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## Integrated one-pot process to fabricate and impregnated starch aerogels in supercritical carbon dioxide

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**TITLE:**

# Integrated One-pot Process to Fabricate and Impregnate Starch Aerogels in Supercritical Carbon Dioxide

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**KEYWORDS:**

Aerogel, starch, one-pot, carbon dioxide ($CO_2$), impregnation, supercritical drying

**SUMMARY:**

This protocol describes the development of a one-pot strategy for the fabrication and impregnation of starch aerogels. Some modifications were made within the traditional fabrication process, which allowed the integration of the three critical steps (gelatinization, retrogradation, and drying) of aerogel fabrication into a single step.

**ABSTRACT:**

The goal of this work was to develop a one-pot strategy for the fabrication and impregnation of starch aerogels with green coffee oil (GCO) in supercritical carbon dioxide ($scCO_2$). For that purpose, different modifications were made to the production process to improve the integration of the three essential steps of aerogel fabrication. A strategy based on supercritical extraction (SCE) was proposed to address the conventional drying process, as well as the utilization of $CO_2$ during the aerogel fabrication steps. The development of a novel drying approach was the most challenging task of this work as it should be performed without any solvent-exchange step. The results show that aerogels with high surface area (95 $m^2.g^{-1}$) could be produced using a continuous flow of $CO_2$/ethanol (20 MPa, 40 °C, 2 $mL.min^{-1}$ $CO_2$ with 11% v/v ethanol). The next step comprised the formation of aerogels in the presence of $CO_2$. The optimal surface area was 185 $m^2.g^{-1}$. Finally, the integration of all the above steps was achieved, and gelatinization, retrogradation, and drying happened sequentially in the same vessel under $CO_2$ atmosphere. This one-pot fabrication was followed by the impregnation with GCO using a high-pressure injection

step. An impregnation efficiency of 24% was obtained with this one-pot strategy confirming that a fully integrated process for the fabrication and impregnation of starch aerogels could be attained.

**INTRODUCTION:**

The fabrication of starch aerogels is often described in three major steps: formation of hydrogels, formation of alcohol gel through solvent exchange step, and drying[1,2]. The formation of hydrogel involves two steps: gelatinization followed by retrogradation. The gelatinization promotes irreversible physical changes[3–5] in the structure of starch and results from the swelling and denaturation of starch granules. Retrogradation promotes the restructuring of free amylose, leading to the formation of a three-dimensional (3D) network. Drying, which is necessary to obtain an aerogel, usually includes a solvent exchange step, wherein water is progressively replaced by ethanol followed by $CO_2$ SCE to remove the ethanol[6]. The solvent exchange step is vital to obtain high-quality aerogels and is usually the focus of different studies to improve the structural properties of these materials[7,8].

For example, Mehling and coworkers[1] have used a multistage solvent exchange process (5 days) followed by supercritical extraction (SCE) to produce aerogels of high surface area (72 and 90 $m^2.g^{-1}$) from potato starch. Following the same concept, Zou and Budtova[9] used a multistage solvent exchange process (4 days) followed by SCE over 5 h. These authors have obtained materials of surface area between 8 and 120 $m^2.g^{-1}$. Furthermore, Santos-Rosales and co-workers[10] have used a multistage solvent exchange (48 h between each stage) followed by SCE over 4 h. This strategy allowed the production of aerogels of high surface area (183−228 $m^2.g^{-1}$). Another good example of starch aerogels with a surface area of 234 $m^2.g^{-1}$ is from the work of García-Gonzales et al.[2] who reported a solvent exchange procedure over 24 h followed by four SCEs. A similar approach was reported by Ubeyitogullari and Ciftci[11] to fabricate wheat starch aerogels with a surface area of 59.7 $m^2.g^{-1}$.

Despite the production of improved aerogel materials in the above studies, all of them describe a complex solvent exchange method that can last between one and five days. In fact, this complex procedure is one of the major bottlenecks in aerogel production. Hence, the improvement of the drying procedure is essential to improve the whole fabrication process, which has encouraged the development of the present protocol. Therefore, the first goal of this study was to integrate the solvent exchange with the SCE in one single step. This strategy presented a clear advantage when compared with the several examples described above, as high-quality materials were obtained by SCE using a mixture of $CO_2$ and ethanol. Only a few studies in the literature describe a similar approach[12–15]. For instance, Comin and co-workers[12] have reported a similar approach to dry starch aerogels involving a two-stage extraction process consisting of a 4 h ethanol extraction (0.3 $mL.min^{-1}$) followed by 1 h of $scCO_2$ extraction (1 $L.min^{-1}$, 15 MPa and 40 °C).

Nevertheless, the materials produced exhibited a low surface area (<10 $m^2.g^{-1}$) and extensive cracking. Moreover, Brown et al.[11] have reported similar problems using a continuous solvent exchange process. Even though both studies produced aerogels with properties suitable for impregnation of bioactive molecules, none of these studies could produce high-quality materials

integrating both solvent exchange and SCE steps. Gurikov et al.[14] reported the preparation of alginate aerogels using a static high-pressure extraction with different $scCO_2$/ethanol/water mixtures. Their results also show that the presence of $scCO_2$ improved the solvent exchange step. More recently, Lebedev et al.[15] also reported a static extraction strategy similar to that of Gurikov et al.[14]. These authors showed that an $scCO_2$/2-propanol/water mixture was able to increase the surface area of alginate aerogels. These examples highlight the novelty and the success of the protocol described herein. Another goal of the present protocol was to evaluate the possibility of using $CO_2$ to improve the properties of the aerogels. In fact, different authors have approached this subject. Francisco and Sivik[16] evaluated the impact of $CO_2$ on the gelatinization of different types of starches. Their results show that starch gelatinization could be attained at a lower temperature in the presence of $scCO_2$. This result was explained by plasticization and hydrostatic effects of $CO_2$ on the structure of starch.

Moreover, Muljana and co-workers[17] also reported a similar effect of $CO_2$ on the gelatinization temperature. Thus, this protocol also introduces the positive effect that $CO_2$ has on the production process. The combination of gelatinization, retrogradation, and drying in a one-pot process not only reduces the overall complexity of aerogel fabrication, but also allows the production of high-quality materials for impregnation with bioactive molecules. Additionally, an impregnation step was also added to this procedure to demonstrate a novel concept for the development of aerogel-based products. For this purpose, GCO was used because of its beneficial impact on human health[18–21]. Thus, the use of this fully integrated protocol for the fabrication/impregnation of aerogels could lead to the development of different, novel nutraceutical products.

**PROTOCOL:**

## 1. Development of a supercritical drying method by using a mixture of supercritical carbon dioxide and ethanol

1.1. Prepare a hydrogel in the traditional way by heating 10 mL of a 15% w/v starch solution in water in a beaker at 120 °C for 20 min with magnetic stirring (600 rpm), as described previously[1,2,22], to promote starch gelatinization.

1.2. For the retrogradation step, transfer 2 mL of this solution to a cylindrical tube, and incubate at 4 °C for 48 h.

1.3. For the supercritical drying step, place the retrograded hydrogel in a 20 mL high-pressure, variable-volume cell. Transfer the cell to a thermostatic water bath at 40 °C and pressurize it with $scCO_2$ until the desired pressure (10–20 MPa).

NOTE: Here, the cell was pressurized at 1 $MPa.min^{-1}$.

1.4. Ensure a continuous flow of $scCO_2$ (2 $mL.min^{-1}$), containing ethanol (11% (v/v) or 22% (v/v), which corresponds to 0.22 or 0.44 $mL.min^{-1}$), through the reactor for 4 h using a continuous high-

pressure pump. Pump the ethanol into the system using a second high-performance liquid chromatography (HPLC) pump.

1.5. After this period, stop the ethanol flow, and start the flow of $scCO_2$ at 2 $mL.min^{-1}$ through the reactor for an additional 2 h. Leave the system to depressurize at a rate of 0.3 $MPa.min^{-1}$.

NOTE: A rapid depressurization process can promote fractures in the structure of the aerogel. The drying apparatus is depicted in **Figure 1**.

[Place **Figure 1** here].

## 2. Fabrication of starch aerogel in the presence of $CO_2$

NOTE: Three different temperatures (40, 90, and 120 °C) were used to evaluate the impact of $CO_2$ on the structural properties of the aerogel.

2.1. Dissolve 0.5 g of starch in 5 mL of water in a 10 mL high-pressure reactor. Heat the reactor to the desired temperature and then pressurize with $scCO_2$ at 1 $MPa.min^{-1}$ to a final pressure of 20 MPa. Stir the pressurized starch solution for 20 min at 600 rpm to promote gelatinization, and then cool the reactor to room temperature.

2.2. For retrogradation, leave the pressurized starch solution in the reactor at 4 °C for 48 h. Then, place the reactor in a thermostatic water bath at 40 °C, and pressurize with $scCO_2$ at 1 $MPa.min^{-1}$ until the pressure reaches 20 MPa.

2.3. Ensure a continuous flow of $scCO_2$ (2 $mL.min^{-1}$) containing 11% v/v/ ethanol through the reactor for 4 h. Use a continuous high-pressure pump to maintain the flow rate of $CO_2$ at 2 $mL.min^{-1}$. Pump the ethanol into the system using a second HPLC pump at 0.22 $mL.min^{-1}$.

2.4. After this period, stop the ethanol flow, and start the flow of $scCO_2$ (2 $mL.min^{-1}$) through the reactor over 2 h. Finally, leave the system to depressurize at a rate of 0.3 $MPa.min^{-1}$. Characterize the materials obtained at the end of this process using the procedures described in section 5.

## 3. Evaluation of impregnation conditions

NOTE: The GCO extract and its characterization have been described previously[23].

3.1. For the supercritical impregnation process, place 0.1 g of the aerogel obtained at the end of the process described in section 1 in a 25 mL high-pressure, variable-volume cell. Leave this cell in a thermostatic bath at 40 °C, and pressurize with $scCO_2$ at 1 $MPa.min^{-1}$ until 10 MPa.

3.2. Leave the aerogel for impregnation for three different periods (6, 12, and 24 h), and depressurize the system at a rate of 0.1 $MPa.min^{-1}$.

NOTE: These impregnation conditions were based on previously reported procedures[24].

3.3. Fill a 0.1 mL loop mounted on a 6-way high-pressure valve (**Figure 2**) with GCO extract using a syringe. Pressurize the loop with $scCO_2$ (flow of 2 $mL.min^{-1}$) until 30 MPa and leave for 10 min to equilibrate. Turn the valve to the injection position to start the flow of $scCO_2$ across the loop until the pressure inside the reactor reaches 30 MPa. Leave the system in this condition for 6, 12, or 24 h.

NOTE: The GCO extract was obtained and characterized using previously reported procedures[23].

[Place **Figure 2** here]

3.4. After this period, depressurize the system at 0.1 $MPa.min^{-1}$, and collect the impregnated material. Determine the amount of impregnated oil using the extraction procedure described in the literature[1, 7, 11].

3.5. Disperse 0.1 g of the impregnated aerogel in 5 mL of chloroform, and perform the extraction for 1 h at 50 °C with magnetic stirring (600 rpm). Filter the extracts using a 0.2 µm syringe filter, and collect the extracts in a 5 mL calibrated round flask. Evaporate the solvent under a flow of nitrogen, and determine the impregnation efficiency in mg of oil per 100 mg of aerogel.

## 4. One-pot process for fabrication and impregnation of starch aerogels

NOTE: This process used the optimized conditions from the previous steps using previously reported procedures[24].

4.1. To fabricate the hydrogel in the presence of $CO_2$, dissolve 0.5 g of starch in 5 mL of water in a 10 mL high-pressure reactor. Heat the reactor to the desired temperature, and then pressurize gently with $scCO_2$ at 1 $MPa.min^{-1}$ to a pressure of 20 MPa. Stir the pressurized starch solution for 20 min at 600 rpm to promote the gelatinization, and then cool the reactor to room temperature.

4.2. For retrogradation, leave the pressurized starch solution at 4 °C for 48 h. Then, place the reactor in a thermostatic water bath at 40 °C, and pressurize with $scCO_2$ at 1 $MPa.min^{-1}$ to a pressure of 20 MPa.

4.3. Continuously flow $scCO_2$ containing 11% v/v ethanol at 2 $mL.min^{-1}$ through the reactor for 4 h. Use a continuous high-pressure pump to maintain the flow rate of $CO_2$ at 2 $mL.min^{-1}$ and a second HPLC pump for the ethanol at 0.22 $mL.min^{-1}$.

4.4. After this period, stop the ethanol flow, and start the flow of $scCO_2$ at 2 $mL.min^{-1}$ through the reactor for 2 h. Decrease the pressure to 10 MPa to allow the supercritical impregnation take place, and maintain the reactor temperature at 40 °C.

4.5. Fill a 0.5 mL loop mounted on a 6-way high-pressure valve (**Figure 2**) with GCO extract using

a syringe. Pressurize the loop with $scCO_2$ to a achieve a pressure of 30 MPa and leave for 10 min to equilibrate. Turn the valve to the injection position, and start the flow of $scCO_2$ across the loop until the pressure inside the reactor reaches 30 MPa; leave the system in this condition for 12 h.

NOTE: The GCO extract was obtained and characterized using previously reported procedures[23].

4.6. Depressurize the system at a rate of 0.1 $MPa.min^{-1}$, and collect the impregnated material. See **Figure 3** for a schematic representation of the one-pot system. Determine the amount of the impregnated oil using the extraction procedure described in the literature[1,7,11].

[Place **Figure 3** here].

4.7. Disperse 0.5 g of the impregnated aerogel in 25 mL of chloroform, and stir for 1 h at 50 °C and 600 rpm for extraction. Use a 0.2 µm syringe filter to filter the extracts, collect them in a calibrated round flask, and evaporate the solvent under a flow of nitrogen. Calculate the extracted mass by determining the difference between the flask weights, and determine the impregnation efficiency in terms of mg of oil per 100 mg of aerogel.

**5. Characterization of aerogels**

5.1. Determine the surface area of the aerogels using an ultra-high-purity nitrogen adsorption and desorption isotherms at 77 K using the Brunauer–Emmett–Teller (BET) method.

5.1.1. Prior to the analysis, heat ~200 mg of sample at 115 °C for 4 h under vacuum.

5.1.2. Evaluate adsorption isotherms in the linear region of the BET plot (at a relative pressure p/p0 in the range of 0.05–0.3) using a multipoint BET for the determination of the surface area.

5.1.3. Estimate both volume and pore size using the Barrett–Joyner–Halenda (BJH) method.

5.2. Characterize aerogel morphology using Field Emission Scanning Electron Microscopy at 5 kV and 15 mA under high vacuum. Cut the materials transversely to obtain 1 mm thickness, place the slices on a double-sided carbon film, and sputter-coat with gold[22].

5.3. Determine the diffraction pattern and the degree of crystallinity by X-ray diffraction.

5.3.1. Before the measurements, crush the samples, and scatter them in the equipment holder. Make sure the system is equipped with a copper target X-ray tube set to 40 kV and 40 mA. Expose the samples to the X-ray beam, and scan from 5 to 40 Å with an angular scanning velocity of $1.267°.min^{-1}$[1,17, 22].

5.3.2. Finally, calculate the degree of crystallinity ($D_C$) using the following equation.

$$D_C = \frac{A_C}{A_C + A_a} x\ 100 \qquad (1)$$

Where $A_C$ is the area under the curve for crystalline reflection, and $A_a$ is the area under the curve for crystalline amorphous[17, 25].

**REPRESENTATIVE RESULTS:**

Traditionally, the process used to transform hydrogels into aerogels involves two major steps: solvent exchange and SCE. The first goal of this protocol was to integrate both steps into a continuous flow process. To that end, the impact of pressure and co-solvent (ethanol) composition on the surface area of the materials was examined. Surface area is an excellent indicator of the quality of the aerogel and allows a direct comparison with literature.

Initial studies demonstrated that the extraction process should be performed for at least 6 h (4 h of $CO_2$/ethanol mixture and 2 h of $CO_2$)[24]. The results of this study are depicted in **Figure 4** and show that high-surface area aerogels could be obtained using a mixture of $CO_2$/ethanol. Optimal results were observed when materials exhibited a surface area of nearly 94 $m^2.g^{-1}$, which agrees with highest values reported in the literature using a solvent exchange step[1, 7, 8, 22]. When compared with the work of Comin et al.[12] who reported surface area of <10 $m^2.g^{-1}$, the integrated approached described here can produce superior materials for the impregnation of different chemical compounds.

[Place **Figure 4** here]

The next stage of the present work was to evaluate the impact of $CO_2$ on the gelatinization and retrogradation steps. Therefore, both gelatinization and retrogradation were carried out in the presence of $CO_2$ at different temperatures (**Figure 5**). The results show the beneficial impact of $CO_2$ on the surface area of the materials. In fact, these results are consistent with the best values reported in literature for starch aerogels (185 $m^2.g^{-1}$)[1, 2, 11,21].

[Place **Figure 5** here]

**Figure 6** and **Figure 7** highlight the importance of temperature for the quality of the materials. For instance, particle formation only occurred at 40 °C (**Figure 6A**).

[Place **Figure 6** here]

Beyond this temperature, all the materials displayed a high degree of agglomeration, and their structures were significantly collapsed. This effect was also confirmed by X-ray diffraction as an increase in temperature promoted a reduction in the crystallinity of the materials (**Figure 7**).

[Place **Figure 7** here]

Even though several studies have reported the impact of $CO_2$ during gelatinization, none of them

mention any effect of $CO_2$ on the retrogradation process[8–12]. The results presented here suggest that $CO_2$ could impact this process as all the materials obtained in the presence of $CO_2$ exhibit a higher surface area. Moreover, several studies report that all the starch crystal structure is destroyed above 70 C[26,27]. Nevertheless, the results of this study show the presence of crystallinity at 120 °C, which confirms the impact of $CO_2$ on the retrogradation process. Therefore, this protocol not only introduces an improvement in the drying process, but could also enhance the properties of the materials produced with this process.

Finally, based on these encouraging results, all these steps were integrated into a one-pot process (**Figure 8**). To accomplish this, gelatinization and retrogradation were performed in the presence of $CO_2$ and dried using the method described above. After this process, the material was impregnated with GCO through the gas phase. Nevertheless, before integrating both steps, the impregnation conditions were evaluated.

[Place **Figure 8** here].

In this case, the starch aerogel was prepared using the procedure described in section 1 and impregnated with the GCO using the procedure described in section 3. This experiment allowed the comparison between a traditional impregnation procedure with the one-pot strategy described here (**Figure 9**).

[Place **Figure 9** here]

**Figure 9** shows that that the impregnation equilibrium could be attained after 12 h with an impregnation efficiency of 40.7%. These results are in agreement with the values reported in literature for the impregnation of similar molecules in starch aerogels[1,3–5]. Furthermore, the one-pot concept exhibited good impregnation efficiency (24%).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Schematic representation of the drying apparatus.** Abbreviations: V-1–5 = valves; RHE1 = refrigerated heat exchanger; CV-1, 2 = check valves; PUMP1 = liquid $CO_2$ pump; PUMP2 = high-performance liquid chromatography co-solvent pump; RPV1 = reactor pressure vessel; TIC = temperature indicator-controller; F1 = filter; PIC = pressure indicator-controller; BPV = Back pressure valve.

**Figure 2: Schematic representation of the 6-Way high-pressure valve.** (**A**) Filling the loop with GCO. (**B**) Injection position. Abbreviations: GCO = green coffee oil; V-4, 5 = valves.

**Figure 3: Schematic representation of the one-pot system.** Abbreviations: V-1–6 = valves; RHE1 = refrigerated heat exchanger; CV-1, 2: check valves; PUMP1 = liquid $CO_2$ pump; PUMP2 = high-performance liquid chromatography co-solvent pump; RPV1 = reactor pressure vessel; 6-wV = 6-way valve; TIC = temperature indicator-controller; F1 = filter; PIC = pressure indicator-controller; BPV: back pressure valve.

**Figure 4: Impact of the drying conditions on aerogel structural properties.** ▪The squares indicate surface area. ● The circles indicate the pore volume. This figure has been modified from Villegas et al.[24].

**Figure 5: Impact of temperature on the aerogel surface area in presence and absence of $CO_2$.** This figure has been modified from Villegas et al.[24].

**Figure 6: Micrographs of the materials obtained with different gelatinization temperatures.** (**A**) 40 °C, (**B**) 90 °C, (**C**) 120 °C. Supercritical drying at 20 MPa and 40 °C with 11% v/v of ethanol. Scale bars = 50 µm. This figure has been modified from Villegas et al.[24].

**Figure 7: Characteristics of the materials obtained at the end of the process.** (**A**) X-ray diffraction patterns of the materials**.** (**B**) Degree of crystallinity of the obtained materials**.** This figure has been modified from Villegas et al.[24].

**Figure 8: Graphical abstract of the one-pot process**. 1. Gelatinization: formation of the gel from the water and starch solution at 40 °C with $scCO_2$. 2. Retrogradation: formation of the 3D network by decreasing temperature in $CO_2$. 3. Supercritical drying: supercritical extraction with $CO_2$ and ethanol. 4. $scCO_2$ impregnation: impregnation of green coffee oil inside the aerogel. This figure has been modified from Villegas et al.[24]. Abbreviations: $scCO_2$ = supercritical carbon dioxide; 3D = three-dimensional.

**Figure 9: Comparison of impregnation efficiency of processes.** Purple circles, Impregnation efficiency of the traditional process. Green square, Impregnation efficiency of the one-pot process**.** This figure has been modified from Villegas et al.[24].

**DISCUSSION:**

One of the critical steps of this protocol is the gradual removal of water in the supercritical drying step. To obtain materials with a good surface area, the flow should be maintained between 2 and 3 $mL.min^{-1}$, otherwise an extensive pore-collapsed material will be obtained. Thus, judicious control of the extraction conditions is essential to obtain materials with the desirable properties for impregnation. Moreover, the size of the hydrogel is also a critical point. Large hydrogels could be difficult to dry and require a longer extraction process.

The improvement of the drying process opened new avenues to enhance the overall aerogel fabrication process as both gelatinization and retrogradation steps could be integrated with drying (one-pot). The gelatinization temperature is the critical point of this one-pot concept. Therefore, to obtain particles like the ones described in **Figure 6A**, the gelatinization temperature should be set at 40 °C to avoid extensive aggregation and dramatic reduction of surface area.

Another critical point of this protocol is related to the pressurization and depressurization steps. Pressurization should be gentle (1 $MPa.min^{-1}$). Faster pressurization could destroy the 3D

structure of aerogel leading to an extensive collapse of the pore structure. Depressurization is also critical, especially after impregnation. Thus, depressurization should be very gentle (0.1–0.3 $MPa.min^{-1}$) to avoid a dramatic decrease in the impregnation efficiency.

The results obtained with this protocol allowed the integration of all aerogel fabrication steps (gelatinization, retrogradation, and drying), making this one-pot concept a very promising tool to develop novel strategies for different applications in the food, nutraceutical, pharmaceutical, or bioenergetic industries.

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**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1. Mehling, T., Smirnova, I., Guenther, U., Neubert, R.H.H. Polysaccharide-based aerogels as drug carriers. *Journal of Non-Crystalline Solids*. **355** (50–51), 2472–2479, doi: 10.1016/j.jnoncrysol.2009.08.038 (2009).
2. García-González, C.A., Camino-Rey, M.C., Alnaief, M., Zetzl, C., Smirnova, I. Supercritical drying of aerogels using CO 2: Effect of extraction time on the end material textural properties. *Journal of Supercritical Fluids*. **66**, 297–306, doi: 10.1016/j.supflu.2012.02.026 (2012).
3. Ring, S.G. *et al.* The gelation and crystallisation of amylopectin. *Carbohydrate Research*. **162** (2), 277–293, doi: 10.1016/0008-6215(87)80223-9 (1987).
4. Debet, M.R., Gidley, M.J. Why do gelatinized starch granules not dissolve completely? Roles for amylose, protein, and lipid in granule "ghost" integrity. *Journal of Agricultural and Food Chemistry*. **55** (12), 4752–4760, doi: 10.1021/jf070004o (2007).
5. Le Corre, D., Bras, J., Dufresne, A. Starch Nanoparticles: A Review. *Biomacromolecules*. **11** (5), 1139–1153, doi: 10.1021/bm901428y (2010).
6. García-González, C.A., Alnaief, M., Smirnova, I. Polysaccharide-based aerogels - Promising biodegradable carriers for drug delivery systems. *Carbohydrate Polymers*. **86** (4), 1425–1438, doi: 10.1016/j.carbpol.2011.06.066 (2011).
7. De Marco, I., Reverchon, E. Starch aerogel loaded with poorly water-soluble vitamins through supercritical CO2 adsorption. *Chemical Engineering Research and Design*. **119**, 221–230, doi: 10.1016/J.CHERD.2017.01.024 (2017).
8. Druel, L., Bardl, R., Vorwerg, W., Budtova, T. Starch Aerogels: A Member of the Family of Thermal Superinsulating Materials. *Biomacromolecules*. **18** (12), 4232–4239, doi: 10.1021/acs.biomac.7b01272 (2017).

9. Zou, F., Budtova, T. Tailoring the morphology and properties of starch aerogels and cryogels via starch source and process parameter. *Carbohydrate Polymers*. doi: https://doi.org/10.1016/j.carbpol.2020.117344 Received 28 August 2020; Received in revised form (2020).
10. Santos-Rosales, V. *et al.* Stability Studies of Starch Aerogel Formulations for Biomedical Applications. *Biomacromolecules*. doi: https://dx.doi.org/10.1021/acs.biomac.0c01414 (2020).
11. Ubeyitogullari, A., Ciftci, O.N. Phytosterol nanoparticles with reduced crystallinity generated using nanoporous starch aerogels. *Roysal Society of Chemistry Advances*. **6** (110), 108319–108327, doi: 10.1039/c6ra20675a (2016).
12. Comin, L.M., Temelli, F., Saldaña, M.D.A. Barley beta-glucan aerogels via supercritical CO 2 drying. *FRIN*. **48** (2), 442–448, doi: 10.1016/j.foodres.2012.05.002 (2012).
13. Brown, Z.K., Fryer, P.J., Norton, I.T., Bridson, R.H. Drying of agar gels using supercritical carbon dioxide. *The Journal of Supercritical Fluids*. **54** (1), 89–95, doi: 10.1016/j.supflu.2010.03.008 (2010).
14. Gurikov, P., Raman, S.P., Weinrich, D., Fricke, M., Smirnova, I. A novel approach to alginate aerogels: carbon dioxide induced gelation. *RSC Advances*. **5** (11), 7812–7818, doi: 10.1039/C4RA14653K (2015).
15. Lebedev, A., Suslova, E., Troyankin, A., Lovskaya, D. Investigation of Aerogel Production Processes: Solvent Exchange under High Pressure Combined with Supercritical Drying in One Apparatus. *Gels*. **7** (1), doi: doi.org/10.3390/gels7010004 (2021).
16. Francisco, J.D.C., Sivik, B. Gelatinization of cassava, potato and wheat starches in supercritical carbon dioxide. *Journal of Supercritical Fluids*. **22** (3), 247–254, doi: 10.1016/S0896-8446(01)00135-8 (2002).
17. Muljana, H., Picchioni, F., Heeres, H.J., Janssen, L.P.B.M. Supercritical carbon dioxide (scCO2) induced gelatinization of potato starch. *Carbohydrate Polymers*. **78** (3), 511–519, doi: 10.1016/j.carbpol.2009.05.005 (2009).
18. Cavin, C., Mace, K., Offord, E.A., Schilter, B. Protective effects of coffee diterpenes against aflatoxin B1-induced genotoxicity: Mechanisms in rat and human cells. *Food and Chemical Toxicology*. **39** (6), 549–556, doi: 10.1016/S0278-6915(00)00168-X (2001).
19. Cavin, C., Holzhaeuser, D., Scharf, G., Constable, A., Huber, W., Schilter, B. Cafestol and kahweol, two coffee specific diterpenes with anticarcinogenic activity. *Food and Chemical Toxicology*. **40** (8), 1155–1163, doi: 10.1016/S0278-6915(02)00029-7 (2002).
20. Lee, K.J., Choi, J.H., Jeong, H.G. Hepatoprotective and antioxidant effects of the coffee diterpenes kahweol and cafestol on carbon tetrachloride-induced liver damage in mice. *Food and Chemical Toxicology*. **45** (11), 2118–2125, doi: 10.1016/j.fct.2007.05.010 (2007).
21. Lee, K.J., Jeong, H.G. Protective effects of kahweol and cafestol against hydrogen peroxide-induced oxidative stress and DNA damage. *Toxicology Letters*. **173** (2), 80–87, doi: 10.1016/j.toxlet.2007.06.008 (2007).
22. Ubeyitogullari, A., Ciftci, O.N. Formation of nanoporous aerogels from wheat starch. *Carbohydrate Polymers*. **147**, 125–132, doi: 10.1016/j.carbpol.2016.03.086 (2016).
23. De Oliveira, P.M.A., De Almeida, R.H., De Oliveira, N.A., Bostyn, S., Gonçalves, C.B., De Oliveira, A.L. Enrichment of diterpenes in green coffee oil using supercritical fluid extraction - Characterization and comparison with green coffee oil from pressing. *Journal*

*of Supercritical Fluids*. **95**, 137–145, doi: 10.1016/j.supflu.2014.08.016 (2014).
24. Villegas, M., Oliveira, A.L., Bazito, R.C., Vidinha, P. Development of an integrated one-pot process for the production and impregnation of starch aerogels in supercritical carbon dioxide. *Journal of Supercritical Fluids*. **154**, 104592, doi: 10.1016/j.supflu.2019.104592 (2019).
25. Nara, S., Komiya, T. Studies on the Relationship Between Water-satured State and Crystallinity by the Diffraction Method for Moistened Potato Starch. *Starch - Stärke*. **35** (12), 407–410, doi: 10.1002/star.19830351202 (1983).
26. Cooke, D., Gidley, M.J. Loss of crystalline and molecular order during starch gelatinisation: origin of the enthalpic transition. *Carbohydrate Research*. **227** (C), 103–112, doi: 10.1016/0008-6215(92)85063-6 (1992).
27. Ratnayake, W.S., Jackson, D.S. A new insight into the gelatinization process of native starches. *Carbohydrate Polymers*. **67** (4), 511–529, doi: 10.1016/j.carbpol.2006.06.025 (2007).

Figure 1



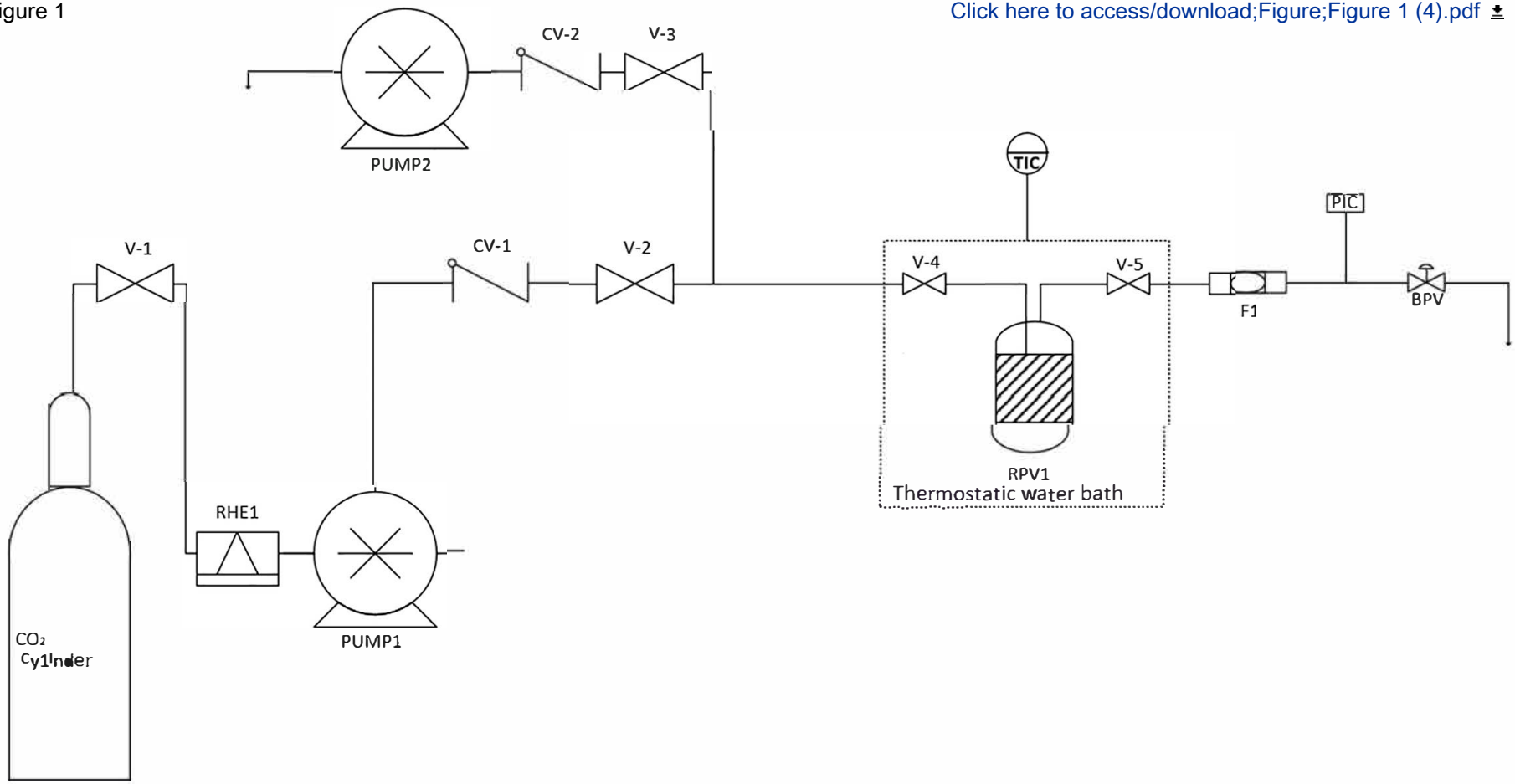


Figure 2



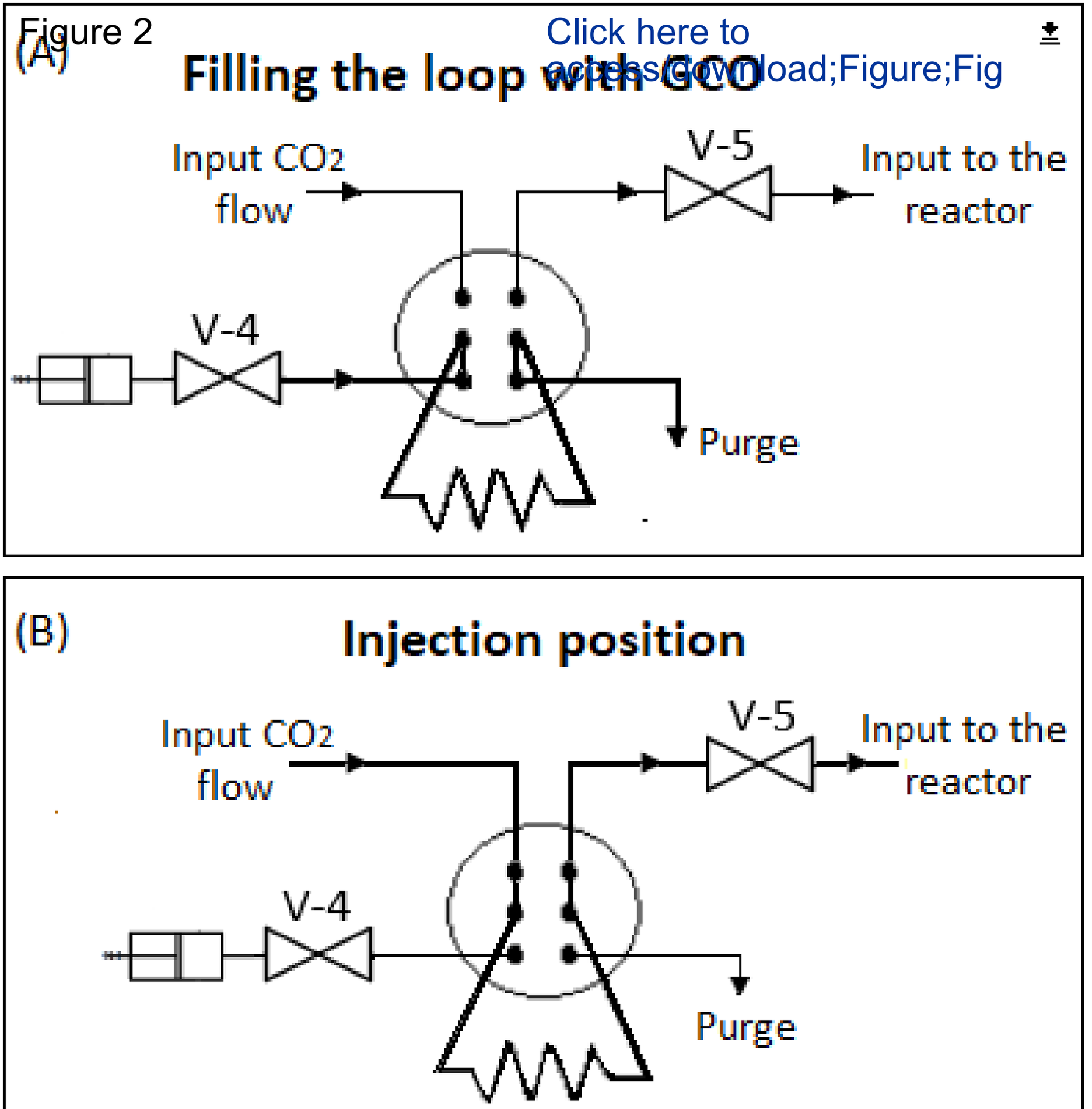


Figure 3



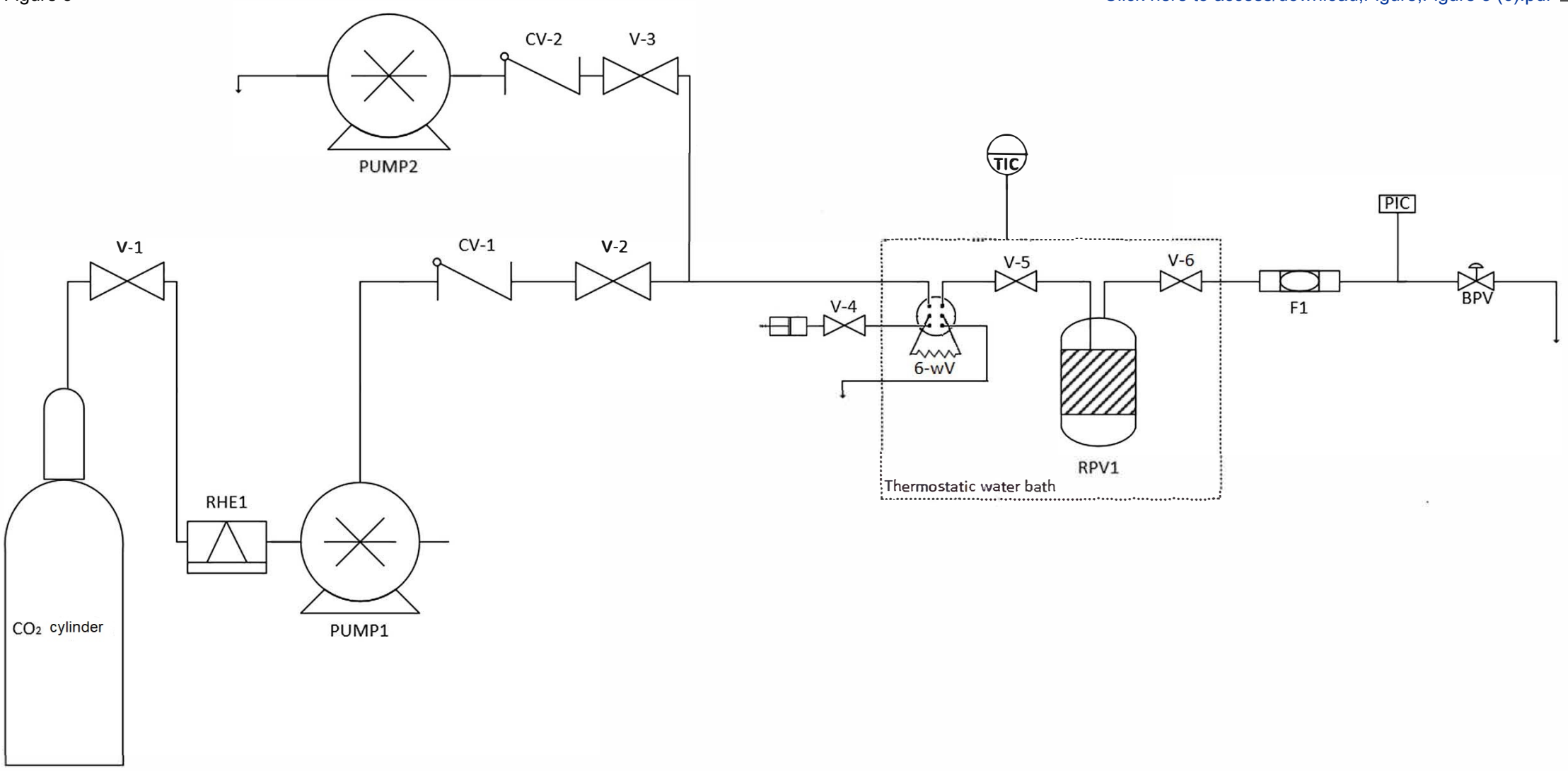


Figure 4



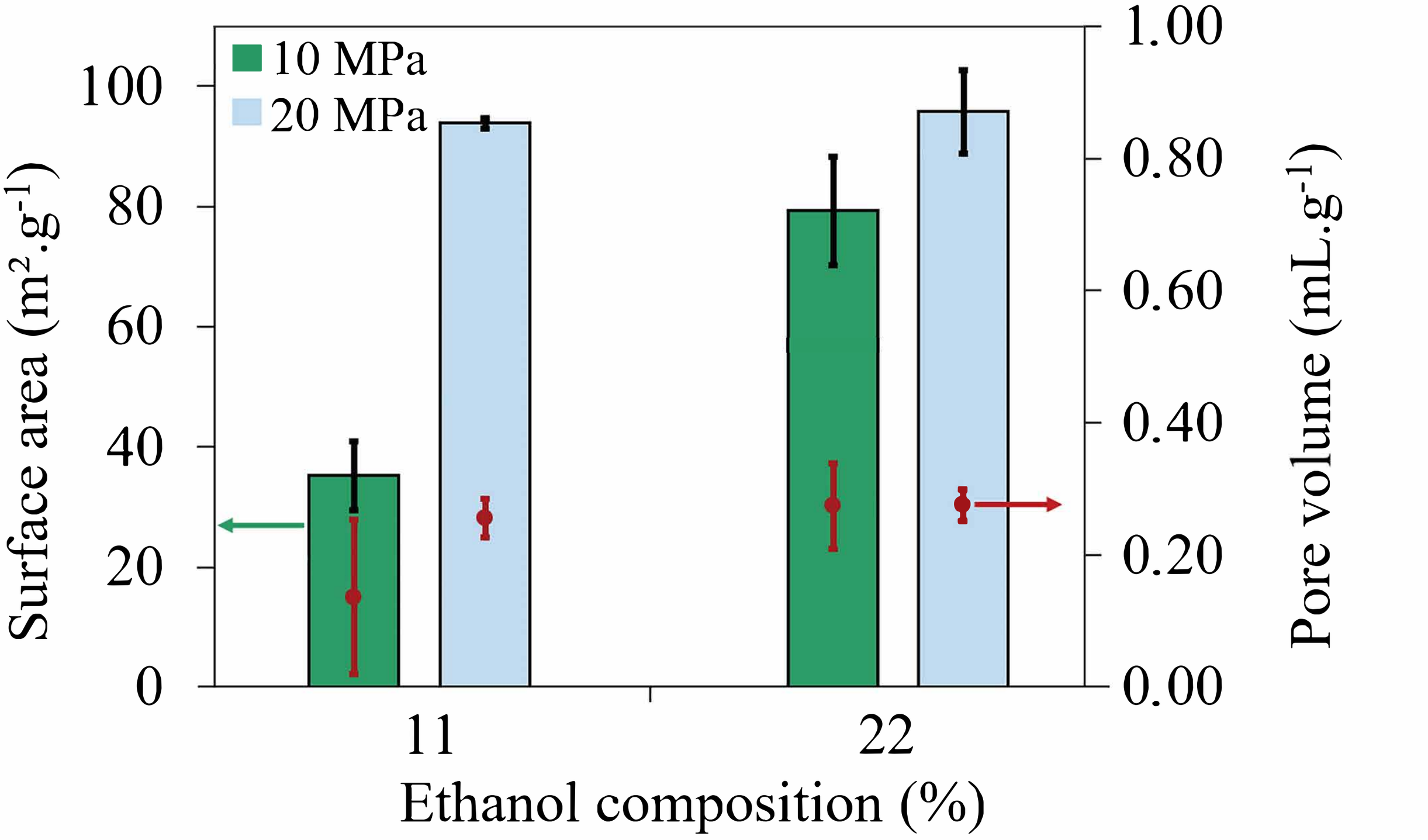


Figure 5



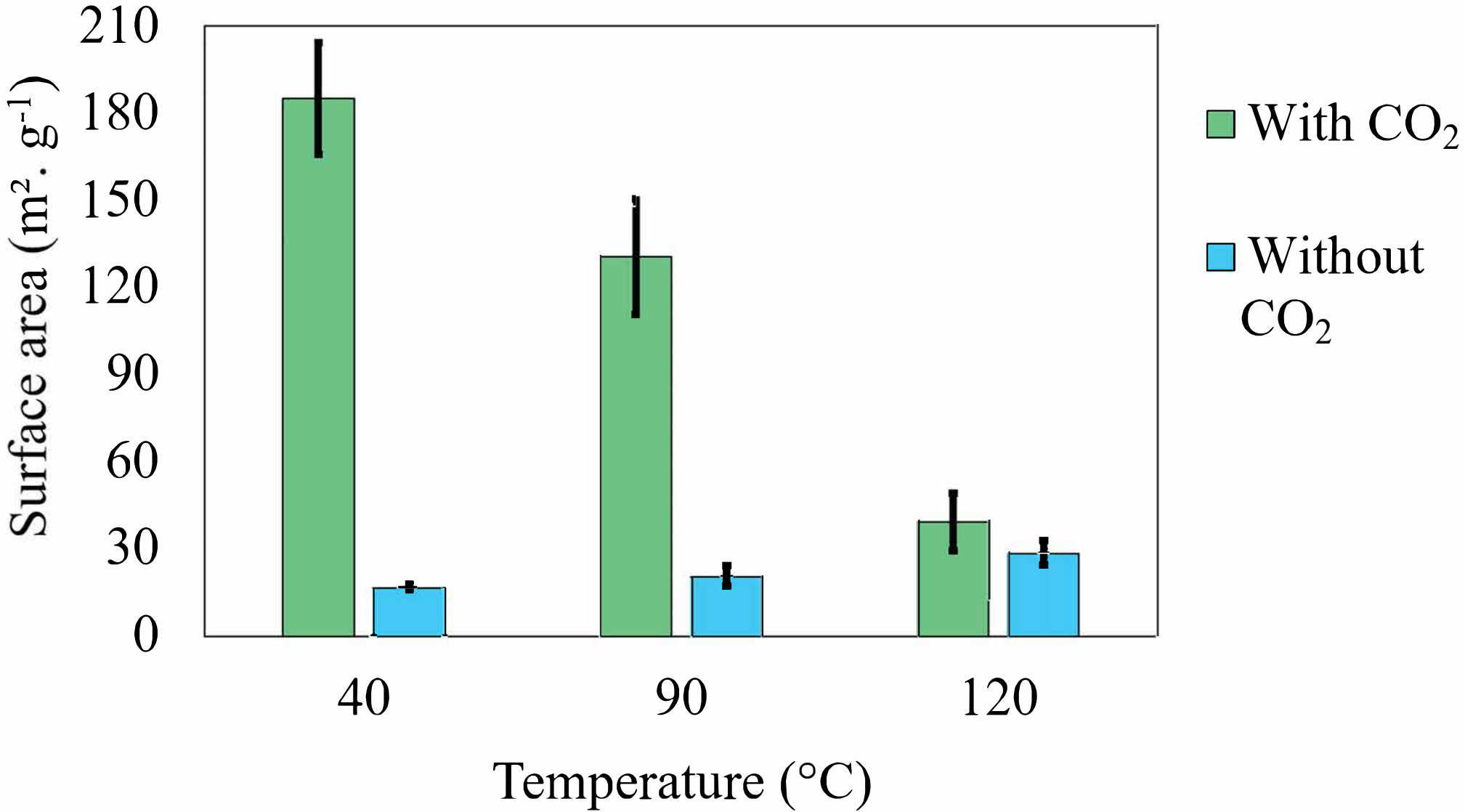


Figure 6



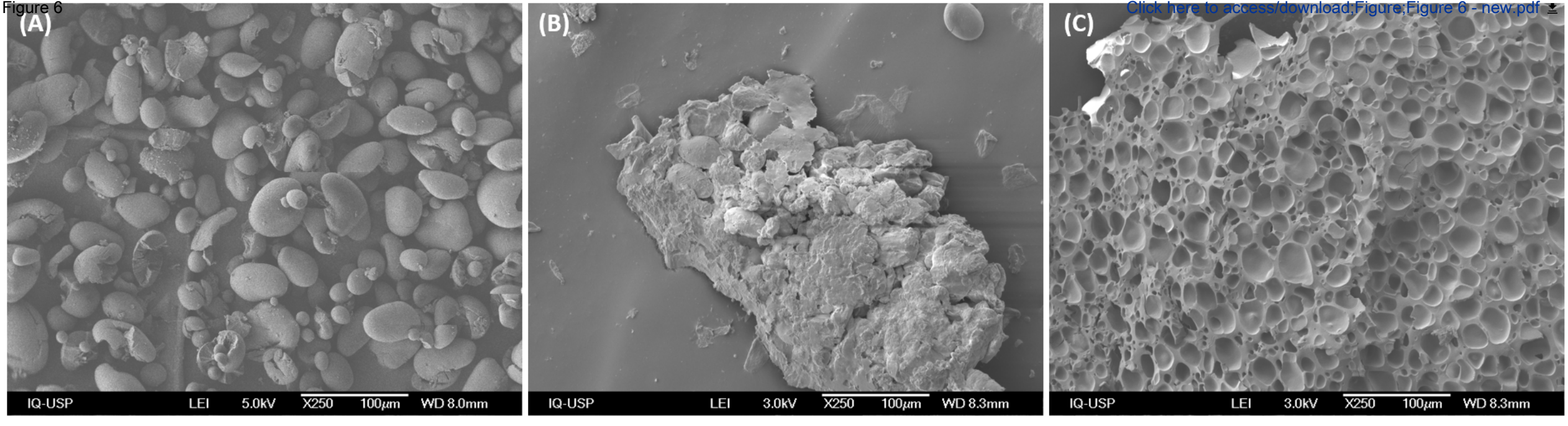
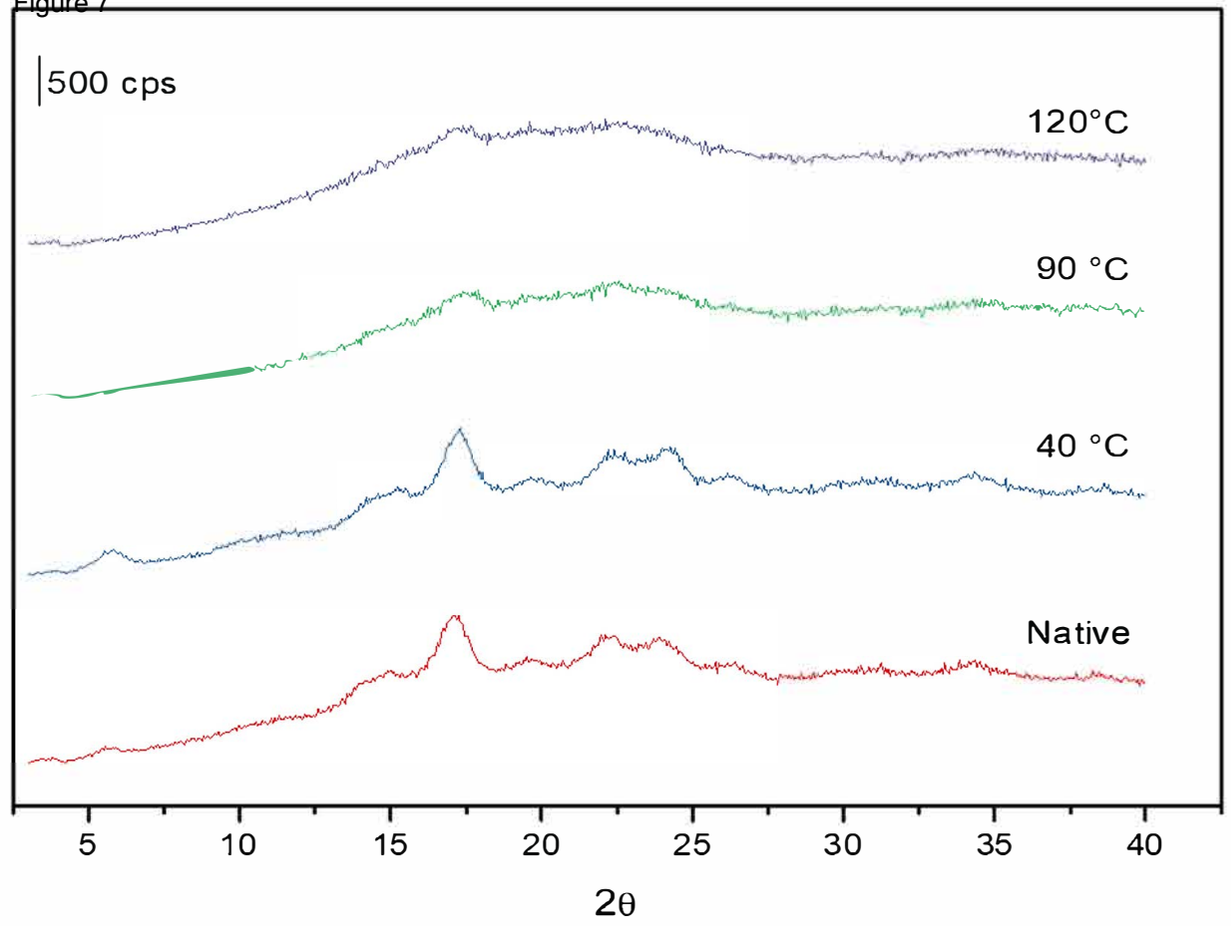


Figure 7





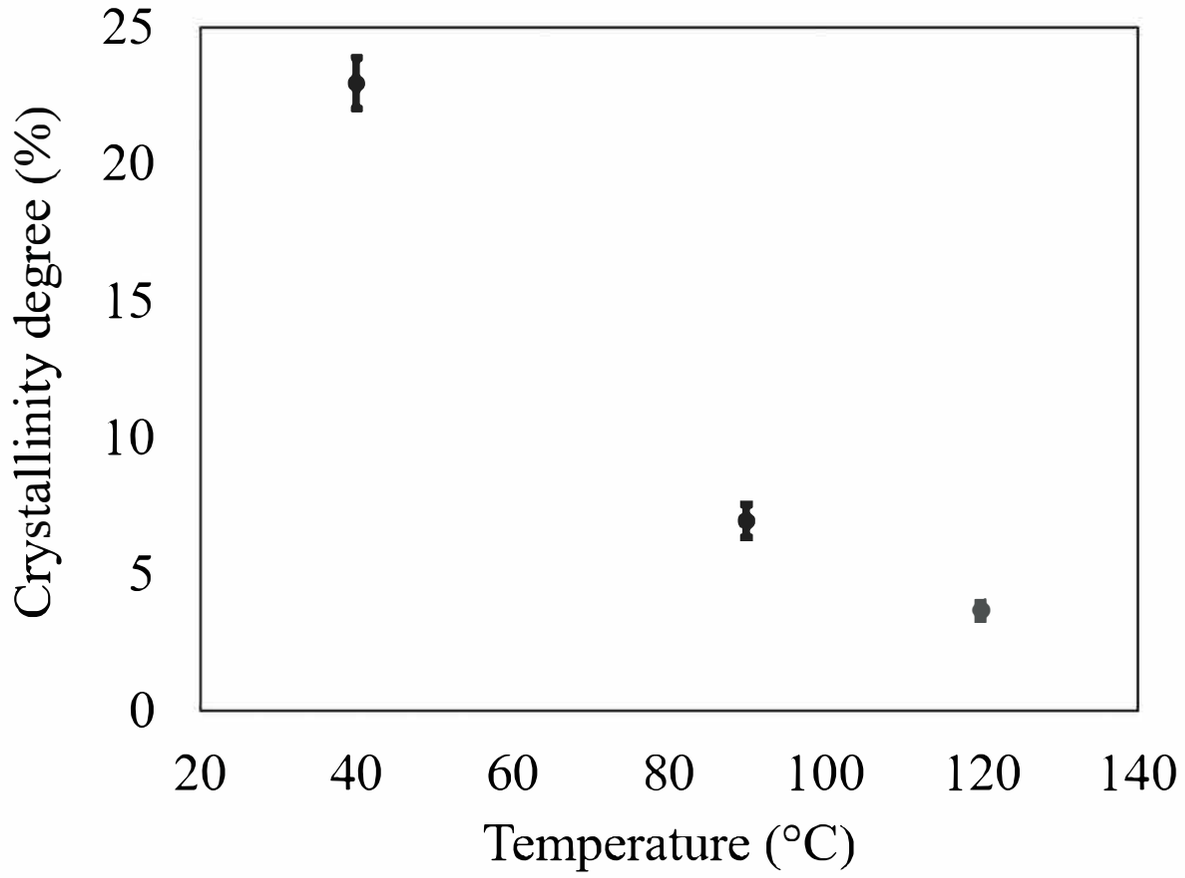



Figure 8

Click here to access/download;Figure;Figure 8 (1).pdf

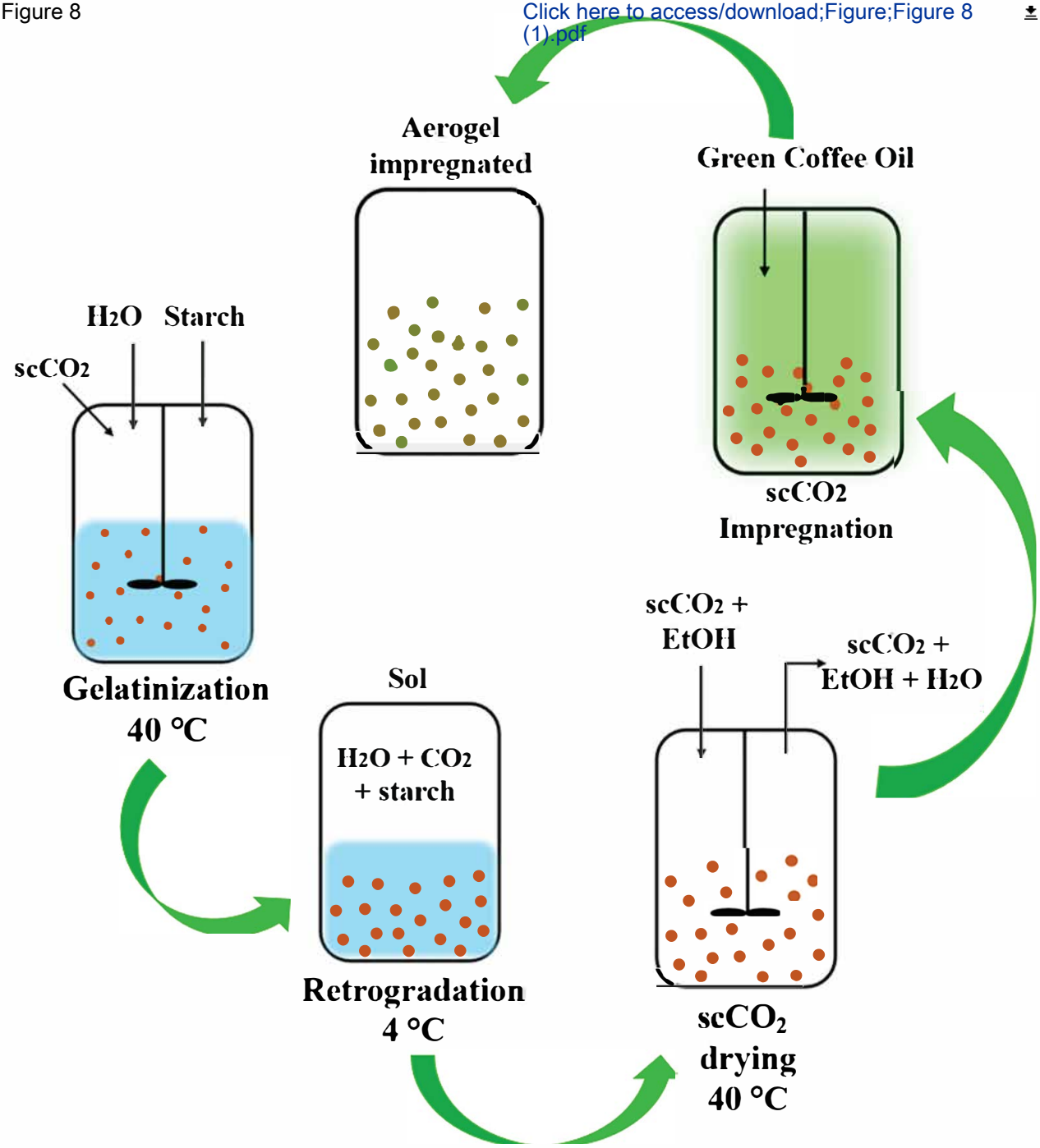
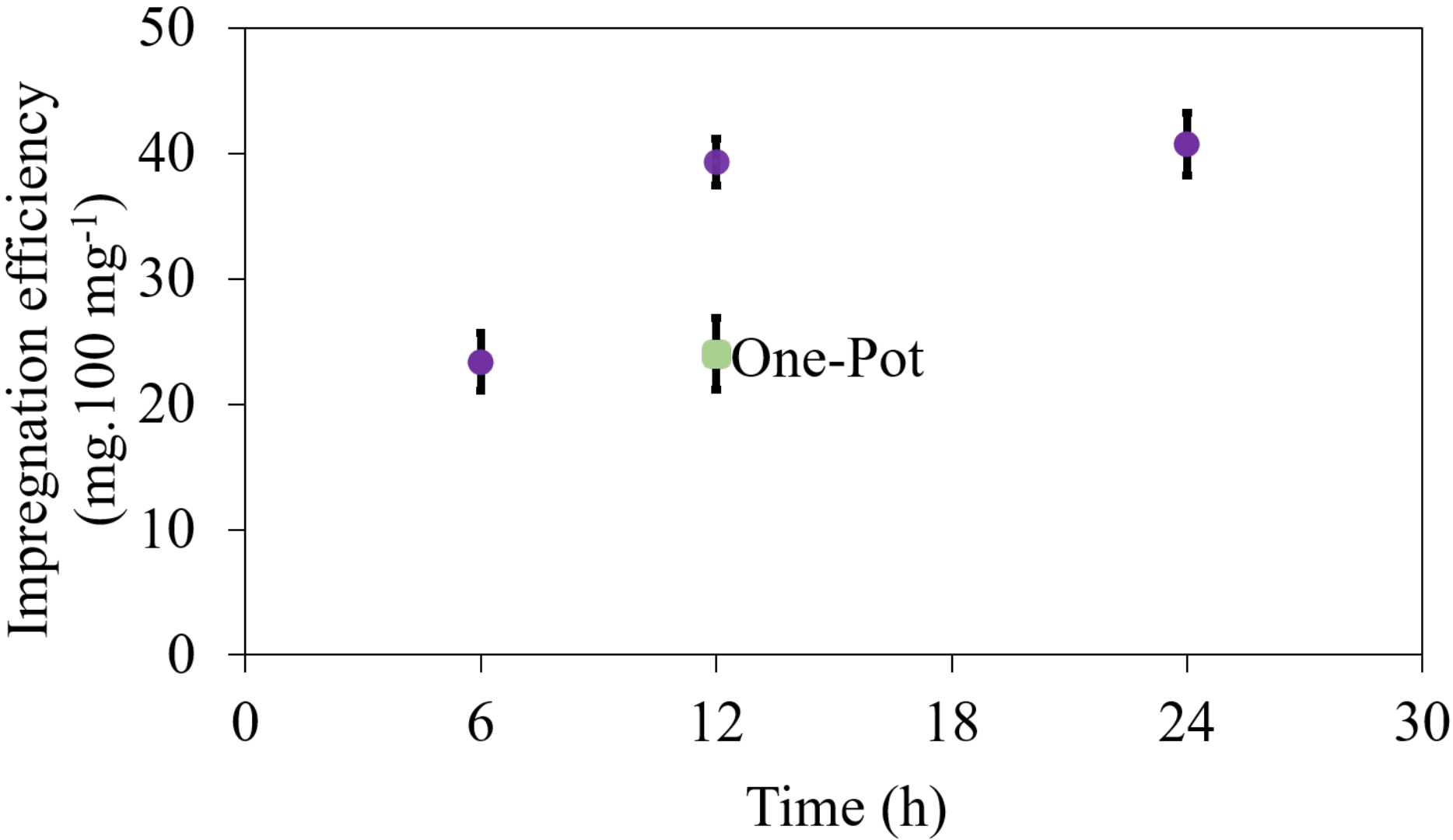


Figure 9





| Name of Material/Equipment Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 6-Way high pressure valve | Rheodyne | | |
| Field Emission Scanning Electron Microscopy | JOEL | JSM-7401 F | |
| High pressure cell 10 mL | Citua (Campinas, Brazil) | Custom made | Set Up construction done by Citua (Campinas, Brazil) |
| High pressure cell 20 mL | Citua (Campinas, Brazil) | Custom made | Set Up construction done by Citua (Campinas, Brazil) |
| High pressure p-series Pump | Thar Technologies | Model Thar SFC, P-50A | |
| HPLC-type pump | Shimadzu | Model LC-10AD Vp | |
| Polypropylene molds (eppendorf) | Eppendorf, Brazil | Safe-Lock Tube 2.0 mL | |
| Surface Area Analyzer | Quantachrome Instruments | Model Nova 1220 | |
| X-Ray Diffraction Instrument | Rigaku | Model Miniflex | Powder X-Ray Diffraction (XRD) Instrument |
| **Chemicals** | | | |
| Ethanol anhydrous, ≥99.5% | Sigma-Aldrich, Brazil | | CAS # 64-17-5 |
| Liquid carbon dioxide, 99.98% | Oxilumen, Brazil | | CAS # 124-38-9 |
| Native potato starch | Vetec, Brazil | | CAS # 9005-25-8 |

**EDITORIAL COMMENTS**:

*Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

***Question 2***. *Please revise the following lines to avoid previously published work: lines: 23-25, lines:30-35, lines: 65-68, lines: 94-99[Maria Villegas, Alessandra L. Oliveira, Reinaldo C. Bazito, Pedro Vidinha. "Development of an integrated one-pot process for the production and impregnation of starch aerogels in supercritical carbon dioxide", The Journal of Supercritical Fluids, 2019301020 doi:10.1016/j.supflu.2019.104592 1-s2.0-S0896844619301020]*

**Answer:**

*lines 23-25; lines:30-35; lines: 65-68 and 94-99*

We accepted the suggestion of the editorial and we changed the text. The changes are highlighted in the manuscript.

***Question 3***. *Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.*

**Answer:** We have corrected the text and the changes are highlighted in the manuscript.

***Question 4***. *Line 105: Please specify the volume of the solution prepared.*

**Answer:** 10 mL, - We have corrected the text and the changes are highlighted in the manuscript

***Question 5***. *Line 106: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).*

**Answer:** In our case, we used a heated magnetic stirrer not a centrifuge.

***Question 6***. *Line 117: Please use standard representation for SI units. "ml" should be written as "mL".*

**Answer:** We have corrected the text and the changes are highlighted in the manuscript

***Question 7**. Line 119: Please mention the rate at which ethanol was pumped.*

**Answer:** 0.22 or 0.44 $mL.min^{-1}$. We have corrected the text and the changes are highlighted in the manuscript

***Question 8**. Line 191: What was the flow rate of scCO2 (please make sure that is mentioned throughout the protocol)*

**Answer:** The flow rate of $scCO_2$ was maintained at 2 $mL.min^{-1}$. We have corrected the text and the changes are highlighted in the manuscript.

***Question 9.** Line 207- 210: Please clarify the statement*

**Answer:** We have corrected the text and the changes are highlighted in the manuscript

***Question 10**. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."*

**Answer:** We attach a pdf file with the copyright permission.

***Question 11**. Please be consistent in labeling the figures. Please make sure that the labels are according to the JoVE format and appear same in both the figure references and legends as well as the figure. Please ensure that each figure is labeled when depicted in multiple panels.*

**Answer:** We have corrected the text and the changes are highlighted in the manuscript.

***Question 12**. Figure 4: Please specify what the red and black error bars represent. Please clarify the right Y-axis*

**Answer:** Both bars represent the standard deviation of the measurements. At least three experiments were performed to obtain these values. The right Y-axis is related with circles which corresponds to the por volume determined by BJH has described in the experimental procedure. We have corrected the figure to become more comprehensive.

***Question 13**. Figure 7: Please clarify the label on the "red XRD pattern"*

**Answer:** This XRD pattern refers to the of "native" starch. This means that this starch did not suffer any modification or thermal treatment.

***Question 14.** Figure 8: Please specify all the necessary parameters.*

**Answer:** We have corrected the figure to become more comprehensive. We have introduced a small text in the legend to explain each step.

***Question 15**. Figure 9: Please ensure that the data presented is labeled properly.*

**Answer:** We have corrected the figure to become more comprehensive. A new legend was added.

---

**REVIEWERS' COMMENTS:**

**Reviewer #1:**

Manuscript Summary:

**Question**: The paper is about a method of making aerogels in supercritical carbon dioxide. It would be interesting to have a video on this topic.

**Answer:** Yes, the video of Jove will provide an excellent way to highlight this topic.

***Question**: Please have your language checked. There is a lot of space to improve the reading.*

**Answer:** We accept the suggestion of the referee and we improved the text.

**Reviewer #2:**

Manuscript Summary:

The manuscript describes a one-pot process for the preparation of starch aerogels and impregnation in one-pot

Major Concerns:

***Question**: In the Introduction section, it is criticized that the current starch aerogel processing methods last more than one day. However, in this work this limitation is not overcome since the starch retrogradation was chosen of 48 hours and the overall process will last around 3 days. Please remove this sentence from the Introduction section*

**Answer:** We believe that there was a miss understanding regarding the reduction of solvent exchange time. The time reduction only applies to the solvent exchange step not to the overall process. In this case, all the examples cited in the manuscript describe a solvent exchange process that last for at least 1 day plus a minimum of four hours of supercritical extraction. Here, we reported an approach that extracts water in 6 hours. Additionally, the retrogradation process described in all the cited examples last at least 48 h. Any way, we change the text to clarify this subject.

Minor Concerns:

**Question** : The references used do not look updated. Recent publications on starch aerogels are suggested:

-https://www.sciencedirect.com/science/article/abs/pii/S0144861720315174

-https://pubs.acs.org/doi/abs/10.1021/acs.biomac.0c01414

**Answer:** We accept the suggestion of the referee and improve the text.

**Reviewer #3**:

Manuscript Summary:

*The manuscript reports on an integrated "one-pot" approach towards starch-based aerogels via dissolution, retrogradation, solvent exchange and supercritical drying all done in CO2 atmosphere. Clear advantages of pressurized CO2 were demonstrated such as increase in surface area and pore volume. Moreover, impregnation step was incorporated into the process scheme as a last step before depressurization. The approach is certainly a step forward towards an efficient processing of starch aerogels. The study design is appropriate, conclusions are fully supported by the presented data.*

Major Concerns:

***Question:** The authors claim that the solvent exchange protocol under high pressure has never resulted in quality materials and cite Comin and Brown works. I am aware of at least two other works where the high-pressure solvent exchange was successfully applied to alginate hydrogels (doi: 10.1039/C4RA14653K and doi: 10.3390/gels7010004). Perhaps there are other attempts I am not aware of. The authors*

*should perform a better literature search on this aspect. Then, to make the Introduction and Discussion parts more comprehensive, the own results should be discussed along with the mentioned publications. Furthermore, to a certain extent the proposed protocol is a supercritical extraction with a co-solvent that has been exemplified many times for extracting essential oils. Such a link made in the manuscript would not compromise novelty, but render the work more effective and comprehensive.*

**Answer:**

We understand the point mentioned by the referee. The novelty of our work was to execute the solvent extraction using a flow continuous process with a mixture of scCO2 and ethanol. The first work coated by the referee (10.1039/C4RA14653K) was a work of Gurikov et al. These authors have first exposed the alginate aerogels to supercritical CO2 (120 bar, 318 K) and then introduced ethanol/water mixtures (30, 60, 90 wt%) to cover gels completely. Instead of immersing hydrogels in grades of ethanol at ambient conditions followed by sc-drying, they first exposed the alginate hydrogels to scCO2 (12 MPa, 318 K) and then introduced ethanol/water mixtures (30, 60, 90 wt%) to cover gels completely. The gels were immersed on these high-pressure mixtures for 2,5 h. After this period, the gels were flushed with sc−CO2/ethanol. Their results show that the solvent exchange at high pressure led to the formation of quality aerogels. This procedure is substantially different from the one we described here. In fact, we cited this work on our publication on journal of supercritical fluids* to justify the novelty of our approach.

The second work mentioned by the referee (10.3390/gels7010004) was published in January of 2021. It was impossible to cite this work during this submission. Nevertheless, this article follows a similar static extraction strategy described by Gurikov. These authors have used a mixture of scCO2/2-propanol/ water to obtained alginate aerogels. Therefore, the concept described here is substantially different from the one reported in this manuscript

Any way we cited both work in the current manuscript.

*( https://www.sciencedirect.com/science/article/pii/S0896844619301020)

Minor Concerns:

***Question****: what types of starch you expect this approach would also be applicable to? Perhaps a short discussion on this point would be helpful for groups working with other starch-based materials.*

**Answer:**

We expect that this strategy could be applied to all the starches that have a similar amylose contend. For instance, corn, maize, wheat, pea and potato. We add a comment on text.

***Question****: explain in more details how the autoclave was maintained at 4 C for 48 hours? Was there a cooling jacket?*

**Answer:** The autoclave was maintained in a refrigerator for 48 h.

***Question:*** *Figures 4 and 5 are difficult to interpret when printed in black-and-white.*

**Answer**: The figures 4 and 5 were corrected

***Question:*** *Figure 7: change "nativo" to "native".*

**Answer:** We have corrected the figure.

Pedro Vidinha

Graduation in Biotechnological Engineering at the Lusofona University of Humanities and Technologies, ULHT, Portugal (2001). Doctor in Chemical Engineering from NOVA University of Lisbon, UNL, Portugal (2007). Post-doctoral at NOVA University of Lisbon, UNL, Portugal (2008 - 2012) and at University of São Paulo, Brazil (2014). Since 2015 is auxiliary professor at Institute of Chemistry at University of São Paulo. He is also currently a researcher at RCGI - Research Center for Gas Innovation (CEPID FAPESP-SHELL), where he coordinates and participates in research projects related to $CO_2$ reduction ("CO2 reduction"). Research interests are related with the development of hybrid catalytical processes based on supercritical fluids for the development of transition fuels and chemical building molecules. This approach is based on the combination of homo, hetero and biocatalytic processes with supercritical $CO_2$ and water. He also as interest in Aerogels and material development using supercritical fluids.

Reinaldo C. Bazito

Bachelor of Chemistry with Technological Assignments from the University of São Paulo (1992), Master of Organic Chemistry from the University of São Paulo (1997), Doctor of Organic Chemistry from the University of São Paulo (2001), Post-doctoral from the University of Wyoming (USA , 2004) and the University of São Paulo (2005). He is auxiliary professor at the Institute of Chemistry at the University of São Paulo since March 2006, where he is part of the Green and Environmental Chemistry Group (GQVA). Develops research in Green Chemistry, especially in the study and applications of supercritical carbon dioxide. The main topics of study for his group are: (i) Surfactants and Amphiphilic Polymers for supercritical CO2 - development of new surfactants and amphiphilic polymers (dendrimers, hyper-branched polymers) for supercritical $CO_2$, aiming at modifying the solubility of substances in this medium or the use of these materials in the encapsulation of drugs and other species of interest; (ii) Reactivity in supercritical carbon dioxide - study of chemical reactions in supercritical CO2 (associated or not with ionic liquids), involving catalysis or organocatalysis, such as catalytic hydrodeschlorination of PCBs, Morita-Bailys-Hillman reactions, condensation aldol, Click Chemistry and others, including mechanistic aspects and process optimization (this in partnership with CESQ - Center for Chemical Systems Engineering at Escola Politécnica - USP). He is a researcher at RCGI - Research Center for Gas Innovation (CEPID FAPESP-SHELL), where he coordinates and participates in research projects related to CO2 reduction ("CO2 abatement"). Develops research, teaching and dissemination work in the area of Chemical Safety, especially in what involves teaching and research laboratories in universities and research centers.

Alessandra Lopes de Oliveira

Graduated in Food Engineering from São Paulo State University “Júlio de Mesquita Filho” / UNESP (1994), master's and doctor in Food Engineering from State University of Campinas/ UNICAMP (1997/2002 respectively). She is an associate professor at the Faculty of Zootechnics and Food Engineering at the University of São Paulo. She has experience in the area of Food Science and Technology, specifically in Food Engineering processes, with an emphasis on systems using supercritical carbon dioxide and extraction with pressurized liquid, acting mainly on the following themes: phase balance, obtaining natural extracts ( fruits and plants) and supercritical extraction. She also has research in the area of chromatographic analysis, sensory analysis and processing of edible ice-cream.

Maria Villegas

Graduated in Agro-industrial Engineering - Popular University of Cesar (2015) and master's degree in Food Sciences from the University of São Paulo (2018). Currently holds a technical position on the Green and environmental chemistry research group at chemistry Institute of University of São Paulo. She currently working on a CO2 reduction project at Research Center for Gas Innovation (RGCI) (CEPID FAPESP-SHELL). She also has research experience in Food Engineering, aerogels fabrication and supercritical $CO_2$

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