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Animal Species Determination in Sausages using an SDS-PAGE technique

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Introduction

In Turkey, one million tons of meat, which plays an important role in human nutrition, are produced per year, 11 % of this amount are consumed as meat products (UĞUR, et al.,1998). For moral and religious reasons, the consumer wants to know the origin of the meat that he is going to eat. For this purpose, studies on the determination of the origin of meat or meat products have been carried out for many years (ERECIN and HASSA, 1952; HITCHCOCK and CRIMES, 1985; HVASS, 1985; PATTERSON and JONES, 1985). In our country, some producers try to make profit by adding meats to meat products which originated from animal species which are not consumed in the country which are comparably cheap. This causes deception of the consumer and a potential risk for public health.

Anatomical and histological characteristics are being used to determine the species of whole carcasses and large pieces of meat (ERECIN and HASSA, 1952; INAL, 1992). For meat cut into small pieces, physical and chemical characteristics are being applied (HITCHCOCK and CRIMES, 1985; OMURTAG, 1959). Because these characteristics do not yield reliable results for meat mixtures and processed meats (HITCHCOCK and CRIMES, 1985; KANG'ETHE et al., 1982), species-specific proteins are being referred to to examine these kinds of products (HITCHCOCK and CRIMES, 1985). Using serological methods reliable results were obtained (DOBERSTEIN and GREUEL, 1985; HVASS, 1985; PATTERSON and SPENCER, 1985). But these methods are generally based on heat-labile proteins and are therefore unsuitable for heated meat and meat products (BABIKER et al., 1981; INAL, 1992; KING, 1984). Although antisera were prepared against heat-stable proteins (KANG'ETHE and GATHUMA, 1987), they did not give satisfactory results because they were present in only low concentrations and sometimes showed cross-reactions between species (KING, 1984; PATTERSON and SPENCER, 1985). For this reason, many researchers have recommended various electrophoretic methods for species identification because of their high sensitivity (BABIKER, 1951; BAUER and KELNER, 1989; PARISI and AGUIARI, 1985; SHERIKAR, 1988).

In this study, the possibility to identify animal species in sausages by means of SDS-PAGE was examined.

Materials and Methods

Material: In this study, sausages prepared from pure beef, pork and horsemeat and from mixtures of two or three of these species were examined. The different ratios used for the sample sausages are given in table 1.

Table 1. **Compositions of the samples**

Experimental groups	Beef (%)	Pork (%)	Horsemeat (%)
1	100	-	-
2	-	100	-
3	-	-	100
4	50	50	-
5	50	-	50
6	60	40	-
7	60	-	40
8	70	30	-
9	70	-	30
10	80	20	-
11	80	-	20
12	90	10	-
13	90	-	10
14	95	5	-
15	95	-	5
16	60	20	20
17	80	10	10

Extraction: The sausages were ground in a blender. Approximately 12 g were mixed with the same amount of distilled water. These mixtures were incubated at 37 °C for one hour, centrifuged at 3000 g/min. for 20 min and filtered through Whatmann No: 3 filter paper. The extracts obtained this way were stored at -18 °C in screw top plastic tubes until examination. The protein concentrations of the extracts were measured by the method of LOWRY et al. (1951).

Electrophoresis: For the experiment, stacking gels with 3 % acrylamide (pH 6.8) and separating gels with 10 % acrylamide were prepared by the method reported by LEE et al. (1976). The extracts were diluted with sample buffer (2 ml 0.625 M Tris-HCl buffer (pH 6.8), 0.2 g. SDS, 5 ml glycerine, 0.5 ml (-mercaptoethanol, 0.1 ml 1 % bromphenol blue). 50 µl of this mixture were pipetted into the wells. A current of 30–32 mA was applied for the stacking gel, then the current was increased to 60–62 mA for the separating gel. Staining was performed with Coomassie Blue 250-G disolved in 225 ml methanol, 22.5 g sulphosalicylic acid, 75 g trichloroacetic acid and 465 ml distilled water for 45 min at 60 °C. A solution containing ethanol, acetic acid and distilled water was used for destaining The gels were immersed in preserving solution consisting of 10 % glycerine in distilled water for an hour before drying The gels were then dried at room temperature for two days on an acetate sheet and evaluated.

Results

In pherograms of sausages made from pure beef, pork and horsemeat some differences in intensity and position of the protein bands were determined (Fig. 1 and 2). Pure pork and beef protein patterns showed three sharp bands in region II, while no bands were ob-

served in this region in horse protein band patterns. In horse protein band patterns an intense and thick band was determined in region IV. Furthermore, the intensity of the bands in region III was lower and the two bands determined in region V were closer to each other compared to beef and pork protein patterns. The band pattern of horsemeat also differed from that of the other species by the appearance of intense and thick bands in region VI and VII. In region VIII, there was only one band in horsemeat protein patterns, while a pair of bands was observed in the same region of pork and beef band patterns. Furthermore, in horse protein patterns two bands were determined in region IX, where the other two species' patterns showed only one band. Pork protein patterns were differentiated from beef protein patterns by the appearance of rather intense bands in region VI. In addition, the bands in region X were closer to each other in pork protein patterns than in beef protein patterns.

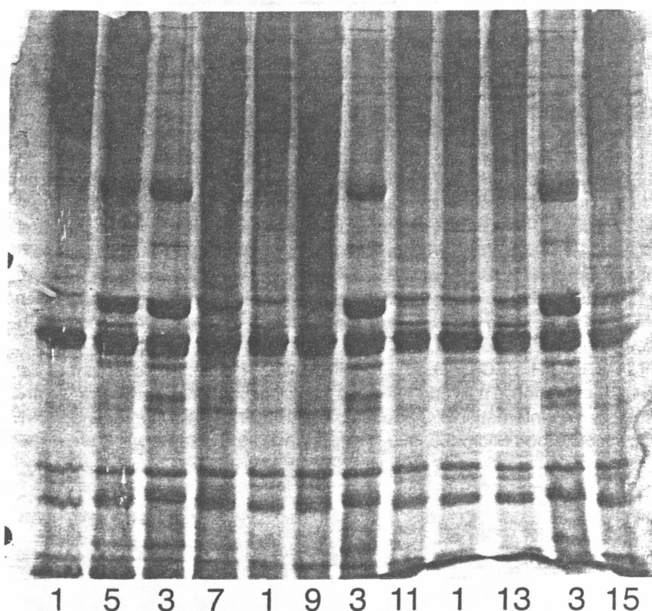

Fig. 3: Pherogram of pure beef (1) and pure horse meat (3) sausages and sausages composed of beef mixed with 50 % (5), 40 % (7), 30 % (9), 20 % (11), 10 % (13), 5 % (15) horsemeat

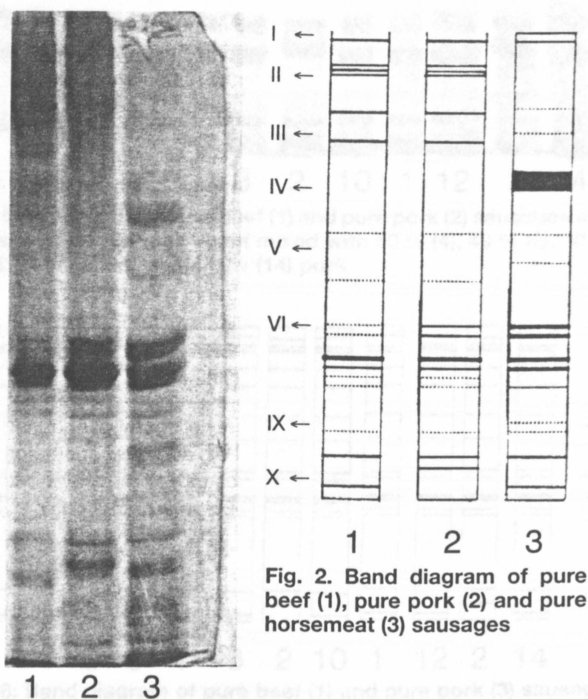

Fig. 1. Pherogram of pure beef (1), pure pork (2) and pure horsemeat (3) sausages

Fig. 2. Band diagram of pure beef (1), pure pork (2) and pure horsemeat (3) sausages

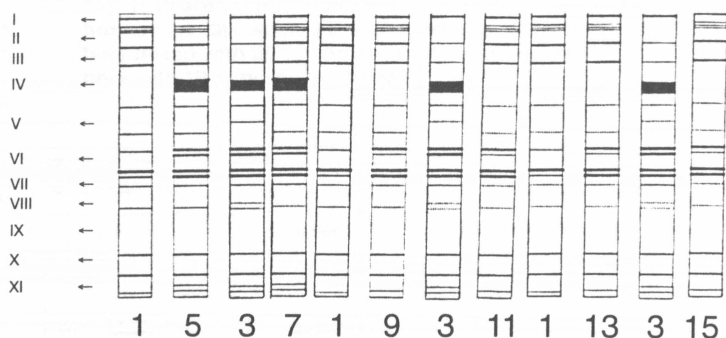

Fig. 4: Band diagram of pure beef (1) and pure horse meat (3) sausages and sausages ccomposed of beef mixed with 50 % (5), 40 % (7), 30 % (9), 20 % (11), 10 % (13), 5 % (15) horsemeat

In pherograms of sausages prepared from mixtures of beef and horsemeat in varying proportions three thin bands similar to those seen in beef sausages were determined in region II in all mixtures of these two species (Fig. 3 and 4). While the intensity of the bands in region III was low in pure horse sausages, the bands in the same region of beef and horsemeat mixtures were observed as intense, like in pure beef sausages. In protein patterns of sausages prepared by mixing horsemeat with beef in various quantities, two weak bands in region V were determined comparably far from each other, similar to beef sausages. In mixtures containing 50 and 40 % horsemeat, the bands of regions VI, VII, VIII appeared in a pattern similar to that of 100 % horsemeat. In addition, in these mixtures band IV was detected, which is characteristic for horsemeat. By reducing the amount of horsemeat in the mixture, band VI, VII and VIII lost their intensity and band IV was not detectable any more. The second band in region VI was determined in mixtures of as little as 5 % horsemeat, yet showed a very low intensity.

In pherograms of pork and beef mixtures, only band V was discovered to be more intense in sausages containing any amount of pork, compared to the bands in the same region of pure beef sausages (Fig. 5 and 6). No further differences between beef and pork mixed beef sausages were observed in the other bands.

In protein patterns of sausages containing 60 % beef, 20 % horsemeat and 20 % pork the observation of band VI revealed an intense colour indicates the presence of horsemeat and pork in the mixture (Fig. 7 and 8). The same results were observed for sausages with 10 % horsemeat and 10 % pork. In these mixtures containing beef, horsemeat and pork no other bands characteristic for horsemeat and pork were determined.

Discussion

In this study, the SDS-PAGE technique was used to determine the meat species in heat processed sausages. Many researchers have claimed that discontinuous gel systems lead to a better separation of proteins compared to homogeneous gels (LEE et al, 1975; RING et al., 1982). In the present study combining this method with the SDS-PAGE technique has improved the separation capability and therefore the sensitivity of determination. LEE et al. (1976) obtained reliable results in their study, in which they used a discontinuous gel system to determine non-meat proteins in meat products. Similar to this, RING et al. (1982) explained that it is possible to distinguish even closer related species with this system.

The different intensity and position of the protein bands in protein patterns of pure beef, pork and horsemeat sausages were considered important criteria. As a matter of fact, the differences in intensity and position of the bands in regions number VI and X of pork sausage band patterns compared to bands of beef sausage, were used to determine the animal species of the sausages in this study. The results of BAYRAMLAR (1992), who detected two closer bands on the anode side of pork pherograms in comparison to beef electrophoretic patterns, showed similarity to our results. In the same way, several other researchers (BAUER and HOFMANN, 1989; HOFMANN, 1989; TINGBERGEN and OLSMAN, 1976) have claimed that they could differentiate pork from beef using different electrophoretic techniques.

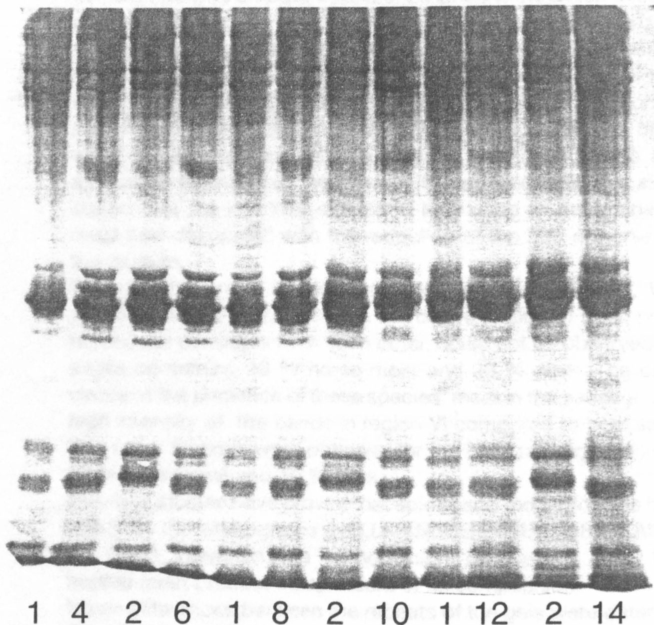

Fig. 5: Pherogram of pure beef (1) and pure pork (2) sausages and sausages composed of beef mixed with 50 % (4), 49 % (6), 30 % (8), 20 % (10), 10 % (12), 5 % (14) pork

Fig. 6: Band diagram of pure beef (1) and pure pork (3) sausages and sausages composed of beef mixed with 50 % (4), 40 % (6), 30 % (8), 20 % (10), 10 % (12), 5 % (14) pork

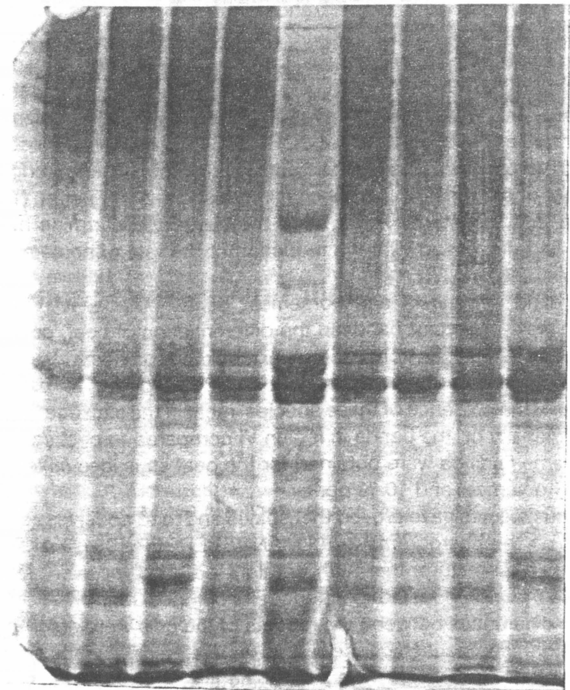

Fig. 7: Pherogram of pure beef (1), pure pork (2), pure horsemeat (3) sausages and sausages consisting of beef mixed with 20 % horsemeat and 20 % pork (16) or beef with 10 % horsemeat and 10 % pork (17)

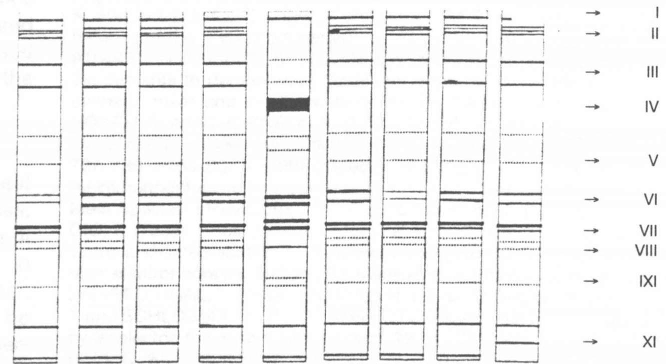

Fig. 8: Band diagram of pure beef (1), pure pork (2), pure horsemeat (3) sausages and sausages consisting of beef mixed with 20 % horsemeat and 20 % pork (16) or beef with 10 % horsemeat and 10 % pork (17)

As indicated in figure 1, the distinction between the band patterns of horsemeat sausage and the band patterns of beef and pork sausages in regions II, III, V, VI, VII, VIII, IX and the existence of band IV which is characteristic for horsemeat has made it possible to identify horsemeat. Similarly, several researchers (HOFMANN, 1989; TINGBERGEN and OLSMAN, 1976) have claimed that they have identified horsemeat by using various electrophoretic techniques.
Many researchers have stated the possibility of identifying raw meat species (CIMEN, 1989; HOFMANN and BAUER, 1986; HOFMANN and BLUECHEL, 1986). HÖYEM and THORNSON (1970) have proved in their study that it is possible to identify raw whale meat, horsemeat and beef using electrophoretic methods. Similarly, SKRÖKKI and HORMI (1994), were able to determine the origin of raw minced meat by detecting species-specific proteins. However, in both studies it is emphasized that the methods were suitable only for unheated products. Besides, further researchers (CRESPO and OCKERMAN, 1974; HOFMANN et al., 1996; LEE et al., 1974; YOWELL and FLURKELEY, 1986) have also reported the negative effect of heat on proteins. CRESPO and OCKERMAN (1977) have investigated the effect of heat on chicken breast and leg meat with SDS-PAGE and have determined that the number and the intensity of the protein bands were reduced when temperatures were increased. YOWELL and FLURKELEY (1986) have reported that 76 % of the protein bands of fish meat disappeared after heating in a microwave oven. However, in our study in the case of the protein patterns of sausages prepared from pure beef, pork and horsemeat which were heated at 75 °C for 15 min, it has been possible to determine that many bands are different in intensity and position, and that it is possible to determine the species by evaluating these differences. Similarly, determining characteristic bands for horsemeat together with bands of beef, it was possible to identify the two species in beef sausages mixed with 50 % and 40 % horsemeat. ZERIFI et al. (1991) have claimed that animal species differentiation is possible with horse meat and beef down to a horsemeat content of 10 %. They have also shown that a weak band used for the identification of horsemeat could be detected in only some gels when the sausages contained only 5 % horsemeat. Corresponding to this, in our experiments we could differentiate horsemeat and pork at amounts of 5 %.

The determination of band number VI, which appeared intense in pure pork, and also showed an intense colour in beef sausages mixed with as little as 5 % pork was used to differentiate pork meat in the mixture. These results were in accordance with those of ZERIFI et al. (1991), who showed that it is possible to determine the animal species in heated mixtures of pork and beef.

In sausages which contained beef and 50 or 40 % horsemeat, the determination of bands in region VI and VII similar to pure horsemeat

protein patterns and the appearance of band IV, which is characteristic for horsemeat, in combination with typical beef bands proved the presence of these two species in the mixture. The intensity of the second band in region VI decreased as the amount of horsemeat was reduced but it was still visible even in sausages with 5 % horsemeat. As a matter of fact, JEMMI and SCHLOSSER (1993) have discovered a relationship between the intensity of protein bands and the amount of the meat of the species in question. ZERIFI et al. (1991) stated that the intensity of a band they used to determine horsemeat had decreased with the reduction of the rate of horsemeat in the mixture.

Pork band number IX and horsemeat bands number IV, VII, VIII, which were important bands in the differentiation of beef, pork and horsemeat sausages from each other, could not be observed in sausages containing 20 % horse meat and 20 % pork. The only evidence of the presence of these species' meat in the sausage was the high intensity of the bands in region VI compared to beef sausage. The same findings were obtained for the beef sausages mixed with 10 % horsemeat and 10 % pork.

Previous studies have proved that spices and food additives have no effect on the pherograms (HELLMANNSBERGER,1981; KAISER et al., 1982). Therefore, the method might be used for every kind of heated meat product independent of its composition.

Minor differences between the repeats of the gels were determined. Therefore, comparison of the results with reference samples directly included in each gel is recommended. Some other researchers have suggested the same solution to this problem (BAUER and KELNER, 1989; CIMEN, 1989; HOFMANN, 1986; IMRE and BILGIC, 1986).

Conclusion: The species-specific position and intensity of the bands in pure beef, pork and horsemeat sausage pherograms were observed. By evaluation of these bands the identification of these three species was possible in sausages heated at 75 °C for 15 min. In sausages with a horsemeat content of 40 and 50 % the presence of horsemeat could be determined as well. In the sausages prepared from a mixture of pork, horsemeat and beef the method used also allowed the determination of the presence of foreign meat in the mixture.

Summary

In this study, the possibility of animal species determination of meat used in heat-treated sausages was demonstrated. For this purpose, sausages made from pure beef, pork and horsemeat and mixtures of two or three of these species were heated at 75 °C for 15 min. In pherograms of sausages made from pure beef, pork and horsemeat, differences that distinguish these three species could easily be determined by their staining densities and positions. It was determined in pherograms of mixtures that were prepared by adding horsemeat and pork to beef in defined rates that protein bands characteristic for beef kept their intensity while the intensities of the bands typical for pork and horsemeat had decreased. In pherograms of sausages that contained a large amount of horsemeat (50 and 40 %), the determination of bands IV and VI, which are characteristic for horse, lead to the identification of horse meat in the mixture. Below these mixture rates, down to 5 % horsemeat and pork mixed into beef sausages, it was concluded that the sausage was not made from only beef by determination of band VI which was darker compared to beef sausage.

Zusammenfassung

In der vorliegenden Arbeit wurde eine Methode zur Tierartenbestimmung von Fleisch in hitzebehandelten Würsten geprüft. Zu diesem Zweck wurden Würste aus reinem Rind-, Schweine- und Pferdefleisch sowie Mischungen aus zwei bis drei dieser Spezies hergestellt und für 15 min auf 75 °C erhitzt. In den Pherogrammen der Würste, die aus reinem Rind-, Schweine- und Pferdefleisch hergestellt waren, konnten diese drei Spezies anhand der Bandenpostition und –farbintensität leicht unterschieden werden. Bei den Pherogrammen der Mischungen, welche durch die definierte Zugabe von Pferde- und Schweinefleisch zu Rindfleisch hergestellt worden waren, behielten die für Rindfleisch charakteristischen Proteinbanden ihre Intensität, wohingegen die für Schweine- und Pferdefleisch charakteristischen Banden eine geringere Intensität aufwiesen. In den Pherogrammen der Würste mit hohem Pferdefleischanteil (50 und 40 %) führte die Bestimmung der pferdecharakteristischen Banden IV und VI zur Identifizierung. Bei Rindfleischwurst-Rezepturen mit Zumischungsraten von bis hinab zu 5 % Pferde- und Schweinefleisch konnte anhand der Bande VI darauf geschlossen werden, daß die Wurst nicht ausschließlich aus Rindfleisch bestand. Die Bande VI war dann dunkler als bei reiner Rindfleischwurst.

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Untersuchungen zur Optimierung des Salmonellennachweises in Milch und Milcherzeugnissen

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1. Einleitung

Bei Anwendung der üblichen kulturellen Methoden zur Isolierung von Salmonellen aus Lebensmitteln (z. B. Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG, Abb. 1) mit den Schritten Voranreicherung — Selektivanreicherung — Nachweis — Bestätigung liegt ein negatives Ergebnis erst am fünften Tag der Untersuchung von. Es gibt zahlreiche Ansätze, dieses Verfahren zu verkürzen. In den vorliegenden Untersuchungen an Milchtrockenprodukten, Rohmilch und Weichkäse wurde versucht, die selektive Anreicherung durch Einbeziehung der Immunomagnetischen Separation bzw. der Immunokonzentration zu umgehen und gleichzeitig den Nachweis auf festen Nährböden durch ein immunologisches Verfahren zu ersetzen. Da mit Salmonellen natürlich kontaminierte Proben nicht in ausreichendem Maße zur Verfügung standen, wurde künstlich kontaminiertes Material verwendet, wobei es im Fall der Milchtrockenprodukte notwendig war, die Keime vor der Kontamination subletal zu schädigen.

2. Material und Methodik

2.1 Material

2.1.1 Bakterienstämme und Untersuchungsmaterial

Die zur künstlichen Kontamination verwendeten fünf *Salmonella*-Serovaren (*S.* I 4,12:D:-; *S.* Derby O:5-; *S.* Infantis; *S.* Livingstone; *S.* Bareilly), Isolate aus Lebensmitteln, wurden der Stammsammlung des Lehrstuhls für Hygiene und Technologie der Milch entnommen. Als Untersuchungsmaterial dienten vier verschiedene Milchtrockenprodukte: Molkenpulver und Süßmolkenpulver unterschiedlichen Demineralisierungsgrades, eiweißangereichertes Molkenpulver sowie Sprühmagermilchpulver. Die Rohmilch bestand zur Hälfte aus Vorzugsmilch des Handels, die andere Hälfte der Proben stammte von einem Milch-ab-Hof erzeugenden Betrieb. Der für die Untersuchungen verwendete Weichkäse setzte sich aus Käse aus wärmebehandelter Milch sowie Rohmilchkäse zusammen, wobei jeweils die Standardsorten Camembert, Brie, Limburger und Münsterkäse verwendet wurden.

2.1.2 Subletale Schädigung der Salmonellen

Hirn-Herz-Bouillon (BHI) (Merck 110493); Phosphate buffered saline (PBS) pH 7,3 [Natriumchlorid zur Analyse 6,79 g (Merck 106404), di-Natriumhydrogenphosphat wasserfrei 1,47 g (Merck 106586), Kaliumdihydrogenphosphat 0,43 g (Merck 104871), Aqua dest. 1 l]; Caseinpepton-Hefeextrakt-Glucose-Agar mit Magermilch (PC-Agar, Oxoid CM 681) und erhöhtem Agar-Agar-Anteil; Reagenzgläser für die subletale Schädigung (Assistent, starkwandig mit rundem Boden und glattem Rand No. 2775/7 ca. 100 x 12 mm Ø).

2.1.3 Referenzverfahren (Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG, L 00.00-20)
Gepuffertes Peptonwasser (GPW) (Oxoid CM 509); Rappaport-Vassiliadis Sojamehlpepton- (RVS) Anreicherungslösung (Oxoid CM 866); Selenit-Cystin-Medium [Selenit-Cystin-Lösung-Basis (Oxoid CM 699), Natriumbiselenit (Oxoid LP 121)]; Mannit-Lysin-Kristallviolett-Brillantgrün (MLCB)-Agar (Oxoid CM 783); Brillantgrün-Phenolrot-Lactose-Saccharose (BPLS)-Agar, modifiziert (Oxoid CM 329).

2.1.4 Kommerzielle Testkits

VIDAS *Salmonella* (SLM) (bioMérieux 30 702) [AOAC Official Method 996.08 *Salmonella* in Foods, Enzyme Linked Immunofluorescent Assay Screening Method (VIDAS *Salmonella* (SLM) Assay), First Action 1996]; VIDAS Immuno-Concentration *Salmonella* (ICS) (bioMérieux 30 435); für die vollautomatische Durchführung wird ein VIDAS-Gerät (miniVIDAS Automated Immunoassay System, bioMérieux) benötigt.
Immunmagnetische Separation (IMS): Dynabeads anti-*Salmonella* (Dynal 710.01); Waschpuffer: 0,15 mol/l NaCl, 0,01 mol/l Natriumphosphatpuffer (pH 7,4) mit 0,05 % Tween 20 (Merck 109280); für die Durchführung der IMS wird ein Magnetic Particle Concentrator für Eppendorf Reaktionsgefäße (Dynal MPC-M 120.09) benötigt.

2.2 Methodik

2.2.1 Subletale Schädigung der Salmonellen

Die Durchführung der subletalen Schädigung findet sich in Abb. 2. Hierbei wurden die Keime zunächst in 5 ml BHI angezüchtet (37 °C, 24 h), wobei regelmäßig Keimzahlen in Höhe von 10^9 KbE/ml erreicht wurden. Daraus wurde eine dezimale Verdünnungsreihe in PBS-Puffer pH 7,3 bis zu einer Verdünnungsstufe von 10^{-6} (das entspricht einer durchschnittlichen Keimzahl von 10^3 KbE/ml) erstellt, davon 4,5 ml in Reagenzgläser überführt und in einem Wasserbad erhitzt, wobei die für die jeweiligen Serovaren charakteristischen Erhitzungstemperaturen und -zeiten zu ermitteln waren. Im Anschluss an die Kühlung in Eis (30 sec) erfolgte die Kontrolle der Schädigung und eine Keimzahlbestimmung der geschädigten Salmonellen. Der Effekt der Schädigung wurde in Anlehnung an EDEL und KAMPELMACHER (1973) beurteilt. Demnach galten die Keime als subletal geschädigt, wenn sie sich nur nach einer Wiederbelebung in gepuffertem Peptonwasser, nicht aber direkt in Rappaport-Vassiliadis-Medium vermehren konnten.

2.2.2 Versuche zur Verkürzung bzw. zur Vereinfachung des Untersuchungsgangs

Verschiedene Ansätze zur Verkürzung bzw. Vereinfachung des Untersuchungsganges wurden, ausgehend von der Voranreicherung des Verfahrens der Amtlichen Sammlung, im Vergleich zu diesem geprüft (Abb. 3). Der Ansatz der Proben erfolgte in GPW. Die Milchtrockenprodukte wurden hierbei mit 1 bis 10 bzw. 10 bis 100 subletal geschädigten Salmonellen pro 25 g kontaminiert. Die Beimpfung der Rohmilch und des Weichkäses erfolgte in gleicher Weise mit ungeschädigten Keimen. Der weitere Untersuchungsgang wurde entsprechend der Methode der Amtlichen Sammlung durchgeführt. Parallel hierzu wurden die folgenden Modifizierungen geprüft:

2.2.2.1 Direktausstrich aus der Voranreicherung

Beim Nachweis aus der Voranreicherung der Amtlichen Sammlung durch Ausstriche auf BPLS- und MLCB-Agar erfolgte die Auswertung nach 24stündiger Inkubation bei 37 °C und anschließender Bestätigung verdächtiger Kolonien durch serologische und biochemische Tests.

2.2.2.2 Nachweis aus der Voranreicherung mit dem VIDAS SLM

Zum Nachweis der Erreger mit dem VIDAS SLM wurden (zur Zeiteinsparung ohne die in der AOAC-Methode vorgesehene 18stündige Nachanreicherung in M-Bouillon) 1 ml der Voranreicherung im Wasserbad in verschlossenen Eppendorf Reaktionsgefäßen erhitzt