METHODS FOR THE DETECTION OF GELATIN ORIGIN IN MEAT PRODUCTS

M. T. Yilmaz[1*], Z. Kesmen[2] and H. Yetim[2]

[1]Department of Food Science, Erciyes University, Safiye Cikrikcioğlu Vocational College, 38039, Kayseri, TURKEY

[2]Erciyes University, Department of Food Engineering, Engineering Faculty, 38039, Kayseri, TURKEY

[*]Corresponding author (Phone: +90 352 437 4901; Fax: +90 352 437 1383; e-mail: mtyilmaz@erciyes.edu.tr)

***Abstract*— The outbreak of BSE in the United Kingdom in 1986 and its extension to other countries have caused the regulatory authorities to make limitations to the use of bovine gelatin for human consumption. In addition, some religious beliefs and convictions have restricted the consumption or the use of porcine derived gelatin in food materials. In this review, we discussed in detail the methods tried to develop for the detection of gelatin origin in meats and other food products, which have been conducted to alleviate any doubts about the origin of gelatin used. Currently, there are no-well-established analytical methods available to confirm the gelatin and the inclusion or exclusion of the certain materials in animal products. In this respect, DNA-based technologies may offer an alternative to overcome the problem. However, extensive target DNA degradation in gelatin is a major challenge for this method and published species-specific PCR systems are not ideal for the use with gelatin DNA templates. Therefore, further species-specific PCR assays that can be applicable to gelatin DNA templates should be established to fill the gap.**

***Index Terms*—Gelatin origin, Bovine, Porcine, Analytical methods.**

I. INTRODUCTION

Gelatin is a protein based product produced by partial denaturation of collagen extracted from connective tissues of animals like cattle and pig. The most striking attribute of the collagen molecule is that its amino acid sequence is composed of a repeated tripeptide unit. This unit could be indicated as Glycine−X−Y where X-Y is generally proline and 4 hydroxy-proline, which causes gelatin to gain exceptional properties like softness, elasticity and forming a reversible gel depending on temperature. Approximately 21 % of aminoacids present in gelatin produced from mammals contains proline and hydroxyproline, whereas the proportion of those in cold-water fish gelatin with low-gelling capacity is about 17 % (Ward, 1958; Veis, 1964). Another case for the primer structure of gelatin is that it contains basic (lysine %2.5, arginin %5) and acidic amino acids (glutamic acid %7.2, aspartic acid %4.7) in high quantities. Therefore, gelatin contains non-polar aminoacids at 80% proportion, mostly in non-polar regions, which causes gelatin to have emulsifying properties throughout the alpha chain (Cole, 2001).

The unique molecule structure gives gelatin many functional properties as well as other properties being almost not affected by denaturing agents such as environmental temperature and alterations in ionic strength. Gelatin is available in capsule and powder form and it is well known for its unique properties in food industry such as foam stabilizer, gelling agent, binding agent, film forming agent, thickener, emulsifier, micro-encapsulation and clarifying agent (Kobayashi, 1996). In addition to these functional properties, gelatin is also of great importance regarding nutritional aspect. For example, gelatin, a protein that can be easily and completely digested in digestive tract, contains 9 of 20 essential aminoacids that should be taken through foods. Combination of gelatin with other proteins could increase their biological value (Baziwane and He, 2003). Furthermore, positive effect of gelatin on protection of bone and joint health was approved by clinical studies (Adam, 1991). For these reasons, it is essential to use gelatin in food products to achieve these properties. Gelatin production was reported to be about 315.000 ton per year around the world, and 46% of raw materials are produced from pig skin and 69 % of that in Europe, as detailed shown in Fig. 1.

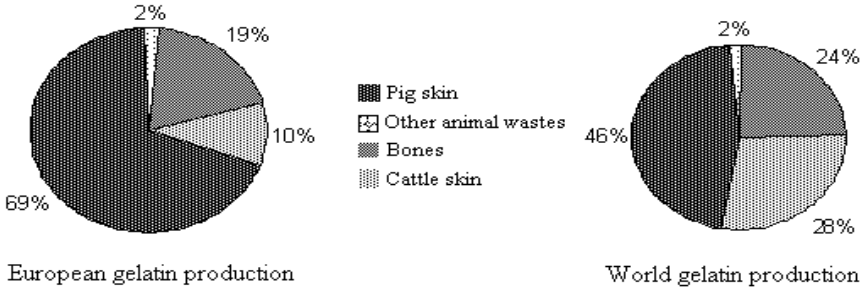

Fig 1. Gelatin production in Europe and World in 2006 (Anon. 2007).

The outbreak of BSE in the United Kingdom in 1986 and its extension to other countries have caused the regulatory authorities to make restrictions to the use of bovine gelatin for human consumption, cosmetic and pharmaceutical products. In addition, some religious beliefs and convictions have restricted the consumption or the use of porcine derived gelatin in food materials. (Venien & Levieux, 2005). For example, in Muslim and Jewish societies, possibility

of using pig origin raw materials in various food products and bovine origin raw materials in the countries where mad cow disease (BSE) broken out has created consumer concerns. In 1989, in fact, Food and Drug Administration (FDA) in the USA banned imports of cattle from the countries where BSE disease took place; however, import of bones and tissues used in the production of gelatin in pharmaceutical quality is permitted by FDA in 1994. Accordingly, to ensure quality and safety of edible gelatin, basic rules in the gelatin production including also certification for production from raw material derived from animals suitable for human consumption are described in directive No. 1999/724/EC of the European Union (Anonymous, 2005). To support edible gelatin documentation, sensitive and reliable analytical methods for determining the species origin of gelatin are important (Tasara, Schumacher, & Stephan, 2005). Therefore, it became necessary to develop analytical methods intended to control the species origin of gelatin (Venien & Levieux, 2005). In literature, such methods have been developed in this respect although their number is limited. In this review, some methods based on chemisorption, chromatographic, immunochemical, mass spectroscopy, spectroscopic and molecular techniques for the detection of gelatin origin in meat products are discussed, and results of the latest analysis are presented.

II. THE METHODS USED IN GELATINE ANALYSES

Species determination methods relying on protein analysis are the well known methods and they have been applied in several food products (Chen & Hsieh, 2000; Skarpeid, Kvaal, & Hidrum, 1998). These approaches to amino acid analysis is based on the technology (Hirs et al., 1954), in which aminoacids with free amino groups are separated by cation-exchange chromatography followed by post-column derivatization (ninhydrin or orthophtaldialdehyde) (Heems, Luck, Fraudeau, &Verette, 1998). However, highly degraded nature of proteins in gelatin due to extreme temperature and pH treatments during production make such methods unsuitable for use with gelatin (Tasara, Schumacher, & Stephan, 2005). To overcome this challenge, some novel methods have also been recently developed. Followings are the examples of these methods applied for differentiation of bovine gelatin from porcine gelatin.

1. Chemisorption method: It was reported by Hunter, Nyburg and Pritzker (1986) that collagen, gelatin, and agarose gels promote the formation of hydroxyapatite (HAP) from amorphous calcium phosphate (ACP). HAP is a compound which is formed by chemisorption of gelatin. Termine, Peckauskas, and Posner (1970) reported that the presence of collagen or gelatin enhanced ACP formation, suggesting that the interaction between gelatin and calcium phosphate precipitation may be used to analyze the source of gelatins. Based on this fact, Hidaka and Liu (2003) presented a new method distinguishing bovine bone gelatin from porcine skin gelatin using the *in vitro* formation of calcium phosphate precipitates. In their study, the reaction of calcium phosphate precipitation was found to be useful in distinguishing bovine gelatin from other gelatin products. However, it was stated that further study is needed to clarify these effects.

2. Chromatographic methods: During the last two decades, interest has been focused on pre-column derivatization followed by reversed-phase (RP) HPLC analysis, which makes the analysis much faster, more efficient and more sensitive, alternative to former amino acid analytical procedures (Kamp, 1997; Heems, Luck, Fraudeau, &Verette, 1998). Nemati, Oveisi, Abdollahi and Sabzevari (2004) carried out differentiation of bovine and porcine gelatins using principal component analysis. In their study, 14 bovine and 5 porcine gelatins were examined with the analysis procedure involved with complete hydrolysis of samples by classic acid hydrolysis in order to release their amino acid residues. Separation and determination of aminoacids was achieved by reversed-phase (RP) HPLC following pre-column derivatization. It was concluded from 20 peaks detected by HPLC analysis that one was very typical in bovine gelatin. Peak height, area, area percentage and width were used to make matrixes. Principal component analysis (PCA) with the MATLAB program was used to differentiate these gelatins. PCA on matrix of height, width and total matrix were resulted in good differentiation between bovine and porcine gelatins. However, they also concluded that more work needs to be done to establish the performance of this technique to classify other proteins and subjects with very high degree of similarity.

3. Immunochemical methods: Due to the large similarity in structure and properties of gelatins from different origins, the aforementioned physicochemical methods based on principal component analysis of amino acid residues obtained after acid hydrolysis (Nemati et al., 2004) and calcium phosphate precipitation test (Hidaka & Liu, 2003) have not been proved yet to be able to detect mixtures of bovine gelatin in porcine gelatin (Venien & Levieux, 2005). On the other hand, immunochemical methods have been found very useful for the control of the species origin of animal tissues in foods (Paraf & Peltre, 1991). Hofmann, Fisher, Mueller and Babel (1999) examined an ELISA kit for its applicability to species identification in gelatin and gelatin-containing products, such as gum confectionery and processed turkey and chicken. However, results were influenced by gelatin type, gelatin quality and concentration used and, in some cases, led to false negative or positive readings. Thus, the authors concluded that this ELISA cannot be reliably used for species identification of commercial gelatins. Due to the very high homology between collagen sequences of mammals, raising species-specific antibodies is a major challenge. Furthermore, the immunogenicity of gelatin is very low and the molecule should be chemically modified for raising antibodies in rabbits (Venien & Levieux, 2005). To overcome the challenge, Venien and Levieux (2005) produced high titre antibodies against tyrosylated bovine and porcine gelatins. However, such antibodies were found to be very sensitive to the alkaline or acidic process used for the gelatin production and not enough species-specific to allow a sensitive detection of mixture of low concentration of bovine

gelatin in porcine gelatin. Therefore, they produced bovine specific antibodies by immunization of rabbits with synthetic peptides mimicking a short putative species-specific sequence of the bovine alpha 1 (I) chain. This allowed them to develop two ELISAs suitable for the differentiation of bovine from porcine gelatins and for the sensitive quantification of their mixtures.

4. Mass spectrometric methods: It has been recently reported that it is difficult to distinguish bovine gelatin from porcine gelatin by the aforementioned conventional spectroscopic methods; moreover, for gelatins derived from mixed sources, the situation is more complex (Zhang et al, 2009). As mentioned above, the physicochemical method based on principal component analysis and calcium phosphate precipitation test have not been proved to be able to differentiate bovine from porcine gelatins (Hidaka & Liu, 2003; Nemati et al., 2004). In addition, the method of Venien and Levieux (2005) has been reported to be inadequate for the differentiation because the method needed repeated results and experience since the sample preparation was very sensitive and rigid (Hashim, Che Man, Norakasha, Shuhaimi, Salmah, & Syahariza, 2010). Furthermore, it was previously indicated by Arbogast, Gunson, and Kefalides (1976) that the hydroxylation of proline plays an important role in determining the antigenicity of collagens. So, immunochemical method might be influenced by the extent of proline hydroxylation in gelatin peptides. Ocana, Neubert, Przyborowsk, Parker, Bramley and Patel (2004) reported that some species specific ions could be detected using mass spectroscopy after bovine gelatin was hydrolyzed with 3 mol/l HCL, which could be used for detection of bovine gelatin. However, the content of target ions might be influenced by the hydrolysis time and temperature. Zhang et al (2009) have developed a new method to differentiate bovine and porcine gelatin based on detection and identification of marker peptides in digested gelatins. In their study, the gelatins were digested by trypsin, and the resulting peptides were analyzed by high performance liquid chromatography/tandem mass spectroscopy (HPLC-MS/MS). The marker peptides specific for bovine and porcine were successfully detected in the digested bovine and porcine gelatin, respectively. It was also found that proline hydroxylation was a key factor affecting the peptide identification. For peptides such as GPPGSAGSPGK and GPPGSAGAPGK detected in digested bovine and porcine gelatin, respectively, the sequence should be verified manually because the mass shift caused by proline hydroxylation can be confused with the mass difference between Ser and Ala residues. They concluded that detection of marker peptides in the digested gelatin sample using HPLC-MS/MS is an effective method to differentiate between bovine and porcine gelatin. However, the methods also have some drawbacks. During MS/MS data processing, the threshold for specific peptide identification might be different from one species to another (Li, Chen, Wang, Ji, & Wu, 2007; Shadforth, Dunkley, Lilley, Crowther, & Bessant, 2005). Zhang et al (2009) reported that the case is more complex for degraded collagens. Due to very high homology between collagen sequences of mammals, detection of marker peptides in the digested collagens is a major challenge. Furthermore, proline hydroxylation makes peptide identification more difficult than for most other proteins. To increase the sequence reliability of the marker peptides in digested gelatin, the searching results should be further verified. They also reported that further experiments should be conducted using bovine and porcine gelatins from different geographical areas.

5. Infrared (IR) spectroscopy methods: Fourier transform infrared (FTIR) spectroscopy has been shown to be a very useful technique for determining a range of adulteration problems in food products such as lard content in cakes and chocolates (Che Man, Syahariza, Mirghani, Selamat, Bakar, 2005), lard in mixture of animal fats (Syahariza, Che Man, Jinap, & Bakar, 2005). It is a powerful analytical technique that provide fast and accurate tool which have potential for discriminating spectra between two samples (Hashim et al., 2010). The FTIR spectroscopy together with attenuated total reflectance (ATR) or transmission accessories has been used to determine gelation and intermolecular cross-linking study of collagen and proteins (Cao & Xu, 2008; Muyonga et al., 2004). Hashim et al. (2010) have used the FTIR spectroscopy for differentiation of bovine and porcine gelatins. In their study, in order to determine unknown gelatin sources, deformation of N-H bonds found in the range 3290-3280 cm^{-1} and 1660-1200 cm^{-1} within infrared spectra of all gelatin samples were analyzed using discriminant analysis. These regions were found to give information about the origin of the gelatin. However, this method should also need repeated results.

6. PCR-Based methods: As discussed in detail, the above methods have some drawbacks due to the mentioned reasons. In this respect, DNA-based technologies offer an alternative. PCR analysis with species-specific primers can be used for rapid and highly sensitive detection of DNA from various species. Several PCR methods were developed for confirmation of food animal species and quantification of various materials in meat products (Tsara, Schumacher, & Stephan, 2005). In the literature, the method of molecular detection of bovine gelatin in porcine gelatin based on PCR techniques is lacking except for the study of Tsara et al. (2005). They evaluated the performance of published species-specific PCR systems using gelatin and tissue samples of known species origin. In their study, most of the species-specific PCR systems tested were able to discern species of origin for tissue-derived DNA, but not for gelatin DNA templates. Although they did not expected this result and the reason was linked to extensive target DNA degradation in gelatin, they envisaged that the majority of species-specific PCR assays applied to other food samples might not be ideal for use with gelatin. Based on their evaluation, only PCR assays designed to detect short regions of highly abundant sequences seem suited for determination of the species of origin for gelatin samples. They reached a conclusion that species-level DNA detection could be achieved only with primers targeting short regions (104 to 134 bp) of 16S rRNA, ATPase 8 subunit, and PRESINE 1 element sequences in the different gelatin samples tested. By targeting the bovine ATPase 8 subunit, they found that bovine material in gelatin can be routinely detected with high

specificity and sensitivity, through application of either conventional or real-time PCR assays to DNA templates isolated from gelatin. They also determined that as little as 0.1 % bovine gelatin can be detected within a pork or fish gelatin background using conventional PCR and as little as 0.001% bovine gelatin can be detected similarly with an even more robust approach of real-time PCR.

III. CONCLUSION

As can be seen from the aforementioned analytical methods, currently, there are no-well-established analytical methods available to confirm the inclusion or exclusion of certain animal materials in gelatin. In this respect, DNA-based technologies could offer an alternative for the purpose. However, extensive target DNA degradation in gelatin is a major challenge for this method and published species-specific PCR systems using gelatin and tissue samples of known species origin are not ideal for use with gelatin DNA templates (Tsara et al. 2005). Therefore, further species specific PCR assays that can be applicable to gelatin DNA templates should be established.

ACKNOWLEDGEMENT

The Authors thank to the Scientific Research Projects (Project no: FBA-10-2895) of Erciyes University Coordinating Office, Kayseri, Turkey for financial support.

REFERENCES

Adam, M. (1991). Osteoarthritis Therapy with Collagen Preparations: Results of a Clinical Study. Vol. 38. Germany: Deutsche Therapiewoche Karlsruhe, 2456-2461.

Anonymous (2005). EU directive 1999/724/EC. Available at: http://www.fsai.ie/legislation /food/eu_docs/Gelatine/Dec%201999.724%20EC.pdf. Accessed 21 February 2005.

Anonymous (2007) Available at: http://www.gelatine.org/en/gelatine/overview/127.htm.

Arbogast, B. W., Gunson, D. E., & Kefalides, N. A. (1976). The role of hydroxylation of proline in the antigenicity of basement membrane collagen. *The Journal of Immunology*, 117(6), 2181–2184.

Baziwane, D., He, Q. (2003). Gelatin: The Paramount Food Additive. *Food Reviews International*, 19(4),423-435.

Cao, H., & Xu, S. (2008). Purification and characterization of type II collagen from chick sterna cartilage. *Food Chemistry*, 108, 439–445.

Che Man, Y. B., Syahariza, Z. A., Mirghani, M. E. S., Selamat, J., & Bakar, J. (2005). Analysis of potential lard adulteration in chocolate and chocolate products using Fourier transform infrared spectroscopy. *Food Chemistry*, 90, 815–819.

Chen, F.C. & Hsieh, Y.H. (2000). Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. *J. AOAC Int.* 83, 79-85.

Cole, C.G.B. (2001). Gelatine: Its Properties and Its Application in Dairy Products. Presented at the Dairy Symposium 2001.

Hashim, D.M., Che man, Y.B., Norakasha, R., Shuhaimi, M., Salmah, Y. & Syahariza, Z.A. (2010). Potential use of Fourier transform infrared spectroscopy for differentiation of bovine and porcine gelatins. *Food Chemistry*, 118, 856-860.

Heems, D., Luck, G., Fraudeau, C., &Verette, E. (1998). *The Journal of Chromatography A*, 798, 9-17.

Hidaka, S., & Liu, S. Y. (2002). Effects of gelatins on calcium phosphate precipitation: A possible application for distinguishing bovine bone gelatin from porcine skin gelatin. *Journal of Food Composition and Analysis*, 16, 477–483.

Hirs, C.H.W., Stein, W.H., & Moore, S. (1954) *The Journal of Biological Chemistry*, 211, 941-950.

Hofmann, K., Fisher, K., Mueller, E., Babel, W. (1999). ELISA-test for cooked meat species identification on gelatine and gelatine products. *Nahrung*, 43, 406-409.

Hunter, G. K., Nyburg, S. C., & Pritzker, K. P. H. (1986). Hydroxyapatite formation in collagen, gelatin, and agarose. *Colloids Related Research*, 6, 229–238.

Kamp, R. (1997). Highly sensitive amino Acid analysis in protein structure analysis. In: R.M. Kamp, T. Choli-Papadopulou, B. Wittman-liebold (Eds.), Springer, Berlin, pp. 231-248.

Kobayashi, T. (1996). Properties and functions of gelatin. *Food Ingredients J.Jpn.* 170:82-88.

Li, B., Chen, F., Wang, X., Ji, B. P., & Wu, Y. N. (2007). Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chemistry*, 102(4), 1135–1143.

Muyonga, J. H., Cole, C. G. B., & Duodu, K. G. (2004a). Fourier transform infrared (FTIR) spectroscopy study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*). *Food Chemistry*, 86, 325–332.

Nemati, M., Oveisi, M. R., Abdollahi, H., & Sabzevari, O. (2004). Differentiation of bovine and porcine gelatins using principal component analysis. *Journal of Pharmaceutical and Biomedical Analysis*, 34, 485-492.

Ocana, M. F., Neubert, H., Przyborowsk, A., Parker, R., Bramley, P., & Patel, R. (2004). BSE control: detection of gelatin-derived peptides in animal feed by mass spectrometry. *Analyst*, 129, 111–115.

Paraf, A. & Peltre, G. (1991). Immunoassays in Food and Agriculture, Kluwer Academic Publishers, Dordrecht.

Shadforth, I., Dunkley, T., Lilley, K., Crowther, D., & Bessant, C. (2005). Confident protein identification using the average peptide score method coupled with search-specific, ab initio thresholds. *Rapid Communications in Mass Spectrometry*, 19(22), 3363–3368.

Skarpeid, H.J., Kvaal, K., & Hidrum, K.I. (1998). Identification of animal species in ground meat mixtures by multivariate analysis of isoelectric focusing protein profiles. *Electrophoresis*, 19, 3103-3109.

Syahariza, Z. A., Che Man, Y. B., Jinap, S., & Bakar, J. (2005). Detection of lard adulteration in cake formulation by Fourier transform infrared (FTIR) spectroscopy. *Food Chemistry*, 92, 365–371.

Tasara, T., Schumacher, S., & Stephan, R. (2005). Conventional and real-time PCR-based approaches for molecular detection and quantification of bovine species material in edible gelatin. *Journal of Food Protection*, 68 (11), 2420-2426.

Termine, J. D., Peckauskas, R. A., & Posner, A. S. (1970). Calcium phosphate formation in vitro II. Effects of environment on amorphous-crystalline transformation. *Archives of Biochemistry and Biophysics*, 140, 318–325.

Veis, A. (1964). The Macromolecular Chemistry of Gelatin. New York and London: Academic Press, p. 196.

Venien, A., & Levieux, D. (2005). Differentiation of bovine from porcine gelatines using polyclonal anti-peptide antibodies in indirect and competitive indirect ELISA. *Journal of Pharmaceutical and Biomedical Analysis*, 39, 418–424.

Ward, A. G. (1958). Conversion of collagen to gelatin, and chemical composition. In: Stainsby, ed. Recent Advances in Gelatin and Glue Research. London, New York, Paris, Los Angeles: Pergamon Press.

Zhang, G., Liu, T., Wang, Q., Chen, L., Lei, Luo, J., Ma, G., & Su, Z. (2009). Mass spectrometric detection of marker peptides in tryptic digests of gelatin: A new method to differentiate between bovine and porcine gelatin. *Food Hydrocolloids*, 23, 2001-2007.