensive double helix association, was proposed more than a decade later by Djabourov et al. (Djabourov et al., 1989). Presently, it is believed that agar fibrils (diameter distribution in the range 3-10 nm) containing six or more double helix compose the percolating 3D networks of agarose gels when sufficient concentration is reached (Chavez et al., 2006; Djabourov et al., 1989). From these early experimental observations (Arnott et al., 1974; Chavez et al., 2006; Djabourov et al., 1989) and based on the known physicochemical properties of the studied agars, we could suspect that for the most dilute medium, NA$_{\text{Wild\_MAE}}$$^{\text{opt}}$ existed mainly as individual molecules while associated double helices were seen in the case of AA. Among these, higher entanglement levels were seen for AA$_{\text{Wild\_MAE\_GS}}$$^{\text{opt}}$. Thus, even if some compression of the samples during the AFM analysis should underestimate the $H_{\text{av}}$ (Morris et al., 2010) the present AFM data clearly support the current vision accepted for agar gelation (Chavez et al., 2006; Djabourov et al., 1989).
In good agreement with the massive aggregation states observed when increasing the polymer concentration, higher $H_{av}$ values were obtained at 50 and 100 µg/mL. The apparent mismatch between the $H_{av}$ estimated for AA_Wild_MAE_Y$^{opt}$ and AA_Wild_MAE_GS$^{opt}$ at 100 µg/mL could be easily explained by the presence of abnormal size aggregates in the AFM image (not shown). The same goes for the AA_Wild_MAE_Y$^{opt}$ data at higher concentrations. Examination of the respective height profiles confirmed this interpretation (not shown).

I.6.2. Concentrated Regime Studies

As concentration was further increased, a significant reinforcement of the NA_Wild_MAE$^{opt}$ and both AAs assemblies was seen (insets of Fig. I.6.6).

![Large deformation profiles of NA_Wild_MAE$^{opt}$ squares] and AA (AA_Wild_MAE_Y$^{opt}$ triangles; AA_Wild_MAE_GS$^{opt}$ circles) gels recorded through penetration tests. The concentration of the hydrogels was 1.5 % wt. **Insets:** CryoSEM pictures of NA_Wild_MAE$^{opt}$ (A), AA_Wild_MAE_Y$^{opt}$ (B) and AA_Wild_MAE_GS$^{opt}$ (C) 1.5% wt hydrogels at 5000 x magnification. The accelerating voltage is 15 kV and working-distances 15 mm in all the cases. These profiles match the GS data listed in Chapter I.4, Table I.4.8, for the same samples.
Table I.6.2 – Failure stress and failure strain data for NA_Wild_MAE$_{opt}$, AA_Wild_MAE$_{Y, opt}$ and AA_Wild_MAE$_{GS, opt}$ (values measured at the point of rupture of the gel structure in Fig. I.6.6).

<table>
<thead>
<tr>
<th>PROPERTIES</th>
<th>NA_Wild_MAE$_{opt}$</th>
<th>AA_Wild_MAE$_{Y, opt}$</th>
<th>AA_Wild_MAE$_{GS, opt}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Failure stress (kPa)</td>
<td>15±5</td>
<td>53±0.1</td>
<td>108±11</td>
</tr>
<tr>
<td>Failure strain ×10$^{-3}$</td>
<td>103±6</td>
<td>101±2</td>
<td>170±3</td>
</tr>
</tbody>
</table>

The large deformation profiles of the equilibrated agar gels (Fig. I.6.6) matched previous ones from agarose, obtained through compression tests (Amici et al., 2000; Aymard et al., 2001; Normand et al., 2000). The shape of the curves showed that the hydrogels broke at small strains, although the stress required for reaching the breakup point was quite large, particularly in the case of AAs.

According to that seen in the literature (Aymard et al., 2001), in the early stage of the test (i.e. linear viscoelastic region) both AAs exhibited similar responses. For large deformations however, a non-linear response was recorded and the systems behaved quite differently. At the breakup point of the gel, the measured stress was, by far, higher for AA_Wild_MAE$_{GS, opt}$ (~108 kPa against ~53 kPa for AA_Wild_MAE$_{Y, opt}$; Table I.6.2). The combination of three factors could account for this behavior: the lower sulfate/LA ratio and higher $M_w$ which consequently, led to more bounded water to the LA units. It is believed that an internal cavity along the helix axis can accommodate water molecules linked to the hydroxyl groups of the agarose chains which are expected to add more stability to the network (Arnott et al., 1974; Chavez et al., 2006). As found previously for pectin gels (Fishman et al., 2007), uniform networks of small pores resulted in better gelling properties. The stronger intermolecular interactions (Boral et al., 2008) and higher connectivity between the AA_Wild_MAE$_{GS, opt}$ molecules (i.e. higher number of junction zones) resulted in a very homogenous, dense and compact 3D network of small pores (Fig. I.6.6, inset C) that seems to be reflected in enhanced gelling properties.

In good agreement with the AFM data, the 3D network of the AA_Wild_MAE$_{Y, opt}$ gel was dense yet, very heterogeneous when compared to AA_Wild_MAE$_{GS, opt}$ (Fig. I.6.6, inset B). The broad distribution of pore sizes could confirm this observation. Thus, the lower stress-at-break of AA_Wild_MAE$_{Y, opt}$ was not surprising. Heterogeneities within gels’ systems are reported to be relevant for the mechanical response at large deformations (Aymard et al., 2001; vanVliet & Walstra, 1995) since the system is more susceptible to
fracture by the ‘weak regions’ (i.e. lower density regions in Fig. I.6.6, inset B) (vanVliet & Walstra, 1995).

The mechanical response of NA_Wild_MAE\textsuperscript{opt} gel showed similar trend but both responses (linear and non-linear) fell significantly below those of the AAs. Thus, at the point of the gel breakup the measured stress was only ~15 kPa. NA exhibited an irregular network with open pores of heterogeneous sizes (Fig. I.6.6, inset A), attributed to the higher number of sulfated monomers which could hamper the perfect alignment of the helices. The low density of the network when compared to AAs suggest less junction zones between helices to “unzip”, thus causing the easier fracture of the gel (lower stress-at-break) (vanVliet & Walstra, 1995).

Gels of AA are expected to exude more water on aging (Matsuhashi, 1990) and for that reason are typically more brittle (lower strain-at-break) than native ones. It has been found that the intensive interhelical aggregation during gel formation forces unbound water to leave the interstitial pores of the networks. Also, a negative correlation is usually expected between the amount of exuded water and the sulfate content of AA gels (Matsuhashi, 1990). Thus, a counterbalance between the above mentioned factors ($M_w$, sulfate content and unbound water) could easily explain the obtained strain-at-break data (Table I.6.2).

I.6.3. Conclusions

Our study suggests that agar shows great self-entanglement capability even in very dilute aqueous media. Depending on the followed MAE route, agars with distinct properties can be obtained thus exhibiting markedly different molecular assemblies in solutions and gels. The ability to modulate the agar skeleton by an alkali modification results in polysaccharides of enhanced gelling capability (lower sulfate/LA ratios). Also, higher molecular mass seems to promote the reinforcement of the polymeric networks, thus favoring gelation. When increasing the polymer concentration, progressive aggregation states were observed; first locally (agar individual molecules → cyclic segments → local networks) with the agar networks becoming coarser (single or multilayer polymeric networks) and reaching a macroscopic level for the concentrated solutions (macroscopic gels). The 2D-shape objects observed by AFM in the dilute media agreed well with the 3D architectures recorded by cryoSEM. A more compact and uniform 3D network resulted in higher resistance to large deformations. Depending on the nature of AA chains, homogenous or heterogeneous assemblies’ growth was seen during network formation. This was relevant for the mechanical
behavior of the hydrogels at large stresses. The present evidence supports well the view proposed for agar gelation, double helix formation followed by intensive interhelical association. Moreover, these findings prove that the molecular assemblies of agars can be tuned via MAE which can be of great interest towards the development of new agar-based materials for emerging applications.

References.


PART II

SUITABILITY OF THE EXTRACTED AGARS FOR THE FABRICATION OF SUSTAINABLE MATERIALS.
II.1 LITERATURE REVIEW

CHAPTER II.1
II.1.1. A factual truth: *We need Plastics!*

The use of plastics has become a true necessity of modern times. Features such as high versatility, durability, low-cost or high volume-to-weight ratio provide great functionality to these man-made polymeric materials used in a wide range of applications. Indeed, if we look around not many of our daily activities are ‘plastic-free’. From the components of our cars, aircrafts or mobile phones to the credit card we use to pay the bills, plastics are everywhere and it is quite reasonable to say they provide great commodity to our existence. Another key benefit of plastics is their usefulness as packaging materials. Almost every kind of products, from food or beverages, to detergents, cosmetics, pharmaceutics, or even chemicals, can be stored in plastic bags and/or containers (Shah et al., 2008).

The plastic industry grew tremendously over the last century to become one of the world leading economic driving forces comprising various converting sectors such as packaging, construction, automotive and others. According to the report *Plastics – The Facts 2011* (published in [www.plasticseurope.org](http://www.plasticseurope.org)), in 2010, approximately 265 million tons of plastics were produced worldwide with China, Europe and the USA being the main contributors. Even though currently facing a global economic crisis, the sector is expected to experience great growth in the coming years much due to emerging economies such as Brazil, the Middle East countries or India. Excellent petrochemical resources and low-cost labor are the main advantages of these countries. India’s extremely low plastics per capita consumption and very high population density add to the country’s growth potential in the global plastic scenario ([www.cipet.gov.in](http://www.cipet.gov.in)).

On the same report by the European plastic industry we can see Germany, France and Italy as the most active plastic producers in Europe. Following the global trend, the packaging (39.0%) was by far the most relevant European converting sector. Synthetic plastics offered a unique platform of development to the packaging sector due to high performances (mechanical as well as water and thermal stabilities), lightness and low-cost when compared with the initially explored cellulose and cellulose-based materials. Some of the most commonly used plastics for packaging include low density polyethylene (LDPE), linear low density polyethylene (LLDPE), and high density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), polyurethane (PU), polystyrene (PS) and polyethylene terephthalate (PET) (Shah et al., 2008).

In 2011, the annual investment of the Portuguese plastic sector for internal demand was ~1.3 million €. Polyethylene (PE~23%), PP and other olefins (~26%) as well as polycarbonates/polyacetals/polyesters (~20%) were the main inputs while PE and PVC...
II.1 LITERATURE REVIEW

comprised most of the country’s outputs (~78%) (data from the Portuguese Association of the Plastic Industry (APIP) in http://www.apip.pt/).

While PE is attractive for the production of plastic bags, food packaging films and water bottles, PP can be shaped in the form of car parts, domestic furniture, bottle caps or disposable syringes. In addition, PP is most adequate for flexible packaging such as yogurt containers, straws or syrup bottles (http://www.lenntech.com/polypropylene.htm). Besides food packaging with efficient barrier to gases, PVC can be used for the manufacture of pipes, medical tools, car seat covers and bottles just to name a few (Filho et al., 2005; Shah et al., 2008) (http://www.institutodopvc.org/).

Indeed, a fair measure of the tremendous success of plastics relies on its outstanding versatility. Besides being shaped into countless commodity and/or durable goods, plastics which can be biocompatible and non-toxic (e.g. PVA, PEO, …) can be processed in the form of beads, capsules, thin films or fibers reaching out to more sensitive applications such as membrane technology, drug delivery systems, enzyme immobilization, electronics and sensors (Bhunia et al., 2013; Caramia et al., 2013; Jankovic et al., 2013; Sin et al., 2012; Toskas et al., 2011).

In view of the above facts, the more than 60 million jobs generated worldwide by the plastic industry cannot be entirely surprising (www.acs.org). The employment numbers from the EU Eurostat report 1.6 million jobs created by the plastics productive and converter sectors (EU Eurostat in www.plasticseurope.org).

II.1.2. Drivers for Bioplastics

Unfortunately, serious environmental problems stem from the massive use of petroleum-based plastics. The greenhouse gases (GHG) emissions into the atmosphere during the production and disposal processes of plastics and the landfill depletion caused by millions of tons of plastic waste that are continuously being generated, cause increasing ecological concerns (Golghate & Pawar, 2011; Queiroz & Collares-Queiroz, 2009). Scientists believe that the GHG effect, mainly driven by the burning of fossil fuels, is responsible for climate changes that are dramatically gaining strength (www.epa.gov). Very recently, at the 18th Conference of the Parties (COP) in Doha, Dr. R. K. Pachauri from the Intergovernmental Panel on Climate Change (IPCC) reinforced “…the seriousness of impacts that would be faced by the world in the years ahead if we do not take timely and adequate action to limit the concentration of GHGs in the earth’s atmosphere, and if we do not adapt to the level of climate change which is now committed to happen.”. At the 2008 G8 summit in Toyako,
Japan, all parties estimated that the CO$_2$ emissions should be reduced by more than 50% when reaching 2050.

The ecosystems are also greatly harmed by the accelerated proliferation of plastics into the environment (Moore, 2008). Plastics have been accumulating over the decades in marine environments and are now commonly ingested by sea turtles, birds and sea mammals (Moore, 2008). In land, several countries are taking actions to stop this threat. For instance, the Mauritania’s governmental authorities recently banned the use of plastic bags since 70% of the cattle and sheep died from eating these end-use products (http://www.bbc.co.uk/news/world-africa-20891539?print=true).

The continuous scale up of oil and gas prices with constant price fluctuations further justify a shift towards renewable resources.

Human health risks associated to plastics exposure is another controversial aspect often debated among the scientific community (Halden, 2010; Mankidy et al., 2013). The migration of toxic plastic components into food products is a current topic concerning the packaging sector (Lagaron & Lopez-Rubio, 2011).

In this unbearable scenario, time has come to make decisions towards sustainable development. In this regard, ‘green-label’ products are getting higher acceptance worldwide.

II.1.3. The Bioplastic Sector

Although it has been developing rapidly during the last decade (approx. 20% annual growth) the bioplastic sector still has a small contribution in the current global plastic scenario.

In 2011, less than 1% of the global annual plastics production was attributed to bioplastics (i.e. approx. 1 million tons) (Institute for Bioplastics and Biocomposites from the University of Hannover – SpecialChem- Oct 12, 2011 published in www.bioplasticseurope.org). The contribution of Portuguese companies to the sector inputs during the same year was even more residual (<0.1% according to the APIP in http://www.apip.pt/). Clearly, there is a need to increase the production and consumption of bioplastics.

In the coming years and despite the economic recession, the bioplastic sector will continue to develop at good speed and is expected to expand greatly, reaching ~3.7 to 5.8 million tons by the year 2016. While Europe and the USA seem to struggle when it comes to production capacity, Asia and South America will continue to reinforce their positions and by
2016 both regions will account for most of the global bioplastic production (>90%; www.bioplasticseurope.org).

**II.1.3.1. The Bioplastic Concept**

Whereas biodegradability was the initial catalyst for the bioplastic sector (regardless of the material origin) currently, plastics from bio-based origin constitute the main focus. Hence, the concept 'bioplastic' can account for both meanings: origin and/or end-of-life of the material. Biodegradability refers to the products disposal and identifies, according to universal standards, materials that are ‘capable of undergoing decomposition into biomass, water, carbon dioxide and/or methane via enzymatic action or microorganisms, in a specified timeframe, reflecting the conditions of the disposal method’ (Song et al., 2009). Bio-based origin means that the plastic comes from 'organic carbon' or 'renewable carbon' which is the case of plant-derived and animal-derived biomass. Concerning the followed route to obtain the bio-based polymer it is possible to identify three main groups (Averous, 2004; Queiroz & Collares-Queiroz, 2009): 1) polymers directly separated or extracted from biomass such as cellulose, starch and SP; 2) polymers produced by microorganisms such as polyhydroxyalkanoates (PHA) and 3) polymers produced from starting bio-based building blocks further synthesized through classical chemical routes. Materials such as polylactic acid (PLA) produced from lactic acid monomers (mainly from starch and sugar) preferentially via ring opening polymerization and non-biodegradable bio-versions of synthetic resins, such as bio-polyethylene (bio-PE) obtained from sugar cane ethanol, comprise this latter category.

In the opposite side of the spectrum, are the plastics that can have a ‘green’ end-of-life option, *i.e.* biodegradable polymers, although being from petrochemical origins (*e.g.* poly-ε-caprolactones (PCL), polyvinyl alcohols (PVA)). EcoFlex® from BASF which is fully biodegradable and compostable even though it comes from fossil fuel origins is another good example of this type of materials (Siegenthaler et al., 2012). Mixing both bio-based and petroleum-based monomers is another approach for producing bioplastics.

Bio-versions of petroleum-based resins also known as ‘drop ins’ (*e.g.* bio-PE, bio-polyethylene terephthalate (bio-PET), bio-polyurethane (bio-PU)) are current trends of the bioplastic market due to identical performances to their synthetic counterparts and ease of processability (*i.e.* produced in conventional machinery) (www.bioplasticseurope.org). Sectors that generate more plastic waste such as packaging are the most receptive to shift from petroleum-based to bio-based plastics. Manufacturers of discardable products such as plastic cups, plastic bottles and plastic bags are good examples. For instance, the 'green PE'
produced by Braskem is fully biobased (*i.e.* made from renewable resources) yet it is neither biodegradable nor compostable ([www.braskem.com.br](http://www.braskem.com.br)). The same goes for the first fully bio-based PET bottle launched by PepsiCo made from plant-derived sources such as switchgrass, pine bark and corn husks ([www.pepsico.com](http://www.pepsico.com)).

Other current outputs of the bioplastic market include PLA, modified starch, cellulose derivatives and PHAs, although in these cases cost, production, or performance issues still narrowstheir commercialization.

PLA can be compostable under specific conditions and thermally processed with currently used processing technologies such as extrusion or thermoforming (Lim *et al.*, 2008). Despite the remarkable accomplishments made so far in terms of price competitiveness, the cost production of PLA is still higher than synthetic plastics. Currently, PLA is mainly used as packaging material for short-life food or single-use products although durable goods (electronics, automotive,...) are also manufactured (Auras *et al.*, 2004; Lim *et al.*, 2008). Ingeo® from Natureworks LLC ([http://www.natureworksllc.com/](http://www.natureworksllc.com/)) or high performance PLA from Purac ([http://www.purac.com/EN/Bioplastics/News-Press-Events/News/Purac-at-Fakuma-2012.aspx](http://www.purac.com/EN/Bioplastics/News-Press-Events/News/Purac-at-Fakuma-2012.aspx)) produced to replace PP, PS and acrylonitrile butadiene styrene (ABS) in demanding applications are self-explanatory of PLA’s placement in the current global market. Most of PLA’s production comes from food feedstock although seaweed are starting to be explored to this end.

Cellulose and starch are very abundant in nature which makes them very cost-attractive. Both have D-glucose as the main building unit in their backbone although with completely different organizations (Averous, 2004). The D-glucose units in cellulose form a microfibrillar structure of semi-crystalline nature making it extremely attractive as reinforcing material for polymer composites. Wood pulp and cotton are the most used raw-materials by cellulose industry due to their respectively, large availability and high cellulose content. Besides the good mechanical performances cellulose fibers present low density which makes them great substitutes for glass fibers in sectors such as the automotive where the cars weight reduction can be very beneficial (Zini & Scandola, 2011). Also, they have low cost, good insulation capacity and are biodegradable. Cellulose can be chemically modified into cellulose acetate which is a thermoplastic material. NatureFlex™ and Cellophane™ from Innovia are two good examples of commercialized cellulose-derived bioplastics.

The granular nature of starch, formed by amylose and amylopectin, is often disrupted by chemical, thermal and/or mechanical processes, for producing bioplastics. Thermoplastic starch (TPS) has high density and lower mechanical performances than synthetic polymers yet, it is relatively cheap, biodegradable and can be processed with conventional technology. Hence, it is widely used in combination with other polymers. 100% bio-based and
biodegradable starch blends include PLA or PHA/PHB combinations with TPS or chemically modified starch while Mater-Bi® from Novamont is only in part bio-based but also biodegradable. Starch blending with petroleum-based polymers (e.g. PE, PP, PU, PCL) is also an attractive possibility. For instance, Mater-Bi® covers an interesting array of applications such as bags for composting waste (with breathable systems), mulch films or even as biofiller in rubber matrices for tires production. Starch is also the first option of leading Portuguese companies in the sector when it comes to bio-based bioplastics. The compostable (EN 13432) cling film from Silvex is mainly sold in external markets such as the Nordic countries, Germany and USA (http://greensavers.sapo.pt/2013/06/06/sacos-de-plastico-portugueses-ecologicos-e-com-prazo-de-validade-com-video/). With a strong R&I component, Silvex seems committed to broaden its options of bio-products and has recently announced the successful fabrication of mulch films from corn starch (http://www.publico.pt/ecosfera/noticia/projecto-cria-plastico-biodegradavel-adaptado-a-agricultura-em-portugal-1595313). Biomind products, from the Portuguese company Cabopol, are processed in conventional machinery. Most of these starch-based materials are approved for contact with food (CE 1935/2004) and are on way to be certified as compostable (EU 13432) (http://www.biomind.pt/). Obviously, bioplastics from petrochemical origins with green end-of-life options such as bi-oriented polypropylene (BOPP) films have greater acceptance by the market. The Portuguese leading company Casfil is a great example of a prominent producer of this kind of packaging films and others with a strong export activity (http://www.casfil.pt/).

PHAs are microbial polyesters produced by several microorganisms. PHAs building monomers can present different compositions thus reflecting distinct properties and functionalities of the final polymer. Currently, only a small variety of PHAs (e.g. polyhydroxybutyrate (PHB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate (PHBH)) are produced at an industrial scale. For instance, PHB has mechanical properties similar to PP yet, the high production cost of these microbial derived polyesters narrows the number of possible applications. Nonetheless, in areas such as medical, PHAs performances well justify the premium fee. PHAs have very efficient end-of-life options being very easily compostable(Averous, 2004; Numata et al., 2009).

II.1.3.2. Current Challenges

The first clear challenge of the bioplastic sector is to broaden the spectrum of products with performances that can compete with conventional plastics. Plastics manufacturers and
converters are willing to replace petroleum-based products by eco-friendly alternatives. Yet if GREEN is IMPORTANT, COST and PERFORMANCE are even MORE IMPORTANT. In the current economic crisis, more than ever in order to meet success, the bioplastic sector must be creative and extremely efficient.

If non-biodegradability was the initial concern of the bioplastic sector (stimulating recycling through mechanical recovery, composting of wastes or energy production through plastic incineration), the need to reduce the dependence on fossil fuel resources turned the focus to plastics from bio-based origin, mainly from food crops. However, these approaches were often neglecting the Carbon footprint of each plastic material (Piemonte, 2011; Piemonte & Gironi, 2011, 2012). Currently, plastics manufacturers are paying attention to the CO₂ balance of the product’s life cycle and the rule is to generate no more CO₂ than we consume.

The support of R&I concerning bioplastics should be promoted and/or reinforced particularly, research programs that can bridge the gap between academic and industry. In Europe, the funding instrument FP7 created by the European Commission is an excellent example of how the governmental authorities can have an active role in the future of the sector. The SEABIOPLAS project, funded by the EU through the EU’s FP7 funds, is just kicking off and comprises 11 partners (including our research group) from 7 European countries working in close partnership to produce biodegradable and bio-based bioplastics using sustainably cultivated seaweeds as feedstock.

At an industrial level, all efforts carried out by companies to shift towards greener materials must be valorized. The development should be supported by the governmental authorities by, for instance, unlocking potential local and renewable raw materials that can be competitive on the market (www.european-bioplastics.org). In the particular case of IMTA approach, proper legislation and incentives would foster industrial implementation as well as break down barriers for products’ marketing.

Another major challenge towards the competitive gain of the sector consists on properly regulating the industry with the goal of ending the abusive use of terms such as bio-based which adds to the consumers’ confusion. The BioPreferred program created by the United States Department of Agriculture (USDA) is a good example. This type of measures can help ending the consumers’ confusion at the moment of discarding the bioplastics.

The current economic conjecture does not make life easier to the bioplastic sector yet, despite all difficulties and uncertainties, it is obviously worth it to invest in the sector. An optimal use of resources through efficient integrated cycles would promote sustainable development.
II.1.4. Seaweeds: a Potential Sustainable Resource for the Bioplastic Industry

Within a scenario of growing environmental concerns, global population increase and expected growth demand for plastics, and with obvious priority being always food production, food origin materials such as PLA, starch or the so-called ‘drop ins’ will fail the goal of sustainable development. This will impel a second generation of feedstock based in non-food crops such as algae and by-products (Colwill et al., 2011). Also, to face the markets’ stiff competition, bioplastics must become price-competitive towards synthetic resins and the massive use of food crops by innumerous sectors has been suggested to have negative impacts on food prices (Rathmann et al., 2010). Moreover, the use of farm machinery and fertilizers needed to grow food crops are known to have a negative impact on the carbon footprint of the plastic material (Hillier et al., 2009). The IMTA approach can mitigate these impacts by continuously supplying the bioplastic sector with non-food and sustainable resources with controlled quality.

The use of seaweeds by the bioplastic sector is far from having met its full potential. Agar, alginate and carrageenan have been the most explored to this end. In recent years, the rich fibrous nature of seaweeds have also motivated its exploitation as biofillers to reinforce bioplastics whether for commodity goods (e.g. packaging) or for more demanding and durable applications (e.g. automotive, construction). Furthermore, bioplastics from SP and/or seaweed blends seem to be promising, where the different compounds of the seaweeds can act either as fillers or as polymeric matrices.

II.1.5. Bioplastics from Seaweed Polysaccharides (SP)

Bioplastics from SP can be made by two mechanisms: dispersing the SP in an appropriate solvent followed by a drying step or melt processing at low moisture conditions. While in the former case, low concentrations of polymer are used, the low moisture environments needed in the latter allow to work with higher SP concentrations. Like most synthetic polymers, SP are rarely used by themselves to obtain bioplastics. The use of additives or blending with other natural polymers can improve the properties of the SP matrix. Also, significant developments in fields such as nanotechnology have proved to be very beneficial in overcoming some of the less attractive properties of SP bioplastics. Finally, SP can act as
biofillers, in the form of fibers or particles, to reinforce polymeric matrices giving rise to biocomposite materials, either aqueous-blended or melt-processed. In the following sections, all these possibilities will be addressed in detail with special emphasis being given to agar applications, which are listed in Table II.1.1, presented at the end of this chapter.

**II.1.5.1. Aqueous-blended Films**

Due to the high water affinity of SP, the use of water as solvent, dispersion medium and plasticizer to produce semi-rigid or flexible bioplastic films and fibers, has long been a common practice. Sectors such as food, agricultural, biomedical and pharmaceutical are the most relevant. Since this hydrophilic nature can lead to unsatisfactory performances of the plastic material, polymer blending and other common strategies are used to enhance the performances of aqueous-blended bioplastics such as edible and/or mulch films and coatings.

**II.1.5.1.1. Edible Films for Food Applications**

As seen previously, sectors that generate more plastic waste, such as food packaging, have been the most receptive to bio-based plastics. Also, the health concerns posed by the migration of toxic components into foods is a controversial subject that has long been fostering the search for ‘greener’ materials in the field (Lagaron & Lopez-Rubio, 2011).

Edible films and coatings are thin layers of material applied onto food products which can be consumed, thus fulfilling the requirements of control directives for waste disposal, and provide great functional benefits towards food preservation (Kester & Fennema, 1986). By creating a modified atmosphere to foodstuff, the polymeric film can further benefit the product during transportation and commercialization. The term ‘coating’ concerns the direct coverage of the food product by the polymeric solution while ‘film’ refers to the dried free-standing sheet wrapped around the food. Particularly in the case of perishable foods, the film can act as a good barrier to moisture, gases (CO₂, O₂) and aroma, avoid mechanical damage or have antimicrobial properties (Falguera et al., 2011). Meat sausage casing and cheese waxes are well-known examples of efficient commercial applications of edible coatings, although in the latter case not all waxes are edible.
SP can be promising alternatives to this end since they can be consumed with the food product (i.e. edible) and are readily degradable. Moreover, when used in the form of film or coating, they generally present good mechanical and moderate gas barrier properties and are also optically clear and transparent which are important features in terms of consumers acceptation (Sousa et al., 2008). Less attractive to this end are the great water affinity and brittle nature of SP.

The final properties of the films and coatings will be determined by the conjugation of several parameters. Specific strategies are chosen considering the desired end-use application that should be optimized in each case. Ultimately, this will allow overcoming or at least minimizing the above mentioned shortcomings.

For instance, the type of SP as well as the followed route to obtain the polysaccharide extracts, i.e. extraction parameters, physiological and environmental factors will be determinant to the final properties of the films (Hiliou et al., 2006a; Hiliou et al., 2006b; Sousa et al., 2008). In this regard, the use of IMTA & MAE sustainable approaches can enable tunability and quality control of the film and coating.

Components such as plasticizers, cross-linking agents, antimicrobials, antioxidants and color or flavor additives are also known to benefit the final properties of the film or coating. Some of these functional compounds, such as antioxidants or antimicrobials, can also be isolated from seaweed-based sources (Barros et al., 2013; Dore et al., 2013; Jin & Kim, 2012). The incorporation of plasticizers such as glycerol is a common practice to increase the films’ flexibility although this often implies significant losses in the mechanical resistance of the material or the plasticization of the matrix turns out insufficient (Gimenez et al., 2013; Sousa et al., 2010). To this end, the combination of multiple additives with distinct and complementary properties can be quite advantageous. The addition of nanoclays can create impermeable shields in the biopolymer matrix when efficiently incorporated, helping to improve the water barrier properties as well as the mechanical properties of SP films (Lim et al., 2010; Rhim, 2012; Rhim et al., 2011; Rhim et al., 2013). When combined with glycerol, the incorporation of nanoclays was favored, particularly at low filler contents (Sanchez-Garcia et al., 2010). The inclusion of green tea extract in agar/gelatin films resulted in lack of mechanical efficiency (i.e. lower tensile strength, TS and elongation-at-break, EB) yet, the polyphenols were bio-accessible during human digestion. In turn, the gelatin addition to the agar-based matrix diminished the release of active compounds although the antimicrobial activity was not affected (de Lacey et al., 2012; Gimenez et al., 2013). Optimal distribution of carbonyl iron was attained up to 20% particles loading on agar films which could be promising for biomedical treatments with external magnetic fields (Diaz-Bleis et al., 2012).
Polymer blending can further improve the properties of the resultant material particularly when synergistic interactions are involved. The addition of arabinoxylan significantly improved moisture barrier properties of agar films while starch increased the film’s deformability (Phan The et al., 2009a). Cross-linking milk-protein with agar led to biodegradable films with improved moisture barrier (Letendre et al., 2002). Agar/κ-carrageenan and agar/κ-carrageenan/glucomannan composite films, plasticized with glycerol, exhibited improved mechanical properties and water resistance upon nanoclay incorporation (Rhim, 2012; Rhim & Wang, 2013). The good adhesion between agar and PVA led to cast films with promising performances for food packaging (Madera-Santana et al., 2011). Good miscibility/compatibility between κ-carrageenan and LBG led to blended films with improved properties at an optimum 40/60 polysaccharide ratio (Martins et al., 2012).

The high hydrophilicity of SP can be undesirable in terms of food dehydration. Yet, in recent years, great technological developments in the field of food research provided several possible strategies to overcome this shortcoming. Hydrophobic compounds such as lipids or other natural polymers can be added through emulsion (i.e. emulsion-based films) or lamination (i.e. multilayer films) to the SP matrix to reduce the moisture sensitivity of the SP films (Phan The et al., 2008; Phan The et al., 2009b; Pinheiro et al., 2012). PLA and agar/κ-carrageenan/glucomannan multilayered films showed better resistance to water than the individual films (Rhim, 2013).

II.1.5.1.1.1. Agar and Locust Bean Gum (LBG)
Synergistic Interactions

As seen in the previous section, polymer blending is one of the strategies commonly used to improve the properties of materials such as hydrogels, edible films and coatings. Synergistic interactions between polysaccharides with different physicochemical properties can be very attractive for the production of materials with improved performances. By varying the composition of the final mixture we can tune the properties of the material to meet the specific needs of the end-use application.

Galactomannans are polysaccharides found in the seed endosperm of terrestrial plants build up on a linear β-(1-4)-D-mannan backbone with side chains of α-(1-6) linked G units (da Silva et al., 1993; Goncalves et al., 2004). Similar to SP, different plant sources will yield galactomannans with distinct properties. Depending on the mannose to galactose (M/G)
ratio, different types of galactomannans can be identified like, tara gum (≈2.7-3; TG), guar gum (≈1.55; GG) and locust bean gum (≈3.5; LBG). The latter has the highest M/G ratio which makes it more prone to intermolecular interactions with other polysaccharides (Srivastava & Kapoor, 2005). Other aspects such as the distribution pattern and orientation of the side chains will play a crucial role on the synergisms of galactomannans (Dea et al., 1986a, b; Wu et al., 2012). Hence, several attempts have been made to obtain a deeper knowledge on the ‘fine structure’ of these plant polysaccharides (Chandrasekaran & Radha, 1997; Daas et al., 2000; Dea et al., 1986a, b; Mazeau & Rinaudo, 2004; McCleary et al., 1985; Petkowicz et al., 1999; Petkowicz et al., 1998; Wu et al., 2012).

In solution, galactomannans exist in the random coil state and upon cooling adopt an ordered two-fold conformation with the molecules being packed together into flat sheets (Morris, 1990). Even though galactomannans are not intrinsically gelling polysaccharides, their very high viscosity at low concentrations can be very attractive to add texture to various products. If exposed to the right freeze-thawing conditions, LBG which has lower G contents can develop a slight gel-like structure (Dea et al., 1977). Like agar, LBG major application is as food additive (E410). It is used to improve texture and the storage life of ice creams, baby foods and pet foods. It is also popular for the production of soups, bakery and dairy products (Copper, 1995). Various forms of interactions were found between LBG and several polysaccharides such as xanthan (Copetti et al., 1997; Schorsch et al., 1997; Zhan et al., 1993), pectin (da Silva et al., 1996; da Silva et al., 1992, 1993, 1994), κ-carrageenan (Andrade et al., 2000; Arnaud et al., 1989; Gonçalves et al., 1997; Pinheiro et al., 2011; Turquois et al., 1992) and furcellaran (Sewall, 1992). Other non-pectic polysaccharides structurally similar to xanthan have shown synergistic peaks when mixed with LBG and other galactomannans (Wu et al., 2009a).

Early studies have also suggested synergistic interactions between agars from Gelidium sp or Pterocladia sp and LBG (Armisen & Galatas, 2000). However, no reports of significant synergisms between these galactomannans and agars from Gracilaria sp were found in literature. The agarose fraction (significantly higher in the former agars) is believed to have a strong interaction with galactomannans via oxygen atom in the anhydride bridge of the LA units and the hydroxyl group at C2 of D-mannan moieties in the plant polysaccharides (Tako & Nakamura, 1988). Gracilaria agars in turn, often exhibit complex substitution patterns. They are typically more sulfated and methylated, and some extracts, may present in their structure pyruvate ketal substituents, all found to weaken or inhibit the interactions between agars and LBG (Dea & Rees, 1987). Apparently, higher levels of substitution in the agar skeleton will result in weaker interactions with galactomannans (Dea & Rees, 1987).
The mechanism underlying the gelation of mixed gels has been subject of intense debate among the scientific community. Obviously, the intrinsic complex nature of natural polymers has much contributed to this fact. If one considers all the controversy around agar gelation, already debated in Part I of the thesis, it seems natural to guess that mixed polysaccharide systems would be an even more intricate topic to find consensual theories. The first model proposed for the synergistic gelation of galactomannan with helix-forming polysaccharides came by Rees and co-workers (Dea et al., 1972). The proposed theoretical principles, based on coupled network assumptions, pointed to the formation of junction zones between the unsubstituted regions of galactomannan chains and the double helix of the helix-forming polysaccharide. The consciousness that this vision could not accurately translate the formation of mixed gels came later with the unforeseen development of high precision techniques. X-ray diffraction data showed no evidence of mixed junctions and polymer incompatibility was pointed out as a more plausible cause to the galactomannan/helix-forming polysaccharide mixed gelation (Cairns et al., 1986, 1988; Cairns et al., 1987; Miles et al., 1984). However, the strong dependence of synergistic gelation on the 'fine structure' and $M_w$ of the galactomannan (Dea et al., 1972) struggles with a pure vision of polymer incompatibility. All in all, the most likely and adequate scenario could be to consider the formation of "mixed junctions" but with intensive interhelical association (Cairns et al., 1987) instead of the individual helices initially defended by Rees and co-workers (Dea et al., 1972; Morris, 1990; Williams et al., 1993) (Fig. II.2.1).

Fig. II.2.1 - ‘Mixed junction zone’ model proposed for synergistic gelation of LBG and agar. This model assumes direct association between the unsubstituted chains of LBG and the LA fraction of intensively associated agar helices (Source: Morris, 1990).

Early studies from Andrade and co-workers have explored the potential synergisms between agarose and GG (Andrade et al., 1997; Garcia & Andrade, 1997; Garcia et al., 1994; Garcia et al., 1992). The authors suggested that the sol-gel transition occurred directly without need
for demixing mechanisms when galactomannan was incorporated in the systems (Garcia et al., 1992) and the resultant gels were stronger (Garcia et al., 1994).

To the best of our knowledge the influence of LBG addition in the properties of agar films has not yet been addressed in literature. The incorporation of LBG in agar-based films can outcome numerous advantages, not only at processing and performance levels (i.e. final product with improved properties and easier to process) but also by lowering the cost-production since galactomannans are cheaper than agar (Kohajdová & Karovičová, 2009).

II.1.5.1.2. Production Methodologies

In terms of production methodologies, casting is the most commonly used technique to produce aqueous blend films. The film forming solution is applied to a suitable solid surface (container) or alternatively, an automatic solution spreader can be used to better control the film thickness (Fig. II.1.2). Although this is usually used for low concentration solutions and when thin layers of films are the target, a minimum value of viscosity should be respected to ensure the formation of homogeneous thin films with uniform thickness (Sousa et al., 2008). The cast or spread film is then dried under specific time, temperature and relative humidity conditions and finally peeled off to obtain the polymeric sheet. The drying step must be well thought out since it is known to affect the final properties of the film. For agars and carrageenans, moderate drying is expected to lead to double helices formation which percolate into 3D polymeric networks upon cooling, resulting in a strong film after solvent evaporation (Karbowiak et al., 2007; Phan The et al., 2009b). Alginate films were found to be significantly affected by extreme drying conditions (Ashikin et al., 2010; da Silva et al., 2012) while optimized starch/κ-carrageenan blends with glycerol showed better mechanical properties for more extreme drying conditions i.e. 50 °C under air forced convection (Moreira et al., 2011).

Fig. II.1.2 – Fabrication of films by ‘knife-coating’ technique (i.e. casting with spreader).
The choice of the method for food coating application must consider important aspects such as viscosity, density and surface tension in order to ensure good adherence of the coating solution to the food surface. Several methodologies such as spraying, dipping, and fluidized bed have been developed for that purpose, with the first being the most commonly used (Andrade et al., 2012). An important feature of edible coatings is that they will be part of the food itself. This means they must not provoke unwanted changes in organoleptic, physical and chemical properties of the coated food. Furthermore, though changes in the internal atmosphere of foods are intended to benefit shelf life by retaining CO$_2$ and reducing O$_2$ permeation (thus reducing the respiration rate), and/or by preventing weight loss, they have to be well controlled to prevent excessive CO$_2$ or too low O$_2$ concentrations. In this case, metabolic problems and anaerobic fermentations can occur, leading to off-flavors and off-smells. Thus, it is important to control the wettability of the coating (that directly influences its thickness, barrier properties and mechanical resistance), to influence gases permeability (water vapor, CO$_2$ and O$_2$), to predict internal atmosphere composition of respiring foods and to monitor the effects of the coating in quality parameters.

When coating cherry tomatoes with agar from *G. vermiculophylla* plasticized with glycerol, gains in firmness and weight loss reduction were only significant for a limited time-frame (Sousa et al., 2010). The glossy appearance of the coated samples compared favorably with the control although the great gelling capacity of the polysaccharide makes difficult its use as coating.

### II.1.5.2. Mulch Films

Agriculture is another industrial segment that generates a large amount of plastic waste. Typically, LDPE synthetic plastics are used for soil mulching in horticulture to reduce water losses from soil, protect plants, improve crop developments as well as decrease agro-chemicals use for weed control (Immirzi et al., 2009). Despite the good mechanical properties and low cost, most of the time LDPE plastics cannot be re-utilized due to soil, organic matter and agro-chemicals contaminations. Mulch films and coatings made from biodegradable polymers such as SP have been extensively studied by researchers as alternatives to synthetic resins since they can be directly integrated into soils through biodegradation, hence not generating plastic wastes. Particularly, SP with the ability to capture divalent ions from the ground and to form polymeric networks when cooled, such as alginates and carrageenans, are the most attractive. The processing technologies of these bioplastics follow the same lines as food films and coatings. The application of the bioplastic solution via spraying technique to form a mulch coating upon drying has the advantage of not
requiring so high mechanical performances as when using extruded mulching films (*e.g.* LDPE, biodegradable starch) where there is the need for plastic handling.

Under the European project BIO.CO.AGRI (*i.e.* Biodegradable Coverages for Sustainable Agriculture), aqueous solutions of sodium alginate were tested as mulch coatings (Immirzi *et al.*, 2009; Malinconico *et al.*, 2006). PCT Int. Appl. WO 2011/128752A1 also refers carrageenan, as possible component for mulch films yet, the preference is greater for alginic SP, which can form irreversible physical gels (Malinconico, 2011). The interaction between the sprayed sodium alginate and the naturally occurring calcium ions from soil guarantee the mulch coating water resistance, while the coating flexibility can be improved by the addition of biodegradable glycerol-based plasticizers (Immirzi *et al.*, 2009).

II.1.6. Melt Processing

Conventional plastics are produced at large scale and low cost by melt processing. Thus, bioplastics chances of a wide spread implementation are highly dependent on their ability of being processed in already existing machinery. Formulation of bioplastics must include at least one polymer able to form a suitable cohesive and continuous matrix. Though SP can be used to form this matrix, its thermal processability is still not competitive with traditional plastic materials. However, plastic compounding often includes additives such as processing aids (*e.g.* plasticizers) or reinforcement agents (such as fillers). Seaweeds seem to be an interesting biofiller and a considerable number of applications are already available, both at research and commercial levels. At lab-scale, thermo-compression of polymers using hydraulic presses is a common practice (Fig. II.1.3).
Fig. II.1.3 – Hydraulic press used to fabricate polymer films by thermo-compression.

II.1.6.1. Biofillers for Melt-processed Biocomposite Films

The incorporation of fillers in polymer matrices to be used in melt-processing technologies has become a common practice among researchers and plastic professionals over the years. When a good filler-matrix interaction takes place, the resultant composite plastic is expected to exhibit enhanced performances when compared to the former bulk polymer, with the filler acting as a reinforcement agent. In any case, even if no properties are altered, the biofiller can significantly lower the price of the final product. The term ‘biocomposite’ is used to designate plastic materials composed by a polymer matrix, whether or not from bio-based origin, and a natural filler agent (i.e. biofiller) in the form of fibers or particles, mostly plant-derived.

Over the last two decades, biocomposites reinforced with natural fibers such as wood, flax, cotton or hemp have received widespread attention as possible substitutes of synthetic fibers like carbon or glass (La Mantia & Morreale, 2011). Main strengths of plant-based fibers include low cost, low density, renewability, non-toxicity and biodegradability while high water affinity and variability in properties are still issues to overcome. Particularly, in sectors where the volume-to-weight ratio is crucial for the product’s performance (e.g. automotive) the use of natural fibers can represent significant energy reductions besides the obvious ecological benefits. Seaweeds are now seen by companies as excellent candidates to reach the goal of moving away from food crops, and especially, aquaculture grown...
seaweeds could help overcome one of the main handicaps pointed out to plant-based fibers which is great variability of quality.

Most scientific papers concerning melt-processing technologies of biocomposites include extrusion followed by injection or compression molding. Mechanical mixing in common domestic mixers can be used prior to thermo-compression instead of extrusion, e.g. for screening purposes, though extrusion final tests are always advisable to reproduce real industrial scenario. Adequate extrusion or mechanical mixing will ensure an efficient filler-matrix blending hence, influencing the performance of the biocomposite plastic. At an industrial scale, extrusion is the most important continuous compounding/melt processing technique and can be coupled to injection molding, film blowing and others. While injection molding is attractive to obtain plastic objects with complex forms, more simple forms such as packaging containers can be produced through thermoforming. Some of the key points of molten-state processing biocomposite plastics are to avoid thermal degradation, ensure efficient blending of components as well as suppress the hydrophilic character of natural fillers. Hence, efficient drying of both biofiller and polymer matrix (when of hydrophilic nature) is critical prior to melt processing to avoid defects in the obtained composite.

II.1.6.1. Seaweeds as Biofillers

Seaweed fibers were shown to have good compatibility with several polymer matrices whether as filler or reinforcement agents. The green seaweed *Ulva amoricana* from the French coasts was positively tested as filler in PVA and PCL matrices (Barghini *et al.*, 2010; Chiellini *et al.*, 2008). Compression molded *Ulva* fibers exhibited good compatibility with both studied matrices; the composites showed increased Young’s Modulus, YM, yet, lower TS and EB. Agricultural and packaging applications were suggested as possibilities. Optimal fibers fabrication of *Gelidium elegance* was accomplished through extraction and bleaching of seaweeds prior to melt-processing (Lee *et al.*, 2008; Sim *et al.*, 2010). The removal of seaweed mucilage and efficient fiber incorporation into poly(butylene succinate), PLA and PP polymer matrices resulted in reinforced biocomposites. Algae fibers with crystalline and thermal properties similar to crystalline cellulose and enhanced flexural and storage moduli up to 40-50% filler contents were obtained hence, proving the great potential of seaweed fibers as reinforcement agents (Lee *et al.*, 2008).
II.1.6.1.2. Seaweed Polysaccharides (SP) as Biofillers

Though the use of seaweeds as fillers is probably simpler and more cost-attractive (i.e. it does not include an extraction step), the use of SP has also proved to be effective in the reinforcement of several polymer matrices. Non-aggregated agar particles from *Gelidium robustum* were successfully incorporated into poly(butylene adipate-co-terephthalate) (PBAT) using extrusion and injection molding to fabricate biodegradable reinforced-composites (Madera-Santana *et al.*, 2009), while lower interfacial adhesion was observed for compression-molded agar/LDPE composites (Madera-Santana *et al.*, 2010). TPS composites with optimal performance were obtained when incorporating hybrid *Gelidium amansii* agar/cotton fibers at a 4:6 polymers ratio by compression molding (Prachayawarakorn *et al.*, 2012).

II.1.7. Fibers of Seaweed Polysaccharides (SP)

As previously mentioned, SP can also be used in the form of fibers, beads and capsules which are of particular interest in fields such as biomedical, pharmaceutical and cosmetic (Rinaudo, 2008). Since the application of SP in the form of fibers was our focus of interest, only this topic will be discussed in detail next.

Materials in the form of ultrathin fibers are amongst the most promising applications for biopolymers. The variety of natural compounds available in nature, with features including biodegradability and biocompatibility, provides endless options for the fabrication of highly functional fibrous materials to be used in the production of biosensors, electronic and optical devices, catalytic supports, membranes, textiles or as reinforcing agents for packaging products (Luo *et al.*, 2012; Peresin *et al.*, 2010; Viswanathan *et al.*, 2006). At an industrial scale however, fibers from synthetic polymers such as, PVA and PEO, continue to be preferred over natural polymers due to the complex structure commonly associated to the latter (Kriegel *et al.*, 2008; Peresin *et al.*, 2010). In fundamental areas such as the biomedical, these biomaterials can be used to fabricate scaffolds that degrade with time and ultimately, are replaced by regenerated tissues (Agarwal *et al.*, 2008; Alhosseini *et al.*, 2012; Toskas *et al.*, 2011). Also, wound dressings and drug delivery systems can be favored by the exquisite properties of these materials (Lee *et al.*, 2009; Rosic *et al.*, 2012; Toskas *et al.*, 2011).
Chitosan and alginate are two of the most explored natural polymers concerning the fabrication of fibers for biomedical applications (Rosic et al., 2012; Toskas et al., 2011). In what SP are concerned, the preference for alginate relies on the same principles as for mulch films *i.e.* its ability to form insoluble polymeric structures in the presence of divalent ions. Alginate fibers for wound dressings can be prepared from spun sodium alginate solutions into a coagulating bath of calcium ions. Different operational conditions will lead to fibers with distinct properties. For instance, small quantities of sodium ions in alginate fibers will result in increased water absorption when compared with pure calcium alginate fibers. Alginate fibers are converted into wound dressings, mostly in nonwoven forms, by textile processes such as needling, pressure rolling and hydroentanglement (Qin, 2006). The addition of chitosan to alginate has proven to benefit the properties of the blended fibers. For instance, alginate/hydroxypropyl chitosan fibers showed enhanced water absorption capacity when compared to alginate fibers alone (Fan et al., 2012).

The use of non-soluble calcium fibrous alginate for wound dressing can offer several advantages over the conventionally used cotton and viscose fibers. The calcium ions in the fibers, exchange with sodium ions from the human body during the healing process forming soluble sodium alginate. This enables easier wound dressing removal without hurting the patient (Fan et al., 2012). Other attractive properties of fibrous forming alginate include excellent biocompatibility, non-toxicity, non-immunogenicity and high absorption capacity (20 to 30 times their own weight). Algisite® M, Algosteril®, AlgiDERM® and Sorbsan® are some of the alginate dressings that can be found in the market (Rinaudo, 2008).

Cellulose, the most abundant natural polymer in nature, is also of particular interest yet great efforts continue to be made to overcome its low solubility in the majority of the available organic and aqueous solvents. The strategies commonly used by researchers include working with modified cellulose (Luo et al., 2013) or using alternative solvents (Meli et al., 2010).

### II.1.7.1. Electrospinning

In recent years, electrospinning technique has become a preferential method for the fabrication of micro- to nano-sized fibers (Greiner & Wendorff, 2007; Luo et al., 2012). Although with lower yields than other typically used processes, electrospinning is a cost-effective method that enables a greater control of the fibers’ final properties and can be applied to almost every solution and polymer melt (Greiner & Wendorff, 2007; Lee et al., 2009). The ability to produce fibers as small as 10-1000 nm permits high surface-to-mass
ratios, enhanced mechanical performances and high porosity (Kriegel et al., 2008). Also, these electrospun fibers are highly functional and the incorporation of enzymes, proteins, viruses or bacteria are common practices (Greiner & Wendorff, 2007).

The concept underlying the electrospinning process is quite simple: high voltage is applied to the polymer solution or melt, held at a tip of a capillary by its surface tension. When the applied electric field is such that overcomes the surface tension of the droplet, a jet is formed and stretched towards the grounded collector with opposite charge, placed at a known distance from the needle tip. If aqueous solvents are used, the solvent will evaporate as the jet travels towards the counter electrode. However, if the process involves non-volatile solvents, the fibers should be recovered through the use of a coagulating/washing bath.

Parameters that govern the electrospinnability include the environment T and R.H., the feed rate of the solution, the applied electric field, and the distance tip-to-collector (Greiner & Wendorff, 2007). Apart from these purely operational requirements, fundamental aspects related to the properties of the polymer solution such as concentration, viscosity, viscoelasticity, electric conductivity and surface tension, also play a crucial role in the overall process. In turn, this will be obviously dependent on the polymer and solvent intrinsic properties such as solubility, melting point, molecular weight and molecular weight distribution, entanglement density or solvent vapor pressure (Greiner & Wendorff, 2007). Hence, despite the apparent simplicity of the overall process, it is evident the intricate conjugation of factors involved in electrospinning. This can be challenging when it comes to producing fibers with controlled morphologies and of very small dimensions. To the best of our knowledge, no studies have been published so far, reporting the successful application of electrospinning to agar. Only one paper was found reporting the fabrication of agar and agarose fibers through wet-spinning using dimethyl sulfoxide (DMSO) aqueous media as solvent. The formed materials presented fiber diameters in the range 74–123 µm (Bao et al., 2010). Producing agar fibers with lower dimensions could be particularly useful since higher surface-to-volume ratios are known to greatly improve the performance of the material.

II.1.7.1.1. Choice of Solvent

Another decisive element when exploring electrospinning of natural polymers is the right choice of solvent. Room temperature ionic liquids (RTIL) have proven to be a great platform for innumerous applications, particularly where volatile solvents seem to be inefficient. In the often complex task of dissolving natural polymers, these most adequately termed “task-specific ILs” (Tang et al., 2012), can be of particular interest since they can be structurally
engineered to meet the specific needs of the compound to be dissolved (Viswanathan et al., 2006). This can be quite advantageous when it comes to fabricate natural fibers through electrospinning.

RTILs arose from the exploitation of high melting salt mixtures, composed of metal salts (*i.e.* zinc, aluminum, tin and iron chlorides) and quaternary ammonium salts. Adding to the exciting tunability feature, unique properties such as high conductivity, very low vapor pressure, low flammability as well as excellent stability, enabled an unforeseen growth of these ionic fluids in a wide array of applications (Meli et al., 2010; Tang et al., 2012).

Considerable investment has been made at research level to develop new materials in the form of ultrathin fibers using RTILs. For instance, non-modified cellulose was successfully spun using 1-butyl-3-methylimidazolium chloride (Viswanathan et al., 2006) and a binary mixture composed of 1-ethyl-3-methylimidazolium acetate and 1-decyl-3-methylimidazolium chloride ionic liquids (Freire et al., 2011). More recently, Barber and co-workers reported the production of chitin fibers by electrospinning chitin directly from a RTIL solution of 1-ethyl-3-methylimidazolium acetate (Barber et al., 2013).

Deep eutectic solvents (DES), also known as IL analogues, form a new family of solvents that are currently being focus of a lot of attention by the scientific community (Zhang et al., 2012). The DES concept arises from the adequate mixture of halide salts with hydrogen bond donors to form eutectics that can be operated at room temperature as fluids. Here, particular emphasis has been given to the quaternary ammonium salt, (2-hydroxyethyl)trimethylammonium chloride (choline chloride, ChCl), due to its very low cost, biodegradability, non-toxicity as well as renewable nature (Zhang et al., 2012). For instance, by mixing urea (melting point, mp=133 °C) with ChCl (mp=302 °C) at 1:2 molar ratio, a eutectic mixture with a freezing point of 12 °C is formed (DES-U; Fig. II.1.4). Computational simulations focusing the molecular dynamics of this eutectics suggest a preferential interaction between the amide groups of urea and the chlorine anion, resulting in the significant depression of the freezing point (Perkins et al., 2013). The successful use of these solvent has been consistently reported whether for the fabrication of starch-based bioplastics (Abbott et al., 2012; Leroy et al., 2012) or, for instance, conducting materials for electronic applications (Ramesh et al., 2012a, b, 2013). Recently, Shamsuri and Daik tested the potential of this eutectics as plasticizer in aqueous agarose films produced by casting (Shamsuri & Daik, 2012). However, the low elongations reported by these authors seemed to suggest an insufficient plasticization of the agarose films. A possible reason could be the rupture of the original molecular complexes that compose the eutectic mixture upon DES-U dilution in water (Nardecchia et al., 2012).
II.1.8. Evaluation of the Properties of Materials

After developing a new material, manufacturers must evaluate their performance under various conditions according to the potential end-use applications. This will give the consumer the needed information to choose the most adequate material based on their needs. Considering the great dependence of natural polymers on the environmental conditions (e.g. R.H., T), the main struggle, when it comes to materials from natural origin, is to convince manufacturers to follow common guidelines for the testing of their products. Currently, most methodologies used to characterize the properties of materials are made at different testing conditions limiting the consumer to relative specifications and making it impossible to actually compare products from different manufacturers. To overcome this issue, certified entities have been created worldwide to develop standardized test methods for the evaluation of the materials properties.

ASTM International, formerly known as The American Chemical Society for Testing and Materials (ASTM; http://www.astm.org/ABOUT/overview.html) and the European Committee for Standardization (http://www.cen.eu/cen/products/en/pages/default.aspx) are two major organizations worldwide focusing on establishing well-defined standard test methods, specifications, guidelines and practices, to be used in benefit of all parties in the material's life-cycle. Depending on the material's form (e.g. films, gels, fibers) and desired end-use, the tests can comprise the assessment of various functional properties. For instance, packaging films must protect sensitive items while being mechanically resistant and extensible in order to keep its integrity throughout processing, handling and storage. It should also present appealing optical properties (e.g. transparency in case of food packaging materials) and all these requirements must be fulfilled at minimum cost.
Table II.1.1 - Studies found in literature concerning agar-based materials, production methods, main results and suggested applications.

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>SOLVENT</th>
<th>PROD. METHOD</th>
<th>STRATEGY</th>
<th>MAIN FINDINGS</th>
<th>SUGGESTED APPLICATION</th>
<th>REFS.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar/glycerol/carbonyl iron films</td>
<td>Water</td>
<td>Casting</td>
<td>Additives Incorporation (plasticizer, magnetic particles)</td>
<td>Optimal particles distribution up to 20% particle loading; Thermal diffusivity and optical absorption coefficient increase w/ particle loading.</td>
<td>Biomedical treatments w/ external magnetic fields</td>
<td>(Diaz-Bleis et al., 2012)</td>
</tr>
<tr>
<td>Agar/silver nanoparticles films</td>
<td>Water</td>
<td>Casting</td>
<td>Additives Incorporation (nano-compound)</td>
<td>Antimicrobial activity; Good mechanical resistance.</td>
<td>Food packaging, water purification, sterile coatings for biomedical devices</td>
<td>(Ghosh et al., 2010)</td>
</tr>
<tr>
<td>PVA/agar (Hydropuntea cornea) films</td>
<td>Water</td>
<td>Casting</td>
<td>Polymer blending</td>
<td>Good adhesion between both polymers; Best mechanical properties at 50-75% agar in the final mixture; Agar from rainy season H. cornea w/ better properties.</td>
<td>Food packaging</td>
<td>(Madera-Santana et al., 2011)</td>
</tr>
<tr>
<td>Agar (Gelidium robustum) films</td>
<td>Water</td>
<td>Casting</td>
<td>Pure agar</td>
<td>Accelerated degradation when exposed to humid tropical climate</td>
<td>Biodegradable plastic</td>
<td>(Freile-Pelegrin et al., 2007)</td>
</tr>
<tr>
<td>Agar (Gelidium robustum)/PBAT composites</td>
<td>n.a.</td>
<td>Extrusion Injection molding</td>
<td>Polymer blending</td>
<td>Good adhesion between both polymers; Enhanced TS and decreased EB w/ agar addition to PBAT;</td>
<td>Reinforcing agent in biocomposites</td>
<td>(Madera-Santana et al., 2009)</td>
</tr>
<tr>
<td>LDPE/agar composites</td>
<td>n.a.</td>
<td>Compression molding</td>
<td>Polymer blending</td>
<td>TS and EB decrease w/ increase in agar %; Stiffer films w/ agar addition.</td>
<td>Biofiller in biocomposites</td>
<td>(Madera-Santana et al., 2010)</td>
</tr>
<tr>
<td>Agar/gelatin/green tea extract films</td>
<td>Water</td>
<td>Casting</td>
<td>Polymer blending</td>
<td>Agar/gelatin films less resistant and more deformable than agar pure films; Decrease in TS and EB w/ green extract addition; Gelatin hindered the release of phenolic compounds.</td>
<td>Edible active packaging</td>
<td>(de Lacey et al., 2012; Gimenez et al., 2013)</td>
</tr>
<tr>
<td>Gelidium corneum agar/gelatin/glycerol/nanoclay films</td>
<td>Water</td>
<td>Casting</td>
<td>Polymer blending</td>
<td>Nanoclay addition improved mechanical resistance; Antimicrobial activity w/ thymol.</td>
<td>Chicken breast packaging</td>
<td>(Jang et al., 2010)</td>
</tr>
<tr>
<td>Agar (Gelidium corneum)/glycerol/catechin films</td>
<td>Water</td>
<td>Casting</td>
<td>Additives incorporation (plasticizer, antimicrobials)</td>
<td>Catechin incorporation improved mechanical resistance and WVP; Antimicrobial and antioxidant activity.</td>
<td>Sausage packaging</td>
<td>(Ku et al., 2008)</td>
</tr>
<tr>
<td>Milk protein/agar/pectin films</td>
<td>Water</td>
<td>Casting</td>
<td>Polymer blending</td>
<td>Improvement of mechanical properties and moisture barrier w/ agar addition.</td>
<td>Food packaging</td>
<td>(Letendre et al., 2002)</td>
</tr>
<tr>
<td>Agar (Gelidium corneum)/glycerol/nanoclay/seed extract or thymol films</td>
<td>Water</td>
<td>Additives incorporation (plasticizer, nano-compound, antimicrobial, antioxidant)</td>
<td>Improvement of antimicrobial activity.</td>
<td>Edible active packaging</td>
<td>(Lim et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>Agar/arabinoxylan/glycerol and agar/starch/glycerol films</td>
<td>Water</td>
<td>Casting</td>
<td>Polymer blending</td>
<td>Phase separation between agar/arabinoxylan and agar/starch; WVP decrease w/ addition of arabinoxylan; Surface wettability improved w/ starch addition; Pure agar films w/ better mechanical properties;</td>
<td>Edible packaging</td>
<td>(Phan et al., 2008)</td>
</tr>
</tbody>
</table>
### II.1 LITERATURE REVIEW

<table>
<thead>
<tr>
<th>Film Composition</th>
<th>Method</th>
<th>Additives</th>
<th>Properties</th>
<th>Applications</th>
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</thead>
<tbody>
<tr>
<td>Agar/glycerol/lipid films</td>
<td>Water Casting w/ spreader Additives incorporation (plasticizer, lipids)</td>
<td>Agar network advantageous for lipid particle entrapment; Improved barrier properties; Good lipid dispersion promoted moisture sorption; Comparable mechanical properties w/ some protein and LDPE films.</td>
<td>Edible packaging</td>
<td>(Phan et al., 2009)</td>
</tr>
<tr>
<td>Shellac/PEG and agar/glycerol bilayer films</td>
<td>Casting w/ spreader Additives incorporation (plasticizer, edible lac/varnish)</td>
<td>Improvement of water barrier properties; Reduction in moisture sorption while maintaining mechanical properties.</td>
<td>Food packaging</td>
<td>(Phan et al., 2008)</td>
</tr>
<tr>
<td>Starch/agar/glycerol/span 80 films</td>
<td>Casting Polymer blending Additives incorporation (plasticizer, surfactant)</td>
<td>Glycerol most influential component in mechanical properties; Surfactant addition decreased WVP.</td>
<td>Edible packaging</td>
<td>(Maran et al., 2013a; Maran et al., 2013b)</td>
</tr>
<tr>
<td>Agar/κ-carrageenan/glycerol/nanoclay films</td>
<td>Water Casting Polymer blending Additives incorporation (plasticizer, nano-compound)</td>
<td>Lower TS, higher WVP, lower swelling ratio and water solubility for pure agar films; Mechanical properties. WVP, water contact angle significantly improved by nanoclay incorporation.</td>
<td>Edible packaging</td>
<td>(Rhim, 2012)</td>
</tr>
<tr>
<td>Agar/κ-carrageenan/konjac glucomannan/nanoclay films</td>
<td>Water Casting Polymer blending Additives incorporation (plasticizer, nano-compound)</td>
<td>Properties of ternary blends between that of individual components; Significant increase of water holding capacity. Antimicrobial activity.</td>
<td>Antifogging packaging for highly respiring agricultural products</td>
<td>(Rhim &amp; Wang, 2013)</td>
</tr>
<tr>
<td>Film Type</td>
<td>Process</td>
<td>Additives Inclusion</td>
<td>Improvement</td>
<td>Application</td>
</tr>
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<td>-----------------------------------------------</td>
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<tr>
<td>Agar/glycerol/silver nanoparticles films</td>
<td>Water Casting</td>
<td>Additives Incorporation (plasticizer, nano-compound)</td>
<td>Improvement in WVP and surface hydrophobicity w/ Ag increase without affecting mechanical properties; Antimicrobial activity.</td>
<td>Antimicrobial food packaging, Wound dressings</td>
</tr>
<tr>
<td>PLA and agar/κ-carrageenan/glycerol/nanoclay multilayered films</td>
<td>Water Casting bylayers</td>
<td>Polymer blending Additives incorporation (plasticizer, nano-compound)</td>
<td>Improved WVP and water resistance when compared to pure films.</td>
<td>Food packaging</td>
</tr>
<tr>
<td>Agar/gelatin/45S5 bioactive glass films</td>
<td>Water Casting</td>
<td>Polymer blending Additives incorporation (antimicrobials)</td>
<td>Improved antimicrobial activity w/ particle loading.</td>
<td>Wound dressing</td>
</tr>
<tr>
<td>Agar (Gracilaria vermiculophylla and commercial)/glycerol films and coatings</td>
<td>Water Casting w/ automatic spreader</td>
<td>Additives Incorporation (plasticizer)</td>
<td>Comparable film properties between Gracilaria and commercial agars; Coatings of Gracilaria agar: effective (weight loss, firmness) within a time-frame; Glossy appearance of coated samples; Difficult to work at low Ts due to agar gelling nature.</td>
<td>Edible packaging Cherry tomatoes coating</td>
</tr>
<tr>
<td>Soy protein/agar/glycerol films</td>
<td>Water (Casting) Thermo-molding</td>
<td>Polymer blending Additives incorporation (plasticizer)</td>
<td>Good compatibility between polymers; Casting films more homogeneous and w/ increased mechanical properties.</td>
<td>Edible packaging</td>
</tr>
<tr>
<td>Material Description</td>
<td>Processing Method</td>
<td>Additives</td>
<td>Properties/Impact</td>
<td></td>
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</tr>
<tr>
<td>Starch/agar/glycerol</td>
<td>Water, Casting</td>
<td>Polymer blending, Additives incorporation (plasticizer)</td>
<td>Improved mechanical properties and WVP at high H.R. upon agar addition. Food packaging (Wu et al., 2009b)</td>
<td></td>
</tr>
<tr>
<td>Agar/glycerol/sorbitol</td>
<td>Water, Casting</td>
<td>Additives incorporation (plasticizers, lipid)</td>
<td>Less attractive properties for cheese coating than other studied polysaccharides. Edible packaging (Cerqueira et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Agar (Gracilaria dura)/silver nanoparticles films</td>
<td>Water, Casting</td>
<td>Additives incorporation (nanoparticles)</td>
<td>Greener synthesis of silver nanoparticles; Antimicrobial films. Food preservation Wounddressing (Shukla et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Agar (Gelidium amansii)/cotton fibers</td>
<td>n.a., Compressional molding</td>
<td>Polymer blending</td>
<td>Optimum TPS composite obtained when incorporating agar/cotton fibers at 4:6 ratio. Biofillers in melt-processed starch composite films (Prachayawarakorn et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Agarose/DES-U films</td>
<td>Water, Casting</td>
<td>Additives incorporation (plasticizer)</td>
<td>Improved mechanical properties at 60% DES-U addition; Smoother surfaces, increased transparency and GTT decrease with DES-U addition. (Shamsuri &amp; Daik, 2012)</td>
<td></td>
</tr>
<tr>
<td>Agarose/talc/BmimCl(Urea at 1:1 molar ratio composite films</td>
<td>Water, Casting</td>
<td>Additives incorporation (filler, coupling agent)</td>
<td>BmimCl/Urea is a good coupling agent for agarose/talc composites; Best mechanical resistance at 8% wt BmimCl/Urea addition; GTT increase by BmimCl/Urea addition. (Shamsuri &amp; Daik, 2013)</td>
<td></td>
</tr>
<tr>
<td>Agarose and agar fibers</td>
<td>DMSO/Water =9:1(v/v), Wet-spinning</td>
<td></td>
<td>Better water swelling capacity for agar fibers; Flexibility flaws caused by agarpectin; Smooth and homogeneous agarose fibers. Wound dressing (agarose fibers) (Bao et al., 2010)</td>
<td></td>
</tr>
</tbody>
</table>

n.a. – not applied; 1-n-butyl-3-methylimidazolium chloride (BmimCl); GTT – Glass transition temperature.
References.


galactomannans, interacting bacterial polysaccharides, and related systems *Carbohydrate Research, 57*, 249-272.


II.2 MATERIALS AND METHODS

CHAPTER II.2
II.2.1. Chemicals

Urea (> 99%; CH\textsubscript{4}N\textsubscript{2}O), choline chloride (> 98%; C\textsubscript{5}H\textsubscript{14}ClNO) and anhydrous glycerol (> 99.5%; C\textsubscript{3}H\textsubscript{8}O\textsubscript{3}) were purchased from Sigma-Aldrich. LBG commercial sample was kindly supplied by Danisco, Faro, Portugal (Meypro-LBG Fleur M-175, batch nr 4760427232) and previously characterized in our lab (>75% galactomannan content; [\eta]= 13.5 dL/g; M\textsubscript{v} ~ 2\times10\textsuperscript{3} kDa). CA was the same as used in Part I (A-7002, St. Louis, MO, (C\textsubscript{12}H\textsubscript{18}O\textsubscript{9})\textsubscript{n}; Sigma-Aldrich Co.) while PVA (average M\textsubscript{w} = 89,000-98,000 Da, 99+% hydrolyzed), used as co-blending polymer for the fabrication of agar-based fibers, was also from Sigma-Aldrich. For the studies concerning agar/LBG aqueous-blended films and hydrogels, NA\textsubscript{IMTA_TWE}\textsuperscript{opt} and AA\textsubscript{IMTA_TWE}\textsuperscript{opt} were chosen as representative samples.

II.2.2. DES Preparation

DES used to fabricate non-aqueous agar films and fibers were prepared by mixing the ChCl, previously dried at 70 °C overnight in an oven, with the appropriate amounts of the H-bond donors i.e. urea and/or anhydrous glycerol at 1:2 molar ratios. The mixtures were heated at 70 °C under stirring until formation of a homogeneous liquid and subsequently left to cool slowly to 25 °C and kept at that temperature (above the solvent's freezing point). For simplification, the shorthand notation DES-G and DES-U was adopted for respectively, ChCl/glycerol and ChCl/urea eutectic mixtures.

II.2.3. Aqueous-blended Films of Agar/Locust Bean Gum (LBG)

For the study concerning aqueous-blended films of agar/LBG, NA\textsubscript{IMTA_TWE}\textsuperscript{opt} and AA\textsubscript{IMTA_TWE}\textsuperscript{opt} were considered as model samples. The addition of LBG to agar film-forming solutions was explored to improve the final properties of the SP films. This case study is presented in Chapter II.3.
II.2.3.1. Preparation of Locust Bean Gum (LBG) Starting Solution

The LBG solution (~1.2 % wt) was prepared by slowly dispersing the polysaccharide in distilled water, under vigorous stirring, in the presence of sodium azide (5 ppm) in order to avoid bacterial growth. The dispersion was left under moderate stirring for 1 h, at room temperature, before being heated at 80 °C for 30 min. After cooling, the solution was centrifuged in a Beckman Coulter centrifuge (Model Alegra 25R) at 21,000 g, for 1h at 25 ºC, to remove the insoluble matter. The final solution concentration was determined as total solids by drying at 105 ºC until constant weight. The solution was stored for at least 12 hours to be properly hydrated before use.

II.2.3.2. Preparation of Agar Starting Solutions

The preparation of the purified agar solutions of 1% wt concentration followed the typical procedure, i.e. heating in boiling distilled water, under stirring, until complete dissolution. Each time, the final polymer concentration was checked by dry weight determination at 105 ºC.

II.2.3.3. Preparation of Agar/ Locust Bean Gum (LBG) Mixtures

Agar/LBG mixtures were prepared at different mass ratios (100/0, 75/25, 50/50, 25/75, 0/100) by mixing the appropriate amounts of each starting solution to achieve a final polysaccharide concentration of 1% wt. LBG solution was stirred at room T for 2 h and heated up to 70 °C prior to mixing with each agar stock solution. Mixing was carried out at 70 °C after which the T was increased to 90 °C and the mixture was left at this temperature for 1h under gentle stirring. Samples were degassed in a vacuum oven (90 ºC for 5 min) to remove air bubbles and subsequently used for film formation and rheological studies. The pure agar and LBG solutions (i.e. 100/0 and 0/100 agar/LBG) were submitted to the same thermal treatment as the mixtures.
II.2.3.4. Rheological Studies of the Film-forming Solutions

The theoretical background concerning the rheological measurements can be found in section I.2.8.2 of Chapter I.2. Dynamic rheological studies were performed on the film-forming solutions (prepared as described in section II.2.3.3) in a stress-controlled rheometer (ARG2, TA Instruments, USA; Fig. I.2.8) using a cone-plate geometry (4 cm diameter, 2° angle and a 54 μm gap). Preliminary strain sweep tests defined the linear viscoelastic region of each sample.

The solutions were loaded on the Peltier plate (pre-heated to 80 °C) and its periphery was coated with paraffin oil to avoid water evaporation. After a conditioning step of 5 minutes, the samples were cooled down to 55 °C (T defined for the spreading of the film forming solutions) at a rate of 1 °C /min, while small amplitude oscillatory shear strain was applied at 6.28 rad/s (1 Hz), allowing to measure the viscoelastic properties as function of temperature. After an equilibration period of 5 minutes at 55 °C (6.28 rad/s), frequency sweeps were performed over the range 0.1 to 100 rad/s.

Finally, flow curves were recorded at 55 °C by using a steady state flow ramp with a considered maximum range of shear rates of 1-1000 s⁻¹. The shear rate was recorded point by point with a maximum point time of 1 min. The curves were built from viscosity data determined for each point as an average of three consecutive measurements with a maximum 5% deviation.

II.2.3.5. Films Fabrication

The hot and degassed solutions prepared as described in II.2.3.3 were cooled down to 55 °C, to be spread over glass plates, using an automatic knife film applicator (Sheen model 1132N, UK) with application speed of 0.3 m/s (Fig. II.2.1). The plates were pre-heated at 55 °C to avoid agar gelation. The spread solutions were allowed to dry in a climate test chamber (environmental test chamber for constant climate Binder KMF 115), at 40 °C and R.H.=30%, for 2-3 hours. Finally, the films were peeled off from the supports and stored in plastic bags.
II.2.3.6. Films Characterization

II.2.3.6.1. Thickness Measurement

Film thickness was measured at five different points on each film using a thickness comparator (Absolute Digimatic Indicator, model ID-F150, Mitutoyo Co., Japan; Fig. II.2.1) with a resolution of 1 μm. Reported thickness values are means of the five measurements.

![Device for measuring the thickness of the films.](image)

II.2.3.6.2. Mechanical Properties

The basic principles of TA can be found in section I.2.8.3.1 of Chapter I.2. As discussed earlier, the mechanical performance of a film is typically evaluated by tensile tests using a texture analyzer. The three parameters commonly evaluated are the tensile strength, $TS$, Young’s modulus, $YM$ and elongation-at-break, $EB$. The tensile strength, $TS$, is defined as the maximum stress the material withstands in the moment of fracture while $YM$, given by the slope in the linear region of the stress-strain curves, is used to evaluate the films’ stiffness (Finkenstadt et al., 2008). To quantify the films’ ability to stretch when exposed to a given stress we measure the $EB$ of the film (Liu et al., 2007) which expresses the percent change of the original length of the specimen between the grips of the texture analyzer.

The mechanical properties of the agar/LBG films were evaluated by tensile tests carried out in a texture analyzer (TA.XT2, Stable 20 Micro Systems, Surrey, UK; Fig. I.2.11) equipped with adequate tensile test attachments (Fig. I.2.12 C). The films were cut into strips.
with equal dimensions (25 × 100 mm) and stored at room T in a desiccator with controlled R.H. (53%) for 7 days, before measurements. The distance between the grips was 9.0 cm and the applied test speed was 0.1 cm/min, while force (N) and deformation (% strain) were recorded. At least five replicates were considered for each film formulation.

These tests were run immediately after removing the specimens from the desiccators, to minimize adsorption/desorption of water by them. At least five replicates were considered for each film formulation.

II.2.3.6.3. Water Resistance

The resistance to water is another crucial aspect when it comes to evaluate the properties of films. This assumes particular relevance when working with natural polymers which are typically very sensitive to moisture. If the goal is to find a good packaging material for sensitive products such as foods, water vapor permeability (WVP) or water sorption isotherms become vital properties to evaluate. Food degradation outcomes from chemical and enzymatic reactions as well as from microbial growth, which are governed by water activity and moisture content of foods.

II.2.3.6.3.1. Water Sorption Isotherms

Based on thermodynamic principles, water sorption determinations allow a better insight of the water retention phenomenon at the primary sites of the polymeric material (i.e. monolayer) when equilibrium is reached, as well as energetic requirements related to differences in the chemical potential of water molecules at the monolayer and upper layers (Souza et al., 2013; Torres et al., 2012).

The water sorption isotherm of a material is defined as the relation between the equilibrium water activity ($a_w$) and the corresponding moisture content (dry basis) of the sample at a given temperature. It provides information on how the material will behave when exposed to different moisture conditions, which is crucial for the development of highly functional and sustainable materials. For food packaging applications, this will show how stable the film will be and to what extent will the food system be susceptible to degradation during processing, handling and storage.
Several mathematical models are used to fit the water sorption data and provide additional thermodynamic information about the studied systems. The well-known Guggenheim-Anderson-de Boer (GAB) model (Anderson, 1946; de Boer, 1968), expressed below (Eq. II.2.1), is commonly used since it is known to successfully fit equilibrium sorption data in a broad range of $a_w$.

$$X_e = \frac{c k X_0 a_w}{(1-k a_w(1-k a_w+c k a_w))}$$

where $X_e$ represents the monolayer moisture content, also on dry basis, i.e. the water content corresponding to saturation of all primary adsorption sites by one water molecule, $C$ is the Guggenheim constant, representing the energy difference between the water molecules attached to the primary sorption sites (i.e. monolayer) and those adsorbed to successive sorption layers (i.e. multilayers), and $k$ is a corrective parameter reflecting properties of multilayer molecules with respect to the bulk liquid.

Water sorption isotherms were obtained gravimetrically as described in Souza et al. (2013). Film samples with approximately 25 mm x 25 mm dimensions, previously dried in a vacuum oven at 50 °C for 48 h, were placed in hermetic chambers with different controlled $a_w$ (in the range 0.11 to 0.90; Table II.2.1), and stored at 25 °C. The $a_w$ of the salt solutions for the temperature of the experiment were obtained from the literature (Greenspan, 1977; Spiess & Wolf, 1983). The samples were weighed periodically, using a balance Sartorius BP211D (Sartorius AG, Germany) with a resolution of 0.01 mg, until they reached constant weight, after which their moisture content was determined by the gravimetric method. The GAB model (Eq. II.2.1) was used to fit experimental sorption data. Duplicate measurements were performed for each film formulation to check the reproducibility of the tests.

Table II.2.1 - Water activities, $a_w$, of the saturated salt solutions at 25 °C obtained from the literature (Greenspan, 1977; Spiess & Wolf, 1983).

<table>
<thead>
<tr>
<th>Salt</th>
<th>$a_w$ of the saturated salt solution at 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium chloride, LiCl</td>
<td>0.112</td>
</tr>
<tr>
<td>Magnesium chloride, MgCl₂</td>
<td>0.328</td>
</tr>
<tr>
<td>Potassium carbonate, K₂CO₃</td>
<td>0.432</td>
</tr>
<tr>
<td>Magnesium nitrate, Mg(NO₃)₂</td>
<td>0.529</td>
</tr>
<tr>
<td>Sodium bromide, NaBr</td>
<td>0.576</td>
</tr>
<tr>
<td>Strontium choride, SrCl</td>
<td>0.709</td>
</tr>
<tr>
<td>Sodium choride, NaCl</td>
<td>0.753</td>
</tr>
<tr>
<td>Barium choride, BaCl₂</td>
<td>0.902</td>
</tr>
</tbody>
</table>
II.2.3.6.3.2. Water Vapor Permeability (WVP)

II.2.3.6.3.2.1. Basic Principles

The majority of polymer films are intrinsically porous structures due to the random nature of the molecular networks. Mass transfer, also known as permeation, refers to the process on which small compounds (i.e. gas or vapor referred to as diffusant or permeant) transfer through a porous material (Duncan et al., 2005). The three main mechanisms involved in permeation are (Fig. II.2.2): i) solubilization of the diffusant in the polymer followed by absorption at the surface of the solid material ii) diffusion of the small molecules through the polymeric material governed by concentration gradients and finally, iii) desorption of the diffusant from the surface of the film (Duncan et al., 2005; Figura & Teixeira, 2007).

![Fig. II.2.2 - Scheme of the permeation process through a polymeric film (adapted from Duncan, 2005).](image)

When the migration of diffusant through the polymer matrix is rapid enough to reach equilibrium concentration at relatively short times (e.g. thin films) the mechanism can be considered steady state permeation. Besides temperature, steady state can only be assumed when the the gradient concentration is constant over time (Figura & Teixeira, 2007).

The initial stage of permeation comprises the solubilization of the small molecules followed by absorption. Solubilization is a thermodynamic phenomenon governed by enthalpic changes which depend on the available volume for the molecules to occupy (Duncan et al., 2005). Ideally, the sorption of gases in a polymer film obeys Henry’s law (Eq. II.2.2),
where $C$ is the concentration of dissolved gas or vapor molecules in $m^3$(STP)/m$^3$, (Note: STP stands for standard temperature and pressure), $p$ the gas partial pressure in Pa and $S$, the solubility parameter in $m^3$(STP)/m$^3$. Pa. $S$ can be defined by the following Arrenhius relationship (Eq. II.2.3):

$$S = S_0 \exp \left( \frac{-\Delta H_S}{RT} \right)$$ (II.2.3)

where $\Delta H_S$ refers to the enthalpic variation caused by the solubilization of the diffusant in the polymer matrix (J/mol$^1$), $T$ is the absolute temperature (K) and $R$, the universal gas constant. Deviations from the ideal Henry’s law may occur resultant from adsorbed molecules into “anomalous” adsorption sites (i.e. micro voids) (Duncan et al., 2005).

The diffusant will then walk randomly through the free volumes within the polymer film (diffusion process). Assuming steady state and expressing the diffusion mechanism only in terms of gradient concentration, Fick’s first law of diffusion is given by (Eq. II.2.4),

$$\frac{dJ}{dt} = -D \frac{dC}{dx}$$ (II.2.4)

where $J$ is the flux of diffusant that transfers through unit area of polymer film (g/m$^2$ or mL/m$^2$), $D$ is the diffusivity constant (m$^2$/s), $C$ is the concentration of permeant (g/m$^3$ or mL/m$^3$), $x$ is the thickness of the film (m) and $t$ is the time (s).

Permeability ($P$) is the property that represents the rate of diffusant transfer through unit area of polymer material per unit thickness (Figura & Teixeira, 2007). At steady state conditions and linear concentration gradient, Eq. II.2.4 can be integrated and rewritten as follows:

$$J = \frac{\Delta m}{At} = -D \frac{C_1-C_2}{x}$$ (II.2.5)
where \( \Delta m \) is the amount of diffusant that transfers through the polymer film (\( g \) or \( mL \)). According to Henry's law (Eq. II.2.2) the difference in concentration of diffusant (C1-C2) is directly proportional to the pressure gradient (\( p_1-p_2 \)):

\[
J = \frac{\Delta m}{At} = -DS\frac{p_1-p_2}{x}
\]

From this relation we can introduce the general concept of permeability, \( P \) ((\( mL \) or \( g \)) \( m/m^2 s \ Pa \)):

\[
P = \frac{\Delta m x}{At \Delta p}
\]

where \( A \) is the area of polymer film accessible to the diffusant (\( m^2 \)).

As for absorption, desorption is also a thermodynamic process whose rates will depend on several aspects including the difference between the concentration at the polymer surface and the atmospheric concentration or the molecular size of the diffusant (Duncan et al., 2005). For these reasons, desorption cannot be seen as an inverse process of absorption since strongly absorbed molecules will take longer times to move away from the polymer film surface.

Factors such as the polymer nature, presence of additives in the polymeric matrix (\( e.g. \) plasticizers, fillers), the sizes of molecules, free volume, defects like pores and voids, temperature and concentration, will influence the overall permeation process (Duncan et al., 2005).

In what edible films and coatings are concerned, it is desirable that the materials form an efficient barrier between the food and the surroundings to prevent the moisture transfer (Martins et al., 2012). The standardised methodology used for WVP determination consists in a simple gravimetric method, known as the ‘cup method’.
II.2.3.6.3.2.2. Experimental Procedure

WVP determinations were carried out according to the ASTM E 96-00. Film samples of 8 cm diameter were tightly sealed to a permeation cell containing an anhydrous salt (calcium chloride, R.H. = 2%) after being left to equilibrate at 25 °C and 50% R.H. for at least 48 h. The cells were placed in a test chamber with controlled temperature (25 °C) and R.H. = 100%. Air convection (approx. 0.3 m/s) ensured uniform conditions throughout the chamber. The permeation cells were weighted periodically in order to obtain the permeability of each film to water vapor (WVP; g·m⁻¹·s⁻¹·Pa⁻¹). According to the general relation of $P$ (Eq. II.2.7) the following equation was used to calculate WVP (Eq. II.2.8):

$$WVP = \frac{\Delta m \cdot x}{A \cdot \Delta t \cdot \Delta P} \quad (II.2.8)$$

where $\Delta m$ is the weight gain (g) for the test cell, $x$ is the film thickness (m), and $A$ is the area ($0.003 \text{ m}^2$) exposed during a time $\Delta t$ (s) to the partial water vapor pressure $\Delta P$ (Pa).

II.2.3.6.4. Scanning Electron Microscopy (SEM)

The basic principles of SEM can be found in section I.2.8.4.1 of Chapter I.2. The surface of the agar/LBG films was examined in the SE mode with a field emission scanning electron microscope (Type FEI Quanta 400 FEG; Fig. I.2.14) under high vacuum at an accelerating voltage of 15 kV and WD between 9.5 and 10.5 mm. Each sample was sputter-coated with Au-Pd prior to the analysis. The experiments were carried out at CEMUP, Porto, Portugal.

II.2.4. Agar/Locust Bean Gum (LBG) Hydrogels

The second tested form of material was agar/LBG hydrogels. The chosen agar samples for this case study were the same as for the aqueous-blended films. LBG and agar starting solutions were prepared as described in sections II.2.3.1 and II.2.3.2, respectively. Agar and
LBG were mixed according to the procedure detailed in section II.2.3.3 to achieve mixtures with a final polymer concentration of 1% wt. This case study will be presented in Chapter II.4.

**II.2.4.1. Rheological Studies**

The rheological properties of agar/LBG hydrogels prepared with different agar/LBG mass ratios were studied under SAOS and LAOS. The studies were conducted in the stress-controlled rheometer (ARG2, TA Instruments, USA; Fig. I.2.8) using a parallel-plate (4 cm diameter and a gap of 1 mm) with a crosshatched steel geometry.

Oscillatory tests were carried out in the linear viscoelastic region of each sample, as follows: agar/LBG solutions were loaded on the Peltier plate (pre-heated to 80 °C) and its periphery was coated with paraffin oil to avoid water evaporation. After a conditioning step of 5 minutes, the gelation process of the sample was monitored by cooling down the system to 25 °C at a rate of 1 °C/min, while small and constant strain was applied at 6.28 rad/s (1 Hz). After letting the agar/LBG systems equilibrate at 25 °C (6.28 rad/s), frequency sweeps were performed over the range 0.1 to 100 rad/s. This allowed measuring the viscoelastic properties as function of ω. Finally, the melting process of the system was followed during a heating ramp carried out from 25 to 90 °C, at a constant rate of 1 °C/min and under the same fixed conditions of strain and frequency. The criteria for T_g and T_m determinations were the same as previously used for agar-pure gels (i.e. tan δ=1).

LAOS experiments consisted in strain sweep tests carried out on agar/LBG hydrogels at 25 °C. Here, the evolution of G’ and G” was measured at 6.28 rad/s until the fracture of the gel.

**II.2.4.2. Penetration Tests**

The penetration tests were performed on equilibrated agar/LBG gels according to the procedure described in section I.2.7.2.2.1 of Chapter I.2. GS was determined as an average value of at least three measurements for each sample.
II.2.4.3. CryoScanning Electron Microscopy (cryoSEM)

The microstructure of the equilibrated agar/LBG hydrogels was analysed by cryoSEM following an identical procedure as described in section I.2.7.3.2 of Chapter I.2.

II.2.5. Non-aqueous Agar Films

The third type of material to be tested was non-aqueous agar films prepared by thermo-compression. For this study, presented in Chapter II.5, CA was used as model sample to find the polymer concentration that led to the best films. The suitability of DES-U and DES-G, as both solvent and plasticizer, was explored for the production of these alternative agar materials. This study was carried out at the ERRC-USDA, in Wyndmoor, PA, USA.

II.2.5.1. Pre-solubilization of Agar in DES

The appropriate amounts of agar (previously dried overnight in a vacuum oven at 60 °C) and DES were weighed according to the desired final concentration (i.e. 2 to 6 % wt). A pre-solubilization of the agar/DES mixtures was carried out under stirring at 140-150 °C and 120-130 °C for, respectively, agar/DES-G and agar/DES-U mixtures, in closed cap vials, using an oil bath. The concentration range and the dissolution temperatures were chosen based on preliminary tests.

II.2.5.2. Thermo-compression

Approximately 10 g of the hot agar/DES mixture, prepared as described in section II.2.5.1., was placed in a square mold (10 x 10 cm) between two Teflon plates covered with aluminum foil and thermo-compressed in a hydraulic press (Carver laboratory press, model B, Fred S. Carver Inc., New Jersey, USA) at 140 °C, 24.5 kN for 20 minutes. The agar/DES system was left to cool down to room temperature for 1 hour, in the press with the load still applied, after which it was left overnight at room temperature. The drying of the films was then
accomplished by immersing the polymer sheet in ethanol, with several changes for 16 hours and subsequent drying in air overnight. Finally, the films were stored at controlled environmental conditions (53% R.H. and room temperature) to carry out the experiments. A shorthand notation was adopted for the different films; for instance, agar film prepared in DES-U at 2% wt polymer concentration will be designated herein, CA2/DES-U while films obtained from 5% wt agar addition in DES-G will be termed CA5/DES-G.

II.2.5.3. Films Characterization

II.2.5.3.1. Scanning Electron Microscopy (SEM)

SEM studies were carried out in a Quanta 200 FEG microscope (SEM, FEI, Hillsboro, OR). Prior to the analysis, film samples were cryo-fractured in liquid N$_2$ and mounted with carbon adhesive tabs (Electron Microscopy Sciences, Hatfield, PA) to specimen stubs, and the edge was painted with colloidal silver adhesive. Afterwards, the samples were coated with a thin film of gold and imaged in the high-vacuum/SE imaging mode SEM using an accelerating voltage of 10 kV and WD of 12.5 mm.

II.2.5.3.2. Thickness Measurement

The thickness of the films was measured using a B.C. Ames comparator, Model 70-1355, Framingham, MA. At least, five measurements were performed in each case.

II.2.4.3.3. Mechanical Properties

The mechanical properties ($TS$, $YM$ and $EB$) of the films were measured using a MTS Insight 5 mechanical property tester, model 1122, equipped with Testwork 4 data acquisition software (MTS Systems Corp., Minneapolis, MN) (Liu et al., 2007). Film specimens with dimensions of $10 \times 50$ mm were tested after being stored at 50% R.H. and 25 °C for at least 48 hours. A 50 mm/min strain rate and a gauge length of 20 mm were used in all experiments. Five replicates were performed in each case.
II.2.4.3.4. Sorption Isotherms

Water sorption isotherms were obtained gravimetrically as described in section II.2.3.6.3.1 for a considered $a_w$ range of 0.11 to 0.84.

II.2.4.3.5. Contact Angle Measurements

A contact angle Automated Goniometer (Ramé-hart Instrument Co., Succasunna, NJ, USA) equipped with a high resolution camera (Model 590 F4 Series) was used to determine the hydrophilicity and wettability of the agar/DES films. These two properties can be correlated with the angle formed between the biopolymer film surface and a tangent drawn on the water droplet surface that passes through the intersection point of the air-water-film phases. Experiments were carried out in air through static sessile drop measurements using ultrapure water as the liquid phase (11±0.6 µL drops). The distance between the needle-tip and the film surface (0.5 cm) was kept constant to ensure consistency between the different measurements. Due to the hydrophilic nature of the studied films, reported angles were recorded after 10 s to avoid as much as possible penetration of water in the films (i.e. absorption phenomena). Determination of the contact angle ($\Theta$), volume (V) and surface area (A) of the droplets were performed using the Dropimage Advanced v2.5 software. These parameters, related to the shape of the water droplet, were used to detect the possible contribution of absorption or spreading phenomena at the surface/liquid interface for each of the studied films (Farris et al., 2011). An additional commercial LDPE sample, of known hydrophobic character, was used as reference. The results presented in Table II.5.2 (Chapter II.5) are averages of five to ten water drops deposited on each film surface.

II.2.5.3.6. Water Vapor Permeability (WVP)

WVP determinations were carried out as described in section II.2.3.6.3.2.2 but using a permeation cell with 0.00196 m² and using beeswax as sealant.
II.2.6. Statistical Analysis

The statistical significance of some of the experimental data concerning the films application was examined using the Student’s t-test. The significance level was set at p<0.01. A non-linear estimation (Quasi-Newton method) was performed using Statistica 8.0 software (StatSoft, Tulsa, OK, USA) to fit the experimental sorption data to Eq. II.2.1 and obtain the correspondent GAB parameters.

II.2.7. Fibers

The final case study of Part II concerns the production of agar fibers by electrospinning. This research was also carried out in the USA. As previously, the best electrospinning conditions were investigated using CA.

II.2.7.1. Preparation of Agar/PVA Spinning Solutions

Agar and PVA powders were pre-dried overnight at 40 °C in a vacuum oven, prior to use. Each starting solution was prepared by dissolving the appropriate amount of polymer in the respective solvent as described next.

II.2.7.1.1. Water as Solvent

For the aqueous media, the concentrations used for the starting solutions were 1% wt for agar and 10% wt for PVA. This choice was based in preliminary tests and considering the intrinsic properties of each polymer. In both cases, the polymers were dispersed in cold water and dissolved at T close to the boiling point of the solvent, under vigorous stirring. Special caution was taken when dissolving the PVA; to avoid the formation of lumps and/or a PVA film at the solution surface a heating bath and stirrers promoting an evenly dispersion of
all PVA particles were used. Both polymers were considered dissolved when a homogeneous transparent solution was obtained which typically occurred after 1h, 1h30m.

Agar/PVA blends with different mass ratios (100/0, 50/50, 40/60, 30/70, 20/80, 0/100) were prepared by weighing the appropriate amounts of each starting solution into closed cap vials. Finally, the mixtures were heated again at the considered dissolution T, under vigorous stirring, until a homogeneous solution was obtained.

II.2.7.1.2. DES-U as Solvent

For the polymer solutions in the DES-U, lower concentrations were considered: 1% wt agar and 3% or 5% wt PVA. This choice was due to the nature of the solvent. The samples were heated in an oil bath, in closed cap vials at 120-130 °C and under vigorous stirring. As for the aqueous media, the SP were dispersed in the solvent at room T under vigorous stirring after which the T was increased. The solubilisation was considered complete when a homogeneous solution of light yellow coloration was obtained. This was typically accomplished after ~20 min at 120-130 °C.

The pure PVA solutions were heated under constant stirring at 120-130 °C until a homogeneous and transparent solution was obtained. This was typically accomplished after 30-40 min. The procedure to prepare the agar/PVA blends (100/0, 50/50, 30/70, 20/80, 0/100) in DES-U was identical to the aqueous media (section II.2.7.1.1), but the mixing was performed at higher T (120 °C).

II.2.7.2. Rheological Measurements

The rheological procedures used to characterize the spinning solutions were the same independently of the used solvent. The tests were performed in the stress-controlled rheometer (ARG2, TA Instruments, USA; Fig. I.2.8) as described next: a cone-and-plate geometry (4 cm diameter, 2° angle and a 54 µm gap) was used in all cases. The samples were loaded while hot into the measuring device after being degassed in a vacuum oven for 5 minutes at 90 °C (water) or 100 °C (DES-U). Each sample was covered with a layer of paraffin oil to prevent evaporation (water) or hydration (DES-U) during the course of the
experiments. Frequency scans were recorded after a cooling ramp from 90 to 50 °C at a cooling rate of 1 °C/min and a fixed frequency of 6.28 rad/s, followed by an equilibration period at 50 °C. Mechanical spectra were recorded at 50 °C, over the range 0.1 - 75 rad/s. The strain conditions were chosen according to the linear viscoelastic region defined during strain sweep tests. A common strain of 1% for the aqueous media and 2% for the solutions prepared in DES-U was selected. Steady shear measurements were carried out at 50 °C (chosen temperature for the electrospinning experiments), in the range 1-200 s⁻¹. Three replicates were performed in each case.

II.2.7.3. Electrospinning

The electrospinning unit used to carry out the experiments was from NaBond Technologies Co., Limited, China. Fig. II.2.3 illustrates the installation setup used for each studied solvent. A tubeless spinneret (NaBond Technologies Co., Limited, China) was attached inside the chamber and connected to the external syringe pump (Veryark, model TCI-IV). This accessory requires a minimum solution volume for each experiment (0.5 mL) and no soft tube is needed for connecting the needle to the syringe. This allows the syringe with the polymer solution to be placed inside the chamber at the temperature defined for the test. The connection between the syringe pump and the tubeless spinneret was accomplished by a soft plastic tube containing water. The flow rate set on the syringe pump forced the piston of the syringe carrying the solution to move. In all cases, the samples were placed in a 5 mL plastic syringe fitted with a metallic needle (outer diameter × length = 0.6 × 30 mm and inner diameter = 0.317 mm). A high voltage power supply device (0-50 kV) was used to generate the high electric fields. For each solution, several operational conditions were tested including flow rate (0.5-16 mL/h), voltages (10-30 kV) and distance tip-to-collector (6-12 cm). The best working temperature was chosen taking into consideration preliminary tests, equipment constraints as well as the solution properties. After several trials, the temperature of the chamber was fixed at 50 °C and the distance tip-to-collector at 8 cm.

Two different collectors were used depending on the solvent. For the aqueous systems, a drum collector covered with an aluminum foil was chosen to collect the electrospun materials (Fig. II.2.3, left image). The spinneret was fixed (null x-axial sliding) and the motor speed of the collector was around 510 rps in all cases.

An ethanol bath with an immersed copper electrode was used as collector for the solutions prepared with the DES-U (Fig. II.2.3, right image). The electrode was kept 1.5 cm
underneath the ethanol surface in all cases. The collected samples were left stirring overnight in ethanol and further washed several times with ethanol to ensure that all DES-U was removed. This prevented the plasticization of the fibers (Freire et al., 2011). Finally, the washed samples were dried in vacuum at 50 °C for a minimum of 24h.

![Fig. II.2.3 - Schematic representation of the electrospinning setup (a- syringe pump; b- tubeless spinneret; d- chamber with T control; e- power supply) when using: (left) water as solvent (c- drum collector); (right) the DES-U (c- coagulating bath).]

II.2.7.4. Scanning Electron Microscopy (SEM)

The morphology and size of agar fibers were investigated in a Quanta 200 FEG scanning electron microscope (SEM, FEI, Hillsboro, OR). The samples were mounted with adhesive to specimen stubs, and the edge was painted with colloidal silver adhesive. Afterwards, the samples were coated with thin film of gold and imaged in the high-vacuum/SE imaging mode scanning electron microscope using an accelerating voltages close to 10 kV and WD around 12.5 mm.

References.


II.3 AQUEOUS-BLENDED FILMS OF AGAR/LOCUST BEAN GUM
II.3.1. Rheological Characterization of the Film-forming Solutions

The rheological properties of agar/LBG mixtures with different compositions (i.e. 100/0, 75/25, 50/50, 25/75, 0/100 mass ratios) were investigated at the temperature at which the film-forming solutions were spread onto the glass plates. Mechanical spectra of representative NA_IMTA_TWE$^{opt}$/LBG and AA_IMTA_TWE$^{opt}$/LBG solutions recorded at 55 °C, are plotted in, respectively, Figs. II.3.1 and II.3.2. In all cases, the systems exhibited typical behavior of macromolecular solutions with $G'' > G'$ in all range of tested angular frequencies, $\omega$. The viscoelastic behavior of the pure LBG solution agreed well with previous studies (da Silva et al., 1993; Sittikijyothin et al., 2010). Another important observation was the frequency-dependence of the viscoelastic response of the systems with LBG, with both moduli describing a monotonic increase over the window of tested $\omega$. For the pure agar systems, the magnitudes of the moduli were very small and it was frequent to acquire negative points (not shown). At high frequencies, it was noticeable the increase of the elastic contribution ($G'$) with the difference between both moduli becoming narrower. For both agars, the addition of LBG decreased the gap between $G'$ and $G''$, consequence of the dominant effect of the non-gelling polysaccharide. In all cases, the cross-over between both moduli, indicative of the beginning of the viscoelastic plateau, was beyond the maximum $\omega$ limit (~63 rad/s) and it was clearly moved to even higher frequencies with the increase of agar content in the final mixture. The limited range of frequencies defined for the mechanical spectra probably did not extend to low/high enough values to reach the end of the terminal zone and beginning of the viscoelastic plateau for the defined experimental conditions. This could suggest low density of interchain entanglements (da Silva et al., 1993). The elasticity of NA_IMTA_TWE$^{opt}$/LBG and AA_IMTA_TWE$^{opt}$/LBG systems, evaluated by the $\tan \delta$ evolution, increased with the increase in LBG concentration in the final mixture for both agars, indicating an elasticity gain of the system.

The main differences between the viscoelastic behaviors of both agars could be seen in the region of low frequencies and particularly for the elastic component. For instance, $G'$ of 25/75 NA_IMTA_TWE$^{opt}$/LBG was higher than that of the pure LBG solution up to ~2.5 rad/s and from then on, it stayed below it (Fig. II.3.1). This behavior was not described by AA_IMTA_TWE$^{opt}$/LBG mixtures whose $G'$ data fell below the curve of the non-gelling gum at all stages of the $\omega$ window (Fig. II.3.2). The moduli of NA_IMTA_TWE$^{opt}$/LBG systems could reach greater magnitudes than AA_IMTA_TWE$^{opt}$/LBG (Fig. II.3.3). For instance, taking as reference the data recorded at 6.28 rad/s, $G'$ and $G''$ of NA_IMTA_TWE$^{opt}$ reached values of respectively, ~2 and ~7.2 Pa, and of ~1 and ~4.6 Pa for AA_IMTA_TWE$^{opt}$ (Table II.3.1). The
different behavior of \( \text{NA\_IMTA\_TWE}^{\text{opt}} \) relative to the \( \text{AA\_IMTA\_TWE}^{\text{opt}} \) could be attributed to chemical (i.e. LA, sulfates and methyl contents) and physical (i.e. \( M_w \)) differences between both SP (Chapter I.4); while the alkali-modified agar possessed low sulfate/LA ratio (~1.37/34 against ~3.39/28 %/%) and total methyl content (~24 mol % against 38%) the native SP exhibited higher \( M_w \) (~532×10³ against ~865×10³ g/mol) (Tables I.4.3 to I.4.6 in Chapter I.4).

Table II.3.1 - Values of \( G' \) and \( \tan \delta \) measured during the mechanical spectra at 55 °C taking as reference 6.28 rad/s for the different agar/LBG mixtures.

<table>
<thead>
<tr>
<th>AGAR/LBG</th>
<th>( G' ) (Pa)</th>
<th>( \tan \delta )</th>
<th>( G' ) (Pa)</th>
<th>( \tan \delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>75/25</td>
<td>0.103(^{1(1)})</td>
<td>6.390(^{1(1)})</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50/50</td>
<td>0.39±0.16</td>
<td>5.1±1.0</td>
<td>0.27±0.05</td>
<td>7.2±1.0</td>
</tr>
<tr>
<td>25/75</td>
<td>1.87±0.94</td>
<td>3.5±1.0</td>
<td>0.94±0.04</td>
<td>4.97±0.20</td>
</tr>
<tr>
<td>0/100</td>
<td>4.2±1.0</td>
<td>3.18±0.25</td>
<td>4.2±1.0</td>
<td>3.18±0.25</td>
</tr>
</tbody>
</table>

\(^{1}\) Only one experiment was considered to give meaningful data; samples with negative values were disregarded.

Fig. II.3.1 - Frequency dependence at 55 °C of elastic (\( G' \); filled symbols) and viscous (\( G'' \); open symbols) moduli of 1 % wt \( \text{NA\_IMTA\_TWE}^{\text{opt}}/\text{LBG} \) solutions (0/100 (squares), 25/75 (triangles), 50/50 (circles)).
Fig. II.3.2 - Frequency dependence at 55 °C of elastic (G'; filled symbols) and viscous (G''; open symbols) moduli of 1 % wt AA_IMTA_TWEopt/LBG solutions (0/100 (squares), 25/75 (triangles), 50/50 (circles)).

Fig. II.3.3 - Mechanical spectra at 55 °C for 25/75 agar/LBG mixtures (NA_IMTA_TWEopt (squares), AA_IMTA_TWEopt (triangles)).

The flow curves of the film-forming solutions were recorded at 55 °C for NA_IMTA_TWEopt (Fig. II.3.4) and AA_IMTA_TWEopt (Fig. II.3.5). Pure agar solutions described Newtonian behavior with NA_IMTA_TWEopt exhibiting higher apparent viscosity, \( \eta_{app} \) (\( \sim 7.8 \times 10^{-3} \) Pa.s) than AA_IMTA_TWEopt (\( \sim 4.4 \times 10^{-3} \) Pa.s). Upon LBG addition, the viscosity of agar solutions increased dramatically. For both agars, the mixtures exhibited intermediate behaviors of the individual components. At higher LBG contents (e.g. 25/75 agar/LBG ratio), shear-thinning behavior with a viscosity plateau region was evident due to the predominant effect of the non-gelling polysaccharide (Figs. II.3.4 and II.3.5). The decrease of viscosity with increase of
shear rate characterizes shear-thinning fluids such as galactomannans solutions (Cerqueira et al., 2011; da Silva et al., 1992; Sittikijyothin et al., 2005). Oppositely, the curves of the mixtures with lower LBG amounts (e.g. 75/25) described a closer behavior to that of the pure agar systems. Shear-thinning behavior, characterized by a decrease in viscosity upon increase of shear rate, reflects significant macromolecular reorganizations induced by the application of relevant shear forces. In the region of low shear rates, the system disrupts some intermolecular entanglements and simultaneously forms new ones enabling the viscosity of the system to stay approximately constant. When a shear rate threshold is attained, the system disturbance becomes too significant and the formation of new entanglements no longer compensates the intermolecular disruptions. At this point, the molecular alignment in the flow direction takes place causing the viscosity of the system to decrease (Bastos et al., 2010).

Fig. II.3.4 - Flow curves at 55 °C for NA.IMTA.TWE-LBG solutions (symbols: 0/100 (squares), 25/75 (triangles), 50/50 (circles), 75/25 (stars) and 100/0 (inverted triangles)).
Several rheological models have been proposed to describe the dependence of apparent viscosity with shear rate for hydrocolloid systems. The Cross (Eq. II.3.1) and Carreau (Eq. II.3.2) models are known to describe well the typical shear-thinning behavior with a zero-shear rate viscosity, followed by a power law dependence (da Silva et al., 1992; Sittikijyothin et al., 2010):

\[ \eta_{app} = \eta_{\infty} + \frac{(\eta_0 - \eta_{\infty})}{1 + (\dot{\gamma})^m} \]  

(II.3.1)

\[ \eta_{app} = \eta_{\infty} + \frac{(\eta_0 - \eta_{\infty})}{1 + (\dot{\gamma})^N} \]  

(II.3.2)

where \( \dot{\gamma} \) is the shear rate (s\(^{-1}\)), \( \eta_{app} \) is the apparent viscosity (Pa.s), \( \eta_0 \) is the zero-shear rate viscosity (Pa.s), \( \eta_{\infty} \) is the infinite-shear rate viscosity (Pa.s), \( a_c \) (s\(^m\)) and \( \lambda_c \) (s) are time constants, and \( m \) and \( N \) are dimensionless constants (da Silva et al., 1992; Sittikijyothin et al., 2010). Both models were used to fit the experimental data recorded during the steady shear experiments (Figs. II.3.4 and II.3.5) taking as valid the assumption that \( \eta_{\infty} \ll \eta_0 \) and so, \( \eta_{\infty} = 0 \). The good fittings of both models to the data of agar/LBG mixtures and LBG-pure solution could be confirmed by the high percentage of explained variances (R\(^2\)) and low relative errors (RE) for these samples (Tables II.3.2 to II.3.5). Naturally, agar-pure systems exhibiting Newtonian viscosity plateau over the entire range of tested shear rates, were not...
so successfully described by the models (Tables II.3.2 and II.3.3). For that reason, the parameters for these samples were not considered. The Cross model predicted higher values of $\eta_0$ as seen previously by other authors (Bastos et al., 2010; da Silva et al., 1992).

Clearly, the magnitude of the estimated parameters for both models exhibited an ascending trend upon LBG addition to agar solutions. The time constants, related to the longest relaxation time of the system were highest for the pure LBG solution ($\alpha_c=0.0321$ s and $\lambda_0=0.1586$ s). The exponents related to the power law region of the curves were also highest for this sample, showing that the shear thinning behavior increased with LBG concentration in the mixture. As expected, the values of $\eta_0$ increased with the increase of LBG content in the final mixture since LBG is much more viscous than agar at comparable concentrations, and apparently, there was not significant incompatibility between both polysaccharides. As seen for other biological systems (Bastos et al., 2010; Sittikijyothin et al., 2010), the transition from Newtonian to shear-thinning behavior shifted to lower shear rates upon increase of LBG concentration in the final mixture. With the addition of LBG, the systems became more viscous and the individual molecules moved less freely. Consequently, longer times were needed to form new entanglements which caused the transition from one state (Newtonian) to another (shear-thinning) to occur at lower shear rates (i.e. higher magnitudes of time constants). It was also noticed that for the same agar/LBG ratio, this shear rate threshold was slightly higher for AA_IMTA_TWE$^{opt}$ as indicated by the lower magnitudes of the time constants estimated for these samples (i.e. Cross and Carreau parameters; Tables II.3.2 a II.3.5). This could be associated with the higher number of LA units (~35 %) of this agar promoting stronger inter- and intramolecular associations when compared to NA_IMTA_TWE$^{opt}$ (~28 %).
FCUP

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Fig. II.3.6 - Comparison between the flow curves at 55 °C of NA_IMTA_TWE\textsuperscript{opt}/LBG (filled symbols) and AA_IMTA_TWE\textsuperscript{opt}/LBG (open symbols) solutions (symbols: 25/75 (triangles), 50/50 (circles), 75/25 (stars) and 100/0 (inverted triangles)). Data of the pure LBG solution (0/100) are represented with crosses. The red lines represent predictions of the Cross model (Eq. II.3.1).

Table II.3.2 - Values of the Cross model parameters (obtained from the fittings of steady shear data at 55 °C to equations Eq. II.3.1) for the NA_IMTA_TWE\textsuperscript{opt}/LBG systems.

<table>
<thead>
<tr>
<th>Agar/LBG</th>
<th>(\eta_0) (Pa.s)</th>
<th>(\alpha_c) (s)</th>
<th>(m)</th>
<th>(R^2)</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0(^{(1)})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>75/25</td>
<td>0.105</td>
<td>0.00266</td>
<td>0.600</td>
<td>0.999</td>
<td>6.384E-3</td>
</tr>
<tr>
<td>50/50</td>
<td>0.337</td>
<td>0.00762</td>
<td>0.700</td>
<td>0.999</td>
<td>7.624E-3</td>
</tr>
<tr>
<td>25/75</td>
<td>1.27</td>
<td>0.0204</td>
<td>0.770</td>
<td>0.999</td>
<td>7.402E-3</td>
</tr>
<tr>
<td>0/100</td>
<td>2.75</td>
<td>0.0321</td>
<td>0.810</td>
<td>0.999</td>
<td>8.656E-3</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Experimental data not so well described by the model (Newtonian behavior).
### Table II.3.3 - Values of the Cross model parameters (obtained from the fittings of steady shear data at 55 °C to equations Eq. II.3.1) for the AA_IMTA_TWE\textsuperscript{opt}/LBG systems.

<table>
<thead>
<tr>
<th>Agar/LBG</th>
<th>$\eta_0$ (Pa.s)</th>
<th>$\alpha_c$ (s)</th>
<th>$m$</th>
<th>$R^2$</th>
<th>$RE$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0\textsuperscript{(1)}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>75/25</td>
<td>0.0517</td>
<td>0.00122</td>
<td>0.654</td>
<td>0.999</td>
<td>5.093E-3</td>
</tr>
<tr>
<td>50/50</td>
<td>0.295</td>
<td>0.0066</td>
<td>0.792</td>
<td>0.998</td>
<td>1.596E-2</td>
</tr>
<tr>
<td>25/75</td>
<td>0.781</td>
<td>0.0118</td>
<td>0.834</td>
<td>0.999</td>
<td>2.075E-2</td>
</tr>
<tr>
<td>0/100</td>
<td>2.75</td>
<td>0.0321</td>
<td>0.810</td>
<td>0.999</td>
<td>1.667E-3</td>
</tr>
</tbody>
</table>

\textsuperscript{(1)} Experimental data not as well described by the model (Newtonian behavior).

### Table II.3.4 - Values of the Carreau model parameters (obtained from the fittings of steady shear data at 55 °C to equations Eq. II.3.2) for the NA.IMTA_TWE\textsuperscript{opt}/LBG systems.

<table>
<thead>
<tr>
<th>Agar/LBG</th>
<th>$\eta_0$ (Pa.s)</th>
<th>$\lambda_0$ (s)</th>
<th>$N$</th>
<th>$R^2$</th>
<th>$RE$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0\textsuperscript{(1)}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>75/25</td>
<td>0.0959</td>
<td>0.0344</td>
<td>0.121</td>
<td>0.988</td>
<td>1.212E-2</td>
</tr>
<tr>
<td>50/50</td>
<td>0.31</td>
<td>0.0496</td>
<td>0.177</td>
<td>0.989</td>
<td>2.393E-2</td>
</tr>
<tr>
<td>25/75</td>
<td>1.14</td>
<td>0.0795</td>
<td>0.231</td>
<td>0.999</td>
<td>3.433E-2</td>
</tr>
<tr>
<td>0/100</td>
<td>2.53</td>
<td>0.159</td>
<td>0.199</td>
<td>0.995</td>
<td>0.3240</td>
</tr>
</tbody>
</table>

\textsuperscript{(1)} Experimental data not as well described by the model (Newtonian behavior).
Table II.3.5 - Values of the Carreau model parameters (obtained from the fittings of steady shear data at 55 °C to equations Eq. II.3.2) for the AA_IMTA_TWE$^{opt}$/LBG systems.

<table>
<thead>
<tr>
<th>Agar/LBG</th>
<th>$\eta_0$ (Pa.s)</th>
<th>$\lambda_0$ (s)</th>
<th>$N$</th>
<th>$R^2$</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0$^{(1)}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>75/25</td>
<td>0.0960</td>
<td>0.0344</td>
<td>0.121</td>
<td>0.988</td>
<td>2.48E-2</td>
</tr>
<tr>
<td>50/50</td>
<td>0.275</td>
<td>0.0341</td>
<td>0.204</td>
<td>0.998</td>
<td>3.909E-2</td>
</tr>
<tr>
<td>25/75</td>
<td>0.732</td>
<td>0.0515</td>
<td>0.231</td>
<td>0.997</td>
<td>5.973E-2</td>
</tr>
<tr>
<td>0/100</td>
<td>2.53</td>
<td>0.159</td>
<td>0.199</td>
<td>0.995</td>
<td>0.1789</td>
</tr>
</tbody>
</table>

$^{(1)}$ - Experimental data not so well described by the model (Newtonian behavior).

In order to check the synergistic or antagonistic effects between agar and LBG, the viscosity data of each individual component obtained by the Cross model (Tables II.3.2 and II.3.3) was used to calculate the predicted viscosities of agar/LBG mixtures according to the following equations,

$$\eta_{mix} = X_A \eta_A + X_B \eta_B \quad \text{(II.3.3)}$$

$$\eta_{mix} = \eta_A^{X_A} + \eta_B^{X_B} \quad \text{(II.3.4)}$$

where $X_A$ and $X_B$ are the weight fractions of polysaccharide A and polysaccharide B, and $\eta_A$ and $\eta_B$ the apparent viscosities (fitted values) of the pure polysaccharide solutions, at equal concentrations. The equation of Kaletunc-Gencer and Peleg, Eq. II.3.3 (Kaletunc-Gencer & Peleg, 1986) is used to calculate viscosities based on simple additivity effect and translates the absence of interactions between both polysaccharides (i.e. no synergism nor antagonism). The logarithmic form of Eq. II.3.4 (Miller & Mann, 1944) was first used with success by Harris (Harris, 1970) and later by Plutchok and Kokini (Plutchok & Kokini, 1986) to correlate the $\eta_0$ of, respectively, binary mixtures of polystyrene and GG with the $\eta_0$ of each individual component.

The logarithmic forms of the predicted vs experimental (fitted) viscosities of the mixtures for each agar, are presented in Fig. II.3.7 (NA_IMTA_TWE$^{opt}$/LBG) and Fig. II.3.8 (AA_IMTA_TWE$^{opt}$/LBG). Due to the poor model predictability of the pure agar solutions, the
viscosities used in the calculations consisted in the average value of the experimental $\eta_{\text{app}}$
(0.00739 Pa.s and 0.00537 Pa.s for respectively, NA\_IMTA\_TWE$^{\text{opt}}$ and AA\_IMTA\_TWE$^{\text{opt}}$). Based on simple additivity (Eq. II.3.3), antagonism was the dominant effect for both type of mixed agar systems ($\eta_{\text{app}}$ calculated $>$ $\eta_{\text{app}}$ experimental; Figs. II.3.7 and II.3.8). Oppositely, the Miller and Mann relation (Eq. II.3.4), which predicts geometric mean viscosities, predicted synergistic interactions for NA\_IMTA\_TWE$^{\text{opt}}$/LBG and AA\_IMTA\_TWE$^{\text{opt}}$/LBG. Similar predictions were found in previous studies concerning high metoxyl pectin and LBG mixtures (da Silva et al., 1992). The prediction of viscosity of mixtures based on simple additivity must outcome from viscosity contributions not massively dominated by one of the components (Amici et al., 2000). In our case however, the viscosity was clearly dominated by the LBG. Hence, the use of such simple relation shouldn’t lead us to rule out completely the existence of synergisms between agar and LBG. In complex gum systems, it is common to observe a nonlinear dependence of viscosity (Cengiz et al., 2013).

![Graph](image)

**Fig. II.3.7** – Magnitudes of experimental and predicted zero-shear rate viscosities ($\eta_0$), at 55 °C, of NA\_IMTA\_TWE$^{\text{opt}}$/LBG mixtures, at equal concentrations of each polysaccharide (blue circles = predicted values with Eq. II.3.3, and orange squares = predicted values with Eq. II.3.4). The linear fittings for each set of data and the respective $R^2$ are also indicated.
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**Fig. II.3.8** – Magnitudes of experimental and predicted zero-shear rate viscosities ($\eta_0$), at 55 °C, of AA\_IMTA\_TWE\textsuperscript{opt}/LBG mixtures, at equal concentrations of each polysaccharide (blue circles = predicted values with Eq. II.3.3, and orange squares = predicted values with Eq. II.3.4). The linear fittings for each set of data and the respective $R^2$ are also indicated.

**Fig. II.3.9** - Combined plot of the magnitude of the complex viscosity, $|\eta^*| (\omega)$ (open squares, open circles), dynamic viscosity, $\eta' (\omega)$ (squares with crosses, black line) and apparent viscosity, $\eta (\dot{\gamma})$ (solid squares, red line) at 55 °C, according to the Cox-Merz rule for the: pure LBG solution (main graphic) and 50/50 NA\_IMTA\_TWE\textsuperscript{opt}/LBG mixture (inset).

In order to correlate the dynamic with the steady shear viscosities, the Cox Merz rule was applied to the experimental data. This rule establishes a correlation between the properties measured in the regime of small and linear deformations (dynamic oscillatory studies) and the properties in the regime of large and non-linear deformations (steady shear tests). Basically, the principle of Cox Merz is the superposition of the shear rate dependence of the
steady shear viscosity, $\eta (\dot{\gamma})$ with the frequency dependence of the magnitude of the complex viscosity, $|\eta^*| (\omega)$, at equal values of $\omega$ and $\dot{\gamma}$,

$$|\eta^*| (\omega) = \eta (\dot{\gamma})_{\omega=\dot{\gamma}} \tag{II.3.4}$$

As seen in previous studies concerning $\beta$-lactoglobulin/LBG (Sittikijyothin et al., 2010) and pectin/LBG (da Silva et al., 1993) mixtures, the dynamic viscosity of the pure agar and LBG solutions and respective mixed systems, $\eta'$, approached the steady-shear viscosity at low $\omega$ and $\dot{\gamma}$. However, when moving towards higher values of $\omega$ and $\dot{\gamma}$, $\eta'$ started to diverge gradually from the flow curve, decreasing more rapidly. This phenomenon is explained by the different molecular motions underlying both types of rheological tests, in the range of high $\omega$ and $\dot{\gamma}$ (Ferry, 1980). As examples, the combined plots of the Cox Merz rule for the pure LBG solution (main graphic) and for 50/50 NA.IMTA.TWE$^{opt}$/LBG are given in Fig. II.3.9. Remaining systems described similar trends (not shown).

In summary, the main conclusions gathered during the rheological characterization of the film-forming solutions were: i) LBG addition increased dramatically the viscosity of agar solutions and significantly decreased the contribution of the elastic component of the systems; ii) for both agars, the mixtures exhibited intermediate behaviors to that of the individual components; also, iii) antagonism was found between each agar and LBG when using the simple linear additivity relation of apparent viscosities while the prediction of geometric mean viscosities pointed out to synergistic effects between the polysaccharides.

II.3.2. Films Preparation

After a short period of degassification, the bubble-free hot-solutions were left to cool down to 55 °C before being spread over a glass plate using an automatic film applicator at a constant application speed of 0.3 mm/s (Fig. II.1.1 of Chapter II.1). Clearly, films with higher LBG contents were easier to process due to the greater viscosity of the film-forming solutions. During the drying stage, the solvent was evaporated leading to a significant reduction of the volume of film-forming solution. In our case, the fabricated films took typically 2h to dry (climate chamber at 40 °C and 30% R.H.), exception made for the pure LBG film that needed 2h30m at the defined drying conditions. All dried films were very easily removed from the glass supports. Blending agar with LBG can be quite advantageous since the
galactomannan increases dramatically the viscosity of agar solutions hence, leading to ‘knife-coating’ films with uniform thickness (Sousa et al., 2008). Overall, agar/LBG films exhibited regular thicknesses (± 1 µm; Table II.3.7). For the pure agar and 75/25 AA IMTA_TWEopt/LBG films, the variability between replicate samples was slightly higher (± 2 µm; Table II.3.7) which could be easily explained by the lower viscosities of these samples (Figs. II.3.4 to II.3.6).

For the same mixture composition, each agar yielded films with different thicknesses. Overall, AA IMTA_TWEopt/LBG mixtures could result in thicker films upon drying (in the range ~5-10 µm against ~4-8 µm for the NA IMTA_TWEopt/LBG films; Table II.3.6). Besides the dry matter content, the thickness of the film is driven by the retraction ratio of the film-forming solution upon drying (Phan et al., 2009a). This phenomenon is particularly relevant for gelling polysaccharides where a combination of factors such as network formation or water syneresis upon gel aging must be considered when interpreting the results. In turn, this will be dependent on the nature of the polysaccharide (i.e. structural and physicochemical properties). As already discussed (e.g. Chapter I.6), upon gelation NA typically present a more irregular network with opened pores of heterogeneous sizes due to the higher number of sulfated monomers which can hinder the perfect alignment of the helices (Sousa et al., 2013). On the other hand, gels of AA are expected to exude more water on aging (Matsuhashi, 1990). In our case, the pure agar films had close final thicknesses (~4-9 µm) which suggested close retractability upon drying of the film-forming solutions. As will be discussed in detail over the next chapter, each agar/LBG system possessed different gelation points, typically bellow the defined drying temperature of the films (40 °C). This should mean different molecular organizations during the drying stage, ultimately reflecting on the final performances of the fabricated materials. Phan and co-workers found agar-based films to have the highest retraction ratio among several tested polysaccharides (Phan et al., 2009a, b).

<table>
<thead>
<tr>
<th>Agar/LBG ratio</th>
<th>NA IMTA_MAEopt d/mm(1)</th>
<th>AA IMTA_MAEopt d/mm(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>0.006±0.002</td>
<td>0.007±0.002</td>
</tr>
<tr>
<td>75/25</td>
<td>0.005±0.001</td>
<td>0.008±0.002</td>
</tr>
<tr>
<td>50/50</td>
<td>0.005±0.001</td>
<td>0.007±0.001</td>
</tr>
<tr>
<td>25/75</td>
<td>0.005±0.001</td>
<td>0.007±0.001</td>
</tr>
<tr>
<td>0/100</td>
<td>0.006±0.001</td>
<td>0.006±0.001</td>
</tr>
</tbody>
</table>

1 Means of all thicknesses measured for the different tests performed on the films.
II.3.3. Films Characterization

II.3.3.1. Films Appearance

The first logical step was the visual inspection of the obtained dried films. The main macroscopic difference between AA\_IMTA\_TWE\textsuperscript{opt} and NA\_IMTA\_TWE\textsuperscript{opt} films relied on the coloration (slight yellowish for the latter and practically colorless for the former). The alkaline treatment and subsequent weak acidic neutralization, performed during the extraction of AA\_IMTA\_TWE\textsuperscript{opt}, could easily explain these differences. Besides the chemical transformation of L6S in LA units, the pre-treatment step can also eliminate impurities present in the NA extracts such as salts, floridean starch and others. As result, AA extracts are typically lighter than NA. Fig. II.3.10 (A) shows the pure agar films placed next to each other to better see this difference. In what agar/LBG blends are concerned, photographs of the films prepared at 25/75 agar/LBG ratio are shown as examples in Figs. II.3.10 (B) and (C). Naturally, the differences in coloration were attenuated by the addition of LBG to the film-forming solution. Overall, each agar yielded films with identical appearance independently of the blend composition. Films made from alkali-modified agar (e.g. Fig. II.3.10 (B)) were colorless and identical to a CA film used as reference (not shown). All films were very homogeneous, transparent, easy to handle and resistant when manipulated by hand. They were also very stable upon storage.
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II.3.3.2. Films Morphology

In order to gain further insight into the films' microstructure, SEM analyses were carried out on the surface of the dried materials. Regardless the agar type or the film composition, the imaged surfaces were very homogeneous and smooth. As seen in Fig. II.3.11 depending on the imaged side of the film, *i.e.* dried against the glass or dried exposed to air, the surfaces were respectively, completely or almost smooth. Pure agar films (*e.g.* AA\_IMTA\_TWE\textsuperscript{opt} (Fig. II.3.11 B) showed similar surfaces to the CA used as reference (Fig. II.3.11 A). The blended films were also very homogeneous and identical. Illustrative examples of 25/75 AA\_IMTA\_TWE\textsuperscript{opt}/LBG and 25/75 NA\_IMTA\_TWE\textsuperscript{opt}/LBG materials are shown in micrographs C and D, respectively. When observed at higher magnifications (E and F) it could be detected large fibrous particles which could be interpreted as large aggregates of LBG chains.

![Fig. II.3.10 - Comparison between pure agar films (A) (NA\_IMTA\_TWE\textsuperscript{opt} (left); AA\_IMTA\_TWE\textsuperscript{opt} (right)). It is evident the slightly yellowish coloration of the film prepared with the non-treated agar. Photograph of the AA\_IMTA\_TWE\textsuperscript{opt}/LBG (B) and NA\_IMTA\_TWE\textsuperscript{opt}/LBG (C) films prepared at 25/75 mass ratio.](image)
II.3.3 AQUEOUS-BLENDED FILMS OF AGAR/LOCUST BEAN GUM

Fig. II.3.11 - Representative SEM pictures of the surface of agar/LBG films at 5000x magnification (A- commercial agar prepared at 1% wt concentration; B- pure AA_IMTA_TWEopt; C- 25/75 NA_IMTA_TWEopt/LBG; D- 25/75 AA_IMTA_TWEopt/LBG). Details of the films represented in images C and D are presented in respectively, E and F at higher magnification (10 000x). Arrows represent larger fibrous particles interpreted as being LBG chains. The accelerating voltage was 10 kV and working-distances 17.1 mm in all cases.

II.3.3.3. Mechanical Properties

The first functional properties to be evaluated were the mechanical. Pure agar films showed close TS (~50-65 MPa) and YM (~30-50 MPa) although AA_IMTA_TWEopt could reach higher values (Figs. II.3.12 and II.3.13). Close data were collected in previous studies focusing agar films from wild G. vermiculophylla (Sousa et al., 2010). Considering the weaker junction zones established between NA_IMTA_TWEopt helices upon gelation, lower TS could
be expected for NA_IMTA_TWE\textsuperscript{opt} films. Other components such as unbound water, salts or other impurities present in the native SP extracts could give some stability to the polymeric networks formed upon drying. However, further tests must be performed to confirm these suggestions. The $T_S$ of the pure LBG film was identical to AA_IMTA_TWE\textsuperscript{opt} although the material was clearly stiffer (\textit{i.e.} higher $YM$, $\sim$64-90 MPa; Fig. II.3.13).

The addition of LBG led to a gradual improvement in the mechanical resistance and stiffness of the blended films, with maximum synergistic peaks for each agar system being observed at 50-75\% LBG content in the final mixture (Figs. II.3.12 and II.3.13; $T_S$ $\sim$80-120 MPa and $YM$ $\sim$67-93 MPa). These findings were somehow surprising since no clear synergisms were observed during the rheological studies performed on the film-forming solutions. Clearly, in the solid-state (films) the contribution of each polysaccharide to the system’s behavior was very significant and different from the solution-state (this topic will be further addressed in the next chapter). This could suggest relevant modifications occurred during the drying stage of the films, resulting in improved properties for agar/LBG films when compared to the pure components. Lafargue and co-workers reported a strong influence of $\kappa$-carrageenan in the rheology of modified starch, in sol and gel states, which was concealed when the mixed systems assumed the film-form (Lafargue \textit{et al.}, 2007). Martins \textit{et al.} found the best film properties at an optimal 40/60 $\kappa$-carrageenan/LBG ratio (Martins \textit{et al.}, 2012). Clearly, the use of NA can be a more attractive option for the fabrication of aqueous-blended films than alkali-treated ones, since they have a less expensive extraction process (no need for alkaline step).

Quite interesting was also the fact that the greater sulfate content of NA_IMTA_TWE\textsuperscript{opt} didn’t affect the overall mechanical performance of the films. This seemed to contradict suggestions from other authors pointing to a more pronounced synergistic effect between SP and galactomannans at lower sulfate contents (Morris, 1990). Nonetheless, the synergisms between polysaccharides may be different depending on the form of the material.
Reflecting the intrinsic brittle nature of agar and LBG, the fabricated films exhibited low EB values (in the range ~0.6-2.8%; Fig. II.3.14). The pure LBG film had the lowest EB (1% maximum). From the EB data, it was also evident the greater brittle nature of the pure AA\_IMTA\_TWE\_opt film when compared to its native counterpart (respectively, ~1.6 and ~2.4% in Fig. II.3.14). This could be consequence of higher amounts of unbound water.
leaving the interstitial pores of the AA.IMTA.TWE\textsuperscript{opt} networks upon the film’s drying. The incorporation of plasticizers is a common used strategy to overcome the intrinsic brittle character of agar (Phan et al., 2009a, b; Sousa et al., 2010) and LBG (Aydinli et al., 2004; Martins et al., 2013; Martins et al., 2012) based films.

<table>
<thead>
<tr>
<th></th>
<th>AA/LBG</th>
<th>NA/LBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75/25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/100</td>
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</tbody>
</table>

Fig. II.3.14 - Elongation-at-break (EB) of AA.IMTA.TWE\textsuperscript{opt}/LBG and NA.IMTA.TWE\textsuperscript{opt}/LBG films.

II.3.3.4. Water Sorption Isotherms

Moisture sorption studies were performed at 25 °C and used to evaluate the moisture sensitivity of the pure and mixed films over a broad range of $a_w$. Experimental sorption data of NA.IMTA.TWE\textsuperscript{opt}/LBG and AA.IMTA.TWE\textsuperscript{opt}/LBG films are represented in Figs. II.3.15 and II.3.16, respectively. When exposed to the same $a_w$, the pure polysaccharide systems behave quite differently. Visibly, NA.IMTA.TWE\textsuperscript{opt} retained the highest water amounts while the pure LBG film was the least hydrophilic. For instance, at $a_w \sim 0.8$, the equilibrium moisture content, $X_e$, of NA.IMTA.TWE\textsuperscript{opt} ($\sim 0.34$) was more than double that of the LBG ($\sim 0.15$) while AA.IMTA.TWE\textsuperscript{opt} exhibited an intermediate retention capability between these two films, $\sim 0.22$ (Fig. II.3.17). The higher hydrophilicity of NA.IMTA.TWE\textsuperscript{opt} can be explained by its higher sulfate content, capable of interacting with water molecules (Torres et al., 2012; Xiao et al., 2012). Oppositely, pure LBG with a neutral backbone and AA.IMTA.TWE\textsuperscript{opt} with lower sulfate/LA ratio than NA.IMTA.TWE\textsuperscript{opt}, had less ability to retain water. For the pure agar and blended systems, $X_e$ increased practically linearly up to $a_w \sim 0.6$ after which it raised
sharply reaching its maximum value at the highest $a_w$ (~0.9). Here, the water retention for NA_IMTA_TWE$^{\text{opt}}$/LBG varied between ~0.26-0.65 (Fig. II.3.15) while for AA_IMTA_TWE$^{\text{opt}}$/LBG films the values fell in the range ~0.26-0.36 (Fig. II.3.16). The addition of LBG to agar gradually decreased the water retention capability of the films. In the range of low $a_w$, $X_e$ values were very low. For instance at $a_w$~0.11, the estimated $X_e$ fell between ~0.018-0.052 for NA_IMTA_TWE$^{\text{opt}}$/LBG films while the range values for AA_IMTA_TWE$^{\text{opt}}$/LBG was narrower, ~0.018-0.035.

Fig. II.3.15 - Experimental sorption data (i.e. equilibrium moisture content, $X_e$ vs water activity, $a_w$; symbols) and respective fit (lines) using the GAB model (Eq. II.2.1) for NA_ IMTA_TWE$^{\text{opt}}$/LBG films (100/0 (inverted triangles), 75/25 (stars), 50/50 (circles), 25/75 (triangles) and 0/100 (squares).
Additional information was tentatively extracted from fitting the experimental sorption data to the GAB model (Eq. II.2.1). In all cases sigmoid curves, typical of hydrophilic materials (Xiao et al., 2012), were obtained. As seen in Table II.3.7, the estimated GAB parameters (C, $X_0$ and k) were very different depending on the agar as well as blend composition. Overall, the model seemed to adequately fit the experimental data as suggested by the high correlation coefficients ($R^2 > 0.99$) and range of estimated k values ($0 < k < 1$). The Guggenheim constant, C, is related with the heat of sorption at the monolayer and can be used to quantify the strength of the established bonds between water molecules and the hydrophilic sites at the monolayer of the polymeric material (Xiao et al., 2012). For the pure films, this parameter followed the trend NA_IMTA_TWE$^{opt}$ > AA_IMTA_TWE$^{opt}$ > LBG; NA_IMTA_TWE$^{opt}$ had clearly the highest C (~20) followed by AA_IMTA_TWE$^{opt}$ whose C was half that of the estimated for NA_IMTA_TWE$^{opt}$ while the pure LBG showed the lowest value (~6.4). This suggested that the interaction at the primary sorption sites was strongest for NA_IMTA_TWE$^{opt}$. Again, this could be easily explained by the higher sulfate content for this sample. Torres et al. found significantly higher C values (~24-41 measured at 20-35 °C) for LBG when studying the water adsorption of gum powders at different temperatures (Torres et al., 2012). Interestingly, the greatest impact of LBG addition was seen for NA_IMTA_TWE$^{opt}$ films. At 50% galactomannan content or higher, the role of LBG seemed to dominate the water sorption activity at the primary sorption sites resulting in a sharp decay of C. The
estimated values for 50/50 and 25/75 NA_IMTA_TWE$^{opt}$/LBG, films with best mechanical properties, were lower than that of the pure LBG film (respectively, ~4.1 and ~3.3 against ~6.4).

The amount of water retained at the primary sorption sites of the polymeric films was quantified on dry basis by $X_0$ (Table II.3.7). This parameter gives a good indication of the best working range of $a_w$ for a given material (Moreira et al., 2008) and is related with the chemical structure and composition of each film (Torres et al., 2012). The estimated $X_0$ was higher for NA_IMTA_TWE$^{opt}$ (~0.07) when compared to AA_IMTA_TWE$^{opt}$ (~0.06). The pure LBG film had clearly the lowest $X_0$ (~0.04) and the predicted value was similar to previous studies (Torres et al., 2012). The LBG addition to the films tended to gradually decrease $X_0$ which seemed to suggest less available bonding sites at the monolayer.

The water in the upper layers was less structured than pure water as indicated by the $k$ range values (0<$k$<1 in Table II.3.7; Xiao et al., 2012). The $k$ values were smaller for systems with higher LBG contents and was consistent with the less pronounced upturn in the $X_a$ vs $a_w$ curves in the region of high $a_w$ (Figs. II.3.15 and II.3.16).

Table II.3.7 - GAB parameters (obtained from the fittings of sorption isotherms experimental data to Eq. II.2.1) for the different agar/LBG films.

<table>
<thead>
<tr>
<th>Agar/LBG ratio</th>
<th>NA_IMTA_TWE$^{opt}$</th>
<th>AA_IMTA_TWE$^{opt}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>$X_0$</td>
</tr>
<tr>
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<td>19.07</td>
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</tr>
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</tr>
<tr>
<td>0/100</td>
<td>6.368</td>
<td>0.0380</td>
</tr>
</tbody>
</table>

II.3.3.5. WVP

As seen previously, the permeation of a given molecule through a polymeric matrix is a thermodynamic process that results from three combined mechanisms: absorption of the
molecule at the polymeric surface, diffusion throughout the material and finally, desorption of the molecule from the surface (George & Thomas, 2001).

WVP data for the pure and blended films are presented in Fig. II.3.17. Pure NA_IMTA_TWE\textsuperscript{opt} and LBG films presented quite close WVP ($\sim 4.1 \times 10^{-11}$ g.m\textsuperscript{-1}.s\textsuperscript{-1}.Pa\textsuperscript{-1}) while the pure AA_IMTA_TWE\textsuperscript{opt} film was a less efficient barrier to water ($\sim 5.7 \times 10^{-11}$ g.m\textsuperscript{-1}.s\textsuperscript{-1}.Pa\textsuperscript{-1}). In general, films made with NA_IMTA_TWE\textsuperscript{opt} were less permeable to water which seemed to suggest that the diffusion path length of water throughout these polymer matrices were larger. The presence of salts in the polymeric matrix is also known to decrease WVP (Martins et al., 2012). The addition of LBG seemed to gradually improve the water barrier properties of the films. For instance, blending NA_IMTA_TWE\textsuperscript{opt} and LBG at 25/75 mass ratio (film with best mechanical properties) yielded a film with improved WVP when compared to the individual components ($\sim 3.6 \times 10^{-11}$ g.m\textsuperscript{-1}.s\textsuperscript{-1}.Pa\textsuperscript{-1}). For the same blend composition, the estimated WVP for AA_IMTA_TWE\textsuperscript{opt} was $\sim 4.63 \times 10^{-11}$ g.m\textsuperscript{-1}.s\textsuperscript{-1}.Pa\textsuperscript{-1}. Again from these results, it was feasible to suggest that relevant interactions took place when agar/LBG systems were in the solid-state. In the next chapter, the molecular organization of NA_IMTA_TWE\textsuperscript{opt}/LBG and AA_IMTA_TWE\textsuperscript{opt}/LBG gel networks will be investigated by cryoSEM. Even though the material is in a different form, some parallels can be drawn between both studies.

![Fig. II.3.17 - Water vapor permeability (WVP) of the NA_IMTA_TWE\textsuperscript{opt}/LBG and AA_IMTA_TWE\textsuperscript{opt}/LBG films.](image-url)
II.3.4. Conclusions

The addition of LBG made the fabrication of agar films easier (dramatic increase of viscosity and decrease in the gelling character of the solutions) and led to a significant improvement in the films’ functional properties. Despite the absence of evident synergisms during the rheological tests (sol-state), significant interactions were found between agar and LBG, in the solid-state. Best films were obtained at 50/50 and 25/75 agar/LBG mass ratios. NA\_IMTA\_TWE$^{opt}$/LBG films exhibited similar or better properties than AA\_IMTA\_TWE$^{opt}$ at comparable blend compositions. This can be cost-attractive since the extraction of NA doesn’t need an alkaline step. Moreover, the addition of LBG at 50-75% could further reduce the cost of the films. *Gracilaria vermiculophylla* from IMTA systems could be a continuous supply with reliable quality for the transformation industries.

References.

II.3 AQUEOUS-BLENDED FILMS OF AGAR/LOCUST BEAN GUM


II.4 AGAR/LOCUST BEAN GUM HYDROGELS

CHAPTER II.4
II.4 AGAR/LOCUST BEAN GUM HYDROGELS

II.4.1. Characterization of Agar/Locust Bean Gum hydrogels

II.4.1.1. Appearance and Microstructure

Details concerning the preparation of the gels can be found in section II.2.4.2 of Chapter II.2. At macroscopic level, equilibrated gels made from the pure and mixed systems of each agar (i.e. NA_IMTA_TWE\textsuperscript{opt} and AA_IMTA_TWE\textsuperscript{opt}) were homogeneous and turbid (Fig. II.4.2). The gel turbidity was gradually attenuated by the incorporation of LBG. Pure NA_IMTA_TWE\textsuperscript{opt} gel presented a slight yellowish coloration (a) when compared to the AA_IMTA_TWE\textsuperscript{opt} sample (e), as seen previously for the films application (section II.3.3.1 of Chapter II.3). When manipulated by hand, AA_IMTA_TWE\textsuperscript{opt} based gels were clearly firmer than the NA_IMTA_TWE\textsuperscript{opt} ones. The NA_IMTA_TWE\textsuperscript{opt} mixed gel with highest LBG content (25/75), didn’t keep its shape after being removed from the cilindric container (h).

In order to gain further insight on the structure of the pure and mixed gels, equilibrated samples were also imaged by cryoSEM after being quickly frozen in liquid N\textsubscript{2} (as described

![Image of AA_IMTA_TWE\textsuperscript{opt}/LBG (a-d) and NA_IMTA_TWE\textsuperscript{opt}/LBG gels (e-h). (From left to right: 100/0, 75/25, 50/50 and 25/75).](image-url)
in section II.2.4.3 of Chapter II.2). Representative micrographs are displayed in Figs. II.4.2 (A-E), II.4.3 (A-C) and II.4.4 (A-D). The pure AA.IMTA_TWEopt gel exhibited by far the smallest pores and densest network (Figs. II.4.2 C and II.4.3 B). Similar images were obtained in early studies on agar gels carried out by our group (Sousa et al., 2013a; Sousa et al., 2013b). The pure LBG system formed fairly homogeneous and regular networks with large pores (Figs. II.4.2 E and II.4.3 C). Despite the inherent non-gelling nature of LBG, the used method for preparing the samples for cryoSEM analysis could have induced the formation of a tenuous gel-like structure for the pure galactomannan (Dea et al., 1977).

Although it remains to enlighten if the imaged structures are truly representative of the networks responsible for the recorded rheological data, other authors have based their discussions on the matter correlating rheological and cryoSEM studies (Dunstan et al., 2001). In any case, the quick plunging in N₂ was carried out to minimize as much as possible the induction of artifacts in the samples. At comparable magnifications, the pores of NA.IMTA_TWEopt pure gel (Fig. II.4.2 A) were comparable in size to those of the pure LBG system (Fig. II.4.2 E), yet the network of the SP was denser, more irregular (regions of large pores intercalated with regions of smaller and heterogenous pores) and apparently with not so fragile junction zones. This can be seen with greater detail in Fig. II.4.3 (A and C). As extensively discussed throughout the various chapters, it was evident that agar gelation was governed by the physicochemical properties of the SP; NA.IMTA_TWEopt with higher sulfate/LA ratio, methylated segments and Mₚ (Tables I.4.3 to I.4.6 in Chapter I.4), had highly branched and less dense networks composed of longer fibers than AA.IMTA_TWEopt (Fig. II.4.3 B and C).

The addition of LBG affected the microstructure of agar gels. As an example, micrographs of the NA.IMTA_TWEopt (B) and AA.IMTA_TWEopt (D) mixed gels prepared at 50/50 mass ratio are presented in Fig. II.4.2, next to the pure polysaccharide systems. The remaining mixtures, developing intermediate structures between these samples, are illustrated in Fig. II.4.4 A-D. When increasing the LBG concentration in the final mixture, different polymeric networks were obtained, depending on the agar type. For the alkali-modified SP, the changes were more significant with the networks becoming much more opened due to the LBG contribution, as seen by the substantial increase in pore sizes (Figs. II.4.2 C and D and Figs. II.4.4 C and D). In the case of NA.IMTA_TWEopt pure gel (Fig. II.4.2 D), whose pores were quite similar in size to LBG, the most visible effect was the reinforcement of the formed networks, seen by the thicker walls of the aggregated structures (Fig. II.4.2 B). The networks of the mixed gels for both SP turned gradually less coarser with the increase of LBG content (e.g. Fig. II.4.2 B and D). This could suggest the formation of coupled networks (Stephen, 1995), at least up to a certain degree of compatibility between the polysaccharides. However, this assumption needs further confirmation. Opposite
observations were reported by Dunstan et al. when imaging κ-carrageenan/LBG gels, whose pore sizes decreased with the incorporation of LBG. In this early report, the gels were formed by large tubular pores of κ-carrageenan with interstitial spaces filled with smaller LBG networks (Dunstan et al., 2001). In the present case, evidence of the formation of such interpenetrating networks (IPNs) seemed to be ruled out from the images observation, at least as dominant mechanism and for the considered scale length. In the particular case of AA_IMTA_TWEopt, the compatibility between polysaccharides seemed even more limited and could lead to a small level of separated networks forming the overall gel structure (Fig. II.4.2 D). However, and as previously, this suggestion would need confirmation. Following the gradual increase of LBG content we could assist to the transition from agar/LBG gels, on which the dominant effect was clearly imposed by the galactan (i.e. 25/75 ratio in Figs. II.4.4 B and D), towards systems where, visibly, the galactomannan shaped the network of the gel (i.e. 75/25 ratio in Figs. II.4.4 A and C). This transition was more evident for AA_IMTA_TWEopt, due to the significant differences between the pure polysaccharide systems.

These first studies seemed to find common points with two of the main views proposed to describe the mechanisms of mixed gelation between galactomannans and helix-forming polysaccharides: no interactions due to polymer incompatibility and interactions between the aggregated helices of the galactan and LBG chains (Cairns et al., 1986, 1988; Cairns et al., 1987; Dea et al., 1972; Morris, 1990; Stephen, 1995). However, no definitive conclusions were taken at this point due to the complex nature of these mechanisms. Additional studies under small and large deformations were carried out on the pure and mixed polysaccharide systems hoping to shed more light into the nature of the synergisms, if any, between agar and LBG, when assuming the hydrogel form.
Fig. II.4.2 - Representative cryoSEM pictures obtained for 1% wt NA_IMTA_TWE\textsuperscript{opt}/LBG (100/0 – A; 50/50 – B), AA_IMTA_TWE\textsuperscript{opt}/LBG (100/0 – C; 50/50 - D) and the pure LBG (0/100 – E) systems at 5 000 x. The accelerating voltage was 15 kV and working-distances 15 mm in all cases.
**Fig. II.4.3** - Higher magnification (10,000 x) cryoSEM pictures obtained for 1% wt NA.IMTA.TWE\textsuperscript{opt} (A), AA.IMTA.TWE\textsuperscript{opt} (B) and LBG (C) pure systems. The accelerating voltage was 15 kV and working-distances 15 mm in all cases.

**Fig. II.4.4** - Representative cryoSEM pictures obtained for 1% wt NA.IMTA.TWE\textsuperscript{opt}/LBG (25/75 – A; 75/25 – B) and AA.IMTA.TWE\textsuperscript{opt}/LBG (25/75 – C; 75/25 - D) systems at 2,000 x. The accelerating voltage was 15 kV and working-distances 15 mm in all cases.
II.4.1.2. Rheological Studies

Mixed gels are an exciting form of materials able to present exquisite properties by simple combination of natural polymers with distinct nature. The viscoelastic behavior of such mixed systems when exposed to low deformations (SAOS) can be very important to know. Under large deformations and fracture (LAOS), the viscoelasticity profile of the mixed gels will guess how the material will act when shaped, handled and/or cut under certain conditions. Steady shear viscosity measured at various shear rates also provides relevant information concerning the performance of the materials at relatively high deformations.

The sol-gel transitions of the pure and mixed agar systems were investigated by monitoring the viscoelastic behavior (G' and G'') during a cooling ramp from 80 to 25 °C, at a constant rate of 1°C/min (as described in section II.2.4.1 of Chapter II.2). Results are presented in Figs. II.4.5 (A-B) and II.4.6 (A-B) for, respectively, NA_IMTA_TWEopt and AA_IMTA_TWEopt. Relevant parameters that could be extracted from the rheological tests are also listed in Table II.4.2. For the pure agar systems, the shapes of the curves were identical to the ones recorded in our earlier studies (Sousa et al., 2013a; Sousa et al., 2013b).

At high temperatures, where the solutions viscosity was low, it was difficult to collect valid data particularly for AA_IMTA_TWEopt, the less viscous sample (Fig. II.4.6 (A)). In this regard, the addition of LBG was highly beneficial since it increased the viscosity of agar solutions. For both SP, G' and G'' were also very small (with G''>G') in the beginning of the cooling step and remained fairly constant up to the moment when the sol-gel transition started to take place, ~42 °C, moment at which both moduli raised sharply matching the beginning of the interhelical association of the helix-forming SP. The increase of G' was even faster than G'' leading both moduli to a crossover point which, for the pure agar solutions occurred sooner in the cooling step. Due to the non-gelling nature of LBG, the formation of the gel networks was considered to be dominated by agar. Taken as valid G'=G'' (or tan δ=1) to define the gelation point, it was noticeable that, despite the rather distinct physicochemical nature, pure agar solutions gelled around the same temperature, ~40-41 °C, and earlier when compared with the mixed systems. The LBG addition moved the gelation threshold gradually to lower temperatures as seen previously for κ-carrageenan/LBG gels (Dunstan et al., 2001). Similar effects were also found for other related mixed systems (da Silva et al., 1996; Rocha et al., 2009; Sittikijyothin et al., 2007). Secondly, the incorporation of LBG decreased significantly the elastic component of the systems and also attenuated its elasticity (i.e. higher tan δ; not shown). At comparable compositions, the differences between
both agars became significant with AA.IMTA.TWE\textsuperscript{opt} mixed gels consistently yielding higher \textit{T}_g than NA.IMTA.TWE\textsuperscript{opt} gels. For instance, mixtures with greater LBG contents (25/75 agar/LBG mass ratio) gelled later for NA.IMTA.TWE\textsuperscript{opt} (during the equilibration step at 25 °C in Fig. II.4.7 A) when compared to AA.IMTA.TWE\textsuperscript{opt}, ~28.6 °C (Fig. II.4.6 A). This could be easily explained by the higher aggregation capability, imparted by the higher LA fraction of AA.IMTA.TWE\textsuperscript{opt} when compared to NA.IMTA.TWE\textsuperscript{opt}. Somewhat unexpected however, were the similar \textit{T}_g estimated for the pure agar systems. The reason behind this observation is unclear to us but could be related to differences in other relevant physicochemical properties such as \textit{M}_w and/or methylation levels. The tendency of \textit{T}_g to decrease with increase of LBG content seemed to agree well with the SEM studies. Norziah and co-workers interpreted the gradual decrease in \textit{T}_g of agar systems when \kappa-carrageenan was added as consequence of segregative phase separation in the mixed systems (Norziah et al., 2006). In the present case however, it was hard to make such assumption due to the different nature of the hydrocolloid system (geling/non-gelling polysaccharide). Other experimental data that will be discussed next also seemed to disagree with this view.

At the end of the cooling period, the \textit{G}' of the systems were still increasing consequence of the networks reinforcement, reaching significantly greater magnitudes in the case of AA.IMTA.TWE\textsuperscript{opt} (at least, one-order of magnitude higher than NA.IMTA.TWE\textsuperscript{opt}; Figs. II.4.5 and II.4.6). The pure LBG (not shown) and 25/75 NA.IMTA.TWE\textsuperscript{opt}/LBG systems did not gel during the cooling step (\textit{G}'' > \textit{G}' in Fig. II.4.5 A).

The time evolution of the viscoelastic behaviors was recorded at 25 °C for all samples. In all cases, \textit{G}' and \textit{G}'' increased slowly and continuously, with each system evolving according to its own kinetics. NA.IMTA.TWE\textsuperscript{opt} mixed systems, whose gelation occurred at lower temperatures, underwent more appreciable changes over this time period when compared to AA.IMTA.TWE\textsuperscript{opt}. This was most significant for 25/75 NA.IMTA.TWE\textsuperscript{opt}/LBG whose evolution from solution to gel state took place during this equilibration step (Fig. II.4.7 A). AA.IMTA.TWE\textsuperscript{opt} mixed gels in turn, described mostly changes in \textit{G}' yet tended to equilibrium faster than samples formed by the native SP. These observations favored the existence of different time kinetics for gelation, most likely dependent on the nature of the SP (e.g. sulfate/LA ratio, \textit{M}_w, methylation degree). \textit{G}' was higher than \textit{G}'' in all cases (only exception being the pure LBG system) and the magnitudes were significantly greater for the alkali-modified SP. This matched the formation of stiffer gels for AA.IMTA.TWE\textsuperscript{opt}. Typical equilibration curves of NA.IMTA.TWE\textsuperscript{opt} (A) and AA.IMTA.TWE\textsuperscript{opt} (B) samples are given in Fig. II.4.7. The viscoelastic behavior of both agars was gradually altered by LBG addition as seen during the cooling step (i.e. gradual \textit{G}' decrease with increase in LBG content). At the end of the time sweep step, even though the
systems were still evolving they could be considered at enough equilibrium to record the mechanical spectra with confidence.

Fig. II.4.5 - Temperature dependence of elastic ($G'$; filled symbols) and viscous ($G''$; open symbols) moduli of 1 % wt NA\_IMTA\_TWE$^{10}$/LBG systems during cooling ramp from 80 to 25 °C (graphic A: 25/75 (squares), 100/0 (triangles); graphic B: 50/50 (circles), 75/25 (stars)). The measurements were recorded in the linear viscoelastic region at 6.28 rad/s.
Fig. II.4.6 - Temperature dependence of elastic (G'; filled symbols) and viscous (G''; open symbols) moduli of 1 % wt AA.IMTA.TWE\textsuperscript{opt}/LBG systems during cooling ramp from 80 to 25 °C (graphic A: 25/75 (squares), 100/0 (triangles); graphic B: 50/50 (circles), 75/25 (stars)). The measurements were recorded in the linear viscoelastic region at 6.28 rad/s.
Table II.4.1 - Thermal hysteresis (ΔT) obtained from gelling (T_g) and melting (T_m) temperatures as well as elastic modulus (G_0') and loss tangent (tan δ) recorded during the frequency sweeps at 25 °C (6.28 rad/s) of NA_IMTA_TWE\textsuperscript{opt} and AA_IMTA_TWE\textsuperscript{opt} pure and mixed systems.

<table>
<thead>
<tr>
<th>Agar/LBG ratio</th>
<th>NA_IMTA_TWE\textsuperscript{opt}</th>
<th>AA_IMTA_TWE\textsuperscript{opt}</th>
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<td>T_m (°C)</td>
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</tr>
</tbody>
</table>

\textsuperscript{(1)} sol-gel transition observed during the equilibration step at 25 °C.
Fig. II.4.7 - Time dependence of elastic (G'; filled symbols) and viscous (G''; open symbols) moduli of 1% wt agar/LBG systems at 25 °C (A - NA_IMTA_TWEopt 25/75 (squares) and 50/50 mixed gels (circles); B - AA_IMTA_TWEopt 25/75 (squares) and 50/50 mixed gels (circles)). The measurements were recorded in the linear viscoelastic region at 6.28 rad/s.

The frequency scans performed on the pure and mixed agar gels were recorded at 25 °C. The curves illustrated in Figs. II.4.8 and II.4.9 constitute a section of the viscoelastic plateau with G' higher than G'' over the defined range of ω. It was evident the greater rigidity (higher G') of AA_IMTA_TWEopt gels (~0.4-14 kPa) when compared to NA_IMTA_TWEopt (~9-680 Pa). This was again related with the physicochemical differences between the SP. For the pure and mixed AA_IMTA_TWEopt gels, G' and G'' were practically frequency independent over the entire range of measured ω (Fig. II.4.9) exception made for 25/75 AA_IMTA_TWEopt/LBG, whose G'' curve described a clear minimum towards the low frequency region. A greater contribution of the non-gelling polysaccharide to the viscoelasticity of the system might explain this result. For the same mixture composition, G'
and $G''$ of NA_IMTA_TWE opt mixed gels were more affected by $\omega$ than those of AA_IMTA_TWE opt, increasing monotonously with $\omega$ (Fig. II.4.8). This was also attributed to differences in the physicochemical properties of both SP. The dependence of the viscoelastic behavior on $\omega$ was more significant at higher LBG contents. This was consequence of the increased chain mobility imparted by the greater number of LBG chains (Norziah et al., 2006). While $G''$ was also increasing for 50/50 NA_IMTA_TWE opt/LBG gel, $G'$ could be considered almost frequency independent (Fig. II.4.8).

Fig. II.4.8 - Frequency dependence at 25 °C of elastic ($G'$; filled symbols) and viscous ($G''$; open symbols) moduli of 1 % wt NA_IMTA_TWE opt/LBG systems (symbols: 100/0 (triangles), 50/50 (circles) and 25/75 (squares)). All measurements were recorded in the linear viscoelastic region of the samples.  

Fig. II.4.9 - Frequency dependence at 25 °C of elastic ($G'$; filled symbols) and viscous ($G''$; open symbols) moduli of 1 % wt AA_IMTA_TWE opt/LBG systems (symbols: 100/0 (triangles), 50/50 (circles) and 25/75 (squares)). All measurements were recorded in the linear viscoelastic region of the samples.
Plotting the $\tan \delta$ data over the considered range of $\omega$ gave us information on the elasticity of the pure and mixed polysaccharide systems (Fig. II.4.10 A and B). The LBG effect in the gels elasticity was dependent on the type of agar as well as system composition. For NA\_IMTA\_TWE$^{\text{opt}}$, the elasticity losses imparted by the LBG addition were significant, even when small amounts of galactomannan were added to the gels (75/25 NA\_IMTA\_TWE$^{\text{opt}}$/LBG in Fig.II.4.10 A). Up to ~2 rad/s, the elasticity of the gel with 25% LBG was close to the pure NA\_IMTA\_TWE$^{\text{opt}}$ gel (~0.016); at higher $\omega$ however, the $\tan \delta$ started to increase monotonously for the former (i.e. loss in elasticity) while remaining fairly constant for the latter. At 50% LBG or higher, the elasticity losses became much more significant and gradually approached the $\tan \delta$-$\omega$ curve of the pure LBG. AA\_IMTA\_TWE$^{\text{opt}}$ mixed gels were typically more elastic NA\_IMTA\_TWE$^{\text{opt}}$ ones (lower $\tan \delta$) and exhibited similar elasticities independent of the agar/LBG formulation, for the considered range of $\omega$ (Fig.II.4.10 B). 25/75 AA\_IMTA\_TWE$^{\text{opt}}$/LBG was the only exception; in the beggining of the frequency scans, $\tan \delta$ values stayed close to those of the remaining samples (~0.024) but started to increase ~0.40 rad/s. This was attributed to a greater influence of LBG in the mixed system. Contrarily to what was previously seen for NA\_IMTA\_TWE$^{\text{opt}}$, in this case, the LBG effect was not so significant which could mean a smaller degree of interaction between both polysaccharides.
In summary, the main effects of LBG addition to the viscoelastic behavior of agar gels were the gradual decrease in gel rigidity ($G'$ decrease) and elasticity ($\tan \delta$ increase), this latter being more significant for NA_IMTA_TWE$^{\text{opt}}$ gels. Taking as reference the viscoelastic properties measured at 6.28 rad/s for the several systems (Table II.4.1), it could be seen that $G'_0$ of the mixed gels fell between the limit values of the pure LBG (~6.4 Pa) and pure agar gels (~720 Pa for NA_IMTA_TWE$^{\text{opt}}$ and ~ 16 kPa for AA_IMTA_TWE$^{\text{opt}}$) and progressively decreased with the LBG addition. The values of $\tan \delta$ were significantly higher for NA_IMTA_TWE$^{\text{opt}}$ mixed gels (lower elasticity) when compared to the correspondent AA_IMTA_TWE$^{\text{opt}}$ samples. For instance, at 25/75 mass ratio, the $\tan \delta$ of NA_IMTA_TWE$^{\text{opt}}$
was ~0.695 while ~0.048 for AA_IMTA_TWE\textsuperscript{opt}. Interestingly, this trend was not observed for the pure agar gels (~0.012 and ~0.019 for respectively, NA_IMTA_TWE\textsuperscript{opt} and AA_IMTA_TWE\textsuperscript{opt}). Also, the viscoelastic properties of NA_IMTA_TWE\textsuperscript{opt} mixed gels seemed to be more affected by LBG addition. The curves for the mixed gels were closer to the pure agar systems suggesting the formation of similar networks most likely arising from a primary structure built from agar gelation (Fernandes \textit{et al.}, 1994).

The ‘true gels’ criterium (\textit{i.e.} $G'$ at least one-order of magnitude higher than $G''$ and both moduli showing little frequency dependence) (Kavanagh & Ross-Murphy, 1998) was obeyed by the pure NA_IMTA_TWE\textsuperscript{opt} and all AA_IMTA_TWE\textsuperscript{opt} gels except 25/75 AA_IMTA_TWE\textsuperscript{opt}/LBG. NA_IMTA_TWE\textsuperscript{opt} gels with increasing LBG amounts evolved from a true-gel like structure to systems approaching the macromolecular solution state, when the galactomannan effect became predominant (\textit{i.e.} 25/75 agar/LBG gel).

In order to evaluate possible synergistic interactions between the polysaccharides forming the mixed systems, the additivity effect of $G_0'$ was determined using Eq.II.4.1 (Rinaudo & Moroni, 2009),

$$G'_{0_{mix}} = X_A G'_{0A} + X_B G'_{0B}$$  \hspace{1cm} \text{(II.4.1)}

where $X_A$ and $X_B$ are the weight fractions of polysaccharide A and polysaccharide B, and $G'_{0A}$, $G'_{0B}$ the elastic modulus of each polysaccharide measured during the frequency scans at 25 °C ($\omega$=6.28 rad/s) and $G'_{0_{mix}}$ the elastic modulus of the mixture. As it can be seen form the results listed in Table II.4.2, $G_0'$ calc > $G_0'$ exp for all the considered NA_IMTA_TWE\textsuperscript{opt}/LBG and AA_IMTA_TWE\textsuperscript{opt}/LBG mixed systems, indicating some polymer incompatibility (Rinaudo & Moroni, 2009). However, as pointed out previously, the use of simple additivity to describe the viscoelastic behavior of the mixtures and the significant difference between the viscoelasticities of the pure polysaccharide systems could have compromised the results. While no synergistic interactions were found between κ-carrageenan and LBG through rheological measurements, large deformation tests performed on the mixed gels followed a maximum synergistic curve up to 30-40% LBG content (Dunstan \textit{et al.}, 2001).
Table II.4.2 - Effect of the composition of agar/LBG mixed gels on $G'_0$ at 25 °C determined by application of Eq. II.4.1 to the experimental $G'(\omega)$ data. Dynamic consistency index, $K$, and dynamic power law factor, $n$, obtained by fitting the $|\eta^*_0(\omega)|$ data to Eq. I.2.5.

<table>
<thead>
<tr>
<th>AGAR/LBG</th>
<th>$G'_0$ exp (Pa)</th>
<th>$G'_0$ calc (Pa)</th>
<th>$K$ (Pa.s)$^{(1)}$</th>
<th>$n^{(1)}$</th>
<th>$G'_0$ exp (Pa)</th>
<th>$G'_0$ calc (Pa)</th>
<th>$K$ (Pa.s)$^{(1)}$</th>
<th>$n^{(1)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>681.4</td>
<td>-</td>
<td>670.8</td>
<td>-0.9917</td>
<td>13780</td>
<td>-</td>
<td>15206</td>
<td>-1.0128</td>
</tr>
<tr>
<td>75/25</td>
<td>276.8</td>
<td>512.7</td>
<td>271.3</td>
<td>-0.9848</td>
<td>9169</td>
<td>11945</td>
<td>10072</td>
<td>-1.0137</td>
</tr>
<tr>
<td>50/50</td>
<td>87.25</td>
<td>343.9</td>
<td>83.0</td>
<td>-0.9332</td>
<td>3414</td>
<td>6893.2</td>
<td>3689</td>
<td>-1.0121</td>
</tr>
<tr>
<td>25/75</td>
<td>20.41</td>
<td>175.2</td>
<td>14.3</td>
<td>-0.663</td>
<td>369.4</td>
<td>3449.8</td>
<td>357.6</td>
<td>-0.9714</td>
</tr>
<tr>
<td>0/100</td>
<td>6.439</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.439</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^{(1)}$ $R^2>0.99$ for all fittings.
Fig. II.4.11 - Frequency dependence at 25 °C of complex viscosity, $|n^*|$ (open squares) of the 1 wt% NA.IMTA.TWE/LBG (A) and AA.IMTA.TWE/LBG (B) mixed gels prepared with 25/75 ratio. The data are presented in bi-logarithmic scales with the respective linear fitting to extract the Power Law parameters (main graphics). Linearity check over the frequency domain for the same gels: viscous modulus ($G''$; open squares) with $G''(\omega)$ recalculated from $G'(\omega)$ data (red lines) using the Tschoegl approximation (Eq.I.2.4). (insets). All measurements were recorded in the linear viscoelastic region of the samples.

The linearity of the viscoelastic behavior is a common concern when performing SAOS of hydrocolloid gels since the structural integrity of the system must be maintained throughout the successive measurements, otherwise the physical meaning of the acquired data will be lost leading to inaccurate interpretations. The system’s response to the induced sinusoidal perturbations should also be sinusoidal and $G'$ and $G''$ should be independent of the strain amplitude, preferentially measured under different $\omega$. Both criteria were respected in the present study. To further check if the frequency scans were actually performed in the region of viscoelastic linearity the first order approximation to Kraemers-Kronig relation (Eq.I.2.4)
was applied to the experimental $G''(\omega)$ of each system. Albeit some punctual dispersion in the numerical derivation data, in all cases the recalculated $G''(\omega)$ were in good agreement with the measured ones thus confirming that the recorded data had physical meaning (Sittikijyothin et al., 2007). Illustrative examples of NA_IMTA_TWE$^{\text{opt}}$ (A) and AA_IMTA_TWE$^{\text{opt}}$ (B) mixed gels prepared at 25/75 mass ratio are given in the inset graphics of Fig. II.4.11. As seen during Part I of the thesis, data of dynamic viscoelastic behavior are often fitted to power law models to extract useful information concerning the rheological profile of the systems. For instance, by plotting the mechanical spectra in terms of $|\eta^*(\omega)|$ data in bi-logarithmic scales (Eq.I.2.5; e.g. 25/75 agar/LBG mixed gels in main graphics of Fig. II.4.11) one could extract the dynamic consistency index, $K$, and the power law factor, $n$ (Table II.4.2). The $K$ values indicated a much less cohesive gel for NA_IMTA_TWE$^{\text{opt}}$ (0.67 kPa.s) when compared to AA_IMTA_TWE$^{\text{opt}}$ (~15.2 kPa.s). The mixed gels followed the same trend and gradually decreased in consistency with the increase of LBG content. Both parameters confirmed the ‘true gel’ behavior of AA_IMTA_TWE$^{\text{opt}}$ systems (Kavanagh & Ross-Murphy, 1998); up to 50% LBG content, $n$ assumed identical values pointing to complete elasticity of the gels (~1.01) while the 25/75 mixed gel fell a little below (~0.97). This matched accordingly with the greater influence of the galactomannan in the gel microstructure. NA_IMTA_TWE$^{\text{opt}}$ in turn, showed $n$ values closer to complete elasticity only for the pure and 75/25 NA_IMTA_TWE$^{\text{opt}}$/LBG gels (~0.99 and -0.98, respectively).

Pure and mixed gels of NA and AA were heated from 25 to 85 or 90 °C at a constant heating rate of 1 °C/min and the correspondent $G'$ and $G''$ variations are plotted in Figs. II.4.12 and II.4.13 for respectively, NA_IMTA_TWE$^{\text{opt}}$ and AA_IMTA_TWE$^{\text{opt}}$. In the beginning of the heating step, the viscoelastic behavior of the gels remained fairly constant with $G'>G''$ for all samples (Figs. II.4.12 and II.4.13). Obviously, the pure LBG continued to exhibit typical viscoelastic behavior of macromolecular solutions, i.e. $G''>G'$ (not shown). Following the trend of previous steps, the magnitudes of both moduli were substantially greater for AA_IMTA_TWE$^{\text{opt}}$ systems. All curves, including the mixtures, described a one-step equilibrium process with the temperature increase contrarily to what was found by Norziah and co-workers when heating κ-carrageenan/agar mixed gels (Norziah et al., 2006). This could be easily explained by the gelling nature of both polymers used in the cited study. The gel-sol transition, marked by the sharp decay in both moduli, started around the same temperature for both agars (~ 60 °C; Fig. II.4.12 A and II.4.13 A for respectively, NA_IMTA_TWE$^{\text{opt}}$ and AA_IMTA_TWE$^{\text{opt}}$). At low LBG contents (25% or less), the temperature window matching the interhelical disruption towards the random coil state was broader and initiated later during the heating step. Systems richer in agar, which is the gelling component of the mixture, should need more time to disrupt the helical aggregates.
hence, presenting higher melting points (Table II.4.1). The higher $T_m$ \text{(NA\_IMTA\_TWE$^{opt}$; in the range 82.5-86 °C; AA\_IMTA\_TWE$^{opt}$: 80.3-81.9 °C)} and $\Delta T$ (NA\_IMTA\_TWE$^{opt}$; in the range 44.6-51.1 °C; AA\_IMTA\_TWE$^{opt}$: 39.9-43.2 °C) estimations for respectively, the pure agar and 75/25 agar/LBG gels favored this interpretation. Inversely, and as seen earlier during the cooling process, the incorporation of LBG attenuated the decay of both moduli while narrowing the window of sol-gel transition. Quite interesting was the fact that this didn’t match a gradual decrease in $\Delta T$ of the NA\_IMTA\_TWE$^{opt}$ mixed gels, contrarily to what was seen for AA\_IMTA\_TWE$^{opt}$ (Table II.4.1). Apparently, the physicochemical differences between the used SP led to different equilibrium states upon heating the systems.

When minimum LBG amount was added (75/25 agar/LBG gel), the melting points were quite different for each agar with $T_m$ of AA\_IMTA\_TWE$^{opt}$ being almost 10 °C higher than the correspondent NA\_IMTA\_TWE$^{opt}$ gel. However, when higher amounts of LBG were considered, both agars yielded gels with close $T_m$ values. For instance, 25/75 NA\_IMTA\_TWE$^{opt}$/LBG had a $T_m$ ~83 °C while ~82 °C was the value estimated for the correspondent AA\_IMTA\_TWE$^{opt}$ gel. Considering that the conformational change \text{(i.e. helix-to-coil transition)} will occur as much later as greater the stability of the formed networks (Labropoulos et al., 2001) and the known weaker nature of NA\_IMTA\_TWE$^{opt}$ and LBG networks, the remarkable increase in $T_m$ seen when increasing the LBG content of NA\_IMTA\_TWE$^{opt}$ gels from 25 to 50%, could reflect some positive interactions between both polysaccharides. These findings seem to disagree with previous views pointing to greater synergisms in agarose and galactomannan and less when the SP is highly substituted (Morris, 1990; Tako & Nakamura, 1988). Also relevant were the significantly higher values of $\Delta T$ estimated for NA\_IMTA\_TWE$^{opt}$ mixed gels when compared to AA\_IMTA\_TWE$^{opt}$. For instance, 75/25 NA\_IMTA\_TWE$^{opt}$/LBG had a $\Delta T$ ~51 °C, around 8 °C higher than the correspondent AA\_IMTA\_TWE$^{opt}$/LBG gel. In all cases, the $T_m$ estimations were quite reproducible giving no suggestion of phase separation as previously seen by Norziah \textit{et al.} for agar/$\kappa$-carrageenan gels (Norziah \textit{et al.}, 2006).
Fig. II.4.12 - Temperature dependence of elastic ($G'$; filled symbols) and viscous ($G''$; open symbols) moduli of 1 % wt NA_IMTA_TWE opt/LBG systems during cooling ramp from 25 to 85 °C (graphic A: 100/0 (triangles) and 25/75 (squares); graphic B: 100/0 (triangles), 75/25 (stars) and 50/50 (circles)). The measurements were recorded at 6.28 rad/s.
Fig. II.4.13 - Temperature dependence of elastic ($G'$; filled symbols) and viscous ($G''$; open symbols) moduli of 1 % wt AA_IMTA_TWE$_{\text{opt}}$/LBG systems during cooling ramp from 25 to 85 °C (graphic A: 100/0 (triangles) and 25/75 (squares); graphic B: 100/0 (triangles), 75/25 (stars) and 50/50 (circles)). The measurements were recorded at 6.28 rad/s.

Overall, the first set of rheological data acquired under SAOS seemed to suggest different degrees of interactions between each agar and LBG. However, further studies must be performed to confirm this suggestion. In summary, as main findings we could point out: i) decrease in gelation point of both agars with LBG addition ii) maximum ΔT found for NA_IMTA_TWE$_{\text{opt}}$/LBG mixed gels (highest value at 50/50 composition) iii) greater rigidity of AA_IMTA_TWE$_{\text{opt}}$/LBG gels (higher $G'$) when compared to NA_IMTA_TWE$_{\text{opt}}$/LBG iv) significant elasticity losses for NA_IMTA_TWE$_{\text{opt}}$/LBG gels with LBG addition contrasting with the neglectable effect observed for AA_IMTA_TWE$_{\text{opt}}$. 
The non-linear properties of the pure and mixed agar gels were studied through rheology and penetration tests performed under large deformations (LAOS). The dependence of the viscoelastic properties on strain ($\gamma$) was followed during rheological tests at 25 °C and 6.28 rad/s. Initially, $G'$ and $G''$ remained fairly constant for all samples and $G'>G''$ up to a $\gamma$ value after which different viscoelastic behaviors started to develop (Fig. II.4.14). This critical strain, $\gamma_c$, defined as the deformation point at which $G'$ varies more than 5% relatively to its initial constant value and after which the viscoelastic linearity is no longer obeyed, was extracted from the strain sweep curves of each sample and is listed in Table II.4.3. As seen during the SAOS experiments, both moduli were greater in magnitudes for AA_IMTA_TWEopt mixed gels.

<table>
<thead>
<tr>
<th>AGAR/LBG</th>
<th>$\gamma_c$ (%)</th>
<th>$G'_\text{max}$ (Pa)</th>
<th>$\gamma_c$ (%)</th>
<th>$G'_\text{max}$ (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>12.2</td>
<td>1797</td>
<td>4.096</td>
<td>15490</td>
</tr>
<tr>
<td>75/25</td>
<td>19.49</td>
<td>714</td>
<td>20.38</td>
<td>9376</td>
</tr>
<tr>
<td>50/50</td>
<td>24.60</td>
<td>449</td>
<td>19.44</td>
<td>3757</td>
</tr>
<tr>
<td>25/75</td>
<td>61.79</td>
<td>85</td>
<td>19.53</td>
<td>1051</td>
</tr>
<tr>
<td>0/100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The $\gamma_c$ was lower for the pure agar gels and increased gradually with the increase of LBG content for NA_IMTA_TWEopt mixed gels. Comparing both agars, $\gamma_c$ was significantly higher for NA_IMTA_TWEopt (~12%) than for AA_IMTA_TWEopt gel (~4%). Depending on the used agar and system composition distinct viscoelastic behaviors were observed. In the case of NA_IMTA_TWEopt, after $\gamma_c$ the $G'$ and $G''$ curves described strain-hardening behavior (i.e. both moduli increased) followed by strain thinning (i.e. both moduli decreased). Visibly, the incorporation of LBG in agar gels added stability to the structure with the irreversible rupture occurring at higher strains (e.g. ~200% for 25/75 NA_IMTA_TWEopt/LBG gel; Fig. II.4.14 A). This ‘strong strain overshoot’ under LAOS (i.e. moduli increase followed by decrease) could be classified according to Hyun et al. as type IV (Hyun et al., 2002). This behavior can be interpreted as follows: at low strains, the formation of new entanglements is able to compensate the disruption of the network junctions and strain hardening is the dominant
behavior. When the disruption rate of the entanglements becomes more significant (higher strains) the new molecular junctions no longer compensates the disruption rate and shear thinning is observed.

In the case of AA_IMTA_TWE$^{opt}$, a distinct viscoelastic behavior was observed after the pure and mixed gels reached the $\gamma_c$ (Fig. II.4.14 B). While $G'$ exhibited shear thinning, $G''$ curve showed strain hardening behavior followed by shear thinning. According to Hyun et al. this 'weak strain overshoot' could be classified as type III (Hyun et al., 2002).

At 25 °C, pure LBG described typical behavior of polymer solutions and melts, i.e. 'strain thinning', which according to Hyun's classification falls into type I category (Hyun et al., 2002). The principles underlying strain thinning are similar to the shear thinning described earlier; chain orientation with ability to form new entanglements simultaneously with disrupted ones in the range of low strains (both moduli constant) while significant entanglements disruption with disentangled polymer chains aligning with the flow reflect the decrease in $G'$ and $G''$ at high strains (Hyun et al., 2002).

Even though the information extracted from LAOS is often disregarded by researchers, in the present study it was extremely relevant. It showed different degrees of interactions between each agar and LBG which were attributed to differences in the physicochemical properties of the SP (e.g. sulfate groups and other substituents, salts, $M_w$). Furthermore, the viscoelastic profiles of NA_IMTA_TWE$^{opt}$/LBG gels (type IV) when submitted to similar strain sweeps could indicate stronger interactions than in the case of AA_IMTA_TWE$^{opt}$ (type III), according to Hyun's classification (Hyun et al., 2002). Yet, additional studies should be conducted to shed more light on this topic. Contrasting with the greater similarity seen during the rheological studies performed in the linear viscoelastic region, data measured under large deformations put in evidence greater differences between the mixed gels formed by each agar.
II.4.1.3. Penetration Tests

Agreeing with the overall trend observed during the rheological studies (i.e. sharp decay in $G'$ upon LBG addition), the GS (and stress-at-break) of NA and AA gels, estimated from penetration tests, seemed to decrease with the addition of LBG. However, the decay in gelling ability was only significant ($p<0.01$) for gels with 50% LBG content or higher (Tables II.4.4 and II.4.5).
For NA gels prepared with higher LBG amounts (50/50 and 25/75 NA_IMTA_TWE\textsuperscript{opt}/LBG in Table II.4.4), the measurements were carried out too close to the limit of the equipment which seemed to suggest the need for a more sensitive load cell. Maximum GS values estimated for NA_IMTA_TWE\textsuperscript{opt}/LBG gels fell in the range ~46-78 g/cm\textsuperscript{2}, matching failure stresses of ~ 3.2-8 kPa and correspondent failure strains of ~0.082-0.104.

AA_IMTA_TWE\textsuperscript{opt}/LBG gel prepared with minimum LBG content yielded the best mechanical properties next to the pure AA_IMTA_TWE\textsuperscript{opt} gel (GS~339-457 g/cm\textsuperscript{2}, failure stress ~33-45 kPa and failure strain ~0.095-0.104; p>0.01 in Table II.4.5). At 50% LBG content or higher, the addition of LBG gradually decreased the mechanical properties evaluated for the gels.

AA_IMTA_TWE\textsuperscript{opt}/LBG gels were firmer (higher GS) than NA_IMTA_TWE\textsuperscript{opt}/LBG gels and became more deformable upon LBG addition (Table II.4.5). This seemed to agree well with the cryoSEM studies performed on the equilibrated gels (Figs. II.4.2 to II.4.4). We could assume that the increase in pore sizes imparted by the addition of LBG to AA_IMTA_TWE\textsuperscript{opt} gel (Fig. II.4.4 C) improved the elasticity of the junction zones between both polysaccharides, reaching its maximum for 25/75 AA_IMTA_TWE\textsuperscript{opt}/LBG (failure strain ~0.081; Table II.4.5). In turn, the less coarse AA_IMTA_TWE\textsuperscript{opt} networks observed upon LBG increase (Figs. II.4.2 D and II.4.4 C-D) could justify the decrease in GS of the mixed gels, particularly at higher LBG contents. Considering the images obtained during the cryoSEM studies (Fig. II.4.4 C), we could assume that the increase in pore sizes imparted by the addition of LBG could have induced an improvement in the elasticity of the junction zones between AA_IMTA_TWE\textsuperscript{opt} and LBG, reaching its maximum at 25/75 mass ratio (~0.081; Table II.4.5). In what GS is concerned, the less coarse networks observed upon LBG addition to AA_IMTA_TWE\textsuperscript{opt} gels could justify the decrease in gelling ability of the systems, particularly for higher LBG contents. Dunstan and co-workers (Dunstan et al., 2001) observed significant synergistic interactions when κ-carrageenan/LBG gels were exposed to large deformations. In the present study, the addition of LBG to AA_IMTA_TWE\textsuperscript{opt} gels led to a significant increase in deformability and didn’t affect significantly the mechanical performance of the gels, at minimum LBG content.

The interactions between agar and LBG were different depending on the material. While films were significantly improved by the addition of LBG (Chapter II.3), this was not the case for gels. In solution (\textit{i.e.} above the gelation point), when galactomannan is added to agar, the molecules of the former can move more freely within the system since the galactan molecules are in the random coil state (even though starting to approach the window of sol-gel transition). This could mean easier entanglements that, when given enough time to

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\textbf{II.4 AGAR/LOCUST BEAN GUM HYDROGELS

\texttt{opt}/LBG in Table II.4.4), the measurements were carried out too close to the limit of the equipment which seemed to suggest the need for a more sensitive load cell. Maximum GS values estimated for NA_IMTA_TWE\textsuperscript{opt}/LBG gels fell in the range ~46-78 g/cm\textsuperscript{2}, matching failure stresses of ~ 3.2-8 kPa and correspondent failure strains of ~0.082-0.104.

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mature, *i.e.* drying of the films performed at conditions above the gelation point (40 °C), could outcome different interactions between both polysaccharides. In the gel state (25 °C), as soon as the system starts to approach the window of sol-gel transition, the movement of LBG molecules becomes more reduced due to the strong interhelical association starting to occur between agar molecules. Moving further in gelation, will further difficult the intermolecular binding between LBG and agar chains with the formed junction zones being weaker than in the solid state. The gradual decrease in gel rigidity under large deformations with increase in LBG content for 50% LBG or higher, could be interpreted as consequence of the large number of agar chains that do not bind directly due to the presence of significant amounts of the non-gelling polysaccharide blocking the interhelical association (da Silva et al., 1996). This will lead to the formation of weaker networks that will fracture when exposed to large deformations primarily through those weaker junction zones.

<table>
<thead>
<tr>
<th>NA_IMTA_TWE$^{opt}$/LBG</th>
<th>Failure stress (kPa)</th>
<th>Failure strain ×10$^{-3}$</th>
<th>GS g/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>6.55±1.33$^a$</td>
<td>90±14$^a$</td>
<td>69.7±8.4$^a$</td>
</tr>
<tr>
<td>75/25</td>
<td>3.98±0.73$^a$</td>
<td>84±2$^a$</td>
<td>54.9±8.8$^a$</td>
</tr>
<tr>
<td>50/50</td>
<td>1.73±0.09$^{1,2}$</td>
<td>97±30$^{1,2}$</td>
<td>23.2±2.5</td>
</tr>
<tr>
<td>25/75</td>
<td>2.48±2.63$^{2}$</td>
<td>91±6$^{2}$</td>
<td>28.1±11.3</td>
</tr>
</tbody>
</table>

1 Sample with typically two peaks in the stress-strain curves; average reports data from the first peak;  
2 Difficulty on measuring reproducible data probably because we were working too close the limit of analysis of the texture analyzer. For this reason, these data were not statistically considered;  
Means in the same column with different letters are significantly different (p<0.01).

<table>
<thead>
<tr>
<th>AA_IMTA_TWE$^{opt}$/LBG</th>
<th>Failure stress (kPa)</th>
<th>Failure strain ×10$^{-3}$</th>
<th>GS g/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>43.3±1.5$^a$</td>
<td>99±4$^a$</td>
<td>441±16$^a$</td>
</tr>
<tr>
<td>75/25</td>
<td>37.6±4.3$^a$</td>
<td>98±6$^a$</td>
<td>383±44$^a$</td>
</tr>
<tr>
<td>50/50</td>
<td>23.3±3.2$^b$</td>
<td>117±10$^b$</td>
<td>238±33$^b$</td>
</tr>
<tr>
<td>25/75</td>
<td>11.0±0.8$^c$</td>
<td>181±11$^c$</td>
<td>113±8$^c$</td>
</tr>
</tbody>
</table>

Means in the same column with different letters are significantly different (p<0.01).
II.4.2. Conclusions

The physicochemical properties of agars (e.g. $M_w$ as well as methyl, LA and sulfate contents) are relevant in the definition of its binding mechanisms with LBG. SAOS and LAOS studies gave different and insightful information on this topic. In the particular case of LAOS, and according to Hyun’s classification (Hyun et al., 2002), the strain sweep profiles seemed to indicate stronger interactions between NA and LBG. Yet, this suggestion must be supported with more studies. In any case, it was clear that NA and AA interacted differently when mixed with LBG to form gels.

The LBG addition to agar, led to mixed gels with various rheological profiles which can be very attractive for the manufacture of new products with interesting textures and mouth feel to the consumers.

References.


II.5 NON-AQUEOUS AGAR FILMS

CHAPTER II.5
All experiments concerning this study were carried out at the ERRC-USDA, in Wyndmoor, Pennsylvania.

II.5.1. Preparation of Agar/DES Films

In the present study, the protocol to fabricate the CA/DES films was adopted from preliminary tests and consisted of a three-step process. Taking into consideration the specifications of the heat press and to prevent polysaccharide degradation, solubilization of the agar in either DES-G or DES-U was carried out in closed cap vials before the thermo-compression process. The samples were slowly cooled down to room temperature and left to set overnight. After this intermediate step, films free of air bubbles and with regular thickness were obtained (Fig. II.5.1). Finally, the DES removal was accomplished by immersing the films in ethanol and subsequently drying in air.

II.5.2. Films Characterization

II.5.2.1. Films Appearance

There were significant differences in the two eutectic films (agar/DES-G and agar/DES-U). In general, DES-G exhibited poor film forming ability. Only the A5/DES-G film showed some consistency after drying (Fig. II.5.1 A) while the remaining concentrations resulted in fragmented films. This suggests there is a critical polymer concentration for the formation of a cohesive agar/DES-G matrix. However upon storage, A5/DES-G showed marked sensitivity to environmental conditions developing unpleasant smells, changing color and shrinking. This could be attributed to some thermal degradation of the polymer during film formation. It should be emphasized that several protocols and conditions were tested for the films’ preparation yet, none of them proved to be more successful.
In contrast, CA/DES-U films were very consistent, uniform, easy to handle and stable upon storage at any polymer concentration (Fig. II.5.1 B). Therefore, only CA/DES-U films are hereafter due to the consistent nature of their film forming ability.

### II.5.2.2. Films Morphology

Cross sections of the cryo-fractured CA/DES-U films observed by SEM are represented in Fig. II.5.2. Some irregularities could be detected on the surface of the cross-sections, however due to the cryo-fracture method were disregarded. More compact and homogeneous microstructures were observed for the films with the lowest (CA2/DES-U; Fig. II.5.2 A) and highest (CA6/DES-U; Fig. II.5.2 E) agar concentrations. This agrees with previous SEM studies from Phan and co-workers on agar aqueous films plasticized with 15% wt glycerol, no pores or voids could be readily seen for these samples, which could be related with the formation of more compact and dense 3D networks upon gel formation (Phan et al., 2005). It is broadly known that in aqueous media, agar gelation leads to 3D networks through double helix formation followed by intensive interhelical association (Sousa et al., 2013a; Sousa et al., 2013b). A perfect arrangement of the helices is supported by strong H-bonds between the polymeric chains and additional H-bonding is expected to occur between water molecules and the agar chains. Agar in DES-U could follow this same mechanism as seen by the cohesiveness of the matrices, particularly for the films containing the lowest and highest concentrations of agar.
Films prepared with agar concentrations in the range 3-5% w/w revealed less cohesive matrices (Figs. II.5.2 B-D) compared to the 2 and 6% w/w samples (respectively, Fig. II.5.2 A and Fig. II.5.2 E). Differences between the fractured surfaces can be seen, especially in Figs. II.5.2 B and D, although cryo-fractured, the surfaces indicate more of a ductile fracture indicated by the high ridges in the fracture surface. The small ridges in Figs. II.5.2 A and II.5.2 E indicate more of a brittle structure with a sheet like pattern. Although it cannot be neglected that the high vacuum conditions and the electron beam used for the SEM experiments could account for the formation of some of these defects (particularly at higher magnifications; (Phan et al., 2009a)), the other measured properties correlated with the microscopic data. The presence of defects (e.g. pores and/or voids caused by air and
cracks) in the polymeric matrix is known to significantly affect the films’ final properties (Duncan et al., 2005). This will be discussed in detail in the following sections.

II.5.2.3. Thickness and Mechanical Properties

The mechanical properties of the CA/DES-U films prepared with different polymer concentrations are presented in Table II.5.1.

<table>
<thead>
<tr>
<th>FILM ID.</th>
<th>d / mm</th>
<th>TS / MPa</th>
<th>EB / %</th>
<th>YM / MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA2/DES-U</td>
<td>0.05±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.3±7.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.3±9.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>656±153&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CA3/DES-U</td>
<td>0.09±0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.83±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.1±5.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.4±3.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CA4/DES-U</td>
<td>0.11±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.49±1.24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.2±1.2&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>179±31.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CA5/DES-U</td>
<td>0.10±0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.26±0.67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>59.2±9.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>55.2±12.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CA6/DES-U</td>
<td>0.12±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.9±4.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.1±9.8&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>754±107&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means in the same column with different letters are significantly different (p<0.01).

In general, an increase in the polymer concentration led to an increase in the thickness (Cerqueira et al., 2013) particularly, for the CA2/DES-U and CA3/DES-U films (respectively, ~0.05 and 0.09 mm). At higher polymer contents (>4% wt) however, the differences in thickness could be considered neglectable (~ 0.11 mm) as it can be confirmed from the SEM images (Figs. II.5.2 A-E).

The highest values of TS and YM were observed for CA6/DES-U, respectively 24.2-33.6 and 647-861 MPa. This is consistent with higher polymer concentrations yielding stronger and more compact networks (Cerqueira et al., 2013). However, we also found that the film with lowest concentration of agar had good mechanical properties (TS of 26.6-42 MPa and YM of 503-809 MPa). These concentrations showed the least sheet like microstructure in the SEM (respectively, Figs. II.5.2 A and E). Between these two concentrations, the films exhibited poor mechanical properties with the minimum values of TS (~3.83 MPa) and YM (~13.4 MPa) being observed at 3% w/w agar concentration. This is
significant and may come from the interplay between agar and DES-U upon partial hydration of the films (Nardecchia et al., 2012). In fact, the films' exposure to different environmental conditions during and after the drying stage (particularly different R.H.) could lead to different molecular reorganizations, consequently affecting the measured properties. Hence, this aspect should be regarded and taken into consideration for future work.

As shown in Table II.5.1, agar/DES-U films with good elasticity were obtained regardless of the polymer concentration. Overall, an inverse correlation between TS and E data was observed. Accordingly, the formulations with the best TS (i.e. CA2/DES-U and CA6/DES-U) revealed the lowest EB (in the range 15.4-38.9%) while CA3/DES-U was the most elastic film (~74%). CA4/DES-U, however, did not follow this trend and showed comparable EB to the films prepared at lowest and highest concentrations of agar, despite having significantly lower TS (Table II.5.1).

Considering typical mechanical data reported by previous investigations for aqueous agar films, the obtained results seem quite promising. For instance, Phan et al. (2005) found comparable TS values (~42 MPa) for aqueous films at 3% wt agar concentration loaded with 15% wt glycerol yet, the EB were significantly lower (~ 6.51%). More elastic films (~62%) were attained by Giménez et al. when adding 66% wt glycerol to 1.5% wt agar aqueous solutions however the TS was clearly below (~18.5 MPa) the values obtained in the present study (i.e. 24.2-42 MPa) (Gimenez et al., 2013). In the work carried out by Shamsuri and Daik, the addition of DES-U to aqueous agarose systems (2.75 % wt) led to an increase in the films’ elasticity (from ~3 to 5.5%) and concomitant decrease in TS (from 66.7 to 6.1 MPa) up to 60% DES-U loading (Shamsuri & Daik, 2012). As previously mentioned, the reported EB seem to indicate an insufficient plasticization of the films probably caused by significant hydration promoting the rupture of the molecular complexes composing the eutectics, upon DES dilution in water (Nardecchia et al., 2012). The properties of the tested films were also comparable to films of other biopolymers such as pectin or pectin/protein composites (Liu et al., 2007). Films made from lignocellulosic materials and conventional imidazole-based ILs showed significantly lower YM (~26-42 MPa) and EB (~1-4%) (Abdulkhani et al., 2013). In the particular case of poplar wood-based films, TS (18 MPa) was clearly below the highest values reported here. By comparison with commercial materials such as PVC (35 MPa; (Callister, 2000)), PS (55 MPa; (Callister, 2000)) or LDPE (9-17 MPa; (Baker, 1986)) the fabricated films appear to possess good mechanical resistance while attaining a high elasticity comparable to cellophane (16-60%; (Phan et al., 2005)).
II.5.2.4. Water Sorption Isotherms

Experimental data was fitted to the GAB equation (II.2.1). The moisture sorption data as well as the respective fittings are represented in Fig. II.5.3. For clarity purposes only results for CA2/DES-U, CA3/DES-U and CA6/DES-U are plotted in Fig. II.5.3 and all of the samples estimated parameters according to the proposed model are listed in Table II.5.2.

![Experimental sorption data (i.e. equilibrium moisture content, $X_e$ vs water activity, $a_w$) and respective fit using the GAB model (Eq. II.2.1) for the CA2/DES-U (blue squares and line), CA3/DES-U (green triangles and line) and CA6/DES-U (red dots and line) films.](image-url)
Table II.5.2 - GAB parameters (obtained from the fittings of sorption isotherms experimental data to Eq. II.2.1) and wettability (obtained from the average contact angle ($\Theta$) formed by sessile water drops) for the different CA/DES-U films.

<table>
<thead>
<tr>
<th>FILM ID.</th>
<th>C</th>
<th>$X_0$</th>
<th>k</th>
<th>$R^2$</th>
<th>$\Theta/\degree$</th>
<th>$\Delta\Theta/\degree$</th>
<th>$\Delta V/\mu L$</th>
<th>$\Delta A/mm^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA2/DES-U</td>
<td>5.248</td>
<td>0.12</td>
<td>0.96</td>
<td>0.992</td>
<td>60±4</td>
<td>-3.66±1.95</td>
<td>-0.25±0.04</td>
<td>0.110±0.066</td>
</tr>
<tr>
<td>CA3/DES-U</td>
<td>1.415</td>
<td>0.15</td>
<td>0.91</td>
<td>0.993</td>
<td>56±2</td>
<td>-0.86±0.35</td>
<td>-0.090±0.044</td>
<td>0.073±0.051</td>
</tr>
<tr>
<td>CA4/DES-U</td>
<td>0.7283</td>
<td>0.11</td>
<td>0.96</td>
<td>0.992</td>
<td>65±2</td>
<td>-1.45±0.64</td>
<td>-0.030±0.017</td>
<td>0.083±0.012</td>
</tr>
<tr>
<td>CA5/DES-U</td>
<td>1.421</td>
<td>0.11</td>
<td>0.94</td>
<td>0.993</td>
<td>63±2</td>
<td>-2.87±0.66</td>
<td>-0.140±0.008</td>
<td>0.270±0.204</td>
</tr>
<tr>
<td>CA6/DES-U</td>
<td>1.956</td>
<td>0.10</td>
<td>0.97</td>
<td>0.994</td>
<td>75±3</td>
<td>-0.90±0.94</td>
<td>-0.072±0.028</td>
<td>0.048±0.288</td>
</tr>
<tr>
<td>LDPE$^a$</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>93±2</td>
<td>-0.077±0.025</td>
<td>-0.033±0.058</td>
<td>-0.037±0.058</td>
</tr>
</tbody>
</table>

n.d. – not determined.
A distinct sorption profile is observed in Fig. II.5.3 for the CA2/DES-U film. The sigmoid shape of the CA2/DES-U curve was more evident, and is commonly observed for aqueous agar/glycerol films (Phan et al., 2005; Phan et al., 2009a; Sousa et al., 2010). This could be confirmed by the estimated GAB parameters; except for CA2/DES-U, low C values (<2) were estimated in all cases, indicating that the applicability of the GAB model to these systems could be restricted (Lewicki, 1997). However, the high correlation coefficients (R²>0.98) and k values (0<k<1) suggested that the model adequately fit the experimental data. For the range of low a_w, X_e was low and increased almost linearly with an increase of a_w. At a_w=0.11 low X_e were observed, with CA2/DES-U reaching a higher X_e (~0.05) when compared to the other films (X_e ~0.03). For higher a_w however, the increase in X_e became much more significant; a sharp rise in X_e seemed to occur around a_w=0.60 and near the saturation point (a_w=0.85) the X_e reached values as high as ~0.48-0.53 or even higher (~0.57 for CA2/DES-U; Fig. II.5.3). Also worth noting is that at the humidity conditions at which the mechanical tests were performed (50%), CA2/DES-U and CA6/DES-U showed distinct water uptakes (X_e ~0.185 against ~0.137).

In general, CA/DES-U films showed higher X_o (in the range 0.10-0.15) than typical agar aqueous films (~0.04-0.08) (Phan et al., 2005; Phan et al., 2009a; Sousa et al., 2010) suggesting enhanced water uptake capacity of the produced materials in the monolayer, at equilibrium conditions. Also, the X_o was higher for CA/DES-U films prepared with lower polymer contents. The decrease in X_o can be explained by a reduction in the number of active sites for water sorption due to chemical and physical changes induced by differences in the polymer concentration as well as DES-U amount in the films.

C was found to be higher in CA2/DES-U (Table II.5.2). This means that the sorption of water by this sample was characterized by a monolayer of molecules, which were strongly bounded to the material (higher C). Also, this value reflects that the subsequent molecules were only slightly or were not structured in the upper layers. In contrast, the remaining film formulations presented lower C values which could suggest that, in this case, the water molecules were not so strongly bounded to the material at the primary sorption sites while a more structured multilayer should be expected. However, due to possible restrictions in the application of the GAB equation no further physical interpretations of the estimated parameters were attempted.
II.5.2.5. Contact Angle Measurements

Understanding the behavior of the film when in contact with liquids is of particular interest when designing new materials. In the case of hydrophilic biopolymers such as agars, this information becomes even more relevant since the material should be able to perform according to the desired end-use which most of the times implies having some resistance to water (i.e. hydrophobicity).

The contact angle (Θ) and contact angle variation (ΔΘ) as well as variations of the droplet volume (ΔV) and droplet surface area (ΔA) for the CA/DES-U films are given in Table II.5.2. The profiles of the water droplets upon contact with the surface of the films were recorded at 25 °C over 10 s. Since the contact angle theory is based on the idea of a true equilibrium between the solid, liquid and vapor interfaces, and in this particular case of hydrophilic biopolymers, these conditions are in reality impossible to achieve, and therefore a short temporal window was chosen to perform the analysis (Farris et al., 2011). Also, to eliminate as much as possible overestimations in the measured data caused by the surface roughness, films were kept flat using special grips and a large number of replications were carried out for each sample in order to obtain reliable data. As expected, the commercial LDPE sample used as reference had the highest contact angle (93±2°) and the least amount of significant changes in the droplet parameters (i.e. lower ΔΘ, ΔV and ΔA). This confirmed the strongest hydrophobic nature of this synthetic material. For the tested CA/DES-U films, an overall inverse correlation could be established between the contact angle and the polymer concentration. The obtained Θ were within the range of values reported by other authors when studying aqueous agar films plasticized with glycerol (Phan et al., 2009a, b; Rhim, 2011), For example, CA6/DES-U was the least hydrophilic film (i.e. 75±3°) exhibiting a quite high Θ despite the agars' known hydrophilicity. It seems rather intuitive that the higher number of established intra and intermolecular bonds between the agar chains at higher polymer concentrations would hamper the interaction with water molecules of the droplet, at least at an early stage of the droplet deposition. Also, the high measured Θ could indicate a higher mobility of the hydroxyl groups in the sugar rings present in agar chains and exposed at the solid/liquid interface, upon formation of the CA/DES-U systems when compared to traditional aqueous agar films (Yasuda et al., 1994). Ultimately, this would confer enhanced reorganization capability to the polymeric matrix upon water deposition similar to what is commonly observed for aqueous gelatin films (Farris et al., 2011). Oppositely, CA2/DES-U and CA3/DES-U exhibited the most hydrophilic surfaces (respectively, 60±4° and 56±2°).
The droplet evolution for the CA2/DES-U film was much more significant than for remaining films. This can be observed from the variations in the droplet parameters (Table II.5.2) and from Fig. II.5.4 A inspection. Significant changes took place upon water droplet deposition on the surface of CA2/DES-U compared to CA6/DES-U (Fig. II.5.4 B) or LDPE (Fig. II.5.4 C) films. Moreover, the data (i.e. $\Delta V<0$ and $\Delta A<0$ in Table II.5.2 and Fig. II.5.4) seemed to suggest significant water penetration in the surface of CA2/DES-U (absorption) during the analysis (Farris et al., 2011) which could mean greater porous irregularity for this film (Bialopiotrowicz & Janczuk, 2002). This agreed well with the higher number of absorption sites suggested from the water sorption studies and the greater variations in the droplet parameters recorded for this sample (Fig. II.5.4 and Table II.5.2). Inversely, other films showed negative variation in volume and positive variation in area ($\Delta V<0$ and $\Delta A>0$ in Table II.5.2), which might suggest a higher contribution from the spreadability phenomena to the surface wettability, at least over the time frame of the analysis (Farris et al., 2011). No significant swelling or migration of film components to the water droplet was observed as indicated by the negative variations in the droplet volume measured in all cases (Phan et al., 2009b).

![Fig. II.5.4 - Water drop shapes after deposition in the CA2/DES-U (A), CA6/DES-U (B) and LDPE (C) films surfaces. Variations in the droplet volume for the CA2/DES-U (green triangles), CA6/DES-U (red dots) and LDPE (blue squares) films over the time frame of the analysis. Area variation for the CA2/DES-U film.](image-url)
II.5.2.6. Water Vapor Permeability (WVP)

Fig. II.5.5 shows the WVP data for the different CA/DES-U films. Agreeing with the trend observed in the mechanical tests, CA2/DES-U showed comparable WVP to CA6/DES-U (5.5-6.8×10⁻¹⁰ g.m⁻¹.s⁻¹.Pa⁻¹) and both were the least permeable films. The slow diffusion rate of water molecules throughout these films suggested smaller interstitial spaces between the polymeric chains (i.e. lower free volume) and seemed in good agreement with the more cohesive matrices imaged by SEM (absence of visible defects: Figs. II.5.2 A and II.5.2 E). At first sight, one might consider this inconsistent with the water sorption and contact angle data. Yet, taking into consideration the three mechanisms underlying the permeation process (i.e. absorption, diffusion and desorption) we could assume that in the case of CA2/DES-U, the absorption of water molecules was thermodynamically favored yet its diffusion and desorption rates were slower. Again, the interplay between polymer concentration and DES amount might explain the observed behavior. This could suggest either, smaller free volumes between CA2/DES-U polymeric chains, as confirmed by SEM (Fig. II.5.2 A), and on the other hand, or water molecules more strongly bonded to the polymer, needing longer times to desorb from the surface (Duncan et al., 2005). Phan and co-workers found no correlation between the WVP and moisture sorption of aqueous agar-based emulsified films (Phan et al., 2009b).

Contrasting with the claims of other authors (Cerqueira et al., 2013; McHugh et al., 1993), thickness was not a determinant factor to the diffusion process. Significantly higher WVP was found by Rhim when studying aqueous agar films plasticized with glycerol (∼2.2×10⁻⁹ g.m⁻¹.s⁻¹.Pa⁻¹; (Rhim, 2011)) while other conventional agar films seemed less permeable to water than the studied CA/DES-U films (Phan et al., 2005; Sousa et al., 2010). However, differences in the followed protocols or the plasticizing effect of the DES-U, suggested by the high $EB$ data, could explain the observed differences. Comparing with the aqueous-blended films studied earlier (Chapter II.3), these non-aqueous materials were much more permeable. It is generally believed that plasticizing agents induce changes in the polymeric matrix creating voids between the polymer chains, hence promoting the water mobility across the film. Ultimately, these larger spaces may give room to the occurrence of water condensation, which can drastically increase the water diffusion (Duncan et al., 2005). CA/DES-U films, with visible cracks in their matrices (Figs. II.5.2 B to II.5.2 D), exhibited higher WVP, in the range 8.4-11.6×10⁻¹⁰ g.m⁻¹.s⁻¹.Pa⁻¹.
By using DES-U, as both solvent and plasticizer, agar films with enhanced stretchability when compared to conventionally-made aqueous films could be obtained without compromising the mechanical properties. The measured properties seemed to result from a complex conjugation of factors related with differences in agar and DES-U contents of the resultant films: i) molecular arrangements upon polymer addition to the DES-U and ii) significant molecular reorganizations upon partial hydration of the agar/DES-U system. Hence, and despite the encouraging results, further research is still needed to gain further insight of the driving mechanisms underlying the formation of these novel materials.

II.5.3. Conclusions

A new approach to fabricate agar films by exploring the role of choline chloride based eutectics as both, solvent and plasticizer was presented. Even at low polymer concentrations, CA/DES-U systems showed good film-forming ability, the obtained films exhibiting good mechanical resistance and enhanced elasticity when compared with typical aqueous agar films. The measured properties seemed to result from a complex conjugation of factors related with different CA/DES-U contents between the tested films: i) molecular arrangements upon polymer addition to the DES-U and ii) significant molecular reorganizations upon partial hydration of the CA/DES-U system. The proposed methodology for the films fabrication seemed to guarantee an efficient plasticization of the materials. Nonetheless, future work is clearly needed to benefit from the potentialities of these novel materials.
materials. Special attention should be given to the water role upon partial hydration of the films. In view of the later achievements of the algae industry, the present results can open a new field of exploration in the discovery of emerging materials based on seaweed polysaccharides. In this regard, agar is a good candidate.

References.


The last study concerning the 2nd stage of the PhD project was the fabrication of agar fibers by the electrospinning technique. All experiments were carried out at the ERRC-USDA, in Wyndmoor, Pennsylvania.

II.6.1. Solubilization of the Polymers

II.6.1.1. In water

In aqueous media, the concentrations used for the starting solutions were 1% wt for agar and 10% wt for PVA. As expected, agar was well dissolved in water, under vigorous stirring, at temperatures close to the boiling point of the solvent. In the case of PVA, the solubilization was not so easily accomplished. The formation of lumps and/or thin PVA films, at the solutions’ surface, could occur if adequate dissolution conditions weren’t used; hence, special caution was taken by using a heating bath and stirrers promoting an evenly dispersion of all PVA particles.

II.6.1.2. In DES-U

Agar and PVA dissolved well in DES-U and homogeneous solutions were being obtained after a short period of time. In this case, the starting solutions were prepared at 1% wt agar and 5% wt PVA concentrations. A PVA solution of 3% wt was also tested. As an example, 1% wt agar solution is shown in Fig. II.6.1. In the case of PVA, DES-U seemed particularly advantageous; no lumps or thin films were formed at the solution surface during the dissolution process, a common problem when working with PVA in aqueous media. The greater capacity for DES to establish hydrogen bonds with the hydroxyl groups present throughout the polymers’ backbones could easily explain the above observations (Zhang et al., 2012).
II.6.2. Rheological Properties of the Spinning Solutions

The vast majority of the available electrospinning studies are essentially supported by data concerning the solution viscosity, concentration or surface tension (Rosic et al., 2012). However, the significance of the viscoelastic behavior of the spinning solution to the overall process cannot be neglected. For instance, Yu and co-workers suggested that the consistency of the polymer jet, imparted by a greater elasticity of the ejected liquid, could effectively prevent beads formation (Yu et al., 2006). Also, chitosan or alginate blends with PEO were found to be more appropriate for electrospinning when showing greater viscous component (Rosic et al., 2012). When dealing with gelling polymers such as agar, this becomes even more relevant since a good insight into the viscoelasticity of the spinning solutions can help to overcome the difficulties commonly associated with processing these gelling systems.

II.6.2.1. Water as Solvent

The first natural step was to explore the spinnability of agar aqueous solutions since water is the preferred solvent when working with SP. For the pure agar system (1% wt) and at 50 °C, the values of G' and G'' as well as apparent viscosity (~0.015 Pa.s) measured over the course of the rheological experiments (dynamic oscillatory and steady shear tests, respectively) were extremely low. For this reason and due to the significant scattering of the viscoelastic data, no curves will be presented for this sample.
The mixed agar/PVA systems were prepared from a 1% wt agar and 10% wt PVA starting solutions, properly mixed according to the desired final composition. The rheological tests were again carried out at 50 °C, temperature at which the electrospinning equipment was operating.

Data from the cooling ramps performed from 90 to 50 °C (e.g. Fig. II.6.2), indicated predominance of the viscous component \(G''\) and fairly constant viscoelasticity when compared with the systems prepared in DES-U (Fig. II.6.6).

The mechanical spectra, recorded at 50 °C, show \(G''>G'\) over the entire range of considered \(\omega\), for all agar/PVA systems (Fig. II.6.3). Here, as well, the elastic component showed some dispersion which was attributed to the low concentration of the samples.

The PVA addition to agar gradually increased the apparent viscosity of the mixed systems, as seen by the flow curves recorded at 50 °C (Fig. II.6.4). This was expected since the PVA concentration was increasing. For instance, at 10 s\(^{-1}\) the \(\eta_{app}\) were ~0.32, ~0.30, ~0.24, ~0.15, ~0.06 Pa.s for respectively, 0/100, 20/80, 30/70, 40/60 and 50/50 agar/PVA mixtures. In the region of low shear rates, an increase of the apparent viscosity with decreasing shear rate is observed for the mixed systems, which can be indicative of some incompatibility between PVA and agar.

**Fig. II.6.2** - Temperature dependence of elastic \((G'; \) filled symbols) and viscous \((G''; \) open symbols) moduli of the 0/100 (inverted triangles) and 50/50 (stars) agar/PVA aqueous mixtures, during a cooling ramp from 90 to 50 °C, recorded at 6.28 rad/s and 1% strain amplitude. The blends were prepared from a 1% wt agar and 10% wt PVA starting solutions.
Fig. II.6.3 - Frequency dependence at 50 °C of elastic ($G'$; filled symbols) and viscous ($G''$; open symbols) moduli of agar/PVA aqueous blends (A) 30/70 (triangles) and 20/80 (circles) mass ratios; and (B) 40/60 (pentagon), 50/50 (stars) and 0/100 (inverted triangles) mass ratios), recorded at 6.28 rad/s and 1% strain amplitude. The blends were prepared from a 1% wt agar and 10% wt PVA starting solutions.
The rheological properties of the pure DES-U were analyzed at 50 °C. The frequency-dependence of the viscoelastic properties showed the predominance of the viscous character ($G'' > G'$) with $G''$ showing a steep increase with $\omega$ (not shown). The higher viscosity of DES-U when compared to water (around 0.1 Pa.s at 50 °C; Fig. II.6.5), could be attributed to the large ion sizes and very small voids within the DES-U system as well as attractive forces between its components (Zhang et al., 2012). Examination of the flow curve also enabled to identify a clear Newtonian behavior in the range of tested shear rates, 1-200 s$^{-1}$ (Fig. II.6.5).
Other relevant properties of the DES-U have been extensively discussed in the reference papers of Abbott (Abbott et al., 2003a; Abbott et al., 2003b) and Zhang (Zhang et al., 2012). For example, the high conductivities reported for this solvent (in the range 2-3 mS/cm at 50 °C) stem from the dissociation of the ionic species in the liquid that can move freely within the formed system.

In Figs. II.6.6 A) and II.6.6 B) are reported the cooling ramps from 90 to 50 °C of the agar (1% wt) and PVA (5% wt) pure solutions as well as agar/PVA blends in the DES-U. Agar pure system was already experiencing significant elasticity gain at the T range defined for the cooling process, indicated by a faster increment of $G'$ in relation to $G''$ (i.e. sharp decrease of the tan δ; not shown). Considering the above results and assuming similar gelation mechanisms for the agar/DES-U and agar/water systems, this could match the initial formation of double helices for the former and random coil state for the latter. The particular architecture of the DES-U, as well as the forces established between its components, could easily explain the observed differences. Taking as valid the commonly used rule $G' = G''$ (or tan δ=1) defined for gelation, one can see the aggregation process leading to gel formation happening much sooner (at higher temperature) for pure agar in the DES-U (~80 °C against ~30 °C in water). Upon PVA addition to the agar solution, the process gradually shifted to lower temperatures (Fig. II.6.6 A). For the 50/50 agar/PVA blend, the gelation occurred later on the cooling step (~67 °C; Fig. II.6.6 A) and, when higher amounts of co-blending polymer were added, no transition in the viscoelasticity of the systems was observed, with the viscous component prevailing over the entire T window ($G'' > G'$). This shift in behavior indicated a concentration threshold for the gelation of agar/PVA systems and was attributed to the
presence of higher number of PVA molecules (non-gelling polymer) hindering the formation of agar 3D networks. After the gelation point, the elastic behavior of agar-pure and 50/50 agar/PVA solutions became predominant ($G' > G''$ or $\tan \delta < 1$) reaching values around respectively, 458 Pa and 63 Pa, at the end of the cooling step. Blends with agar contents between these two limits showed intermediate viscoelastic responses (not shown). As temperature was further reduced, $G'$ and $G''$ continued to increase suggesting progressive structural reinforcement; at 50 °C, both moduli were still increasing. Early studies from Kumar and his group described remarkably lower $T_g$ (~17-40 °C) for native and regenerated agarose at 2-5% wt concentrations, in various RTILs (Singh et al., 2010; Trivedi et al., 2012). It was concluded that the presence of large ions would interact with the agarose molecules hindering the double helical conformation.
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Fig. II.6.6 - Temperature dependence of elastic (\(G'\); filled symbols) and viscous (\(G''\); open symbols) moduli of the agar/PVA blends in the DES-U (A) 100/0 (squares), 50/50 (stars) and 0/100 (inverted triangles); B) 30/70 (triangles) and 20/80 (circles) mass ratios) during cooling ramp from 90 to 50 °C, recorded at 6.28 rad/s and 2% strain amplitude. The blends were prepared from a 1% wt agar and 5% wt PVA starting solutions.

The equilibration curve of the systems recorded at 50 °C, confirmed the predominance of the viscous component (i.e. \(G'' > G'\)) for the agar/PVA blends with mass ratios 30/70, 20/80 and 0/100, whereas higher agar amounts led to a more pronounced elastic behavior (\(G' > G''\); Fig. II.6.7). In any case, the viscoelastic response of the systems was not undergoing appreciable changes that could compromise acquiring the mechanical spectra.

Fig. II.6.7 - Time evolution of elastic (\(G'\)) and viscous (\(G''\)) moduli at 50 °C of the agar/PVA blends in the DES-U (100/0 (squares) 50/50 (stars) and 20/80 (circles) mass ratios), recorded at 6.28 rad/s and 2% strain amplitude. The blends were prepared from a 1% wt agar and 5% wt PVA starting solutions.
Fig. II.6.8 - Frequency dependence at 50 °C of elastic ($G'$; filled symbols) and viscous ($G''$; open symbols) moduli of agar/PVA blends in the DES-U (A) 100/0 (squares), 50/50 (stars) and 0/100 (inverted triangles); (B) 100/0 (squares), 30/70 (triangles) and 20/80 (circles) mass ratios), recorded at 6.28 rad/s and 2% strain amplitude. Inset of A): Frequency dependence at 50 °C of the complex viscosity, $\eta^*$ (squares) of agar 1% wt in DES-U. The solid line in B) represents $G''(\omega)$ recalculated from $G'(\omega)$ data using the Tschoegel approximation (Eq.I.2.4) for sample 30/70 agar/PVA. The blends were prepared from a 1% wt agar and 5% wt PVA starting solutions.
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Fig. II.6.9 - Frequency dependence at 50 °C of tan δ of agar/PVA blends in the DES-U (100/0 (squares), 50/50 (stars), 30/70 (triangles), 20/80 (circles) and 0/100 (inverted triangles) mass ratios), recorded at 6.28 rad/s and 2% strain amplitude. The blends were prepared from a 1% wt agar and 5% wt PVA starting solutions.

Results displayed in Figs. II.6.8 show the viscoelastic properties of the pure polymers and polymer blends measured for ω in the range ~1-75 rad/s. The Tschoegel approximation (G''(ω) recalculated from G'(ω) data using Eq.I.2.4) was used to check that the data were acquired below the linearity limit of viscoelastic response (e.g. 30/70 agar/PVA in Fig. II.6.8 B). The G' of the pure agar in DES-U was, at least, one-order of magnitude higher than G" and both moduli showed little frequency dependence (‘true’ gel behavior; (Kavanagh & Ross-Murphy, 1998)). This behavior was not observed in aqueous agar solutions of the same concentration, as referred above (section II.6.2.1). The consistency of the system was followed by plotting the mechanical spectra in terms of |η*(ω)| logarithmic data (Eq.I.2.5; inset of Fig. II.6.8 A)). Here, the estimated dynamic consistency index, K and the power law factor, n, were respectively, ~564 Pa.s and -0.93, ascribing significant elasticity of the system, especially if one considers the temperature at which the data were collected (50 °C).

A noticeable difference was found for the viscoelastic behaviour of pure PVA where G'' was higher than G' in all range of considered ω (not shown). The mechanical spectra of agar/PVA blends exhibited intermediate behaviours between those described by the pure polymer solutions. The PVA addition had two visible effects in the gelled systems, decrease of gel rigidity (G' decrease) and decrease of gel elasticity (tan δ increase). For the 30/70 and 20/80 agar/PVA blends, the behavior of the co-blending polymer becomes clearly predominant with G'' higher than G' over the entire ω scale (Fig. II.6.8 B). Accordingly, the elastic character of agar/PVA systems is attenuated; for instance, taking as reference the elastic storage modulus measured at 6.28 rad/s (G')), the following trend could be observed
for the agar/PVA blends: 100/0 > 50/50 > 30/70 > 20/80 > 0/100 respectively, ~620, ~57, ~6, ~1.7 and ~0.86 Pa. The evolution from systems with predominance of agar behavior towards PVA-dominant character could be followed from the observation of the tan δ dependence with ω (Fig. II.6.9).

Plotting the flow curves of the agar/PVA systems with predominant viscous component at 50 °C, showed explicitly an increase in viscosity with the PVA addition (Fig. II.6.10). As seen previously for the aqueous media, this indicates appreciable structural modifications of the systems upon PVA addition. The measured viscosities were significantly higher (roughly ~4-6 Pa.s at 10 s⁻¹) when compared to agar aqueous media (~0.32 Pa.s or lower at 10 s⁻¹; Fig. II.6.4), which could be attributed to the nature of the DES-U solvent (Fig. II.6.5). The shape of the viscosity curves shows decreasing viscosity values with increasing shear rate indicating that all solutions behave as non-Newtonian, shear-thinning fluids. A Newtonian plateau zone, at intermediate shear rates, is observed for the pure PVA solution while, for the mixed solutions, this plateau is ill-defined. For these mixed solutions, an increase in viscosity is observed for lower shear rates, the upturn in the flow curve being more pronounced for the mixture with higher agar content (30/70), which may be indicative of some incompatibility between both polymers. Rošic and co-workers reported similar behaviors for alginate/PEO and chitosan/PEO aqueous blends (Rosic et al., 2012).

![Image](image.png)

**Fig. II.6.10** - Shear-dependence of the apparent viscosity of agar/PVA blends in the DES-U (0/100 (inverted triangles), 20/80 (circles) and 30/70 (triangles) mass ratios) at 50 °C. The blends were prepared from a 1% wt agar and 5% wt PVA starting solutions.
II.6.3. Electrospinning

II.6.3.1. Production of Agar Fibers Using Water as Solvent

As starting point for the electrospinning experiments we fixed the temperature at 50 °C (maximum working T of the apparatus), the sample volume at 3 mL and the distance tip-to-collector at 8 cm. Tables II.6.1 and II.6.2 detail the operational parameters used in each run as well as relevant observations. The working distance was fixed after several tests and chosen according to the stability and direction of the polymer jet towards the counter electrode. Other parameters (working T, concentration) were defined according to the intrinsic nature of the polymers. In order to keep the spinning solution warm during the process, a tubeless spinneret was placed inside the chamber and connected to a syringe pump placed outside the chamber using a soft plastic tube. The experimental setup is described in detail in section II.2.7.2, Chapter II.2. It should be stressed out that, due to the used setup the flow rates that could effectively pull out the spinning solution from the needle tip towards the counter electrode, were above the values commonly reported for other natural polymers, ~0.1-3 mL/h (Freire et al., 2011; Martinez-Sanz et al., 2012; Toskas et al., 2011; Viswanathan et al., 2006). Finally, the needle size was fixed at the lowest possible available (inner diameter = 0.317 mm).

The first trials were carried out using water as solvent. The detailed operational conditions at which the fibers were obtained can be found in Table II.6.1. Agar pure aqueous solution (1% wt) was impossible to be successfully spun at any set of electrospinning parameters. Droplets fell down from the needle tip in all cases revealing inadequate viscoelasticity of the solution (Nie et al., 2008). Rheological data confirmed this interpretation.

In order to overcome this problem, PVA was added to the pure agar solution. PVA shows interesting properties to this end because it's non-toxic and biocompatible, being used in sensitive fields such as pharmaceutical and biomedical while exhibiting great spinnability (Rosic et al., 2012; Toskas et al., 2011). A PVA starting solution of 10% wt concentration was considered adequate to obtain agar/PVA blends with satisfactory spinnability. Micrographs of the agar/PVA fibers prepared at various process conditions and agar/PVA ratios, are represented in Fig. II.6.11. For each agar/PVA ratio, the operational parameters summarized in Table II.6.1 were chosen based on the best possible polymer jet (i.e. uniform spinning or ejection).

Blends with higher agar contents (40% or more) were harder to process. At these ratios, the needle would be obstructed in the early stage of the spinning process and the
experiment stopped. When using water as solvent, shower jets intercalated with droplets were mostly observed. The droplets persisted, even after mixing both polymers under heat for a significant time-frame, which could indicate limited compatibility between agar and PVA in aqueous media. Based on the jets of the pure polymer solutions, the droplets were attributed to agar rich-phase regions on the spun solution.

Tests of the 50/50 agar/PVA blend were carried out between 14-24 kV, range at which a polymer jet was pulled out from the needle. The ejected solutions were more uniform at low flow rates, hence the material was collected at 0.7 mL/h (E.1 conditions in Table II.6.1).

Discontinuous fibrous mats were observed for the 50/50 and 40/60 agar/PVA blends (Figs. II.6.11 A and II.6.11 B) which might indicate insufficient entanglement density (i.e. low concentration) or excess voltage causing inadequate instabilities during the jet formation (Barber et al., 2013). The frequency scans and flow curves recorded for these samples seemed to agree well with this view (Figs. II.6.3 B and II.6.4). The irregularity in shape was significant, with fibers and beaded fibers of variable diameters being formed. For the 40/60 blend, higher flow rates were the most adequate (E.2 conditions in Table II.6.1).

When increasing the PVA concentration in the final mixture, the morphology of the collected fibers was significantly improved (i.e. 30/70, 20/80 and 0/100 agar/PVA blends; Figs. II.5.7 C-F). This matched an increase in apparent viscosity measured through the steady state shear tests (Fig. II.6.4) due to a greater influence of PVA to the systems behavior. Individual nanofibers were seen in majority although some fiber doublets or partially fused fibers as well as fibers exhibiting branching could be seldom identified (Fig. II.6.11 D; Peresin et al., 2010). A close inspection of Table II.6.1 and Figs. II.6.11 D/II.6.11 E point out to the effectiveness of higher flow rates in preventing the formation of beaded fibers at 20/80 agar/PVA ratio (E.4 vs E.5 conditions). Of particular interest were the E.4 conditions where the homogeneity of the obtained fibrous mat was quite evident (Fig. II.6.12 A).
Fig. II.6.11 - Representative SEM pictures of the agar/PVA fibers produced by electrospinning and using water as solvent, at 25 000x magnification (agar 1% wt / PVA 10% wt ratios: A- 50/50; B- 40/60; C- 30/70; D- 20/80 obtained with E.4 conditions; E- 20/80 obtained with E.5 conditions; F- 0/100). Details concerning the process conditions are given in Table II.6.1.
Fig. II.6.12 - SEM picture of the 20/80 agar/PVA fibers displayed in Figs. II.6.11 D (obtained with E.4 conditions) and II.6.11 E (obtained with E.5 conditions) at lower magnification (5 000x).

Agar gelation occurs gradually, the change in viscoelastic properties happening over a broad temperature window, ~43-30 °C (Sousa et al., 2013). Even though the equipment was setup to operate at 50 °C, for longer processing times the samples would approach the region of sol-gel transition causing the obstruction of the needle. Moreover, constraints related with the T controller (variable T profile inside the chamber) could have also contributed to agar gelation over the course of the experiments.

All together, these results indicate that high flow rates (14 mL/h), coupled with high voltages (24-30 kV), seemed more effective at producing agar/PVA nanofibers when using water as solvent. Discarding the processing difficulties, agar/PVA nanofibers were successfully spun, particularly at higher PVA contents (70% or higher). The nature of the polymer jets seemed to indicate some phase separation between both polymers in aqueous media, particularly at higher agar contents, as we pointed out in the rheological measurements. However, this is a suggestion that needs to be confirmed.
Table II.6.1 – Details of the electrospinning experiments concerning the fabrication of agar/PVA fibers using water as solvent. The following conditions were fixed in all experiments: temperature of 50 °C, initial sample volume of 3 mL and distance tip-to-collector of 8 cm. A drum collector was used in all cases. For more details concerning the overall process please see the experimental section.

<table>
<thead>
<tr>
<th>Figure Id.</th>
<th>Agar conc. (% wt)</th>
<th>PVA conc. (% wt)</th>
<th>Agar/PVA ratio</th>
<th>Conditions Id.</th>
<th>Volume (mL)</th>
<th>Flow rate (mL/h)</th>
<th>Voltage (kV)</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.6.11 A</td>
<td>1</td>
<td>10</td>
<td>50/50(^{(1)})</td>
<td>E.1</td>
<td>3</td>
<td>0.7</td>
<td>24</td>
<td>Shower jet intercalated with droplets</td>
</tr>
<tr>
<td>II.6.11 B</td>
<td>1</td>
<td>10</td>
<td>40/60(^{(1)})</td>
<td>E.2</td>
<td>3</td>
<td>6</td>
<td>20</td>
<td>Shower jet intercalated with droplets</td>
</tr>
<tr>
<td>II.6.11 C</td>
<td>1</td>
<td>10</td>
<td>30/70</td>
<td>E.3</td>
<td>3</td>
<td>14</td>
<td>24</td>
<td>Shower jet intercalated with droplets</td>
</tr>
<tr>
<td>II.6.11 D</td>
<td>1</td>
<td>10</td>
<td>20/80</td>
<td>E.4</td>
<td>3</td>
<td>14</td>
<td>24</td>
<td>Shower jet intercalated with a few droplets</td>
</tr>
<tr>
<td>II.6.12 A</td>
<td>1</td>
<td>10</td>
<td>20/80</td>
<td>E.5</td>
<td>3</td>
<td>6</td>
<td>30</td>
<td>Shower jet intercalated with droplets</td>
</tr>
<tr>
<td>II.6.11 F</td>
<td>1</td>
<td>10</td>
<td>0/100</td>
<td>E.6</td>
<td>3</td>
<td>14</td>
<td>24</td>
<td>Shower jet</td>
</tr>
</tbody>
</table>

\(^{(1)}\) At some point the needle was obstructed due to agar gelation.
II.6.3.2. Production of Agar Fibers using DES-U

Aiming at improving the spinnability of agar solutions, water was replaced by the DES-U. As observed for the majority of the eutectic mixtures, this solvent presented higher viscosity than water which consequently led to electrospun solutions with greater consistency. A detailed description of the used operational parameters to carry out the experiments can be found in Table II.6.2.

As first attempt, we tried to electrospin 1% wt agar pure solution in DES-U. The temperature and distance tip-to-collector were again fixed at respectively, 50 °C and 8 cm. Far beyond the gel transition, the solution possessed good spinnability forming branched nanofibers (indicated by an arrow in Fig. II.6.13) when applying a 10 kV starting voltage and a flow rate of 5 mL/h (E.1 conditions in Table II.6.2). After sufficient spinning time, however, the needle would be obstructed due to an increase in the solution viscosity and gelation (Pirzada et al., 2012). In our point of view, these phenomena resulted in the formation of the micron-sized material seen in Fig. II.6.13. Taking into account that the spinning solution was placed inside the chamber at temperatures well above 50 °C, the significant changes in the viscoelastic properties undergone by the pure agar solution during the cooling step agreed well with these observations (Fig. II.6.6 A). Nonetheless, the use of a more accurate T controller in the equipment, with an enlarged range of working T would enable the collection of higher amounts of nanofibers and with greater structural regularity. Compared with previous studies (Bao et al., 2010), electrospinning technique can be a more advantageous method particularly if one considers the relevance of the surface-to-volume ratio in many applications. Viswanathan and co-workers (2006) found similar branched morphologies when electrospinning a 10% wt cellulose solution in the RTIL 1-butyl-3-methylimidazolium chloride. The higher viscosity of the RTIL could explain the branching of the fibrous material (Viswanathan et al., 2006). In the present study this feature seemed to promote sufficient entanglement density even at 1% polymer concentration.
To overcome this initial processing difficulties, PVA was added as a co-blending polymer to agar solution and the resultant blends tentatively electrospun. Based on the properties of the chosen solvent, a low PVA concentration (3% wt) was first tested. At this concentration however, all tested agar/PVA blends seemed ineffective at producing fibers with satisfactory consistency. Despite the good fluidity of the polymer jets, well oriented to the center of the coagulating bath, the formed entangled fiber webs were ultrathin, and mostly, hard to collect. A picture of the web formed by the pure PVA (3% wt) ejected solution when using E.8 set of conditions (Table II.6.2) is displayed in Fig. II.6.14 A. This was one of the few collectable materials; however, mostly collapsed structures with punctual fibrous material could be identified after drying (indicated with arrows in Fig. II.6.14 B). In order to examine the materials by SEM, the remaining samples were tentatively recovered from the coagulating bath through vacuum filtration, using a filter with 0.2 µm porosity. To keep as much as possible the integrity of the electrospun material, the vacuum was slowly applied and the samples washed thoroughly with pure ethanol. Also, the vacuum was stopped at an early stage after the last wash, to minimize significant structural damage. Micrographs of the 20/80 agar/PVA blend prepared from a 3% wt PVA starting solution are given as example in Fig. II.6.15 (E.9 conditions in Table II.6.2). The filtered materials were structurally similar to the one collected directly from the ethanol bath indicating that the damaged, aggregated and/or fused structures, were mostly due to insufficient polymer concentration (below the threshold of entanglement density needed to attain cohesive fibers). Nonetheless, nano-sized fibers could be identified (Fig. II.6.15 B). The same kind of structures were observed for the other agar/PVA blends (not shown).
II.6 AGAR FIBERS

In the following section, the electrospinning process is utilized to create agar fibers. Fig. II.6.14 shows an ultrathin fiber web formed by the electrospun 3% wt PVA solution when using DES-U (A). Representative SEM picture of the respective material upon drying (B). Despite the evident collapsed structures, fibers could be detected (indicated with arrows).

Fig. II.6.15 - Representative SEM pictures of the electrospun material obtained with a 20/80 3% wt PVA/1% wt agar solution when using DES-U. The material was recovered from the coagulating ethanol bath through vacuum filtration. Despite the evident collapsed structures, fibers could be detected (indicated with arrows).

To improve the consistency of the electrospun fibers, the concentration of the PVA starting solution was increased to 5% wt. Again, all experiments were performed at 50 °C and using a distance tip-to-collector of 8 cm. Here, it should be emphasized that longer distances were tested, yet the polymer jets described highly unstable paths which, considering the used voltages, could pose safety issues. The polymer jets were less fluid than what was observed previously for the 3% wt PVA solution which could be attributed to the increase in viscosity.

At adequate processing conditions, set after preliminary experiments, the electrospun materials could be easily collected from the coagulating bath and showed good mechanical resistance when manipulated with hands, upon drying. Another observation that stands out immediately from the SEM images (Figs. II.6.16, II.5.17 e II.5.19) was the greater
homogeneity of the composite fibers when using the DES-U. This seemed to indicate these eutectics as more appropriate media for blending agar and PVA. The majority of the collected materials were at sub-micron scale, yet this could be easily attributed to the higher viscosity of the DES-U when compared to water (Viswanathan et al., 2006).

Fig. II.6.16 - Representative SEM pictures of the agar/PVA fibers produced by electrospinning and using DES-U, at 250x and 2500x magnifications (agar 1% wt / PVA 5% wt at 50/50 mass ratio). The operational conditions used to obtain each sample (i.e. A/B; C/D; E/F) are described in detail in Table II.6.2.

Fig. II.6.16 represents the micrographs of the collected materials of the 50/50 agar/PVA blend at various sets of electrospinning conditions (E.10 to E.12 in Table II.6.2). The majority of the fibers were in the range of low micron sizes. Higher flow rates seemed to favor the
formation of thinner fibers with smoother surfaces (E.10 conditions in Figs. II.6.16 A/B vs E.12 conditions in Figs. II.6.16 E/F). At fixed flow rate, higher voltages would decrease the fibers diameter (E.11 conditions in Fig. II.6.16 C/D vs Fig. II.6.16 E/F) meaning improved jet instabilities at higher electric fields (Barber et al., 2013).

It is worth pointing out that, when lower voltages were applied, the feed rate had to be often readjusted for the solution to continue to come out of the needle. Again, this could be caused by the temperature decrease inside the chamber leading to the systems’ gelation. Also, the surface roughness of the fibers seemed greater at higher agar contents. This could indicate phase separation of agar and PVA during the fabrication of the fibers (Viswanathan et al., 2006). Possible reasons for these findings include differences in the mixtures composition, $M_w$ and fiber sizes. Also, the critical step of solvent removal could cause the observed differences since the washing rates couldn’t be reproduced from one sample to another (Viswanathan et al., 2006).

During the course of the experiments, the 50/50 agar/PVA blend would often gel and obstruct the needle. Agreeing with the rheological profile recorded during the cooling step (Fig. II.6.6. A) this happened at lower temperatures than for the agar pure solution. The viscoelastic behavior of the 50/50 blend led to good processability; continuous and well orientated polymer jets were formed at various sets of electrospinning conditions. Again, if the mixture was kept well above the $T_w$ window at which the sol-gel transitions occurred (i.e. $G''>G'$), the samples would be easily electrospun, uninterruptedly.

Discontinuous spinnability associated with an increase in the solution’s viscosity was occasionally observed for blends with higher PVA contents (30/70 and 20/80 agar/PVA). Also, these samples tended to form fibers with smoother surfaces (Figs. II.6.17, II.6.19 e II.6.20) (Cho et al., 2010).

The spinnability of the 30/70 agar/PVA blend was favored by higher flow rates and voltages (E.13 vs E.14 conditions in Table II.6.2). The greater uniformity in sizes was evident when increasing the applied electric field, yet only micron-sized fibers were formed (Fig. II.6.17 E/F). Often, two distinct types of electrospun materials were formed in the coagulating bath for this blend (e.g. E.16 conditions in Table II.6.2); one, of higher dimensions, easily collected from the ethanol and other, composed of extremely small particles, only collectable through filtration (respectively, Figs. II.6.17 E/F and II.6.18 C/D). In opposition to what was seen for the blends prepared from a 3% wt PVA starting solution, the filtered materials showed some consistency (Fig. II.6.18); several fibers at nano- scale could be observed even after the recovery process (Figs. II.6.18 C/D). Despite the irregular morphologies, the sample obtained with E.18 conditions wasn’t affected by the applied vacuum, exhibiting significant mechanical integrity (Figs. II.6.18 E/F). Oppositely, when lower flow rates and
electric fields were considered the collectable material was highly irregular and of greater dimensions (not shown). Nonetheless, nano-scale fibers could be recovered after filtration (Fig. II.6.18 A/B).

Fig. II.6.17 - Representative SEM pictures of electrospun agar/PVA mixtures at 30/70 mass ratio (agar 1% wt / PVA 5% wt) using DES-U. The electropsinning conditions used to obtain each sample are described in Table II.6.2.
In Figs. II.6.19 are reported the best 20/80 agar/PVA fibers obtained for the tested electrospinning conditions (E.19 and E.20 conditions in Table II.6.2). Even though the process was not affected by coagulating phenomena we noted that it was more difficult to find conditions able to pull out the solution from the needle. The morphology of the collected materials, at this polymer ratio, was quite interesting. When beaded materials were avoided, “noodle-shape” fibers were formed (Fig. II.6.19 A/B). The predominant effect of PVA on the
viscoelasticity of the 20/80 agar/PVA blend could explain the porous regions observed throughout the structure of these “noodle-shape” materials (Fig. II.6.20). Yet, this is a suggestion that needs further confirmation. Another interesting fact was the significant number of beads formed by the polymer jet with, apparently, best spinnability (E.20 conditions in Table II.6.2; Fig. II.6.19 C/D).

**Fig. II.6.19** - Representative SEM pictures of the agar/PVA fibers produced by electrospinning when using DES-U, at 250x (A), 2500x (B, C) and 10 000x (D) magnifications (agar 1% wt / PVA 5% wt at 20/80 mass ratio). The operational conditions used to obtain the fibers are described in Table II.6.2.

**Fig. II.6.20** - Detail of the porous regions formed for the 20/80 agar/PVA fibers illustrated in Figs. II.6.19 A/B.
When increasing the PVA concentration from 3 to 5% wt, fibers with greater consistency were formed. However, the 5% wt PVA pure solution in DES-U didn’t lead to satisfactory results, when using the several sets of electrospinning conditions listed in Table II.6.2. The greater viscosity of this sample could hinder the solution spinnability. Also, other relevant aspects known to affect the electrospinning process (i.e. surface tension, conductivity), not covered in the present study, could account for these observations. In any case, the motivation of the present study was the fabrication of agar fibers and so, no further PVA experiments in the DES-U were carried out. Moreover, PVA nanofibers can be easily produced using water as solvent.

Clearly, agar pure nanofibers could be successfully spun when using the DES-U if working at temperatures far beyond the sol-gel transition. The SEM studies reveal that the PVA addition changed greatly the morphology of the fibers. Overall, the rheological and SEM data were in good agreement. All in all, higher viscosity was advantageous for electrospinning (aqueous vs DES-U systems), although an improvement in the spinnability of the agar/PVA blends in DES-U was noticed, when the solution viscosity was decreased.
Table II.6.2 – Electrospinning conditions used for the fabrication of agar and agar/PVA fibers in DES-U. In all experiments an ethanol bath was used as collector. For more details concerning the overall process please see the experimental section.

<table>
<thead>
<tr>
<th>Figure id.</th>
<th>Agar conc. (% wt)</th>
<th>PVA conc. (% wt)</th>
<th>Agar/PVA ratio</th>
<th>Conditions Id.</th>
<th>Flow rate (mL/h)</th>
<th>Voltage (kV)</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.6.13</td>
<td>1</td>
<td>-</td>
<td>100/0</td>
<td>E.7</td>
<td>5</td>
<td>10 (1)</td>
<td>Continuous, uniform and oriented polymer jet</td>
</tr>
<tr>
<td>II.6.14</td>
<td>-</td>
<td>3</td>
<td>0/100</td>
<td>E.8</td>
<td>14</td>
<td>18</td>
<td>Continuous, uniform and oriented polymer jet; very fluid</td>
</tr>
<tr>
<td>II.6.15</td>
<td>1</td>
<td>3</td>
<td>20/80</td>
<td>E.9</td>
<td>6</td>
<td>12</td>
<td>Discontinuous, uniform and oriented jet</td>
</tr>
<tr>
<td>II.6.16 A/B</td>
<td>1</td>
<td>5</td>
<td>50/50</td>
<td>E.10</td>
<td>14</td>
<td>18</td>
<td>Continuous, uniform and oriented polymer jet</td>
</tr>
<tr>
<td>II.6.16 C/D</td>
<td>1</td>
<td>5</td>
<td>50/50</td>
<td>E.11</td>
<td>6</td>
<td>14</td>
<td>Continuous, uniform and oriented polymer jet</td>
</tr>
<tr>
<td>II.6.16 E/F</td>
<td>1</td>
<td>5</td>
<td>50/50</td>
<td>E.12</td>
<td>6</td>
<td>18</td>
<td>Continuous, uniform and oriented polymer jet</td>
</tr>
<tr>
<td>II.6.17 A/B</td>
<td>1</td>
<td>5</td>
<td>30/70</td>
<td>E.13</td>
<td>14</td>
<td>18</td>
<td>Discontinuous, uniform and chaotic polymer jet</td>
</tr>
<tr>
<td>n.a.</td>
<td>1</td>
<td>5</td>
<td>30/70</td>
<td>E.14</td>
<td>11</td>
<td>11</td>
<td>Didn’t form collectable material</td>
</tr>
<tr>
<td>II.6.17 C/D</td>
<td>1</td>
<td>5</td>
<td>30/70</td>
<td>E.15</td>
<td>6</td>
<td>20</td>
<td>Continuous, uniform and chaotic polymer jet; two distinct electrospun materials recovered from the coagulating bath (2)</td>
</tr>
<tr>
<td>II.6.17 E/F; II.6.18 C/D</td>
<td>1</td>
<td>5</td>
<td>30/70</td>
<td>E.16</td>
<td>6</td>
<td>26</td>
<td>Continuous, thin and stretched polymer jet; two distinct electrospun materials collected from the bath (2)</td>
</tr>
</tbody>
</table>

1 increased up to 20 kV during the course of the experiment; 2 See discussion for details; n.a.- not applied.
Table II.6.2 – (continuation).

<table>
<thead>
<tr>
<th>Figure id.</th>
<th>Agar conc. (% wt)</th>
<th>PVA conc. (% wt)</th>
<th>Agar/PVA ratio</th>
<th>Conditions Id.</th>
<th>Flow rate (mL/h)</th>
<th>Voltage (kV)</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.6.18 A/B</td>
<td>1</td>
<td>5</td>
<td>30/70</td>
<td>E.17</td>
<td>6</td>
<td>14</td>
<td>Discontinuous and oriented polymer jet; two distinct electrospun materials collected from the coagulating bath(^2)</td>
</tr>
<tr>
<td>II.6.18 E/F</td>
<td>1</td>
<td>5</td>
<td>30/70</td>
<td>E.18</td>
<td>6</td>
<td>28</td>
<td>Discontinuous, short and oriented jet; one electrospun material collected from the bath</td>
</tr>
<tr>
<td>II.6.19A/B; II.6.20</td>
<td>1</td>
<td>5</td>
<td>20/80</td>
<td>E.19</td>
<td>11</td>
<td>11</td>
<td>Continuous jet and oriented jet. A collectable thin fiber web was formed. Very resistant</td>
</tr>
<tr>
<td>II.6.19 C/D</td>
<td>1</td>
<td>5</td>
<td>20/80</td>
<td>E.20</td>
<td>14</td>
<td>18</td>
<td>Continuous and oriented bath. A collectable thin fiber web was formed. Not too resistant</td>
</tr>
</tbody>
</table>

* See discussion for details.
II.6.4. Conclusions

Novel agar-pure and agar composite fibers were successfully fabricated by electrospinning using water and DES-U as solvents.

The low viscosity of agar/PVA aqueous blends resulted in poorer spinnability. Pure agar nanofibers could only be collected at 1% wt concentration when replacing water by the DES-U. The morphology of agar/PVA fibers could be tailored by manipulation of the final blend composition and the electrospinning conditions. The combination of a co-blending polymer and a DES could further improve agar spinnability and variability in the fibers morphologies. This could broaden the potential niches of applications of these novel materials.

It is our hope that the present study will provide good foundations for future researches towards the development of new agar-based micro and nanofibers. Deep eutectic solvents (DES) can be very promising tools to this end. The successful use of the ChCl/Urea eutectics at 1:2 molar ratio for the fabrication of electrospun agar fibers will most likely stimulate investigations focusing on other natural polymers.

In order to explore the full potential of agar-pure fibers, the electrospinning apparatus is currently being adapted to allow a wider range of processing temperatures. In this first approach, a CA sample was used but the encouraging results attained will definitely move us to explore alternatively extracted agars from sustainable resources. This can be of great interest from a perspective of sustainable development.

References.


FINAL CONCLUSIONS & REMARKS
In the first stage of the PhD thesis, it was clearly demonstrated that, by applying MAE, higher yields and reproducibility as well as AA with the most desirable GS were achieved when compared to TWE.

Given the advantages of MAE, future work focusing on the degradation process of NA under microwaves must be conducted, since this type of agars seemed to be more sensitive to microwaves than AA (higher yields were obtained but the resulting agars were more degraded which was reflected in their lower GS). Nevertheless, a strict control of MAE conditions can produce NA with GS appropriate to soft-texture food products applications, while spending much less time, energy and solvent in the process.

This work suggests the feasibility of exploitation of *G. vermiculophylla*, wild or cultivated in IMTA systems, for production of agar gels with superior quality. Ultimately, the exploitation of a national underused resource such as *G. vermiculophylla*, could help maintain the coastal ecosystems equilibrium. Considering the seasonal variation on the abundance and quality of the biomass harvested from wild populations, the production of seaweeds in IMTA systems can offer a continuous supply of raw material with reliable quality to the transformation industries.

At molecular level, MAE can shape agar assemblies which can be of great interest towards the development of new agar-based materials for emerging applications. MAE agars showed greater capacity for self-association than TWE agars, at comparable conditions, which seemed in accordance with their higher GS, consistency and thermal stability. The 3D gel network of MAE agars were compact and regular contrasting with the more open structures of TWE agars. The image studies support well the view proposed for agar gelation, *double helix* formation followed by intensive interhelical association.

During the second stage of the PhD project, it was clearly demonstrated that agar can be processed in innumerous forms to fabricate materials with various functional properties and with the most desirable performances to be used in a broad spectrum of end-use applications.

Interesting molecular interactions can outcome from mixing agars from *G. vermiculophylla* with LBG, which will manifest differently, depending on the form of the material. The incorporation of LBG in agar films made the process fabrication of the materials easier (dramatic increase of viscosity and decrease in the gelling character of the solutions) and led to a significant improvement in the films' functional properties. Also, our study seems to indicate that NA can be used to fabricate agar-based films with comparable or even better
properties than AA. This can be very attractive for the transformation industries since NA are cheaper to produce than AA (no need for pre-treatment prior to the extraction process). Moreover, the addition of LBG, at percentages as high as 50-75%, will further reduce the cost of the films since this polysaccharide is cheaper than agar. In the hydrogel form, various and complex rheological textures were obtained, which can be very important for the fabrication of food products with appealing 'mouth feel' for the consumers. Both SAOS and LAOS gave important and complementary information about the rheological agar/LBG systems, and clearly, evidenced differences in the rheological behavior of NA and AA mixed systems. These findings could be related with the unique physicochemical nature of each galactan; however, further studies must be conducted to shed more light on this topic.

By replacing water by DES-U, new agar-based materials, with interesting functional properties, could be obtained. Elastic (non-aqueous) films, with good mechanical resistance, were produced by the proposed three-step methodology: pre-solubilization of agar in DES-U, thermo-compression of agar/DES-U systems and drying. The unusual trends in the studied properties seemed to result from a complex conjugation of factors related with the differences in polymer and DES-U contents of the resultant film most likely, associated with processing limitations. Hence, further work is clearly needed to improve the processing conditions of these materials, which will enable a stricter control of the materials’ functional properties. Ultimately, this will allow us to take advantage of the true potentialities of these new materials.

Agar-based fibers were successfully produced by electrospinning, at 50 ºC, using water and DES-U as solvents. Constraints in processability due to agar gelation could be minimized, by using a tubeless spinneret placed inside the electrospinning chamber. The low viscosity of agar/PVA aqueous blends resulted in poorer spinnability. Pure agar nanofibers were successfully electrospun when using DES-U yet, the temperature decrease inside the chamber, on the course of experiments, led to agar gelation. The morphology of agar/PVA fibers could be tailored by manipulation of the final blend composition and the electrospinning conditions. The combination of a co-blending polymer, such as PVA, and a DES, could further improve agar spinnability and lead to various fiber morphologies.

In summary, the MAE approach supports sustainable development, as it requires less energy and solvent than conventional processes, while generating fewer wastes. MAE compared favorably with conventional methodologies used to extract agars and the excellent gelling properties obtained, particularly in the case of AA, seems definitely worth it to explore. Considering that one major issue of the hydrocolloid industry is the outdated machineries often lacking efficiency used by most manufacturers, the development of new and more
competitive extraction processes would definitely help companies to keep competitiveness. The successful application of MAE technique in agar recovery seems to have excellent receptiveness from companies in the field. Throughout these 4 years, the feedback we had from people working with agar and other related polysaccharides was very positive. The implementation of agar MAE at an industrial scale is most likely to become a reality in a near future since there is already ongoing work focusing on the process scale-up.

At R&D level, a deeper investigation focusing on the stability of NA backbone when exposed to microwaves could be of great interest to companies since NA are more cost-attractive and showed comparable or even better performances than AA in some applications (aqueous-blended films).

Wild and farmed G. vermiculophylla constitute sustainable national underexploited resources that can be used to extract agars of commercial grade. This is particularly relevant since the main commercial use of agar is, by far, in the gel form. The use of controlled grown IMTA seaweeds can be more attractive since it enables tunability of the SP final properties avoiding common variability problems affecting wild biomass (physiological and environmental factors). This can be crucial for emergent transformation industries such as the bioplastics. Also, recent attempts of seaweed exploitation for the production of PLA show that the best for the marine-based bioplastics is still yet to come.

The benefits of using seaweeds and SP to produce bioplastics are obvious. Their high versatility and great functionality place them as frontrunners when it comes to find a second generation of non-food crops that meets the goals of sustainable development for the bioplastic sector. Strategic plans such as Horizon 2020 or incentives given to emerging industries such as the bio-refinery will most likely place algae and algae industry in the spotlights for the years ahead. Moreover, the increased interest in other related sectors relying on marine products can further project this industry in the global industrial scenario.

At the moment, the bioplastic market seems very receptive to melt-processed biocomposites reinforced with seaweed fillers. For instance, biopropylene® 109D plastic, is a biocomposite filled with 20% algae matter resulting from industrial processing to extract specialty chemicals (http://www.cereplast.com/wp-content/uploads/Biopropylene-109D-TDS-12-11-12.pdf) recently launched by Cereplast Inc.. Besides competitive performances in relation to its synthetic counterpart, biopropylene® 109D can be processed in conventional processing machinery such as injection molding while reducing the carbon footprint. In the automotive sector, during the next decade, the Japanese company Toyota expects to launch its first seaweed-fiber reinforced car, taking advantage of the abundant marine resources of the country. Other good example comes from the two commercial products algopack® and algoblend® made from seaweeds of a different family than Ulva to be used in a wide array of
end-use applications such as packaging, supports, signs and others (http://www.algopack.com/algopackgb.php).

Yet, in order to grow on solid ground and be sustainable, this bioplastic sector based on marine products should be competitive (i.e. cost and performance-attractive) and always think ahead, in order to respond to the market needs at all stages. From a material developer’s perspective, the priority should be the development of marine-based bioplastics that can be used in already implemented operation lines such as melt-processed composites. Other key point is to ensure controlled quality of marine-based crops that meet the performance requirements of plastic converters and manufactures. In this regard, aquaculture grown seaweeds can be an excellent option. Also, it should promote the use of optimized cascade schemes involving several segments of algae industry with efficient life-cycles of the materials hence, complying with the sustainability requirements. In this regard, the market is already giving some very positive signs. For instance, Algix LLC adds value to by-product biomass from agriculture and other industrial segments through bioremediation, which can be subsequently used as quality-controlled raw material for the production of bioplastics (http://www.algaeindustrymagazine.com/algix-developing-algal-based-plastics/; http://algixllc.com/).

The outstanding gelling capacity of agar when compared to similar biopolymers can help revolutionize emergent fields. Materials made from SP such as films, hydrogels or fibers can conquer specific market niches where the final applications justify the cost productions. The possibility of tuning the growth conditions of seaweeds to improve its yield in a targeted polysaccharide with tailored properties, can also be a promising route for these more specific marine-based materials.

Blending agar with LBG can be a good strategy to obtain various and complex textures (hydrogels) as well as materials with enhanced functional properties (films). The interactions between Gracilaria agars and LBG seem undermined and should be explored more thoroughly, particularly the NA option which are cheaper alternatives to AA.

For specific applications, choline-derived IL analogues such as DES-U can hold the potential to unlock some of the processing difficulties associated to gelling polymers when processed in water. Agar-based materials with completely different shapes and functionalities can be produced by simply replacing water by DES-U. Yet, to leap forward from R&D to industrial level, further studies must be conducted to gain more insight on the true green nature of these DES.
Meanwhile, the promising results obtained with the electrospinning studies made us pursue this line of research; currently, we are working to improve the processing conditions of the electrospinning equipment particularly, in what the temperature control is concerned.

Despite the final goal of this project wasn’t fully accomplished (there is still ongoing research focusing on the production of materials using MAE agars) we strongly feel that the results gathered throughout these 4 years are very encouraging and makes us believe we chose the right line of research when we outlined this project. With strategic plans such as Horizon 2020 pointing sustainability as the only possible road, agar, which is a highly versatile biopolymer that can be processed in several forms, is an excellent candidate to explore, particularly if sustainable seaweed resources and extraction methodologies such as MAE are considered.

Hence, it seems quite reasonable to say that the future seems very bright for this new generation of materials based on SP obtained from sustainable grids of production.