Sara Raquel Gonçalves Cândido

Licenciada em Ciências da Engenharia Química e Bioquímica

Design and Characterization of Novel Biopolymeric Structures using Biocompatible Ionic Liquids

Dissertação para obtenção do Grau de Mestre em
Engenharia Química e Bioquímica

Orientador: Doutora Luísa Alexandra Graça Neves,
Post-Doctoral Researcher, FCT-UNL
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Palavras-chave

Biopolímero, FucoPol, Líquido iónico biocompatível, Estruturas poliméricas

Resumo

O principal objetivo desta tese foi o desenvolvimento de estruturas poliméricas a partir da dissolução de Fucopol, um exopolissacárido (EPS) bacteriano, num líquido iónico biocompatível, o acetato de colina.

O FucoPol foi produzido pela bactéria *Enterobacter* A47, utilizando glicerol como fonte de carbono a temperatura e pH controlados (30ºC e 7, respetivamente). Ao final de 3 dias obteve--se uma produção de 7 g/L de FucoPol. O rendimento de produto face à fonte de carbono ($Y_{P/S}$) foi de 0.22 g/g e a produtividade máxima de 2.37 g/L.d.

Este polímero foi caracterizado em relação à composição em açúcares e grupos acilo (por Cromatografia Líquida de Alta Eficiência - HPLC), contendo fucose (35% mol), galactose (21% mol), glucose (29% mol), ramnose (3% mol) e ácido glucurónico (12% mol), assim como acetato (14.28% mol), piruvato (2.15% mol) e succinato (1,80% mol). O seu conteúdo em água e cinzas foi de 15% p/p e 2% p/p, respetivamente, e as ligações químicas (determinadas por Espectrocopia de Infravermelhor – FT-IR) apresentam-se coerentes face às relatadas na literatura. No entanto, por limitações no equipamento de Calorimetria Diferencial de Varrimento (DSC) não foi possível determinar a temperatura de transição vítrea. Por sua vez o líquido iónico apresentou um comportamento típico de um fluido Newtoniano, temperatura de transição vítrea (determinada por DSC) de -98.03ºC e densidade de 1.1031 g/cm^3. O estudo das ligações químicas, através de FT-IR demonstrou que a quantidade de água existente neste (8.80%) influenciou a visualização das bandas previstas face à sua estrutura química.

Após a dissolução do FucoPol no líquido iónico a diferentes temperaturas (50, 60, 80 e 100 ºC), promoveu-se à remoção deste através do método de inversão de fase, utilizando água como solvente, seguido de secagem em estufa a 70ºC. As misturas antes e após o método de inversão de fase foram caracterizadas através dos estudos referidos anteriormente.

Como forma de explorar possíveis campos de aplicação foram feitos testes de biocompatibilidade e de colagem em madeira do tipo balsa.

Verificou-se que a lavagem com água realizada pelo método de inversão de fase não se mostrou totalmente eficiente na remoção do líquido iónico biocompatível, uma vez que as todas as misturas de FucoPol e Líquido iónico continham líquido iónico na sua composição como se pode aferir pelos resultados de DSC e FT-IR. Para além disso, a lavagem com água alterou significativamente a composição do FucoPol. No entanto, estas misturas, que desenvolveram um comportamento viscoso e típico de um fluído não Newtoniano (reofluidificante), têm potencial para serem aplicadas no campo biomédico bem como em colas biológicas.

Keywords

Biopolymer, FucoPol, Biocompatible Ionic Liquid, Polymeric Structures

Abstract

The main objective of this thesis was the development of polymeric structures from the dissolution of FucoPol, a bacterial exopolysaccharide (EPS), in a biocompatible ionic liquid, choline acetate.

The FucoPol was produced by the bacteria Enterobacter A47 using glycerol as carbon source at controlled temperature and pH (30ºC and 7, respectively). At the end of 3 days it was produced 7 g/L of FucoPol. The net yield of Fucopol in glycerol ($Y_{P/S}$) was 0.22 g/g and the maximum productivity 2.37 g/L.d

This polymer was characterized about its composition in sugars and acyl groups (by High-Performance Liquid Chromatography - HPLC), containing fucose (35 % mol), galactose (21 % mol), glucose (29 % mol), rhamnose (3% mol) and glucuronic acid (12% mol) as well as acetate (14.28 % mol), pyruvate (2.15 % mol) and succinate (1.80 % mol). Its content of water and ash was 15% p/p and 2% p/p, respectively, and the chemical bonds (determined by Infrared Spectroscopy - FT-IR) are consistent to the literature reports. However, due to limitations in Differential Scanning Calorimetry (DSC) equipment it was not possible to determine the glass transition temperature. In turn, the ionic liquid showed the typical behavior of a Newtonian fluid, glass transition temperature (determined by DSC) -98.03ºC and density 1.1031 g/cm^3. The study of chemical bonds by FT-IR showed that amount of water (8.80%) influenced the visualization of the bands predicted to in view of their chemical structure.

After the dissolution of the FucoPol in the ionic liquid at different temperatures (50, 60, 80 and 100 ° C) it was promoted the removal of this by the phase inversion method using deionized water as a solvent, followed by drying in an oven at 70 ° C. The mixtures before and after the phase inversion method were characterized through the studies mentioned above.

In order to explore possible application field’s biocompatibility assays and collage on balsa wood tests were performed.

It was found that the process of washing with water by the phase inversion method was not totally effective in removing the biocompatible ionic liquid, since all FucoPol – IL mixtures still contained ionic liquid in their composition as can be seen by the DSC results and FT-IR. In addition, washing the mixtures with water significantly altered the composition of FucoPol. However, these mixtures, that developed a viscous behavior typical of a non-Newtonian fluid (shear-thinning), have the potential to be applied in the biomedical field as well as biological glues.

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Abbreviations

$Y_{P/s}$ – Yield of EPS on glycerol (g EPS/g glycerol)

$Y_{x/s}$ – Yield of biomass on glycerol (g cell/g glycerol)

[C_2MIM][Acetate] – 1-ethyl-3-methylimidazolium acetate

[AMIM][Br] – 1-allyl-3-methylimidazolium bromide

[BMIM][Ac] – 1-butyl-3-methylimidazolium acetate

[BMIM][Cl] – 1-butyl-3-methylimidazolium chloride

CDW – Cell Dry Weight (g/L)

CGC – Chitin Glucan Complex

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethylsulfoxide

DO – Dissolved oxygen (%)

DSC – Differential Scanning Calorimetry

Ea – Activation energy (kJ/mol)

EPS – Exopolysaccharide

FBS – Fetal Bovine Serum

FCT – Faculdade de Ciências e Tecnologia

FT-IR – Fourier Transform Infrared Spectroscopy

G' – Storage Module or Elastic Module (Pa)

G'' – Loss Module or Viscous Module (Pa)

HPLC – High-Performance Liquid Chromatography

Huh-7 – Transformed Human Liver Cells

ILs – Ionic Liquids

IR – Infrared

ISA – Ionic Strength Adjuster

MTT – (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)

N – Normal unit

nm - nanometers

OD – Optical Density

PES – Polyethersulfone

rpm – rotation per minute

RTILs – Room Temperature Ionic Liquids

TCA – Trichloroacetic acid

TFA – Trifluoracetic acid

Tg – Glass Transition Temperature (ºC)

v/v – volume per volume

vvm – volume of air per volume of reactor per minute

1 Introduction

1.1 Polysaccharides

Polysaccharides ($C_6(H_{10}O_5)_n$) are polymeric carbohydrates molecules composed of more than ten monosaccharides units joined by glycosidic bonds which may form repeating units and different degrees of branching. They have a high molecular weight (10^4 – 10^7) and are mainly composed of carbohydrates, namely glucose, galactose and mannose, uronic acids (e.g. glucuronic acid and galacturonic acid) and aminosugars (e.g. N-acetyl-glucosamine and N-acetylgalactosamine) [1,2]. Furthermore, many polysaccharides have inorganic (e.g. phosphate) and organic groups (e.g. pyruvyl, acetyl and succinyl) [1].

The polysaccharides may be divided into two groups: homopolysaccharides, composed of only one type of monosaccharide and heteropolysaccharides having different monosaccharides [3].

In recent years the demand for natural polymers produced by sustainable processes and renewable resources, as well as those that combine the good product yield and environmental conditions, have increased. Polysaccharides may be obtained from animals (e.g. chitin), algae (e.g. alginate and carrageen), plants (e.g. cellulose) and produced by microorganisms (e.g. xanthan, gellan) [4,5].

Some properties of most polysaccharides, such as non-Newtonian behaviour, biocompatibility and the capacity to be thickening, stabilizing, binding and structuring agents, lead to their use in several areas such as food, pharmaceutical and biomedicine [1]. In food industry, this group of sugar has been used as hydrocolloids or gums which is an area that has a market value higher than 4 million US$ (2008 data) or for packaging (e.g. vessels for agro-food applications) due to the ability of certain polysaccharides (e.g. starch and cellulose) to form biodegradable films. In turn, in the medical field, the biopolymers are used as matrices for wound dressing (e.g. chitin) and drug delivery [1,6].

Despite the variety of polysaccharide sources, those derived from plants and algae (e.g. pectin) dominate the market when compared to the small fraction occupied by microbial polysaccharides (e.g. xanthan) (6% of the total market value that it was more than 4 million US$ in 2008) [1,7].

Microbial polysaccharides, are renewable, biodegradable and biocompatible polymers. These polysaccharides are competitive with other natural polysaccharides and with synthetic products because they have new or improved properties. Furthermore, the process of obtaining these polymers is easier and faster, their production is independent of weather conditions, the materials used as carbon sources may be wastes or byproducts and the required production space is relatively small [4]. Although there are common features in all biopolymers, some properties depend on other variables including, the strain used in the process. In terms of physical properties, they depend on the manner in which the sequences of sugars are arranged and how these simple polysaccharide chains associate with each other. There is also a relationship between the glycosidic linkage, the geometry of the polysaccharide and its conformation. Microbial polysaccharides can be divided into intracellular polysaccharides (e.g. glycogen), structural polysaccharides (e.g. chitin) and extracellular polysaccharides or exopolysaccharides (EPS) (e.g. gellan). There are two types of exopolysaccharides, capsular, also known as CPS or K-antigens (e.g. K30 antigens), that are bound to the cell surface with covalent bonds, and slime polysaccharides which do not have such a strong connection (e.g. xanthan) [4,6].

Recent studies show that some EPS can be competitive with polysaccharides extracted from algae (e.g. alginate) and plants (e.g. gellan gum), because they have new and improved properties [1,8].These properties include the resistance to solvents, rheological characteristics (e.g. stability to a wide range of pH, temperature and high viscosity when polymer concentration is low), biological activity (e.g. anticarcinogenic) and higher emulsifying and flocculating activities [2,4].

For the discovery and application of new exopolysaccharides, it is also necessary to search new biosynthetic and genetic mechanisms for their synthesis. All processes depend on enzymes which are located at different positions in the microbial cells, some are intracellular and others are located outside the cell. Generally, macromolecules are synthesized and transported to the cell membrane to the outside of the cytoplasmic membrane, but there are exceptions as levans, alternans and dextrans, which are synthesized by an extracellular process [1].

In addition to the genetic mechanisms for synthesis, it is important to know the influence of each parameter, in order to control them and prevent changes in the final product characteristics. To obtain a high yield of exopolysaccharide production, each microorganism has different needs in terms of carbon, nitrogen, minerals, pH and temperature [4].

A greater production of EPS is usually reached with excess of carbon source and residual concentration of other nutrients, such as nitrogen and oxygen (e.g. GalactoPol and FucoPol). Besides the amount of EPS produced, polysaccharide size can also depend on this parameter [1,4]. The most common carbon sources are sucrose, glucose, lactose, maltose and sugar concentrates (as Neosorb™ or Cerelose™) while the most common source of nitrogen for the production of exopolysaccharide are ammonium sulfate, peptone, sodium nitrate, urea and yeast extract [1,4,9].

The pH and temperature are also parameters that directly influence culture growth. In most organisms the maximum production of the biopolymer is achieved by buffering the pH to neutral, and keeping it constant (e.g. *Rhizobium fredii*[10]), but there are microorganisms that belong to a small minority whose maximum production is reached at acid pH (e.g. *Neisseria meningitides*[4]) . In turn, the incubation temperature below the optimum growth temperature results in greater productions of exopolysaccharide. Typically, a lower incubation temperature may cause a decrease in growth rate and cell mass as compared to elevated temperatures, as the case of *Lactobacillus delbrueckii subsp. Bulgaricus*. Nonetheless, there are exceptions to this behavior, as the case of *Propionibacterium acidipropionici* [4,11,12].

However, there are drawbacks in the implementation of exopolysaccharide production processes in industry, mainly due to the high production cost. That value corresponds mainly to the high price of the substrate, approximately 40% of total process costs, and the downstream or recuperation phase [1,9]. To improve this situation it is possible to use agricultural and industrial wastes or byproducts as substrates for microbial cultivation instead of the common substrates that are more expensive. Assuming common substrates as sugars and starch, its values varies between 350-518 US$/ton and 279-310 US$/ton, respectively, while for byproducts such as glycerol from the biodiesel industry is 0.88-1.07 US$/ton [1,9].

Despite the cost reduction it is necessary to consider that the use of this type of substrates have some problems associated with their different nutrient composition and the presence of contaminants that may reduce biopolymer production or lead to the synthesis of different polymers and byproducts, as a result of the choice of different cellular metabolic pathways. The non-reacted components are also a problem because they can accumulate in the culture broth and become inhibitors, reducing process yield [1,9].

Other solutions to reduce the cost of the process, without compromising the quality of the substrate, are to increase product yield by optimizing fermentation conditions or the development of higher yielding strains, using metabolic engineering. For this it is possible to manipulate the genes encoding the enzymes of the process or change the regulatory pathways, which will result in a different gene regulation and enzymatic activity. However, although these methods have already been applied in production of xanthan, gellan, bacterial cellulose and levan, in most cases it lead to a change in the biopolymer composition [4].

To obtain the polysaccharides after fermentation, a recovery process occurs, also known as downstream. This phase includes extraction and polysaccharide purification. Before the choice of procedures it is necessary to create a balance between the recovery of polymers, its purity and the impact on their properties. Usually, the cells are removed by centrifugation followed by a purification step, but another process consist in addition of a precipitating agent, usually a water-miscible solvent but immiscible with the polymer, so that the precipitation of the polymer occurs in the cell-free supernatant. The final stage is drying the precipitate or the purified polysaccharide solution. In laboratory scale, freeze-drying is normally used, while in the industrial scale, drum drying is used [4].

In this thesis a polymer called FucoPol is used, which is a fucose-containing exopolysaccharide produced by *Enterobacter* strain *A47*, using as carbon source glycerol from the biodiesel industry [13,14]. FucoPol is a biopolymer with high molecular weight (10^4-10^7)[1], composed of sugar residues, including fucose, galactose, glucose and glucuronic acid, and it also contains acyl groups, namely pyruvate, succinate and acetate [8,14]. Due to its properties (e.g. rheological properties in aqueous medium, emulsion forming, stabilizing capacity and flocculating capacity), it can be applied in the pharmaceutical, food, cosmetic, textile, paper and petroleum industries [14].

An application example is the production of biodegradable films for food packaging [15]. For its preparation, it was used FucoPol capacity to dissolve in water and properties of citric acid as the crosslinking and plasticizer agent [16]. The prepared films are transparent and flexible, having a slight brownish tinge. They are completely soluble in water indicating that citric acid does not promote crosslinking reactions [15]. Another study with this polymer was the preparation of composite and homogeneous membranes for ethanol dehydration by pervaporation. The homogeneous membranes were obtained directly from the fermentation broth by adding trichloroacetic acid (TCA). In turn, the composite membranes occurred by depositions of four EPS layers on a polyethersulfone (PES) support. These membranes showed a high affinity to water and good chemical resistance towards organic solvents that may compete with commercial hydrophilic pervaporation membranes for dehydration of ethanol. To this end, have to be fulfilled conditions, as the elimination of the use acetone or any other hazardous organic solvents as precipitation agents [17].

1.2 Biocompatible Ionic Liquids

Ionic liquids (ILs) are salts entirely composed of ions, with an inorganic cation and inorganic or organic anion. They have a melting point below 100ºC or even at room temperature due to the poor coordination of their ions [18,19]. These compounds are also called Room Temperature Ionic Liquids (RTILs) distinguishing them from traditional salts, which melt at much higher temperatures and are classified as molten salts [18,19].

ILs have been widely studied due to their unique properties such as chemical and thermal stability, their negligible vapor pressure that make them non-volatile compounds, high ionic conductivity and possibility of recycling [13,20-22]. Another characteristic of ILs is the possibility of obtaining desired physicochemical properties by selecting different cations and anions or by adding functional groups [18,23].

Due to these properties, ILs have become ideal candidates for different types of applications such as organic synthesis, catalysis, and as a media for extraction processes, decreasing costs and to improve the reaction activity and selectivity performance processes [24]. They are also found as a polymerization media in various kinds of processes (e.g. free radical polymerization or ionic and coordination polymerizations), in polymeric matrixes (such as polymer gels) and templates for porous polymers [25]. Currently, they are associated with various areas of high value, such as energy (e.g. battery, fuel cells, thermo fluids) [26], biotechnology (e.g. biocatalysis, protein purification and stability) [26], coat (e.g. surfactant, lubricant, metal deposition) [26] as well as in gas separation processes (e.g. preparation of supported ionic liquid membranes for CO_2/N_2 and CO_2/CH_4 separation)[27]. In addition to these applications, ionic liquids have shown positive results in the dissolution of biomolecules with many hydrogen bonds in their chains (e.g. polysaccharides as cellulose dissolved in 1-ethyl-3-methylimidazolium diethylphosphonate [28], starch dissolved in 1-butyl-3-methylimizazolium chloride

or 1-allyl-3-methylimidazolium chloride [29] and chitosan dissolved in 1,3-dimethylimidazolium chloride or 1-h-3-methylimidazolium chloride [30]) [31].
Since 1934, by Graenacher, there is registration that molten N-ethylpyridinium chloride, in the presence of nitrogen bases, dissolves cellulose, however, this time the definition of ionic liquid was not yet known. More recently, in 2002, Rogers et al, found that 1-butyl-3-methylimidazolium chloride [BMIM][Cl] dissolve cellulose in relatively high concentrations obtaining a gel or a film [20 32]. Also dissolution of chitin with an ionic liquid was tested in 2009, in order to obtain a weak gel[33]. Another polymer studied was chitosan, where films were developed by dissolving chitosan with 1-butyl-3-methylimidazolium acetate [BMIM][Ac] ionic liquid.[34].

Based on the good results obtained, tests with polymer blends were initiated. In 2014, a mixture of cellulose and xanthan was studied by adding [BMIM][Cl]. After the dissolution of the mixture, a composite gel was obtained, that transforms into a hydrogel when submerged in water, or in a film after a Soxhlet extraction with ethanol[35]. In addition to gels, chitin is also capable of forming films, for example with cellulose and 1-alyl-3-methylimidazolium bromide [AMIM][Br][36].

Even though ILs proved to dissolve a variety of polysaccharides, their acceptance in the industry is still not complete because of possible problems with their degradation, persistence and toxicity in the environment. The toxicity of these liquids depends on its raw materials, mainly from the oil industry, and the ions present. It has been reported that the type of anion do not have any impact on the toxicity, while the effect of the cation alkyl chain length is more evident. Thus, it was necessary to find strategies to lower the toxicity and increase the biodegradability, such as the use of cations and anions based on biomolecules (e.g. natural amino acids, lactic acid, choline and fructose) [21,37].

The ionic liquid used in this thesis is the choline acetate (Figure 1-1) and it is an example of this strategy. This ionic liquid has low toxicity, excellent biodegradability and low cost synthesis when compared with organic solvents but especially compared with other ILs [38,39].

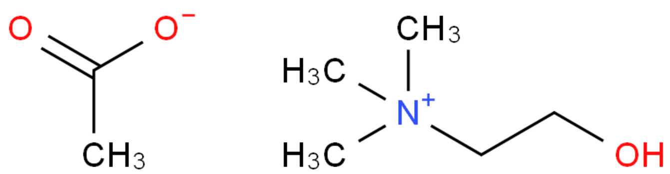


Figure 1-1- Molecular Structure of Choline Acetate Ionic Liquid, adapted from [23]

The capacity of this IL to dissolve biomolecules has already been proven recently by its use with Chitin Glucan Complex (CGC) to prepare membranes for wound dressing materials [40].

The use of biocompatible ILs to dissolve EPS represents a novelty in this thesis when compared with literature reports for EPS.

1.3 Objective of this thesis

In this thesis, the design and characterization of novel polymeric structures, especially hydrogels, based on FucoPol biopolymer, produced in FCT – Universidade Nova de Lisboa, was studied. For this purpose, a new approach was performed in order to explore possible changes in FucoPol properties using ecological and biocompatible solvents such as ionic liquids. Thus, in this study it is expected to obtain polymeric structures such as gels that maintain the biocompatible characteristics and can be used, for example, in the biomedical field.

To this end, the production of EPS was carried out using a protocol previously developed and the characterization of the polymer was made in terms of its composition, water and ash content, glass transition temperature, chemical bonds and biocompatibility.

For the development of the polymeric structures, the polymer was dissolved in Choline Acetate at temperatures of 50ºC, 60ºC, 80ºC and 100ºC and at a concentration of 2.5% (w/w). These mixtures, as well as those that were obtained after the phase inversion method using water, were characterized based on its composition, glass transition temperature, chemical bonds, solution properties and biocompatibility.

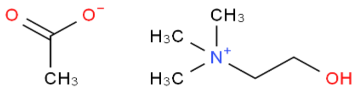
In order to explore the field of mixtures applications, it was also studied its potential as biological glues.

2 Materials and Methods

FucoPol was produced by *Enterobacter* strain A47 using glycerol (ReagenPlus 86-88% w/w Scharlau) as carbon source. The EPS produced was then purified by diafiltration as explained below.

The biocompatible ionic liquid used to dissolve this exopolysaccharide was Choline Acetate ($C_7H_{17}NO_3$), supplied by Faculdade de Farmácia, Universidade de Lisboa. The principal properties of this ionic liquid are shown in Table 2-1 [23].

Table 2-1 - Ionic Liquid properties

Ionic Liquid	Molecular Formula	Chemical structure [23]	Molecular Weight (g/mol)	Water content (%) [a]	Density (g/cm^3) [b]
Choline Acetate	$C_7H_{17}NO_3$		163.21	8.80	1.1031

The other materials and reagents used during the experiment, are described below.

2.1 FucoPol production

Enterobacter strain *A47* was grown on Medium E* (pH 7.0) with the following composition 4.64 g/L of dipotassium phosphate (K_2HPO_4), 2.96 g/L of monopotassium phosphate (KH_2PO_4) and 2.64 g/L of diammonium hydrogen phosphate [$(NH_4)_2HPO_4$]. For the 10 L reactor used in this work were added 80 mL of magnesium sulfate ($MgSO_4$) and 80 mL of mineral solution. The first solution was prepared by adding 12.5 g of this compound and 500 mL of deionized water. In turn, the mineral solution was prepared in two steps, the first consisted in a solution of 500 mL of hydrochloric acid (1 N) while the second it was added 1.39 g of Iron (II) Sulfate Heptahydrate ($FeSO_4.7H_2O$) 0.9927 g of Manganese (II) Chloride Tetrahydrate ($MnCl_2.4H_2O$), 1.405 g of Cobalt (II) Sulfate Heptahydrate ($CoSO_4.7H_2O$), 0.835 g of Calcium Chloride Dihydrate ($CaCl_2.2H_2O$), 0.085 g of Copper (II) Chloride Dihydrate ($CuCl_2.2H_2O$) and 0.145 g of $ZnSO_4.7H_2O$ (Zinc Sulfate Heptahydrate).

Inoculums for bioreactor experiments were prepared by incubating the culture in Medium E* supplemented with glycerol, in shake flasks for 72 h at 30ºC, in an incubator shaker (150 rpm).

[a] Measured by Karl-Fisher (831 KF Coulometer da Metrohm) (Protocol in Appendix 2)
[b] Measured by using a pycnometer (Protocol in Appendix 1)

2.1.1 Bioreactor operation

Enterobacter strain *A47* was cultivated using glycerol as the sole carbon source in a 10 L bioreactor (BioStat B-plus, Sartorius) with controlled temperature and pH of 30ºC and 7, respectively.

In the first 12 hours of cultivation, the bioreactor was operated in a batch mode, then a fed-batch mode started by supplying the bioreactor with cultivation Medium E*, with a glycerol concentration of 400 g/L, at a constant rate of 20 mL/h. The aeration rate (0.125 vvm, volume of air per volume of reactor per minute) was kept constant throughout the cultivation and the dissolved oxygen concentration (DO) was controlled at 10% air saturation by automatic variation of the stirrer speed (300-800 rpm) provided by two 6-blade impellers.

Throughout the cultivation, culture broth samples were recovery from the bioreactor and centrifuged (8 000 rpm, 10 minutes) for cell separation. The cell-free supernatant was stored at -20ºC for determination of glycerol and ammonium concentrations.

2.1.2 Analytical Techniques

2.1.2.1 Determination of Cell Dry Weigh (CDW)

The Cell Dry Weigh was determined by measuring the optical density (wavelength = 410 nm) of the broth samples taken from the reactor (no treatment). With this measuring and knowing the calibration curve equation (Equation 2-1) it was possible to determine the desired value.

CDW = 0.26 x OD *Equation 2-1*

2.1.2.2 Broth Viscosity Measurement

The measurement of the viscosity of the broth was performed with samples collected from the reactor without treatment, using a viscometer. To this end, the LCP and L1 mode were used (L1 used when the viscosity increased) and the rotational speed was varied between 100 and 0.3 rpm.

The target value was given for the equipment in cP.

2.1.2.3 Glycerol Consumption

Glycerol consumption was determined by high-performance liquid chromatography (HPLC) with an Aminex HPX-87H column (BioRad) coupled to an infrared (IR) detector. The analysis were performed at 50ºC, with sulphuric acid (H_2SO_4 0.01 N) as eluent at a flow rate of 0.6 mL/min.

The cell-free supernatant was diluted in H_2SO_4 (0.01N) and filtered with Vectra Spin Micro Polysulphone filters (0.2 µm) (Whatman), at 15 000 rpm for 10 minutes. Glycerol (ReagentPlus 86-88% w/w Scharlau) standards were prepared at a concentration of 1 g/L successively diluted at concentrations of 0.5 g/L, 0.1 g/L, 0.05 g/L and 0.01 g/L while all samples, from the reactor after treatment, were diluted (1:50) ensuring that its concentration was less than 1 g/L (calibration curve in Appendix 3).

2.1.2.4 Ammonium Consumption

Ammonium concentration was evaluated using a potentiometric sensor (Thermo Electron corporation, Orion 9512). Cell-free supernatant samples (1mL) were mixed with 20 µL of ISA (Ionic Strength Adjuster) solution and the electric potential was measured after 5 minutes. A calibration curve (Appendix 4) was constructed using NH_4CL solutions (100 - 0.03 mM).

2.1.3 FucoPol Extraction

2.1.3.1 FucoPol quantification

FucoPol production across cultivation run was evaluated by dialysis. This process is intended to separate molecules such as salts and polysaccharides, using the difference between the diffusion rate across a permeable membrane (Figure 2-1) [3].

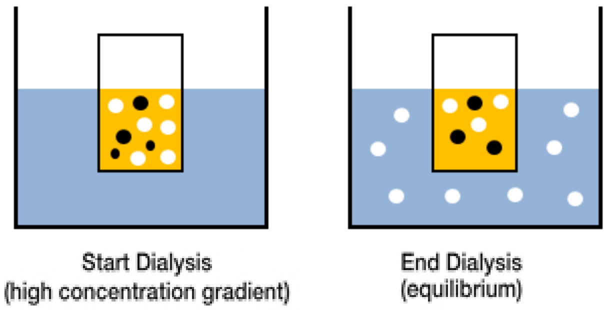


Figure 2-1 - Dialysis process engine, adapted from [3]

The cell-free supernatant samples were dialyzed with 12 000 -14 000 MWCO membrane (Zellutrans Roth and Nadir – Dialysis Tubling Cellulosehydrat), against deionized water for 48 hours (changing the water each day) (Figure 2-2). 10 ppm of sodium azide (Riedel-deHaen) were added to avoid cellular growth. Then, the dialyzed samples were freeze dried (in Scanvac, CoolSafe).

Figure 2-2 - Dialysis process

2.1.3.2 FucoPol Purification

At the end of cultivation, the culture broth was recovered and diluted (1:4 v/v) in order to lower the viscosity. Then, the bacterial cells were removed by centrifugation (8 000 rpm during 45 minutes). A thermal treatment (1h, 70ºC), to inactivate bacterial enzymes that may cause FucoPol degradation, was performed. A second centrifugation (8 000 rpm during 45 minutes) was performed to remove any remaining cell debris and denatured proteins. The supernatant was then submitted to a diafiltration process using a slice cassette membrane (Sartocon Slice Hydrosart) with a 100 000 Dalton cut-off and a surface area of 6000 cm^2, operated at a transmembranar pressure below 0.7 bar, to remove low molecular contaminants (e.g. salts, glycerol and proteins). After impurities removal, the treated supernatant containing FucoPol was concentrated using the same membrane module, switching to an ultrafiltration process mode. The obtained solution was freeze dried (in Scanvac, CoolSafe) during 48 hours, and the obtained FucoPol was stored at room temperature (Figure 2-3).

Figure 2-3 - FucoPol polymer

2.1.4 FucoPol Characterization

2.1.4.1 Sugar and Acyl Groups Composition

FucoPol samples (5 to 6 mg) were dissolved in 5 ml of deionized water and hydrolyzed with 100 µL of trifluoracetic acid (TFA 99%), from Sigma Aldrich-German, for 2 hours at 120ºC.

The acid hydrolysate was used for identification and quantification of the constituent monosaccharides by high-performance liquid chromatography (HPLC), using a CarboPac PA 10 column (DIONEX) equipped with an amperometric detector (Figure 2-4). The analyzes were performed at 30ºC with the sodium hydroxide (NaOH 4mM) as eluent and a flow rate of 0.9 mL/min. Galactose, Glucose, Fucose, Rhamnose, Mannose and glucuronic acid were used as standards (50 – 1 ppm) (calibration curves in Appendix 5) .

Figure 2-4 - Ion Chromatography - DIONEX, model ICS-3000

The group substituents (acyl groups) were also identified and quantified using the acid hydrolysate. The analyzes were also performed in HPLC, with an IonPac ICE_AS1 9x250mm column (Dionex), coupled to a Photodiode Array PDA ICS series (Dionex), using sulphuric acid (H_2SO_4 0.01N) as eluent, at 30ºC, with a flow rate of 0.6 ml/min. The detection was performed at 210 nm. Standards of acetate, succinate and pyruvate solutions, with a concentration ranging from 1 to 100 ppm, were used (calibration curves in Appendix 6).

2.1.4.2 Water Content

FucoPol water content was evaluated by subjecting it to 100ºC for 24h. The biopolymer was weighted before and after 100ºC.

2.1.4.3 Ash Content

The total ash content was determined by subjecting the biopolymer to pyrolysis at a temperature of 550ºC, for 12h.

2.1.4.4 Differential Scanning Calorimetry (DSC)

To determine the thermal properties and stability of FucoPol at high temperatures, differential scanning calorimetry (DSC) analysis was performed. This is a thermal technique that records the heat energy flow that is associated with transitions in materials as a function of temperature. For this purpose, a Setaram calorimeter (France, Model DSC 131) was used (Figure 2-5), using a scan rate of 10ºC/min in the temperature range of -130ºC to 100ºC and 30ºC to 300ºC. The heating was done under a stream of nitrogen and the sample (10 mg) was placed in a hermetically insulated aluminum crucible while the reference was an empty crucible.

Figure 2-5 - Differential Scanning Calorimeter, France, model DSC 131

2.1.4.5 Fourier Transform Infrared Spectroscopy (FT-IR)

The infrared spectra of the FucoPol was acquired using a Nicolet Nexus spectrophotometer interfaced with a Continumm microscope, using a MCT-A detector cooled by the liquid nitrogen.

All the spectra were obtained in the transmission mode, using a Thermo diamond anvil compression cell. The spectra were obtained in a 100 µm x 100 µm area, with a resolution of 4 cm^{-1} and 128 scans. They are shown here as acquired for the removal or the CO_2 absorption at approximately 2300-2400 cm^{-1}.

2.2 Characterization of the ionic liquid

Choline acetate ionic liquid was characterized with respect to its glass transition temperature (by DSC), chemical bonds (by FT-IR), viscosity at different temperatures (by rheology test) and biocompatibility evaluation.

2.2.1 Differential Scanning Calorimetry (DSC)

The ionic liquid was first characterized in terms of its glass transition temperature using the method previously described in sub section 2.1.4.4.

2.2.2 Fourier Transform Infrared Spectroscopy (FT-IR)

The chemical bonds of ionic liquid were determined by FT-IR. The method was the same as described in sub section 2.1.4.5.

2.2.3 Rheological properties

Rheological studies were performed in a controlled stress rheometer (Haake RS-75, Thermo Scientific, Germany) (Figure 2-6). This equipment has a Peltier liquid temperature control unit and does not work without the use of a cone and a plate, in this case having a diameter of 3.5 cm and 2º angle. Each sample was placed in the plate and the shearing geometry embedded in paraffin oil to prevent water loss.

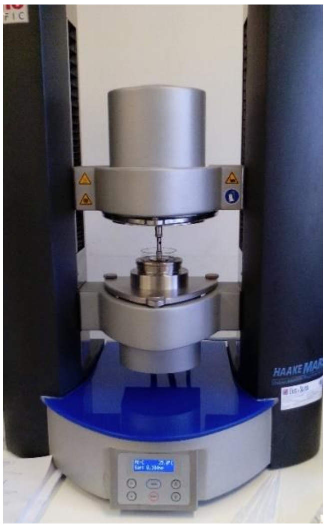


Figure 2-6 – Rheometer Haake RS-75, Thermo Scientific, Germany

The steady-state flow of choline acetate was studied at different temperatures (30ºC, 50ºC, 60ºC, 80ºC and 100ºC). The samples were equilibrated at the respective temperature (30ºC, 50ºC, 60ºC, 80ºC and 100ºC), for 10 minutes. Flow curves were determined using a steady-state flow ramp in the range of shear rate from 0.01 to 1000 s^{-1} for all the temperatures tested.

2.2.4 Biocompatibility evaluation

The measurement of cytotoxic effects of the ionic liquid was performed using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. This method is based on the conversion of MTT into formazan crystals as result of mitochondrial activity. Knowing that this is associated with the number of viable cells it is then possible to determine the desired results by measuring the optical density (OD).

For this assay, transformed human liver cells (Huh7 – JCRB 0403) were used. These were cultivated in fetal bovine serum (FBS) supplemented with Dulbecco's Modified Eagle Medium (DMEM) and placed in an incubator at 37ºC under controlled atmosphere (95% air and 5% carbon dioxide).

Initially, the cells were harvested, and trypsin was used to remove adherent cells. Then a new cell resuspension was made to $1x10^6$ cell/mL. After 24 hours, the samples were added in a 96-well microplate (Greiner, Bio-one, Alphen a/d Rijin, Netherlands) with a culture medium and were performed on serial dilutions from $1x10^6$ to $1x10^3$ cell/mL. The previously cultivated cells were placed in a new microplate and 50 µL of dilutions made previously were placed into each well. In this step it was necessary to account duplicate and control wells, making it necessary to use two microplates.

The cells were then put to incubate for 72 hours at 37ºC. After such time, 20 µL of MTT reagent (5 mg/mL) were added in each well including the controls ones, and the cells return to incubation for 4 hours.

When the purple precipitate was visible, the medium was removed and added to 100 µL of dimethylsulfoxide (DMSO) in order to dissolve the formazan crystals. After gentle agitation, the optical density was quantified at 540 nm and the background signal at 720 nm, which was measured in Spectra Max 340, Molecular Devices, Sunnyvale, CA, USA.

2.3 FucoPol Dissolution in Ionic Liquid

FucoPol was dissolved in choline acetate ionic liquid (2.5 wt%, 5 wt% and 10 wt%), at different temperatures. The sample was agitated continuously (200 rpm) with the aid of a magnetic stirred, for 24 hours. The dissolution temperatures studied were 50ºC, 60ºC, 80ºC and 100ºC. For this, the beaker glass was placed in an oil bath and the temperature was controlled by a digital thermo-regulator connected to the heating magnetic stirrer from Velp Scientific, Italy (Figure 2-7).

Figure 2-7 – Dissolution of FucoPol polymer

The mixtures prepared were characterized with respect to its composition, glass transition temperature (by DSC), chemical bonds (by FT-IR) and viscosity at different temperatures (by rheology test).

2.3.1 Sugar and Acyl Groups Composition

The composition of sugars and acyl groups of mixtures FucoPol – IL was determined by the method previously described in sub section 2.1.4.1.

2.3.2 Differential Scanning Calorimetry (DSC)

The mixtures were characterized in terms of its glass transition temperature using the method previously described in sub section 2.1.4.4.

2.3.3 Fourier Transform Infrared Spectrocopy (FT-IR)

For determining the chemical bonds existing in mixtures, FT-IR was conducted by the method described in sub section 2.1.4.5.

2.3.4 Rheological studies

The steady state of the FucoPol - IL mixtures were studied at 25ºC. The method used was the same as described in the section 2.2.3. Stress sweeps were performed at a constant frequency (*f*=1Hz) in order to ensure that the frequency sweeps were performed within the linear viscoelastic region. A tension of 1 Pa was used in all frequency sweeps performed, determining the dependence of the storage (G') and loss (G'') modules in relation to the frequency.

2.4 Phase Inversion Method

The mixture FucoPol - Ionic liquid was cast in a teflon plate and submerged in deionized water for 24 hours (Figurre 2-8). This procedure is commonly referred as the phase inversion method, were the objective of this step is to remove the maximum quantity of ionic liquid while maintaining the composition of FucoPol. Water was selected due to the high solubility of choline acetate in water. After this time, the water was discarded and the remaining content was placed in as oven at 70ºC for 24 hours. It should be noted that drying tests were also made at oven temperatures of 80 ° C to 90 ° C, as well as tests with longer periods of time (Up to 4 days).

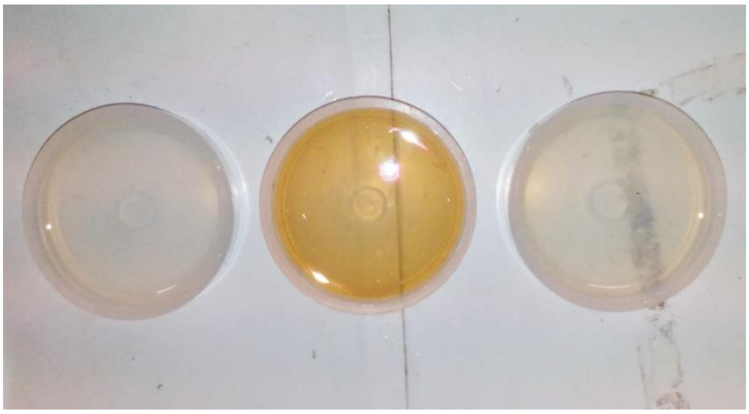


Figure 2-8 - Inversion Phase Method

After the drying procedure, the final structure obtained was characterized in terms of its sugar and acyl groups composition, glass transition temperature, chemical bonds, viscosity and biocompatibility. The objective of the determination of these properties after the phase inversion method is to analyze the influence of ionic liquid removal on the polymeric structures here developed, and try to infer if the ionic liquid was completely removed.

2.4.1 Sugar and Acyl Groups Composition

The determination of sugars and acyl groups present in the mixture after phase inversion method were determined by the method described in sub section 2.1.4.1.

2.4.2 Differential Scanning Calorimetry (DSC)

The glass transition temperature was determined by DSC by the method described in sub section 2.1.4.4.

2.4.3 Fourier Transform Infrared Spectrocopy (FT-IR)

The chemical bonds were evaluated by FT-IR by the procedure described in sub section 2.1.4.5.

2.4.4 Rheological studies

The solution properties of the mixtures after phase inversion were studied as described in sub section 2.3.4.

2.4.5 Biocompatibility evaluation

To the mixture after the phase inversion method was carried out a study of biocompatibility, similar to the process performed for the ionic liquids (sub section 2.2.4).

2.4.6 Biological Glue – Compression Test

In order to test an application against the viscous characteristics of the mixture, it was preceded to gluing balsa wood.

To this, 0.3 g of each sample after the phase inversion method was placed on the balsa wood and pressed by springs placed at the ends. Half of them were left at room temperature (which had been drying at 70ºC) and the other half dried at 100ºC for 24 hours.

To evaluate the bond strength a compression test was performed using a texturometer (TA.xt.Plus) and a probe (Craft Knife Blade). The process occurs at a rate of 0.5 mm/sec and in a safety distance of 4.5 mm (distance traveled by the probe where the blade acts only).

3 Results and Discussion

3.1 Fucose-containing EPS production

EPS production was carried out in a 10 L bioreactor fed with Medium E* and glycerol as the sole carbon source. After an adaptation period (lag phase) of about 4 hours, *Enterobacter* A47 entered an exponential growth phase that lasted around 12 hours, after which the concentration of ammonium has become too low (under 0.19 g $NH4^+$/L). Then the fed-batch phase was initiated with the addition of mineral medium solution with a concentration of 400 g/ L glycerol, while the dissolved oxygen concentration was controlled at 10% by the automatic variation of the stirring speed between 300 and 800 rpm. Data for the cultivation run of *Enterobacter* A47 is shown in the Figure 3-1, in which cell growth on glycerol and EPS productions are shown over time.

The maximum cell dry weight (CDW) attained by the culture was 6.76 g/L with a specific growth rate of 0.11 h^{-1} and a net yield of biomass in glycerol ($Y_{x/s}$) of 0.13 g/g. Concomitant with the cell growth, the glycerol concentration in the culture broth decreased from the initial 48 g/L to 23 g/L by the time that the fed-batch phase was initiated. Considering the glycerol consumption (25 g/L), the result is analogous to the study performed by Alves et al in similar conditions, wherein the glycerol concentration dropped from 25.74 g/L to 4.67 g/L, achieving a biomass concentration of 6.00 g/L (Figure 3-1).

EPS synthesis was initiated during cell growth, but increased production was observed during the stationary growth phase (fed-batch phase), promoted by the high glycerol concentration (400 g/L) fed to the reactor and low ammonium concentration (0.9 g NH_4^+/L). Thus the maximum EPS production attained was 7 g/L. Considering the EPS production between 45.57 and 69.83 hours, during which there was a significant production of polymer, the yield of EPS on glycerol $Y_{P/s}$ was 0.22 g/g and the maximum volumetric productivity of 2.37 g/L.d. In terms of yield of EPS on glycerol the amount is substantially lower than the previous study ($Y_{P/s}$ 0,47 g/g). Since the substrate consumption is equivalent, the productivity is also slightly lower compared to 3.64 g/L.day obtained by Alves et al.. This difference in results is due to the use of other purification method, since the work of Alves et al. the polymer was obtained by precipitation with acetone. This has as a consequence a less pure polymer, thus the obtained amount of EPS was not real, seen that contains a larger number of proteins and inorganic residues as compared to the thesis process.

However, productivity is in the range of xanthan gum (1.46-2.4 g/L.d)[41] production and above the values linked to bacterial alginate (0.43-1.53 g/L.d) [42] production and the results for $Y_{P/s}$ (0.24 g/g)[8] are close to those obtained for the production of the same polymer with a similar process.

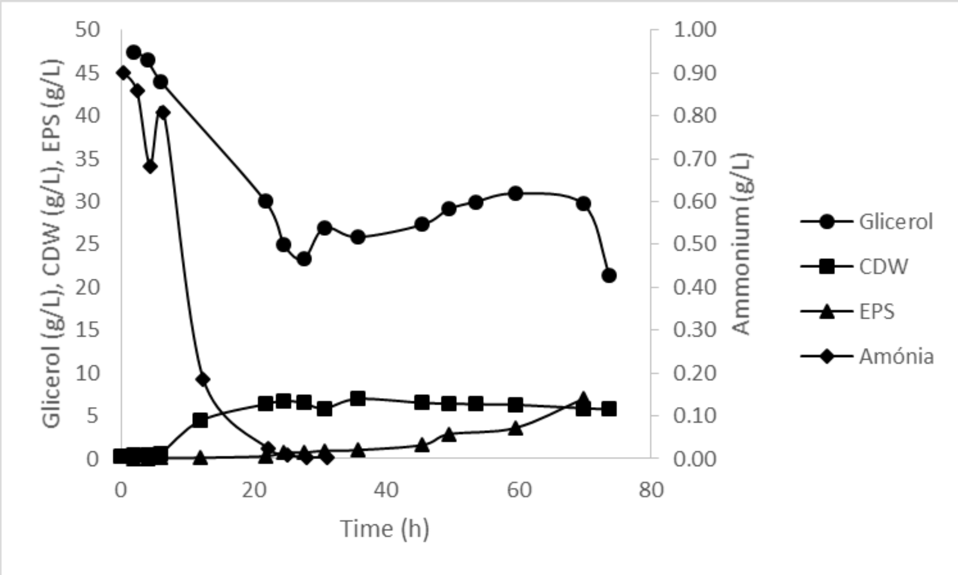


Figure 3-1 - Cultivation run of *Enterobacter* A47

3.1.1 Culture Broth Viscosity

Broth viscosity increased during the cultivation of *Enterobacter* A47, as shown in previous work[13].

In the first two days of the process, there is no large increase in viscosity, however, on the following days there was a gradual increase. This behavior may be related to the increased concentration of EPS in the broth, as well as changes in the composition and molecular weight of the polymer. The most significant increase of broth's viscosity was observed in the transition from the 2nd to the last day of the cultivation run (Figure 3-2). In addition to the aspects mentioned above, this sharp increase may be related to the strong interactions that occur among EPS molecules or between the polymer chains and the other cultivation broth components [8,13,14].

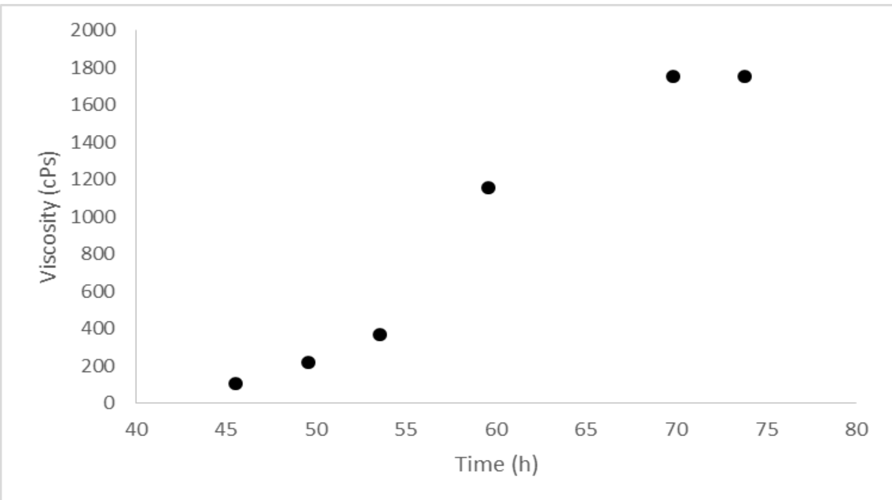


Figure 3-2 - Culture Broth Viscosity

This increase of viscosity is observed in various microbial cultures (e.g. *Pseudomonas oleovorans* [43] *and Rhizobium hedysari* [44]) for the production of extracellular polysaccharides and it determines the end of the cultivation run due to a loss of bulk homogeneity of the culture broth in terms of mixing, mass and heat transfer [13,43,45].

3.2 FucoPol Physicochemical Characterization

The purified FucoPol has a water and ash content of 15 wt% and 2 wt%, respectively.

It is also considered a heteropolysaccharide which is composed of fucose, galactose, rhamnose, glucose and glucuronic acid (Figure 3-3).

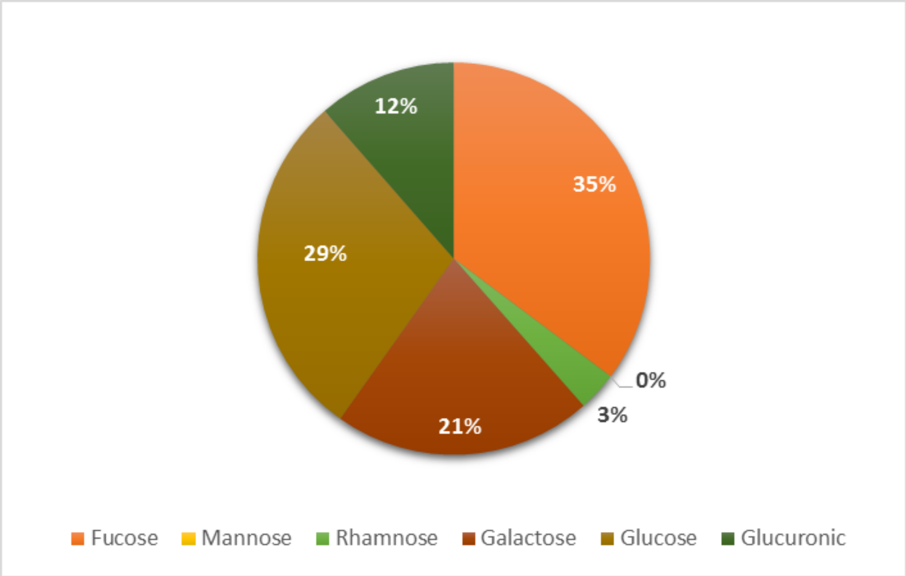


Figure 3-3 - FucoPol sugar composition (% mol)

The EPS produced in this work is slightly different to the one presented in Freitas et al [14] and Torres et al [8], since it has rhamnose (3% mol) in it composition[14]. Compared to earlier work done, this monomer had a null value. These results can be explained by the altered bacterial metabolism during the process which consequently modifies the composition of sugars over time[8]. Thus since the process of this work has a shorter duration than those in the literature (3 days compared to 4 and 7 days)[8,14] , the rhamnose may not have been processed in order to obtain the residual percentages referred to in other studies [8,13,14].

The presence of these sugars, particularly fucose, galactose and glucose are common in exopolysaccharides produced by *Enterobacteriaceae* family [46].

FucoPol is also composed of acyl groups, namely acetate (14.28% mol), pyruvate (2.15% mol) and succinate (1.80% mol).

3.3 Ionic Liquid Characterization

3.3.1 Rheological Study

In figure 3-4 it is represented the flow curves for the ionic liquid at 30ºC, 50ºC, 60ºC, 80ºC and 100ºC.

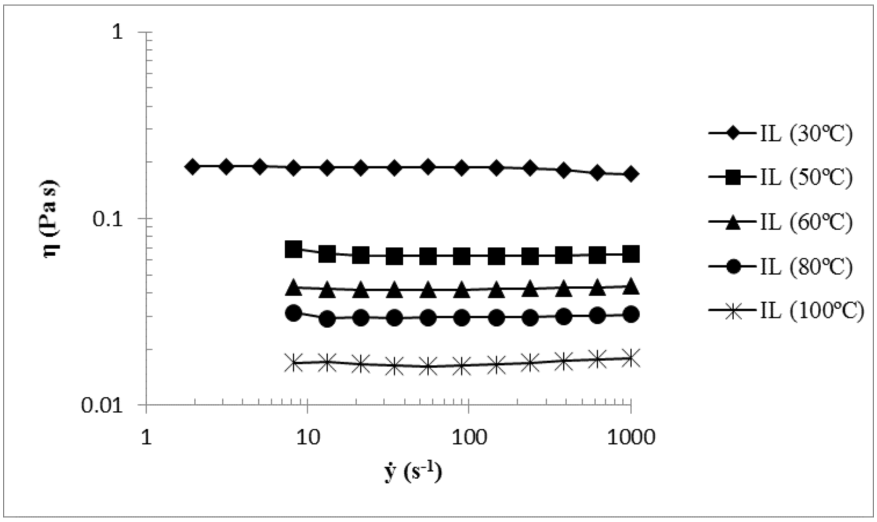


Figure 3-4 - Flow curve of the choline acetate ionic liquid for different temperatures

Note that the viscosity is directly related to the temperature. With an increase in temperature it is observed a decrease in ionic liquid viscosity. On the other hand, the ionic liquid viscosity is constant with the variation of shear rate (ẏ), acting as a Newtonian fluid, which is a common behavior of other ionic liquids (e.g. $[C_2mim][NTf_2]$) [47].

The viscosity value obtained in this work at 30ºC (0.186 Pa.s) is somewhat higher than the one obtained in other work [40], using the same ionic liquid at 25ºC (0.130 Pa.s). This value is beyond the expected results given the observed relationship between the behavior of viscosity and the temperature change. However, this variation may occur since despite being the same ionic liquid the batches used were different and, for example a slight change in the water content will be enough to change the viscosity behavior, causing its decrease when the amount of water increases.

The experimental viscosity values were fitted as a function of temperature, using Arrhenius Law described in Equation 3-1 [48].

$$\eta_{(T)} = \eta_0 . e^{-\frac{E_a}{RT}}$$ *Equation 3-1*

Where η is the apparent viscosity at a specific shear rate (mPa.s), η_0 is the frequency factor (mPa.s), E_a is the activation energy (kJ/mol^{-1}), R is the gas constant (kJ/mol.K) and T is temperature (K).

To determine the activation energy it was used a logarithmic equation based on Arrhenius model, described by the Equation 3-2:

$$ln(\eta) = ln\left(\eta_0\right) - \frac{E_a}{RT} \qquad \textit{Equation 3-2}$$

The graphical representation of the equation 3-2 is shown in Figure 3-5.

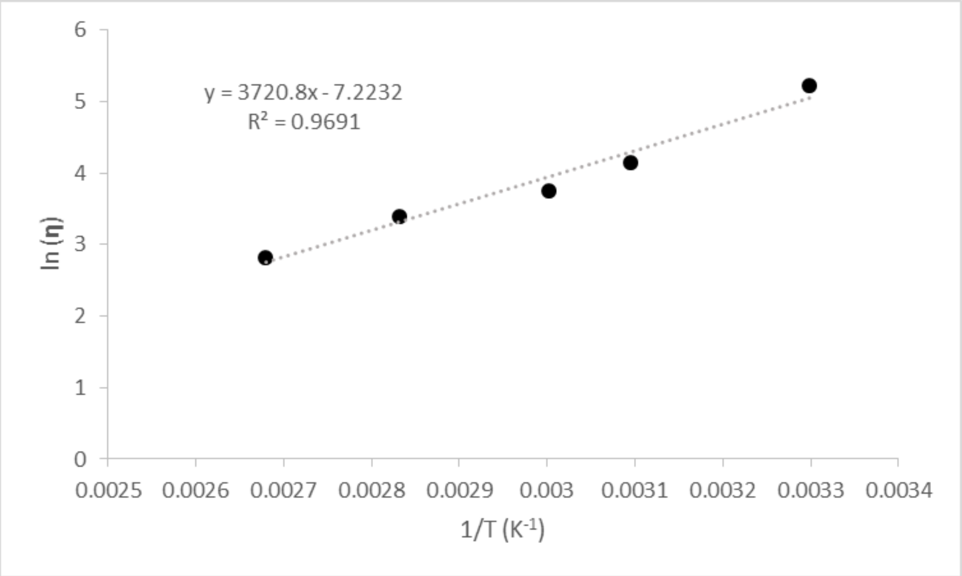


Figure 3-5 – Representation of ionic liquid viscosity as a function of temperature.

The obtained value of E_a was 30.94 kJ/mol. Comparing the obtained value with the one of another ionic liquid with de same anion, namely 1-ethyl-3-methylimidazolium acetate [C_2*MIM*][*Acetate*], (37 kJ/mol [49]), it appears that the choline acetate has a lower activation energy. Although interaction of the molecules of said ionic liquids are not measurable, this result indicates that the choline acetate has an increased mobility of ions due to the weaker molecular interactions, as compared to the other ionic liquid [49].

3.4 FucoPol and Choline Acetate Mixtures

The mixtures were prepared following the procedure described in section 2.3 of this thesis. However regardless of the applied dissolution temperature it was found that for concentrations of 5

%wt and 10 %wt the polymer did not dissolve completely. Since there was no dissolution, the process time was increased, but the measure has proved to be inefficient.

Thus, for all subsequent studies mixtures with improved results were considered, ie those with a concentration of 2.5 %wt and dissolution temperature of 50, 60, 80 and 100ºC.

Also the temperature and time drying in an oven was changed, as stated in section 2.4. In this case, the increase of each parameter caused no changes in the end aspect of the mixtures, ie, in the end, these had not dried. Thus, it was considered drying temperature at 70ºC for the following items analysis.

In this work is referred as S1, S2, S3 and S4 the samples that correspond to the dissolution temperatures of 50, 60, 80 and 100 ° C, respectively (Figure 3-6). In turn the samples after the phase inversion method are referred to as SI1, SI2, SI3 and SI4 (Figure 3-7), where the number refers to the original sample (before the phase inversion method).

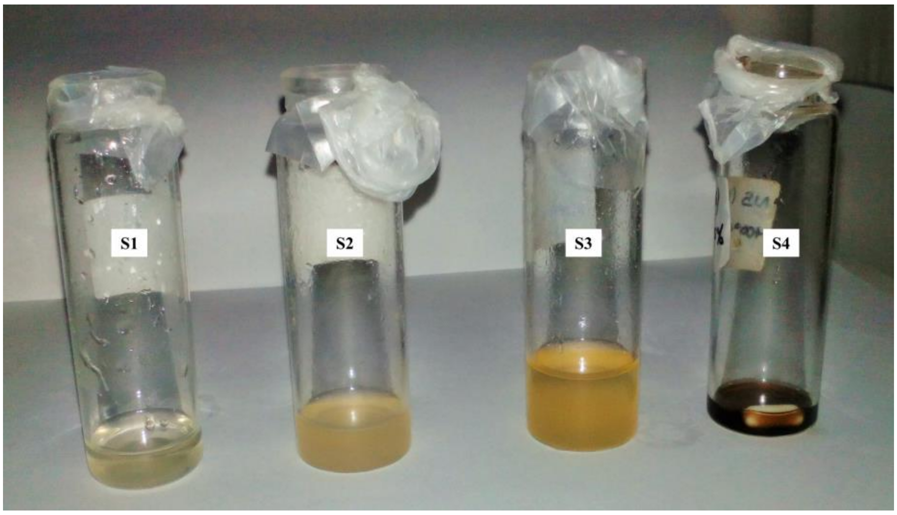


Figure 3-6 – Mixtures: Before the Phase Inversion Method

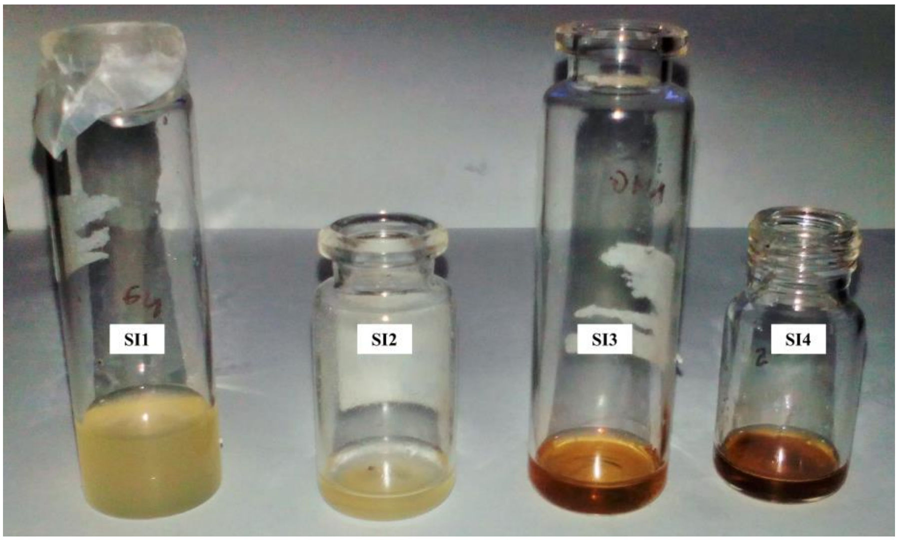


Figure 3-7 – Mixtures: After the Phase Inversion Method

3.4.1 Sugar and Acyl Groups Composition

The study of composition of sugars was also performed for each FucoPol and choline acetate mixture before and after the phase inversion method. The results after the phase inversion method are shown in Table 3-1.

Table 3-1 – FucoPol and choline acetate mixtures composition

	ppm					
	Fucose	**Rhamnose**	**Galactose**	**Glucose**	**Glucuronic Acid**	**Total**
FucoPol	11.76	1.08	7.81	10.53	4.51	35.69
SI1	1.29	0.21	0.30	1.06	1.52	4.37
SI2	2.40	0.33	1.13	2.24	1.73	7.84
SI3	2.66	0.34	1.41	2.60	1.99	8.99
SI4	1.69	0.24	0.65	1.61	1.74	5.92

Mixtures before phase inversion method have a similar sugar amount (data not shown) to the FucoPol sample (Table 3-1). However, after the phase inversion method, the sugar amount has a great decrease, which may be related to the hydrolysis that happens during the dissolution at high temperatures. Furthermore, the phase inversion method was performed with water, which may also contributed to the loss of some sugars, since FucoPol is a hydrophilic molecule.

Regarding the acyl groups (data not shown), after phase inversion method the pyruvate and succinate disappear, which is also related to the hydrolysis. However, the result for the acetate is quite different, with the appearance of a quantity 10 times greater than in the EPS sample, being influenced by the presence of choline acetate in the mixture.

3.4.2 Thermal Properties

Differential scanning calorimetry (DSC) was used in this work to determine the glass transition temperature (T_g) of FucoPol polymer, choline acetate ionic liquid as well as the mixtures FucoPol-IL before and after the phase inversion method. Glass transition temperature (T_g) was taken at half-height of heat capacity increment. However, during the DSC measurement, the T_g of FucoPol could not be observed due to the limitation of the instrument, however through the analysis of this parameter for other biopolymers such as chitosan (T_g=140ºC-150ºC), dextran (T_g=223ºC) and alginate (T_g=113.56ºC), it is expected that the Tg of the polymer used in this work lies in a range between 100 and 300ºC.

The thermograms obtained are presented in Appendix 7, while the average glass transition temperature of ionic liquid and mixtures are shown in Table 3-2.

Table 3-2 – Average Glass transition temperature (T_g) of FucoPol, ionic liquid and mixtures

Samples	Dissolution Temperature (ºC)	Average Glass Transition Temperature (T_g)
Fucopol	-	ND [c]
Choline Acetate	-	-98.03
S1	50	-88.30
S2	60	-85.22
S3	80	-85.30
S4	100	-84.21
SI1	-	-91.27
SI2	-	-95.93
SI3	-	-83.18
SI4	-	-88.93

The T_g determined in this work for the ionic liquid is slightly lower when compared with those obtained in previous studies (-88.34ºC [40] and -96.64ºC [50]). As mentioned previously in rheology studies (section 3.3.1), this difference in behavior can be explained by slight variations in the characteristics of the ionic liquid, such as water content, since it is know that the higher is its content the lower is the T_g.

From these results it can be seen that the glass transition temperature of the FucoPol-IL mixtures before the phase inversion method, although higher, is close to the value determined for the ionic liquid. These results indicate that the T_g value obtained for the mixtures is masked by the presence of the ionic liquid. For these samples (S1, S2, S3 and S4) it is found that with an increase in the dissolution temperature, the higher is the value of T_g. Thus, one can conclude that the heat that the mixture is subjected promotes the arrangement of the polymer chains, increasing the rigidity of the samples, as well as their crystallinity. [51].

Regarding the T_g for FucoPol-IL mixtures after phase inversion method, the same behavior is observed, which is an indication that the phase inversion method was not efficient for the full removal of the ionic liquid, however, there were still sugars present, since the values are not exactly equal to the choline acetate. This situation was also observed during the formation of membranes from chitin-glucan complex and with the same ionic liquid [50].

As mentioned, higher T_g values are associated with greater crystallinity of the samples, thus the SI3 sample is presented as the most crystalline, while the ionic liquid is amorphous [48] [52]. However, since all values are negative it can be stated that both the ionic liquid and EPS – IL mixtures have an amorphous behavior (at room temperature).

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[c] Meaning of ND: not determined

3.4.3 Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectrum of FucoPol, choline acetate and the FucoPol-IL mixture (dissolved at 50ºC) before and after the phase inversion method is shown in 3-8.

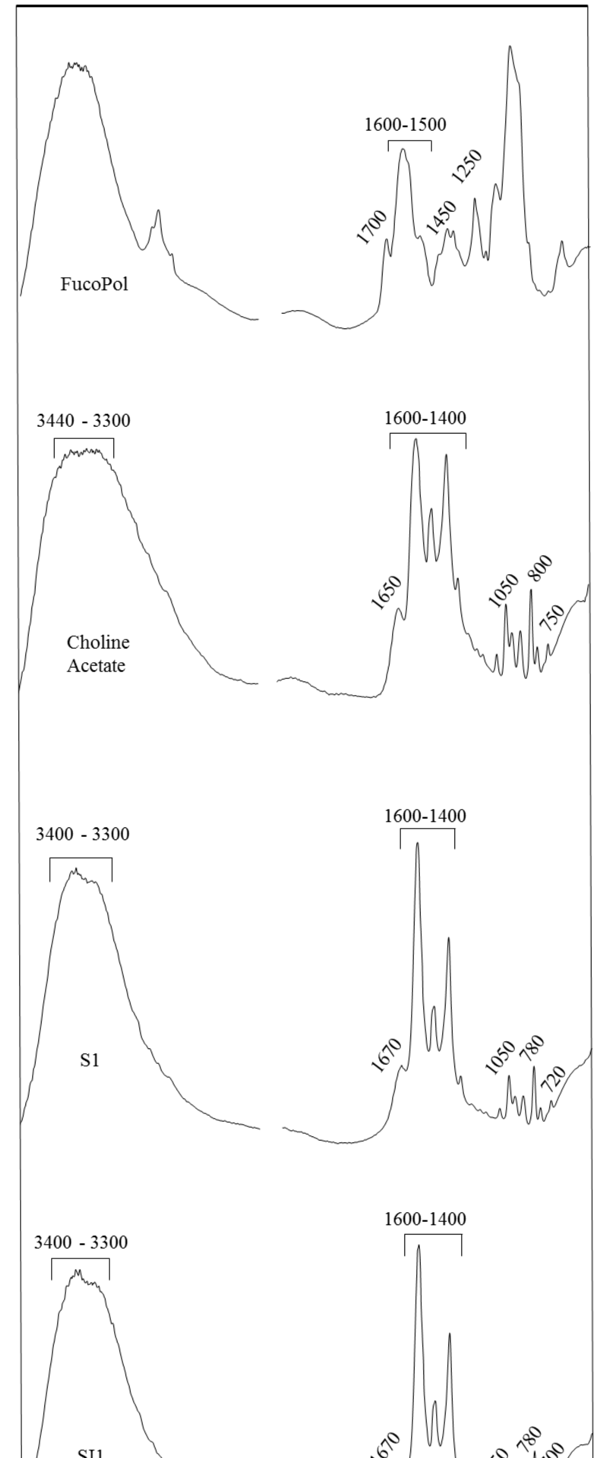

Figure 3-8 - Comparative FT-IR spectrum for Fucopol, choline acetate and mixtures

In FT-IR spectrum of FucoPol, a peak for values around 3500 cm^{-1} is observed. This is common to all polysaccharides and represents O-H stretching of hydroxyls and bound water. This is followed by another band, which occurs at around 2900 cm^{-1}, which is associated with C-H stretching peak of CH_2 groups [53].

The band detected at 1700 cm^{-1}, is associated to the C=O stretching of carbonyls in acyl groups [54]. There are also two bands in the range between 1600 and 1500 cm^{-1} [53]. These are ascribed to the asymmetric and symmetric stretching of carboxylates, respectively. This range is also to highlight the detected peak around 1450 cm^{-1}, which are associated to the C=O anti-symmetric stretching vibrations of succinate [55].

The band detected at 1250 cm^{-1}, may be related to the C-O-C of acyls. Finally, between 1200 and 900 cm^{-1}, there is the existence of a band in the form of representing C-O and C-C vibration bands of glycosidic bonds pyranoid ring [53].

All submitted peaks are similar in a previous study by Freitas et. al [14]. However in some cases it is noted a slight variation in the intensity of the bands. This may be due to small variations in the level of the polymer production process.

For the spectrum of choline acetate and taking into account its chemical structure present in Table 2.1, some of the peaks are the same as those observed in FucoPol, namely the band between 3440 and 3300 cm^{-1} which can be associated with the O-H bond of water [53]. Also in this case there was a peak in the region of 1650 cm^{-1}, which as in the previous case corresponds to the C=O bond [54] and another peak at 1540 cm^{-1} related to the N-O bond. With a wavenumber of 1050 cm^{-1} it can be related to the existence of another peak in the C-O band. The peak at 800 cm^{-1}, corresponding to the C-C bond [53]. However, it would be expect to find a peak at around 2940 cm^{-1}, corresponding to the CH_2 bond, but such is not the case, as well as the band corresponding to the CH_3 group [56]. These may be masked by the presence of a high amount of water.

In addition to these links is also detected a peak at 750 cm^{-1} which corresponds to the C-N connection [56].

The spectrum obtained for the Fucopol-IL mixtures, before and after the phase inversion method is similar to the ionic liquid. After the study of the glass transition temperature (section 3.4.2) this result was expected, since the mixtures contain ionic liquid in a higher quantity when compared with EPS concentration.

3.4.4 Rheological Studies

The figures 3-9 and 3-10 represent the flow curves for the studied temperatures (before and after the phase inversion method). Sample S1 corresponds to a dissolution at 50ºC, S2 corresponds to 60ºC, S3 belongs to 80ºC and finally S4 is associated with a dissolution temperature of 100ºC. SI1, SI2, SI3, SI4 are the respective samples after the phase inversion method.

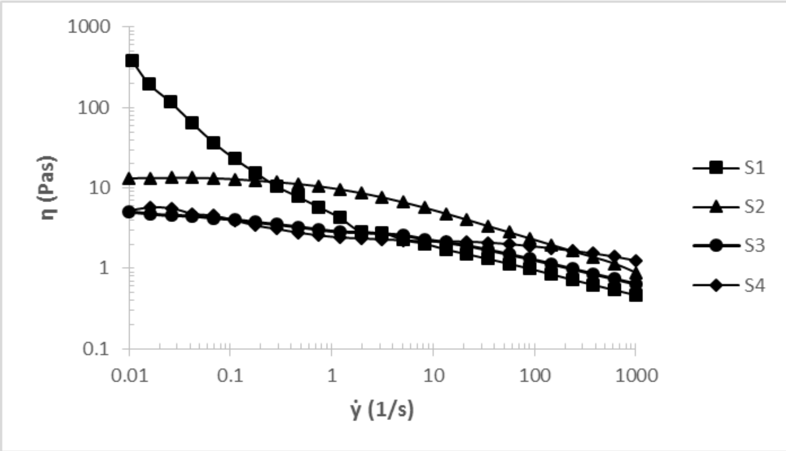

Figure 3-9 - Flow Curve for FucoPol-IL mixtures (before phase inversion method)

Figure 3-9 shows that the higher the dissolution temperature the lower is the viscosity. This situation is similar to the one observed for the ionic liquid, however this is noteworthy because the viscosity decreases with increasing shear rate, in the case of a non-Newtonian shear-thinning behavior. Nevertheless, the result obtained for the sample S1 is not the expected. This may be due to placing an excessive amount of sample at the time the test was performed. It would be expected that the flow curve submit a viscosity slightly higher than for the other temperatures tested, but with a similar progress. The flow curves behavior are probably related with the degree of hydrolysis (increased with increasing temperature) and with possible protein degradation (FucoPol protein content is less than 5% [14]).

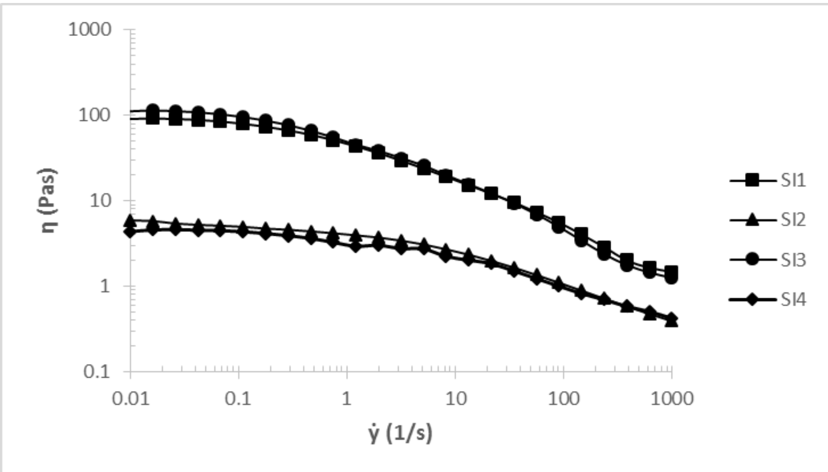

Figure 3-10 - Flow Curve for FucoPol-IL mixtures (after phase inversion method)

After the phase inversion method, the behavior of samples changes, despite the maintenance of the non-Newtonian behavior. To SI1 samples is assumed that probably the viscosity are no largely changed, reasoning considering the previously predicted. In the SI3 sample there is also an increase of viscosity, while for the sample SI2 the viscosity decreases and SI4 maintain similar values to the previously observed. These results may be due to the change in the sugar concentration.

In order to confirm the behavior described above, it was applied to the Power Law (equation 3-3) for all mixtures, relating shear stress (τ, Pa) with shear rate $(\dot{y}, s^{-1})$.

$$\tau = k\, \dot{y}^{n} \qquad \textit{Equation 3-3}$$

Where k is the consistency index (Pa s^n) and n is the power law index. This indicates the degree of non-Newtonian behavior. If $n = 1$, the fluid is Newtonian, while if $n < 1$ it is considered shear-thinning. The Power Law parameters are presented in Table 3-4.

Table 3-3 - Power Law parameters for mixtures

Sample	Dissolution Temperature (ºC)	η (Pa.s)[d]	k (Pa s^n)	n
Before the Phase Inversion Method				
S1	50	377.9	3.91	0.69
S2	60	13.19	10.45	0.61
S3	80	5.07	2.81	0.82
S4	100	5.24	2.97	0.88
After the Phase Inversion Method				
SI1	50	90.77	61.82	0.45
SI2	60	5.88	3.47	0.85
SI3	80	111.8	51.39	0.52
SI4	100	4.36	3.19	0.34

[d)] Viscosity values for a shear rate of 0.1 s^{-1}

In Table 3-3 it can be seen that the viscosity of the mixtures decreases after the phase inversion method. The exception to this behavior is SI3 sample which has a much higher viscosity than before phase inversion method. This result can be associated with a non-homogeneous solution, where it was observed a liquid phase separated from the viscous solution.

For most cases (exception to sample S2) the value of *k* decreases with the decrease in viscosity (Table 3-4). Concerning the value of n, as can be seen in the flow curves (Figure 3-7 and 3-8), the blends have a Non-Newtonian shear-thinning behavior (n < 1) [57], confirming the results obtained from the flow curves. The presence of this kind of behavior in the mixtures, indicates, as assessed by studying of the T_g, that there are still sugars in the composition, otherwise the behavior of a Newtonian fluid would be observed for the ionic liquid. However, when the dissolutions temperature are higher the mixture is closer to the behavior of a Newtonian fluid, i.e. solution had a higher n value (S3 and S4) (Table 3-3). After the phase inversion method the mixtures are more distant from a Newtonian behavior.

In this study, the strain sweep measurements were carried out to estimate the linear viscosity region at a relative low frequency. Referring to this result, the dynamic frequency sweep measurements were carried out in linear response region for all samples.

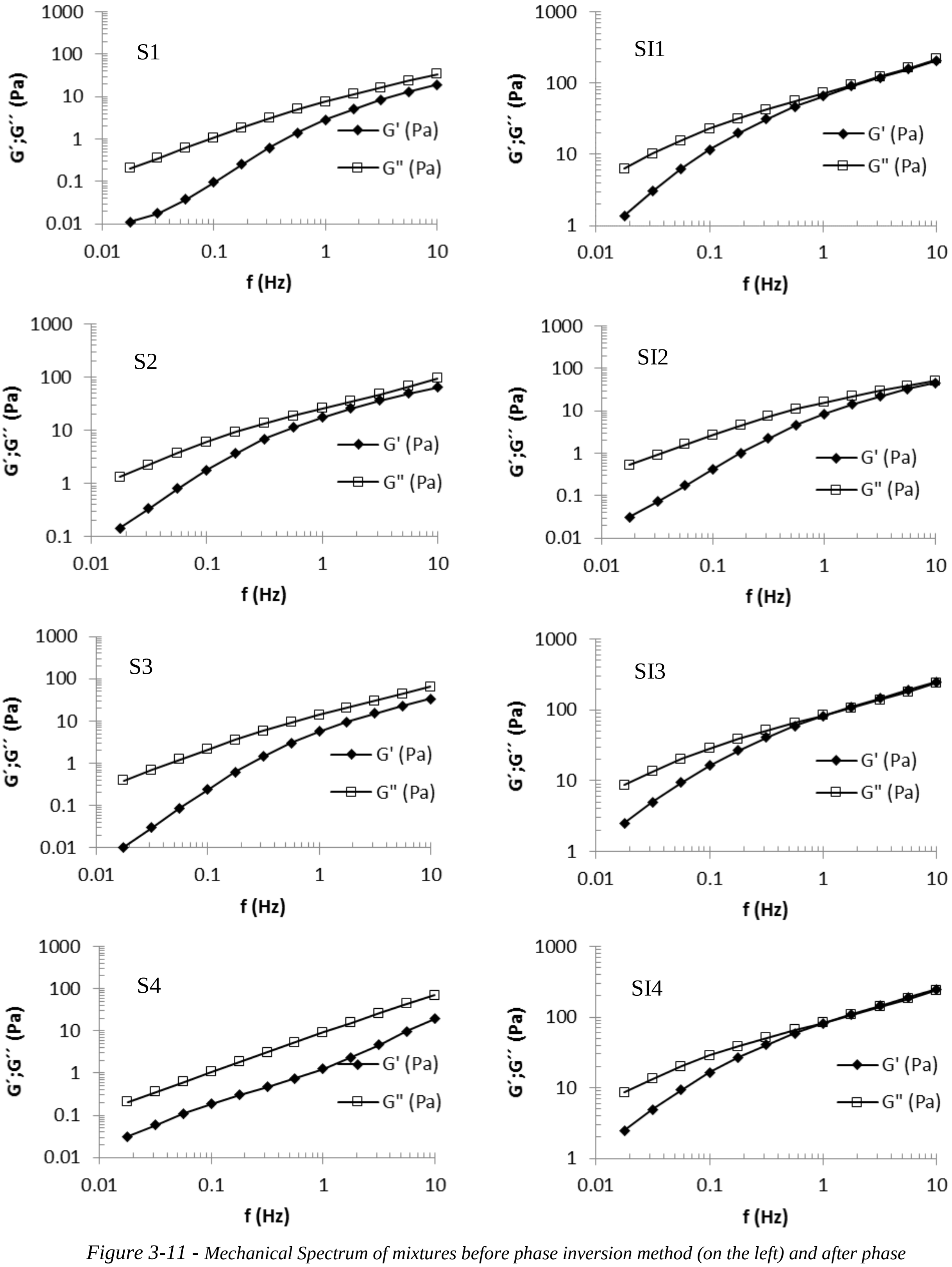

Figure 3-11 - Mechanical Spectrum of mixtures before phase inversion method (on the left) and after phase inversion method (on the right)

In Figure 3-11 it can be seen that, for all samples, viscous modulus (G'') are higher than the elastic modulus (G'). Furthermore, both G' and G'' values increase gradually. In the samples before the phase inversion method the viscous modulus (G'') and the elastic modulus (G') never crossover for the entire frequency range, while the curves of the remaining samples presented a crossover around 1Hz (SI1, SI3 and SI4) and 9 Hz (SI2). This behavior may be related to the action that the water has on the mixture, since the samples has about 50% water, it may act as a plasticizer or lubricant [52]. Thus, the frequency dependence nature of the samples is typical of a viscous solutions with entangled polymer chains.

Also comparing G' and G'' at different temperatures, it can be concluded that the change of this parameter has little influence on the internal structure of the mixture. Such behavior has been observed in previous studies on the dissolution of chitin with the same ionic liquid [50].

However, the sample S4, seems to be less structured, since despite the viscous modulus (G´´) being higher than the elastic modulus (G´), the distance between both keep the same for all the frequency range.

3.4.5 Biocompatible Evaluation

In order to evaluate the biocompatibility of FucoPol, choline acetate (ionic liquid) and FucoPol-IL mixtures after phase inversion method, the response for cell viability assessment was studied using a human cell line Huh-7 (transformed human liver cells). For this purpose, these cells were cultured in the presence of the samples.

The tested samples concentration range was determined by evaluating which concentration had no effect on cell culture medium pH and osmolarity. Regarding the pH, it was found that this does not influence the samples and only osmolarity has an impact in all cases. These results are obtained by measuring each of these parameters. The greater the deviation from the baseline value (i.e. the value of the test cells placed at the edge of the plate) the greater the impact (Appendix 7 and Appendix 8).

Huh-7 cells were cultured in the presence of different samples concentrations, and after 72 hours, cell viability was determined using MTT assay by assessing mitochondrial integrity.

In Figure 3-12 is shown a dose response curves for cell viability assessment.

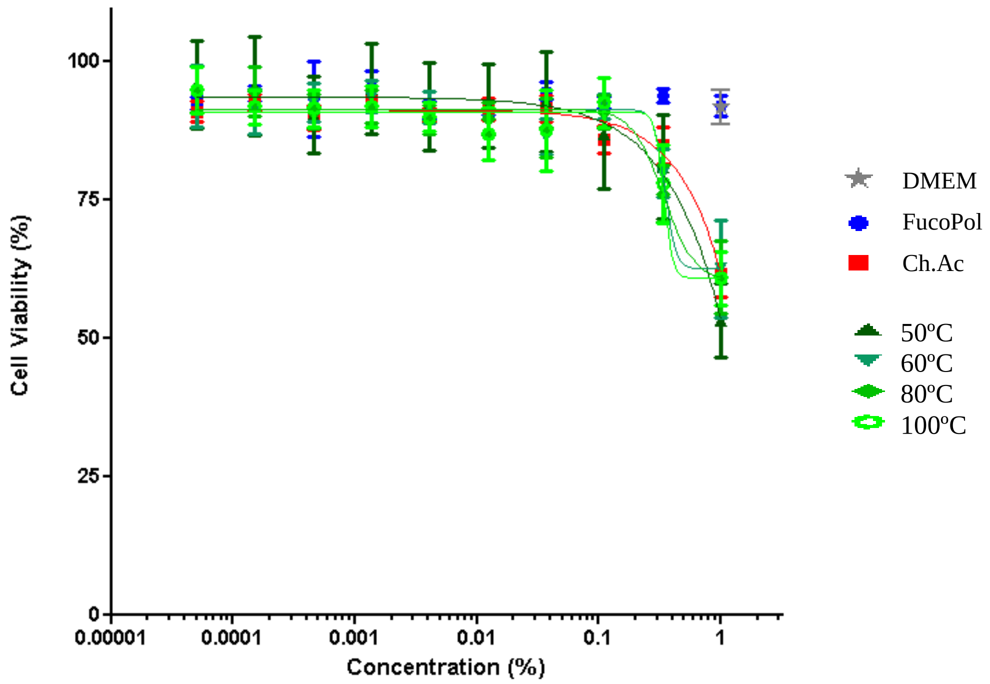

Figure 3-12 – Dose response curves for cell viability assessment in Huh-7 cells after 72 hours in culture (DMEM – Dulbecco's Modified Eagle's medium; FucoPol polymer, Ch. Ac – Choline Acetate; 50ºC, 60ºC, 80ºC and 100ºC are dissolution temperature before inversion phase method).

In all cases, the results showed that FucoPol, the ionic liquid and FucoPol-IL mixtures are biocompatible. This conclusion is drawn by observing the percentage of viable cells. The smaller this value is, the lower the resistance of cells against the action of the mixtures added, in turn, the higher the value, lower is the harmful effect on cells. For the purposes of biomedical applications a cell viability of 100% is desirable.

This biocompatibility was already expected for the ionic liquid [39], which adding the results of the remaining samples makes its application in biomedical and pharmaceutical field feasible. However, at a concentration of 1% it is found that cell viability is lower in comparison with the remaining values. As previously mentioned the osmolarity may have an influence in the samples, and these values are associated with this parameter and not by the presence of toxic characteristics. It is also observed that the behavior of each mixture after phase inversion method is similar to that obtained for the ionic liquid.

3.4.6 Biological Glue – Compression Test

All FucoPol-IL mixtures after phase inversion method were tested for balsa wood adhesive use. To that end 0.05 g/cm^2 of FucoPol-IL mixtures were applied in the wood in their original form and dried at room temperature and at 100ºC for 24h.

For the wood collage performed at room temperature it was found that sticking did not occur. The SI1 samples did not glue for any of the cases studied.

Thus, the strength tests for the remaining samples were performed (Figure 3-11).

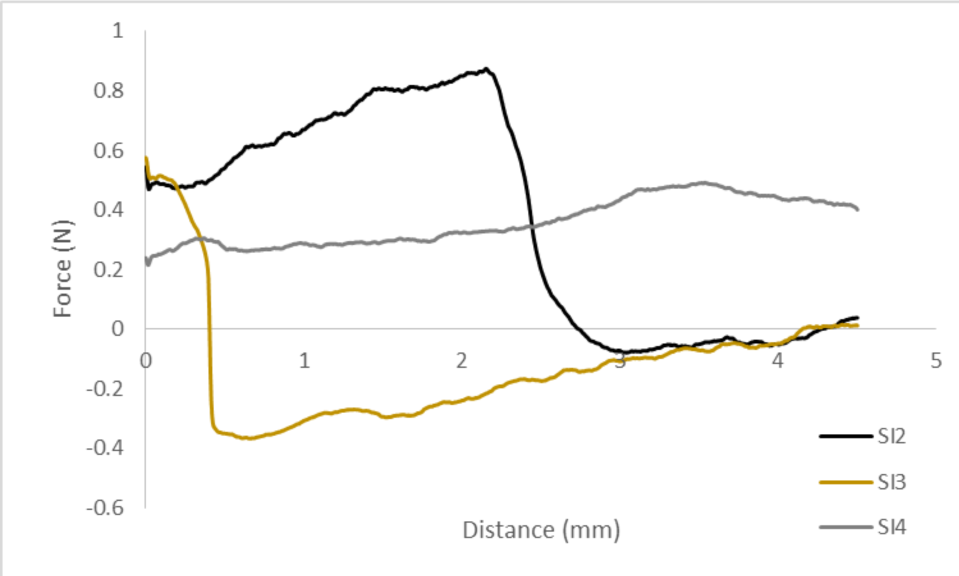

Figure 3-13 – Test of strength for bonding with balsa wood

Figure 3-13 shows that the force required to detach the timber is quite low. All samples show a similar behavior, however the sample SI2 is the one that can withstand the application of a higher strength (close to 0.8 N). The sample SI3 support a value close to 0.5 N but the probe only covered a distance of approximately 0.5 mm, this, this sample shows a lower resistance compared to others, because in addition to supporting less force also separates earlier.

Apparently the SI4 sample is never taken off, however this is not true. For on-site observation, it was found that the sample took off with the simple initial contact with the blade.

At this stage it can be said that the bonding is only made when removing water from mixtures. Moreover, the mixtures can be used as wood adhesives despite the quite low strength applied. In order to improve the mixtures adhesive properties it would be required to perform a greater number of tests, for example stress, tension, shear and cleavage tests[58]. For this purpose, it could be tested the use of crosslinking agents (e.g. polyethylene glycol, glycerol, sorbitol and other biopolymers such as alginate and carrageenan)[58] to promote the interconnection of polymer chains, as well as lower the amount of ionic liquid in the mixtures promoting the increase in viscosity or increase the amount of glue used.

4 Conclusions and Future Work

The main objective of this study was to develop and characterize biopolymeric structures by dissolving the FucoPol biopolymer in choline acetate ionic liquid.

FucoPol was produced by the bacterium *Enterobacter* A47 in batch mode during 12 hours followed by a fed-batch mode. The maximum CDW achieved was 6.75 g/L while the polymer concentration was 7 g/L, with 25 g/L of glycerol consumption. The sugar content of the polymer was 32.25% mol of fucose, 3.25% mol of rhamnose, 21.32% mol of galactose, 28.76% mol of glucose and 11.42% mol of glucuronic acid, while water and ash content were 15 wt.% and 2 wt.%, respectively.

The characterization of the choline acetate ionic liquid showed that it contains a high percentage of water (8.80% w/w) and a density of 1.1031 g/cm^3: It has also been observed that the ionic liquid behaves like a Newtonian fluid, where the viscosity decreases with increasing temperature.

The dissolution of 2.5% (w/w) of EPS with choline acetate was carried out for temperatures of 50ºC, 60ºC, 80ºC and 100ºC. After the dissolution process, the samples were subjected to the phase inversion method using water as non-solvent, in order to eliminate the amount of ionic liquid present in the resulting polymeric structure. However, this process has not proved to be effective. By the analysis of DSC and FT-IR it has been observed that the ionic liquid remains in FucoPol – IL mixture after phase inversion method.

By HPLC analysis it was found that after the phase inversion method the sugar composition decreases compared to the one that exists in the original polymer, there is also a loss of acyl groups resulting from hydrolysis. Acetate is the only exception whose value is highly affected by the ionic liquid (a value 10 times higher than that of the polymer).

The FucoPol-Il mixtures glass transition temperatures are also similar to the ionic liquid with no significant difference between the mixtures before and after the phase inversion method, and all have an amorphous behavior at room temperature.

All mixtures exhibit a non-Newtonian fluid behavior. The viscoelastic properties studied can confirm that the mixtures behave as viscous liquids, since G'' is always higher than G'. In the samples after the phase inversion method there was a crossover of both modules, and when evaluating the water content this may be due to the plasticizing effect than it acquires.

In order to increase the application range of the polymeric structures developed in this thesis, their biocompatibility was tested proving the feasibility and possibility to use such structures for biomedical applications.

It was also tested the application of mixtures for wood paste. Despite the positive results, where some mixtures glued, when dried at 100ºC, compression tests showed that the bonding strength is small, so the potential of these mixtures is only for weak glue.

For future work, there are various procedures that can be performed to improve the results obtained as well as in field applications. For example the process of water washing in the phase inversion method so as to enhance the removal of the ionic liquid mixture. To improve this, the samples may be subjected to a washing with other solvents where the FucoPol is not soluble (e.g methanol and dichloromethane).

As a biocompatibility test was accomplished it also make sense to do an antibacterial test, to assess the ability of the mixtures to withstand pathogens (e.g.: bacteria).

For the application of biological glues it should be promoted the addition of substances to increase their strength as crosslinking agents (e.g. polyethylene glycol, glycerol, sorbitol and other biopolymers such as alginate and carrageenan) and test other materials (e.g.: metal and glass).

In addition to all this suggestions, procedures performed in this study could be repeated using a biocompatible ionic liquid more hydrophobic. As the choline acetate increases its water content very easily on contact with air, the use of a liquid with the suggested characteristics could reduce the water content in the structure, promoting the formation of films that could help the adhesive potential.

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6 Appendices

Appendice 1 - Density Measurement of the ionic liquid – Protocol

The density at 30ºC of the ionic liquid used was determined using a pycnometer. This utensil made of glass, has a cap with a capillary hole and permits to obtain a precise volume (Figure 6-1). Consequently, it is possible to determine the density by using an analytical balance and compare it with a reference fluid, in this case distilled water, for which the density at different temperatures is known [43].

Figure 6-1 - Pycnometer

It is necessary to determine the weight of the pycnometer (mpic) with 5 mL capacity and its weight with distilled water (mH2Opic) and ionic liquid (mILpic). Knowing the volume (V) formula (equation 6-3), and assuming that is the same for both fluids it is possible to obtain the unknown density through:

$$V = \frac{m_{fluid}}{\rho_{fluid}} \qquad \text{Equation 6-1}$$

$$V_{H2O} = V_{IL} \leftrightarrow \qquad \text{Equation 6-2}$$

$$\leftrightarrow \frac{m_{H2O}}{\rho_{H2O}} = \frac{m_{IL}}{\rho_{IL}} \leftrightarrow \qquad \text{Equation 6-3}$$

$$\leftrightarrow \rho_{IL} = \frac{m_{IL}}{m_{H2O}} \times \rho_{H2O} \qquad \text{Equation 6-4}$$

However, for the weight of the ionic liquid (mIL) and water (mH2O) it was necessary to reduce the portion of the pycnometer. Thus the density was determined mathematically by:

$$\rho_{IL} = \frac{m_{ILpic} - m_{pic}}{m_{H2Opic} - m_{pic}} \times \rho_{H2O} \qquad \text{Equation 6-5}$$

Appendice 2 – Measurement of the water content of the ionic liquid - Protocol

In order to determine the ionic liquid water content, a Karl Fischer (831 KF Coulometer, Metrohm) was used (Figure 6-2).

Figure 6-2 - Karl Fischer (831 KF Coulometer, Metrohm)

Appendice 3 – Glycerol Calibration Curve

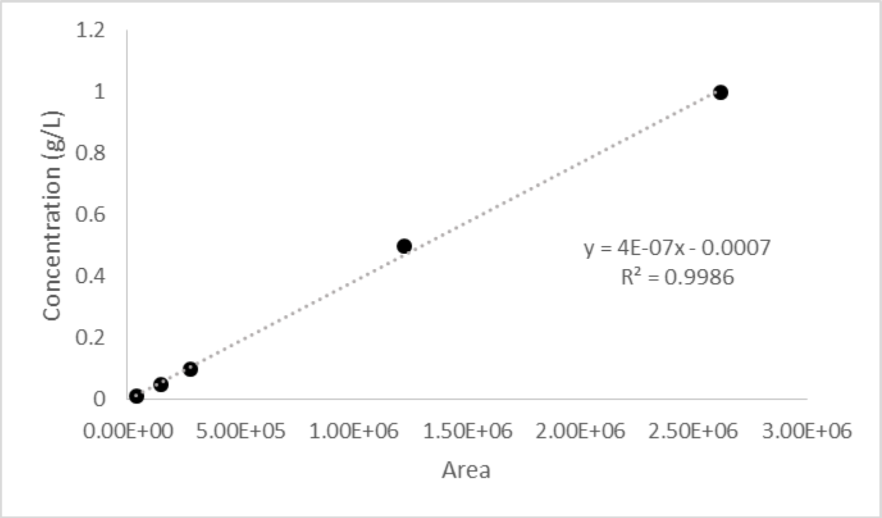

Figure 6-3 - Glycerol Calibration Curve

Appendice 4 – Ammonium Calibration Curve

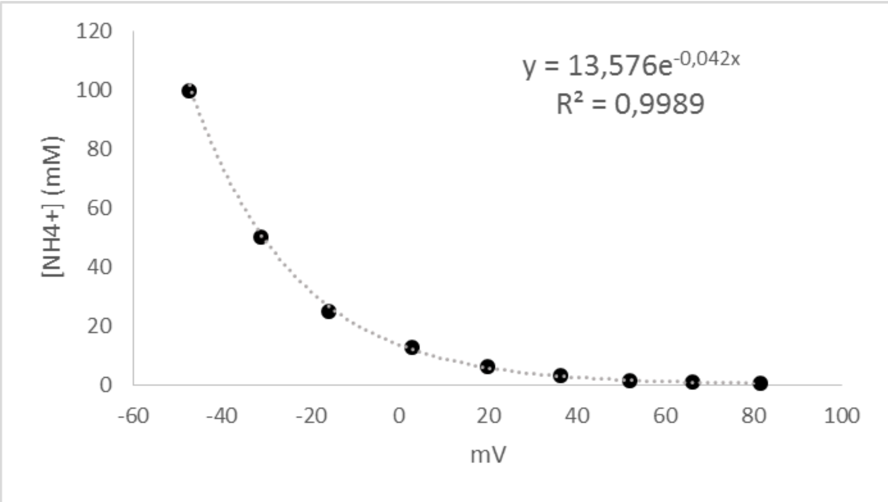

Figure 6-4 - Ammonium Calibration Curve

Appendice 5 – Sugars Calibration Curves

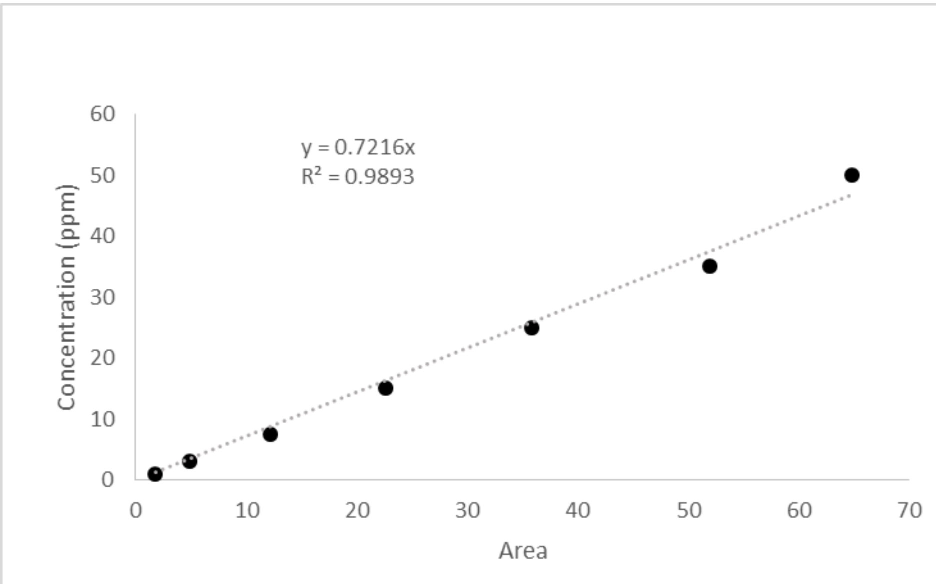

Figure 6-5 - Rhamnose Calibration Curve

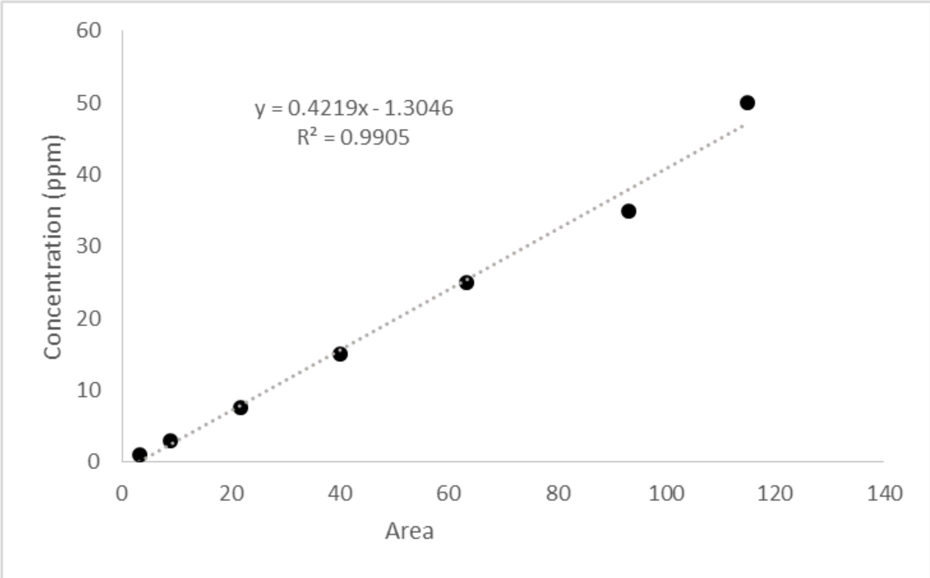

Figure 6-6 - Galactose Calibration Curve

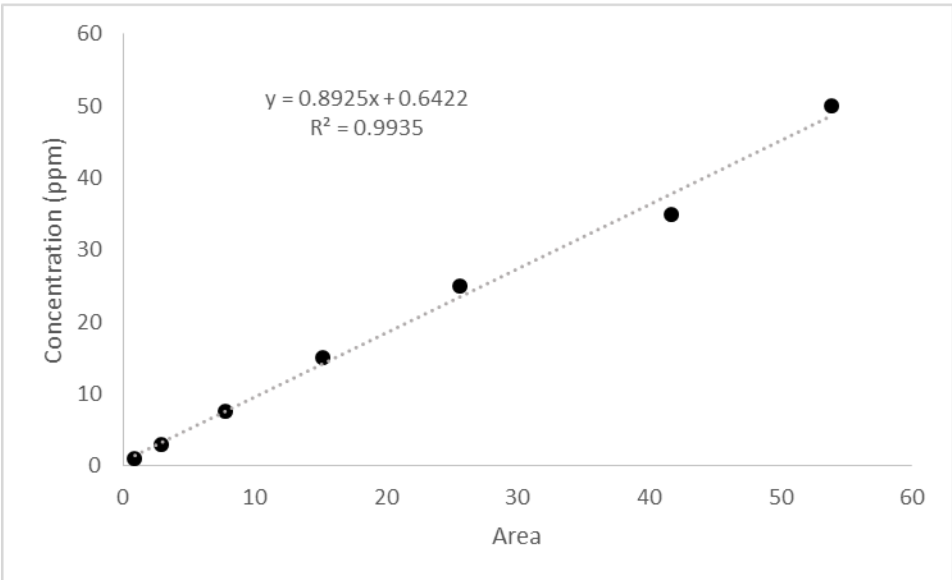

Figure 6-7 – Glucuronic Acid Calibration Curve

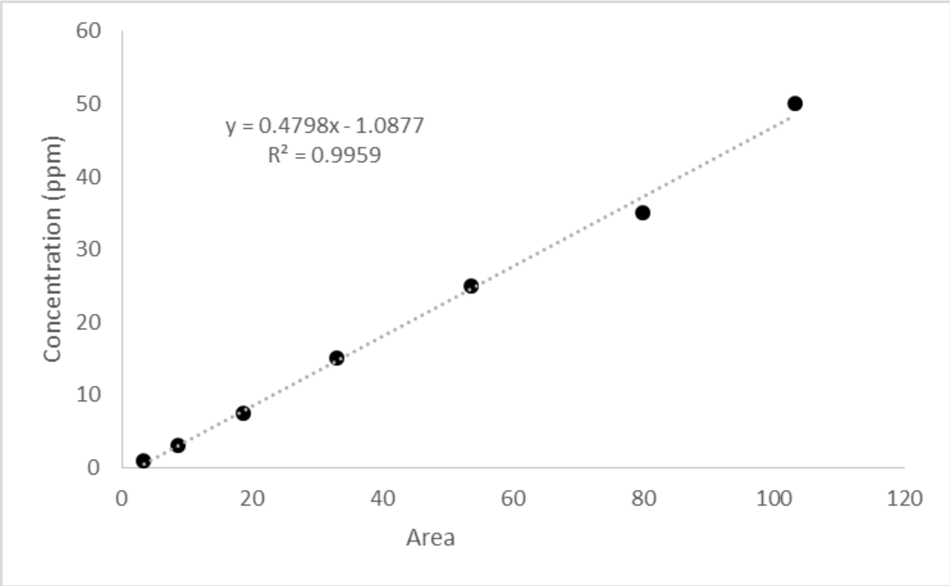

Figure 6-8 - Glucose Calibration Curve

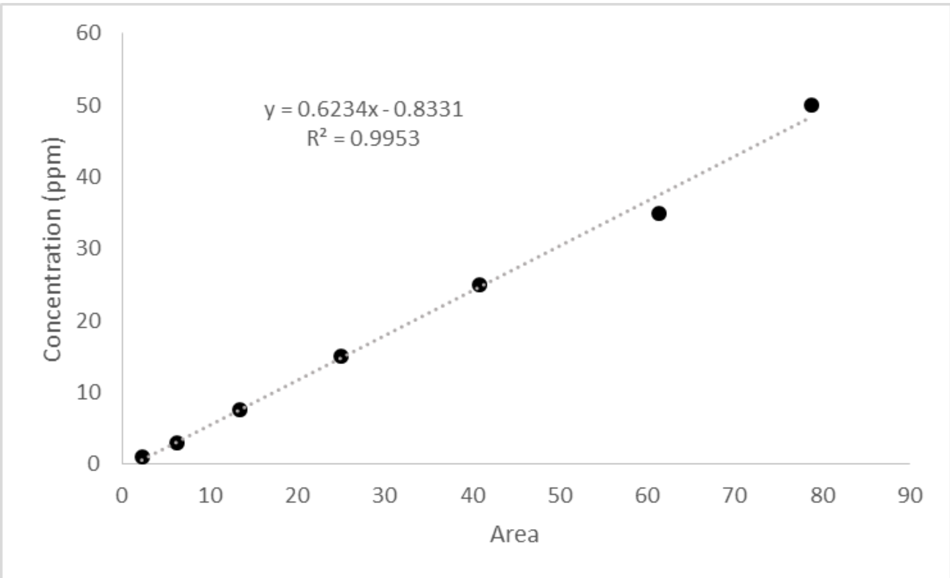

Figure 6-9 - Mannose Calibration Curve

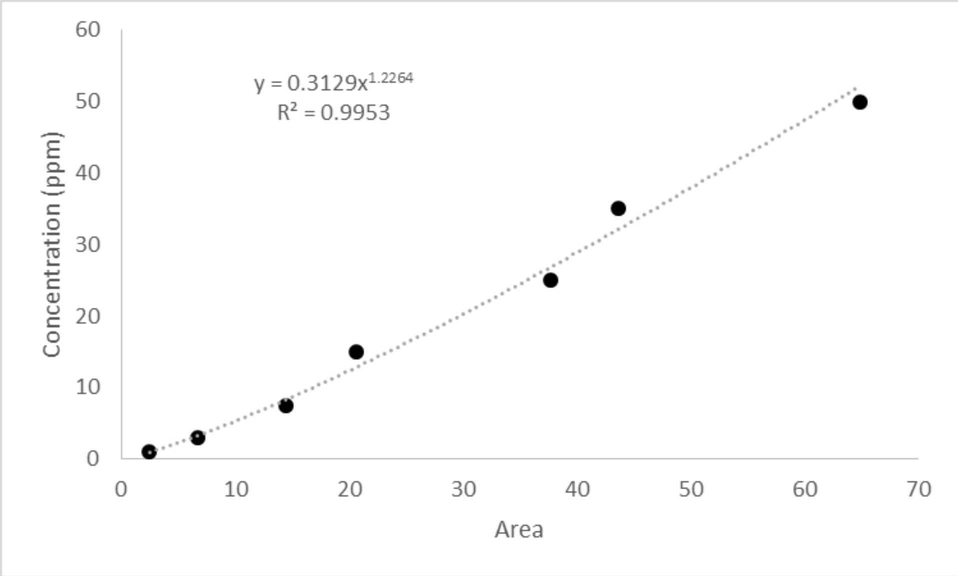

Figure 6-10 - Fucose Calibration Curve

Appendice 6 – Acyl Groups Calibration Curve

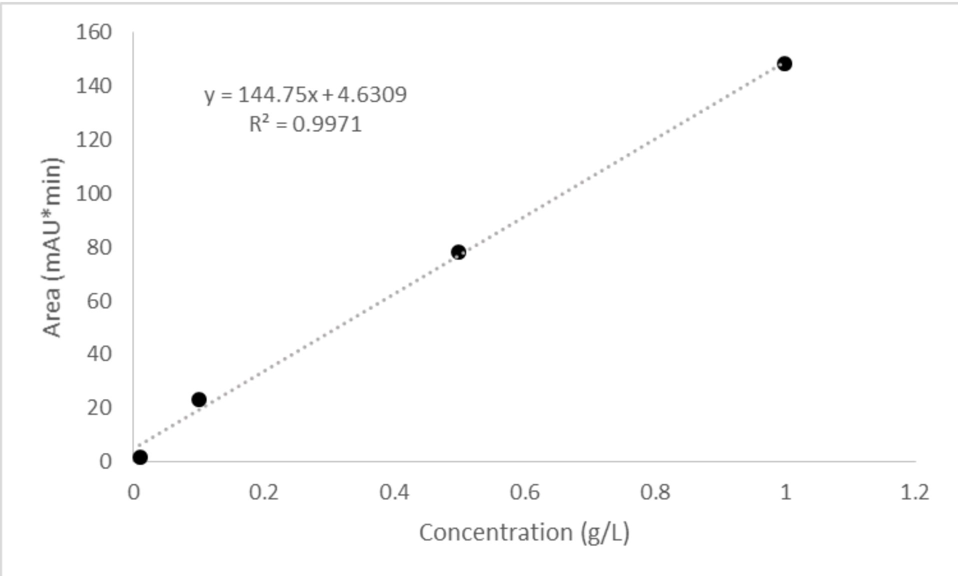

Figure 6-11 - Pyruvate Calibration Curve

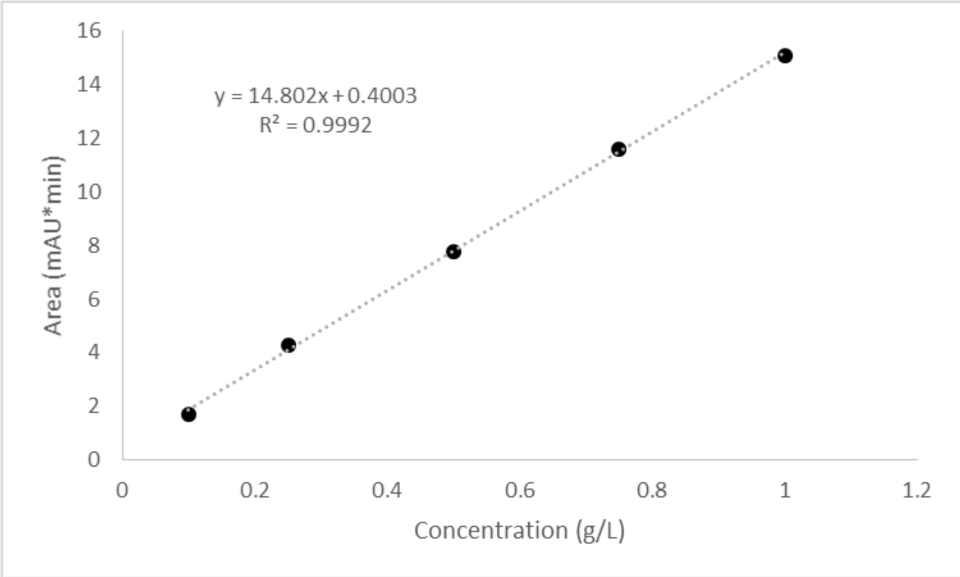

Figure 6-12 - Succinate Calibration Curve

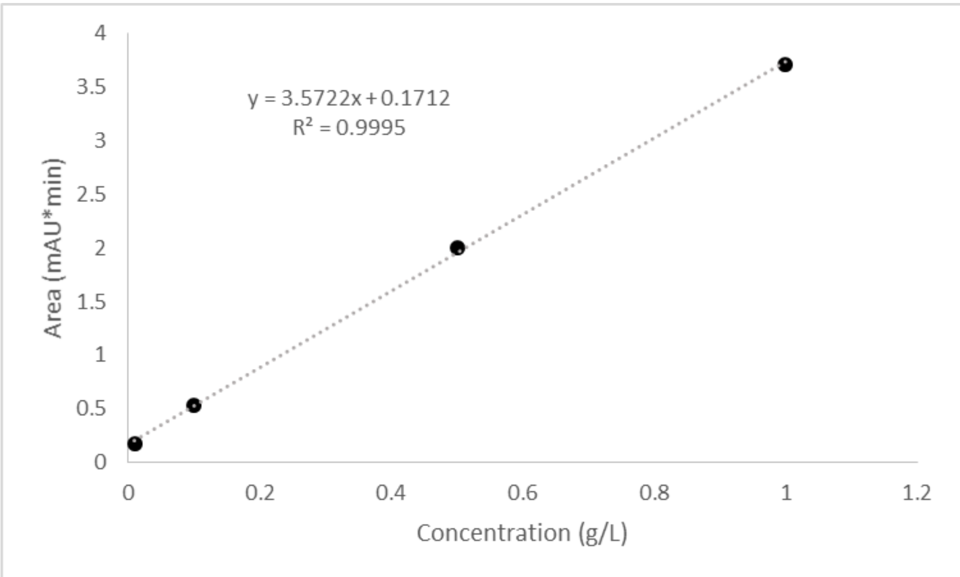

Figure 6-13 - Acetate Calibration Curve

Appendice 7 – DSC Analyses

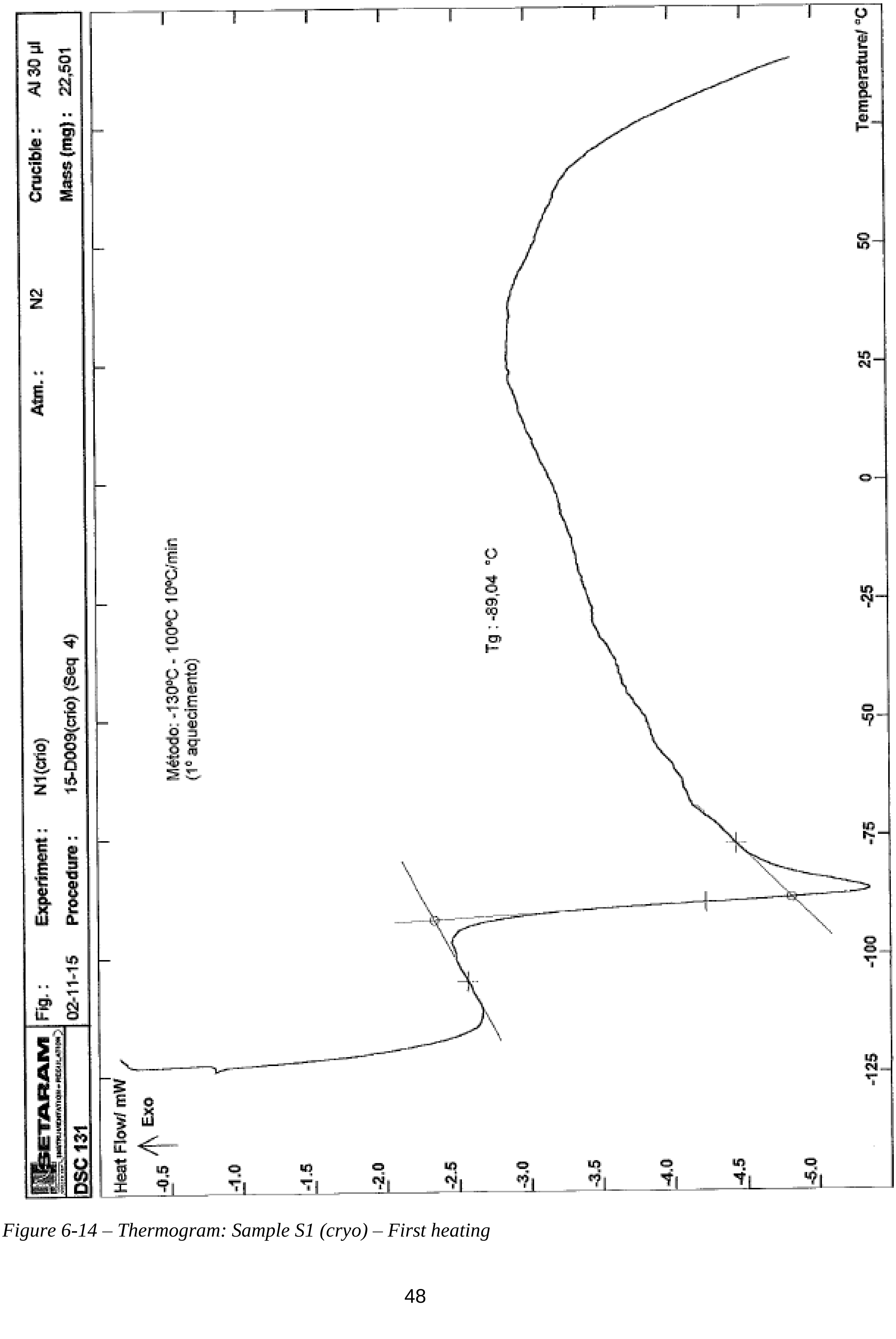

Figure 6-14 – Thermogram: Sample S1 (cryo) – First heating

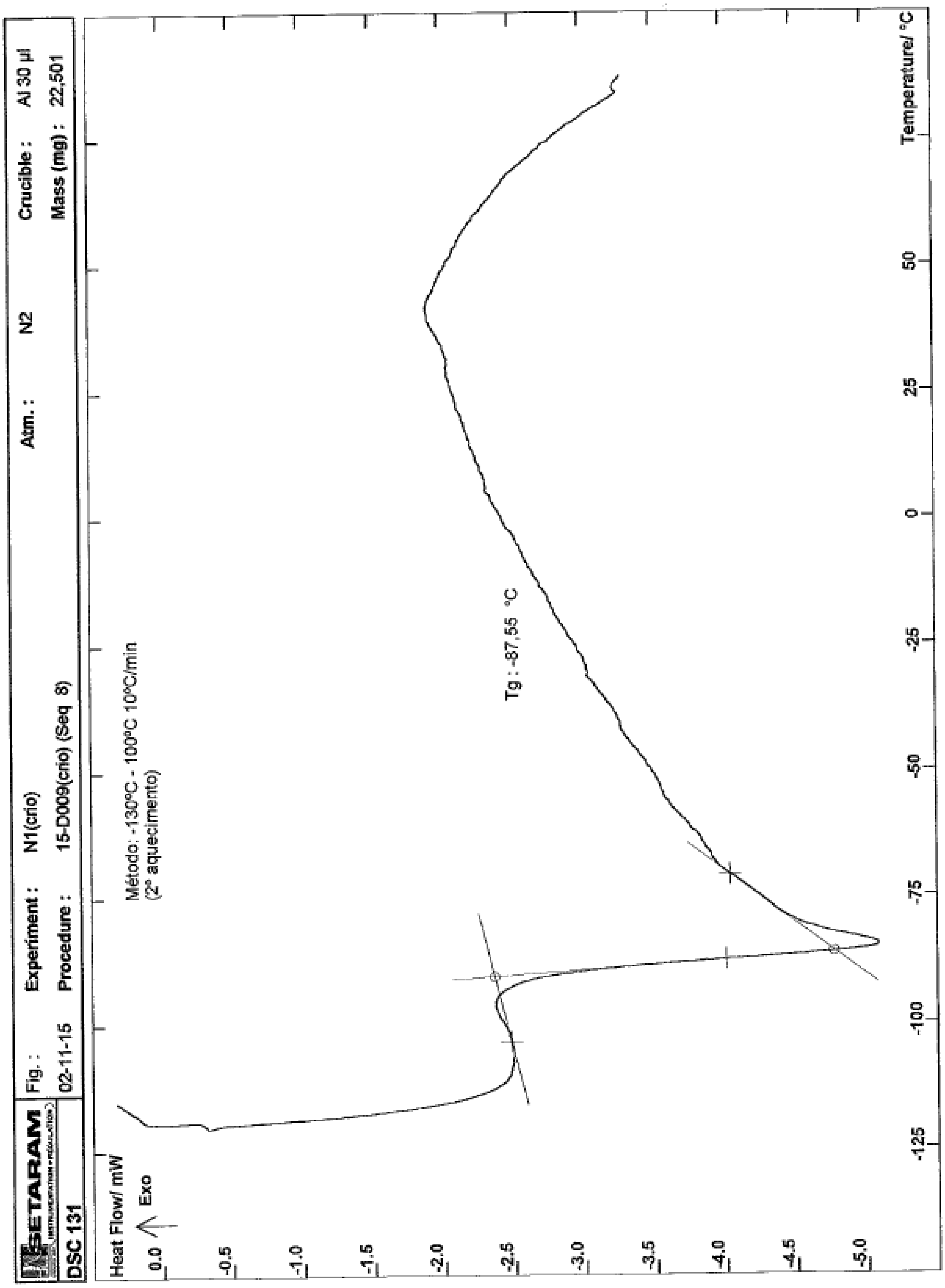

Figure 6-15 - Thermogram: Sample S1 (cryo) – Second heating

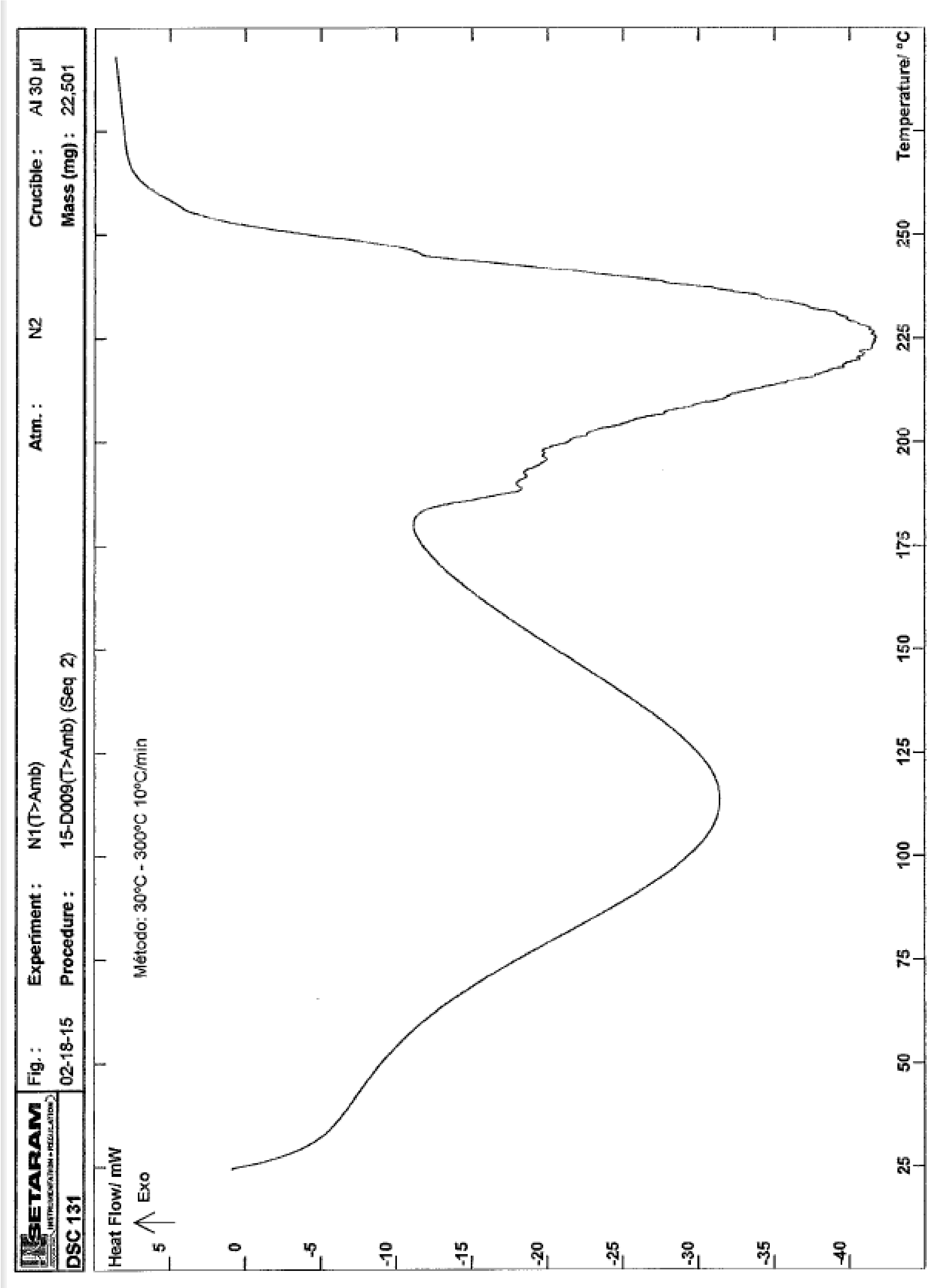

Figure 6-16 - Thermogram: Sample S1 (T>Troom)

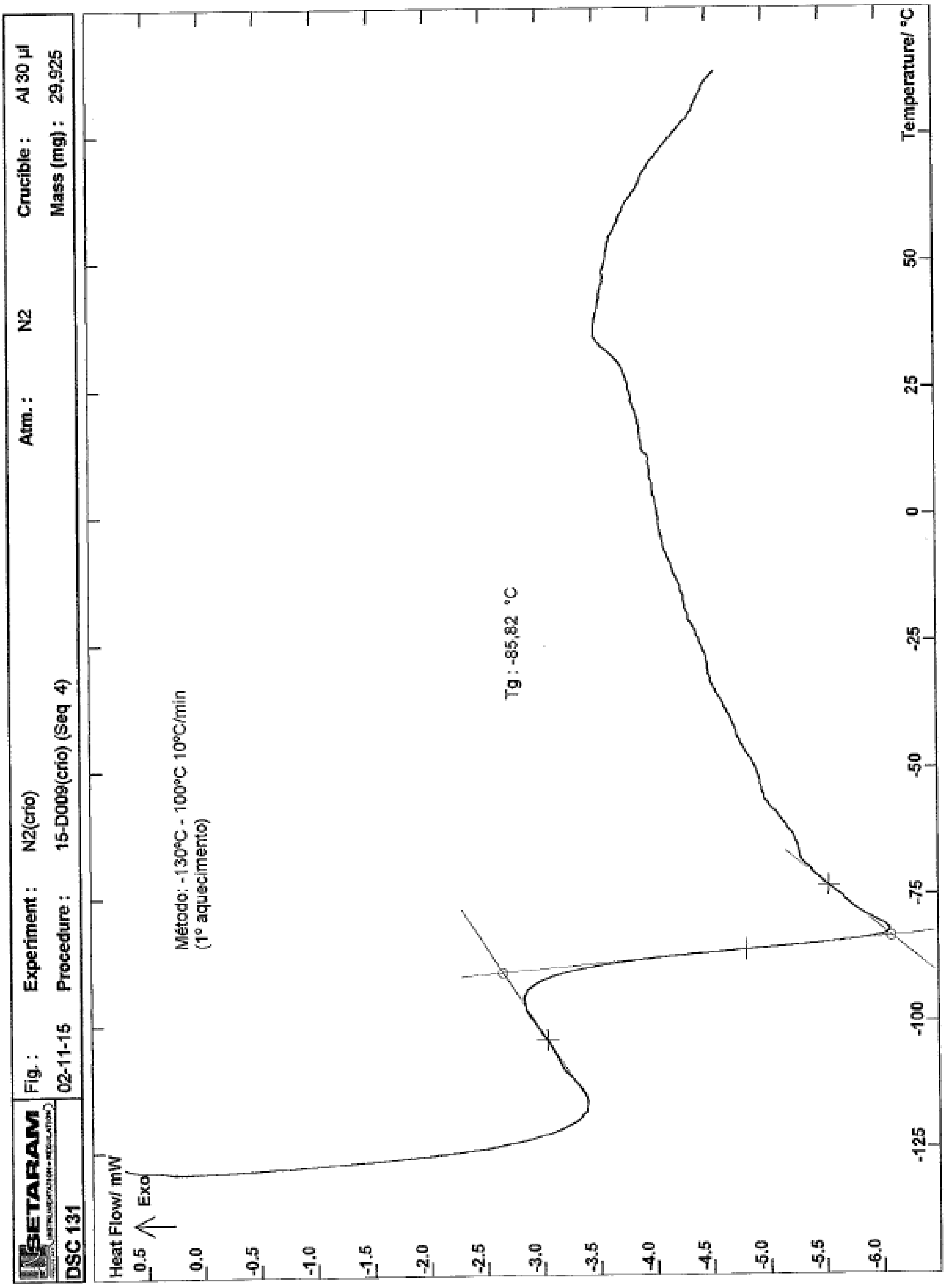

Figure 6-17 - Thermogram: Sample S2 (cryo) - First heating

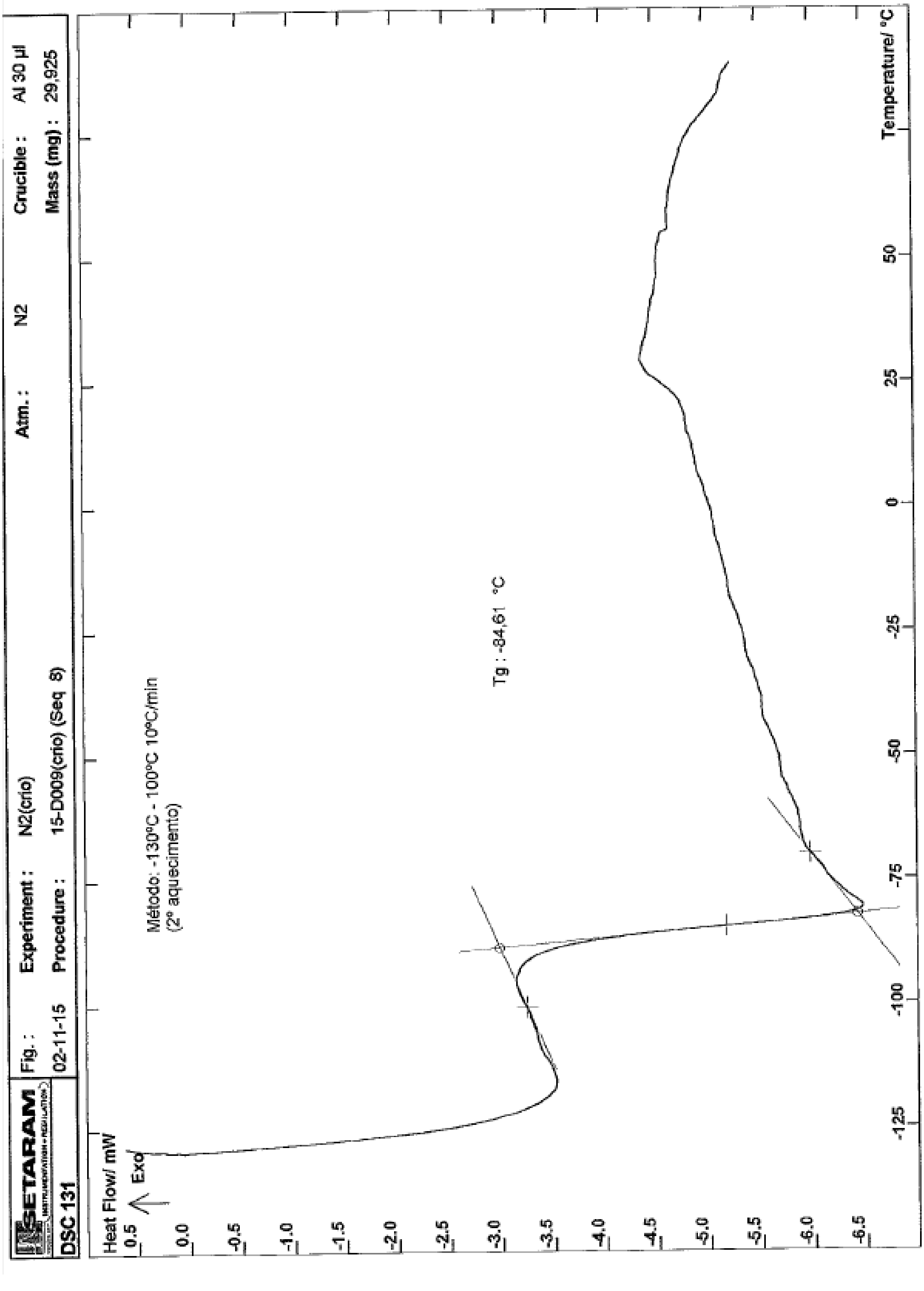

Figure 6-18 - Thermogram: Sample S2 (cryo) - Second heating

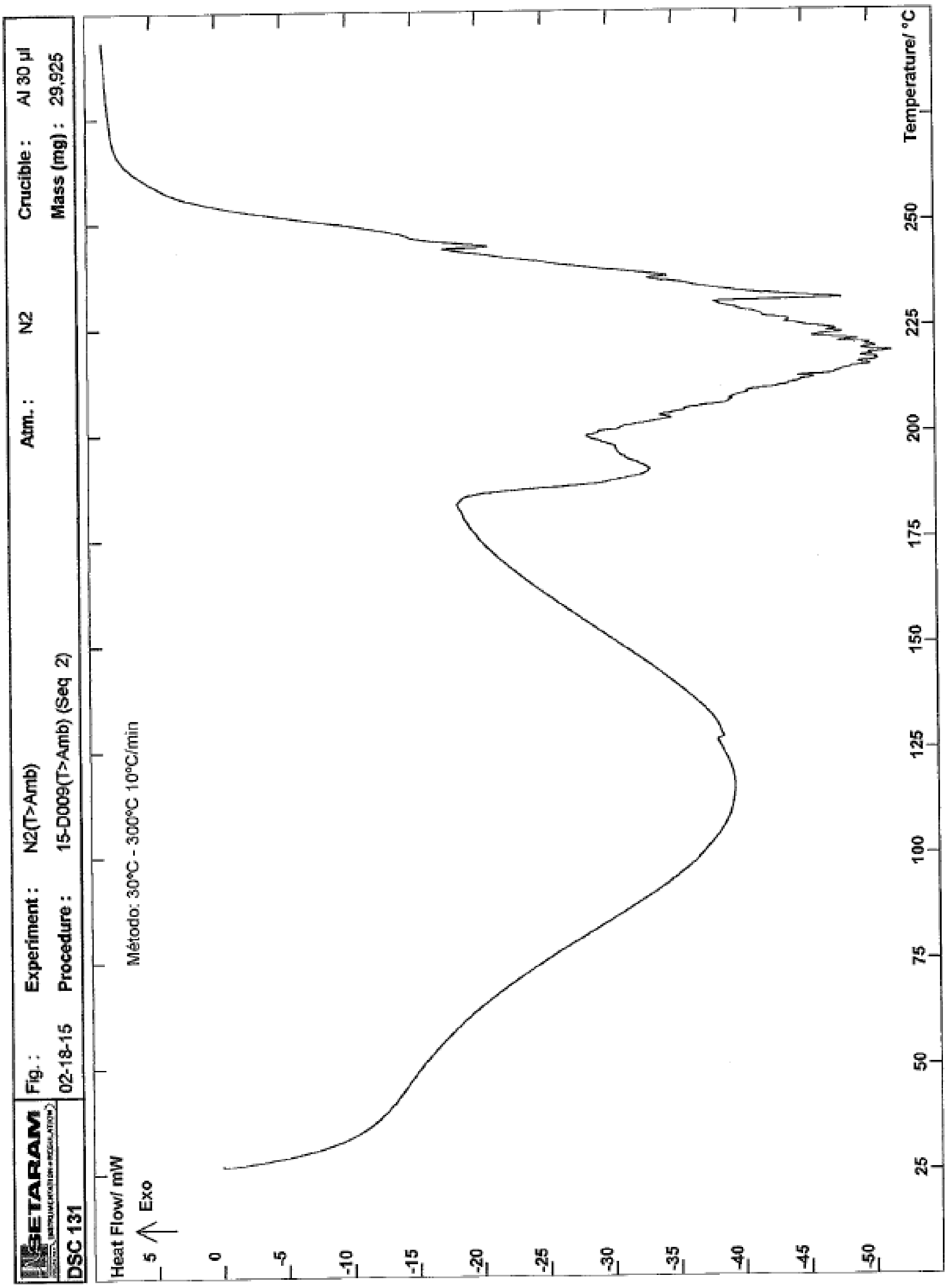

Figure 6-19 - Thermogram: Sample S2 (T>Troom)

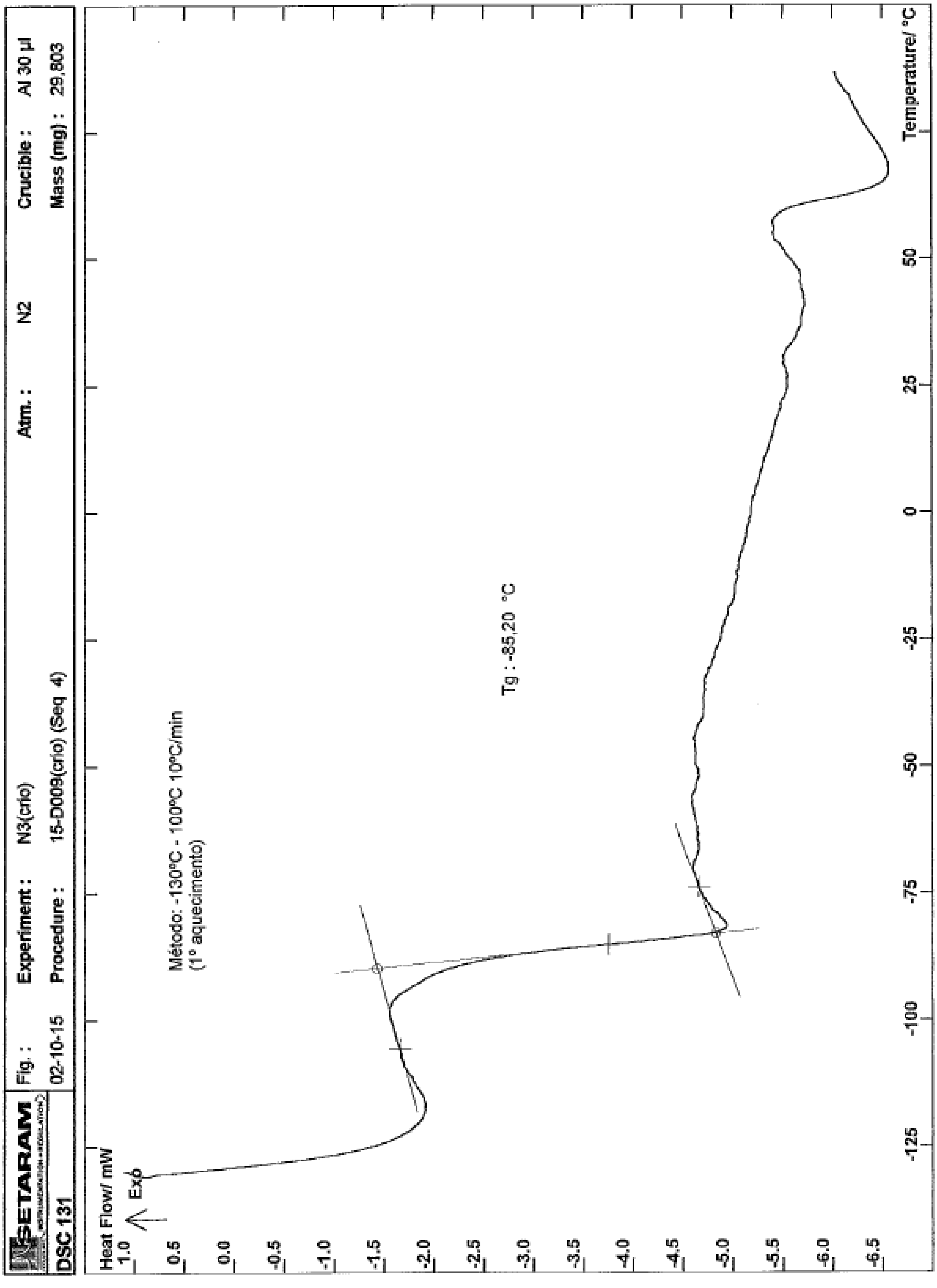

Figure 6-20 - Thermogram: Sample S3 (cryo) - First heating

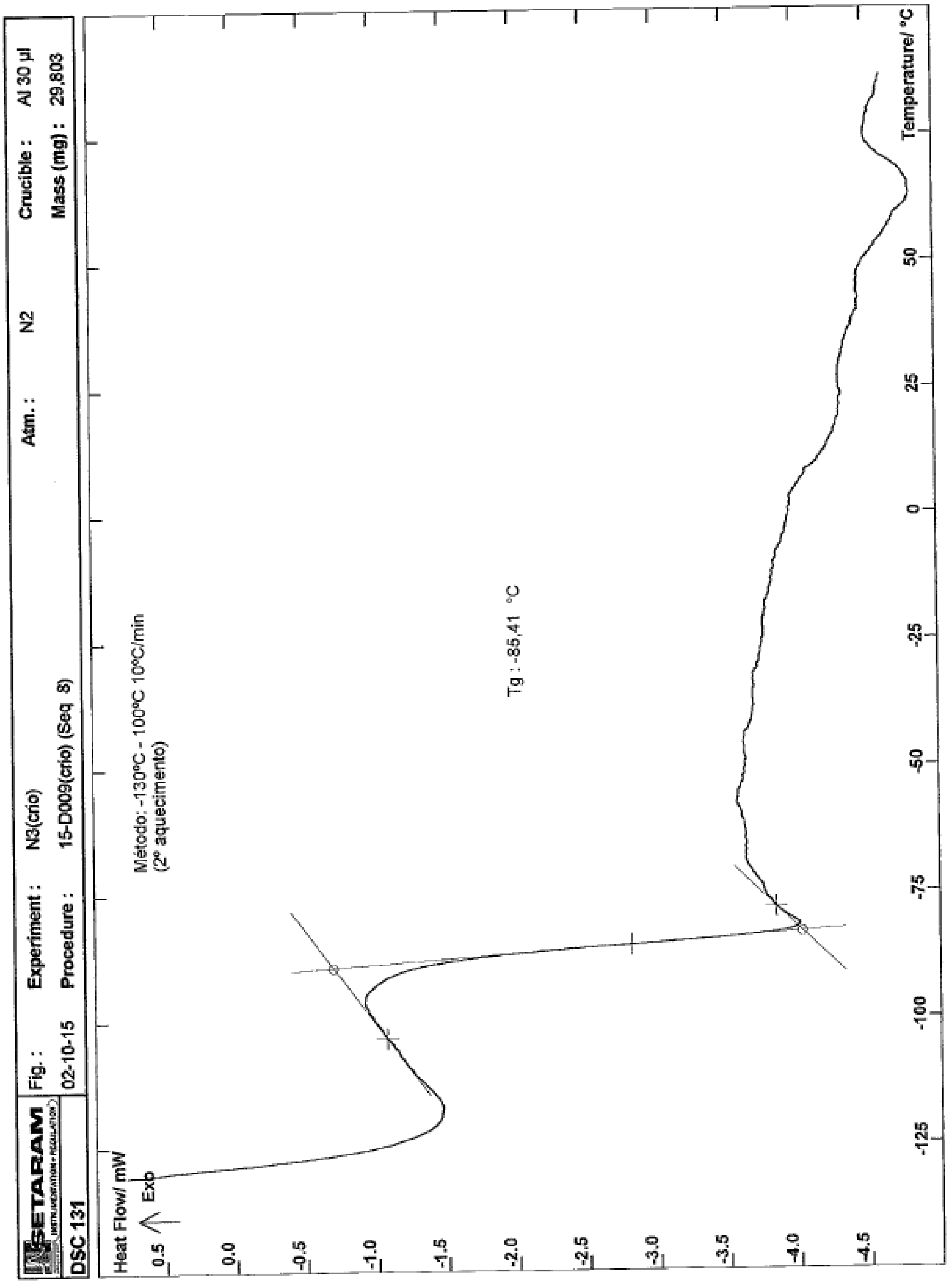

Figure 6-21 - Thermogram: Sample S3 (cryo) - Second heating

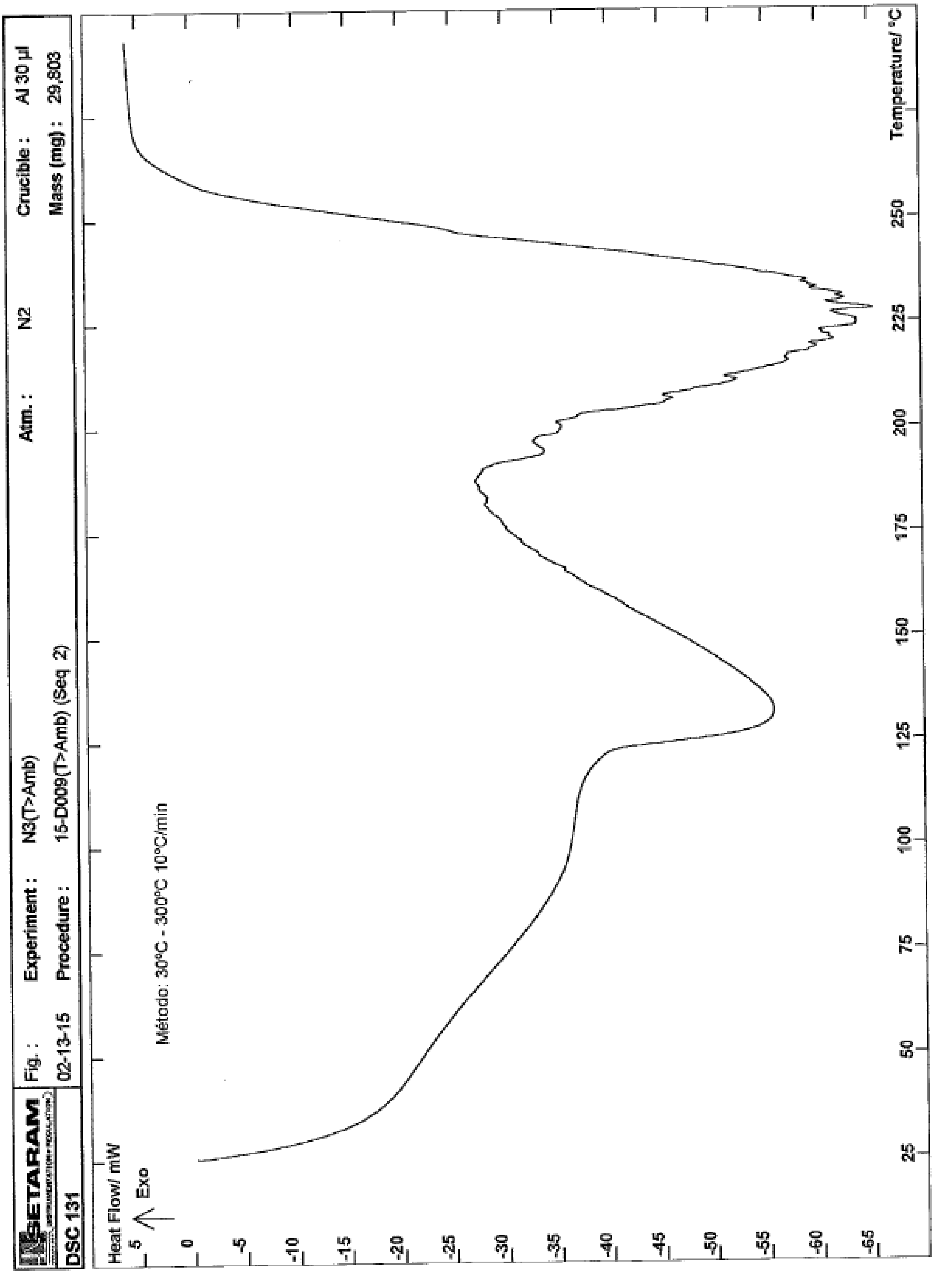

Figure 6-22 - Thermogram: Sample S3 (T>Troom)

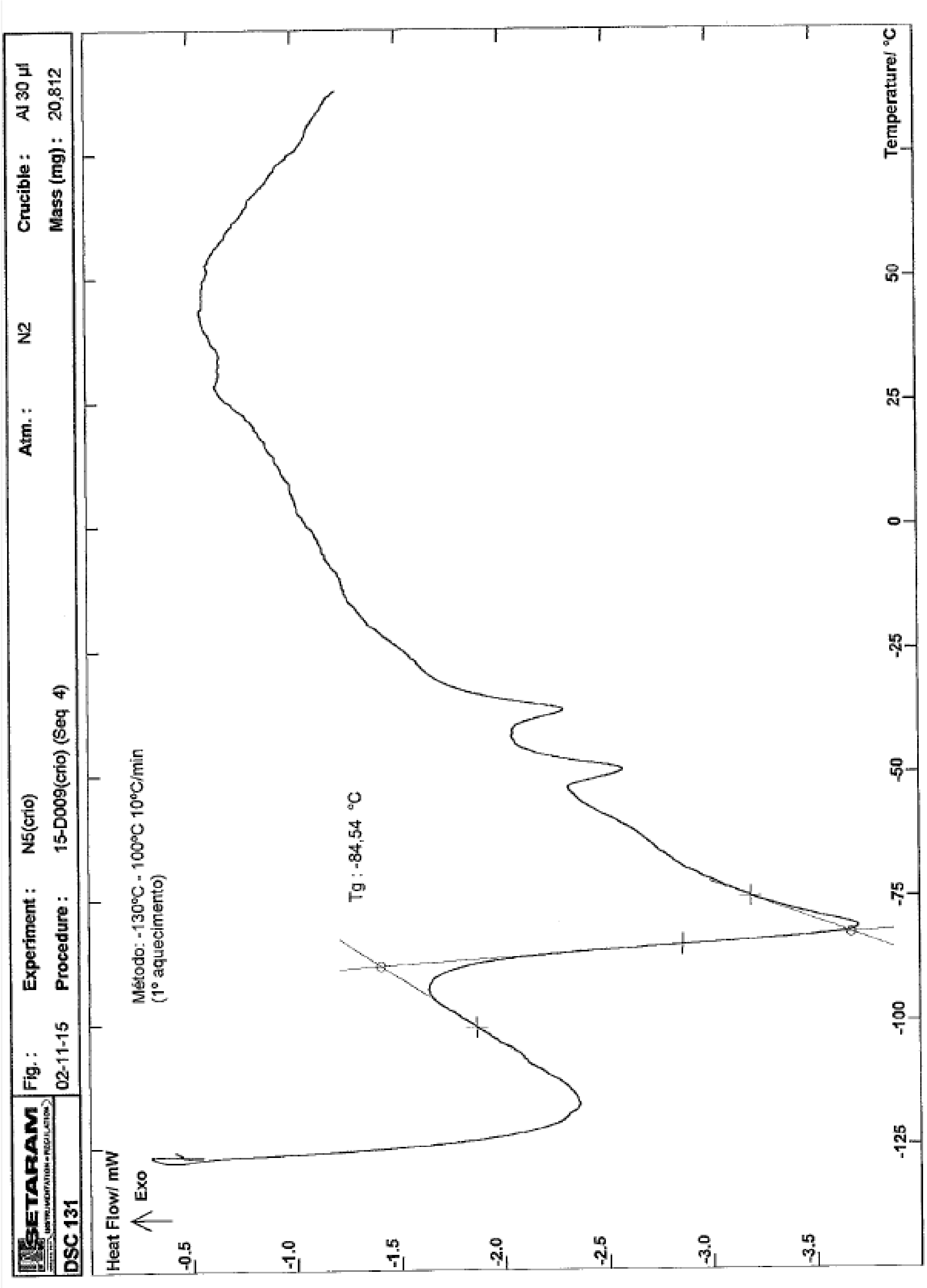

Figure 6-23 - Thermogram: Sample S4 (cryo) - First heating

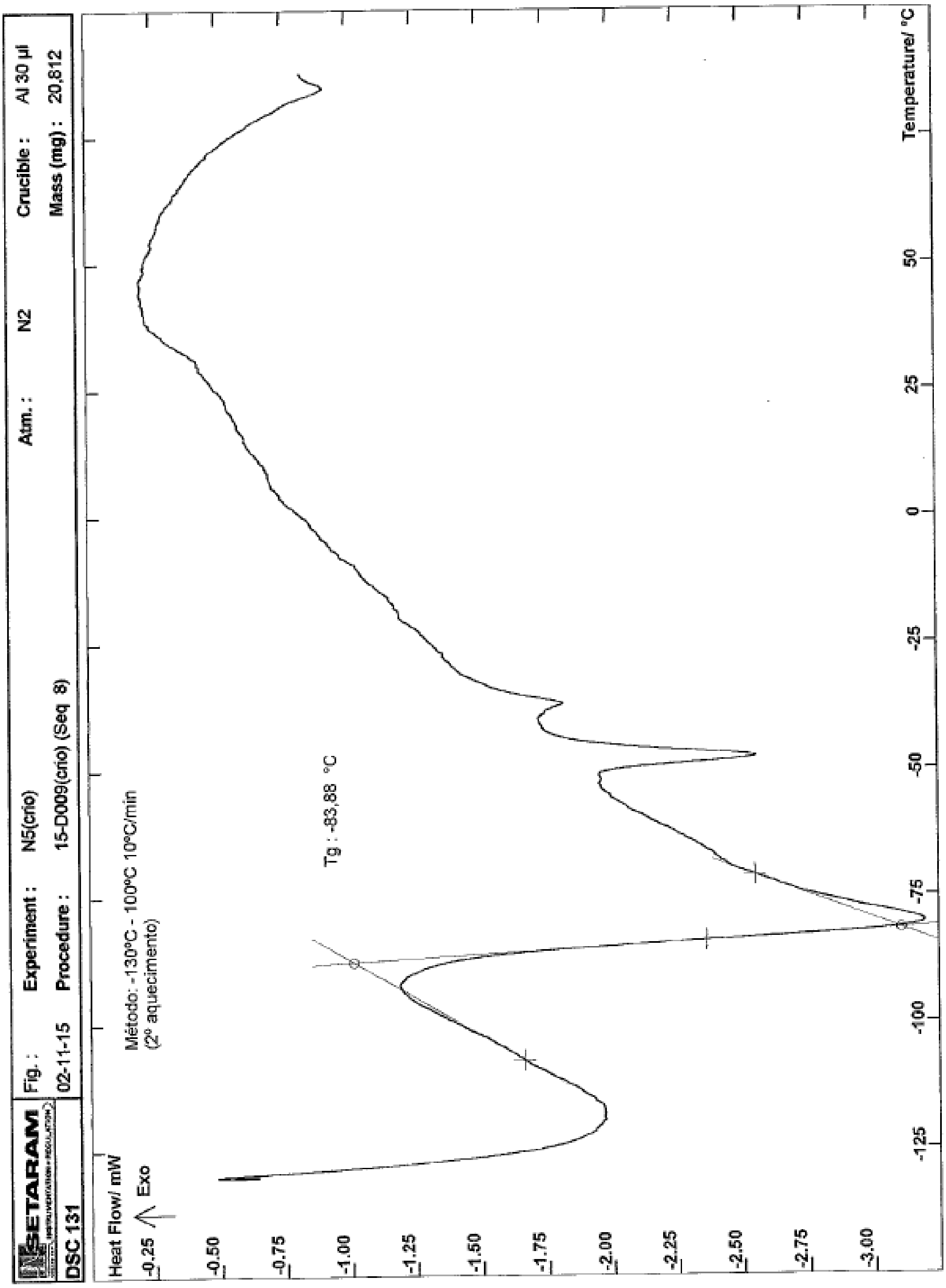

Figure 6-24 - Thermogram: Sample S4 (cryo) - Second heating

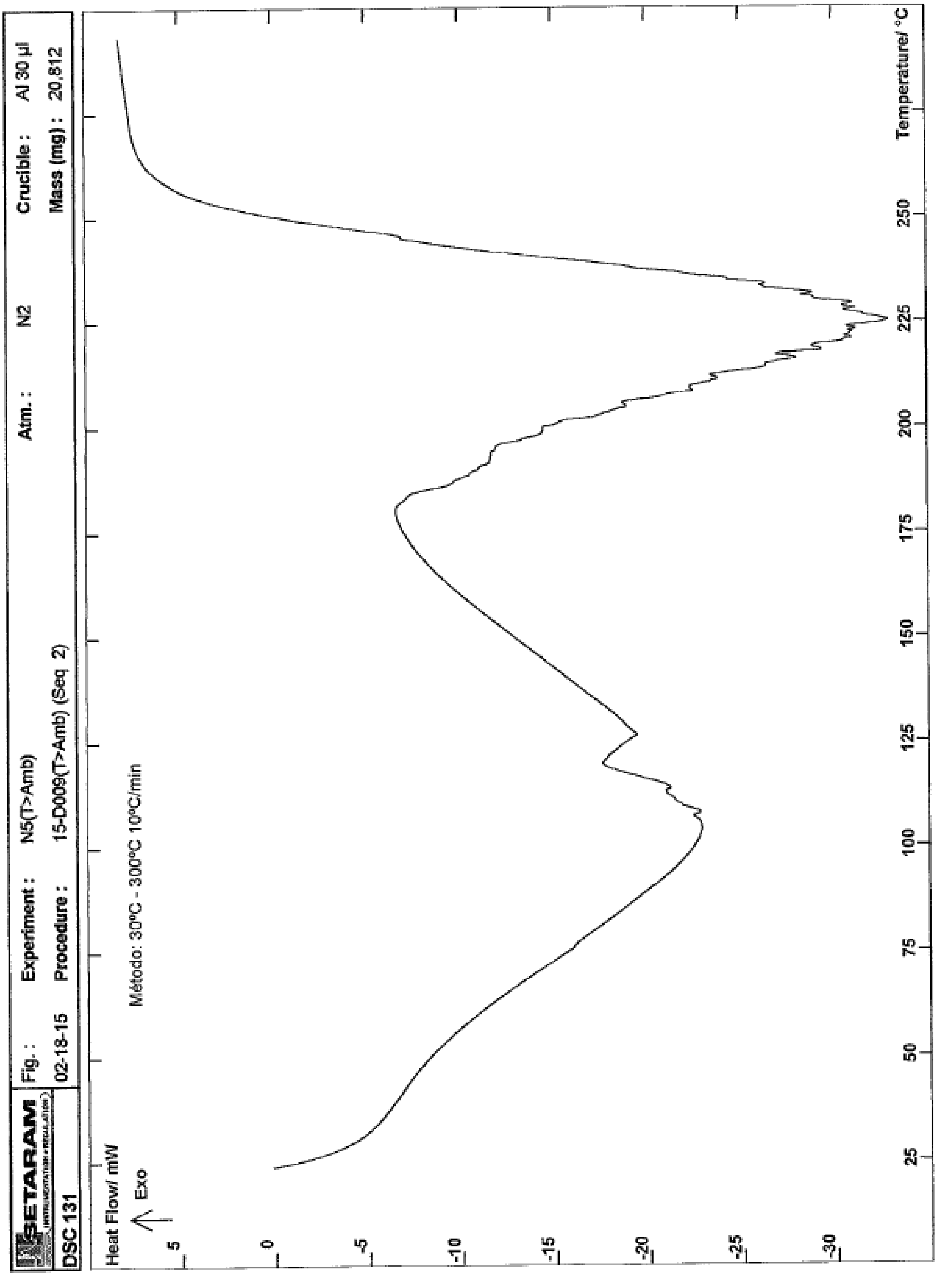

Figure 6-25 - Thermogram: Sample S5 (T>Troom)

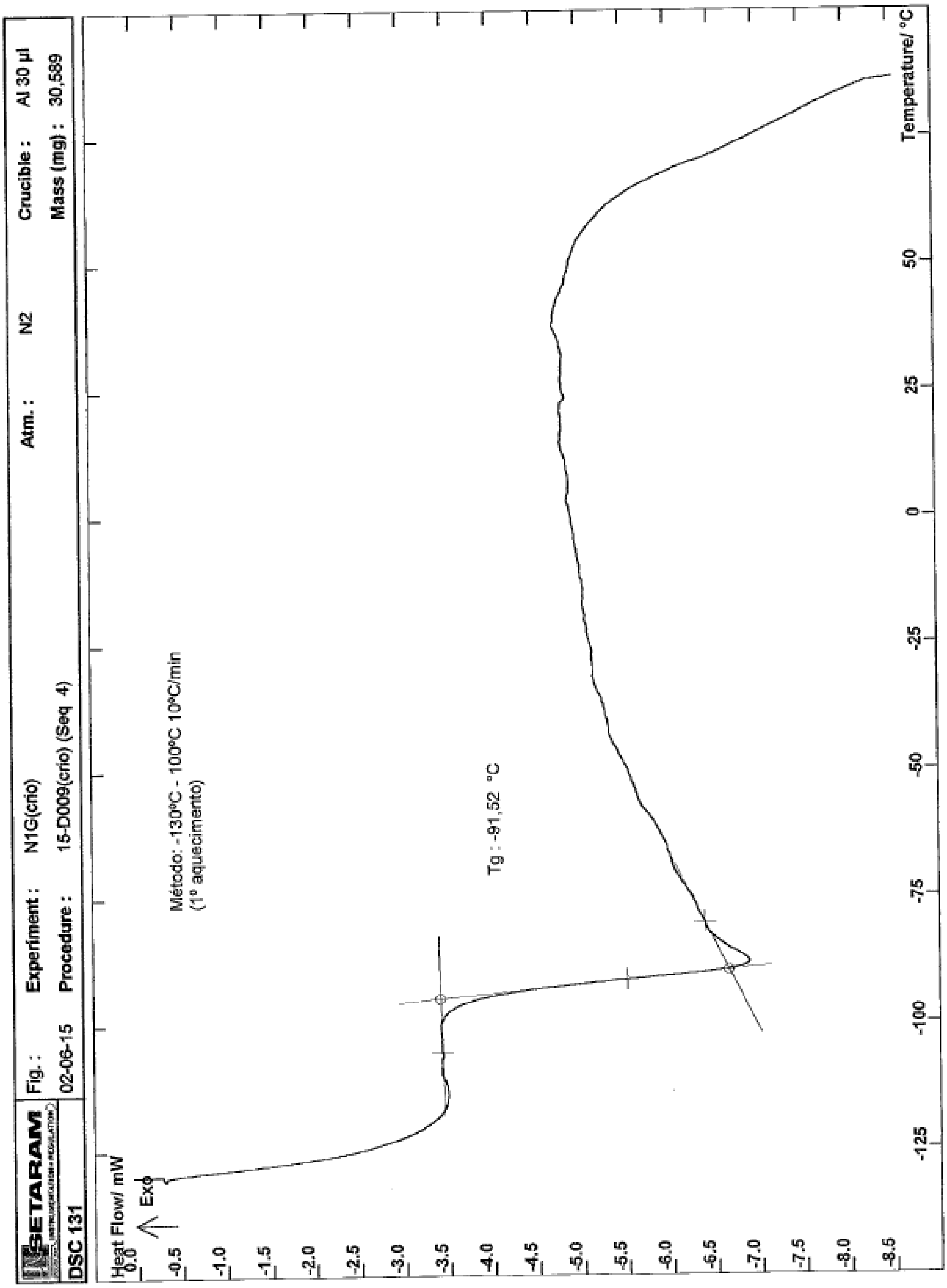

Figure 6-26 - Thermogram: Sample SI1 (cryo) - First heating

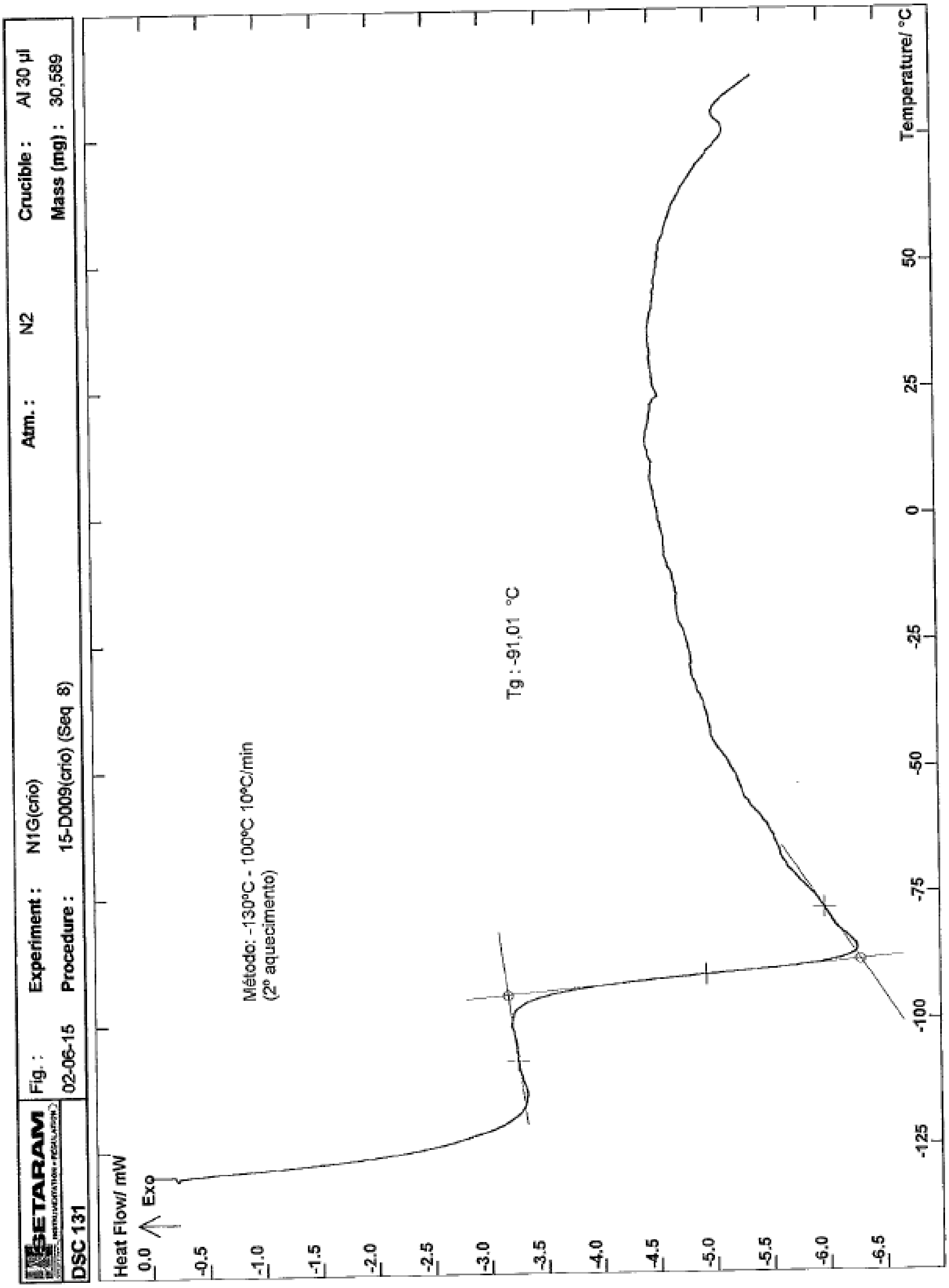

Figure 6-27 - Thermogram: Sample SI1 (cryo) - Second heating

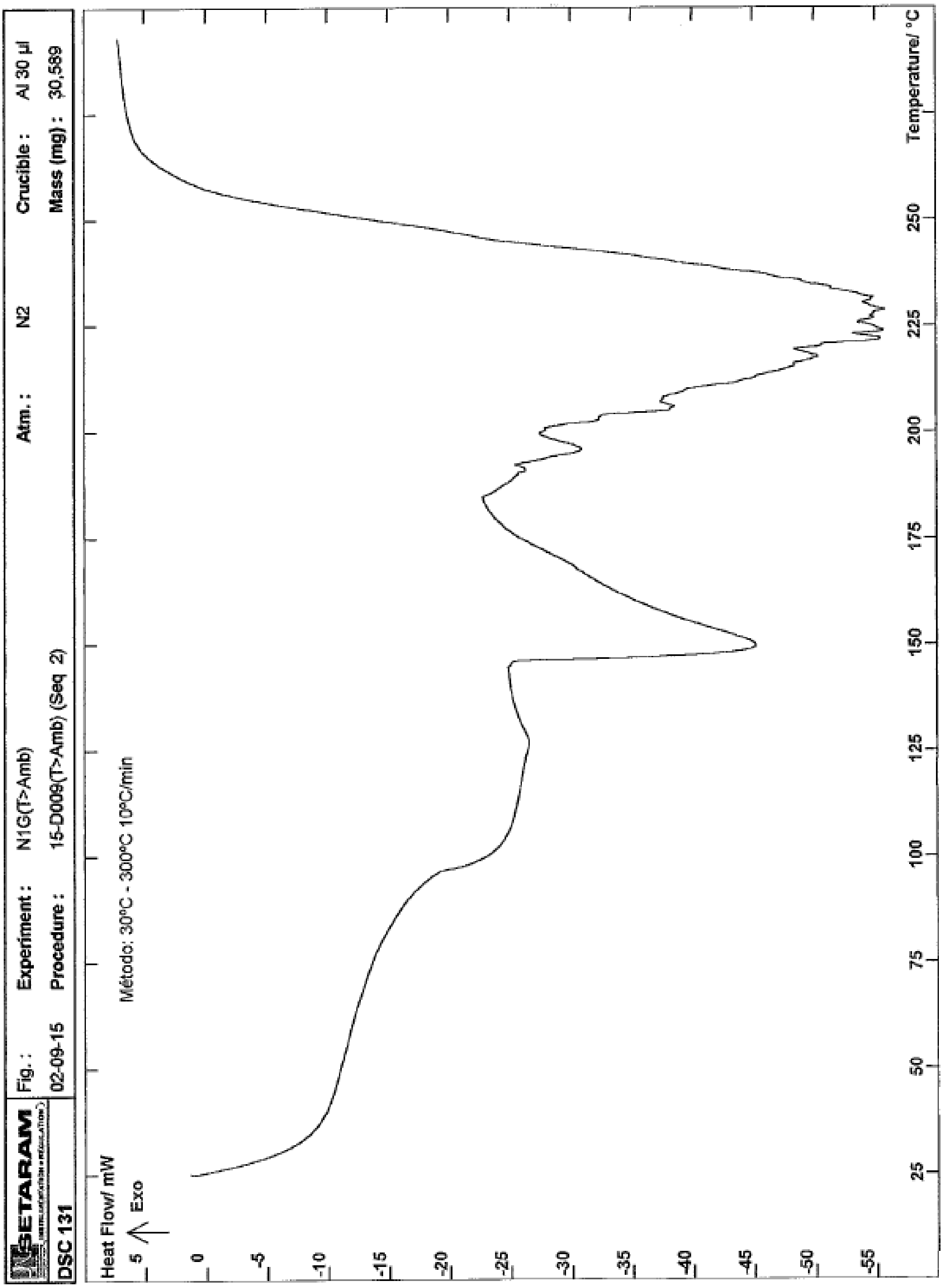

Figure 6-28 - Thermogram: Sample SI1 (T>Troom)

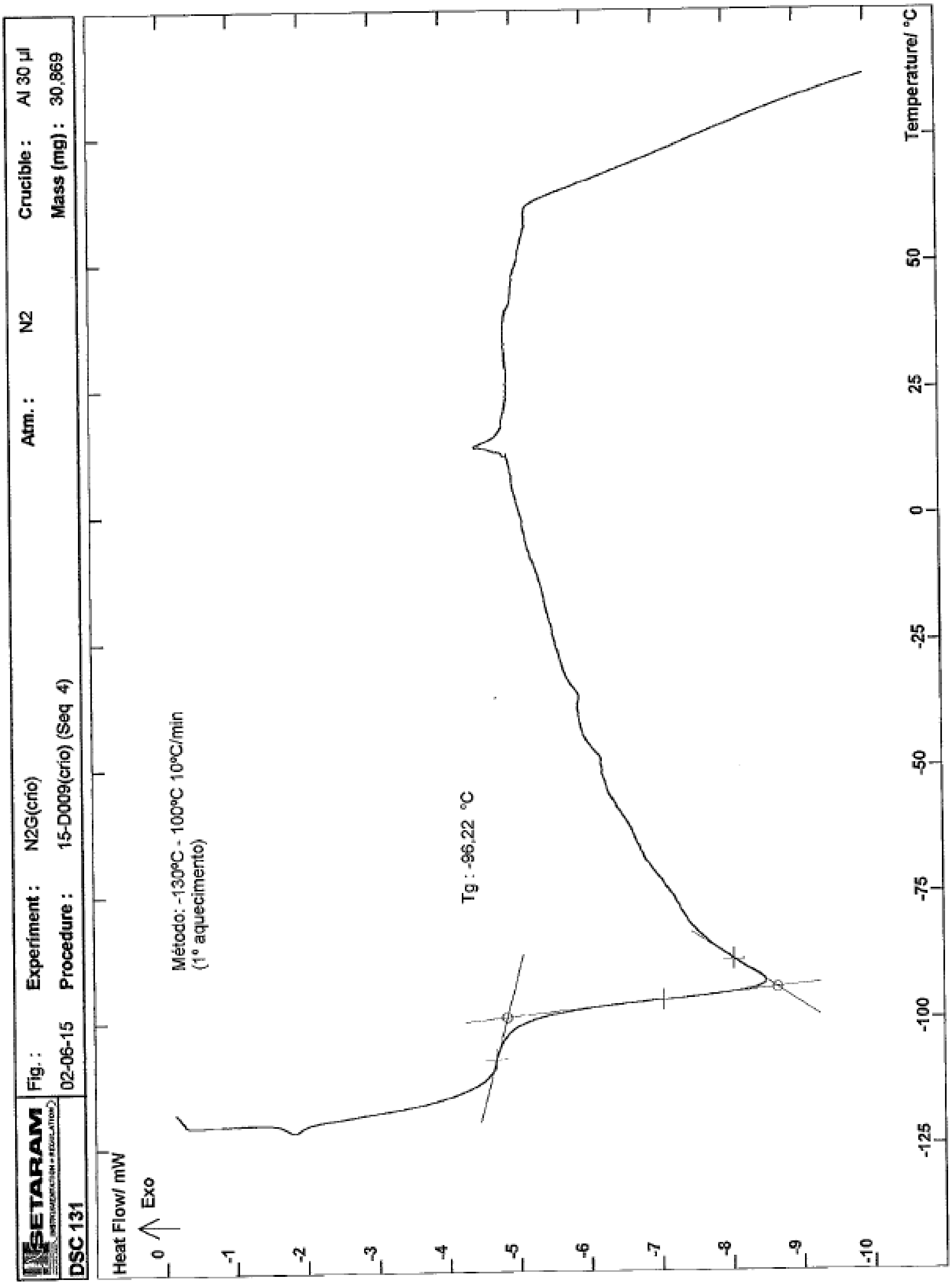

Figure 6-29 - Thermogram: Sample SI2 (cryo) - First heating

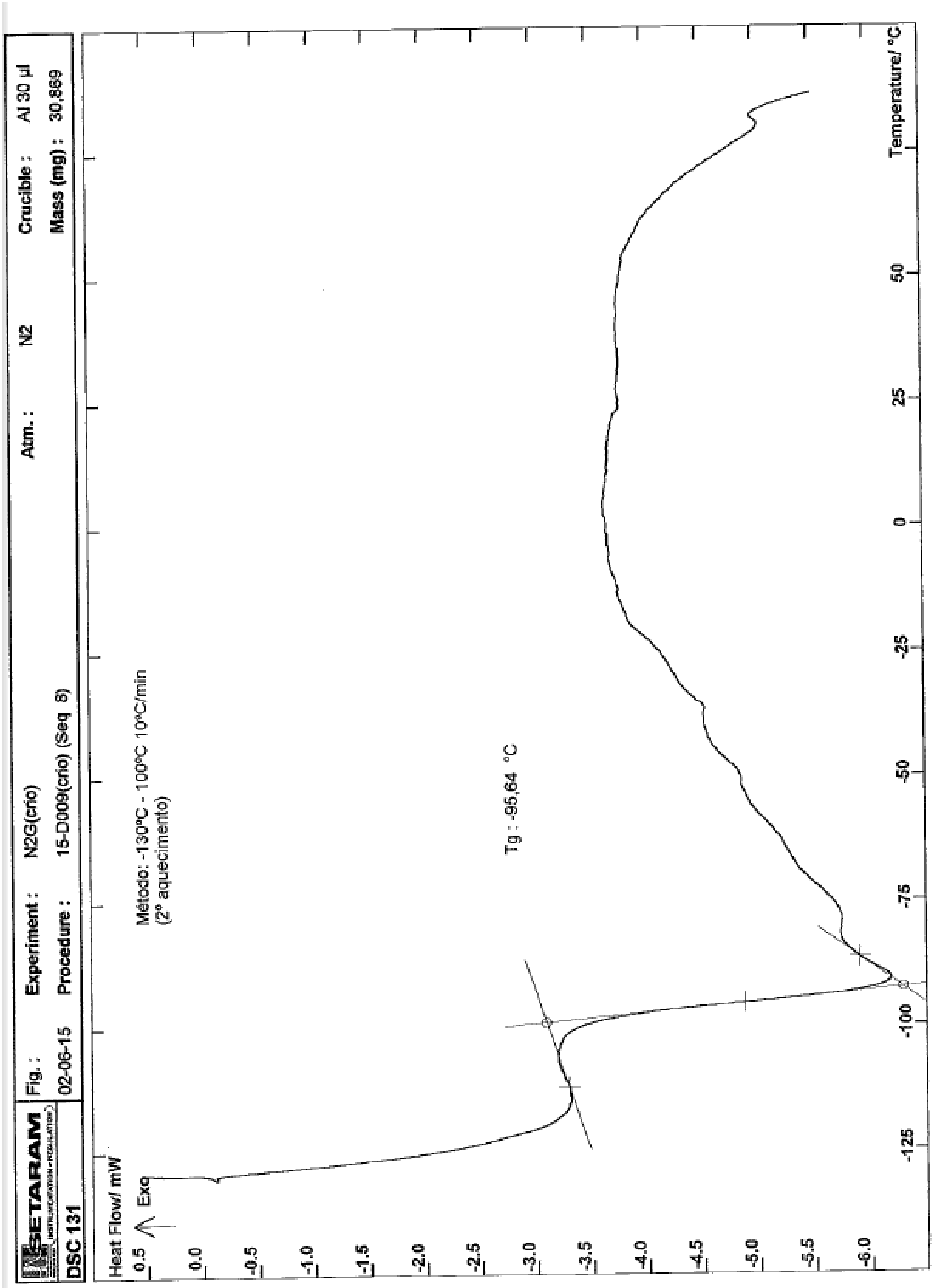

Figure 6-30 - Thermogram: Sample SI2 (cryo) - Second heating

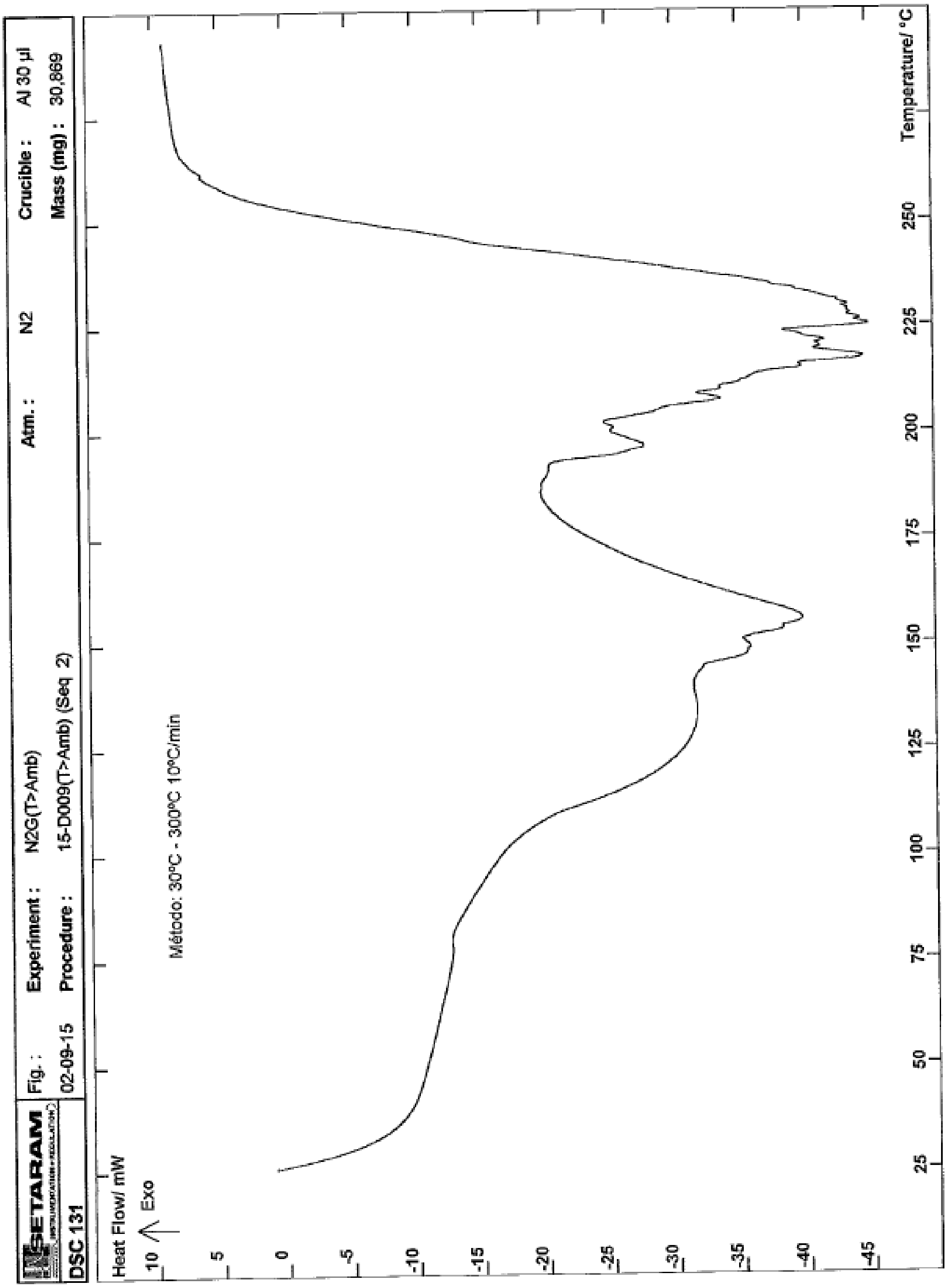

Figure 6-31 - Thermogram: Sample SI2 (T>Troom)

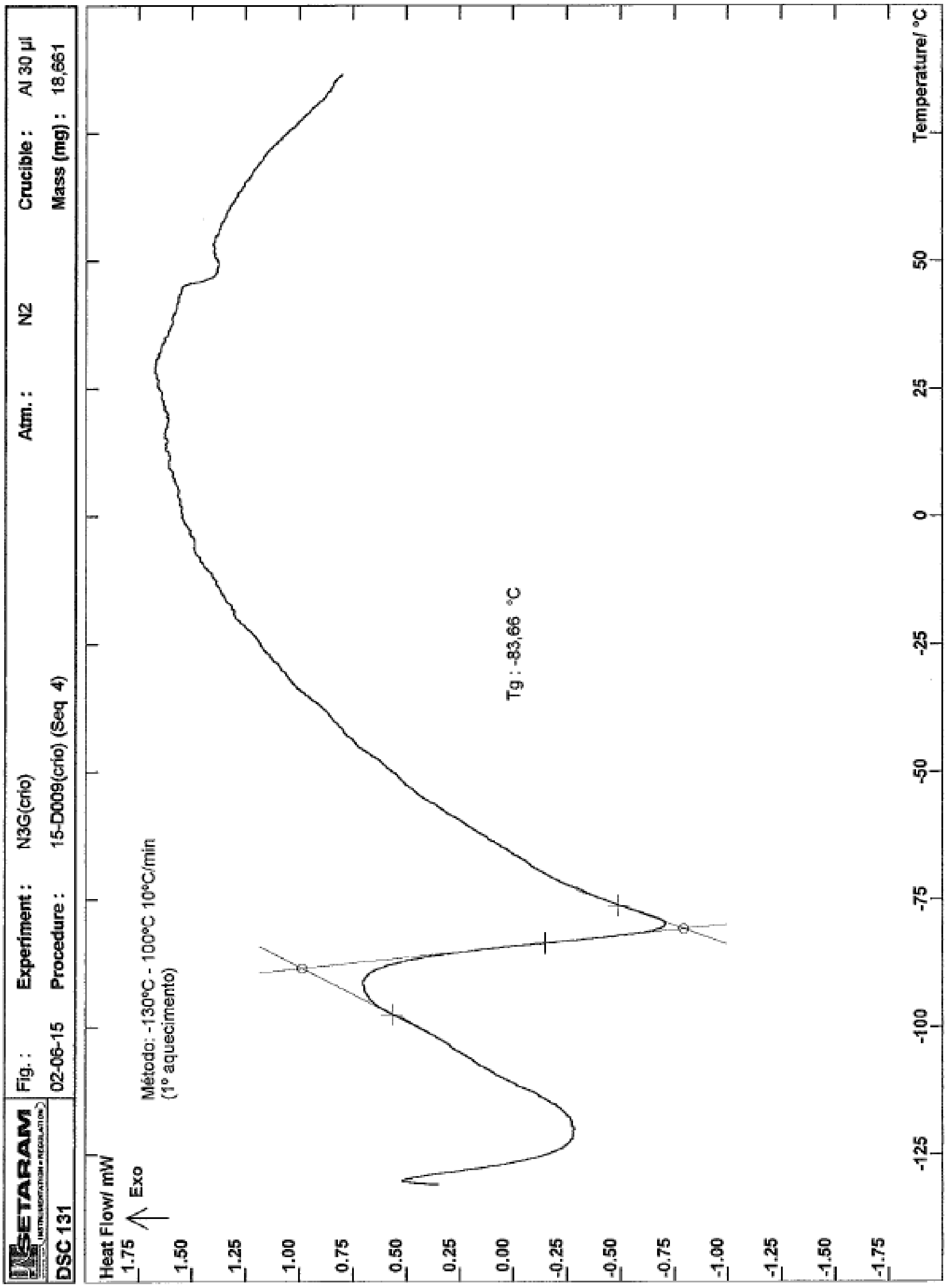

Figure 6-32 - Thermogram: Sample SI3 (cryo) - First heating

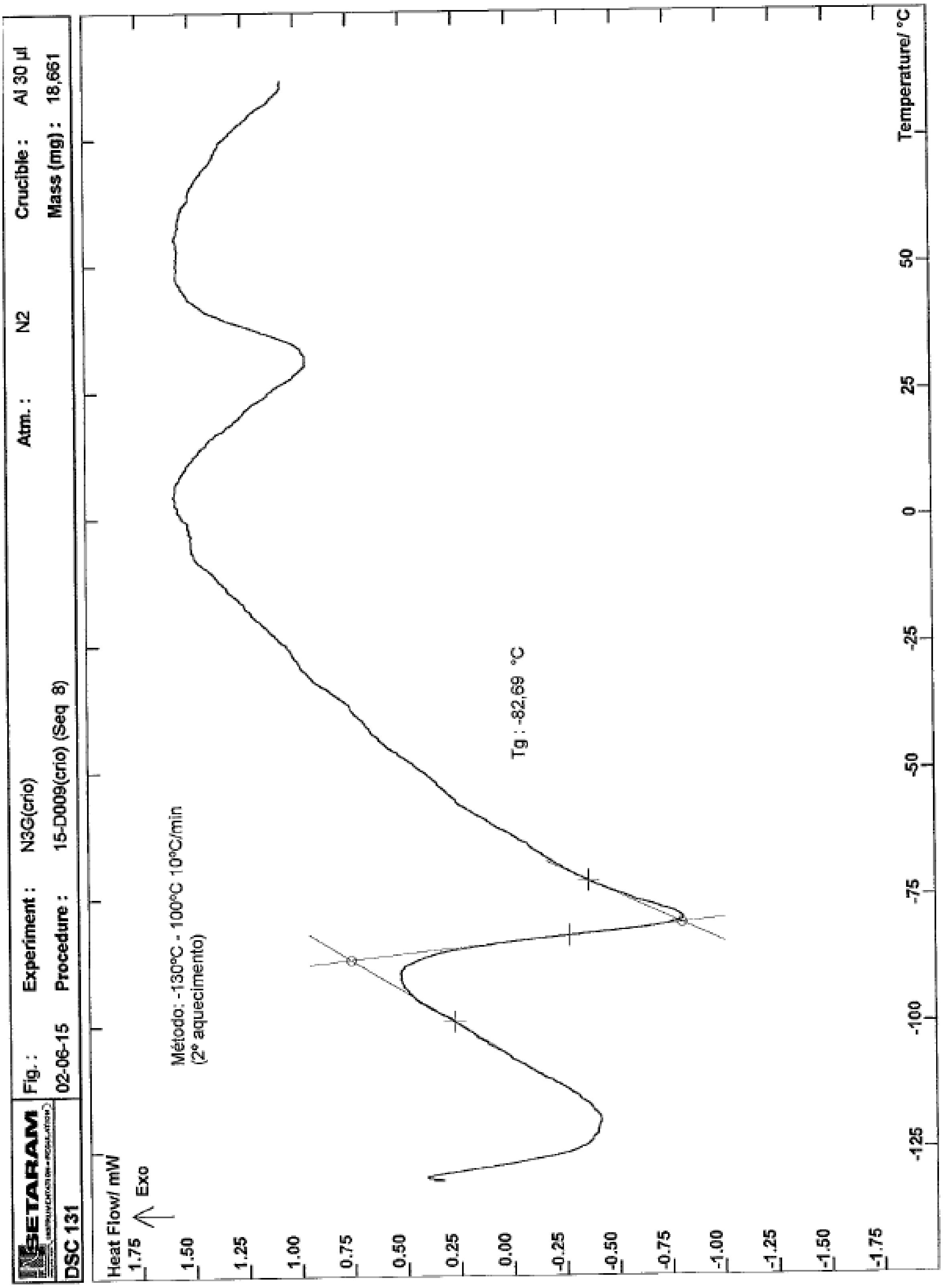

Figure 6-33 - Thermogram: Sample SI3 (cryo) – Second heating

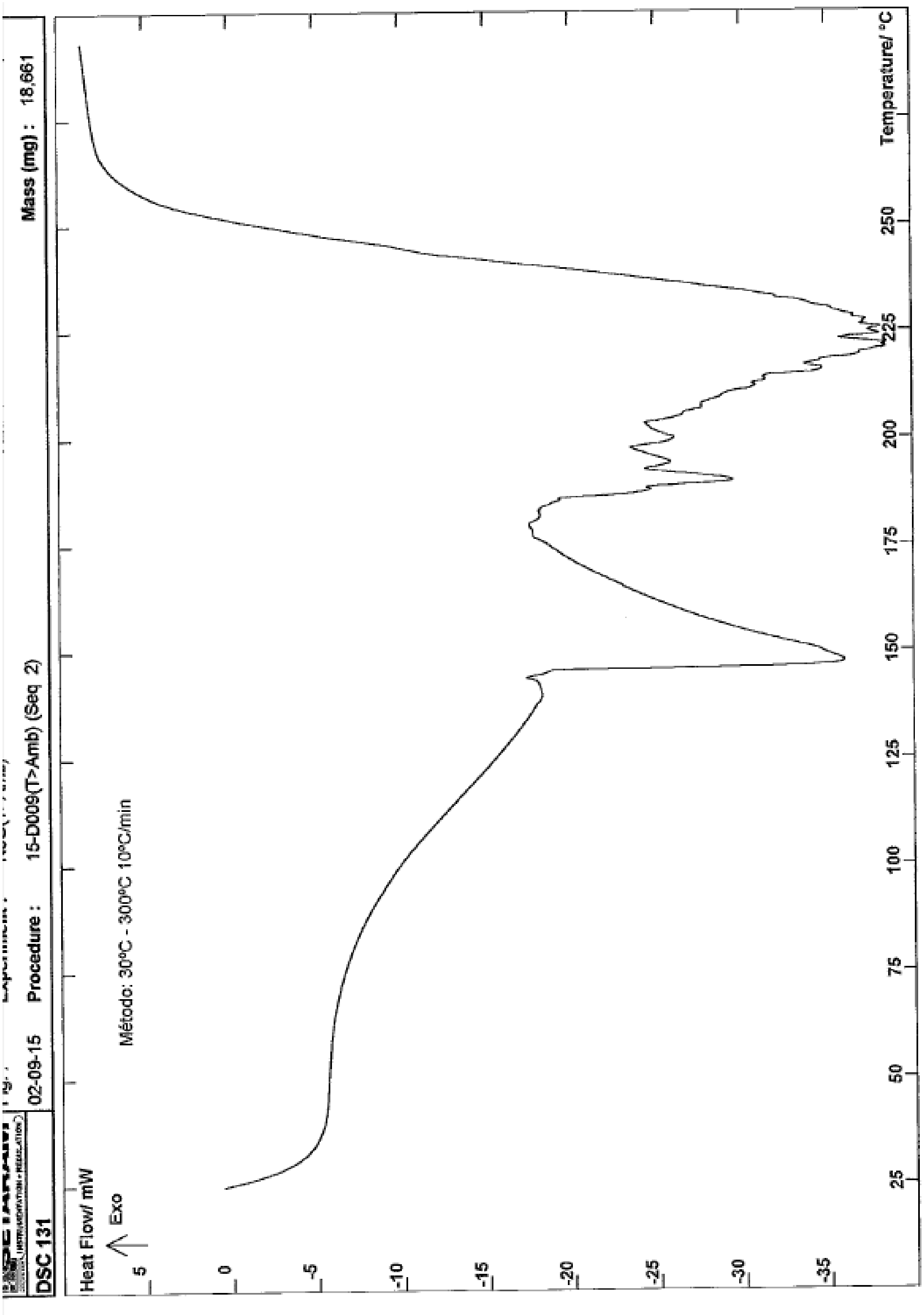

Figure 6-34 - Thermogram: Sample SI3 (T>Troom)

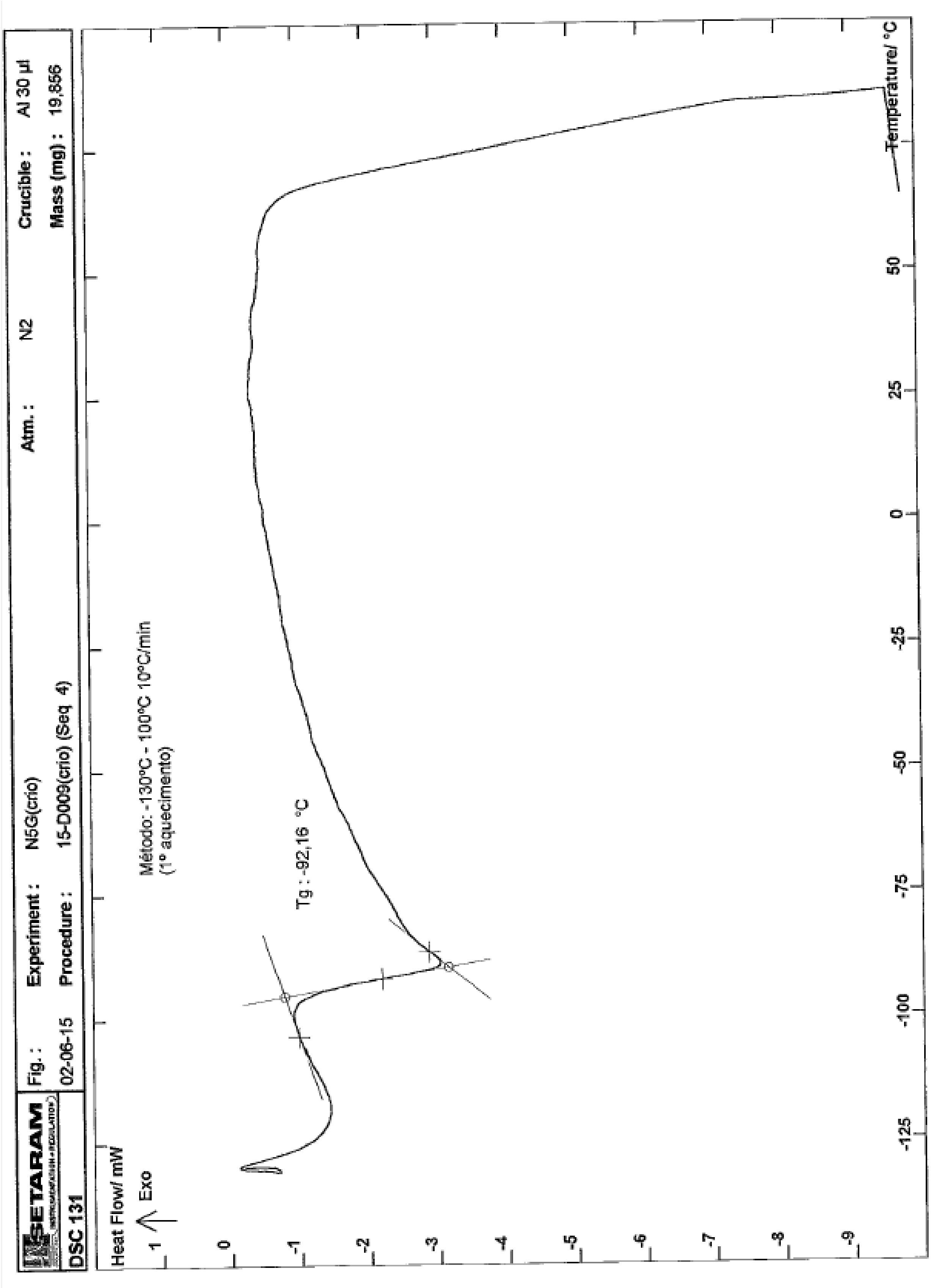

Figure 6-35 - Thermogram: Sample SI4 (cryo) - First heating

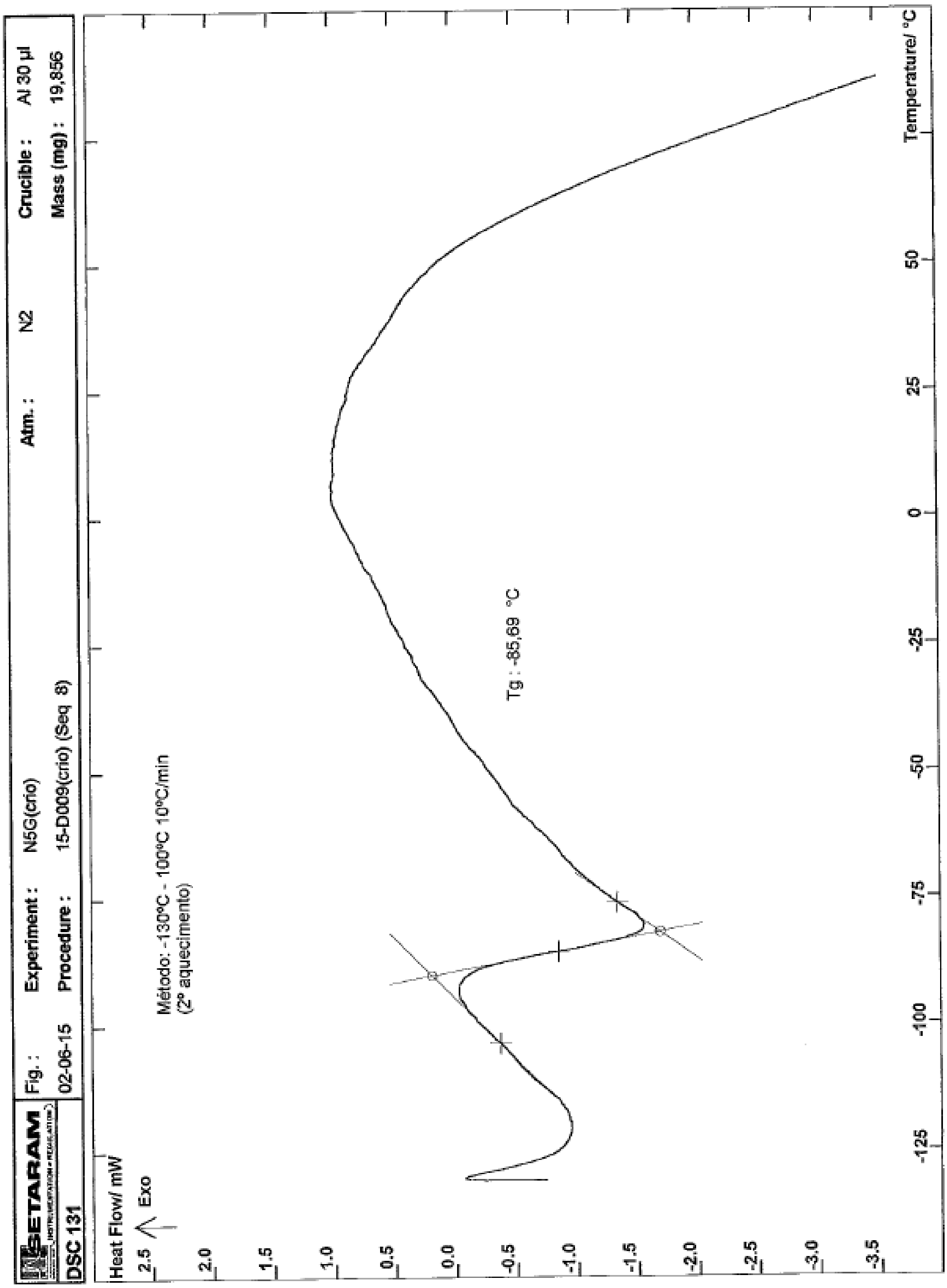

Figure 6-36 - Thermogram: Sample SI4 (cryo) - Second heating

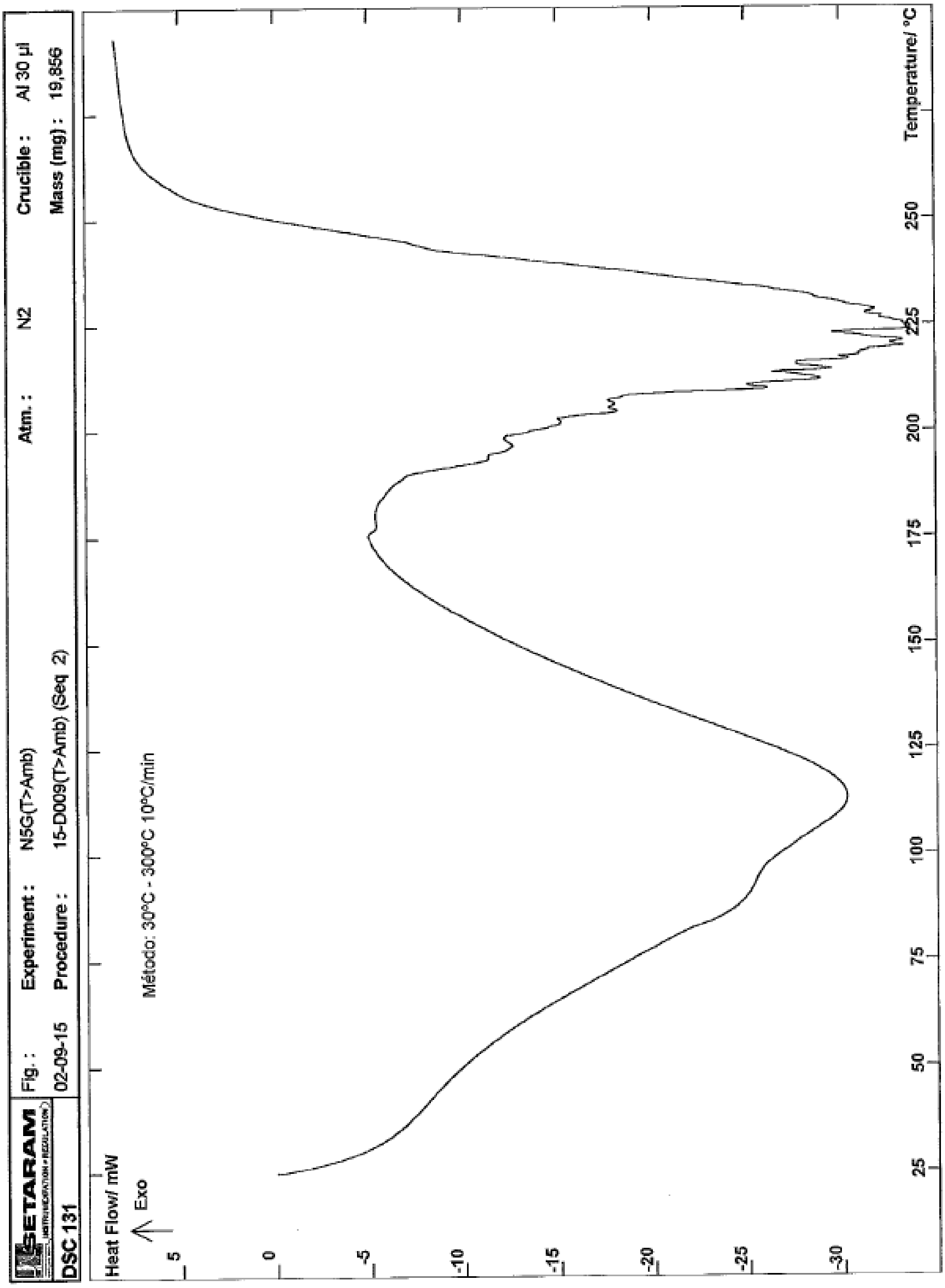

Figure 6-37 - Thermogram: Sample SI4 (T>Troom)

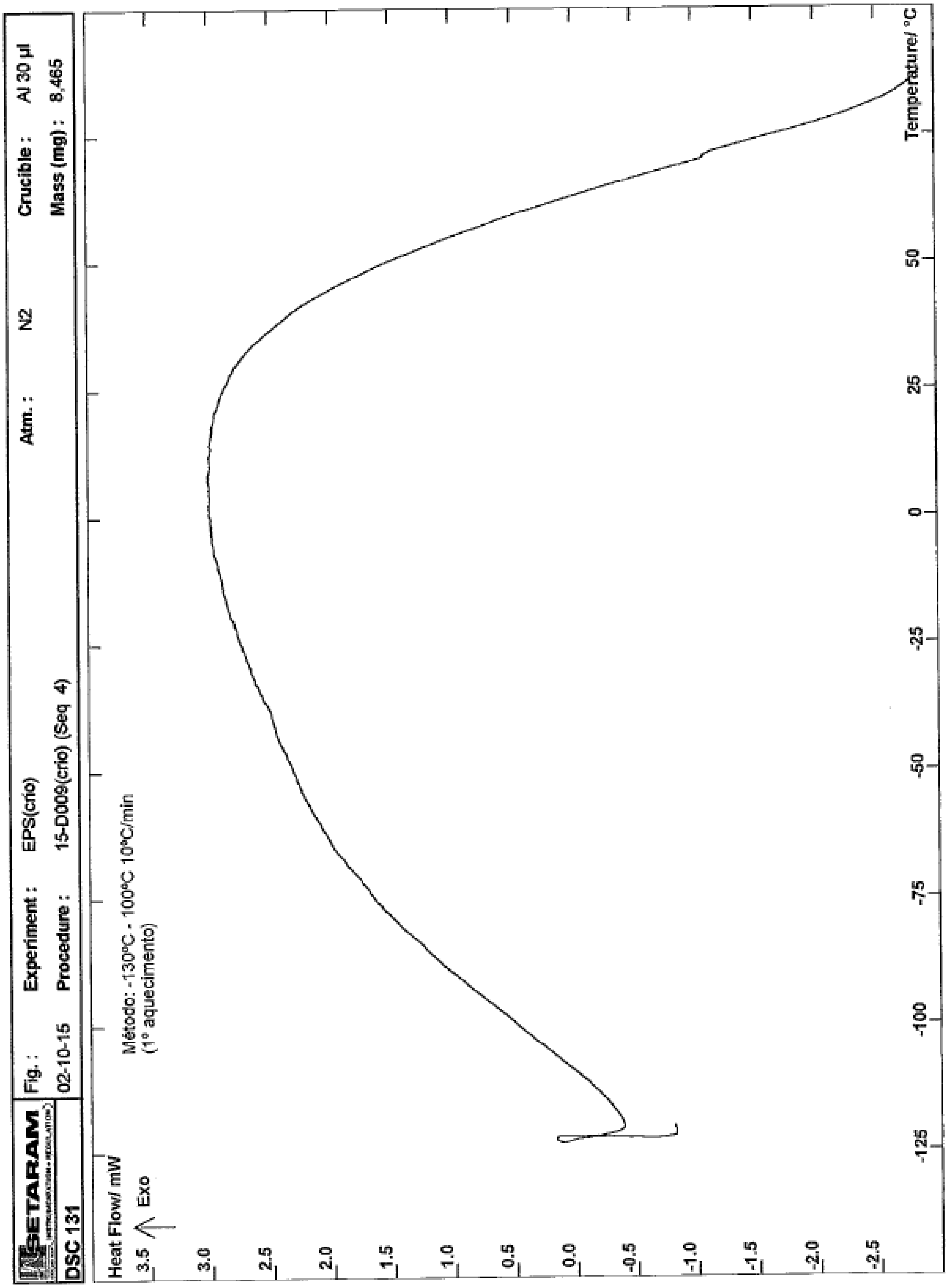

Figure 6-38 - Thermogram: FucoPol (cryo) - First heating

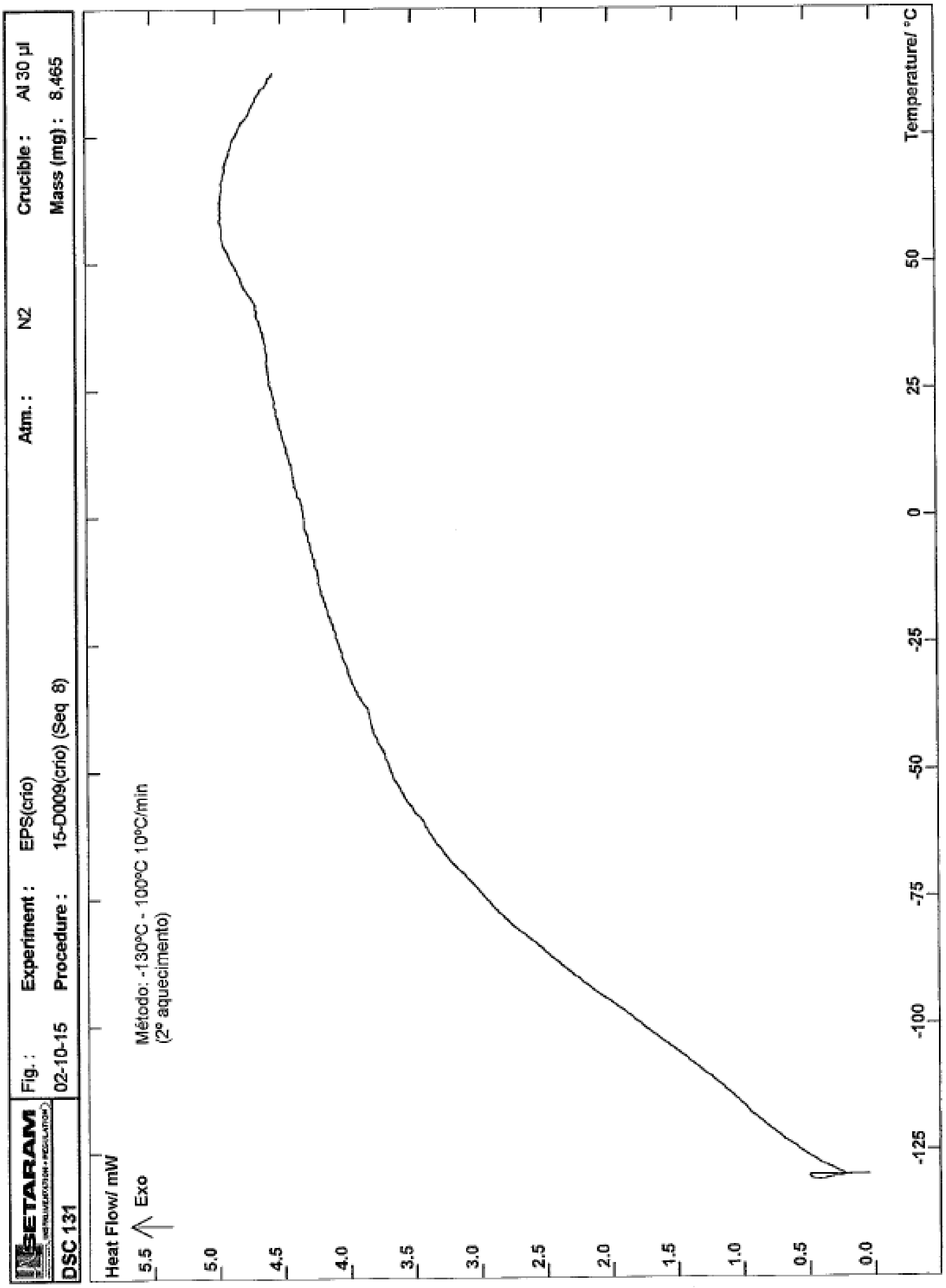

Figure 6-39 - Thermogram: FucoPol(cryo) - Second heating

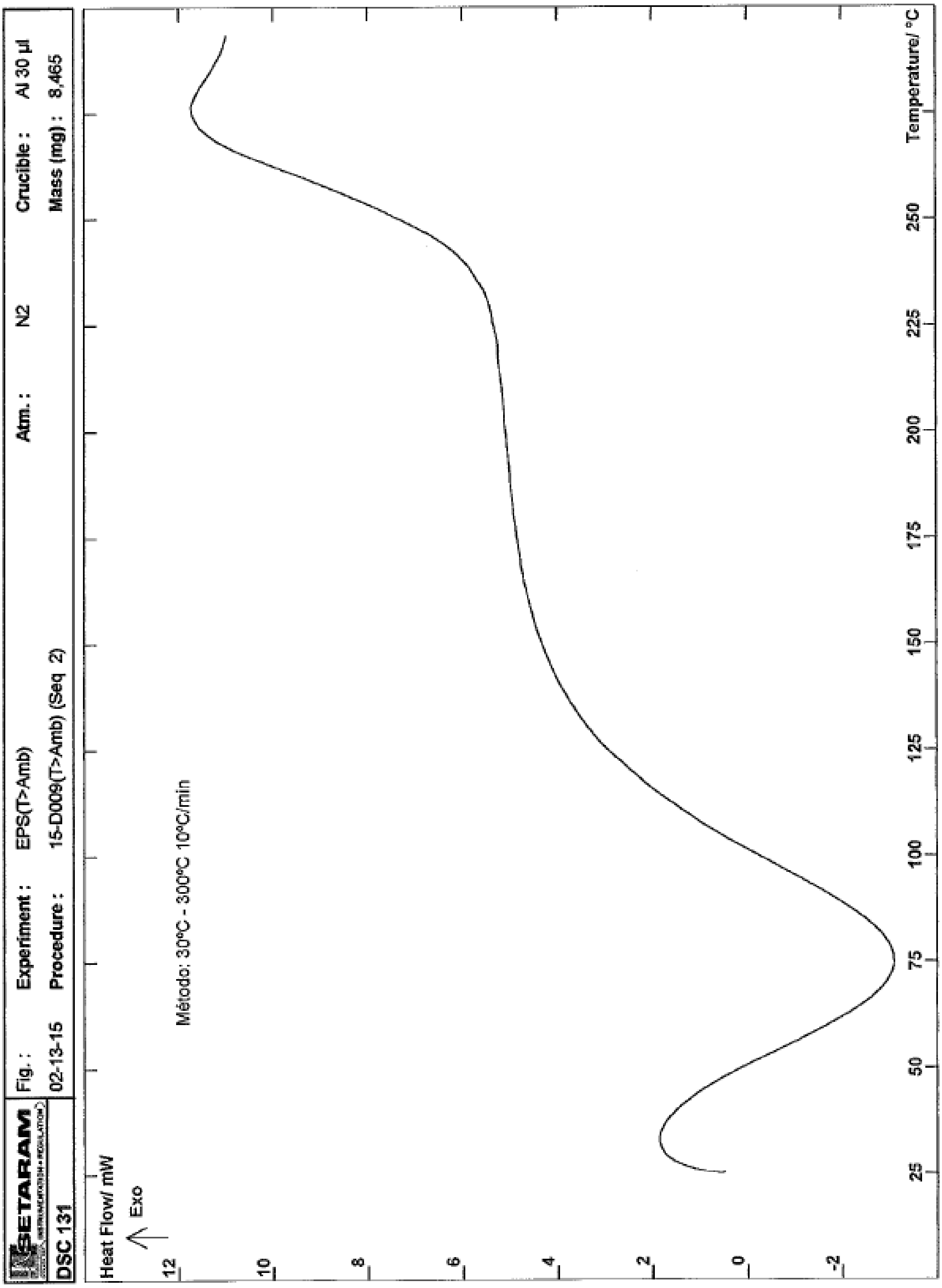

Figure 6-40 - Thermogram: FucoPol (T>Troom)

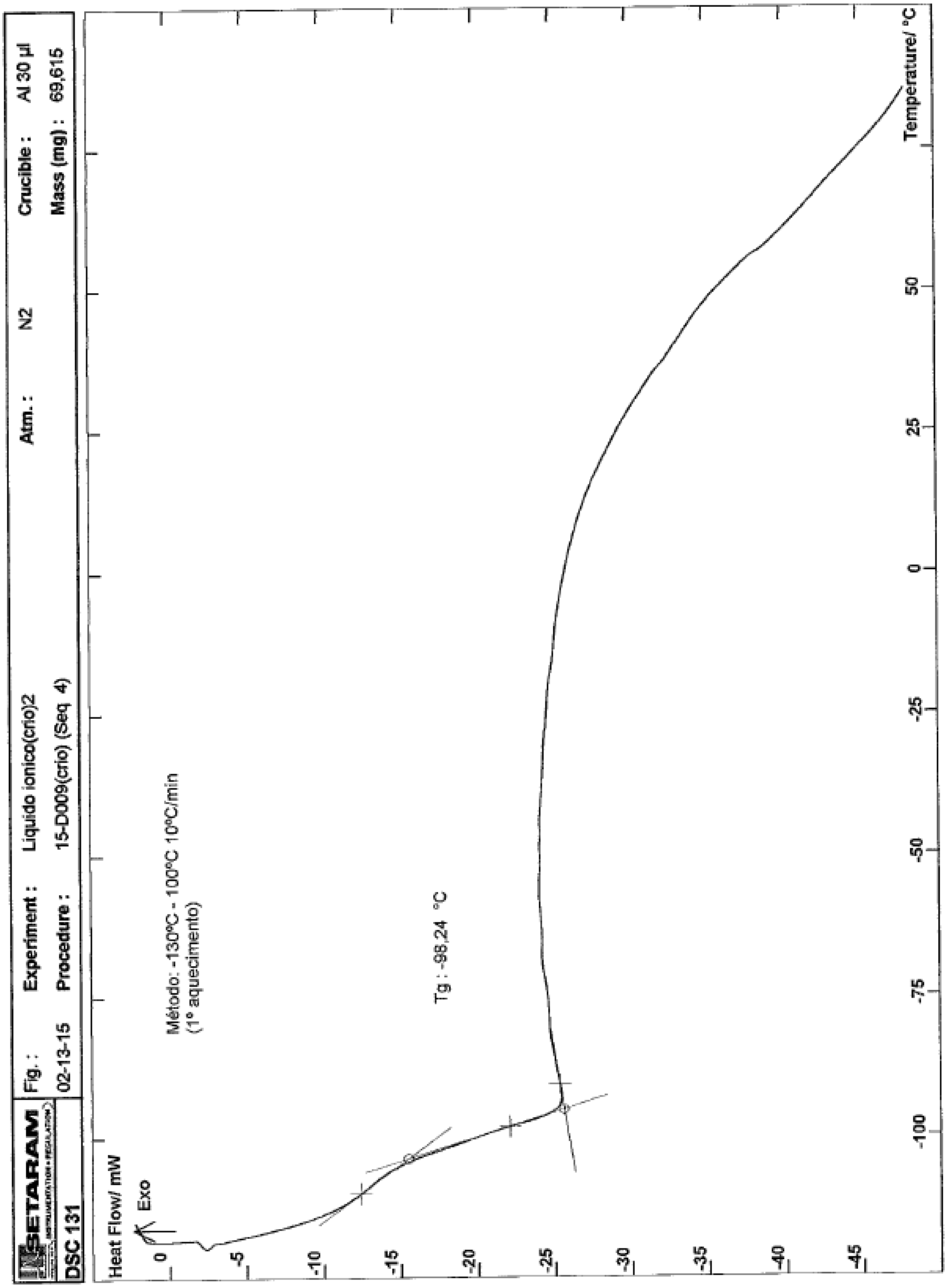

Figure 6-41 - Thermogram: Choline Acetate (cryo) - First heating

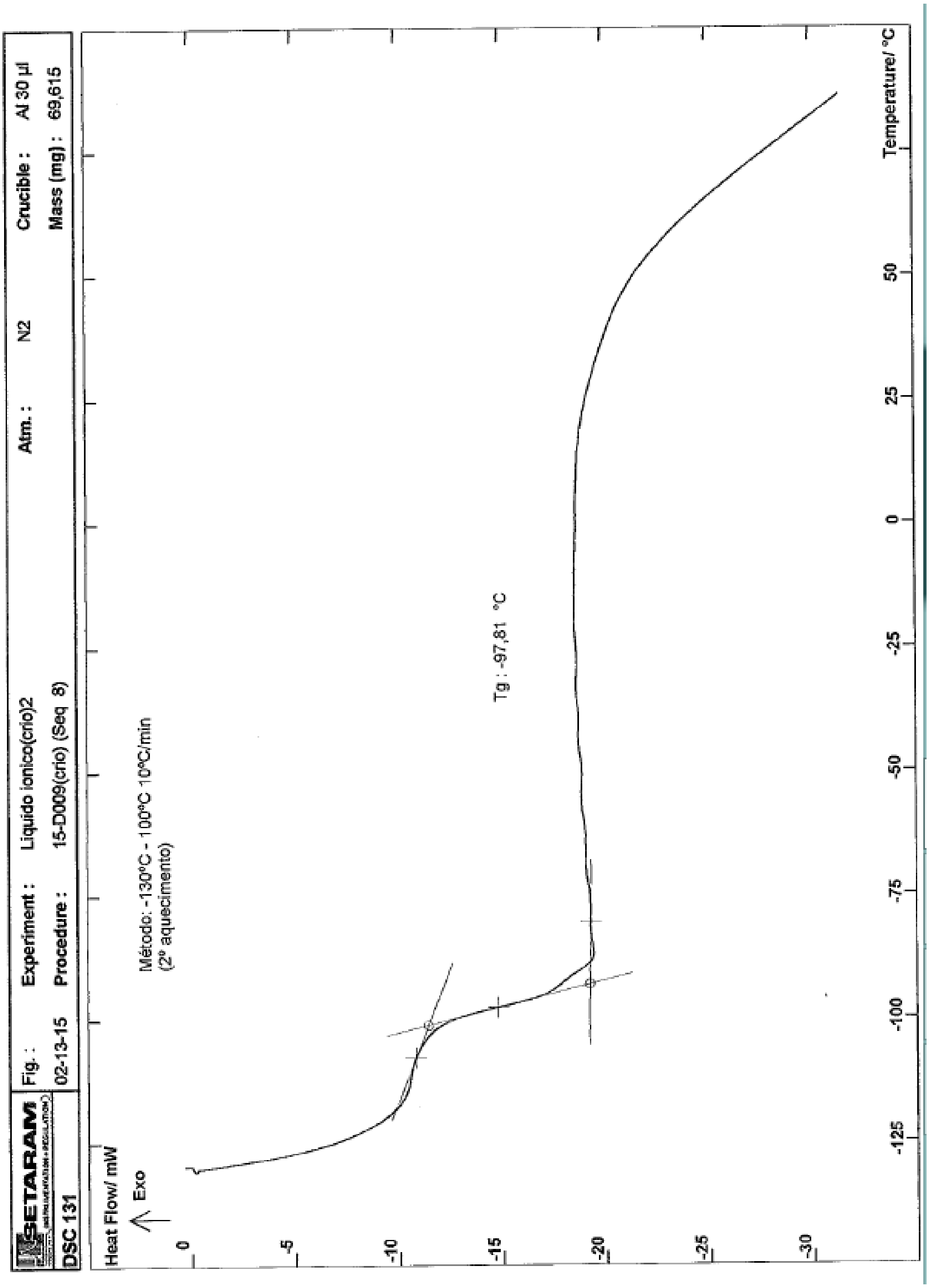

Figure 6-42 - Thermogram: Choline Acetate (cryo) - Second heating

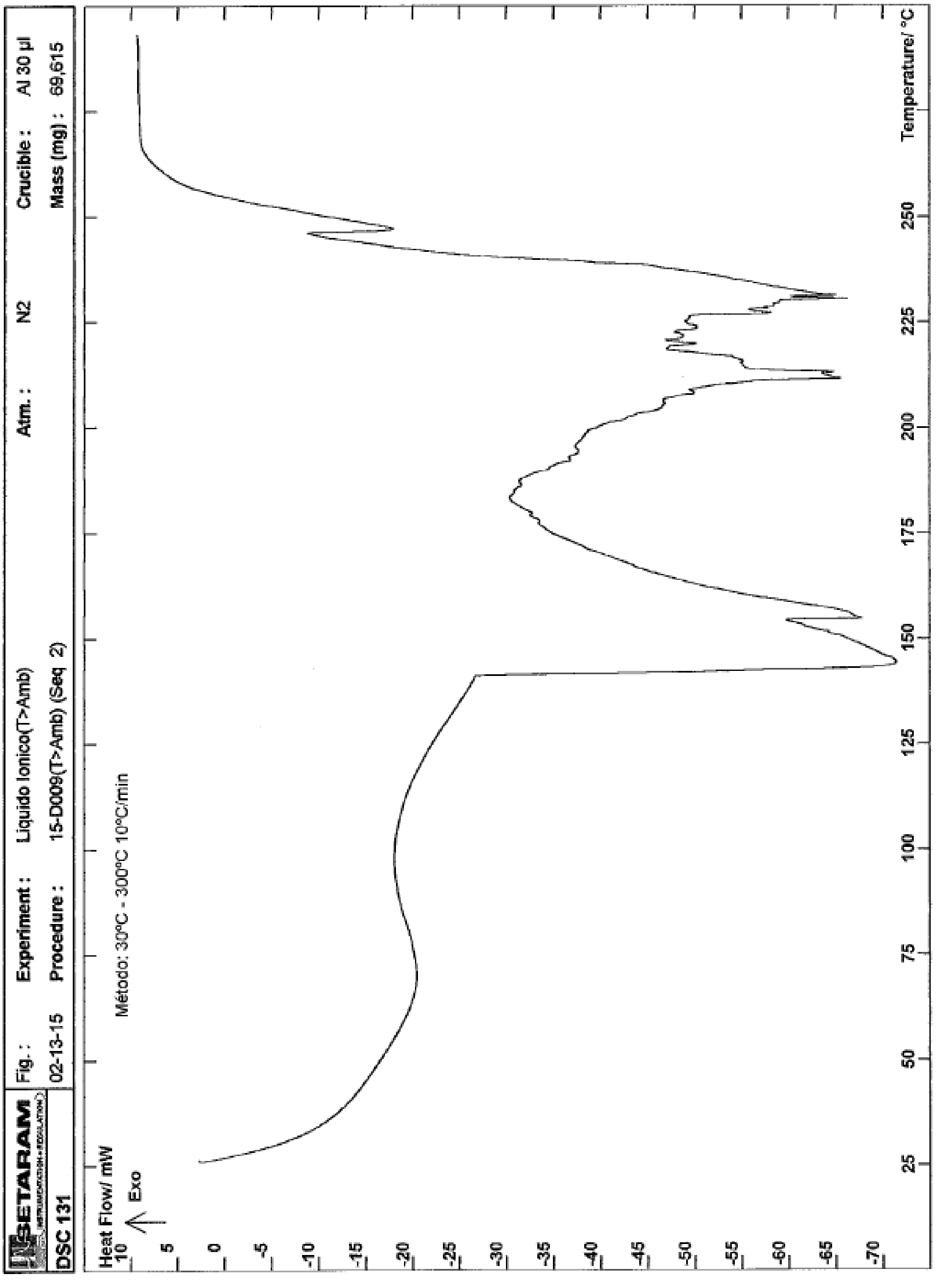

Figure 6-43 - Thermogram: Choline Acetate (T>Troom)

Appendice 8 – Osmolarity

Table 6-1 – Measurement of the osmolarity - samples subjected to the biocompatibility test

Sample	Test	Osmolarity (osmol)
Control	1	363
	2	366
FucoPol	1	333
	2	355
	3	358
	4	358
Choline Acetate	1	1905
	2	478
	3	362
	4	348
	5	347
SI1	1	1182
	2	423
	3	353
	4	348
SI2	1	1050
	2	410
	3	354
	4	346
SI3	1	1031
	2	495
	3	367
	4	349
SI4	1	1144
	2	578
	3	370
	4	350

Appendice 9 – Measurement of pH

Table 6-2 – Measurement of the pH - samples subjected to the biocompatibility test

Sample	Test	pH
Control	1	8.69
	2	8.69
	3	8.66
FucoPol	1	8.81
	2	8.54
	3	8.48
	4	8.50
Choline Acetate	1	6.27
	2	8.22
	3	8.36
	4	8.41
SI1	1	8.75
	2	8.45
	3	8.41
	4	8.40
SI2	1	8.81
	2	8.50
	3	8.42
	4	8.42
SI3	1	8.10
	2	8.52
	3	8.43
	4	8.46
SI4	1	8.49
	2	8.58
	3	8.47
	4	8.57