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Searching for protein biomarkers related to pre-slaughter stress using liquid isoelectric focusing

C. Fuente-García[1,2], N. Aldai[1], E. Sentandreu[2], M. Oliván[3], F. Díaz[3] and M.A. Sentandreu[2]

[1]University of the Basque Country (UPV-EHU), Lactiker Research Group, Department of Pharmacy and Food Sciences, Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain, [2]Institute of Agrochemistry and Food Technology (IATA-CSIC), Carrer Catedràtic Agustín Escardino Benlloch 7, 46980 Paterna (Valencia), Spain, [3]Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Apdo 13, 33300 Villaviciosa (Asturias), Spain; claudia.fuente@ehu.eus

Proteome changes derived from animals that have suffered pre-slaughter stress are a fact although the discovery of associated biomarkers is still a challenge. In this study, Proteomic analysis was carried out on 16 loin samples of beef from Asturiana de los Valles breed and crossbreds animals previously classified as normal and DFD meat at 24 h post-mortem using pH measurements. Sarcoplasmic sub-proteome of *Longissimus thoracis* muscle was fractionated by the use of liquid isoelectric focusing (OFFGEL) in the pH range of 3 to 10, followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of each retrieved fraction. The obtained protein separation profile showed high reproducibility along the different samples. Five different bands showed significant statistical differences ($P<0.01$) in both sample groups, which allowed to compare and distinguish normal and DFD meat. Some proteins present in these bands, which were identified by liquid chromatography coupled to tandem mass spectrometry, were phosphoglucomutase-1 and alpha-crystallin B. The significance of this study relies on the optimization of the OFFGEL technology. This method separates proteins along different fractions according to their isoelectric point; then the obtained fractions can be further separated by SDS-PAGE. This achievement stands out as an alternative to the use of two dimensional gel electrophoresis, enabling a higher resolution in protein separation and shorter analysis times.

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European Federation of Animal Science

SEARCHING FOR PROTEIN BIOMARKERS RELATED TO PRE-SLAUGHTER STRESS USING LIQUID ISOELECTRIC FOCUSING

C. Fuente-García, N. Aldai, E. Sentandreu, M. Oliván, F. Díaz, M.A. Sentandreu

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Universidad del País Vasco
Euskal Herriko Unibertsitatea

Lactiker

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Instituto de Agroquímica y Tecnología de Alimentos

SERIDA

Background

MEAT QUALITY

"Muscle to meat" molecular events and technological transformations: The proteomics insight ☆

Gianluca Paredi[a,b], Samanta Raboni[a,b], Emøke Bendixen[d,e], André M. de Almeida[f,g], Andrea Mozzarelli[a,b,c,]*

Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved

Ahmed Ouali [a], Mohammed Gagaoua [a, b], Yasmine Boudida [b], Samira Becila [b], Abdelghani Boudjellal [b], Carlos H. Herrera-Mendez [a], Miguel A. Sentandreu [c]

What about pre-slaughter stress?

Tackling proteome changes in the *longissimus thoracis* bovine muscle in response to pre-slaughter stress

Daniel Franco[a], Ariadna Mato[b], Francisco J. Salgado[c], María López-Pedrouso[b], Mónica Carrera[d], Susana Bravo[e], María Parrado[b], José M. Gallardo[d], Carlos Zapata[b,]*

Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants?

D.M. Ferguson [a,], R.D. Warner [b]*

Background

Extrinsic

Intrinsic

Sex, age, genetics, physiology

Handling activities, human presence, water deprivation

Pre-slaughter stress

Changes in meat quality

Normal Animal

Under anoxic conditions after slaughter

glycolysis

Glycogen Glucose → Lactic acid → pH drop

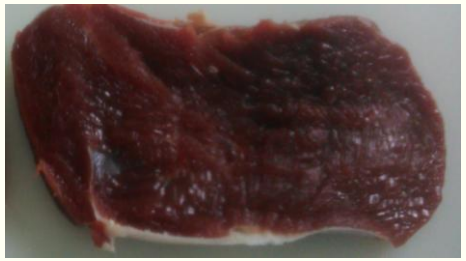
ADP → ATP + Pi

Normal

Stressed Animal

pH (>6.0)

Glycogen Glucose



Dry, Firm, Dark

Objective

Study of sarcoplasmic sub-proteome from pre-slaughter stressed animals in comparison to normal animals using liquid isoelectric focusing (OFFGEL) in order to elucidate which proteins and biochemical pathways could be involved in pre-slaughter stress (PSS)

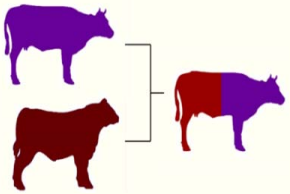
Materials and methods

Asturiana de los Valles breed

n=8

Crossbred

n=8

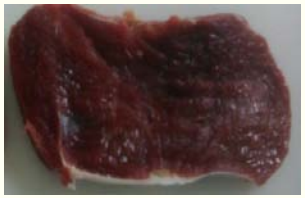


pH measurement

Normal

(pH<6)

n=10



DFD

(pH>6)

n=6

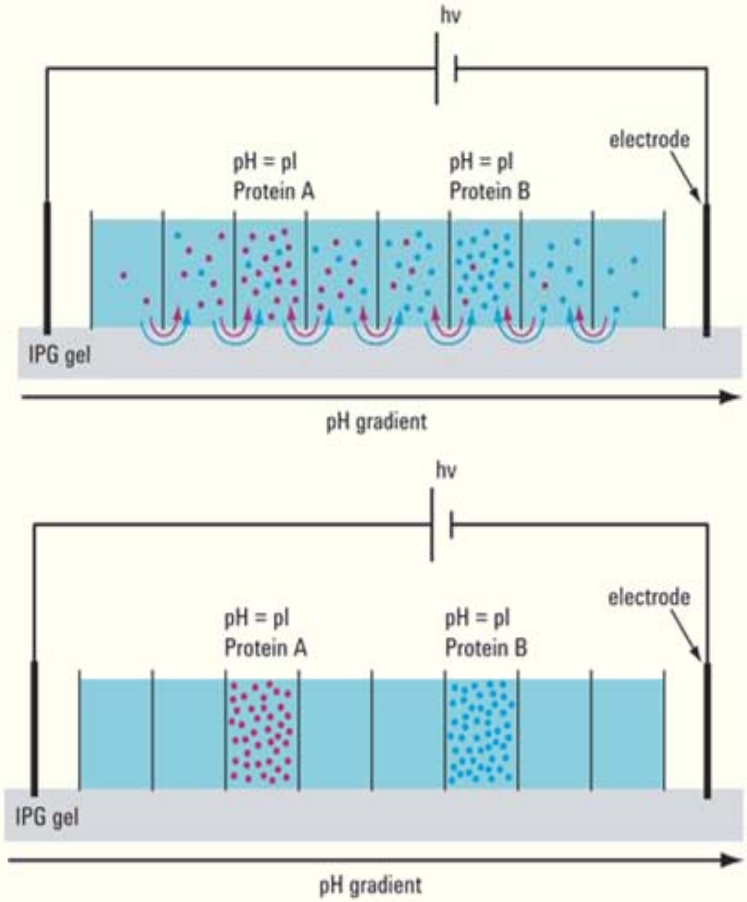
Proteome analysis

- ✓ Extraction
- ✓ Fractionation
- ✓ Quantification
- ✓ Identification

Materials and methods

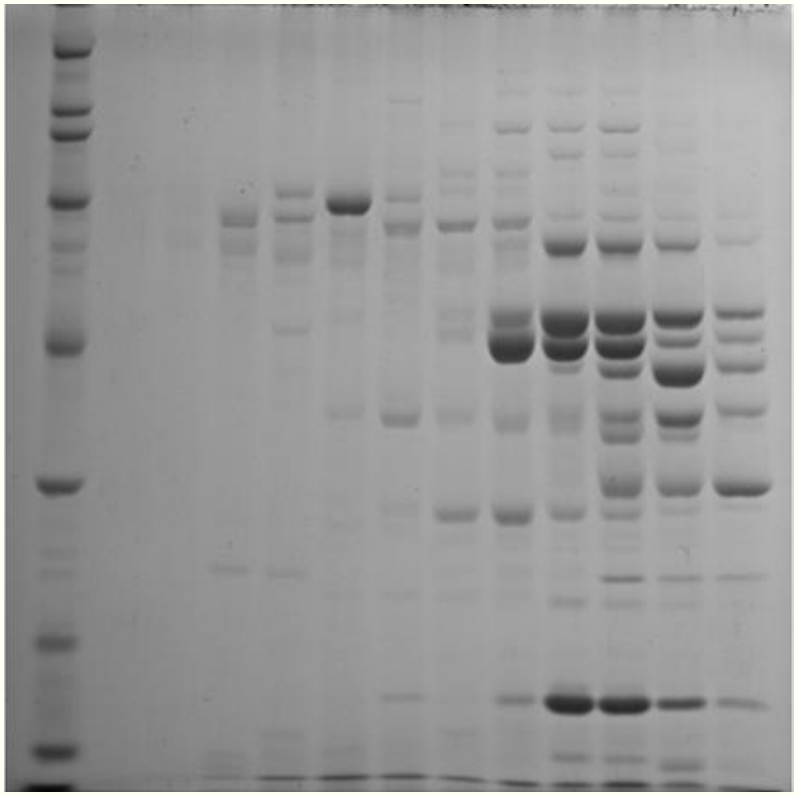
1 OFFGEL technology

✓Separation through their p*I*

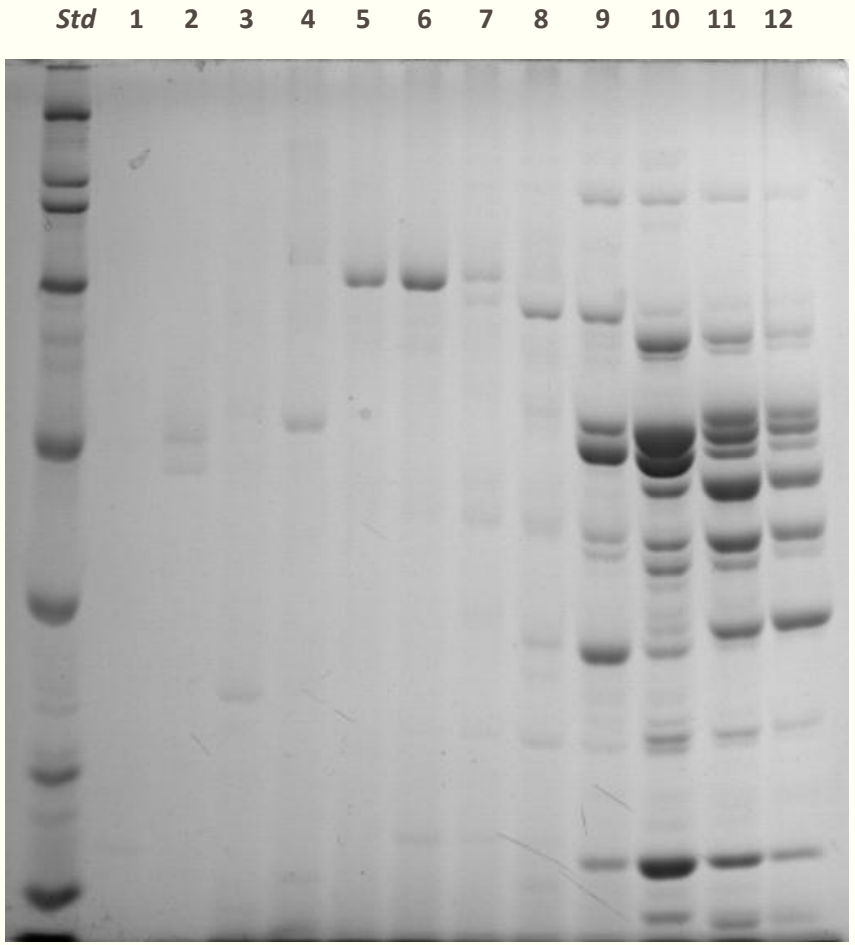


2 SDS-PAGE

✓Separation through their Mr



Materials and methods



SDS-PAGE

OFFGEL plus SDS-PAGE

Materials and methods

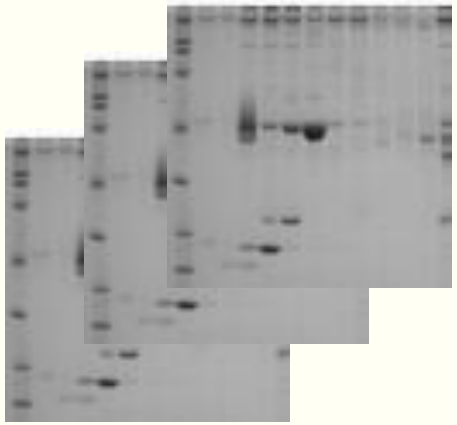
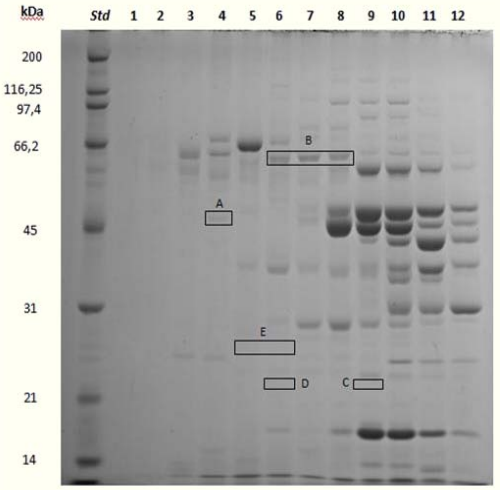


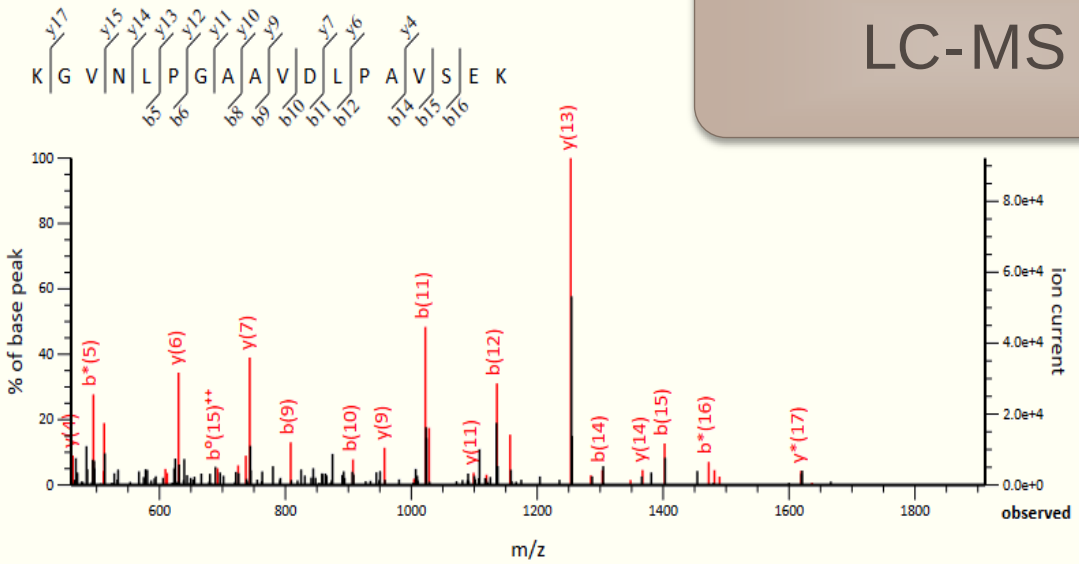
Image Analysis

Statistical Analysis



Protein Selection

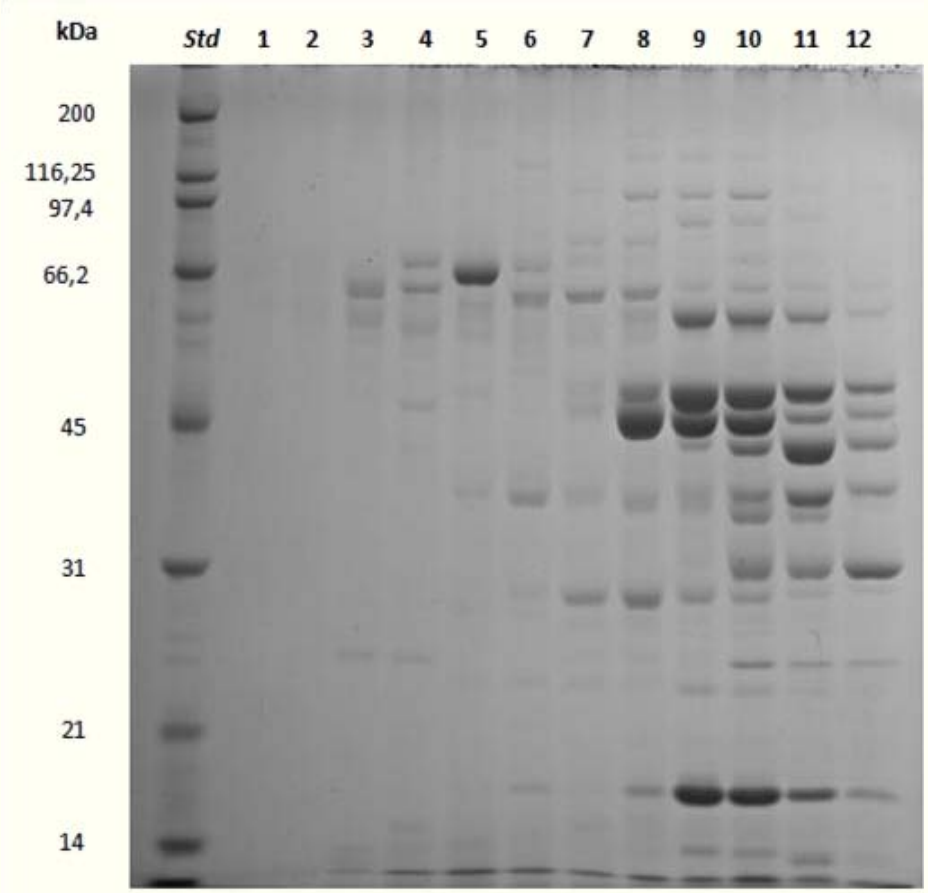
LC-MS



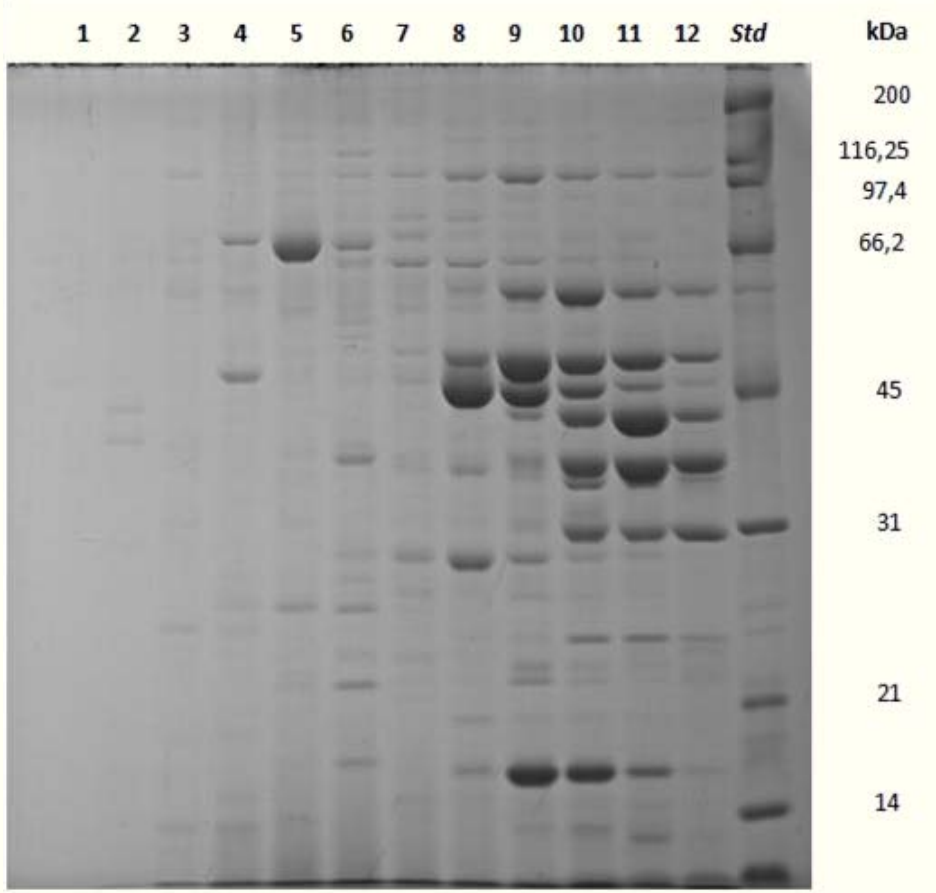
Protein Identification

Results

Sarcoplasmic Proteome



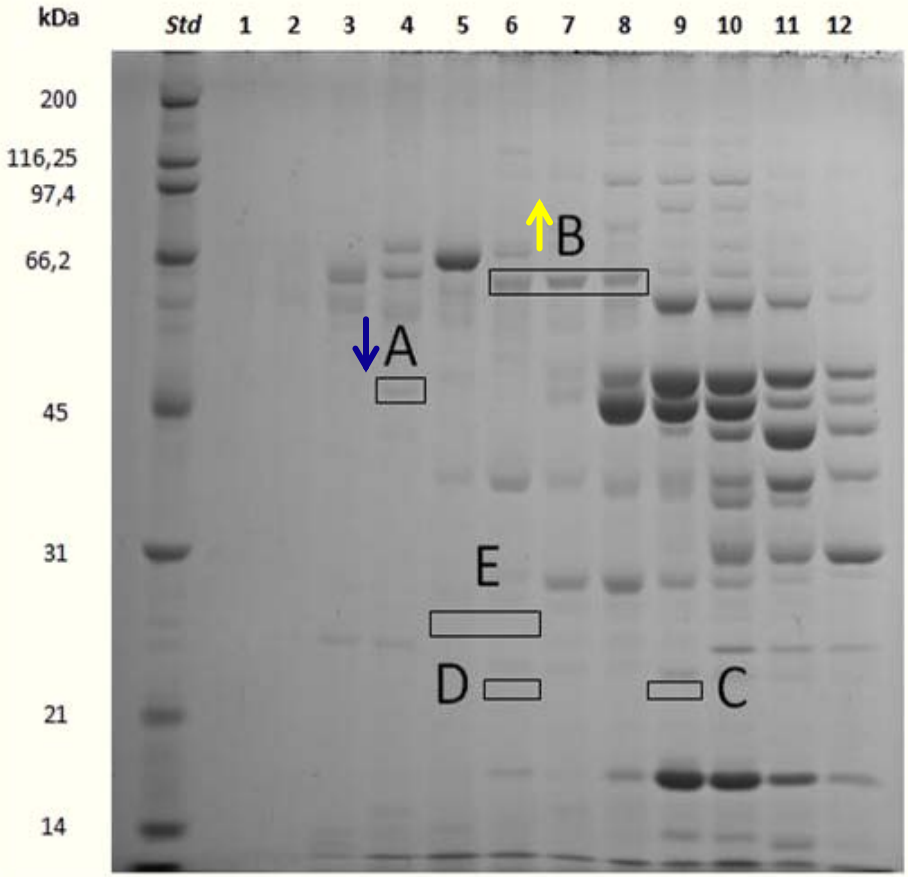
a) NORMAL



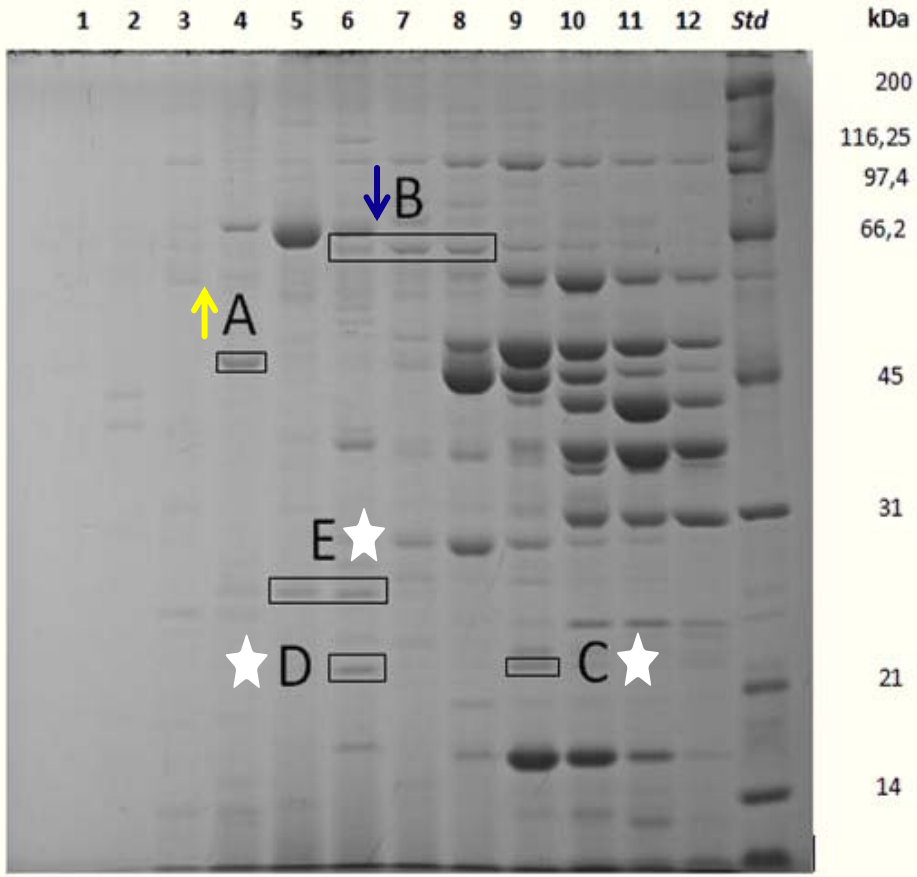
b) DFD

Results

Sarcoplasmic Proteome

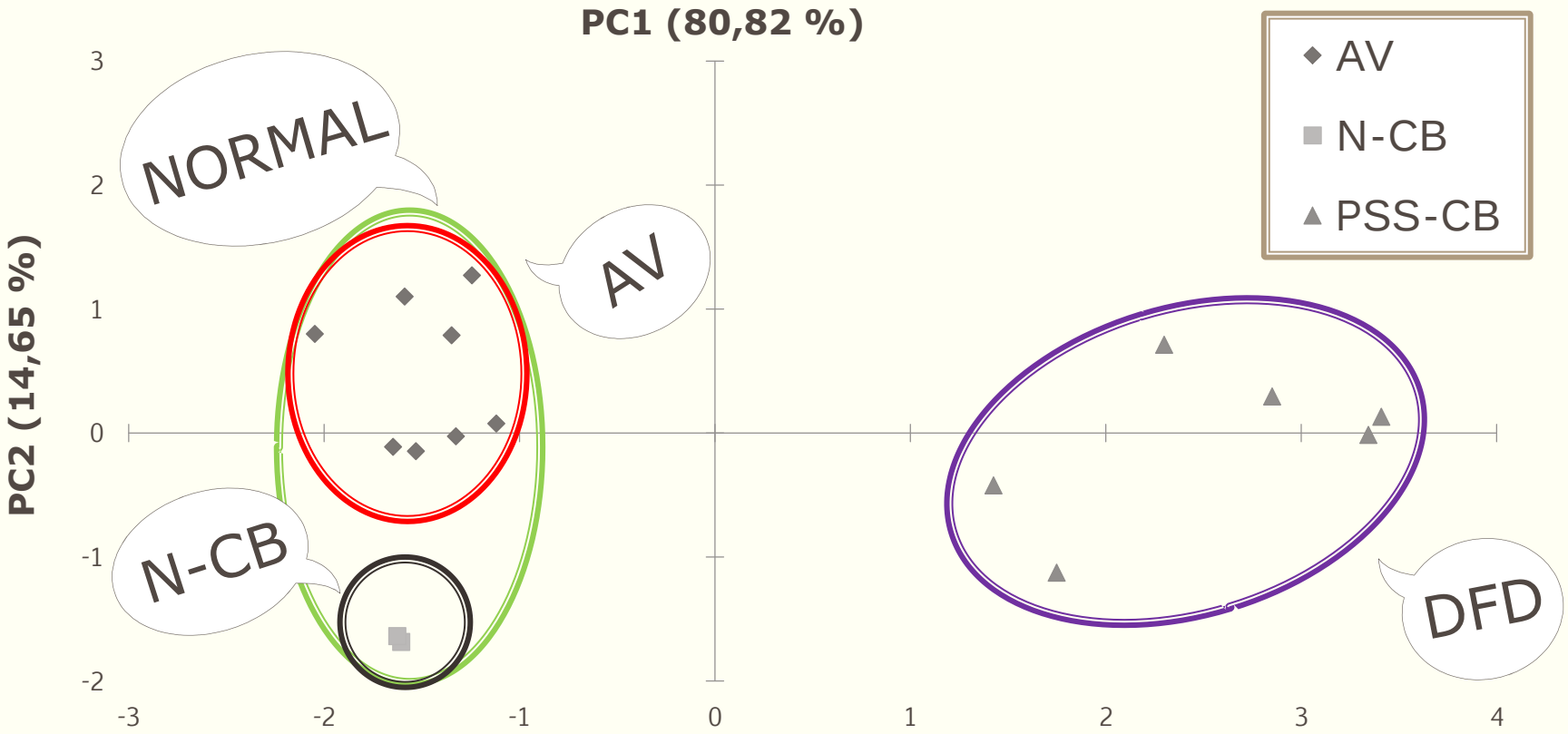


a) NORMAL



b) DFD

Principal component analysis (PCA)

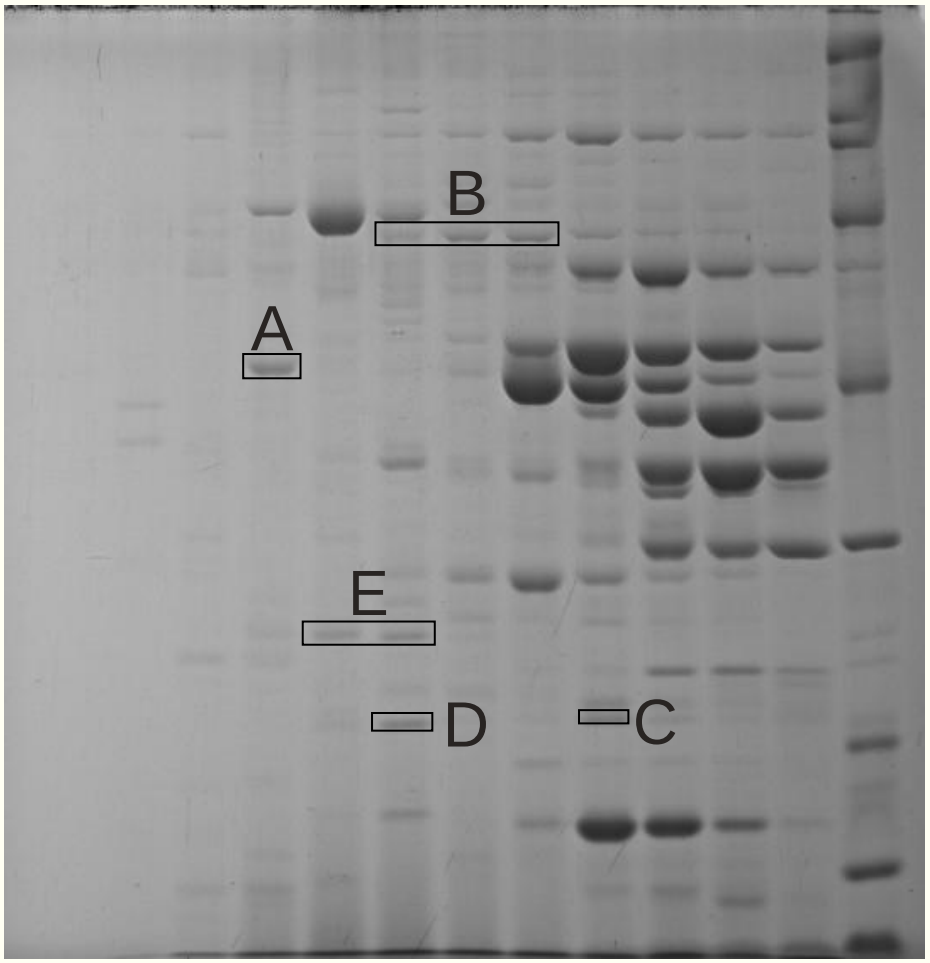


Animal sample distribution on the PCA. Each point represents an individual sample (AV: Asturiana de los Valles animals; N-CB: normal crossbred animals; PSS-CB: pre-slaughter stressed crossbred animals).

PC1: Separation between normal (AV and N-CB) and DFD (PSS-CB) groups

PC2: discriminate between AV and N-CB samples showing higher values for AV

Indentified proteins



b) DFD

Band **A**
Actin

Band **B**
Phosphoglucomutase-1

Band **C**
Alpha-crystallin B

Band **D**
Heat shock protein beta-6

Band **E**
Heat shock protein beta-1

Identified proteins

Band	Protein Identification	Biological function
A	Actin	Structural maintenance
B	Phosphoglucomutase-1	Regulation of glycogen metabolism
C	**Alpha-crystallin B**	Stress resistance
D	**Heat shock protein beta-6**	Stress resistance
E	**Heat shock protein beta-1**	Stress resistance and actin organization

Only appeared in DFD samples

$p < 0.05$

Conclusions

The comparison of sarcoplasmic sub-proteomes was successfully implemented using liquid isoelectric focusing (OFFGEL) as fractionation strategy.

The abundances of actin, alpha-crystallin B, heat shock protein B6 and heat shock protein B1 were higher in DFD samples, whereas phosphoglucomutase was over-represented in normal samples.

The identified proteins could be used as reliable biomarkers of pre-slaughter stress (PSS) through high throughput analytical approaches.

THANK YOU FOR YOUR ATTENTION

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The Department of Economic Development & Competitiveness of the Basque Government

Spanish Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (RTA2014-00034-C04) and FRIBIN S.A.T. (Binéfar, Huesca)

SEARCHING FOR PROTEIN BIOMARKERS RELATED TO PRE-SLAUGHTER STRESS USING LIQUID ISOELECTRIC FOCUSING

C. Fuente-García[1,2], N. Aldai[2], E. Sentandreu[1], M. Oliván[3], F.Díaz[3] and M.A. Sentandreu[1]

[1] Instituto de Agroquímica y Tecnología de Alimentos (CSIC). 46980 Paterna (Valencia), Spain

[2] Lactiker Research Group, Department of Pharmacy and Food Sciences, University of the Basque Country (UPV/EHU). 01006 Vitoria- Gasteiz, Spain

[3]Servicio Regional de Investigación y Desarrollo Alimentario (SERIDA). 33300 Villaviciosa (Asturias), Spain

Abstract - Proteome changes derived from animals that have suffered pre-slaughter stress are a fact although the discovery of associated biomarkers is still a challenge. In this study, Proteomic analysis was carried out on 16 loin samples of beef from Asturiana de los Valles breed and crossbreds animals previously classified as normal and DFD meat at 24 h post-mortem using pH measurements. Sarcoplasmic sub-proteome of *Longissimus thoracis* muscle was fractionated by the use of liquid isoelectric focusing (OFFGEL) in the pH range of 3 to 10, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of each retrieved fraction.

The obtained protein separation profile showed high reproducibility along the different samples. Five different bands showed significant statistical differences ($p<0.01$) in both sample groups, which allowed to compare and distinguish normal and DFD meat.

The significance of this study relies on the optimization of the OFFGEL technology. This method separates proteins along different fractions according to their isoelectric point (pI); then the obtained fractions can be further separated by SDS-PAGE. This achievement stands out as an alternative to the use of two dimensional gel electrophoresis (2-DE), enabling a higher resolution in protein separation and shorter analysis times.

Key Words: DFD meat, pre-slaughter stress, OFFGEL, biomarkers, sarcoplasmic proteins, Proteomics.

I. INTRODUCTION

Pre-slaughter stress condition (PSC) arises as a consequence of several factors that are both intrinsic and extrinsic to the animal. Among the intrinsic factors, the more decisive ones are physiology, age, sex and genetics. Regarding extrinsic stressors, such as temperature, handling activities, human presence, lairage time or feed and water deprivation in abattoir [1], these are not only prevalent but also manageable. Before slaughter, animals exposed to PSC consume their glycogen reservoirs. This perturbs glycolysis after slaughter, lowering its rate due to the prior reduction in glycogen content and generating less lactic acid and increasing pH, which concludes in an abnormal conversion from muscle to meat [2].

PSC produces dark, firm and dry meat, also known as DFD meat, which causes huge economical losses in the meat industry because of its poor quality attributes. Examples of such undesirable attributes are changes in tenderness during aging, high water holding capacity or fast spoilage due to microbial contamination.

Proteomics and other omic disciplines have thoroughly sought a deep understanding about how proteins are expressed or modified in different conditions and how they interact between each other or between other compounds. Moreover, they can help to study the biochemical pathways that take place in several scenarios related to meat aging and PSC.

Traditionally, the most commonly used technique to separate proteins has been 2-DE [3], closely followed by SDS-PAGE [4]. In spite of the spread of these techniques, new ones are being developed, such as OFFGEL fractionation. This latter separates proteins according to their isoelectric point within a defined pH range into liquid fractions that are easily recovered by pipetting.

This study aims at finding and comparing proteome changes in the sarcoplasmic protein fractions in order to elucidate which proteins and biochemical mechanisms can be involved in PSC. To that end, bovine *Longissimus thoracis* (LT) muscle from normal and DFD meat has been analysed by means of OFFGEL fractionation, followed by SDS-PAGE analysis of the retrieved fractions.

II. MATERIALS AND METHODS

Half gram of LT muscle sampled at 24 h post-mortem from 16 Asturiana de los Valles and crossbred animals from northern Spain, which were previously classified as normal and DFD meats according to their pH value, were homogenized in 4 mL of extraction buffer (10 mM Tris pH 7.6, 1mM EDTA, 0.25M Sucrose) using an Ultra-Turrax DI 25 Basic (Ika, USA). The homogenate was then centrifuged at 20000 *g* during 20 minutes at 4 °C (Beckman Coulter, USA). Protein quantification for each sample was assessed using the Bio-Rad Protein Assay Kit (Bio-Rad, USA) following the Bradford method ranging from 0.05 to 0.8 mg/mL (Bio-Rad, USA). Bovine serum albumin was used as a protein standard to get a calibration curve. Sarcoplasmic proteins were focused based on their isoelectric point into 12 liquid fractions using 13 cm immobilized pH gradient gel (IPG) strips with a linear pH gradient in the range from 3 to 10 (GE healthcare, Sweden). One mg of each sarcoplasmic extract was employed for protein fractionation using an Agilent 3100 OFFGEL fractionator (Agilent, USA). The protein content of each one of the 12 obtained fractions was further separated by SDS-PAGE on 12% polyacrylamide gels. After electrophoresis, gels were stained with colloidal Blue Coomasie [5] overnight. The volume of each band was quantitatively assessed using Gel Analyzer 2010 software (Biosoft, United Kingdom).

Man-Whitney non-parametric test was applied to find significant differences between normal and DFD samples ($p<0.01$) using SPSS 23.0.0 software (SPSS Inc., USA).

III. RESULTS AND DISCUSSION

Samples analysed in this work correspond to meat cuts from sixteen bovine animals classified as previously described according to their pH value (data not shown). The main aim of this study was to compare normal and pre-slaughter stressed animals' proteome using liquid isoelectric focusing coupled to SDS-PAGE.

After OFFGEL fractionation, 12 liquid fractions were obtained for each animal sample along the assessed pH range (3-10), then being further separated by SDS-PAGE.

Figures 1 and 2 are representative examples of the distribution of sarcoplasmic sub-proteome from both studied groups. It can be observed that proteins distribution profile after OFFGEL fractionation was highly reproducible over the whole analysed samples, thus enabling to search for differences between normal and DFD groups.

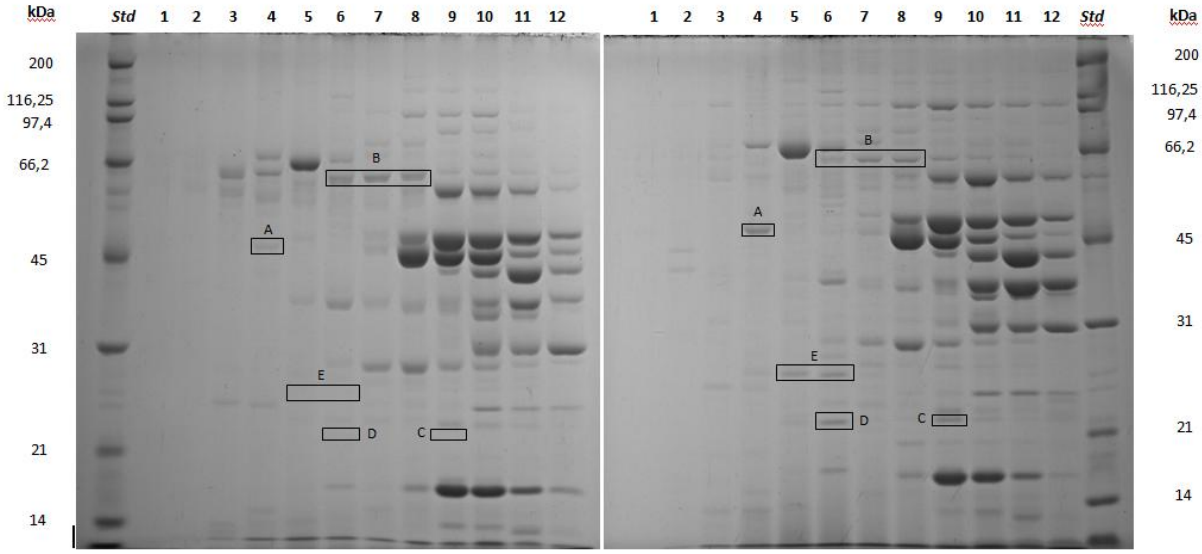

Figure 1 and 2. **12% SDS-PAGE gel of fraction obtained after OFFGEL isoelectric focusing from two representative samples: normal (on the left side) and DFD (on the right side) in the pH range 3-10**

According to the densitometry analysis, the most noticeable changes in protein abundance between the two types of meat were observed in bands A to E. Furthermore, it was confirmed by statistical tests that these spots (A to E) had significant statistical differences ($p<0.01$) between the two groups. These spots are indicated by letters on both gels and summarized in **Figure 3**. The abundance of spot B was higher in normal meat samples, whereas spot A were significantly less abundant in the normal group in comparison to DFD meats. Otherwise, it was observed that spots C, D and E appeared only in DFD meat samples. Therefore it could be hold that these spots are exclusive of DFD meat.

In the future, the next step will be to identify the bands present in different abundances (Figure 3) by means of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). This technique will provide a deeper knowledge about which proteins are differently expressed as a result of a pre-slaughter stress condition and which biochemical mechanisms lead to the development of DFD meat.

In this regard, there are many biochemical parameters that can be measured to evaluate the animal stress status [6], however, these cannot provide further information about meat quality or proteins changes due to stress. Therefore, Proteomics has become a powerful tool to further understand aforementioned changes.

The use of Proteomics represents a valuable tool to study biomarkers related to meat tenderness [7]. However, fewer research efforts have been put towards pre-slaughter stress biomarkers [8]. Franco *et al.* (2015) reported the use of 2-DE for separating the muscle proteome followed by the identification of the most relevant spots by LC-MS/MS. By this way, they were able to identify some structural-contractile proteins

and metabolism enzymes from beef, which showed statistically significant differential abundance between normal and DFD samples [8].

Other authors have studied the expression of proteins that are directly related to stress and defense status. Xing *et al*. (2017) measured the level of different enzymes in plasma and performed a *Western blot* to evaluate the influence of the transport duration of broilers on the expression of those proteins [9]. These authors were also able to differentiate between normal meat and meat derived from stressed animals, being able to relate those changes to meat quality.

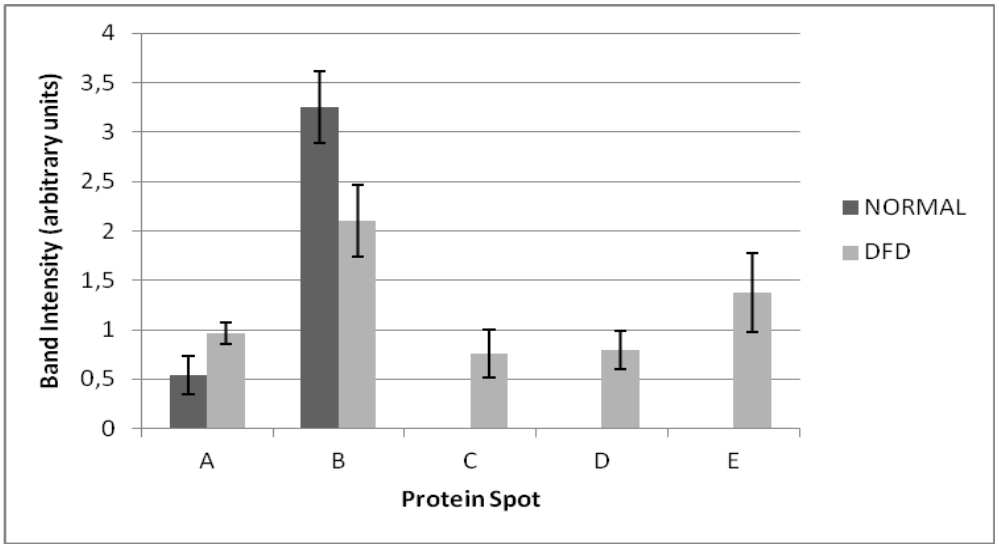

Figure 3. Mean values of abundances (and standard deviations) of the five selected bands in normal (dark) and DFD (light) meat sample groups.

The method described in the present work constitutes a novel alternative to the use of 2-DE, requiring less manual work and shorter time of analysis. Moreover, once the different OFFGEL fractions have been obtained, proteins contained in each one of them could be identified directly by LC-MS/MS avoiding a previous gel separation step. Many studies have reported promising results after using this technology [10]. For example, Naveena *et al*. (2017) studied the high efficiency of OFFGEL fractionation in comparison to DNA-based PCR method for meat species identification from raw and cooked ground meat [11].

IV. CONCLUSION

The proteome study of *Longissimus thoracis* muscle using liquid isoelectric focusing allows a high reproducibility to differentiate and to compare the protein profile between normal and DFD meat samples. The obtained results would not be possible using only the measurements of biochemical biomarkers established in the literature, which can only supply quantitative data but do not report proteome changes related to pre-slaughter stress condition. According to the data obtained in the present work, by means of OFFGEL fractionation, five protein bands showed statistically significant differences in abundances between the studied sample groups (normal and DFD). In the near future, the nature of these bands will be elucidated in order to discover new and reliable pre-slaughter stress biomarkers.

ACKNOWLEDGEMENTS

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