Title: A METHOD TO DIFFERENTIATE GELATIN CAPSULE OF THE PORCINE SOURCE FROM THE BOVINE SOURCE

Abstract: A method to differentiate a gelatin capsule of a porcine source from a bovine source which comprising the steps of: (a) extracting a protein from the gelatin capsule using ammonium sulphate precipitation, wherein the amount of sample used is between 150mg to 400mg, preferably 150mg, which is to be dissolved in a 0.9mL to HmL, preferably 10mL, of a liquid such as distilled water at a temperature of between 35°C to 50°C, preferably 40°C; and, (b) separating the protein using gel electrophoresis technique, 7.5% SDS-PAGE, which runs at 200V.
A METHOD TO DIFFERENTIATE GELATIN CAPSULE OF THE PORCINE SOURCE FROM THE BOVINE SOURCE

FIELD OF INVENTION
The present invention relates to a method to differentiate a gelatin capsule of a porcine source from that of a bovine source. This method comprises extracting proteins from said gelatin capsule and separating said proteins according to their molecular weights using a gel electrophoresis technique.

BACKGROUND OF INVENTION
Islam is a way of life, a comprehensive religion guiding the lives of its followers through sets of rules governing the personal, social and public aspects. Driven by the fast-growing Muslim population, halal products are headed towards wider popularity. Thus, this depiction also shows that the needs for halal products in the future will also be increasing. In the medical field for example, there are a lot of capsulated medications and supplements that are put in the market utilize gelatin for the capsules. Since the gelatin may be derived from a porcine or bovine source, Muslims and other non-pork consumers will usually try to avoid such capsulated medications or supplements unless they are confident that the capsules are not from a porcine source.

There have been some known techniques used to determine whether the gelatin capsules are either of porcine or bovine source. Such techniques include principal component analysis (PCA), enzyme-linked immunosorbent assay (ELISA) and calcium phosphate precipitation (CCP). However, these techniques have their own setbacks which limit their effectiveness and reliability. The PCA involves a lengthy and costly procedure, where gelatin will undergo acid hydrolysis to form amino acids, the amino acids will then derivatize prior to HPLC separation. Time needed for the whole procedure is ~24 hours. In addition, PCA reduces the dimensionality of data set and this may affect its effectiveness in analyzing the data. The ELISA is a very costly technique as it requires the use of specific antibody. CCP technique is based on the formation of calcium phosphate
precipitation measured by the pH drop method, this technique depend strongly on the known concentration of pure bovine or porcine gelatin and therefore in the situation where the source of gelatin is not pure, the technique cannot be used to differentiate between the bovine and porcine source of gelatin.

Thus, it is the objective of the present invention to provide a method to differentiate the gelatin capsule of porcine source and that of a bovine source in capsulated medicine and supplements whereby the method desirable is simple, rapid, reliable and economical as compared to other known methods in the literature as described above.

It shall be noted herein that although a non-porcine source of gelatin may not be the sole factor to determine whether the gelatin is halal, as halal is also determined by other factor i.e. slaughtering of the animals has to be in line with the Syarak Law, this method can lower the risk of Muslims and non-pork consumers from consuming gelatin capsules from the porcine source.

SUMMARY OF INVENTION
According to the object of the invention, there is provided a method to differentiate a gelatin capsule of a porcine source from that of a bovine source comprising the steps of:
(a) extracting proteins from said gelatin capsule using ammonium sulphate precipitation; and,
(b) separating said proteins according to their molecular weights using a gel electrophoresis technique.

In accordance with the present invention, proteins from a bovine source gelatin produce a first band at molecular weights 130-140kDa and a second band at molecular weights 120-130kDa and, proteins from a porcine source gelatin produce a first band at molecular weights 235-265kDa, a second band at molecular weights 115-130kDa and a third band at molecular weights 105-105kDa.
The present invention consists of several novel features and a combination of parts hereinafter fully described and illustrated in the accompanying description and drawing, it being understood that various changes in the details may be made without departing from the scope of the invention or sacrificing any of the advantages of the present invention.

**BRIEF DESCRIPTION OF THE DRAWING**

The present invention will be fully understood from the detailed description given herein below and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, wherein:

Figure 1 is an image obtained from an analysis of four different gelatin capsule samples and four standards using the method of the present invention.

Figure 2 is an image obtained from an analysis of another one gelatin capsule sample and three standards using the method of the present invention.

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

The present invention is basically based on the principle that gelatin of different source consists of different mixture of proteins. With this principle in mind, the inventor created a method to extract the proteins from the gelatin capsule using ammonium sulphate precipitation. These proteins will then undergo separation according to their molecular weights using gel electrophoresis (i.e. 7.5% SDS-PAGE, which runs at 200V). As different proteins have different mobility in SDS-PAGE, the different profiles of protein from bovine and porcine gelatin can be used to determine the source of the gelatin by comparing to those from the standard bovine and porcine gelatin.

The method in accordance with the present invention comprises the steps of:

(a) Gelatin capsule was cut using a clean scissors, its content was removed and the capsule was cleaned using a piece of blotter paper.

(b) 150mg of capsule and 0.835g of ammonium sulphate were weighed.
(c) The capsule was dissolved in 10 mL of distilled water in a 100mL beaker that was maintained in a water bath at 40°C. This is essential as gelatin usually dissolves at temperature above 30°C.

(d) After the gelatin dissolved, ammonium sulphate salt was added little by little into the solution that was under constant stirring using a magnetic bar.

(e) After added all the salt, the solution was left for 30 minutes under constant stirring.

(f) After 30 minutes, 1 mL of the supernatant was collected into 1mL microcentrifuge tube. The solution must be under constant stirring when the supernatant is pipetted out. This is important to ensure there is no precipitated protein sedimented to the base of the beaker.

(g) The tube was then centrifuged at 14000rpm for 20 minutes at 35°C. This is to spin down the precipitated protein.

(h) After centrifugation, the supernatant was decanted from the tube. Pellet may or may not be visible at the bottom or side of the tube as it depends on the amount of protein in the capsule. No pellet seen may indicate that only a minute protein has been spin down.

(i) The pellet was then re-suspended with 70µL of TSE buffer. This allows the protein to dissolve in the buffer. Note: The volume of TSE buffer needed may differ from samples to samples in order to be visible in SDS-PAGE.

(j) After re-suspension, the tube was vortexed for about 10 seconds to mix the protein in the buffer homogeneously. Note: If the pellets did not dissolve completely in the buffer e.g. in coloured hard gelatin capsules, centrifuge the solution at 500rpm for 4 minutes at 35°C to spin down the un-dissolved impurities. Collect the supernatant.

(k) 20µL of solution was then transferred from the tube into a new microcentrifuge tube containing 5µL of sample buffer. The mixture was vortexed briefly and then heated at 95°C for 4 minutes.

(l) Steps (a) to (k) were repeated for both standard bovine and porcine.

(m) After step (k), 15µL of each sample and standards were loaded into the wells of the 7.5% 8cm x 10cm polyacrylamide gel.
After all samples and standards were loaded, the gel was subjected to electrophoresis separation at 200V for 50 minutes.

Once the run ended, the gel was transferred immediately into a container and immersed in Commassie blue staining solution for 1 hour.

After 1 hour, the staining solution was discarded and the gel was soaked in the de-staining solution for 30 minutes. It was then replaced with fresh de-staining solution for another 30 minutes and finally the gel was soaked in distilled water.

The image of the gel was then captured using VersaDoc Imaging System and analysed with the Quantity One 1-D Analysis software (Bio-Rad, USA).

The method as described above used most preferred parameters. However, in this invention, a suitable amount of gelatin capsule pieces to extract the protein from the sample in step (b) ranges between 150mg to 400mg and, the suitable amount of distilled water used to dissolve said gelatin capsule pieces may be between 9mL to 10mL. The distilled water was to be kept at constant temperature in a water bath at a temperature between 35°C to 50°C to dissolve the gelatin as gelatin usually dissolves in a temperature above 30°C.

In the above steps, (a) - (k) comprises the steps to extract protein from the gelatin capsule samples and the standards and preparing the same to be used in gel electrophoresis. The gel electrophoresis, i.e. steps (m) - (p), is to separate said proteins according to their molecular weights. The band images of the separated proteins in the gel are then used to determine the source of the gelatin capsule.
In accordance with the method of present invention, below is the formulations for:

1. **TSE buffer**
   - 40mM TRIS 12.5mL
   - 10% (w/v) SDS, pH 8.8 5mL
   - 1mM EDTA 0.01861g
   - Distilled water 32.5mL
   - Total volume 50mL

2. **Sample Buffer**
   - Distilled water 3.8mL
   - 0.5M Tris-HCl, pH 6.8 1.0mL
   - Glycerol 0.80mL
   - 10% (w/v) SDS 1.6mL
   - 2β-mercaptoethanol 0.4mL
   - 0.05% (w/v) bromophenol blue 0.4mL
   - Total volume 8.0mL
   - *2β-mercaptoethanol is to be added freshly*

3. **Staining Solution**
   - Distilled water 500mL
   - 0.1% (w/v) Coomassie Blue R250 1.0g
   - Methanol (40% v/v) 400mL
   - Acetic acid (10% v/v) 100mL
   - Total volume 1.0L

4. **De-staining Solution**
   - Distilled water 500mL
   - Methanol (40% v/v) 400mL
   - Acetic acid (10% v/v) 100mL
   - Total volume 1.0L
The gel electrophoresis, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (7.5% SDS-PAGE, which runs at 200V) is performed as described by the modified method of Laemmli (1970). The preparation of 7.5% Polyacrylamide Gel (Size 8cm x 10cm x 1.0mm) is as follows:

(i) Resolving Gel Preparation:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>4.85</td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
<td>2.5</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>100</td>
</tr>
<tr>
<td>30% (w/v) acrylamide/ bis-acrylamide</td>
<td>2.5</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>50</td>
</tr>
<tr>
<td>TEMED</td>
<td>5</td>
</tr>
<tr>
<td>Total monomer</td>
<td>10</td>
</tr>
</tbody>
</table>

The resolving gel solution was prepared by adding and mixing all the reagents shown in the formula above except for ammonium persulfate and TEMED which was added only after all the other reagents have been added, the mixture was swirled gently and transferred quickly into gel casting glass plates to a level of 5cm from the top. Then, a small volume of water saturated in butanol solution (water: butanol 1:1) was filled on the surface of the solution to level the gel surface layer. When the gel had completely polymerized (20 minutes), the water saturated butanol solution was removed and the gel was rinsed with distilled water.

(ii) Stacking Gel Preparation:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.03</td>
</tr>
<tr>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>1.25</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>50</td>
</tr>
<tr>
<td>30% (w/v) acrylamide/ bis-acrylamide</td>
<td>665</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>25</td>
</tr>
<tr>
<td>TEMED</td>
<td>5</td>
</tr>
<tr>
<td>Total monomer</td>
<td>5</td>
</tr>
</tbody>
</table>
The stacking gel solution was prepared by adding and mixing all the reagents shown in the above formula except for ammonium persulfate and TEMED, which was added after all the other reagents were added. After the addition of ammonium persulfate and TEMED, the mixture was swirled gently and transferred into the polymerized resolving gel (mentioned earlier) until the brim. Then, a well forming comb was inserted immediately. After the stacking gel had polymerized completely (50 minutes), the comb was removed. The wells formed were filled with electrode buffer before samples were loaded.

(iii) **Electrode buffer (Running Buffer)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Tris base (FW 121.1)</td>
<td>3.03g</td>
</tr>
<tr>
<td>Glycine (FW 75.07)</td>
<td>14.4g</td>
</tr>
<tr>
<td>SDS (FW 288.38)</td>
<td>1.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>q.s.</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>100OmL</td>
</tr>
</tbody>
</table>

An analysis has been carried out on five different products (samples) available on the shelf using the above-stated method and the images as in Figure 1 and Figure 2 were obtained wherein in the samples, bands (3) and (4) indicate bovine softgel, (6) and (7) indicate bovine hard gelatin and (11) indicates porcine hard gelatin whereas in the standard, band (1) and band (13) indicate a protein marker (unstained), band (5) and band (9) indicate bovine gelatin standard (from Halagel) and bands (2), (8) and (12) indicate porcine gelatin standard (manufactured by Sigma). Band (10) indicate the mixture of bovine and porcine standards in a similar proportion, which the profile is more similar to porcine.

The images of Figure 1 and Figure 2 showed the markers used to differentiate bovine and porcine gelatin. In bovine, there are two bands at molecular weights 130-140kDa and 120-130kDa (circle) whereas in porcine, there is a band at MW 235-265kDa (rectangular box) and there are two bands at MW 115-130kDa and 105-110kDa (circle). The four samples in FIG.1, i.e. (3), (4), (6), and (7) showed bands which are similar to those of bovine standards (5) and (9). Hence, it is
concluded that all these four capsules are from the bovine source. On the other hand, one sample in Figure 2, i.e. (11) showed bands which are similar to those of porcine standard, (2), (8), (10) and (12). Hence, it is concluded that this sample (11) is from porcine source.

As described above, the present invention relates to a method to differentiate the gelatin capsule of the porcine source from the bovine source wherein this description above describes the present invention according to its preferred embodiments. However, it is to be understood that limiting the description to the preferred embodiments of the invention is merely to facilitate discussion of the present invention and it is envisioned that those skilled in the art may devise various modifications and equivalents without departing from the scope of the appended claims.
CLAIMS

1. A method to differentiate a gelatin capsule of a porcine source from that of a bovine source comprising the steps of:
   (a) extracting proteins from said gelatin capsule using ammonium sulphate precipitation; and,
   (b) separating said proteins according to their molecular weights using a gel electrophoresis technique.

2. A method according to Claim 1 comprising the steps of:
   (a) dissolving 150-400mg said gelatin capsule in 9-11 mL of distilled water that is maintained in water bath at a temperature 35°C to 50°C;
   (b) adding in small amounts of a total of 0.835g ammonium sulphate salt into said capsule solution in step (a) while keeping the mixture solution under constant stirring;
   (c) leaving the mixture solution in step (b) to rest under constant stirring for about 30 minutes;
   (d) pipetting 1 mL of supernatant from said solution mixture in step (c) into a 1mL microcentrifuge tube while keeping said mixture solution under constant stirring;
   (e) centrifuging said supernatant in step (d) at 14000rpm for 20 minutes at 35°C to spin down precipitated protein;
   (f) decanting said supernatant to collect said precipitated protein;
   (g) re-suspending said protein in TSE buffer to completely dissolve said protein in said buffer and vortexing said mixture for about 10 seconds to homogeneously mix said protein in said buffer;
   (h) transferring 20µL of said solution in step (g) into a new microcentrifuge tube containing 5µL of sample buffer, vortexing said mixture briefly and heating said mixture at 95°C for 4 minutes;
(i) repeating steps (a) - (h) for both standard bovine and porcine;

(J) loading 15µL of each sample and standards from step (h) and (i) into wells of 7.5% 8cm x 10cm polyacrylamide gel and subjecting said gel to electrophoresis separation at 200V for 50 minutes;

(k) immediately transferring said gel into a container and immersing said gel in Commassie blue staining solution for 1 hour;

(l) discarding said staining solution and soaking said gel in destaining solution for 30 minutes;

(m) replacing said destaining solution with fresh destaining solution and soaking said gel further for another 30 minutes;

(n) soaking said gel in distilled water before capturing the image of said gel using VersaDoc Imaging System; and

(o) analyzing said image with the Quantity One 1-D Analysis software (Bio-Rad, USA).

3. A method according to Claim 2 wherein said gelatin capsule in step (a) is 150 mg.

4. A method according to Claim 2 wherein said distilled water in step (a) is 10 mL.

5. A method according to Claim 2 wherein said water bath in step (a) is 40°C.

6. A method according to Claim 2 wherein said mixture in step (h) is heated in boiling water for 4 minutes before being loaded into said polyacrylamide gel wells.

7. A method according to Claim 2 wherein said TSE buffer in step (g) is 70µL.
8. A method according to Claim 2 or Claim 7 wherein said TSE buffer includes 12.5mL of 40mM TRIS, 5mL of 10% (w/v) SDS, pH 8.8, 0.01861g of 1mM EDTA and 32.5mL of distilled water, with a total volume of 50mL.

9. A method according to Claim 2 wherein said sample buffer in step (h) includes 3.8mL of distilled water, 10mL of 0.5M Tris-HCl, pH 6.8, 0.8mL of glycerol, 1.6mL of 10% (w/v) SDS, 0.4mL of 2β-mercaptoethanol and 0.4mL of 0.05% (w/v) bromophenol blue, with a total volume of 8.0mL.

10. A method according to Claim 9 wherein said 2β-mercaptoethanol is added freshly.

11. A method according to Claim 2 wherein said Commassie blue staining solution in step (k) includes 500mL of distilled water, 1.0g of 0.1% (w/v) Commassie Blue R250, 400mL of methanol (40%v/v) and 100mL of acetic acid (10%v/v), with a total volume of 1.0L.

12. A method according to Claim 2 wherein said de-staining solution in step (l) includes 500mL of distilled water, 400mL of methanol (40%v/v) and 100mL of acetic acid (10%v/v), with a total volume of 1.0L.

13. A method according to Claim 2 wherein said polyacrylamide gel in step (k) comprises resolving gel of 4.85mL of distilled water, 2.5mL of 1.5M Tris-HCl, pH 8.8, 100µL of 10% (w/v) SDS, 2.5mL of 30% (w/v) acrylamide/ bis-acrylamide, 50µL of 10% ammonium persulphate and 5µL of TEMED, with a total volume of monomer of 10mL.

14. A method according to Claim 2 wherein said mixture in step (g) is centrifuged further at 500 rpm for 4 minutes at 35°C to spin down undissolved impurities, and resulting supernatant is collected to be used in
the subsequent step (h), if said protein does not dissolve completely in said TSE buffer.

15. A method according to Claim 1 or Claim 2 wherein proteins from a bovine source gelatin produce a first band at molecular weights 130-140kDa and a second band at molecular weights 120-130kDa and, proteins from a porcine source gelatin produce a first band at molecular weights 235-265kDa, a second band at molecular weights 115-130kDa and a third band at molecular weights 105-110kDa.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

GOIN27/447 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum classification of subject matter (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

EPOQUE, WPI, CAPLUS, FSTA, AGRICOLA, keywords search: gelatin+, collagen, pork, porcine, pig, capsul+, gel 3w electrophor+, ammonium 2w sul(ph)ate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<td>MCBAIN, J. W. et al &quot;The Salting Out of Gelatin into Two Liquid Layers with Sodium Chloride And Other Salts&quot; Journal of General Physiology (1928) pages 1-15</td>
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* Special categories of cited documents:

'A' document defining the general state of the art which is not considered to be of particular relevance

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'P' document published prior to the international filing date but later than the priority date claimed

'I' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

'&' document member of the same patent family

Date of the actual completion of the international search: 15 July 2010

Date of mailing of the international search report: 06 AUG 2010

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