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VALIDATION OF TISSUE HYDROLYSIS METHODS FOR QUANTIFICATION OF COLLAGEN IN BOVINE PERICARDIUM

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ABSTRACT: Collagen is the most abundant protein in the extracellular matrix of animals and is mainly composed of amino acids glycine, proline and hydroxyproline.Collagen has been used in haemostatic sponges, dermal equivalents, injectables, and as a vehicle for drug delivery. The quantity and properties of the collagen present in biomaterials must be thoroughly evaluated as the collagen may influence the properties of biomaterials. Hydroxyproline (Hyp) plays key role in collagen stability by permitting the sharp twisting of collagen helix and quantification of Hyp is an indicator of collagen content. The main objective of this study is to validate different tissue hydrolysis methods in order achieve simple, fast and reproducible method for quantification of collagen in biomaterials. Bovine pericardium used in cardiovascular surgery was used as a source material for collagen in the present study. We estimated amounts of collagen present in the bovine pericardium by hydrolysing with collagenase type 1, trypsin, sodium hydroxide (NaOH), hydrochloric acid (HCl) and sulphuric acid (H_2SO_4) singularly. We have observed 13.5 mg of collagen/100 mg of in pericardial tissue hydrolysed by collagenase type 1 treatment and similarly 4.45 mg by trypsin, 16.9 mg by NaOH, 7.55 mg by HCl and 29.45 mg of collagen / 100 mg tissue by H_2SO_4 was quantified. Simultaneously we have tested the robustness and reproducibility of H_2SO_4 hydrolysis method. Our study shows tissue hydrolysis by H_2SO_4 is more efficient, simple and fast for estimation of collagen in biomaterials.

KEY WORDS: Collagen, Hydroxyproline, Bovine pericardium, Sulphuric acid, Hydrolysis and Biomaterials

INTRODUCTION:

Collagen is the primary structural material of vertebrates and is the most abundant mammalian protein accounting for about 20–30% of total protein mass. [1] Collagen accounts for about one third of the protein and three fourth of the dry weight of skin in humans.[2] Collagen is a long and fibrous structural protein that contains three peptide chains, which form a triple helical structure by intra-molecular hydrogen bonds between glycine and hydroxyproline (Hyp) in adjacent chains. [3] Around 28

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types of collagen have so far been identified and, among these, type I collagen is the most prevalent type found in the extracellular matrix. [4] Evidence suggests that collagen's unique characteristics provide normal tissue with great tensile strength maintains integrity, structure and supports the re-growth of joint tissue**.** [5] The molecular structure of collagen has been understood very well from previous studies such as amino acid composition analysis, X-ray diffraction studies, electron microscopy and physicochemical properties of collagen.[6] Hyp is a major component of collagen in the Y position of repeating tripeptide Gly-X-Y, where X is proline. Hyp can be widely used as an indicator to determine the presence and metabolism of collagen. [7] The hydroxyl group of Hyp plays an important role in stabilising the triple helix of collagen. [8] Biomaterials composed of collagen rich extracellular matrices are commonly used for a variety of reconstructive surgical applications and are increasingly used in regenerative medicine strategies. Thumann et al. proved that collagen is non-toxic by morphology, viability and differentiation analysis using retinal pigment epithelial cells.[9] Collagen has been widely employed in the construction of artificial skin substitutes used in the management of severe burns, and several commercial products from collagen have been marketed. [10] Depending on how it is processed, collagen can potentially cause alteration of cell behaviour, have inappropriate mechanical properties, or undergo shrinkage. Collagen is mechanically stable, and could be fabricated into 3-D scaffolds by chemical cross-linking techniques, forming a porous structure. [11, 12] Collagen is used as a biomaterial for many tissue engineering applications because of its excellent biocompatibility, negligible immunogenicity, and high bio-absorbability. [13], [14] Collagen has been widely used in biomedical applications [15], as surgical suture [16], haemostatic agent [17], and matrix for cell culture systems [18] and replacement/substitutes for artificial blood vessels and valves. [19] In the food industry, collagen is widely used to improve the elasticity, consistency and stability of foods [20] in a wide variety of products, such as drinks, soups, noodles, candies and meat products. [21] Collagen has also acquired importance in protein supplements, where they have been shown to be useful in maintaining the nitrogen balance in older people. [22] The analysis of collagen can be an aid in meat authentication because different amounts of collagen are present in different meat cuts [23] and collagen content has also been used as an index of the quality for meat sausages. [24] The quantity and type of the collagen present in biomaterials must be thoroughly evaluated as collagen plays an important role in refining cellular behaviour and tissue function. Bovine pericardial patches have been mostly used for cardiovascular applications, i.e. vascular grafts and heart valves. [25] Bovine pericardium (Bp) is used especially in cardiovascular surgery for repair of atrial septal defect, ventricular septal defect, soft tissue repair, and strengthening the suture line during general surgical procedures etc. Additionally, Bp has also been used for the construction of bio prosthesis in non-cardiac treatments such as patches for vaginal or abdominal wall repair, dural repair and tracheoplasty. In the present study bovine pericardium was used as a source material for collagen.

Many techniques to estimate collagen content has been described. Capillary electrophoresis is used to detect

adulteration associated with the addition of collagen to milk and dairy products. [26] Ion exchange high-performance chromatography with pulsed ampere-metric detection (HPIEC-PAD) associated with microwave-assisted hydrolysis of collagen was used to analyse meat products. High-performance liquid chromatography (HPLC) with fluorometric detection was applied to determine the amount of collagen in meat products. [27, 28] Picro-sirius red dyes are widely used due to their specific reactivity and have been largely employed for quantitative estimation. [29] However these techniques require sophisticated equipment and high maintenance and more suitable for purified collagens, not for complex tissues containing collagen and other extracellular matrix components.

An accurate and high-throughput assay for collagen is essential for collagen research and development of collagen products. Hyp is routinely assayed to provide a measurement for collagen and preparation of samples for analysis has routinely been carried out using acid or alkaline hydrolysis before the colorimetric assay quantification. The time required for sample preparation and the substantially large amount of starting material required for chemical hydrolysis are the major limitations of the standard assay. The main objective of this study is to estimate the collagen content by comparing different hydrolysis treatments and to compare the efficiency of tissue digestion protocols based on the amount of collagen released by each method. A modification of the ISO method of Hyp determination has been devised and to develop a simple and reproducible procedure for the determination of collagen in biomaterials with basic instrumentation. [30] The major modification consists of the hydrolysis of proteins in 3M sulphuric acid of 105°C for 3 hrs instead of 16 hrs and a smaller quantity of starting material (50 mg) required for chemical hydrolysis.

MATERIALS:

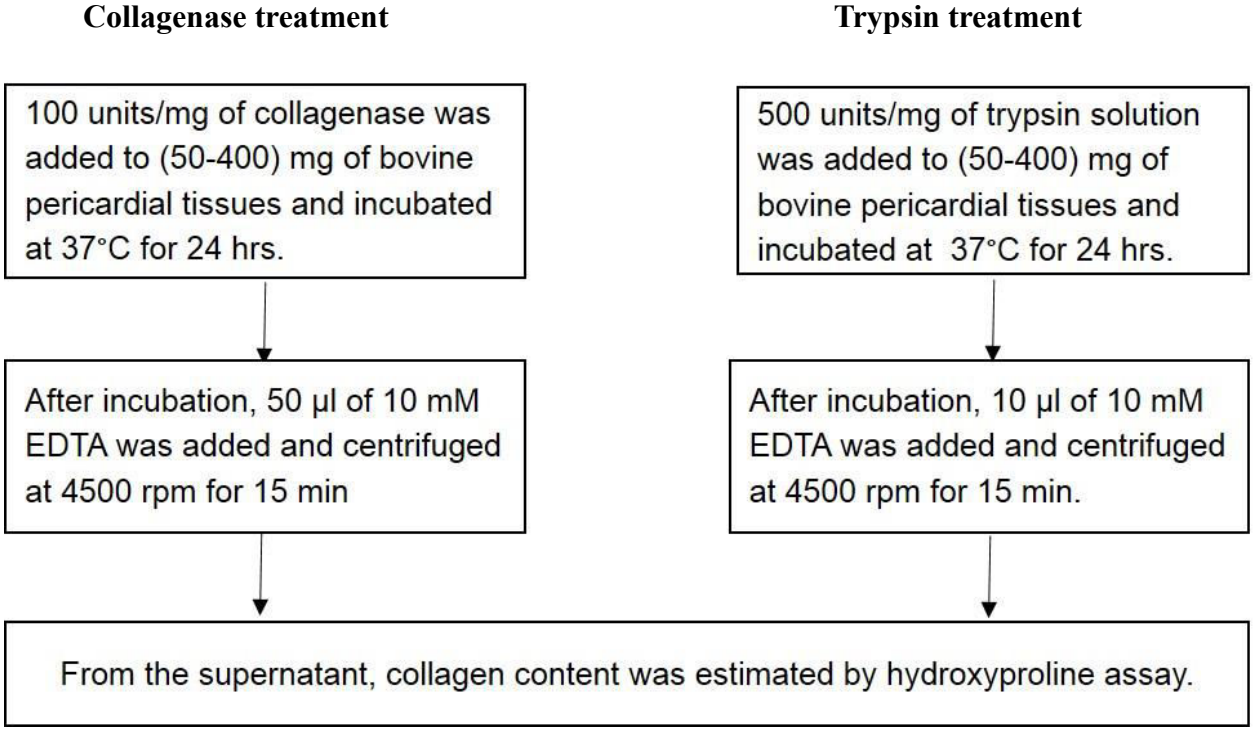
Reagents used: Sodium acetate trihydrate, citric acid monohydrate, glacial acetic acid, isopropyl alcohol, perchloric acid, hydrochloric acid, sulphuric acid, sodium hydroxide , ethylenediaminetetraaceticacid (Loba Chemie); chloramine , Hyp, p-dimethylaminobenzaldehyde, collagenase type 1, trypsin (Sigma Aldrich. Co)

METHODS:

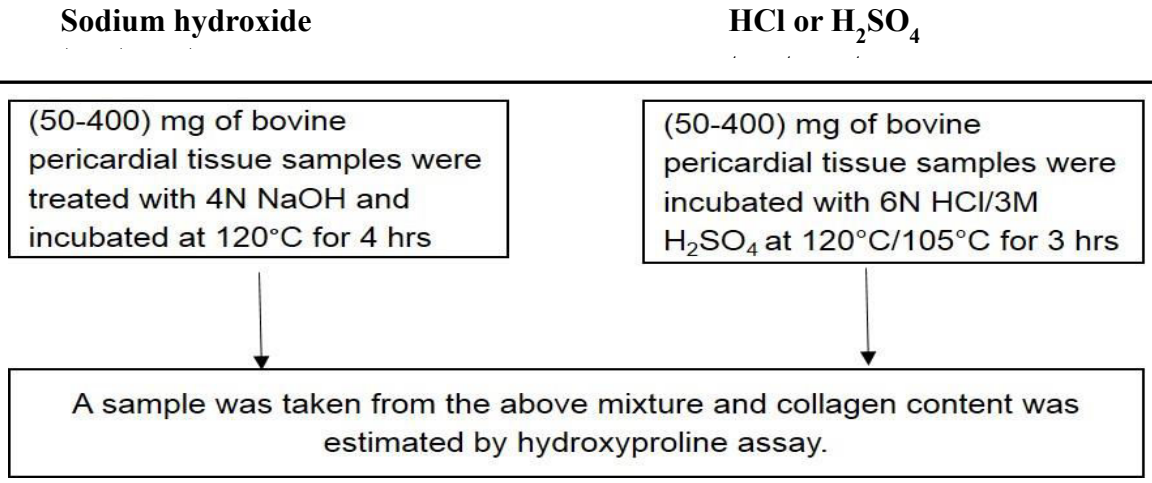
Hyp assay: In 1989, a collaboration involving 18 laboratories resulted in an International Standard method for the quantification of collagen in meat and meat products (ISO 3496:1994).Hyp assay was performed as described in ISO standard for determination of Hyp content.[30] Briefly Hyp standards (1-100 µg/ml) were prepared from the stock solution of 1 mg/ml concentration. 500 µL of chloramine T reagent was added to the standards and mixed well and oxidation was allowed to happen for 25 min at room temperature. 500 µL of Ehrlich's reagent was added to the standards and mixed well and chromophore was developed by incubating the samples at 65˚C in a water bath for 20 min. With a spectrophotometer, absorbance of each sample was measured at 560 nm and the graph was plotted by taking the concentration of Hyp on X axis and absorbance on Y axis. The collagen content was calculated by multiplying the amount of Hyp with factor 8.[30]

Optimisation of tissue hydrolysis: The Bp from adult buffalo was harvested directly from an inspected abattoir and transported within 48 hrs to the laboratory. The adherent fat was carefully removed, and the pericardium was cut into pieces of 2 cm × 2 cm. [25] Bovine pericardial tissue hydrolysis was done by enzymatic, acid and alkaline methods as mentioned below.

Enzymatic Hydrolysis: Tissue was subjected to hydrolysis with collagenase type I or trypsin as given in the flow chart below.



Alkali hydrolysis: Tissue was treated with NaOH for alkaline hydrolysis and with hydrochloric or sulphuric acid for acid hydrolysis.



Test for robustness: Robustness test was performed by hydrolyzing different quantity of tissue samples with H_2SO_4as given below. a) Bovine pericardial tissue samples of (50-600 mg) as separate test specimens were weighed and incubated with 3M H_2SO_4 at 90°C/105°C for 3 hrs b) a sample was taken from the above mixture and collagen content was measured by Hyp assay.

Statistical analysis: The experiments were conducted in triplicate and averages were presented as mean ± standard deviation (SD). Standard curves were fit between measured absorbance values and standards of known Hyp concentrations by least squares linear regression for all repetitions of the assay. Coefficients of determination (R^2) were calculated for each standard curve. Differences were determined by using the paired *t*-test and a *p*- value of < 0.05 was considered statistically significant.

RESULTS:

Test for linearity: Hyp assay was initially tested using standard concentrations of Hyp. The graph plotted with concentration against absorbance at 560 nm shows all the values were in linear range as indicated by R^2 value. R^2 value was 0.9972, an indicator of a good fit (Fig 1).

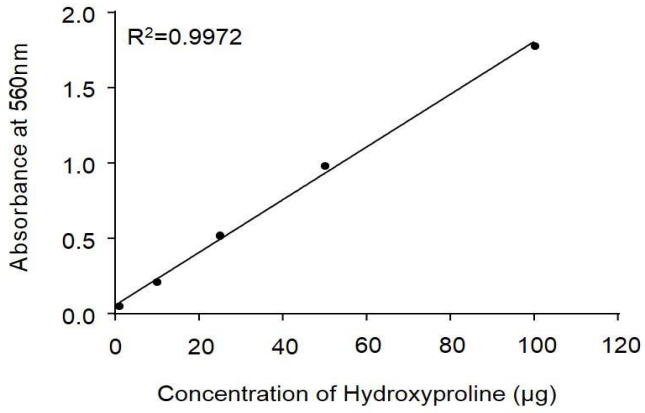


Figure 1: Representative calibration curve for the assay of Hyp: six different concentrations of standard Hyp plotted against their absorbance at 560 nm shows a linear fit with increasing concentrations (n=3).

Comparison of tissue hydrolysis methods for collagen content measurement:

We have hydrolysed pericardial tissue with enzymatic or chemical treatments and compared the amount of collagen released using Hyp assay. The pericardial tissue was mostly resistant to enzymatic hydrolysis and this method was quite expensive. Subsequently we applied chemical hydrolysis to produce hydrolysed collagen in less time by using bovine pericardium as raw material. Several parameters were evaluated such as duration of assay, collagen content released in order to compare chemical hydrolysis and enzymatic proteolysis for the estimation of collagen present in pericardial tissues. We observed that 13.5 mg of collagen/100 mg of pericardial tissue was hydrolysed by collagenase type 1 treatment. Similarly 4.45 mg by trypsin, 16.9 mg by NaOH, 7.55 mg

by HCl and 29.45 mg of collagen/100 mg tissue by H_2SO_4 was quantified. When the tissue was treated with collagenase type 1 and trypsin for 24 hrs the amount of collagen released was comparatively lesser than H_2SO_4 treatment. The hydrolysis with NaOH or HCl also resulted in lesser collagen content as compared to H_2SO_4 hydrolysis. With varying amounts of tissue the amount of collagen release measured is higher after hydrolysis with H_2SO_4 for 3 hrs at 105°C. (Table 1)

Table 1: Data summary of collagen content quantified from collagenase type 1, trypsin, NaOH, HCl and H_2SO_4 treatments. Incubation time of tissue samples with the respective treatments is shown in the table.

Tissue (mg)	Collagen (mg) Collagenase-24 hrs	Collagen (mg) Trypsin-24 hrs	Collagen (mg) NaOH-4 hrs	Collagen (mg) HCl-3 hrs	Collagen (mg) H_2SO_4-3hrs
50	3.75	3	6.75	3.25	14
100	13.5	4.45	16.9	7.55	29.45
200	18.8	9	36.7	16.75	43
400	36.1	27.1	52.5	36.75	98.3

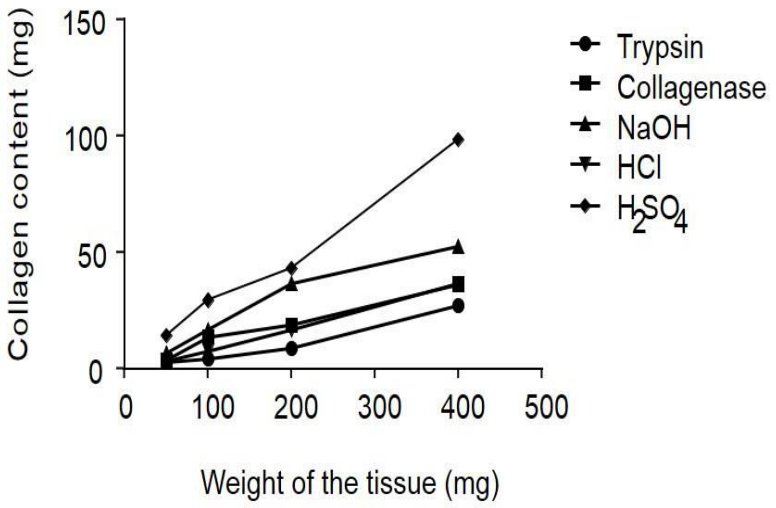


Figure 2: Comparison curve of hydrolysis treatments: Tissue samples of different weights were subjected to five various hydrolysis treatments singularly and plotted against the quantity of collagen obtained.

Test for robustness: The robustness of the tissue hydrolysis method was analyzed by altering the reaction conditions given in the ISO standard reference [30]. We changed the incubation temperature for H_2SO_4 tissue hydrolysis from 105°C to 90°C, incubation time in Hyp assay from 25 min to 20 min after chloramine T addition and incubation time from 20 min to 15 min after Ehrlich's regent addition and found that these changes had no significant effect on the amount of collagen quantified. We also observed the amount of collagen released with varying amounts of tissue weight and found linearity with the increasing weight of tissue (Fig 3). With the *p*-value less than 0.05 these results show that the H_2SO_4 hydrolysis conditions are robust.

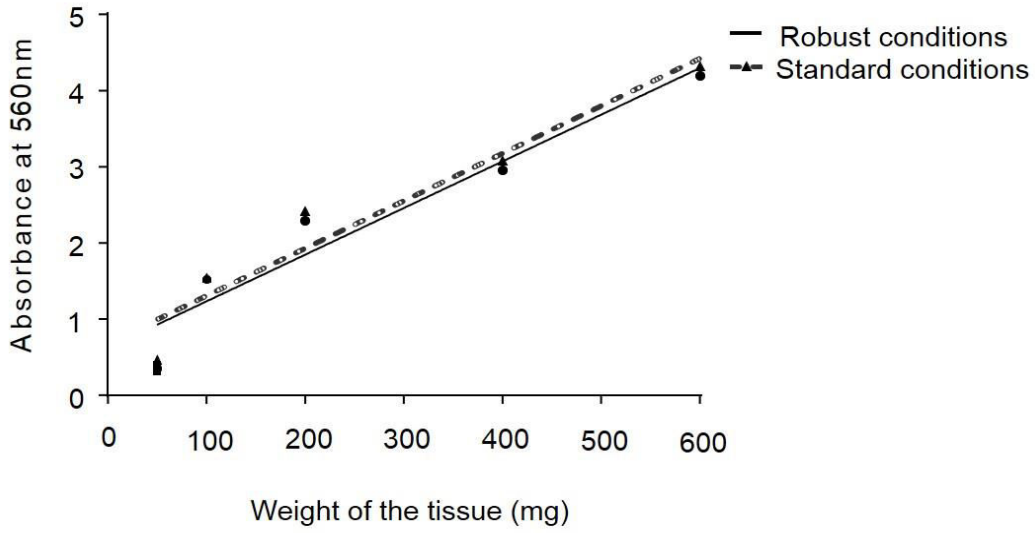


Figure 3: Comparison of tissue samples when subjected to H_2SO_4 hydrolysis method against robust conditions and plotted against the absorbance at 560 nm. The absorbance values directly relate to the amount of collagen.

DISCUSSION:

Collagen is the most abundant connective tissue in vertebrates. The metabolism of collagen and its regulation are of interest in many clinically important diseases that are characterized by accumulation or loss of tissue collagen. Commonly used assays indirectly measure collagen content in tissue samples by exploiting its unique amino acid sequence, which can over or underestimate the true sample collagen content. [31] Using Hyp to measure collagen in a sample relies on the repeating amino acid sequence.The proportion of Hyp by mass is highly conserved within a given type of collagen and ranges from 11.3% in type I collagen to 15% in type III collagen. [31] Other methods of quantifying collagen include sirius red dye binding and ELISA, each associated with their own advantages and disadvantages. Sirius red dye binds to the highly basic residues of solubilised fibrillary collagen amino acid chains and does not bind mostly to non-collagenous proteins that are high in Hyp content. [31] The

Sirius red binding assay also eliminates the need for hydrolysis of collagen to individual amino acids and allows for quantification of different collagen fractions based on their solubility. [31] Assays based on Hyp or Sirius red binding do not distinguish between different types of collagen, in such conditions ELISA is the best choice for quantification. ELISA can detect different types of collagen from the same tissue; the main disadvantage of ELISA is that it's more expensive. Chromatographic purification standardizes conditions of the assay, and increases the yield in a number of routine assays.

To understand the critical role of collagen in various patho-physiological conditions several methods were developed for the determination of Hyp in various tissues and fluids such as plasma. In this study we compared the yield of collagen by hydrolysing the bovine pericardial tissue by different treatments. The Bp was chosen to be studied in the present work because it is a versatile biomaterial used in multiple surgical applications. [25] The investigated process variables were substrate concentration, hydrolysis temperature, and hydrolysis time and the amount of collagen released was measured using Hyp assay. We have noticed that hydrolysis of bovine pericardial tissue with enzymes such as collagenase type 1 and trypsin for 24 hrs yielded significantly lesser collagen content compared to chemical hydrolysis of the tissue. In the present study we have also hydrolysed tissue with strong alkali such as NaOH for 3 hrs and strong acid such as HCl for 3 hrs and found that the amount of collagen hydrolysed was comparatively lesser than H_2SO_4 hydrolysis. According to our findings, treatment of pericardial tissue with 3M H_2SO_4 at 105°C for 3 hrs yielded high amount of collagen measured by Hyp assay and the yields or protein contents of the hydrolysates produced by different processes significantly differed. The 3 hrs acid hydrolysis time was chosen, for shorter time (2 hrs) led to incompletely hydrolysed products. We have tested the robustness of the H_2SO_4 hydrolysis by changing the reaction temperature and time which showed no significant difference with the standard H_2SO_4 hydrolysis treatment. Our method required relatively small amounts of tissue, for Hyp quantification compared to ISO standard method. [30] We have tested the H_2SO_4 hydrolysis starting with minimum weight of 50 mg pericardial tissue and a maximum of 400 mg pericardial tissue.

The present study highlights that the hydrolysis of pericardial tissue with collagenase type 1 and trypsin requires more reaction time and expensive. Whereas treatment of tissue with strong alkali like NaOH for hydrolysis requires less time but efficiency is the limitation. We have found hydrolysis of tissue with H_2SO_4 is more effective with respect to reaction time, cost and collagen content yield. The discrepancy in the data from the several methods highlights the technical challenge of the Hyp measurement. We have evaluated this assay using a range of collagen samples including calf skin collagen (Sigma), fish scale and calf skin collagen produced in our laboratory and observed similar efficiency (data not shown). This data also shows that H_2SO_4 method of tissue hydrolysis can also be effectively applied for other tissues. The optimization of the hydrolysis for this method was done by following the general principles. Chemical hydrolysis was tested between 90°C to 105°C between 2 hrs to 20 hrs. These alterations were tested empirically in small steps. Our results suggest that, the quantification of samples of collagen from a particular source should be carefully validated to estimate the Hyp content of the material tested. The primary objective of this experiment was to ensure Hyp assays, using acid hydrolysis, yield comparable results for various tissue explants and tissue-engineered constructs. The method described in the present study was found to be effective for quantifying Hyp with the required sensitivity for utility in characterization of biomaterials. [32] It also eliminates some of the troublesome issues such as duration of assay and amount of starting material of other methodologies.

CONCLUSION:

In this study we have successfully optimised the efficient tissue hydrolysis method for collagen quantification in biological tissues. This method of H_2SO_4 based tissue hydrolysis may be applied for both clinical and research applications. In conclusion a simple, fast and cost effective method was established for the quantification of collagen present in biological tissues.This modified method would help manufacturers, and researchers to quantify collagen present in biomaterials.

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