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Mona Soltani, Claire Bordes, Dounia Ariba, Mustapha Majdoub, Hatem Majdoub, et al.. Emulsifying properties of biopolymer extracts from *Opuntia ficus indica* cladodes. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2024, 683, pp.133005. 10.1016/j.colsurfa.2023.133005 . hal-04365731

HAL Id: hal-04365731

<https://hal.science/hal-04365731>

Submitted on 28 Dec 2023

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Emulsifying properties of biopolymer extracts from *Opuntia ficus indica* cladodes

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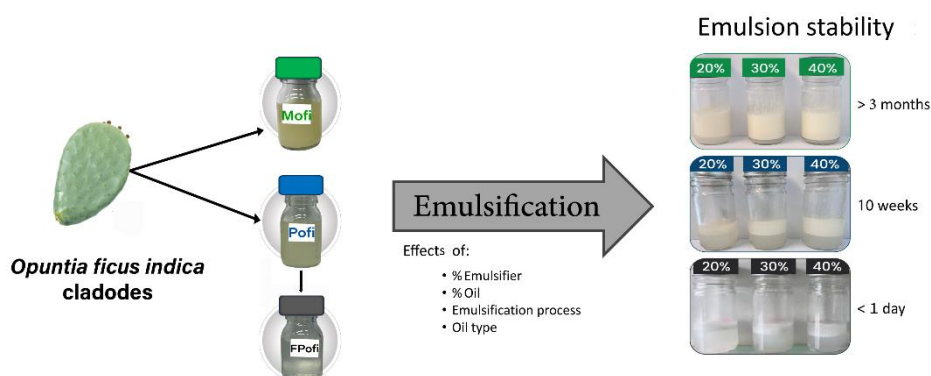
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Highlights

- Mucilage from *Opuntia ficus indica* allows the stabilization of o/w emulsions
- The insoluble part of the mucilage is the actual stabilizing agent
- The soluble part made of purified pectin cannot stabilize emulsions
- A Pickering effect stabilizes the emulsions

Graphical Abstract



Abstract

The use of natural extracts in the field of cosmetics and pharmaceuticals is increasing due to the growing demand for sustainable and safe products, together with their potential therapeutic benefits. Bioextracts from the parenchyma of *Opuntia ficus indica* cladodes were characterized and evaluated as emulsifying agents. They contain pectin together with variable amounts of arabinogalactan-protein complex (AGP), as in many polysaccharides of natural origin. Their protein content varies according to the purification steps: 11.4 wt% for the raw mucilage (Mofi), 4.7 wt% for the pectin fraction (Pofi) and less than 0.5 wt% for the filtrated pectin fraction (FPofi). Part of the AGP contained in the Mofi and Pofi extracts precipitates as particles with a $\sim 5 \mu\text{m}$ diameter. Mofi at 1.5 wt% concentration is an efficient emulsifier of oil-in-water emulsions of medium-chain triglycerides or sunflower oil with stability against coalescence for more than three months. Pofi is less efficient and FPofi does not allow the stabilization of emulsions. The performance of emulsifiers is directly related to the protein content of the extracts and the role of the insoluble particles is essential. The insoluble particles of AGP are responsible for the stabilization through a Pickering effect. The emulsions have the characteristic properties of Pickering emulsions.

Keywords: *Opuntia ficus indica*, biopolymers, mucilage, emulsifier, emulsion

1. Introduction

Consumer demand for “clean” labels has increased over the past decade, favoring biomass-derived products with fewer chemical treatments/modifications and reduced energy consumption, to ensure their accessibility, safety and sustainability.¹ In the field of cosmetics, synthetic excipients are increasingly being replaced by natural ones, especially emulsifiers used to stabilize the many emulsion-based systems.² As surface active ingredients, synthetic emulsifiers can cause health problems and toxic symptoms, as well as environmental concerns due to their poor biodegradability.³ Therefore, replacing them with natural products from renewable sources has become a challenge, especially in the food, cosmetic and pharmaceutical industries to improve sustainability and ensure better consumer’s safety.⁴ In this context, biopolymers are attracting much attention considering their renewable source, biocompatibility, biodegradability, eco-friendliness and non-toxicity.⁵ Their rich and diverse structures give them several interesting functions such as thickeners, foam stabilizers, fixatives and emulsifiers.⁶

Emulsifying properties are thought to arise from proteins associated with polysaccharides that are not themselves surface active. This can be achieved by nature, as many gums and mucilages consist of polysaccharides naturally conjugated with proteins, or by mixing polysaccharides with proteins to form surface active associations or precipitation of complex coacervates.⁷ The emulsion stabilizer role of the protein part of polysaccharides extracted from plants is a common idea, mostly based on experimental observations of emulsion stabilization. This has recently been experimentally confirmed in the case of Acacia gum showing the preferential adsorption of the gum fraction with higher protein content and higher molar mass on o/w emulsion droplets.⁸ Combining the environmental stability and physicochemical properties of polysaccharides with the emulsifying effect of amphiphilic proteins may result in effective and safe bioemulsifiers.^{9,10} Emulsion stabilization can also be achieved using associations of biopolymers,¹¹ like gelatin and gum Arabic¹² or whey protein and pectin^{13,14}. It is also possible to extract natural polysaccharide-protein complexes from plant mucilage.^{15,16} Combination of polysaccharides and proteins allow the encapsulation of o/w emulsion droplets by complex coacervation in the design of drug delivery systems.¹⁷

Mucilage is a physiological product of plants¹⁸ and is formed in plant cells as a thick sticky substance that swells on contact with water. It contains mainly carbohydrates, proteins, and minerals. The presence of carbohydrates and proteins enables the mucilage to form three-dimensional networks that are potentially useful as encapsulating agents.¹⁹ Mucilage is mainly extracted from seeds,²⁰ although it is also found in other parts of plants. For example, in cactus plants (*Opuntia spp.*), the mucilage is distributed in the cladodes and the fruits.¹⁸

A common plant source is the species *Opuntia ficus indica* (Ofi) (called prickly pear). It is found in many countries around the world;²¹ it is abundant in the countryside of Tunisia. The mucilage is found in the parenchyma tissue inside the cladodes. The cladodes of *Opuntia ficus indica* have gained attention in recent years as an interesting source of bioactive compounds.²² It has been investigated in terms of composition and potential biological activities: the composition of the mucilage, its structural characteristics and physicochemical

properties vary according to the growing region, climate, soil type, harvesting period/time, age of the cladodes and finally on the type of extraction process.^{23,24,25} The molar mass of pectin extracted from Ofi mucilage ranges from 2×10^5 to 6×10^6 g·mol⁻¹.^{26, 27} Its monosaccharide content consists of rhamnose, arabinose, galactose, galacturonic acid, xylose, mannose and glucose with the following respective mass percentages: 46.0, 15.0, 11.0, 10.2, 9.1, 4.1 and 1.9%.²⁶ Mucilage proteins belong to the albumin family.²⁸ In addition, mucilage has many interesting biological activities such as antioxidant,^{29,30} wound, burn and ulcer healing,³¹ hypoglycemic effect³² and others.³³ Due to its rheological properties and low toxicity, it has been proposed as an effective pharmaceutical excipient.³⁴

A few papers have disclosed the emulsifying properties of Ofi extracts in combination with xanthan gum³⁵ or sodium caseinate.³⁶ Interestingly, the stabilization of emulsions against coalescence was improved by cross-linking beet sugar pectin with genipin.³⁷

The aim of this study was to investigate the emulsifying properties of three different biopolymer extracts from Ofi mucilage obtained by using a simple, environmentally friendly and safe extraction process. The first extract corresponded to lightly purified crude mucilage (Mofi), the second was a lightly purified polysaccharide extract (Pofi), and the last one (FPofi) was obtained by filtration of Pofi. The physicochemical properties of the three extracts were investigated, and their stabilizing capacities for o/w emulsions were evaluated for different oil types and biopolymer concentrations.

2. Materials and methods

2.1. Materials

Opuntia ficus indica cladodes were collected in the region of Hajeb Laayoun Kairouan (Tunisia). Medium chain triglycerides (MCT), Myritol 318, was a gift from BASF, France. Sunflower oil (SFO) was purchased from a local grocery store. Deionized water of 18 MΩ·cm resistivity was used all along.

2.2. Preparation of Ofi extracts

Three different extracts were isolated from Ofi pulp and were designated as Xofi, where X = M for the mucilage, X = P for the polysaccharide extract and X = FP for the filtered polysaccharide extract.

The Ofi cladodes were washed, despined and peeled to recover the pulp (Ofi), also called parenchyma, which is rich in mucilage. It was then diced and stored at -15 °C until freezing. The frozen pulp was left to thaw and the crude mucilage was collected. The retrieved hydrocolloid was diluted once with purified water and centrifuged for 5 min at 3500 rpm to eliminate solid residues and impurities. The supernatant was dialyzed using dialysis bag of molar mass cut-off 7 kDa. The dialysate was centrifuged (3000 rpm, 10 min) to eliminate insoluble or degraded fractions, and the supernatant was lyophilized to obtain the mucilage extract (Mofi). Solids content was determined by drying known volumes of the crude mucilage at 50 °C until a constant mass was reached. The yield of Mofi was calculated as:

$$\%Mofi = m_{Mofi} / m_{CMofi}$$

where m_{Mofi} is the mass of the obtained freeze-dried Mofi and m_{CMofi} is the mass of the crude mucilage used for the extraction.

The remaining pulp was used for polysaccharides extraction; it was macerated in deionized water (500 g in 1.5 L) for 24 h, and a conventional pectin extraction was performed as described by Jiang et al.³⁸ with some modifications: the mixture was acidified with hydrochloric acid (1 M) down to a pH of 2.6³⁹, it was then heated to 75 °C for 3 h under mechanical stirring (500 rpm). The mixture was then neutralized up to a pH 6 and cooled down to room temperature. Solid residues were eliminated by filtration and the filtrate was centrifuged at 4500 rpm for 10 min. The supernatant was precipitated with 3 volumes of ethanol 96%. The precipitate was dissolved in deionized water and dialyzed for 72 h via a 7 kDa cut-off dialysis membrane. The dialysate was finally centrifuged. The supernatant was freeze-dried to provide the second extract (Pofi). The extraction yield was determined as follow:

$$\%Pofi = (m_{Pofi} / m_{Ofi}) \times 100$$

where m_{Pofi} is the mass of freeze-dried Pofi and m_{Ofi} is the mass of remaining Ofi pulp after mucilage removal.

Aqueous solutions of 1.5% Pofi were centrifuged at 10000 rpm at 4 °C for 2 h. The resulting pellet was dried and weighted as Residue 1 (m_1). The supernatant was filtered through 0.45 µm syringe filter. The mass difference before and after filtration was Residue 2 (m_2). The transparent extract obtained after filtration was designated as FPofi.

The amount of insoluble particles is $m_1 + m_2$, and the percentage of solid residue in Pofi was determined by:

$$\%SR = (m_1 + m_2) / m_{pofi} \times 100$$

2.3. Characterizations of *Opuntia ficus indica* extracts

Elemental analyses for carbon, hydrogen and nitrogen were performed on two replicates using a FlashSmart™CHNS/O elemental analyzer (Thermo Fisher). The samples have been dried under reduced pressure at 50 °C before their analyses. The protein contents were calculated from total nitrogen content using the value of the nitrogen/protein conversion factor of 6.0 taken as an average of factors calculated from the amino acid sequences of several proteins.^{40,41}

Size Exclusion Chromatography (SEC) analyzes of a FPofi aqueous solution were performed using a Wyatt instrument equipped with PL Aquagel OH mixed M8 micro and PL Aquagel OH mixed H8 micro columns and with refractive index, UV absorbance at 280 nm and multi-angle light scattering (MALS) detectors. 100 µL of FPofi solution was injected into a mobile phase of 0.1 M NaNO₃ at pH 7 flowing at 0.5 mL·min⁻¹.

Particle size distributions of the extracts and emulsions were measured at 25 °C by small-angle light scattering with a MasterSizer 3000 instrument (Panalytical Malvern, UK). The emulsions were diluted with water till the obscuration reached 5–7%. The scattering pattern was interpreted in terms of particle size distribution by the Mie theory using 1.33, 1.45, 1.47 and 1.60 for the refractive indices of water, MCT, SFO and proteins. The refractive index of proteins

(that the exact composition is not known) was taken as 1.60, which is an average value for albumins and many other proteins.⁴² The droplet size distributions of emulsions were expressed through their median diameter $D(50)$ and $Span = [D(90) - D(10)]/D(50)$.

Optical microscopy pictures were taken using a Leica DMLM (Germany) microscope equipped with a video camera. One drop of the emulsion spread between a glass plate and a cover slip was observed in transmission mode. Images were analyzed with the ImageJ® software to determine the droplet size distribution.

Zeta potentials were measured by electrophoresis with a NanoZS instrument (Panalytical Malvern, UK). 1.5 wt% solutions of every extract were prepared beforehand, then diluted 20 folds in 10^{-3} M NaCl aqueous solution. The pH was adjusted with either sodium hydroxide or hydrochloric acid. Measurements were performed in DTS1070 U-shaped tubes equipped with electrodes. The zeta potential was calculated from the electrophoretic mobility using the Henry law under the Smoluchowski approximation valid for moderate and high ionic strengths.

Rheological experiments were performed using a rheometer MCR 302 (Anton Paar) equipped with a CP-50 cone-plate geometry (50 mm diameter and cone angle of 1°).

Interfacial tension between aqueous extracts and MCT (Myritol 318) was measured by the pendant drop method using a DSA10Mk2 instrument (Krüss, Germany). A pendant drop of aqueous extract at the tip of a syringe needle of 0.5 mm diameter was immersed in a MCT medium. The image of the drop was analyzed with the DSA10Mk2 software using the densities of water and MCT respectively equal to $0.9982 \text{ g}\cdot\text{cm}^{-3}$ and $0.947 \text{ g}\cdot\text{cm}^{-3}$ at 20°C .

2.4. Emulsifying properties

2.4.1. Preparation of emulsions

Aqueous solutions of Xofi (at 0.5, 1.0 and 1.5 wt%) were prepared in advance and left stirring overnight for completion of solubilization. Emulsions containing 20, 30, 40 or 50% (v/v) MCT were prepared by mixing defined amounts of both phases at 24000 rpm for 10 min with a rotor-stator device Ultra-Turrax® T25 equipped with a S25N-18G shaft (IKA, Germany). Emulsions were then stored 24 h at room temperature before further analysis.

To evaluate the effect of the emulsification process, similar emulsions were prepared at 14500 rpm with an Ultra-Turrax® T10 equipped with a S10N-10G shaft (IKA, Germany).

For comparison purpose, the applied shear strain rates were calculated. The geometry of an Ultra-Turrax rotor-stator mixer is the same as for a Couette geometry of a rheometer made of coaxial cylinders of internal and external radii R_{int} and R_{ext} . The gap is $R_{\text{ext}} - R_{\text{int}}$. For a gap narrow compared to these radii, the shear strain rate is:

$$\dot{\gamma} = \frac{d\gamma}{dt} = \omega \frac{R_{\text{ext}} + R_{\text{int}}}{2(R_{\text{ext}} - R_{\text{int}})}$$

where ω is the angular velocity defined by $\omega = \frac{2\pi}{60} \times N$, N being the rotation rate in rpm. The shear strain rates in the gap were 107652 s^{-1} and 58460 s^{-1} for the Ultra-Turrax® T25 with a S25N-18G shaft and the Ultra-Turrax® T10 with a S10N-10G shaft respectively.

2.4.2. Stability of the emulsions

The emulsions were observed visually and the oil droplet size distributions were measured by small-angle laser light scattering. The average droplet size was characterized by the volume median diameter $D(50)$, which is the particle diameter at 50% cumulative volume.⁴³ These droplet size measurements were confirmed by optical microscopy. For emulsions showing an already formed oil layer on top at the end of the emulsification process, a simple upside-down shake was performed to homogenize the samples prior to size analysis.

Stability was first assessed by visual observations, which mostly gave information on creaming. Droplet size measurements at different storage times allowed the identification of the less stable emulsions. For the most stable emulsions where no coalescence could be visually observed, accelerated destabilization by centrifugation was performed by using a Dispersion Analyzer LUMiSizer® (LUM GmbH, Berlin, Germany). This analytical centrifuge measures the time evolution of the transmitted light over the full depth of the samples leading to the detection of the coalescence, creaming or sedimentation processes. Emulsions were centrifuged at 3000 rpm in the measurement cell of the LUMiSizer® at 25 °C for 14 h. 170 transmittance profiles were recorded at time intervals of 5 s during the first minute, then 10 s between 1 and 2.5 min, 20 s between 2.5 and 6 min, 30 s between 6 and 20 min, 4 min between 20 and 2 h, and finally 8 min between 2 h and 14 h.

3. Results and discussion

3.1. Biopolymers extraction

The extraction process was designed with the quality of the final extract and its suitability for cosmetic and pharmaceutical formulations in mind. Only very necessary and green purification steps were used. Precipitation was carried out in the environmentally friendly solvent ethanol.⁴⁴

The steps for the preparation of the different extracts are shown in [Figure 1](#). Fresh Ofi juice was a greenish hydrocolloid with a slimy and slightly sticky texture. Ofi was freeze-thawed to facilitate the collection of the mucilage without additional solvent. As the freezing of liquid water to ice caused a volume expansion, cell walls were broken and cell contents were released. This process yielded a liquid fraction and a solid residue. Low speed centrifugation of the liquid fraction was used to remove the largest pieces of insoluble materials. Dialysis was performed to remove low molar mass compounds and salts, so as to ensure that the evaluated emulsifying properties were actually due to the biopolymers. This stage yielded the Mofi extract, which is the “raw mucilage”.

Pofi was prepared from the solid residue by a conventional extraction of polysaccharides. A few mild purification steps involved a prolonged exposure to a warm acidic medium that denatured the proteins and eliminated a maximum amount of them together with other water-insoluble compounds without resorting to enzymatic treatment or extraction with organic solvents. Polysaccharides are often extracted under acidic conditions that help at opening the cell walls, leading to improved dissolution in the extraction solvents and better extraction yields.⁴⁵ At a pH below 2, acid hydrolysis caused the degradation of polysaccharides

and this effect increased with decreasing pH.⁴⁶ The medium was neutralized at the end to stop the hydrolysis of the protopectin acid chains, allowing the recovery of most of the pectin fractions.⁴⁷ Precipitation with ethanol followed by dialysis and centrifugation of the aqueous solution yielded the Pofi extract. Finally, the turbid Pofi sample was filtered through a 0.45 µm syringe filter to yield a clear solution called FPofi.

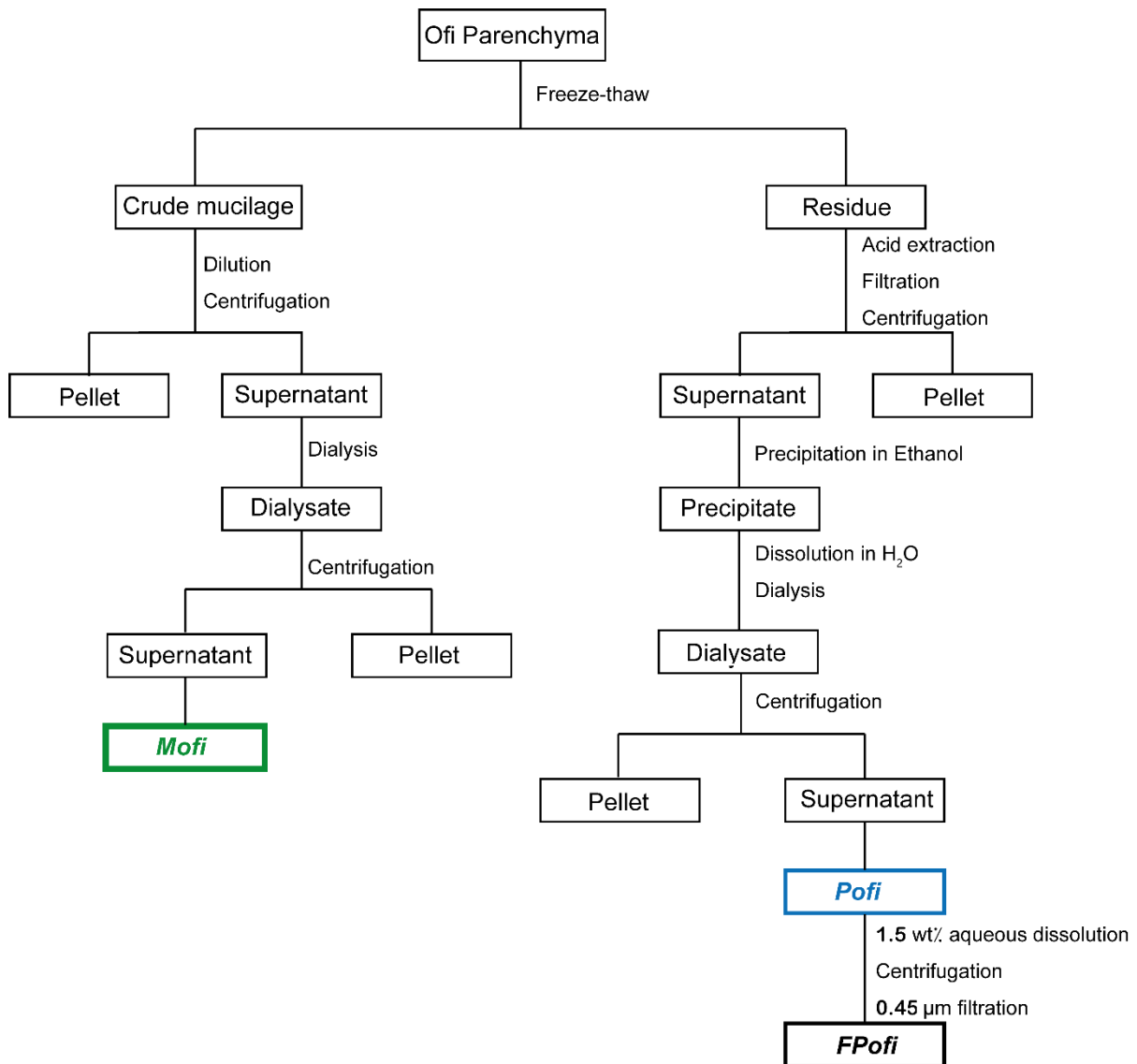


Figure 1. Extraction and purification steps yielding the Ofi extracts: preparation of Mofi, Pofi and FPofi samples.

The yield of Mofi was calculated on the basis of the dry mass of the crude mucilage extracted from Ofi pulp. A low yield of 6.1 ± 0.3 wt% was obtained, probably due to the presence of a high proportion of solid residues and plant impurities contained in the crude mucilage before its filtration. The extraction of Pofi from the parenchyma residue gave a yield of 0.16 ± 0.01 %. This value was in the highest range of previous reports of comparable extraction procedures (0.06–0.18 wt%).^{27,39} It has been reported that extraction yields from Ofi cladodes based on fresh mass are generally less than 2% (and close to 19% expressed as dry mass).⁴⁸ Pofi

solutions were clarified by filtration to yield FPofi. The amount of insoluble particles removed was 10.9 ± 0.6 wt%. Overall, the successive purification steps of Xofi aqueous "solutions" reduced the content of suspended microparticles and finally produced a clear FPofi solution (Figure 2).

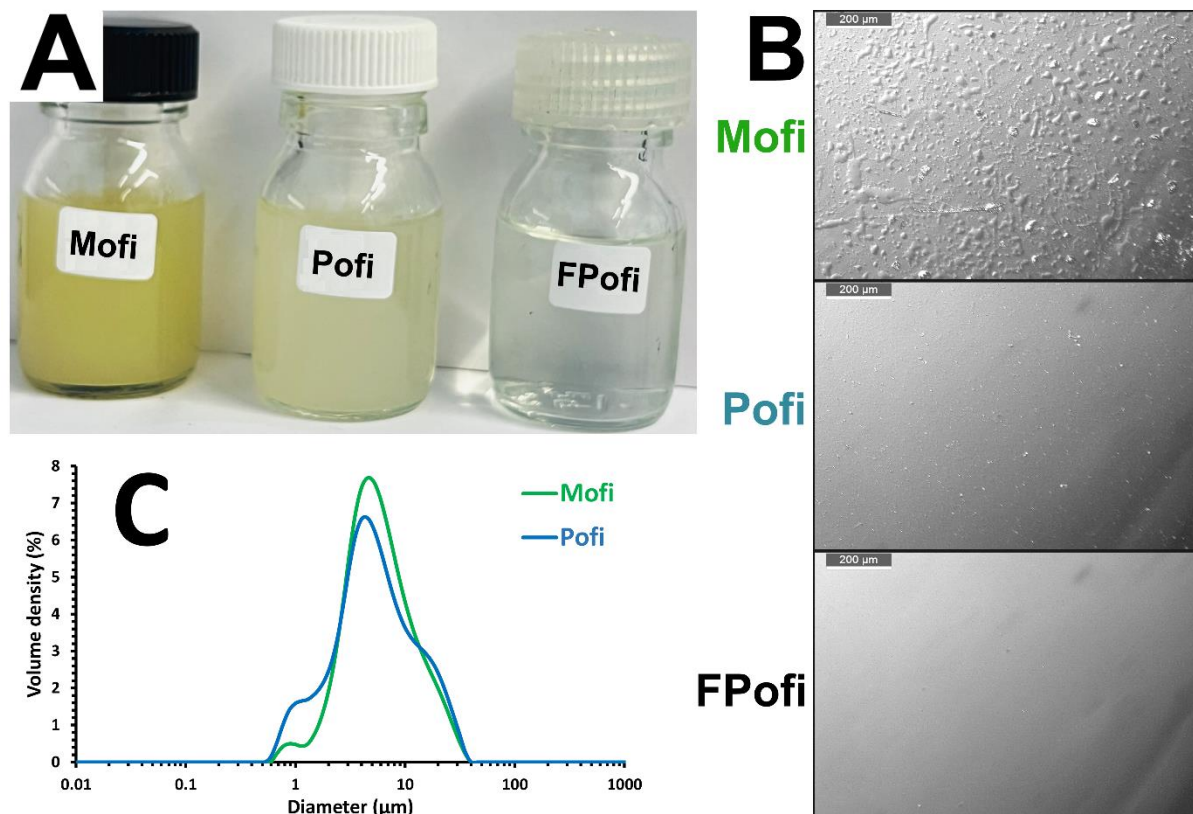


Figure 2. Pictures of the Mofi, Pofi and FPofi samples (A), optical microscopy images of 1.5 wt% Xofi samples (B, the scale bars are 200 μm), and particle size distributions of Mofi and Pofi aqueous extracts measured by small-angle light scattering (C).

3.2. Biopolymers characterization

The aspect of aqueous solutions of the different extracted fractions is shown in Figure 2A. Mofi, Pofi and FPofi samples in water were respectively opaque, translucent and transparent, which was consistent with the purification degree of each.

Elemental analyses for C, H and N were performed on the dry samples (Table 1). Completion of drying was ensured by checking that the measured sample mass did not vary with prolonged drying. The results of elemental analyses also indicated the absence of moisture. Thus, the C/H ratio of 6.6 for all samples is the expected value for dry organic materials. As the main elements are C, H, N and O, the remainder to 100% after C, H, N analyses was considered as coming from oxygen. Its mean value slightly above 50% matches the oxygen content of carbohydrates having ~6% hydrogen content (1 mole of O for 2 moles of H). Indeed carbohydrates are the main components of the samples as indicated by the moderately low protein contents. The protein contents of the three extracts were deduced from their

elemental nitrogen analyses taking a multiplicative factor of 6.0.^{40,41} The protein content of Mofi was quite high. It was lower in Pofi and below the limit of detection of elemental analysis of nitrogen (0.3 %) for FPofi.

Table 1. Elemental analyses for C, H and N and the corresponding protein contents.

Sample	% elements	Protein content (wt%)
Mofi	C 40.8 ± 0.5	11.4 ± 0.4
	H 6.2 ± 0.2	
	N 1.9 ± 0.1	
Pofi	C 40.7 ± 0.3	4.7 ± 0.6
	H 6.1 ± 0.2	
	N 0.8 ± 0.1	
FPofi	C 40.5 ± 0.5	smaller than the LOD
	H 6.2 ± 0.2	
	N < 0.3	

The ¹H NMR spectra of Mofi, Pofi and FPofi in D₂O solution were identical, showing that the soluble fractions were the same. The contents of galacturonic acid and its methyl ester in the soluble FPofi were determined by pH-metric titrations reported in the [Supplementary Information](#) file. The total galacturonic acid content was 26.4 mol%, divided into 11.4 mol% for the free acid form and 15.0 mol% for the methyl ester form. The pK_a of FPofi was 3.7, in agreement with the literature value for general pectin (3.5).^{49,50}

SEC analysis of a 1 mg·mL⁻¹ FPofi aqueous solution was performed using multiple detections of refractive index, UV absorbance and MALS ([Figure 3](#)). The peak between 20 and 25 min retention time was used to calculate the FPofi molar masses. The refractive index detection allowed the determination of the specific refractive index increment $dn/dc = 0.150 \text{ mL}\cdot\text{g}^{-1}$. The number and mass-average molar masses of FPofi were determined from the Zimm plot of MALS. The calculated number-average and weight-average molar masses were $M_N = 5.8 \times 10^6 \text{ g}\cdot\text{mol}^{-1}$ and $M_W = 7.8 \times 10^6 \text{ g}\cdot\text{mol}^{-1}$ (polydispersity index $M_W/M_N = 1.3$). The very high molar mass of the soluble fraction was in agreement with previous measurements on Ofi extracts,²⁷ indicating that the extraction treatment did not significantly break the polymer chains of pectin. A significant UV absorbance signal was detected at the retention times of FPofi, showing that a fraction of proteins was still attached to the polysaccharide after filtration of Pofi.

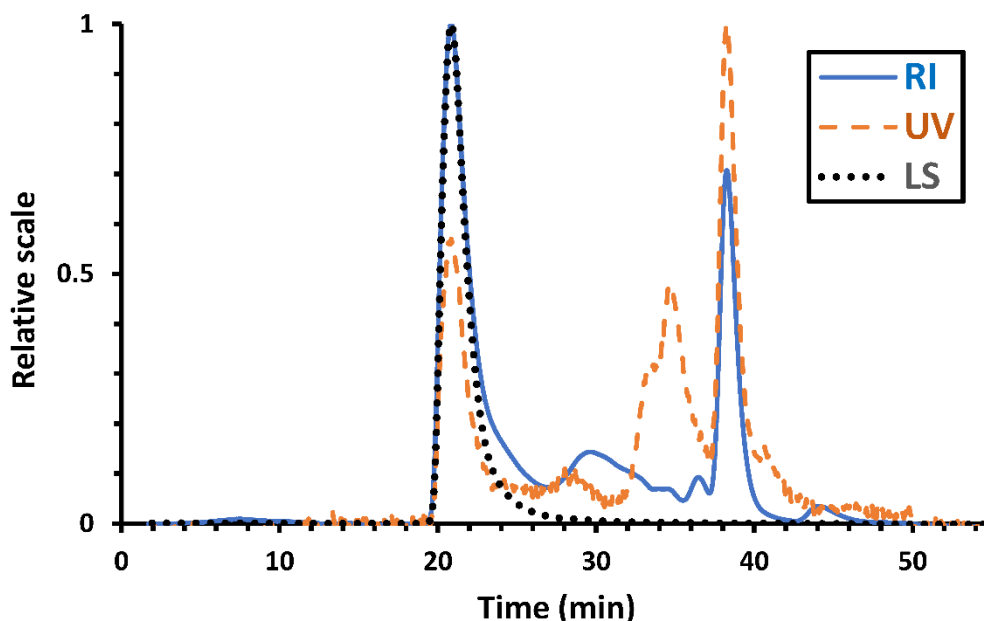


Figure 3. Size exclusion chromatography refractive index (RI, full line), UV absorbance (UV, dashed line) and light scattering (LS, dotted line) signals of a $1 \text{ mg}\cdot\text{mL}^{-1}$ FPofi aqueous solution.

Optical microscopy images of Mofi (Figure 2B) showed the presence of aggregated microparticles, responsible for the opacity of the samples. Such water-insoluble compounds were less abundant in Pofi samples, and they were not detected in FPofi. This was consistent with the protein contents derived from nitrogen elemental analyses: $11.4 \pm 0.4\%$ for Mofi and $4.7 \pm 0.6\%$ for Pofi. The loss of protein content with successive purification steps indicated that the insoluble particles mostly contained proteins.

Both Mofi and Pofi extracts showed similar particle size distributions centered at $D(50) = 5 \mu\text{m}$ (Figure 2C). No particles were detected in FPofi, suggesting that the water-insoluble compounds were eliminated to completion by the filtration process.

The zeta potential variations with pH (Figure 4) were similar for the different extracts. Increasing the pH deprotonated the acidic groups of the uronic acids, resulting in negative surface charges revealed through the zeta potential assuming more negative values as the pH was increased. The isoelectric point was at pH 2. Above pH 5.5 (red dots in Figure 4), the zeta potential remained relatively constant for each extract at about -30 , -28 and -25 mV (for Mofi, Pofi and FPofi, respectively). The zeta potential profiles were in accordance with a previous study.⁵¹

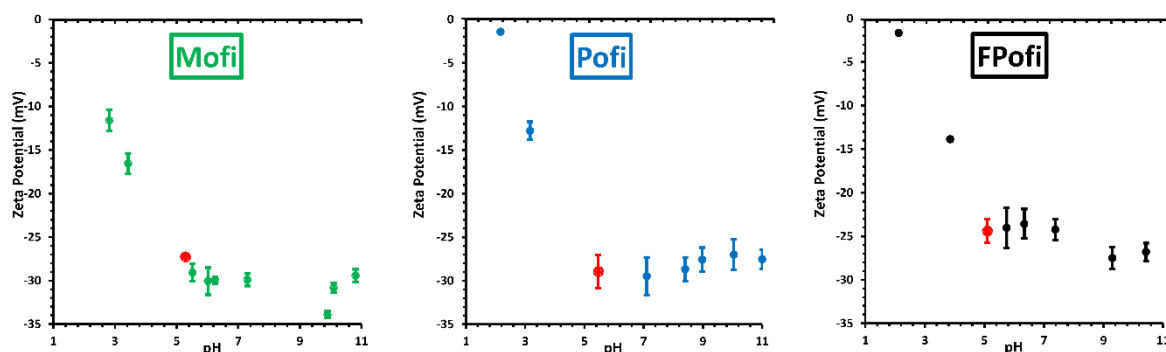


Figure 4. Zeta potential of Mofi, Pofi and FPofi samples as a function of pH. The red dots correspond to measurements at the natural pH of extracts. Results are presented as mean \pm sem ($n = 3$).

The rheological measurements of the 1.5 wt% extracts (Figure 5A) showed a shear-thinning behavior. A large difference of viscosity between Mofi and Pofi was observed at low shear strain rates. The viscosities were quite high showing the thickening properties of the pectin of high molar mass. The presence of insoluble particles had a thickening effect since further purification of Pofi to FPofi by a simple filtration decreased the viscosity of the FPofi solution, although the soluble part of these samples was the same and the only difference was the full elimination of insoluble particles.

Interface stabilization is one of the determining factors in the stabilization of emulsions. The adsorption of molecules at the oil/water interface reduces the interfacial tension and facilitates the fragmentation of oil droplets into smaller ones during emulsification. Figure 5B shows the evolution of interfacial tension over 60 min. At the start of the experiment, the interfacial tension decreased very fast by $\sim 10\text{--}14 \text{ mN}\cdot\text{m}^{-1}$ from the value of the bare MCT/water interface ($26 \text{ mN}\cdot\text{m}^{-1}$). The interfacial tension reached after a short delay required for mechanical equilibration of the drop at the end the syringe needle was the lowest for Mofi ($12.3 \text{ mN}\cdot\text{m}^{-1}$) followed by Pofi and FPofi (14.4 and $16 \text{ mN}\cdot\text{m}^{-1}$, respectively). This showed that the surface activity was related to the amount of protein in each sample. The slow decrease of the interfacial tension over time was related to the adsorption kinetics of particles that are the slowest adsorbing species.

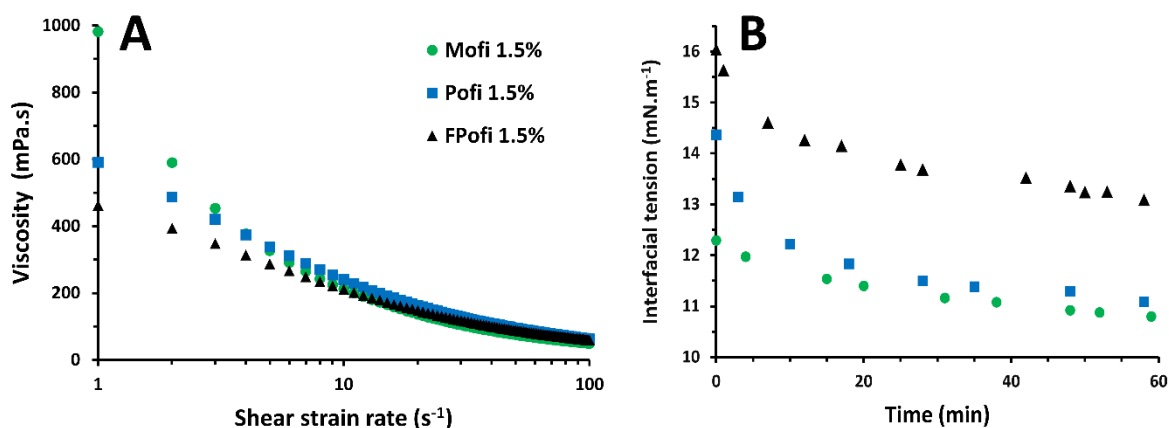


Figure 5. Physicochemical properties of the aqueous extracts containing 1.5 wt% of Mofi (green circles), Pofi (blue squares) or FPofi (black triangles). Viscosity as a function of the shear strain rate (A), and dynamic interfacial tension at the interface between Xofi samples and MCT oil (B).

3.3. Emulsifying properties of Mofi, Pofi and FPofi extracts

O/w emulsions have been successfully prepared with MCT as the oil using an Ultra-Turrax T25 rotating at 24000 rpm. But their stabilities were quite different depending on whether the stabilizer was Mofi, Pofi or FPofi. Because the densities of the aqueous and oil phases were high, a creaming phenomenon occurred after emulsification. When emulsified/stabilized oil droplets migrated to the top surface without coalescing, the size of oil droplets was not changed. The appearance of a layer of pure oil on top of the samples indicated the onset of coalescence. All these observations are shown in [Figure 6](#).

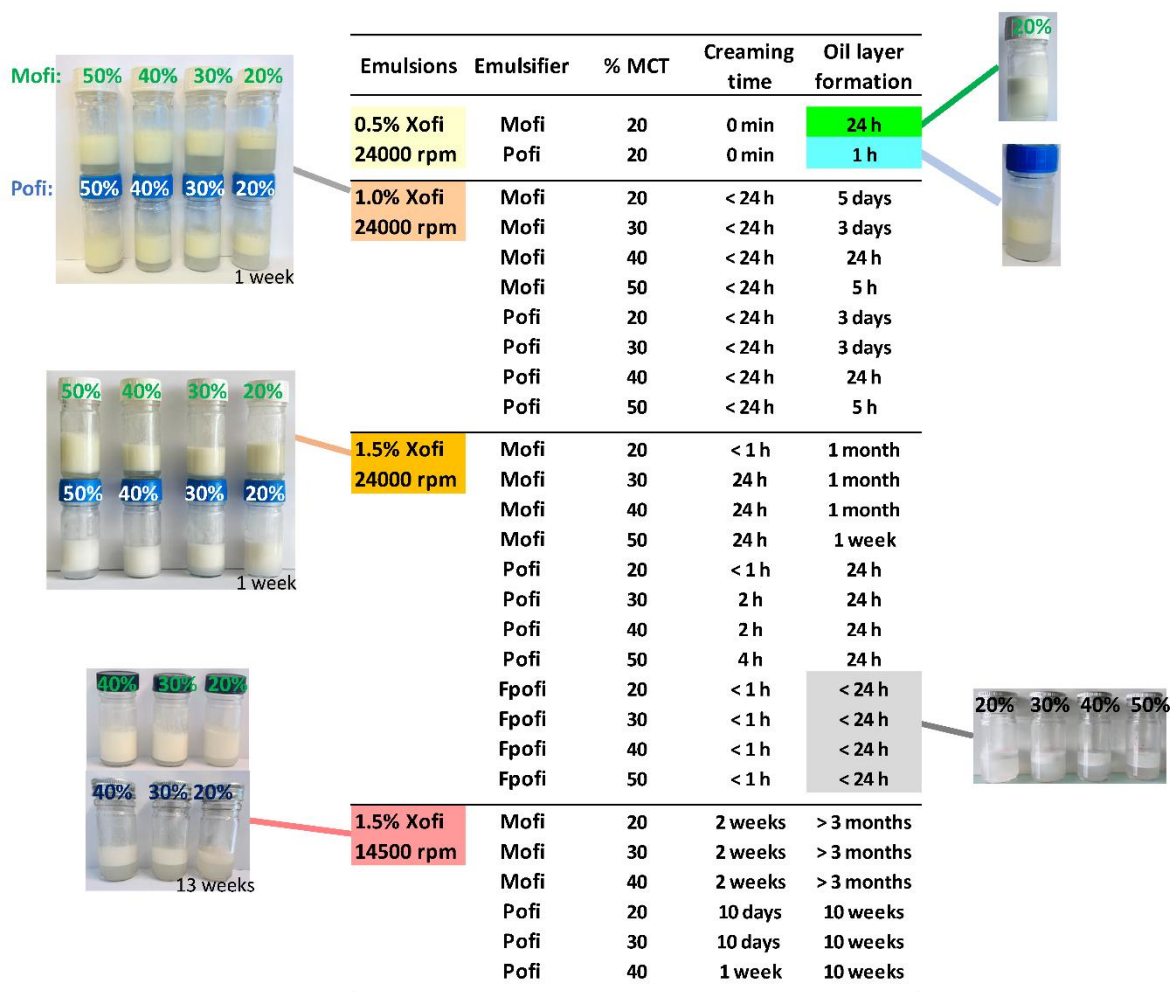


Figure 6. Visual observations and emulsion characteristics in terms of creaming dynamics.

3.3.1. Emulsion stabilization by Mofi, Pofi and FPofi extracts

According to visual observations reported in Figure 6, creaming and coalescence were slower for higher concentrations of Xofi extracts. This is an obvious result. Emulsions stabilized with FPofi showed immediate creaming and the release of an upper phase of pure oil started a few hours after the end of the emulsification process. Completion of phase separation was observed within a week, whatever the oil content. Clearly, FPofi did not have the ability to stabilize emulsions, whatever the oil content. Emulsions with 1.0% Xofi showed slower creaming phenomenon (< 24 h) and slower oil release (> 24 h) than 0.5% Xofi emulsions. At 1.5% Xofi, emulsions were even more stable as the oil layer appeared on top of the emulsion after 24 h for Pofi and 1 month for Mofi. In all cases, the stabilization by Mofi was better than by Pofi.

Early detection of the release of pure oil by coalescence was achieved by accelerated aging 1.5% Xofi emulsions containing different amounts of MCT oil in the analytical centrifuge LUMiSizer (Figure 7). Centrifugation caused faster creaming and forced coalescence of oil droplets at mutual contact within the creamed layer. Such centrifugation experiments allowed discriminating the stability of emulsions that were quite stable under earth gravity and would

require experiments during very long storage times. The time evolution of the transmission profiles is shown in [Figure 7A](#), with the bottom of the tube is on the right side and the top of the sample on the left. Initially, the milky emulsion was spanning the entire sample, resulting in low transmission over the full height. As creaming progressed, the transmission increased at the bottom of the sample and a layer of low transmission remained, corresponding to the creamed layer. A sharp boundary separated the creamed layer above the residual emulsion that became increasingly clear. The kinetics of creaming might be assessed from this part of the transmission profile. However, creaming is not an actual destabilization of emulsions because oil droplets are still present inside the creamed layer. Destabilization by coalescence was detected by the presence of a layer of pure oil with high transmittance at the top of the sample (on top of the creamed emulsion layer corresponding to the region close to the meniscus at 108 mm). For example, in 1.5% Mofi emulsion with 20% MCT, there was initially no transmitted signal: the first red transmission profile was close to zero throughout the whole creamed layer and rose sharply to a maximum value in air above the top of the emulsion. At the end of the experiment, the final green profile indicated a creamed emulsion standing above a large volume of clear water. The height of the creamed layer relative to that of the whole sample corresponding to its volume fraction was ~27%. The oil content within the creamed layer was then $27/20 = 72\%$. The creamed layer is just a highly concentrated emulsion. A clear layer of pure oil (high transmittance) did not appear on top of the sample, indicating a high stability of the creamed emulsion against coalescence. Increasing the oil content caused larger volume of creamed emulsion and a faster creaming because the oil droplets were larger. Coalescence was detected as pure oil separated on top. The results obtained with 1.5% Pofi showed the same trend with a smaller volume of the creamed layer indicating a higher oil concentration inside it. The volume of the clear layer of pure oil on top increased progressively with time. It was faster for Pofi than Mofi and at higher oil contents. Stability against coalescence was assessed from the kinetics of transmittance on top of the sample, i.e. in the 2 mm thick top layer just below the meniscus. The mean transmittance of the layer increased progressively as the transparent part of the layer of released pure oil became thicker, until it reached a constant value when the layer was completely filled with released pure oil. The slope of the transmittance against time is the rate of oil release. Such a measurement is not actually the rate of coalescence because some of the large droplets coming from coalescence are got jammed for a while inside the highly viscous creamed layer. The measured rate is a combination of rates of coalescence and of release of the large coalesced droplets. The measured slopes were 0.00015 min^{-1} for Mofi 1% - 20% MCT emulsions, 0.0012 min^{-1} for Mofi 1% - 30% MCT, 0.0008 min^{-1} for Pofi 1% - 20% MCT emulsions, and 0.0014 min^{-1} for Pofi 1% - 30% MCT. These rates clearly show the much higher stability of 20% MCT emulsions stabilized by Mofi and the better stabilizing efficiency of Mofi than Pofi.

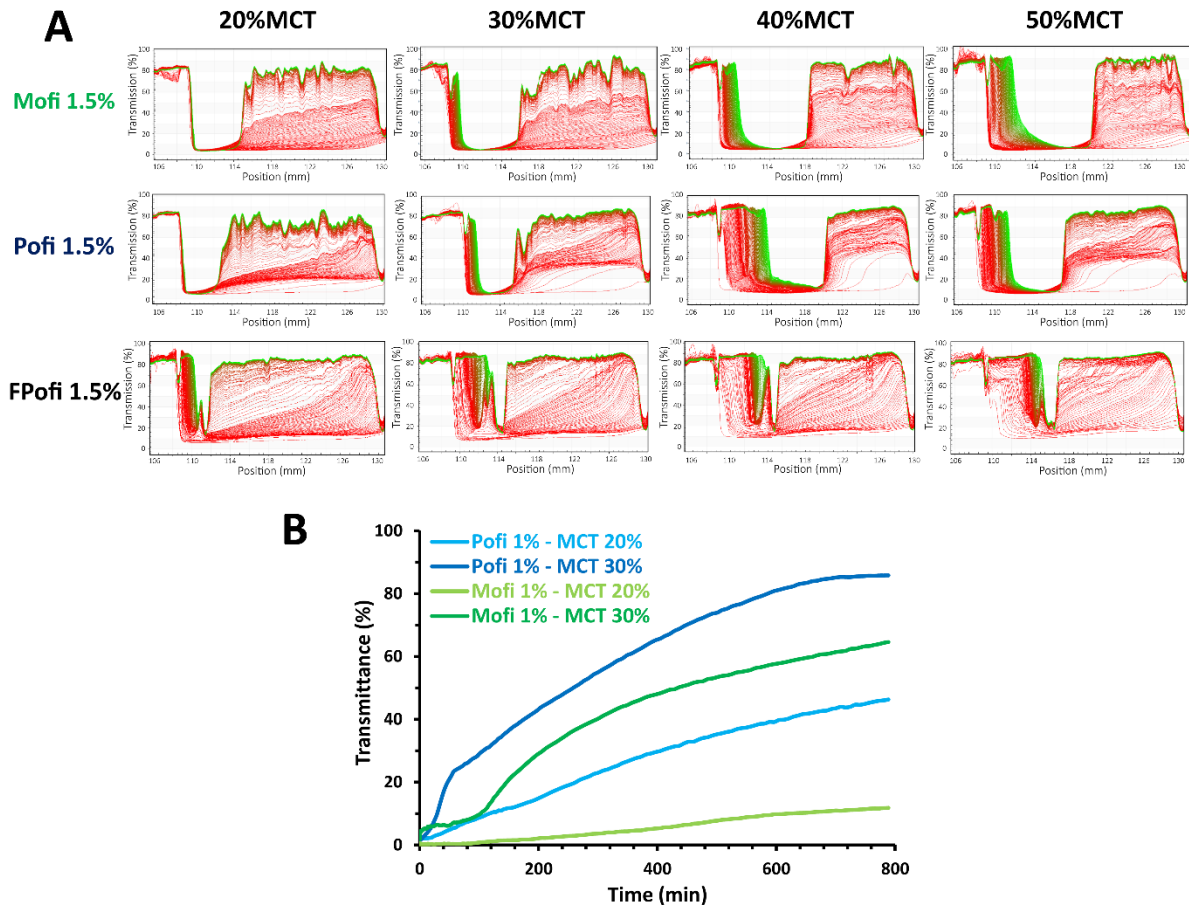


Figure 7. A: Time evolution of transmission profiles measured during centrifugation at 3000 rpm of 1.5% Xofi emulsions prepared with different oil contents (20, 30, 40 and 50%). B: Mean transmittance in the upmost 2 mm thick layer.

To summarize the previous results, creaming and forced coalescence phenomena were more pronounced in emulsions with 1.0% Xofi than with 1.5% Xofi, and they were faster at higher oil contents. Emulsions stabilized with 1.5% Mofi resisted coalescence better than those stabilized with 1.5% Pofi. It is worth noting that only 20% MCT emulsions prepared with 1.5% Mofi remained stable after 1 month storage. A higher concentration of Mofi would be required for the stabilization of emulsions containing higher oil content.

In addition, the stability of the resulting emulsions was estimated from measurements of droplet size over time until a layer of pure oil began to form on top. [Figure 8](#) shows the evolution of mean droplet diameter during 1 week storage for emulsions prepared with 1.5 wt% of the three extracts. Although the majority of the oil phase had been released from the FPofi emulsions after 24 hours, the remaining emulsified droplets kept coalescing as their mean droplet size increased during storage. The stability derived from the droplet growth was depending on the degree of purification of the extracts. The smallest size and most stable droplets were obtained for Mofi emulsions, followed by Pofi and finally FPofi. The size of the Mofi emulsion droplets did not increase significantly during the duration of the experiments (1 week). These results demonstrated the important role of the insoluble fraction of the

extracts. That insoluble particles had a stabilizing effect showed that they acted as Pickering emulsifiers.^{52,53} The water-soluble biopolymers alone as in FPofi were ineffective at the stabilization of the oil-water interface. In the case of Mofi, the amount of insoluble particles was greater than in the other extracts. At 1.5%, the use of Mofi extract was effective in preventing coalescence of droplets in emulsions, whatever the MCT oil content between 20% and 50%. This clearly demonstrated the potential of such an extract as an emulsifier.

The mean droplet size of all Xofi emulsions increased with the oil content. For example, after one week storage, $D(50)$ values were 29 ± 1 , 62 ± 1 and $133 \pm 10 \mu\text{m}$ for 30% MCT emulsions stabilized with Mofi, Pofi and FPofi, respectively, against 64 ± 1 , 158 ± 1 and $285 \pm 24 \mu\text{m}$ for 50% MCT emulsions.

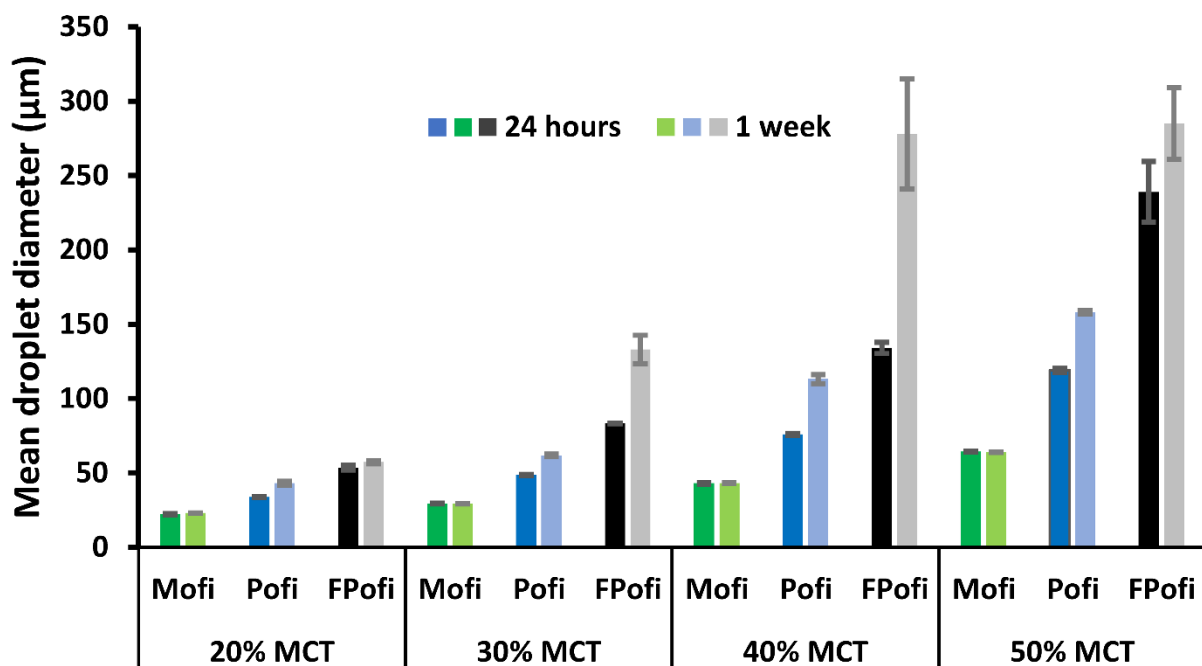


Figure 8. Droplet median diameter of emulsions prepared with 1.5 wt% Mofi, Pofi and FPofi at different oil contents, measured after 24 hours and 1 week storage.

3.3.2. Influence of biopolymer concentration

The droplet size and stability of o/w emulsions was investigated over one month storage for the three Xofi concentrations 0.5, 1.0 and 1.5% (Figure 9). All emulsions prepared with 0.5% Xofi were unstable. Creaming was immediate, and a top oil layer appeared after one hour and 24 hours for Pofi and Mofi emulsions, respectively. A Xofi concentration of 0.5% was not sufficient to stabilize the oil-water interface and prevent coalescence of oil droplets.

The droplet sizes stabilized by 1.0% Xofi were always larger than with 1.5% Xofi (Figure 9). This was an expected trend as a higher concentration of emulsifier allows stabilization of a larger surface area of droplets and therefore smaller droplets.⁵⁴

In all instances, the droplet sizes of emulsions stabilized by Mofi were smaller than for Pofi and the droplet growth by coalescence was slower. As previously pointed out from visual observations, Mofi appeared more efficient as emulsifier than Pofi. This again highlighted the

role of the greater amount of insoluble particles acting as Pickering stabilizing agents in the Mofi extracts. The present droplet sizes and their variation against emulsifier concentration were consistent with those previously found for emulsions prepared with 20% sunflower oil and an Ofi extract as emulsifier, where the median droplet sizes were of 36.3, 36.2, 38.0 and 64.8 μm for emulsifier concentrations of 0.5, 1.0, 1.5 and 4.5%.⁵⁵ However, it was not reported whether particles of insoluble materials were present in the Ofi extracts and the "stability" was only assessed by creaming measurements. The Ofi extract at 4.5% concentration achieved similar emulsion stability as guar gum that is a fully water-soluble polysaccharide. One reason for this similarity may be a switch to a stabilization mechanism by its thickening effect on the aqueous phase coming from the high concentration of polysaccharides.

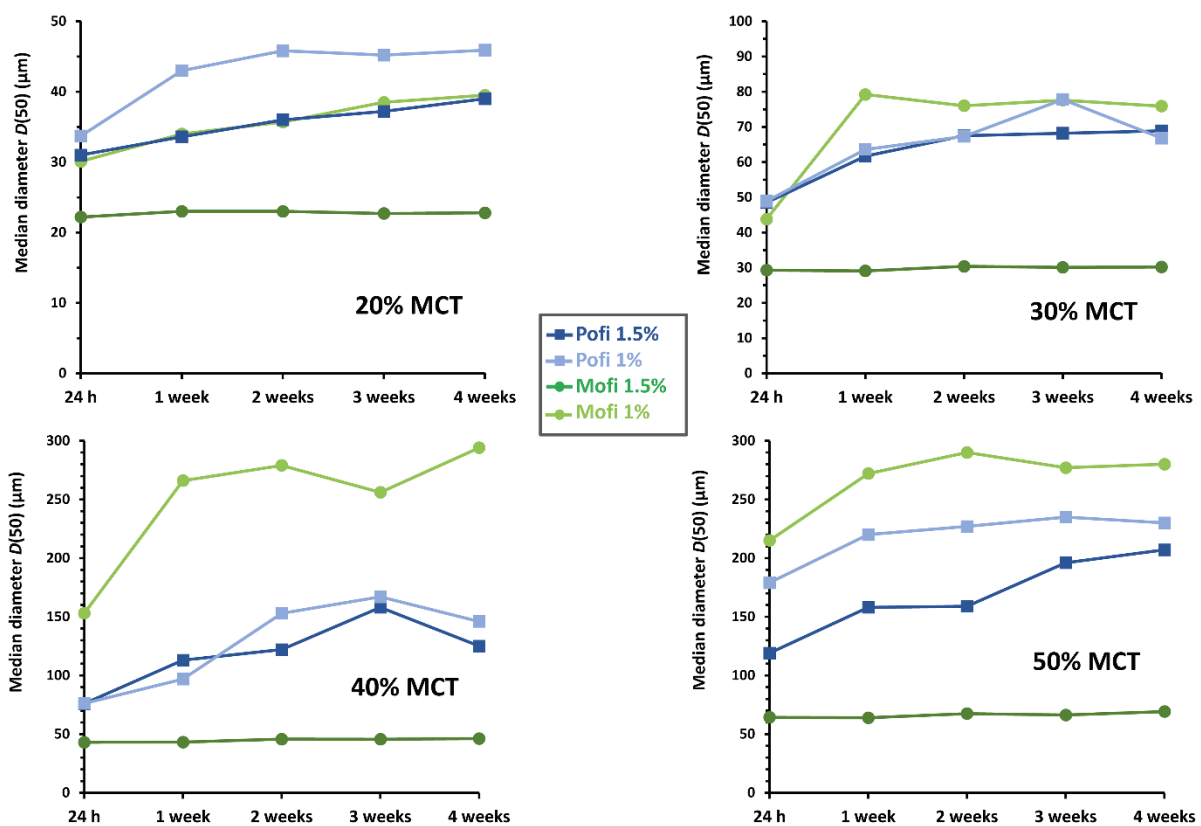


Figure 9. Droplet median diameters $D(50)$ of emulsions of MCT prepared with different Xofi concentrations and MCT contents during one month storage. Clear green circles: 1.0% Mofi, dark green circles: 1.5% Mofi, clear blue squares: 1.0% Pofi, dark blue squares: 1.5% Pofi.

The droplet size distributions provided additional information on the structure and stability over 1 month storage for emulsions prepared with 1.0% and 1.5% Xofi (Figure 10). After 24 h storage, the droplet size distribution of 20% MCT emulsions showed three distinct populations, the main one centered at 30–40 μm . Two additional populations were also detected.

A population of large particles centered on 150 μm was probably due to droplets coagulation and/or coalescence. For 1.0% Mofi, 1.0% Pofi and 1.5% Pofi, the population centered on

150 μm increased within 1 month due to the merging of oil droplets by coagulation and coalescence. The stabilizing power of 1.0% Mofi and Pofi was the worst. Stabilization by 1.5% Mofi was much more efficient as no significant change in size was observed during one month storage.

A very weak population of small particles centered on 5 μm was detected, especially for emulsions containing 1.5% Xofi after 24 h. It was probably due to the presence of free (non-adsorbed) particles of Xofi extracts considering their similarity to that of Mofi and Pofi extracts (Figure 2C). This last population was more important at the highest Xofi concentration investigated, 1.5%, because more non-adsorbed particles remained present in the continuous phase of the emulsion. This assumption was supported by the disappearance of this smaller size population when the amount of oil was increased from 20% up to 50%. This population weakened at longer times (4 weeks) because the non-adsorbed particles were slowly adsorbed during a long storage time. The fraction of non-adsorbed particles was negligibly small in all cases, and it was assumed in the following that all particles were adsorbed on the surface of emulsion droplets.

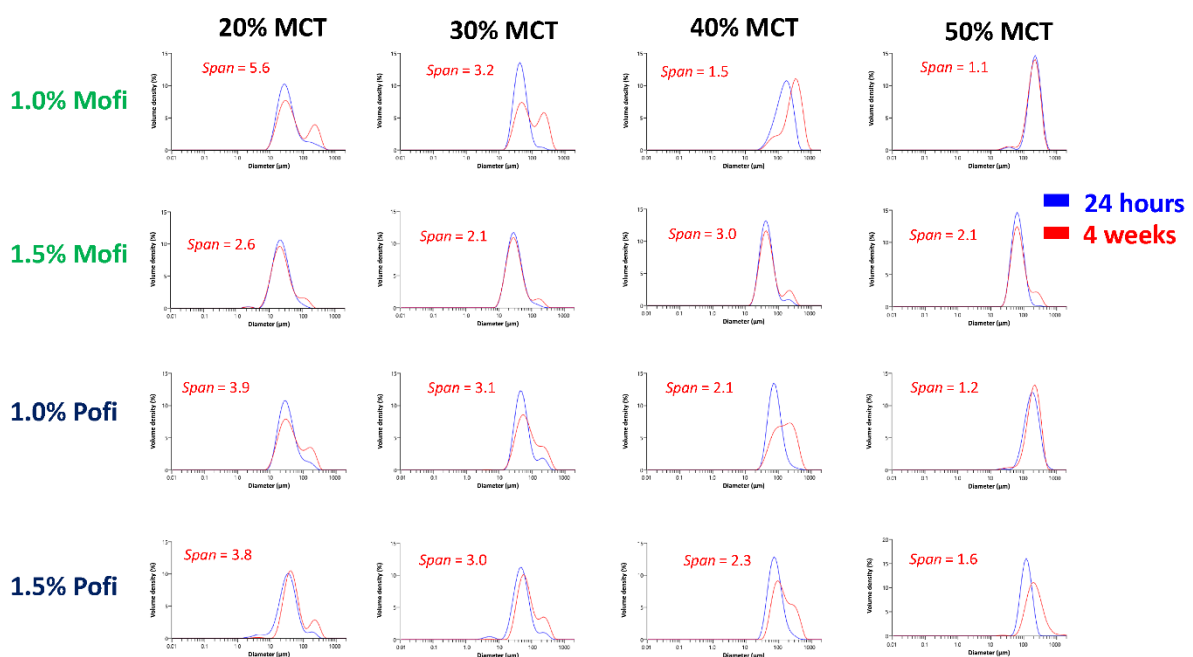


Figure 10. Droplet size distributions of 1.0% and 1.5% Xofi emulsions for different MCT concentrations after 24 h and 4 weeks storage. *Span* values written in red indicate the distribution width after 4 weeks.

3.3.3. Influence of the emulsification process

The parameters of the emulsification process such as duration and stirring speed have an important influence on the properties of the emulsions.⁵⁶ In order to evaluate the effect of the emulsification process, the emulsifier (Xofi) concentration was set at 1.5 wt%, as it gave the most stable emulsions, and a different rotor-stator emulsification device (Ultra-Turrax® T10 with a S10N-10G shaft) was used, achieving a shear strain rate of 58460 s^{-1} , which was

approximately half of the shear strain rate previously used (107652 s^{-1} for the Ultra-Turrax® T25 equipped with a S25N-18G shaft).

Reducing the shear strain rate produces larger droplets. However, when there is not enough emulsifier to cover the full available surface area of droplets, limited coalescence may occur until the decrease of interfacial area caused by droplet growth reaches a stable value corresponding to a dense coverage by adsorbed solid particles.^{57,58} When limited coalescence is operating, the droplet size is controlled by the concentration of solid particles; in other words, the droplet size should not depend on the strain rate of the emulsification device. In fact, the droplet size actually did depend on the strain rate in several instances. For example, 1.5% Pofi emulsions with 30% MCT produced larger droplets size of $137\text{ }\mu\text{m}$ at 58460 s^{-1} shear strain rate against $49\text{ }\mu\text{m}$ at 107652 s^{-1} . On the contrary, the droplet size of 1.5% Mofi emulsions of 30% MCT did not depend significantly on the emulsification process ($26\text{ }\mu\text{m}$ and $29\text{ }\mu\text{m}$ respectively at the low and high shear strain rate) and $D(50)$ was constant for at least 13 weeks. A consequence of a larger size was a slightly better stability against coalescence revealed by a slower growth of oil droplets stabilized by Pofi (Figure 11) because the smaller interfacial area of larger droplets was more densely covered by solid particles. It was noted that the median diameter $D(50)$ of emulsions of 20% MCT stabilized by Pofi appeared lower than expected, probably because the droplet size distribution was broader ($Span \approx 1.5$) than for 30% and 40% MCT ($Span = 1.1\text{--}1.2$).

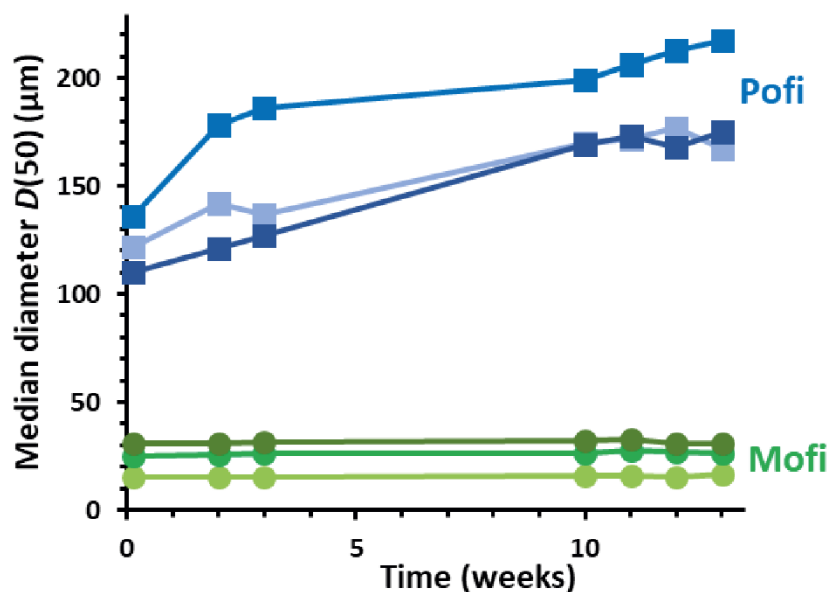


Figure 11. Droplet median diameters $D(50)$ of emulsions of MCT prepared with 1.5% Xofi concentrations and different MCT contents during 13 weeks storage. From clear to dark green circles: Mofi 20%, 30% and 40% MCT; from clear to dark blue squares: Pofi 20%, 30% and 40% MCT.

The control of droplet size was different at low and high shear strain rate. The same size of the Mofi emulsion droplets at low and high shear strain rate indicated that their size was not

controlled by the emulsification process, but by the concentration of solid particles. A limited coalescence phenomenon may have been operating. On the contrary, the size of the emulsion droplets stabilized by Pofi depended on the strain rate, indicating that the adsorption of solid particles onto the oil droplets was not complete. In other words, the adsorption of the solid particles was slower than the fragmentation of the oil droplets by the shear. The adsorption of the Pofi particles was slower than that of the Mofi particles because the concentration of the particles was smaller in the Pofi extract than that in the Mofi. For emulsions stabilized by Mofi, the droplet size increased with respect to the oil content because the interfacial area is the same at all oil contents (the concentration of Mofi is the same). The opposite trend was observed for emulsions stabilized by Pofi because the particle adsorption rate was faster at high oil contents. The actual emulsifier concentration (particle concentration) in the extract was a key parameter in controlling the oil droplet size.

3.3.4. Emulsions of vegetable oil

As a complementary evaluation of the emulsifying properties of Xofi extracts, sunflower oil was used as the oil phase to prepare new emulsions mimicking food products. Taking into account the previous results, emulsification conditions were set as follows: a concentration of 1.5% for Xofi emulsifiers, a shear strain rate of 58460 s^{-1} , and oil contents of 20, 30 and 40%. [Figure 12A](#) shows the time evolution of the droplet size distribution for the different emulsions. Droplet coalescence was observed for emulsions prepared with Pofi, as revealed by a growing population of large droplets ($\sim 200 \mu\text{m}$). This was due to the poor emulsifier properties of the Pofi extract. The mean droplet diameters of the 40% SFO emulsion stabilized with 1.5% Pofi increased from $57 \mu\text{m}$ up to $196 \mu\text{m}$ over 3 months whereas stabilization by Mofi kept the droplet size small and constant (non-significant increase from 22.3 to $25.6 \mu\text{m}$) ([Figure 12B](#)). Optical microscopy pictures of 20% SFO and 20% MCT emulsions stabilized with 1.5% Mofi or Pofi ([Figure 12C](#)) confirmed the size measurements by small-angle light scattering: small droplets ($\sim 10 \mu\text{m}$) for stabilization by Mofi and large droplets with a broad size distribution for stabilization by Pofi.

As observed for Mofi - MCT emulsions, Mofi - SFO emulsions remained stable over 3 months. Smaller emulsion droplets were obtained with SFO (respectively 16 and $26 \mu\text{m}$ for emulsions containing 30% and 40% oil) than with MCT (26 and $33 \mu\text{m}$). Sunflower oil is a mixture of triglycerides containing small amounts of surface-active di- and mono-glycerides together with free fatty acids. The presence of such surface-active compounds could lead to fast droplet stabilization during the emulsification process, resulting in a reduction of droplet size. The long-term stability of SFO emulsions was due to the insoluble fractions of Mofi and Pofi as in the case of MCT emulsions.

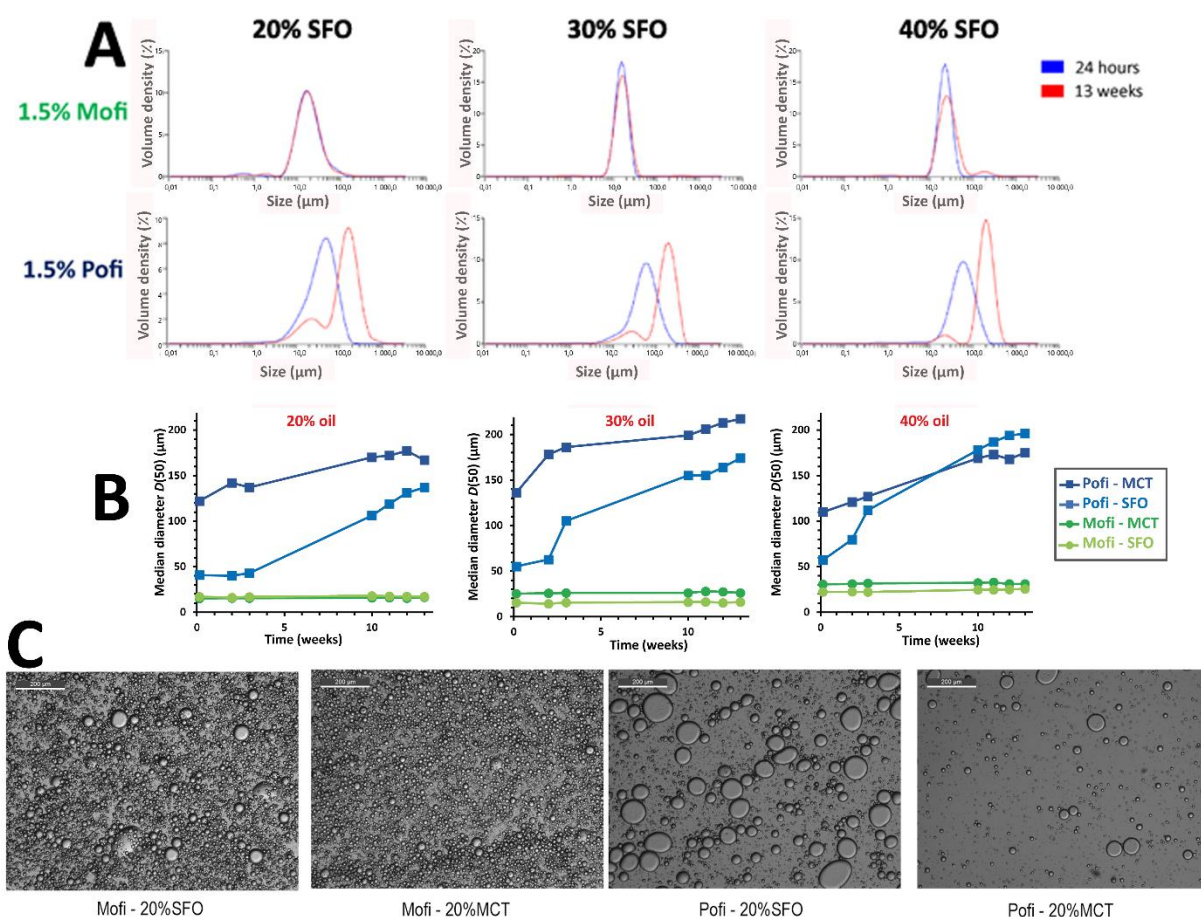


Figure 12. (A): Droplet size distribution of 1.5% Xofi emulsions for different SFO contents, after 24 h and 13 weeks storage. (B): Droplet median diameter $D(50)$ of emulsions prepared with 1.5% Xofi with different MCT or SFO contents after 24 h and 13 weeks. (C): Optical microscopy observations of Xofi emulsions prepared with 20% MCT or SFO after 13 weeks storage (the scale bars are 200 μm).

4. General discussion

The use of polysaccharides for the stabilization of emulsions is developing because of the demand for "naturalness" of ingredients in many products, especially in cosmetics. Those polymers of natural origin stabilize emulsions by two very different mechanisms.¹⁰ They can act as surfactants if they are able to adsorb at the water-oil interface. Besides, the polymers of high molar mass can act as thickeners of the aqueous phase, slowing down all destabilization processes because the continuous aqueous phase of o/w emulsions is viscous. Many polysaccharides containing protein parts are thought responsible for their surface-active properties.⁸ Pure polysaccharides (free of protein) act only as thickeners. Rough assessment of emulsion stability by creaming measurements does not allow these two mechanisms to be discriminated.

- The present investigation of *Opuntia ficus indica* extracts has clarified this point by showing that the stability of o/w emulsions was correlates with the protein content of extracts containing mainly polysaccharides (pectin) because the emulsion stability was not only

assessed through the creaming phenomenon, but mostly by considering the droplets coalescence.

- In addition, there was a definite stabilizing contribution from insoluble particles coming from an incomplete purification of the raw pulp. Such insoluble protein-rich particles provided a long term stability of o/w emulsions, whereas the FPofi fully soluble fraction obtained by filtration (0.45 μm) did not stabilize emulsions. The present work disclosed that such emulsions stabilized by solid particles are Pickering emulsions.

The utilization of natural extracts as stabilizers of Pickering emulsions is an innovative approach, especially for applications to the fields of cosmetics and pharmaceuticals.⁵³ In Pickering emulsions, solid particles adsorb at the oil-water interface, building a densely packed particle layer that acts as a physical barrier preventing coalescence. The choice of particles, including their size, hydrophilicity, and concentration, influences the efficiency of the stabilization process. As Pickering emulsifiers, common stabilizing particles include various types of nanoparticles,⁵² mineral substances (clays⁵⁹, silica⁶⁰), synthetic polymers,⁶¹ and, increasingly, biocompatible and biodegradable particles coming from the biomass.⁵³

The role of particles of insoluble materials in the stabilization of o/w emulsions has been disclosed in the present study. The presumed mechanism of such stabilization is the adsorption of these particles on the surface of oil droplets, corresponding to the formation of Pickering emulsions.^{52,53} In general, the main components of mucilages are water-soluble polysaccharides and proteins. The proteins are bound to the polysaccharides as presumably water-soluble associations. In the present case, the strong turbidity of the mucilage (Mofi) and its Pofi extract clearly indicated the presence of particles in aqueous suspension. Filtration of the Pofi extract yielded a clear solution (FPofi). Mofi contained a soluble fraction consisting of soluble polysaccharides with a few proteins. The Pofi extract was still a turbid aqueous dispersion. Its water-soluble fraction contained pectin and part of the proteins. The insoluble parts in Mofi and Pofi were probably rich in proteins. The FPofi solution contains the soluble part of Pofi. It contained soluble proteins as shown by its elemental analysis of nitrogen and UV absorbance in the SEC analysis. Therefore, the soluble fraction of Pofi also contained soluble proteins.

The most remarkable point was that the FPofi solution did not stabilize the emulsions. This clearly showed that the stabilization of the emulsions came from the insoluble fractions of Mofi and Pofi. However, the adsorption of the particles responsible for emulsion stabilization could not be observed by optical microscopy. It is not clear whether these are solid particles or soft particles of water-swollen hydrogel. Pickering emulsions can be effectively stabilized by both solid particles and soft particles as well.⁶² In the case of extensive swelling of insoluble particles by water, their observation by optical microscopy in emulsion samples is difficult because of the lack of contrast with the aqueous continuous phase. The same difficulty applies to electron microscopy (Cryo-SEM or Cryo-TEM). Such a clear evidence of particles adsorption on the surface of oil droplets could not be obtained. The main clues indicating a Pickering stabilization are given below.

- The difference between the extracts lies in the amount of insoluble particles; the soluble pectin fraction was the same in all Xofi extracts. FPofi, which was free of insoluble particles, did not stabilize emulsions. The Mofi extract definitely stabilized emulsions with a higher efficiency than Pofi because Mofi contains a higher amount of insoluble particles. Emulsions of 20% MCT or SFO oils prepared with the crude mucilage extract (Mofi) showed remarkable stability, maintaining their properties for a period of more than three months.
- The size of the emulsion droplets also indicated the stabilization of the emulsions during the emulsification process. Smaller droplet sizes with Mofi showed better stabilization. For example, the median droplet sizes $D(50)$ of 40% MCT emulsions stabilized with Mofi, Pofi and FPofi respectively were 43, 113 and 278 μm after one week storage.
- The particle size distributions measured by small-angle light scattering did not disclose a significant amount of free particles. Some of the samples did show a very small amount of them, showing that their detection was possible. The adsorption of insoluble particles reached completion because of the very high adsorption free energy of particles at the oil-water interface. This is a feature of Pickering emulsions.

According to the literature, the emulsifying capacity of mucilages and natural gums derives from their protein-rich fraction.^{8,10,63} This has been most extensively documented in the case of Acacia gum. The distribution of the macromolecular species of Acacia gum has been divided into two parts, both containing polysaccharide/protein complexes in which the proteins are attached to the polysaccharide by chemical bonds:⁶⁴ *i)* The main one, called arabinogalactan (AG), is made of polysaccharide/protein complexes with a low protein content of less than 1%. *ii)* The other, called arabinogalactan-protein complex (AGP), contains a high amount of proteins (~12%) bound to polysaccharides of high molar mass. These latter polysaccharide-based compounds have hydrophilic and hydrophobic parts that allow them to adsorb at the oil-water interface⁸ and reduce the oil-water interfacial tension that determines the emulsion formation and stability.⁶⁵ Indeed, the present dynamic interfacial tension measurements have shown that the protein content contributes to the rate of adsorption at the oil-water interface (Figure 5B).

The actual protein content of Mofi was 11.4%, much higher than the average protein content of Acacia gum (~2%), and close to the protein content of the AGP fraction of Acacia gum. Part of the Mofi extract was insoluble because of its high concentration of AGP. The concentration of the APG fraction of Mofi exceeded its low solubility. Acacia gum is fully soluble in water because its fraction of AGP is low. Owing to its high content of AGP, the mucilage of Ofi appeared as a special type of polysaccharide-based hydrocolloid. One consequence is the formation of small particles of insoluble materials upon dissolution in water; these insoluble particles in Mofi act as Pickering emulsifiers and enhance the stability of the o/w emulsion compared to Pofi and FPofi.

5. Conclusions

The use of naturally derived particles offers many benefits including biocompatibility and biodegradability,^{53,66} which have a great impact on the consumer's perception of the product; consumers are increasingly drawn to products that align with their environmental values. In addition, the sensory dimensions of natural ingredients, such as pleasant scents and textures, further enhance the product experience.⁶⁷

Pickering emulsions stabilized by natural water-insoluble protein particles have already been produced with emulsifiers extracted from zein,^{68,69} flaxseed⁷⁰ and others.⁷¹ However, it is worth highlighting that the majority of cases relied on complicated extraction procedures and purification processes including delipidation, fractionation, solvent extraction, etc. While these approaches are effective in refining particle properties for emulsion stability, they can have significant implications for both consumer safety and environmental considerations. The use of raw materials as natural particles for Pickering applications have not been widely investigated.⁷² In our case, the crude mucilage with the highest content of water-insoluble particles proved to be the most efficient emulsifier of the three extracts studied.

Our study showed the greater potential of the crude extract as a natural emulsifier compared to the purified extracts. Emulsion stability was improved by a higher fraction of water-insoluble particles. Indeed, the "unpurified" extract Mofi successfully stabilized emulsions for more than three months, whereas the most purified extract FPofi could not stabilize emulsions for more than a few hours. Correspondingly, droplet sizes were smaller in the presence of higher amounts of insoluble particles, indicating that the latter particles acted as Pickering emulsifiers. In similar manners, the formation of insoluble particles of pectin by cross-linking beet sugar pectin with genipin³⁷ or by complex coacervation of low-methoxy pectin with sodium caseinate³⁶ improved the stability of emulsions against coalescence.

Acknowledgments

This study was supported by a scholarship to MS from the Tunisian Ministry of Higher Education and Scientific Research. We are grateful to Agnès Crépet (IMP, University of Lyon 1) for the SEC analyses of FPofi and to Kai-Chung Szeto (CP2M, University of Lyon 1) for the elemental analyses of C, H and N.

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SUPPLEMENTARY INFORMATION

Emulsifying properties of biopolymer extracts from *Opuntia ficus indica* cladodes

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SI1. ^1H NMR spectra of Mofi, Pofi and FPofi

^1H NMR spectra of $5\text{ mg}\cdot\text{mL}^{-1}$ aqueous solutions of Mofi, Pofi and FPofi were run on a Bruker AVL300 spectrometer operating at a Larmor frequency of 300 MHz. FPofi was soluble in D_2O . Mofi and Pofi contained an insoluble fraction that was not observed with the NMR spectrometer designed for liquid samples; only the soluble fraction was observed.

The ^1H NMR spectra of Mofi, Pofi and FPofi were identical, showing that the soluble fractions were the same (Figure SI1). The spectra were typical of polysaccharides with all their lines between 3.0 and 5.4 ppm.

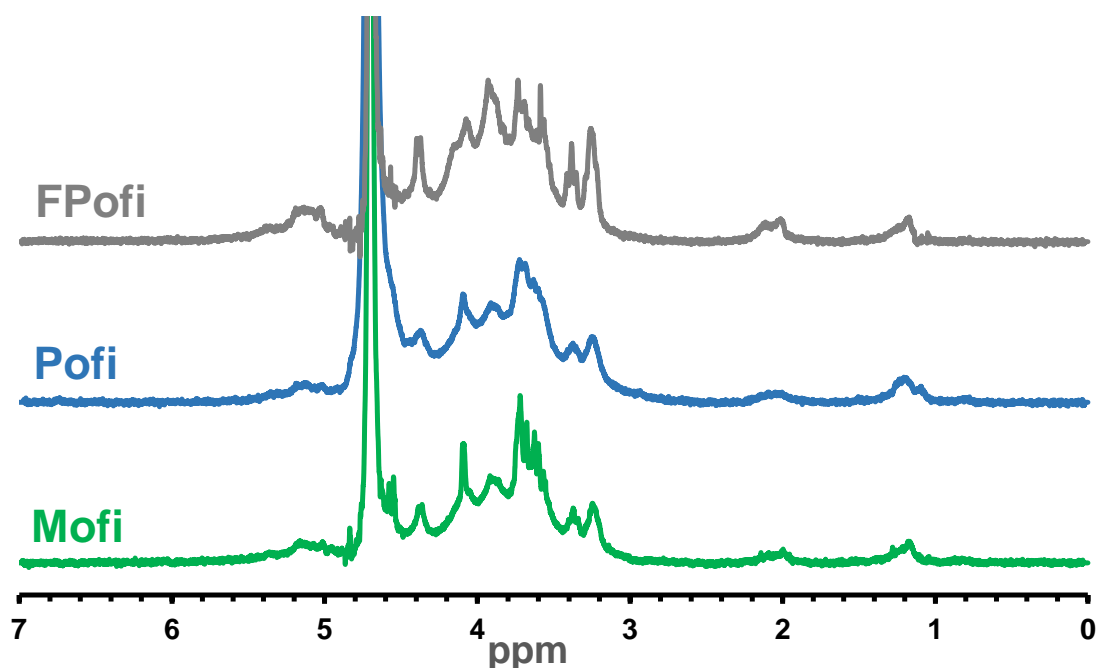


Figure SI1. ^1H NMR spectra of Mofi, Pofi and FPofi $5\text{ mg}\cdot\text{mL}^{-1}$ in D_2O .

SI2. Determination of the galacturonic acid and its ester by pH-metric titration

5 mL of a $4\text{ g}\cdot\text{L}^{-1}$ FPofi solution was titrated in several steps as follows (Figure SI2). The initial pH of this solution was 4.82.

The FPofi solution was first titrated with HCl 0.1 M up to a volume of 0.4 mL. It was then back-titrated with NaOH 0.1 M up to exceed the equivalent volume and reach pH 11.27. These titrations allowed the calculation of the concentration of acid groups derived from galacturonic

acid in its free carboxylic acid form, as detailed below. The pH of this solution was raised up to 12.3 by adding 0.1 mL of NaOH 1 M and the solution was kept at ambient temperature for 30 min to allow the hydrolysis of the methyl esters of galacturonic acid to proceed. The hydrolysis of the methyl esters to carboxylic acids was considered complete because this time exceeded those reported in the literature ¹⁻⁴. After the hydrolysis process, 0.1 mL of HCl 1 M was added to restore the pH to 11.27, and the solution was titrated with HCl 0.1 M up to a volume of 1.0 mL, allowing the determination of the total galacturonic acid content (free carboxylic acid + methyl ester).

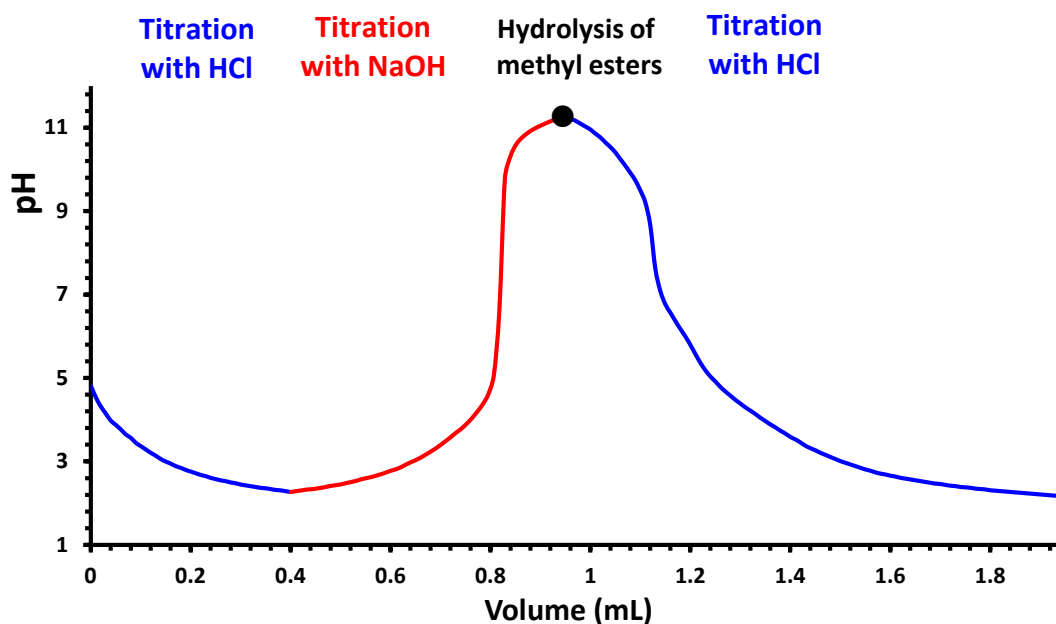


Figure SI2. Successive titrations with HCl, NaOH and HCl.

The first titration step with HCl and the second one with NaOH were nicely superimposed when the titration by addition of increasing volume of NaOH is plotted as a decreasing volume of HCl (Figure SI3). There was no visible equivalent point corresponding to the full protonation of the carboxylate groups. Because HCl is a strong acid and the galacturonic acid is a weak acid ($pK_a = 3.5$)^{5,6}, the first addition of HCl was used to protonate the carboxylate groups, and this was followed by an accumulation of HCl in excess of the end point. The concentration of excess HCl is given by the value of the pH at which the dissociation of the galacturonic carboxylic acid is negligible. The dissociation of the carboxylic acids is $\sim 10\%$ at a pH of $pK_a - 1$, and 1% at a pH of $pK_a - 2$, so that it can be neglected at pH 2.27 reached at the end of the NaOH addition ($V(\text{NaOH}) = 0.4 \text{ mL}$). The concentration of excess HCl is $10^{-\text{pH}}$, corresponding to a volume of added HCl of 0.29 mL. The end point for the protonation of carboxylate groups was therefore 0.11 mL.

The pH variation calculated as a function of the volume of added HCl fitted quite accurately the experimental data for $pK_a = 3.7$, which is close to the reported value of 3.5 for pectin ^{5,6}.

The volume of the end point of the back-titration with NaOH was 0.42 mL, corresponding to an equivalent volume of 0.13 mL after subtracting the volume of NaOH required to neutralize the excess HCl. The derived galacturonic acid content was $0.65 \text{ meq}\cdot\text{g}^{-1}$. Taking the same molar mass of the galacturonic acid unit ($176.12 \text{ g}\cdot\text{mol}^{-1}$) for all the saccharide units of pectin, the

galacturonic acid content in FPofi was 11.4 mol%. The galacturonic acid groups of FPofi were 85% under their acidic form, giving a pH of 4.82 for the FPofi solution in pure water.

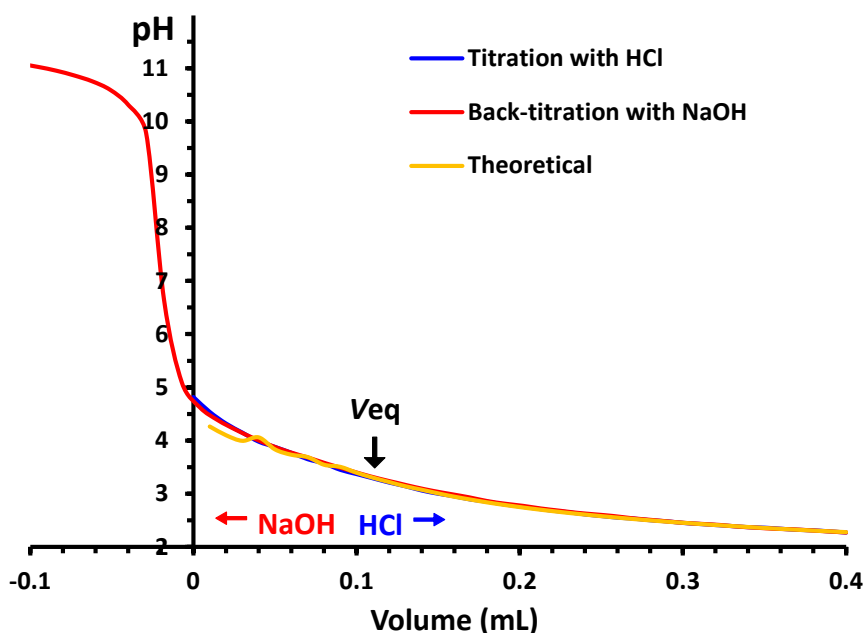


Figure SI3. Titration with HCl (blue) and back titration with NaOH (red). The theoretical calculation fitting the experimental data for $pK_a = 3.7$ is given in yellow.

After hydrolysis of the methyl esters in strongly basic medium, the FPofi solution was titrated with HCl. The volume for complete neutralization of the excess NaOH was unambiguously determined and the end point for complete conversion of the carboxylates to carboxylic acids was determined from the pH value in excess HCl in the same way as for the first titration with HCl. The total content of galacturonic acid was $1.50 \text{ meq}\cdot\text{g}^{-1}$, corresponding to 26.4 mol%, and the pK_a of the hydrolyzed form was 4.1.

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