

# Master in Chemical Engineering

## *"Measuring the Degradation of Elastin and Collagen Throughout a Chemical Elastin Isolation Process"*

### Master's Thesis

Of

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University of Maryland, Baltimore County



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## Abstract

The aorta is the largest artery in the human body, and over time changes occur in the functionality of the aorta. The age-related changes in mechanical behavior depend on biochemical changes in the elastin and collagen in the arteries. It is necessary to understand how various processes affect the mechanical response of arteries and isolated collagen and elastin components. In particular, mechanical properties like elastic modulus change as a function of age.

The overall goal of this work is to study the elastin isolation process to determine if the method currently used to isolate elastin modifies the elastin protein. The technique used to isolate elastin from arteries required tissue digestion by exposure to cyanogen bromide in formic acid for different time durations, and then performing uniaxial mechanical tests to examine changes in mechanical behavior. It was demonstrated that the Elastic Modulus (E) at low stress does not change significantly with different exposure times during the isolation process.

To measure the mass of elastin and collagen in pig aorta samples, we used colorimetric methods: for elastin we used a commercial kit “Fastin™ Elastin Assay” and for collagen we use a commercial kit “Sircol™ Soluble Collagen” purchased by Biocolor Ltd.

The results obtained by the collagen assay shows that most of the collagen was removed after 2 hours of exposure to the cyanogen bromide/formic acid, only a residual amount was found at the end of the process (19 hours), about 90 % was removed. For the elastin, the quantity of elastin did not change significantly during the process. The initial elastin:collagen ratio obtained in the porcine aorta was  $1.41 \pm 0.02$ , this value is in agreement with the finding obtained by Basu et al. in the aorta was  $1.18 \pm 0.02$ .

These results indicate that the amount of elastin is not changed by the isolation process, and that the mechanical properties (E) did not change; however these results are not enough to confirm that the process does not chemically damage the elastin.

**Keywords:** Aorta; Elastin; Collagen; Cyanogen Bromide; Mechanical Properties; Colorimetry

## Resumo

A aorta é a maior artéria do corpo humano e ao longo do tempo sucedem mudanças na funcionalidade da aorta. São principalmente estudadas, nas artérias, as mudanças no comportamento mecânico relacionadas à idade da elastina dessas mesmas. É imprescindível compreender como vários processos afectam a resposta mecânica das artérias e individualmente do colágeno e da elastina. Em particular, as propriedades mecânicas como o módulo de elasticidade em função da idade.

A finalidade geral deste trabalho é estudar o processo de isolamento da elastina e procurar saber se o actual método usado para isolar a elastina altera a proteína. A técnica usada para isolar a elastina das artérias requiere digestão do tecido com brometo de cianogénio em ácido fórmico para diferentes tempos, e em seguida, o sistema de teste uniaxiais para realizar ensaios mecânicos analisando as mudanças no comportamento mecânico. Com as ferramentas disponíveis no BiMaTS, demonstrou-se que o módulo de elasticidade (E) a baixa força/stress não se modifica significativamente com o processo de isolamento.

Para quantificar a elastina e o colágeno nas amostras de aorta de suínos foi utilizado um método colorimétrico. Para a elastina foi utilizado um kit comercial "Fastin™ Assay Elastin" e para o colágeno, um kit comercial "Sircol™ Soluble Collagen", fornecidos por Bicolor Ltd. Os resultados obtidos pelo ensaio de colágeneo mostram que a maior parte do colágeneo é eliminado após 2 horas de isolamento com brometo *de cianogénio*/ácido fórmico. Apenas uma quantidade residual foi encontrada no final do processo (19 horas) e cerca de 90% foi retirado. Para os testes de elastina foi observado que a quantidade de elastina não se altera significativamente ao longo do processo. A razão de elastina:colagénio obtida na aorta suína foi de  $1.41 \pm 0.02$ , este valor encontra-se em consenso com os valores publicados por Basu et al. na aorta,  $1.18 \pm 0.02$ .

Estes resultados indicam que a quantidade de elastina não se altera durante o processo de isolamento, assim como a propriedade mecânica (E). No entanto, não são suficientes para confirmar que o processo não degrada quimicamente a elastina.

**Palavras-Chave:** Aorta; Elastina; Colagénio; brometo de cianogénio; Propriedades Mecânicas; Colorimetria



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## Notation and Glossary

$A$	Área	$m^2$
$E$	Elastic modulus	kPa
$L$	Initial lenght	m
$P$	Load	N
$l$	Lenght	m
$t$	Thickness	m
$w$	Width	m
$m$	Mass	g
$V$	Volume	L
$C$	Concentration	g/L

### *Greek letters*

$\sigma$	Stretch	m/m
$\sigma_e$	Transition Stretch	m/m
$\sigma_r$	Breaking Stretch	m/m
$\lambda$	Stress	kPa
$\lambda_e$	Transition Stress	kPa
$\lambda_r$	Breaking Stress	kPa

### *List of Acronyms*

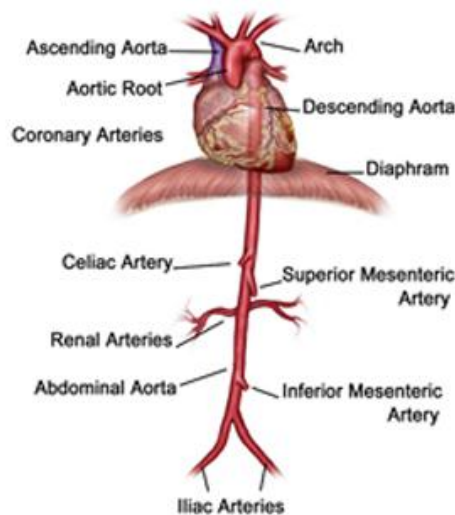
BiMaTS	Biological Materials Testing System
Met	Methionine
PBS	Phosphate Buffered Saline
LM	Light Microscopical
SEM	Scanning Electron Micrographs
TEM	Transmission Electron Micrographs
HyPro	Hydroxyproline
SCA	Sircol Collagen Assay
TPPS	5,10,15,20-tetraphenyl-21,23-porphine tetrasulfonate
Abs	Absorbance

# 1 Introduction

## 1.1 Background and Project Presentation

In the last few years there has been a significant growth in interest in the mechanical properties of biological tissue, in particular the mechanics of arterial tissue. The arteries have an important role in human health: they carry the blood from the heart through the body to provide oxygen and other nutrients. Cardiovascular disease, i.e., when arteries do not function properly, can lead to stroke, heart attack, and death.

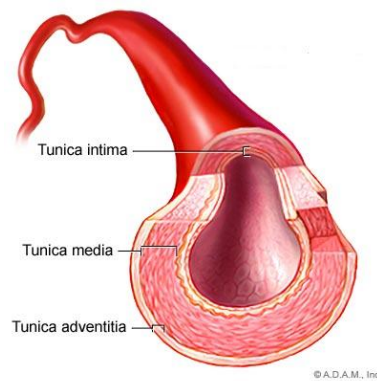
The aorta is the largest artery in the body. It ascends from the left ventricle of the heart to an arch where blood vessels branch off to supply blood to the arms and head. It then begins to descend through the chest and into the abdomen, where it splits into two iliac arteries that provide blood flow to the legs. Along its descent, more small arteries branch out to supply blood to the stomach, intestine, colon, kidneys, and the spinal cord, see Figure 1.



*Figure 1* - Structure of aorta [1].

The aorta not only conducts the blood to the different organs, but is also a major component to regulate the biomechanics of the circulatory system. The aorta distends when the blood pressure rises during systole and then recoils when the blood pressure falls during diastole.

The walls of the aorta consist of three layers of tissue that allow the blood vessel to support the high pressure that is generated when the heart pumps the blood to the body. The inside thin layer in contact with blood is called the tunica intima and has endothelial cells; the middle layer is called the tunica media and consists mostly of elastin and some collagen; the external layer has mostly collagen and some elastin, and is called the tunica adventitia, *Figure 2*.



**Figure 2** - Arterial wall layers [2].

There are two blood pressure numbers, the systolic and the diastolic pressures. Systolic blood pressure is the pressure when the heart beats while pumping blood. Diastolic blood pressure is the pressure when the heart is at rest between beats. The normal blood pressure numbers are 120/80 mmHg [3]. The elastin and collagen allows the aorta to stretch to prevent over-expansion due to the pressure that the blood flow exerts on the walls.

Biomechanical properties of the aorta have been intensively studied by many researchers over the past decades and are still the subject of a growing interest to better understand cardiovascular diseases. It is well known that the elastic properties of human aorta decrease gradually with age; the changes in mechanical properties could be caused by a decrease of elastin [4], so elastin has been isolated by hot alkaline extraction from the aortic connective tissue from subjects of different ages, and their amino acid composition has been studied.

Vascular diseases are associated with changes in the mechanical properties of the arterial wall. Degradation of elastin in the artery has been assumed to be a major cause that alters the stress distribution produced by blood pressure [5]. Elastin degradation affects the mechanical properties of blood vessels. The experimental tools available today permit the calculation of stress in the wall of aorta. In a typical experiment, tissue strips of longitudinal or circumferential orientations are stretched to failure. However, the mechanical properties

of elastin, and how those properties change with age, are not fully understood, so mechanical testing of tissue specimens is one of the methods to gain this knowledge.

One of the projects of Dr. Topoleski is looking at age related changes in the mechanical behavior of arterial elastin, trying to understand the stress-stretch response of the artery, and how this response changes over the time in healthy and diseased arteries. To do this, Dr. Topoleski's lab isolates the elastin by dissolving away the other materials that make up the artery (collagen, smooth muscle cells, and ground substance). They do this using a formic acid - cyanogen bromide digestion. The procedure takes about 19 hours to complete [6].

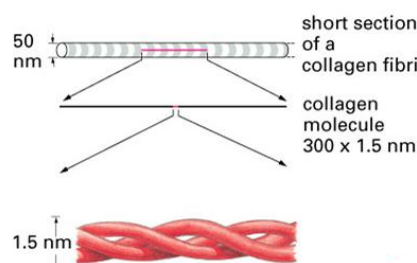
The majority of procedures used to obtain purified elastin are drastic enough that breakdown of elastin occurs [7]. Our goal is to determine whether the formic acid solution produces changes in the elastin protein. Therefore, this project will focus on the method to isolate elastin, and investigate whether the elastin has the same properties before and after the isolation process.

## 1.2 Collagen and Elastin

Collagen, the main protein of connective tissues, is an inextensible fibrous protein, which can be found at the level of connective tissues in heart, vessels, skin, cornea, cartilage, ligaments, tendons, bone, and teeth. Collagen comprises about 20-30 % of the total body protein [8] and there are twenty-eight known types of collagens [9]. Is the most abundant protein in vertebrates and accounts for 66 % of all proteins in humans [10].

Collagen is a triple helix protein composed of three chains that wrap around one another, see

**Figure 3.**



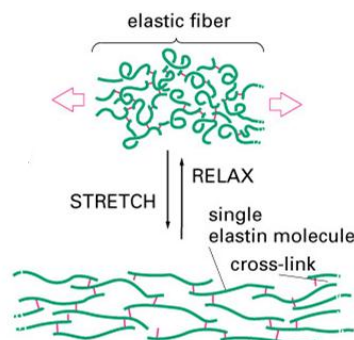
**Figure 3-** Structure of Collagen.



The primary structure of collagen is the polypeptide chain, a linear array of  $\alpha$ -amino acids, mainly comprising the repeating tripeptide unit Gly-X-Y (Gly = glycine). The nature of the X and Y amino acid residues can vary but X is mostly proline (Pro) and Y is mostly hydroxyl-proline (Hyp), other common amino acid found in collagen is hydroxylysine [11].

Elastic fibers are composed of two primary elements: amorphous elastin and microfibrils. Elastin is an extracellular matrix protein and is formed from the cross-linking of many peptides, but the structure is not organized as in the collagen triple helix; the loose fitting peptides give elastin its elastic qualities. Common amino acids are non-polar glycine, valine, alanine as well as proline and lysine.

Elastin, as its name implies, is a protein with an elastic quality: it will return to its original state after stress, whether it is compressed or stretched. Is one of nature's most apolar proteins and is highly insoluble and has a large number of inter- and intra-chain crosslinks in the protein *Figure 4*. Because of these properties elastin, is a protein with poor dissolution even when exposed to strong protein denaturants, hot alkali treatment, or repeated autoclaving.

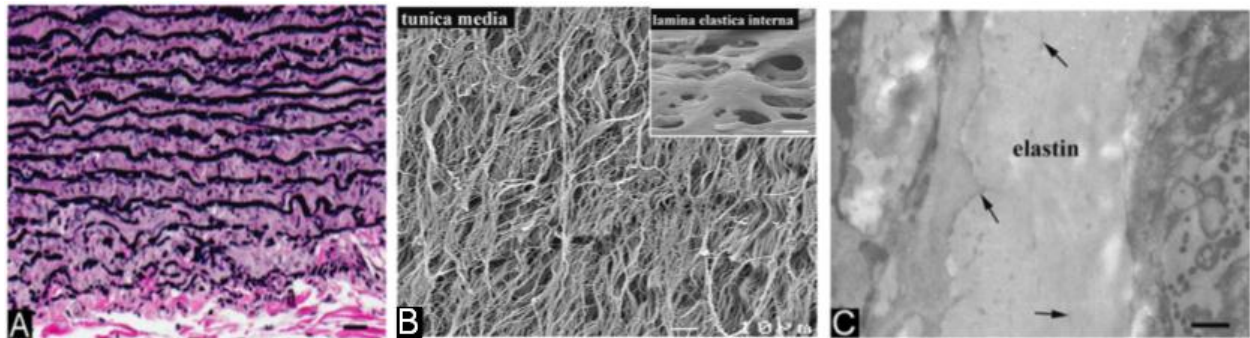


**Figure 4** - Structure of Elastin [12].

Therefore, elastin is most abundant in organs where elastic properties are of major importance, like in blood vessels, which stretch and relax more than a billion times during life, in elastic ligaments, and in skin. Elastin comprises up to 70 % of the dry weight in elastic ligaments, about 50% in large arteries, 30 % in lung, and 2-4 % in skin. In general, elastic fibers are present as rope-like structures like in ligaments, in the media of elastic arteries and in skin [13]. The elastin degradation rate strongly depends on age, amount of hormones, stress, smoking and UV exposure [14].

Collagen and elastin support the body's tissues; they give body tissue form and provide firmness and strength. Collagen provides tensile strength and elastin gives flexibility that the tissues need. Elastin provides for structural integrity and for the compliance of the vessel at low pressure, whereas collagen gives the tensile resistance required at high pressures.

Images of bovine aorta are shown in *Figure 5*, A-from a light microscope (LM) show elastic fibers in black and collagenous material in pink; B,C-scanning/transmission electron microscopes, respectively, of partly purified bovine aorta show elastic fibers ultrastructure.



**Figure 5** - Pictures of bovine aorta: (A) - Light Microscopic (LM) staining (Verhoeff-Van Gieson) show elastic fibers in black and collagenous material in pink; (B) - Scanning Electron Micrographs (SEM); (C) - Transmission Electron Micrographs (TEM) [13].

SEM reveals internal elastic lamina of aorta, a layer that is formed of mainly longitudinally directed elastic fibers, *Figure 5A* in the corner of the picture. The internal lamina elastic layer separates the media from the intima.

### 1.3 Contribution of this research

It is important to study the biomechanics of normal and diseased tissue function to understand the behavior throughout a person's lifetime. One important motivation for such studies is the belief that mechanical factors may be important in atherosclerosis, also known as arteriosclerotic vascular disease or ASVD, the major cause of Human mortality in the western world [15]. Research in biomechanics has already led to the discovery of treatments and improvements in many fields of medicine, including cardiology. Is essential to understand the changes of structure in Human arteries that occur as a result of aging or disease, and responses to interventions used to treat heart diseases.

Elastin is a protein that needs considerable study to understand how aging affects the protein and thus the function of the arteries. To study the mechanical age-related changes in the arteries induced by elastin is necessary to have specimens of arterial elastin to test.

The overall aim of this work is to understand if the elastin in specimens isolated for the mechanical testing of elastin are the same as the untreated elastin in arteries.

## 1.4 Thesis organization

This thesis is organized in several sections.

The first Chapter is the introduction with the presentation of the project.

The second Chapter is a review of the State of the Art, a description of the current state of the art of the problem under the present work.

The third Chapter is the technical description of the work conducted. Here all the methods and protocols used are described in detail.

The fourth Chapter is the Results and Discussion section, which is one of the most important sections of this thesis, as it is where the results obtained from the experiments conducted are presented and discussed.

The fifth and final Chapter presents the conclusions of this project, followed by a general evaluation of the work conducted, which includes a commentary on the objectives accomplished as well as some suggestions for future work to be performed.

In the Appendixes, additional information can be found as well as all of the data and graphs from the BiMaTS in Appendix I, the data and from the collagen assay in Appendix II, and from elastin assay in Appendix III. Statistical results are presented in Appendix IV, and details on the protocol for the elastin and collagen assays provided by the manufacturer are presented in Appendix V and VI respectively. And finally, the material safety data sheet is presented in Appendix VII.

## 2 State of the Art

The Young's modulus of single elastic fibers isolated from cows was, for the first time, determined by Aaron, et al. [16] using a micro test apparatus attached to a polarizing microscope and was in the range of 400 - 1200 kPa. The Young's modulus of elastic fiber-rich tissue samples of purified dog or sheep aorta was determined to be in the range of 130 - 650 kPa [17]. Lillie, et al. determined the Young's modulus to be between 100 - 800 kPa for tissue samples of purified pig aorta enriched with elastic fibers [18].

Yu Zou, et al., discovered that the isolated elastin appears to be a more porous structure; the isolation of elastin increases the elastin concentration by removing SMCs, collagen, and other ECM components. The tangent modulus in the circumferential direction at low tensile strains was shown to vary from 100 to 350 kPa. The tangent modulus in the longitudinal direction is significantly lower than that in the circumferential direction [19].

Healthy thoracic aorta has been studied by Vorp [20] and no difference was found between the two orientations, circumferential or longitudinal. Also he demonstrated that elastic modulus from ascending thoracic aortic aneurysm (ATAA) is 4.67 MPA for circumferential orientation and 4.48 MPA for longitudinal orientation.

Ferraresi, et al. [21] performed uniaxial testing of porcine aortic tissue and reported the circumferentially-oriented tissues to be stiffer than their longitudinal counterparts.

The histological structure of the wall, especially the orientation and integrity of elastin and collagen fibers, is related to the issue of anisotropy, and was studied by He and Roach in aneurysmal and healthy abdominal aortas [22]. They found that in healthy aorta, the media is organized in lamellar units composed of elastin layers, while in aneurysmal abdominal aortas, the media lamellar units were damaged and the elastin fragmented. These results suggest that healthy tissue is anisotropic and that, theoretically, the anisotropy is partially or completely lost in aneurysmal tissue.

Q. Lu et al. [6] published the first report that shows the use of CNBr to cleave aorta for tissue engineering applications. This report demonstrated that treatment with CNBr completely removed cells and collagen from the porcine aorta, resulting in elastin scaffolds. This study did not detect cells and collagen by H&E and Masson's trichrome staining, and SEM analysis of elastin scaffolds revealed a well-oriented 3-D network of elastic fibers, indicating that CNBr treatment effectively removed aortic cells and collagen fibers, obtaining highly

porous elastin scaffolds. DNA assays showed that scaffold preparation steps removed more than 98% of DNA compared to fresh aorta.

Basu et al reported the elastin:collagen ratio in the aorta was  $1.18 \pm .02$ ; in the carotid artery the ratio was  $1.03 \pm 0.4$ , whereas in the femoral artery and vena cava it was  $0.61 \pm 0.1$  and  $0.57 \pm 0.1$ , respectively [23].

Many different procedures have been published for purifying elastin, and all have their relative advantages and disadvantages. Hot alkali and autoclaving are the most used procedures.

Daamen et al reported comparison of six procedures: one procedure is based on treatment with 0.1 M NaOH, another on treatment with cyanogen bromide. Three other procedures are based on combinations of extraction steps and enzyme digestions. The procedure involving extractions/enzymes combined with an early application of 2-mercaptoethanol and cyanogen bromide gives a highly pure elastin preparation [24]. The method with cyanogen bromide (CNBr) treatment obtains elastin from tissues containing low amounts of elastin as compared to tissues like the aorta [25].

Even with the increased interest in studying the behavior of the arteries, currently little information exists about the elastin in human arteries.

## 3 Technical Description

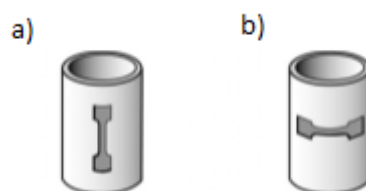
### 3.1 Tissue harvesting

Segments of pig aortic were obtained from a local abattoir, and were dissected, cleaned of adherent tissues and fat and rinsed in cold sterile saline, Figure 6 .



**Figure 6** - Pig aorta (a) before cleaning; (b) after cleaning.

The specimens were cut into standard dumbbell shaped testing specimens with 10.9mm x 30.8 mm cross-section for tests along circumferential direction. Healthy thoracic aorta has been studied by Vorp [20] and no difference was found between the two orientations, circumferential or longitudinal, *Figure 7*.



**Figure 7** - Orientation of aortic wall samples: a) longitudinal; b) circumferential [26].

### 3.2 Preparation of aortic elastin scaffolds

To isolate the elastin in aortic samples, we use the protocol of Q. Lu et al [6]. This treatment used cyanogen bromide (CNBr) to remove cells, collagen and other components except elastic fibers.

The samples were treated with 50 mg/ml of CNBr in 70 % formic acid (8 ml/cm<sup>2</sup>) under a fume hood with gentle stirring at 20 °C for nineteen hours. Then the samples are

heated to 60 °C for one hour followed by five minutes in a large beaker with boiling water to inactivate CNBr.

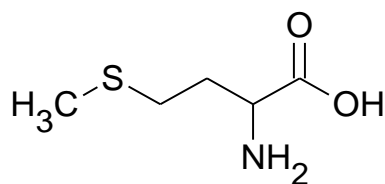
This process works by cleaving methionine from proteins. Collagen and other proteins in arteries tend to be methionine rich whereas elastin tends to be methionine poor, which is why it survives the process.

To investigate the possible effects of the elastin isolation process on the biomechanical behavior and chemical make up, different specimens were exposed to the the formic acid/CNBr digestion technique for different time periods, up to the full nineteen hours specified in Q. Lu et al [6]. After each time period, specimens were biomechanically and chemically examined.

### 3.2.1 Isolation of elastin

Because elastin is more resistant to chemical treatments than other proteins it should be an easy protein to purify under extreme conditions of pH and high temperature. Most proteins are denatured and solubilized under these condition, but not elastin. In fact, extraction with hot alkali has long been the method of choice for obtaining what is regarded as highly purified protein from elastin tissues [27]. The extraction of soluble  $\alpha$ -elastin using hot alkali treatment was first described in 1901 [28]. Otherwise some hot alkali treatments cause fragmentation of the elastic network.

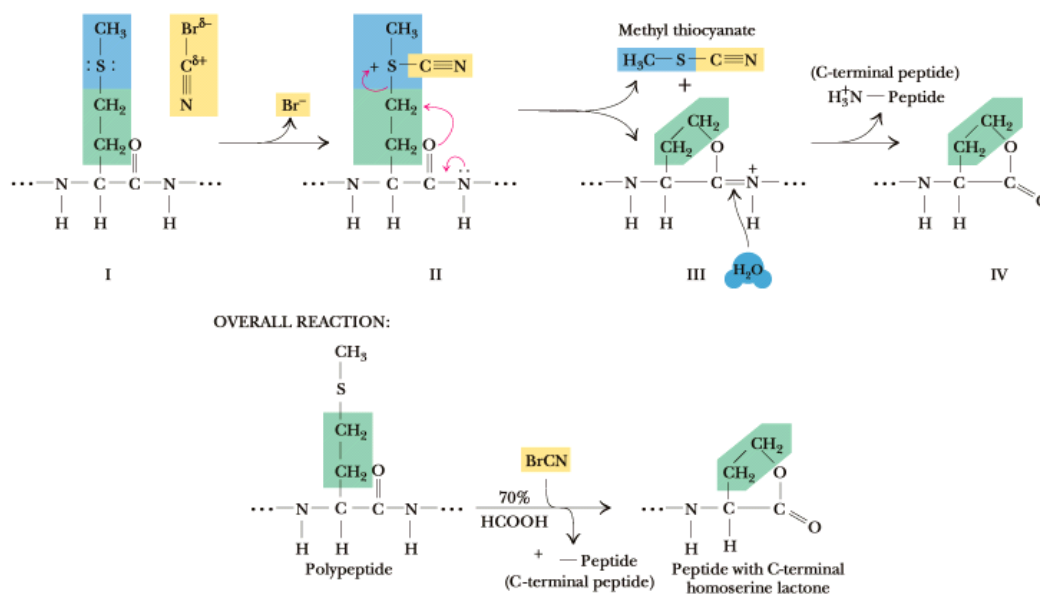
Cyanogen bromide - formic acid was chosen because of its effectiveness in removing non-elastin components from arterial tissues; other procedures using hot alkali or autoclaving were rejected because of possible fragmentation and incomplete elastin purification [29]. It is demonstrated by Q. Lu et al that the treatment with cyanogen bromide (CNBr) in formic acid (HCOOH) removed collagen and cells from the porcine aorta, resulting in elastin scaffolds [6]. This treatment is known to cleave proteins at methionine residues. The methionine or 2-amino-4-(methylthio)butanoic acid (abbreviated as Met), is an amino acid with the chemical formula  $\text{HO}_2\text{CCH}(\text{NH}_2)\text{CH}_2\text{CH}_2\text{SCH}_3$  - *Figure 8*. The methionine is present in most part of proteins including collagen, but absent in elastin. The conformation of elastin confers resistance to cleavage, and the absence of methionine allows the treatment with CNBr to isolate the elastin.



2-amino-4-(methylsulfanyl)butanoic acid

**Figure 8** - Chemical structure of methionine.

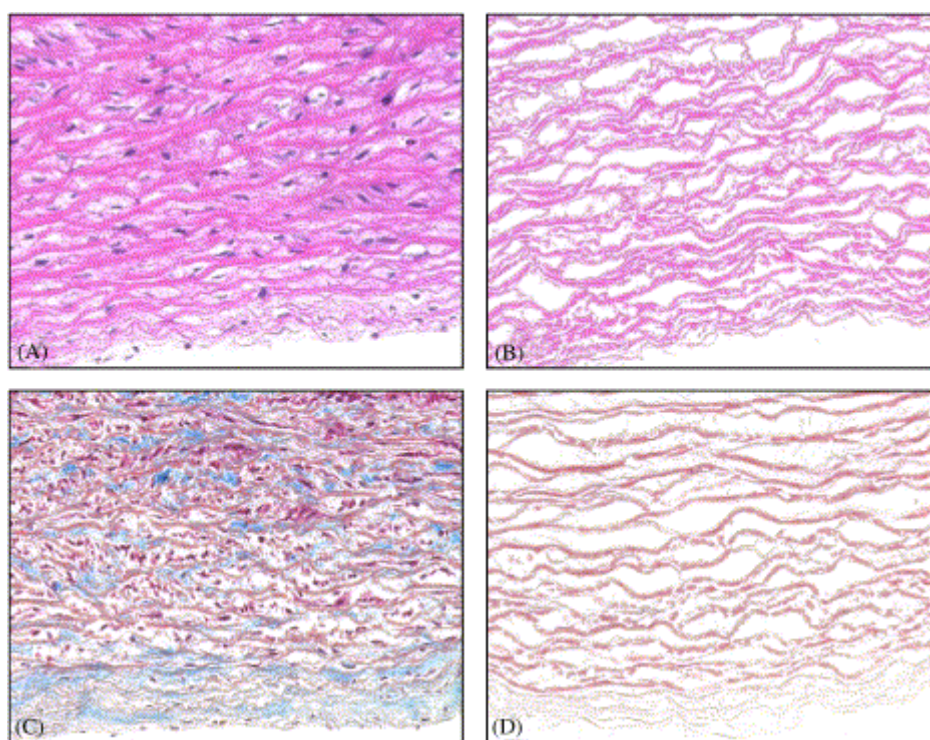
The mechanism of cleavage begins with a nucleophile acyl substitution reaction in which bromide ion (bromine) present in CNBr is ultimately replaced by the sulfur in methionine. This happens because in the CNBr the electron density is shifted away from the carbon atom, leaving the carbon vulnerable to attack by a nucleophile. This attack is followed by a rapid intermolecular rearrangement to form a ring due to the nucleophile attack by the Met carboxyl oxygen atom. The cyclic derivate is unstable in aqueous solution; water hydrolyzes/cleaves the Met peptide bond C-terminal, generating peptide fragments and homoserine residues. This mechanism of the CNBr in formic acid is demonstrated in *Figure 9*.

**Figure 9** - Mechanism of protein isolation with CNBr in acid formic [30].

This mechanism is applied usually for nineteen hours and then followed by one hour at 60 °C where the protein is denatured by breaking the hydrogen bonds. Finally, specimens are placed in a large beaker with boiling water for five minutes to inactivate CNBr.



The pure elastin fibers have less mechanical strength than the native vessel because is the collagen that provides strength and durability. The hot alkali extraction process typically resulted in a pure elastin fibrous matrix that is mechanically fragile and fully hydrated. In Figure 10 is one of the results from Q. Lu et al. after the CNBr digestion with H&E stain (hematoxylin and eosin stain) and Masson's trichrome staining, where it is evident that collagen and cells are digested after the procedure. Fresh aorta stained with H&E and Masson's trichrome staining show a dense structure of smooth muscle cells surrounded by collagen and elastic fibers, **Figure 10 A and C**. After CNBr treatment, cells and collagen could no longer be detected by H&E and Masson's trichrome staining **Figure 10 B and D**, obtaining highly porous elastin scaffolds.



**Figure 10** - Picture from fresh porcine aorta before (A, C) and after (B, D) the CNBr extraction. The picture A and B was used H&E stain (cell nuclei are dark and extracellular matrix is pink); C and D are stained with Masson's trichrome, in which collagen fibrils stain blue and elastic fibers red. Original magnification,  $\times 200$  [6].

### 3.3 Characterization of elastin scaffolds

The Biological Materials Testing System (BiMaTS) in the Laboratory for Implantable Materials and Biomechanics at UMBC was used to analyze the properties of elastin scaffolds by mechanical tests to examine changes in mechanical behavior. This involves a uniaxial tension test and creating a stress-strain curve from the load-deformation data. Then to quantify the amounts of collagen and elastin in the samples a quantitative dye-binding method was used to

measures the protein using a spectrophometer, giving an indication of the purity of the samples. The “Sircol™ Soluble Collagen Assay” and “Fastin™ Elastin Assay” kits (Biocolor Ltd.,UK) were used.

### 3.3.1 Experimental System - BiMaTS

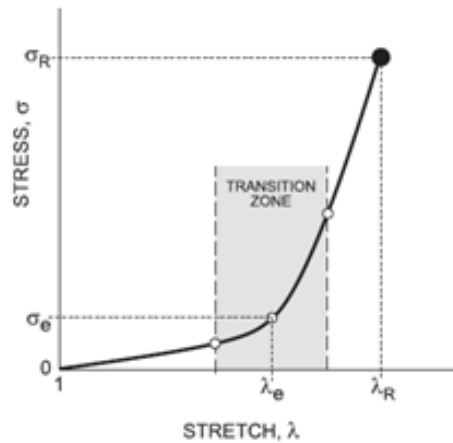
The Biological Materials Testing System, BiMaTS, shown in Figure 11, is a custom built, computer-controlled uniaxial test system. The equipment consists of a linear position table, micro-stepper motor, linear optical encoder and load cell. The samples are assembled on a precision machined rigid L-frame. A tension/compression load cell is attached to the mounting bracket and the X-Y stage is attached to the horizontal arm of the L-frame [31].



**Figure 11** - Biological Materials Testing System, BiMaTS.

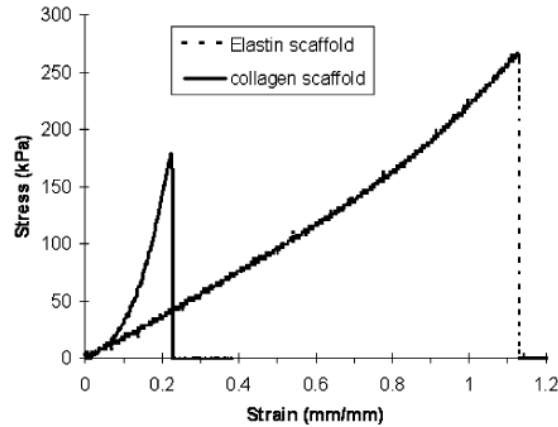
The aortic specimens, either circumferential or longitudinal, display a nonlinear stress-stretch curve, depicted in *Figure 12*. For small values of the applied load, the aortic tissue shows the characteristic behavior of elastin, and going through a transition point at  $(\lambda_e, \sigma_e)$ , and with higher values of applied strength the response is a consequence of collagen. The stretch and stress at the breaking point  $(\lambda_r, \sigma_r)$ , is when the tissue breaks. Stress is fundamental to describe the mechanical behavior of a material, and is given in units of force/area and is a measure that is independent of the amount of a material [32].

As is advisable when testing viscoelastic soft tissues, each specimen was preconditioned by fifteen loading-unloading cycles at a constant speed of 0.25 mm/s to eliminate the difference in stress-strain relation between loadings and on-loadings, is a property of viscoelastic materials, effect of hysteresis.



**Figure 12** - Stress-Stretch curve of aortic tissue and mechanical parameters [26].

Roach and Burton have demonstrated that under very low levels of stress, the elastic modulus of the arterial wall is equal to the elastic modulus of elastin, since little or no collagen bears stress under this condition [33]. By breaking down the original stretch-stress relation to split the elastin and collagen stretch-stress relations, it was found that the participation of the collagen fibers in supporting the load of the aortic wall starts when the stretch-stress relation no longer fits a linear mathematical model [34]. This indicates that collagen fibers recruitment increases as the artery distended. Q. Lu et al. results indicated that removal of the collagen network reduced tensile strength of scaffolds but maintained their distensibility. The rupture tensile strength of the elastin scaffold showed values of  $269 \pm 9$  kPa, which was about 5 times lower than that of fresh aorta ( $1132 \pm 85$  kPa), Figure 13.



**Figure 13-** Stress-strain analysis [6].

The elastic modulus, or Young modulus,  $E$ , is the most common mechanical parameter used to describe the behavior of elastic material. However, aortic tissue is a nonlinear material, and a single value of elastic modulus does not represent the continuously varying response of the tissue.

To evaluate the circumferential behavior of the aortic wall, sets with at least three samples each were obtained.

Engineering stress,  $\sigma$ , versus stretch,  $\lambda$ , relationships for all the tests were derived from experimental load-displacement curves. The stretch was obtained by dividing the current length,  $l$ , by the initial length,  $L$ , of the specimen, equation (3.1), and the true stress was calculated as the applied load,  $P$ , divided by the current cross-sectional area,  $A$ , equation (3.2).

$$\sigma(Pa) = \frac{Load}{Area} = \frac{P(N)}{A(m^2)} \quad (3.1)$$

$$\lambda(m/m) = \frac{lengt_{time}}{lengt_{initial}} = \frac{l(m)}{L(m)} \quad (3.2)$$

The elastic modulus,  $E$ , is the slope of the linear Stress-Stretch curve and indicates how much a material stretches when it is subjected to an applied stress.

In Mosora's experiments [35] the Young's modulus of the thoracic ascending aorta was 2 MPa to 6.5MPa. Based on the area equation present in Watanable's studies, the young's modulus of the intima, media, and adventia layers is 2.98 MPa, 8.95 MPa, and 2.98 MPa respectively [36].

### 3.3.2 Quantification of Soluble Collagen by a Colorimetric Method [37]

#### (“Sircol™ Soluble Collagen Assay”)

Collagen contains the amino acid hydroxyproline (HyPro), which is involved in the stabilization of this triple helical molecule. Normally, the concentration of HyPro is used to calculate the total collagen content in the samples. Radiolabelling, chromatographic and calorimetric assays have been developed over the years for the accurate determination of collagen content through HyPro estimation. Recently, the Sircol Collagen Assay has been almost exclusively adopted as the fastest and simplest colorimetric method for the determination of collagen concentration in complex protein solutions. The dye Sirius Red in picric acid solution is a specific collagen stain for tissues, interacting by means of ion binding with the basic groups of the collagen molecule.

Pepsin treatment of insoluble tissue in 0.5 M acetic acid results has been utilized successfully to obtain collagen from several tissues [38]. The Sircol Collagen Assay (SCA) is a dye-binding method specific for acid soluble collagens extracted from mammalian and collagens into culture medium by mammalian cells during in vitro culture.

The components of the Sircol assay Kit are the dye reagent that contains Sirius Red and has been formulated for specific binding to collagen, the alkali reagent that contains 0.5 M sodium hydroxide, the salt soluble collagen precipitating reagent, contains L-lysine monohydrochloride and the collagen standard with concentration 1 mg/ml.

The Sircol dye reagent contains Sirius Red that is an anionic dye with sulphonic acid side chains groups, these groups react with the side chain groups of the basic amino acid present in collagen. The manufacturer’s protocol for SCA was followed - Appendix II.

- Test sample preparation - Pepsin soluble collagen

The pepsin is dissolved in 0.5 M acid acetic, normally is used about a 1:10 ratio of pepsin: tissue wet weight. This needs to stay overnight at room temperature in aseptic conditions with vigorously stirring. This method produces a larger yield of collagen than when 0.5 M acetic acid is used alone. The pepsin should have a good activity and is dissolved in 0.5 M acetic acid. This is due to pepsin cleavage of part of its C-terminal non-helical alpha chain that makes up the triple helix of tropocollagen.

Pepsin digestion is the most useful method to prepare a soluble collagen and quantify the collagen chains of different types. Fujimoto (1968) found that collagen could be solubilized by pepsin treatment, and Bannister & Burns (1972) have since applied the same technique. [39]

- Set up assay

For the assay it was necessary run duplicate 1.5 ml microcentrifuge tubes with collagen standards, test sample and reagent blanks. The volume in all the microcentrifuge tubes must be 100  $\mu$ l.

To produce a calibration curve with the spectrophotometer, the Reference Collagen Standard was used at seven known different concentrations.

For the measurement of collagen it must be dye-binding, so to all tubes was added 1.0 ml of Sircol dye reagent and the *Clay Adams® Brand Nutator* was used for continuous gentle mixing during 30 minutes, and then the tubes were centrifuged at 10,000 x g for 10 minutes.



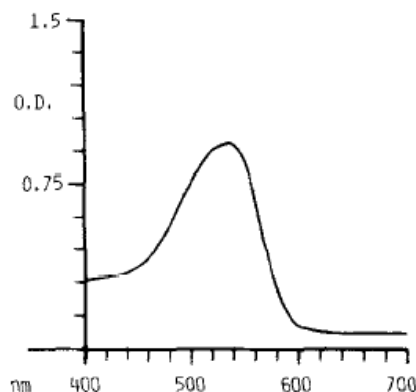
**Figure 14** - Collagen-dye pellet for standard collagen concentration: left - 40  $\mu$ g; 7.5  $\mu$ g.

The supernatants were discarded and the collagen-dye was added, to the precipitated - *Figure 14*. 1.0ml of Alkali reagent and mixed in a vortex tube mixer tube to bring the collagen-dye back into a solution, *Figure 15*. The Alkali reagent contains 0.5 M sodium hydroxide solution which serves to release the Dye Sircol collagen-dye complex.



**Figure 15** - Collagen-dye in solution for different standard collagen concentration: left - 40  $\mu$ g; middle - 25  $\mu$ g; right - 7.5  $\mu$ g.

As the absorbance maximum for Sirius Red dye is at 540nm the wavelength was set at 540 nm on a UV-Vis Spectrophotometer, *Spectronic GENESYS 20* Thermo Fisher, see **Figure 16**.



**Figure 16-** Absorption spectrum of Sirius Red solution in 0.5 M acetic acid [40].

The samples were ready to do the measurement with the spectrophotometer, Figure 17. The values from the test samples were determined based in the standard graph composed from collagen standard supplied with the kit.



**Figure 17 -** Collagen standard in cuvettes for different concentration: left - 40 µg; middle - 25 µg; right - 7.5 µg.

Mass values from the test samples were determined based in the standard graph composed from collagen standard supplied with the kit.

### 3.3.3 Quantification of Elastin by a Colorimetric Method [41]

(“Fastin™ Elastin Assay”)

The elastin content in intact aorta and isolated elastin scaffolds was measured using Fastin™ elastin assay following manufacture’s protocols. This method quantifies soluble

tropoelastin and insoluble elastin made soluble after treatment with hot water solution of oxalic acid.

The components of the Fastin Assay Kit are the Fastin Dye Reagent contains 5,10,15,20-tetraphenyl-21,23-porphine tetrasulfonate (TPPS) in a citrate-phosphate buffer, that also contains ammonium sulphate and anti-microbial agents; the Elastin Precipitating Reagent contains trichloroacetic acid and hydrochloric acid; the Elastin Standard is a high molecular weight fraction of  $\alpha$ -elastin prepared from bovine neck ligament elastin at concentration of 1 mg/ml supplied as a sterile solution, in 0.25 M oxalic acid; the Dye Dissociation Reagent contains guanidine HCl and propan-1-ol.

The dye reagent containing a synthetic porphrine TPPS contains four sulfonate groups what makes it water soluble. Its affinity for elastin was first observed when used as a “vital stain” on live animals. Most tissues initially took up the dye, but with time only elastin retained its molecules. The mode of action of TPPS with elastin remains uncertain. It may be due to shape-and-fit with the acidic dye being firmly retained by the basic amino acid chain residues of elastin [41]. The manufacturer’s protocol for Fastin elastin assay was followed - Appendix III.

- Test sample preparation

It is necessary convert insoluble elastin to water soluble  $\alpha$ -elastin with 0.25 M oxalic acid. The tissue samples were weighed, placed into a glass centrifuges tubes, and ~20 volumes of 0.25 M oxalic acid were added. These tubes were placed into a boiling water-bath at 100 °C. After 60 minutes the tubes were cooled to room temperature and then centrifuged at low speed of ~3000 rpm for 10 minutes. The liquid was pipetted off and retained. More oxalic acid was added to the residual tissue in the tubes (~20 volumes) and again heated and centrifuged. Three extractions were sufficient to complete solubilisation of elastin. The extracts from each tissue were pooled.

- Set up assay:

To duplicate, the following solution was added to the labelled 1.5 ml microcentrifuge tubes: 100  $\mu$ L of 0.25 M oxalic acid use as reagent blank, 12.5, 25.0, 75.0  $\mu$ L aliquots of elastin standards and test samples. The volumes were adjusted with 0.25 M oxalic acid.

For the precipitation of elastin to each tube 100  $\mu$ L of the cold Fastin Precipitating Reagent was added. This reagent was pre-cooled. The tubes were capped and the contents mixed by inversion and the held for about 10 minutes. Following the centrifugation at 10,000



x g for 10 minutes, the supernatants were drained off carefully. The elastin precipitate occurs as a translucent gel, which was hard to see.

The Next step was the reaction of elastin with the Fastin Dye 1.0 ml that was added to each tube. The tubes were capped and a vortex mixer was used to bring the elastin gel precipitate into a solution, then the elastin and the dye reagent were allowed to interact for 90 minutes with gentle mechanical mixing.

The elastin-dye complex was separated with centrifugation at 10,000 x g for 10 minutes, and then the supernatant was discarded carefully, Figure 18.



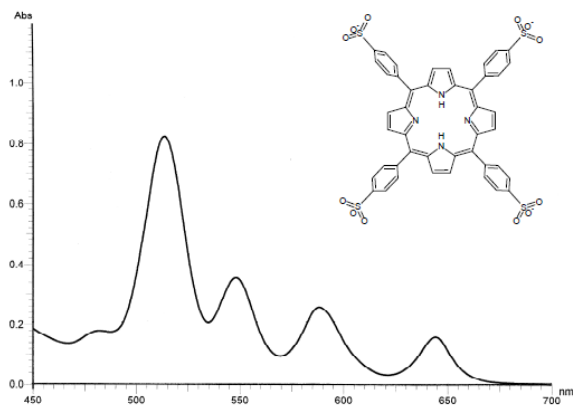
**Figure 18** - Elastin-dye complex pellet for standard elastin concentration: left - 25  $\mu\text{g}$ ; 40  $\mu\text{g}$ .

To release of the elastin bound dye 500  $\mu\text{l}$  of Fastin Dissociation Reagent was added and a vortex mixer was used to bring the elastin-bound dye into solution. The samples were ready to do the measurement with the spectrophotometer, with the wavelength set at 513 nm on a UV-Vis Spectrophotometer, *Spectronic GENESYS 20* Thermo Fisher, Figure 19.



**Figure 19** - Elastin samples in cuvettes for different concentration: Blank; Standard concentration 25  $\mu\text{g}$ ; Sample of aorta; Sample of aorta with 6h isolation; Sample of aorta with 19h isolation.

The elastin content of the assayed samples is determined by amount of bound dye released from the elastin. The absorbance peak of TPPS occurs at 513 nm, Figure 20 , so the spectrophotometer was used by setting to 513 nm and the blank was used to adjust the zero. The elastin standards were used to produce the calibration curve.



**Figure 20** - The visible absorbance spectrum and structural form of TPPS [41].



## 4 Discussion of Results

### 4.1.1 Experimental System - BiMaTS

The porcine aorta received by the laboratory were cleaned with a bistoury to remove the fat present, and then cut into dumbbell shaped test specimens with the specific equipment. The samples have approximately the follow dimensions: 10.9 mm x 30.8 mm.

To do the isolation it was necessary to use 8 ml/cm<sup>2</sup> of 50 mg/ml CNBr in 70 % formic acid, and each experiment time had four samples, so a total of 216 ml of CNBr in 70 % formic acid was used, based on the calculation shown below.

$$\text{Sample Area} = 10.9 \times 30.8 = 335.72\text{mm}^2 = 3.36 \frac{\text{cm}^2}{\text{sample}} \quad (4.1)$$

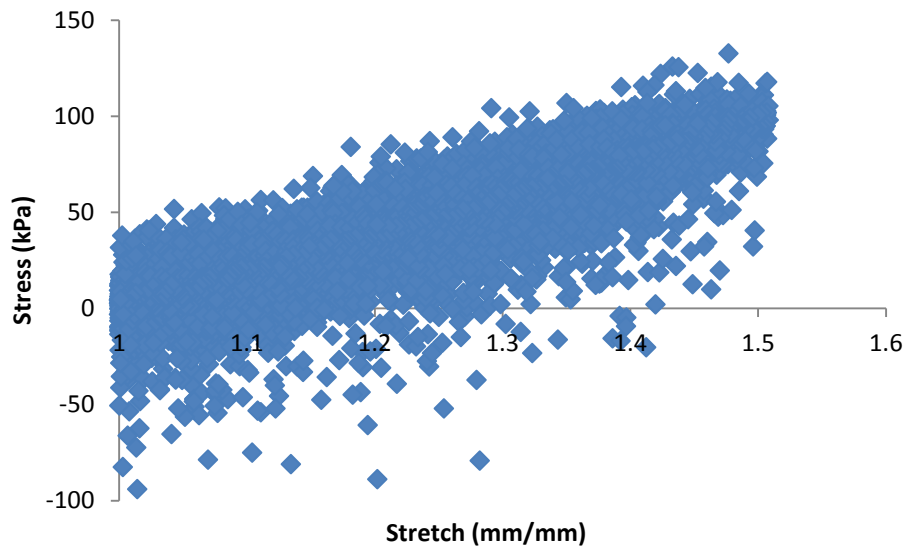
$$\text{Sample Surface Area} = 3.36 \frac{\text{cm}^2}{\text{sample}} \times 2 \text{ sides} = 6.12 \frac{\text{cm}^2}{\text{sample}} \quad (4.2)$$

$$\text{Quantity of CNBr/sample} = 6.12 \frac{\text{cm}^2}{\text{sample}} \times 8 \frac{\text{ml}}{\text{cm}^2} = 54 \frac{\text{ml}}{\text{sample}} \quad (4.3)$$

$$\text{Quantity of CNBr for 4 samples} = 54 \times 4 = 216 \text{ ml} \quad (4.4)$$

After the isolation process, the samples were placed into a test tube with deionized water and are placed in a shaker table for ten minutes; these steps were repeated three times. Finally the samples were stored in phosphate buffered saline (PBS), this helps to maintain a constant pH. All procedures involving CNBr must be completed with nitrile gloves, and all laboratory material cleaned with 10 % bleach after with 4 % NaOH.

The next step was the biomechanical tests. Experiments were carried out using the tensile testing machine, the BiMaTS. The sample was measured, thickness and width, and each sample was preconditioned by fifteen loading-unloading cycles at a constant speed of 0.25 mm/s to ensure that the sample behavior was consistent, Figure 21 shows a representative graph for one sample. This step may also reduce the hysteresis effects, due the viscoelastic properties.



**Figure 21** - Preconditioned test: Stress vs. Stretch for two hours isolation - 5.20 X 1.60 mm.

After preconditioning, the sample underwent the uniaxial tensile testing with continuous recording of tensile force in grams and extension at constant speed 0.05 mm/s until failure.

The parameters of preconditioning and tensile testing are in Table 1. Load and stretch were continuously recorded by the data acquisition software.

**Table 1** - Parameters of BiMaTS.

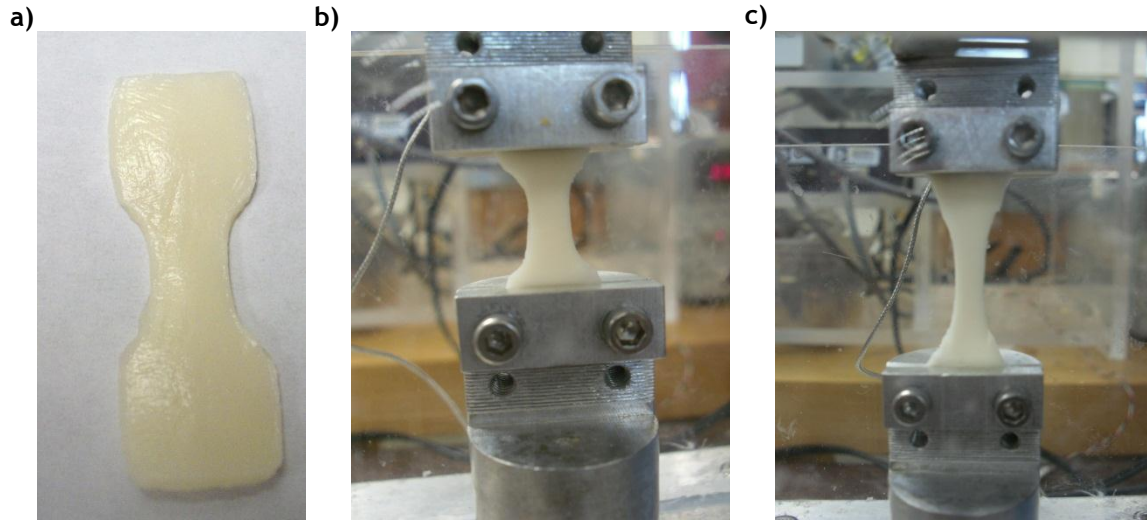
	Precondition test	Tensile test
Velocity	0.25 mm/s	0.05mm/s
N° Cycle	15	-
Max-Position	10 mm	
High Force	100 mm	
Monotonic Position	18 mm	

The BiMaTS data gives the load in grams and the displacement or distance in mm. To obtain the stress, equation (4.5), it is necessary to convert the load into Newtons and calculate the cross-sectional area of each sample, equation (4.6).

$$Stress(Pa) = \frac{Force(N)}{Area(m^2)} \quad (4.5)$$

$$Area(m^2) = width \times thickness \quad (4.6)$$

The stretch is the displacement of the sample compared to the initial length (=12 mm), Figure 22.



**Figure 22** - Freshly prepared porcine aorta sample (a) specimen with an initial length of 12mm; (b) specimen mounted in the BiMaTS; the initial position with no force; (c) specimen stretched in BiMaTS.

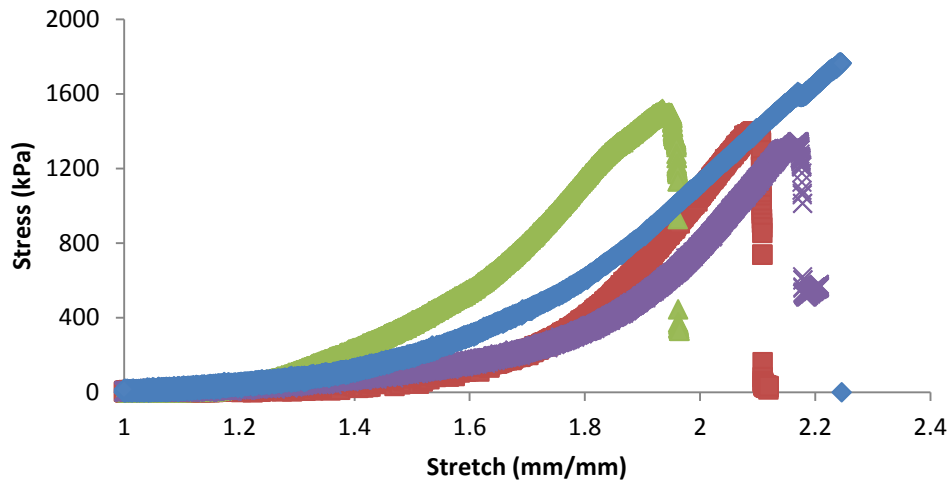
Equation (4.7) demonstrates how to obtain the stretch, where the length at each time is given by equation (4.8).

$$Stretch = \frac{lenght_{time}}{lenght_{initial}} \quad (4.7)$$

$$lenght_{time} = (displacement \times -100) + lenght_{initial} \quad (4.8)$$

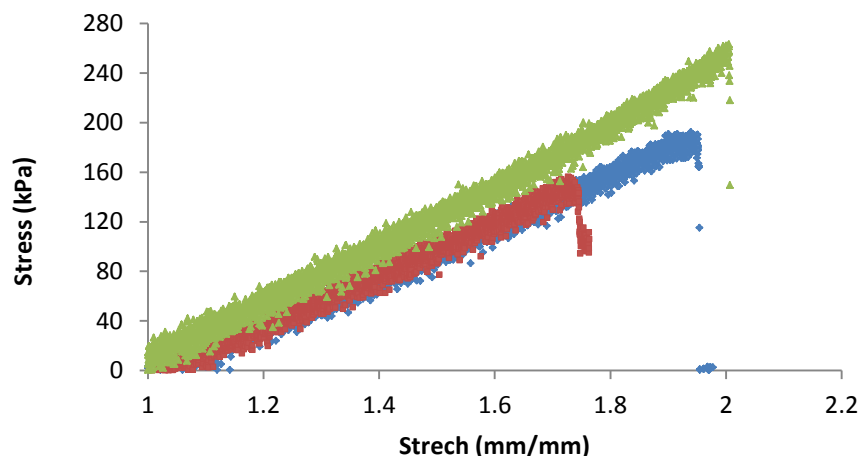
The Modulus of Elasticity is the ratio of stress to stretch, it can be experimentally determined from the slope of a linear section of the stress-stretch curve. The Modulus of Elasticity is a measure of the stiffness of an elastic material, and can be used to predict the elongation or compression of an object as long as the stress is less than the yield strength of the material.

Figure 23 shows representative stress-stretch curves obtained for intact porcine aortas without isolation process. The average elastic modulus obtained was  $236 \pm 33$  kPa, and the maximum stress average was  $1507 \pm 188$  kPa, equivalent to  $2.15 \pm 0.2$  as an average maximum of stretch.



**Figure 23** - Stress vs. Stretch for pig aorta: blue plot, specimen cross section: 5.25 x 2.11 mm; red plot, specimen cross section: 5.07 x 2.15 mm; green plot, specimen cross section: 5.00 x 2.11 mm; violet plot, specimen cross section: 5.05 x 1.98 mm.

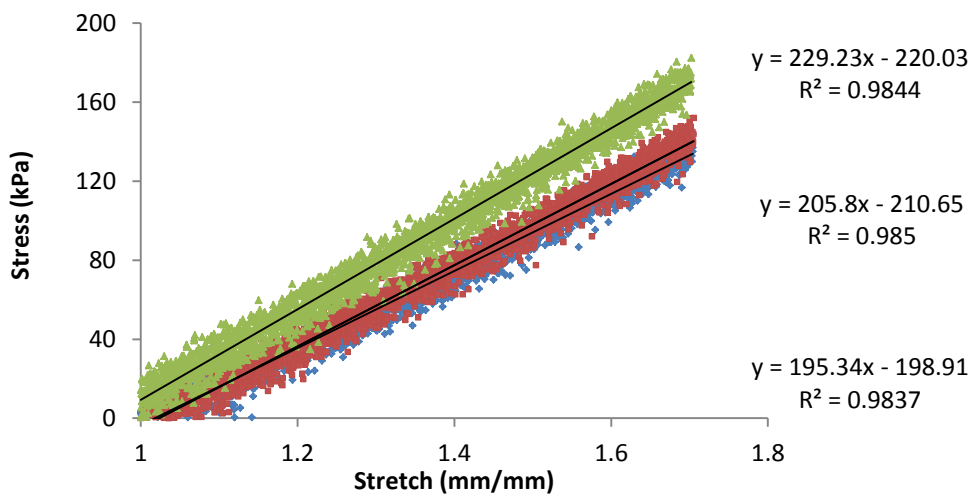
The results for two hours of chemical isolation treatment are represented by the graph in Figure 24. Appendix I includes all the graphs for the different isolation times.



**Figure 24** - Stress vs. Stretch for two hours isolation: blue plot, specimen cross section: 5.2 x 1.67 mm; red plot, specimen cross section: 5.17 x 1.52 mm; green plot, specimen cross section: 4.96 x 1.38 mm.

An important aspect to remark upon is that with only two hours of insolation, the maximum stress was reduced by about 86 % of the intact sample of aorta. For two hours of isolation of the maximum average stress is equal to  $204 \pm 44$  kPa.

Because the elastic modulus is the slope of the linear portion of the stress-stretch curve, a linear least-squares trend line was calculated for each data plot to determine the elastic modulus, Figure 25.



**Figure 25** - Stress vs. Stretch for two hours isolation: blue plot, modulus of elasticity = 195 kPa; red plot, modulus of elasticity = 205 kPa; green plot, modulus of elasticity = 229 kPa.

The average maximum stress, maximum stretch and elastic modulus values are given in Table 2 for the different processing times. The values are calculated by each set (n).

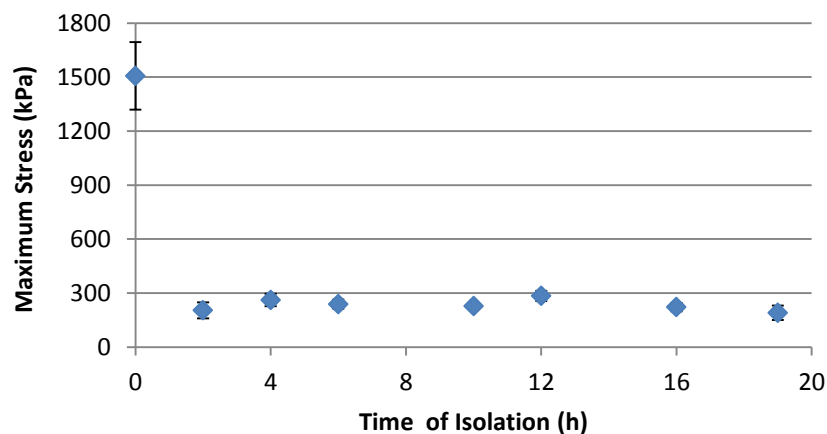


**Table 2** - Average Values of Elastic Modulus, Maximum of Stress and Stretch.

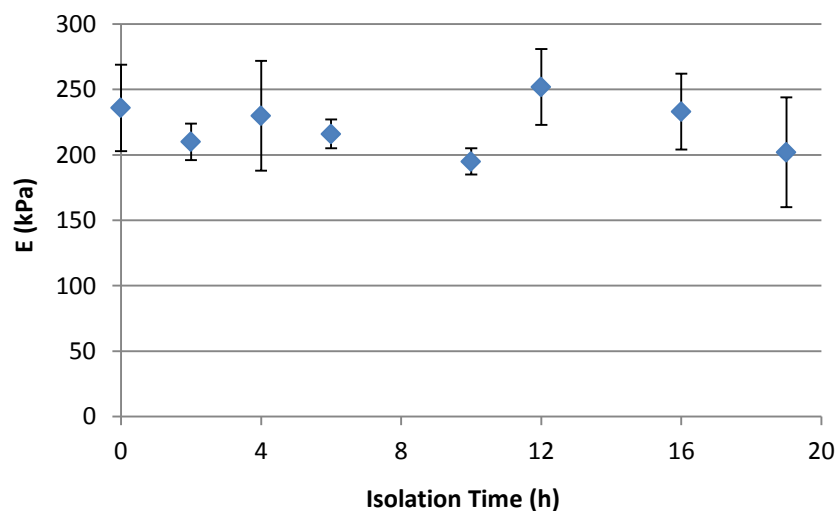
Isolation time	n	Elastic Modulus (kPa)	Maximum Stress (kPa)	Maximum Stretch
0 hours	4	236 ± 33	1507 ± 188	2.2 ± 0.2
2 hours	3	210 ± 14	204 ± 44	1.9 ± 0.1
4 hours	5	229 ± 42	262 ± 35	2.1 ± 0.1
6 hours	4	213 ± 11	238 ± 26	1.9 ± 0.1
10 hours	3	195 ± 10	228 ± 5	2.1 ± 0.1
12 hours	3	252 ± 29	284 ± 29	2.1 ± 0.1
16 hours	4	233 ± 29	222 ± 23	1.93 ± 0.07
19 hours	3	202 ± 42	190 ± 40	1.920 ± 0.008

The data presented in Table 2 indicate that the cleavage with CNBr in formic acid decreased the maximum stress, Figure 26. The decrease occurred because it is the collagen that provides strength and durability, and the hot alkali extraction process typically destroys the collagen, resulting in a pure elastin fibrous matrix that is more mechanically fragile and fully hydrated. These facts are consistent with studies of Q. Lu et al. where they demonstrated that elastin scaffolds had an average maximum stress of  $269 \pm 9$  kPa and the intact aorta had an average maximum stress of  $1132 \pm 85$  kPa.

After only two hours of isolation, the maximum stress was drastically decreased, and then remained approximately constant, regardless of the exposure time, Figure 26. This may be an indicator that almost all of the collagen is removed at two hours of isolation processing.

**Figure 26** - Maximum Stress versus time of isolation processing.

Although the maximum stress changed after two hours of processing, the initial elastic modulus for the different times of isolation processing did not change significantly. Figure 27 shows the elastic modulus as a function of time of isolation of elastin with the standard error. These data demonstrate that the process of elastin isolation described by Q. Lu et al [6] does not appreciably change the elasticity of the samples. Note that the average value for the initial elastic modulus does not consistently increase or decrease with isolation process time, but apparently randomly varies to either side of approximately 225 kPa.



**Figure 27** - Modulus of elasticity as a function of elastin isolation time.

The initial elastic modulus in the circumferential direction at low stress was shown to vary from 154 to 293 kPa, and are consistent with Yu Zou et al studies where they obtained a range of values for elastic modulus of 100 to 350 kPa [19].

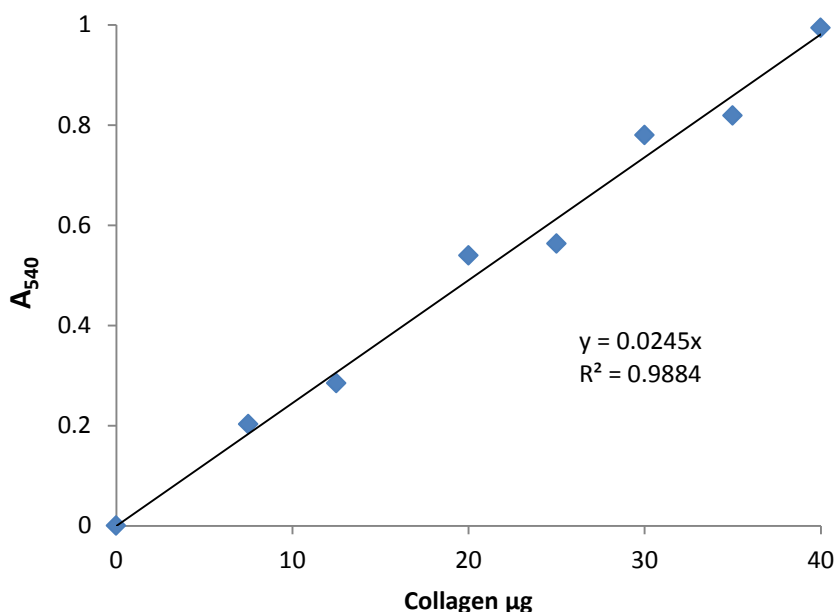
A 1-Way ANOVA test was used to compare groups in the experiment using the F distribution; the one-way ANOVA test can help to determine if the means for each group are significantly different from one another or if they are statistically the same.

The samples were considered statistically different when  $p < 0.05$  for a 95% confidence test. The significance, or p-value, is the probability that an effect at least as extreme as the current observation has occurred by chance. The F value is the ratio of variance of the group means and the mean of the within-group variances.

The significance value of the elastic modulus media for different times is p-value = 0.1419, therefore there is no significant difference between the groups because the significance is more than 0.05. The detailed results obtained from the 1-Way ANOVA are in table VI.1 Appendix IV.

#### 4.1.2 Sircol™ Soluble Collagen Assay

For the construction of the calibration curve was made using seven different concentrations, in duplicate, of standard collagen. *Figure 28* is the calibration graph with the trend line. In Appendix II all the results used in the construction of calibration curve are presented the table II.1.



**Figure 28** - Collagen Calibration Curve.

For each time of isolation was used a minimum of two tissue samples with different weights, present in Appendix II, tables II.2 & II.3, and presented in *Figure 29*. The amount of pepsin added was approximately ten times less than the mass of tissue, as the manufacturer's protocol mentions, assuming the density of tissue is equal to  $1 \text{ g.cm}^{-3}$ . Using the calibration curve of *Figure 28*, it was possible to calculate the mass of collagen in the diluted samples, see equation (4.9) where Abs is the absorbance.

$$m_{\text{collagen samples diluted}} (\mu\text{g}) = \frac{\text{Abs}_{\text{average}}}{0.0245} \quad (4.9)$$

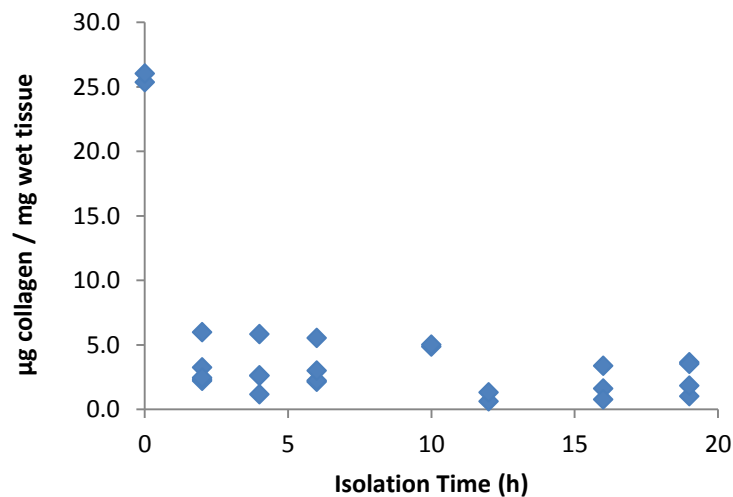
The concentration of the sample was calculated by equation (4.10), the volume total was  $100 \mu\text{L}$  and the volume of the sample  $100$  or  $50 \mu\text{L}$ .

$$C_{collagen\ sample} \left( \frac{\mu g}{\mu L} \right) = \frac{m_{collagen\ samples\ diluted} (\mu g)}{V_{total} (\mu L)} \times \frac{V_{total} (\mu L)}{V_{sample} (\mu L)} \quad (4.10)$$

The mass of collagen extracted from the tissue sample was calculated with equation 4.11, where the volume of acetic acid for extraction was 5 ml.

$$m_{collagen\ tissue} (\mu g) = \frac{C_{collagen\ sample} \left( \frac{\mu g}{\mu L} \right)}{V_{extraction} (\mu L)} \quad (4.11)$$

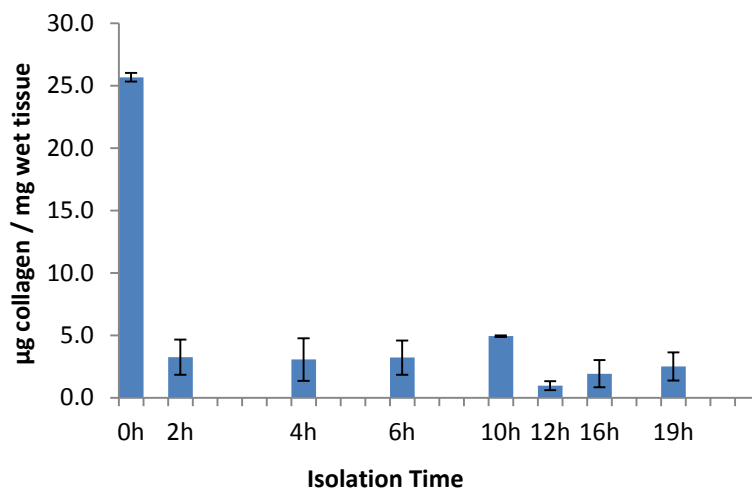
The data in *Figure 29* verify that the mass of collagen decreased with time of elastin isolation processing as it was predicted.



**Figure 29** - Quantification of collagen mass in test sample with different isolation time.

It is noted that at sixteen hours and nineteen hours the difference of collagen's concentration is minimal. On the other hand, can be said that the collagen does not completely disappear during the isolation process; there is a quantity of collagen, even if negligible.

For clarity, the average values of collagen mass for different processing times are shown in *Figure 30*.

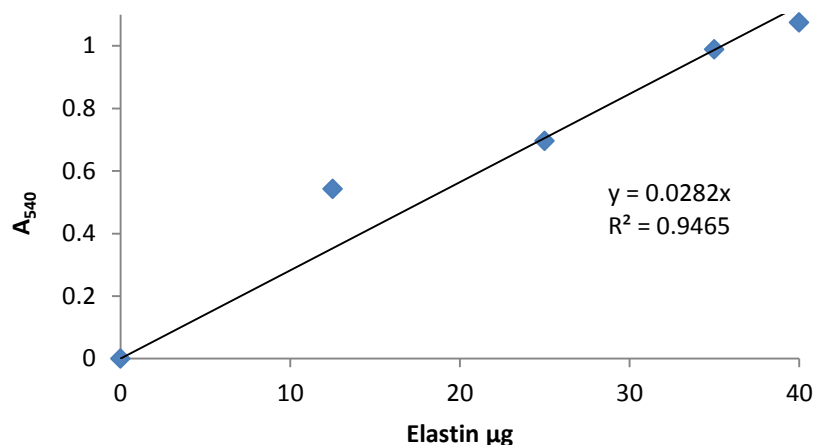


**Figure 30** - Average of collagen in test sample for different times of isolation.

The graph shows that the concentration of collagen decreased as the isolation processing time increased, and the cleavage of collagen assumed a negative exponential form. An interesting fact is, that at two hours of isolation, the amount of collagen in the sample decreased notably; after two hours of processing time, the amount of collagen decreased only by a trace amount. The 1-way ANOVA gives a p-value = 0.2336 for the results from 2h to 19h, table IV.2 in Appendix IV, which means there is no significant change in collagen concentration after two hours of isolation.

### 4.1.3 Fastin™ Elastin Assay

The calculations required for the construction of an elastin calibration curve are in Appendix III. We used duplicates of four known concentrations of the standard solution provided by Biocolor Ltd. The Figure 31 is the calibration curve of elastin mass as a function of isolation processing time.



**Figure 31** - Elastin Calibration curve.

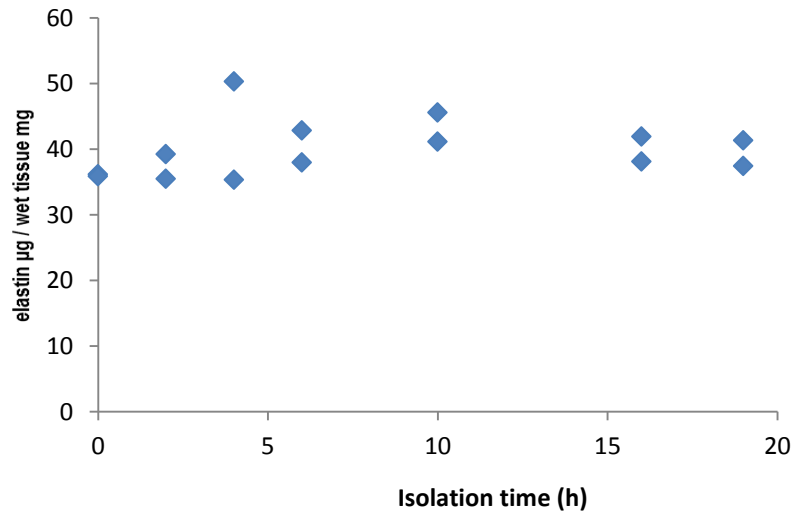
As mentioned, the precipitate is formed with the Fastin Precipitating Reagent is a translucent gel and at low concentration is difficult to process.

With the trend line of the calibration curve we calculated the mass of elastin in each sample for different isolation processing times, equation (4.12). The volume of extraction is the amount of oxalic acid used in each extraction of elastin.

$$m_{\text{elastin in tissue}}(\mu\text{g}) = \frac{\frac{\text{Absaverage}/0.0283}{V_{\text{sample}}(\mu\text{L})}}{V_{\text{extraction}}(\mu\text{L})} \quad (4.12)$$

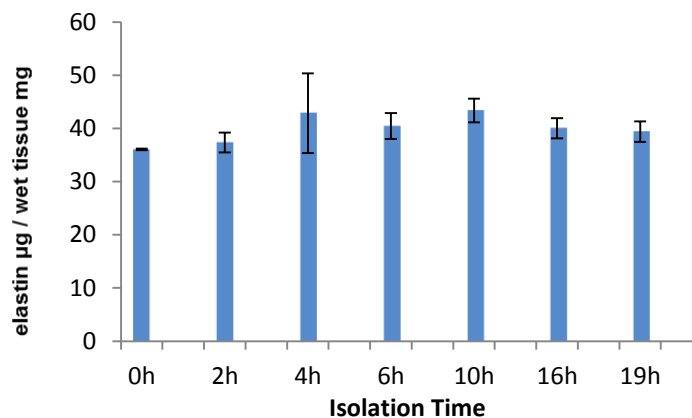
Initially, in the conversion of insoluble elastin to water soluble  $\alpha$ -elastin, the oxalic acid extracts were retained separately and analyzed., It was established that three extraction were necessary, Figure III.1 in Appendix III.

The mass of elastin results, obtained with three extractions for two different weights of tissue, are presented in Figure 32, with their standard deviations.



**Figure 32** - Quantification of elastin in test samples for different isolation time.

With the observation of these data, it was conclude that elastin content did not vary significantly. In Figure 33 are presented the average results and their standard deviation.

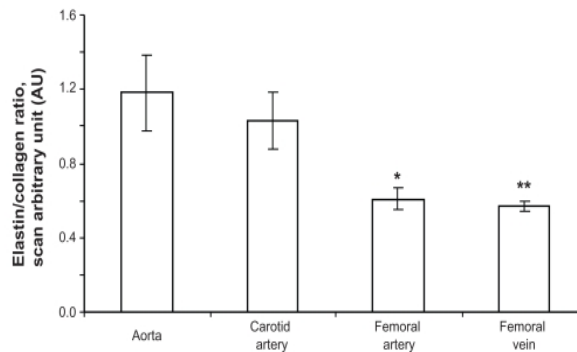


**Figure 33**- Average of elastin in test sample for different times of isolation.

The observed variance can be caused by errors in removing the liquid from the precipitate, or by the amount of water existent in initial samples. We can say that no significance exists along the isolation time, p-value = 0.6987. To better analyze the results it will be necessary to quantify the protein existent in each sample.

These results, however, give preliminary conclusions about the isolation process: the process is highly efficient to remove the collagen in the samples more than 90 % is removed.

The initial elastin:collagen ratio obtained in the porcine aorta was  $1.41 \pm 0.02$ , this value is in agreement with the finding obtained by Basu et al. [23] in the aorta was  $1.18 \pm 0.02$ , Figure 34.



**Figure 34** - Elastin:collagen ratio obtained by Basu et al [23].

In Basu et al. the elastin:collagen ratio, measured by expression of elastin and collagen in the aorta, carotid artery, and femoral artery and vein showed a high ratio in the carotid artery and aorta, whereas the ratio was significantly lower in the femoral artery and vein. These results indicate that arteries near to the heart have more elastin compliance, probably because of their high blood flow rate and blood pressure. Therefore, the ratio of elastin and collagen tends to decrease with increasing distance from the heart [23].





## 5 Conclusions

The results from uniaxial tests demonstrated that the cleavage with CNBr in formic acid decreased the maximum stress. This happened because the collagen provides strength and durability in the tissue. When we performed the isolation process, the collagen is cleaved in the methionine with the CNBr. However, the elastic modulus at low stress for the different times of isolation processing did not change significantly,  $p = 0.1419$ . It was expected at low stress the stress-stretch curve is controlled by elastin, because the collagen has not yet been stretched, and does not carry any load. These facts are in agreement with other studies testing elastin scaffolds. The elastic modulus in the circumferential direction at low stress was shown to vary from 154 to 293 kPa.

The collagen in tissue samples has been measured using the colorimetric analysis. According to this method, the Sirius red dye is eluted in alkali solution and measured using the spectrophotometer after digestion with pepsin. The quantification of collagen demonstrated that almost all the collagen is cleaved. At two hours of isolation processing, the total of collagen in the sample decreased significantly, and at processing times greater than two hours, the amount of collagen decreased only by a trace amount. At the final time, nineteen hours, there is still a residual mass of collagen.

In the elastin assay, we used a dye reagent containing a synthetic porphine TTPS. The results indicated that that the amount of elastin remained approximately constant during the isolation process,  $p\text{-value} = 0.6987$ .

## 6 Evaluation of Work Conducted

### 6.1 Accomplished Objectives

The objectives of the present work were:

1) Perform mechanical tests (BiMaTS) for the elastin scaffolds at different times of elastin isolation processing. The tests were completed for seven different times: [0, 2, 4, 6, 12, 16, 19] hours. The results proved that the elastic modulus of pig aorta do not change during the process of elastin isolation.

2) Quantify the collagen in the elastin scaffolds during the process of cleavage - With the Collagen Assay from Biocolor Ltd, we demonstrated that collagen is removed during the elastin isolation process. After sixteen hours of processing time, there is no significant cleavage of collagen, because the concentration at sixteen hours and nineteen hours of isolation are about the same.

3) Quantify the elastin during isolation process - We used the Fastin Elastin Assay and it was concluded that the amount of elastin remains fairly constant during the isolation process; however it would be necessary more research in this point.

### 6.2 Limitations and Future Work

We have faced several limitations while conducting this work.

The most important was the fact that we only received pig aorta on Tuesday of each week, and once the biological tissue is received, the elastin isolation process should be started immediately. Therefore, we were limited a one or two isolation times per week.

We have also faced a shortage of supplies, for example, the first Fastin Elastin Assay did not work because the reference standard was damaged. Also, the kits for the Elastin and Collagen assay have a limited number of tests each (120), so it was not possible make more tests.

We were not able to use a mass spectrophotometer to verify whether there were changes in the elastin, because the necessary equipment was not working. There is one PhD student that is working on this problem and studying this technique, but we were not able to use the equipment in this study in the time-frame available.

As future work there is another viable option to quantify the elastin: high-performance liquid chromatography (HPLC), that was used by Honório et al. [42]. The HPLC analysis was carried out in a reversed phase system with a binary gradient and detection by UV at 254 nm to quantify desmosine and isodesmosine, as the elastin cross links, in the studied samples.

Also the high-performance liquid chromatography (HPLC) involving the oxidation of free hydroxyproline with chloramine could be future work for the determination of collagen to reinforce the Sircol Assay results. Collagen contains the unique amino acid hydroxyproline (HyPro), which is involved in the stabilization of this triple helical molecule. The concentration of HyPro is customarily used to calculate the total collagen.

We presented the results of the amount of elastin and collagen compared of wet mass tissue, and will be more correct to present compared how much total soluble protein is in the tissue. Honório et al. [42] determined the total protein based on Peterson's modification (1977) of the Lowry method (1951), according to the Protein Assay Kit purchased by Sigma® (St. Louis, MO, USA).

Also it will be necessary do the same study for longitudinal direction of samples to see if there are differences in results compared with circumferential orientation.

### **6.3 Final Comment**

Most of the objectives were accomplished during the time-frame of this project. While conducting the experiments, the main objective of the project, was to know if elastin will change along the isolation process. Looking at the results of the Fastin Elastin Assay, the concentration of elastin is practically the same during the entire elastin isolation process. However, a further evaluation will be necessary to validate the results obtained.

This work has significance because the elastin scaffolds have a greater importance in the biomaterials studies and it is the major importance to prove the elastin remains the same during the isolation treatment with cyanogen bromide. Otherwise, the studies using elastin scaffolds are not consistent.



## 7 References

- [1] Cedars-Sinai Health System Los Angeles, 2011.
- [2] J. Arnold, D.M. Oz, Sharecare, 2009.
- [3] R.E. Kowalski, *The Blood Pressure Cure: 8 Weeks to Lower Blood Pressure without Prescription Drugs*, John Wiley & Sons, Inc, 2007.
- [4] M. Watanabe, T. Sawai, H. Nagura, K. Suyama, Age-related alteration of cross-linking amino acids of elastin in human aorta, *Journal of Experimental Medicine* 180 (1996) 115-130.
- [5] Y. Zou, Y. Zhang, *The orthotropic viscoelastic behavior of aortic elastin*, *Biomechanics and Modeling in Mechanobiology* (2010).
- [6] Q. Lu, K. Ganesan, D.T. Simionescu, N.R. Vyavahare, Novel porous aortic elastin and collagen scaffolds for tissue engineering, *Biomaterials* 25 (2004) 5227-5237.
- [7] B.L. Rasmussen, E. Bruenger, L.B. Sandberg, A New Method for Purification of Mature Elastin, *Analytical Biochemistry* 64 (1975) 255-259.
- [8] R.R. Lareu, D.I. Zeugolis, M. Abu-Rub, A. Pandit, M. Raghunath, Essential modification of the Sircol Collagen Assay for the accurate quantification of collagen content in complex protein solutions, *Acta Biomaterialia* 6 (2010) 3146-3151.
- [9] G. Veit, B. Kobbe, D.R. Keene, M. Paulsson, M. Koch, R. Wagener, Collagen XXVIII, a Novel von Willebrand Factor A Domain-containing Protein with Many Imperfections in the Collagenous Domain, *The Journal of Biological Chemistry* 281 (2006) 3494-3504.
- [10] G. Faury, Function-structure relationship of elastic arteries in evolution: from microfibrils to elastin and elastic fibres, *Pathologie Biologie* 49 (2001) 310-325.
- [11] P. AV, R. JAM, K. A, B. B, Amino acid propensities for the collagen triple-helix, *Biochemistry* 39 (2000) 14960-14967.
- [12] B. Alberts, D. Bray, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, *Essential Cell Biology*, 2<sup>a</sup> ed., 2004.
- [13] W.F. Daamen, J.H. Veerkamp, J.C.M. van Hest, T.H. van Kuppevelt, Elastin as a biomaterial for tissue engineering, *Biomaterials* 28 (2007) 4378-4398.
- [14] J. Albona, W.S.S. Karwatowskib, D.L. Eastyc, T.J. Simsd, V.C. Duance, Age related changes in the non-collagenous components of the extracellular matrix of the human lamina cribrosa, *Br J Ophthalmol* 84 (2000) 311-317.
- [15] G. Holzapfel, T. Gasser, R. Ogden, A New Constitutive Framework for Arterial Wall Mechanics and a Comparative Study of Material Models, *Journal of Elasticity* 61 (2000) 1-48.
- [16] B.B. Aaron, J.M. Gosline, Elastin as a random-network elastomer - a mechanical and optical analysis of single elastin fibers, *Biopolymers* 20 (1981) 1247-1260.
- [17] S. MH, S. SH, R. MR, Mechanical anisotropy of purified elastin from the thoracic aorta of dog and sheep, *Can. J. Physiol. Pharmacol* 61 (1983) 539-545.

- [18] L. MA, D. GJ, G. JM, Mechanical role of elastin-associated microfibrils in pig aortic elastic tissue, *Connect. Tissue Res* 37 (1998) 121-141.
- [19] Y. Zou, Y. Zhang, An Experimental and Theoretical Study on the Anisotropy of Elastin Network *Annals of Biomedical engineering* 37 (2009) 1572-1583.
- [20] V. DA, S. BJ, E. MP, J. TS, E. MA, G. BP, Effect of aneurysm on the tensile strength and biomechanical behavior of the ascending thoracic aorta, *The Annals of Thoracic Surgery* 15 (2003) 1210-1214.
- [21] C. Ferraresi, A. Bertetto, L. Mazza, D. Maffiodo, W. Franco, One-dimensional experimental mechanical characterisation of porcine aortic root wall, *Medical and Biological Engineering and Computing* 37 (1999) 202-207.
- [22] C.M. He, M.R. Roach, The composition and mechanical properties of abdominal aortic aneurysms, *J Vasc Surg* 20 (1994) 6-13.
- [23] P. Basu, U. Sen, N. Tyagi, Blood flow interplays with elastin: collagen and MMP: TIMP ratios to maintain healthy vascular structure and function *Vascular Health and Risk Management* 6 (2010) 215-228
- [24] W.F. Daamen, T. Hafmans, J.H. Veerkamp, T.H.v. Kuppevelt, Comparison of five procedures for the purification of insoluble elastin, *Biomaterials* 22 (2001) 1997-2005.
- [25] N. Gundiaha, M.B. Ratcliffea, L.A. Pruittc, Determination of strain energy function for arterial elastin: Experiments using histology and mechanical tests, *Journal of Biomechanics* 40 (2007) 568-594.
- [26] G.V. Guinea, J.M. Atienza, F.J. Rojo, C.M. García-Herrera, L. Yiqun, E. Claes, J.M. Goicolea, C. García-Montero, R.L. Burgos, F.J. Goicolea, M. Elices, Factors influencing the mechanical behaviour of healthy human descending thoracic aorta, *Physiological Measurement Journal (PMea)* 31 (2010) 1553-1565.
- [27] R.P. Mecham, *Methods in elastic tissue biology: Elastin isolation and purification*, *Methods* 45 (2008) 32-41.
- [28] C. Campbell, A. Bazar, P.-C. Wu, K. McKenna, M. Henrie, K. Troi, K. Gregory, A Simple Apparatus and Process to Produce Novel Elastin Biomaterials, *Trends Biomater. Artif. Organs* 19 (2005) 1-6.
- [29] S.R. Lammers, P.H. Kao, H.J. Qi, K. Hunter, C. Lanning, J. Albiertz, S. Hofmeister, R. Mecham, K.R. Stenmark, R. Shandas, Changes in the structure-function relationship of elastin and its impact on the proximal pulmonary arterial mechanics of hypertensive calves, *Heart and Circulatory Physiology* 295 (2008).
- [30] R.H. Garret, C.M. Grisham, *Biochemistry*, Fourth ed., Mary Finch, 2010.
- [31] L.D.T. Topoleski, N.V. Salunke, J.D. Humphrey, W.J. Mergner, Composition- and history-dependent radial compressive behavior of human atherosclerotic plaque, *Journal of Biomedical Materials Research* 35 (1997) 117-127.
- [32] P. Lanzer, E.J. Topol, *PanVascular Medicine: integrated clinical management*, Springer-Verlag New York, LLC, 2002.
- [33] A.J. Bank, R.F. Wilson, S.H. Kubo, J.E. Holte, T.J. Dresing, H. Wang, Direct Effects of Smooth Muscle Relaxation and Contraction on In Vivo Human Brachial Artery Elastic Properties, *Circulation Research* 77 (1995).
- [34] B. JG, A. RL, L. J, F. EI, P. RH, S. A., Assessment of smooth muscle contribution to descending thoracic aortic elastic mechanics in conscious dogs, *Circulation Research* 73 (1993) 1040-1050.
- [35] M. F., H. A., B. C., F. A., P. T., J. J., C. S., Modelling the arterial wall by finite elements, *Arch Int Physiol Biochim Biophys* 101 (1993) 185-191.

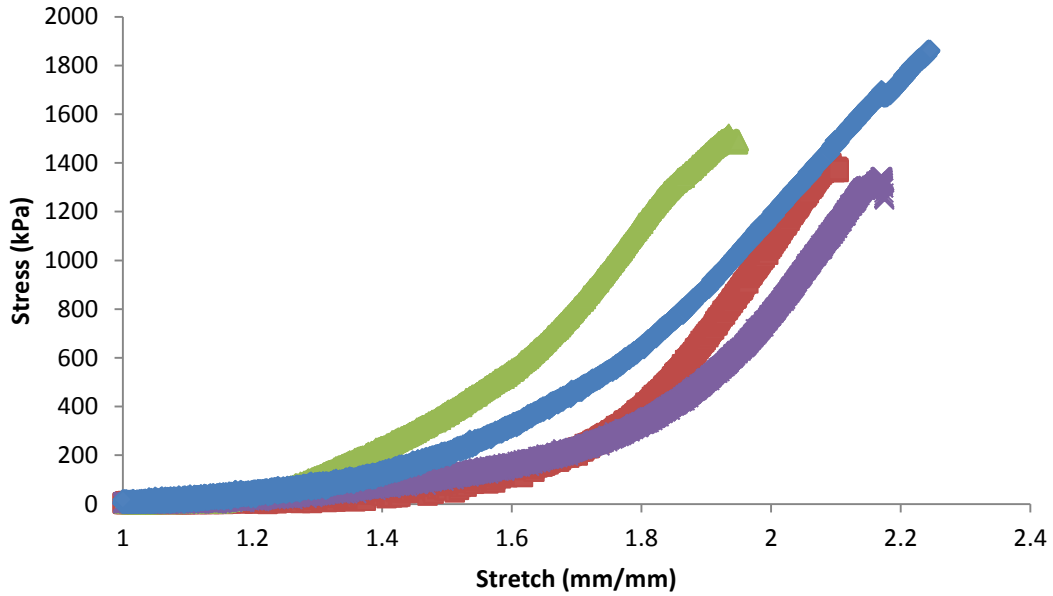
- [36] F. Gao, M. Watanabe, T. Matsuzawa, Stress analysis in a layered aortic arch model under pulsatile blood, *BioMedical Engineering OnLine* 5 (2006).
- [37] B. Ltd, *Sircol Collagen Assay Manual*, Biocolor Ltd, 2002.
- [38] J.B. Lian, S. Morris, B. Faris, J. Albright, C. Franzblau, The effects of acetic acid and pepsin on the crosslinkages and ultrastructure of corneal collagen, *Biochimica et Biophysica Acta (BBA) - Protein Structure* 328 (1973) 193-204.
- [39] D.W. Bannister, A.B. Burns, Pepsin Treatment of Avian Skin Collagen, *Biochem. J.* 129 (1972) 677-681.
- [40] M. Marotta, G. Martino, Sensitive spectrophotometric method for the quantitative estimation of collagen, *Analytical Biochemistry* 150 (1985) 86-90.
- [41] B. Ltd, *Fastin Elastin Assay Manual*, Biocolor Ltd, 2002.
- [42] A. Honório, M.d.L. Pinto, C. Gonçalves, V. Bairos, Elastin in the Avian Lungs, *The Open Chemical and Biomedical Methods Journal* 2 (2009) 18-23.



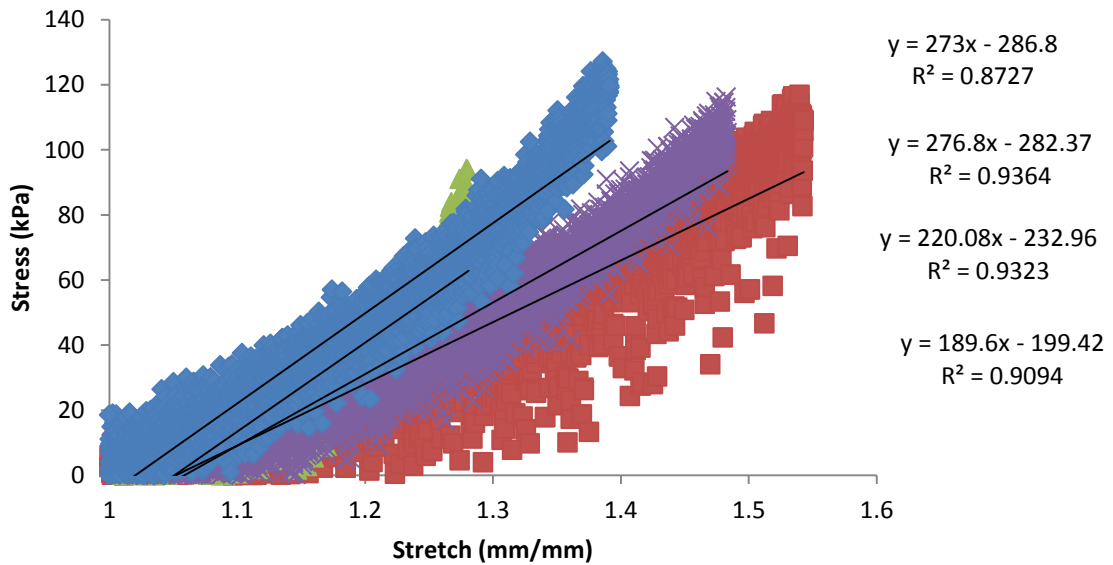


# Appendix I Mechanical Testing Data from BiMaTS

- Zero hours of isolation

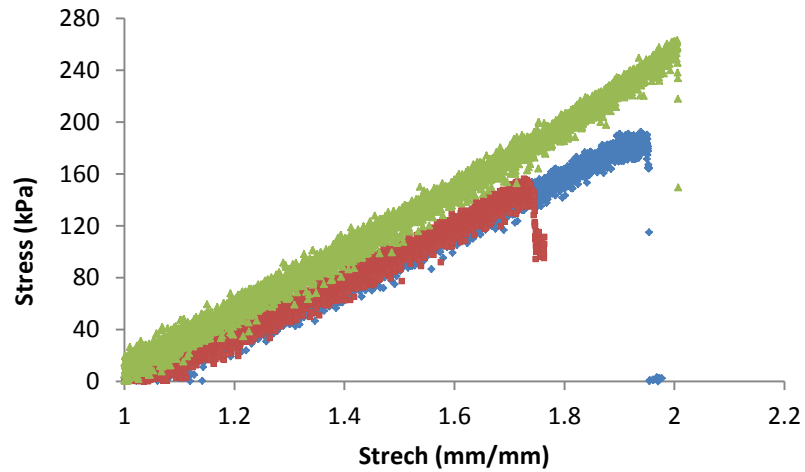


**Figure 1.1** - Stress vs. Stretch: green plot, cross section = 5.00 mm x 2.11 mm; blue plot, cross section = 5.25 mm x 2.11 mm; red plot, cross section = 5.07 mm x 2.15 mm; violet plot, cross section = 5.05 mm x 1.98 mm.

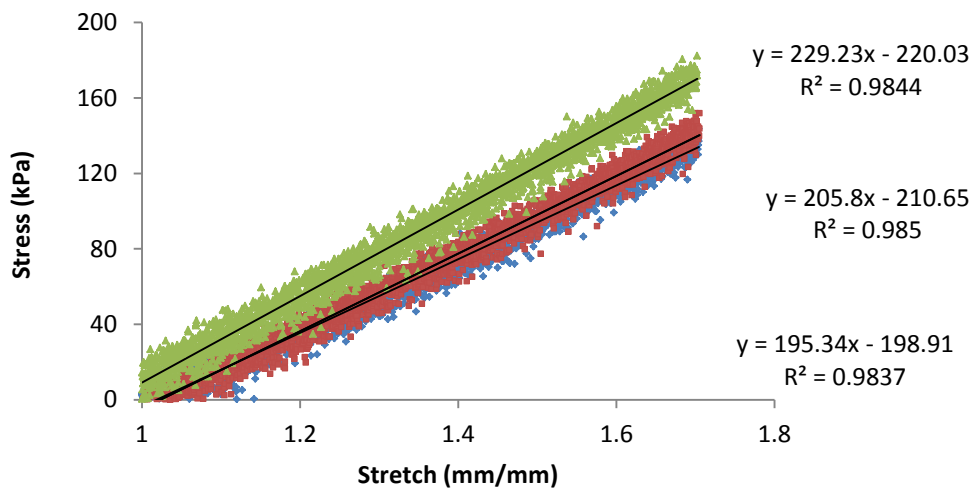


**Figure 1.2** - Stress vs. Stretch: green plot - 273 kPa; blue plot - 277 kPa; red plot - 190 kPa; violet plot - 220 kPa.

- Two hours of isolation

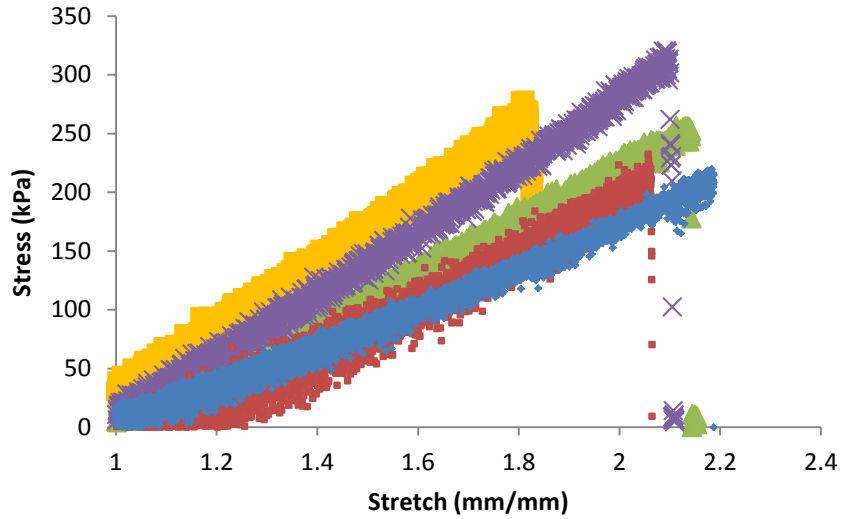


**Figure 1.3** - Stress vs. Stretch: blue plot, cross section = 5.2 mm x 1.67 mm; red plot, cross section = 5.17 mm x 1.52 mm; green plot, cross section = 4.96 mm x 1.38 mm.

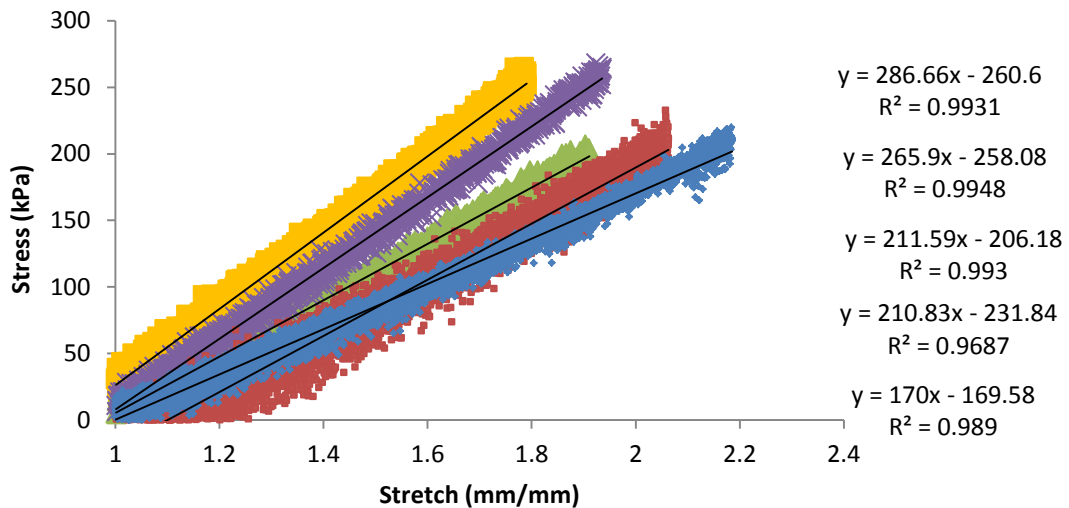


**Figure 1.4** - Stress vs. Stretch: blue plot 195 kPa; red plot 206 kPa; green plot - 229 kPa.

- Four hours of isolation



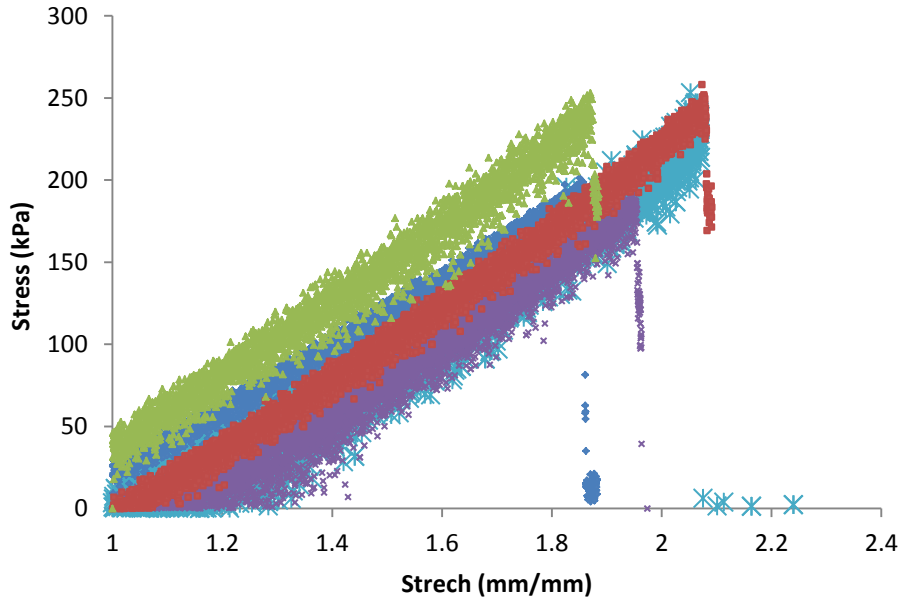
**Figure 1.5** - Stress vs. Stretch: orange plot, cross section = 4.55 mm x 0.85 mm; violet plot, cross section = 4.35 mm x 1.24 mm; red plot, cross section = 4.86 mm x 1.27 mm; green plot, cross section = 5.29 mm x 1.78 mm.



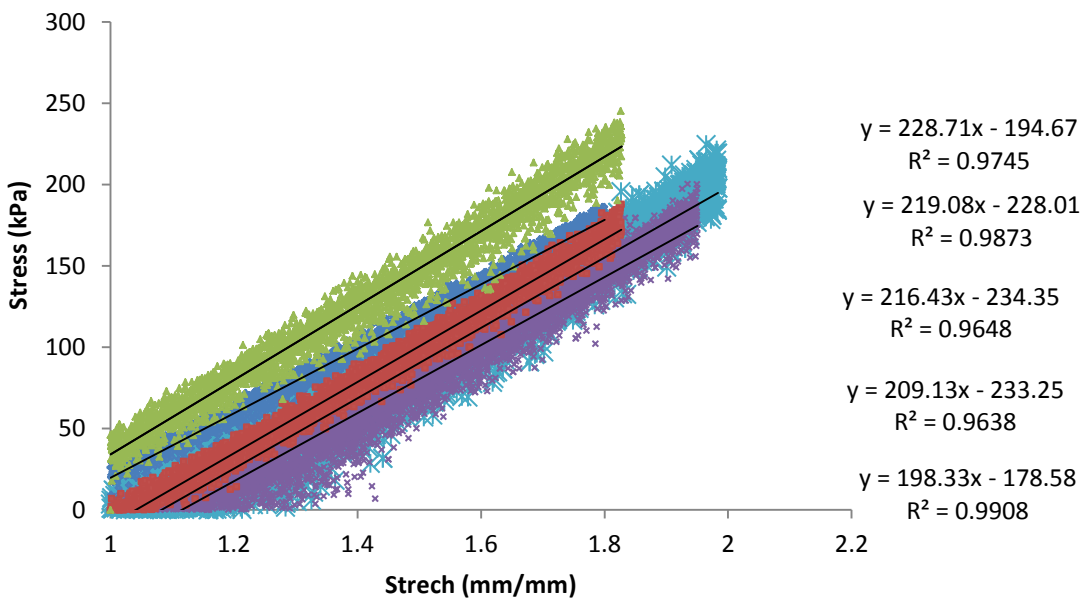
**Figure 1.6** - Stress vs. Stretch: orange plot - 287 kPa; violet plot - 267 kPa; red plot 2.11 kPa; green plot - 212 kPa, azul plot - 170 kPa.

- Six hours of isolation

For 6 hours of isolation the stress-stretch curve is represented in figure 9.

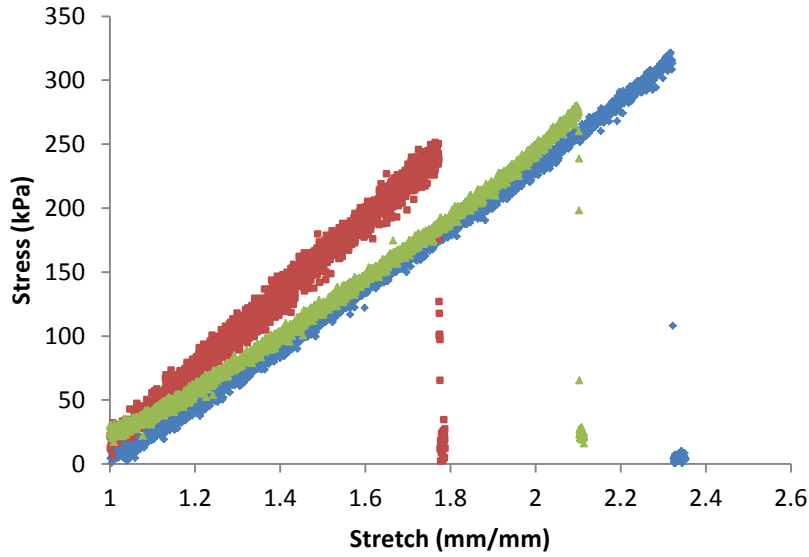


**Figure 1.7** - Stress vs. Stretch: blue plot, cross section = 5.24 mm x 1.18 mm; red plot, cross section = 5.15 mm x 1.3 mm; green plot, cross section = 4.85 mm x 1.15 mm; light blue plot, cross section = 4.94 mm x 1.27 mm; violet plot, cross section = 5.1 mm x 1.38 mm.

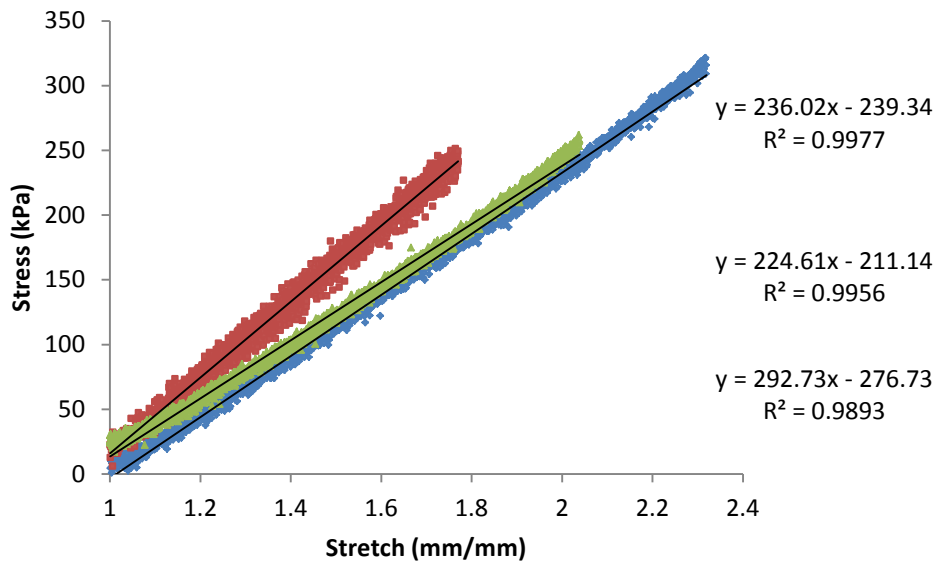


**Figure 1.8** - Stress vs. Stretch: blue plot 198 kPa; red plot 219 kPa; green plot - 229 kPa; light blue plot - 216 kPa; violet plot - 209 kPa.

- Twelve hours of isolation

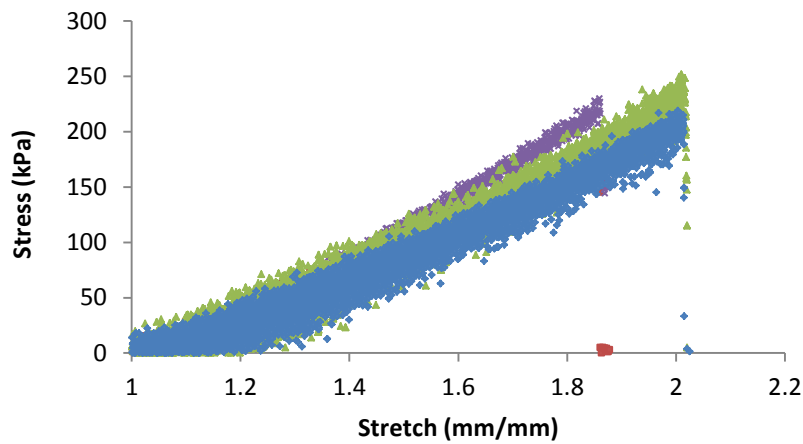


**Figure I.9** - Stress vs. Stretch: blue plot, cross section = 5.00 mm x 1.51 mm; red plot, cross section = 4.89 mm x 1.05 mm; green plot, cross section = 5.12 mm x 1.52 mm

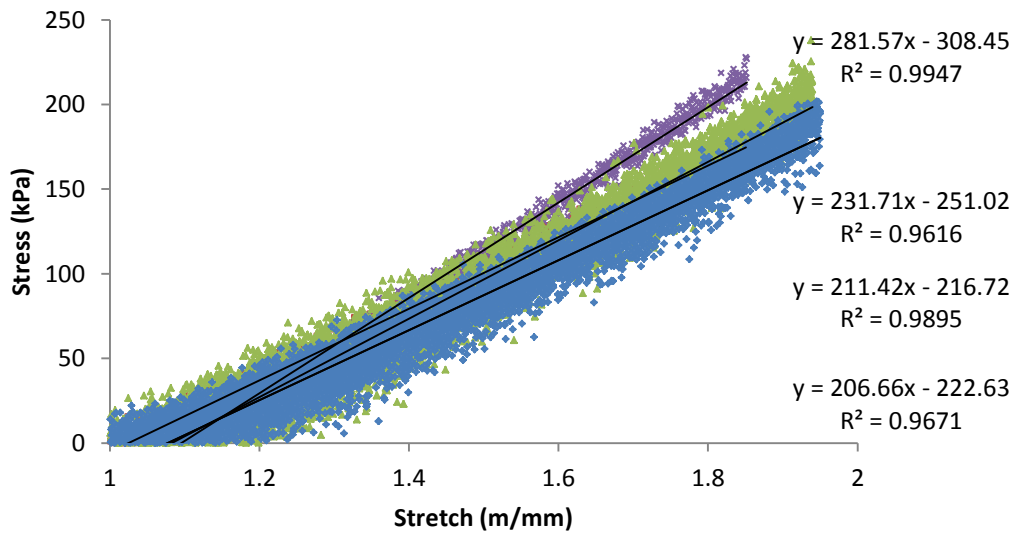


**Figure I.10** - Stress vs. Stretch: blue plot - 236 kPa; red plot - 293 kPa; green plot - 225 kPa.

- Sixteen hours of isolation

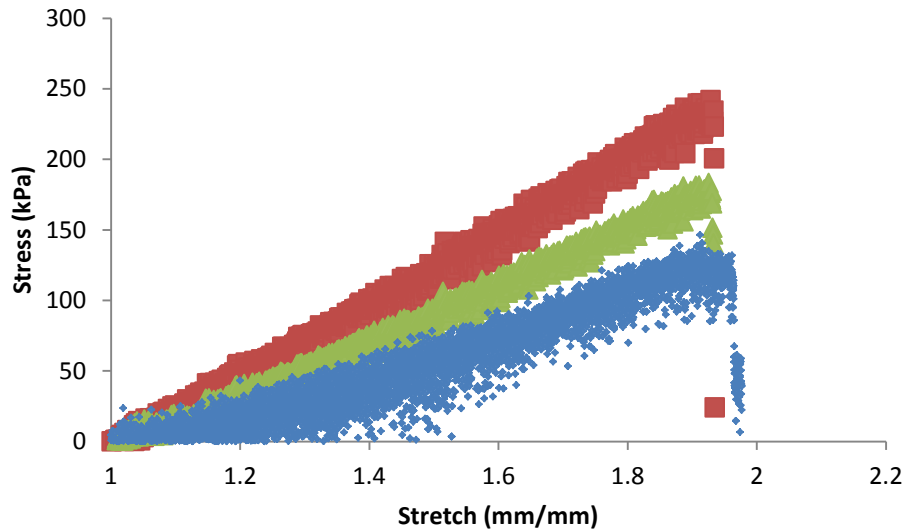


**Figure I.11** - Stress vs. Stretch: blue plot, cross section = 5.04 mm x 1.42 mm; red plot, cross section = 5.00 mm x 1.17 mm; green plot, cross section = 4.87 mm x 1.15mm; violet plot, cross section = 5.04 mm x 1.07 mm.

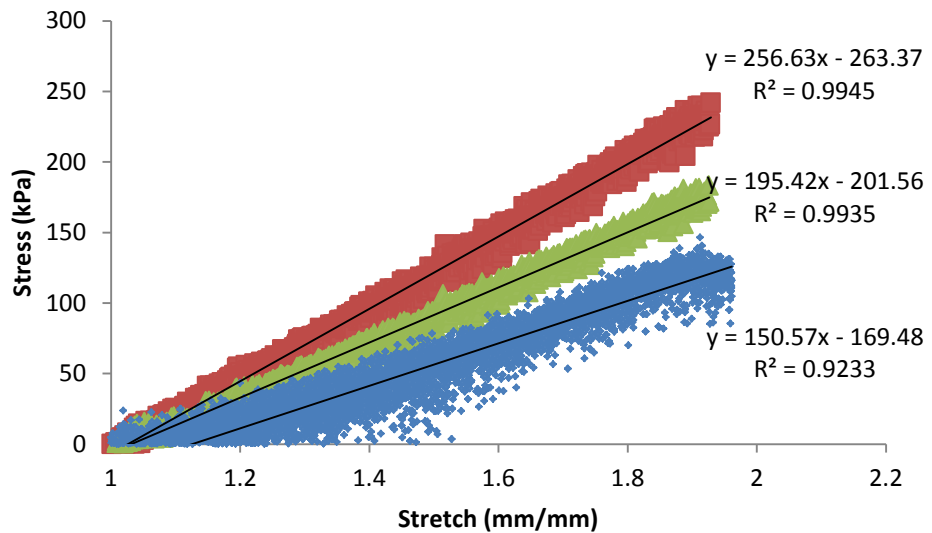


**Figure I.12** - Stress vs. Stretch: blue plot - 209 kPa; red plot - 211 kPa; green plot - 234 kPa; violet plot - 281 kPa.

- Nineteen hours of isolation



**Figure I.13** - Stress vs. Stretch: blue plot, cross section = 4.86 mm x 1.32 mm; red plot, cross section = 4.99mm x 1.18mm; green plot, cross section = 5.06mm x 1.52mm.



**Figure I.14** - Stress vs. Stretch: blue plot 151 kPa; red plot 195 kPa; green plot - 256 kPa.





## Appendix II Collagen Assay Results

*Table II.1* - Values of collagen calibration curve.

V (total) $\mu\text{L}$	V (sample) $\mu\text{L}$	C(standard) $\mu\text{g}/\mu\text{L}$	m collagen (standard) $\mu\text{g}$	Abs	Abs Average	Standard Deviation
100	0	0	0	0	0	0
	7.5	0.075	7.5	0.203 0.202	0.2025	0.0005
	12.5	0.125	12.5	0.284 0.285	0.2845	0.0005
	20	0.2	20	0.544 0.535	0.5395	0.005
	25	0.25	25	0.563 0.568 0.559	0.563333	0.004
	30	30	30	0.757 0.802	0.7795	2.3
	35	0.35	35	0.804 0.833	0.8185	1.5
	40	0.4	40	0.99 0.997	0.9935	0.4

Abs - Absorbance



Table II.2 - Values of collagen for test samples.

Isolation time / h	Wet tissue/mg	pepsin/mg	V (sample) / $\mu$ L	collagen diluted sample / $\mu$ g	Concentration Collagen ( $\mu$ g/ $\mu$ L)	Collagen / $\mu$ g	Abs	Abs average	Standard deviation	$\frac{\mu\text{g collagen}}{\text{mg wet tissue}}$
0	80.64	9.39	50	20.439	0.409	2044	0.501 0.552 0.544	0.501	0.3	25.3
	190.51	1.995	35	34.704	0.992	4958	0.888 0.813	0.850	0.04	26.0
2	93.34	9.997	100	11.173	0.112	559	0.219 0.329	0.274	0.06	6.0
	64.85	6.53	100	3.010	0.0301	151	0.064 0.084	0.074	0.01	2.3
	64.85	6.53	50	1.439	0.0288	144	0.041 0.030	0.035	0.006	2.2
	100.32	1.024	50	3.255	0.0651	326	0.064 0.096	0.080	0.02	3.2
	93.34	9.97	50	2.296	0.0459	230	0.105 0.008	0.056	0.05	2.5
	71.24	7.41	50	1.867	0.0373	187	0.039 0.053	0.046	0.007	2.6
4	77	7.96	50	0.898	0.0180	90	0.028 0.016	0.022	0.006	1.2
	71.24	7.41	100	3.704	0.0370	185	0.138 0.044	0.091	0.05	2.6
	189.53	1.853	50	11.071	0.221	1107	0.248 0.295	0.271	0.02	5.8
6	41.79	4.11	50	0.888	0.0178	89	0.010 0.034	0.022	0.01	2.1
			100	1.867	0.0187	93	0.038 0.054	0.046	0.008	2.2

Table II.3 - Continuation values of collagen for test samples.

Isolation time / h	Wet tissue / mg	pepsin/ mg	V (sample) / $\mu$ L	collagen diluted sample / $\mu$ g	Concentration Collagen ( $\mu$ g/ $\mu$ L)	Collagen / $\mu$ g	Abs	Abs average	Standard deviation	$\frac{\mu\text{g collagen}}{\text{mg wet tissue}}$
6	64.43	6.73	50	3.561	0.0712	356	0.092 0.083	0.087	0.005	5.5
	64.43	6.73	50	1.929	0.0386	193	0.051 0.044	0.047	0.004	3.0
10	87.06	8.86	50	4.235	0.0847	423	0.120 0.087	0.104	0.02	4.9
	87.06	8.86	50	4.357	0.0871	436	0.091 0.123	0.107	0.02	5.0
12	54.7	5.77	100	0.663	0.00663	33	0.029 0.004	0.016	0.01	0.6
	54.7	5.77	50	0.724	0.0145	72	0.012 0.024	0.018	0.006	1.3
16	45.21	4.61	50	0.724	0.0145	72	0.010 0.026	0.018	0.008	1.6
	45.21	4.61	100	0.704	0.00704	35	0.025 0.010	0.017	0.008	0.8
	76.29	8.09	50	2.582	0.0516	258	0.084 0.043	0.063	0.02	3.4
19	77.41	8.25	50	0.786	0.0157	79	0.026 0.013	0.019	0.007	1.0
	77.41	8.25	100	2.847	0.0285	142	0.082 0.058	0.070	0.01	1.8
	131.48	1.363	50	4.806	0.0961	481	0.119 0.117	0.118	0.001	3.7
	131.48	1.363	50	4.622	0.0924	462	0.121 0.106	0.113	0.008	3.5

## Appendix III Elastin Assay Results

Table III.1 - Values of elastin calibration curve.

V (total) $\mu\text{L}$	V (sample) $\mu\text{L}$	C(standard) $\mu\text{g}/\mu\text{L}$	m elastin (standard) $\mu\text{g}$	ABS	Abs average	Standard deviaton
100	0	0	0	0	0	0
	12.5	0.125	12.5	0.522	0.542	0.01
				0.542		
				0.564		
				0.541		
	25	0.25	25	0.692	0.695	0.02
				0.724		
	35	0.35	35	0.67	0.988	0.01
				0.978		
	40	0.4	40	0.998	1.074	0.02
1.053						
				1.095		

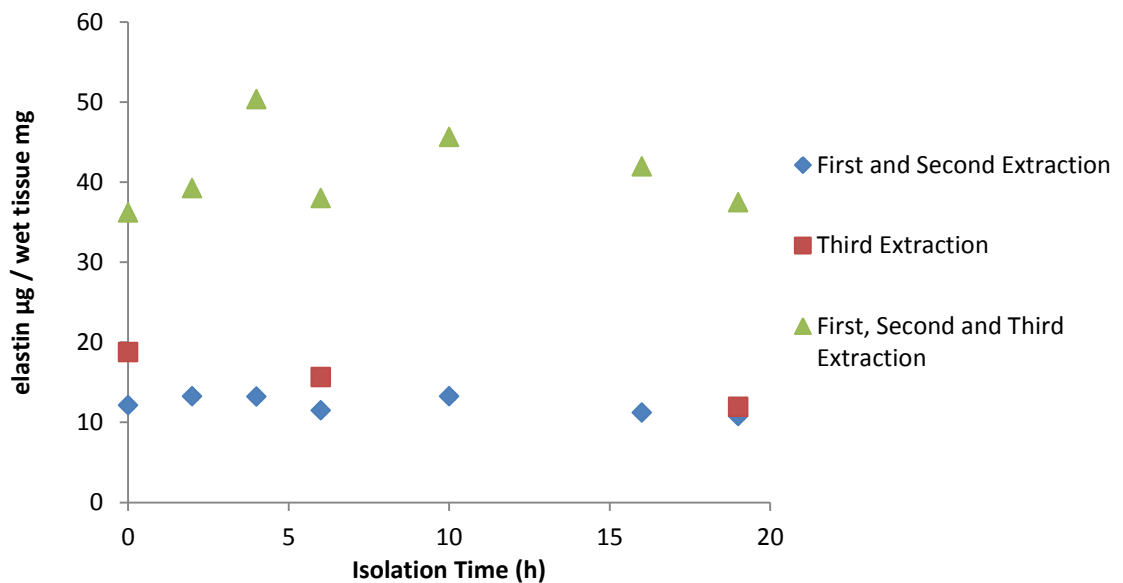


Figure III.1 - Ratio of mass elastin as function of isolation time for different number of extractions.



**Table III.2** - Values of elastin for test samples.

Isolation Time /h	Wet tissue /mg	V (extraction) / $\mu$ l	V (sample) / $\mu$ l	Abs	Abs average	Standard deviation	Elastin diluted sample / $\mu$ g	Elastin / $\mu$ g/ $\mu$ L	Elastin sample / $\mu$ g	$\frac{\mu\text{g elastin}}{\text{mg wet tissue}}$
0	120.43	10000	50	0.523	0.617	0.07	21.775	0.436	4355	36
				0.644						
0	56.89	3390	50	0.683	0.849	0.01	30.1066	0.602	2041	36
				0.838						
2	75.65	6000	50	0.860	0.700	0.02	24.823	0.496	2979	39
				0.717						
2	63.32	3690	50	0.683	0.859	0.1	30.461	0.609	2248	36
				0.759						
4	59.57	4800	50	0.959	0.884	0.007	31.348	0.623	3009	47
				0.877						
4	5413	3300	50	0.891	0.818	0.06	29.008	0.580	1914	35
				0.757						
6	66.53	5200	50	0.879	0.69	0.05	24.379	0.488	2535	38
				0.642						
6	49.63	3000	50	0.733	1.00	0.001	35.461	0.709	2128	43
				1.001						
10	57.48	3420	50	0.999	0.975	0.01	34.575	0.691	2365	41
				0.987						
10	50.76	4800	50	0.963	0.68	0.01	24.202	0.484	2323	46
				0.692						
				0.673						
				0.682						



Table III.2 continuation - Values of elastin for test samples.

Isolation Time /h	Wet tissue /mg	V (extraction) / $\mu$ l	V (sample) / $\mu$ l	Abs	Abs average	Standard deviation	Elastin diluted sample / $\mu$ g	Elastin / $\mu$ g/ $\mu$ L	Elastin sample / $\mu$ g	$\frac{\mu\text{g elastin}}{\text{mg wet tissue}}$
16	46.52	4800	50	0.641 0.509	0.58	0.07	20.390	0.408	1957	42
	62.34	3720	50	0.879 0.923	0.901	0.02	31.950	0.639	2377	38
19	53.66	3300	50	0.938 0.957	0.948	0.01	33.599	0.672	2218	41
	90.01	7200	50	0.643 0.682	0.66	0.02	23.493	0.470	3383	38

## Appendix IV Statistical Calculation

- Elastic Modulus

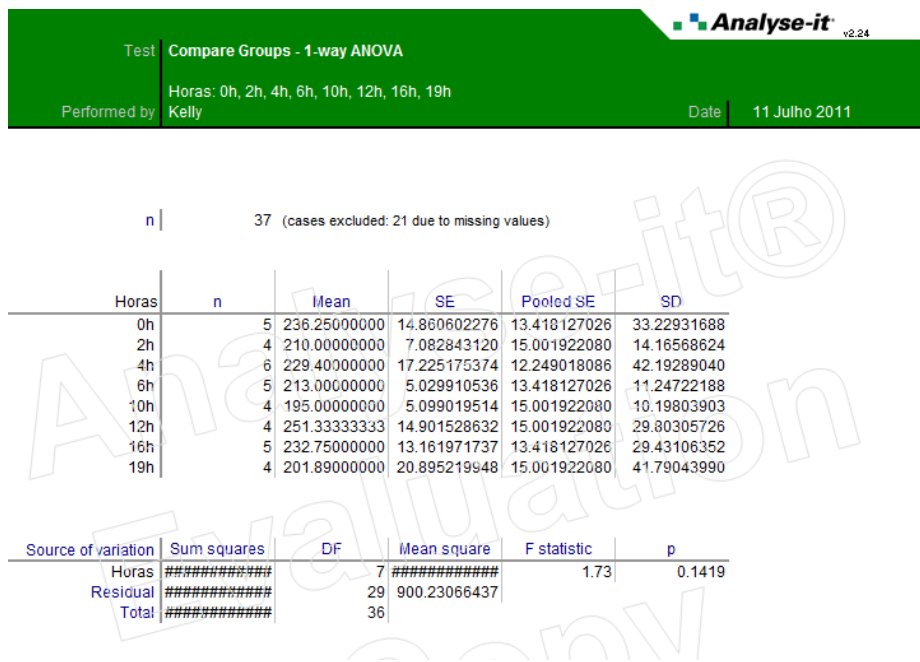


Figure IV.1 - 1-Way ANOVA results for elastic modulus at different times of isolation process.

Collagen Assay from 2h until 19h

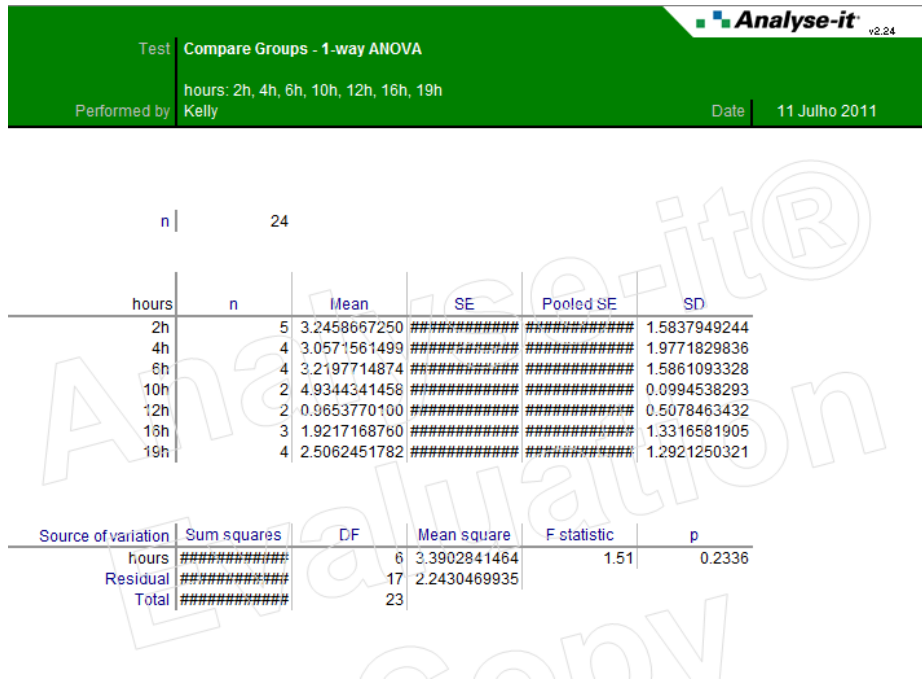


Figure IV.2 - 1-Way ANOVA results for amount of collagen from 2h until 19h of isolation.

Elastin Assay

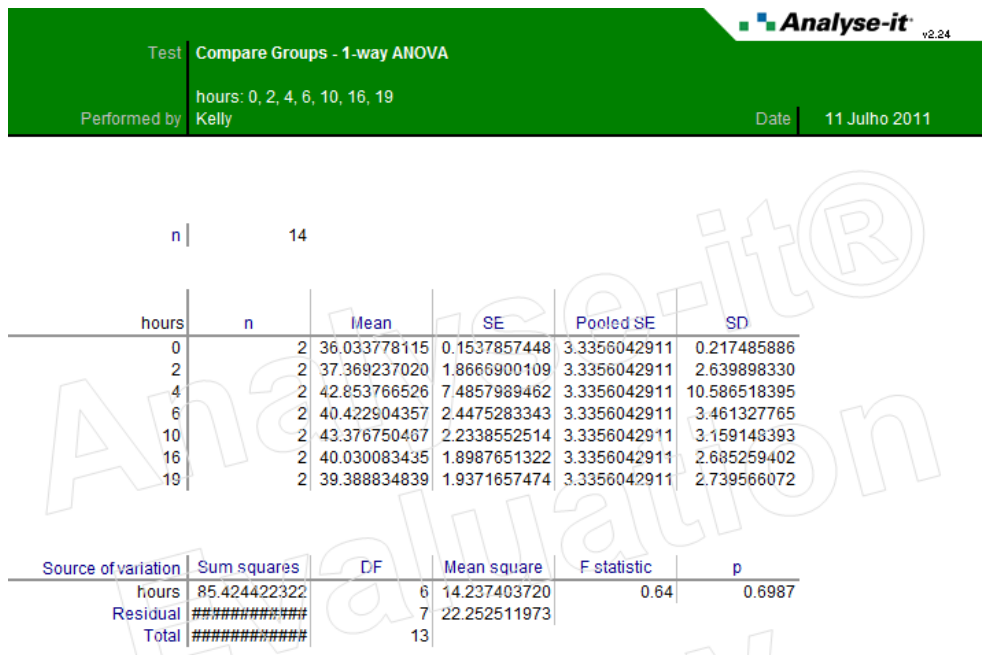
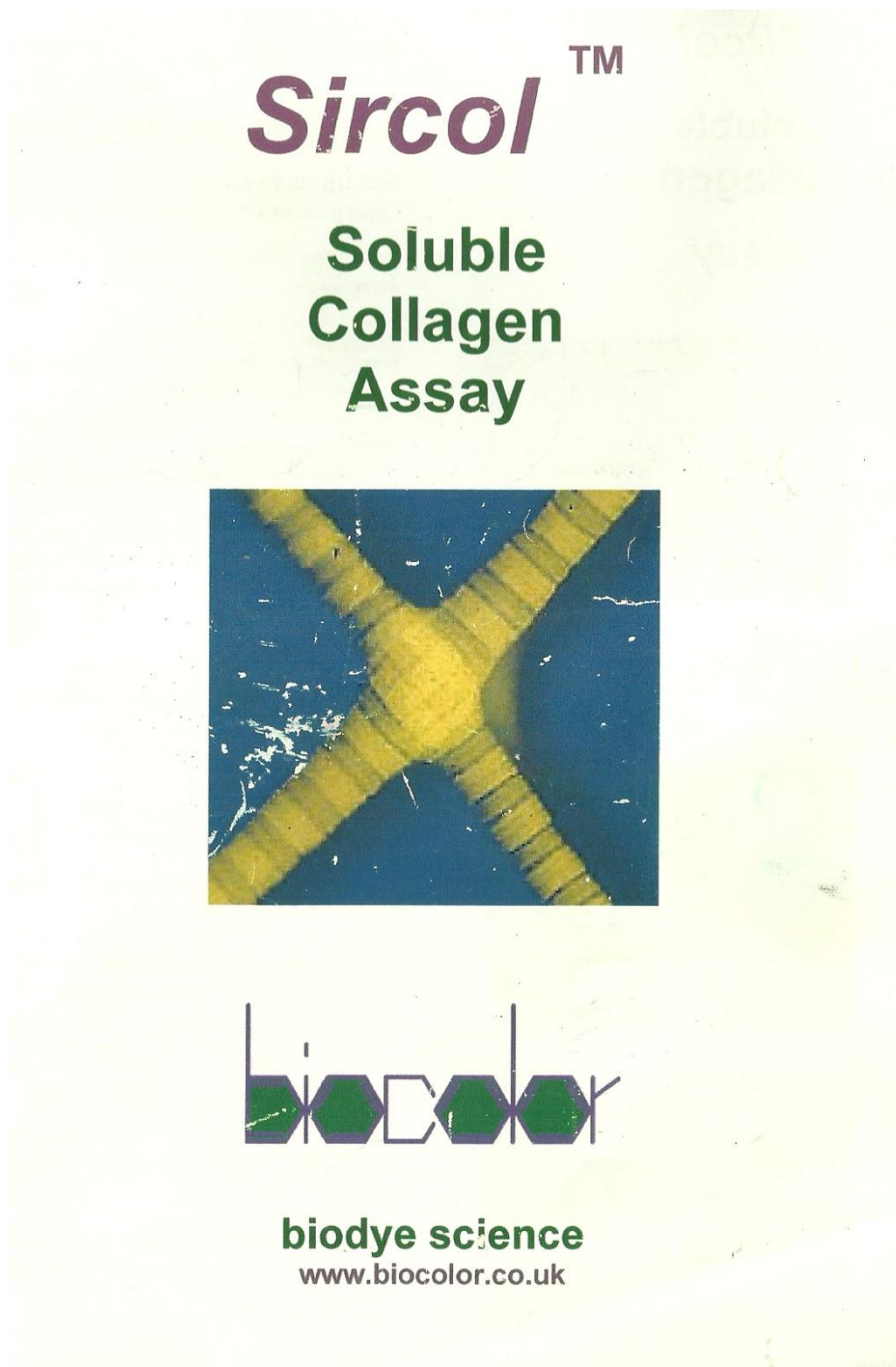


Figure IV.1 - 1-Way ANOVA results for amount of elastin at different times of isolation process.

## Appendix V Collagen Assay Manual



## Assay Snapshots



**+30 min**

Mix Sircol reagent  
and collagen  
reference  
standard



**+40 min**

Centrifuge and  
remove the  
supernatant  
carefully



**+50 min**

Add Alkali reagent

\* Picture obtained using 0-  
50 µg collagen reference  
standard

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**Sircol™  
Collagen  
Assay Kit**

Options

<b>Standard Assay Kit</b>	<b>Product Code: S1000</b>
<b>Components of the assay kit:</b>	<b>(120 assays)</b>
Sircol Dye Reagent	120 ml
Alkali Reagent	120 ml
Salt-soluble collagen Precipitating Reagent	15 ml
Collagen [Type I]	5ml
1mg/ml standard in 0.5 M acetic acid, (Bovine; produced from USA disease free animals)	
Sircol Assay Manual	24 pages
(also available to download from our website )	

<b>Assay Large Economy Pack</b>	<b>Product Code: S5000</b>
<b>Components of this assay kit, as S1000</b>	<b>(475 assays)</b>

**Supplementary Unit Packs**

**Sircol Dye Reagent only** [3 x 120 ml]  
**Product Code: S1005**

**Collagen Standard** [3 x 5 ml ampoules]  
**Product Code: S1010**  
Sterile bovine acid-soluble collagen; [Type I]  
Concentration: 1 mg/ml in 0.5M acetic acid.

**Collagen Standard** [3 x 5 ml ampoules]  
**Product Code: S1011**  
Sterile rat tail acid-soluble collagen; [Type I]  
Concentration: 0.5 mg/ml in 0.5 M acetic acid.

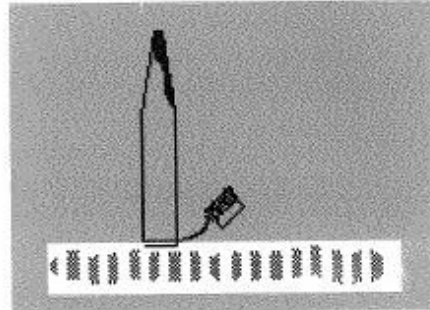
**Gelatin Standard** [4 x 10ml ampoules]  
**Product Code: S1020**

### Draining:

The unbound dye solution is removed by carefully inverting and draining the tubes.

Any remaining droplets can be removed from the tubes by *gently* tapping the inverted tube on a paper tissue, or a cotton wool bud can be used for removing droplets of dye from the rim of tubes.

Do not attempt to remove any fluid that is in close contact to the deposit.



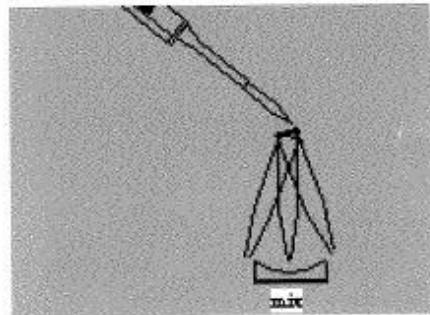
### Release of bound dye:

To each tube add 1 ml of the Alkali reagent.

Re-cap the tubes and release the bound dye into solution. A vortex mixer is suitable.

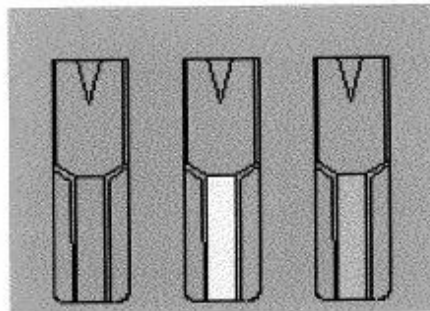
When the bound dye has been dissolved, usually within 10 minutes, the samples are ready for measurement.

The colour is light stable, but should be read within 2 to 3 hours. Keep the tubes capped, until ready for measurement.



### Measurement:

- (a) Spectrophotometer, set wavelength to 540 nm. Use semi-micro glass, or plastic disposable, cuvettes.
- (b) Colorimeter, set using a blue-green filter.
- (c) Multiwell plate reader, set using a blue-green filter. Transfer 200  $\mu$ l aliquots of samples from tubes to the wells of a 96 well, multiwell plate.
- (d) Set the above instruments to zero using water. Measure absorbance of reagent blanks, collagen standards and the test samples.
- (e) Subtract the reagent blank reading from the standard and test sample readings. Check duplicates are within  $\pm 10\%$ .
- (f) Plot standards on graph and use the graph to calculate the collagen content of the test sample.



### Footnote:

Where \* occurs, see the Sircol manual for more detailed information and/or some suggested modifications

The Sircol Assay has been designed for  
*in vitro* research work only

Handle the Sircol Assay Kit  
using

**GOOD LABORATORY PRACTICE**

Read Manual before use

**Sircol Manual**

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[www.biocolor.co.uk](http://www.biocolor.co.uk)



**Sircol™  
Collagen Assay  
Manual**

**Intended Applications:**

The Sircol Collagen Assay is a quantitative dye-binding method designed for the analysis of acid-soluble collagens extracted from mammalian tissues and collagens released into culture medium by mammalian cells during *in vitro* culture.

**Collagen forms that can be measured:**

- [i] salt-soluble collagens (1 M NaCl in 0.05M Tris, pH 7.5.)
- [ii] acid-soluble collagens (0.01 to 1.0 M acetic acid)
- [iii] pepsin-soluble collagens (0.5 M acetic acid + pepsin)
- [iv] total soluble collagens (composed of [i],[ii] & [iii])

**Collagen Types that can be measured:**

- [1] Mammalian collagens, Types I to V, can be measured
- [2] Collagen Types VI to XIV can be assayed, but have not been calibrated due to insufficient purified material available for the preparation of standard curves
- [3] Sircol dye binding does not discriminate between collagen types
- [4] The dye reagent binds specifically to the [Gly-X-Y]<sub>n</sub> helical structure found in all collagens
- [5] Sircol dye binding decreases, gradually with the thermal denaturation of collagen (collagen to gelatin)
- [6] Non-vertebrate collagens bind less dye, due in part to lower denaturation temperature and less hydroxyproline residues, and would require species matched purified preparations for standard calibration curves

**Sircol Assay Kit components:**

- [1] The dye reagent contains Sirius Red in picric acid and has been formulated for specific binding to collagen under the conditions defined in the Sircol Assay Manual.

[2] Alkali reagent contains 0.5 M sodium hydroxide, for releasing Sircol Dye from the collagen-dye complex.

[3] Salt soluble collagen precipitating reagent; contains L-lysine monohydrochloride.

[4] Collagen standard, acid soluble Type I, supplied as a sterile solution in 0.5 M acetic acid within a sealed vial. Concentration: 1 mg/ml.

This is sterile bovine skin collagen that has been imported from the USA, and was obtained from disease free animals.

In countries that inhibit the importation of bovine derived material, a rat tail Collagen standard, acid soluble Type I, is supplied as a sterile solution in 0.5 M acetic acid within a sealed vial. Concentration: 0.5 mg/ml.

[5] This Sircol Assay Manual; (further copies of the Manual can be downloaded from Biocolor's website, [www.biocolor.co.uk](http://www.biocolor.co.uk))

**Other components required, but not supplied:**

[a] Capped 1.5ml capacity microcentrifuge tubes.

[b] Variable volume micropipettors and pipette tips.

[c] A mechanical mixer for the microcentrifuge tubes. Any equipment that provides consistent shaking, rolling or rotation of the tubes is suitable.

[d] A centrifuge with a 1.5ml tube rotor head and capable of at least 10,000 x g, to firmly pack the collagen-dye pellet.

[e] A spectrophotometer, a colorimeter or a microwell plate reader with a blue-green filter.

**Recommended storage conditions for components:**

**Unopened;** All components have been prepared for long term stability (at least 12 months), when stored at room temperature.

Do not freeze as complete re-solubilisation may not occur.

**Opened;** The assay components will retain their shelf-life, providing the glass vials of collagen standard and lysine solution are:

- (i) stored at +4°C when not in use
- (ii) the metal seal is not removed

The contents of both vials are best sampled as follows:

Remove the centre metal disc only from the vial tops. Obtain aliquots from the vials, when required, using a plastic syringe fitted with a sterile hypodermic needle. The butyl rubber seal on the vial has a thin centre disc, suitable for needle insertion into the vial. Discard vial if solution contents become turbid.

**Mode of action of the Sircol dye reagent with soluble collagens:**

The Sircol dye reagent contains Sirius Red. The Colour Commission name is Direct Red 80. The molecular structure of the dye is shown below (Fig. 1).

**Mechanism by which the dye reacts with collagen:**

Sirius Red is an anionic dye with sulphonic acid side chain groups. These groups react with the side chain groups of the basic amino acids present in collagen.

The specific affinity of the dye for collagen, under the assay conditions is due to the elongated dye molecules becoming aligned parallel to the long, rigid structure of native collagens that have intact triple helix organisation.

Dye affinity is much reduced when collagen is heat denatured ( $>45^{\circ}\text{C}$  to the form of random chains of gelatin) and the triple helix unwinds.

Dye binding is gradually lost when collagen (and gelatin) are exposed to bacterial collagenases.

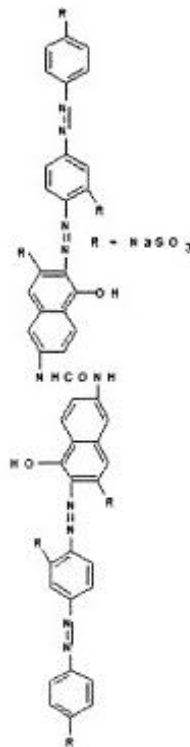


Fig.1 The molecular structure of the Sircol Dye

**Assay – Test sample compositional requirements:**

As the Sircol Assay is a colorimetric procedure, samples for analysis should be free of any particulate material (cell debris and insoluble extracellular matrix).

- [1] The test sample can be in a salt buffer solution, acetic acid or culture medium.
- [2] If a surfactant has been used during tissue extraction, it is recommended that this extraction solution is Sircol tested with the Reference Collagen Standard to check that it has no adverse effects on collagen-dye binding.
- [3] The presence of soluble proteins in samples, including proteoglycans, tropoelastin and other soluble ECM materials, does not interfere with the Sircol Assay.
- [4] Cell culture medium with foetal calf serum supplements of up to 5% does not interfere with the collagen assay. When higher serum supplements have been used, the increasing bulk of serum proteins, relative to the amount of collagen present, can cause problems. In this latter case the following options could be examined:
  - [a] reduce the serum supplement to 5%, either after cell attachment has occurred, or reduce serum to 5% for the culture medium that will be collected and used for assay;
  - [b] selectively remove the bulk of the serum albumin from the test sample by affinity chromatography (Blue-Sepharose CL-6B; Pharmacia Biotechnology);
  - [c] precipitate the collagen out of solution, adding NaCl to a concentration of 4 M for samples with neutral pH; or 2 M NaCl for samples in acetic acid. Centrifuge, drain well and re-solubilize the collagen pellet in 0.5 M acetic acid.

**Assay of test solutions containing < 25 µg/ml collagen, without prior concentration:**

**Method** The Sircol Dye Reagent contains sufficient dye to permit test sample volumes to be increased up to 200 µl, before adding 1.0ml of Dye Reagent.

**Note 1** It is not recommended that more than 200 µl of sample volumes be used with 1.0 ml of Dye Reagent. Excessive dye dilution, by increasing sample volumes above 200 µl, can result in loss of collagen dye saturation.

**Note 2** For test samples with collagen levels less than 25 µg/ml, but with more than 5 µg/ml, it is possible to directly obtain assay results without sample concentration by *increasing* both sample and dye volumes, using larger volume conical centrifuge tubes.

Add 1000 µl of test solution, followed by 5.00ml of Dye Reagent, contained in a 15 ml capacity conical centrifuge tube. The collagen bound dye complex, recovered after centrifugation, is then solubilised in 1.00 ml of the Alkali Reagent.

No multiplication factor is required, when using 1.00 ml test samples/5.00 ml dye volumes, to express the results as µg/ml.

**Set up assay:**

To duplicate 1.5 ml microcentrifuge tubes, add sample volumes of between 10 and 100  $\mu$ l:

- (a) collagen standards
  - (b) test samples and reagent blanks (100  $\mu$ l of 0.5 M acetic acid, extraction buffer or fresh unused tissue culture medium).
- Adjust the volume in all tubes to 100  $\mu$ l.

**Working Standards:**

It is recommended that the collagen standard is initially run, in duplicate, at three concentrations; using 12.5, 25 and 50  $\mu$ l aliquots of the supplied Reference Collagen Standard.

The standards, with the reagent blanks, are used to produce a calibration curve with the selected spectrophotometer, colorimeter or microwell plate reader.

In subsequent assay batches a minimum requirement is duplicates of a mid-range collagen standard and reagent blanks.

In repeated assays these secondary standard and reagent blanks should give absorption values, at 540 nm, to within  $\pm 5\%$  of that defined by the initial standard curve.

**Test samples:**

With test samples, where the approximate collagen concentrations are as yet unknown (collagen absorption at 280 nm is very low due to the limited number of aromatic amino acids and, therefore, cannot be used as a guide), 50  $\mu$ l aliquots are suggested for the first run.

If required use more or less, in the next batch of assays to bring all test sample readings within the concentration range covered by the standard curve.

**Dye reagent and mixing:**

Add 1.0 ml Sircol dye reagent to all tubes. Cap tubes and mix contents. First by inversion, as the density/viscosity difference between the sample and the dye reagent can differ considerably. Then gently mix tube contents at room temperature for  $30 \pm 5$  minutes.

The same mechanical mixer, at the same setting, should if possible be used for all assay batches to minimise experimental variations.

**Centrifuge to recover collagen-dye complex:**

Transfer tubes to a microcentrifuge and centrifuge at 10,000 x g for 10 minutes. This operation is to pack the collagen-dye pellet at the bottom of the tubes.

A minimum RCF of 10,000 x g is required; higher values, if available, can be used as this will force more unbound dye out of the pellet. This also reduces the risk of any pelleted material being lost when the tube contents are decanted.

**Removal of unbound dye:**

Remove tubes from centrifuge and uncap. The supernatants are drained off and discarded. The collagen bound dye remains as a pellet at the bottom of the tubes.

While the tube is still inverted, use an absorbent paper tissue or a cotton bud to remove any dye solution from the top end of the tube wall. Do not remove any beads of fluid close to the collagen-dye precipitate on the side wall of the tube.

The Sircol dye reagent contains a surfactant to aid draining from the non-wettable plastic tubes. A wash step is **not** recommended, but may be considered necessary for low concentration test samples. Wash with 500  $\mu$ l of ethanol (99% pure; and methanol free).

**Do not wash with water.** An ethanol wash step can reduce reagent blank readings close to zero, but increases the experimental variation between duplicate samples to above that obtainable without the wash step. This is due to the dye precipitate not packing as firmly after the ethanol wash, which causes a loss of some bound dye during the second draining step.

**Recovery of collagen bound dye:**

To the collagen-dye pellet add 1.0 ml of the Alkali reagent and then cap the tubes. Bring the collagen bound dye back into solution; a vortex tube mixer is most convenient, but holding the top of the tube in one hand while flicking the bottom of the tube with a finger of the other hand is also effective.

The dye should be in solution within 5 minutes. Pellets that are centrifuged at a high RCF may take a little longer. Gelatin samples can require several mixing operations to fully release gelatin-bound dye.

**Measurement of collagen bound dye:**

The alkaline dye solution is stable to indoor light, but should be measured within 3 hours. Ensure tubes remain capped, to avoid loss due to evaporation, until ready for reading.

**Spectrophotometer;**

Use semi-micro glass, or plastic disposable cuvettes. Set the instrument wavelength to 540 nm and use water to set the absorbance reading to zero. Read and record the absorbance values of assay blanks, standards and test solutions.

**Colorimeter;**

The instrument should have a sample cell or cuvette suitable for reading test volumes within 1 ml, and a 1 cm light path length.

The range of filters in colorimeters varies. A blue-green filter, often labelled 500, 510 or 550 nm, will usually be found suitable. To confirm that the filter selected is suitable, use the three concentrations of collagen standards. Ensure that these readings produce a straight line standard curve, that passes through zero.

**Microwell plate colorimeter;**

Transfer 200  $\mu$ l aliquots of the alkali dye solutions from the assay tubes to the wells of a microwell plate.

The selection and testing of a suitable colour filter should follow the recommendations given for the colorimeter above.

**Calculation of collagen concentration in test samples:**

Subtract the reagent blank reading from all the standard and test readings. The reagent blank value should be between 0.15 and 0.18 (a guide range only – based on the equipment used). Higher values (>0.25) indicate that the draining and removal of unbound dye technique could be improved.

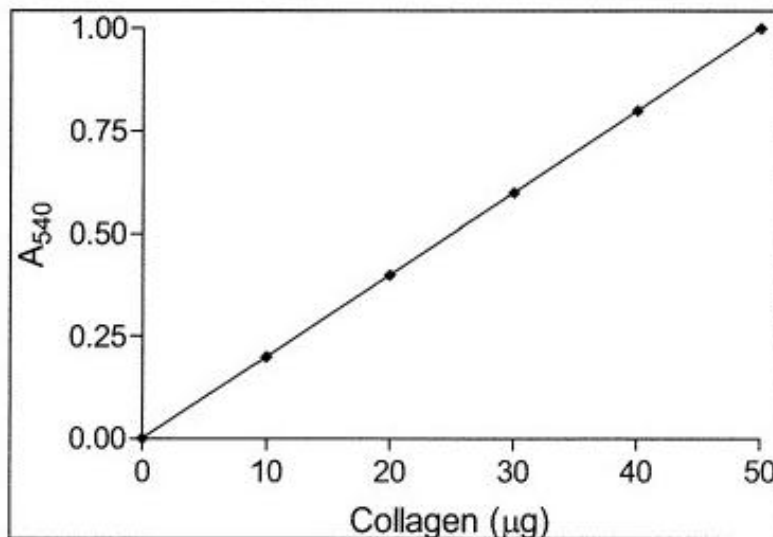
When low reagent blank values are obtained consistently, it may be more convenient to set the measuring instrument to zero with the reagent blank; thus avoiding the need to subtract the background value from the test samples.

Monitor the variation in absorbance readings between duplicate samples. Initially some wide variations may occur. Assuming that this is not due to a pipetting error, the most likely source of error is the draining step. A little practice with draining and drying of the top of the tubes leads to a consistent mode of practice, and the ability to bring experimental error of duplicate samples to within  $\pm 5\%$  of the mean.

Using a computer spreadsheet with graphical output, plot the three Collagen Reference Standard absorbance means against their known collagen concentrations. On joining the points, these should produce a straight line graph which can then be extended to pass through zero (absorbance and concentration).

Test sample absorbance values can now be read off the graph to determine their collagen concentration. Readings below 0.05 and higher than 1.00 are unreliable and should be re-assayed – after either concentration or dilution of the test material.

Where test material contains uncommonly used salts, detergents or biological molecules, it may be necessary to initially evaluate their suitability with the assay. This can be performed by adding known amounts of the Collagen Reference Standard to the test samples and checking collagen recovery, following completion of the assay.



*Fig. 2 A typical straight line calibration curve for dye bound by acid soluble collagen.*

*The above calibration graph was prepared using aliquots of the collagen standard solution; according to the assay procedure outlined on the inside cover.*

## TEST SAMPLE PREPARATION

### General comments

Collagen is ubiquitous to all animals and is found within, or surrounding, all tissues and organs. Collagen is the most abundant animal protein, accounting for about one third of the total protein of mammals.

The range of collagen types and their functional roles ensures that they play a key role in all aspects of growth, maturity, pregnancy and ageing. Because of their widespread occurrence, changes to collagens occur in most chronic and some acute diseases.

Given the diversity of animal species and tissue material that are used to study the above processes, there can be no single universal sample preparation procedure. Some of the more commonly used preparations and extractions are described below. A further valuable source of information is in published research papers (see page 18 for recent research papers that have used the Sircol Assay).

### IN VITRO STUDIES

#### Application for low collagen concentrations in cell culture medium:

Common practice during cell culture is to employ the culture medium at a volume of  $\sim 0.25$  ml/cm<sup>2</sup> surface area of the selected culture flask, dish or plate. This culture medium ratio has been found to provide an acceptable balance between providing sufficient medium to meet the cells nutritional requirement, while avoiding excessive concentrations of cellular metabolites that accumulate during culture. The 0.25 ml/cm<sup>2</sup> ratio also ensures that culture medium volume, above anchorage dependent cells, does not unduly restrict exchange of CO<sub>2</sub> and O<sub>2</sub>.

When monitoring the secretion of collagen the 0.25 ml/cm<sup>2</sup> culture medium ratio can frequently produce collagen concentrations of  $\sim 5$   $\mu$ g/100  $\mu$ l (50  $\mu$ g/ml) in the culture medium.

The amount and rate of production of collagen can vary considerable from this 'average' value under various test conditions; collagen synthesis is often found to increase as the cell population nears confluence.

Examination of the 'standard curve' in the Sircol manual reveals that for test samples with collagen concentrations of  $\sim 5$   $\mu$ g/100  $\mu$ l the absorbance reading is too close to the reagent blank absorbance value to provide confidence in the collagen value obtained.

The need to concentrate test samples, prior to the assay, would cause a substantial delay and additional work before results can be obtained. A delay that detracts from a major benefit of using the Sircol Assay – the convenience of obtaining results in one hour.

For direct procedures to measure test samples with collagen concentrations below 25  $\mu$ g/ml, and more than 5  $\mu$ g/ml, see page 6.



### ***IN VIVO* STUDIES**

#### **Extraction of soluble collagens from tissue, cartilage and organs:**

Samples for analysis should be collected under aseptic conditions, where possible. Material sampled post-mortem should be collected as soon after death as possible. Briefly wash the external surface with sterile water or saline to remove any debris and blood stains. If the sample contains attached adipose tissue this should also be trimmed off using a scalpel.

If extraction is not to be carried out immediately, then the samples should be placed into labelled, sealable, plastic envelopes and frozen as quickly as possible (weigh prior to freezing). Do not store at 0 to 5°C, even if extraction is to be performed the following day. The major risk at this early stage of preparation is proteases; these are released by dead cells and by contaminating bacterial enzymes (many active at low temperatures).

Stored frozen samples are best 'thawed-out', in the plastic envelope, within a refrigerator at 5°C. Decide if collagen content of the test samples is to be expressed as 'dry weight' or 'wet weight'. If dry weight, it will be necessary to take a representative sample, obtain its wet weight and then dry the sample in a heated, or un-heated, drying cabinet containing drying granules. The samples are weighed daily until a constant dry weight value is obtained; most tissues and cartilages are ~ 70% water.

To optimise collagen extraction the tissue sample should be 'diced' into small cubes, using a sharp scalpel. Avoid producing cubes of less than 2 to 3 mm as the 'squeezing' of these small tissue particles can result in fluid being lost from the cut surfaces. Weigh the prepared samples into sterile flasks or beakers. Use as large a weight sample as possible. You cannot have too much collagen and larger sample sizes also reduce variation, due to non-homogeneous collagen distribution within the tissue.

#### **[1] Salt soluble collagens**

The salt soluble collagen fraction represents the most recent collagen secreted by the cell. Within a few hours in the extracellular matrix (or in the cell culture medium) this salt soluble collagen (tropocollagen monomers) will 'crystallise' into collagen fibrils and become salt insoluble. The salt soluble collagen fraction will be small, needing a large sample weight to produce Sircol detectable amounts of collagen (>2.5 µg). The salt soluble collagen fraction is also the most vulnerable to protease degradation.

**Extraction:** Salt soluble collagen solvent is a 0.05 M Tris buffer, pH 7.5, containing 1.0 M sodium chloride. This solvent should also include a 'Protease Inhibitor Cocktail' (ready to use cocktail mixtures are available from Sigma-Aldrich; see P8340).

**Beware** if preparing a DIY cocktail from dry components, as many of these agents are **toxic**.

Extraction solvent volumes will depend on the material being extracted. A 10 volumes of solvent to wet tissue weight ratio is suggested. The sample should be stirred overnight at 0 to 5°C. To obtain a transparent solution, containing the salt soluble collagen, centrifuge at 15000 x g for 60 minutes. As the Sircol Assay is a colorimetric assay, turbid or translucent extracts are not suitable for analysis. If centrifugation does not produce a transparent supernatant, consider filtering this solution through a 0.4 or 0.8 µm filter unit. Initial trials should be performed on non-essential tissue; to determine test sample weights, weight to solvent ratio and whether a second extraction of the residue is required for quantitative extraction.

### [2] Acid soluble collagens

Dilute acetic acid (0.5 M) solubilises non-cross linked, and some cross linked forms of collagen. The pH of 0.5 M acetic acid is ~3.0, so as with salt extraction a protease inhibitor cocktail is recommended. The solvent to tissue ratio and extraction times are also similar to salt extraction.

This method of extraction represents the quickest and simplest procedure for recovering the recently synthesised collagen pool from tissues. The yield of acid soluble collagen recovered will be dependent on the age of the animal (more collagen is synthesised during early growth).

In adult animals most of the collagens have long term stability, usually exceeding the life span of the animal. Increases in acid soluble collagens, however, are found in various disease processes; where the extracellular matrix is being destroyed or collagen is being laid down to replace cell loss in tissues and organs.

### [3] Pepsin soluble collagens

This extraction procedure is usually the 'method of choice' for recovering the recently synthesised collagen pool from tissues. It produces a larger yield of collagen than when 0.5 M acetic acid is used alone.

This is due to pepsin cleaving off part of the C- terminal, non-helical region of the alpha-chains that make up the triple helix of tropocollagen. This C- terminal non-helical region contains the initial covalent cross link between the alpha-chains. This cross-link aligns the three tightly wound left handed helices, permitting them to form the right handed super helix of tropocollagen. In the ECM further cross-linking occurs between adjacent alpha-chains and other tropocollagen molecules that are packed into the forming collagen fibrils.

The pepsin (EC 3.4.23.1) should have good activity, and is dissolved in 0.5 M acetic acid. As a general rule, use about a 1:10 ratio of pepsin: tissue wet weight. Aseptic conditions should apply during overnight extraction at room temperature. Stir vigorously during this time period.

#### **Treatment of Extracts prior to Assay:**

The Sircol Assay is a colorimetric assay and it is essential that test samples are transparent. Opalescence or turbidity will result in the non-specific attachment of Sircol Dye to suspended material. Coloured transparent solutions are suitable. It is not uncommon to have reddish-brown extracts due to the presence of haemoglobin and/or myoglobin. These soluble proteins do not cause interference with the Sircol Assay.

When turbidity occurs, it should be removed prior to assay. High speed centrifugation is often effective. Filtering a small representative aliquot of the test solution through a 0.4 or 0.8  $\mu\text{m}$  filter unit (attached to a 2 or 5 ml syringe) is also usually effective.

**Final Note:** Beware of microbial protease activity; assay samples as soon as possible following extraction.

**Cross-linked insoluble collagen:**

The fraction of collagen remaining in the tissue residue after salt, acid and pepsin extraction is insoluble, covalently cross-linked collagen. Extraction treatments that can solubilise this collagen do so by either causing peptide cleavage into fragments or by denaturation of the collagen to gelatin.

Gelatin can be measured by the Sircol assay, but the degree of protein denaturation effects how much dye will be bound. If heat is used to solubilise the insoluble collagen, then providing the temperature/time are standardized, and similarly treated insoluble collagen standards are included as controls, the assay can be used to measure insoluble collagen.

**Extraction procedure:**

Insoluble collagen when suspended in water and heated at temperatures above 60°C will be gradually converted into water soluble gelatin. The time required will depend, in part, on the nature and frequency of cross-links and the collagen: water ratio. However, the major factor that the time will depend on is the temperature used for extraction. The higher the temperature the shorter the time required, but also the more collagen that is denatured. The more collagen that is denatured the less Sircol dye that can be bound by the gelatin.

The time-temperature effect on dye binding by gelatin is shown below. A temperature of 80°C is recommended, as this permits accurate temperature control by using a water-bath with a thermostat and a lid to reduce evaporation loss. It is important to ensure that all of the insoluble collagen has been solubilised from the test samples. Use small samples of test material and run similar weights of insoluble collagen standards as controls. Insoluble collagens are readily available from biochemical suppliers at low cost.

The heat extraction procedure, although prolonged, can after the initial calibrations be performed with limited supervision.

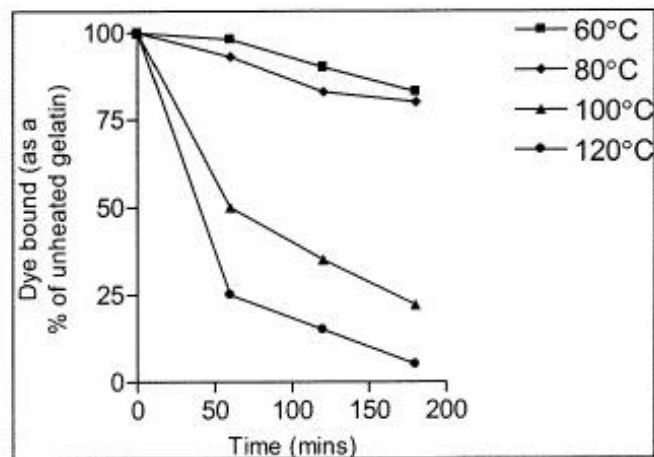
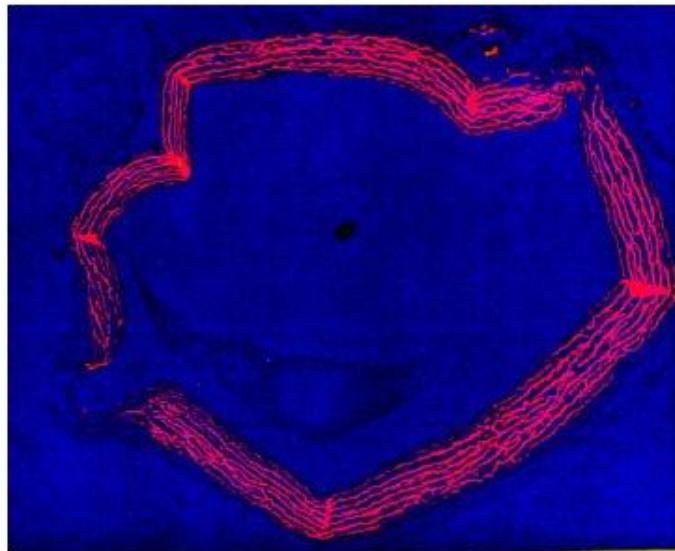


Fig. 3 The effect of temperature/time on gelatin, and the subsequent dye-binding of the Sircol Dye

## Appendix VI Elastin Manual Assay

# Fastin™ ELASTIN Assay



biodye science

[www.biocolor.co.uk](http://www.biocolor.co.uk)



The Fastin Assay has been designed  
~~for~~ in vitro research work only

Handle the  
Fastin Assay Kit  
~~using~~

GOOD LABORATORY PRACTICE

Read Manual before use

Fastin Manual

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Northern Ireland.

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## Fastin Elastin Assay Manual

### Intended Applications:

The Fastin Elastin Assay is a quantitative dye-binding method for the analysis of *elastin* extracted from biological materials and of elastin released into cell culture medium.

The dye label employed is 5, 10, 15, 20-terraphenyl-21, 23-porphine *trifluoromethane* (TFPS). For the structural form of the dye see Fig. 1 on page 6.

### Test sample material:

Tissue extracts, cell culture medium and protein chromatography fractions.

*Elastolytic* enzymes recovered from cell extracts and tissue.

Elastin forms that can be measured by the Fastin Assay:

- (i) *soluble elastin*
- (ii) *heterogenic elastin*
- (iii) *insoluble elastin*, as stabilized elastin polypeptides, (*alpha-elastin*; *gamma-elastin*)

It is also possible to use the assay to detect and/or measure:

- (iv) *elastase* activity, using elastin as the enzyme substrate.

All mammalian *elastins*, so far examined, can be assayed. The dye reagent binds to the 'basic' and *non-polar amino acid sequences found in mammalian elastin*.

Due to the difficulty of obtaining sufficient quantities of *elastin* the assay development was aided, not using *alpha-elastin*.

### Test sample quantities:

A sample volume of between 5 and 100  $\mu$ l is required, containing not less than 5  $\mu$ g and not more than 75  $\mu$ g elastin.

Samples with elastin of >75  $\mu$ g/10  $\mu$ l should be diluted with water, dilute buffer or salt solution.

Samples with elastin of <5  $\mu$ g/100  $\mu$ l should be concentrated by freeze-drying or with the aid of an ultrafiltration membrane.



**Test sample composition:**

For analysis of soluble elastin, samples should be free of any particulate material (cell debris, insoluble extracellular matrix material). The presence of other soluble proteins or of complex carbohydrates, including collagens and proteoglycans, does not interfere with the Fastin Assay.

Test solutions must have a pH between pH 6.0 and pH 8.0. If the pH is outside this range, adjust pH using acetic acid or NaOH.

Test samples obtained by extracting elastin from tissues with oxalic acid (see page 13) should be adjusted to ~ pH 7.0, using 1.0 M NaOH.

Cell culture medium, with fetal calf serum supplements of up to 5%, do not interfere with the Fastin Assay. When higher serum supplements have been used, the increasing bulk of serum proteins, relative to the microgram amounts of elastin present, can cause elastin-dye contact problems.

In samples where serum levels are high two options are available:

- [1] Reduce the serum supplement to 5%, either after cell attachment has occurred, or reduce the serum supplement in the medium that will be collected and used for assay.
- [2] Selectively remove the major serum protein, albumin, from the test samples by using dye-affinity chromatography (Blue-Sepharose CL-6B, Pharmacia Biotechnology)

**Fastin Assay Kit components:**

- [1] The dye reagent contains 5,10,15,20-tetraphenyl-21,23-porphine (TPPS) in a citrate-phosphate buffer, also containing surfactants and anti-microbial agents. The reagent has been formulated for specific binding to elastin under the assay conditions.
- [2] Elastin precipitating reagent contains trichloroacetic acid and arginine
- [3] Elastin standard is a high molecular weight fraction of  $\alpha$ -elastin prepared from bovine neck ligament elastin. The  $\alpha$ -elastin standard is supplied as a sterile solution, at a concentration of 1 mg/ml.
- [4] Elastin-dye complexing reagent is a 90% saturated solution of ammonium sulfate, supplied as a sterile solution.
- [5] Fastin Dissociation reagent is prepared from guanidine HCl and propan-1-ol

**Recommended storage conditions for Assay Kit components:**

**Unopened:**

All of the reagents have long term stability (at least 6 months), when stored at room temperature. Do not freeze as complete desiccation may not occur on thawing. Avoid prolonged exposure to direct sunlight.



Opened;

**Reference Standard:** When stored at +4°C the  $\alpha$ -clastin standard is a clear transparent solution. On heating at room temperature the solution may be observed to become opalescent. This is due to the characteristic concentration property of soluble elastin. On cooling, the process is reversible and the clear solution again becomes transparent.

As concentration is a two-phase system, errors in sampling could occur if used in this form. Therefore, always take sample aliquots from cold solutions (both for standards and test materials).

The metal seal should not be removed from the vial; the contents can be sampled as follows:

- [1] Remove the metal disc only from the vial top.
- [2] Obtain aliquots from the vial by using a syringe fitted with a sterile hypodermic needle. The rubber seal on the vial has a thin metal disc.

The  $\alpha$ -clastin standard should be discarded if the solution becomes turbid.

**Faslin Dye Reagent:** The pH of this reagent is pH7.0 and the dye label, TPPS, is probably a source for some forms of microbial organisms. Microbial contamination can be accidentally introduced to the Dye Reagent from using non-sterile pipette tips, or when unused aliquots of reagent are returned to the bottle.

To limit microbial growth, inhibitors have been added to the reagent. These agents, benzalkonium and sorbic acid, are compatible with the Faslin Assay, but are not 'universal' microbial inhibitors. Good laboratory practice and the storage of an opened Faslin Dye Reagent bottle at 4°C can extend the life of the reagent. **DO NOT FREEZE.**

The Faslin Dye Reagent active component is TPPS. In opened bottles this compound will gradually precipitate as the air space inside the bottle increases during usage. This does not alter elastin binding. The insoluble form of TPPS, however, is less soluble and this is observed in higher Reagent Blank values. This can be corrected, if desired, by filtering the Faslin Dye Reagent before use, so as to remove any insoluble material (a general lab grade filter paper is suitable). This should not be necessary within three months of opening the reagent. The loss of soluble TPPS does not alter the Standard Curve. In old, repeatedly filtered reagent, a calibration check can be run to confirm that TPPS has not become substrate limiting at the higher elastin concentrations.

**Other components required, but not supplied:**

Capped 1.5 ml microcentrifuge tubes and variable volume micropipettes, with matching pipette tips.

A mechanical mixer for the microcentrifuge tubes. Any equipment that provides consistent shaking, rolling or rotation of the tubes is suitable.

A centrifuge, fitted with a 1.5 ml rotor head and capable of at least 10,000 x g, to firmly pack the elastin-dye pellet.

A spectrophotometer, colorimeter or plate reader, with suitable filters (absorbance peaks at 513 nm and 405 nm).





Mode of action of the Fastin dye reagent with elastins:

The Fastin Dye Reagent contains a synthetic porphyrin, 5,10,15,20-tetraphenyl-21,23-porphine, in a water soluble form as the tetrasulfate. The majority of the TPPS molecules contain three or four sulfate groups.

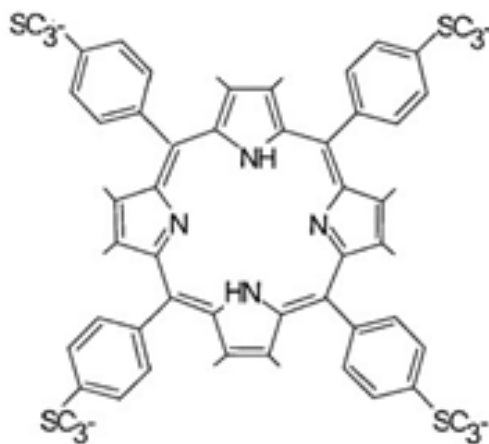


Fig. 1 The structural form of 5,10,15,20-tetraphenyl-21,23-porphine, tetrasulfate.

The affinity of TPPS for elastin was first observed when used as a 'vital stain' on live animals. Most tissues initially took up the dye, but with time only elastin retained the TPPS molecules. [Weissman, J. (1962), Cancer Res. 22, 589-596; [Weissman, J. & Spicer, S. (1962), Stain Technol. 37, 303-305].

The mode of action of TPPS with elastin remains uncertain. It may be due to shape-and-fit with the dye being firmly retained by the basic amino acid side chain residues of elastin.

At pH 7.0 and 20°C, TPPS has been reported to occur as a dimer, producing an out-of-plane type conformational change [Schneider, W. (1973) Struct. Bond. 23, 123].




---

METHOD FOR THE MEASUREMENT OF TOTAL SOLUBLE ELASTIN

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Set up assay:

To duplicate, ~~labeled~~ 1.5 ml microcentrifuge tubes add between 5 and 100  $\mu$ l of test samples, ~~standards~~ standards and reagent blanks.

Adjust the fluid volume in all tubes to 100  $\mu$ l, using water or with the buffer/salt solution ~~used~~ used for test samples.

---

Working standards:

It is recommended that with each Fastin Assay Kit, the elastin standard is initially run in ~~duplicate~~ duplicate at four concentrations; using 12.5, 25.0, 50.0 and 75.0  $\mu$ l aliquots.

The standards along with the reagent blank can then be used to produce a straight line ~~calibration~~ calibration curve from a selected spectrophotometer, colorimeter or ~~microplate~~ microplate reader.

In subsequent assay batches, a minimum calibration requirement is duplicate 25  $\mu$ l aliquots of the elastin standard and the reagent blank. This secondary standard should give ~~absorbance~~ absorbance values to within  $\pm$ 5% of that defined by the standard curve.

When using a different measuring system, or following instrument servicing, a new standard ~~curve~~ curve should be prepared.

---

Test samples:

With test samples where the approximate elastin concentrations are as yet unknown, initially ~~try~~ try single 50  $\mu$ l aliquots for the first trial (also see notes at top of page 4).

If the absorbance readings, at 513 nm, are found to be  $>1.5$ , take 100 or 200  $\mu$ l aliquots of ~~the~~ the deep pink solution and dilute to 1 ml with dissociation reagent and again measure the ~~absorbance~~ absorbance at 513 nm.

If your initial trial aliquots produced absorbance values of  $<0.05$  (at 513nm), after ~~subtraction~~ subtraction of the reagent blank value, the test sample contains less than 5  $\mu$ g elastin and ~~will~~ will require concentration before being re-assayed.

A freeze drier (e.g. ~~SpeedVac~~ SpeedVac, Savant Instrument Inc) can quickly dry 1 ml of sample within  $\pm$  1.5 ml microcentrifuge tube; the sample can then be reconstituted in 5 to 25  $\mu$ l of water or ~~buffer~~ buffer (but beware of drying samples containing excessive amounts of salts as these will also ~~be~~ be concentrated; therefore, prior desalting may also be required).

For reliable and accurate results all test samples should have their absorbance readings within ~~the~~ the range of the Standards that were plotted on the calibration curve.



**START ASSAY:**

---

**Elastin Isolation: [This Is a Temperature Critical Step]**

The Precipitating Reagent has been developed to perform this fractionation; it should not be diluted with sample volumes greater than 1:10, or used at temperatures above 5°C.

The reagent should be pre-cooled to -4°C (it is convenient to store this reagent in the refrigerator so that it is ready for use).

- (1) To each tube add 1.0 ml of the Cold Ficin Precipitating Reagent.
- (2) Tubes are capped and the contents mixed, then placed into an ice/water mixture and left in a refrigerator overnight – to keep the samples at 0°C.

The following morning replacement ice should be added to the container and returned to the refrigerator for about 30 minutes. This will ensure that the tube contents are at 0°C, prior to centrifuging.

---

**Recovery of elastin:**

- (3) Following the precipitation of the elastin, the microcentrifuge tubes are centrifuged (while the contents are still cold) at >10,000 x g for 10 minutes, to pack the precipitated elastin (a centrifuge while useful is not essential; see [2]).
- (4) Remove tubes from the centrifuge, uncap and carefully invert, to drain the liquid contents into a waste beaker. It is important to remove the acid solution from the tubes.

While inverted remove any remaining fluid from the top of the tubes by gently tapping the tubes onto an absorbent paper towel. The tubes now contain only the precipitated elastin.

This elastin is difficult to 'see' as it occurs as a weak translucent gel. A photograph of this gel is shown on the outside back cover of this manual. 1 mg of elastin was required to obtain this image, where a small pellet can just be seen through the wall of the white translucent microcentrifuge tube. Subsequent assay steps can now be carried out at room temperature.

---




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Reaction of the elastin with the Fastin dye:

- [5] Cap the tubes and use a vortex mixer to bring the elastin gel precipitate into solution with the dye reagent.
- [6 a] To each tube add 100µl of 90% saturated ammonium sulfate.
- [6 b] Then add 1.0ml of the Fastin Dye Reagent to each tube.

If high g forces were used during centrifugation (>12,000 xg) the precipitate will have been compacted and may require two or three short periods with the vortex mixer to bring the elastin into solution.

Low g forces (<8,000 xg) produce less well packed precipitates, but have the risk of causing partial loss of the precipitate during the removal of the supernatant.

- [7] The elastin and the dye reagent are allowed to interact for 60 min.

Gentle mechanical mixing is recommended during this period to ensure maximum elastin dye binding. Any form of mixer, to which the microcentrifuge tubes or rack can be attached is suitable. If no suitable mixer is available, then mix the tube contents at ~10 min intervals by manual inversion or, using a vortex mixer.

For optimum results the above conditions should be ~~supplied~~. Time periods of less than 1 hour for reacting elastin with the TPPS are not recommended.

---

Recovery of the elastin-dye complex:

Following the dye binding step the elastin-dye complex formed becomes insoluble in the presence of the added ammonium sulfate.

- [8] The elastin-dye complex is separated from the remaining soluble unbound dye by centrifuging the tubes (>10,000 x g for 10 minutes).
- [9] The tubes are uncapped and the supernatant discarded. Any remaining fluid is removed by gentle tapping of the inverted tubes onto a paper towel.

Visual inspection should reveal a brown-red residue within the elastin standard tubes and, hopefully, also in the test sample tubes. The reagent blank tubes will also have a smaller deposit (see photograph on the outside back cover of this manual).

---

If it is possible to stop the assay at this stage, cap the tubes and store in the dark; avoid allowing the residues to dry.




---

**Release of the elastin bound dye:**

[10] To each tube add 1.0 ml Elastin Dissociation Reagent.

Cap the tubes and use a vortex mixer to bring the elastin-bound dye into solution. Two short mixing periods are usually more effective than one long mixing period.

Traces of ammonium sulfate still present in the tubes may cause turbidity; this can be removed by centrifugation.

Tubes should not be uncapped until absorbance measurement. The dye solutions are stable for several hours, but if readings are delayed store the tube rack in a light-proof box or cupboard.

---

**Elastin measurement:**

The elastin content of the assayed samples is determined by the amount of bound dye released from the elastin.

The absorbance peak of TPDS occurs at 513 nm.

**[a] Measurement using a spectrophotometer;**

Set wavelength to 513 nm, adjust absorbance to zero using a semi-micro cuvette (capacity 1.0 to 1.5 ml) containing water.

Although the instrument can be set to zero using the reagent blank, it is usually better to determine the absorbance of the blank as a quality control check of the assay.

Duplicates (blanks, standards and test samples) should not exceed +5% of their respective means. With practice the analyst can usually obtain this after processing one or two batches of assays.

When any set of duplicate test sample readings exceed +10% of their mean value the sample should be checked for clarity and re-assayed.

**[b] Measurement using a colorimeter;**

Check the colorimeter filter options that are available for the colorimeter; a blue green filter will probably be found to be suitable.

Initially also try the filters on either side of the selected filter; read the blank and standards with each of the three filters.

Plot standard curves for each of the filters and select the optimum curve based on that which gave the highest absorbance readings of the standards, and also produced a straight line for the standard readings.



[c] Measurement using a microwell plate reader (colorimeter);

The filter selection, as described for a tube/cuvette colorimeter, also applies to the plate reader.

100  $\mu$ l aliquots of the Dissociation Reagent are added to the wells of a 96-well plate.

When elastin levels are low, the amount of TPFS present in the 100  $\mu$ l aliquots can give unacceptably low absorbance readings when using some microwell plate readers.

In these cases use 250  $\mu$ l aliquots or, if a suitable filter is available, the aliquots can be read at 405 nm. Some stronger coloured aliquots may require dilution with Fastin Dissociation Reagent.

Elastin concentration determination:

The duplicate absorbance readings of the reagent blank and the three or more sets of elastin standards are used to produce a standard curve.

Subtract the reagent blank mean absorbance value from the absorbance means of each duplicate set of elastin standards.

Plot the elastin standard values; vertical axis 'Absorbance at 515 nm' and horizontal axis; 'Elastin concentration;  $\mu$ g'.

Obtain the best fit line through these plotted points.

The line should pass through zero (0.0 absorbance, 0.0  $\mu$ g elastin).

Do not extend the line beyond the highest plotted point. All of the points should be within  $\pm 5\%$  (absorbance) of the fitted line.

The concentration of elastin present in the test samples can now be determined.

Take the mean of each duplicate test sample and subtract the reagent blank value.

The equivalent elastin concentration for the absorbance value obtained is then read from the standard curve.

The quantity of elastin in the aliquot taken for analysis is then multiplied to give the elastin concentration present, either in 100  $\mu$ l or 1ml of the test sample.



Standard curve; obtained by measuring the amount of elastin bound dye

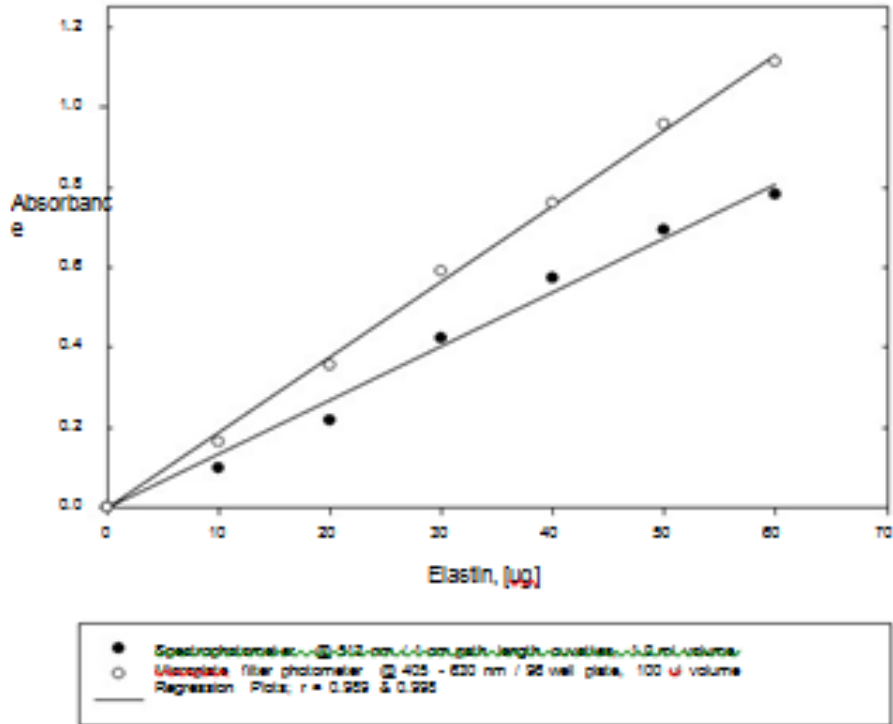


Fig. 2 Elastin Standard Curve.



## MEASUREMENT OF INSOLUBLE ELASTIN

The Fastin Elastin Assay can be used to measure insoluble, cross-linked elastin. The insoluble elastin is extracted from tissue in the form of high molecular weight cross-linked polypeptides.

The insoluble elastin will be either  $\alpha$ -elastin or  $\beta$ -elastin. After extraction, or removal, of the cross-linking acid/alkali, the samples can be assayed by the procedure previously described for soluble elastin (pages 7 - 12).

### Conversion of insoluble elastin to soluble $\alpha$ -elastin:

- (a) The tissue samples are weighed (decide if the elastin content is to be expressed as wet weight or as dry weight,  $\mu\text{g}$  elastin/mg tissue). Place the weighed samples into glass centrifuge tubes ( $\sim 10 \times 2$  cm) and add  $\sim 20$  volumes of  $0.25$  M oxalic acid (assuming tissue density is  $1.00$  g/cm<sup>3</sup>).
- (b) The tubes are then placed into a boiling water-bath (or a metal heating block, with the thermostat set at  $95^\circ\text{C}$ ) for  $60$  min.
- (c) Remove the tubes from the heat and cool quickly to room temperature. Centrifuge at low speed of  $\sim 3000$  rpm for  $10$  min. Pipette off the liquid and retain this extract in labelled containers.
- (d) To the residual tissue in the tubes add a further  $20$  volumes of  $0.25$  M oxalic acid and again heat for  $60$  min. Up to four heat extractions should be initially employed to ensure complete solubilisation of the tissue elastin.
- (e) Some tissue material, such as that from foetus or immature animals, can be fully solubilised after one or two extractions. Tissue from mature or old animals, including humans, may require up to four extractions.
- (f) Initially when using new test material retain each of the oxalic acid extracts separately and assay each for elastin, to establish that elastin extraction was quantitative. The last extract should contain no elastin. Elastin extracted from mature tissue will often produce yellow coloured extracts.
- (g) The individual extracts from each tissue sample are pooled and placed in either dialysis tubing or in pressure filtration cells, fitted with low molecular weight cut-off membranes ( $\sim 15,000$  MW).
- (h) For rapid processing, aliquots of pooled extracts can be quickly cleared of acid and concentrated by using a microcentrifuge tube fitted with a filter membrane and centrifuging at  $10,000 \times g$ .





- (d) The oxalic acid free extract contains the tissue elastin in the form of  $\alpha$ -elastin which has an average molecular weight of ~80,000 MW. The extract can now be assayed using the procedure described for soluble elastin. Retain a record of the extract volumes so as to calculate the tissue elastin content.

Some of the elastin extracted by oxalic acid will be < 5,000 MW. ~~soluble~~ elastin is lost during removal of the oxalic acid.

This form of

#### Conversion of insoluble elastin to soluble $\kappa$ -elastin:

In this method, insoluble elastin is ~~solubilized~~ using aqueous potassium hydroxide (1 M KOH in 80% ethanol).

The elastin ~~solubilization~~ reaction is carried out at 37°C for three (or more) one hour periods. The excess alkali in the pooled extracts is then ~~neutralized~~ by titration, using concentrated phosphoric acid and the alcohol removed by distillation (using a rotary glass evaporator, under reduced pressure).

The ~~solubilized~~ elastin recovered has been termed Kappa-elastin ( $\kappa$ -elastin). This high molecular weight fraction is composed of elastin polypeptides that have 'similar' properties to that of  $\alpha$ -elastin.

Further details on how to prepare  $\kappa$ -elastin can be found in the following reference sources:

- (a) Kornfeld-Rovinsky, N & Robert, L. (1988) Bull. Soc. Chim. Biol. 50, 238.  
 (b) Moore, M., Moore, E. & Robert, L. (1979). Conn. Tiss. Res. 6, 207.  
 (c) Moore, M., Moore, E. & Robert, L. (1980). Frontiers Matrix Biol. 8, 174.



***Fastin™***  
**Elastin**  
**Assay Kit**

Options

~~Standard Assay Kit~~ ~~Product Code: F2000~~  
Components of the assay kit (120 assays)  
Fastin Dye Reagent 120 ml

Elastin Precipitating Reagent	120 ml
Dye Complexing Reagent	25 ml
Fastin Dissociation Reagent	120ml

Elastin, [a high MW -elastin preparation] 5ml  
1 mg/ml standard in water, sterile vial,  
(bovine elastin, from USA disease free animals)

Fastin Assay Manual 24 pages  
(available to view or as a download from our website)

~~Assay Large Economy Kit Pack~~ ~~Product Code: F4000~~  
Components of this assay kit as F2000 (475 assays)

*Supplementary Unit Packs*

Fastin Dye Reagent only [3 x 120 ml]  
Product Code: F2300

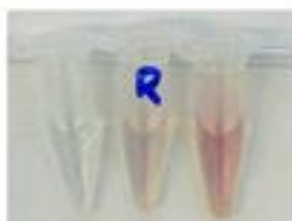
Elastin Standard [3 x 5 ml ampoules]  
Product Code: F2010  
Sterile bovine Elastin [a high MW -elastin preparation,  
water, and salt soluble]. Concentration: 1 mg/ml in water.



- [1] Cold precipitated elastin (1 mg overnight), then centrifuged and drain dried



- [2] Elastin-dye complex after centrifugation and removal of unbound dye  
Left: Reagent Blank  
Right: Standard, 50 µg



- [3] Recovery of elastin bound dye, after adding Dissociation Reagent and centrifugation, to remove turbidity  
Left: Solvent/ buffer Blank  
Middle: Reagent Blank  
Right: Standard, 50 µg

**Bioscolor Ltd.**  
Valley Business Centre, 67 Church Road  
Newtownabbey, BT36 7LS Northern Ireland  
Telephone: +44 (0) 2890 551 732  
Fax: +44 (0) 2890 551 733  
E-mail: [info@bioscolor.co.uk](mailto:info@bioscolor.co.uk)

## Appendix VII Material Safety Data Sheet

- Pepsin

Company:

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Fisher Scientific  
One Reagent Lane  
Fair Lawn, NJ 07410  
United States



Product information:

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Chemical Stability: Stable under normal temperatures and pressures.

Conditions to Avoid: Incompatible materials, dust generation, excess heat.

Incompatibilities with Other Materials Strong oxidizing agents, alcohols, alkalies, heavy metal salts.

Hazardous Decomposition Products Carbon monoxide, carbon dioxide.

Handling and Storage:

---

Handling: Minimize dust generation and accumulation. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale. Use only in a chemical fume hood.

Storage: Store in a tightly closed container. Store in a dry area. Keep refrigerated. (Store below 4°C/39°F.)

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▪ **Acid Formic**

Company:

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EMD Chemicals, Inc.  
480 S. Democrat Road  
08027 Gibbstown  
United States

Product information:

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Synonyms: Methanoic Acid, Aminic Acid

Chemical formula: HCOOH

Molar mass: 46.03

CAS number: 64-18-6

Hazard symbols: C - Corrosive; F - Flammable



Specifications: 98 %

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▪ **PBS**

Company:

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Thermo Fisher Scientific  
Pierce Biotechnology  
P.O. Box 117  
Rockford, IL 61105  
United States



Product information:

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Product name: Dulbecco's Phosphate Buffered Saline

Synonyms: Wash Buffer Powder

Physical state: Solid - Crystalline powder

Hazard symbols: Xi - Irritant



WARNING: Causes respiratory tract, eye and skin irritation. May be harmful if swallowed. Can cause target organ damage.

Composition/information on ingredients:

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Sodium Chloride 80 % - 95 %

Sodium Phosphate 10 % - 20 %

Potassium Chloride 1 % - 3 %

Potassium Phosphate, Monobasic 1 % - 3 %.

Physical and chemical properties:

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Physical state: Solid. [Crystalline powder.]

pH: 7.2 to 7.6 [Conc. (% w/w): 1%]

Solubility: Easily soluble in the following materials: cold water and hot water.

Color: white

Handling and Storage:

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Store between the following temperatures: 20 to 25°C (68 to 77°F). Store in accordance with local regulations. Store in original container protected from direct sunlight in a dry, cool and well-ventilated area.

Handling: Put on appropriate personal protective equipment. Eating, drinking and smoking should be prohibited in areas where this material is handled, stored and processed. Workers should wash hands and face before eating, drinking and smoking. Do not breathe dust. Do not ingest. Avoid contact with eyes, skin and clothing. Use only with adequate ventilation. Wear appropriate respirator when ventilation is inadequate. Keep in the original container or an approved alternative made from a compatible material, kept tightly closed when not in use. Empty containers retain product residue and can be hazardous. Do not reuse container.

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▪ **Acetic Acid**

Company:

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Fisher Scientific  
One Reagent Lane  
Fair Lawn, NJ 07410  
United States



Product information:

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Synonyms: Ethanoic acid; Glacial acetic acid; Methanecarboxylic acid

Appearance: Colorless

Physical State: Liquid

Odor: vinegar-like

Hazard symbols: Hazard symbols: C - Corrosive; F - Flammable

Hazards Identification:

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Flammable liquid and vapor. Causes severe burns by all exposure routes.

Target Organs Eyes, Respiratory system, Skin, Teeth, Gastrointestinal tract (GI), Liver, Kidney, Blood

This material is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200).

WARNING: CAUSES RESPIRATORY TRACT, EYE AND SKIN IRRITATION. MAY BE HARMFUL IF SWALLOWED. CAN CAUSE TARGET ORGAN DAMAGE.

Physical and chemical properties:

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Physical: State Liquid

Appearance: Colorless

Odor: vinegar-like

Odor: Threshold No information available.

pH: < 2.5 10 g/L aq.sol.

Vapor Pressure: 1.52 kPa @ 20 °C

Viscosity: 1.53 mPa.s @ 25 °C

Boiling Point/Range: 117 - 118°C / 242.6 - 244.4°F

Melting Point/Range: 16 - 16.5°C / 60.8 - 61.7°F

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Flash Point: 40° C / 104° F

Evaporation Rate: 0.97 (Butyl Acetate = 1.0)

Specific Gravity: 1.048

Solubility: Soluble in water

Molecular Weight: 60.05

Molecular Formula: C<sub>2</sub> H<sub>4</sub> O<sub>2</sub>

Handling and Storage:

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Handling: Use only under a chemical fume hood. Use explosion-proof equipment. Keep away from open flames, hot surfaces and sources of ignition. Do not breathe vapors or spray mist. Do not get in eyes, on skin, or on clothing. Take precautionary measures against static discharges.

Storage: Keep containers tightly closed in a dry, cool and well-ventilated place. Keep away from open flames, hot surfaces and sources of ignition. Flammables area.

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