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Studiul profilelor termodinamice în sistem izocoric pentru cele mai importante substanțe crioprezervante

Study of thermodynamic profiles in isochoric conditions for the most important cryoprotective substances

REZUMAT / ABSTRACT

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List of contents

3. LIST OF FIGURES

4. LIST OF TABLES

5. NOTATION LIST

Q	Heat exchanged by a thermodynamic system with the outside environment	[J]
W	Mechanical work	[W]
ΔU	Internal energy variation	[-]
P	Pressure	[Pa]
V	Volume	[m³]
Pi	Initial pressure	[Pa]
Pf	Final pressure	[Pa]
T	Temperature	[K] [°C]
m_0	Mass in initial conditions	[kg]
m_1	Ice mass	[kg]
m_2	Water mass	[kg]
V_0	Volume under initial conditions	[m³]
V_1	Ice volume	[m³]
V_2	Water volume	[m³]
Z	Quality	[-]
υ_0	Water volume	[m³/kg]
υ_1	Specific volume of ice	[m³/kg]
υ_2	Specific volume of water	[m³/kg]
β	Compressibility coefficient	[-]
α	Coefficient of thermal expansion	[-]
T_{ph}	Phase change temperature, isochor, for mixture	[K] [°C]

T^0	Freezing temperature of water at pressure of 1 atm	[K] [°C]
ΔT(P)	Temperature drop caused by increased pressure P	[Pa]
ΔT(c)	Temperature drop as a function of mixture c concentration	[K] [°C]
c	Concentration	[mol/m³]
MW	Molecular weight	[g]
M	Molarity	[mol/l]
$c_{solvent}$	Solvent concentration	[mol/m³]
c_{solvat}	Solute concentration	[mol/m³]

6. DICTIONARY

biological materials for the purposes of this work	Cells: Cells are one of the most common biological substances studied in isochoric cryopreservation. They can be harvested from different tissues, such as blood, connective tissues or organs, and can be preserved for use in medical research or therapeutic purposes. Tissues: Biological tissues, such as muscle tissue, bone tissue, or nervous tissue, can be studied in isochoric cryopreservation. These samples can be used to research disease development or find new treatments. Microorganism: Micro-organisms, such as bacteria, fungi or viruses, can be studied in isochoric cryopreservation. This technique can be used to preserve pure cultures of microorganisms for biological research or drug production. Plants: Plants, such as seeds or buds, can be preserved in isochoric cryopreservation. This technique can be used to preserve plant biodiversity and preserve rare or important varieties

Isochoric preservation	Crioprezervare izocoră, Criostocare izocoră Isochoric cryopreservation is a technique for preserving biological materials at extremely low temperatures without volumetric deformation of the storage medium
Cryoprotective solution	Cryoprotective solution, cryoprotective agent A cryoprotective agent is a substance added to biological samples prior to the cryopreservation process in order to protect cells and tissues from damage caused by freezing. These substances help reduce the formation of ice crystals in cells and protect cell membranes and their internal structures, thereby allowing cells to survive the frost and be preserved for future use.
DMSO	Dimetilsulfoxid
Physiological saline	Saline 0.9%
Mixtures	Mixtures
Criopreservation	Cryopreservation, isochoric cooling
Isochoric reactor	Hermetically sealed metal container
Cryopreservants	Cryoprotectants
Temperature threshold	Temperature level

7. ISOCHORIC SYSTEMS

7.1. Introduction

The desire of humanity to preserve various biological materials has existed since antiquity. Artificial cooling is part of our species' concerns to create better living conditions.[1] The preservation of various biological materials dates back to ancient times, as perfectly preserved animal bodies (especially mammoths) have been found in Arctic areas.[2]

Beyond these early and rudimentary discoveries of using artificial cold for use in different branches of comfort, through various very simple procedures, it is appropriate to mention that the real boom of artificial cooling was achieved with the discovery of refrigeration mixtures in the sixteenth and seventeenth centuries [1] and the principles of thermodynamics in the nineteenth century.

7.2. Theoretical basis

$W=P\Delta V$, where

W – Mechanical work

P – Pressure

V– Volume

As can be seen from the above relationship, in the case of an isochoric process, the work of the system is 0, the only difference in the case of heat transfer can only be given by the pressure variation inside. If we plot the process in a pressure-volume diagram, it would look like this (Figura 1) [3] :

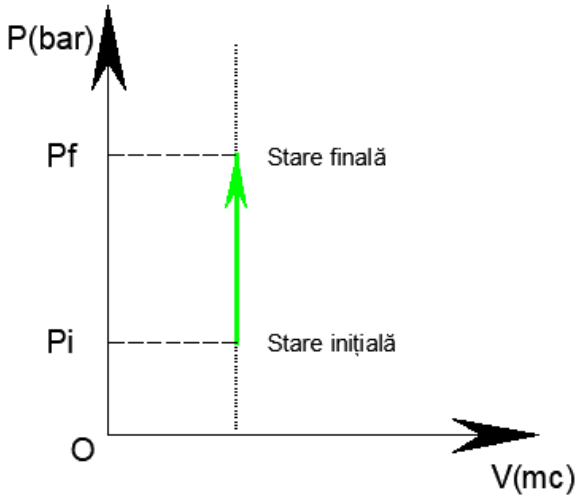


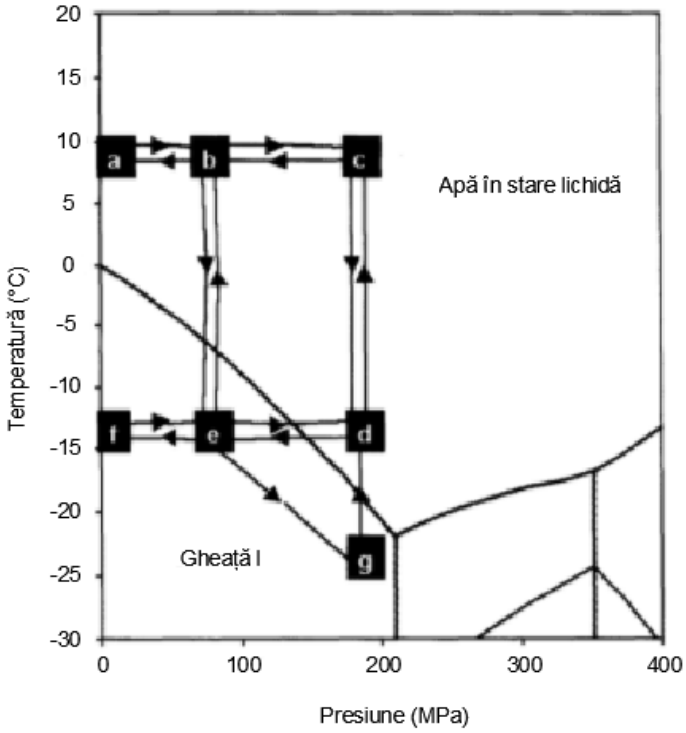
Figura 1.Volume pressure diagram in isochoric,
where V – volume; P- pressure; V=constant, Pi>Pf;

7.3. Etymology of the word "isochor"

From the point of view of the definition of the word "isochor" or in English "isochor", it means " a line representing the variation of pressure as a function of temperature when the volume of the substance under study is constant "[4] .

7.4. History of preservation in isochor

The study of the behavior of liquids at high pressures and in isocor regime gained momentum in 1998 when a synthesis of research done in this field by Knorr D., Schluetler O. and Heinz V. was published.[5] . They first defined conservation types:



1. Pressure – assisted freezing : phase change occurs at constant volume
   - Pressure – shift freezing : The phase change occurs due to a change in pressure
   - Pressure - induced freezing : The phase change is initiated by pressure change and is continued at constant volume.

In (Figura 2) we can observe the transposition of their theory on the phase diagram of water.

Figura 2– Water phase change models theory

(ACDF) phase change occurs due to a change in pressure; (abef) – phase change occurs at constant volume; (FDCA) – phase change (melting) starts by pressure change and continues at constant volume. [5]

7.5. Intermediate conclusions

All these studies culminated in the paper "The thermodinamic principles of isochoric preservation", published in 2005. [6] This paper proposed in the specialized literature the thermodynamic principles of cryopreservation in isochoric conditions Through the mathematical model shown in it, we can calculate the amount of ice that forms in a constant-volume system,

both for water and for different mixtures of water with other solutions. Theoretical data are supported by a number of practical determinations to demonstrate the veracity of the presented methods.
In this paper are also defined a series of fundamental principles of the cryopreservation process:

- It takes place in a two-phase thermodynamic system, in which water and ice coexist and are in thermodynamic equilibrium
- In a two-phase, equilibrium thermodynamic system, temperature and pressure are interdependent
- In an isochoric system the volume is constant
- In a two-phase isochoric system at a certain pressure and temperature, the only variable that can adjust to be the system in equilibrium is the "quality" of the system, the relative percentage of ice and water in the system
- The temperature or pressure and quality at each temperature or pressure complete the isochoric cryopreservation process.

In addition to these, we can add some recent conclusions:

- We can have a predictable amount of liquid and ice in the isochoric system, depending on the liquid (cryopreservant solution) we use. As we decrease the temperature, theoretically, the amount of crystals will increase and it is important to study with each type of cryopreservant solution, which is the optimal temperature to which we will need to cool it so that we have as little pressure as possible and ice crystals as few as possible. [7]
- We can end up in a situation where we have supercooled liquid, without having ice crystals in the composition. Some solutions are very stable in terms of microscopic disturbances and nucleation effectively does not occur during cooling, in this case transport protocols can be developed, without crystallization occurring, very stable and safe.[8]
- In the case of some mixtures of liquids (water + cryopreservants), we can reach vitrification, without ice crystals forming during transformation. We can also greatly reduce the pressure at which this transformation takes place by optimally mixing the component solutions.[9]

7.6. **Mechanisms of tissue destruction**

We will mention the mechanisms of tissue destruction in preservation under isobaric conditions. Ice cannot contain dissolved preservatives and as tissue freezes, solutes are rejected and concentrated around cells. This results in what is known as damage during frost, and consists of:
Dehydration of cells [10]
Precipitation of the solution [10]
Changes in pH [10]

Chemical destruction[10]
The limitations of using cryopreservant solutions under isobaric conditions are also negative, making isochoric conservation a viable method:
Large organs are difficult to preserve in isobaric because heat transfer from liquid to organ takes a very long time
Cryopreservant solutions can, in themselves, be dangerous to organs intended to be preserved.
These aspects can be avoided if isocoric preservation is used. [10]

8. CRYOPRESERVANTS

8.1. Introduction

Cryopreservants have revolutionized the way we look at conservation. The fact that we can preserve a multitude of prokaryotic and eukaryotic organisms and use them later, after cooling them to a temperature of -196 °C was a huge step for many branches of science. We can list here: biology, chemistry or medicine. However, this would not be possible without the presence of cryopreservatives/cryoprotectants. These are aqueous or water-soluble solutions, which, when mixed with water or other solvent, can influence the temperature at which the mixture crystallizes.

8.2. Definition

The best definition of cryoprotectants was published in 1974 by Armand Karow: "Any additive that can be added to cells before they are preserved by freezing, thus increasing their survival rate after thawing compared to adding nothing."[11][12], can be named cryoprotectant."
Cryoprotectants are used to protect the integrity of cell membranes and their interiors. They also reduce the risk of destruction of membrane lipids, proteins and nucleic acid.[13]

8.3. How it works

The way cryoprotective agents work is very varied, they can function as intracellular or extracellular solutions. All of them, however, have the property of being very hydrophilic and can form very close bonds with hydrogen in water. This allows them to greatly delay ice formation, even if the necessary temperature conditions and pressures are met (for the studied solvents (which can be simple or compounds)) under normal atmospheric conditions.
Mixtures consist of two or more basic substances and are obtained as a result of physical phenomena. The phases disperse with each other, but the chemical bonds do not break. The chemical properties of the components remain unchanged, but the physical properties of the mixture generally differ from the physical properties of the individual components.[14]

8.4. History

The first concepts of cryopreservation appeared at the end of the XIX century, when Hans Molisch studied the freezing of plants with a cryomicroscope using an incipient technology. He concluded that the composition and concentration of substances in the cytoplasm of plants have a defining role in their survival rate after freezing. [15]

In discussions H. Molisch had with his contemporary, Hermann Muller-Thurgau, they concluded that exposing plants to negative temperatures leads to an accumulation of sugars in them, but they did not make a connection between this and the fact that sugar could function as an inhibitor of crystallization.[16]

The successful use of cryoprotectants began in 1949 when the benefit of using glycerol as a cryoprotectant was first shown. In the study conducted by G. Polge, A.U. Smith and A.S. Parkes on living biological matter (red blood cells), its beneficial properties used for preservation in isobarium were found. Their work was continued and J.E. Lovelock's research published results for DMSO in 1954, along with other low molecular weight aqueous solutions such as methanol or acetamide.[17]

Interestingly, out of the 127 studies reported with the 56 solutions with cryopreservative potential, the following were highlighted at an early stage of the studies: Glycerol (15 studies); Dimethylsulfoxide – DMSO- (9 studies); Glucose (7 studies); Sucrose (8 studies); Methanol (5 studies);

In 1986, "Mechanism of cryoprotectant action" was published by Ashwood-Smith MJ. In this paper, based on studies between 1969 and 1986, just over 20 effective cryopreservative solutions remained. [18]

Karow's "list of 56" remained, however, still viable in other branches of science, such as aquaculture. In the paper published in 1996 "Cryopreservation of finfish and shellfish sperms" by Nai Hsien-Chao, there are 52 cryocondom solutions still being studied in aquaculture.[19] Of the cryoprotective solutions presented in 1969, many are still under study. (Tabelul 1)

Tabel 1- Cryoprotective solutions 1996 – aquaculture and other fields [19]

Acetamid	Ethylene glycol	Mannose	Serin
L. Alanine	Formamid	Methanol	Sodium bromide
Albumin	Glucose	Methyl acetamid	Sodium chloride
Ammonium acetate	Glycerol	Methyl formamid	Sodium iodide
Cloroform	Glicerol monoacetat	Methyl urea	Sodium nitrate
Coline	Glicin	Phenol	Sodium sulphate
Dextrans	Hidroxietilic starch	Pluronic polyols	Sorbitol
Diethylene glycol	Inositol	Polyethylene glycol	Sucrose
Dimethyl acetamid	Lactose	Polyvinyl pyrrolidonă	Triethylene glycol
Dimethyl formamid	Magnesium chloride	Praline	Trimethylamină acetate
Dimetil sulfoxid (DMSO)	Magnesium sulfate	Propilen glicol	Urea
Eritritol	Maltose	Pyridine-N-oxide	Valine
Ethanol	Manitol	Ribose	Xilose

Among the solutions presented, they proved high efficacy (relative to the solutions in the table): Dextrans, ethylene glycol (relative to fish sperm), hydroxyethyl starch, methanol, polyethylene glycol, polyvinyl pyrrolidone and sucrose. [19]
From this list we can extract 8 viable solutions.
In the paper "Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures" by Gloria D., Shangping Wang, Barry J. Fuller from 2017, studies in the field were synthesized. The following have been reported as viable[20]. (Tabelul 2)

Tabel 2- Cryoprotective agents studied and considered viable in 2017[20]

Alcohols and derivatives	Sugars and Fortified Sugars	Polymers	Sulphides and amides	Amines
Methanol - 2	Glucose - 2	Polyethylene glycol (PEG) - 3	Dimethyl sulfoxid - 5	Proline - 3
Ethanol - 1	Galactose - 2	Polivinil pirolidin (PVP) - 2	Acetamide- 2	Glutamine – 2
Glycerol - 4	Lactose – 1	Dextrans - 3	Formamide - 2	Betaine - 2
Propylene glycol - 3	Sucrose – 1,3	Ficoll - 3	Dimethyl Acetamide - 1	
Ethylene glycol - 3	Trehalose- 3	Hidroexitilic starch - 3		
	Rafinose- 3	Serum proteins (complex mixture) - 3		
	Manitol – 1,2	Lactic protein (complex mixture) – 1,2		
	Sorbitol – 1	Peptone - 1		
1 – Effective on prokaryotic cells 2 – Effective on a small scale in eukaryotic cells 3 - Poorly effective on eukaryotic cells. Often mixed. 4 – Very effective on a clearly defined number of cells 5 – Very effective on all cell types				

The authors determined that DMSO is the most effective cryoprotectant available at that time.
In 2021, in the paper "The need for novel cryoprotectants and cryopreservation protocols: Insights into the importance of biophysical investigation and cell permeability" by Rekha Rajua, Saffron J. Bryanta, Brendan L. Wilkinsonb, Gary Bryanta concluded that the most commonly used cryocondom solutions at this time, multidisciplinar, are DMSO and glycerol. [21]

We can also add dextrans, ethylene glycol (relative to fish sperm), hydroxyethyl starch, methanol, polyethylene glycol, polyvinyl pyrrolidone and sucrose. [19]

8.5. Presentation of the most commonly used cryopreservation solutions

8.5.1. Dimethyl sulfoxyd (DMSO)

Synthesized in 1866[22]. It is an organic sulfur, having the chemical formula $(CH_3)_2SO$. It is an aprotic polar solvent, which can dissolve polar and non-polar compositions. It is miscible with water and a wide range of organic solvents. [23] DMSO is used in various applications such as in veterinary and human medicine, in the electronics industry and in organic synthesis, it is considered one of the most widely used cryopreservant solutions nowadays.[21] DMSO has a very interesting property, it freezes at 18.5 °C, this property making it extremely easy to use.[23]

8.5.1.1. Behaviour of mixtures of aqueous solutions and DMSO in isobaric cooling

In 1966[24] , The first diagram for the behavior of mixture in isobari was published.
At the same time, based on it, the authors also made a table with freezing temperatures, depending on the molar concentration.
In 1968, the first phase diagram for this mixture, in isobar, was published.

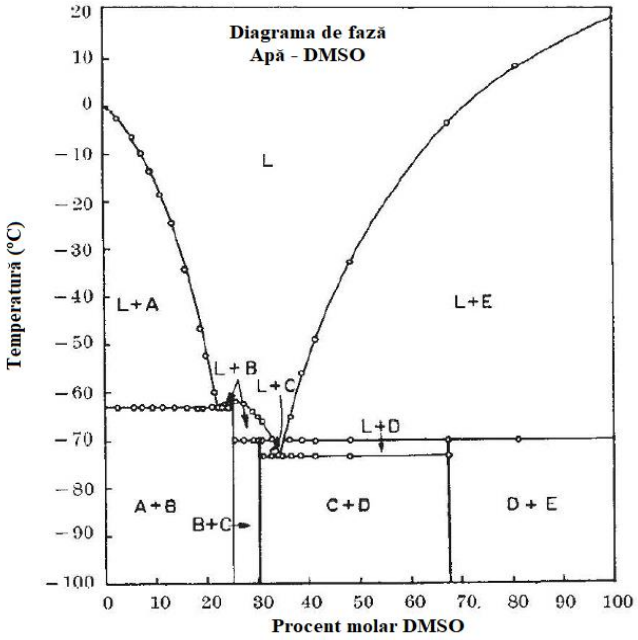


Figura 3- Isobaric phase diagram for mixtures of DMSO and water based on molar percentage.

L - Liquid phase, A - Water, B - DMSO 3 Water, C - DMSO 2.5 Water, D - DMSO 1/2 Water, E - DMSO [25]

As we can see, this mixture has an anomalous behavior even in isobars. Research into this phenomenon continues to this day. [26][27]

8.5.2. **Glycerol**

Also called glycerin($C_3H_8O_3$), aceasta formează legături cu hidrogenul din apă. This makes it difficult for ice crystals to form when mixed with water at concentrations of 70% glycerol and 30%, up to temperatures of -37.8 °C.[23] It acts as osmolite, solvent, detergent, human metabolite and preservant solution.

8.5.2.1. **Crystallization of glycerin-aqueous mixtures in isobaric cooling**

Glycerin is liquid in high concentrations and at temperatures generally encountered in storage. Undesirable crystallization, which adversely affects the appearance of a product, does not occur with glycerin at room temperature. If, for example, glycerin is exposed in a dry atmosphere, it will not become "stony" like sugar, for example.[28]

Due to these properties, glycerin was the first type of antifreeze for car radiator cooling systems. Although later replaced by ethylene glycol for this application, combinations of alcohol or glycol and glycerin are still used for this purpose.

8.5.3. **Etihylen glycol**

It was first prepared in 1856 by Charles Adolphe Wurtz. It weakens hydrogen bonds in water when mixed with it. The pure solution has a freezing temperature of -12 °C, but when we mix it with water (40% ethylene glycol, 60% water), the freezing temperature drops to -45 °C. This property makes it very suitable for cryopreservation, but it is considered quite toxic. [23]

8.5.3.1. **Behaviour of ethylene glycol and water mixtures in isobaric cooling**

Their behavior has been studied since the 1950s [29], with the widespread introduction of the use of this cryopreservant agent.

8.5.4. **Glucose**

Glucose is a sugar-based, natural protective agent. In nature we find examples of plant protection in winter by producing glucose. [30][31] It has been observed that mixing glucose with methanol leads to an increase in cell survival rate after preservation.[30]

Glucose is used in cryomicrobiology in concentrations of 1-18% (average 4%). [32] A higher survival rate of some microbial cultures using aqueous mixtures with glucose at temperatures of -20°C has been observed for a very long time.[33] . Over time, glucose has proven its

effectiveness in a number of researches, such as the T4 bacterium virus [34], E-aerogens [35], yeast[36] or P.Berghei in the blood .

8.5.4.1. **Behavior of water-glucose mixtures in isobaric cooling**

The higher the concentration of glucose we have in the studied mixture, the lower the temperature at which our mixture will freeze.

8.5.5. **Dyethylene glycol**

Produced from the partial hydrolysis of ethylene oxide, diethylene glycol is an organic composition with the chemical formula $(HOCH_2CH_2)_2O$. Its diluted solution with water can be used as a cryoprotectant [23]. Diethylene glycol is considered dangerous for human consumption, and from this point of view its use is limited.[37]

8.5.5.1. **Behaviour of mixtures of diethylene glycol and water in isobaric cooling**

In the literature, information on the behaviour of mixtures is limited. From the information found, diethylene glycol is in the shadow of ethylene glycol, with the caveat that it "behaves similarly". [38][39][40]

The interesting facts that emerge from the literature are that diethylene glycol and ethylene glycol indeed behave similarly when used as cryoprotectant mixed with water. When we mix ethylene glycol or diethylene glycol with water in a concentration of 60%-80% with water, and cool it to a temperature below -50 °C, then they freeze.

8.5.6. **Trehalose**

Until the 2000s it was an extremely expensive cryoprotectant and difficult to produce. It is a sugar that consists of two glucose molecules having the chemical formula $C_{12}H_{22}O_{11}$. It naturally accumulates in some fungi, bacteria, invertebrate animals or plants that use it as an energy source. [41] It can be used as a cryoprotectant for a wide range of applications such as gene banks [42], carrots or tobacco cells[43] or human sperm [44].

8.5.6.1. **Behaviour of mixtures of trehalose and water in isobaric cooling**

Recent studies show that with the cooling of mixtures of water and trehalose, they do not crystallize, but form an amorphous glass, which is responsible for their preservation properties. [45]

8.6. Classification of cryoprotectants

By permeability, cryoprotectants can be divided into 2 groups:

Nonpermeable – large molecule protectors [46]-(do not penetrate inside cells, are extracellular): saccharules (trehalose and sucrose); starch (hydroxyethyl); PVP or polyethylene oxide.

They are mainly used if the cryopreservation process proceeds very quickly. These solutions turn the liquid into glass during cooling. The flow of water between the inside and outside of the cells will be interrupted because of this.[13]

Permeable – small molecule protectants, generally polymers [46]- (penetrating inside cells, are intracellular): DMSO (Dimethhilsulfoxide), glycerol, ethylene glycol, or propylene glycol.

They are mainly used if the cryopreservation process proceeds slowly. Due to this, there is enough time for the water in the cells to evacuate due to osmotic pressure that concludes with a decrease in cell volume.[13]

Although numerous studies on cryoprotectants have been conducted, the basis of current research has not undergone significant changes since 1969. In some cases, mixing cryoprotectants has improved their effectiveness over their separate use [21] but there hasn't been a major discovery in 60 years. The studies conducted have provided a lot of results in different fields and on various types of cells, but none of them led to the development of a universally accepted cryoprotection solution used multidisciplinary. Consequently, the need to develop new cryoprotective methods and solutions remains crucial for multidisciplinary research and progress in this field.

9. PHYSICO-MATHEMATICAL MODELING OF CONSERVATION IN ISOCHOR

The thermodynamic model, with which one can express the phenomenon of cooling in isochore for mixtures in the liquid state, is still in its infancy. If we reduce the discussion to explaining the basis of the phenomenon for water, we can draw a number of conclusions from it.

Several theories have been developed, each supporting the results obtained through experiments. The first notable work in the field was published in 2005 by Boris Rubinsky et. al "The thermodynamic principles of isochoric cryopreservation" [6] .

9.1. Classification of cryoprotectants

The phase change temperature in isochoric of a simple system consisting of a pure substance in a gravitational field is a unique function of pressure. For any pressure, there is a corresponding temperature, at which the phase change occurs.

A regression curve was defined to facilitate the relationship between pressure and temperature during phase change from liquid to intersection with Ice I and III. This curve has also been validated experimentally and is extracted from the phase diagram of water in isochore[47]

$$P = -0.1461T^2 - 12.58T + 0.1013 \ \#\ (1)$$

T - expressed in °C

P – expressed in MPa

Therefore, the percentage of ice or water in an isochoric system can be calculated in a system for each temperature and the system can be fully specified.

We need to introduce "Quality Z" – This is the percentage by mass of water related to the total weight of the mixture of water and ice at a given pressure and temperature.

"Z-quality" is obtained from the equation of conservation of mass and volume in isochoric.

$$m_0 = m_1 + m_2 \ \#(2)$$

$$m_0 - \text{mass in initial conditions } [\text{kg}]$$
$$m_1 - \text{Ice mass}[\text{kg}]$$
$$m_2 - \text{Water mass}[\text{kg}]$$

$$V_0 = V_1 + V_2 \# \ (3)$$

$$V_0 - \text{volume in initial conditions } [\text{m}^3]$$
$$V_1 - \text{Ice volume } [\text{m}^3]$$
$$V_2 - \text{Water volume}[\text{m}^3]$$

From these, "quality Z" is expressed as,

$$Z = \frac{m_2}{m_0} = \frac{m_2}{m_1 + m_2} \# \ (4)$$

Subsequently, from the theory of conservation of mass in constant volume and from the definition of specific volume, we will have

$$\upsilon_0 = \frac{V_0}{m_0} = \frac{V_1 + V_2}{m_0} = \frac{m_1 * V_1}{m_0 * m_1} + \frac{m_2 * V_2}{m_0 * m_2} = \frac{(m_0 - m_2)}{m_0} * \upsilon_1 + Z * \upsilon_2 = (1 - Z) * \upsilon_1 + Z * \upsilon_2 \# \ (5)$$

$$\upsilon_0 - \text{specific volume under initial conditions } [\text{m}^3/\text{kg}]$$
$$\upsilon_1 - \text{specific volume of ice } [\text{m}^3/\text{kg}]$$
$$\upsilon_2 - \text{specific volume of water } [\text{m}^3/\text{kg}]$$

If we reorganize the terms in the equation, a general expression of quality will result, during cooling in isocor by specific volumes of ice and unfrozen water:

$$Z = \frac{\upsilon_0 - \upsilon_1}{\upsilon_2 - \upsilon_1} \# \ (6)$$

In general terms, the specific volume of water or ice can be determined by solving for the first-rank Taylor polynomial, entering values for equivalent pressure and temperature.[48]

$$dv = \frac{\delta_v}{\delta_P} dP = \frac{\delta_v}{\delta_T} \# (7)$$

from it, according to the principle of chemical equilibrium[49]

$$\beta = \frac{1}{v} \frac{\delta_v}{\delta_P} \# (8)$$

$$\alpha = \frac{1}{v} \frac{\delta_v}{\delta_T} \# (9)$$

The specific volume for ice as a function of temperature and pressure calculates by the following equations:

$$\upsilon_1 = \upsilon_{10} \exp\left[- \int_{P_0}^{P} \beta_{T_1}(P', T) dP' + \int_{T_0}^{T} \alpha_{T_1}(P_0, T') dT' \right] \# (10)$$

$\beta -$ compressibility coefficient
$\alpha -$ coeficcient of thermal expansion
indicele 0 reprezintă proprietăţile apei la punctul de îngheţ la o presiune de 1 [atm]
These can be found in the paper "Freezing processes in high-pressure domains" published by P.D. Sanz et. al. [50]

$$\alpha_{T_1}(P_o, T) = A_1 + A_2 T + A_3 T^2 + A_4 T^3 \# (11)$$

$A_1 = 1.5756 * 10^{-4}$
$A_2 = 5.556 * 10^{-7}$
$A_3 = 2.655 * 10^{-8}$
$A_4 = 7.11 * 10^{-10}$
The unit of measurement for temperature is [°C]
The values are taken from the paper "Thermodynamic Properties of Ice, Water and Their Mixture Under High Pressure, Glaciers– Ocean– Atmosphere Interactions" by V. E. Chizhov et. al. [51]

$$\beta_{T_1}(P, T) = \frac{\beta^o_{T_1}}{1 + m_1 \beta^o_{T_1} P} \# (12)$$

$$\beta^0_{T_1} = \frac{\beta_1}{1 - \beta_2 T}$$
$\beta_1 = 1.827 * 10^{-5}$

$$\beta_2 = 1.418 * 10^{-3}$$
$$m_1 = 5$$

The unit of measurement for pressure is [bar]
Values are taken from the work "Thermodynamic Properties of Ice, Water and Their Mixture Under High Pressure, Glaciers– Ocean– Atmosphere Interactions" by V. E. Chizhov et. al. [51]

The specific volume for water depending on temperature and pressure calculates by the following Equations:

$$\upsilon_2 = \upsilon_{10} \exp\left[-\int_{P_0}^{P} \beta_{T_2}(P', T)dP' + \int_{T_{k_0}}^{T_k} \alpha_{T_2}(P_0, T')dT'\right] \# (13)$$

The coefficients of compressibility and thermal expansion are:

$$\beta_{T_2}(P, T) = \left(\sum_{i=0}^{4} b_i P^i\right) * 10^{-4} \# (14)$$

$$\alpha_{T_2} = \left(A + \frac{B}{C + \Gamma}\right) * 10^{-4} \# (15)$$

The temperature functions A, B, C and Γ are expressed by:

$$A = a_1 + a_2 T_K + a_3 T_K^2 \# (16)$$

$$B = a_4 + a_5 T_K + a_6 T_K^2 + a_7 T_K \Gamma + a_8 \Gamma \# (17)$$

$$C = a_8 + a_9 T_K + a_{10} T_K^2 + a_{11} T_K^3 \# (18)$$

$$\Gamma = P + a_{13} P^2 + a_{14} P^3 \# (19)$$

where,
$$a_1 = 4.7856 * 10^1$$
$$a_2 = -8.12847 * 10^{-2}$$
$$a_3 = 8.49849 * 10^{-5}$$
$$a_4 = 5.56047 * 10^5$$
$$a_5 = -3.76355 * 10^3$$
$$a_6 = 5.56395$$
$$a_7 = 5.59682 * 10^{-3}$$
$$a_8 = -2.76522 * 10^1$$
$$a_9 = -4.28067 * 10^3$$
$$a_{10} = -3.39150 * 10^1$$
$$a_{11} = 3.65873 * 10^{-1}$$

$$a_{12} = -5.89617 * 10^{-4}$$
$$a_{13} = 3.28892 * 10^{-4}$$
$$a_{14} = 2.65933 * 10^{-8}$$

These constants are found in the work"Thermodynamic properties of water under pressure up to 5 kb(kilobar), and between 28 and 120 ℃. Estimations in the supercooled region down to − 40 ℃" by L. T. Minnasian et. al. [52]

Knowing the initial specific volume of water and the specific volumes of water and ice at the new conditions of thermodynamic equilibrium, we can obtain from Ec. (20) the percentage weight of water relative to the percentage weight of the mixture in an isochore system, at each temperature and pressure.

The percentage of ice in the mixture of water and ice is given by the equation:

$$\%\mathrm{ICE} = (1 - \mathrm{Z}) * 100\# \ (20)$$

9.2. The case of mixed aqueous solutions

From the fundamental concepts of thermodynamics, we can state that the phase change temperature of a mixture is a function of pressure and composition of the mixture under study. [6]

$$T_{ph}(P, c) = T^0 + \Delta T(P) + \Delta T(C)\# \ (21)$$

$\mathrm{T_{ph}} -$ phase change temperature, in isochoric, for the mixture
$\mathrm{T}^0 -$ freezing temperature of water at pressure of 1 atm
$\Delta \mathrm{T\ (P)} -$ temperature drop caused by increased pressure P
$\Delta \mathrm{T(c)} -$ temperature decrease depending on the concentration of mixture c

9.2.1. Pressure

$$\Delta T(P) = -4E - 10P^3 - 3E - 7P^2 - 0.00809P + 0.00819547826\# \ (22)$$

9.2.2. Concentration

$$\Delta T(c) = \Delta T_{solvent}(c_{solvent}) + \Delta T_{solvat}(c_{solvat})\# \ (23)$$

$$c = MW * M * v\# \ (24)$$

where,

c – concentration [mol/m^3]
MW – molecular weight [g]
M – molarity [mol/l]
$\upsilon -$ specific volume of solution $[m^3/kg]$

We can say that $\Delta T(c)$ It can be a linear combination incorporating the freezing points of several solutes, in the form of:

$$\Delta T(c) = \Delta T(c_1) + \Delta T(c_2) + \cdots + \Delta T(c_n) \# (25)$$

Data for different types of mixtures, can be found in the paper „CRC Handbook of Tables for Applied Engineering Science" published by R.E. Bolz et.al. [53]
In this case we must bear in mind that we have at least two mixed aqueous solutions, each with different composition and osmolality. Starting from equations (21-25) mixtures are separated by a theoretical boundary, which is impermeable to matter but completely malleable and can transmit heat and pressure. And the mixture has the same pressure and temperature. $\Gamma P\ T$[54] , A graphical presentation can be found in Figura 4.
a)General figure, pressure-temperature, of the 3 stages through which the mixture passes
b)In the first stage of cooling, we consider that the solute has a higher freezing temperature than the solvent, and therefore begins to crystallize. The limit is an imaginary one (b)Γ
c)In the second stage of cooling, the solute freezes completely (c)
d)In the third stage of cooling, the solvent also begins to freeze (d)

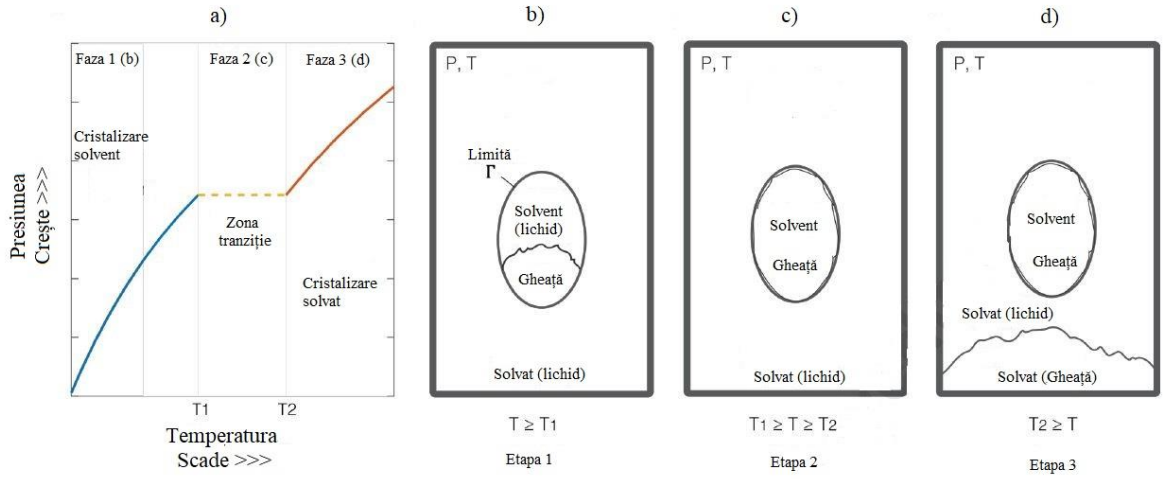


Figura 4-General figure, pressure-temperature, of the 3 stages through which the mixture passes[54].

For simplicity we have introduced the terms $\boldsymbol{c_{solvent}}$ and $\boldsymbol{c_{solvat}}$
If we assume that $\boldsymbol{c_{solvat}}$ has a freezing temperature higher than $\boldsymbol{c_{solvent}}$ then the phase change in $\boldsymbol{c_{solvat}}$ The phase diagram for the solution itself will follow. (except for some conditions not yet specified in the literature)

At the same time, if we assume that as $\boldsymbol{c_{solvent}}$ has a freezing temperature higher than $\boldsymbol{c_{solvat}}$ then the phase change in $\boldsymbol{c_{solvent}}$ The phase diagram for the solution itself will follow. (except for some conditions not yet specified in the literature)
The principle is a theoretically separate treatment of the two components of the mixture.

$$c_{solvent} = \frac{c_0}{Z} \# (26)$$

If we substitute equation (26) into equation (23), and iteratively recalculate the values for we arrive at convergence $T, P \; şi \; Z$[54] , and we will be able to explain the process of freezing the mixture in isochor.

10. COMPUTER-ASSISTED MODELING OF WATER COOLING IN ISOCHOR. (TESTING OF REACTOR FOR RESISTANCE TO MAXIMUM PRESSURES IN LITERATURE)

Through the computational models present in the literature so far, we can obtain quasi-static results. The lack of continuity in thermotechnical models fragments the achievement of complete assisted modeling of the isochore freezing process.

10.1. General settings in the simulation program

In the first stage we will simulate the behavior of the isoric reactor, during the maximum pressure recorded during the experiments, respectively for the mixture glucose 1M and saline solution 0.9% - where the maximum pressure recorded was 212.8 MPa at an outside temperature of -22. Since the outside temperature and pressure remain constant and the system reaches thermodynamic equilibrium, it is assumed that the temperature inside the reactor is -22.℃℃
The simulation was performed steady-state using an Axisymmetric 2D model.
For this, the Thermal Stress module was used.

10.1.1. Setting the model in the simulation program

After opening the program we will select 2D Axisymmetric.

10.1.2. Setting of the geometry

A simplified reactor model was defined to shorten the convergence time of the results obtained.
As can be seen in Figure 5, it complies with the dimensions of the original MS-02 reactors.

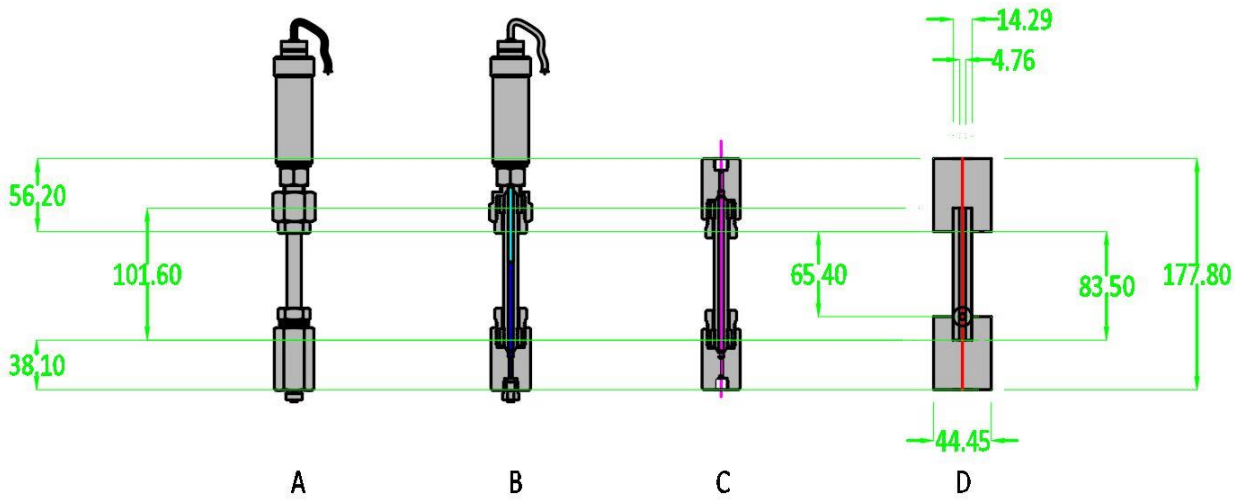


Figura 5– A) model reactor MS-1. B) reactor section MS-1 C) simplified reactor variant MS 1 D) model introduced in the simulation program

10.2. **Add physical sizes**

We will add the defined physical quantities to the parameters.

To be added: Internal pressure (Pi), A fixed point[-], Indoor temperature[°C] and Outside temperature[°C] according to Figure 6

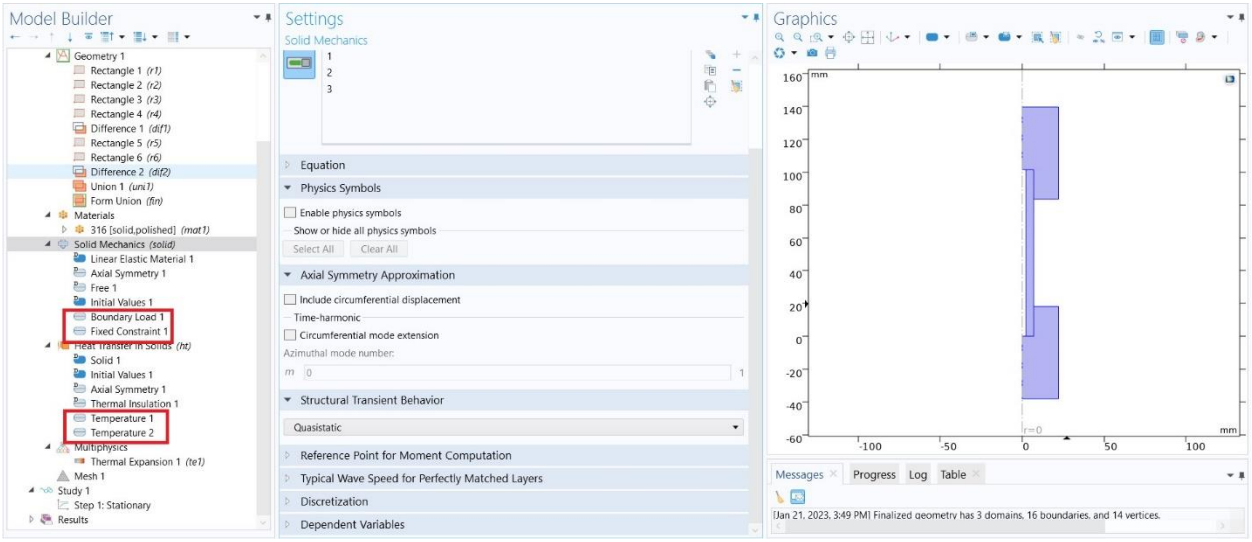


Figura 6– Adding physical sizes

10.2.1. **Model meshing**

Mesh or meshing is necessary to replace the continuum with a finite set of points. For surfaces, the finite element method is used, and for volumes, the finite volumes method.

10.3. **Results**

10.3.1.1. **Von Misses tension in 2D.**

This tension is a theoretical tension with which we can find out the breaking point of the material. The criterion for verifying this voltage assumes that if the Von Misses voltage of a material under load is greater than or equal to that material at the flow limit of the uniaxial voltage, it will fail.

$$\sigma_{VM} = \sqrt{\frac{\sigma_1 + \sigma_2^2 + \sigma_2 - \sigma_3^2 + \sigma_3 - \sigma_1^2}{2}}$$

Unde

σ_1-is the normal component of tension x

σ_2-is the normal component of tension y

σ_3-is the shear effort

From the literature, the strength limit for steel alloy is 690 MPa. [55]

From the results obtained by us, the maximum value is 487 MPa, which indicates a safe use of the reactor during experiments. In figure 34 and figure 35 we have a screenshot from the simulation program in 2D and 3D respectively with the results:

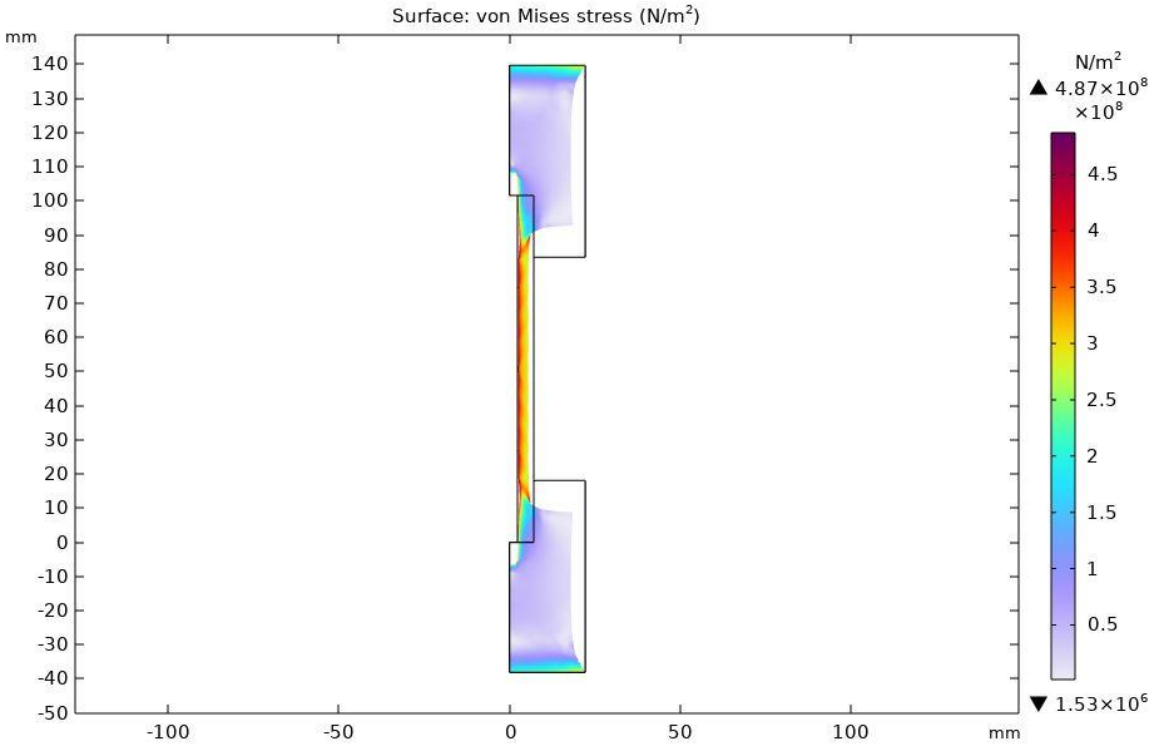


Figura 7– Graphical results – in section – Von Mises tension

Following simulations, a maximum von Mises voltage of 487 MPa was recorded during the reactor cooling process. This value represents the highest equivalent voltage in the reactor material during the experiments.

10.3.1.2. **Pressure**

From the pressures added to the program, no deformation occurred, as expected, and as can be seen in the Figure extracted from the program.

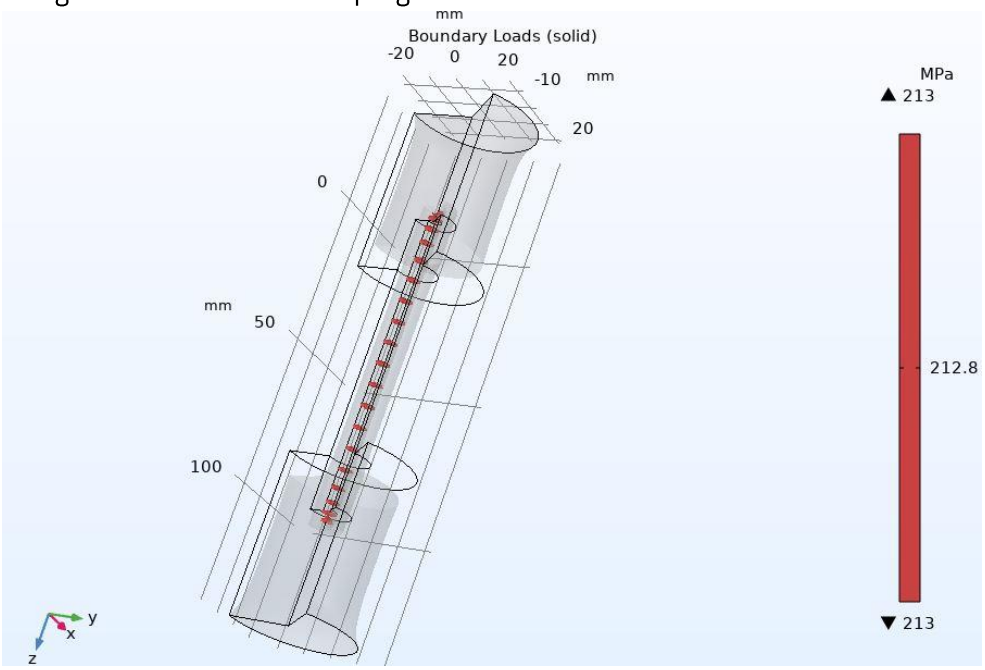


Figura 8– Deformation caused by pressure in the reactor

10.3.1.3. **Temperature**

The simulation was performed at equilibrium temperature, i.e. -25°C . We can extract isotherms inside the reactor from the program according to Figure

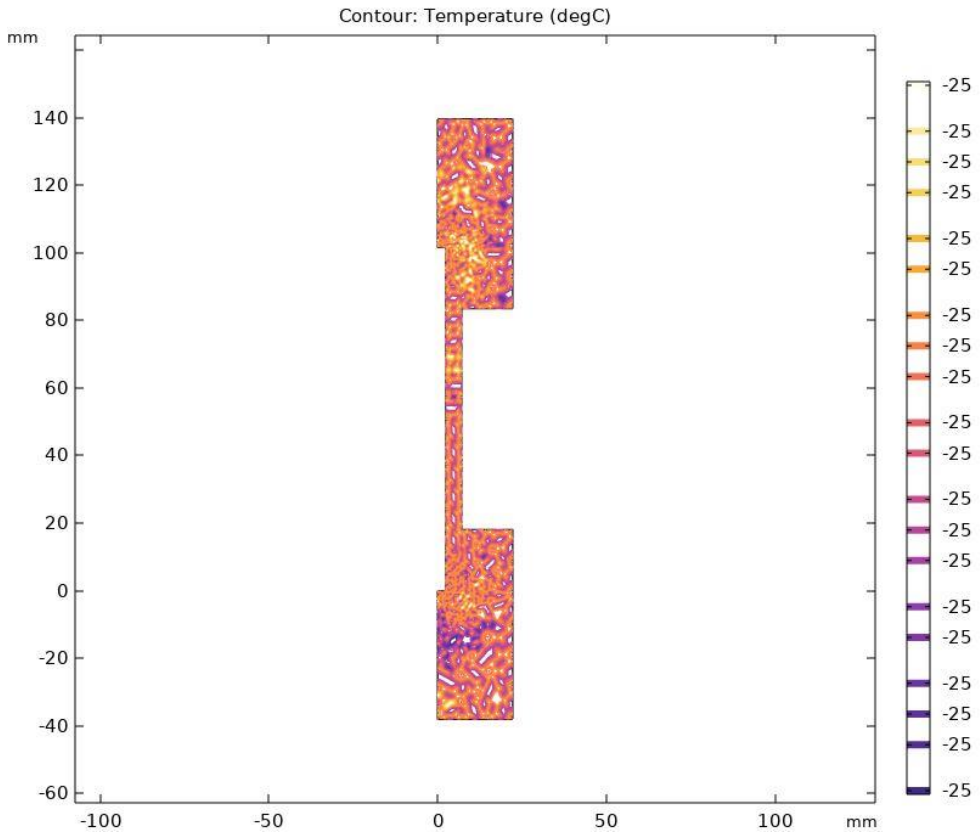


Figura 9– Izotermele din interiorul reactorului

10.4. **Simulation of water behavior during isochoric cooling by pressure calculation**

To reduce convergence times, we set a 2D model, as shown in the figure below

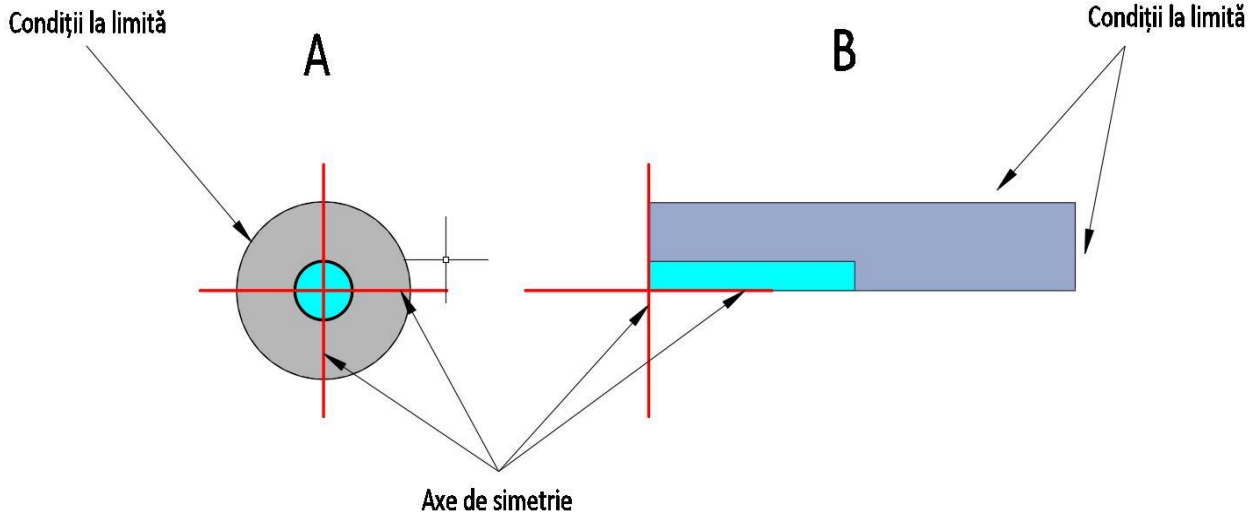


Figura 10– Model set in simulation program

Constant volume pressure can be calculated using continuous media mechanics or phase change correlations. The literature has shown that using both methods, we can achieve correct results.[56]

10.4.1. **Setări generale.**

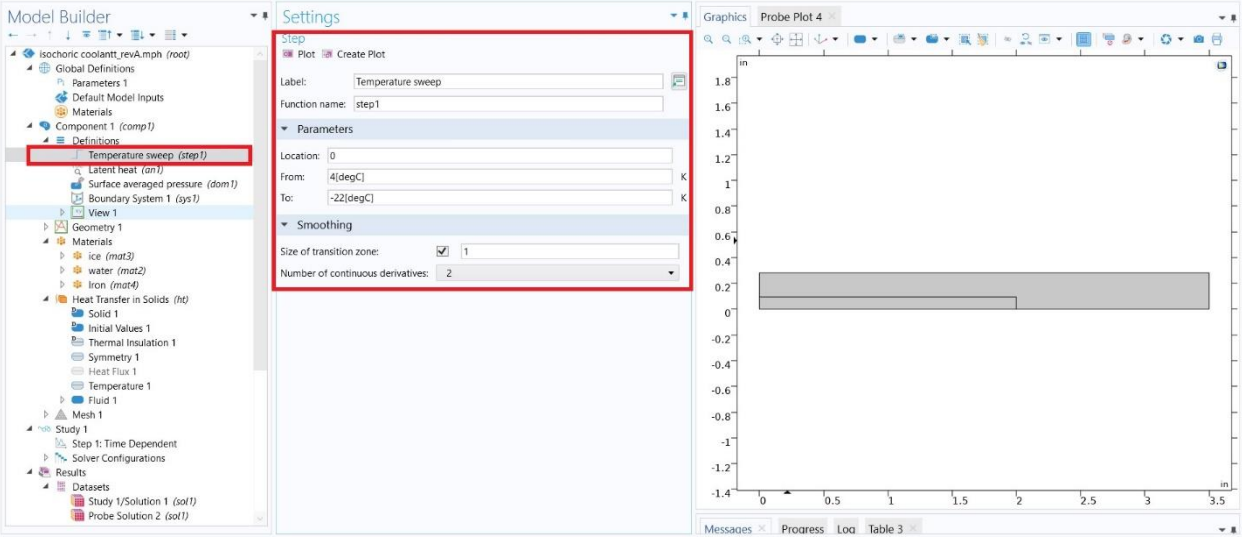


Figura 11– Simulation program settings, transient

We will also add latent heat, in the form of an analytical function:

$$L(T_m) = 1.617 * 10^{-4} * T_m^4 - 1.711 * 10^{-1} * T_m^3 + 67.94 * T_m^2 - 1.198 * 10^4 * T_m + 7.926 * 10^5 \ [kJ/kg]$$

Where T_m represents the phase change temperature for water[K] [57] [51]

We will add a domain probe.

$$P = 8.78 * 10^2 * IPm^4 + 2.75 * 10^2 * IPm^3 + 3.24 * 10^2 * IPm^2 + 1.97 * 10^2 * IPm + 0.31 \ [Pa]$$
$$\text{where, IPm is the indicator of phase change from 1 , water. to 0(ice)}$$

10.4.2. **Model meshing.**

In this case we will discretize our model with 2 types of sizes.

1. In the area where we have the reactor (Stainless Steel 316) we will use free meshing
2. In the area where we have water and where the phase change will take place, for the necessary results we will use meshing with a maximum element size of 0.6 [mm]

10.5. **Results**

10.5.1. **Temperature** °C

The programme captures in the figures below (ABCDEFG) represent the temperature values resulting from the simulation at min (0,5,10,15,20,25,30)

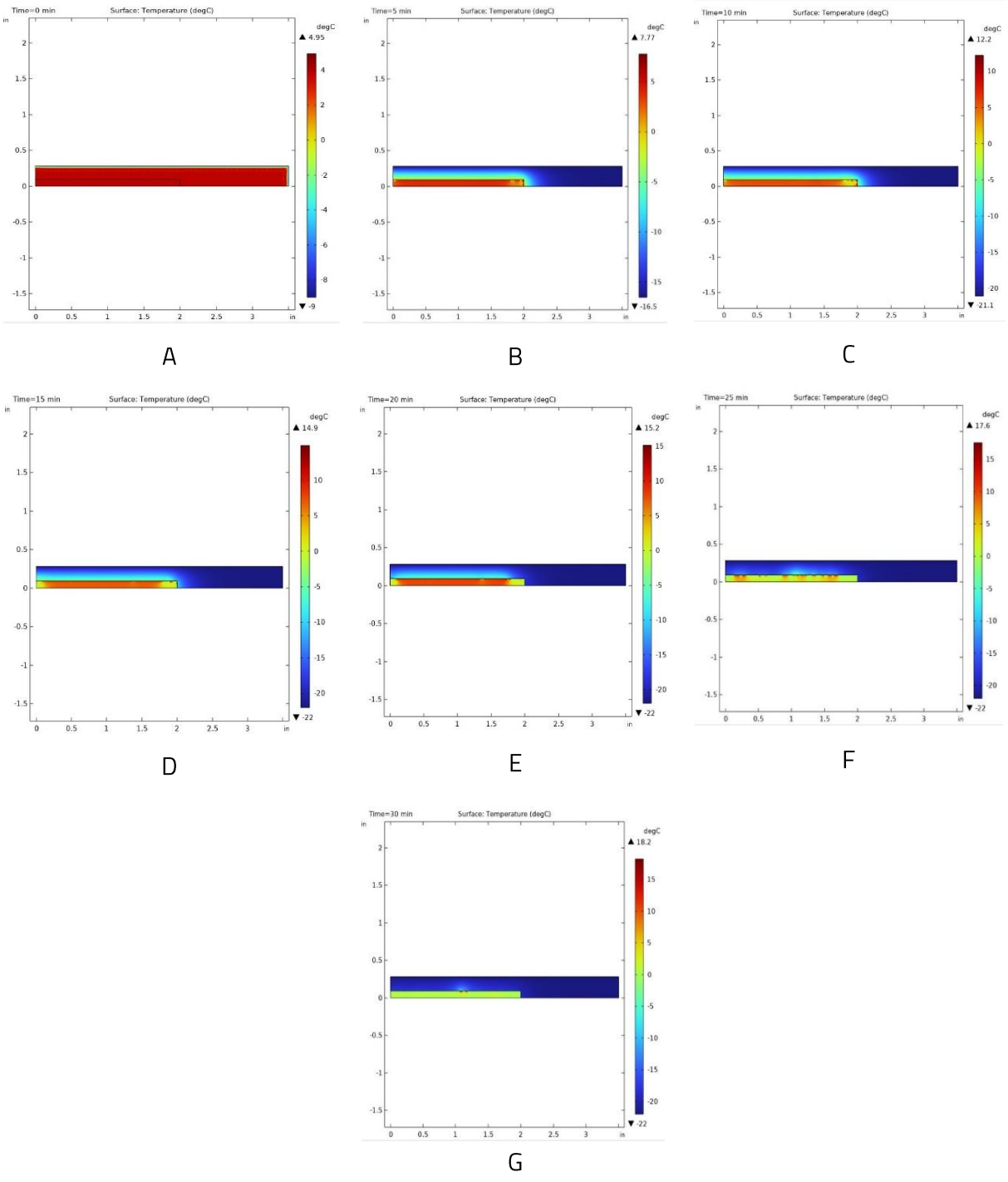


Figura 12– The results obtained with the simulation program in the minutes [A=0,B=5,C=10,D=15,E=20,F=25,G=30] – temperature °C.

10.5.2. **Phase change**

Program captures (ABCDEFG) represent the phase change model resulting from running the simulation program at min (A=0,B=5,C=10,D=15,E=20,F=25,G=30)

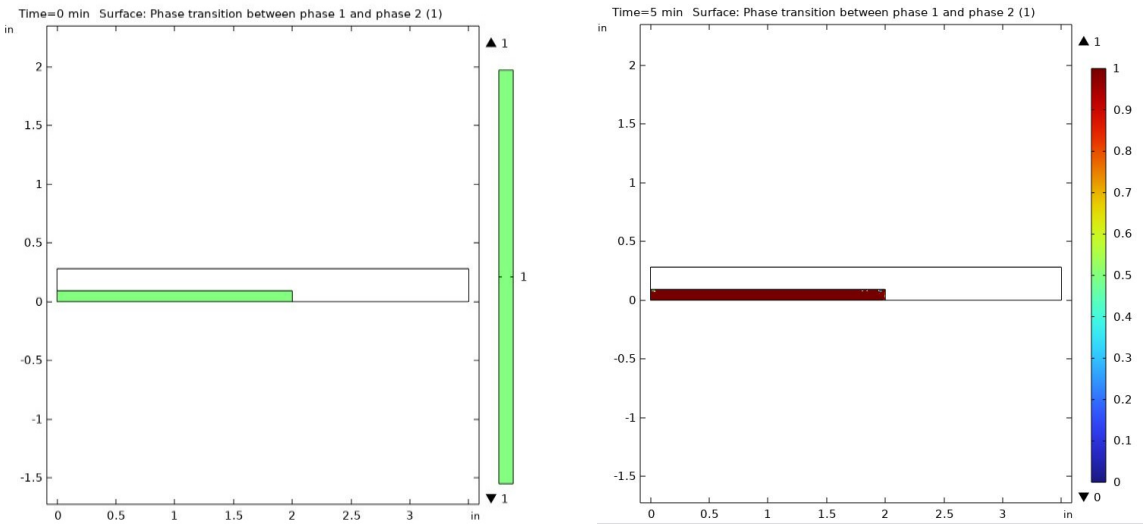


Figura 13 - Results obtained with the simulation program in minutes [A=0,B=5] - phase change

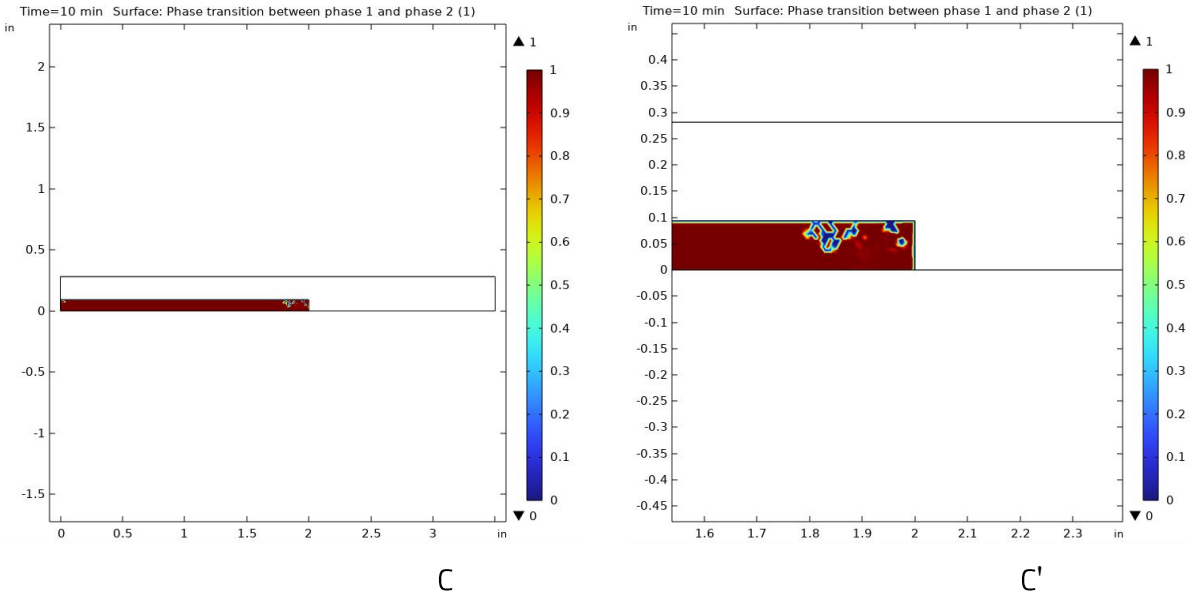
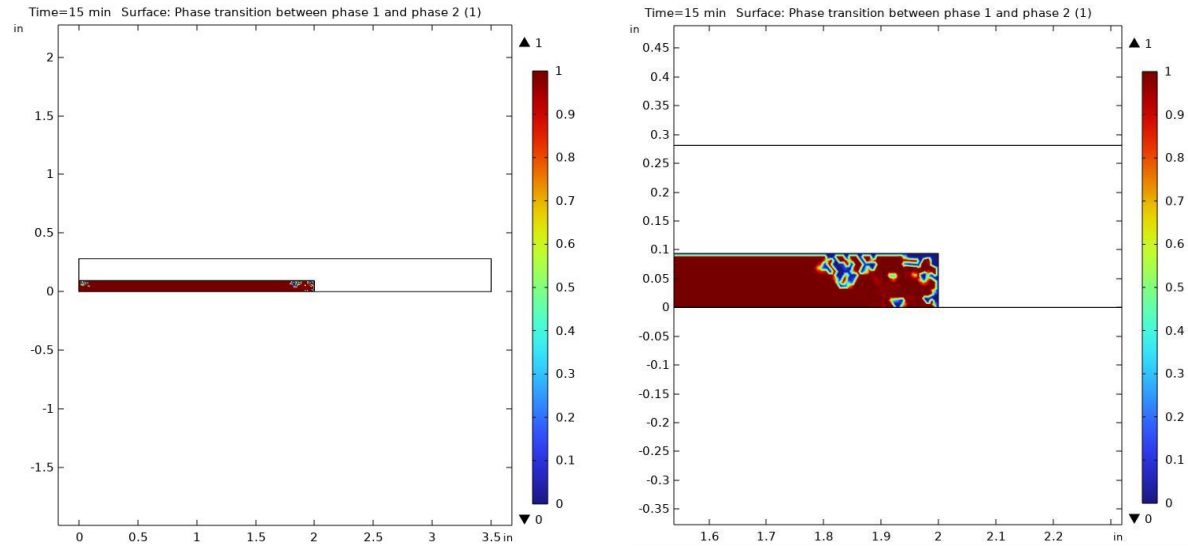
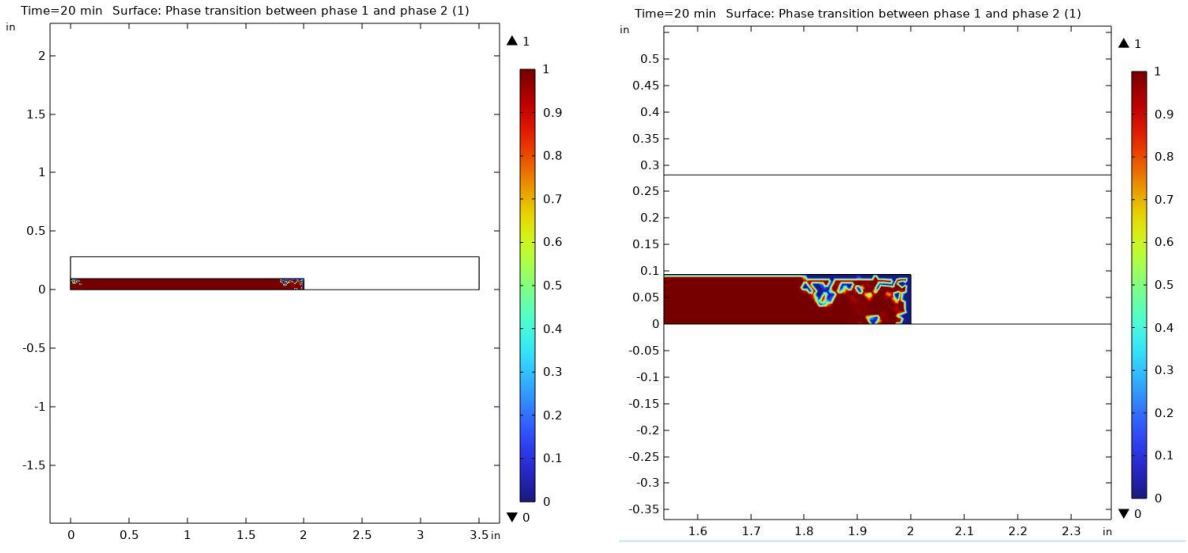


Figura 14 - The results obtained with the simulation program in minute [C=10] – phase change – catch ', represent increased catch



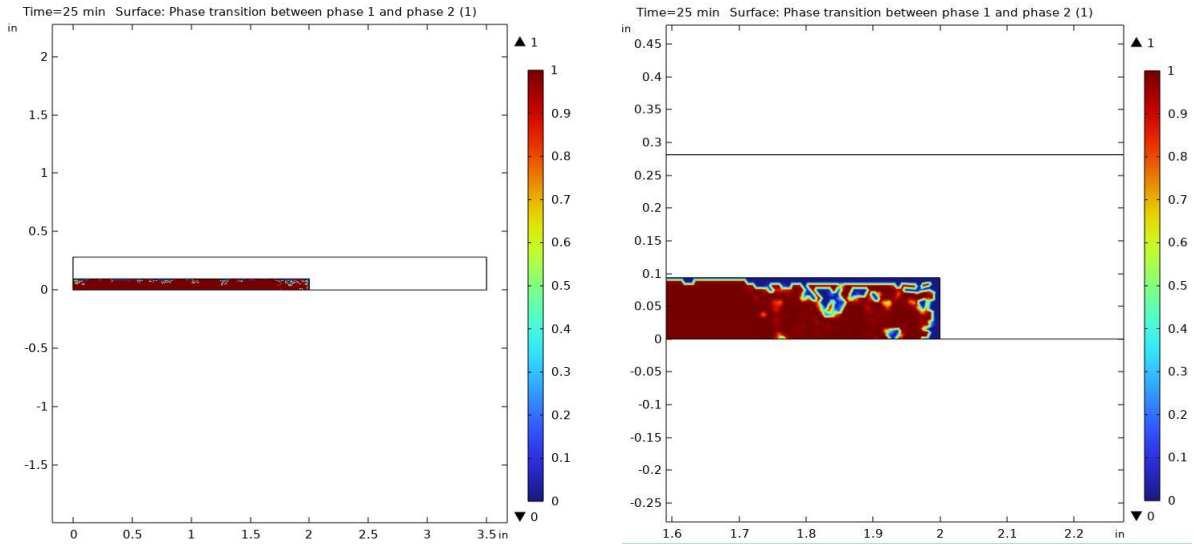
D D'

Figura 15 - The results obtained with the simulation program at minute [D=15] – phase change – capture ', represent increased capture



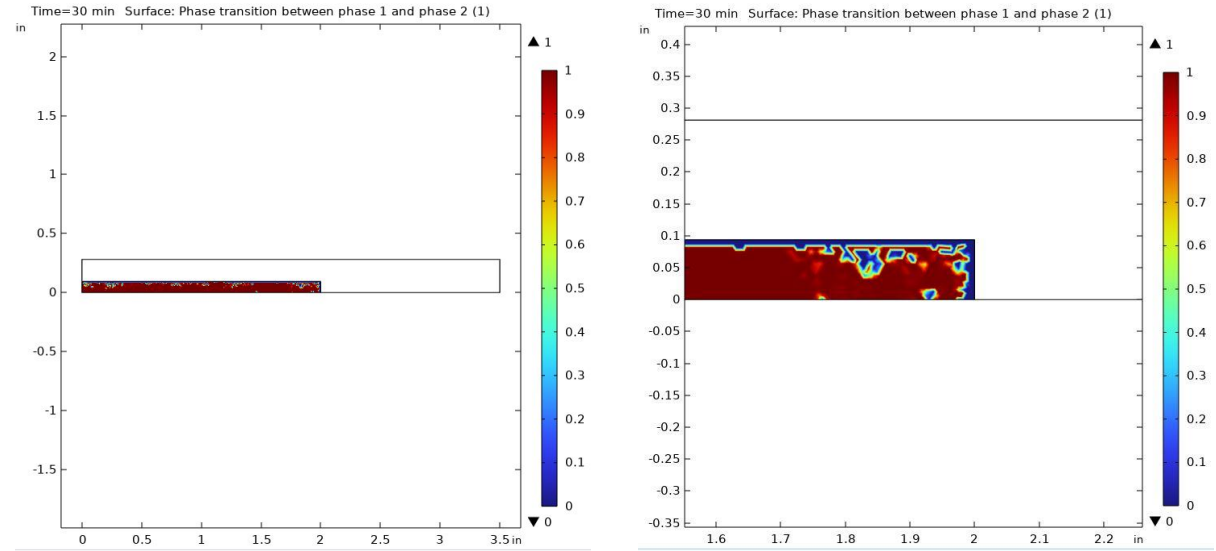
E E'

Figura 16 - The results obtained using the simulation program at minute [E=20] – phase change – catch ', represent increased capture



F F'

Figura 17 - The results obtained using the simulation program in minute [F=25] – phase change – capture ', represent increased catch



G G'

Figura 18 - The results obtained using the simulation program at minute [G=30] – phase change – catch ', represent increased capture

11. MATERIALS AND METHODS

To obtain the results from this work, a unitary experimental model was used for each determination.

11.1. System components

The microreactor used is one of the MS-1 type produced by High-Pressure Equipment Company (Erie, PA, USA). It is made of stainless steel 316 and is designed in such a way that it can withstand the pressure generated by the mixtures studied during the experiments without any expansions. [58]

The pressure transducer is connected to the isochoric reactor using a USB cable. It is manufactured by ESI Technology Ltd company and is GD4200-USB model, 0-5000 bar (0-72,519 psi).

The USB cable – USB CAB 2 – is manufactured by ESI Technology Ltd, has a length of 2 m and is used to connect the pressure sensor to the laptop.

11.1.1. Data analysis and storage program

For real-time analysis, storage and viewing of results, the software "ESI – USB Dynamic Interface" was used

11.1.2. Laptop

The laptop used to run the data acquisition program is an HP ProBook 6570b. It comes with Intel I5 processors with processing speed up to 3.2 GHz, internal DDR3 8 Gb RAM and has a storage space of 250 GB.
The laptop's performance allows the data acquisition program to run without affecting the accuracy of the collected data.

11.1.3. Cooling bath

The cooling bath used to carry out the experiments is Lauda RE 1225 S produced by LAUDA DR. R. WOBSER GMBH and CO. KG, Lauda-Königshofen,

A picture of the fully assembled system used to conduct the experiments.

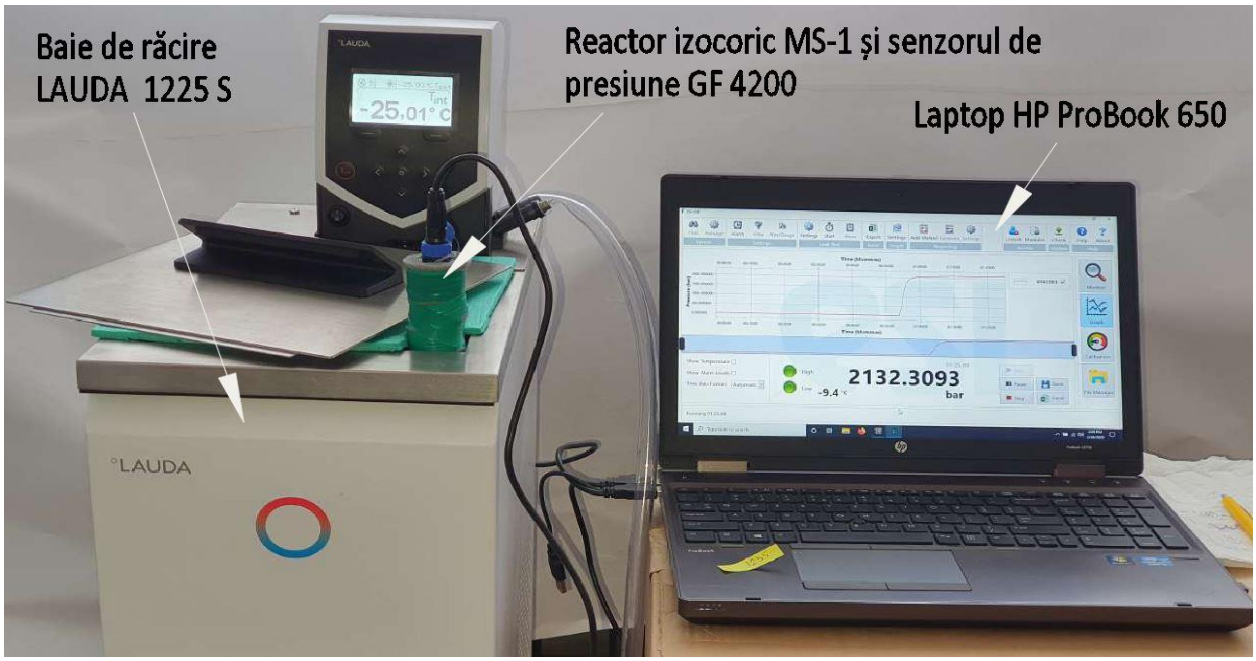


Figura 19- Equipment used to carry out experiments

11.2. **MATERIALS**

Saline solution with 9 grams sodium chloride (3.54g Na+ = 154.0 mmol/l and 5.46 g Ca- = 154.0 mmol/l) infusion solution packed in 1000 ml bags, with an osmolality of 308 mOsm/l, produced by STADA HEMOFARM SRL, Timisoara, Romania

Steam distilled water manufactured by European Drinks (14-16 Libertatea Street, Oradea, Bihor)

Glycerol for molecular biology (≥99% - G5516-100 ml manufactured by SIGMA-ALDRICH Co.3050 Spruce Street, St. Louis, MO, USA)

Glucose D-(+)-Solution Glucose 100 g/L in H2O, sterile filtrate, BioXtra, suitable for cell cultures (G8644-100 mL – SIGMAALDRICH Co.).

Ethylene - glycol anhydrous, 99.8%, manufactured by SIGMA-ALDRICH Co., USA.

Diethylene - BioUltra glycol, ≥99.0%, manufactured by SIGMA-ALDRICH Co., USA

Dimetil - sulfoxid (DMSO) fabricat de EMPLURA® - EMD Millipore Co., SUA

Trehalose – in powder form manufactured by (SOSA Ingredients S.L., Spain.).

11.3. **Experimental protocol**

For defining the thermodynamic profiles, mixtures of saline solution as solvent and cryopreservative solutions as solutes were chosen. The concentration of solutions in all cases to obtain comparable results was 1M, 2M, 3M, 4M and 5M. [59][60].

During the experiments, the pressure in each reactor and the temperature on the outer walls of the reactor were measured in parallel, these values being recorded. The temperature of the

antifreeze inside the cooling bath was measured using sensors built into the machine, and it was also recorded.
Each measurement was repeated between 3 and 5 times.

12. Results of experiments

12.1. Behavior of glycerin-saline mixtures 0.9% in isochor

The following solutions were used in this study: 0.9% physiological saline and glycerol
1 M, 2 M, 3 M, 4 M, 5 M. Each experimental protocol was repeated three to five times.[59]
The pressure in the isochoric system was measured throughout all experiments and recorded simultaneously with the temperature measured on the reactor wall.
In the table below, we find all pressures measured at equilibrium temperatures for each concentration of saline and glycerol studied separately.
At the same time, in the table, shaded with green color, we find the recommended interval, in which using this mixture for the conservation of different biological materials, we can obtain viable results, given the experiments validated until the time of writing this paper.
In the green box on the right-hand side of the table, the temperatures up to which supercooling has been observed for each concentration are shown.
The maximum pressure up to which promising results have been achieved so far is [40] MPa. [7]

Tabel 3- Equilibrium temperature-pressure correlations in isochoric for different concentrations of saline and glycerin .[59]

Mixture	Pressure at -5°C [MPa]	Pressure at -10°C [MPa]	Pressure at -15°C [MPa]	Pressure at -20°C [MPa]	Pressure at -25°C [MPa]	Supercooling (°C)
Saline 0.9%, 0.308M						
cooling	48.8±0.05	98.1±0.09	141.3±0.07	179.7±0.09	214.4±0.14	n.a.
heating	49.1±0.06	98.3±0.06	141.4±0.09	179.9±0.12	214.1±0.22	
Glicerol						
1M cooling	-	59.2±0.00	97.9±2.35	132.0±2.86	161.9±3.37	-11.44±1.41
1M heating	14.4±1.70	59.3±2.00	98.0±2.35	132.1±2.76	161.9±3.37	
2M cooling	-	33.7±0.00	70.1±3.72	101.5±4.31	129.0±4.81	-12.55±2.28
2M heating	-	34.2±3.01	70.3±3.67	101.7±4.31	129.0±4.81	
3M cooling	-	-	33.4±4.51	61.2±4.25	85.8±4.81	-14.65±0.97
3M heating	-	2.2±2.49	33.3±3.61	61.7±4.64	85.8±4.81	
4M cooling	-	-	-	21.5±0.66	43.1±0.61	-18.63±0.72
4M heating	-	-	-	21.8±0.75	43.1±0.61	
5M cooling	-	-	-	-	2.7±0.06	-24.74±0.46
5 M heating	-	-	-	-	2.7±0.06	

12.2. **Behavior of glucose-saline mixtures 0.9% in isochor**

The following solutions were used in this study: 0.9% saline and glucose 1 M, 2 M, 3 M, 4 M, 5 M. Each experimental protocol was repeated three to five times.[59]
In the table below we find all pressures measured at equilibrium temperatures for each concentration of saline and glucose studied separately.
At the same time, in the table, marked with green color, we find the recommended interval, in which using this mixture for the conservation of different biological materials, we can obtain viable results, given the experiments validated until the moment of writing this paper.
The maximum pressure up to which promising results have been achieved so far is [40] MPa. [7]

Tabel 4- Temperature-pressure correlations at equilibrium in isochoric for different concentrations of saline and glucose .[59]

Mixture	Pressure at -5°C [MPa]	Pressure at -10°C [MPa]	Pressure at -15°C [MPa]	Pressure at -20°C [MPa]	Pressure at -25°C [MPa]	Supercooling (°C)
Saline 0.9%, 0.308M						
cooling	48.8±0.05	98.1±0.09	141.3±0.07	179.7±0.09	214.4±0.14	n.a.
heating	49.1±0.06	98.3±0.06	141.4±0.09	179.9±0.12	214.1±0.22	
Glucose						
1M cooling	46.9±0.00	96.1±0.15	138.7±0.10	176.7±0.00	210.9±0.15	-7.26±2.46
1M heating	47.5±0.10	96.5±0.10	139.3±0.15	177.2±0.10	210.9±0.15	
2M cooling	45.3±0.00	93.9±0.44	136.3±0.63	173.5±1.16	205.3±3.05	-7.96±2.81
2M heating	45.0±1.05	93.5±1.50	135.8±1.93	173.5±2.32	205.3±3.05	
3M cooling	-	91.4±1.13	133.4±1.37	170.7±1.69	202.9±2.44	-9.48±0.85
3M heating	43.6±0.64	91.7±0.96	133.8±1.25	171.1±1.63	202.9±2.44	
4M cooling	-	90.0±0.40	131.3±1.11	168.4±1.36	201.5±1.49	-9.58±0.34
4M heating	41.6±0.61	89.8±0.75	131.8±1.01	168.8±1.16	201.5±1.49	
5M cooling	-	86.9±0.75	128.3±1.12	164.8±1.55	196.9±2.97	-9.73±0.71
5 M heating	40.0±0.34	87.4±0.70	128.9±0.97	165.6±1.28	196.9±2.97	

12.3. **Behavior of DMSO-saline mixtures 0.9% in isochor**

The following mixtures were used for this study: 0.9% saline and DMSO 1 M, 2 M, 3 M, 4 M. Each experimental protocol was repeated three to five times. At a concentration of 5M DMSO in saline, the mixture no longer nucleated.

In the table below we find all pressures measured at equilibrium temperatures for each concentration of saline and DMSO studied separately.
At the same time, in the table, marked with green color, we find the recommended interval, in which using this mixture for the conservation of different biological materials, we can obtain viable results, given the experiments validated until the moment of writing this paper.
The maximum pressure up to which promising results have been achieved so far is [40] MPa. [7]

Tabel 5- Temperature-pressure correlations at equilibrium in isochor for different concentrations of DMSO and saline 0.9%

Mixture	Pressure at -5°C [MPa]	Pressure at -10°C [MPa]	Pressure at -15°C [MPa]	Pressure at -20°C [MPa]	Pressure at -25°C [MPa]	Supercooling (°C)
Saline 0.9%, 0.308M						
cooling	48.8±0.05	98.1±0.09	141.3±0.07	179.7±0.09	214.4±0.14	n.a.
heating	49.1±0.06	98.3±0.06	141.4±0.09	179.9±0.12	214.1±0.22	-
DMSO						
1M cooling	-	-	104.2±1.69	139.3±1.88	169.2±3.22	-12.82±1.73
1M heating	19.2±0.96	64.9±1.34	104.5±1.62	139.7±1.85	169.2±3.22	-
2M cooling	-	-	63.0±2.35	93.9±2.59	120.5±3.02	-13.59±2.70
2M heating	-	28.9±1.75	63.9±2.22	94.3±2.59	120.5±3.02	-
3M cooling	-	-	22.6±3.68	68.5±3.72	68.5±3.72	-16.53±2.85
3M heating		-	23.1±2.54	68.5±3.13	68.5±3.72	-
4M cooling	-	-	-	-	13.9±0.38	-23.85±1.18
4M heating	-	-	-	-	13.9±0.38	-

12.4. **Behavior of Ethylen - Glycol-saline 0.9% mixtures in isochor**

During this study, the following mixtures were used: physiological saline 0.9% and ethylene glycol 1 M, 2 M, 3 M, 4 M, 5 M. Each experimental protocol was repeated three to five times. The mixture nucleated at all concentrations studied.[60]

To view the data easily and completely, they have been entered into the table below.
In the table we find all pressures measured at equilibrium temperatures for each concentration of saline and EG studied separately.
At the same time, in the table, marked with green color, we find the recommended interval, in which using this mixture for the conservation of different biological materials, we can obtain viable results, given the experiments validated until the moment of writing this paper.
The maximum pressure up to which promising results have been achieved so far is [40] MPa. [7]

Tabel 6- Temperature-pressure correlations at equilibrium in isocor for different mixtures of ethylene glycol and saline 0.9%

Mixture	Pressure at -5°C [MPa]	Pressure at -10°C [MPa]	Pressure at -15°C [MPa]	Pressure at -20°C [MPa]	Pressure at -25°C [MPa]	Supercooling (°C)
Saline 0.9%, 0.308M						
cooling	48.8±0.05	98.1±0.09	141.3±0.07	179.7±0.09	214.4±0.14	n.a.
heating	49.1±0.06	98.3±0.06	141.4±0.09	179.9±0.12	214.1±0.22	
Ethylene Glycol						
1M cooling			104.5±0.89	139.6±1.23	168.7±2.55	-10.36±3.06
1M heating	19.2±0.28	65.0±0.53	104.8±0.79	139.7±1.30	168.7±2.55	
2M cooling	-	-	75.1±1.60	107.7±2.15	136.0±2.92	-13.05±2.21
2M heating	-	38.6±1.33	75.5±1.79	107.8±2.40	136.0±2.92	
3M cooling	-	-	-	72.6±1.13	97.8±1.62	-18.00±0.34
3M heating	-	9.6±0.64	43.2±0.90	73.1±1.07	97.8±1.62	
4M cooling	-	-	-	37.2±0.00	59.1±0.18	-21.65±3.03
4M heating	-	-	11.6±0.22	37.2±0.24	59.1±0.18	
5M cooling	-	-	-	-	14.2±0.08	-22.14±1.59
5 M heating	-	-	-	-	14.2±0.08	

12.5. **Behaviour of mixtures of diethylene glycol and saline 0,9 % in isochoric conditions**

At this stage of the study, the following mixtures were used: 0.9% saline and diethylene glycol 1 M, 2 M, 3 M, 4 M. Each experimental protocol was repeated three to five times. At a concentration of 5M diethylene glycol in saline, the mixture no longer nucleated.

In the table we find all pressures measured at equilibrium temperatures for each concentration of saline and DEG studied separately.

At the same time, in the table, marked with green color, we find the recommended interval, in which using this mixture for the conservation of different biological materials, we can obtain viable results, given the experiments validated until the moment of writing this paper.

The maximum pressure up to which promising results have been achieved so far is [40] MPa. [7]

Tabel 7- Temperature-pressure correlations at equilibrium in isochoric conditions for different mixtures of diethylene glycol and saline 0.9% [60]

Mixture	Pressure at -5°C [MPa]	Pressure at -10°C [MPa]	Pressure at -15°C [MPa]	Pressure at -20°C [MPa]	Pressure at -25°C [MPa]	Supercooling (°C)
Saline 0.9%, 0.308M						
cooling	48.8±0.05	98.1±0.09	141.3±0.07	179.7±0.09	214.4±0.14	n.a.
heating	49.1±0.06	98.3±0.06	141.4±0.09	179.9±0.12	214.1±0.22	
Dyethylene Glycol						
1M cooling	-	-	102.7±1.30	137.2±1.65	167.6±2.00	-13.36±0.47
1M heating	18.4±0.70	63.6±0.90	102.9±1.20	137.4±1.55	167.6±2.00	
2M cooling	-	-	53.7±2.62	82.7±1.60	107.7±1.84	-16.10±1.28
2M heating	-	21.2±1.11	54.4±1.37	83.2±1.60	107.7±1.84	
3M cooling	-	-	-	28.2±0.00	47.9±0.21	-19.29±2.93
3M heating	-	-	5.9±0.25	28.6±0.26	47.9±0.21	
4M cooling	-	-	-	-	3.4±0.22	-23.45±0.21
4M heating	-	-	-	-	3.4±0.22	

12.6. **Behaviour of trehalose and saline mixtures 0,9 % in isochoric conditions**

The mixtures used to conduct these experiments were: 0.9% saline and trehalose 10%, 20%, 30%, 40% and 50%. Compared to previous experiments (where the solutes were in liquid form), the trehalose used was in powder form, and its use as a percentage by mass was much easier to interpret.[60].

To view the data easily and completely, they have been entered into the table below.
In the table we find, all pressures measured at equilibrium temperatures for each concentration of 0.9% saline solution and trehalose studied separately.
At the same time, in the table, marked with green color, we find the recommended interval, in which using this mixture for the conservation of different biological materials, we can obtain viable results, given the experiments validated until the moment of writing this paper.
The maximum pressure up to which promising results have been achieved so far is [40] MPa. [7]

Tabel 8- Temperature-pressure correlations at equilibrium in isochoric conditions for different trehalose and saline 0.9% mixtures [60]

Mixture	Pressure at -5°C [MPa]	Pressure at -10°C [MPa]	Pressure at -15°C [MPa]	Pressure at -20°C [MPa]	Pressure at -25°C [MPa]	Supercooling (°C)
Saline 0.9%, 0.308M						
cooling	48.8±0.05	98.1±0.09	141.3±0.07	179.7±0.09	214.4±0.14	n.a.
heating	49.1±0.06	98.3±0.06	141.4±0.09	179.9±0.12	214.1±0.22	
Trehalose + saline 0.9%						
10% cooling		85.4±0.00	127.2±1.41	164.0±1.74	196.4±2.54	-10.72±0.85
10% heating	38.6±0.57	86.2±0.91	127.9±1.27	164.4±1.65	196.4±2.54	
20% cooling		72.8±0.00	113.1±0.64	147.8±0.81	178.5±0.78	-11.24±1.76
20% heating	27.9±0.17	73.5±0.38	113.6±0.44	148.2±0.67	178.5±0.78	
30% cooling			96.3±0.00	128.4±1.51	156.9±1.25	-15.60±0.56
30% heating	14.4±0.84	58.4±1.01	95.9±1.27	128.6±1.40	156.9±1.25	
40% cooling		40.2±0.00	74.3±0.38	103.1±1.33	129.4±1.49	-14.20±1.76
40% heating	0.5±0.14	39.4±1.38	73.8±1.32	103.6±1.33	129.4±1.49	
50% cooling					165.8±0.71	-11.36±1.12
50% heating	15.9±0.76	65.3±0.84	105.8±0.65	138.5±0.58	165.8±0.71	

12.7. **Behaviour of mixtures of trehalose and distilled water in isochoric conditions**

The mixtures used for this experiment were: distilled water and trehalose 10%, 20%, 30%, 40% and 50%. Compared to previous experiments (where the solutes were in liquid form), the trehalose used was in powder form, and using it as a mass percentage was much easier

To view the data easily and completely, it has been entered into the Table below.
In the table we find all pressures measured at equilibrium temperatures for each concentration of distilled water and trehalose studied.
At the same time, in the table, marked with green color, we find the recommended interval, in which using this mixture for the conservation of different biological materials, we can obtain viable results, given the experiments validated until the moment of writing this paper.
The maximum pressure up to which promising results have been achieved so far is [40] MPa. [7]

Tabel 9- Temperature-pressure correlates at equilibrium in isocor for different trehalose and distilled water mixtures [60]

Mixture	Pressure at -5°C [MPa]	Pressure at -10°C [MPa]	Pressure at -15°C [MPa]	Pressure at -20°C [MPa]	Pressure at -25°C [MPa]	Supercooling (°C)
Saline 0.9%, 0.308M						
cooling	48.8±0.05	98.1±0.09	141.3±0.07	179.7±0.09	214.4±0.14	n.a.
heating	49.1±0.06	98.3±0.06	141.4±0.09	179.9±0.12	214.1±0.22	
Trehalose + distilled water						
10% cooling		96.3±0.42	138.0±0.72	174.8±0.84	206.6±1.75	-10.20±1.23
10% heating	47.9±0.15	96.2±0.29	138.3±0.43	175.3±0.69	206.6±1.75	
20% cooling		81.9±0.57	122.8±1.27	157.9±1.70	187.6±3.34	-10.71±1.52
20% heating	36.5±0.53	83.0±0.90	123.5±1.00	158.6±1.39	187.6±3.34	
30% cooling		66.4±0.44	104.2±0.62	136.8±0.60	163.2±0.84	-9.96±0.10
30% heating	22.8±0.29	66.8±0.43	104.5±0.42	137.1±0.44	163.2±0.84	
40% cooling			90.9±0.48	121.9±0.55	147.1±1.34	-11.63±0.21
40% heating	13.0±0.22	55.1±0.29	91.3±0.35	122.2±0.49	147.1±1.34	
50% cooling					173.0±2.72	-13.48±1.00
50% heating	25.2±0.16	76.0±0.21	115.9±2.92	147.1±3.26	173.0±2.72	

13. General conclusion

Previous research, found in the literature, showed that biological matter survived preservation up to temperatures of about -4 °C and pressures of 40 MPa [61][62][63][64][65][66][7][67], after which the detrimental effect of increased pressure, outweighs the beneficial effects of storage at low temperatures in the absence of ice (Figure 20).[7]

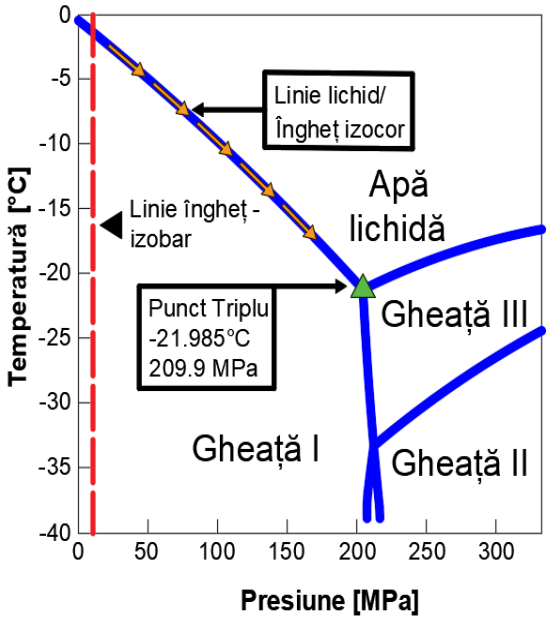


Figura 20-Phase change diagam of water in isochoric conditions[59]

The results presented in the literature showed that common cryoprotectants, used for preservation in isobaria, could be used below their freezing point (in isobar), to preserve biological matter. During the study, by cooling mixtures of cryoprotectants in different concentrations and saline 0.9%, in most cases viable ranges were found, in the negative temperature range < 0 [°C], in which the pressure does not reach 40 MPa, avoiding ice formation and the harmful effect of increased pressure, thus increasing the temperature range viable in the survival of biological matter, even alive.

As far as we know, the data obtained are new in the specialized literature, thermodynamic profiles for glucose and saline 0.9% mixtures, glycerol and saline 0.9%, dimethylsulfoxide and saline 0.9%, ethylene glycol and saline 0.9%, diethylene glycol and saline 0.9%, trehalose and saline 0.9%, trehalose and distilled water can therefore be used in the realization of new cryopreservation protocols.

Given that all studies were conducted with 0.9% saline solution (except for the mixture of trehalose and distilled water), we can also say that the study also shows the behavior of the latter, during cooling in isocor, mixed with various chemical agents.

In the table below were centralized the data obtained for all mixtures investigated in this paper.

Tabel 10– Centralizer of the studied mixtures and minimum viable temperatures (maximum pressure 40 MPa)

Mixture	Temperature to 40 MPa [°C]
Saline 0.9% + 1M Glucose	n.a
Saline 0.9% + 2M Glucose	n.a
Saline 0.9% + 3M Glucose	n.a
Saline 0.9% + 4M Glucose	n.a
Saline 0.9% + 5M Glucose	-5
Saline solution 0.9% + 1M Glycerol	-8
Saline solution 0.9% + 2M Glycerol	-11
Saline solution 0.9% + 3M Glycerol	-16.5
Saline solution 0.9% + 4M Glycerol	-24
Saline solution 0.9% + 5M Glycerol	<-25
Saline solution 0.9% + 1M DMSO	-7
Saline solution 0.9% + 2M DMSO	-13
Saline solution 0.9% + 3M DMSO	-20.5
Saline solution 0.9% + 4M DMSO	<-25
Saline solution 0.9% + 5M DMSO	<<-25
Saline solution 0.9% + 1M ethylene glycol	-7.2
Saline solution 0.9% + 2M ethylene glycol	-10
Saline solution 0.9% + 3M ethylene glycol	-14.5
Saline solution 0.9% + 4M ethylene glycol	-20.5
Saline solution 0.9% + 5M ethylene glycol	<-25
Saline solution 0.9% + 1M Diethylene glycol	-7.2
Saline solution 0.9% + 2M Diethylene glycol	-12.9
Saline solution 0.9% + 3M Diethylene glycol	-22.8
Saline solution 0.9% + 4M Diethylene glycol	<-25
Saline solution 0.9% + 5M Diethylene glycol	<<-25
Saline 0.9% + 10% Trehalose	-5.1
Saline 0.9% + 20% Trehalose	-6.2
Saline 0.9% + 30% Trehalose	-7.9
Saline 0.9% + 40% Trehalose	-10.1
Saline 0.9% + 50% Trehalose	-7.7
Distilled water + 10% Trehalose	n.a
Distilled water + 20% Trehalose	-5.2
Distilled water + 30% Trehalose	-6.9
Distilled water + 40% Trehalose	-8.1
Distilled water + 50% Trehalose	-6.5

Similar behaviour was observed in all mixtures studied except trehalose mixtures. With the increase in the concentration of cryoprotectant solution studied in the composition of the mixture, an increase in osmolarity and implicitly a decrease in the freezing point was reached.

This behavior can be explained by the fact that cryoprotective solutions prevent water crystallization by forming a molecular micropackaging around water molecules, which reduces the tendency of water to form crystals at low temperatures. However, as the concentration of the cryoprotective solution increases, the greater the number of cryoprotective molecules in the solution, and this leads to an increase in osmolarity and implicitly to a decrease in the freezing point.

The results obtained in this study open new perspectives regarding the conservation of biological materials at low temperatures below freezing, in constant volume, introducing for the first time, correlated with the specialized literature, viable study intervals. Even though their continuation must be treated from many other perspectives, such as toxicology or chemical composition.

The thermodynamic profiles obtained in this study can be used to optimize conservation conditions and improve the efficiency of cryopreservation methods. These results can also contribute to the development of new conservation protocols for a variety of biological materials, with significant implications for engineering and biotechnology.

We could add some areas where the results can be used:

1. Developing safer food products – The study of these mixtures can help develop food and medicines that are safer for consumers by using effective combinations of preservatives.
2. Reducing losses in the food and pharmaceutical industry - Thanks to the study of mixtures of preservatives and 0.9% saline in isocore systems, the food and pharmaceutical industry can reduce the losses of products that degrade due to contamination, which can lead to significant savings
3. Contribution to the development of policies and standards - The study of mixtures of preservatives and saline 0.9% in isocore systems can contribute to the development of policies and standards on food and pharmaceutical safety, thus ensuring better protection for consumers
4. Developing more natural and healthier products – The study of mixtures of preservatives and 0.9% saline in isocore systems can help identify effective combinations of natural preservatives and saline, enabling the development of more natural and healthier products

The study of mixtures of cryopreserving solutions and 0.9% saline in isochore systems is a research area of crucial importance for various applications, from food and drug preservation to cell and tissue storage in regenerative medicine. Research in this area has led to the development of effective and safer cryopreservative solutions that protect biological samples against degradation and loss of functionality over time. In addition, this research has shown that cryopreservative solutions can be successfully used in various applications, such as storing cells and tissues for later use in regenerative medicine or preserving vaccines and drugs to make them more accessible in areas where resources are limited. Studies in isocor contribute to the

identification of the composition and effects of mixtures of cryopreservative agents and to the development of mixtures optimized for different applications. Furthermore, studies in this area can help develop more advanced technologies for storing and preserving biological samples and other temperature-sensitive products, with significant effects on medicine and the food industry. In addition, this research can bring new perspectives on the development of sustainable and environmentally friendly solutions for product storage and conservation of natural resources. Research in the field of mixtures of cryopreservative solutions in isocore systems is essential for the development of innovative and effective solutions for the preservation and protection of temperature-sensitive products, with significant benefits for medicine, food industry and the environment. This research can bring new perspectives and contribute to the development of more sustainable and efficient solutions for storing and preserving products, with significant effects on society and the environment. Studies have shown that, depending on the concentration and combination used, these mixtures may have different effects on the survival and functionality of cryopreservation, cells and tissues. For example, DMSO and glycerol have been found to be effective in protecting cells and tissues against freezing, while glucose and trehalose can improve cell viability by reducing osmotic stress. Recent research has also shown that adding antioxidants and cellular protection agents, such as vitamin C or N-acetylcysteine, to saline and cryopreservative mixtures can further improve cell viability and cryopreserved tissue.

In conclusion, the study of 0.9% saline mixtures with cryopreservatives represents an important area of research for medical and pharmaceutical science, with potentially significant implications in the development of better and more effective cryopreservation solutions for clinical and therapeutic use.

14. Personal contributions

During the research and experiments underlying the writing of this thesis, I consider that I had the following personal contributions:

- Detailed bibliographic research both from the perspective of the large number of evaluated publications and from a qualitative point of view. This led to a very well elaborated state of knowledge and which was also published in a journal: Cryoletters, ISSN - 0143-2044.
- The mathematical modeling elaborated in the paper starts from a series of fundamental principles underlying cryopreservation phenomena in isochoric systems and then they are observed in practical determinations.
- Modeling of phenomena occurring inside an isocoric reactor with the help of a finite element analysis, solving and simulation software package for various physics and engineering applications, especially coupled phenomena and multiphysics. Original paper published in IOP Conference Series: Materials Science and Engineering, ISSN - 1757-899X
- Participation as a member and active involvement in a national project won in the competition, project code PN-III-P4-ID-PCE-2020-1706 Contract no. PCE230202021,

named after the title of this thesis: "Study of thermodynamic profiles in isochoric regime for the most important cryoprotective substances", within the type of project: Exploratory research projects.

- Development and extension of a new field of research within the Department of Building Installations by creating an experimental stand in the laboratory of Refrigeration and Cryogenic Installations. With the help of this stand, research can be carried out and further expanded, and also the stand can be used for teaching purposes for students, and with portable measuring equipment and instruments, measurements can be made outside the laboratory.
- Based on the experimental results, polynomial equations of degree 2 were proposed for each substance and for each concentration analysed. With the help of these newly introduced equations, interpolations can be easily made to find intermediate pressure values, or even propose and then experimentally confirm values outside the range.
- Participation and active involvement in writing and reviewing articles submitted for publication to scientific journals.
- Contributions to expanding experimental knowledge based on temperature-pressure measurements for some of the most widely used cryopreservatives. Publication of two papers in the field, in the journals Biochemical and biophysical research communications, ISSN 0006-291X and Cryobiology, ISSN - 0011-2240.
- Propose polynomial equations based on trend lines, for each substance analysed and for each concentration analysed.
- Active participation in the study of the phenomenon of random nucleation in isochorus. Publication of article (co-author) in AIP Advances journal, ISSN 2158-3226.
- Active participation in the study of fruit preservation in isocor. Publication of article (co-author) in the journal Heliyon, ISSN 2405-8440.
- I propose further the following directions of study, taking into account the experience of writing this thesis:
    1. Analysis of the behavior of substances studied at cryogenic temperatures
    2. Analysis of the behaviour of these substances and for other concentrations
    3. Use of results from this thesis in experiments with biological material for comparative investigation of fresh biological material - preserved biological material

Concluding succinctly, through the experiments carried out and included in this paper, we managed to measure and subsequently graphically present the thermodynamic profiles for 5 mixtures of cryopreservatives and 0.9% saline solution along with a mixture of cryopreservative and distilled water. The method, and especially the temperature range studied for thermodynamic profiling, includes intervals from studies conducted so far in the field [68], [69], [64], [7], [66], [62], [63], [61].

The method used to determine profiles is accepted and used in common practice in this field [8], [70], [71], [65] .

The uniqueness of this work lies in standardizing the profiles both graphically and in terms of studied temperatures, and presenting them in a simple and concise way, so that in experimental practice, future researchers who will want to conduct experiments in the field, can find the information easily, quickly and grouped in a single paper.

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Scurt rezumat (română /engleză)

The thesis " Study of thermodynamic profiles in isochoric conditions for the most important cryoprotective substancesanalyzes the behavior of mixtures of glucose and saline 0.9%, glycerin and physiological saline 0.9%, DMSO and saline 0.9%, ethylene glycol and saline 0.9%, diethylene glycol and saline 0.9%, trehalose and saline 0.9, trehalose and distilled water. All solutes are studied in concentrations of 1-5 M, the study interval is between 0 °C and – 21 °C and all experiments are performed in isochoric conditions (in constant volume).

The uniqueness of the thesis lies in compressing all the results obtained by identical methods for the range of mixtures mentioned above and by defining polynomial curves and equations of degree two, unique for each mixture and concentration separately.

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