INVALID IMAGE ANALYSIS AND INAPPROPRIATE STATISTICAL ANALYSIS INDUCE MISLEADING RESULTS OF TYPE I AND III COLLAGEN CONTENTS WITHIN MUSCLES.

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Background

The tenderness of cooked meat is influenced by many factors including the quantity and solubility of muscle collagen (the major component of muscular connective tissue). The solubility of collagen is determined by several parameters including the types of collagen present in intramuscular connective tissue. In mammalian muscle, several collagen types have been detected, the two major types being types I and III. Although Bailey et al. (1979) have shown that tough muscles have higher proportions of type III collagen, other authors have reported opposite results (Burson and Hunt, 1986) and others have found no relationship between toughness and type III collagen content (Light, 1987). However, it is of paramount importance to estimate type I and III collagen contents properly to establish their putative relationship with meat quality. Several methods may be used for such a purpose. For muscles with collagen composed of 90% type I and III collagen, CNBr digestion followed by separation of CNBr peptides on SDS-PAGE was considered as the best method (Light, 1982).

Objectives

By using the above method, we noticed that results were not so reproducible and highly variable. Our objective was thus to develop appropriate procedures of image analysis and statistics, crucial to ensure valid results.

Material and Methods

Animals and samples

Two groups of Salers (n = 12) and Holstein (n = 12) cull cows (non pregnant and non lactating) of similar age, conditions of breeding and fatness, were used. The animals were slaughtered at the INRA slaughter-house in compliance with the current ethical guidelines for animal welfare. *Semimembranosus* (SM) muscles were excised from the carcass immediately after slaughter. The rest of the sample was cut into pieces (1 cm cross-section), vacuum-sealed, and stored at -20°C. The frozen meat was homogenised in a household cutter, lyophilised for 48 h, then pulverised in a horizontal blade mill and stored at 4°C in stoppered plastic flasks until analysed.

Digestion of collagen samples with cyanogen bromide (CNBr)

One ml of 70% formic acid was added to 25 mg of the powdered dried tissue. Nitrogen gas was then bubbled through the homogenate and 40 µl of a 1 g/ml solution of cyanogen bromide was added. The reaction was allowed to proceed for 4 h at 30°C under agitation and the solution was then dried under vacuum at 40°C in a Speed-Vac.

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gels electrophoresis (SDS-PAGE) were performed on plates of 160*160*1.5 mm. The resolving gel was a 12% polyacrylamide gel and the stacking gel a 3.5% polyacrylamide gel. The dry pellet (8 µg of total hydroxyproline) obtained after CNBr digestion was dissolved under agitation for 30 min at room temperature, then 10 min at 100°C, within 56 µl of a solution containing SDS, 1M Tris (pH 6.8), glycerol, pyronin Y and β-mercaptoethanol. Migration was performed at constant amperage, at a temperature of 4°C. For uniform penetration of the proteins in the stacking gel, migration was started at 15 mA for 30 min and then continued for 40 mA for 4 h. Proteins were stained in a 0.2% solution of R250 Coomassie Blue.

Quantification of type I and III collagen contents in muscle samples

Image analysis was performed on wet gels using the ChemiImager 5000 and the AlphaEase FluorChem software (Alpha Innotech Corporation, San Leandro, California, U.S.A.). All the proteins from the top to the bottom of the gel were quantified together with a background line drawn from valley to valley between the peaks. α1(I)CB8 peptide from type I collagen and α1(III)CB5 peptide from type III collagen were chosen for quantification as none of these peptides is involved in collagen cross-linking (for review, see Bailey & Light, 1989). Their positions were established on polyacrylamide gel using purified type I and III collagen standards (5 µg) from Chemicon (CA, U.S.A.). Type I and type III collagen contents were determined by quantifying these two specific peptides within the same analysis and with the same background line as for total protein.

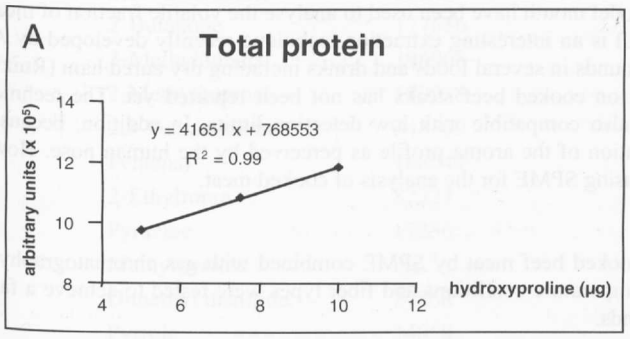
Results and discussion

The means and standard deviations of the amounts of total protein, type I and type III collagens analysed in three different experiments were calculated for 24 different samples. The coefficients of variation were high : 34%, 27% and 36% for total protein content, type I and type III collagen contents respectively. This means that the reproducibility of the analysis was low. Densitometric values for total proteins of the same samples obtained in two different experiments were plotted. No correlations between the results of the two different experiments were observed although similar amounts of hydroxyproline were loaded in each lane. This again underlines the low reproducibility of the analysis. Several hypothesis may explain this discordance : (i) heterogeneity of the samples, (ii) low accuracy in the loading sample, (iii) high variability among samples in the penetration of proteins within the gel, (iv) high variability among samples in dying proteins, or (v) low reproducibility in the image analysis procedure between samples. Whatever the reason, we concluded from these observations that the contents of type I collagen or type III collagen must be corrected for the total protein content determined on each lane and within the same experimental procedure (from electrophoresis to image analysis). Additional experiments were performed to choose the most appropriate calculation for this correction.

When we loaded increasing amounts of total hydoxyproline of the same sample on the gel, increasing densitometric values of total proteins (Fig. 1A) and type I and type III collagen contents were observed (Fig. 1B) and the relationships between the amount of the loaded samples and the results were high (r^2 = 0.95 to 0.99). This demonstrated the linearity of the method for the quantification of total protein or of the two collagen types. This also indicated that densitometric values for type I and type III collagens were linearly proportional to total protein

content quantified on the same lanes during the same procedure. Therefore, type I and III collagen contents can be indeed corrected for variations in total protein quantifiable on the gels.

However, the intercept of the regression line with the y-axis was different from zero (Fig. 1A and 1B). Consequently, the relationship between collagen type I or type III (y) and the amount of loaded proteins (x) follows the equation : $y = ax + b$ with b being equal to the y-intercept of the linear slope which is different from zero. In other words, it is impossible to simply divide values for type I and type III collagens by total protein values for correction purposes. A regression method should be preferred. In practice, analysis of type I and III collagens with total protein amount as covariable is the best way to achieve this goal (Hocquette and Brandstetter, 2002).



To demonstrate these theoretical considerations by practical experiments, *semimembranosus* samples from two groups of 12 culled cows from two cattle breeds (Holstein and Salers) were analysed in three different experiments. A significant difference was detected between the three experiments for the quantification of total protein ($P < 0.0001$), but the effect was greater for Holstein cows than for Salers cows as revealed by a significant interaction between breed and experiment effects ($P < 0.0001$). The ratios of type I and type III contents to total hydroxyproline content were analysed by the same model of variance analysis. For both ratios, the experiment effect was significant as well as the interaction between experiment and breed effects as for total protein content. This significant interaction indicates that the differences between breeds in these two ratios is either in favour of Salers or in favour of Holstein depending on the experiment. Thus, the reproducibility of the analysis was low.

As indicated above, the last model of variance analysis tested was the introduction of total protein content as a covariable. When type I and type III collagen contents were analysed by this model, the interaction between the breed and the experiment effects was not more significant, but the covariable effect was highly significant ($P < 0.0001$). We conclude that the covariable effect explained a great part of the variability of type I and type III collagen contents, especially the breed x experiment interaction effect in the previous analysis. As a consequence, the breed effect was reproducible and significant for type III collagen content (Table I) taking into account variability between experiments.

Table I. Variance analysis of the variables (X) by the following model : X = mean + total protein effect + Breed Effect + Animal Effect + Experiment Effect + Breed x Experiment Interaction

	Effects of (P <)				
Variables	**Total protein**	**Breed**	**Animal**	**Experiment**	**Breed x Experiment**
Type I collagen content	**0.0001**	0.40	0.10	**0.005**	0.19
Type III collagen content	**0.0001**	**0.03**	0.51	**0.01**	0.60

In conclusion, we have demonstrated a great variability in crude values of type I and type III collagen contents in muscle samples and a low reproducibility of type I and type III collagen analysis by electrophoresis. Thus, a normalisation of the results is required for an accurate quantification of type I and type III collagen contents within muscle samples. The results indicate that the variability in total protein contents detected and quantified on the gels is very high. Thus, using this parameter for data-normalization appeared to be a key point in data analysis. Indeed, no data-normalization induces a methodological bias. However, dividing crude results of type I and type III collagen contents by total protein content induces a statistical bias. The recommended procedure is to use total protein content as a covariable in the model. Otherwise, misleading biological conclusions may be provided regarding type I and type III collagen amounts within muscle samples. Similar results were obtained in other experiments with different muscle type.

Pertinent literature

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