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"Quantitative analysis of residual detergents in decellularized biological matrices"

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ABSTRACT:

The growing demand for organ transplantation is a major problem for all diseases that lead to a loss or functional failure of tissues and organs. There is still a significant gap between the number of organ donors and recipients that declares a serious organ shortage problem. Tissue engineering offers an alternative solution with biological organs devoid of donor cells. The decellularization of biological tissues, to be used as a bioprosthesis, is, therefore, a fundamental process for the solution of the lack of tissues, especially in the case of xenogenic tissues. Decellularization is a treatment that allows the removal of antigenic factors and avoids the rejection phases by attenuating inflammatory processes; most decellularization methods use treatments with one or more detergents. The data on the implantation in humans of xenogenic valve substitutes treated with detergents are contradictory but highlight cases of low activity of the cells that repopulate the scaffold. Determining the residues of detergents that remain "trapped" in the extracellular matrix is fundamental since their desorption, even in very long times, could be cytotoxic or induce inflammation and therefore could contribute to the poor functionality of the bioprosthesis. Up to now, the determination of the residual detergents has been carried out almost exclusively on the washes of the decellularized matrix. The possibility of a direct analysis of the residual amount of detergent shows that the detergents are not completely removed from the matrices. In this work, a procedure that involves the complete digestion of the decellularized tissue and the analysis of the digested product by an HPLC equipped with a mass detector is used for direct quantification of detergent residues (SDS, Sodium Cast, Tergitol 15-s- 9 and Triton X-100) “trapped” in decellularized extracellular matrices of different origins (porcine larynx, porcine small intestinal submucosa, bovine pericardium). The method, compared to a previously developed one by the same research group, has proved to be applicable to practically all types of detergents.

Abbreviations:

TAH total artificial heart

TE tissue engineering

RM regenerative medicine

ECM extracellular matrix

SDS sodium dodecyl sulfate

COL sodium cholate

SIS small intestine submucosa

MW molecular weight

HPLC high-performance liquid chromatography

1 Introduction:

Organ transplantation currently represents the gold standard treatment for all diseases leading to irreversible organ failure. Despite efforts to increase the supply pool of suitable organs for transplantation, a significant gap still exists between the numbers of organ donors and recipients, highlighting the major problem of organ shortage, as shown in the Figure 1.1.

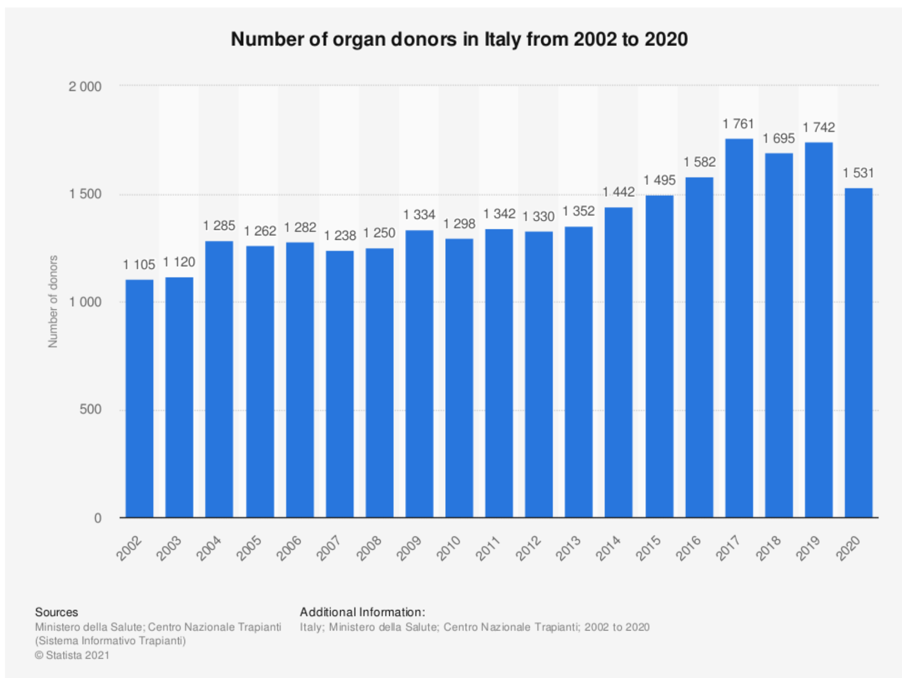


Figure 1.1 Number of organ donors in Italy from 2002 to 2020 *[1]*

The availability of artificial organs could become a viable alternative to organ transplantation.

1. One way of producing artificial organs is the self-therapy and/or tissue engineering (Figure 1.2) techniques approach. An organ not taken from the cadaver but built in the laboratory, thanks to the use of a bioreactor. A still biological organ seeded with the cells harvested from the patient (autologous cells). This is important to avoid any rejection action, which means to avoid the organ being recognized as foreign by the receiver with the consequent failure of the implant.

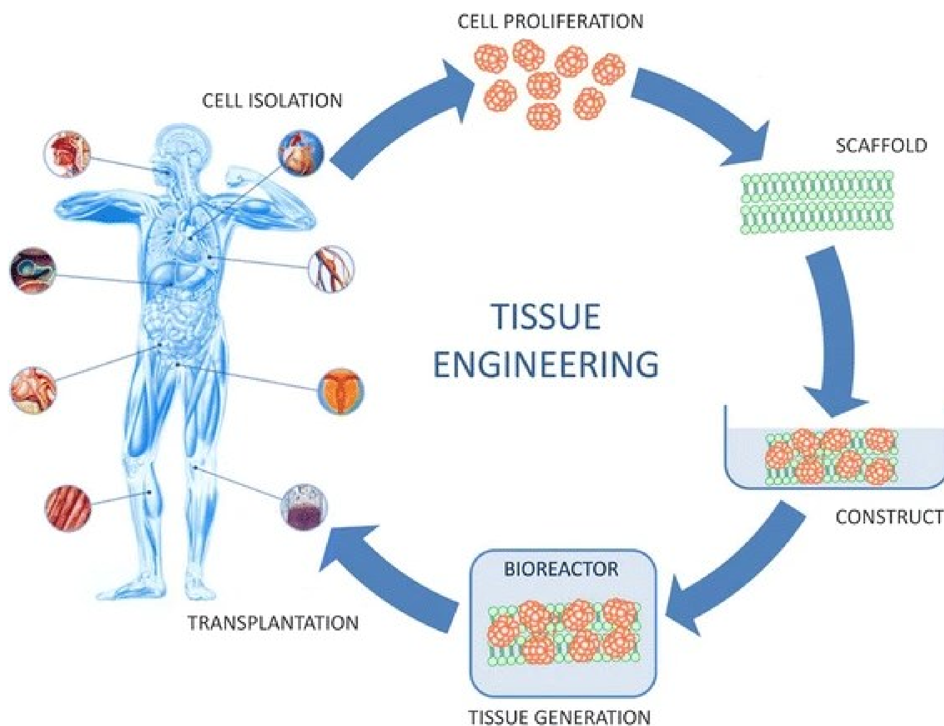


Figure 1.2 Tissue engineering *[2]*

2. The other solution is a mechanical organ. It consists in the use of completely artificial organs built in the laboratory. An example is shown below in Figure 1.3.

Figure 1.3 An employee of the company Carmat shows a total artificial heart (TAH) *[3]*

There is not the best choice. They are two hard and innovative solutions. Technology is advancing so that even a heart can be replaced by a mechanical one. On the other hand, tissue engineering techniques have also reached a good level of sophistication that it is possible to think about developing a totally biological heart *in vitro* in the laboratory.

A mechanical organ needs to work for a long time, which is approximate to the life of the patient, and it has to work in a special mood: with the absence of possible maintenance.
The biological one has to be produced so that once implanted, it behaves exactly as the real native organ.
Both techniques involve margins of risk.

1.1 Tissue engineering.

On 7 January 2022, a 57-year-old man with terminal heart disease received a successful xeno-transplant of a genetically modified pig heart. The historic surgery was conducted by the University of Maryland School of Medicine (UMSOM) in Baltimore, U.S.A. [4]
Xenografts are grafts harvested from a species different from that of the recipient. [5]

The concept of tissue engineering dates back to the 1930s when Charles Lindbergh, who was better known for his aerial activities, went to Rockefeller University and began to study the culturing of organs *ex vivo* in order to repair or replace damaged or diseased organs. [6]
It was only in the late 1980s that scientists, engineers, and clinicians began to conceptualize the possibility of *de novo* tissue generation to address the acute shortage of organs. Tissue engineering technology is envisaged for the generation of simple tissues at present to the future generation of organs in attempts to potentially confront the transplantation crisis. Regardless of the tissue or organ to be engineered, the basic concept for tissue engineering technology involves cell sourcing, isolation, and proliferation, fabrication of biodegradable scaffolds or polymers, seeding of cells on scaffolds to create constructs and the application of *in vitro* or *in vivo* bioreactor options. [2]
Tissue engineering and regenerative medicine (TE/RM) share the same ultimate target: the creation of functional tissues or whole organs and their use as ‘replacement parts’ for the human body [7]. Successful achievement of this goal will play a ground-breaking role in clinical transplantation [8].
A common approach of TE/RM is to create a structural and molecular environment that accurately mimics the properties (mechanical, geometrical, and biological) of the native organ in order to support the recipient’s cells and create an autologous tissue/organ.
Although there have been several attempts to produce synthetic scaffolds, they have produced only constructs that partially mimic the natural vascular network. Recently, a new technology

was introduced to overcome this problem by using whole organ decellularization to create a three-dimensional (3D) extracellular matrix (ECM) that preserves the native tissue architecture, including the vasculature. [9]

The patient becomes an autologous donor, because thanks to a biopsy, as reported in Figure 1.4, tissue is taken from the patient, from which the cells, separated from the matrix, are harvested. Only the cells that are cultured and proliferated *in vitro* are preserved, so that they can be used to repopulate a scaffold, cells are needed in abundance. Then, cells are seeded on a matrix enriched with biochemical signals and stimulated with mechanical stimuli, in order to obtain a functional construct. The final engineered construct can be implanted in the patient to replace the damaged tissue/organ.

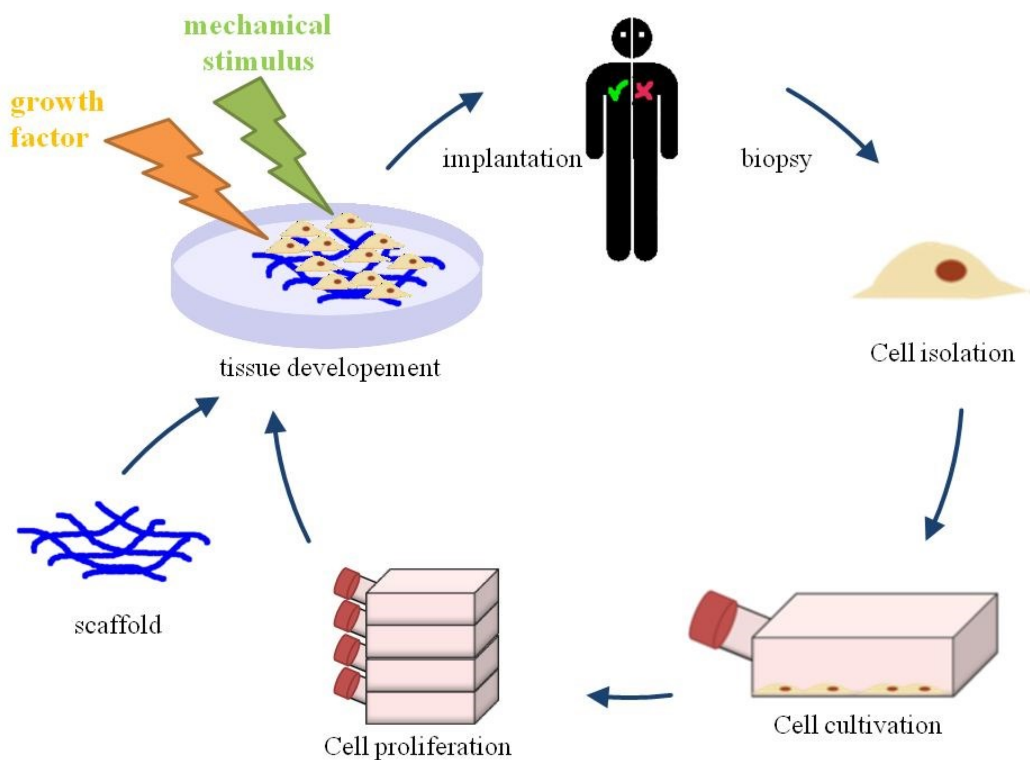


Figure 1.4 Iter of tissue engineering *[10]*

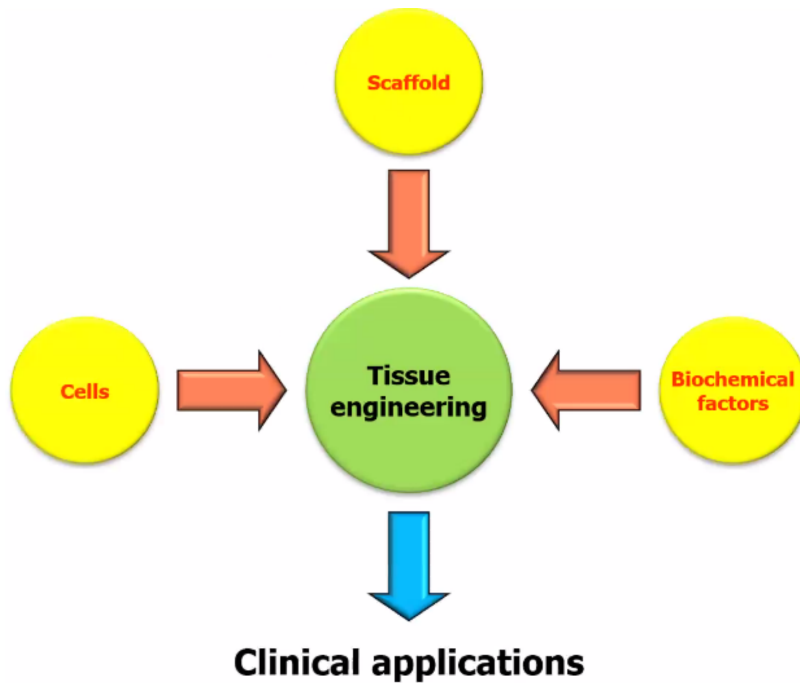


Figure 1.5 The three basic elements are: cells, biomaterials as scaffolds and biomolecules as growth factors. In reality, there are three other important components linked to tissue engineering: the overall engineering of the process (the bioreactor), the biomechanical aspects of the design (it is important to understand, for example, how much a decellularization treatment can damage the tissue and organ in terms of mechanical resistance) and the informatics aspects of TE, bioinformatics is useful for studying the genome/proteome, the ability of certain cells to respond to certain stimuli.

The type of scaffold used for TE application and constituted by extracellular matrix, changes according to:

- starting tissue (pericardium, valve, larynx, small intestine submucosa)
- species of origin (porcine, bovine)
- decellularization methods
- sterilization methods.

1.2 Decellularization.

The goal of a decellularization protocol is to efficiently remove all cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining extracellular matrix (ECM). Any processing step intended to remove cells will alter the native three-dimensional architecture of the ECM.

Tissues are composed of both cellular material and ECM arranged in variable degrees of compactness depending on the source of the tissue. The ECM must be adequately disrupted during the decellularization process to allow for adequate cellular exposure to the chaotropic agents and to provide a path for cells to be removed from the tissue. The intent of most

decellularization processes is to minimize the disruption and thus retain native mechanical properties and biologic properties. [11]

The matrix is an extracellular entity composed mainly of collagen, elastin, proteoglycans and glycosaminoglycans; in each tissue these components are organised differently but in a very precise way (Figure 1.6). A compromise has to be found between the total removal of the cellular component and the attempt to preserve the integrity of the extracellular matrix remaining from the decellularization treatment. The ECM contains growth factors and other bio-inductive factors, which facilitate cell attachment, tissue integration, remodelling, and development. The ECM also provides organ-specific physical, biochemical, and biomechanical properties.

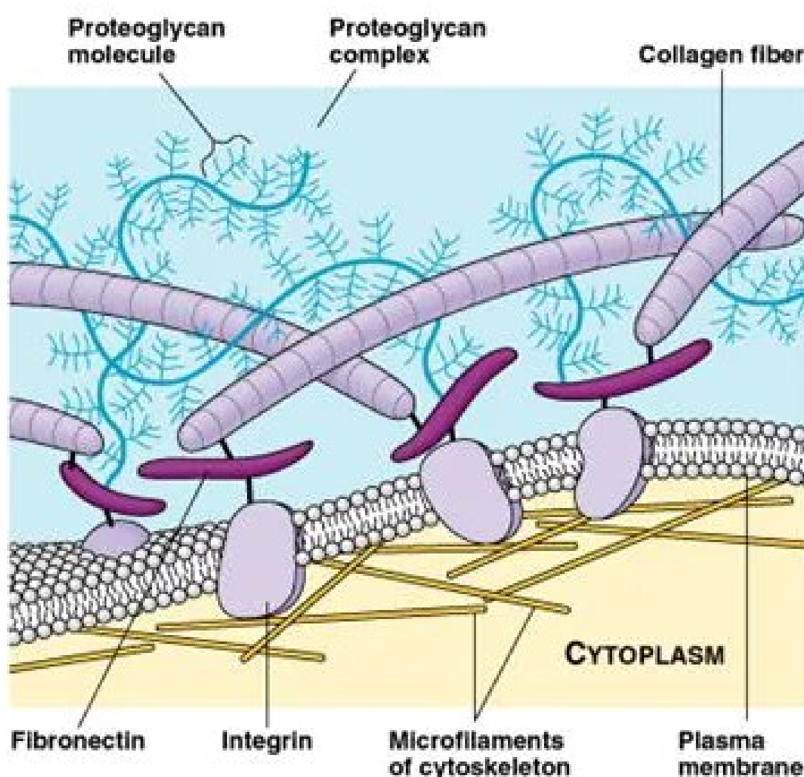


Figure 1.6 Interaction between a cell and ECM

Effective decellularization depends on many factors, including tissue density (greater or lesser cell density), thickness (pericardium is thin, while the heart muscle is much thicker), cellularity (ratio of cell number to extracellular matrix), and lipid content (brain vs. urinary bladder). [12] It is, therefore, necessary to choose the appropriate decellularization protocol in order to preserve as much as possible the physical, chemical, mechanical and biological characteristics of the tissue to be treated. Several decellularization methods are used for this purpose, essentially grouped into three categories: physical methods, chemical methods and biological

methods. It is important to emphasize that a combination of these procedures is usually used with the aim of maximising the effectiveness of decellularization.

1.2.1. Physical methods.

The physical treatments can include agitation or sonication, mechanical massage or pressure, freezing and thawing, and irreversible electroporation.

Mechanical agitation and sonication have been utilized simultaneously with a chemical treatment to assist in cell lysis and removal of cellular debris. Mechanical agitation can be applied by using a magnetic stir plate, an orbital shaker, or a low-profile roller. There are no studies on the optimal magnitude or frequency of sonication for cell disruption, but a standard ultrasonic cleaner appears to be as effective at removing cellular material as placing the tissue on an orbital shaker. For all these procedures, the optimal speed, volume of reagent, and duration of mechanical agitation are dependent on the composition, volume, and density of the tissue. These methods disrupt the cell membrane, release cell contents, and facilitate subsequent rinsing and cell contents removal from the ECM.

Cells on the surface of a tissue or organ (for example urinary bladder, small intestine, skin, amnion) can be effectively removed by mechanical abrasion in combination with enzymes, hypertonic saline, or chelating agents, all of which facilitate the dissociation of cells from their subjacent basement membrane. However, underlying ultrastructure and basement membrane integrity are invariably damaged by any direct application of mechanical force. Hydrostatic pressure requires relatively little time and can be more effective than detergents or enzymes for removing cells from a blood vessel and corneal tissues, although the baric formation of ice crystals may disrupt ECM ultrastructure. The increased temperature during pressure decellularization prevents ice crystal formation but may disrupt ECM due to the associated increase in entropy, which can be mitigated by colloids such as dextran.

Freeze-thaw processing effectively lyses cells within tissues and organs, but the resulting membranous and intracellular contents remain unless removed by subsequent processing. A single freeze-thaw cycle can reduce adverse immune responses such as leukocyte infiltration in vascular ECM scaffolds. Multiple freeze-thaw cycles may be used during decellularization without significantly increasing the loss of ECM proteins from tissue. Freeze-thaw processing does produce minor disruptions of the ECM ultrastructure and should therefore be used only

when such effects are acceptable in the final ECM product. The effect of freeze-thaw processing on mechanical properties is minimal for load-bearing, mechanically robust tissues.
Rapid freezing results in the formation of ice microcrystals within cytoplasmic membranes, causing their destruction and subsequent cell lysis. In this case, it is essential to control the temperature change to avoid possible damage to the ECM, and the process must be coupled with appropriate methods for removing residual cellular material from the tissue. [11]
Non-thermal irreversible electroporation (NTIRE) is a process in which electrical impulses lasting a few microseconds are applied using probes. These impulses act by altering the balance of the membrane potential, causing the formation of micropores on the membrane that lead to the loss of homeostasis and consequent cell death. Despite the good prospects for this technique, it has some limitations: the size of the probes is still rather small, thereby limiting the size of the tissue that can be decellularized. In addition, since it has been suggested that the mechanism of cell removal is mediated by the immune system, the ideal use of this technique is *in vivo*, thus limiting its applications. However, one recent study has investigated the *in vitro* use of NTIRE combined with vascular perfusion to decellularize porcine liver. [9]
These physical treatments are generally insufficient to achieve complete decellularization and must be combined with a chemical treatment.

1.2.2. Chemical methods.

Chemical reagents used for decellularization are:

- Acids and bases
- Hypotonic and hypertonic solutions
- Detergents: Ionic, Non-ionic and Zwitterionic
- Solvents

Acids and bases cause or catalyze hydrolytic degradation of biomolecules. Peracetic acid is a common disinfection agent which also acts as a decellularization agent by removing residual nucleic acids with minimal effect on the ECM composition and structure. Acetic acid damages and removes collagens with a corresponding reduction in ECM strength, but it does not affect sulfated glycosaminoglycans (sGAG).
Bases (for example calcium hydroxide, sodium sulfide, and sodium hydroxide) are harsh enough and commonly used to remove hair from dermis samples during the early stages of decellularization. However, bases can completely eliminate growth factors from the matrix and decrease ECM mechanical properties more significantly than other chemicals and enzymatic

agents. The primary mechanism by which bases such as sodium hydroxide reduce mechanical properties is the cleavage of collagen fibrils and disruption of collagen crosslinks.
Hypotonic solutions can readily cause cell lysis by simple osmotic effects with minimal changes in matrix molecules and architecture. For maximum osmotic effect, it is common for the tissues to be immersed alternately in hyper- and hypotonic solutions through several cycles. Hypertonic and hypotonic solutions also help rinse cell residues from the tissue.

DETERGENTS:

Detergents are amphipathic molecules, consisting of a polar head group and a hydrophobic chain (or tail), and exhibit unique properties in aqueous solutions in which they spontaneously form micellar structures. Membrane proteins are frequently soluble in micelles formed by amphiphilic detergents. Detergents solubilize membrane proteins by mimicking the natural cellular lipid bilayer normally inhabited by the protein. [13]
Detergents are commonly used in the decellularization of organs and tissues. Ionic, non-ionic, and zwitterionic detergents are able to solubilize cell membranes and dissociate DNA from proteins, therefore, they are effective in removing cellular material from tissue.
However, these agents also disrupt and dissociate ECM proteins as evidenced by their use in protein extraction procedures in tissue proteomics. The removal of ECM proteins and DNA by detergents increases with exposure time and varies with organ subunit, tissue type, and donor age. [14]
Combining multiple detergents increases ECM components loss which are important for the final scaffold, but also allows for more complete decellularization process.

A) **Non-ionic detergents** have been used extensively in decellularization protocols because of their relatively mild effects upon tissue structure. [11]
   Non-ionic detergents disrupt lipid–lipid and lipid–protein interactions but leave protein–protein interactions intact so that proteins within a tissue or organ following non-ionic detergent treatment should be left in a functional conformation.
   Triton X-100 (Figure 1.7) is one of the most studied non-ionic detergents, which has a hydrophilic polyethylene oxide chain and an aromatic hydrocarbon lipophilic or hydrophobic group. It can effectively remove cell residues from thicker tissues such as valve conduits where enzymatic and osmotic methods are insufficient, with concomitant ECM protein loss accompanied by a decreased adverse immune response *in vivo*. [9]

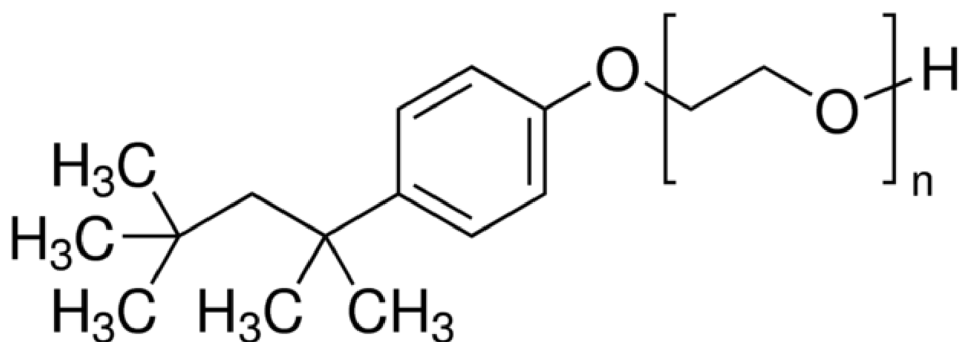


Figure 1.7 Triton X-100

Triton X-100 has recently been included in the list of compounds that the European Chemicals Agency (ECHA) defines as high-risk and whose sale and/or use is illegal from 4 January 2021 (except for specific authorizations). For this reason, research is under way into possible substitutes, including Tergitol, which could bring satisfactory results.

Tergitol 15-S-9 (Figure 1.8) is a linear non-ionic surfactant that is secondary ethoxylated alcohol. This clear liquid surfactant is compatible with anionic, cationic and other non-ionic surfactants.

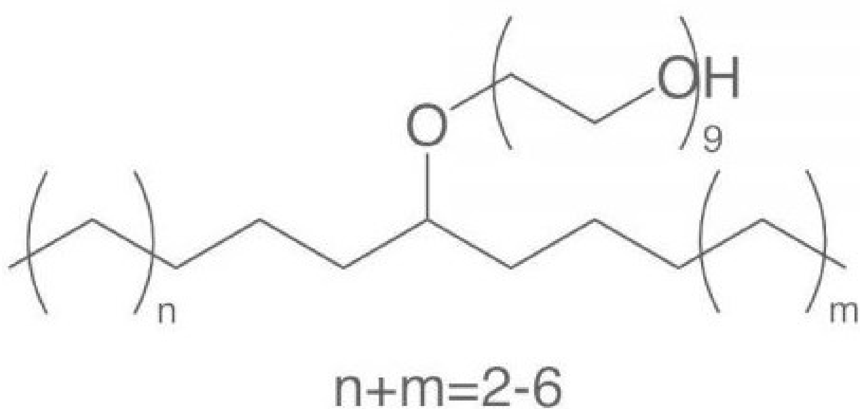


Figure 1.8 Tergitol 15-S-9

B) **Ionic detergents** contain a head group with a net charge that can be either cationic or anionic. They also contain a hydrophobic hydrocarbon chain or steroidal backbone. The critical micelle concentration of an ionic detergent is determined by the combined effect of the head group repulsive forces and the hydrophobic interactions of the tails. [13]

Ionic detergents are also effective in solubilising the cytoplasmic membrane but tend to denature proteins by destroying the interactions they form with each other. [11]
It is therefore necessary to analyse and dose their use carefully.
Among the best known are sodium dodecyl sulfate (SDS), sodium deoxycholate (SD), Triton X-200 and Sodium Cholate (COL).
SDS appears more effective than Triton X-100 for removing nuclei from dense tissues and organs such as the kidney and temporomandibular joint while preserving tissue mechanics. The addition of a detergent such as SDS to a decellularization protocol can make the difference between complete and incomplete cell nuclei removal but has the associated drawback of ultrastructure disruption and growth factor elimination. SDS (Figure 1.9) is typically more effective for removing cell residues from tissue compared to other detergents but is also more disruptive to ECM. It is an anionic surfactant used in many cleaning and hygiene products. Its hydrocarbon tail combined with a polar "headgroup" gives the compound amphiphilic properties and so makes it useful as a detergent. [9]

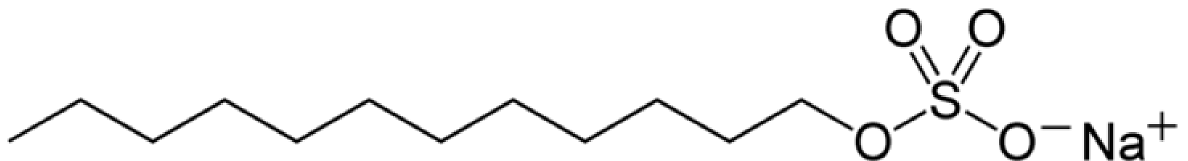


Figure 1.9 Sodium Dodecyl sulfate

Sodium deoxycholate also results in satisfactory removal of nuclear material, but at the same time it appears to cause more damage to the ECM structure than SDS. Sodium deoxycholate has been used in combination with other zwitterionic detergents with the intention of decellularizing the nerve tissue, however a combination of these and Triton X-200 is more effective in achieving full decellularization. Triton X-200 is very effective in removing cells in thin tissues, but with a certain level of damage to the infrastructure.
Sodium Cholate (Figure 1.10) belongs to the class of ionic detergents which act by solubilising nuclear and cytoplasmic membranes, acting in particular on phospholipid complexes and solubilising membrane proteins. This removes cell debris still present in the tissue that has resisted the action of the non-ionic detergent.
It is also used because it enables the solubilisation of sphingolipid-cholesterol complexes, which are responsible for the insolubility of alkaline phosphatase, an

enzyme believed to be responsible for triggering the process of the valve calcification process.

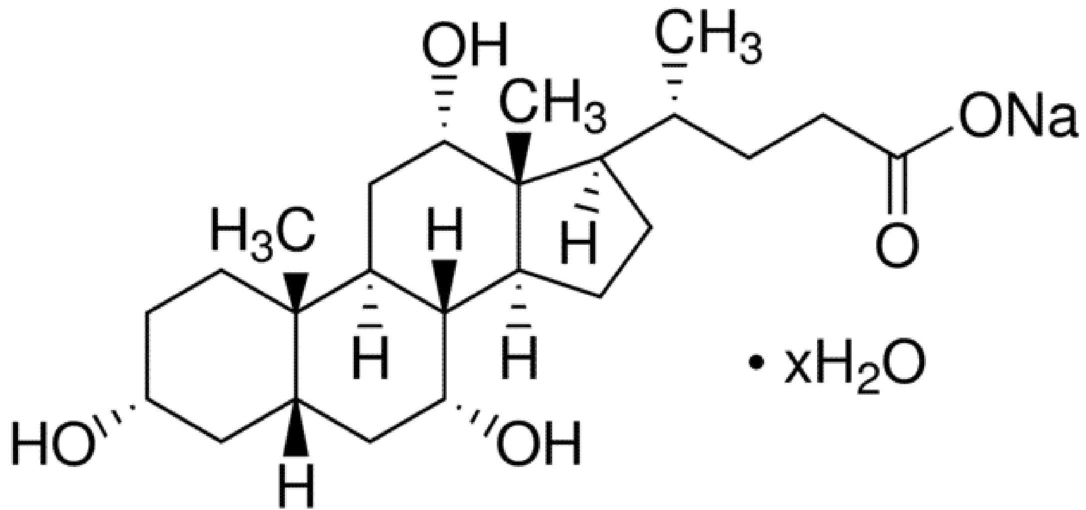


Figure 1.10 Sodium Cholate

C) Zwitterionic detergents combine the properties of ionic and non-ionic detergents and are in general more deactivating than non-ionic detergents. They have, however, found uses in structural studies of membrane proteins. The zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonium]- 1-propanesulfonate (CHAPS) is most effective for cell removal from thinner tissues such as lung [9] and may be ineffective for decellularization of thicker tissues, even when used in combination with SDS or alone for relatively acellular tissues.

Care must be taken to flush residual chemicals from ECM after decellularization, particularly detergents such as SDS that penetrate into thick or dense tissues. Cytotoxicity is possible even at reduced agent concentrations and will inhibit or completely negate the beneficial properties of a cell-free ECM scaffold. Even thin tissues such as valve leaflets require multiple (more than six) agitated washes to completely remove detergents.

Alcohols such as glycerol aid in tissue decellularization by dehydrating and lysing cells. Phospholipids in valve leaflets and conduits contribute to prosthesis calcification and failure and can be extracted using alcohols. In fact, alcohols such as isopropanol, ethanol, and methanol are more effective than lipase in removing lipids from tissue and are capable of rendering adipose tissue lipid-free in a relatively brief period. Methanol in combination with chloroform has been used during delipidization of tissues. Caution should be used in treating tissues with

alcohols such as ethanol and methanol due to their use as tissue fixatives in histology, their ability to precipitate proteins, and the damage they cause to ECM ultrastructure.
Acetone can also be used to remove lipids during decellularization. However, like alcohols, the use of acetone as a tissue fixative and its damage of ECM ultrastructure warrants conservative use, especially for biological scaffolds used in load-bearing clinical applications. In comparison to detergent treatments, acetone crosslinks ECM to produce stiffer scaffolds with mechanical properties further removed from those of native tissue. Tributyl phosphate (TBP) is an organic solvent with viricidal properties. For decellularization of dense tissues such as tendon, TBP appears to be more effective than detergents such as Triton X-100 and SDS, with varying effects on retention of ECM constituent and native mechanical properties. [9]

1.2.3 Biologic agents.

Enzymatic agents:
Enzymes reported in tissue decellularization protocols include nucleases, trypsin, collagenase, lipase, dispase, thermolysin, and α-galactosidase. Enzymes can provide high specificity for removal of cell residues or undesirable ECM constituents. However, complete cell removal by enzymatic treatment alone is difficult and enzyme residues may impair recellularization or evoke an adverse immune response. Nucleases (DNases and RNases) cleave nucleic acid sequences and can therefore aid in removal of nucleotides after cell lysis in tissues.
Endonucleases such as benzonase may be more effective than exonucleases because they cleave nucleotides mid-sequence and thereby more effectively fragment DNA in preparation for its removal. Likewise, non-restriction endonucleases will more effectively fragment DNA compared to their sequence-dependent counterparts. Trypsin is a serine protease commonly used as an enzymatic decellularization agent. However, ECM proteins such as collagens have limited resistance to trypsin cleavage and tissue exposure to trypsin should therefore be used with caution. In comparison to detergents, trypsin is more disruptive to elastin and collagen and slower to remove cells but shows better preservation of GAG content.
Trypsin disruption of ECM can be correlated to changes in mechanical properties. Removal of cells and ECM constituents by trypsin is time-dependent, and complete decellularization by trypsin alone may require lengthy incubation even for thinner tissues such as valve leaflets. Trypsin can be used effectively to disrupt tissue ultrastructure and improve penetration of subsequent decellularization agents; therefore, exposure to trypsin as the initial step in a tissue decellularization protocol may be desirable or even necessary, particularly for complete

removal of cell nuclei from dense tissues. Collagenase may be used during decellularization, but only when ultrastructure preservation and maximum collagen retention are not critical to the intended clinical application of the resultant ECM.
Lipase aids in delipidization but is typically insufficient to remove all lipids when used alone. After delipidization of dermis, direct comparison of trypsin and dispase treatments showed superior decellularization by dispase accompanied by increased ECM disruption. The same study also showed greater cell infiltration in dispase-treated tissue after four weeks of subcutaneous implantation. Dispase and trypsin can be used successively to improve cell removal from thicker tissues such as dermis if used in combination with detergents, and repeated treatments with dispase may further improve decellularization.
Using an enzyme such as dispase or thermolysin as the sole decellularization agent is only effective for removing cells on the surface of a tissue and is likely to require mechanical abrasion for complete cell removal. With regard to the underlying basement membrane and ECM, thermolysin is less disruptive compared to dispase. Decellularized xenogeneic tissues can be treated with α-galactosidase to reduce the immunogenic cell surface antigen galactose-a-(1,3)-galactose (Gal epitope), although the immunomodulatory effect of Gal epitope does not adversely affect in vivo remodelling of xenogeneic ECM. [9]

Non-enzymatic agents:

Chelating agents such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetra acetic acid (EGTA) aid in cell dissociation from ECM proteins by sequestering metal ions. It is likely that chelating agents contribute to subtle disruptions in protein-protein interactions by the same mechanism. Chelating agents alone are insufficient for superficial cell removal even with agitation and they are therefore typically used in combination with enzymes such as trypsin or detergents. The combined efficacy of chelating agents and simple hyper- or hypotonic solutions for decellularization is unknown.
Antibiotics and antimycotics such as penicillin, streptomycin, amphotericin B, and sodium azide may be used to minimize microbial contamination during decellularization but also present a potential regulatory hurdle for clinical biologic scaffolds. [9]

A decellularization protocol generally begins with lysis of the cell membrane using physical treatments or ionic solutions, followed by separation of cellular components from the ECM using enzymatic treatments, solubilization of cytoplasmic and nuclear cellular components

using detergents, and finally removal of cellular debris from the tissue. These steps can be coupled with mechanical agitation to increase their effectiveness. Following decellularization, all residual chemicals must be removed to avoid an adverse host tissue response to the chemical. It is important to understand how one can then evaluate the goodness of the treatment applied to decellularize a tissue/organ and one has to apply sequential techniques/assays to evaluate, for example how much DNA is still contained within the organ, whether one has been able to remove it effectively or whether there are still DNA residues, which may lead to an adverse response from the tissue when repopulated with the recipient's autologous cells.

1.3 Biological matrices

Clinically successful extracellular matrix (ECM) materials predominantly consist of allogeneic and xenogeneic decellularized tissues. Those tissues commonly in use are urinary bladder matrix, skin, pericardium and small intestine submucosa (SIS), which have all been used for a variety of clinical applications and are derived from human, bovine and porcine sources. [15]

1.3.1 Porcine larynx

The porcine larynx is a tubular structure whose walls are made of cartilage, perichondrium, striated muscle, connective tissue, and mucosa. Anatomical subdivisions were extrapolated from humans and are the supraglottis (which includes the epiglottis), glottis, and subglottis. The gross anatomy is very similar to that of the human larynx. One study confirms that the pig larynx provides a valuable preclinical model for laryngeal transplantation. [16]
All the tubular structures that make up the porcine apparatus are lined by pseudostratified ciliated epithelium with calico cells (Figure 1.11). It is responsible for one of the main defence systems of the respiratory mucosa, the mucociliary apparatus. This system is made up of the cilia of the respiratory epithelium cells, together with the secretions of the calico cells and seromucous glands that appear underneath the respiratory mucosa and their main function is to eliminate particles that enter through the inhaled air. [17]

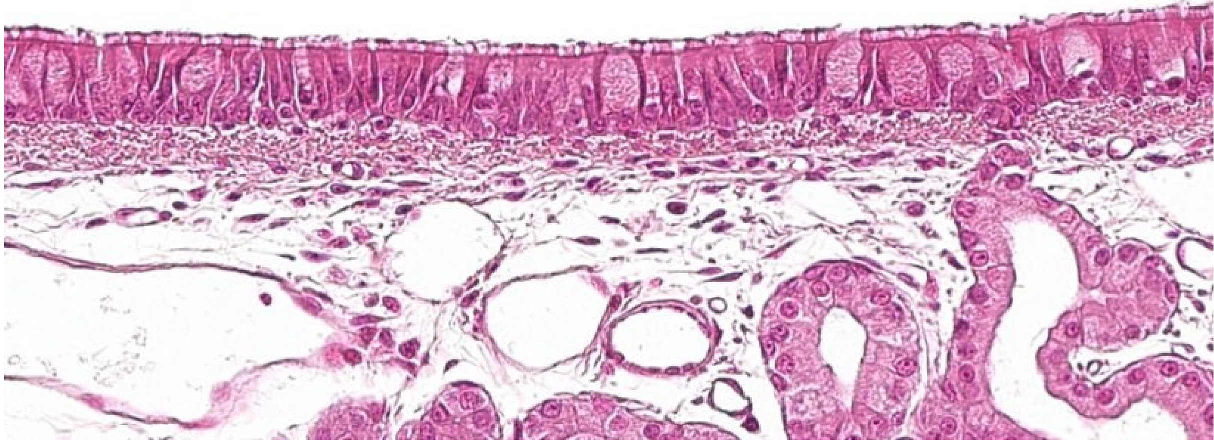


Figure 1.11 Pseudostratified ciliated epithelium with calyciform cells characteristic of the porcine larynx *[17]*

1.3.2 Porcine Small Intestinal Submucosa (pSIS)

Among all the decellularized matrices, small intestinal submucosa (SIS) is one of the earliest and most widely researched and applied biomaterials. [18]

SIS, produced from submucosal layer of porcine intestine, is an acellular, naturally occurring collagenous ECM material. [19] More than 90% of SIS is collagen, which is also the main component of several tissues (Figure 1.12)

SIS also contains preserved bioactive factors, including glycosaminoglycans, glycoproteins (fibronectin), and abundant growth factors such as basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1). [20]

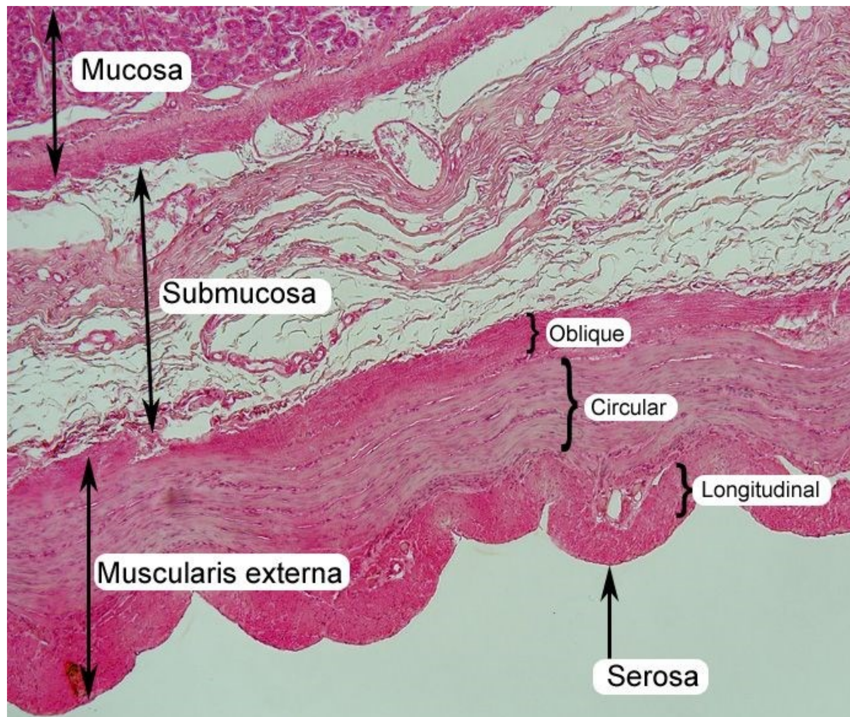


Figure 1.12 Histological structure of stomach

Due to the excellent angiogenic activity, SIS is widely used in tissue engineering for tissue remodelling and repair. [21] The microstructure of SIS provides the cellular microenvironment for adhesion, proliferation and survival; and the intrinsic growth factors or cytokines would guide directional differentiation of the cells, tissue remodelling and repair. All these processes depend on appropriate decellularization treatments. There are plenty of articles and reports about the preparation of decellularization of SIS. [22] The advantages of SIS include relatively rapid degradation by non-immunogenic means and a restricted immune response, in conjunction with the release of growth factors which can contribute towards constructive tissue remodelling and new tissue formation. [23]

SIS of xenogenic origin has been proved to be nonimmunogenic *in vivo*. [24]

One of the major limitations of ECM materials such as SIS is that they do not possess the mechanical properties for relatively large tissue reconstruction roles or for resisting the stresses present in applications like rotator-cuff injury repair, where clinical trials have been unsuccessful. [25]

1.3.3 Bovine pericardium

The pericardium (from the Greek περί, 'around', and καρδία, 'heart') is the fibrous-serous sheath that surrounds the heart, it is mainly composed by two layers, the fibrous pericardium and the serous pericardium, as shown in Figure 1.13.

The fibrous pericardium is a thick membrane that covers the outside of the heart muscle, forming a sort of the outside of the cardiac muscle, forming a sort of cone-shaped sac whose apex is connected to the root of the great vessels, at the base of the heart. [26]

The serous pericardium, on the other hand, consists of a thin membrane that folds back on itself at the level of the great vessels, thus forming two leaflets: the parietal leaflet (which adheres to the fibrous pericardium) and the visceral leaflet. Between these two layers is enclosed the pericardial cavity, a lumen containing up to 50 mL of pericardial fluid [27] whose function is to reduce friction and rubbing between the layers that make up the pericardium itself.

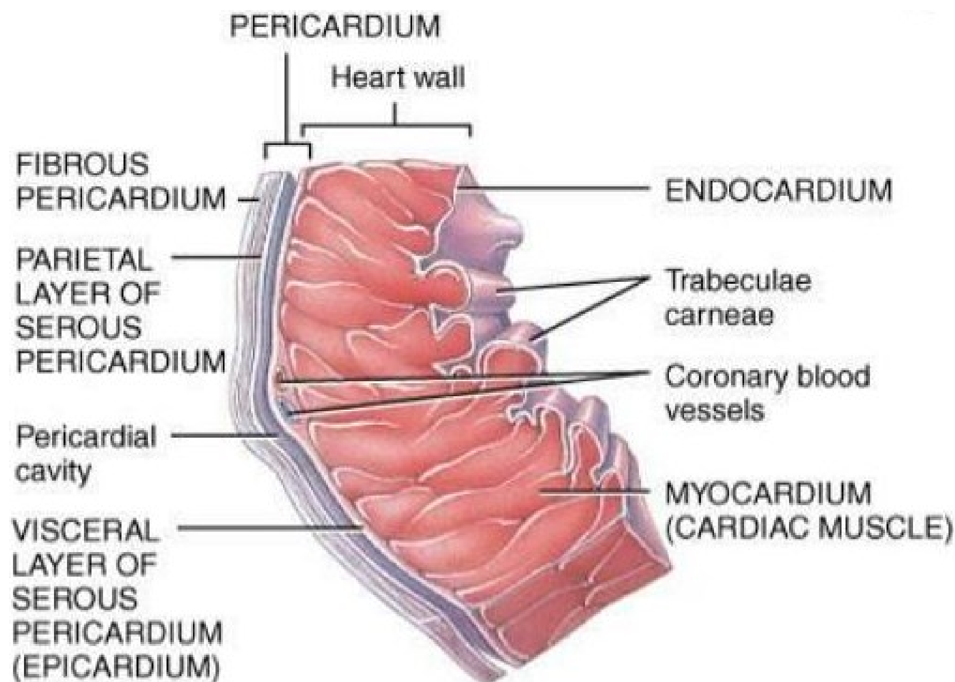


Figure 1.13 Anatomical structure of the heart envelope

The pericardium is not essential for survival and its absence due to congenital causes or surgical removal does not imply adverse reactions [27]; however, it performs important and delicate tasks that can be quickly divided into mechanical, reflex, membrane, metabolic and ligamentous functions.

1.4 AIM OF THE THESIS:

Organ transplantation is the standard goal for the treatment of many diseases. Despite efforts to increase the number of donated organs, there is a gap between the number of donors and the number of patients on the waiting list. The combined approach of tissue engineering and regenerative medicine techniques would like to create functional tissue and organ replacements to address this gap. If this can be achieved, we will have a revolutionary role in transplantology. We want to create an environment that from a structural and molecular point of view, as a chemical composition, can come close to natural organs using autologous cells. The vasculature is still missing from synthetic scaffolds. Decellularization of biological tissues is, therefore, a fundamental process for the solution of the lack of tissues, especially in the case of xenogenic tissues. We want to decellularize organs and tissues in order to already have the ECM we need, using chemicals, detergents in contact with which the cellular component of tissues and organs is removed, as long as the extracellular matrix is not affected. [12]

Determining the residues of detergents that remain "trapped" in the extracellular matrix is fundamental since their desorption could be cytotoxic or induce inflammation and therefore could contribute to the poor functionality of the bioprosthesis.

To date, different methods have been developed for the quantification of residual detergents in decellularized extracellular matrices. Many of these involved indirect determination of detergents by analysing solutions from the matrix washes themselves. A direct determination from the tissue, previously digested into papain, with high-performance liquid chromatography (HPLC) with mass spectrometric (MS) detection was considered. The difficulty in determination arises from the great complexity of the digested mixture and the low analyte content.

The proposed decellularization methods differ in the type and number of detergents, treatment times and washing methods. It is in any case important, for each protocol, to define the efficiency in the removal of antigenic detergents but also to evaluate structural modification induced by decellularization that will be reflected on the biological and mechanical properties of the prosthesis.

The aim is to assess whether the decellularization method used is suitable for future use by humans. Residual concentrations of detergents can be cytotoxic or induce inflammation and therefore calcification or produce protein denaturation of collagen and prevent cell recolonisation.

2 Experimental part:

In this chapter are listed and described all the materials mainly used in this study and all the methods exploited for the obtaining of the results.

2.1 Materials.

2.1.1 Reagents:

Acetonitrile MW 41.05 [g/mol], produced by Carlo Erba (France)
Formic Acid MW 46.03 [g/mol], produced by Sigma-Aldrich (Germany)
Methanol MW 32.04 [g/mol], produced by Sigma-Aldrich (Germany)
Chloroform MW 119.38 [g/mol], produced by Carlo Erba (Milano)
Cysteine (L-Cys) MW 157.62 [g/mol], produced by Fluka Biochemika - Merk (Germany)
Dimethyl sulfoxide (DMSO) MW 78.13 [g/mol], produced by Sigma-Aldrich (Germany)
Phenylmethylsulphonyl fluoride (PMSF) MW 174.19 [g/mol], produced by Merk (Germany)
N-Ethylmaleimide (NEM) MW 125.13 [g/mol], produced by Sigma- Aldrich (Germany)
Benzamidine Hydrochloride MW 174.63 [g/mol], produced by Sigma-Aldrich (Germany)
Iodoacetamide MW 184.96 [g/mol], produced by Sigma- Aldrich (Germany)
Sodium Ascorbate MW 198 [g/mol], produced by Sigma-Aldrich (Germany)
Ethylenediamine tetra-acetic acid (EDTA) MW 372.24 [g/mol], produced by Sigma-Aldrich (Germany)
Phosphate buffered saline (PBS) pH 7.4, produced by Sigma-Aldrich (Germany)
Sodium Hydroxide (NaOH) MW 39.99 [g/mol], produced by Sigma-Aldrich (Germany)
Sodium Chloride (NaCl) MW 58.44 [g/mol], produced by Sigma-Aldrich (Germany)
Tris-hydrochloride (Tris-HCL) MW 157.60 [g/mol], produced by Sigma-Aldrich (Germany)
Magnesium Chloride ($MgCl_2$) MW 95.21 [g/mol], produced by Sigma-Aldrich (Germany)
Benzonase Nuclease, produced by Sigma-Aldrich (Germany)
Penicillin Streptomycin MW 581.57 [g/mol], produced by Gibco (U.S.A.)
Amphotericin B X100 produced by Carlo Erba (France)
Vancomycin, produced by Sigma-Aldrich (Germany)
Gentamicin, produced by Sigma-Aldrich (Germany)
Cefoxitin, produced by Sigma-Aldrich (Germany)

Peracetic acid MW 76.05 [g/mol], produced by Sigma-Aldrich (Germany)
Papain from papaya latex MW 23.4 [kDa], produced by Sigma-Aldrich (Germany)

2.1.2 Detergents:

Sodium Cholate MW 408 [g/mol], produced by Sigma-Aldrich (Germany)
Triton X-100 MW 625 [g/mol], produced by Sigma-Aldrich (Germany)
Sodium dodecyl sulfate MW 265 [g/mol], produced by Sigma-Aldrich (Germany)
Tergitol 15-s-9 MW 596 [g/mol], produced by Sigma-Aldrich (Germany)

2.1.3 Instrumentation.

For the development of the experimental part of this thesis work, various chemical laboratory equipment was used.

HPLC:

HPLC is the high-performance liquid chromatography (once meant high-pressure).
Chromatography, from the Greek 'khrôma' which means colour, is a separation technique of compounds which constitute a mixture based on the distribution of these compounds in two phases, the stationary phase and the mobile phase that moves along a defined direction.[28]
With the term 'chromatography' are generally indicated all the various separative techniques, applied to mixture of substances and based on the distribution of two phases in which it is used the same principle, the different velocity with which different compounds of a mixture migrate in a stationary phase with the influence of a mobile phase, that has the role of drag along the system all the solutes that constitute the mixture under consideration.
The detector, which is at the end of the device, records the passage of the eluted substance, it processes the data in a chromatogram. The chromatogram is a graphic which represents the intensity of the signal related to the detected substance that is proportional to its quantity, as a function of time. Here depicted an example (Figure 2.1).

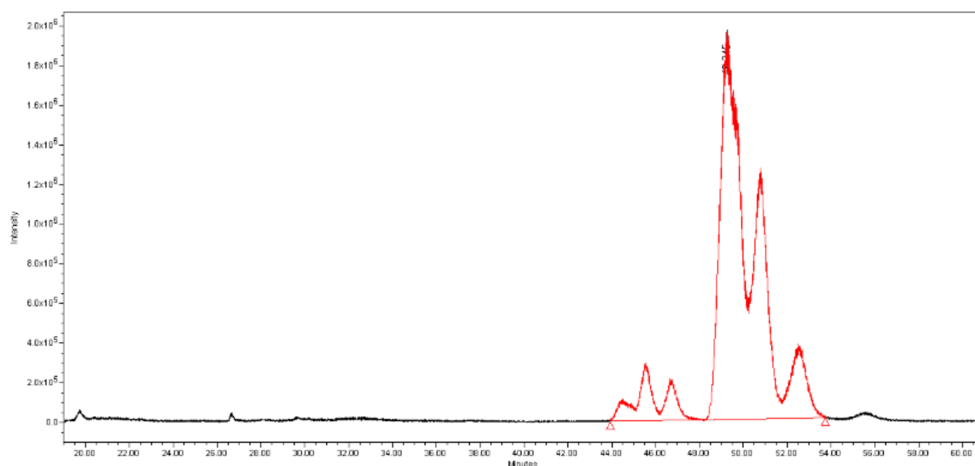


Figure 2.14 Example of Tergitol chromatogram

Every time that a substance is detected, the detector records a higher or lower peak depending on the quantity of the analyte. In order to obtain a reliable chromatogram, it has to have a good resolution. ‘Resolution’ is a parameter that relates efficiency, selectivity and the retention factor. In addition to quantitative considerations, from the chromatogram it is possible to make qualitative considerations, related to the different times when the peaks appear.

The retention time is the time necessary to the injected substance to be eluted from the beginning to the end of the column.

There are many types of chromatography, they are divided in liquid chromatography, gas chromatography and supercritical fluid chromatography, it depends on the mobile phase (eluent) and the stationary phase (eluate). [29]

The liquid chromatography (LC) is the set of analytic techniques. The analytical technique is used to examine or identify one specific material. The aim of the analytical technique is to get the material characteristics through the determination of the chemical/physical properties or through its chemical/physical behaviour.

There are two types of liquid chromatography: high-performance liquid chromatography and classic liquid chromatography (for preparatory purpose).

Type	Stationary phase	Mobile phase
Gas chromatography (GC)	solid or liquid supported on solid	gas
Liquid chromatography (LC)	solid or liquid supported on solid	liquid
Thin layer chromatography (TLC)	Solid or liquid supported on solid	liquid
Ion exchange chromatography (IEC)	solid	liquid
Molecular exclusion chromatography (EC)	solid	liquid

Table 2.1: Different types of chromatography

The high-performance liquid chromatography (HPLC) is a type of liquid chromatography which represents the instrumental evolution of the liquid chromatography with classic columns. It is a method exploited for its potentialities in quantitative analysis. It is a chromatographic technique which allows to divide two or more compounds present in a solvent by exploiting the affinity balance between a stationary phase placed inside the chromatographic column and the mobile phase which is flowing through it. A substance with more affinity to the stationary phase with respect to the mobile phase takes longer time to cover the chromatographic column length (retention time), compared to a substance with low affinity for the stationary phase and high affinity for the mobile phase.

The sample, that has to be analysed, is injected at the beginning of the chromatographic column, where it is pushed through the stationary phase from the mobile phase, applying pressures in the order of hundreds of atmospheres. To achieve high separation efficiency, a very small particles size is necessary (diameters between 3 and 10 µm), for this reason it is essential to apply high pressure if a reasonable eluent flow rate has to be maintained and thus adequate analysis time.

The chromatographic column is the main element of the HPLC system that usually consists of a steel tube inside which there is a porous material consisting of particles between 0.2 and 0.5 µm in size.

The other elements, as showed in Figure 2.2, are the pumps, usually one for each line of eluent, that manage the flow of eluents, which must always be controlled and constant because the column is sensitive and must not undergo major flow changes. To avoid blockage of the pumps, the eluents must always be degassed to remove any air bubbles that could enter the lines and damage the instrument.

Another element of the system is the injector or autosampler, capable of injecting small amounts of compound into the column.

The carousel is the place where all the samples are positioned. It is composed by plastic/glass vials, which are the containers where the autosampler takes samples.
At one end, the column is connected to a detector (IR, UV/Visible, Mass Detector), an instrument necessary for data collection, and to a computer, allowing continuous analysis of the column output. Thanks to this configuration it is possible to quantify the injected substances through the relative chromatogram.
Optimization of liquid chromatography occurs thanks to the dynamic composition variation of the liquid phase used to elute the analyte.
This type of elution is called gradient elution, and differs from the isocratic elution, which uses the same eluent for the entire run. The gradient is obtained by changing over time the percentage of different eluents in the mobile phase.
The gradient elution is essential to obtain a better separation of the resulting peaks on the chromatogram.

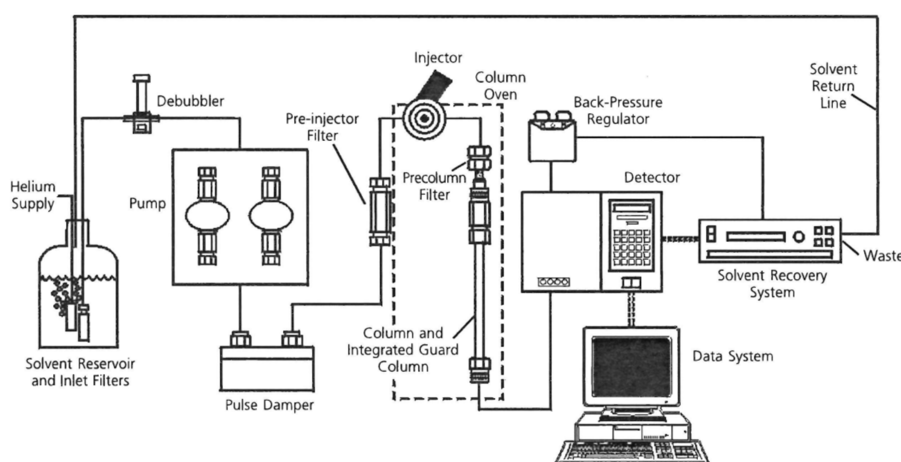


Figure 2.15 HPLC scheme

Mass Detector:

The mass spectrometer is an analytical technique applied both in unknown substances identification and trace analysis of substances. Commonly it is used combined with separative techniques, like liquid chromatography. The principle on which mass spectrometry is based is the possibility to split an ion mixture according to the ratio mass/charge (m/z). This principle is called electrospray ionization (ESI). It uses electrospray by applying a high voltage to a liquid to produce an aerosol. Due to relatively fragile biomacromolecules, their structures are easily destroyed during the process of dissociation and ionization. ESI overcomes the tendency of these molecules to fragment upon ionization. ESI differs from other atmospheric pressure

ionization processes, in which it may produce multiple charge ions, effectively extending the analyser's mass range to accommodate the magnitude of kDa-mDa observed by proteins and their associated peptides.
ESI applies a high voltage at the outlet of the capillary, and the high electric field generated atomizes the liquid flowing out of the capillary into tiny charged droplets. As the solvent evaporates, the charge intensity on the surface of the droplet gradually increases, and finally the droplet splits into one or a plurality of charged ions, allowing the analyte to enter the gas phase in the form of a single charge or multiple charges and become a gas phase ion.
There are two explanations for the mechanism (Figure 2.3) of gas phase ion generation: the ion evaporation model (IEM) proposed by Thomsona and lribarne, and the charged residue model (CRM) advocated by Dole and Rllgen. In the two models, the ions to be analysed are not excited by external energy and do not generate debris during the process of becoming a gas phase ion.

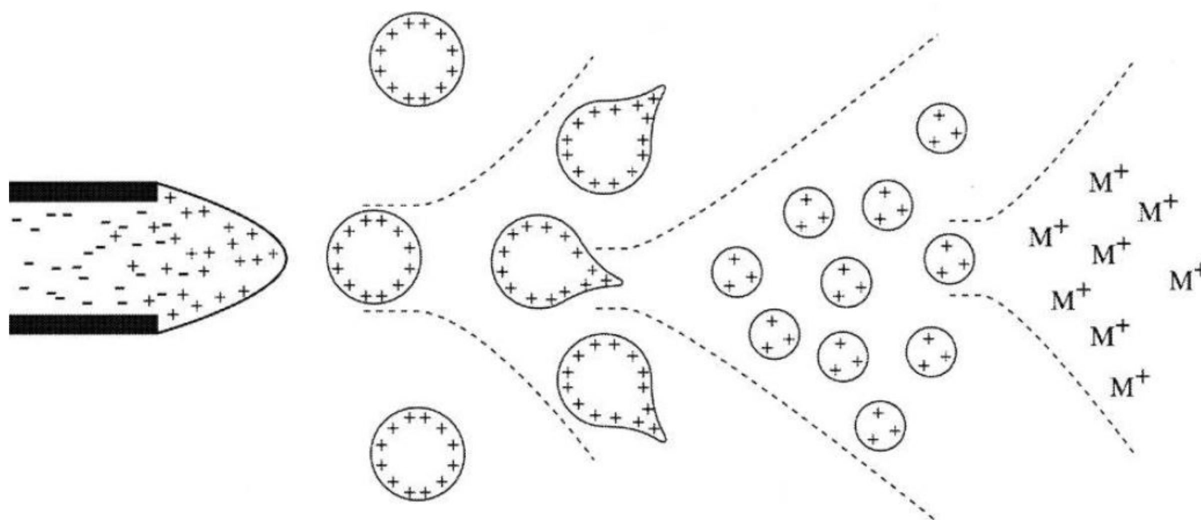


Figure 2.16 Mechanism of electrospray ionization *[30]*

The diagram, which reports the abundance of each ion, according to the mass/charge ratio, is the so-called mass spectrum, which is typical for every compound because it is directly related to its chemical structure and to its conditions of ionization.
The difference from other spectrometric techniques is that this analysis method is destructive, the molecule is not going to remain the original size after the analysis; and this technique is not based on the interaction between radiations and matter. [31]

2.2 Methods.

2.2.1 Development of the Chromatographic method.

Chromatographic method for native tissue:

Fundamental was the development of a method capable of directly quantifying residual detergents from three types of matrices: i) portion of thyroid cartilage porcine larynx ii) pSIS = porcine small intestinal submucosa iii) bovine pericardium.
At the beginning, known amounts of the investigated analytes (detergents) were added to the digests of defatted native tissues in order to build up calibration curves, one for each detergent, under conditions similar to those expected in the determination of the specific residual detergent after decellularization. Creating a calibration curve involves the statistical theory of fitting straight line based on the method of the least squares.[32]
Thanks to the HPLC and the software Empower (Waters, Milford, MA, U.S.A.), the peak of the mass of a specific detergent is recorded during the analysis.
The calibration curve has in abscissas the area of the chromatogram peak corresponding to the detergent, in ordinates the concentration (molar one or mass one, expressed in mol/L or mg/ml respectively).
The calibration curve has to be obtained for every detergent that is used in combination with the others. Every detergent has its own calibration curve.
HPLC is used to develop a technique for direct determination of residual detergents. In early studies of detergents determination, they were detected by the combination of HPLC and UV/Vis detector, but the main problem was that not all detergents possess a chromophore group, which would allow their identification by UV detectors. Therefore, to overcome this problem it was decided to use an RP-HPLC connected to a mass detector.
In this project the HPLC Waters e2695 with 2489 UV/Vis detector and Acquity QDa detector was used as chromatographic system.
Initially, the detergents were analysed in water to study their retention time and how they behaved during the analysis with HPLC and mass detector.
The column with silica gel functionalized with linear hydrocarbon chains of 18 carbon atoms Vydac 218TP C18 (produced by Grace Columbia, MD, U.S.A.) 250 mm x 4.6 mm, 300 Å pore size and 5 µm particle size, was used as the stationary phase during the chromatographic analyses. Initially two eluents were used as mobile phase, Eluent A: 0.05% trifluoroacetic acid

(TFA) in milliQ water and Eluent B: 0.05% TFA in acetonitrile. However, it was discovered that the use of TFA in the eluents does not allow a good ionization of the molecules even for lower percentages of acid. Therefore, several tests with difluoro acetic acid (DFA) and formic acid (FA) at different percentages were performed, then it resulted that a 0.05% of formic acid in both eluents was the best performing concentration for mass detector.

The eluents finally employed for all analyses were: eluent A: milliQ water + 0.05% FA; eluent B: acetonitrile + 0.05% FA.

To prepare the samples, a 1 mg/mL solution in milliQ water were initially prepared for each detergent of interest, solutions at lower concentrations were then obtained by successive dilutions. The highest concentration that can be injected into the mass detector is 0.01 mg/mL. All solutions were filtered through PVDF filters of 0.45 μm before being injected into HPLC.

The analyses performed for each detergent identified its retention time, related to the gradient, and its value of mass/charge ratio (m/z) as reported in Table 2.2 [33].

Detergent	Gradient	Retention Time (related to %B)	m/z [Da]
Sodium Cholate	From 40 to 55%B in 15 minutes	t_R= 6 minutes, 46% B	408
Sodium Dodecyl sulfate	From 45 to 60 %B in 15 minutes	t_R= 5 minutes, 46% B	265
Tergitol 15-s-9	From 62 to 77 %B in 15 minutes	t_R= 10 minutes, 46% B	614.5
Triton X 100	From 50 to 70 %B in 20 minutes	t_R= 10 minutes, 46% B	576.5

Table 2.2 Analyte retention time and m/z ratio

In order to find the right value of mass, expressed in Dalton, which must be added before starting the analysis, an ESI-TOF mass analysis is performed at the Department of Pharmacy. It was used to determine the m/z values of Tergitol and Triton X-100. This analysis revealed a characteristic molecular mass profile for these two detergents: a Gaussian curve mass distribution profile, as visible in Figure 2.4.

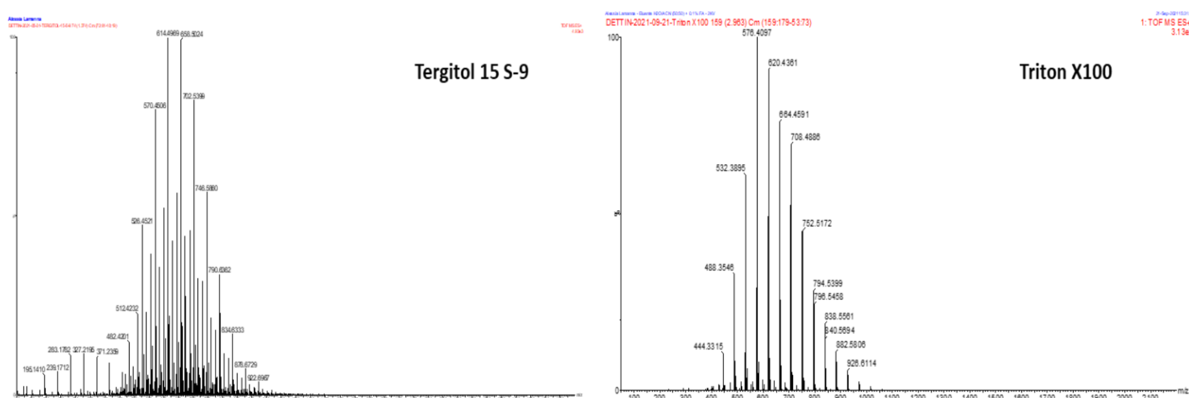


Figure 2.4 ESI-TOF mass analysis on the left of Tergitol 15-s-9, on the right of Triton X-100

By considering the area of the peaks obtained from HPLC combined with mass detection of each detergent at different concentrations in water, it was possible to determine the calibration curves.

From considering the detergents in water to considering the detergents in digested biological tissues must, it should be considered that the biological matrices are multicomponent and contain very high concentrations of protein fragments and consequently if during the analysis all the eluate is sent to the mass detector, a clogging and saturation could occur. The initial idea was therefore to aspirate the digested sample and perform a first part of the analysis using the UV detector to visualize the chromatogram peaks related to all protein fragments and then, after the elution of all the digested tissue the HPLC column was then connected to the mass detector for the detection of all residual detergents.

i) The portion of **thyroid cartilage porcine larynx** was the first native tissue to be analysed. The defatting is not executed for this specific biologic matrix since it is mainly composed by gristle, without any lipids. For this reason, the porcine larynx only requires the digestion with papain.

   After this treatment the digested tissue is ready to be enriched with a specific quantity of detergent, known to be used for the decellularization protocol.

   The analysis of the native tissue was performed on 1 mL of digested sample. Multiple injections were necessary as the maximum injection volume of the HPLC

for each analysis is 150 μL. To reduce errors on the injected volume, it was decided to perform 10 injections each of 100 μL, as reported in Figure 2.5.

Active sample set : 1

	Vial	Inj Vol (uL)	# of Injs	Label	SampleName	Level	Function	Method Set / Report Method	Label Reference	Processing	Run Time (Minutes)	Data Start (Minutes)	Next Inj. Delay (Minutes)
1							Equilibrate	Injection			10.00	0.00	0.00
2	73	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
3	74	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
4	75	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
5	76	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
6	77	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
7	78	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
8	79	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
9	80	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
10	81	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
11	82	100.0	1		1		Inject Samples	Injection		Normal	0.50	0.00	0.50
12							Equilibrate	Injection			6.00	0.00	0.00
13	82	0.1	1		PA DX41 (3)		Inject Samples	PA method		Normal	61.00	0.00	0.00

Figure 2.5 Example of Empower settings for one analysis of porcine larynx

Before the injections, the column was conditioned for 10 minutes at 100% A at a flow rate of 1 mL/min. The flow rate was unchanged until the end of the analysis. After all injections there was a 6-minute isocratic phase at 100% A to elute all the hydrophilic part of the matrix. During column conditioning, multiple injections, and 6 minutes of isocratic the mass detector is not connected from the HPLC system.

Based on the retention time of each detergent, an initial gradient was set to elute all the residual hydrophilic components of the mixture at low percentages of eluent B: from 0% B to 36% B in 18 minutes. The first gradient was set up in a way that anything that elutes below 36% B could not enter the mass detector, to avoid the above-mentioned problems of clogging, etc. After the elution of the hydrophilic components, the column was connected to the mass detector, and the detergents (hydrophobic components), was eluted with a second gradient: from 36% B to 77% in 41 minutes.

The gradient method was set as follows:

Time [minutes]	Flow [mL/min]	% A	% B
0	1	100%	0%
18	1	64%	36%
19*	0.5	64%	36%
20	1	64%	36%
61	1	23%	77%
62	1	20%	80%
72	1	20%	80%
73	0	20%	80%

Table 2.3 Gradient method for porcine larynx

*The decrease in flow rate (from 1 mL/min to 0.5 mL/min) at minute 19 is needed to allow the connection of the column to the mass detector during the analysis.

At the end of the analysis the gradient is brought to a concentration of 80% B that washed of the column from all possible hydrophobic residual, in order to prepare it for further analysis. After the washing is finished, the flow is brought to 0 mL/min in 1 minute.

A specific operative option was set for the Acquity QDa mass detector during the slow second gradient. Selected Ion Recording (SIR) was selected in order to record a specific mass with a specific polarity (Positive or Negative) in a specific range of time, which depends on the chemical composition of the specific detergent used (Figure 2.6).

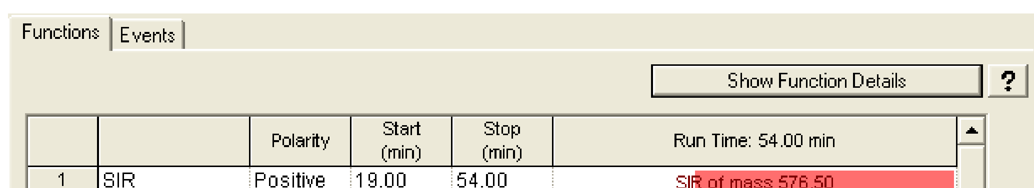


Figure 2.6 Example of SIR for porcine larynx: Positive SIR of 576.5 Dalton from minute 19 to minute 54

Another option consists in the use of the Full scan (Scan), which is able to record all the big range of masses that are eluted during the second gradient, and it requires to set the lower mass and the higher mass to be recorded, the polarity and the range of time of the analysis. This option was used during the first-time analysis when the mass of a specific compound was still unknown. For the porcine larynx, to be able

to identify and quantify the three detergents we set: three SIR, one for each detergent and two scan covering a large range (one positive and one negative).

ii) The second native tissue needed to be defatted since the **porcine small intestinal submucosa is a fat tissue, and then it was digested with papain. Two** similar gradient methods depending on the decellularization method were used: one for Triton X-100 and Sodium Cholate, and another one for Tergitol and Sodium Cholate.
Tissue enriched with Tergitol and Sodium Cholate was analysed with the gradient method reported in Table 2.4:

Time [minutes]	Flow [mL/min]	% A	% B
0	1	100%	0%
19	1	64%	36%
20	0.5	64%	36%
21	1	64%	36%
62	1	23%	77%
63	1	20%	80%
73	1	20%	80%
74	0	20%	80%

Table 2.4 Gradient method for porcine SIS decellularized with Tergitol and Sodium Cholate

Tissue enriched with Triton X-100 and Sodium Cholate was analysed with the gradient method reported in Table 2.5:

Time [minutes]	Flow [mL/min]	% A	% B
0	1	100%	0%
19	1	64%	36%
20	0.5	64%	36%
21	1	64%	36%
55	1	30%	70%
56	1	20%	80%
66	1	20%	80%
67	0	20%	80%

Table 2.5 Gradient method for 'TriCol' decellularization

The second gradient is shorter because Triton X-100 has a lower retention time than Tergitol. Three SIR mass operative methods were imposed for Sodium Cholate,

Tergitol and Triton X-100. And two Scan were imposed for 300 Da to 700 Da mass range, one with positive polarity and one with negative polarity.

iii) For the **native bovine pericardium**, the same gradient method of porcine larynx was used.

Chromatographic method for decellularized tissue:

Porcine larynx decellularized is analysed with the same gradient method used for the native tissue.

For the decellularized porcine SIS the main problem was that the signal of the samples of SIS was very low and comparable with background noise. After many attempts, 3 mL of sample divided into 30 multiple injections of 100 μL each, were injected. A significative signal was finally achieved. There was a lot of porcine SIS decellularized with the method with Triton X-100 and Sodium Cholate available, so the HPLC analysis was made with 40 multiple injections in order to inject in the column 4 mL.

Decellularized pericardium is analysed with the same gradient method used for the native tissue.

2.2.2 Defatting or delipidization.

The aim of this procedure is to remove the lipid component of the cell membrane in order to eliminate as much as possible the production of signals classified as 'background noise'. It is important to remove lipids from biological native matrix because they are hydrophobic compounds, so they can compromise instrumental investigations on detergents.

The delipidization method involves different steps in which the biological tissue is immersed in solutions with increasing concentrations of ethanol (EtOH): 40%, 80% and then 100% (w/v); followed by solutions of chloroform/methanol in different ratios (2/1 and 3/1 v/v respectively). EtOH draws water from the cell, dehydrating it, and organic solvents solubilize membrane lipids. [34]

Then rehydration of the tissue starts through the passage in solution at decreasing concentrations of EtOH (100%, 80% and 40%) and finally in water overnight. Each of these steps is made under gentle agitation for subsequent 1 hour periods at 4°C. [33]

2.2.3 Digestion with Papain.

The tissues to be analysed were subjected to digestion with papain (from papaya latex), which is a proteolytic enzyme belonging to the class of hydrolases which catalyses hydrolysis of proteins, in order to degrade the protein scaffold and free any residual detergent remaining within the extracellular matrix.
Before the digestion of the tissue, it is important to prepare the digestion buffer: 5 mM of L-Cysteine (L-Cys), which is the amino acid that is able to establish disulphide bridges[35]; 5 mM of **ethylenediamine tetra-acetic acid** (EDTA), which is an amino poly carboxylic acid white and water-soluble solid widely used to bind iron and calcium ions; both diluted in milliQ water. In the obtained buffer, 6.0-6.1 pH, is dissolved papain (7 u/mL, 2.34 u/mg). 5 mL of solution are used each 15-18 mg of wet tissue[33], which has to be weighted after drying with blotting paper. Digestion then takes place over 24 hours in a 60°C oven with a rotary shaker (Figure 2.7).

Figure 2.7 Four samples of tissue in buffer with papain placed in a rotary shaker in the oven at 60°C

2.2.4 Decellularization

Porcine larynx decellularization: this tissue was analysed in collaboration with Professor Astolfi's research group with the help of Dott. Erica Gentilin.

The first protocol used to decellularize PA40SX and PA40DX samples involved two freeze/thaw cycles followed by washing in 0.1% peracetic acid for 45 minutes. Subsequently, the tissues were incubated in SDS 1% for 6 hours, Tergitol 1% for 24 hours and colic acid, sodium cholate 10 mM for 4 hours. All steps were performed at room temperature. Between steps, samples were washed. At the end of the protocol, the samples were sterilised with 0.005% peracetic acid for 2 hours. At the end of all this, a 3-day washing step is planned.
The second protocol with which PA41SX and PA41DX samples were decellularized involved a single freeze/thaw cycle and the use of SDS 1% for 6 hours, Tergitol 1% for 24 hours and colic acid, 10 mM Sodium Cholate for 4 hours. All steps were performed at room temperature. Between steps, samples were washed. At the end of the protocol the samples were sterilised with peracetic acid 0.1% for 2 hours. At the end of all this, a 3-day washing step is planned.
The third protocol with which PA43SX and PA43DX samples were decellularized is identical to the previous one with the only difference that the final washes are done for 5 days.
The last protocol with which PA44SX and PA44DX samples were decellularized is identical to the previous one with the only difference that the final washes are done for 6 days.

Porcine SIS decellularization: this tissue was analysed in collaboration with Professor Gerosa's research group with the help of Dott. Tiziana Palmosi. This research group used the same decellularization protocols that were used for the bovine pericardium for both Tergitol/Sodium Cholate and Triton X-100/Sodium Cholate detergents, as written below.

Bovine pericardium decellularization with Tergitol and Sodium Cholate:
Pericardium was taken in the slaughterhouse and transported to the laboratory for cell removal treatment. It was put in physiological saline buffer (0.9% NaCl in distilled water) to store it. The first step consisted in roughly remove the superficial adipose component that is bound to the pericardial flaps using scissors (Figure 2.8).

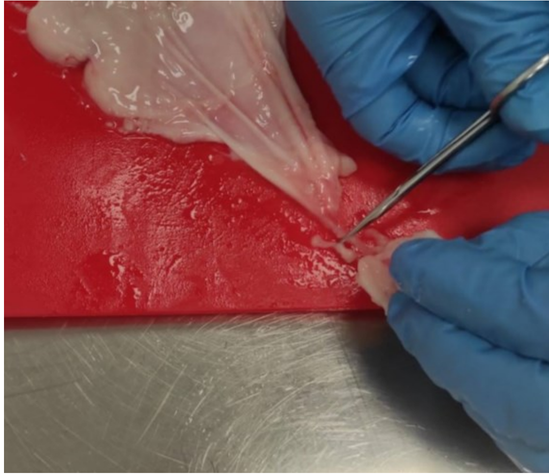


Figure 2.8 Removing fat with scissors

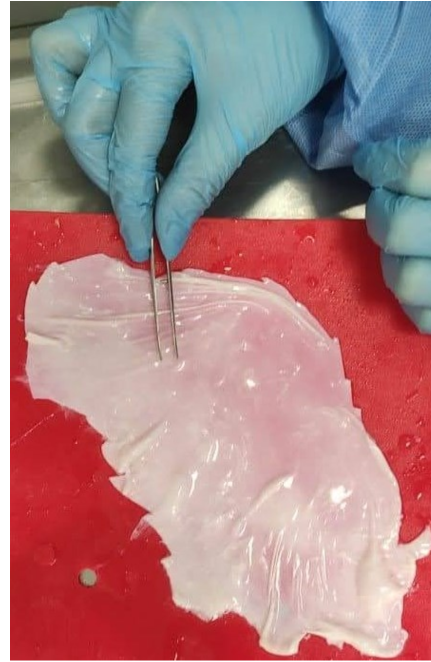


Figure 2.9 Removing fat with tweezers

Once the fat was removed from the edges, the second step involved the use of tweezers (Figure 2.9). The tissue was covered with physiologic saline because wetting it facilitates fat removal, which was performed in a very meticulous manner. The tissue was put under agitation at 4°C for few minutes, then it was cut and put in the final container before decellularization.

Decellularization with TERGICOL protocol:

This method is named after the two detergents used for this protocol: Tergitol (non-ionic) and Sodium Cholate (ionic). The same treatment called 'TriCol' is based on the use of Sodium Cholate (ionic) and Triton X-100 (non-ionic), which is replacing Tergitol 15-s-9.

This decellularization process used is able to effectively remove cellular components, xenogenic plasma membrane residues and nuclei, while keeping the architecture of the extracellular matrix intact.

The following solutions were prepared prior to cell removal:

- Buffer A: 50 mM EDTA (ethylenediaminetetraacetic acid), which is a chelating agent and therefore promotes cell detachment from the ECM, 100 mM sodium ascorbate in PBS, which promotes cell motility. The solution containing both agents is brought to physiological pH (7.4) and stored in the dark at 4°C. Before use, the solution is flushed with nitrogen to prevent oxidation of the sodium ascorbate;
- PBS 1x (phosphate buffer saline).

The TERGICOL protocol involves the use of two protease inhibitor solutions:

- Protease inhibitor A: 400 mM phenylmethylsulfonyl fluoride (PMSF); 1 M N-Ethylmaleimide (NEM) in dimethylsulfoxide (DMSO);
- Protease inhibitor B: 1 M benzamidine; 200 mM iodoacetamide in DMSO.

Protease inhibitors are molecules that inhibit cellular proteases released as a result of cell lysis during decellularization and preserve the matrix from protein degradation.

The TERGITOL decellularization method lasts for 5 days and consists of the following steps:

- Step 1: Protease inhibitors (8h at 4°C) (10% buffer A, 10% DMSO, 0.5% v/v protease inhibitor A and 0.5% protease inhibitor B in milliQ water). DMSO is added as first solvent because it helps the dissolution of the inhibitors.
- Step 2: wash with hypotonic solution (8h-overnight, under stirring at 4°C): 10% buffer A in milliQ water.
- Step 3: protease inhibitors are used (8h at 4°C) 10% buffer A, 1% v/v Tergitol, 0.5% protease inhibitor A and 0.5% protease inhibitor B in milliQ water.
- Step 4: washing (8 h, under stirring, at 4°C) 10% buffer A, 0.1% Tergitol in milliQ water.
- Step 5: wash with hypertonic solution (8h under shaking at 4°C) 10% buffer A, 0.5 M NaCl, 0.1% Tergitol in PBS. The wash should be repeated twice.
- Step 6: Wash 1 (1.5 h under shaking at room temperature) 10% buffer A, 0.1% Tergitol in milliQ water. The wash should be repeated twice.
- Step 7: Wash 2 (1.5 h under shaking at room temperature) 10% buffer A, 0.1% Tergitol in PBS. The wash should be repeated twice.
- Step 8: addition of Sodium Cholate (16h, under stirring, at room temperature in the dark, pH 7.4) 10% buffer A, 10 mM Sodium Cholate in PBS. This step should be performed twice. The first time lasts 2h and the second time overnight. Sodium Cholate powder is very dangerous if inhaled, so a specific mask is worn to be safe.
- Step 9: washing (1.5 h, under agitation, at room temperature) in PBS. the washing should be repeated twice, the first time at room temperature and the second time at 4°C.
- Step 10: Wash (30 min, under agitation, at 4°C) physiological solution 0.9% NaCl
- Step 11: Wash (1h under agitation at 4°C) physiological solution 0.9% NaCl, 10% isopropanol. The process should be repeated twice.
- Step 12: Wash (1.5 h under agitation at 4°C) 0.9% NaCl, 10% isopropanol saline solution. Washing should be repeated twice. The first wash lasts 30 minutes, the second lasts 1 hour.

Enzymatic treatment of the tissue is carried out using benzonase. Benzonase, which amount is decided according to the wet weight of the tissue, has the capability to attack and degrade all forms of DNA and RNA. This non-specific, recombinant endonuclease cleaves all kinds of DNA and RNA variants into fragments that comprise < 8 soluble base pairs.
In order to optimise the action of this enzyme a sterile equilibration buffer is prepared. It consists of:
- Tris-HCl 50 mM
- MgCl_2 1 mM in milliQ water
To prepare 1 L of equilibration buffer use 6.057 g of tris-HCl, 0.009521 g of MgCl_2 and add 1 litre of milliQ water, then measure the pH. Since benzonase works well at a basic pH, the buffer is brought to a pH of 8-8.5, using HCl. The equilibration buffer is filtered under sterile conditions with a 0.20 μm filter (GVS, Filter Technology, U.S.A.).
After 46 hours the tissue is washed with physiologic saline solution two times (30 minutes under agitation, room temperature) and then sterilization is completed using Amphotericin b, Vancomycin, Gentamicin and Cefoxitin, in order to avoid the growth of fungi and bacteria. Eventually, antibiotics are rinsed off using sterilized PBS two times and then a solution with peracetic acid is added. The quantity of peracetic acid depends on the quantity of solution that is necessary to use, which is based on the tissue sample quantity. Peracetic acid acts as an antiseptic and disinfectant and it has a removal effect on residual nucleic acids. For this reason, it is used in the final phase of the cell removal treatment. After three hours peracetic acid was removed with two washes of PBS. Finally, the tissue was ready to be digested with papain before being analysed with the HPLC system.
The method appears to be effective, but it needs a long period of time to be completed. The pro is that it is a simple method, the con concerns the presence of the epitope α - gal, which is ubiquitously presented in non-primate mammals, but absent in human. Humans have a distinct anti -α – gal reactivity which is responsible for hyperacute rejection of organ transplanted from α – gal donors. A further step could be required, such as the use of the enzyme alpha-galactosidase.

3 Results:

3.1 Chromatography:

The analysis of remnant detergents starts with an isocratic wash of 100% aqueous eluent (Figure 3.1), which allowed the column to elute hydrophilic components contained in the digested tissue mixture. During this phase the mass detector is disconnected and only the UV detector works. The chromatogram shows how the absorbance decreases with time as the hydrophilic components contained in the digested mixture, and not retained from the column, flow into the column first, while the hydrophobic components such as detergents remain trapped inside until the gradient begins.

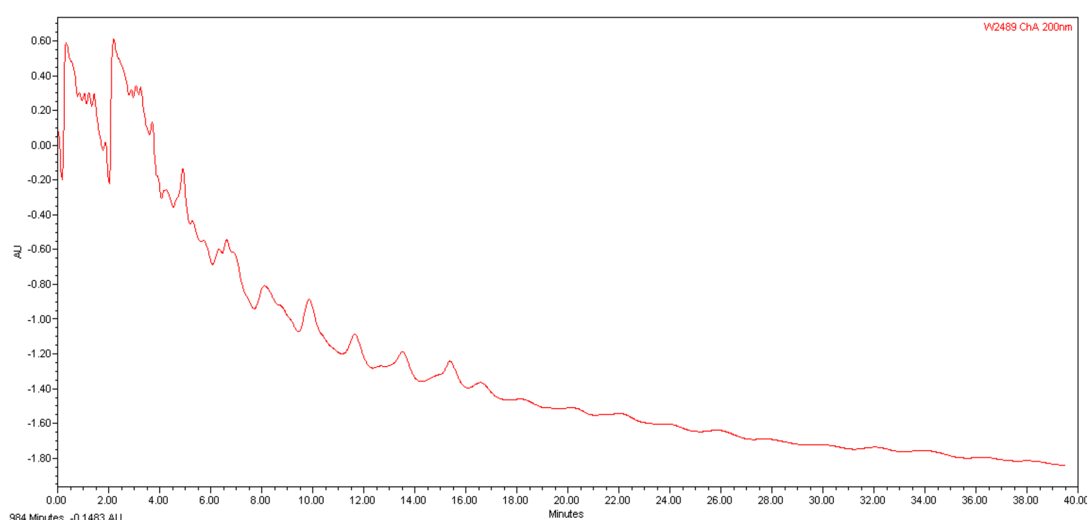


Figure 3.17 Chromatogram of the isocratic washing after sample injection

After this washing, the gradient starts (from 0% B to 70% B in 55 minutes). The chromatogram reported in Figure 3.2 shows a very high peak of 20 minutes retention time due to hydrophilic components of the digested tissue. The mass detector is excluded to avoid its saturation. In the last part of the gradient, where the most hydrophobic analytes elute, the mass detector can be used.

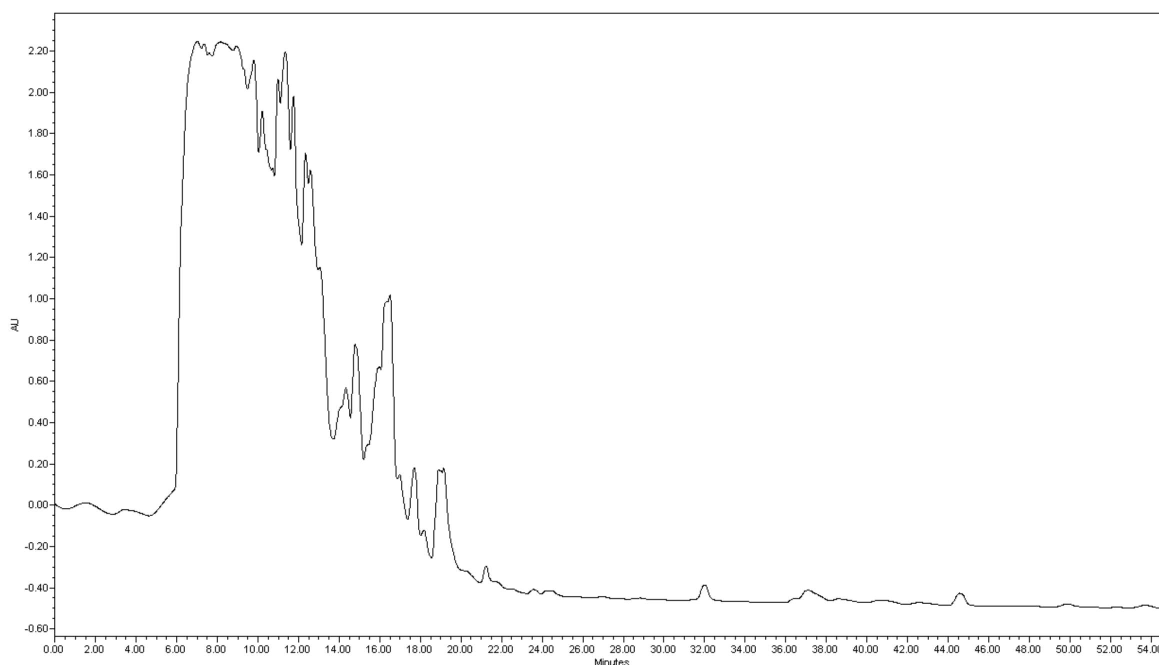


Figure 3.18 Chromatogram of the gradient from 0% B to 70 % B in 55 minutes

3.1.1 Reproducibility of the results

As one specific example, the overlay of chromatograms of the three runs for Sodium Cholate detection, carried out by injecting subsequently into the column 1 mL of each of porcine larynx digested, is reported in Figure 3.3. To express the precision and repeatability of the assay, the coefficient of variation (CV) of the Sodium Cholate analysis was calculated. It is the ratio of standard deviation and the arithmetical media. An average value of 0,1369 is found. The closeness of CV to zero indicates a low-variance distribution of measures.

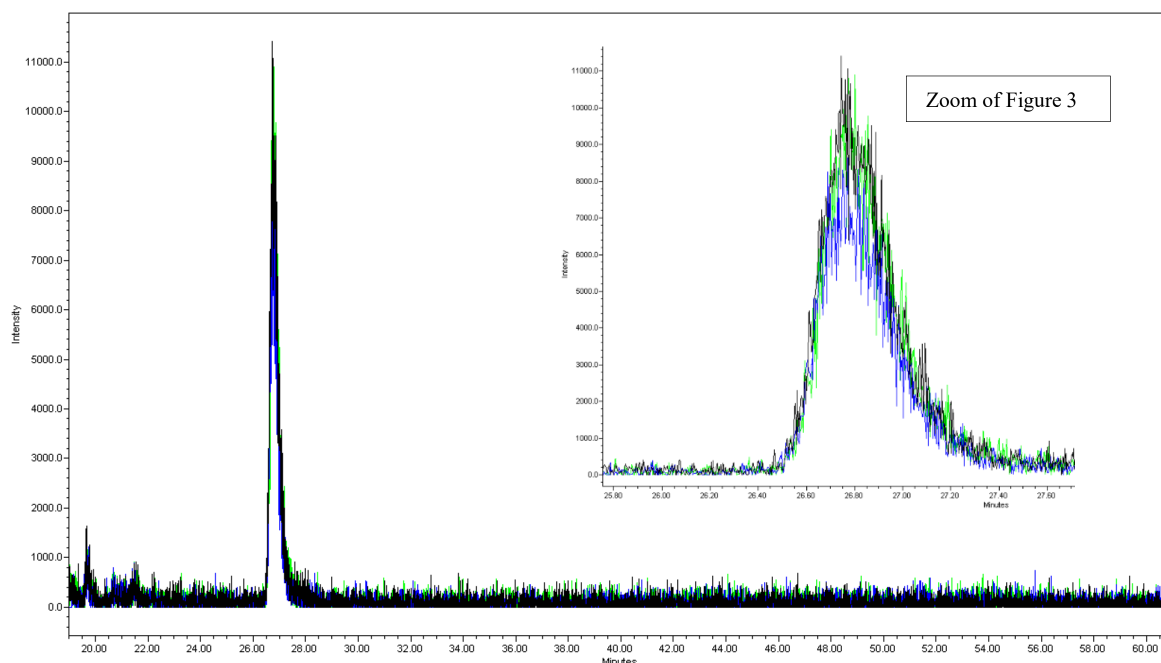


Figure 3.19 Overlay of the chromatographic traces of Sodium Cholate of three different runs of an identical sample of porcine larynx decellularized with SDS and Sodium Cholate (peak at minute 26-27)

Another example shows the overlay of chromatograms of the three runs for Tergitol 15-s-9 detection, carried out by injecting subsequently into the column 1 mL of each of porcine smooth SIS digested, is reported in Figure 3.4. To express the precision and repeatability of the assay, the coefficient of variation (CV) of the Tergitol 15-s-9 analysis was calculated. It is the ratio of standard deviation and the arithmetical media. An average value of 0,078367 is found. The closeness of CV to zero indicates a low-variance distribution of measures.

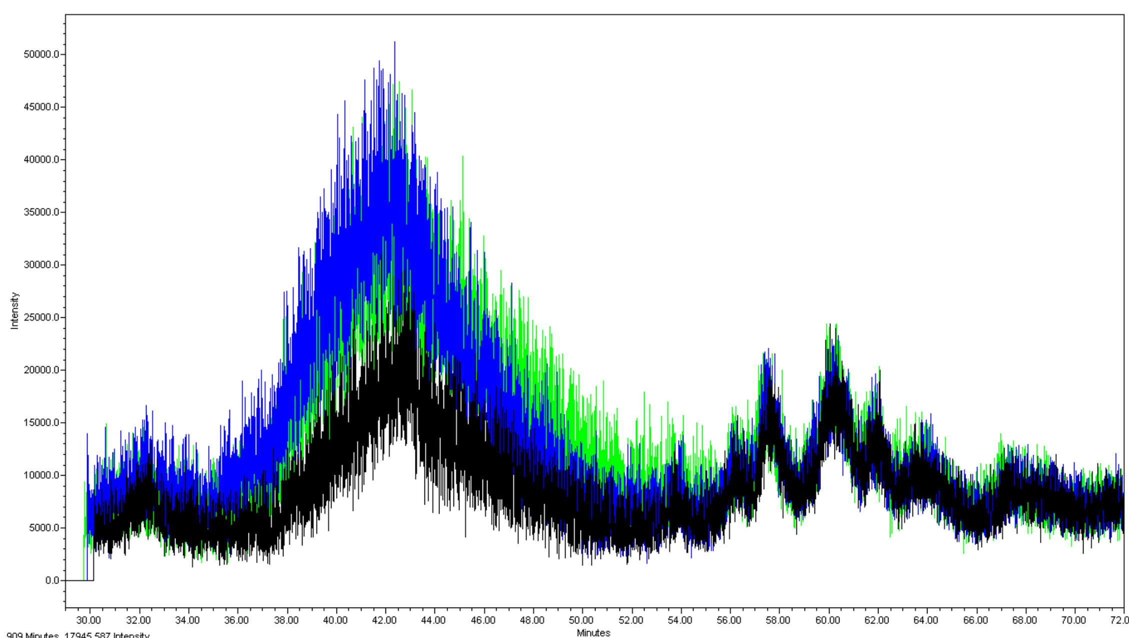


Figure 3.4 Overlay of the chromatographic traces of Tergitol 15-s-9 of three different runs of an identical sample of porcine smooth SIS decellularized with Tergitol 15-s-9 and Sodium Cholate (peak at minute 42)

The overlay of chromatograms of the three runs for Triton X-100 detection, carried out by injecting subsequently into the column 1 mL of each of porcine rough SIS digested, is reported in Figure 3.5. To express the precision and repeatability of the assay, the coefficient of variation (CV) of the Triton X-100 analysis was calculated. It is the ratio of standard deviation and the arithmetical media. An average value of 0,064075 is found. The closeness of CV to zero indicates a low-variance distribution of measures.

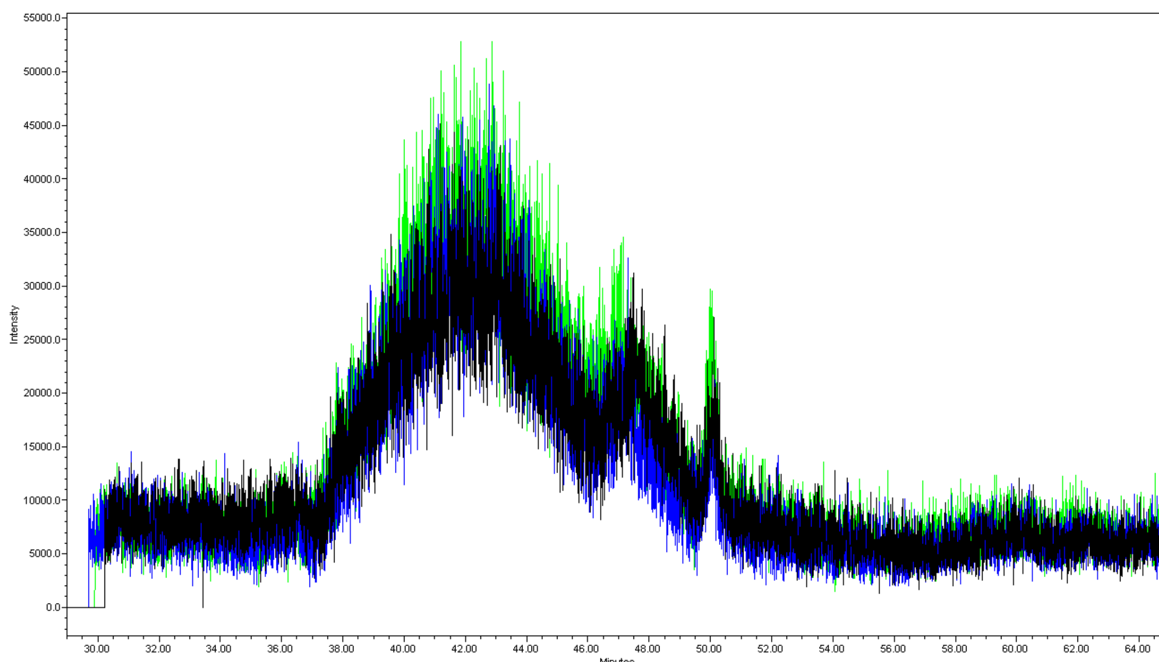


Figure 3.5 Overlay of the chromatographic traces of Triton X-100 of three different runs of an identical sample of porcine rough SIS decellularized with Triton X-100 and Sodium Cholate (peak at minute 42)

The overlay of chromatograms of the three runs for SDS detection, carried out by injecting subsequently into the column 1 mL of each of porcine larynx digested, is reported in Figure 3.6. To express the precision and repeatability of the assay, the coefficient of variation (CV) of the SDS analysis was calculated. It is the ratio of standard deviation and the arithmetical media. An average value of 0,101608 is found. The closeness of CV to zero indicates a low-variance distribution of measures.

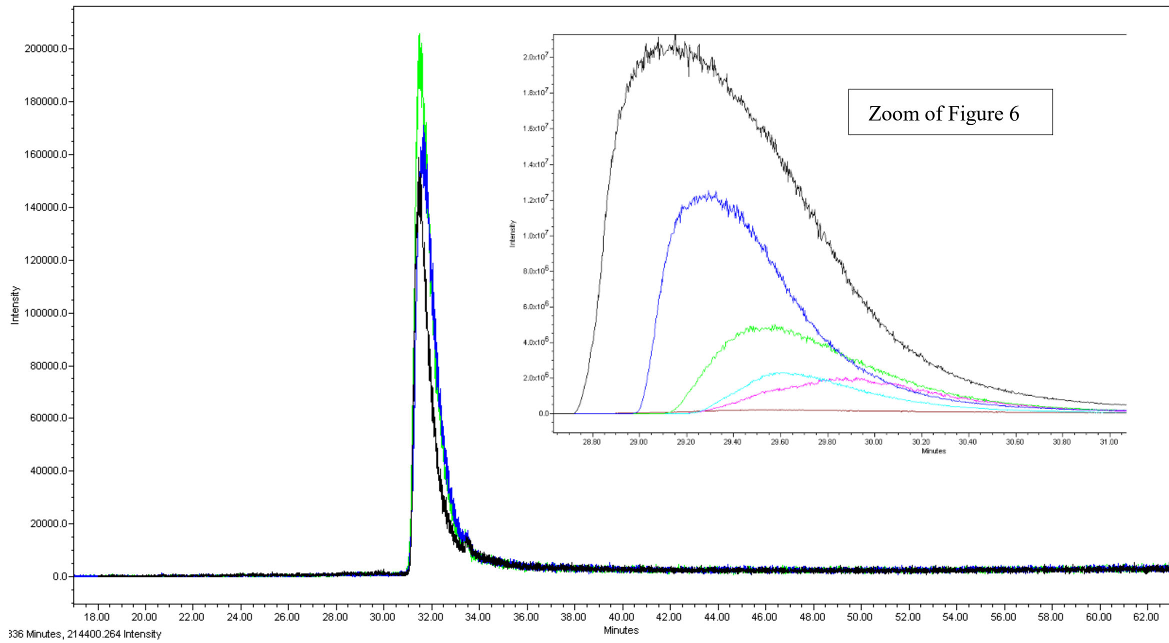


Figure 3.6 Overlay of the chromatographic traces of SDS of three different runs of an identical sample of porcine larynx decellularized with SDS, Tergitol 15-s-9 and Sodium Cholate (peak at minute 31-32)

The chromatograms used to formulate the calibration curves with respect to Tergitol 15-s-9 are shown below in Figure 3.7. These are the superposition of the five points on the line showing increasing area trends in ascending order (black, blue, green, light blue, violet).

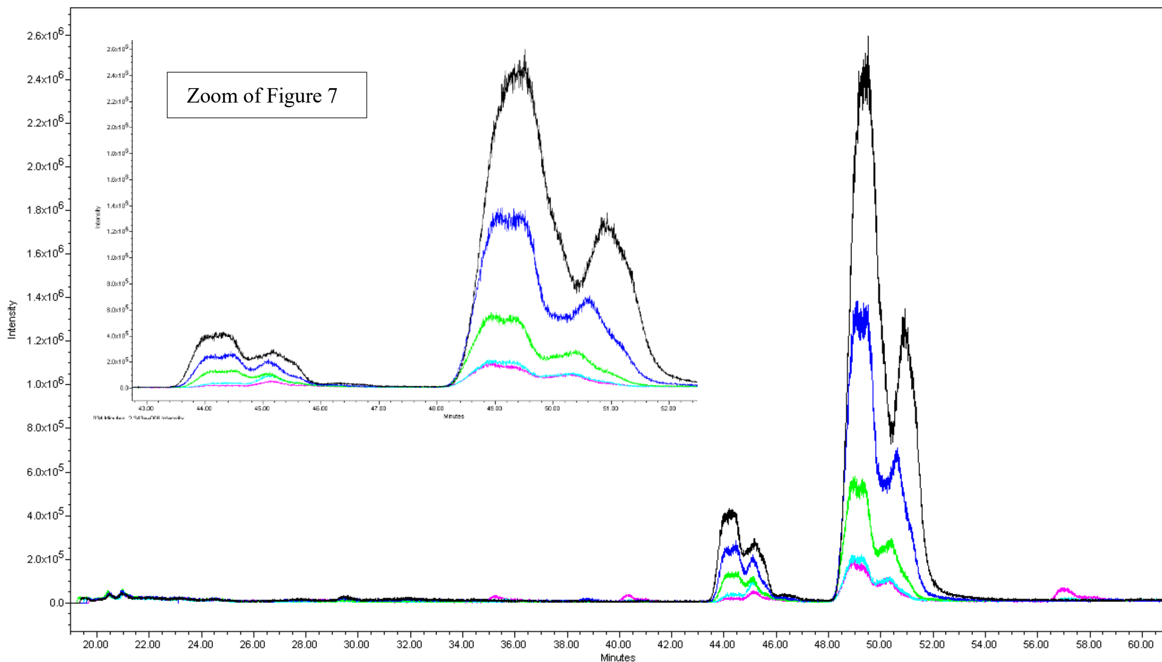


Figure 3.7 Overlay of chromatograms for the calibration curve of Tergitol, used in conjunction with Sodium Cholate to decellularize the pericardium.

The chromatograms used to formulate the calibration curves with respect to Triton X-100 are shown below in Figure 3.8. These are the superposition of the five points on the line showing increasing area trends in ascending order (black, blue, green, light blue, violet).

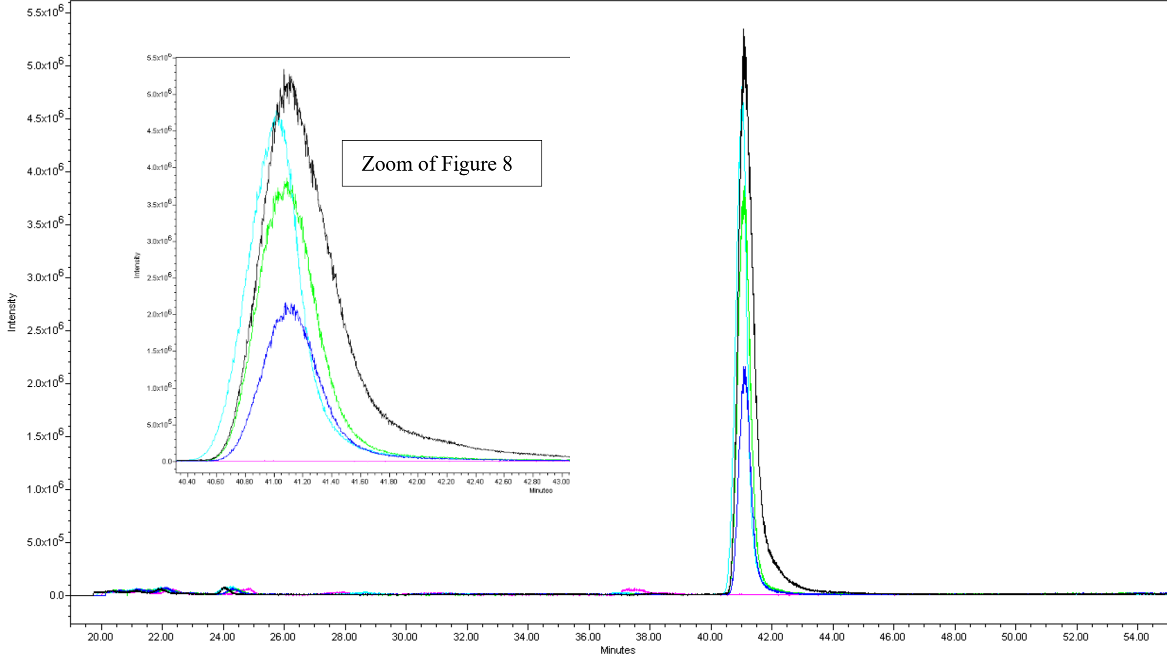


Figure 3.8 Overlay of chromatograms for the calibration curve of Triton X-100, used in conjunction with Sodium Cholate to decellularize the porcine SIS.

The chromatograms used to formulate the calibration curves with respect to Sodium Cholate are shown below in Figure 3.9. These are the superposition of the five points on the line showing increasing area trends in ascending order (black, blue, green, light blue, violet).

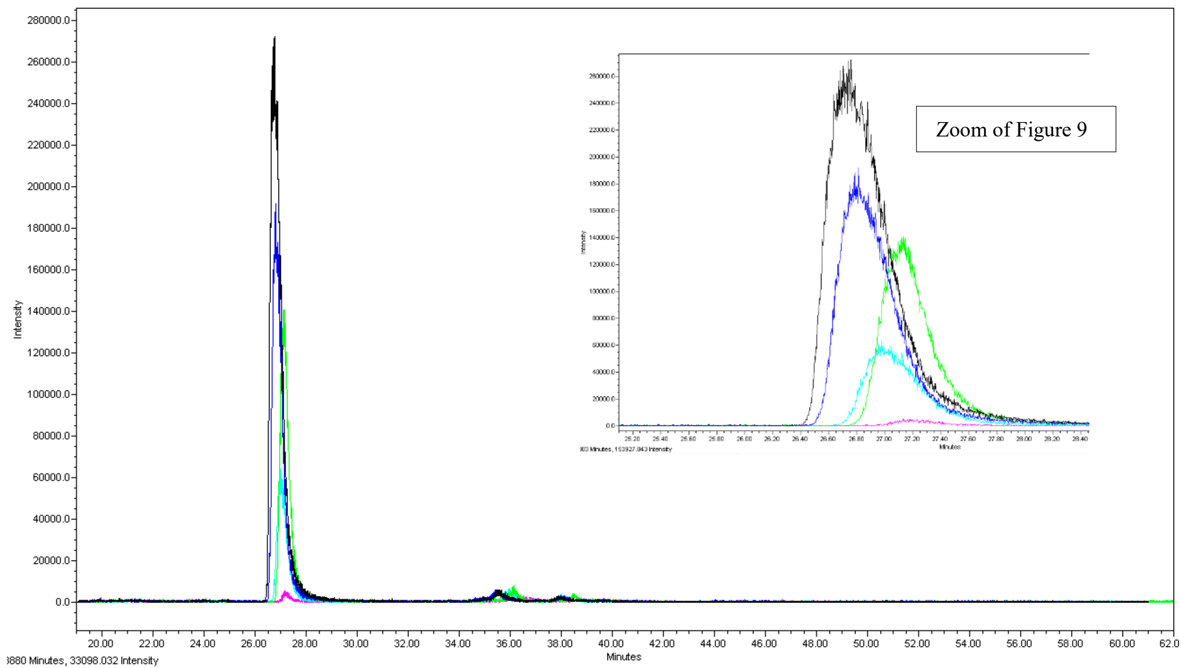


Figure 3.9 Overlay of chromatograms for the calibration curve of Sodium Cholate, used in conjunction with Tergitol 15-s-9 to decellularize the porcine SIS.

The chromatograms used to formulate the calibration curves with respect to SDS are shown below in Figure 3.10. These are the superposition of the five points on the line showing increasing area trends in ascending order (black, blue, green, light blue, violet, brown).

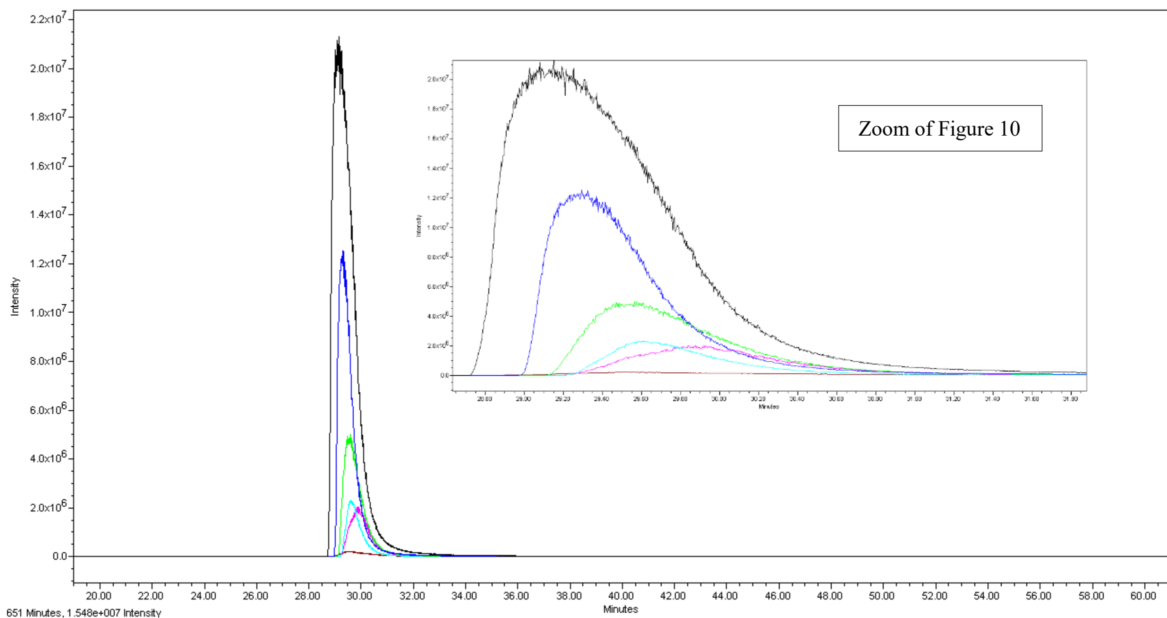
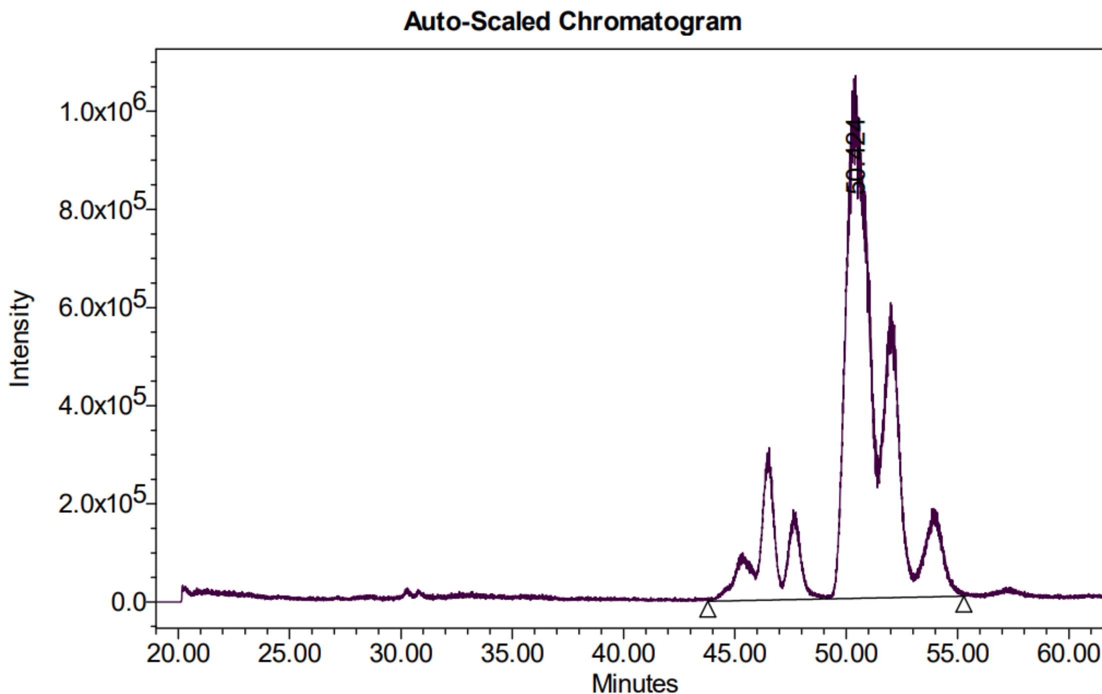


Figure 3.10 Overlay of chromatograms for the calibration curve of SDS, used in conjunction with Tergitol 15-s-9 and Sodium Cholate to decellularize the pericardium.

For each analysis and for each detergent, a mass spectrum is produced. An integration operation returns the subtended area, as an example shown in Figure 3.11.



Peak Results

	Name	RT	Area	Height	Amount	Units
1		50.424	130977179	1044381		

Figure 3.11 Integration to derive the Tergitol area in the smooth pSIS tissue

3.2 Calibration curves:

Initially analyses were done by injecting a known concentration of detergents in order to individuate the detergent retention time, as written in chapter 2.

In order to build calibration curves, native tissue is enriched with a known concentration of detergent. The calibration curve has in abscissa the area, in ordinates the concentration (molar one, expressed in mol/L or mg/ml, respectively).

The calibration curve of each detergent is the result of at least four/five analyses involving various concentrations of each detergent. At least four/five points on the calibration line thus obtained shall be obtained and passed through the origin. This will be the reference for quantifying the residual detergent in the decellularized tissue.

Each calibration curve is presented with its characteristic equation ($y = m*x$) and the coefficient of determination R^2, which is the index measuring the link between the variability of the data and the correctness of the statistical model used.

3.2.1 Calibration curves for porcine larynx.

Three calibration curves (Figure 5,6,7) are built up for porcine larynx, one for each detergent (Sodium Cholate, Sodium Dodecyl sulfate and Tergitol 15-s-9) which interact with the others. Decellularization protocol with Sodium Cholate, SDS and Tergitol 15-s-9.
On the top right side, the equation of the calibration curve is reported with the value of the determination coefficient.

Sodium Cholate:

Concentration [mM]	Area
0,0000875	47842
0,000175	123696
0,00035	1033162
0,0007	2501143
0,0028	3661479
0,0056	7529842
0,0084	11302654

Concentration [mg/mL]	Area
0,0000377	47842
0,000075	123696
0,00015	1033162
0,0003	2501143
0,0012	3661479
0,0024	7529842
0,0036	11302654

Table 3.1 Data for the calibration curve of Sodium Cholate, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

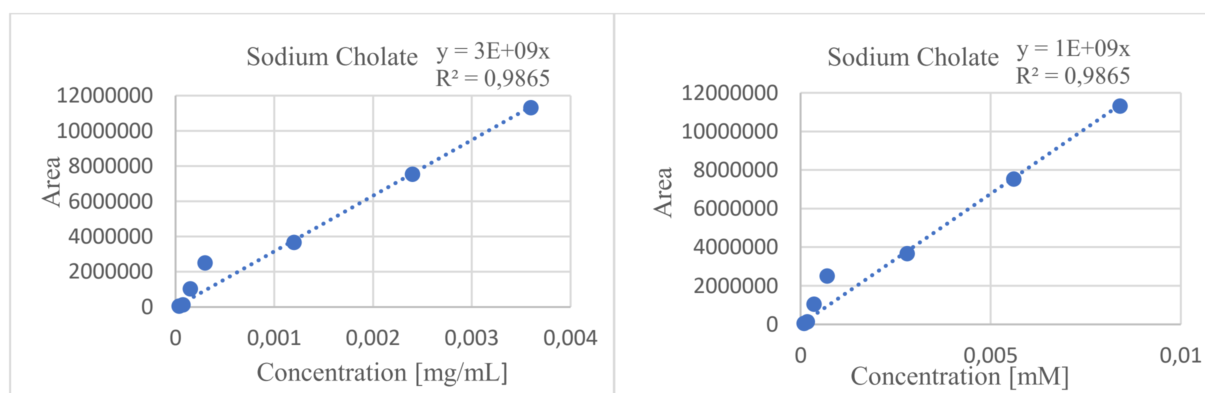


Figure 3.12 Calibration curve of Sodium Cholate, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

SDS:

Concentration [mM]	Area
0,0000875	7397094
0,000175	17797191
0,00035	93758465
0,0007	94246440
0,0014	243017700
0,0028	493558503
0,0056	956647622

Concentration [mg/mL]	Area
0,000025	7397094
0,00005	17797191
0,0001	93758465
0,0002	94246440
0,0004	243017700
0,00081	493558503
0,0016	956647622

Table 3.2 Data for the calibration curve of Sodium Dodecyl sulfate, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

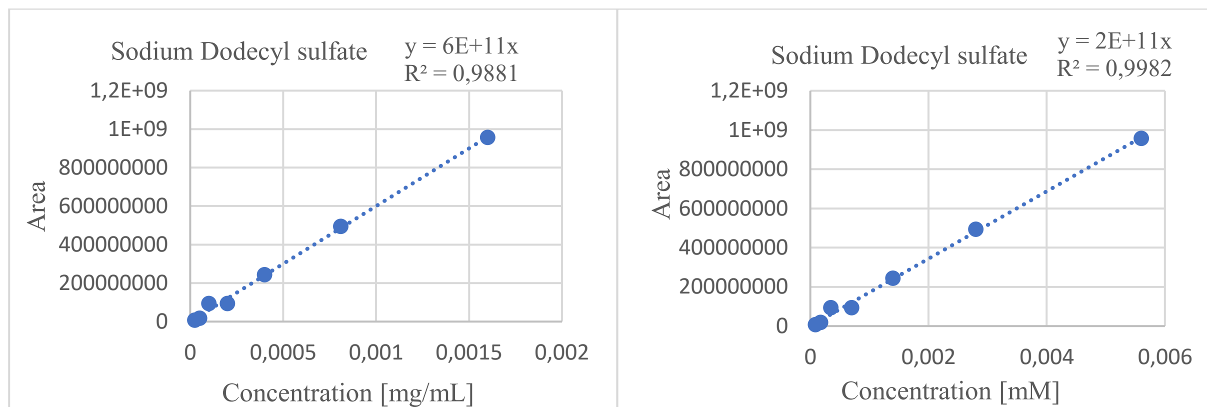


Figure 3.13 Calibration curve of Sodium Dodecyl sulfate, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Tergitol:

Concentration [mM]	Area
0,0007	33518372
0,0014	67874771
0,0028	130635009
0,0056	244126400

Concentration [mg/mL]	Area
0,00042	33518372
0,00083	67874771
0,0017	130635009
0,0033	244126400
0,005	291153994

Table 3.3 Data for the calibration curve of Tergitol 15-s-9, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

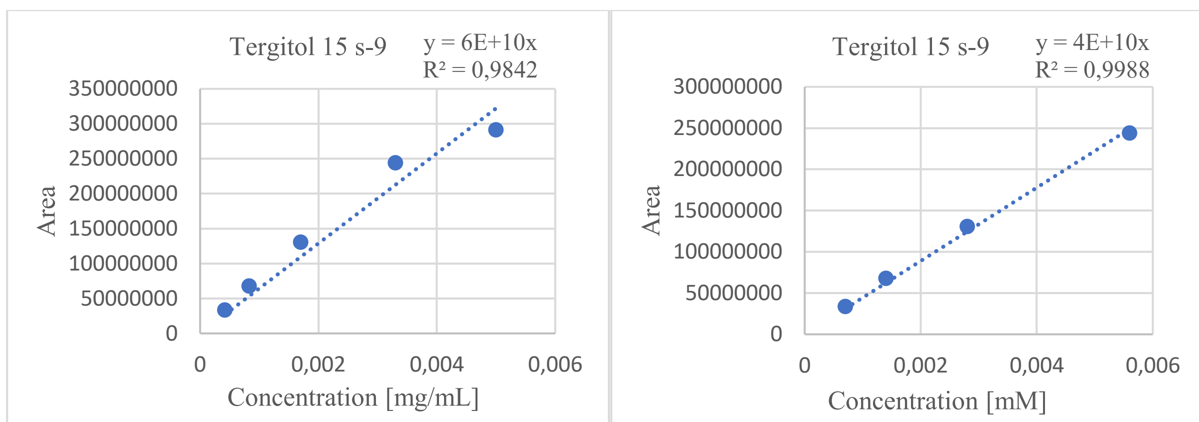


Figure 3.14 Calibration curve of Tergitol, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

3.2.2 Calibration curves for porcine Small Intestinal Submucosa.

For the analysis of the remnant detergents in pSIS, two titration curves have been formulated, considered the smooth part and rough part of this porcine tissue. The aim of these analyses is to observe whether the combination of detergents Tergitol 15-s-9 and Sodium cholate can be as satisfactory as Triton x-100 and Sodium Cholate one. This is fundamental because Triton X-100 has been taken off the market because it is environment harmful and was used in the previous decellularization methods.

For this reason, four calibration curves are built up for, one for each couple of detergent: Triton X-100 and Sodium Cholate, Tergitol 15-s-9 and Sodium Cholate. Eight calibration curves in total were formulated (Figure 8,9,10,11,12,13,14,15).

On the top right side, the equation of the calibration curve is reported with the value of the determination coefficient.

Smooth pSIS:

Decellularization protocol with Sodium Cholate and Tergitol 15-s-9.

Sodium Cholate:

Concentration [mM]	Area
0,000175	513990
0,00035	1668534
0,0007	3467365
0,0014	5087684
0,0028	8087768

Concentration [mg/mL]	Area
0,000075	513990
0,00015	1668534
0,0003	3467365
0,006	5087684
0,0012	8087768

Table 3.4 Data for the calibration curve of Sodium Cholate, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

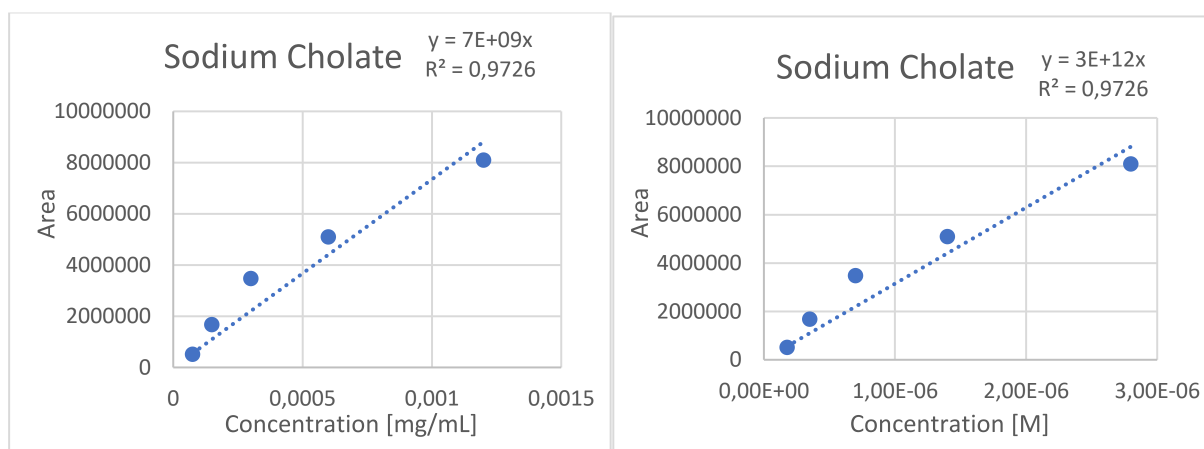


Figure 3.15 Calibration curve of Sodium Cholate, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Tergitol:

Concentration [mM]	Area
0,000175	14039168
0,00035	32395762
0,0007	84103743
0,0014	130977179
0,0028	219414621

Concentration [mg/mL]	Area
0,00001	14039168
0,00021	32395762
0,00042	84103743
0,00083	130977179
0,0017	219414621

Table 3.5 Data for the calibration curve of Tergitol, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

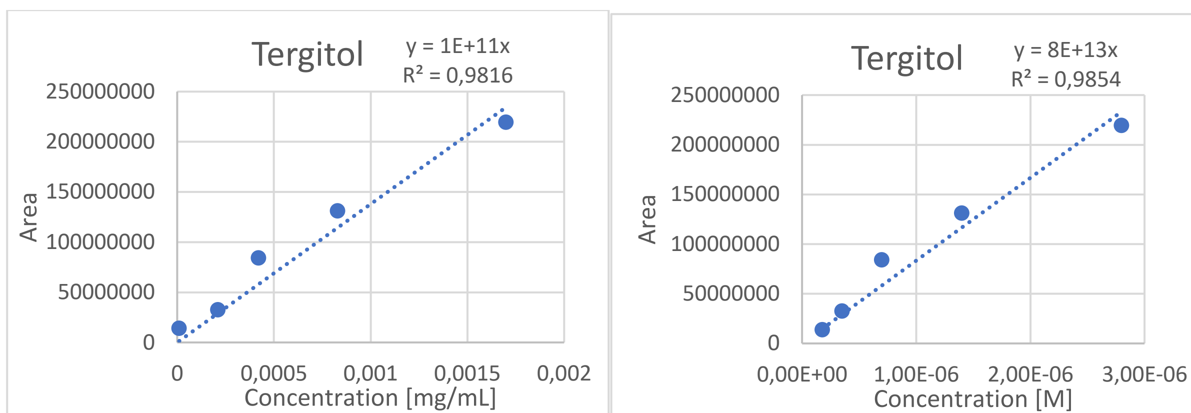


Figure 3.16 Calibration curve of Tergitol, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Decellularization protocol with Sodium Cholate and Triton X-100.
Sodium Cholate:

Concentration [mM]	Area
0,000175	616709
0,00035	1642741
0,0007	2841390
0,0014	5093074
0,0028	7948642

Concentration [mg/mL]	Area
0,000075	616709
0,00015	1642741
0,0003	2841390
0,0006	5093074
0,0012	7948642

Table 3.6 Data for the calibration curve of Sodium Cholate, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

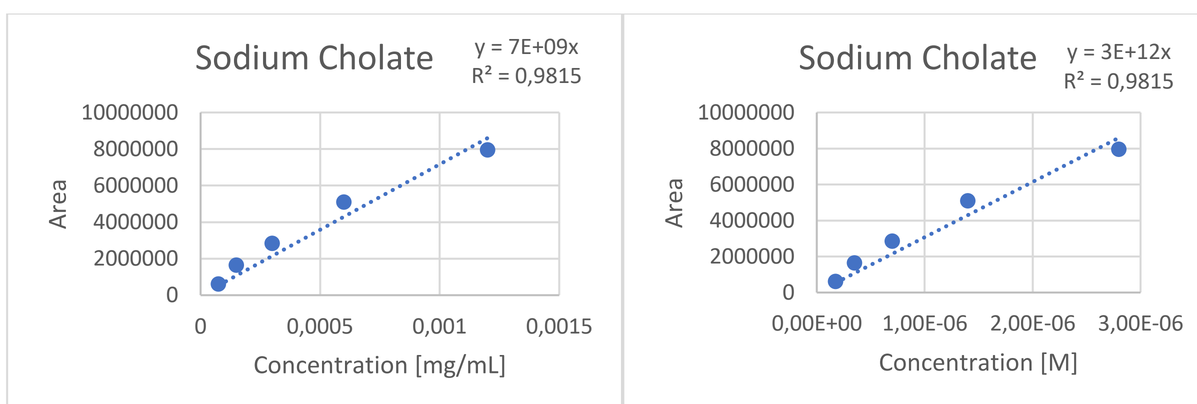


Figure 3.17 Calibration curve of Sodium Cholate, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Triton X-100:

Concentration [mM]	Area
0,000175	37179797
0,00035	52442977
0,0007	71527705
0,0014	93321902
0,0028	241698431

Concentration [mg/mL]	Area
0,00011	37179797
0,00022	52442977
0,00044	71527705
0,00088	93321902
0,00175	241698431

Table 3.7 Data for the calibration curve of Triton X-100, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

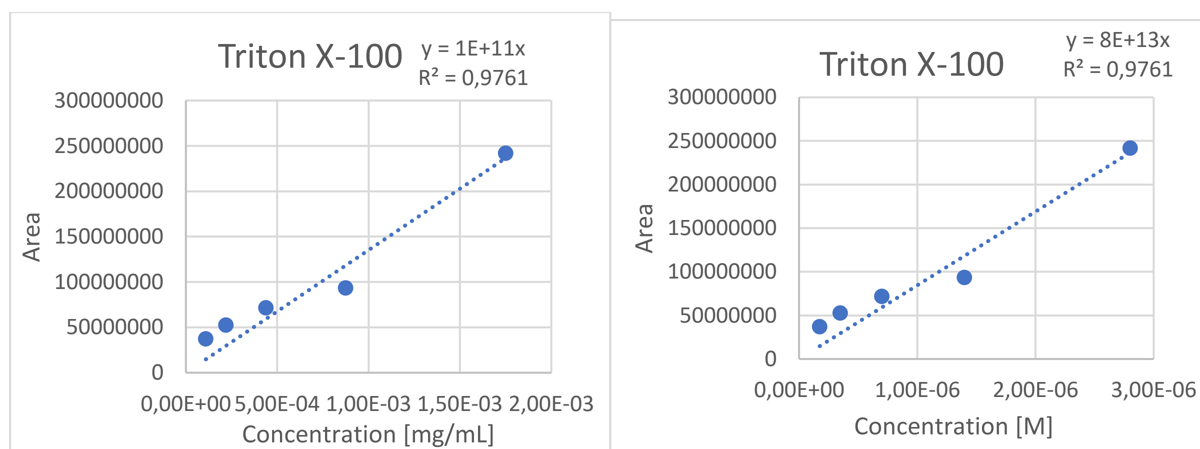


Figure 3.18 Calibration curve of Triton X-100, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Rough pSIS:

Decellularization protocol with Sodium Cholate and Tergitol 15-s-9.

Sodium Cholate:

Concentration [mM]	Area
0,000175	370972
0,00035	899922
0,0007	3016722
0,0014	4868616
0,0028	8256724

Concentration [mg/mL]	Area
0,000075	370972
0,00015	899922
0,0003	3016722
0,0006	4868616
0,0012	8256724

Table 3.8 Data for the calibration curve of Sodium Cholate, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

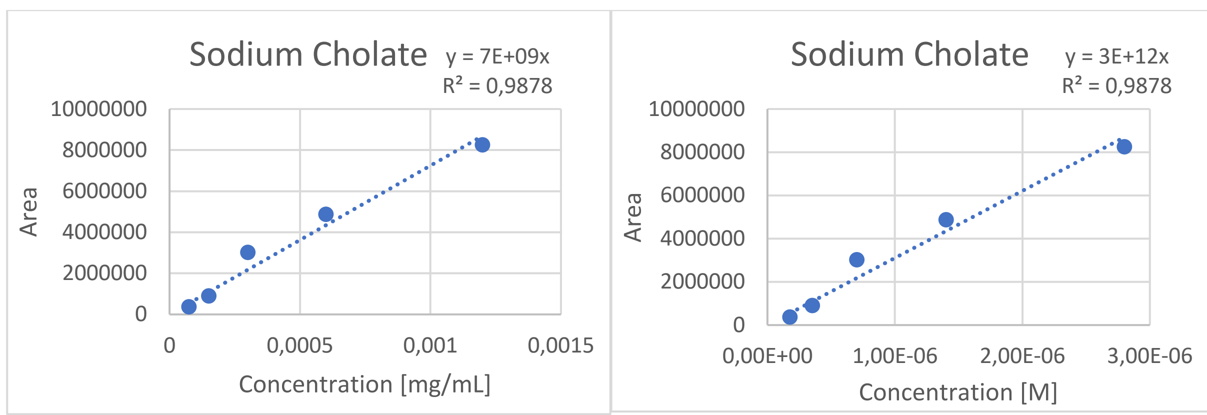


Figure 3.19 Calibration curve of Sodium Cholate, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Tergitol:

Concentration [mM]	Area
0,000175	13349433
0,00035	38761429
0,0007	63837303
0,0014	110502039
0,0028	189596974

Concentration [mg/mL]	Area
0,00001	13349433
0,00021	38761429
0,00042	63837303
0,00083	110502039
0,0017	189596974

Table 3.9 Data for the calibration curve of Tergitol, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

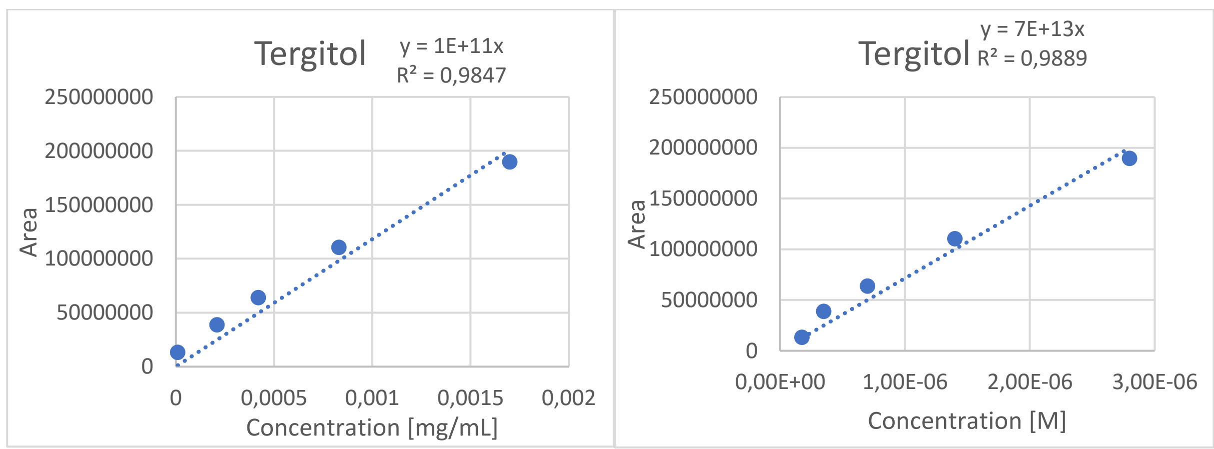


Figure 3.20 Calibration curve of Tergitol, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Decellularization protocol with Sodium Cholate and Triton X-100.
Sodium Cholate:

Concentration [mM]	Area
0,000275	1053971
0,00035	1304577
0,0007	2909242
0,00085	2664696
0,00105	3917291
0,0014	6366988

Concentration [mg/mL]	Area
0,000118	1053971
0,00015	1304577
0,0003	2909242
0,00037	2664696
0,00045	3917291
0,0006	6366988

Table 3.10 Data for the calibration curve of Sodium Cholate, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

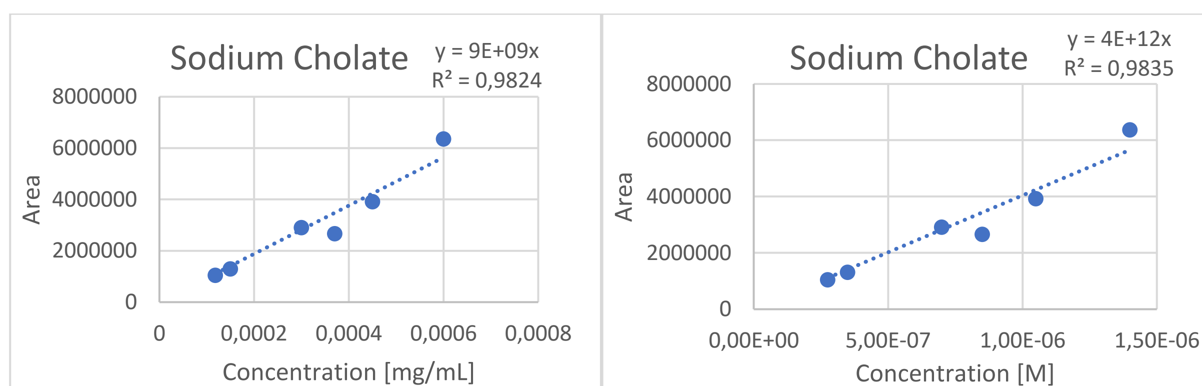


Figure 3.21 Calibration curve of Sodium Cholate, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Triton X-100:

Concentration [mM]	Area
0,000275	94619358
0,00035	127073548
0,0007	112731855
0,00085	70877344
0,00105	289757204
0,0014	209995916

Concentration [mg/mL]	Area
0,00017	94619358
0,00022	127073548
0,00044	112731855
0,00053	70877344
0,000656	289757204
0,000875	209995916

Table 3.11 Data for the calibration curve of Triton X-100, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

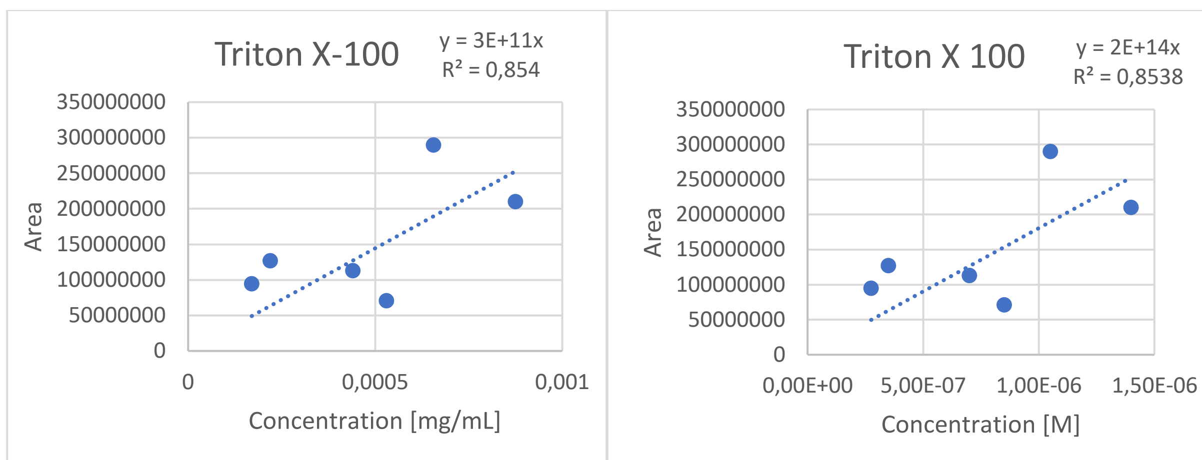


Figure 3.22 Calibration curve of Triton X-100, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

3.2.3 Calibration curves for bovine Pericardium.

Two calibration curves are built up with the native bovine pericardium, one for each detergent: Tergitol 15-s-9 and Sodium Cholate (Figure 16 and 17).

On the top right side, the equation of the calibration curve is reported with the value of the determination coefficient.

Decellularization protocol with Sodium Cholate and Tergitol 15-s-9.

Sodium Cholate:

Concentration [mM]	Area
0,000175	688732
0,00035	937164
0,0007	2083799
0,0014	2531431
0,0028	4371936

Concentration [mg/mL]	Area
0,00007	688732
0,00015	937164
0,0003	2083799
0,0006	2531431
0,0012	4371936

Table 3.12 Data for the calibration curve of Sodium Cholate, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

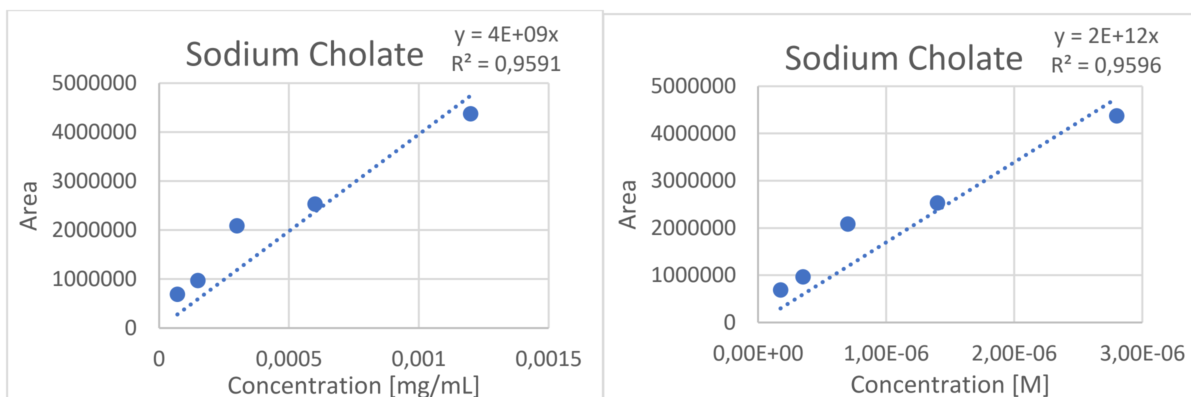


Figure 3.23 Calibration curve of Sodium Cholate, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

Tergitol:

Concentration [mM]	Area
0,000175	21348973
0,00035	26213720
0,0007	62396286
0,0014	156804389
0,0028	293640140

Concentration [mg/mL]	Area
0,00001	21348973
0,00021	26213720
0,00042	62396286
0,00083	156804389
0,0017	293640140

Table 3.13 Data for the calibration curve of Tergitol, on the right the concentration is expressed in mg/mL, on the left the concentration is expressed in mM.

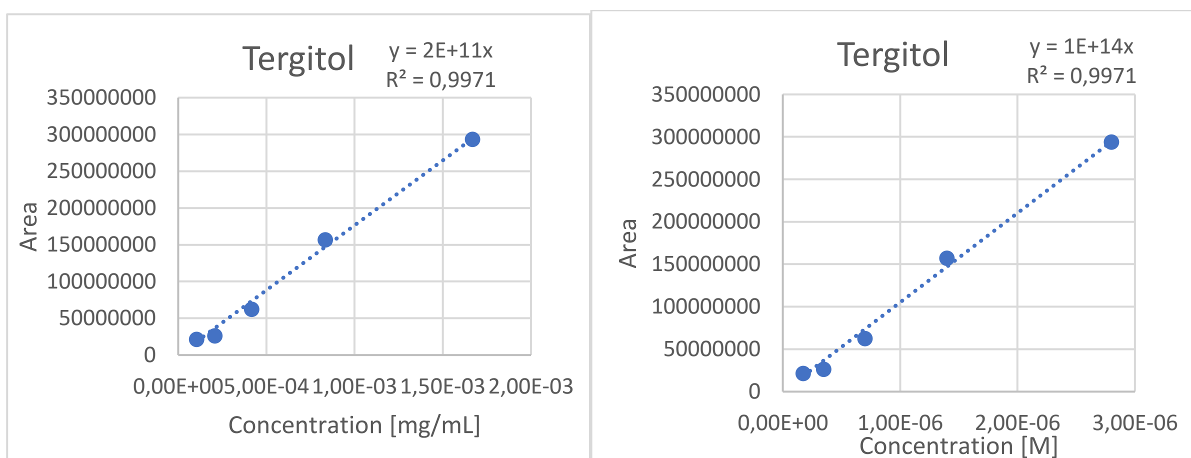


Figure 3.24 Calibration curve of Tergitol, on the left the concentration is expressed in mg/mL, on the right the concentration is expressed in mM.

3.3 Decellularized tissues:

The chromatographic runs for all the samples performed after tissue decellularization treatments and digestion were carried out in triplicate by separate injection into the column of 1 mL (3 or 4 mL for the pSIS tissue) of the previously filtered proteolytic digest.
The calibration curve is $y = m \cdot x + q$, where y is the area of the peak detected by HPLC + mass spectrometry, x is the unknown quantity of residual detergent, q is the intercept, here it is considered null because the calibration curves are imposed passing through the origin, m is the parameter, derived from the calibration curves, used to find the concentration of each detergent.

3.3.1 Porcine larynx samples.

Every sample (for example PA 41SX) is decellularized with a different protocol of decellularization.
Every analysis involves the injection of 1 mL in the chromatographic column. Every sample that the research group provided is with 40 mg of decellularized tissue for 10 mL of digestion buffer.

PA 40SX	**Concentration [mg/mL]**	**Concentration [mM]**
Sodium Cholate	$6,19 * 10^{-5}$	$19,1 * 10^{-5}$
Sodium Dodecyl sulfate	$1,61 * 10^{-5}$	$4,16 * 10^{-5}$
Tergitol 15-s-9	Too low to quantify	Too low to quantify

PA 40DX	**Concentration [mg/mL]**	**Concentration [mM]**
Sodium Cholate	$7,68 * 10^{-5}$	$23,0 * 10^{-5}$
Sodium Dodecyl sulfate	$2,19 * 10^{-5}$	$5,46 * 10^{-5}$
Tergitol 15-s-9	Too low to quantify	Too low to quantify

PA 41SX	**Concentration [mg/mL]**	**Concentration [mM]**
Sodium Cholate	$4,98 * 10^{-5}$	$14,9 * 10^{-5}$
Sodium Dodecyl sulfate	$1,24 * 10^{-5}$	$3,10 * 10^{-5}$
Tergitol 15-s-9	Too low to quantify	Too low to quantify

PA 41DX	Concentration [mg/mL]	Concentration [mM]
Sodium Cholate	$5{,}22 * 10^{-5}$	$15{,}7 * 10^{-5}$
Sodium Dodecyl sulfate	$1{,}05 * 10^{-5}$	$2{,}64 * 10^{-5}$
Tergitol 15-s-9	Too low to quantify	Too low to quantify

PA 43SX	Concentration [mg/mL]	Concentration [mM]
Sodium Cholate	$0{,}902 * 10^{-5}$	$2{,}71 * 10^{-5}$
Sodium Dodecyl sulfate	$2{,}07 * 10^{-5}$	$5{,}17 * 10^{-5}$
Tergitol 15-s-9	Too low to quantify	Too low to quantify

PA 43DX	Concentration [mg/mL]	Concentration [mM]
Sodium Cholate	$0{,}336 * 10^{-5}$	$1{,}01 * 10^{-5}$
Sodium Dodecyl sulfate	$0{,}832 * 10^{-5}$	$2{,}08 * 10^{-5}$
Tergitol 15-s-9	Too low to quantify	Too low to quantify

PA 44SX	Concentration [mg/mL]	Concentration [mM]
Sodium Cholate	$0{,}248 * 10^{-5}$	$0{,}743 * 10^{-5}$
Sodium Dodecyl sulfate	$0{,}895 * 10^{-5}$	$2{,}24 * 10^{-5}$
Tergitol 15-s-9	Too low to quantify	Too low to quantify

PA 44DX	Concentration [mg/mL]	Concentration [mM]
Sodium Cholate	$0{,}428 * 10^{-5}$	$1{,}28 * 10^{-5}$
Sodium Dodecyl sulfate	$1{,}38 * 10^{-5}$	$3{,}45 * 10^{-5}$
Tergitol 15-s-9	Too low to quantify	Too low to quantify

3.3.2 Porcine Small Intestinal Submucosa samples (pSIS).

Every analysis involves the injection of 3 mL (4 mL for the decellularized tissue with Triton X-100) in the chromatographic column. Every sample, that the research group provided, is with 15-18 mg of decellularized tissue for 5 mL of digestion buffer.

Smooth pSIS	Concentration [mg/mL]	Concentration [mM]
Sodium Cholate	$0{,}146 * 10^{-5}$	$0{,}340 * 10^{-5}$
Tergitol 15-s-9	$1{,}49 * 10^{-5}$	$2{,}50 * 10^{-5}$

Smooth pSIS	**Concentration [mg/mL]**	**Concentration [mM]**
Sodium Cholate	$0,212 * 10^{-5}$	$0,492 * 10^{-5}$
Triton X-100	$0,0760 * 10^{-5}$	$0,127 * 10^{-5}$

Rough pSIS	**Concentration [mg/mL]**	**Concentration [mM]**
Sodium Cholate	$0,213 * 10^{-5}$	$0,494 * 10^{-5}$
Tergitol 15-s-9	$1,50 * 10^{-5}$	$2,51 * 10^{-5}$

Rough pSIS	**Concentration [mg/mL]**	**Concentration [mM]**
Sodium Cholate	$0,196 * 10^{-5}$	$0,455 * 10^{-5}$
Triton X-100	$0,0349 * 10^{-5}$	$0,0586 * 10^{-5}$

3.3.3 Bovine pericardium samples.

Every analysis involves the injection of 1 mL in the chromatographic column. Each sample is the result of 15-18 mg of decellularized tissue digested in 5 mL of buffer.
These results are preliminary as there are two runs with the decellularized pericardium and therefore no triplicate with statistical significance is obtained.

Pericardium	**Concentration [mg/mL]**	**Concentration [mM]**
Sodium Cholate*	$12,7 * 10^{-5}$	$25,5 * 10^{-5}$
Tergitol 15-s-9*	$6,22 * 10^{-5}$	$12,4 * 10^{-5}$

3.4 Discussion.

The decellularization of biological tissues, for bioprostheses application aim, is a treatment that allows the removal of antigenic determinants and avoids rejection phases by attenuating inflammatory processes. Determining the fate of the detergents used in this treatment is particularly important since their deabsorption from the scaffold may be cytotoxic or induce inflammation and subsequent calcification of the bioprosthesis.
In addition, the determination of residual concentrations of detergents in the scaffold is important to study denaturing effects on the ECM that could be reflected in a difficult post-implant scaffold cell repopulation.

To date, many studies in the literature only report the determination of detergent concentrations in the washing solutions (undirect methods) of the bioprosthesis undergoing decellularization: studies on the cytotoxicity of detergents are carried out by seeding cells directly into these solutions. Quantification of detergents in washings included in decellularization methods are not an accurate tool for check whether quantities of detergents that may be harmful are present or not. [36] [37] [38]

The few publications reporting a determination of detergents entrapped in the scaffold use methods in which the detergent forms an ion pair with methylene blue and the salt obtained is extracted with chloroform and quantified spectrophotometrically.

The possibility of direct analysis of residual amount of detergent shows that detergents are not completely removed from the matrices. Direct chromatographic analysis of the detergents in the digest without any sample manipulation (concentration, elution in cartridges, extraction in the organic phase) has enabled significantly reduce sources of error.

The practicability of this method is confirmed by the linearity of the measurements, it gives results proportional to the analyte concentration in a given range.

3.4.1 Coefficient of determination R^2 of calibration curves.

The coefficient of determination R^2 is calculated for each calibration curve.

For the porcine larynx, the coefficient of determination is close to the unit (0,99 and 0,98), this highlights the reliable proportionality between the peak area and the amount of the analyte added to the defatted sample digest.

For the porcine SIS, the coefficient of determination is less close to the unit in the case of the decellularization with Triton X-100 and Sodium Cholate, in all the other cases it is close to the unit (0,99 0,98 0,97).

For the bovine pericardium, the coefficient of determination of the Tergitol 15-s-9 is close to the unit (0,997), while the R^2 of the Sodium Cholate is a bit worse (0,959).

3.4.2 Discussion about porcine larynx samples.

Comparing samples PA40SX and PA40DX with Pa41SX and PA41DX shows that there is a lower amount of Sodium Cholate and SDS in the latter samples. Therefore, the decellularization method involving a single freeze/thaw cycle and a single final step with peracetic acid is better.

Comparing samples PA41SX and PA41DX with Pa43SX and PA43DX, it can be deduced that there is a lower amount of Sodium Cholate in the latter samples. Therefore, the decellularization method involving two additional days of washing is better.
Comparing samples PA43SX and PA43DX with Pa44SX and PA44DX, it can be deduced that the method with the final 6 days of washing is not much better than the decellularization protocol of 5 days.

3.4.3 Comparison between Tergitol 15-s-9 and Triton X-100.

Triton X-100 was extensively used in the past to perform tissue decellularization, but recently it was retired from the commerce because it is considered environment harmful.
The main purpose of the analysis with the porcine SIS is the comparison between the residual detergents Tergitol 15-s-9 and Triton X-100. Both for the smooth and rough tissue, the concentration of Triton X-100 is less than Tergitol 15-s-9 by an order of magnitude.
A further comparison can be done between smooth and rough SIS porcine tissue. For tissues treated with Sodium Cholate and Triton X-100 there is a lower detergent content in the rough samples and vice versa for decellularisation with Sodium Cholate and Tergitol. There is therefore an influence due to the roughness of the surface or the composition of the two sides (amount and type of biomolecules composing it).

4 Conclusions:

In this thesis work, three extracellular matrices were taken into consideration, which were decellularized using a total of four different detergents. The porcine larynx decellularized with SDS, Tergitol and Sodium Cholate; the porcine SIS which is deprived of cells by two different methods: TriCol (Triton-X and Sodium Cholate) and TergiCol (Tergitol 15-s-9 and Sodium Cholate); decellularized bovine pericardium which is analysed for residues of Tergitol 15-s-9 and Sodium Cholate.

The calibration curves obtained allow to estimate, and in one case compare, the residual amounts of SDS, Sodium Cholate, Triton X-100 and Tergitol 15-s-9.

The different treatments analysed, aimed at removing the cellular component, show different levels of effectiveness in removing detergent residues from the tissue.

After overcoming various difficulties and carrying out a great deal of experimentation in the search for a method, it was possible to find one that finally determines the amount of detergent directly in the matrix with good linearity and reproducibility. The main advantage of this method is that it is no longer possible to determine not only the detergents that can be seen at the UV, but all detergents with the mass detector from their molecular mass. A small step that opens a big door to research in Tissue Engineering world.

Future development of this study will be directed towards the examination of detergent residues in different extracellular matrices, analysing new decellularization protocols.

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