

# Elastin in the Avian Lungs

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**Abstract:** Elastic fibres play an important role in the pulmonary development process. The overall goal of this work was to quantify elastin in chicken lungs, using domestic fowl (*Gallus gallus*) as an experimental model, from the 14<sup>th</sup> day of hatching until the 42<sup>nd</sup> after hatching. As analytical methodologies we used high-performance liquid chromatography (HPLC) and a colorimetric method based on the use of a stain that has been only applied in histological techniques. 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. The colorimetric method was used by applying a commercial kit ("Fastin™ Elastin Assay") which quantifies soluble tropoelastin and insoluble elastin made soluble after treatment with hot water solution of oxalic acid. The results obtained by HPLC were statistically different if comparing the incubation period with the after-hatching days ( $p < 0.001$ ). The determinations by the colorimetric method provided results without statistical difference between both analyzed periods ( $p = 0.581$ ). These facts are in agreement with the immaturity of elastin in the period of incubation, without the presence of cross-linkings. The scope, limitations, and choice between both methods have been discussed.

**Keywords:** Elastin, tropoelastin, HPLC, colorimetry, avian lungs.

## 1. INTRODUCTION

Elastic fibres are major insoluble extracellular matrix assemblies, providing elasticity and resilience to tissues that require the ability to deform repetitively and reversibly. They comprise a central crosslinked core of an amorphous component, consisting in the protein elastin (~90% of the fibre) surrounded by a sheath of microfibrils. Microfibril bundles form a template for the deposition of tropoelastin (the soluble ca. 64 kDa polypeptide monomer precursor of elastin) in the extracellular space. Deposits of elastin first appear within microfibril bundles, and then coalesce to form the central crosslinked elastin core of mature elastic fibres. Mature elastic fibres have an outer mantle of microfibrils and some microfibrils appear to be embedded within the elastin core.

In mammals, elastic fibres play a key role in the alveolization process, being responsible for the initiation and maturation of the primitive alveolar sacs into true alveoli [1]. Among vertebrates, the avian respiratory system is of exceptional morphological complexity and remarkably efficient. The lungs are compact and virtually non-expandable [2] while their functional units, the parabronchi, are continuously and unidirectionally ventilated in a caudocranial direction by the synchronized action of the air sacs [3]. Besides, a cross-current arrangement occurs between the parabronchial

air-flow and the venous blood, while there is a counter-current design between the air and blood capillaries [4, 5]. These are some of the unique features that contribute to the efficiency of the avian respiratory system and support the high metabolic rates of flight [6].

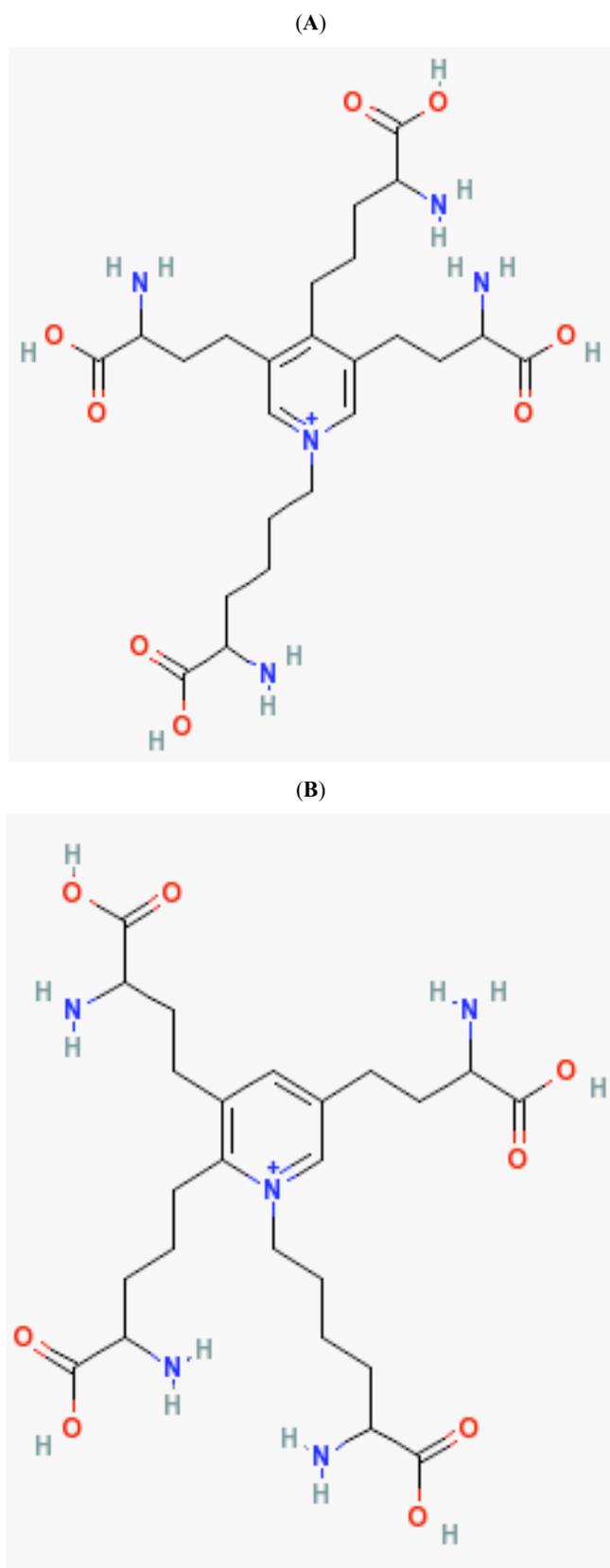
Elastogenesis during the development of the chick embryo's lungs has been studied both with reference to the localization of the various elastic fibre components, and the successive formation of the different microfibrillar and amorphous structures [7-9]. In the avian lung, elastic fibres are present in the bronchial tubes, major blood vessels, pleura and within the parabronchial units, where they are most abundant [7].

In the mammalian lung, the spatial organization of the elastic fibres [10] as well as both the total elastin contents and the area occupied by the elastic fibres in the alveoli have already been determined [1, 11]. The study on elastic fibres distribution, and the quantification of the fractional area occupied by these elements in the functional units of the avian lung was recently carried out by showing the important morphological changes occurring during lung development [12].

In this work the authors present a study on the quantification of elastin in the avian lung, using domestic fowl as an experimental model and making use of two biomedical methods – high-performance liquid chromatography (HPLC) and a colorimetric method based on the use of a stain that has been applied only in histological techniques. The determination of elastin in the avian lung was made from the 14<sup>th</sup> day of incubation until the 42<sup>nd</sup> day after hatching. In both

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methods the amount of elastin or of both desmosines (see Scheme 1 for structure) is related to the total protein contents in the analyzed sample.



**Scheme 1.** Structure of compound determined by HPLC method: desmosine (A), and isodesmosine (B).

## 2. MATERIALS AND METHODOLOGY

### 2.1. Animals

Fertilized ‘Sásso 44’ chicken eggs (*Gallus gallus*) were incubated under normoxic conditions at 37°C in 60% humidity in an incubator with an automatic turner. The chicks received balanced food and water *ad libitum*. Chick embryo lungs were collected on the 14<sup>th</sup>, 16<sup>th</sup>, 18<sup>th</sup>, and 20<sup>th</sup> days of incubation and chick lungs on the 1<sup>st</sup>, 7<sup>th</sup>, 14<sup>th</sup>, 35<sup>th</sup>, and 42<sup>nd</sup> days after hatching. Four chicken embryos and four chickens were used for each age group on the quantification of elastin by both methods to study. The chickens were anaesthetized with an intraperitoneal injection of sodium pentobarbital (22 mg/kg). Their coelomic cavities were opened; the lungs were collected and immediately frozen at -20°C. All the experiments described fulfilled accepted national and international animal well-fare regulations.

### 2.2. Quantification of Desmosine and Isodesmosine by HPLC

#### 2.2.1. Chemicals

Crystalline dansyl chloride (DNS-Cl) and cellulose powder were purchased from Sigma<sup>®</sup> (St. Louis, MO, USA). Pure desmosine, isodesmosine and elastin from bovine ligamentum nuchae were supplied by Elastin Products Co. Inc. (Owensville, MI, USA). Trichloroacetic acid (TCA), sodium hydrogencarbonate, potassium dihydrogenphosphate and *n*-butanol were analytical-reagent grade products from Merck<sup>®</sup> (Darmstadt, Germany). Hydrochloric acid and potassium hydroxide were obtained from BDH<sup>®</sup> Chemicals (Poole, UK). Acetone was supplied by Riedel-deHaën<sup>®</sup> (Seelze, Germany) and acetic acid from Panreac<sup>®</sup> (Quimica SAU, Barcelona, Spain). HPLC-grade acetonitrile was purchased from SDS<sup>®</sup> (Barcelona, Spain) and ultra-pure water was obtained through a Sation<sup>®</sup> 9000 system (Sation, Barcelona, Spain).

#### 2.2.2. Sample Preparation

Lung samples (usually 50-100 mg) were homogenized in 2 mL of water for 30 seconds at +4°C. One half of this emulsion (1 mL) was separated and stored at -20°C for future determination of total protein. To the remaining homogenate it was added 1 mL of water and the resulting 2 mL were mixed with an equal volume of 10% (w/v) cold TCA solution and centrifuged at 9000 g for 10 minutes at +4°C. The supernatant was discarded and the residue treated with 5 mL of 5% TCA at +90°C for 30 minutes for the collagen to be extracted [13]. This mixture was centrifuged at 9000 g for 10 minutes at +4°C and the supernatant discarded. The resulting residue was washed twice in 2.5 mL acetone by centrifugation and dried at +60°C under vacuum.

The dried material was suspended in 2 mL of 6 M hydrochloric acid; after flushing with argon, the vial was flame-sealed and the sample was hydrolysed for 48 hours at +120°C. The isolation of desmosine and isodesmosine in an almost pure form was achieved by using a cellulose packed short column [14]. Minicolumns were made from disposable plastic Pasteur pipettes by cutting off the top hemisphere of the bulb, which became a 4-mL solvent reservoir. The pipette tip was plugged with a small piece of fibre glass. A slurry was prepared by mixing cellulose (1 g) with 20 mL of

the mobile phase, *n*-butanol:acetic acid:water (4:1:1, v/v). The previous hydrolysed sample was mixed in order of addition with acetic acid (0.5 mL), cellulose slurry (0.5 mL), and *n*-butanol (2 mL). This mixture was introduced in the previously prepared column and the vial was washed with 1.5 mL of freshly prepared mobile phase, which was also transferred to the column, and then eluted, with 2 mL of mobile phase. Elution was continued with five times column volume. The desmosine and isodesmosine were eluted from the column with 5 mL of pure water. The aqueous fraction containing the both desmosines was lyophilized to remove residual *n*-butanol and acetic acid and to concentrate the sample.

The previously lyophilized sample was dissolved in 1 mL of 0.01 *N* hydrochloric acid. To 100  $\mu$ L of the sample solution were added 100  $\mu$ L of 0.5 *M* sodium hydrogencarbonate in HPLC-grade water and 100  $\mu$ L of 20 *mM* DNS-Cl in acetone. This mixture was submitted to derivatization for 40 minutes at +65°C in the dark and then diluted to 1 mL with the HPLC mobile phase [15]. The same derivatization procedure was carried out for the working standard solution. In this way, samples and standard were ready to start chromatographic analysis.

### 2.2.3. High-Performance Liquid Chromatography

Chromatographic separations were performed using an HPLC apparatus including two pumps models 305 and 302, a dynamic mixer model 811B, a manometric module model 805, and a UV detector model 115, all from Gilson® (Roissy, France), an injection valve model 7125 with a 100  $\mu$ L loop from Rheodyne® (Phenomenex, Torrance, CA, USA) using a Nucleosil® 120-5 C18 pre-column (Machery-Nagel, Duren, Germany) and a Spherisorb® S5 ODS2 column (150  $\times$  4.6 mm I.D.) from Waters® (Milford, MA, USA). Chromatographic profiles and peak areas were determined by using Unipoint™ v1.9 data station system software (Gilson®, Roissy, France).

Chromatographic analysis was performed with a binary gradient elution as described below. Mobile phase A was a mixture of 25 *mM* potassium dihydrogenphosphate - 25 *mM* acetic acid and acetonitrile (85:15, v/v); mobile phase B was the same mixture but in the proportions 40:60 (v/v); the mobile phases were degassed under reduced pressure and the pH adjusted to 7.2 with 2 *mM* potassium hydroxide solution. The column was first equilibrated with mobile phase mixture: A (70%) and B (30%) at room temperature and a flow-rate of 1.2 mL/min for 15 minutes. Elution was carried out with a linear gradient from 30% to 60% B in 20 minutes. At the end of each run, the column was conditioned with mobile phase mixture: A (70%) and B (30%) for 5 minutes.

Quantification was performed by external calibration. The analyte concentration was determined by comparison of the peak area with that of a known standard. A calibration graph was obtained by applying aliquots of working standard solutions containing scalar amounts of desmosine and isodesmosine (DID) from 100 to 500 pmol to the column.

## 2.3. Quantification of Elastin by a Colorimetric Method (“Fastin™ Elastin Assay”)

### 2.3.1. Chemicals

“Fastin™ Elastin Assay” is a kit purchased by Biocolor Ltd. (Carrickfergus, UK). Fastin Assay Kit components: 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, in a solution concentration of 1 mg/mL in 0.25 *M* oxalic acid; the *Dye Dissociation Reagent* contains guanidine HCl and propan-1-ol. Oxalic acid was purchased by Merck® (Darmstadt, Germany).

### 2.3.2. Sample Preparation

The first step in sample preparation was the conversion of insoluble elastin to water soluble  $\alpha$ -elastin. Tissue samples were weighted and cut into small fragments; these pieces were placed into centrifuge tubes and well mixed with 2 mL of water using a Vortex mixer. One half of this emulsion (1 mL) was separated and stored at -20°C for future determination of total protein. To the remaining homogenate it was added 1 mL of 0.50 *M* oxalic acid so as to obtain 2 mL of 0.25 *M* oxalic acid solution. The tubes were then placed into a boiling water-bath for 60 minutes. After removing from heat the tubes were cooled to room temperature and centrifuged at ~3000 rpm for 10 minutes. The liquid extract was pipetted off and retained. To the residual tissue in the tubes further 2 mL of 0.25 *M* oxalic acid were added and again heated for 60 minutes. Up to three heat extractions were performed until complete solubilisation of the tissue elastin has occurred. The extract volumes were recorded to permit calculation of the tissue elastin content. Next, the extracts were directly assayed. Thus, in labelled micro centrifuge tubes following solutions were placed:

- 100  $\mu$ L of sample extract;
- 12.5, 25 and 50  $\mu$ L aliquots of the *Elastin Standard*; in each tube the volume of 100  $\mu$ L was adjusted with 0.25 *M* oxalic acid;
- 100  $\mu$ L 0.25 *M* oxalic acid to use as reagent blank.

### 2.3.3. Elastin Isolation

To the tubes containing sample extract, elastin standards and blank, 100  $\mu$ L of *Elastin Precipitating Reagent* were added. The tubes were capped; the contents were mixed by inversion and then held for 10 minutes.

### 2.3.4. Recovery of Elastin

Following the precipitation of the elastin, the micro centrifuge tubes were centrifuged for 10 000 g for 10 minutes, to pack the precipitated elastin. After removing from the centrifuge, the tubes were uncapped and carefully inverted to drain off the liquid contents, by tapping onto an absorbent paper towel. The elastin precipitate occurs as a translucent gel.

### 2.3.5. Reaction of the Elastin with the Fastin Dye

To each tube 1 mL of the *Fastin Dye Reagent* was added. The tubes were capped and a vortex mixer was used to bring the elastin gel precipitate into solution with the *Dye Reagent*. The elastin and the *Dye Reagent* were allowed to interact for 90 minutes, with gentle mechanical mixing during this period to ensure maximum elastin dye binding.

### 2.3.6. Recovery of the Elastin-Dye Complex

Following the dye binding step the elastin-dye complex formed becomes insoluble in the presence of ammonium sulphate within the *Fastin Dye Reagent*. So, the elastin-dye complex is separated from the remaining soluble unbound dye by centrifuging the tubes for 10 000 g for 10 minutes. After centrifugation the tubes were uncapped and the supernatants discarded. Any remaining fluid was removed by firm tapping of the inverted tubes onto a paper towel. Visual inspection revealed a red residue within the elastin standard tubes and of course also in the test sample tubes.

### 2.3.7. Release of the Elastin Bound Dye

To each tube 500  $\mu$ L of *Dye Dissociation Reagent* were added. The tubes were capped and the elastin-bound dye was brought into solution using a vortex mixer.

### 2.3.8. Elastin Measurement

The elastin content of the assayed samples is determined by the amount of bound dye released from the elastin. As the absorbance peak of TPPS in the *Dye Dissociation Reagent* occurs at 513 nm, a spectrophotometer was used by setting wavelength to 513 nm and using the blank for adjusting absorbance to zero. The solutions of 12.5, 25 and 50  $\mu$ L of the Elastin Standard were used to produce a standard curve, by plotting the elastin standard values: vertical axis "Absorbance at 513 nm" and horizontal axis "Elastin concentration ( $\mu$ g/mL)". The concentration of elastin present in the test samples was determined by the absorbance value obtained and read from the standard curve.

## 2.4. Determination of Total Soluble Protein

The determination of the total protein was based on Peterson's modification (1977) of the Lowry method (1951), according to the *Protein Assay Kit* purchased by Sigma<sup>®</sup> (St. Louis, MO, USA).

## 2.5. Statistical Analysis

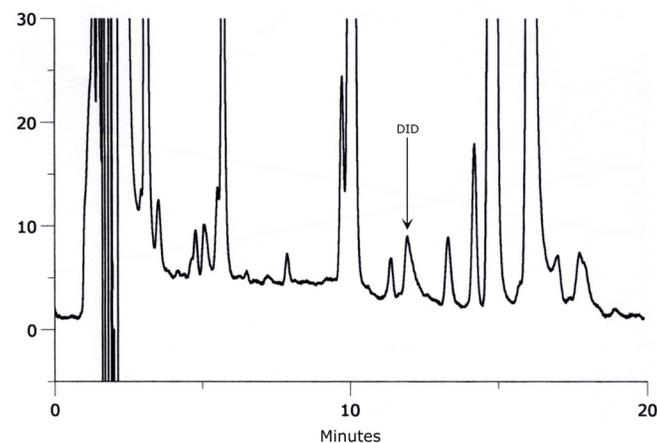
The comparison between the results in both periods (incubation and after hatching) was studied using Mann-Whitney test, both for the HPLC method and for the colorimetric method. The evaluation of any correlation between both methods was carried out by applying the Spearman correlation coefficient.

## 3. RESULTS

### 3.1. Quantification of Desmosine and Isodesmosine by HPLC

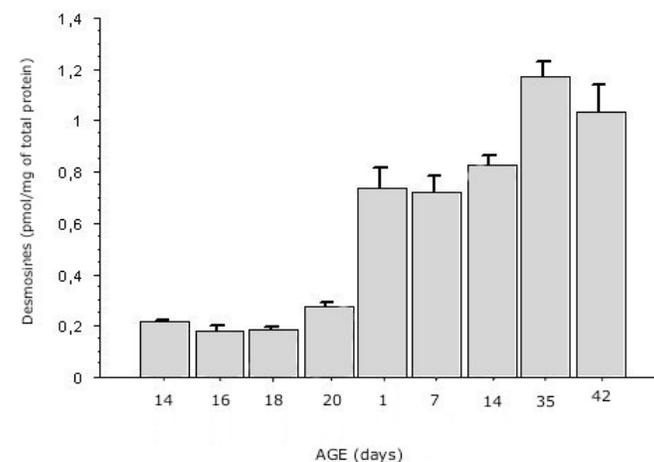
Through the methodology of high-performance liquid chromatography (HPLC), we were able to quantify both desmosine and isodesmosine in the chick lungs from the 14<sup>th</sup>

day of incubation until the 42<sup>nd</sup> day after hatching. With the chromatographic conditions described in "Materials and Methodology", the retention time was observed at 11.8 minutes, with desmosine and isodesmosine (DID) detected as a single signal (Fig. 1). A calibration curve was constructed by analysis of standard solutions containing scalar amounts of desmosines from 100 to 500 pmol/mL ( $r^2 = 0.9935$ ).



**Fig. (1).** Chromatogram representing the separation of DID by HPLC.

The quantification of desmosine and isodesmosine is shown in Fig. (2). The values are expressed in pmols of



**Fig. (2).** Quantification by HPLC of both desmosines concentrations (per mg of total protein) in chick lungs from the 14<sup>th</sup> day of incubation until the 42<sup>nd</sup> day after hatching. Data for each day is the mean  $\pm$  SE value of 3 consecutive determinations ( $n = 12$ ), as shown in Table 1.

desmosine and isodesmosine per mg of total protein. By the graph we can notice that along incubation period the values

**Table 1. Results Obtained for Desmosines Concentrations (Per mg of Total Protein) in Chick Lungs from the 14<sup>th</sup> Day of Incubation Until the 42<sup>nd</sup> Day After Hatching**

Incubation				After-Hatching				
14 <sup>th</sup> Day	16 <sup>th</sup> Day	18 <sup>th</sup> Day	20 <sup>th</sup> Day	1 Day	7 Days	14 Days	35 Days	42 Days
0,218 (0,001)	0,182 (0,015)	0,188 (0,006)	0,277 (0,009)	0,735 (0,078)	0,719 (0,059)	0,825 (0,033)	1,223 (0,007)	1,032 (0,057)

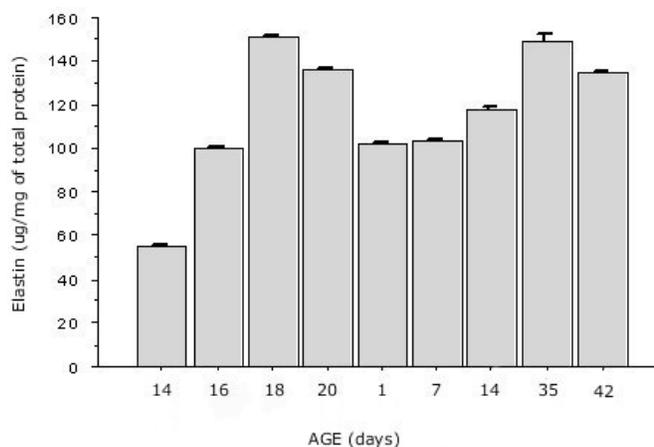
Data for each day is the mean  $\pm$  SE value of 3 consecutive determinations ( $n = 12$ ).

for desmosines are very low if we will compare them with those obtained for after-hatching period. So we decided to consider those two periods separately.

The comparison test revealed significant differences ( $p < 0.001$ ) between values of both analyzed periods (incubation and after-hatching). If we will consider the difference between each analyzed day, we will find  $p = 0.020$  (statistically significant) for the 14<sup>th</sup> and the 16<sup>th</sup> day of incubation as well as for the 14<sup>th</sup> and the 35<sup>th</sup> after-hatching. Between the 18<sup>th</sup> and the 20<sup>th</sup> day of incubation as well as between the 35<sup>th</sup> and the 42<sup>nd</sup> day after-hatching the difference is only tendentially significant ( $p = 0.050$ ).

### 3.2. Quantification of Elastin by a Colorimetric Method (“Fastin™ Elastin Assay”)

With this method we can quantify elastin under several forms from tropoelastin until insoluble elastin since this will be transformed into solubilized polypeptides. A calibration curve was constructed by analysis of standard solutions containing scalar amounts of  $\alpha$ -elastin in oxalic acid 0.25M from 25 to 100  $\mu\text{g/mL}$  ( $r^2 = 0.9998$ ). The quantification of elastin was shown in Fig. (3). The values were expressed in  $\mu\text{g}$  of elastin per mg of total protein. With this method the results show a profile different from that obtained by HPLC. In fact, in the incubation period we observe now a clear increase between the 14<sup>th</sup> and the 18<sup>th</sup> day, with a slight decline until hatching, followed by a new increase that will remain stationary at adult age. When applying the comparison test over the



**Fig. (3).** Quantification elastin concentrations (per mg of total protein) in chick lungs from the 14<sup>th</sup> day of incubation until the 42<sup>nd</sup> day after hatching. Data for each day is the mean  $\pm$  SE value of 3 consecutive determinations ( $n = 12$ ), as shown in Table 2.

results obtained by this method, we can remark that with colorimetric method the results for the incubation period and for

the after-hatching period present very similar values ( $p = 0.581$ ). By relating each analyzed day there is no statistically significant difference between any of the incubation days. In the after-hatching period the unique difference with statistical significance is between the 14<sup>th</sup> and the 35<sup>th</sup> days ( $p = 0.020$ ).

## 4. DISCUSSION AND CONCLUSIONS

Unlike mammalian lungs, avian lungs are relatively rigid, due to the absence of a functional diaphragm, and require the action of respiratory muscles to move air through the lungs [9]. The contraction of inspiratory muscles forces air to enter the larynx and ultimately to pass through the lungs into thin-walled air sacs that emanate from various bronchi and fill most of the body cavity not occupied by other viscera. This cycle reverses when the expiratory muscles contract. During both inspiration and expiration, air moves unidirectionally, assuring that stale air doesn't accumulate or mix with fresh air. In addition, air flows through the tertiary bronchi at right angles to the flow of blood in the blood capillaries. This arrangement can be described as a cross-current exchange system and is inherently more efficient than the uniform pool system that exists in the mammalian lung [9].

The avian lung acquires its rigid structure already during embryonic development in the egg by its fusion with the walls of the pleural cavity. During embryonic lung development not only the number of all secondary bronchi and their side branches is fixed, but also the exact topographical position, and also the number and exact course of all parabronchi [16]. This means that an important developmental peculiarity of the lung-air sac system of birds is that at hatching (i.e., at the end of the embryonic life), it is practically mature while in the mammalian lung, intensive growth of the terminal airways and important structural changes occur postnatally [17]. So, we may presume that the functional structure of birds' respiratory system will be concluded at the hatching, and consequently its elastic system will be in late maturation.

The aim of this work was to quantify elastin in the domestic fowl lung, by using two kinds of methods. The purpose was to contribute for a better understanding of what will be the best approach for quantifying elastin. So we used two different methods to quantify the elastin contents in the domestic fowl lung, since the incubation period until the adult age. On the one hand we determined the desmosine and isodesmosine proportions in all the samples, by using HPLC. With this procedure we could know the mature elastin contents seeing that we have quantified the amino acids responsible for the cross-linking in the mature elastin. In spite of the precision degree of this method, it has been pointed as showing some drawbacks. In fact fairly large amounts of sample are needed just because desmosine and isodesmosine are the meanest portion of the total residues of mature elastin; in some species lung elastic fibres, the desmosines contents remains below 0.2 % of total

**Table 2.** Results Obtained for Elastin Concentrations (Per mg of Total Protein) in Chick Lungs from the 14<sup>th</sup> Day of Incubation Until the 42<sup>nd</sup> Day After Hatching

Incubation				After-Hatching				
14 <sup>th</sup> Day	16 <sup>th</sup> Day	18 <sup>th</sup> Day	20 <sup>th</sup> Day	1 Day	7 Days	14 Days	35 Days	42 Days
55,417 (0,095)	100,154 (0,134)	151,208 (0,045)	135,650 (0,133)	102,253 (0,061)	103,377 (0,030)	117,717 (0,217)	149,431 (2,355)	134,683 (0,071)

Data for each day is the mean  $\pm$  SE value of 3 consecutive determinations ( $n = 12$ ).

mature elastin. Besides, as the cross-linking assembly process is very slow, their values are very low in developing organs because in this case the elastic fibres are still in formation and maturation process [18-20].

On the other hand we used a method that determines both insoluble elastin that must be made soluble by hot oxalic acid and soluble elastin ( $\alpha$ -elastin) before evolution forward an effective maturation condition. In this method it is used a dye reagent containing a synthetic porphrine TPPS. This molecule 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 [21].

By analyzing the results obtained through both methods, we observed first of all that both analyzed periods (incubation and after-hatching) behaviour differently according to the applied methodology. So we studied them separately, concerning each one of the methods. During the after-hatching period there is a positive and significant correlation between both methods ( $p < 0.001$ ), while this is not observed in the incubation period ( $p = 0.618$ ).

In fact, both methods detect different moments of the elastin development itself. While with HPLC we determine the desmosines that are evident only in a mature stage of elastin, with the colorimetric method we can determine several elastin stages since insoluble elastin, soluble polypeptides ( $\alpha$ -elastin) and even tropoelastin. In a premature stage of elastic fibres formation it is possible to detect all the elastin shapes if we use the "Fastin™ Elastin Assay". Otherwise if we use HPLC we can detect only the mature elastin content.

The amino acid composition is significantly different between soluble and mature elastin, concerning chiefly the cross links. Considering the cross-linking amino acid residues per total 850 amino acid residues of elastin, we can find 25-30 residues expressed as lysine equivalents of cross links in mature elastin and a very low amount in soluble elastin [22]. Tropoelastin has the amino acid composition of the  $\alpha$ -elastin, lacking the cross links desmosine and isodesmosine; instead, it has high lysine content. Lysine is the precursor of the desmosines cross links [23, 24]. After all this we may conclude that both studied methods are not alternative; none can substitute the other. Meanwhile they turn out to be complementary according to the aim of the study to be done.

## ABBREVIATIONS

DID	=	Desmosine and isodesmosine
DNS-Cl	=	Dansyl chloride
HPLC	=	High-performance liquid chromatography
TCA	=	Trichloroacetic acid
TPPS	=	5,10,15,20-Tetraphenyl-21,23-porphrine

## REFERENCES

- [1] Gonçalves, C.; Barros, J.; Honório, A.; Rodrigues, P.; Bairos, V. Quantification of elastin from the mouse lung during postnatal development. *Exp. Lung Res.*, **2001**, *27*, 533.
- [2] Jones, J.H.; Effmann, E.L.; Schmidt-Nielsen, K. Lung volume changes during respiration in ducks. *Respir. Physiol.*, **1985**, *59*, 15.
- [3] Scheid, P. Mechanisms of gas exchange in bird lungs. *Rev. Physiol. Biochem. Pharmacol.*, **1979**, *86*, 137.
- [4] Scheid, P.; Piiper, J. Analysis of gas exchange in the avian lung: theory and experiments in the domestic fowl. *Respir. Physiol.*, **1970**, *9*, 246.
- [5] Scheid, P.; Piiper, J. Cross-current gas exchange in avian lungs: effects of reversed parabronchial air flow in ducks. *Respir. Physiol.*, **1972**, *16*, 304.
- [6] Maina, J.N. What it takes to fly: the structural and functional respiratory refinements in birds and bats. *J. Exp. Biol.*, **2000**, *203*, 3045.
- [7] Jones, A.W.; Barson, A.J. Elastogenesis in the developing chick lung: a light and electron microscopical study. *J. Anat.*, **1971**, *110*, 1.
- [8] Batzan, T.; Klika, E. Development of the elastic fibres of chick embryo lungs. *Folia Morphol.*, **1983**, *31*, 94.
- [9] James, M.F.; Rich, C.B.; Trinkaus-Randall, V.; Rosenbloom, J.; Foster, J.A. Elastogenesis in the developing chick lung is transcriptionally regulated. *Dev. Dyn.*, **1998**, *213*, 170.
- [10] Gonçalves, C.A.; Figueiredo, M.H.; Bairos, V.A. Three-dimensional organization of the elastic fibres in the rat lung. *Anat. Rec.*, **1995**, *243*, 63.
- [11] Rodrigues, P.; Gonçalves, C.; Honório, A.; Barros, J.; Bairos, V. Quantification of mouse lung elastin during prenatal development. *Open Resp. Med. J.*, **2008**, *2*, 46.
- [12] Pinto, M.L.; Gonçalves, C.; Rodrigues, P.; Bairos, V.A. Quantification by image analysis of the gallus gallus lung elastic fibres from embryonic to adult birds. *Anat. Histol. Embryol.*, **2006**, *35*, 293.
- [13] Fitch, S.M.; Harkness, M.L.R.; Harkness, R.D. Extraction of collagen from tissues. *Nature*, **1955**, *176*, 163.
- [14] Skinner, S.J.M. Rapid method for the purification of the elastin cross-links, desmosine and isodesmosine. *J. Chromatogr.*, **1982**, *229*, 200.
- [15] Guida, E.; Godini, M.; Palmerini, C.A.; Fini, C.; Lucarelli, C.; Floridi, A. Development and validation of a high-performance liquid chromatographic method for the determination of desmosines in tissues. *J. Chromatogr.*, **1990**, *507*, 51.
- [16] Duncker, H.-R. Vertebrate lungs: structure, topography and mechanics. A comparative perspective of the progressive integration of respiratory system, locomotor apparatus and ontogenetic development. *Respir. Physiol. Neurobiol.*, **2004**, *144*, 111.
- [17] Maina, J.N. Developmental dynamics of the bronchial (airway) and air sac systems of the avian respiratory system from day 3 to day 26 of life: a scanning electron microscopic study of the domestic fowl, Gallus gallus variant domesticus. *Anat. Embryol.*, **2003**, *207*, 119.
- [18] Partridge, S.M.; Elsdon, D.F.; Thomas, J. Biosynthesis of the desmosine and isodesmosine cross-bridges in elastin. *Biochem. J.*, **1964**, *93*, 30C.
- [19] Partridge, S.M.; Elsdon, D.F.; Thomas, J.; Dofman, A.; Telsner, A.; Ho, P.-L. Incorporation of labelled lysine into the desmosine cross-bridges in elastin. *Nature*, **1966**, *209*, 399.
- [20] Campagnone, R.; Regan, J.; Rich, C.B.; Miller, M.; Keene, D.R.; Sakai, L.; Foster, J.A. Pulmonary fibroblasts: a model system for studying elastin synthesis. *Lab. Invest.*, **1987**, *56*, 224.
- [21] *Fastin™ Elastin Assay*, Biocolor® - Biodye Science: 5<sup>th</sup> ed.; Newtownabbey, Northern Ireland, **2006**.
- [22] Gray, W.R.; Sandberg, L.B.; Foster, J.A. Molecular model for elastin structure and function. *Nature*, **1973**, *246*, 461.
- [23] Tamawski, R.; Kasperczyk, J.; Drózd, M. Carbon-13 NMR studies of alpha-elastin. *Uppsala J. Med. Sci.*, **1993**, *98*, 53.
- [24] Chung, M.I.S.; Miao, M.; Stahl, R.J.; Chan, E.; Parkinson, J.; Keely, F.W. Sequences and domain structures of mammalian, avian, amphibian and teleost tropoelastins: Clues to the evolutionary history of elastins. *Matrix Biol.*, **2006**, *25*, 492.