TECHNICAL REPORT

SOLUBILIZATION TESTS TO DETERMINE THE NATURE OF MACROMOLECULES BY MEANS OF SOLVENTS IN POLARITY GRADIENT AND THEIR RELATIONSHIP WITH THE TRITURABILITY OF THEIR STRUCTURE

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CONTEXT OF THE STUDY

The solubility analysis of a sample provides specific results of its physical characteristics, the grindability of a sample, in this case of a vitreous biomaterial - obtained through Powder House's Vitreous Transformation Process of grape pomace - can be defined as the fragility that it has to mechanical action, this is a very variable characteristic and empirical tests have been conducted to determine the force necessary for the grindability of this vitreous biomaterial. It can also be defined as the relative amount of work required to pulverize the vitreous biomaterial. In this same context, porosity is an important element to analyze to supplement the phenomenon of high grindability of the vitreous biomaterial, therefore the lower the porosity, the higher the pressure or force required to grind the sample.

Preliminary studies performed by SEM scanning electron microscopy indicate through micrographs that the vitreous biomaterial is an inhomogeneous, disintegrated and easily fracturable structure, since it has a low structural integrity of support. In reference to this study, it can also be stated that different non-cohesive domains are observed which allow a high grindability of the samples, which are susceptible to mechanical compression due to the semi-conserved and heterometric structures.

With respect to porosity, this measure does not have an absolute value and is assessed qualitatively as the ratio between the voids and the skeleton of the structure. A very important characteristic related to the porosity that can account for the grindability or crushability of the vitreous biomaterial is the permeability, that is, the ease of passage of fluids through the material, but this entry of the fluid into the sample depends on the fluid, in the case of this test depends on the type of solvent, and depends on the type of macromolecule that is able to be diluted in this solvent and pass through the layers of the vitreous biomaterial.

The realization of solubility tests for vitreous biomaterial has been used to study different processes of obtaining vitreous biomaterial, some authors indicate that vitreous biomaterials with a more homogeneous structure, which provides more hardness and less crushability, can absorb less water than other vitreous biomaterials with a less conserved ultra-structure, which allows the entry of water or other solvents in an easy way to the sample.

The higher hardness of the formulations is strongly associated with the decrease in the ability to absorb water and other solvents and this correlated with the feasibility of the solvation of the internal macromolecules of the formulation and their release to the medium in different solvents. That is to say, the hardness and low grindability of a vitreous biomaterial can be related to the difficulty of solvating molecules and releasing them to the medium, the harder vitreous biomaterial or microstructurally more compact, the less ease or freedom of movement the solvent will have to collide inside the vitreous biomaterial with the macromolecules, dissolve them -separate them from the matrix- and release them to the medium.

OBJECTIVE

In order to directly relate the hardness or low grindability of biomaterials with the solubility, studies were carried out on the vitreous biomaterial from grape pomace and to establish the most appropriate solvent for this material, as well as to determine which type of molecules are more bioavailable in this type of structure after having undergone the Powder House's Vitreous Transformation Process of grape pomace.

If the biomaterial is very compact, the solvation of the molecules will not be high due to the lack of effective collisions between the solvent and its sample, on the contrary, a lower degree of compactness allows the facilitated entry of the solvent, the interaction with the macromolecules, their solvation and release to the medium, in a facilitated way, this indicates that the ultra-structure of the vitreous biomaterial has heterogeneous characteristics which generates fracture points and high triturability.

EXPERIMENTAL PROCEDURE

The tests were carried out with samples of 1 gram of vitreous biomaterial and 4 solvents were used, apolar, partially polar and polar; Hexane, Ethanol, Acetone and Water, in volumes of 25 mL. Each mixture was placed in 50 mL Erlenmeyer flasks and kept under constant agitation for 24 hours at 25°C with a stirring speed of 1,300 rpm.

Once the agitation time had elapsed, each sample was filtered and the sediment that remained insoluble was weighed on an analytical balance. The tests with each solvent were carried out with 5 replicates so that the results were representative.

RESULTS OBTAINED

After 24 hours the assays were reviewed considering that there are different levels of solubility of the macromolecules depending on their functional groups in different solvents of apolar or polar character. As a simple explanation, a macromolecule is more or less soluble if the functional groups that compose it are polar in nature.

For the description of the images below show the vitreous biomaterial samples after 24 hours of solubilization, Fig. 1, is representative of the initial color of the 4 solvents used.

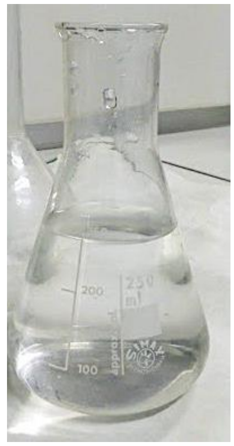

Fig. 1, *Reference image of a flask with the four solvents used, none of them shows color or turbidity before the test.*

It was observed that in different solvents the expected solubilities for the macromolecules were:

WATER: mainly polysaccharides and minor sugars are solubilized, it is also expected to find low molecular weight proteins soluble in water. According to analyses previously carried out by another team, the amount of total protein in the vitreous biomaterial is reported to be 10.8 g in 100 g of sample. This finding is very important since it ratifies the proximal analyses previously performed.

On the other hand, the coloration found in the water-solubilized vitreous biomaterial sample indicates a high degree of solubility of phenolic acids, various phenylpropanoid compounds, flavonoids and quinones. The strong purple color of the solubilized sample, Figure 2, can be seen mainly to the anthocyanins in the vitreous biomaterial since it is derived from grape pomace. Preliminary studies indicate total Polyphenol values of 2,693 mg GAE/100 g sample. This solubility analysis links the chemical results of the amount of polyphenols in the sample and SEM analysis of the vitreous biomaterial 's ultrastructure.

Fig. 2 Vitreous biomaterial *sample solubilized in water for 24 hours with constant agitation, the characteristic purple color of the anthocyanins of the red polyphenols can be observed.*

ETHANOL: ethanol is the universal solvent for secondary plant metabolites such as phenolic compounds, due to the polar structure of these molecules whose rings are highly polar and soluble in ethanol.

Figure 3 shows the test performed on the vitreous biomaterial solubilized in ethanol for 24 hours, a reddish color characteristic of total phenols that are passively released into solution can be observed.

For this particular sample the term solubility can be used to describe qualitatively the dissolution process of macromolecules into the sample depending on the nature of the solvent, ethanol, and its solute, in this case total phenols. The interaction between the secondary metabolites inside the vitreous biomaterial and the molecules of the solvent, clear ethanol, suggests a high level of solvation of the sample.

Fig. 3. *Sample treated for 24 hours at constant temperature and agitation with ethanol. The red or purple color of the solvent shows the release of different polyphenols.*

ACETONE: acetone was chosen as one of the solvents to be analyzed for the vitreous biomaterial sample since it is capable of dissolving large molecules of cellulose or resins, unlike water and ethanol, acetone is a solvent with a low polarity and does not exert dipole-dipole forces, therefore acetone is an excellent solubilizer or solvator of lipids, whether they are large or small molecules. In general, many terpenes are soluble in acetone and some non-phenolic secondary metabolites. Figure 4 shows the test performed for the vitreous biomaterial with acetone and a yellow coloration typical of dissolved lipids, oils or fats is clearly seen.

Fig. 4. *Sample treated for 24 hours at constant temperature and agitation with acetone. It is possible to be seen in contrast with figure 1 the slight yellow tone of the sample product of the solubilized lipids.*

HEXANE: this solvent is used industrially to solubilize oils and fats because it is apolar and has no possibility of forming, for example, hydrogen bridge type bonds but can only show induced dipole type interactions, which make fats whose nature is apolar highly soluble in hexane, also waxes and structurally more complex lipids are soluble in hexane. Figure 5 shows the vitreous biomaterial solubilized in this apolar solvent.

Along with apolar waxes and fats, there are also water-insoluble phenolic compounds such as condensed tannins, lignin and hydroxycinnamic acid, which is bound to the cell wall of plant cells. The yellow color of the final solution shows the release of fats and some non-colored phenylpropanoid compounds.

Fig. 5. *Sample treated for 24 hours with hexane at constant temperature and agitation. In contrast to Figure 1, the faint yellow coloration of the sample is seen, probably the product of the extraction or only the location of waxes and fats.*

Finally, this study of solubility contemplates the analysis of the dissolution of the vitreous biomaterial during 24 hours in constant agitation in the different solvents, the results can be seen in table 1 where it indicates the solubility or the difference of initial weight versus final weight of the vitreous biomaterial submitted to different solvents, water, universal solvent shows the greater amount of solubilization or hydration of the sample reaching 18% of solubilization of the sample. These results are expected according to the different polarities of the solvents tested.

Table 1. Solubilization percentages of 1 g of vitreous biomaterial in different solvents during 24 hours at 25° with constant agitation.

Solvent	Hexane	Ethanol	Acetone	Water
Solubility of the vitreous biomaterial	7,07%	14,56%	8,74%	17,85%

The final objective of the solubility tests is to make a study of the homogeneity of the ultra-structure of the vitreous biomaterial, Fig 6, based on its macro molecular components and the polarity of these macromolecules, according to this, they are soluble or insoluble in polar or apolar solvents studied in this report.

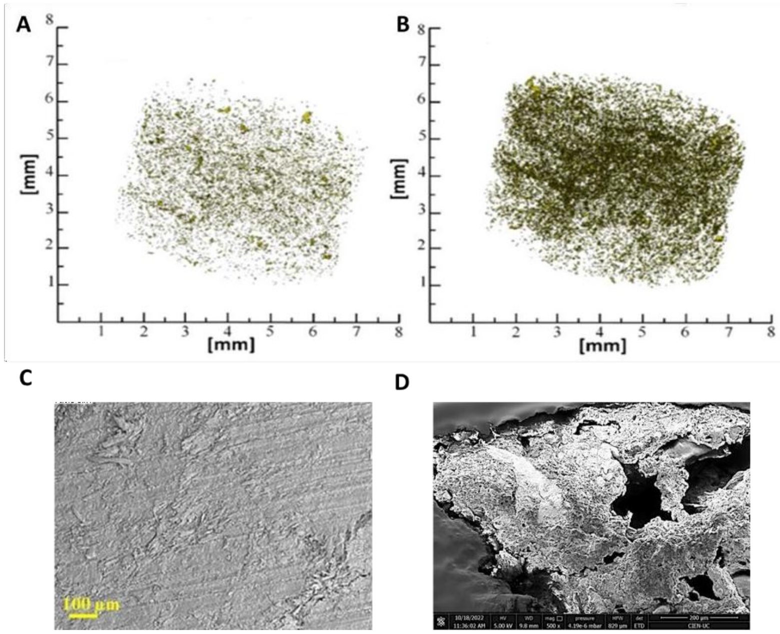

Fig. 6. *Comparison of vitreous biomaterial structures with different degrees of porosity (A) Comparative plot of vitreous biomaterial with a low amount of pores and (B) one with a high amount of pores (each dot represents one pore). The increase of pores in the vitreous biomaterial manifests itself as a series of channels in its interior that increase the solubilization surface and facilitate its crushability. This can be seen in the SEM microscopy images, which compare (C) a compact wood vitreous biomaterial with very few perforations or cracks with (D) the vitreous biomaterial analyzed in this solubility study (grape pomace vitreous biomaterial surface), which presents a surface with irregularities and large cracks, making it more susceptible to destruction. Images A, B and C were taken from the study of Cutz et al. 2021), and related to image D taken from the SEM microscopy study of the samples.*

Our analyses show that the solubility of the vitreous biomaterial is higher in water than in other solvents with lower polarity, the choice of the appropriate solvent for the vitreous biomaterial dissolution will depend on the volume of the solvent to be used and the primary or secondary metabolites to be recovered.

The nature of the group to which the dissolved or solvated macromolecule belongs can be determined, the chemical structures of the macromolecules present in the sample are surrounded by the solvent and separated from their matrix to be released to the medium, this test allows to affirm that the sample at least has macromolecules such as polysaccharides, minor sugars, phenolic compounds, or polyphenols, terpenes, other secondary metabolites, fats, waxes and lipids with their molecular structure intact.

CONCLUSION

The study of solubility is applied to a vitreous biomaterial and structures that are not ideal solids, but are media with different porosities, which facilitates or hinders their triturability by conventional means or with low energy, therefore, a porous medium or structure has a property that conditions the permeability of a solvent -liquid- in a matrix -solid- as the vitreous biomaterial. It can be inferred that a material or sample is permeable when it has continuous voids, the circulation of a solvent (water or other) through the mass (vitreous biomaterial) obeys physicochemical laws that relate permeability or solubility to the degree of compactness or homogeneity of the sample.

The structural properties of the biomaterials are important since they determine the physical quality parameters, as well as their millability or grindability. One of the properties of greatest interest for this physical-chemical phenomenon is porosity, since it determines the capacity to absorb or eliminate substances from the matrix.

To determine the porosity there are different methods reported in literature, according to the experimental technique used varies the range of measurement, as an example, the most frequently used techniques to measure the porosity in materials are the scanning electron microscopy SEM, transmission electron microscopy TEM, which allows measuring the geometry and distribution of the particles in the vitreous biomaterial, liquid or solvent instruction in the sample and determining by means of solubility tests the output of macromolecules, techniques such as X-ray diffraction that can determine the surface area and distribution of the ultra-structure, among others.

This report complements the results of SEM micrographs analysis that show that the vitreous biomaterial is an irregular formation with internal galleries connected to the external medium that allow the entry and exit of the solvent and other macromolecules due to its non-compact structure, which finally leads to have a vitreous biomaterial with high grindability and crushability requiring low energy for its pulverization.