Paper A6

Collagenic biopolymer isolation from bovine hides for its use in medical applications.

M. Catalina[1,*], J. Cot[1], P. Celma[2], A. Manich[1]
[1] Institut de Química Avançada de Catalunya (IQAC) - Consejo Superior de Investigaciones Científicas (CSIC); C/ Jordi Girona, 18-26, Barcelona, 08034, Spain. Tlf: +34-93-4006153; e-mail: merche.catalina@iqac.csic.es
[2] Instituto Químico Sarriá (IQS), Ramon Llull University, Vía Augusta, 390; Barcelona, 08017, Spain

Abstract: The current project stems from an investigation of years from an innovative research line in the treatment of collagenic wastes (hides) generated by the tanning industry worldwide. In the beginning, this research was based on chrome-tanned waste, with the aim of removing and recovering chromium and also to obtain a gelatin suitable for the manufacture of auxiliary products for tanning, textile, paper and wood industries. Subsequently, non-tanned wastes (not stabilized) were included in the investigation, obtaining gelatin or "pseudo-collagen" with technical applications in agriculture (manure, fertilizer), or photography. The evolution of this investigation leads to the production of collagenic biopolymers with high added value.

The conventional process of tanning, which aims to obtain a material, "leather" (with its subsequent manufacture into articles for clothing, footwear, upholstery, etc.) is diversified and an important alternative is found, with both economical and ecological benefits. The new path for the treatment of high quality raw hides: obtaining collagenic biopolymers for potential applications in various fields such as medicine and veterinary.

Worth mentioning the possible future applications in the field of medicine, such as, for example, treatment of ulcers, burns of various degrees, suture, etc. Especially interesting is to remember that the number of patients with pressure ulcers is more than 100,000 per day in Spain and the annual cost of treating these ulcers in 2007 was 461 million Euros.

Therefore, this paper's primary goal is obtaining a suitable biopolymer for use in medical applications, being indispensable to find the optimal extraction process of the biopolymers from high quality animal hides. For this purpose, three types of bovine hides were studied as raw material: thick splits, thin splits and crust. The experimental design is based on the Box and Behnken factorial model. This design selects the data on 3 levels and sets the response from a second degree polynomial. The influence of hydrolytic agent (acetic acid, ammonia and water) as well as time and temperature of the extraction process, were studied. The size of hide fibre is set at 0.25mm (previous studies demonstrated the importance of fibre size, being the reaction optimal when the size is the smallest).

The optimum is determined from the results of swelling, molecular weight and ability to form fibres and films. Collagenic biopolymers can easily be produced as gel, film, fibre and/or sponges.

1. Introduction

This paper's primary goal is obtaining a suitable biopolymer for use in medical applications, being indispensable to find the optimal extraction process of the biopolymers from high quality animal hides.

The extraction of high-added value products from hides and its medical application fits perfectly the idea of “sustainable development”, the rational use of resources and improvement of life quality. Raw material such as hides, have some potential value since they contain collagen which could be recycled and reused. Collagen is a very versatile and special high-added value protein and the most abundant and ubiquitous in vertebrates[1, 2]. This collagenic nature of the tannery solid waste permits us to think about treatments for obtaining biopolymers of reconstituted collagen, and their use in a wide range of potential applications.

Up to now, "low cost" biomaterials have been obtained. Their main applications have been: as filler, re-tanning agents and finishing agents in the tanning industry itself; and as a binder in the paper industry, partially substituting casein (much more expensive). The objective of the present work is the extraction and application of new "Tailor-made" smart biopolymers with high-added value, finding a new and feasible link between solid tannery waste and the rising market of tissue engineering

Tissue engineering can be defined as an interdisciplinary field which applies, for one side, the principles of tissue engineering and, for another side, the sciences of life, with the aim of obtaining "Biological Structures" in order to regenerate and/or improve the tissue function[3]. Although lots of synthetics biodegradable or bio-stable polymers have been employed on these "special structures", biologically derived materials are advantageous in that they contain information that facilitates cell attachment and function, whereas synthetics may not interact with cells in the desired manner[4].

The importance and special appeal of collagen as a biomaterial is based on the fact that collagen is a natural material and therefore it is assimilated by the human/animal body as a normal constituent and not as a foreign material, subjected to rejection. A great competitiveness of reconstituted collagen fibres in the field of regenerative medicine (tissues and/or organs) has been found in literature[2, 4].

Biopolymers are polymers generated from renewable resources, often biodegradable and from non-toxic production. They can be produced from biological systems or chemically synthesised from biological raw materials. They are an alternative to the petrol-based polymers. The main problems of biopolymers are bio-compatibility, mechanical properties and adaptability. Collagenic biopolymers present huge possibilities due to the possibility of manufacture, and application, in different ways, forms and shapes, with well determined characteristics. We can talk about "Taylor-made" biopolymers: it is possible to produce easily said biopolymers as gel, film, fibres, tissue and/or sponges, using techniques such as freeze drying/lyophilisation, extrusion, or electro-spinning for nano-fibres formation.

Previous work[5], using splits as a raw material, has demonstrated the feasibility of obtaining gels, films and fibres of collagenic biopolymers, however, new raw materials (thin splits and crust) have been investigated in order to optimise the process and product. In addition, some applications of the biopolymers as a "healer" (treatment of wounds) will be carried out in the future.

The use of mathematical experimental designs permits to study the degree of significance of the different variables and the corresponding interaction between them in the different processes for obtaining those collagenic biopolymers. This ensures that the experimentation can be rationalised and a mathematical equation controlling the whole process can be defined, this will determine the optimum in each case, being able to achieve a controlled production of "Tailor-made" biopolymers for each specific application.

2. Materials and methods

Two types of bovine hides were studied as raw material: splits of pickled hides (thin) and crust, which were supplied by the Leather Technology School of Igualada. Acetic acid (99.5% PS) and ammonia (25% PA) were supplied by Panreac. Standard marker for SDS-PAGE (from 6.5 to 205 kDa) was supplied from Bio-Rad. Analytical grade chemicals were used for fibre formation: the phosphate buffer comprised disodium phosphate heptahydrate and monosodium phosphate monohydrate, supplied by Riedel-de Haen and Fluka, respectively. Polyethylene glycol Mw 8000 and sodium chloride were supplied by Sigma and Carlo Erba, respectively.

Biopolymer extraction: The basis for the preparation of biopolymer was the degradation of collagen by hydrolysis. The dried hides were cut manually in small pieces and then ground in a grinder rotor mill (Retsch SR-01). Ground bovine hide in a concentration of 50 g hide per liter of hydrolytic solution, were mixed by mechanical stirring (Heidolph stirrer). A temperature controlled bath (Lauda E100) with a through-flow cooler attached (Lauda DLK10) was used at a fixed temperature for a determined period of time.

Experimental design: is based on the Box and Behnken factorial model. This design selects the data on 3 levels and sets the response from a second degree polynomial. The influence of hydrolytic agent (acetic acid, ammonia and water) as well as time (8, 16 and 24 hours) and temperature (5, 15 and 25ºC) of the extraction process for each of the hides selected (split and crust), were studied. The experimental design variables are shown in Table I.

Table I: experimental design applied for the different variables in study.

Sample	Hydrolytic agent	Time (h)	Temperature (ºC)
1	H_2O	16	5
2	CH_3COOH	8	25
3	H_2O	24	15
4	H_2O	16	25
5	CH_3COOH	24	25
6	CH_3COOH	8	5
7	NH_3	24	15
8	NH_3	8	15
9	CH_3COOH	24	5
10	NH_3	16	25
11	NH_3	16	5
12	H_2O	8	15
13	CH_3COOH	16	15
14	CH_3COOH	16	15
15	CH_3COOH	16	15

Fibre formation (extrusion): The process for fibre formation was based on previous work[4] with slight modifications. A syringe was loaded with biopolymer solution and placed on a syringe pump system supplied by KDScientific (model no: KDS-100-CE). One end of a silicone tube was connected to the syringe and a needle was fitted at the other end and then placed at the bottom of a container. The fibres were extruded into a "Fibre Formation Buffer" (FFB) remaining there for 30 minutes and then transferred into a "Fibre Incubation Buffer" (FIB) for another 10 minutes. Finally, the fibres were air-dried under the tension of their own weight at room temperature. The "Fibre Formation Buffer" comprised 118 mM phosphate buffer and 20% of polyethylene glycol (Mw 8000) at pH 7.55 and 37 ºC. The "Fibre Incubation Buffer" comprised 6 mM phosphate buffer and 75 mM sodium chloride at pH 7.10 and 37 ºC.

Film formation: Aliquot of the extracted biopolymer (10 ml) was placed in a small Petri dish and allowed to air dry at a constant temperature (20ºC) and relative humidity (60%).

Percentage of centrifuged: all the samples were centrifuged at 5000rpm for 15 minutes. The supernatant was poured out and percentage of centrifuged biopolymer was calculated as follows: Centrifuged (%) = $100(W_{centrifuged\ residue})/W_{initial\ sample}$; where $W_{centrifuged\ residue}$ is the weight of the sample after pouring out the supernatant and $W_{centrifuged\ residue}$ is the initial weight of the sample prior centrifugation.

Swelling[6-8]: The films were weighed and then immersed in a phosphate buffered saline (PBS) solution for different periods of time. Wet samples were blotted with filter paper to remove the surface water not taken into the gel, and re-weighed. The percentage of swelling was calculated as follows: Swelling (%) = $100(W_{wet} - W_{dried})/W_{dried}$; where W_{wet} is the weight of the film after being immersed in PBS solution for a determined period of time and W_{dried} is the initial weight of the film.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): Aliquots of 50 mg of gelatin were dissolved in 1 ml of sample buffer. The samples then were denatured at 90ºC for 5 minutes, and loaded in appropriate volumes onto a vertical acrylamide gel (4% (v/v) stacking gel, 7.5% (v/v) resolving gel). A standard marker, from 6.5 to 205 kDa was loaded with the samples. The gels were run at 0.01 mA/gel, stained overnight with Coomassie Brilliant Blue solution, and then destained prior to analysis.

2. Results and discussion

According with the statistics studies, the variables with significant influence ($p<0.005$; ANOVA) on the yield (percentage of centrifuged) of the biopolymer extraction and the swelling of the biopolymer films obtained was the hydrolytic agent, being acetic acid the optimum agent for the extraction of collagenic biopolymers.

Figures 1, and 3 represent the effect of the variables time and temperature on the swelling (after 5 and 90 min) and percentage of centrifuged of the extracted biopolymer, respectively; using acetic acid as hydrolytic agent.

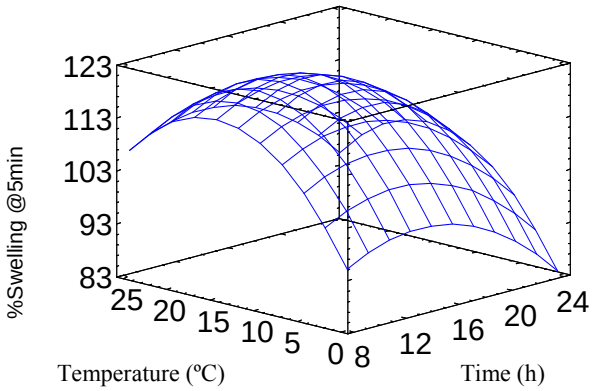


Fig. 1 Surface plot of the effect of time and temperature on the percentage of swelling (after 5min) of extracted biopolymer. Hydrolytic agent: CH_3COOH

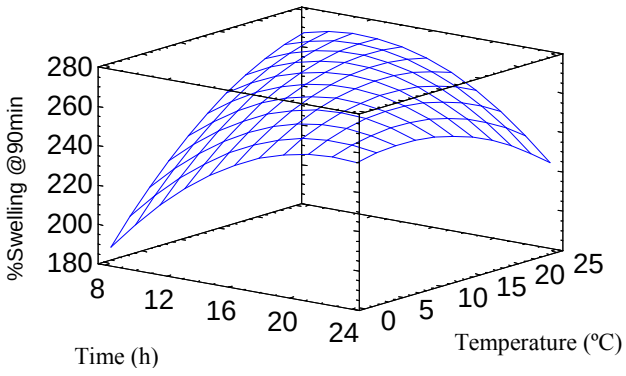


Fig. 2 Surface plot of the effect of time and temperature on the percentage of swelling (after 90min) of extracted biopolymer. Hydrolytic agent: CH_3COOH

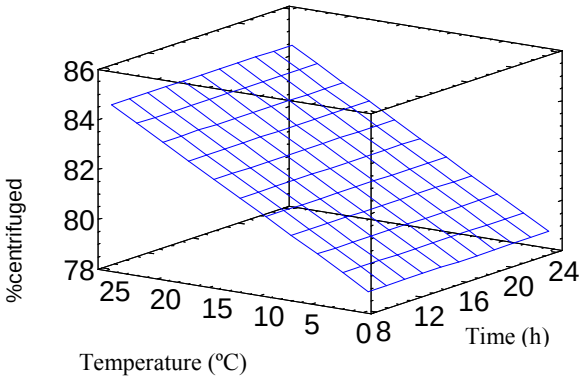
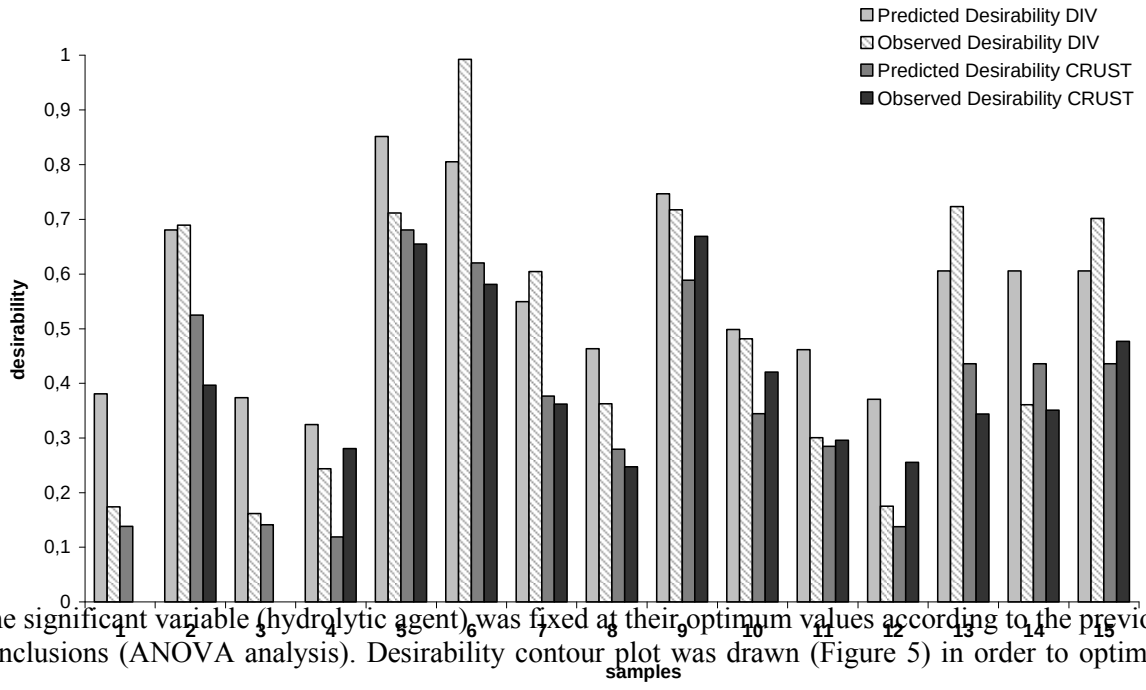


Fig. 3 Surface plot of the effect of time and temperature on the centrifuged percentage of extracted biopolymer. Hydrolytic agent: CH_3COOH

The results were analysed by Multiple Response Optimization, a function that determines the combination of experimental factors that simultaneously optimize several response variables; the goal of the function is to maximize a desirability function. The general approach of the desirability function is to first convert each response into an individual desirability function that varies over the range 0-1 where, if the response is at its goal, then the desirability value is 1, however, if the response is outside an acceptable region, desirability value is 0^9. The design variables are chosen to maximise the overall desirability from the geometric average of individual desirabilities.

The desired responses are to maximise the percentage of centrifuged (yield) and minimise the percentage of swelling. The capacity of forming films has been not analysed because all the samples formed films.

Figure 4 shows the desirability (predicted and observed) for all the samples in study. It can be observed that samples obtained from splits (DIV) presented better desirability than the samples extracted from crust. The sample with higher observed-desirability was sample DIV-6 (CH_3COOH, 8h, 5ºC), however, the higher predicted-desirability was sample DIV-5 (CH_3COOH, 24h, 25ºC).



The significant variable (hydrolytic agent) was fixed at their optimum values according to the previous conclusions (ANOVA analysis). Desirability contour plot was drawn (Figure 5) in order to optimise

Fig. 4 Observed/predicted desirabilites for the study of the effect of hydrolytic agent, time, temperature and raw material on the extraction of collagenic biopolymers.

the response of the other two variables in study, temperature and time. The black dot indicates the optimum, the desirability was maximised at high temperature for a long period of time, or at low temperature for a short period of time (corresponding to samples DIV-5 and DIV-6, respectively; as seen in previous Figure 4).

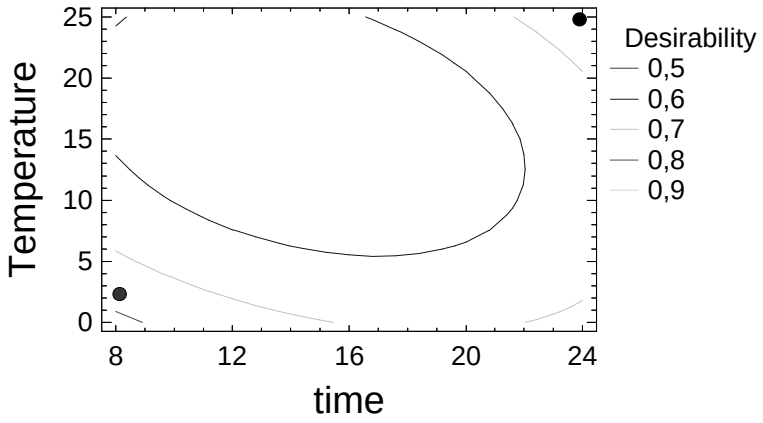


Fig. 5 Desirability contour plot of the effect of time and temperature on the biopolymer extraction procedure (acetic acid as hydrolytic agent). ● indicates the optimum.

Table II summarizes the optimum values for each variable to optimise each response as well as the overall optimisation. It has been found that the biopolymer with optimum properties can be obtained using splits as a raw material (DIV), and carrying out the extraction with acetic acid during 24 hours at 25ºC.

Tab. II Optimum values of variables for biopolymer extraction procedure (study3_around the optimum).

Variable	Optimum (maximise yield)	Optimum (minimise % swelling (5min)	Optimum (minimise % swelling (90min)	Optimum
Time (h)	24	26	26	24
Temperature (ºC)	5	14	14	25
Hydrolytic agent	CH_3COOH	CH_3COOH	CH_3COOH	CH_3COOH

Collagen isolated from various tissues has a molecular weight of about 300kDa[2]. For collagen derivatives, the molecular weight usually ranges within limits of 15-50kDa for hydrolysates[10] and 50-200 kDa for gelatin[11]. From figures 6 and 7, it can be observed that samples from split hides showed a distinctive band in the range of 150-100 kDa, for all the samples. However, crust samples showed some differences between ammonia (CR-7, CR-8, CR-10 and CR-11) samples and the rest. Ammonia derived samples did not show the distinctive band at 150 kDa, but a band within the whole range of molecular weights (CR-7 and CR-10) and below 75 kDa (CR-8 and CR-11).

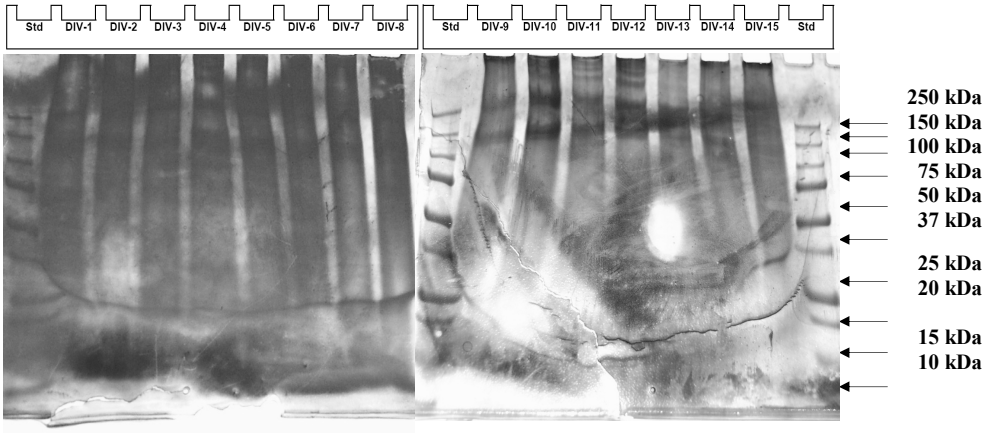


Fig. 6 Molecular weight analysis on the biopolymer from samples extracted from splits (DIV).

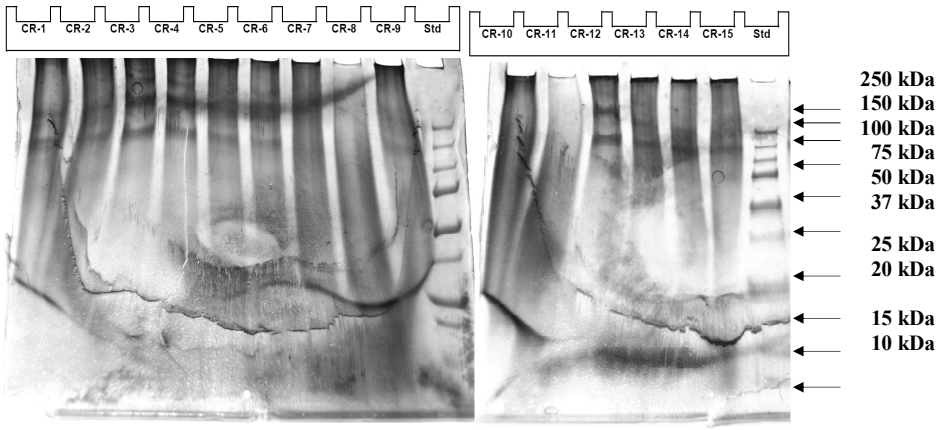


Fig. 7 Molecular weight analysis on the biopolymer from samples extracted from crust.

4. Conclusions

The possibility of obtaining new high-added value biomaterials from solid leather waste, more specifically from bovine hides (splits), has been demonstrated.

A complete methodology for the extraction of new biopolymers from tannery solid waste with optimum properties has been developed. It has been demonstrated that acetic acid permitted to extract biopolymers with the desired properties. In addition, a comparison between two hides has been carried out showing that splits worked better as raw material than crust leather.

The process and product have been optimised, and the product will be tested as a "healer" (treatment of wounds) in new applications to be carried out in the future.

Therefore, a new potential future market in the fields of cosmetics, medicine or veterinary, with both important economical and environmental benefits, has been opened up.

5. References

1. Gelse, K., Pöschl, E. and Aigner, T., *Advanced Drug Delivery Reviews,* **55,** 1531-1546, 2003
2. Reis, R. L., Neves, N. M., Mano, J. F., Gomes, M. E., A.P.Marques and Azevedo, H. S. Woodhead Publishing, CRC Press. 2008
3. Langer, R. and Vacanti, J. P., *Science,* **260,** 920-926, 1993
4. Zeugolis, D., Paul, R. G. and Atenburrow, G., *Journal of Biomedical Research Part A,* **86A,** 892-904, 2008
5. Catalina, M., Celma, J., Cot, J., Manich, A. and Marsal, A., *J Am Leather Chem As,* 2011
6. Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N. and Rubini, K., *Biomaterial,* **23,** 4827-4832, 2002
7. Bigi, A., Panzavolta, S. and Rubini, K., *Biomaterial,* **25,** 5675-5680, 2004
8. Zeugolis, D. I., Paul, R. G. and Attenburrow, G., *J Biomed Mat Res A,* **89,** 865-908, 2009
9. Vílchez, S., Manich, A. M., Jovancic, P. and Erra, P., *Carbohydrate Polymers,* **71,** 515-523, 2008
10. Langmaier, F., Mokrejs, P., Karnas, R., Mládek, M. and Kolomazník, K., *Journal of the Society of Leather Technologists and Chemists,* **90,** 29-34, 2006
11. Brown, E., Thompson, C. and Taylor, M. M., *Journal of the American Leather Chemists Association,* **89,** 215-220, 1994

6. Acknowledgments

The authors are indebted to the Spanish CTM2009-07435 Project, from the National R&D&I Plan of the Ministry of Science and Innovation.