International Symposium

on

"Food and feed safety in the context of prion diseases"

organised

in Namur, Belgium

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by **CRA-W, JRC-IRMM, AFSCA and Agrobiopôle**

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STRATFEED

"Strategies and methods to detect and quantify mammalian tissues in feedingstuffs"

Agenda and Abstracts

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(G. Lopez-Galvez, JRC-IRMM, BE)

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(J. van der Veen, EFPRA, NL)

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Prevention and technical aspects

L.18.- Overview of current and alternative slaughter practices - Klaus Troeger, Federal research centre for nutrition and food , D

L.19.- Overview of European rendering technology - Steve Woodgate, EFPRA, UK

L.20.- Requirements of markers in the frame of the EU animal by-product regulation - Gloria López-Gálvez, JRC-IRMM, BE

L.21.- Inactivation efficiency of the TSE agent in rendering processes - Radulf Oberthür, EFPRA, D

L.22.- Fat processing - Johan van der Veen, EFPRA, NL

L.1.- Current status of TSE related problems

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Transmissible spongiform encephalopathies (TSE) encompass subacute neurological degenerative diseases for which the prototypes are scrapie in sheep and some forms of Creutzfeldt-Jakob disease in man. The emergence of a new form of TSE in cattle in Great Britain since 1986, namely bovine spongiform encephalopathy (BSE), sharply increased the interest for these diseases, especially because of the epidemic nature of BSE in Great Britain and the later discovery of its zoonotic character.

The number of measures of veterinary public health taken to control the disease and to prevent its spread to animals and human beings increased in time and culminated by the total feed ban. Indeed, since the beginning of 2001, feed containing proteins of animal origin is prohibited for the feeding of production animals, including ruminants and monogastric species.

The effect of the total feed ban needs to be evaluated. The incidence of BSE has a trend to decrease in Great Britain and the other European member states. However, as it is a rare event distributed in a large bovine population, it is difficult to state unambiguously whether this trend is significant. Furthermore, the evaluation of this measure will be only effective at least five years after its introduction, since this period is the mean incubation time of BSE.

The main concern is currently the eradication of BSE in the infected countries. Additionally, the control of scrapie is also carried out due to the possible contamination of sheep with the BSE agent. These actions must take into account several new facts: the recent discovery of BSE cases in countries with a low geographical risk as Japan, Canada and United States of America (USA); the identification of cases in young cattle in Japan; the growing incidence of chronic wasting disease, a spongiform encephalopathy observed in deer in USA; the characterization of a new pattern of bovine amyloidotic spongiform encephalopathy in Italy and atypical scrapie and BSE cases in Europe; the efficacy of sheep selection based on scrapie resistant genotypes.

TSEs are still emerging diseases. Although the scientific knowledge is steadily increasing, many aspects of the pathogenesis and the epidemiology of these diseases remain to be elucidated. However, efficient control measures were enforced in most of the European member states. Every measure which can improve the quality and the respect of the feed ban is a step towards eradication of BSE in cattle.

Keywords

Transmissible spongiform encephalopathy, prion, scrapie, bovine, sheep

L.2.- BSE overview: consumer-Risk assessment

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The bovine spongiform encephalopathy (BSE) epizooty has been monitored in France since the end of 1990, whereas BSE was described first in 1987 en Great-Britain (GB). A risk analysis undertaken in France in 1990 led to the conclusion that BSE might have spread to France via import of British meat and bone meal (MBM) (Savey et al. 1990). Epidemic surveillance was therefore set up at the end of 1990 with different steps:

- BSE is a notifiable disease (June 1990);
- Clinical surveillance based on a mandatory reporting system (December 1990);
- Complementary programs as checking for BSE each animal suspected of rabies (1998)…;
- Pilot screening program using rapid tests for the detection of PrPres in BSE infected animals (June 2000) in the North-Western France;
- Since January 2001, every cow aged 30 months and more is tested at slaughter (24 months and more since July 2001).

Regulation measures

The mean measures in France have been :

- June 1989 : ban on the import from GB of live cattle and ban on MBM;
- July 1990 : ban on MBM for cattle feed;
- December 1994 : ban on MBM for all ruminant feed;
- August 1996 : removal of specified risk materials (SRM) and cadavers from MBM ;
- November 2000 : ban on MBM and on certain animal fats for all farmed animals.

The two major measures (feed ban and removal of SRM) were set up at different dates in other European countries : 1988 and 1990 respectively in GB, whereas in all other countries these measures were not implemented until 2000.

French Food Safety Agency (Afssa)

Afssa was created in 1999. The government asked the Agency to evaluate the preventive system against BSE. From 2000 to 2002, the Agency has performed several (and when possible, quantitative) risk assessments about different topics in order to increase the level of consumer's protection:

- Nutritional and zootechnic consequences of the ban ;
- Risk of pitching for slaughtering;
- Evaluation of different strategies for slaughter policies;
- Implementation of the testing programs in collaboration with the French administrations;
- Update of the SRM list according to the scientific state of the art;
- Risks assessments for the "embargo" concerning GB and Portugal;
- Evaluation of the residual risk for the French consumer.

Since the middle of 2002, based on the decreasing incidence of BSE, the Agency has opened some "bolts" such as the re-introduction of thymus in human food chain, of some animal fats in animal feed, or the slaughtering policy based on a "cohort" strategy etc….
Compared to the European Union regulation, the French one is more restrictive in the following fields: SRM list, ban concerning certain animal fats, age of testing healthy animals but these discrepancies are going to decrease.

The epidemiological status of BSE epizooty in 2003 / 2004

In France

A decreasing number of cases has been observed : 276 in 2001, 240 in 2002, 142 in 2003 and 27 in may 2004. Between August 2001 and December 2003, the overall prevalence was divided by 4 and the median age increased: 5.9 years in 2000, 7.5 years in 2003.
Whereas more than 80 cases were notified as "Born after the second ban – BASB" (animals born after 1996) : the meaning of these reports needs further evaluation.

In European countries

More ten millions tests were performed in 2003 (8 700 000 in healthy animals). 78 % of the cases were detected by the tests.

The number of cases and the overall prevalence decreased by 36 % and 33 % respectively between 2002 and 2003. The number of cases in young animals (under 5 years) decreased and the mean age of cases increased. Both observations support the effectiveness of the preventive measures.
No case was reported in Finland, Sweden , Austria, Greece, and Luxembourg. Concerning the new European countries, cases were reported in Poland, Czech Republic, Slovenia and Slovak Republic.

Keywords

BSE, French food safety, regulation measures

L.3.- TSE Legislation in the EU: aspects in feed

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Epidemiological studies, rendering studies and the effect of feed bans in countries with BSE very clearly support the hypothesis that the recycling of inadequately heat-treated ruminant proteins into feed for ruminants has been the main source of the BSE epidemic. Therefore, a ban on the use of mammalian tissues in the feeding of ruminants was introduced in 1994 and a treatment at 133°C/20 minutes/3 bars/50 millimetres particle size for mammalian by-products intended for use in feed, except certain derogations, became mandatory on 1 April 1997.

The detection of BSE in cattle born after 1994 and even after April 1997 demonstrated that the above measures were not fully effective mainly because of cross-contamination of ruminant feed by feed intended for non-ruminants and containing animal proteins. An addition problem to control the ban was the difficulty to differentiate heat treated ruminant and non-ruminant proteins. These observations resulted in the extended feed ban introduced in January 2001, prohibiting the use of processed animal proteins, except some derogated ones, in the feeding of farmed animals which are kept, fattened or bred for the production of food.

The provisions of extended feed ban are currently laid down in Regulation (EC) No 999/2001 as amended by Regulation (EC) No 1234/2003. Milk, milk products, colostrums, eggs, egg products and gelatine of non-ruminants are derogated. Under strict conditions the use of fishmeal, hydrolysed proteins derived from hides, skins or non-ruminant tissues, dicalcium phosphate and tricalcium phosphate is also authorised in the feeding of non-ruminants and the use of blood meal and blood products is authorised in the feeding of fish.

In addition, Regulation (EC) No 1774/2002 lays down health rules for the collection, transport, storage, handling, processing and use of animal by-products. In accordance with this Regulation, only so called Category 3 by-products (by-products derived from animals fit for human consumption) can be used in feed and the feeding of a species with processed animal proteins derived from the same species is prohibited (prohibition of cannibalism).

The use of animal proteins may be reconsidered in the future. The main conditions for such revision are at first the correct implementation of the relevant current provisions in Regulation (EC) No 999/2001 (the extended feed ban) and in Regulation (EC) No 1774/2002, and secondly the availability of validated control methods in feed. Such methods should not only be able to detect ruminant proteins in feed containing non-ruminant proteins but also to exclude the use of non-ruminant proteins in feed intended for the same species (e.g. exclusion of poultry proteins in poultry feed containing fishmeal or meat-and-bone meal derived from pigs).

During the last years, the European Commissions has supported research and ring trials to improve methods and to increase the performance of laboratories to detect species-specific proteins in feed. If the results are favourable a reauthorisation of fishmeal in the feeding of ruminants might be considered.

Keywords :

BSE, regulation, feed, ruminant protein

L.4.- TSE Legislation in the EU: aspects in food

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On 22 May 2001, the European Parliament and Council adopted Regulation (EC) 999/2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies, which is known as the 'TSE Regulation'. This Regulation is applicable as of 1 July 2001.

The TSE Regulation provides measures targeting all animal and public health risks resulting from all animal TSE, and governing the entire chain of production and placing on the market of live animals and products of animal origin. It consolidated much of the existing legislation on BSE or TSE, including rules for the monitoring of TSE in bovine, ovine and caprine animals, removal of specified risk material and prohibitions concerning animal feeding. It also introduced new legislation for areas which are not yet covered by European Union (EU) rules such as eradication of TSE and trade rules covering the domestic market, intra-community trade, import and export. Furthermore it provides for the procedure, criteria and categories for the classification of countries according to BSE status. Pending the final categorisation of countries according their BSE risk, transitional measures apply until 1 July 2005.

The removal of the specified risk material is the most important measure to protect the health of the consumers against the risk related to BSE. Specified risk materials (SRM) are defined as the animal tissues being most at risk of harbouring the TSE agent. By way of precaution, these tissues must be removed from the food and feed chains to avoid the risk of recycling the TSE agent. They are separately collected at slaughterhouses and disposed of by direct incineration or after pre processing. The Commission keeps SRM measures under regular review and has requested on a number of occasions scientific advice on the appropriate measures to be taken in relation to TSE risk in cattle and sheep.

The TSE Regulation also establishes the rules for the surveillance and the monitoring of TSE in bovine, ovine and caprine animals. These rules include two elements: a passive surveillance in animals with clinical symptoms compatible with BSE and an active surveillance (monitoring), which was introduced in 2001 at EU level and is based on the use of rapid post mortem tests.

The main purpose of the monitoring programme is to provide a reliable insight into the prevelance of BSE in the Member States. At the same time it also ensures that no BSE cases are being slaughtered for human consumption. This increases beef safety in combination with other measures such as the removal and destruction of specified risk materials. The compilation of Member State data is important to enhance the understanding of the epidemiology of TSEs and allows us to better identify the future direction policies which should be taken to protect animal and human health.

Keywords :
TSE regulation, SRM, BSE risk

L.5.- Point of view from USA

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The United States (US) Food and Drug Administration's (FDA) main strategy for preventing the establishment and spread of BSE in the United States is to control feed intended for ruminant animals. FDA regulation Title 21 Code of Federal Regulations (CFR) 589.2000 prohibits with some exceptions the use of mammalian proteins in feed intended for cattle and other ruminants. In response to the December 2003, diagnosis of BSE in a cow presented for slaughter in Washington State, the US government developed strategies that would provide additional protection to the US consumers. While some measures have been introduced, others are being considered. For example, FDA announced its intention to amend 21 CFR 589.2000 to reduce further the potential for BSE to amplify in the US cattle herd. Compliance with feed regulations is essential to the prevention of the disease. Feed tests capable of detecting prohibited ingredients, would greatly facilitate assessing compliance with feed regulations. Currently available tests are based on analyses of DNA, bone, or protein. None of the tests is definitive, meaning that the primary way of enforcing feed regulations continues to be through facility and record inspections at renderers, feed mills, and other handlers of prohibited ingredients. Chronic Wasting Disease (CWD) is another transmissible spongiform encephalopathy, affecting the American deer and elk populations. Because the potential risks from CWD to humans or non-cervid animals, such as poultry and swine, are not well understood, and because CWD's route of transmission is poorly understood, FDA has recommended that material from CWD-positive animals not be used in any animal feed or feed ingredients. Any animal feed containing such materials would be considered adulterated and subject to recall or other types of removal from the marketplace.

Keywords

BSE, FDA, animal feed, testing, CW

L.6.- Evaluation of rapid BSE and scrapie tests

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Transmissible spongiform encephalopathies (TSEs) comprise several fatal neurodegenerative diseases of mammals, including bovine spongiforme encephalopathy (BSE) in cattle and scrapie in small ruminants. Testing for TSE in cattle and small ruminants populations is compulsory in the European Union since 2001 and 2002, respectively. Diagnostic methods to detect BSE and scrapie include the classical post mortem examination of central nervous tissue by histopathology or immunohistochemistry and, more recently, the use of rapid immunoassays. The difficulty in diagnosing TSE, however, is the early detection of abnormally folded prion protein (PrPsc) and the uneven distribution of PrPsc only in certain tissues. Both aspect require the use of highly sensitive tests to allow a detection. Nowadays, rapid tests for the diagnosis of BSE in cattle, and scrapie in small ruminants are used worldwide more than 12 million times per year. The Institute for Reference Materials and Measurements (IRMM) of the European Commission has evaluated in the past nine different rapid BSE tests, five of which have been formally approved for large scale testing under regulation EC 999/2001. During these evaluations many critical points for both, the design of evaluation schemes and quality control protocols could be identified and were considered for further evaluations of rapid TSE tests. In addition to BSE test evaluations in 1999, 2001 and 2004, IRMM has recently carried out an evaluation of rapid tests for the detection of scrapie in sheep. Data on the outcome of 20 BSE test 6 scrapie test evaluations as well as results on the development of reference materials for the quality control of rapid post mortem TSE tests will be presented.

Keywords

Bovine spongiform encephalopathy, scrapie, rapid test, diagnosis, evaluation

L.7.- Prion test in practice in Belgium

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In order to guarantee the safety of consumers, the European Union has decided to organize the surveillance and the control of BSE in various ways.
In July 1994, the ban on the feeding of mammalian specific risk materials to ruminants (cattle, sheep and goats) was effective. The elimination of SRMs from the food chain is compulsory from 1 October 2000 and the Commission Decision of 29 November 2000 (2000/764/EC) imposes to all Member States the obligation of testing for BSE of all slaughtered animals aged over 30 months.

In this context, Belgium has been obliged to implement this directive in a very short delay. In mid-December, the Food Security Agency after receiving scientific advice from the reference laboratory decided to use the ELISA test finalized in the CEA at Gift sur Yvette (France) and commercialized by BIO-RAD.

The urgency of the situation pushed the Agency to ask accredited laboratories using ELISA technique in the agro-feed sector for help. On 2 January 2001, four laboratories had the necessary equipment to begin the test. From mid-January the number of laboratories raised to 18.

The repartition of the samples to be analyzed is daily organised by the BSE data bank which gives each laboratory the name of the slaughterhouse and the number of samples. The routine test used by the laboratories up to this day is the Platelia-BSE test from BIO-RAD.

All the documents used for the identification of the samples are standard. The same files are used for sending the results to the data bank. The laboratory has 30 hours at its disposal between the notification and the transmission of the results to the slaughterhouse and to the Agency in Brussels. The samples which are positive must be analyzed a second time before being sent to the reference laboratory for confirmation by other methods (histopathology, immunohistology, electron microscopy and Western Blotting).The results for 2001 and 2002 together with their repartition will be presented.

Keywords :
SRM, ELISA test, BSE

L.8.- Methods to detect central nervous DNA using specific DNA

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For the detection of non-muscle tissues in meat samples, to comply with BSE and QUID legislation, we have sought to develop a method in which DNA is extracted from a processed sample, modified by a chemical treatment process to preserve the methylation status of the DNA, then non-muscle tissue derived DNA is detected by PCR amplification. In order to differentiate between different tissue types we have exploited the fact that not all tissues express the same sets of genes and that such differences in expression can be controlled by site-specific methylation. The promoter regions of selected genes that are expressed in the target tissue of interest were isolated and key differences in the methylation patterns of CpG dinucleotides between these and those from the corresponding skeletal muscle identified. In this case we were looking for residues where the target tissue form (non-muscle) of the gene is unmethylated and the non-target (muscle) form is methylated. These differences were then used to design a PCR assay exploiting Methylation Specific PCR (MSP) to specifically amplify the target tissue derived (unmethylated) sequence and thereby identify the presence of that tissue in mixed samples. Examples of the various detection formats that have been utilised for these types of assays will be given.

Keywords :
PCR, methylation specific PCR (MSP), central nervous DNA

L.9.- Methods for detection of central nervous tissue in meat products

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The main infectivity in transmissible spongiform encephalopathies (TSE) can be found in tissues of the central nervous system. The most efficient measure for preventing contamination of the food and feed chain with TSE agents is the removal and destruction of specified risk materials (SRM) in combination with post-mortem testing of cattle. In addition, the European Directive 2001/101/EC prescribes the listing of all ingredients of food products where meat is defined only as skeletal attached muscle. Other animal parts for human consumption such as heart, liver, or fat have to be labelled as such but not as meat. Brain or CNS tissues – if not specified as SRM – i.e. brain of calves younger than 12 months, pigs, or poultry may still be used as an ingredient in meat products but must be labelled indicating also the animal species.

In order to enforce legislation, there is a strong need for reliable analytical methods for the detection and determination of CNS tissues in meat products such as sausages, and to indicate failure of complying with the ban on SRM.

Several methods for the detection of CNS tissues have been described so far in the literature. Due to technological processes, CNS material can be morphologically destroyed, rendering it undetectable by food histology and conventional neuro-histological staining [1]. Immunochemical methods using neurone specific enolase (NSE) or glial fibrillary acidic protein (GFAP) as markers for detection have been developed [2-4] and have been recently validated [5-7]. In addition a surface plasmon resonance bio-sensor has been developed [8]. A high cholesterol content could also be a possible indicator of the presence of CNS tissue. Although cholesterol shows no absolute specificity with reference to NSE or GFAP, it might be used within the scope of being a low-cost and fast screening procedure [2]. As nervonic acid (15-rtetracosenoic acid) is a quantitatively contained constituent of brain and characteristic part of CNS tissue, it has been proposed as an alternative specific marker to reveal brain material in meat-derived food [9]. Analytical methods based on solid-phase extraction (SPE) clean up and gas chromatography – mass spectrometry (GC-MS) have recently been published for the determination of CNS material in meat products suing marker fatty acids including nervonic acid from sphingolipids and phospholipids [9-12]. An on-line liquid chromatography (LC) – GC method has been recently developed for the determination of markers of CNS tissue in meat products at trace levels [13].

The methods described here and results of their validation (as far as available) will be presented in more detail.

[1] S. Wenisch et al., Nutr. Res. 19, 1165-1172 (1999)
[2] E. Luecker et al., J. Food Protect. 62, 268-276 (1999)
[3] E. Luecker et al., J. Food Protect. 63, 258-263 (2000)
[4] G.R. Schmidt et al, J. Food Protect. 62, 394-398 (1999)
[5] M.E. Agazzi et al., Eur. Food Res. Technol. 215, 334-339 (2002)
[6] M.E. Agazzi et al., Food Control 15, 297-301 (2004)
[7] E. Hughson et al., Food Add. Contam. 20, 1034-1043 (2003)
[8] A.A. Bergwerff et al., Proceedings of 1st Internat. Symp. on Recent Advances in Food Analysis, Prague 2003
[9] M. Niederer et al., Mitt. Lebensm. Hyg. 92, 133-144 (2001)
[10] W. Biedermann et al., Berl. Muench. Tieraerztl. Wschr. 115, 131-1434 (2002)
[11] M.E. Agazzi et al., Anal. Bioanal. Chem. 376, 360-365 (2003)
[12] W. Biedermann et al., results submitted for publication (2004)
[13] R. Barcarolo et al., J. Sep. Sci. 26, 1347-1352 (2003)

Key words

Tissue of the central nervous system, Meat products, Methods, Validation

L.10.- Determining the origin of meat

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The determination of the origin of meat can be separated into three categories: geographical origin, production origin and species origin.

True markers of for the determination of geographical origin in food must be influenced by the local environment in which it was grown. Trace elements and stable isotope ratios are the most commonly used markers as they have a direct relationship to geology and climate. Stable isotope ratios of hydrogen (2H/1H) and oxygen (18O/16O) in food are in influenced by local ground water that was used in the food's production. Similarly stable isotope ratios of strontium (87Sr/86Sr) combined with trace elements can provide information relating to the local geology.

Production processes inevitable effect food composition and resulting markers can be exploited using analytical methods. Again stable isotopes 13C/12C, 15N/14N, 34S/32S combined with trace elements are being used to provide information on the diet regime and investigations into their use as markers of organic production are being undertaken. Chemical markers have been developed for use in identifying natural hormone abuse.

The identification of meat and meat products throughout the food chain relies of the genetic identification of the species and the ability to relate the species to a population group or breed. At the simplest level a piece of meat can be identified as a particular animal species on the basis of mitochondrial cytochrome B sequence. Oligo arrays have the capacity to identify multiple species, and have come to the market place as a commercial product. The ability to trace an animal back to an origin relies on micro satellite analysis, and an understanding of population genetics. For the species in question primers are developed that allow the specific separation of alleles. The characteristic pattern produced by a number of alleles can define a particular breed or sub population and hence the country of origin.

A review of methods for determining the origin of meat will be presented together with an outline of future strategies that are currently being proposed. Select the field and type or paste the text.

Keywords

Meat, geographical origin, stable isotopes, micro satellite

L.11.- Stratfeed project overview

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To contribute to the implementation of the Commission decisions and to reduce the economic impact of a total ban on the use of MBM, it was clear that a research project at the European level had to be built to develop new tools and methods for the detection and the quantification of meat and bone meal in the feedingstuffs: STRATFEED answered to those European needs. The STRATFEED project was submitted by CRA-W in 1999 within the Measurement and Testing generic activity of the GROWTH programme FP5. It has been launched since the 1st January 2001 for a period of 42 months. The STRATFEED consortium was coordinated by the Walloon Agricultural Research Centre in Belgium and gathered 10 partners including official laboratories, research centres, universities and private companies (CRA-W, FUSAGx, AFSCA-FVLT, JRC-IRMM, NUTRECO, RIKILT, SAC, UCO, LAGC and ISS) from 5 EU countries involved in the control of compound feeds, the development of new methods, the validation of new methods and the application of the methods to the industry. During the project, it was decided to extend the consortium and three new institutes (ALP, LUFA and DPD) were invited to join the STRATFEED project to share the experience in classical microscopy of their country not yet represented in the consortium. Different collaborators have been involved too for carrying out specific tasks.

The STRATFEED project structure was centred around 7 workpackages. One workpackage (WP1) was devoted to the management of the project, one workpackage (WP2) to the constitution of a sample bank and to the preparation of the sample sets analysed in the project, 3 workpackages (WP3, WP4 and WP5) were devoted to the improvement of the existing official method (i.e. Classical microscopy) and to the development of new methods (i.e. Polymerase Chain Reaction (PCR), Near-infrared spectroscopy (NIRS) and Near-infrared microscopy (NIRM)), one workpackage (WP6) was dedicated to constructing an Internet site and a European database. The project included a well-established validation plan as well (WP7). The tasks undertaken in workpackage 7 (following-up the method development, the method validation and the organisation of collaborative studies) have ensured the development of robust methods. During the progress of the project, the European legislation was modified a lot moving from a ruminant ban to a total ban and recently considering the species ban. The STRATFEED consortium has taken this evolution in consideration and has continuously adapted the development of the methods in order to fit the new analytical challenge.

In terms of management, during those three years, six internal project meetings were organised in the partners countries and several extraordinary meetings were planned by the workpackage leaders. Beside those project meetings, others were organized with the European commission or local authorities and the STRATFEED partners also attended some international scientific meetings to report about the work produced in the project. All the work was reported in 13 newsletters and 6 reports.

To disseminate the results of the project, an international symposium entitled “Food and feed safety in the frame of TSE” was planned in Namur (Belgium) on the 16th, 17th and 18th June 2004. The publication of the proceedings will be published in the Journal BASE, Biotechnology, Agronomy, Society and Environment Vol 8(4). The other main ways of spreading the results are the website (http://stratfeed.cra.wallonie.be), the publications and a lot of actions linked to each workpackage, described in the electronic technology implementation plan.

Key words

Project management, consortium, feed domain, FP5, website, symposium

L.12.- Microscopic detection of animal proteins

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In the framework of the European Union funded project Stratfeed the microscopic method for detection of animal proteins in animal feeds has been improved, starting with the obligated method as published in Directive 88/1998. Major achievements are the enhancements of the sedimentation procedure, now based on the results of comparative experiments, a better described detection and evaluation process and more information on embedding agents, especially with respect to different viscosities. Tests by the partners and a validation study showed a major improvement in terms of reliability and repeatability. The new Directive 126/2003/EC is primarily based on the Stratfeed protocol, with some additions: the procedure is allowed to start with 5 grams of material instead of 10 grams, a sediment beaker is allowed as well, and more different embedding agents can be used. The French method with two solvents is prohibited for the time being.

Pure material of ruminant, pig, sheep and poultry has been produced in a pilot plant in a completely controlled and dedicated process. In addition, animal meals have been selected from the large sample bank, collected in the framework of the project, and stating a pure origin. Based on these materials the characteristics of bones of mammalian, avian and different fish types has been established. Literature sources has been used as reference and several odd or deviating descriptions were identified. A special case are some long bones of birds that show an appearance more or less similar to that of mammals. This causes considerable difficulties when mixtures of different vertebrate classes are assumed to be present in a feed. A new character has been developed based on muscle fibre information (muscle ratio: fibre width divided by sarcomere length). There are gradual differences between mammalian, avian and fish fibres, which can support a presumed identification based on bones. The statistics of the fibres also provide information on the distribution (standard deviation, frequency plot), which can help to identify mixtures of materials. This type of information is urgently needed for the discrimination between the different groups of animals (support of the species-to-species ban).

All information collected has been used to develop a Decision Support System, called ARIES (Animal Remains Identification and Evaluation System). This system can be applied for training and as support to the actual process of detection of presumed animal proteins in feeds. It provides three different modules for step-wise identification, a glossary, a gallery with additional series of images, a range of literature and information on legislation. ARIES will be made available as stand-alone system, and after proving sufficient interest from a market analysis as an internet-based system. A quick identification system called STRATFEED-DSS has been developed for internet application, based on 14 parameters divided in four different categories: macroscopic vs. microscopic and bone vs. additional features.

Key words

Animal proteins, sedimentation procedure, microscopic detection, identification, Directive 126/2003/EC, species-to-species ban, muscle ratio.

L.13.- PCR as tool to identify taxon-specific processed animal proteins

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There is the consolidated evidence that the use of feeds containing specific TSE risk materials in animal nutrition contributed to the spread of BSE among cattle. Because the difficulty to detect directly the causative agents in feeds, the preventive measures have been diverted to the detection and the identification of those animal constituent from those species that could harbour the causative agent.

To this respect, methods based on DNA identification are generally proved to be effective in tracing the origin of specimens. The PCR techniques contribute to amplify the target DNA sequence, thus allowing the detection of small copies of DNA, as in the case of that from heat and pressure stressed animal proteins, the so called PAPs (Processed Animal Proteins). In this work we illustrate the results achieved within the STRATFEED project in developing PCR techniques for a reliable taxon-specific detection of PAPs. The first attention has been focused on the suitability of mitochondrial vs nuclear DNA, and on the length of target amplicons; then, a comparison between classical vs real time PCRs have been made, in order to achieve lower detection limits. To this respect, rather than the total amount of DNA extracted from MBM and feeds, plays a key role its purity, to reduce possible matrix-induced inhibitory effects. In this light, the addition of plasmids as quality control could help the setting of sharper cut-off values for a better discrimination between compliant/non compliant samples. At <1.0% >0.5% MBM contamination level, it seems mandatory to perform different replicates from the same extract, to draw any conclusion about compliance. The PCR approach can allow a taxonomic modulation of the research, at level of ruminants as well as level of single species identification. Reinforcements in the forensic evidence could consist in performing PCRs using two independent and not DNA overlapping target sequences primers, thus achieving more than one information (identification point) on the same sample tested. Nevertheless, the technique requests a strict control of the environmental requirements, to avoid possible cross-contamination and carry-over phenomena during all the procedure, from samples handling to PCR runs. Dedicated rooms and facilities are required, as well Real Time PCR devices.

Keywords :
Real Time PCR, MBM, PAP

L.14.- Application of Near Infrared Spectroscopy (NIRS) to detection of Mammalian Meat and Bone Meal (MBM) in Feeds

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Reflectance spectra of feeds are easily acquired and provide an established means of quality control in the feed industry. It is appropriate then that the same technology could be used to screen feeds for mammalian protein that must be specifically excluded from the food chain because of the risk of transmission of BSE and vCJD to cattle and humans respectively.

Within Work Package 5 of the Stratfeed project NIR reflectance was studied extensively by partners in five laboratories using cloned spectrometers. Three sets (C, T and D) of laboratory prepared mixtures of compound feed containing meat and bone meal (MBM) at levels of 0, 1, 3, 5, 7 and 9 % (w/w) respectively were scanned in triplicate re-packing to acquire 2106 spectra from 700 specimens. Calibration models were constructed using quantitative MPLS and qualitative PLS2 discriminant analysis provided by the WinISI v 1.5 software of Shenk and Westerhaus.

Individual laboratories could achieve excellent calibration models having standard errors of calibration (SEC) and cross validation (SECV) typically in the range 1.0 to 1.5 % MBM. However performance usually degraded when standardised spectra were pooled across laboratories. This was attributed to the diversity of spectra of background feed matrices (GH statistic outliers) accumulated and some over-fitting by MPLS models having typically 16 or more factors. Some poorly mixed samples (t statistic outliers) were also implicated. Much better discriminant results were found if feeds were restricted in composition by segregating ruminant and non-ruminant feeds.

Nevertheless when the spectra of the laboratory prepared mixtures were combined with the spectra of 'real' processed compound feeds containing MBM, acquired by partner UCO prior to the ban, performance was apparently much improved. A model developed from this combined set of 1005 spectra (SECV = 0.94 % MBM; R^2 = 0.97) correctly assigned 8 samples in the validation set as well as giving acceptable performance in 54 compound feeds supplied by the co-ordination team for validation. Combining laboratory prepared specimens with 'real' specimens seemed to 'customise' the calibration to cope with real samples.

Although implementation of NIRS in industry is beyond the scope of WP5, our research suggests that NIRS is most appropriately implemented as a screening method within industry where there is an established product spectra library with a QC timeline for a particular product with running mean and SD. In this more restricted product range it may be possible to likewise customise the manufacturer's spectra database by incorporating reference spectra having a range of added MBM.

In this situation the main disadvantage of NIRS in having to acquire a large spectra library may be resolved at a stroke by taking advantage of the archive of spectra that already exists. Legislation could demand that product timeline surveillance becomes a regular facet of good manufacturing practice. This would benefit responsible manufacturers, their clients and consumers while lifting the burden on regulatory authorities allowing them to concentrate on referred suspect specimens that really warrant inspection.

Keywords :
Spectroscopy, NIRS, MBM, MPLS, PLS2, screening

L.15.- Review of the possibilities offered by the near infrared microscope (NIRM) and near infrared camera (NIR Camera) for the detection of MBM

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Recent technological developments have lead to instrument combinations coupling for instance a near infrared spectrometer to a microscope (NIRM), and instruments based on the use of the near infrared camera technology (IR camera). With these instruments, spectra of up to hundred or thousand particles can be obtained from the analysis of one feed ingredient or one compound feed[1]. In the NIRM instrument, thank to the help of a dedicated microscope the infrared beam is focused on each particle of a sample spread on a sample holder and the near-infrared spectrum is collected. The result of the sample analysis is a successive collection of hundreds of spectra, each one being the molecular near infrared signature of a particle from one of the feed ingredients used in the formulation of the compound feed. The spectral features measured in the near-infrared region (1100 – 2500 nm) of the electromagnetic spectrum can be used to identify and to quantify the used ingredients. With a near-infrared camera (also called near-infrared imaging system) pictures of a pre-defined sample area are taken sequentially at different wavelengths are collected. This technique allows the analysis of about 500 particles in 5 minutes.[2]

Already gathered results show the high potential of near-infrared microscopy and infrared camera for the detection of meat and bone meal in feedingstuffs with analyses either on the raw material or on the sediment fraction. In the framework of STRATFEED[3] and Belgian RCS[4] projects different studies have been conducted: (i) comparison with classical microscopy and alternative methods (NIRS, PCR, ELISA), (ii) transfer of the method, (iii) discrimination between particles from different sources (e.g. fish versus terrestrial material), (iv) repeatability study. Moreover, a strategy based on the combination of the infrared microscopic methods (NIRM & IR camera) and polymerase chain reaction method (PCR)[5] has been proposed as an elegant solution for the control of the presence of animal products and the identification of their origin at species level.

Keywords :

Spectroscopy, microscopy, NIRM, Camera IR, PCR, hyphenated techniques MBM, fish meal.

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[1] Baeten V. & Dardenne P. (2002). - Spectroscopy: Developments in instrumentation and analysis. Grasas y Aceites, 53(1), 45-63.

[2] G. Gizzi, L.W.D. van Raamsdonck, V. Baeten, I. Murray, G. Berben, G. Brambilla, C. von Holst, *An overview of tests for animal tissues in animal feeds used in the public health response against BSE*. Sci. Tech. Rev., In press.

[3] Public Federal Service (RCS-S6112, P01/03(376)-C03/07, P01/03(376)-C03/08) (2001-2005)

[4] UE STRATFEED project G6RD-2000-CT-00414, *Strategies and Methods to detect and quantify mammalian tissues in feedingstuffs*, www.stratfeed.cra.wallonie.be. (2001-2004).

[5] Dubois M., Fumière O., von Holst C. & Berben G. (2002). - Meat and bone meal detection in feed by search of specific animal DNA segments. 181st meeting of the Belgian Society of Biochemistry and Molecular Biology , 4th of May 2002, Katholieke Universiteit Leuven (KUL), Heverlee, Belgium, abstract nr. 7, (*http://www.biochemistry.be/4may2002/abstracts_1_13.htm#dubois*).

L.16.- Counter Immuno Electrophoresis: Methodology for confirmation of positive results on Immunoassay and DNA based tests through detection of milk based proteins

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Following the outbreak of Bovine Spongiform Encephalopathy (BSE) the inclusion of animal proteins in compound animal feedstuffs was restricted. Banned proteins are generally associated with rendered material in which most proteins are significantly disrupted and denatured. Some animal proteins are permitted e.g. milk products. These are not subjected to the same heat exposure as rendered material and retain aspects of their neo-protein structure. Problems arise when permitted feed ingredients generate positive reactions on immuno assay and DNA based tests. i.e. a positive bovine result could be due to the presence of meat and bonemeal contamination but could also result from the presence of permitted milk products.Testing milk products using a counter-immuno electrophoresis technique, with antisera raised against non-heat treated serum based proteins, confirmed that non-denatured proteins were present. This was not the case when testing rendered material where the protein structure is altered far beyond the detection capability of a serum based test. Non-denatured proteins, such as those derived from milk products, can be extracted from within a compound feed matrix using an ammonium sulphate precipitation process (precipitation band 40% to 77% w/v). Problems associated with the liberation of proteins from within feed pellets can be overcome by soaking samples in an buffer heated to 50°C (this temperature and time exposure isn't sufficient to inactivate the test). Extracted feed pellets are tested using counter-immuno electrophoresis. This test is based on electro-osmosis in an alkaline gel (pH 8.6) and tested using species specific antisera.This test can be used alongside assays for the detection of rendered materials (ELISA or PCR) in order to rule out milk powders as the cause of positive reactions, e.g. a positive ELISA test coupled with a negative CIE test would strongly suggest that a positive result would not be a consequence of permitted material within the feedstuff. Data is presented outlining test development, sensitivity and application of this method.

Keywords :
Counter Immuno Electrophoresis

L.17.- Meat and bone meals in feed: Results from recent interlaboratory studies for the validation of methods and for the evaluation of the proficiency of laboratories

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Conducting intercomparison studies is an important means to obtain information about the performance of a method or the proficiency of the laboratory. In either cases laboratories analyse the same batch of samples but in the case of a method validation study the laboratories apply the same protocol, whereas in a proficiency test they can select a method of their choice. In the frame of the detection of meat and bone meal in feed we organised intercomparison studies for both purposes. Important method performance characteristics for the detection of meat and bone (MBM) are (1) the sensitivity expressed in terms of false negative results of feed samples containing traces of MBM and (2) the specificity expressed in terms of false positive results of samples without MBM. Microscopic allows for the detection of MBM in general, MBM from terrestrial animals and – under certain conditions- also MBM from mammals.

In the proficiency test carried out in 2003 we investigated the capability of European control laboratories of classifying correctly feed samples with unknown content of meat and processed animal proteins (PAPs) from other species. Although these laboratories utilised microscopy, quite different variants of this method were applied. The majority of the official laboratory detected 0.1 % MBM, but experienced problems when analysing samples that also contained fishmeal. However, a subset of the laboratories that applied a more harmonised variant of the method produced very good results. Also the experience of the laboratory turned out to be very important. In the same study other laboratories applied different methods such as immunoassays and polymerase chain reaction (PCR). Whilst PCR showed in general very poor results, immunoassays scored much better than PCR.

A validation study conducted by RIKILT Wageningen and focusing on the detection of meat and bone meal in feed in the presence of fishmeal showed that laboratories could detect 0.1% MBM if they applied a harmonised microscopic protocol and if they had enough experience of microscopic analysis.

In the validation study of the Stratfeed project the laboratories utilized the microscopic method as developed within the Stratfeed project. This method also complies with current protocol according to European legislation [1]. In addition, as sub-group of the laboratories applied the computer supported decision support system ARIES developed by the Stratfeed project. The results demonstrated that the detection of 0.1% MBM in feed in the presence of fishmeal is still a challenging task. Comparing these results with the results from the other showed that training of the laboratories would most likely lead to an improvement of the results. The use of ARIES helped significantly in detecting mammalians MBM in the presence of poultry and fishmeal.

Acknowledgement: I am extremely grateful to Gisèle Gizzi, Stefan Strathmann, Leo van Raamsdonk, Vincent Baeten and Gilbert Berben for their contributions to the various intercomparison studies conducted by the IRMM

[1] EC/2003/126 Commission Directive of 23 December 2003 on the analytical method for the determination of constituents of animal origin for the official control of feedingstuffs. Official Journal of the European Communities L 339, 24.12.2003, 78-84

Keywords

Validation, intercomparison study, meat and bone meal in feed

L.18.- Overview of current and alternative slaughter practices

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The conventional slaughtering of cattle includes some critical process stages where a carry over of Specified Risk Material (SRM: brain, spinal cord) on the meat and meat contact surfaces can occur. These processes are captive bolt stunning, cut off the head and first of all carcass splitting (sawing the spine lengthways). Captive bolt stunning results in massive brain tissue damage with bleeding, and in some cases brain tissue also emerges from the hole made by the bolt. As the heart is still functioning, there is a risk of brain tissue particles being transferred via the blood flow to heart and lungs or even in the whole carcass. This contamination risk is actually assessed to be low, but a continuing leakage of CNS material from the captive bolt aperture in the further slaughter process may lead to direct and indirect contamination to carcass meat and equipment. Therefore alternative stunning methods like electrical stunning or concussion stunning are under investigation or already installed (electrical stunning). A further critical point is the treatment of the head. When the head is removed, the spinal cord is cut with a knife. There is a danger of cross contamination due to spinal protein that may adhere to the knife and because of liquid cerebralis, which leaks from the foramen occipitale magnum. Further head cleaning with hand-held hoses following skinning also includes the danger of cross contamination from cleaning water or aerosol. The most critical point in terms of contamination of the meat surface with SRM is the currently common practise of sawing the spine vertically in the middle with hand-guided belt-type saws. A mixture of sawing residues and rinsing water (“sawing sludge”) collects in the housing of the saw, and if it contains infectious material this leads to contamination of the subsequent carcasses. The most promising methods available at present for minimising this risk appear to be in manual cattle slaughtering boning the entire (not split) carcass, either still warm or refrigerated and in industrial beef cattle slaughtering extraction of the spinal cord by vacuum from the whole carcass followed by conventional sawing or completely sawing out the spine including spinal ganglia.

Keywords

Cattle slaughtering, Specified Risk Material, Captive bolt stunning, Carcass splitting

L.19. Overview of European rendering technology

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Introduction:

The rendering process will be described as it relates to the activities of the European Fat Processors and Renderers Association [EFPRA]. The focus of the paper will relate to animal by-products not intended for human consumption and the position of edible fats will be covered only briefly, as this subject is covered by my colleague, Johan van der Veen.

The Rendering Industry:

The role of rendering within the EU Animal By-Product Regulation [ABPR 1774/2002] will be explained in detail. Key aspects of the ABPR will be explained in terms of raw material inputs, the processing systems approved for use and the product outputs.
TSE risk reduction will be dealt with briefly, in relation to the category of raw material, the specific approved processes and the control of the products. Further details relating to TSE risk reduction will be considered by my colleague, Radulf Oberthur.

Applications for rendered products:

The major products of rendering are processed animal proteins (PAP) and processed animal fat (PAF). Applications for these products will be discussed in terms of the hierarchy of value. These applications will include use of rendered products as fuels, in construction products, as fertilisers and as ingredients in pet foods and animal feeds.

Legislation and controls:

The controls pertaining to the processing and application of the products will be discussed and the initiatives being developed by EFPRA to facilitate the future wider use of rendered products will be highlighted. Particular reference will be made to
a) control systems for materials that require "marking" with coloured dyes or indelible compounds and
b) identification methods for determining the species identity of processed animal proteins either as pure PAP or as an ingredient in animal feed.

Conclusions:

EU Rendering systems will be reviewed in relation to the Animal By-Products Regulation.
[ABPR 1774/2002] and the consequences of this regulation as it relates to animal by-products not intended for human consumption will be shown with reference to current and future control systems. The position of the rendering industry in a sustainable livestock production system will be confirmed.

Key words

Animal By-Products; Rendering; ABPR (1774) ; Sustainable

L.20.- Requirements of markers in the frame of the EU animal by-product regulation

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Within the European Union the safe use of animal by-products that are not intended for human consumption is laid down in the Regulation (EC) No 1774/2002. The main aim of the Regulation is to prevent animal by-products (ABP) from presenting a risk to animal or public health through the transmission of diseases. This aim is achieved by rules for the collection, transport, storage, handling, processing and use or disposal of animal by-products and the placing on the market, export and transit of animal by-products and certain products derived from them.

The Regulation divides animal by-products into three categories, with risk decreasing from category 1 to category 3. The Regulation stipulates that each category of ABP must be identified and kept separate during collection and transportation. Also it specifies that the processed products derived from category 1 and category 2 (with some exceptions) must be permanently marked, where technically possible with smell, using a system approved by the competent authority.

As a request of DG SANCO, the Joint Research Centre is searching for a valid marker for the mentioned ABP items. The marker has to comply with certain technical requirements such as being visible and olfactory detectable, non toxic, safe for handlers, commercially available, inexpensive, stable, recoverable and easy to analyse, etc. A survey was done throughout the EU Member States to assess the state-of-the-art of the use of markers for the different ABP categories. It was concluded that both colours and odours are used for marking ABPs in the slaughterhouses. But none of the presently used markers would survive to the required legal processing conditions in a rendering plant.

In 2001 the JRC proposed the artificial marker Glyceroltriheptonate (GTH), which is a triglyceride containing three heptanoic acids. This marker would allow for traceability of the ABPs from the slaughterhouse to the processed products such as meat and bone meal because this substance withstands severe sterilisation conditions as required in European legislation. Furthermore, the GTH has been used for several years as anhydrous butter marker, and therefore it meets safety and toxicity requirements. Both JRC and industry have already conducted some experiments using the GTH as ABP marker and found that:

- it mixes well with lipophilic dyes and can be added to raw material,
- it proves to be stable under sterilisation conditions in an autoclave,
- it can be recovered from both rendered fat and meat and bone meal (MBM).

Experimentation is still being carried out to test water soluble dyes to mix with the GTH, to validate the analytical method for the GTH, the dye and the smell, and to test the practical application in slaughterhouses and rendering plants, taking into account current marking practices in Member States.
The GTH marker used as complement of a colour/odour system, in both ABPs in the slaughterhouse and those processed products derived from them, would ensure identification and traceability of products intended for disposal, avoiding possible risk of fraud and reassuring consumers of the safety of the food/feed chain. The principle is that any material (MBM or fat) containing GTH will not be considered suitable for use in food or feedingstuffs. Currently the JRC is working together with DG SANCO, Member States' representatives and the industry on the implementation of the proposed marker system

Key words

Regulation (EC) No 1774/2002, Animal By-Products, Marker, GTH, Dye

L.21.- Inactivation efficiency of the TSE agent in rendering processes

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In 1990 and 1991 inactivation trials of both the BSE or the scapie agent in spiked rendering raw material in pilot rendering plants (100 kg scale) have been performed in England in order to demonstrate the inactivation efficiency of thermal inactivation of the TSE agent in all the different rendering processes operating in the EU at that time. The results of these trials available in 1994 and 1996 became the guideline for the EU legislation on process conditions in animal by-product processing.

In 1993 until 1995 inactivation trials in small vials (20 g scale) of sterilized rendering raw material spiked with both the BSE or the scrapie agent were performed for different temperature/time ranges in the Netherlands. The results obtained in 1997 led to the removal of SRM from the rendering raw material in the Netherlands.

The results of both trials, however, did not fit simple first order inactivation kinetics as used for bacterial inactivation description.

In 1996 a programme was developed in the Netherlands to approach mathemetically the temperature/time history of a raw material particle entering a rendering process. Two years later epidemiological calculations based on the reproduction number of the proliferation of BSE has been performed in Lelystad to show what efficiency a reduction of the infectivity in a rendering process would have on the proliferation of BSE.

In 2000 new inactivation studies were performed with the 301V strain of BSE in Edinburgh and with BSE in Lelystad using a stirred autoclave (70 g scale). The results together with the known results from literature led to the development of a kinetic model for the thermal inactivation of a TSE agent including a competition between a protecting and an inactivating reaction as a function of temperature. This model explains the existing data for TSE inactivation by heat but with thermodynamically different parameters for the different TSE-strains. The 22C scrapie strain appears to be the most thermolabile and BSE the most thermostable strain.

Introduction of this kinetic model into the improved simulation programme for the time/temperature history of a particle in a rendering process using the technical data for heat supply and water loss within the process as auxillary parameters lead to satisfactory explanation of the inactivation data obtained in the pilot plant inactivation experiments done in 1990/1991 in England together with the experiments done in the Netherlands.

This kinetic model, however, has some important consequences: (1) fast heating is much more efficient than slow heating, (2) prolongated heating has no additional effect, (3) adding a second heating step to a first heating step has no additional effect, (4) heating of the bulk through a superheated wall is especially efficient in rendering processes even at atmospheric pressure or below atmospheric pressure.

Keywords

TSE inactivation, rendering processes, inactivation kinetics, technical process simulation, inactivation prediction for rendering processes

L.22.- Fat Processing

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Lipids are of vital importance. Without lipids there is no life, we can't think, and we experience no sense. The main categories of lipids are fats. Besides a sustainable source of energy, fats have many biological functions, such as storage of fat-soluble vitamins, supply of essential fatty acids and protection of organs. Fatty acids are essential as part of the synthesis of polar lipids for lipid bilayers and intracellular messengers. In spite of all these positive biological functions, animal fats have a negative reputation regarding obesity and increasing "bad" LDL-cholesterol. However so far there is no conclusive proof that a high consumption of animal fat is the cause. Even the contribution of saturated fats with regard to coronary heart disease has lead to extensive scientific disagreement. Putting into perspective a recent meta-analysis reported that the consumption of trans-fatty acids has a 7.3 more adverse effect compared to saturated fats. For food safety aspects in the context of prion diseases we should question ourselves continuously on the current risk for humans; do we still have to fear BSE?

In a balanced diet animal fat is a valuable source of concentrated energy and (essential) fatty acids needed for growth and development. For hundreds of years animal fats are used for cooking. Lard is applied in bread making to assist the leavening process and to soften the crumb. The soft consistency and crystalline character makes lard the most suitable shortening for pastry contributing to colour, flakiness, flavour and tenderness. For feed manufacturers the fatty acid profile and melting point of animal fats are important features for feed production as well as for producing tendered and tasteful meat. Along with nutritional aspects and digestibility, feed producers prefer animal fat on account of the positive crystallisation characteristics for calfmilk replacers and the formation of firm feed pallets with a high feed performance. Better economics are also relevant.

The business of fat processors is historically determined by the processing of slaughter by-products exclusively from approved animals fit for human consumption. Edible fat processing is traditionally associated with species-specific processing, high-grade animal fats and mild processing conditions which are essential to supply specific markets for food and feed. Therefore the commercial specifications of animal fats derived by fat processors are traditionally much stricter than the legal specifications. The animal proteins produced are wet frozen or dried for the use in foodstuffs or as an ingredient for petfood. The quality and food safety system applied in the fat processing industry is aimed at integrated chain control and based on the food safety principles of HACCP. Accordingly the used raw materials are subject to the same food safety inspections and monitoring program which is compulsory for meat. Finally and essential for slaughterhouses is that fat processors are in the position to pay for raw materials, therefore creating an added value for the animal chain.

Keywords

Fat processing, animal fat, animal protein, slaughter by-products

Posters list

General aspects: TSE overview, legislation

Current status of TSE related problems

Analytical methods for food

Prion test in practice

Determining the origin of meat

Analytical methods for feed focusing an the detection of banned meat and bone meal

Overview of the STRATFEED project

Microscopy

PCR – Polymerase Chain Reaction

IR – Infrared methods

Immunological methods

HPLC – High Performance Liquid Chromatography

P.44.- Analysis of products of animal origin in feeds by determination of Carnosine and related dipeptides by High Performance Liquid Chromatography – LUFA - Germany

Electronic nose

P.45.- Potential application of electronic nose in PAP detection in feedstuffs, University of Milan – Italy

Comparison of methods

P.46.- Technological Development for Detection of Animal Materials in Feed in Japan - Japan

P.47.- Determination of animal constituents in feedingstuffs: Evaluation of diverse methods and their results – LUFA – Germany

P.48.- Studies on the detection and monitoring for the contamination of land animal protein in domestic and imported fish meals as prophylactic for BSE – RDA - Korea

P.49.- Methods for species identification – CCL Research – The Netherlands

P.50.- Detection of animal DNA in feedstuffs by polymerase chain reaction compared with the official microscopic method – IZS - Italy

P.51.- Possibilities of FT-IR and PCR to discriminate species by animal fats – CRA-W - Belgium

Prevention and technical aspects

Rendering process

P.52.- Relationship between rendering process temperatures and DNA degradation in Meat and Bone Meals by Real Time PCR assay – ISS - Italy

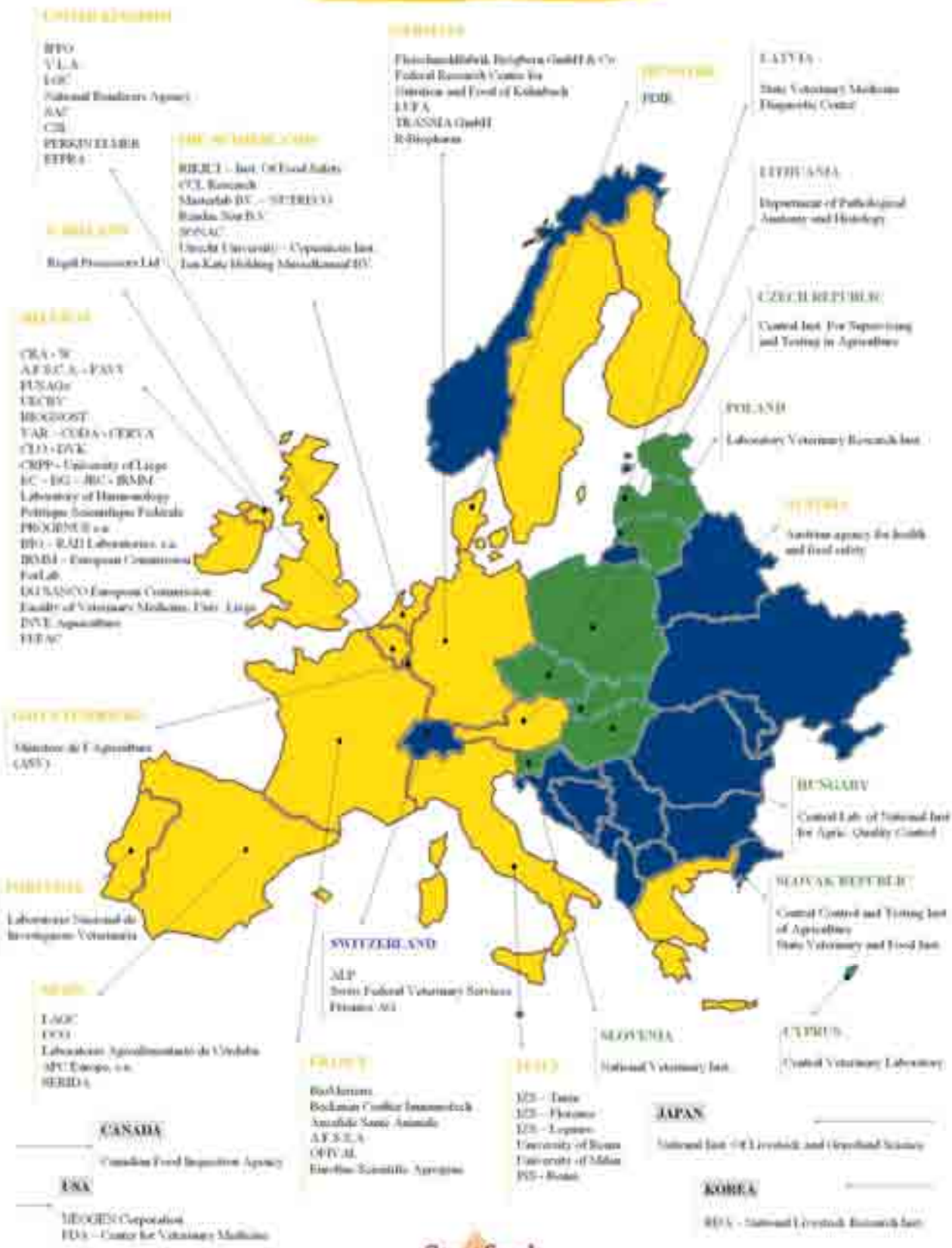
Markers

P.53.- Glyceroltriheptanoate as marker for category 1 and 2 animal by-products – CCL Research – The Netherlands

INTERNATIONAL SYMPOSIUM
« FOOD AND FEED SAFETY IN THE CONTEXT OF PRION DISEASES »

16-18 June 2004 Namur, Belgium

Organised by CRA-W, JRC-IRMM, AFSCA and Agrobiopole



P.1. – International symposium “ Food and feed safety in the context of prion diseases”, 16-18 June 2004, Namur, Belgium, organised by CRA-W, JRC-IRMM and Agrobiopôle.

BSE ESB

BSE IN SWITZERLAND

Chronology of Events and Measures

Activities of the BSE-Unit

agroscope

1986: → First BSE in GB

1988: → No more import licences given for MBM from GB

1989: → Veterinarian trained and research encouraged

1990: → First BSE in CH = First BSE in continental Europe

MBM banned for ruminants

Official import ban for MBM and ruminants from GB

Elimination of risk material from animals > 6 month

1993: → **MBM Treatment 133°C, 20 min, 3 bar**

1996: → Elimination of risk material from animal-based feed

Elimination of offspring from BSE-animals

1998: → Additional measures against risk material in food and by-products

Registration of cohort animals

1999: → Elimination of cohort animals

Blood-meal banned for ruminants

Active surveillance (fast BSE-tests)

2001: → MBM and extraction fats feed ban for all farm animals

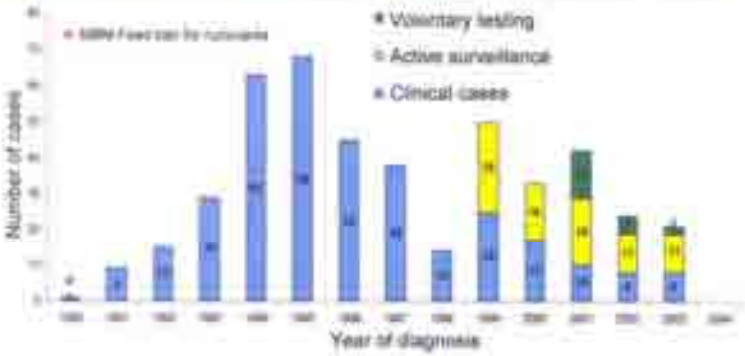
"Zero-tolerance" for MBM in feed for all farm animals

Fish-meal banned for ruminants

Creation of the BSE-Unit

2002-4: → Intensified Schooling, Training and Inspections by the BSE-Unit

Cases of BSE per year



TSE in Switzerland:

-In animals:

- sheep: 8 cases of scrapies (1991 to 1999)
- goats: 2 cases of scrapies (1981 + 1993)
- cats: 2 cases of FSE (2001 + 2003)

-In humans:

- 0 cases of vCJD

Total fast BSE-tests

1999:	• 18'808
2000:	• 25'784
2001:	• 165'896
2002:	• 101'500
2003:	• 179'455

BSE Unit

General and Specific Tasks

- Eradication of BSE
- Consumer Health Protection
- Consistent implementation of Regulations
  - Standards
  - Advisory inspections
  - Instruction and training

Legal Mandate

- Animal Health Law
- Agriculture Law
- Epidemic Law
- Food Law

→ *Confederation: Supervision*

→ *3 State Departments: Public Health, Agriculture, Veterinary Affairs*

Concept: 6 Sections

Animal Feed: *MBM Residue Analyses, Feed Plant Inspections, Sanctions,...*

Livestock: *Identification, Selection, BSE Suspicion Cases, Notification, Surveillance Programme, ...*

Abattoirs: *Slaughter Process, Specified Risk Material, Identification, Sampling, Storage, ...*

Food / Cosmetics: *Specified risk material, production processes, contaminations, import, ...*

Animal Waste Disposal: *Separation, transport of waste, waste material flow, quantity balance, ...*

Analysis and Testing: *Laboratory inspections, evaluation of tests, examination of test results, ...*

Inspections in 2003

Animal feed producers: 535
Abattoirs: 78
Meat cutting plants: 54
Animal Waste Disposal: 9
Processing plants for liquid feed for pigs: 3
Waste transport companies: 8
Collection centre for fallen stock: 9
Burning installations for MBM: 7
Pet-food producers: 2
Food industries: 8
Cosmetic companies: 6
BSE-Test laboratories: 9

P.2. - BSE in Switzerland, Chronology of Events and Measures, Activities of the BSE-Unit.

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This presentation gives an overview of the most important events and measures taken in the field of BSE since the first reported case in Great Britain in 1986 and also describes the concept of surveillance and inspections developed to reach two main goals: Eradication of BSE and Consumer Health Protection. Measures such as MBM ban and elimination of risk material from the feed and food chain were taken rapidly to prevent the spreading of the disease. Training and research were intensified to detect possible cases, and the first case in Switzerland was described in 1990. Statistics of the number of cases of BSE per year show that a rapid increase of the cases took place up to a maximum of 68 in 1995, and a delay of five years was observeable before the measures taken had an effect on the number of cases. After a second smaller wave of cases in the years 1999 to 2001 was observed, the decrease shown is now steady and no cases have yet been reported in 2004.

Switzerland has used the fast BSE-tests since 1999 for epidemiological reasons, and the number of these tests per year is presented.
Information on TSE in Switzerland is also given and the number and type of inspections done by the BSE-Unit in 2003 is enumerated.

Internet Links: www.alp.admin.ch and www.bse-einheit.ch

Keywords

Switzerland, BSE cases, TSE, BSE-tests, Inspections

www.bse-einheit.ch

BSE IN SWITZERLAND

Feed Measures and Results of Feed Inspection

www.alp.admin.ch

Daniel Guidon[1], Geneviève Frick[1], Claude Chaubert[1], Paul Boss[2].
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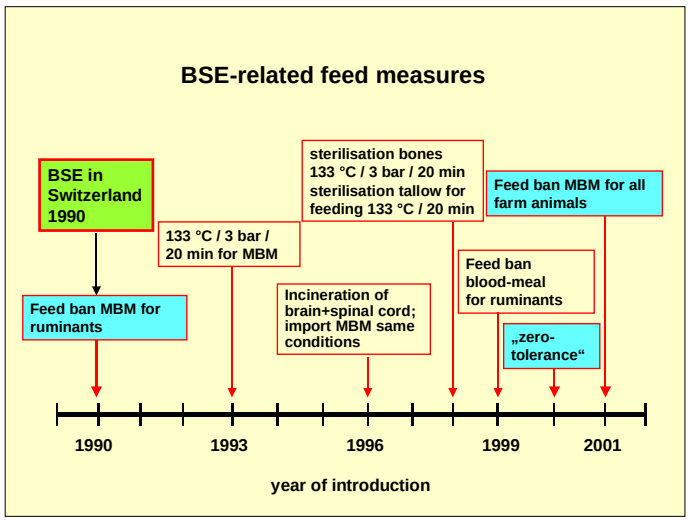
[2] BSE Unit of Switzerland, Schwarzenburgstrasse 161, CH-3003 Bern-Liebefeld



main measures

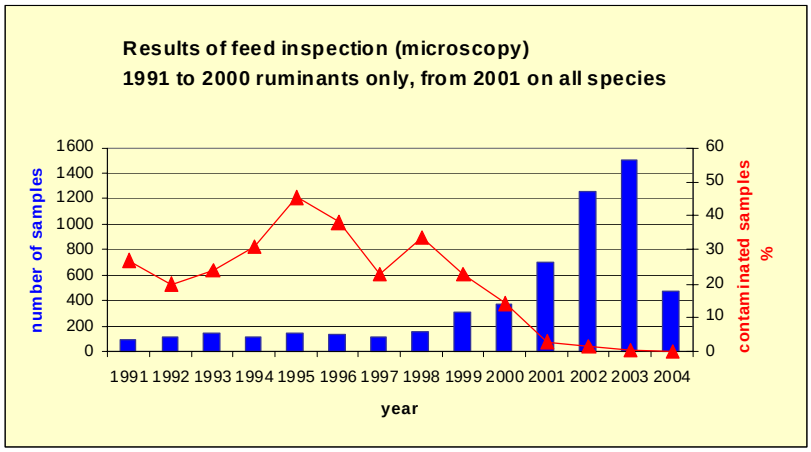
1990 MBM (meat and bone meal = several animal by-products) removed from ruminant formulas, MBM still in pig and poultry feed

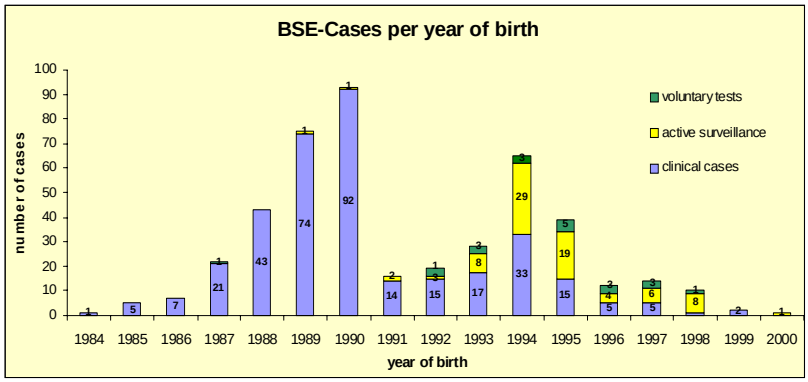
2000 so called „zero-tolerance“: all microscopically detectable traces are considered

2001 MBM removed from all formulas for farm animals, no more MBM in the feed circle, all MBM has to be burnt

several hurdles following each other, introduced according to the **increasing scientific knowledge**:

- removal of MBM out **of ruminant feed**
- all **BSE-suspicious cows** have to be **burnt**
- **heat/pressure/time treatment** of MBM
- removal of **specific risk material** out of MBM
- **complete ban** of MBM for all farm animals
- **all MBM** has to be **burnt**





important findings

almost all positive samples contained only very small amounts of MBM ➔ no illegal use of MBM in compound feedstuffs for ruminants

most effective measure: removal of MBM from ruminant feed (1990: 93 BSE-cows, 1991: 16 BSE-cows)

smallest amounts of MBM have to be considered: 0.1 g of contaminated MBM is able to transfer BSE ➔ introduction of the so called “zero-tolerance”, because of the cross-contaminations in feed mills

not only feed formulas but also manufacturing equipment and above all MBM storage silos have to be considered ➔ smallest amounts of MBM may contaminate equipment during years

creation of the Swiss BSE-Unit ➔ increased number of samples analysed from 2000 on

no more MBM at all in the feed industry ➔ striking decrease of positive samples from 2001 on

no BSE-cows and no positive feed samples in 2004 (end of April)

P.3.- BSE in Switzerland, Feed Measures and Results of Feed Inspections

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The first BSE-cow in Switzerland was detected in November 1990. Subsequently numerous measures had to be introduced, an important part of them related to feedstuffs. With the increase of scientific knowledge these measures were adapted and reinforced.

The most important measures were

- The ban of MBM (meat and bone meal = several animal by-products) in formulas of compound feedstuffs for ruminants
- The ban of all microscopically detectable traces of MBM in compound feedstuffs for ruminants
- The complete ban of MBM in compound feedstuffs for all farm animal species

Additional measures were taken such as the burning of all suspicious BSE-cows, the heat/pressure/time treatment (133 °C/3 bar/20 min) of MBM for poultry and pigs and the removal of all specific risk material out of MBM for poultry and pigs. The most effective measure was the feed ban for ruminants. In this way the number of BSE-cows born in 1990 could be reduced from 93 to 16 born in 1991. When it was realized that even smallest amounts of infectious MBM are able to transfer the disease (0.1 g), the so called “zero-tolerance” had to be introduced. Cross-contaminations in feed mills, manufacturing compound feedstuffs for ruminants and for poultry/pigs had therefore to be considered in the official feed inspection. After the complete ban, manufacturing equipment and storage silos once used for MBM, were the last remaining sources of contaminations in some single cases.

From January 1991 to April 2004, 5637 feed samples were analysed microscopically for the presence of MBM. Only two cases of illegal use of MBM in compound feedstuffs could be detected: 5 % MBM in a complete feedstuff for laying hens and 2.5 % MBM in a complementary feedstuff for fattening bulls.

In the year 2004 (end of April) no BSE-cows (46’272 animals tested) and no contaminated feedstuffs (558 samples) could be found.

Keywords

Switzerland, BSE, Feedstuff, Feed Inspection

PORTUGUESE OFFICIAL INSPECTION IN THE FIELD OF ANIMAL NUTRITION CONCERNING THE DETECTION OF BANNED MEAT AND BONE MEAL

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INTRODUCTION

Since 1990 that Portuguese competent authorities have developed an official inspection in the field of animal nutrition, helping safeguard of animal health, human health and environment, as well as to ensure the fairness of commercial transactions and protect the interests of consumers. Starting with the detection of the presence of illegal anabolic agents in animal feed, several changes have been added to the strategy control, such as the raw data composition in order to evaluate the compliance of feedingstuffs labelling, additives content, as well as undesirable and forbidden substances determination.

All the routine methods are EU official methods of analysis, European or International Standards or based in recognised scientific papers.

Due to the problem of BSE, with the first Portuguese diagnosed case in 1990, and in view to accomplish European and National legislation requisites aimed, a new official method was included in our control plan. The method based on the guidelines for microscopic examination established by Directive 96/25/CE, was implemented after a training course in Rome in 1998 and the participation in a proficiency test (organised by European Commission), and allows the qualitative identification of animal origin constituents in animal nutrition.

Since then, we have analysed more than ten thousands samples for constituents of animal origin, and quality control is assured by internal procedures and the participation in available intercomparison tests.

2003 ANALYTICAL ACTIVITIES CONCERNING THE DETECTION OF BANNED MEAT AND BONE MEAL

During 2003 the inspection program comprised three official plans with different but also specific targets: manufacturers of compound feedingstuffs and mineral premixes - PICAGA; livestock farms, commercialisers and distributors of compound feedingstuffs, premixes and feed additives - COMPLEMENTARY PLAN; fish meal manufacturers - AUTOCONTROL PLAN.

The analysed matrices were animal feedingstuffs, raw materials (vegetable and animal origin) and premixes.

EVOLUTION ACTIVITY CHART DURING THE LAST FOUR YEARS

RESULTS AND CONCLUSIONS

So far, only eight samples were considered positive for the presence of animal origin constituents: three raw materials in 1999 and three raw materials and one dairy feed in 2000 revealed the presence of mammalian bone structures, and one dairy feed in 2004 showed the presence of fish meal. All these feedingstuffs samples came from livestock farms.

Microscopic method proves to be a good tool for animal constituents detection qualitative analysis, although the existence of minor difficulties on the distinction between animal species origin of the observed structures *

* This was the case of some particles observed in fish meal, which were similar to mammalian bones. However when comparing microscopic structure with the ones of those particles, some differences were showed. The confirmation of the results were done with collaboration of DGCCRF, Rennes, France

P.4.- Portuguese Official Inspection in the field of animal nutrition concerning the detection of banned meat and bone meal

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Since 1993 that Portuguese competent authorities have developed an official inspection in the field of animal nutrition, helping safeguard of animal health, human health and environment, as well as to ensure the fairness of commercial transactions and protect the interests of consumers. Starting with the detection of the presence of illegal anabolic agents in animal feed, several changes have been added to the strategy control, such as the raw data composition in order to evaluate the compliance of feedingstuffs labelling, additives content, as well as undesirable and forbidden substances determination. All the routine methods are EU official methods of analysis, European or International Standards or based in recognised scientific papers.

Due to the problem of BSE, with the first Portuguese diagnosed case in 1990, and in view to accomplish European and national legislation meanwhile adopted, a new official method was included to the control plan. The method based on the guidelines for microscopic examination established by Directive 98/88/CE, was implemented after a training course in Rome in 1998 and the participation in an proficiency test (organised by European Commission), and allows the qualitative identification of animal origin constituents in animal nutrition.

Actually the inspection program comprises four official plans with different business operator targets: manufacturers of compound feedingstuffs and onfarm mixers (PNCACA); livestock farms, intermediates and distributors of compound feedingstuffs, premixes and feed additives (complementary plan); fish meal manufacturers, (autocontrol plan) and 3rd countries importation of fish meal. The matrices analysed are animal feedingstuffs, raw materials (vegetables and animals origin) and premixes.

So far, we have analysed more than ten thousand samples and only eight of them were considered positive: three in 1999, four in 2000, and one in 2004, all observed in the feedingstuffs samples from livestock farms.

Keywords

Official control, animal feed, microscopic method.

FISH MEAL RUMINANT/MULTI-SPECIES MILL BAN – THE LOSSES: IS THERE A RISK?

Ian H. Pike
IFFO

THE BAN AND ITS CONSEQUENCES

- Fish meal use primarily for health benefits – especially for young animals (starter) on unmedicated feeds
- Ruminant ban stopped use in many pig+poultry starter diets – as most produced in multi-species mills
- Mistaken image of fish meal that it is not a safe food, but
- What are the risks?

SOURCES OF RISK

- Contamination with land animal protein
  - Fish meal produced in factories dedicated to fish processing – controlled by animal by-products Directive 1774/2002
  - Risk of cross contamination low – similar to that in vegetable proteins
  - New method available to detect land animal tissue in feeds containing fish meal down to 0.1% content (Directive 2003/126)
- Prions in fish very different to those in land animals

EFFECTS OF BAN ON FISH MEAL INDUSTRY AND EU FISH MEAL USE

ESTIMATED LOSS OF EU FISH MEAL MARKET BASED ON USE UP TO 2000 (EU Fish meal use in 2000 – 1056 TT)

	Thousand Tonnes (TT) per annum
Ruminants	**100**
Pigs and poultry use in multi-species mills	**200**
New EU states (mainly multi-species mills)	**50**
TOTAL	**350**

ESTIMATED LOSS IN VALUE OF FISH MEAL AS A RESULT OF RUMINANT BAN

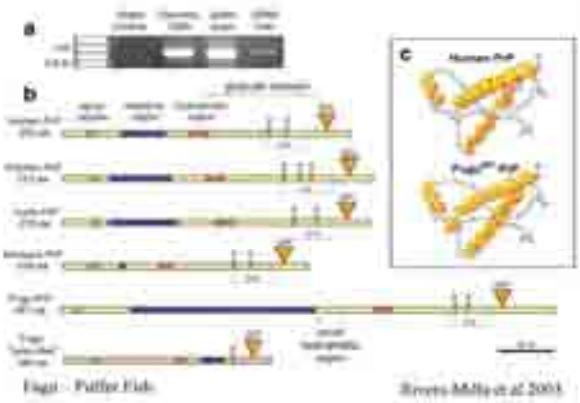
- Effect of ban on price, due to loss of 350TT market – price reduced by €30/tonne
- This caused by the displaced 350TT having to be sold in lower value markets
- Loss to EU fish meal producers

Production by EU members in 2002	575TT
Loss to EU producers @ €30/T	€17 million p.a.
Loss to producers in Peru and Chile	€84 million p.a.
Loss to all producers in world	€192 million p.a.

PRION PROTEINS IN FISH – A LINK TO DISEASE?

Edward Málaga-Trillo
University of Konstanz

The First Fish Prion Protein



Fugu – Puffer Fish

Rivera-Milla et al 2003

CONCLUSIONS

- Fish have normal prion proteins (PrPs)
- Fish prion proteins are highly diverse (in contrast to land animals)
- Each group of vertebrates rapidly evolved its own type of prion protein: pathogenicity is probably restricted to specific environments
- Similar predicted folds suggest similar functions of fish and mammalian prion proteins
- Fish prion proteins are required for proper brain development
- Fish PrPs are markedly different to those in land animals.
- Therefore unlikely that rogue prions, even if produced in fish, would transmit encephalopathy disease to land animals.
- Rogue prions not found in fish. So far, no evidence for their existence.
- Risk of cross infection of land animals with encephalopathies from fish has not been demonstrated.

P.5.- Fish meal ruminant/multi-species mill ban – the losses: is there a risk?

Ian H. Pike

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Atypical case of bovine spongiform encephalopathy in an East-Flemish cow in Belgium.

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Introduction

Bovine spongiform encephalopathy (BSE) is a prion disease with a fatal neurodegenerative pathogenesis. It is characterized by the accumulation of an abnormal protein (PrP^{res}), which is formed posttranslationally from the normal isoform (PrP^{c}). Research in mice showed the existence of different sheep scrapie strains. Scrapie strain discrimination is currently based upon biological typing in a panel of inbred mice, using incubation time and brain pathology scoring as criteria. However, no large scale studies of the molecular features of PrP^{res} have been reported for bovine BSE to date. Till now, the BSE strain seemed to maintain constant biological and molecular properties even after experimental or accidental passages into different species (mice, humans, primates & sheep). Very recently, variant forms of BSE have been reported in Japan, Italy and France. These forms were characterized by atypical histopathological, immunohistochemical and/or biochemical phenotype compared to the classical BSE strain. The present case describes the first Belgian atypical BSE case.

Materials & Methods

Since January 1st 2001, all cattle older than 30 months is tested for TSE via a rapid test (the NRL uses the TeSeE-kit of Bio-Rad, Nazareth, Belgium) following EC regulation 999/2001. Samples positive according to the ELISA screening are further subjected to scrapie associated fibrils (SAF), histopathology, immunohistochemistry and Western blot (WB) at the NRL.

Results

A positive ELISA sample from a 64 month-old East-Flemish or Belgian white and red cow (inset) was presented at the NRL for confirmation. The animal was reported healthy before slaughter. The optical density (OD) titers were positive at the local laboratorium (2.324 & 2.116) and at the NRL (0.953 & 0.708). The histopathology of the obex, pons and midbrain showed no spongiform changes; immunohistochemistry of the brainstem revealed no signal of PrP^{res} accumulation typical for BSE and SAF was negative. However, WB analysis (Bovine WB, Bio-Rad, France –antibodies 12F10 and SAF60) of the same homogenate that was prepared from the obex region (contralateral side) for ELISA revealed a small amount of PrP^{res} with an electrophoretic profile different from that of typical BSE-associated PrP^{res}. The band on the gel of the non-glycosylated form of PrP^{res} of the present case clearly showed a lower migration pattern compared to that of a typical BSE case (Fig. 1).

Discussion

For many years it was assumed that there was only one BSE strain. Only very recently, reports of atypical BSE cases were announced in Japan, Italy and France. The Japanese case describes a very young bull (23 months) characterized by the absence of vacuolation changes and PrP^{res} deposits immunohistochemically. The WB analysis revealed an electrophoretic profile different from that of classical BSE, characterized by a low content of the di-glycosylated molecular form of PrP^{res} and a faster migration of the non-glycosylated form of PrP^{res}. The Italians observed two BSE affected cattle with a previously unrecognized neuropathological profile and PrP^{res} type. These cases were determined by a different staining pattern on immunohistochemistry, a difference in size and glycoform ratio of PrP^{res} on immunoblot and a difference in regional distribution of lesions. The French two cases showed variant molecular features with a different PrP^{res} electrophoretic profile from other BSE cases, mainly characterized by a higher molecular mass of the non-glycosylated PrP^{res}. The present case shows the most similarities with the Japanese case (except for the age).

The fact that these strains were detected worldwide and in several breeds suggest that there is no local or breed dependent feature involved. It could be that the WB techniques have become more specifique within the last year in the detection of minor differences in di-, mono- and non-glycosylated molecular forms of PrP^{res}. Infection of cattle by scrapie could also be considered since scrapie can be transmitted by direct contact between animals and/or through environmental contamination.

In conclusion, continued research on BSE reveals nowadays different BSE strains in analogy with the different sheep scrapie strains. Further research is necessary about the epidemiological significance of these new strains. Atypical BSE cases may question the significance and efficiency of the BSE epidemio-surveillance protocol and the validation of the confirmatory tests.

Reference

H. De Bosschere, S. Roels, E. Vanopdenbosch (2004) Atypical case of bovine spongiform encephalopathy in an East-Flemish cow in Belgium. The Journal of Applied Research in Veterinary Medicine , vol 2 (1), 52-54

P.6.- Atypical case of bovine spongiform encephalopathy in an East-Flemish cow in Belgium.

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Bovine spongiform encephalopathy (BSE) is a prion disease with a fatal neurodegenerative pathogenesis. It is characterized by the accumulation of an abnormal protein (PrPres), formed posttranslationally from the normal isoform (PrPc). Research in mice showed the existence of different sheep scrapie strains. Scrapie strain discrimination is currently based upon biological typing in a panel of inbred mice. However, no large scale studies of the molecular features of PrPres have been reported for bovine BSE to date. Till now, the BSE strain seemed to maintain constant biological and molecular properties even after experimental or accidental passages into different species. Very recently, variant forms of BSE have been reported in Japan, Italy and France. These forms were characterized by atypical histopathological, immunohistochemical and/or biochemical phenotype compared to the classical BSE strain. The present case describes the first Belgian atypical BSE case. Since January 1st 2001, all cattle older than 30 months is tested for TSE via a rapid test following EC regulation 999/2001. Samples positive according to the ELISA screening are further subjected to scrapie associated fibrils (SAF), histopathology, immunohistochemistry and Western blot (WB) at the NRL. A positive ELISA sample from a 64 month-old East-Flemish or Belgian white and red cow was presented at the NRL for confirmation. The histopathology of the obex, pons and midbrain was negative, immunohistochemistry and SAF were also negative. However, WB analysis was positive with an electrophoretic profile different from that of a typical BSE case. The band on the gel of the non-glycosylated form of PrPres of the present case clearly showed a lower migratrion pattern compared to that of a typical BSE case. For many years it was assumed that there was only one BSE strain. Only very recently, reports of atypical BSE cases were announced in Japan, Italy and France. The Japanese case describes a very young bull (23 months) negative on histopathology and immunohistochemistry and a WB electrophoretic profile different from that of classical BSE. The Italians observed two BSE affected cattle with a a different staining pattern on immunohistochemistry, a difference in size and glycoform ratio of PrPres on WB and a difference in regional distribution of lesions. The French two cases showed variant molecular features with a different electrophoretic profile from other BSE cases. The present case shows the most similarities with the Japanese case (except for the age). The fact that these strains were detected worldwide and in several breeds suggest that there is no local or breed dependent feature involved. It could be that the WB techniques have become more specifique within the last year or infection of cattle by scrapie could also be considered. In conclusion, continued research on BSE reveals nowadays different BSE strains in analogy with the different sheep scrapie strains. Atypical BSE cases may question the significance and efficiency of the BSE epidemio-surveillance protocol and the validation of the confirmatory tests.

Keywords

Bovine spongiform encephalopathy, BSE, Western Blot, atypical BSE

First belgian Nor98 scrapie case diagnosed via active surveillance

De Bosschere H. [1], Roels S. [1], Benestad S. L. [2], Vanopdenbosch E. [1]

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Introduction

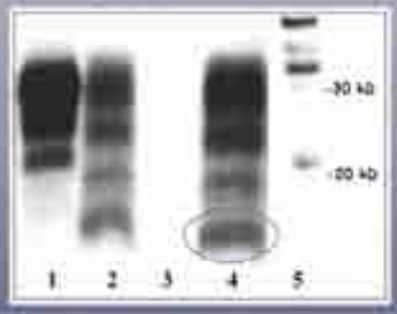
Scrapie is a fatal transmissible spongiform encephalopathy (TSE) caused by prions, similar to bovine spongiform encephalopathy (BSE) in cattle. Typical features of these diseases are a long incubation period and the gradual vacuolation of brain neurons and neuropil. The pathogenesis of the diseases is believed to due to the conversion of the normal protease-sensitive prion protein, PrP^{c}, into a partly protease-resistant isoform, PrP^{res}, which accumulates progressively in the central nervous system of the affected animals. Several (classical) scrapie strains have been described. However, the present case corresponded very well with the unusual Nor98 strain detected in Norway.

Materials & Methods

In Belgium, the active TSE surveillance program (EC regulation 999/2001) is implemented. In case of a positive rapid test, Classical tests (such as histopathology, immunohistochemistry, scrapie associated fibrils (SAF) and western blot (WB) are used for confirmation.

Results

In 2002, 8 scrapie outbreaks have been detected in Belgium. Five of these outbreaks showed a classical scrapie lesion profile, but the 6th (present case) showed special features. This apparently healthy ewe was presented for slaughter. According to the active TSE surveillance program only part of the medulla oblongata around the region of the obex is available for testing. The sample repeatedly tested positive with the Bio-Rad rapid test. Histopathology as well as immunochemistry (polyclonal R524-7) and SAF were negative. PrP^{res} WB analysis (Bio-Rad protocol) with antibodies 12F10 & SAF60 and BAR228 & SAF60 gave clear positive result showing a PrP^{res} glycoprofile with a strongly marked lower band at ca 12 kDa, compared to a classical scrapie glycoprofile (Fig. 1). This "special" glycoprofile of the present case was confirmed by Benestad S. et al. in Norway. The whole flock (n=55) was culled and examined, but no other animal tested positive with any test. The sheep's genotype was ARQ/ARQ.



Discussion

The unusual characteristics of the present case are:

1/ only one (of the 55) animals of the flock was affected;

2/ no lesions were present in the brainstem (obex) as compared to classical scrapie cases;

3/ the absence of PrP^{res} immunolabeling in the area of the obex;

4/ the PrP^{res} glycoprofile differed clearly from the glycoprofiles of classical scrapie strains and the BSE strain.

All these features corresponded very well with those reported of the unusual Nor98 strain detected in Norway. Nor98 cases are characterized by ataxia, the onset of disease at older age, the presence of neuropil vacuolization and PrP^{res} deposits mainly localized in the cerebellar and cerebral cortices respectively on histopathology and immunohistochemistry.

In the present case, histopathology, immunohistochemistry and SAF were all negative. Only the WB confirmed the scrapie positivity of the present case and the diagnosis of scrapie could therefore have been overlooked. Nor98 cases may question the significance and efficiency of the scrapie active epidemiu-surveillance protocol, because from a diagnostic point of view, the positive results obtained with a rapid test require to be confirmed by standard methods like the histopathological examination and the immunohistochemical detection of PrP^{res} at the level of the obex.

Reference

H. De Bosschere, S. Roels, S. L. Benestad, E. Vanopdenbosch (2004). Scrapie case similar to Nor98 diagnosed in Belgium via active surveillance. Veterinary Record (in press).

P.7.- First Belgian Nor98 scrapie case diagnosed via active surveillance.

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Scrapie is a fatal transmissible spongiform encephalopathy caused by prions. Typical features of these diseases are a long incubation period and the gradual vacuolation of brain neurons and neuropil. Several (classical) scrapie strains have been described based on lesion profiling in mice. In Belgium, since April 2002, all sheep older than 18 months are tested with a rapid test (Bio-Rad) through the active TSE surveillance program (EC regulation 999/2001). Five of the 6 outbreaks in 2002 showed a classical scrapie lesion profile, but in one case the positive sheep showed special features. The ewe was apparently healthy and presented for slaughter. According to the active epidemio-surveillance protocol only part of the medulla oblongata around the region of the obex is taken out. The sample was repeatedly tested positive with the rapid test. The histopathological investigation revealed no vacuolar lesions neither in neurons and in neuropil in the region of the obex. There was no detectable PrPSc as revealed by immunohistochemistry in the obex region and tonsils. The detection of scrapie associated fibrils (SAFs) was also negative. PrPSc Western blot (WB) analysis was positive showing a PrPsc glycoprofile with a strongly marked lower band at ~ 12 kDa, compared to a classical scrapie glycoprofile. The “special” glycoprofile of the present case was confirmed by Benestad S. and coworkers in Norway. According to the surveillance protocol, the whole flock was culled and the brains of all animals older than 18 months were examined, but no other animal of the flock tested positive with the rapid test.. The sheep PrP genotype was A136R154Q171 homozygous, analyzed via denaturizing gradient gel electrophoresis.

The unusual characteristics of the present case are: 1/ only one (of the 55) animals of the flock was affected; 2/ no lesions were present in the brainstem (obex) as compared to the described lesion profiles of classical scrapie cases; 3/ the absence of PrPsc immunolabelling in the area of the obex; 4/ the PrPsc glycoprofile of the present case differed clearly from the glycoprofiles found in isolates of classical scrapie strains and the BSE strain, and is not distinguishable from the Nor98 glycoprofile. All these features corresponded very well with those reported of the unusual Nor98 strain detected in Norway. This type of scrapie, described for the first time outside Norway, may question the scrapie active epidemio-surveillance protocol because: from a diagnostic point of view, the positive results obtained with a rapid test require to be confirmed by standard methods like the histopathological examination and the immunohistochemical detection of PrPsc at the level of the obex. In the present case the official Belgian recognized confirmation tests (histology, immunohistochemistry and SAF) were all negative. Only the WB confirmed the scrapie positivity of the present case and the diagnosis of scrapie could therefore have been overlooked. This may be of significance for future sampling in scrapie surveillance programs and confirmation tests.

Keywords

Sheep, NOR98, scrapie, TSE

Evaluation of a new test for genotyping codon 136-154-171 of the ovine prionic protein (PRNP) gene through reverse hybridization

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Introduction

Scrapie is the oldest transmissible spongiform encephalopathy (TSE) and is described in literature since 2.5 centuries. It is a fatal neurodegenerative disease that occurs naturally in sheep and goats. Currently, the etiology is considered as an infectious disease with a maternal and horizontal contagious transmission, where host genetic factors play a central role. The scrapie natural incidence in sheep is associated with prion protein gene polymorphism, in particular regarding the codons 136, 154, 171. At present, as no rapid difference can be made between scrapie and bovine spongiform encephalopathy (BSE), all prion diseases in sheep are determined as TSE. Genotyping of sheep has become very important for the eradication strategy of TSE and the breeding program towards TSE resistance (Common Decision 1803/2002). Only laborious techniques such as denaturizing gradient gel electrophoresis (DGGE) were until recently available. Recently, genotyping via real-time polymerase chain reaction (Fontaine and others, in preparation) became possible. The purpose of this paper is to describe the test results of the newly developed 'Ovine PrP Gene Test' of Nuclear Lase Medicine. The technique is based on reverse hybridization and appears to be competitive with other tests available for genotyping of sheep.

Materials & Methods

The test consists of three phases :

1) DNA isolation from non-coagulated blood (EDTA or citrate as anticoagulant) through a coloumn DNA purification system. However, although the ' Ovine PrP Gene Test ' is not originally designed for use on brain tissue, we also evaluated this test with brain tissue of sheep.

2) Amplification of the relevant ovine prionic protein (PRNP) gene sequences in the target PrP region with biotinilated primers and

3) Hybridization of biotinilated amplicons on strips coated with allele-specific probes. The hybrids are further revealed through streptavidin conjungated with alkaline phosphatase and an appropriate substrate.

The test is presented as a kit, with all necessary buffers and solution.

Results

100 blood and 28 brain samples were tested. Finally all samples gave satisfactory results. The results obtained with the "Ovine PrP Gene Test" corresponded with those obtained via DGGE and RT-PCR. The signal on the strips obtained using brain tissue appeared much stronger than the signal on the strips obtained using non-coagulated blood.

Discussion

The major advantages of this new 'Ovine PrP Gene Test' are its rapidity, with results within 5 hours, its easiness in use and manipulation, its need of only a restricted amount of blood (20µl) and its rather restricted investigation costs. Our evaluation showed also that it could be used on older blood samples (there were the DNA is already denaturating) and even on brain tissue. For this last application however, a special kit has to be used for the extraction of the DNA that is not available in the standard kit. Nevertheless, we encountered some problems with 'phantom lines'. These false positive signals could, as mentioned in the trouble shooting guide included in the test, mainly be contributed to the temperature during hybridisation and stringent wash steps.

The new test can be a powerfull tool in the EU selection program of sheep breeding towards TSE resistance and in the frame of the TSE-related safety of certain sheep products.

Reference

H. De Bosschere, S. Roels, C. Renard, T. Briers, J. De Sloovere, E. Vanopdenbosch. Evaluation of a new test for genotyping codon 136-154-171 of the ovine prionic protein gene through reverse hybridization (submitted for publication)

P.8.- Evaluation of a new test for genotyping codon 136-154-171 of the ovine prionic protein (PRNP) gene through reverse hybridization.

S. Roels[1] , H. De Bosschere[2], E. Vanopdenbosch[3] , C. Renard[4], J. De Sloovere[5] , T Briers[6]

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A new test for genotyping codon 136-154-171 of the ovine prionic protein (PrP) gene via the detection of mutations of the PrP gene through reverse hybridization has been evaluated on non-coagulated blood and brain tissue. A total of 100 sheep blood samples and 28 brain tissue samples were tested. These samples were also analyzed with denaturating gradient gel electrophoresis and real-time polymerase chain reaction to confirm the results obtained via the present test. The results obtained via these three tests corresponded perfectly. The poster briefly describes the new test.

The new test can be a powerfull tool in the EU selection program of sheep breeding towards TSE resistance and in the frame of the TSE-related safety of certain sheep products.

Keywords

PRNP genotyping, ovine, reverse hybridisation

P.9.- Evaluation of Prionics Check – LIA Test for the screening of PrPSc in the lymphoreticular system of sheep.

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The aim of the study was to investigate the presence of PrPSc in the lymphoreticular system (LRS) of italian Scrapie infected sheep. For this purpose, we tested the performance of a luminescence immunoassay (Prionics check LIA), already approved from EU for the active surveillance of TSE, on lymphoid tissue. We first applied this test to lymph nodes, positive for PrPsc with a highly sensitive Western blot (WB) based on NaPTA precipitation, of six sheep coming from different Italian Scrapie outbreaks. All animals were positive by confirmatory tests performed on central nervous tissue (CNS). Subsequently, the Prionics check LIA was used for the screening of a Piedmont scrapie outbreak, comparing the results obtained with NaPTA WB. The trial was carried out on 101 Biellese sheep with ARQ/ARQ (74), ARR/ARQ (13), ARQ/AHQ (9), ARQ/VRQ (4), ARQ/ARH (1) genotypes, four of which Scrapie positive. For the screening of the LRS lymph nodes, tonsil, spleen, ileum and ileumcaecal valve were analyzed. To warrant comparable results, the samples of spleen and ileum were cut in small pieces by two scalpels without any buffer, untill the tissue appeared homogeneous. The resulting homogenate was split in two aliquots for the two methods, while lymph nodes and tonsils were simply cut in half part and used at random.

The results showed a complete agreement between Prionics-Check LIA and NaPTA WB (that can reach an analytical sensitivity 3 logs more then routine WB). It was however noted that critical points on phases of homogenation and resuspension of pellet can imply a decrease in luminescence unit values. LRS positivity and CNS negativity, due to a preclinical status of the animals, was never detected while; among four sheep positive at CNS (genotype ARQ/ARQ), only 3 resulted positive to LRS. Our results show that Prionics-Check LIA test is a reliable tool for the screening of the disease-specific form of PrP in ovine lymphoid tissue.

Keywords

Evaluation, rapid tests, TSE, lymphoreticular system, sheep.

The Development of a Diagnostic Test for Scrapie Infection in Sheep: ^{1}H-NMR Spectroscopy and Trace Element Profiling

Abstract

The metabolite profiles of blood plasma from scrapie infected (pre-clinical and post clinical) and control sheep were analysed by ^{1}H-Nuclear magnetic resonance spectroscopy (^{1}H-NMR). The results indicate that comparison of the citrate, lactate and 3-D-hydroxybutyrate concentrations enabled successful discrimination between blood plasma samples from control, and pre-clinical and post-clinical animals. Trace element analysis by inductively coupled plasma mass-spectroscopy (ICPMS) identified significant differences between the concentrations of strontium, calcium, copper, magnesium, phosphorous and zinc in the pre-clinical and post-clinical samples. Interestingly the concentrations of copper and manganese, which are elements normally associated with scrapie, did not differ significantly in the samples analysed.

Samples

All animals are of the same breed (Cheviot) and were reared under identical experimental conditions.

- Blood plasma samples analysed by ^{1}H-NMR and ICPMS:
  - 10 uninfected sheep, 10 pre-clinical sheep and 10 post-clinical sheep.

^{1}H-NMR Spectroscopy

- Proteins and high mass lipids removed. Remaining low molecular weight metabolites analysed (Figure 1)
- Comparison of metabolite concentrations in a three dimensional plot (Figure 2) reveals a clear distinction between the control and infected samples
- Analysis of the non-polar fraction of post-clinical samples exhibits increased intensity in three regions (Figure 3)

Figure 1. ^{1}H-NMR spectra of whole blood plasma (A) and blood plasma following centrifugal filtration (B)

Figure 2. A 3D plot of the concentrations of citrate, lactate and 3-hydroxybutyrate for all control, pre-clinical and post-clinical samples.

Figure 3. NMR spectra of blood plasma following centrifugal filtration and extraction with chloroform. Differences in peak intensities can be observed between 0.5 and 2 ppm

Multi-elemental Profiling

- Trace element analysis of blood plasma samples by inductively coupled plasma mass-spectroscopy (ICPMS) identified several elements of interest
- Figure 4 shows a distinct separation between the pre-clinical and control samples and the post clinical samples

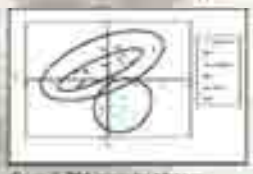

Figure 4. PCA scores for 12 trace elements with 95% confidence boundaries for each sample type

Conclusions

- Citric acid, lactic acid and 3-D-hydroxybutyric acid concentrations have enabled discrimination between scrapie infected and control sheep
- Multi-element profiling enables identification of post-clinical sheep
- Metabolite and elemental profiling identifies changes in the biochemistry of scrapie infected animals aiding pre-clinical diagnosis of the disease

References

(1) Nicholson, JK.; Foxall, P.J.D. Anal. Chem. **1995**, 76, 793-811

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Acknowledgement

We would like to thank the UK Food Standards Agency for funding this work and also ADAS for supplying the experimental samples.

P.10.- The Development of a Diagnostic Test for Scrapie Infection in Sheep: ^{1}H-NMR Spectroscopy and Trace Element Profiling

A. J. Charlton, S. Jones, J. Lewis, N. Langford, R. Macarthur, J. Dennis & P. A. Brereton

Central Science Laboratory, Sand Hutton, York, Y041 1LZ, UK.

The metabolite profiles of blood plasma from scrapie infected (pre-clinical and post clinical) and control sheep were analysed by ^{1}H-Nuclear magnetic resonance spectroscopy (^{1}H-NMR). The results indicate that comparison of the citrate, lactate and 3-D-hydroxybutyrate concentrations enabled successful discrimination between blood plasma samples from control, pre-clinical and post-clinical animals. Trace element analysis by inductively coupled plasma mass-spectroscopy (ICPMS) identified significant differences between the concentrations of strontium, calcium, copper, magnesium, phosphorous and zinc in the pre-clinical and post-clinical samples. Interestingly the concentrations of copper and manganese, which are elements normally associated with scrapie, did not differ significantly in the samples analysed.

Keywords

Scrapie, NMR, ICPMS, Diagnostic test

A new sensitive assay for the detection of ovine and caprine PrP

TeSeE® sheep/goat

Pending E.C. Approval

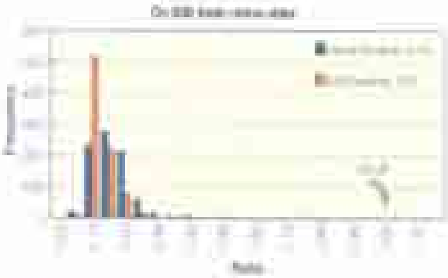
Introduction

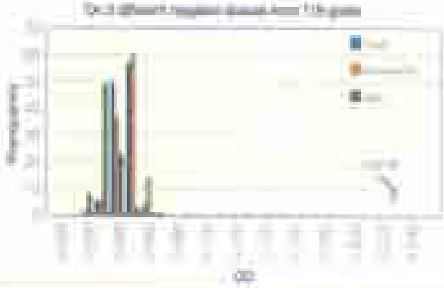
Performances

Comparative performances of TeSeE® sheep/goat and current TeSeE® assays on 5 diluted positive ovine samples

Comparative performances of TeSeE® sheep/goat and current assays on animals from flocks with TSE infected sheep

Comparative performances of TeSeE® sheep/goat and current TeSeE® assays on different tissues from a flock with TSE infected goats





Conclusions

BIO-RAD

P.11.- A new sensitive assay for the detection of ovine and caprine PrPres

M. Feyssaguet, J. L. Morize, A. Compoint

Bio-Rad R&D TSE - 3 bd Raymond Poincaré - 92430 Marnes-la-Coquette - France

TeSeE™ Sheep/Goat is a new rapid assay for the post mortem diagnosis of TSEs in small ruminants (sheep and goat). This new Bio-Rad ELISA kit is based on the same assay procedure and interpretation criteria than the TeSeE™ assay actively used in most laboratories for the detection of BSE in cattle. The new TeSeE™ Sheep/Goat assay has the capacity to detect PrPSc in different type of tissues from infected animals. The presented data were obtained when comparing the performances of the two TeSeE™ and TeSeE™ Sheep/Goat assays, with different ovine and goat tissue samples (nervous tissues, lymph nodes tissues, …).Assays were performed either manually or semi automatically with the TeSeE™ NSP system. With the same assay procedure for both sample processing and immunometric detection, TeSeE™ Sheep/Goat assay can be easily adapted to all TSEs screening laboratories. The assay sensitivity and specificity are improved by the specifically designed buffer A composition. Newly selected monoclonal antibodies enhance the specific recognition of ovine and caprine PrPSc in various tissues from infected animals, with a very low affinity with bovine PrPres. For better adaptability of the test to the laboratory activity, the sample purification stage of the TeSeE™ Sheep/Goat assay has been adapted and validated on the TeSeE™ NSP System. Thus, assay reproducibility is improved even when large series of samples are tested.

Keywords

Evaluation, rapid tests, TSE, sheep.

Ovine TSE confirmatory testing with the new TeSeE® Sheep/Goat Western Blot assay

[redacted]

TeSeE® Sheep/Goat Western Blot assay protocol

Glycoprofile of the Nor98 type of scrapie with the Bio-Rad TeSeE® Sheep/Goat Western Blot assay

BIO-RAD

P.12.- Ovine TSE confirmatory testing with the new TeSeE Sheep/Goat Western Blot assay

S. L. Benestad[1], J. M. Bilheude[2], J. P. Bourgeois[2]

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[2] Bio-Rad R&D TSE - 3 bd Raymond Poincaré - 92430 Marnes-la-Coquette - France

The active surveillance program conduced by the EC for sheep and goats has resulted in the identification of increased numbers of TSE positive cases.

The Bio-Rad TeSeE™ screening rapid assay has been evaluated and approved by the commission for diagnosis of BSE in cattle and was also recommended for TSE testing in sheep and goats.

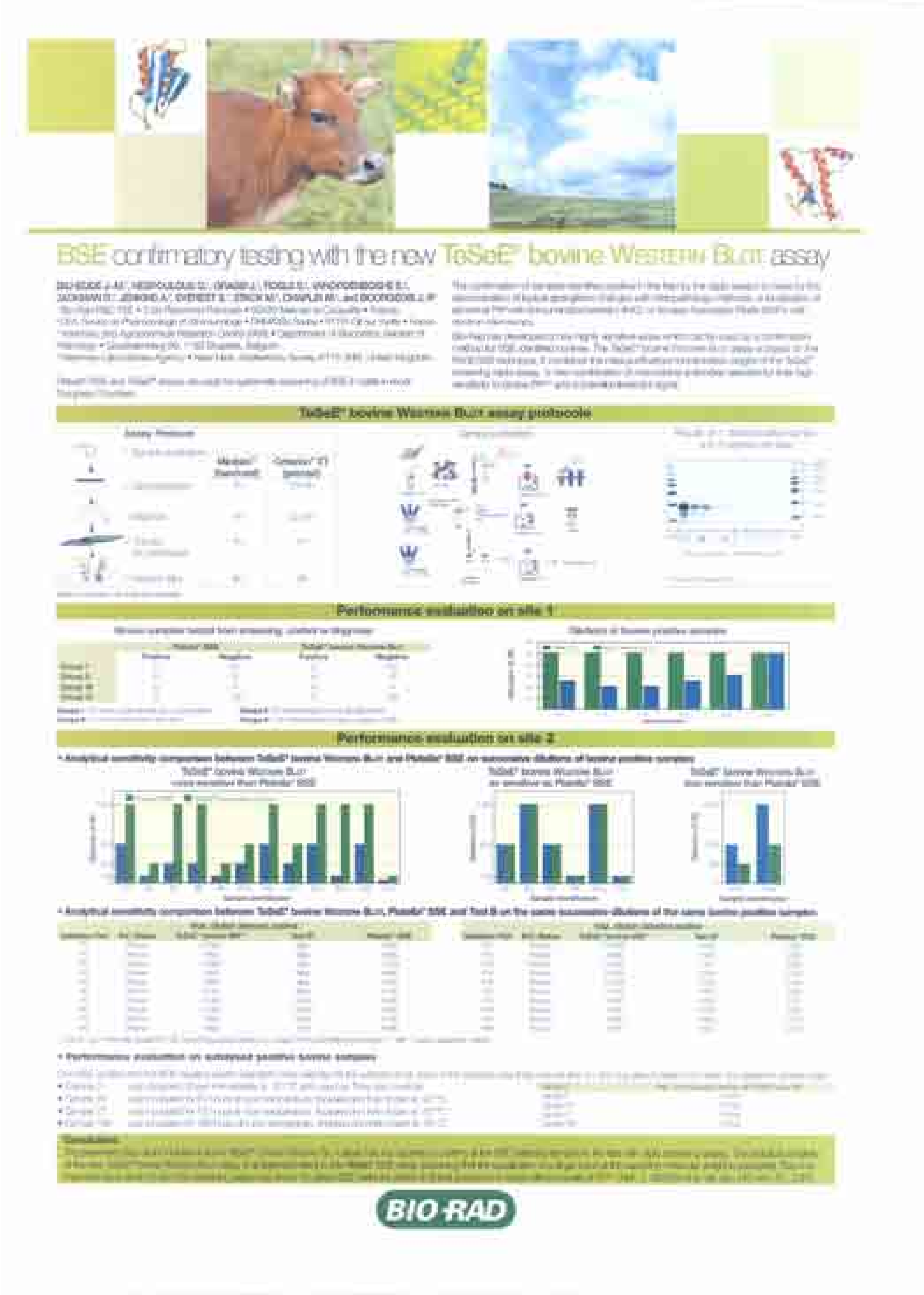
Positive samples identified during this surveillance program are systematically confirmed as TSE-affected by the demonstration of typical spongiform changes by histopathology, or by the detection of abnormal PrP by Immunohistochemistry (IHC), or of Scrapie Associated Fibrils (SAFs) by electron microscopy.

Bio-Rad has developed a new highly sensitive assay, which can be used as a confirmatory method in sheep and goats. The TeSeE™ Sheep/Goat WESTERN BLOT assay is based on the PAGE/WBtechnique.

It consists of the initial purification/concentration stages of the TeSeE™ screening rapid assay, a new combination of monoclonal antibodies selected for their high affinity to sheep and goat PrPSc and a chemiluminescent signal.

Keywords

Evaluation, rapid tests, TSE, sheep.


BSE confirmatory testing with the new TeSeE® bovine Western Blot assay
TeSeE® bovine Western Blot assay protocols
Performance evaluation on site 1
Performance evaluation on site 2
BIO-RAD

P.13.- BSE confirmatory testing with the new TeSeE bovine Western Blot assay

J. M. Bilheude[1], G. Nespoulous[1], J. Grassi[2]

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[2] CEA, Service de Pharmacologie et d'Immunologie - DRM/DSV, Saday - 91191 Gif-sur-Yvette - France

Platelia¨ BSE and TeSeE™ assays are used for systematic screening of BSE in cattle in most European Countries. The confirmation of samples identified positive in the field by the rapid assays is made by the demonstration of typical spongiform changes with histopathology methods, or localisation of abnormal PrP with Immunohistochemistry (IHC), or Scrapie Associated Fibrils (SAFs) with electron microscopy.

Bio-Rad has developed a new highly sensitive assay which can be used as a confirmatory method for confirmation of bovines detected BSE positives. The TeSeE™ bovine WESTERN BLOT assay is based on the PAGE/WB technique. It combines the initial purification/concentration stages of the TeSeE™ screening rapid assay, a new combination of monoclonal antibodies selected for their high sensitivity to bovine PrPres and a chemiluminescent signal.

The presented data clearly indicate that the TeSeE™ bovine WESTERN BLOT assay has the capacity to confirm all the BSE detected samples in the field with rapid screening assays.

The analytical sensitivity of the new TeSeE™ bovine WESTERN BLOT assay is at least equivalent to the Platelia¨ BSE assay (assuming that the visualization of a single band at the expecting molecular weight is accepted).

This is an important issue since the Bio-Rad screening assay was shown to detect BSE before the onset of clinical symptoms in cases with low levels of PrPres. (Ref.: J. GRASSI et al. Vet. rec.,145, nov. 10th, 2001).]

Keywords

Evaluation, rapid tests, TSE, bovine.


Comparative evaluation of the Bio-Rad TeSeE® and Platelia® assay formats
Comparison of the Platelia® and TeSeE® assay procedures
Performance of the TeSeE® assay with bovine and ovine samples
Conclusion
BIO-RAD

P.14.- Comparative evaluation of the Bio-Rad TeSeE and Platelia assay formats

J. M. Bilheude, G. Allain, G. Sauvage

Bio-Rad R&D TSE - 3 bd Raymond Poincaré - 92430 Marnes-la-Coquette - France

The practicability of the Bio-Rad BSE screening assay procedure has been improved. The new TeSeE™ assay format can be easily automated.

The following modifications were introduced:

• Packaging: 2 kits (TeSeE™ Purification and TeSeE™ Detection kits) for 2x 96determinations instead of 3 kits (BSE Purification, PK and Platelia BSE) for 1x96 determinations.

• Reactional volumes: decreasing reactional volume of reagents A, B and C to perform the purification steps in micro-test tube or in Deepwell Microplate format to partially or fully automate the different steps of the procedure.

The presented data where obtained during comparative studies performed on positive and negative sample panels tested manually or semi-automatically, with the Platelia¨ and TeSeE™ assay formats.

Comparative evaluation performed between the Platelia¨ and TeSeE™ assay formats demonstrated similar results in term of sensitivity when tested with all panels of positive sample dilutions (2 bovine panels and one ovine panel).

The specificity was evaluated on a population of 5343 negative bovine samples. No 1st intention false positive results were observed when testing the samples with the semi automatic system (TeSeE™ NSP). The OD distribution of the negative samples was also improved with the semi automated protocol.

On the 1058 samples manually tested with both Platelia¨ and TeSeE™ assays, similar results and OD repartition were observed (1st intention results).

This comparative evaluation clearly demonstrated that the performances were equivalent between the TeSeE™ and the Platelia¨ BSE kits. Based on the data obtained during the evaluation, it was also clear that the performance level was equal when testing the samples manually or with the semi automatic system (TeSeE™ NSP and DW40).

Note: The TeSeE™ assay format has been tested and validated with obex, retropharyngean lymph node from mule deer and elk animals.]

Keywords

Evaluation, rapid tests, TSE

Affinity capture by immunomagnetic beads : an original approach for the detection of prion proteins in environmental biofilms

B ElMoualij*, D Zorzi, 0. Pierard, E. Heinen and W Zorzi

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INTRODUCTION

Several data support the hypothesis that the environment could be a reservoir for the pathological infectious agent in Transmissible Spongiform Encephalopathies (TSEs). Some experiments reported previously show that healthy sheep have contracted TSEs upon introduction into fields and enclosures previously occupied by infected animals. It was also demonstrated that residual infectivity was retained in garden soil in prion scrapie-infected hamster brain material interred for 3 years (1). The maintenance of the resistance properties of TSE agent in the environment may be due to its adsorption to components of the soil matrices. In this study, we are investigating the potential contamination by TSEs agents of well defined complex environment samples such as bacterial biofilms.

Methods

We focussed on the detection of the spiked prion proteins in environmental matrices (activated sludge originating from waste water station) by combining two approaches. The first technique was based on the use of magnetic beads and allowed the selective extraction of the prion proteins from complex matrices, the elimination of all contaminants and also the concentration of the sample before analysis (figure 1). The second technique called the microextraction patented in our laboratory (2) is a physicochemical procedure which alters the structure of environmental samples and makes it suitable for the immunodetection analysis.

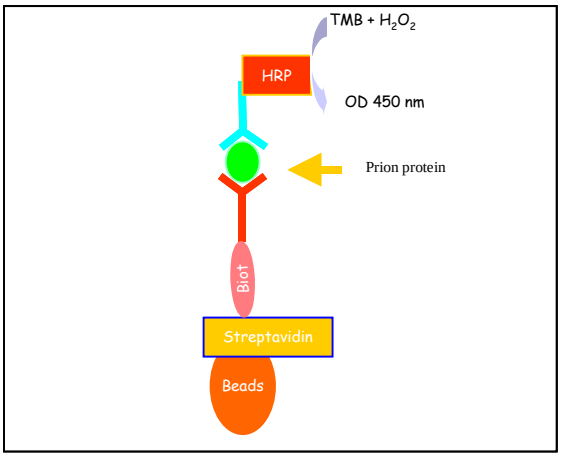

Figure 1: Magnetic Beads ELISA

RESULTS

By combining the two techniques and using a magnet, it was very easy to recover the prions proteins linked to immunomagnetic beads (figure 2). The successful immunocapture of the prion proteins has been evaluated using classical ELISA *(Enzyme-Linked Immunosorbent Assay)*. As the immunomagnetic capture ELISA method is simple and easy to apply, this technique could be useful for the detection of prion protein in environmental samples.

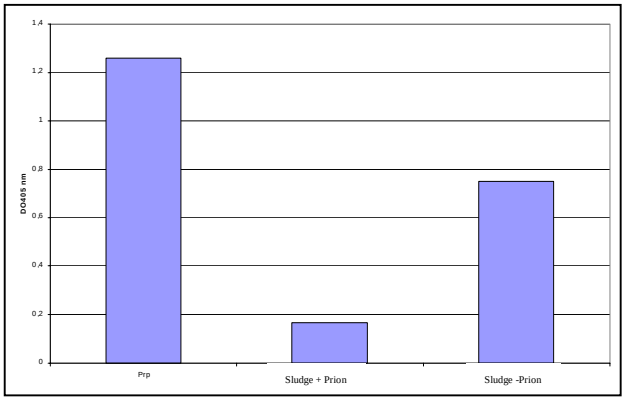

Figure 2: Magnetic Beads ELISA Comparaisons of prions proteins in buffer and spiked in activated sludge after microextractions procedures (2)

References:

1- Brown P, Gajdusek DC, Lancet. 1991 Feb 2;337(8736):269-70

2- ZORZI WILLY, EL MOUALIJ BENAISSA, ZORZI DANIELE; HEINEN ERNST; MELEN LAURENCE, Procédé d'extraction de micro-organismes intacts à partir de boues ou de biofilms, PCT/BE/00179, WO 02/31184 A2, 2002-05-18.

This work is supported by the Région Wallone (R.Rwal. 0744 "BSE Clean").

P.15.- Affinity capture by immunomagnetic beads : an original approach for the detection of prion proteins in environmental biofilms

B. El Moualij, D. Zorzi, O. Pierard, E. Heinen, W. Zorzi

Laboratory of Human Histology- Center of research on Prion Proteins (CRPP), Institute of Pharmacy-CHU, Avenue de l'Hôpital, 1, Sart Tilman, 4000, Liège, Belgium. (b.elmoualij@ulg.ac.be)

Several data support the hypothesis that the environment could be a reservoir for the pathological infectious agent in Transmissible Spongiform Encephalopathies (TSEs). Some experiments reported previously show that healthy sheep have contracted TSEs upon introduction into fields and enclosures previously occupied by infected animals. It was also demonstrated that residual infectivity was retained in garden soil in prion scrapie-infected hamster brain material interred for 3 years (1). The maintenance of the resistance properties of TSE agent in the environment may be due to its adsorption to components of the soil matrices. In this study, we are investigating the potential contamination by TSEs agents of well defined complex environment samples such as bacterial biofilms.

Methods:
We focussed on the detection of the spiked prion proteins in environmental matrices (activated sludge originating from waste water station) by combining two approaches. The first technique was based on the use of magnetic beads and allowed the selective extraction of the prion proteins from complex matrices, the elimination of all contaminants and also the concentration of the sample before analysis. The second technique called the microextraction patented in our laboratory (2) is a physicochemical procedure which alters the structure of environmental samples and makes it suitable for the immuno-detection analysis.

Results & Discussion**:**
By combining the two techniques and using a magnet, it was very easy to recover the prions proteins linked to immunomagnetic beads. The successful immunocapture of the prion proteins has been evaluated using classical ELISA *(Enzyme-Linked Immunosorbent Assay).* As the immunomagnetic capture ELISA method is simple and easy to apply, this technique could be useful for the detection of prion protein in environmental samples.

Keywords

Immunomagnetic bead, prion

Immuno-quantitative PCR :

a new way for detection of resistant prion protein

Stéphanie GOFFLOT[1], Benaïssa EL MOUALIJ[1], Danièle ZORZI[1], Stefan ROELS[2], Dominique QUATPERS[3], Jacques GRASSI[4], Ernst HEINEN[1] and Willy ZORZI[1].

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[2]VAR-CERVA-CODA, Veterinary and Agrochemical Research Center, Department of Biocontrol - Section of Pathology, National Reference Laboratory for Veterinary TSE (Belgium & Luxemburg), Groeselenberg 99, B - 1180 Brussels, Belgium.

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INTRODUCTION

Diagnosis tests approved today for the bovine spongiform encephalopathy allow to detect proteinase K resistant form of the prion protein (PrP^{Res}) present in the brain only. It would be interesting to detect at low concentration to find infectious agent in body fluids and then at ante mortem stage.

We have developed a new method associating the advantages of two advanced techniques : high-specificity ELISA-based immuno-detection and highly sensitive, quantitative real-time PCR. We have called our new technique ***immuno-quantitative PCR*** (Patent WO0131056).

IMMUNO QUANTITATIVE PCR

The first step to perform was a proteinase K digestion to keep only the infectious form of the prion protein. The resistant form was captured and detected by monoclonal antibodies (generous gift of J. Grassi from CEA). The reporter biotinylated DNA to amplify was linked to the biotinylated antibody by a streptavidin.

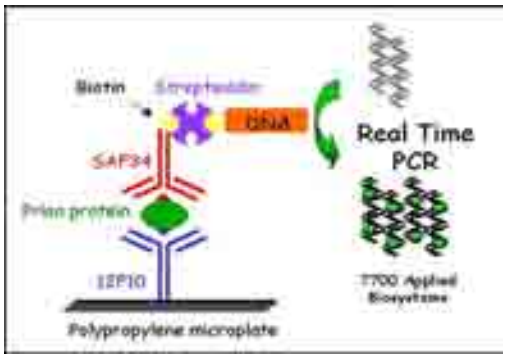

Figure 1: immuno quantitative PCR

RESULTS

Optical density signal in a healthy brain was detected at dilution 8 (--) and completely disappeared following PK treatment (--). The BSE infected brain PK treated was detected between dilution 32 and 64 (--).

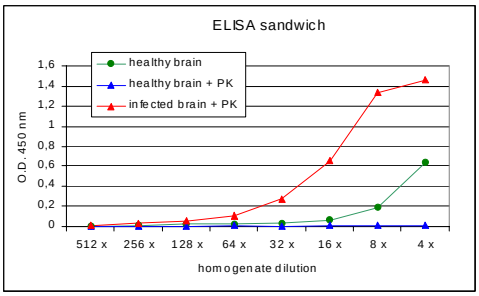

Figure 2 : sandwich ELISA results where OD at 450 nm is plotted versus brain homogenate dilution.

The results obtained with iqPCR gave a lower limit of detection. The figure 3 illustrated detection of infected brain PK treated. The limit of detection is between 100-500.

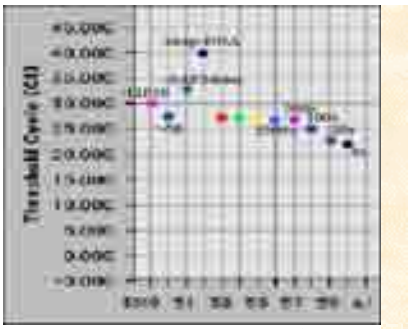

Figure 3 : immuno quantitative PCR results where threshold cycle (intersection between baseline and fluorescent curve) is plotted versus the well position.

CONCLUSION

The immuno quantitative PCR is more sensitive than classical ELISA. This new technology open new ways for diagnosis tests development.

REFERENCES

(1) WO0131056, 2001-05-03: Detection method by PCR, Zorzi Willy (BE); El Moualij Benaïssa (BE); Zorzi Danièle (BE); Heinen Ernst (BE); Melen Laurence (BE).

This work is supported by the "Région Wallonne" : contract 14531, iPCRq.

P.16.- Immuno-quantitative PCR : a new way for detection of resistant prion protein

S. Gofflot[1], B. El Moualij[1], D. Zorzi[1], S. Roels[2], D. Quatpers[3], J. Grassi[4], E. Heinen[1], W. Zorzi[1].

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[2] VAR-CERVA-CODA, Veterinary and Agrochemical Research Center, Department of Biocontrol - Section of Pathology, National Reference Laboratory for Veterinary TSE (Belgium & Luxemburg), Groeselenberg 99, B - 1180 Brussels, Belgium.
[3] CRPP-Laboratoire ULg BSE, Tour de Pharmacie, Avenue de l'Hôpital 1, B-4000 Liège, Belgium.
[4] CEA, Service de Pharmacologie et d'Immunologie, CEA Saclay, 91191 Gif sur Yvette cedex, France.

I n biology today, methods for detecting organic molecules or infectious agents are diversified and very sensitive. Yet the need to lower detection limits persists, and in many cases quantitative estimates are required. Immuno-PCR is an extremely sensitive detection method combining the specificity of antibody detection and the sensitivity of PCR. We have developed an immuno-quantitative PCR (iqPCR*) exploiting real-time PCR technology in order to improve this immuno-detection method and make it quantitative.

To illustrate the advantages of iqPCR, we have compared it with a conventional ELISA technique in experiments aimed at detecting the cellular and the resistant form of prion protein in bovine brain extract. The iqPCR technique proved to be more sensitive than ELISA, so it could be a technique of choice for the diagnosis of infected animals both at an ante mortem and post mortem stage.

* Patent WO0131056, 2001-05-03: Detection method by PCR, Zorzi Willy (BE); El Moualij Benaïssa (BE); Zorzi Danièle (BE); Heinen Ernst (BE); Melen Laurence (BE).

Keywords

Immono-PCR, prion

The Development of Rapid Immunoassays for Specified Risk Material Through the Use of Bioinformatics

A novel approach, involving bioinformatics, is being investigated in an EU funded project (http://www.smitest.com) to address the challenge of developing a test kit to detect specified risk material, specifically brain, tonsil, eye and ileum, in cooked meat products.

The approach involves database mining for proteins specific to the target tissue, in silico digestion of these proteins followed by Blast searching of the resulting peptides identifies peptides which are unique to the parent protein and hence the tissue.

Proteins specific to a specified risk material (according to the literature) were short listed. Bioinformatics software MassPep (Figure 1) was developed at CSL and the digestion of the short listed proteins was simulated for a range of enzymes by the software. The generated peptide sequences were then interrogated to determine whether they also contain any degree of tissue specificity. Chemical characteristics including theoretical isoelectric points (pI), hydrophobic indices and mass values for the peptides were also generated by MassPep.

Figure 1. Screen shot of Java application showing a simulated Trypsin digest of proteins from different organisms, size range of peptides 500 - 2000 daltons

Twelve peptides were selected from the sequences of 37 proteins identified as specific for the 4 SRM tissues. These were synthesised and antibodies are currently being raised in order to develop immunoassays. Preliminary results on the first peptide selected from brain confirm that the monoclonal antibody raised is specific to this brain peptide (Figure 2)

Figure 2. Cross-reactivity data for anti-bovine monoclonal antibody

showing there is no cross-reactivity between the brain monoclonal antibody and other peptides

Current work is focused on developing a method to solubilise heat processed specified risk material using the enzymes used for in Silico digestion. The project aims to solubilise proteins into fragments with no secondary structure (~4kDa) enabling the antibodies to freely access their linear epitopes. A combination of enzymic and chemical agents is currently being investigated using Size Exclusion HPLC to monitor the size of the digest products (Figure 3).

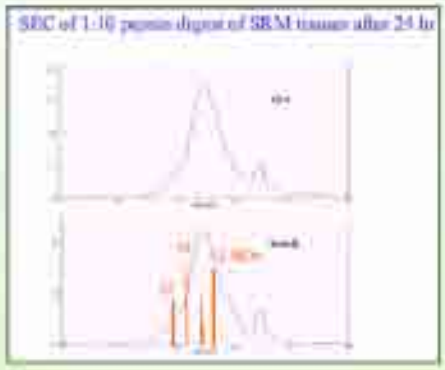

Figure SEC of 1:10 pepsin digest of SRM tissues after 24 hr

The final output of the project will be a rapid immunotest format (Lateral flow device) which will have been validated in a pan European inter laboratory trial to be used to screen European cooked meat products for the presence of specified risk materials.

Central Science Laboratory

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Acknowledgement

Funding for this project from the European Commission 5th framework Growth programme (contract GERD-CT-2002-00889) and the UK Food Standards Agency BSE Division is gratefully acknowledged

P.17.- The Development of Rapid Immunoassays for Specified Risk Material through the Use of Bioinformatics

H. Cameron[1], P. Reece[2], R. Stones[3], V. Tomkies[4], W. Lubbe[5], S. Baumgartner[6], C. Hemetsberger[7], M. Bremmer[8]

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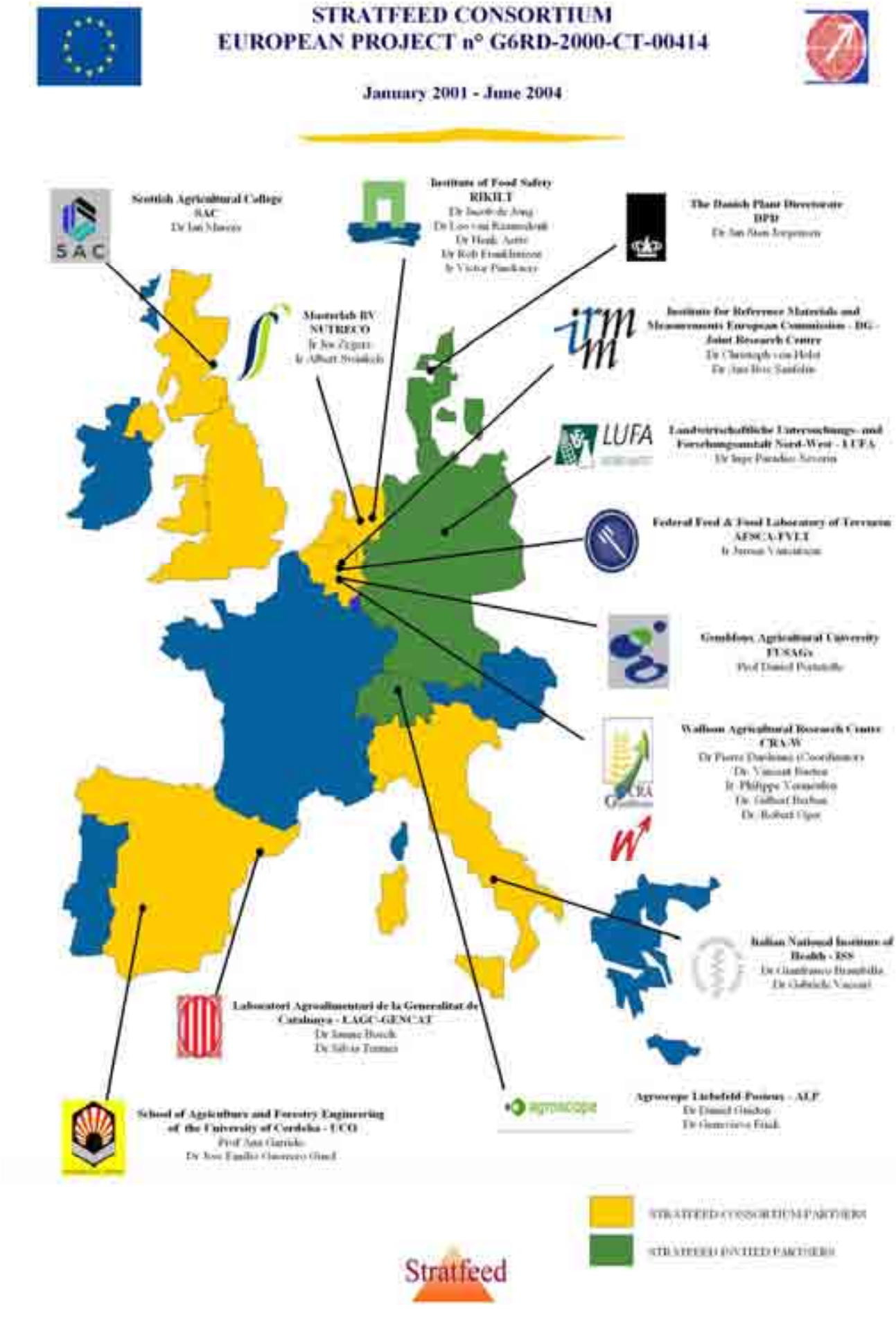
A novel approach to the development of immunotests for Specified Risk Material (SRM) in cooked meat products is being investigated in which protein sequence databases are being interrogated to identify the sequence of tissue-specific proteins using in silico protease digestion. Short peptide sequences within these tissue specific proteins will be identified which are unique to each protein. These peptides will be created through in silico protease digestion of the target proteins. The peptides will be synthesised and used to raise monoclonal antibodies for use in immunoassays. The assays will be carried out by first digestion of the cooked meat samples with the protease identified in the in silico studies followed by evalaution of the proteolytic digests using the antibodies incorporated into lateral flow devices. To date the bioinformatic studies have identified twelve peptide sequences in nine proteins that are theoretically specific to four SRM tissues, brain, eye, ileum and tonsil (lymphoid). These peptides have been synthesized and are being used to raise monoclonal antibodies. Four of the peptide sequences are theoretically specific to ruminant species and two specific to bovine therefore the final test kits may provide both tissue and species specificity. A complementary approach is simultaneously being investigated, in which monoclonal antibodies are raised against bovine SRM protease digests that have been affinity purified using polyvlonal antibodies raised against avian brain and bovine muscle digests. For this part of the study, enzyme digestion procedures have been developed that rapidly solubilise 60-95% of the protein in cooked meat samples, the resultant peptides having a mean molecular mass of approximately 5kDa. More rigorous digestion procedures are being developed for the exposure of the peptide sequences identified as specific to the target tissues. Ultimately these will be used in conjunction with the monoclonal antibodies raised to the synthetic peptides, to provide a test kit for SRM in cooked meat products. The optimized assays will be validated in an interlaboratory trial, using cooked meat containing known amounts of SRM and subsequently used in a European survey of cooked meat products. This research project is being supported by the EU 5th Framework ‘Competitive and Sustainable Growth’ Programme (1998-2002) and by the UK Food Standards Agency. Additonal information can be obtained from the project website (www.srmtest.info)

Keywords

Specified Risk Material, Bioinformatics, Lateral flow devices, Proteomics, Proteolytic digestion


STRATFEED CONSORTIUM
EUROPEAN PROJECT n° G6RD-2000-CT-00414
January 2001 - June 2004
Scottish Agricultural College
SAC
Institute of Food Safety
RIKILT
The Danish Plant Directorate
DPD
Masterlab BV
NUTRECO
Institute for Reference Materials and Measurements European Commission - DG - Joint Research Centre
LUFA
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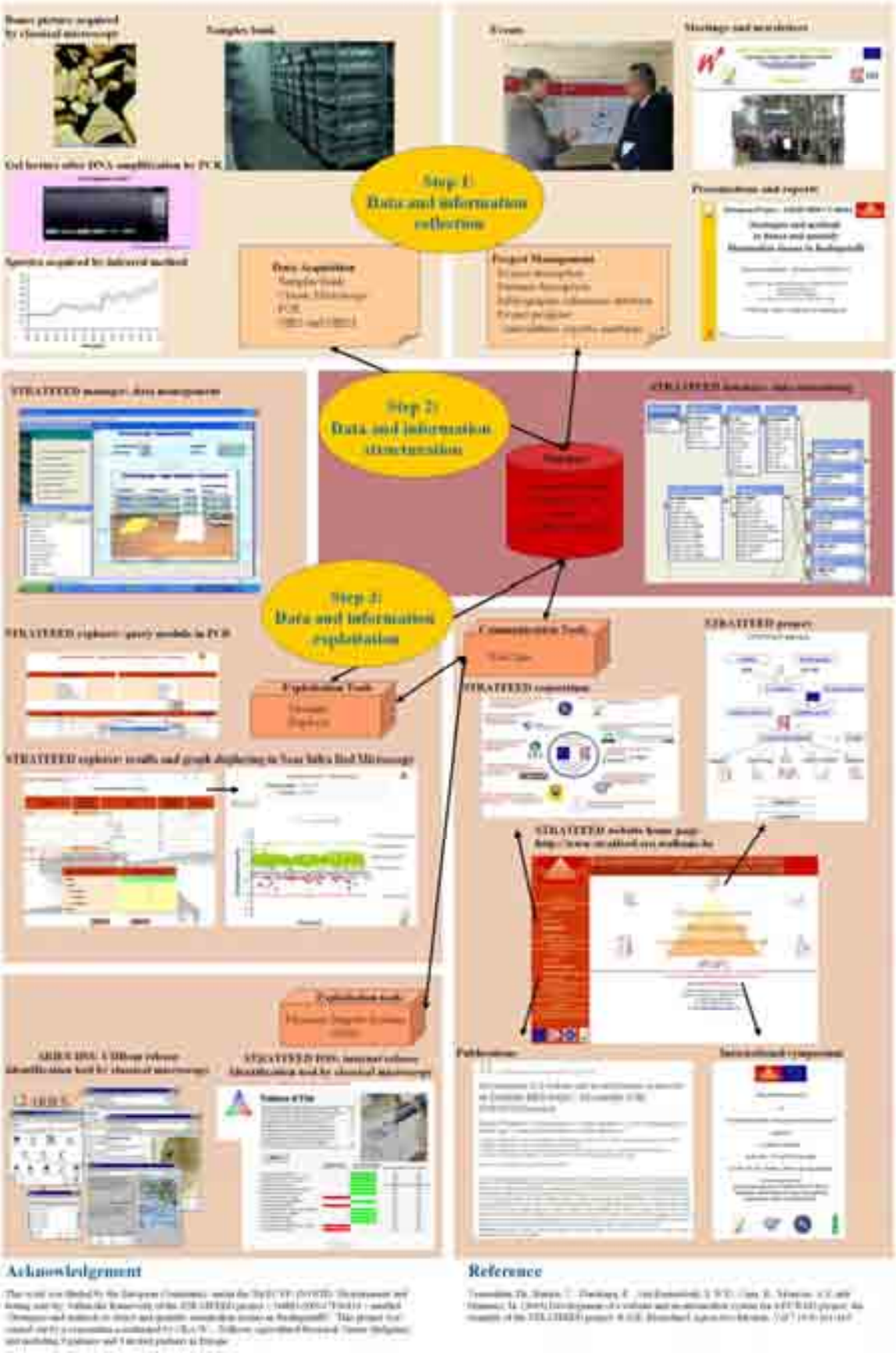
FUSAGx
Walloon Agricultural Research Centre
CRA-W
Italian National Institute of Health - ISS
Laboratori Agroalimentari de la Generalitat de Catalunya - LAGC-GENCAT
School of Agriculture and Forestry Engineering of the University of Cordoba - UCO
agroscope
Agroscope Liebefeld-Posieux - ALP
STRATFEED CONSORTIUM PARTNERS
STRATFEED INVITED PARTNERS
Stratfeed

P.18.- Stratfeed consortium
European project n° G6RD-2000-CT-00414

Development of a website and an information system for an EU R&D project: the example of the STRATFEED project

Philippe Vanmeulebrouck, Vincent Baeten, Pierre Carolienne, Leo van Rasmenhoven, Robert Ogier, Anne-Sophie Manjule and Michel Martinez



Acknowledgment

Reference

P.19.- Development of a website and an information system for an EU R&D project: the example of the STRATFEED project

P. Vermeulen[1], V. Baeten[1], P. Dardenne[1] , L. van Raamsdonk[3] , R. Oger[2] , A. S. Monjoie[2] & M. Martinez[2]

[1]Quality Department of Agro-food Products**,** Walloon Agricultural Research Centre (CRA-W), Chaussée de Namur, 24, B-5030 Gembloux, Belgium (vermeulen@cra.wallonie.be)
[2]Biometry Department**,** Walloon Agricultural Research Centre (CRA-W), Rue de Liroux, 9, B-5030 Gembloux, Belgium (oger@cra.wallonie.be)
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The multidisciplinary nature, the international partnership and the large amount of information to be managed in the European project STRATFEED, required the development of an information management system in three facets.

The first part concerns the data and information collection. On the one hand, the different labs produce a lot of data concerning the identification of the samples and regarding analyses by classical microscopy, polymerase chain reaction (PCR), near infrared spectroscopy (NIRS) or near infrared microscopy (NIRM). On the other hand, each participating team provides information that is useful for the management of the project. Such information includes, for instance, the project description, details of the partners, bibliographic references, newsletters, events, reports, lectures from meetings and EC documents. All of these types of information and data are available in different formats.

The second part of the computer system, and one of the project's objectives, is to build a database to gather and store all the relevant data and information in a dedicated structure. The MS *Access* platform has been selected to implement the database and to manage both numbers and text as well as figures and files.

From a well-defined and structured database, any application can be developed; this is the third part of the flowchart. In the frame of the project, two types of application have been developed: an information communication tool using the website to facilitate exchange between the partners and data exploitation tools including a manager specifically for the use of the database administrator, an explorer dedicated to the user to enable exploration of the available data, and two decision support systems designed for the classical microscopy. The STRATFEED Internet site is based on an environment for development (*Cold fusion web application server*) that enables the user to create dynamic web pages.

This attractive website with those different applications built and improved during 3 years is used at the end of the project, as the main tool for the dissemination and the valorisation of the results about the detection of MBM in feedingstuffs.The website will be maintained by CRA-W at least 3 years. It will also facilitate the follow-up on the project. The applications will be maintained and updated as often as necessary, depending on the development of the BSE problem and on the research. Other tools may also be developed around the database. The modular structure of the system, according to the different topics of the project, facilitates updating and favours the development of further tools. The concept developed for the STRATFEED project can be used for any other project and can easily be adapted to meet new requirements.The example of the STRATFEED project can be accessed at: http://stratfeed.cra.wallonie.be.

This work was funded by the European Community, under the 5th EC FP, DG RTD, Measurement and testing activity, within the framework of the STRATFEED project – G6RD-2000-CT00414 – entitled "Strategies and methods to detect and quantify mammalian tissues in feedingstuffs". This project was carried out by a consortium coordinated by CRA-W – Walloon Agricultural Research Centre (Belgium) and including including 9 partners and 3 invited partners.

Reference

Vermeulen, Ph., Baeten, V., Dardenne, P. , van Raamsdonk, L.W.D., Oger, R., Monjoie, A.S. and Martinez, M. (2003).Development of a website and an information system for a EU R&D project: the example of the STRATFEED project. BASE, Biotechnol.Agron.Soc.Environ. Vol 7 (3-4) 161-169.

Keywords

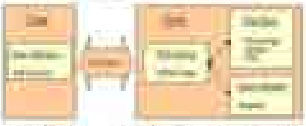
Databases, internet, project management, decision support, knowledge based systems, computer systems (applications), information systems

Stratfeed

Development of an internet based data explorer : the example of the STRATFEED explorer

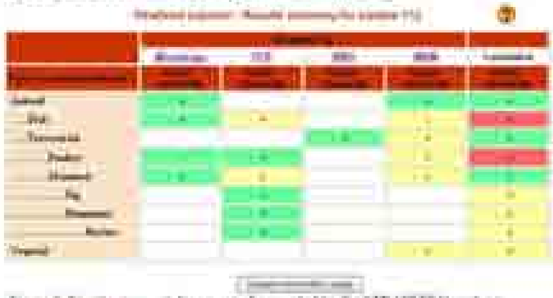
Introduction

Concept



Queries modules

Results display



Conclusion

Acknowledgement

Reference

Walloon Agricultural Research Centre

P.20.- Development of an internet based data explorer: the example of the STRATFEED explorer

Philippe Vermeulen[1], Vincent Baeten[1], Pierre Dardenne[1], Leo van Raamsdonk[3], Robert Oger[2], Anne-Sophie Monjoie[2] and Michel Martinez[2]

[1]Quality Department of Agro-food Products**,** Walloon Agricultural Research Centre (CRA-W), Chaussée de Namur, 24, B-5030 Gembloux, Belgium (vermeulen@cra.wallonie.be)
[2]Biometry Department**,** Walloon Agricultural Research Centre (CRA-W), Rue de Liroux, 9, B-5030 Gembloux, Belgium (oger@cra.wallonie.be)

In the framework of the European project STRATFEED, the building of a sample bank was a key point into development and validation of analytical methods for the detection of animal meals in feedingstuffs. To manage all the samples, another main objective was the building of a database to store all the data in a specific structure that allows the easy retrieval of specified parts of the data via the applications. The MS *Access* platform has been selected to implement the database and to manage both numbers and text as well as figures and files. In order to have maximum flexibility for development, updating and dissemination, different tables have been created to suit each topic in the project. The formulae of samples gathered in the sample bank have been described according to the EC guidelines of the Commission Directive 98/67/EC which amended Council Directive 96/25/EC. The incorporation of data and the updating of the database, is facilitated by the STRATFEED manager which is a management tool dedicated to the database administrator. In order to valorise this first reference database gathering so much information on feed samples adulterated or not with animal tissue, an internet based data explorer was developed to give to each lab or manufacturer working in the feed sector the opportunity to request the database.

The development of this tool is based on client-server architecture using the Internet to provide the linkage between the two sides. With this application, on the one hand, the user can quickly get an overview of the "STRATFEED database" through predefined queries and, on the other hand, a user with more expertise can build his own query using different query modules which correspond to the different techniques.
For each method, criteria have been defined by the respective work packages.Those criteria including text, pictures or graphs have been defined to identify the laboratory and to describe the analyses. Queries modules for each topic, based on those criteria were developed.

The results of each analysis are displayed by sample, by method and by laboratory. The conclusions are presented according to those 3 levels: the laboratory level, the method level and the sample level. Those conclusions are expressed for each taxon or species detected, by a result and by the reliability of this result.
Today, the STRATFEED explorer can be run from the public website http://stratfeed.cra.wallonie.be on a database reduced to the sets of samples used by the different work packages for the development of the methods. On the private part, the STRATFEED partner can explore the complete database including 2500 samples. To help the user in the exploring, each webpage is described by a help file. The modular structure of the system, according to the different topics of the project, facilitates updating and favours the development of further tools for others techniques. The concept developed for the STRATFEED project can be used for the samples management of any other project and can easily be adapted to meet new requirements.

This work was funded by the European Community, under the 5th EC FP, DG RTD, Measurement and testing activity, within the framework of the STRATFEED project – G6RD-2000-CT00414 – entitled "Strategies and methods to detect and quantify mammalian tissues in feedingstuffs". This project was carried out by a consortium coordinated by CRA-W – Walloon Agricultural Research Centre (Belgium) and including including 9 partners and 3 invited partners.

Reference

Vermeulen, Ph., Baeten, V., Dardenne, P. , van Raamsdonk, L.W.D., Oger, R., Monjoie, A.S. and Martinez, M. (2003).Development of a website and an information system for a EU R&D project: the example of the STRATFEED project. BASE, Biotechnol.Agron.Soc.Environ. Vol 7 (3-4) 161-169.

Keywords

Databases, internet, computer systems (applications), information systems, data explorer, samples management

The decision support system ARIES

L.W.D. van Raamsdonk [1]

Identification of animal proteins in feeds

The Decision Support System ARIES (Animal Remains Identification and Evaluation System) aims at the support of the detection and identification of animal proteins in animal feeds. It provides a full range of animal meal descriptions as well as a range of plant parts and minerals that can be confused with animal material. The package can be used to support and document the actual identification in common practice and is capable of being used as a training system. ARIES will be made available as a stand-alone system (CD-ROM).

Control process

Collected information can be used for browsing in the module Sample Types with descriptions and images. Sample info can also be used in the modules for identification: Text Key, Picture Key or IdentifyIt. These modules assist step-by-step the process of identification. The additional modules Glossary and Literature are available for supporting the recognition of particles. After reaching a conclusion verification can be achieved by using the Gallery module.

Training tool

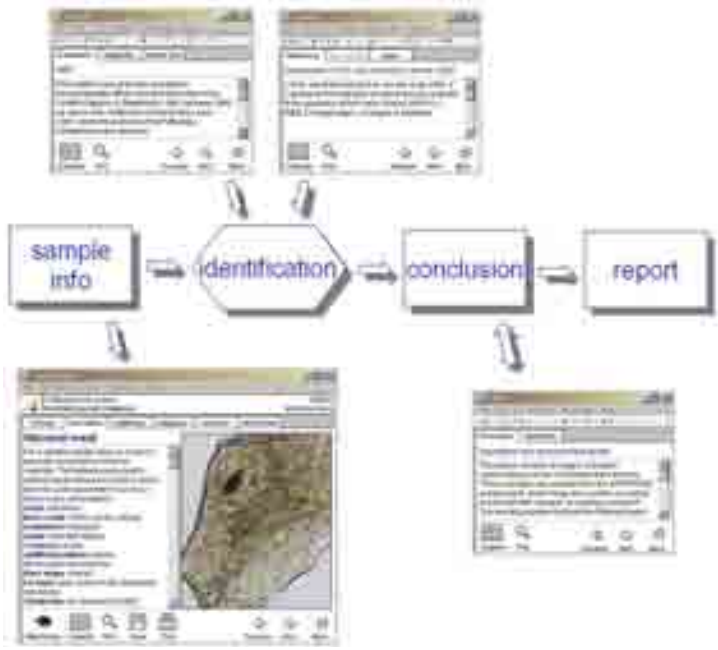
Basic information on animal materials and on a range of confusing particles is available by browsing in the modules Sample Types and the Gallery. The types of ingredients are classified in different Categories for easier organization of the information. The identification modules keep records of the choices made by the trainee, which supports the training of the tutor.

Framework

STRATFEED project: strategies and methods to detect and quantify mammalian tissues in feedingstuffs, granted by the European Commision under contract G6RD-2000-CT00414. website: http://stratfeed.cra.wallonie.be

ARIES is developed in the program package Linnaeus II produced by ETI, Amsterdam, the Netherlands: www.eti.uva.nl

Copies of ARIES can be ordered from the author: leo.vanraamsdonk@wur.nl



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P.21.- The decision support system ARIES

L.W.D. van Raamsdonk

RIKILT- Institute of food safety, Wageningen, the Netherlands (leo.vanraamsdonk@wur.nl)

The Decision Support System ARIES (Animal Remains Identification and Evaluation System) aims at the support of the detection and identification of animal proteins in animal feeds. It provides a full range of animal meal descriptions as developed in Stratfeed, including shell fish and a range of plant parts and minerals that can be confused with animal material. Three different modules for step-wise identification are being developed, and a glossary, a gallery with additional series of images, a range of literature and information on legislation is included. All modules can be entered from the main Contents tab in the Navigator. Bookmarks to the main pages are available as well. The package can be used to support and document the actual identification in common practice and is capable of being used as a training system. ARIES will be made available at first as a stand-alone system (CD-ROM) and depending on the result of a market analysis in second instance as web-based system.

The system provides full information on the protocols, laboratory equipment and chemicals in the module Methods. Basic information on animal materials and on a range of confusing particles is available by browsing in the modules Sample Types and the Gallery. The types of ingredients are classified in different Categories. Each class (animal, non-animal origin) is divided in several groups, which are divided in subgroups when appropriate. In this way a logical structure to the information is given.

The procedure for identification starts with the sample under study. Sample information will be collected from different fractions of the sample and from slides. The collected information can be used for browsing in the module Sample Types. This can be a time consuming process. The collected information can also be used in the modules for identification: Text Key, Picture Key or IdentifyIt. These modules assist step-by-step the process of identification. Several additional modules are available for supporting the recognition of particles: Glossary, Literature and Legislation. After reaching a conclusion verification can be achieved by using the Gallery module.

ARIES has been developed in the framework of the STRATFEED project: strategies and methods to detect and quantify mammalian tissues in feedingstuffs, granted by the European Commision under contract G6RD-2000-CT00414.The program package Linnaeus II as produced by ETI, Amsterdam, the Netherlands was used as platform: www.eti.uva.nl.

Copies of ARIES are available from the author.

Keywords

Decision support systems, animal proteins, sedimentation procedure, microscopic detection, identification, training.

MICROSCOPIC METHOD IN PAP IDENTIFICATION IN FEED: APPLICATION OF IMAGE ANALYSIS

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Background

•The microscopic method may be adequate for enforcing the EU's total ban on MBM in ruminant feeds, and is usually able to distinguish fish from land animal material

•The classic microscopic method is often unable to distinguish between land (terrestrial) animals (i.e. poultry and mammals), due to the fact that lacunae from mammals and birds are not always distinguishable

•Several morphological features have been described in order to solve this problem, proving to the analyst different approaches that should be followed in decision tree scheme

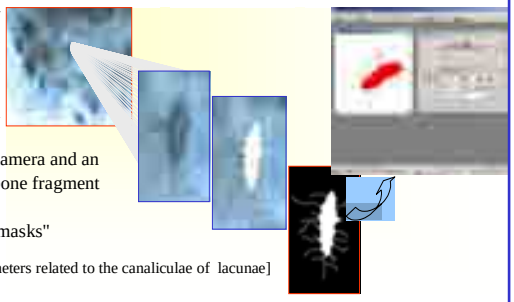
•However, in several cases knowledge as well as both experience and practice of the analyst are not enough, and additional tools in making a response and diagnosis are required

Aim

The aim of this study was to evaluate the potential application of image analysis in PAP identification in feedstuffs

Materials and Methods

•Samples: four reference samples containing poultry meals (ECB s.p.a., Bergamo, Italy; VSA, University of the Studies in Milan) and four reference samples containing mammalian meat and bone meals (Agricultural Research Centre of Gembloux, Belgium, STRATFEED Project; VSA, University of the Studies in Milan) were used. Bone fragments characterized by similar morphological features (colours, shape, lacunae shape, lacunae distribution, ect.) that made difficult to distinguish between poultry and mammals were selected



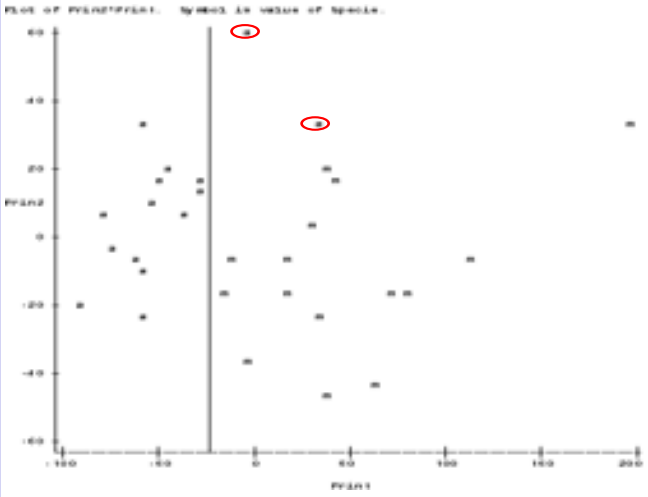
•Analitical procedure: Each samples was analyzed using the microscopic method (98/88/EC). By a digital camera and an image analysis software (Image-for Plus 4.5.1, Media Cybernetics Inc., Silver Springs, USA) a total of 30 bone fragment lacunae images at X400 were obtained

•Images processing: Images have been elaborated/manipulated obtaining for each lacunae a monochrome "masks"

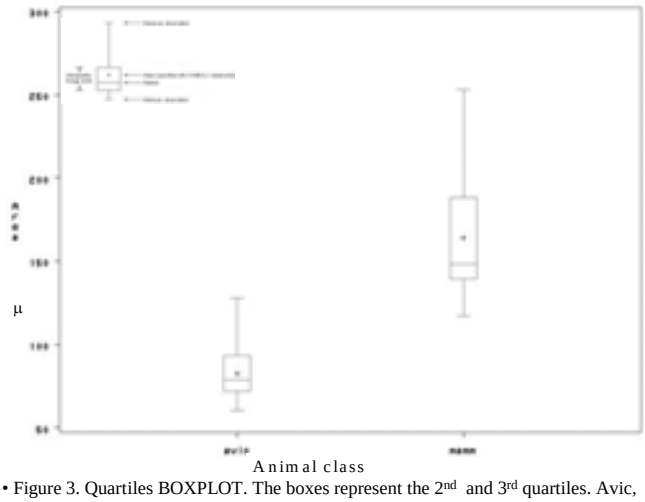
•Measurements: for each image 32 descriptors [29 geometric parameters related to the lacunae and 3 geometric parameters related to the canaliculae of lacunae] were measured using the image analysis software to provide 960 observation

•Statistical analysis: data were analysed using the PRINCOM, ANOVA procedures and BOXPLOT of SAS, (2001)

Results & Discussion



•Figure 2. Results of PRINCOMP. Prin 1, area of the lacunae; prin 2, perimeter of the lacunae .



• Figure 3. Quartiles BOXPLOT. The boxes represent the 2nd and 3rd quartiles. Avic, poultry; Mam, mammals.

•Analysis of the principal component have shown that of 32 descriptors, two principal component were able to explain 96.15% of the total variability of the data

- •The first principal component was lacunae area (83.97% of the total variability of the data)
- •The second principal component was lacunae perimeter (12.18% of the variability of the data)
- •The sum of all the other 30 descriptors, covered the remainder 3.85%

•The descriptor "area" (P<0.001) was more informative compare to the descriptor "perimeter" (P<0.0165)

•Higher variability in the lacunae area recorded for mammals compare to poultry

Conclusions

·Image processing, integrated with morphometric measurements (particle number, area, radius, diameter, and their structural relations) can provide accurate and reliable results that can be very usful to the analyst for feedstuffs characterisation, analysis and control

·Image analysis approach can generate precise descriptive data providing several benefits and drawbacks that can support and or facilitate the analyst in an objective assessment of the sample.

·On the basis of the results here presented, it can be concluded that image analysis represents a promising potential tool in PAP identification in feedstuffs

Keywords: PAP determination, Electronic nose.

P.22.- Microscopic method in PAP identification in feed : Applications of image analysis

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Processed animal proteins (PAPs) detection and identification in feedstuffs can be difficult in distinguishing among land animal, i.e. poultry and mammals. Thus the, aim of this study was to evaluate the potential application of image analysis in PAPs identification. For this purpose four reference samples containing poultry meals and four reference samples containing mammalian meat and bone meals were used. Each samples was analyzed using the microscopic method (88/98/EC). Bone fragments characterized by similar morphological features (colours, shape, lacunae shape, lacunae distribution, ect.) that made difficult to distinguish between poultry and mammals were selected. Though a digital camera and an image analysis software a total of 30 bone fragment lacunae images at X400 were obtained. For each image 29 geometric parameters related to the lacunae and 3 geometric parameters related to the canaliculae of lacunae, were measured using the image analysis software obtaining 960 observations. Obtained data were analysed using the PRINCOMP, ANOVA, and BOXPLOT procedures of SAS/STAT. Of the 32 descriptors used two, the area of the lacunae and their perimeter, were able to explain 96.15% of the total variability of the data, even though their contribution was different (83.97% vs. 12.18, respectively). These results were also supported by the variance analysis (ANOVA) for the two variables, that showed how descriptor "area poly" ($P<0.001$) was more informative than descriptor "perimeter" ($P<0.0165$). Through these two descriptors it was possible distinguish between mammalian and poultry lacunae, except in two cases (6.6%), in which poultry lacunae were wrongly classified as mammalian. This latter can be related with higher variability in the lacunae area recorded for mammals compare to poultry. On the basis of the present study, it can be concluded that image analysis represents a promising potential tool in PAPs identification, that may provide accurate and reliable results in feedstuffs characterisation, analysis and control.

Keywords

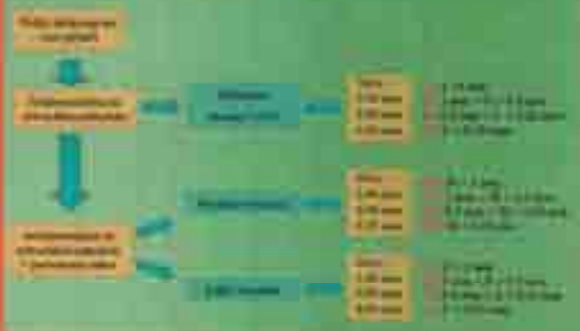
Processed animal proteins (PAPs), official microscopic method, image analysis

IMPROVEMENT OF THE STRATFEED MICROSCOPY METHOD FOR DETERMINATION OF ANIMAL ORIGIN CONSTITUENTS IN FEEDSTUFFS CONTROL

INTRODUCTION

THE STRATFEED METHOD

THE IMPROVEMENT METHOD



CONCLUSIONS: THE IMPROVEMENTS

P.23.- Improvement of the STRATFEED microscopy method for determination of animal origin constituents on feedstuffs control

F. Calero[1,2], P. Pascual[1,2], C. Gálvez[1]

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[2]Desarrollo Agrario y Pesquero (D.A.P.). Junta de Andalucía.

After the 90's BSE crisis, official methods for feedstuffs control must be suggested in the European Union to observe the fulfilment of law controls against this disease and the use of animal tissues as feed components.
Currently, the microscopy method has been shown as the most effective method to carry out with the European controls due to its easy use in any laboratory, low cost, accuracy and detection limit down to 0.1%, as shown in several interlaboratory assays
Due to the need to establish a common method in the European Union, the Stratfeed project has proposed a common protocol to assay it and, If it is the case, to adopt it as the only European microscopy method. Recently the JRC have developed a validation study with this method in which we have participated.
However, in our view, this method presents some lacks when is compared to the analytical method developed in our laboratory. Our method is been used under accredited system by ENAC (Spanish National Accreditation Agency) since 2001 to official feedstuffs sample control in Andalusia region.
Hence, we suggest the next modifications to improve the proposed protocol by the Stratfeed project:

a) Do not grind the samples before they were studied under stereoscopic microscope.
b) Separate the samples in three fractions (light, medium and sediment) instead of two fractions by the proposed method.
c) Separate each of the above fractions through three sieves of 1, 0.5 and 0.25 Ø mm in four subfractions.
d) Examine all the obtained fractions with the stereoscopic microscope and confirm the suspicious particles using the compound microscope.

As result of these modifications, the next improvement could be succeed in:

a) Increase the same class particles number for each fraction.
b) Avoid the cross contamination among samples by mill use.
c) Assure very low detection limits (0.05 to 0.5%).
d) Easier microscopic analysis.
e) In some cases is possible to distinguish between mammal and poultry bones.

Keywords

Animal tissues identification, microscopic methods, MBM.

Institut für Futtermittel

Detection of animal material in feeding stuff with PCR

Michael Egert[1] and Sven Pecoraro[2]

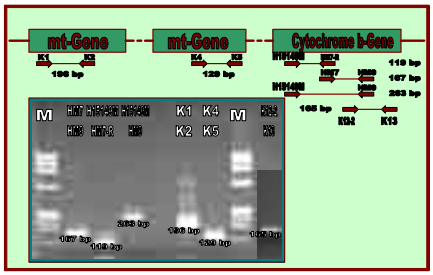
1. Introduction

The first case of Bovine Spongiphorme Encephalopathy (BSE) in Germany in the year 2000 led to a total ban of animal material derived from land living endotherms and fishes in feed for farm animals. (Exception: Fish derived protein products and fats in feed for non ruminants). The ban caused a demand for analytical methods for the detection of animal material in feed. Microscopy constitutes a well established and official method in that field. With microscopy characteristic structures such as bone fragments, muscle fibres, feathers, scales etc. are distinguishable. Limitations of this method are met with material which shows no visible structure like bowels, fat etc. The allocation of microscopically detectable muscle fibres to an animal species is not possible by this technique. Molecular biological methods can expand und support microscopy as DNA based analytical methods are independent from visible structures.
The development of a PCR-Based method for the detection of animal material in feed is presented.

2. Choice of PCR primers

Most important for the specifity of a PCR assay is the choice of appropriate primers for DNA amplification. With universal primers ubiquitous DNA sequences like parts of the mitochondrial cytochrome b gene can be amplified. Animal meal is usually heated under pressure (3 bar) to 133 °C. DNA is degraded by this process. To successfully find even traces of heavily degraded DNA in a sample, it is crucial to chose primers that will amplify short (< 300 bp) DNA fragments. For primer design sequence homology studies of the cytb gene of 54 different animal species (including all relevant farm animals and domestic animals) were applied.Six primers were tested in detail (Figure 1). The primer pairs HM15149/HM9 and K12-2/K13 showed best results for the detection of animal DNA in feed.

Figure 1: **Position of some tested primersystems and size of their amplicons**



3. Results

Primers H15149M/HM9 generated an amplicon of 263 bp with DNA from 22 animal species. Primers K12-2/K13 generated amplicons with 165 bp in seize. (Figure 2)

With 11 tested plants including plants common in feeding stuffs like maize, soy, canola, wheat, barley and oat no amplicons were generated.

With both primer pairs animal derived DNA was detected in different animal meal samples from poultry, fish and different animals (Figure 3). Various tests with feeding stuff samples which are typically investigated in official feed control were successfully done.
Primer pairs HM15149/HM9 und K12-2/K13 were tested in two ring trails. Samples were both animal meal (100% fish, 100 % poultry, 100 % different animals) and defined mixed samples with an animal meal content of 0,1 %, 0,5 % and 3 %. In total some 130 feeding stuff samples with different contents of defined produced animal material from cattle, pig, chicken and sheep were tested. Feed samples were based on feed for dairy cows. Additionally animal DNA could be detected in mixed samples each containing 0,1 % of the mentioned species (Figure 4).

The identification of a certain species like cattle was possible by enzymatic cleavage of the amplicons generated with both primer pairs in a restriction fragment polymorphism (RFLP) analysis. (Figure 5)

Figure 2:

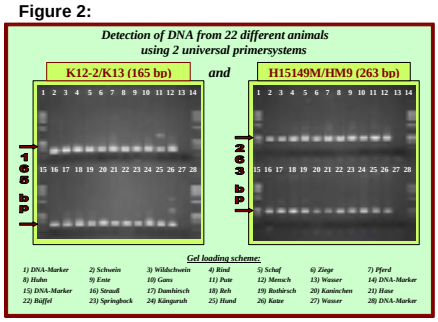


Figure 3:

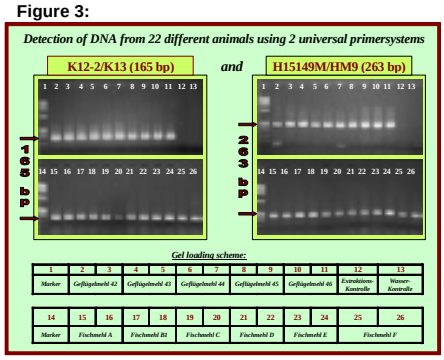


Figure 4:

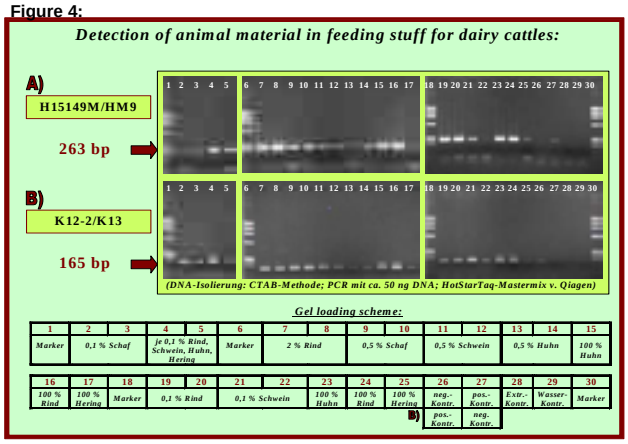
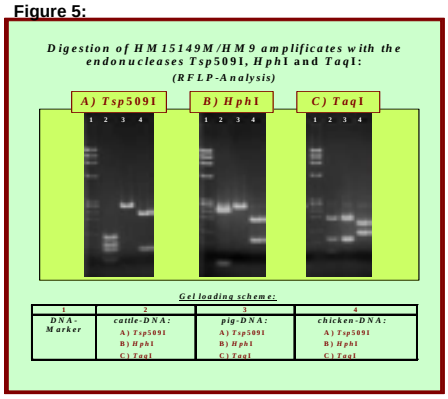


Figure 5:



4. Summary

Primer pairs HM15149/HM9 und K12-2/K13 are applicative primers for the universal detection of animal material in feed. They admit the detection of traces of animal DNA in highly heated samples by PCR.
With this method a portion of 0,1 % animal material in feeding stuff could be detected.
It is recimmended to use both primer systems in parallel.
The detection of animal constituents with primer pairs HM15149/HM9 and K12-2/K13 is part of the method „Molecularbiological determination of animal ingredients - PCR-method“ that was validated by 10 German laboratories and now is deposited in the method collection of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA), Germany.

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2) Dr. Sven Pecoraro, Bavarian Health an Food Safety Authority, Veterinärstr. 2, D-85764 Oberschleißheim, Germany, phone: #49-89-31560-451, e-mail: sven.pecoraro@lgl.bayern.de

P.24.- Detection of animal material in feeding stuff with PCR

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The first case of Bovine Spongiphorme Encephalopathy (BSE) in Germany in the year 2000 led to a total ban of animal material derived from land living endotherms and fishes in feed for farm animals. (Exception: Fish derived protein products and fats in feed for non ruminants). The ban caused a demand for analytical methods for the detection of animal material in feed. Microscopy constitutes a well established and official method in that field. With microscopy characteristic structures such as bone fragments, muscle fibres, feathers, scales etc. are distinguishable. Limitations of this method are met with material which shows no visible morphological structures like bowels, fat etc. The allocation of microscopically detectable muscle fibres to an animal species is not possible by this technique. Molecular biological methods can expand and support microscopy as DNA based analytical methods are independent from visible structures. The development of a PCR-based method for the detection of animal material in feed is presented. Most important for the specificity of a PCR assay is the choice of appropriate primers for DNA amplification. With universal primers ubiquitous animal DNA sequences like parts of the mito-chondrial cytochrome b gene can be amplified. Animal meal is usually heated under pressure (3 bar) to 133 °C. DNA is degraded by this process. To detect even traces of heavily degraded DNA in a sample, it is crucial to chose primers that will amplify short (< 300 bp) DNA fragments. For primer design sequence homology studies of the cytb gene of 54 different animal species (including all relevant farm animals and domestic animals) were applied. Six newely designed primer pairs were tested in detail, whereof two primer pairs showed best results. With DNA from 22 different animal species these primer pairs amplified a 263 bp and 165 bp fragment. With eleven tested plants including plants common in feeding stuffs no amplicons were generated. With both primer pairs animal derived DNA was detected in different animal meal samples (meal from poultry, fish and different animals). Various tests with spiked feeding stuff samples which are typically investigated in official feed control were successfully done. The two primer pairs were tested in two ring trails. Samples investigated were pure animal meal (100% fish, 100 % poultry, 100 % different animals) and defined mixed feed samples with an animal meal content of 0,1 %, 0,5 % and 3 %. In total some 130 feeding stuff samples with different contents of defined produced animal material from cattle, pig, chicken and sheep were tested. Additionally animal DNA was detected in mixed samples with 0,1 % of the mentioned species. Certain species like cattle were identified by enzymatic cleavage of the amplicons generated with both primer pairs in RFLP-analysis. The designated primer pairs HM15149/HM9 und K12-2/K13 are applicative for the universal detection of animal material in feed admitting the detection of even animal DNA-traces in highly heated samples. The method is part of the „Molecularbiological determination of animal ingredients - PCR-method“ that was validated by 10 German laboratories and now is deposited in the method collection of the German VDLUFA.

Keywords

DNA technology (PCR), universal primer pairs for the detection of animal ingredients

PCR analysis can help microscopist to identify animal species present in the sediment fraction of compound feed

Fumière O.[1], Osmanaj I.[1], Chiappini B.[2], Brambilla G.[2], Aarts H.[3], Vaccari G.[2] and Berben G.[1]

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Introduction

The optical microscopy is the only official method of detection of meat and bone meal (MBM) in animal feed. The method is nevertheless limited to the differentiation of large taxonomic groups (e.g. terrestrial animal or fish) and depends on the presence of bone fragments. In some cases the poultry meal can hide the presence of mammalian meal. Taking into account the ban on intra-species recycling due to the Regulation (EC) N°1774/2002, there is a crucial need of species-specific control methods. The combination of microscopy and PCR could answer to this need.

Material and methods

Samples

Sediment fractions prepared according to the Dutch method were obtained from 6 feed samples spiked with animal meals. The composition of the meals and the level of contamination are described in Table 1.

Table 1 : Percentages and composition of animal meals present in the feed samples

Sample number	Composition
1	5 % fishmeal
2	0.5 % MBM & 5 % poultry meal
3	5 % poultry meal
4	0.1 % MBM
5	0.5 % MBM & 5 % poultry meal
6	0 % MBM

DNA extraction

CRA-W used a classical phenol-chloroform method for the DNA extraction on the sediment fraction;
ISS used the Wizard® Magnetic DNA Purification System For Food (Promega).

Real Time PCR

The Real Time PCR protocols developed for the detection of ruminant and bovine material in MBM and feedingstuffs were tested. They used mitochondrial targets.

Results and comments

Table 2 : Real Time PCR results obtained with two bovine targets and one ruminant target

Sample number	Bovine target CRA-W	Bovine target ISS	Ruminant target ISS
1	-	-	-
2	+[a]	+[b]	+[b]
3	-	-	-
4	+	+	+
5	+	+	+
6	-	-	-

-Despite sample manipulation with tetrachloroethylene ($Cl_2C=CCl_2$) during sedimentation step, DNA can still be extracted and amplified efficiently by PCR ;
-no false positive was observed ;
-the 2 samples containing 0.5% of MBM (samples nr.2 and 5) were detected. Nevertheless, CRA-W observed delayed signals[a] and ISS obtained only 50% of positive results[b] with sample nr. 2. A higher heat treatment of the MBM incorporated in this sample could explain these PCR results;
-the sample containing 0.1% of MBM was detected with all the targets ;
-several dilutions had to be tested. Indeed PCR inhibition was observed with the DNA extracts of CRA-W.

Conclusion

These first results show that a PCR analysis of the sediment fraction is still possible. The PCR can confirm the conclusion of analyses by optical microscopy and give additional information on the animal species present in the sediment (e.g. detection of mammalian material in presence of an excess of poultry meal).
The analysis of sediment fraction essentially composed of animal materials seems to increase the sensitivity of the PCR to 0.1% level but this should be checked on a wider set of samples.

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P.25.- PCR analysis can help microscopist to identify animal species present in the sediment fraction of compound feed

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[3]State Institute for Quality Control of Agricultural Products (RIKILT), Institute for Food Research, Wageningen, The Netherlands.

Nowadays, the official method for the detection of meat and bone meal (MBM) in animal feed is optical microscopy[1]. Successfully validated, the method is limited to the differentiation of large taxonomic groups e.g. terrestrial animal or fish. In some cases, it is possible to distinguish mammalian bones from poultry bones. Nevertheless, it is experimentally observed that the detection of 0.5% of mammalian MBM (MMBM) in a feed containing 5% of poultry meal is very hard even in the sediment fraction which concentrates bone particles. During the STRATFEED project[2] Real Time PCR protocols able to detect ruminants as well as bovine or pig material were developed and successfully tested on samples such as pure MBMs or feedingstuffs containing MBM. The combination of the two techniques (microscopy and PCR) could answer to the need of control methods allowing for species differentiation required for the ban of intra-species recycling[3].

The present work describes the first PCR results obtained with cattle and ruminant targets on DNA extracted from sediment portions of 6 feed samples containing respectively 5% of fish meal, 5% of poultry meal, 5% of poultry meal and 0.5% of MMBM (2 different samples), 0.1% of MMBM and a blank feed. With this limited sample set, some observations can be stated : a) despite sample manipulation with an organic solvent (tetrachloroethylene - $Cl_2C=CCl_2$) during sedimentation step, DNA can still be extracted and amplified by PCR ; b) several dilutions have to be tested as PCR inhibition can occur ; c) the presence of mammalian material in problematic samples for microscopy (feedingstuffs with 0.5% of MMBM and 5% of poultry meal) can be detected by PCR ; d) even on the sediment portion, the detection at a level of 0.1% of MMBM remains difficult but is possible.

From these preliminary promising results, we can conclude that PCR could be a useful complementary tool to microscopy to determine whether mammalian material is present in a sample containing poultry meal. The PCR analysis of the sediment portion as a way to increase its sensitivity is not evident. Therefore before applying this protocol in routine analyses, it should first be checked on a wider set of representative samples and may probably need some improvements.

References

1 Gizzi G., van Raamsdonk LWD., Baeten V., Murray I., Berben G., Brambilla G., von Holst C. (2003). An overview of tests for animal tissues in feeds applied in response to public health concerns regarding bovine spongiform encephalopathy. *Rev. Sci. Tech. Off. Int. Epiz.* **22** (1), 311-331.

1 UE STRATFEED project G6RD-2000-CT-00414, Strategies and Methods to detect and quantify mammalian tissues in feedingstuffs, www.stratfeed.cra.wallonie.be (2001-2004).

1 Regulation (EC) N°1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. *Off. J. Eur. Comm.* L273, 10/10/2002, 1-95.

Keywords

PCR, microscopy, sediment, species, MBM

An original strategy coupling NIRM and PCR for detection and species identification of MBM particles

Fumière O.[1], André Ch.[2], Berben G.[1], Dardenne P.[1] and Baeten V.[1]

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Introduction

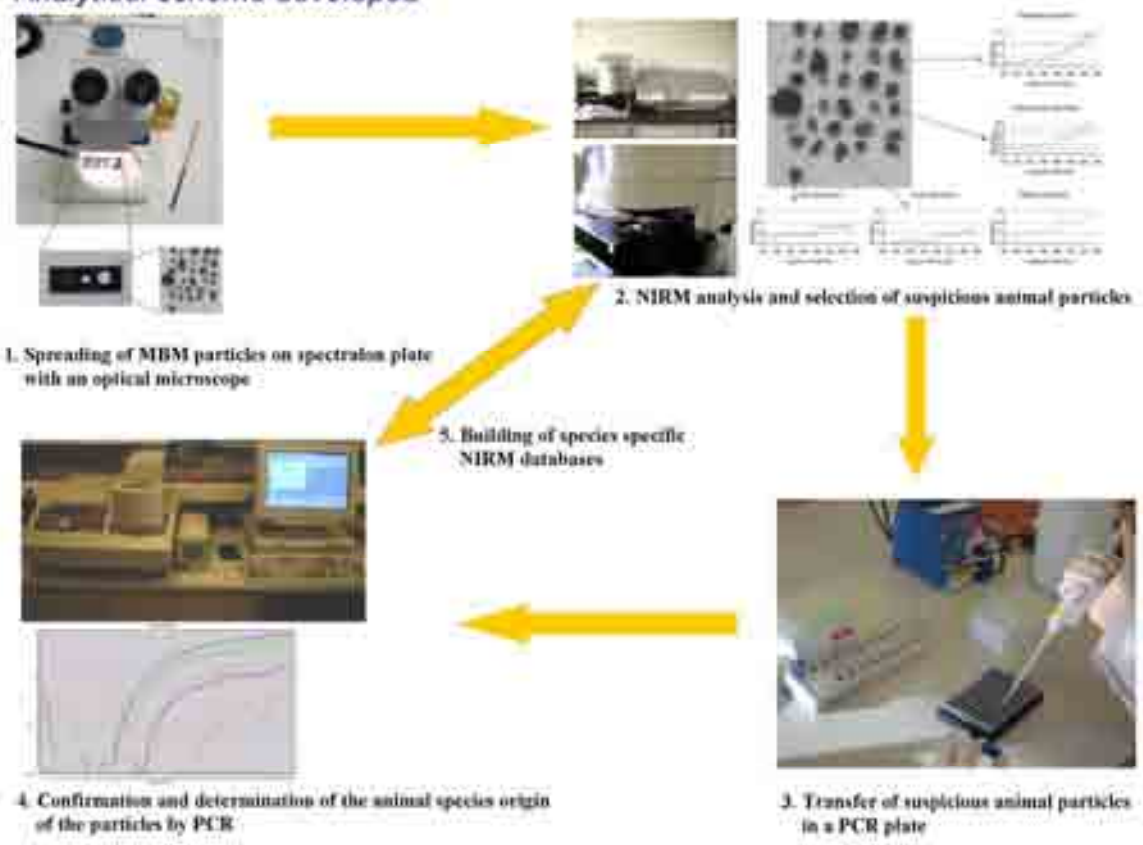
Due to the Regulation (EC) N° 1774/2002 which extends the current ruminant intra-species recycling ban to other species, there is a crucial need of methods able to determine the species origin of meat and bone meals (MBM).

Near-infrared microscopy (NIRM) has demonstrated its potential to detect MBM in feed. Thanks to discrimination models built on wide spectral libraries, the particles can be assigned to one of six defined groups (1. vegetal; 2. animal; 3. terrestrial animal; 4. fish; 5. bovine & pig; 6. poultry). Up to now, discrimination between bovine and pig particles remains impossible by NIRM.

Polymerase Chain Reaction (PCR) is able to detect the presence of animal sources of DNA even in heat treated samples using short targets but is unable to distinguish forbidden materials (MBM) from authorised ones (e.g. milk powder or blood).

The coupling of the two techniques could improve the capabilities and remove the drawbacks of each technique.

Analytical scheme developed



Conclusion

A single particle can be used as 'template' for DNA amplification by PCR. Future improvements of the protocol will allow the test of more than one species on the same particle. The confirmation of the genetic origin of a particle will help NIRM to build species specific spectral databases and to develop a discriminant model able to differentiate cattle and pig particles. On the other hand, NIRM will submit to PCR, only particles of a well-defined tissue origin (meat or bone).

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P.26.- An original strategy coupling NIRM and PCR for detection and species identification of MBM particles

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The potential of near-infrared microscopy (NIRM) for the detection of meat and bone meals in feed was demonstrated by researchers of CRA-W. Wide spectral libraries including thousands spectra from particles of animal, vegetal and mineral ingredients used for the preparation of compound feeds were built and discriminant models have been developed to predict the origin of unknown particles. Five discriminant equations (between 2 and 4 used simultaneously) decide about the group assignation of the particles with a success rate of more than 90 %. Defined groups are : 1) vegetal ; 2) animal ; 3) terrestrial animal ; 4) fish ; 5) bovine & pig ; 6) poultry[1]. Up to now, discrimination between bovine and pig particles remains impossible by NIRM. Moreover, a positive detection obtained by NIRM always needs confirmation with a forensic method. As the NIRM methodology is a non destructive analysis, the particles classified as animal origin can be selected and analysed by another technique like PCR. Certified pure species animal meals were used to test the potential of PCR to amplify DNA targets directly from singel particles identified as belonging to terrestrial animal.

In the analytical procedure, animal meat and bone particles were spread on a spectralon plate and presented to the NIR microscope for spectrometric analysis. Then some particles were recovered and put each separately into the wells of the PCR plate. Real Time PCR protocol developed at CRA-W targetting short mitochondrial DNA fragments[2] was used to confirm the species origin of these particles. The results obtained showed that a single particle can be used directly as 'template' for PCR with an interesting rate of success. In that case, great care should be taken to the environment during the preparation of the PCR plate to avoid any air contamination. Efforts will be made to improve again the rate of successful amplification and to develop a system allowing to test more than one species on the same particle. Tracing back the species origin of the particle by PCR will also allow the building of species specific spectral databases. Based on such libraries, it should be possible to develop models of discrimination between bovine and pig particles. On the other hand, as NIRM is able to discriminate some authorised animal components (e.g. blood and milk powder) from MBM particules, it can help PCR to determine which is the source of the detected DNA.

1 Baeten V., Michotte Renier A., Sinnaeve G. & Dardenne P. (2001). Analyse of feedingstuffs by near-infrared microscopy (NIRM) : detection and quantification of meat and bone meal (MBM). In *Proc. of the sixth International Symposium on food authenticity and safety*, 28-30 November 2000, Nantes, 1-11.
2 Dubois M., Fumière O., von Holst C. & Berben G. (2002). - Meat and bone meal detection in feed by search of specific animal DNA segments. *181st meeting of the Belgian Society of Biochemistry and Molecular Biology*, 4th of May, Katholieke Universiteit Leuven (KUL), Heverlee (Belgium) abstract nr. 7, (*http://www.biochemistry.be/4may2002/abstracts_1_13.htm#dubois*).

Keywords

NIRM, PCR, particles, MBM

Molecular multi-detection for speciation testing in feed products. How to address the open question: "What animal species are in this product ?"

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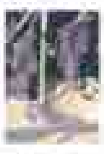
THE CHALLENGE

History of the intra species recycling ban and the need to analyse MBM

A SOLUTION



MATERIALS

Reference materials

METHODS

DNA extraction

DNA amplification and transcription

RNA hybridization on the chip

Signal analysis

RESULTS

DISCUSSION

Key references

P.27.- Molecular multi-detection for speciation testing in feed products. How to address the open question: "What animal species are in this product?"

O. Babola[1], S. Desvarenne[2], B. Lacroix[3], C. Mabilat[4], Z. Billinghurst[5]

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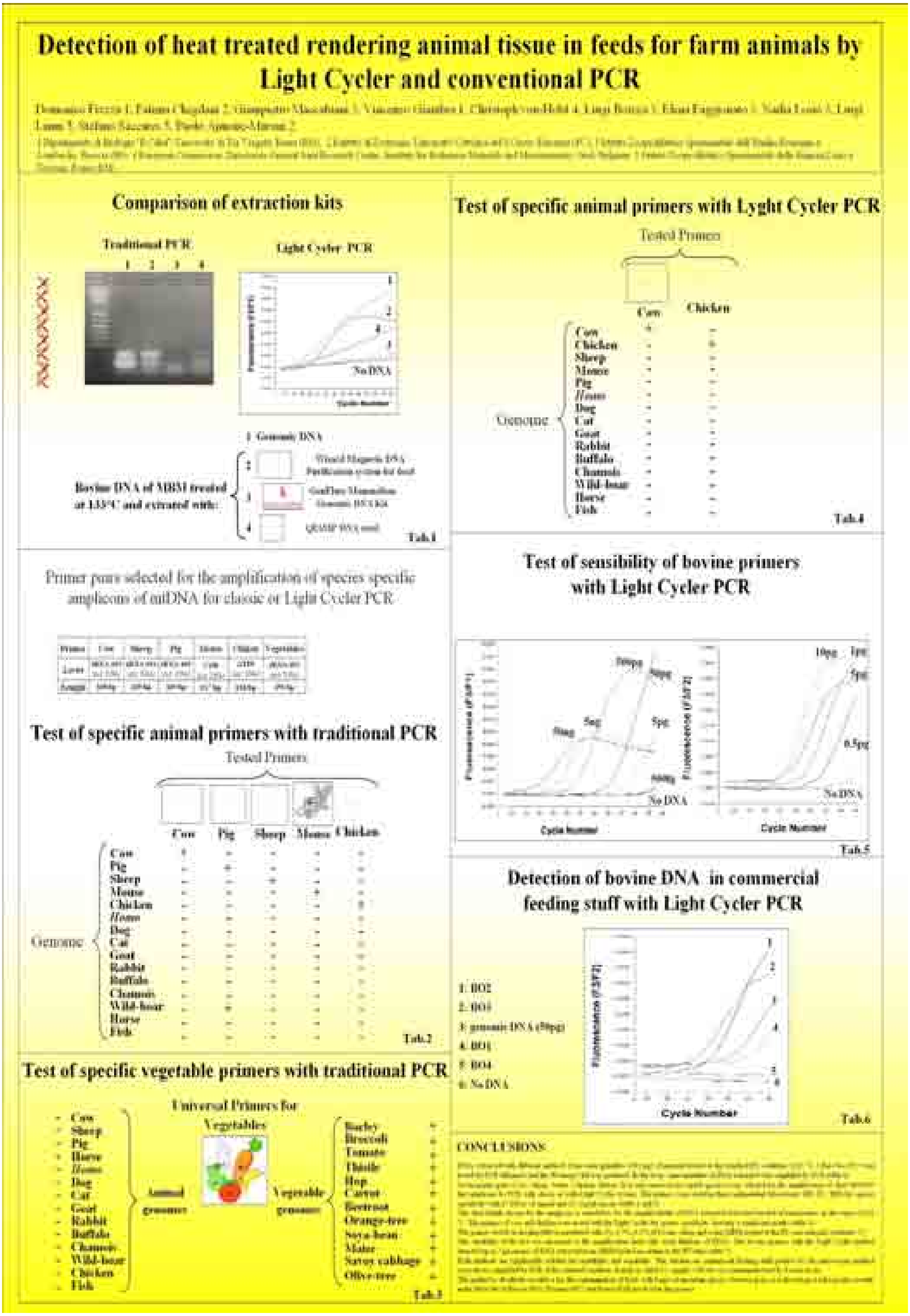
The challenge in the animal feed industry today is driven by the desire and need to produce safe feed for farmed animals. In order to safely and naturally feed farmed animals the composition of the feed must be known at the species level, and this is the challenge. We present a breakthrough technology that has the potential, in conjunction with PCR expertise available in the feed testing industry, to meet this challenge. A high Density DNA chip which supports over 80,000 probes with the capacity to identify over 25 species as well as the classes bird, mammal and fish. The ability to detect these two taxonomic levels is a unique feature of this tool. The probes have been designed based on vertebrate *cytochrome B* sequences to be specific and sensitive. The upstream process involves DNA extraction, PCR, transcription and labelling before the DNA chip system is introduced for hybridisation, scanning and analysis with a simple user-interface. This presentation will present the technology and results of analysis of feed sample analysis. These include samples heated to over 133°C, pre and post pressure cooked, mixed and pure. Finally, we will discuss the potential applications for this tool for feed analysis.

Keywords

GeneChip, Meat and Bone Meal, Species, PCR, Animal Feed


Detection of heat treated rendering animal tissue in feeds for farm animals by Light Cycler and conventional PCR
Comparison of extraction kits
Traditional PCR
Light Cycler PCR
No DNA
1 Genomic DNA
Bovine DNA of MBM treated at 133°C and extracted with:
Tab.1
Test of specific animal primers with Lyght Cycler PCR
Tested Primers
Cow
Chicken
Genome
Cow
Chicken
Sheep
Mouse
Pig
Homo
Dog
Cat
Goat
Rabbit
Buffalo
Chamois
Wild-boar
Horse
Fish
Tab.4
Primer pairs selected for the amplification of species specific amplicons of mtDNA for classic or Light Cycler PCR
Test of specific animal primers with traditional PCR
Tested Primers
Cow
Pig
Sheep
Mouse
Chicken
Genome
Tab.2
Test of sensibility of bovine primers with Light Cycler PCR
No DNA
Cycle Number
Tab.5
Detection of bovine DNA in commercial feeding stuff with Light Cycler PCR
1: BO2
2: BO3
3: genomic DNA (50pg)
4: BO1
5: BO4
6: No DNA
Cycle Number
Tab.6
Test of specific vegetable primers with traditional PCR
Universal Primers for Vegetables
Animal genomes
Vegetable genomes
Cow
Sheep
Pig
Horse
Homo
Dog
Cat
Goat
Rabbit
Buffalo
Chamois
Wild-boar
Chicken
Fish
Barley
Broccoli
Tomato
Thistle
Hop
Carrot
Beetroot
Orange-tree
Soya-bean
Maize
Savoy cabbage
Olive-tree
Tab.3
CONCLUSIONS

P.28.- Detection of heat treated rendering animal tissue in feeds for farm animals by Light Cycler and conventional PCR

D. Frezza [1], F. Chegdani [2], G. Maccabiani [3], V. Giambra [1], Ch. Von Holst [4], L. Boizza [3], E. Faggionato [3], N. Losio [3], L Lanni [5], S. Saccares [5], P. Ajmone-Marsan [2]

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5 Istituto Zooprofilattico Sperimentale del Lazio e Toscana, via Appia Nuova 1411, 00178 Roma, Italy (lannilu@tin.it)

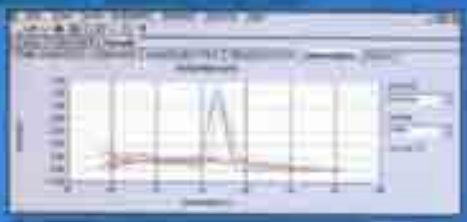
The ban of animal proteins from ruminant feedingstuff has been an effective measure to stop the spreading of the BSE epidemy from the centres of infection. The search for bone residues by optical microscopy is actually the official method for identification of animal material in feeds. This method has difficulties in the determination of the contaminating animal species and in the detection and quantification of the contamination when caused by bone free rendering material. The PCR and real time PCR assays may complement the classic method, permitting a quantitative analysis of contaminants, detecting the species of origin, increasing sensitivity, reducing the time and increasing the automation of analyses. We designed species-specific primer pairs and PCR protocols for the detection of bovine, ovine, pig, poultry, dog and mouse materials in feeds. The target amplicons were identified in the mitochondrial genome present in thousand of copies per cell and short enough (around 120 bp), to obtain amplification products also in heat treated food and feed. Species specificity was tested across 35 different animal and plant species. Methods have been tested on commercial feeds artificially contaminated by animal tissue previously autoclaved according to the European Conditions (133C, 20', 3 bar). The detection limit of the assays is lower than 0.1 % (1 kg / ton). Moreover for bovine, ovine, pig and poultry we set up a method for quantitative analysis of meat products by Light Cycler PCR.

Keywords

BSE prevention, feedingstuff safety, tissue contamination, species detection, quantitative PCR.

TRACEABILITY AND IDENTIFICATION OF ANIMAL TISSUES BY QRT-PCR

UNIVERSIDAD DE CORDOBA



P.29.- Traceability and identification of animal tissues by QRT-PCR

B. Alcaide[3], P. Pascual[1,2], F. Calero[1,2], C. Gálvez[1], G. Dorado, G[3]

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Different animal tissue components, as bones, blood, fat and others, have been widely used in feedstuffs until the Bovine Spongiform Encephalopathy (BSE) crisis in the 90's. Nowadays, only the fish tissue components are allowed for this industrial use in pig feeding. It is uncertain if some mammalian tissues could be used as feedingstuffs in the future.

Hence, it is absolutely necessary to develop and validate an analytical method to determine the origin of feedingstuffs and: a) to prevent the cannibalism in industrial mammalian feeding; b) to avoid the fraud or accidental contamination in fish meal of mammalian meat and bone meal (MMBM); and c) the use of ruminant MBM to prevent the BSE prion transmission.

DNA-based techniques have become very important and are widely used nowadays. Advantages of DNA-analyzing methods are manifold. Thus, DNA is a relatively stable molecule, it is present in MBM samples, and can be easily detected and analyzed. Besides, DNA fingerprinting is the method of choice to identify species. The Polymerase Chain Reaction (PCR) proved to be an adequate technique for detection of small amounts of DNA, specifically amplifying a target region of template DNA in a rapid and sensitive manner. The species-specific genetic differences can be exploited to amplify selected sequences of DNA, which allows the identification or species or animal groups (eg., birds, mammals and fishes) and individual species. We have designed primers using mitochondrial DNA-specific regions, available at the GenBank database. It is important to note that the success of this approach in directly depending on such primer design. Thus, such primers should amplify only the target sequence. In particular, problems of cross-hybridization, specially between species more closely related (eg., sheep-goat and chicken-turkey) should be avoided.

In this work we have developed an strategy through DNA-based methods using our own primer design to identify 6 species (cow, goat, sheep, pig, chicken and turkey). We are also carrying out experiments to identify other 17 species (6 birds, 6 mammals and 5 fishes) present in MBM and fat. Our goal is to develop and validate a protocol to be included in the accreditation system (ENAC) in which we are involved.

Furthermore this technology will be also used to detect frauds and to guarantee food authenticity in processed food of special interest in meal products from the Andalusia region (Southern of Spain, EU).

Acknowledgments: This work was supported by the grant "Desarrollo de técnicas de ADN para el control de calidad de piensos, alimentos y bebidas y la detección de transgénicos" from the "Consejería de Agricultura y Pesca, Junta de Andalucía" (Spain) .

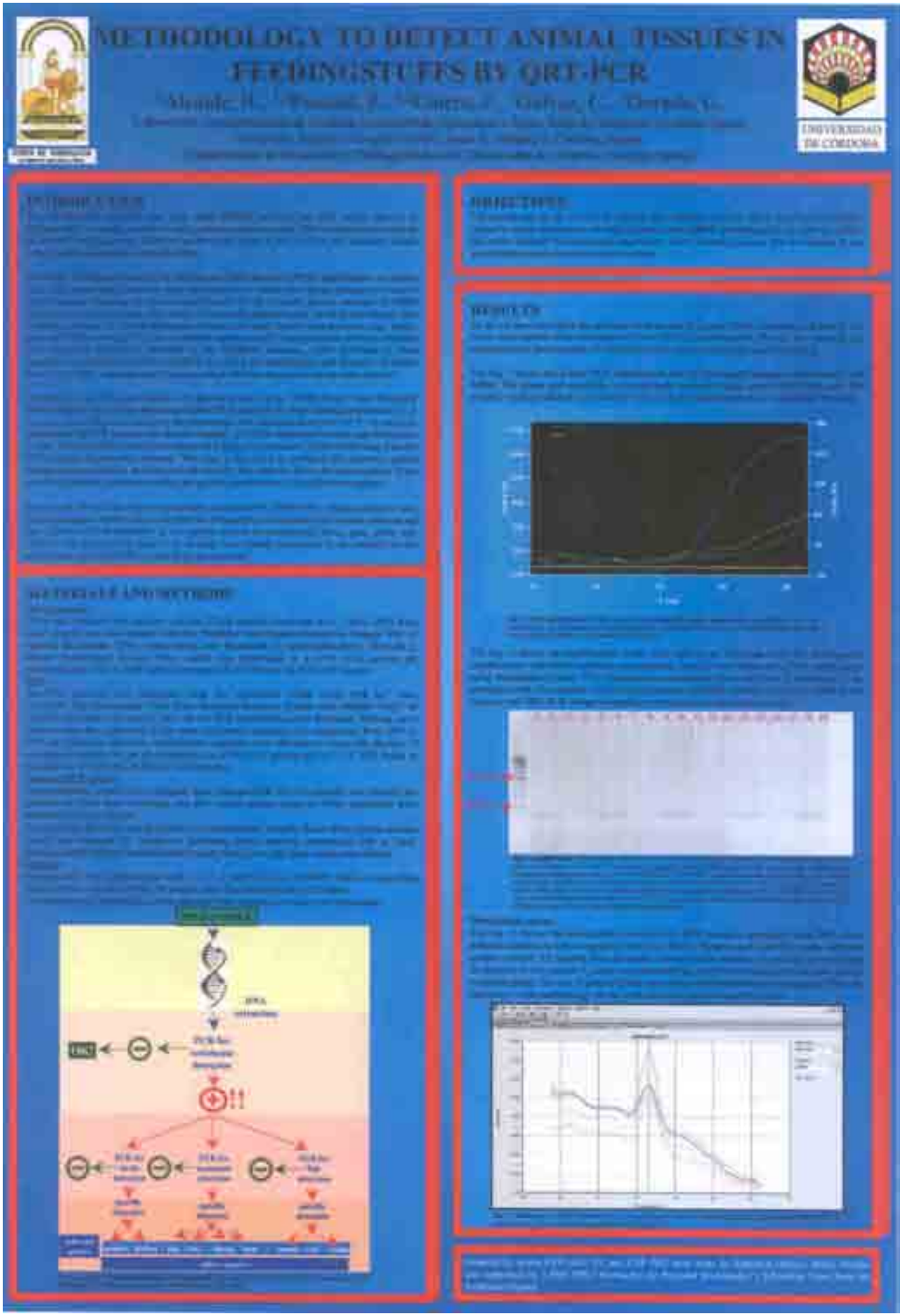
Belén Alcaide was supported by a grant from the "Consejería de Agricultura y Pesca, Junta de Andalucía".

Samples were provided by the "Departamento de Producción Animal, ETSIAM, Universidad de Córdoba" (Spain).

Keywords

Species-specific identification, PCR, MBM

METHODOLOGY TO DETECT ANIMAL TISSUES IN FEEDINGSTUFFS BY QRT-PCR



P.30.- Methodology to detect animal tissues in feedingstuffs by QRT-PCR

B. Alcaide[3], P. Pascual[1,2], F. Calero[1,2], C. Gálvez[1], G. Dorado, G[3]

[1]Laboratorio Agroalimentario de Córdoba. Consejería de Agricultura y Pesca, Junta de Andalucía. (candido.galvez@juntadeandalucia.es)
[2]Desarrollo Agrario y Pesquero (DAP.), Junta de Andalucía.
[3]Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba.

The current procedures of meat and bone meal (MBM) and species identification are carried out mainly using microscopic methods. This is based on the analysis of animal bone fragments, which are cumbersome, need a lot of effort and specially trained experts, being susceptible to subjective bias.

With the development of molecular biology, new approaches emerged, based on genetic differences between species. In particular, molecular techniques based on the Polymerase Chain Reaction (PCR) offer advantages in that they have the possibility to detect even minute amounts or traces of animal tissues and thus identify the different species included in MBM samples (raw and processed).

The quality of the results are depending on primers design, specially to avoid problems of cross-hybridization between species more closely related (eg., sheep-goat and chicken-turkey). We have developed species-specific oligonucleotide primers, designed from sequence information available in the GenBank database, which hybridize to short sequences of mtDNA and allow the amplification and detect of animal groups (eg., birds, mammals and fishes) and different species during the same analysis.

As a laboratory for the official control of feedingstuffs in the Andalusia region (Southern of Spain), we use methods based on the PCR technique to confirm the results that we obtained by an accredited microscopic method and thus obtain additional information.

The overall aim of our work is to develop and validate a method which could be successfully applied to detect the presence of meat and bone meal (MBM) in feeding stuffs in order to confirm the results obtained by microscopic observation. The establishment of this protocol will be included in the accreditation system in which we are involved.

Following our protocol, we have identified the presence of three animal groups (birds, mammals and fishes) and seven main species (5 mammals and 2 birds) in feedingstuffs. We are also carrying out procedures to detect another 17 extra species (6 birds, 6 mammals and 5 fishes).

Acknowledgments: This work was supported by the grant "Desarrollo de técnicas de ADN para el control de calidad de piensos, alimentos y bebidas y la detección de transgénicos" from the "Consejería de Agricultura y Pesca, Junta de Andalucía" (Spain).
Belén Alcaide was supported by a grant from the "Consejería de Agricultura y Pesca, Junta de Andalucía".
Samples were provided by the "Departamento de Producción Animal, ETSIAM, Universidad de Córdoba" (Spain).

Keywords

Species-specific identification, PCR, microscopic methods, MBM

INFRARED SPECTROSCOPY: AN INNOVATIVE SOLUTION TO IDENTIFY ANIMAL CONSTITUENTS IN FEED

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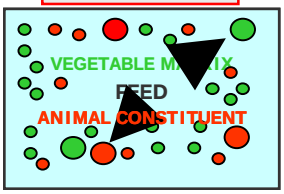
2) Department of Chemistry IFM – University of Turin – via P. Giuria 7, 10125 Torino

The system



The problem

Identification of animal constituents (bone fragments)

Which animal species are?

A tool for a possible solution: IR SPECTROSCOPY

Why? High sensitivity to molecular or reticular materials structure

MIR: fundamental absorption of all chemical bonds

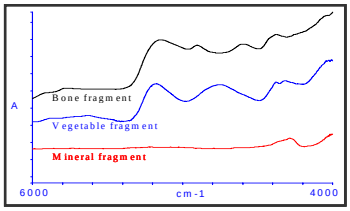
NIR: overtones of stretching -CH, -OH, -NH, -SH bonds and combination bands of these ones and of C=O e C=C bonds

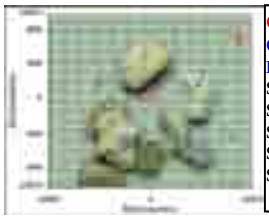
Bone fragments detection:

NIR: bands due to collagen

MIR: bands due to mineral part (aphatite)

1. Fragments identification: FT-NIR Microscopy





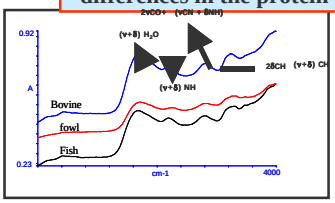
COMPARE (ε) : Bone fragment analysis

$$\varepsilon = \frac{\Sigma w_i A_i B_i}{(\Sigma w_i A_i A_i)^{1/2} \times (\Sigma w_i B_i B_i)^{1/2}}$$

Compare - Reference File	Correlation	
Sample2.sp	0.956	Bone fragments
Sample1.sp	0.951	
Sample3.sp	0.727	Vegetable fragments
Sample4.sp	0.776	
Sample5.sp	0.240.	Mineral fragments

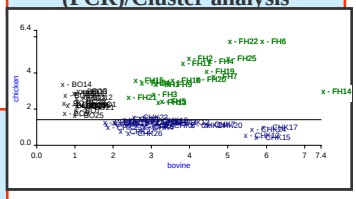
2. Animal species differentiation

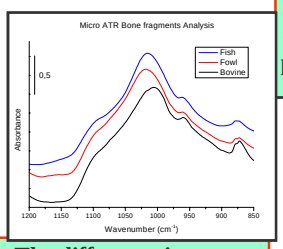
Diffuse reflectance NIR Studies: differences in the protein absorption bands



Spectral pattern affected by specular reflectance phenomena: prevention of the validation of the method

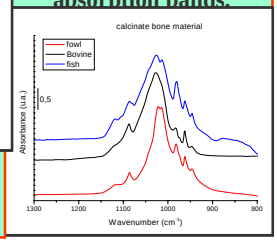
Chemometric analysis: Principal components regression (PCR)/Cluster analysis





The difference is more marked in calcined materials

ATR-MIR Studies: differences in the zone of phosphate and carbonate absorption bands.



Conclusion

IR spectroscopy allows:

NIR: identification of bone fragments from vegetables and mineral ones

MIR: identification of the animal species

REFERENCE -W. Fred McClure, Donald L. Stanfield " Near-infrared Spectroscopy of Biomaterials" (2002), North Carolina State University, Raleigh, N.C., USA, 212-228. – Abete M.C., Andruetto S., Cazzola P.L., Pavino D., Proceedings of "11th International conference on Near Infrared Spectroscopy", Cordoba, 6-11 Aprile 2003.

ACKNOWLEDGEMENT to the Piedmont Region for the financial support

P.31.- Infrared Spectroscopy: an innovative solution to identify animal constituents in feed

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The target of our research has been the developing of a method for the detection of bone fragments in animal feed with an improved efficacy with respect to the official one, based on optical microscopy observation. To this aim, we used IR Spectroscopy, in both NIR (1000-4000 cm-1), where the vibrational features of bone collagen are observed, and MIR (4000-400 cm-1), where the vibrational absorptions due to the mineral part of bone tissues fall. As for NIR spectroscopy, a microscope Autoimage was employed, allowing the recording of the spectrum, in diffuse reflectance mode, of single objects of micrometric size, as the bone fragments possibly present in animal feed are. In the MIR range, the spectra of the single bone fragments were recorded by using a single reflection ATR cell.

The recognition of bone fragments among other constituents was made treating their vibrational spectra by a mathematics algorithm based on least-squares resulting from the comparison between the spectrum of unknown sample and spectra of reference materials. By setting a 95% level of similarity as acceptance threshold, good results were obtained in the detection of bone fragments mixed with vegetable and mineral particles.

Moreover, EC legislation prohibits the addition of transformed animal proteins to cattle for bovine and the addition of bovine meal for all animal breeding, but permits the use of meals of different animal species as fish for several animal breeding (non ruminants). Thus, besides the animal/non animal recognition, it is very important to distinguish bone tissues of different zoological origin. In this respect, Principal Component Analysis (PCA), Principal Components Regression (PCR) and cluster analysis (carried out by using a soft independent modelling of class analogies-SIMCA) were used. About 25 spectra of bone tissue (single fragments) of different zoological origin were considered, and the best separation between the spectra of bone fragments from different animal species was obtained with PCR and cluster analysis.

Keywords

Vibration spectroscopy, feed, bone fragments, PCA.

FT-NIR MICROSCOPY TECHNIQUE APPLIED TO THE ANALYSIS OF BONE PARTICLES IN THE SEDIMENT FRACTION OF FEED

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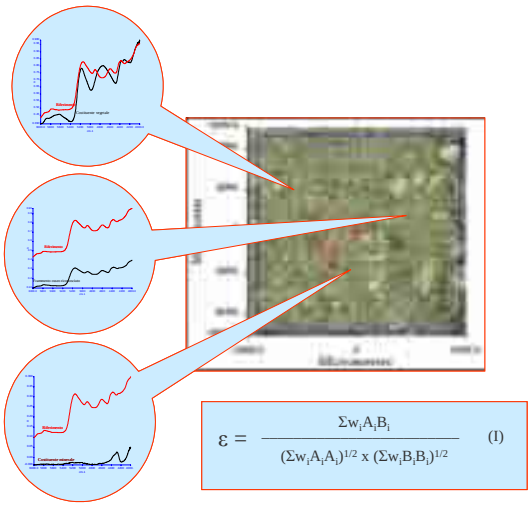
AIM AND OBJECTIVES

The risk of bovine spongiform encephalopathy (BSE) spreading in cattle, induced the European Union to prohibit the administration of feed containing banned meals to cattle for human consumption (2000/766/EC). contaminated feeds are commonly assetted as the main transmission of the mad cow disease in the european bovine herds. In this corcern the control of feeds for the presence of animal meals has an high priority. The official methods (Directive 98/88/EC and Directive 2003/126 EC) is a classical optical microscopy analysis in which the detection of bone particle are done by microscopy observation after a sample pretreatment for the separation of the sediment.
The objective of this research is to test an alternative analytical method for the detection of animal meals in feeds based on the FT-NIR microscopy technique. This new vibrational spectroscopy imaging generates spatially localized chemical and morphological information. The data obtained should be further elaborated by using a mapping procedure.The FT-NIR spectra has been collected on the sediment obtained in the same way as the official method.



$$\varepsilon = \frac{\Sigma w_i A_i B_i}{(\Sigma w_i A_i A_i)^{1/2} \times (\Sigma w_i B_i B_i)^{1/2}} \quad (I)$$

Fig. 1 – The figure shows the microscopic image of a selected area of the sample in which FT-NIR spectra should be aquired. These spectra are compared with those obtained with reference samples and for the correlation the function Ɛ has been used.

RESULTS AND DISCUSSION

In table 1 are reported the results obtained with sediments prepared in four different laboratory with samples spiked at three different concentration levels.
For all analysed samples bone particles was found in quantities proportional to the added meal concentrations. The comparison and the identification of the unknown spectra of the sample with spectra of reference materials (Fig 1) was made via a mathematical function based on the least square method (Equation I). Previous studies have allowed us to set the correlation coefficient ε value egual to 0.95 (95% similarity).
In Fig 2 is reported an example of the mapping procedure in which different coulor correspond to different types of particles (bone, mineral, vegetal).
In table 2 are reported the data relative to ten replicate at a concentration level of 0,1 %. In any case the method allowed us to detect bone particles.
The standard deviation of the percentage of sediment is equal to 0.04, instead the standard deviation of the ratio between the number of bone particle with the total particle analysed is equal to 4.04. These data show a better reproducibility for percentage of sediment compare to that obtained for the number of particle detected. These should be correlated to a poor homogeneity of the commercial animal meal used.

CONCLUSION

By using FT-NIR spectroscopy technique it has been possible to detect the presence of ban meals in all samples in a range of concentration between 0.1% and 3%. Further experimental data are necessary to define reproducibility and repeatability. These preliminary results are very promising for the possibility to apply this new technique as screening method for the detection of ban meals in feeds.

MATERIALS AND METHODS

The samples are treated as in the official method for the separation of the sediments. A commercial feed has been spiked with animal meal at three different concentration levels: 0.5 %, 1% and 3 %. These samples have been analysed in 4 different laboratories by using the official method and the sediments are further analised with the FT-NIR technique.
Another commercial feed has been spiked at a concentration level of 0.1% with animal meals and the sediment separated (10 replicates) and subsequently analised with the FT-NIR technique.
The spectra are collected in the range between 6000-4000 cm^{-1} with 8 cm^{-1} of resolution by using the Spectrometer Spectrum ONE NTS coupled with the AUTOIMAGE microscopy, both purchased by Perkin Elmer.

Laboratories	N° of particles analized	N° of bone particles (sample 1)	N° of bone particles (sample 2)	N° of bone particles (sample 3)
1	144	9	16	30
2	144	9	20	41
3	144	7	14	30
4	144	3	20	52

TAB. 1 – In the table is reported for the four different laboratories, the number of bones particles detected in the samples spiked with animal meals (sample 1 fortified at 0.5% level; sample 2 fortified at 1% level and sample 3 fortified at 3% level)

Number of samples	% of sediments	N_0	N_0/N_t
1	0.305	23	16
2	0.291	9	8.2
3	0.282	8	5.6
4	0.274	9	6.3
5	0.230	15	8.9
6	0.250	7	4.9
7	0.363	22	15.3
8	0.298	10	6.9
9	0.244	10	6.9
10	0.298	7	4.9

TAB.2 – In the table are reported the number of sample analysed, at 0.1% level of fortification, the percentage of sediment obtained, the number of bones particles (N_0) and the ratio between N_0 and N_t (number of particle analysed)

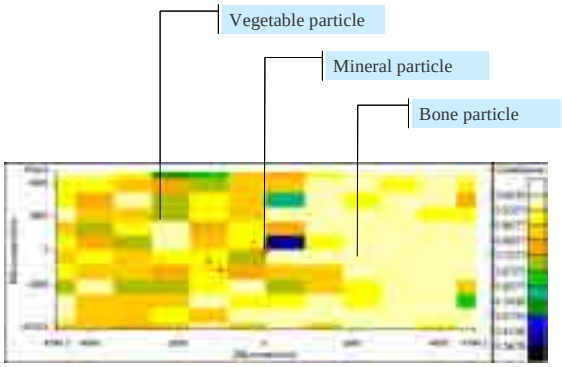


Fig. 2 – Mapping of a sediment sample. Each square is a spectrum of material acqiured at a precise point within the mapped area. The colour refers to the correlation coefficient arising from equation I.

REFERENCE -W. Fred McClure, Donald L. Stanfield " Near-infrared Spectroscopy of Biomaterials" (2002), North Carolina State University, Raleigh, N.C., USA, 212-228. – Douglas A. Skoog, James J. Leary, "Chimica Analitica Strumentale", (2000) EdiSES. – Abete M.C., Andruetto S., Cazzola P.L., Pavino D., Atti del convegno "11th International conference on Near Infrared Spectroscopy", Cordoba, 6-11 Aprile 2003

ACKNOWLEDGEMENT to the italian Minister of the Health for the financial support

P.32.- FT-NIR Microscopy technique applied to the analysis of bone particles in the sediment fraction of feed

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The ban on using processed animal proteins in feed is an important measure to prevent the spread of Transmissible Spongiform Encephalopathies and it is regulated by Council Decision 2000/766/EC. More recently regulation EC 1774/2002 is imposing a ban on feeding animals with protein of the same species. The application of such legislation requires the availability of reliable analytical methods. From 1998 the classical optical microscopy method is the official one for the determination of processed animal protein in feed. In this method the detection of bone particles are done by microscopic observation after a sample pretreatment for the separation of the sediment. In the present study we test an alternative analytical method based on the FT-NIR microscopy technique; this new vibrational spectroscopy imaging generates spatially localized chemical and morphological information. On this point of view this technique seems to be suitable for the detection of the presence of bone meals in the sediment fraction of feed. Preliminary results obtained by using the mapping procedure are reported . Commercial feed spiked with animal meal in concentration range between 0,1% to 3% are analysed.

Keywords

FT-NIR Microscopy, Animal Proteins, Feed, Bone meals

Detection of meat and bone meal in hydrolysed feather protein using Near InfraRed Spectroscopy (NIRS)

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The use of hydrolysed feather protein (HFP) and meat and bone meal (MBM) in animal feed is forbidden

It is anticipated that, under strict conditions, the use of HFP in feed will be allowed in the future. Than, screening techniques are needed to prevent *accidental* of *fraudulent* contamination of HFP with MBM.

Materials and methods

NIR models are validated with 8 commercial HFPs (Sonac, Son, The Netherlands; Sonac, Burgum, The Netherlands; Saria, Isse, France; De Vries Protein, Barneveld, The Netherlands; PDM [samples 31, 32 and 60], United Kingdom; A&L, Diepholz, Germany) and 4 MBMs (poultry: Sonac, Burgum, The Netherlands; Sonac België, Denderleeuw, Belgium; mammalian: SNP, Lingen, Germany; Sonac, Burgum, The Netherlands). All the materials are from different locations and vary considerable in both colour and texture. 328 mixtures of MBM and HFPs are used for the validation. First a model was formulated using 2/3rd of the samples. This model was subsequently tested on the remaining samples (see figure 1).
The spectra (1100 – 2500 nm) of 328 mixtures of MBM and HFP have been collected using a Foss NIRSystem 6500.

Results

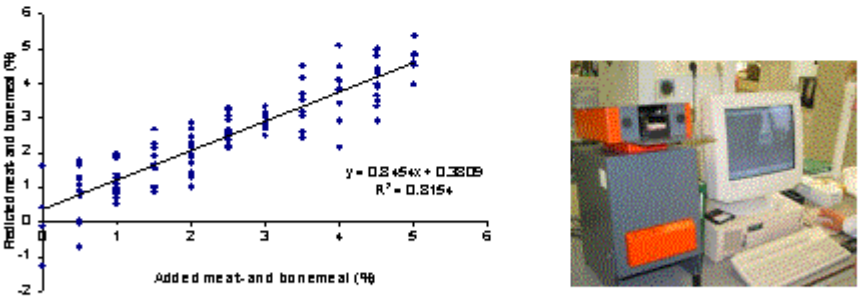

Figure 1. The relationship between added and predicted percentage MBM in HFP.

Conclusions

On the basis of these (preliminary) results it appears possible to detect accidental or fraudulent addition of MBM to HFP with an uncertainty of about 2%.
Based on limited information, the detection limit is roughly 3% when an unknown MBM is the contaminant. These models can probably be made even more robust by inclusion of more different HFPs and more MBM samples.

Acknowledgements
This study is a collaboration of the European Fat Processors and Renderers Association (EFPRA). It is funded by the Natural Organic Products group of Sobel (Best, the Netherlands).

P.33.- Detection of MBM in hydrolysed feather protein using Near Infra Red Spectroscopy (NIRS).

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At present, the use of hydrolysed feather protein (HFP) and meat- and bone meal (MBM) in animal feed is forbidden. It is anticipated that, under strict conditions, the use of HFP in animal feed will be allowed in the future. To prevent accidental or fraudulent contamination with meat- and bone meal, screening techniques are needed.

NIRS models are validated with eight commercial HFPs and four MBMs. All the materials are from different locations. The MBMs were added to the HFPs in concentrations ranging from 0 to 5% with 0.5% increments (328 samples in total). The spectra (1100 to 2500 nm) of these mixtures were collected using a Foss NIRSystem 6500. NIRS models were formulated for seven out of eight HFP and tested on the HFP not used for the model. Simularly, NIRS models were formulated using HFP contaminated with three out of four MBMs and tested on HFP contaminated with MBM not used for the model.

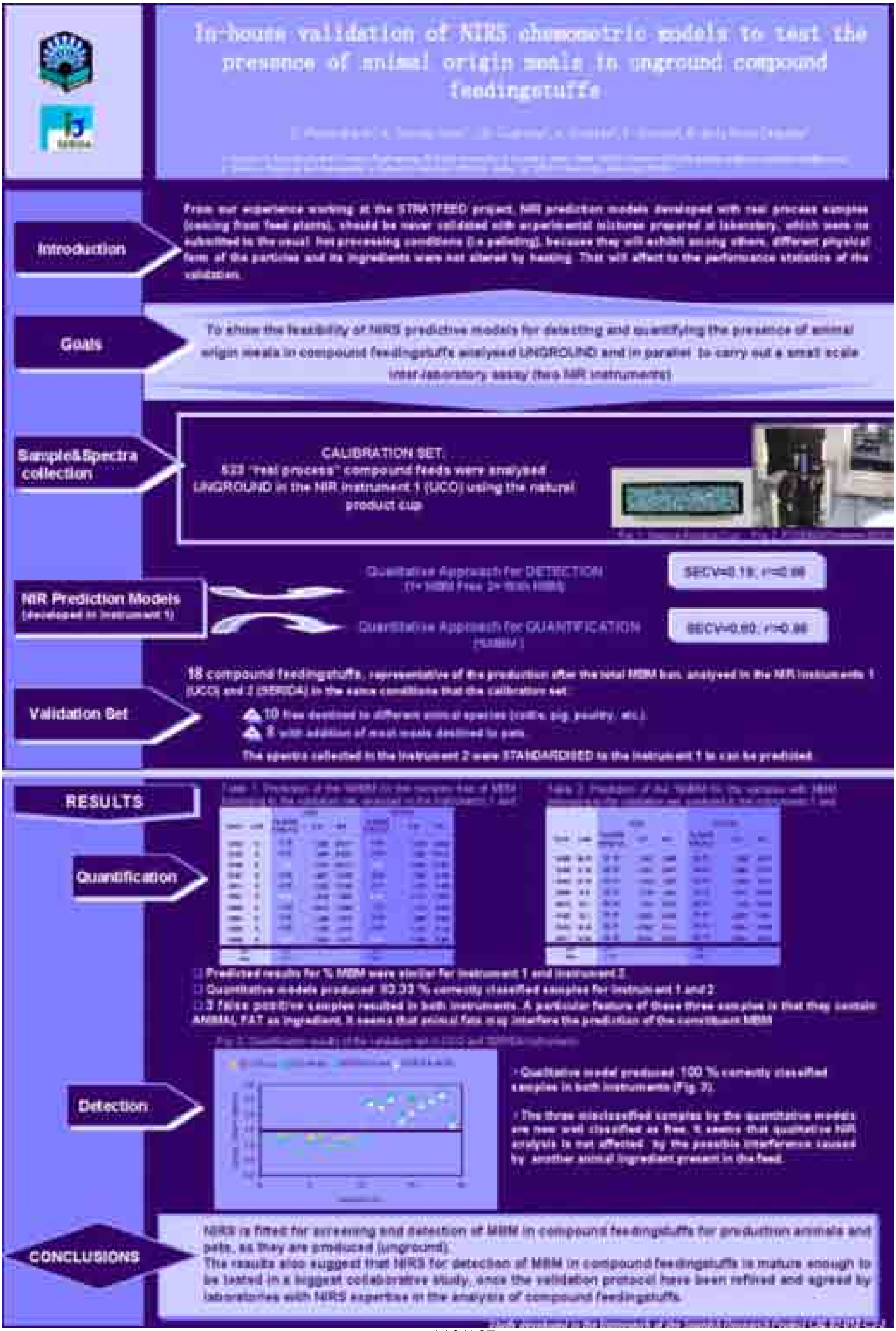
The results clearly show that the type of HFP has a large effect on the results. It is clear that for a reliable estimate a NIRS model should contain spectra of the HFP involved. The models appear to be less sensitive to the type of MBM.

NIRS can be used to detect accidental or fraudulent addition of MBM to HFP. A predictive model based on a number of HFPs and MBM can achieve estimations with an uncertainty of 2%.

Based on limited information, the detection limit is roughly 3% when an unknown MBM is the contaminant.

Keywords

NIRS, hydrolysed feather protein, HFP, MBM, fraude, contamination


In-house validation of NIRS chemometric models to test the presence of animal origin meals in unground compound feedingstuffs
Introduction
From our experience working at the STRATFEED project, NIR prediction models developed with real process samples (coming from feed plants), should be never validated with experimental mixtures prepared at laboratory, which were no submitted to the usual hot processing conditions (i.e pelleting), because they will exhibit among others, different physical form of the particles and its ingredients were not altered by heating. That will affect to the performance statistics of the validation.
Goals
To show the feasibility of NIRS predictive models for detecting and quantifying the presence of animal origin meals in compound feedingstuffs analysed UNGROUND and in parallel to carry out a small scale inter-laboratory assay (two NIR instruments)
Sample&Spectra collection
CALIBRATION SET.
523 "real process" compound feeds were analysed UNGROUND in the NIR instrument 1 (UCO) using the natural product cup
NIR Prediction Models
(developed in instrument 1)
Qualitative Approach for DETECTION
Quantitative Approach for QUANTIFICATION
Validation Set
18 compound feedingstuffs, representative of the production after the total MBM ban, analysed in the NIR instruments 1 (UCO) and 2 (SERIDA) in the same conditions that the calibration set
The spectra collected in the instrument 2 were STANDARDISED to the instrument 1 to can be predicted.
RESULTS
Quantification
Predicted results for % MBM were similar for instrument 1 and instrument 2.
Quantitative models produced 83.33 % correctly classified samples for instrument 1 and 2.
3 false positive samples resulted in both instruments. A particular feature of these three samples is that they contain ANIMAL FAT as ingredient. It seems that animal fats may interfere the prediction of the constituent MBM
Detection
Qualitative model produced 100 % correctly classified samples in both instruments (Fig. 3).
The three misclassified samples by the quantitative models are now well classified as free. It seems that qualitative NIR analysis is not affected by the possible interference caused by another animal ingredient present in the feed.
CONCLUSIONS
NIRS is fitted for screening and detection of MBM in compound feedingstuffs for production animals and pets, as they are produced (unground).
The results also suggest that NIRS for detection of MBM in compound feedingstuffs is mature enough to be tested in a biggest collaborative study, once the validation protocol have been refined and agreed by laboratories with NIRS expertise in the analysis of compound feedingstuffs.

P.34.- In-house validation of NIRS chemometric models to test the presence of animal origin meals in unground compound feedingstuffs.

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The ban of the use of animal origin meals in compound feeds is one of the measures carried out in the EU to stop the spread of the Bovine Spongiform Encephalophathy (BSE) and to prevent its re-occurrence. It is now clear that the ban will be only lifted if there are available analytical methods that ensure its enforcement.

NIR spectroscopy is likely to be the most rapid method of testing feedingstuffs, allowing a substantial increase of the number of controlled samples and providing an instantaneous response to detect adulterated samples. However, the traditional application of NIRS involves grinding of samples. That sample preparation is some times one of the most critical steps in the implementation of NIRS technology.

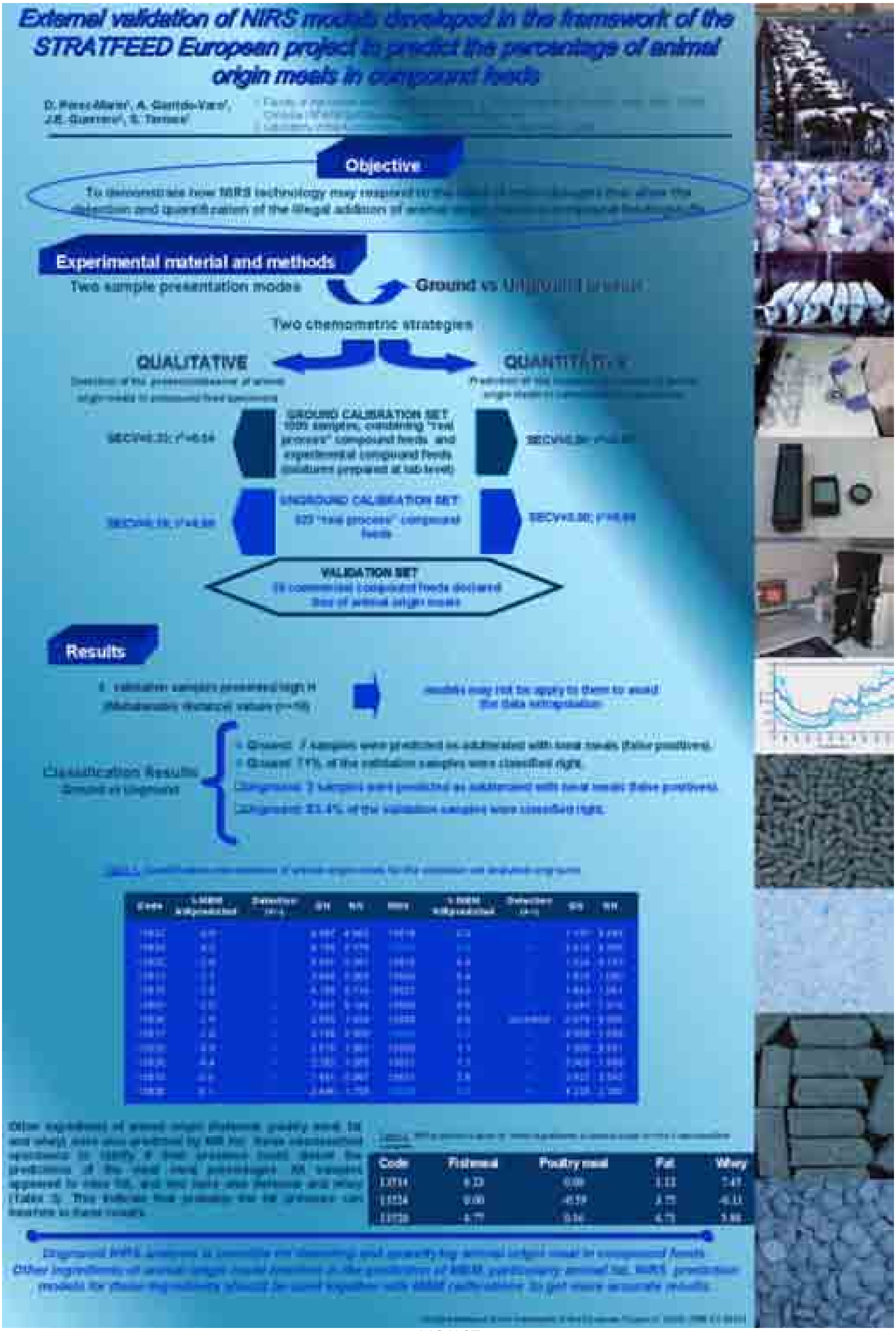
The goals of the present work is to show the feasibility of NIRS predictive models for detecting and quantifying the presence of animal origin meals in compound feedingstuffs analysed unground and, moreover, to demonstrate the ability for transferring the calibration models between two instruments. For the purpose of this research, two complementary chemometric strategies were evaluated. First, the use of a qualitative discriminant PLS equation (n= 523; SECV=0.19; r^2=0.86), which uses dummy variables with values 1 (free) or 2 (with), to determine whether a compound feedingstuff has been contaminated or not with meat meal. Second, a quantitative PLS calibration equation for the prediction of the inclusion percentage of meat meals in compound feedingstuffs (n= 523; SECV= 0.80%; r^2=0.98).

An in-house validation of these two models was carried out with a blind test set of 18 compound feedingstuffs (10 free and 8 contaminated with meat meals) produced during 2003 and being representative of those produced after the publication of the total ban. This validation set was scanned using the natural cup, in two cloned NIR instruments equipped with transport module. The results show that the qualitative model gave 100% classification rate for the blind samples analysed in both, the master and the host instruments. For the quantitative prediction of meat meal percentage, the results obtained in both matching instruments are quite similar; all the samples contaminated and seven samples free are well predicted, resulting the other three as false positive samples. The origin of that mis-classification may be found when considering the composition of the three samples on other animal by-products (i.e. animal fats).

The results demonstrate that NIR can be used as a screening method to accomplish the fulfilment of regulations concerning the production of compound feedingstuffs. NIRS must therefore be considered an indispensable tool and an integral part of Food Safety programs.

Keywords

Compound feedingstuffs, NIR, animal origin meal, unground analysis.


Objective
Experimental material and methods
Two sample presentation modes
Two chemometric strategies
QUALITATIVE
GROUND CALIBRATION SET
UNGROUND CALIBRATION SET
VALIDATION SET
Results
Classification Results

P.35- External validation of NIRS models developed in the framework of the STRATFEED European project to predict the percentage of animal origin meals in compound feeds

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The main goal of this work is to demonstrate how NIRS technology may respond to the need of methodologies that allow the detection and quantification of the illegal addition of animal origin meals to compound feedingstuffs.

Different NIRS prediction models have been developed in the framework of the STRATFEED project (European Project nº G6RD-2000-CT-00414), using different strategies with regard to the calibration sets, reference data and chemometric algorithms used.

Two strategies are validated together, one quantitative and other qualitative. For quantification, i.e. for predicting the inclusion percentage of animal origin meals in compound feed specimens, the calibration evaluated in this work (SECV=0.86; r^2=0.98) was obtained using as calibration set a combined set of "real process" compound feed samples (supplied by several feed manufacturers) and experimental compound feeds (prepared at labortatory). For the qualitative approach, i.e. for detecting presence/absence of animal origin meals in compound feed specimens, the models developed (SECV=0.33; r^2=0.54) used the same training set described. All these samples were analysed **ground.** Moreover, in parallel, the "real process" compound feed samples were also scanned **unground**, and new chemometric models with this sample presentation mode were performed for the detection (SECV=0.19; r^2=0.86) and the quantification (SECV=0.80; r^2=0.98) of meat meal.

The validation of the prediction models obtained were carried out using a set of 28 commercial compound feeds declared as free of animal origin meals. These samples were scanned ground and unground. Although the calibrations obtained with ground material classified most of the validation samples as free, nevertheless there were seven samples predicted as adulterated with meat meals. The prediction models performed with unground material showed better classification results. Thus, for the qualitative approach, only three samples are classify as positive in meat meal presence. Moreover, to clarify if other ingredients of animal origin could interfere and distort the results for these misclassified specimens, the inclusion percentage of fish meal, poultry meal, animal fat and whey were predicted using NIR calibrations obtained in a previous research project. The results indicated that samples predicted with high levels of fish meal and whey were correctly classified as free of meat meals; while samples with presence of fat were identified as false positive in meat meal.

Keywords

NIRS, validation, compound feed, animal origin meal, quantification, detection, adulteration, unground analysis

THE USE OF NIR SPECTRAL SIGNATURES TO DIFFERENTIATE AMONG CATEGORIES OF INEDIBLE FATS AND OILS

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INTRODUCTION

Inexpensive, fast, reliable and automated methodologies are needed for the complete traceability of feed grade fats and oils. That it is crucial not only for enforcement of the present EU legislation concerning its use as feed, but also when looking for new alternatives of use of feed grade fats and oils.

OBJECTIVE

To show how the NIR spectra of animal and vegetable fats together with relevant chemical and technical processing conditions may offer an automatic and instantaneous method of fats classification according its chemical composition/origin/class.

SAMPLE BANK AND SPECTRAL LIBRARIES METHODOLOGY

PRELIMINARY QUANTITATIVE AND QUALITATIVE NIRS RESULTS

CALIBRATION STATISTICS FOR THE QUANTITATIVE PREDICTION OF FATTY ACIDS N= 57

Calibrations are also being developed for: Free acidity, MIU, oxidative stability, % pork, poultry, ruminant non-ruminant....

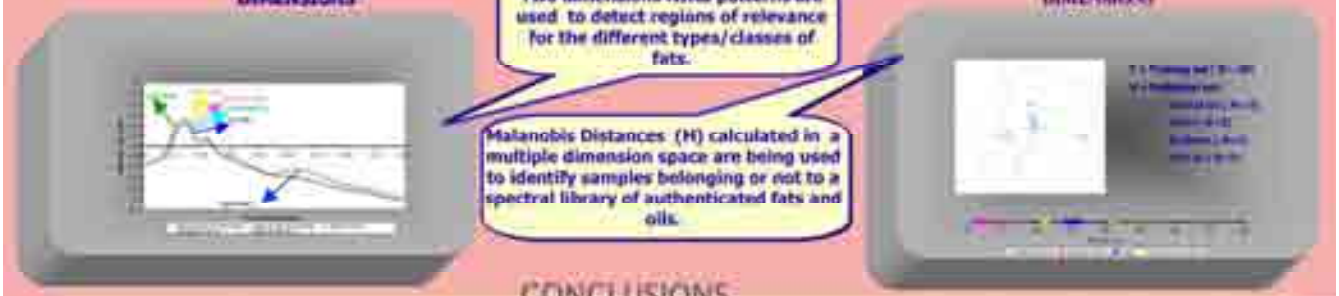
PLS2 DISCRIMINANT ANALYSIS FOR CLASSIFICATION INTO ANIMAL SPECIE CATEGORIES (N=77)

Discriminant analysis and other Pattern Recognition Methods are also being developed to classify into: ruminant/non-ruminant, vegetal/animal....

NIRS PATTERNS OF FATS AND OILS IN TWO DIMENSIONS

Two dimensions NIRS patterns are used to detect regions of relevance for the different types/classes of fats.

NIRS PATTERNS OF FATS AND OILS IN MULTIPLE DIMENSIONS



Malanobis Distances (H) calculated in a multiple dimension space are being used to identify samples belonging or not to a spectral library of authenticated fats and oils.

CONCLUSIONS

- In order to obtain robust NIRS predictive models it is essential to use high-quality spectral libraries. The use of algorithms designed to correct factors affecting spectral repeatability of fats and oils it is of great importance for model robustness.
- The success of a traceability system based on NIRS technology is clearly dependent of the collaboration of producers and fats producers in the building of authenticated library files.

P.36.- The use of NIR spectral signature to differentiate among categories of inedible fats and oils.

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The EU Regulation EC Nº 1774/2002 governing Animal Processed By-Products (ABPs) addressed the possible risk inherent in recycling potential BSE infectivity due to the absence of barrier within species and claimed to exclude the cannibalism, which may be induced by the intra-species recycling. This Regulation opened discussions about a possible ban of ruminant fats in feedinsgtuffs and about the importance of differentiate among categories of animal fats and oils. Therefore, it is urgent to have methods analytical with can help to enforce legislation concerning the traceability of animal and vegetable fats and oils. The Near Infrared Spectra of fats and oils seems to be an affordable spectral signature for identification fats and oils, however a pre-requisite for its implementation in practice is to have well authenticated samples to be used as NIRS spectral libraries and chemometric models from which the spectra of unknown samples may be identified. In the framework of a PhD thesis which is being undertaken at the University of Córdoba and funded by a national R & D project, a sample bank of 473 fat and oil samples have been created, in collaboration with several Spanish rendering plants and vegetable fat producers.

The objective of the present work is to show preliminary results of the use of NIRS library files for identification of unknown animal and vegetable fats and oils. A set of 40 authenticated rendered fats (training set) identified by the providers as being from pure poultry (n= 16), pure pork (n = 5) and mixture of ruminants and non-ruminants (n = 19) were scanned in a FOSS NIRSystems 6500 from October 2002 to October 2003. Latter on, from January to April 2004 , 20 unknown samples of fats and oils were also scanned (N = 8 animal fats, N = 2 soybean oils and 5 vegetable oleins and N = 5 fish oils). Principal component analysis of the training set reduced the multivariate spectral space into 10 new coordinates. Once, the unknown samples were projected into that new coordinate space, the Mahalanobis Distances (H) of each unknown sample from the center of the training set were calculated. The H distances ranged from 1 to 4 for the 8 animal fats, 11 to 20 for the 7 oils and oleins and from 21 to 34 for the 5 fish oils.

The preliminary results open great expectations for the use of the NIRS spectral data for classification of fats and oils according its origin. The success of a traceability system for animal fats and oils based on NIRS technology, is clearly dependent of the collaboration of fats and oils producers in the building of well authenticated spectral library files.

Keywords

NIR spectroscopy, animal fat, fish, vegetable, robust calibration, traceability

NEAR INFRARED REFLECTANCE SPECTROSCOPT (NIRS) FOR IDENTIFICATION OF THE SPECIE IN ANIMAL PROTEIN PROCESSED BY-PRODUCTS.

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1

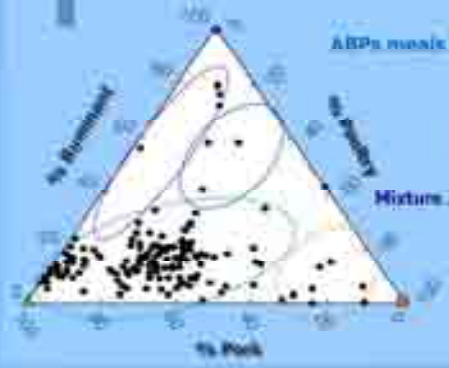
Due to the Regulation (EC) nº 1774/2002 governing Animal By-Products (ABPs) there is an urgent need for developing methods of analysis which allows to identify the animal specie in protein ABPs.

2 MIGHT NIRS TECHNOLOGY BE USEFUL TO IDENTIFY THE SPECIE IN ANIMAL PROTEIN BY-PRODUCTS ?

3 SAMPLE BANK AND NIRS ANALYSIS

- Sample Bank created with samples provided by different renders and feed manufacturers.
- From a total of 280 samples the specie is known.
- The NIRS spectra of the 280 samples have been collected and different quantitative and qualitative chemometrics models have been developed.

4 STRUCTURE OF THE SAMPLE BANK (N = 280)



Pure poultry	56
Pure pork	16
Pure cattle	1
poultry-cattle	1
poultry-pork	3
cattle-pork	3
poultry-cattle-pork	189

5 CALIBRATION RESULTS

Quantitative analysis			Discriminant analysis
Constituent (eqa)	SECV	r²	(1 = ruminant; 2 = non ruminant)
% Poultry meal (nº 1)	8.73	0.94	
% Pork meal (nº 2)	8.12	0.93	
% Cattle meal (nº 3)	3.45	0.81	r^2 = 0.82
% Ruminant meal (nº 4)	3.93	0.76	SECV = 0.19
% Non-Ruminant meal (nº 5)	3.79	0.78	(eqa nº6)

SECV = Standard Error of Cross Validation.
R² = Coefficient of Determination (% of the variance explained by the model)

6 EXTERNAL VALIDATION

- 29 blind ABPs samples provided by EFPRA were predicted with chemometrics models developed with the ABPs meals of the Sample Bank (SB). All the samples were far away from the centre of the Sample Bank population (Mahalanobis distance, GH>3-4)
- The NIR predicted results for 16 EFPRA samples which obtained GH values between 3 and 7 are shown in Fig 1

Figure 1

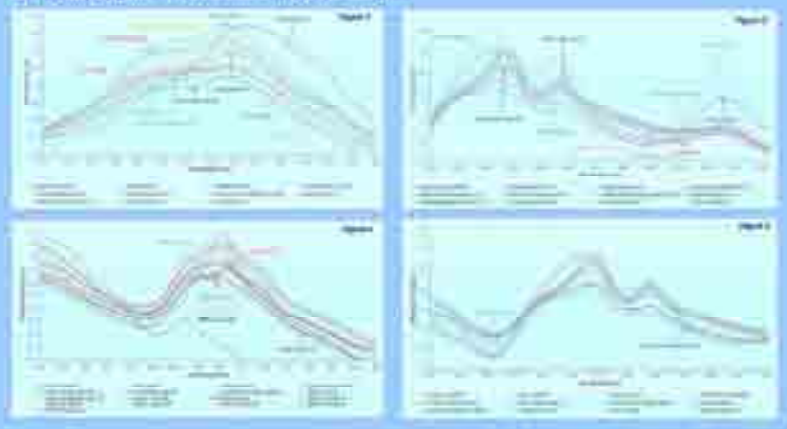
Sample Bank Reference	EFPRA Reference	% Poultry (eqa nº1)	% Pork (eqa nº2)	% Cattle (eqa nº3)	% Ruminant (eqa nº4)	% Non Ruminant (eqa nº5)	PLS2 (eqa nº6) (1=R; 2=NR)	GH
13655	5	++++	+/-	-	-	++++	2.36	7.725*
13656	6	++++	+/-	-	-	++++	2.00	3.766*
13657	7	-	++++	+	+	++++	1.42	5.568*
13658	8	-	++++	?	+	++++	1.66	4.531*
13659	17	-	++++	?	+	++++	1.43	4.908*
13660	14	-	++++	+	+	++++	0.93	4.410*
13661	15	-	++++	+	+	++++	0.69	4.092*
13662	16	-	++++	?	+	++++	0.88	4.344*
13663	17	+	+++	+	+	++++	0.95	2.577
13664	18	?	++++	+	+	++++	0.89	3.920*
13665	19	+	+++	?	+	++++	0.74	7.224*
13666	20	?	+++	?	+	++++	1.43	4.027*
13667	21	++	+++	?	+	++++	0.66	4.087*
13668	22	+	+++	+	+	++++	0.99	3.106*
13671	29	-	++++	?	+	++++	1.04	5.307*
13673	31	++++	-	+	+	++++	1.76	5.884*

+ : Clearly positive on the presence of animal ingredient. It has divided as follows:
+ : 0-25% ; ++ : 26-50%
++ : 51-75% ; +++ : 76-100%
+/- : Slightly positive on the presence of animal ingredient
- : Negative on the presence of animal ingredient
? : No clear result

The two samples classified as free of ruminant by the equations nº 3 and 4 were classified as non-ruminant by equation nº5 and they were also classified as no containing ruminant by the PLS discriminant model (eqa nº 6)

- Another 13 EFPRA samples had GH values higher than 10.
- However, by comparison of their spectra with the spectra of well identified samples from the Sample Bank (SB) (Fig 2 and 3), the 13 samples were classified as indicated in figures 4 and 5

Fig 2 to 5. Relevant absorption features of ABPs spectra from samples including in the Sample Bank versus blind EFPRA samples



7 CONCLUSIONS

The NIR spectra of animal protein by-products contain relevant information related to the animal specie from which they were obtained.

The results obtained may not be accurate enough, because EFPRA ABP samples are not represented in the present calibration models.

Collaboration to enlarge the Sample Bank is crucial for increasing NIRS models robustness.

P.37.-Near Infrared Reflectance Spectroscopy (NIRS) for identification of the specie in animal protein processed by-products

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From 3 October 2003 the EU has adopted Regulation (EC) nº 1774/2002 governing Animal By-Products (ABPs) and it adresses the possible risk inherent in recycling potential infectivity due to the absence of barrier within species and excludes the cannibalism, which may be induced by the intra-species recycling. The main aim of the present work was to develop and validate quantitative and qualitative NIRS models to identify the animal specie in ABPs.

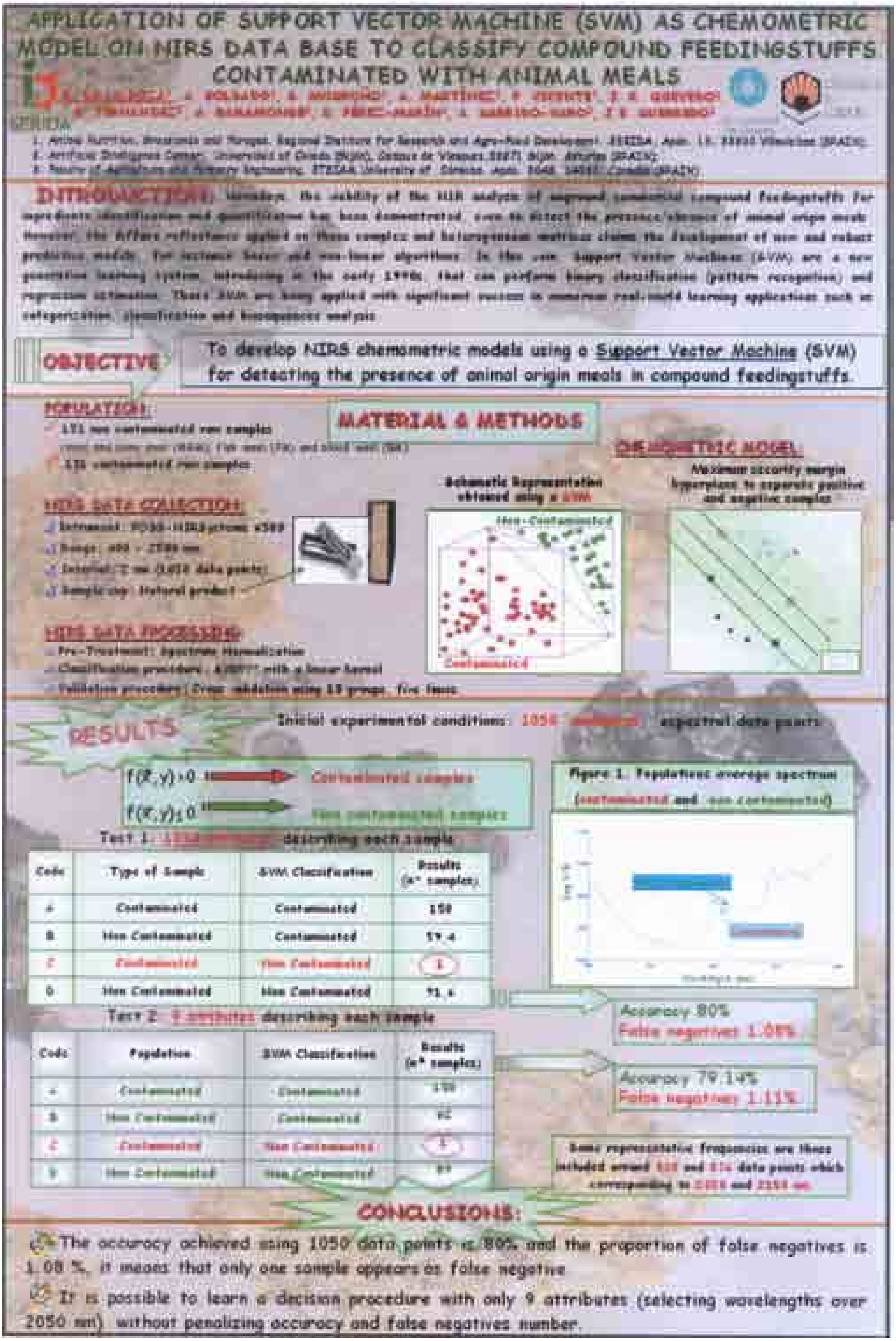
A total of 280 samples of protein animal meals were collected from October 2002 to April 2004 from different providers in the framework of two projects[1,2]. The animal meals samples were identified by the providers as being from pure poultry (n=56), pure cattle (n=1), mixture cattle-poultry (n=1), mixture cattle-pork (n=3), mixture poultry-pork (n=3), blood (n=1), fish (n=8), hydrolised feather meal (n=1), feather meal (n=1) and mixture cattle-ewe-pork and poultry. The spectra of the meals samples were recorded in a FOSS NIRSystems 6500 instrument and thus, a NIRS spectral library of identified ABPs was build (training set). Modified PLS quantitative models were developed to predict the percentage of poultry, pork, cattle, ruminant and non-ruminant meat meals in protein ABPs and they explained 94%, 93%, 81%, 76% and 78% of the variance existing for the parameter "specie" in the complete spectral library. PLS2 discriminant models were also developed, using dummy variables (1=ruminant; 2=non-ruminant). The best model obtained explained the 82.7% of the variance and it had a SECV value of 0.19.

The NIRS chemometrics models were applied to the spectra of 29 samples provided by EFPRA as a blind set for external validation. All of these samples were far away (H > 3) of the center of the training set, measured by the Mahalanobis distance (H). The Mahalanobis distance of 13 of these samples was higher than 10; this means that the spectra of this population is far for the average spectrum of the training set. Despite of that, the predicted values obtained from the NIRS quantitative and qualitative models used together with the comparison of the spectra with other samples from the training sets provided the following information: 2 samples look as poultry greaves; 1 sample was identified as blood meal; 2 samples seem to be fish meals; 5 samples were rather similars to feather meal; 1 sample looks as mixture of poultry and cattle meals and 2 samples were mixture meals greaves. Another group of 16 unknown samples had lowest H values (between 3 and 7).These samples were identified as follow: 5 samples as mixture of poultry and pork meal, 8 samples as mixture of pork and cattle meals, 2 samples were identified as mixture of the three species (poultry, pork and cattle) and, finally, 1 sample was classified as hidrolysed feather meal.

This study shows the potential of NIRS technology to identify the animal specie in protein animal by-products. Collaboration with EFPRA is needed for a final evaluation of this out-house validation exercise. Further co-operation with renderers is important for enlarging the library file in order to build models which can be applied to all the rendered protein meals circulating at intra and inter-European Community level.

Keywords

Protein by-products, cannibalism, specie identification, discriminant analysis, partial least squares, NIRS technology


APPLICATION OF SUPPORT VECTOR MACHINE (SVM) AS CHEMOMETRIC MODEL ON NIRS DATA BASE TO CLASSIFY COMPOUND FEEDINGSTUFFS CONTAMINATED WITH ANIMAL MEALS
INTRODUCTION
OBJECTIVE
To develop NIRS chemometric models using a Support Vector Machine (SVM) for detecting the presence of animal origin meals in compound feedingstuffs.
POPULATION:
MATERIAL & METHODS
CHEMOMETRIC MODEL:
NIRS DATA COLLECTION:
NIRS DATA PROCESSING:
RESULTS
Initial experimental conditions: 1050
f(x,y)>0
f(x,y)≤0
Contaminated samples
Non contaminated samples
Figure 1. Populations average spectrum
Test 1
Test 2
Code
Type of Sample
SVM Classification
Population
Contaminated
Non Contaminated
Accuracy 80%
False negatives 1.08%
Accuracy 79.14%
False negatives 1.11%
CONCLUSIONS:
The accuracy achieved using 1050 data points is 80% and the proportion of false negatives is 1.08 %, it means that only one sample appears as false negative.
It is possible to learn a decision procedure with only 9 attributes (selecting wavelengths over 2050 nm) without penalizing accuracy and false negatives number.

P.38.- Application of Support Vector Machine (SVM) as chemometric model on NIRS data base to classify compound feedingstuffs contaminated with animal meals

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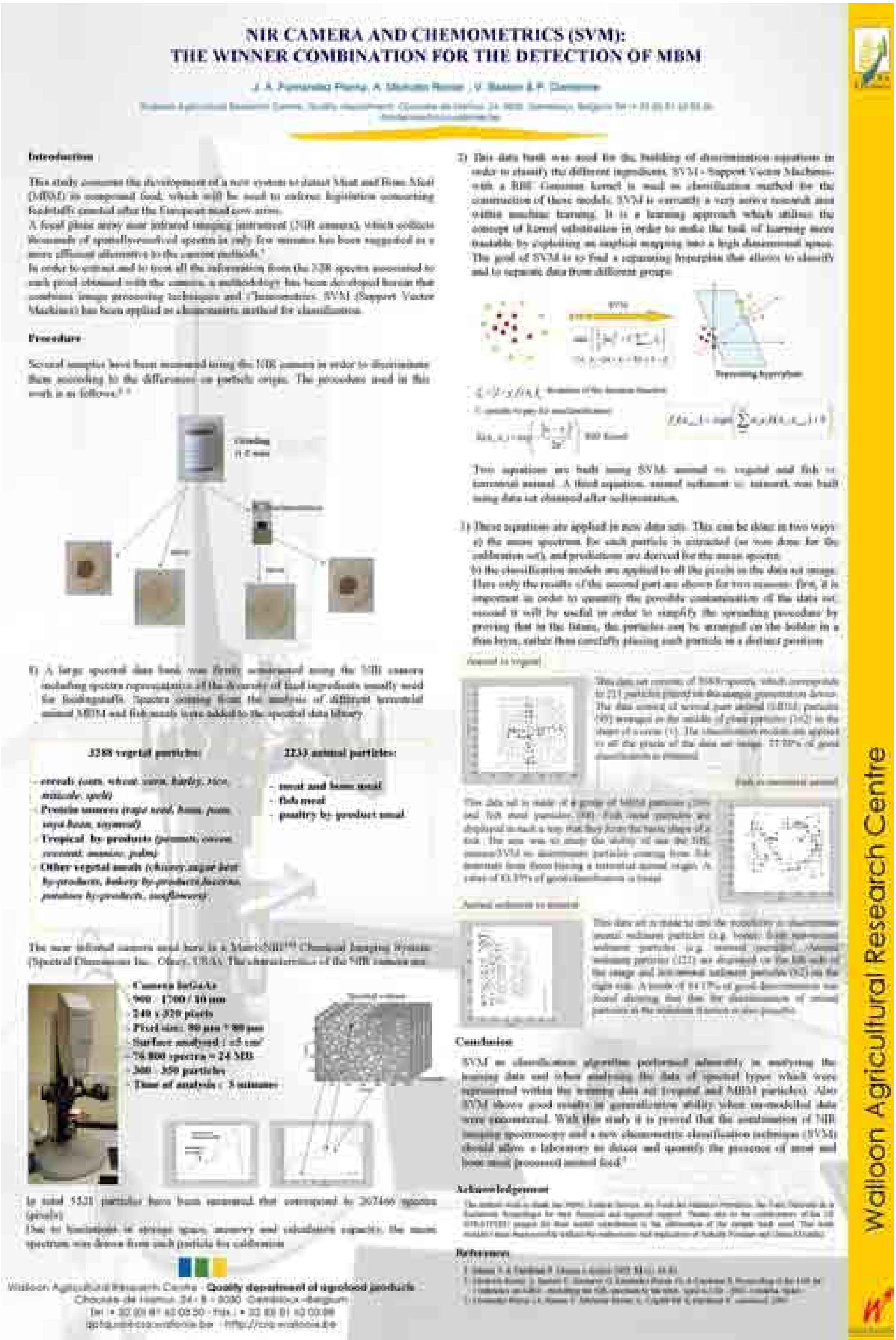
Near Infrared Spectroscopy is demonstrating considerable potential for screening of compound feedingstuffs in order to detect the presence/absence of meat and bone meal. Nowadays NIRS applications on feeds ingredients and compound feedingstuffs are going beyond the limits of the theory of diffuse reflectance, of agro-food products, which claims the need for a fine milling particularly for so heterogeneous matrices as compound feedingstuffs. Several papers have demonstrated the viability of the NIR analysis of unground commercial compound feedingstuffs for ingredients identification and quantification. However, robustness of the predictive models may be evaluated using linear and non-linear algorithms. Support Vector Machines are learning machines that can perform binary classification (pattern recognition) and regression estimation and are being applied with significant success in numerous real-world learning applications. The aim of the present work is the development of NIRS chemometric models using a Support Vector Machine (SVM) for detecting the presence of animal origin meals in compound feedingstuffs.

In this study were considering two populations: population A containing 151 contaminated samples, with different proportions of meat and bone meal (MBM), fish meal (FM) and blood meal (BM), and population B with 151 non-contaminated. The two populations were scanned in unground form using a FOSS NIRSystems 6500. The log 1/R from 400 to 2500 nm, every 2nm, have been used as input for developing a classification method induced with a SVM : a state-of-the-art technique of artificial intelligence specially devised to obtain accuracy classifiers in data sets whose objects are described by a large set of attributes (absorbances). Prior to start with the development of the NIRS chemometrics models it was needed to normalize all the spectrum data.
Using the training samples labeled either "yes" (containing MBM, FM or BM) or "no" (free of animal meals), the algorithms are able to learn a maximum-margin hyperplane which separates the region of positive and negative samples. We used Joachims' SVM^{light} with a linear kernel, thus the resulting classification procedure consists in checking the sign of an inner product, and its implementation is straightforward in any computer platform. In all the experiments reported, we used a cross validation procedure with 10 groups. In our experiment, 1050 data points were used as inputs to describe each sample belonging to the training set. In that case, the classification accuracy achieved was 80% when we stressed algorithm to reduce as much as possible the proportion of false negatives that was only of 1.08%. It is necessary to remark that the most relevant wavelengths range in order to classify the samples is those above 2050 nm. In fact, it is possible to learn a decision procedure with only 9 attributes without penalizing the classification scores. Thus, in this case, the global accuracy is 79.14% while the proportion of false negatives is just 1.11%.

Keywords

Compound feedingstuffs, NIR, animal origin meal, classification algorithms.


NIR CAMERA AND CHEMOMETRICS (SVM):
THE WINNER COMBINATION FOR THE DETECTION OF MBM
Introduction
Procedure
Conclusion
Walloon Agricultural Research Centre

P.39.- IR camera and Chemometrics (SVM): the winner combination for the detection of MBM

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This study concerns the development of a new system to detect Meat and Bone Meal (MBM) in compound feed, which will be used to enforce legislation concerning feedstuffs enacted after the European mad cow crisis. Focal plane array near infrared imaging spectroscopy (NIR camera), which collects thousands of spatially-resolved spectra in a massively-parallel fashion, has been suggested as a more efficient alternative to the current methods, that are tedious and require significant expert human analysis. This camera allows to acquire in few minutes 76800 NIR spectra corresponding to the information of about 400-600 particles. The instrument includes a camera coupled to a wavelength selector (Liquid Crystal Filters) that allows the reflected energy for the wavelengths to be passed through. The activity range of the camera is 900-1700 nm. For each of these wavelengths one image of the analysed region is recorded. Then, the spectrum for each pixel can be reconstructed by joining together the absorbance at each wavelength. The spectra for all pixel constitutes the spectral volume.

In order to extract and to treat all the useful information from the spectra associated to each pixel, a methodology has been developed herein that combines image processing techniques and Chemometrics. SVM (Support Vector Machines) has been applied as new Chemometric method for classification. Several samples have been measured using the NIR camera in order to discriminate them according to the differences on particle origin. The procedure used in this work is as follows: 1) A large spectral data bank was firstly constructed using the NIR camera including spectra representative of the diversity of feed ingredients usually used for feedingstuffs. Spectra coming from the analysis of different terrestrial animal MBM and fish meals were added to the spectral data library. 2) This data bank was used in order to build the discrimination equations using SVM in order to classify the different ingredients. 3) These equations are applied in new data sets.

SVM as classification algorithm performed admirably in analysing the training data and when analysing the data of spectral types which were represented within the training data set (vegetal and MBM particles). Also SVM shows good results in generalisation ability when un-modelled data were encountered. With this study it was proved that the combination of NIR imaging spectroscopy and a non-linear chemometric classification technique (SVM) should allow a laboratory to detect and quantify the presence of meat and bone meal in processed animal feed.

Keywords

Meat and Bone Meal, NIR camera, Chemometrics, Support Vector Machines

Stratfeed

In-house validation of the near infrared microscopy (NIRM) technique for the detection of animal meal in feedingstuffs.

Vincent Baeten[1], Antonio Michotte Renier[1], Christoph von Holst[2], Isabelle Fissiaux[2], Ian Murray[3] & Pierre Dardenne[1]

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NIRM analysis of the raw and sediment fractions

Since 1998, near infrared microscopy (NIRM) has been proposed by the Walloon Agricultural Research Centre (CRA-W) as an attractive alternative method for the detection and the quantification of meat and bone meal (MBM). Spectra library including more 20 000 spectra of particles coming from allowed and forbidden (MBM, Poultry meal, Fish meal and diverse animal by-products) feed ingredients has been constructed. The NIRM analyses can be done on the raw and sediment fractions of the samples (Figure 1).

Table 1 presents the classification results obtained for the particles used in the training stage (=construction of the discriminant models) of the study. The number of particles, the classification percentage for the different groups, the percentage of the particles not classified i.e. outside the specified limits of one of the equations and the percentage of the particles correctly classified (i.e. number of the particles classified in the correct group over the total number of the particles classified in one of the groups) are included in Table 1.

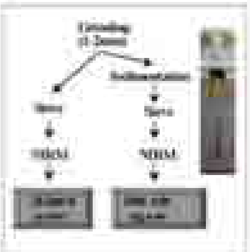

Figure 1. Schema presenting the NIRM protocol.

PARTICLE ORIGIN						
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 1. Classification results (round figures) of the particles spectra from the training stage.

Test of the protocol on pure animal meals and compound feeds

Table 2 presents the results of the NIRM analysis of the raw fraction of 21 samples feed contaminated with animal meal at different concentrations and pure animal meal. The analysed samples, the animal particles detected percentage, an evaluation of the presence of each species of particle and the number of particles from the raw fraction analysed by NIRM are included in the table. Between 28 and 900 particles of each sample have been analysed. The samples were analysed in blind. The discrimination of spectra was correct. Indeed, pure animal meal spectra were correctly classified. Contaminated feed were also correctly detected. Concerning the discrimination of the different animal species, the unexpected contaminations were in accordance with the results obtained with the optical microscopy and PCR methods.

Table 2. Results of the NIRM analysis of the raw fraction of 21 samples contaminated with animal meal at different concentrations and pure animal meal.

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Detection of MBM in fishmeals

Table 3 summarises the results of the NIRM analyses of the 20 samples. All the spiked samples have been detected as positive. A correlation of 88.6 % between the theoretical percentage and the percentage calculated by NIRM have been observed. These results show that the NIRM method can be used at least as semi-quantitative method. Two about ten of the free MBM samples have been detected as spiked at low level with MBM. One and three particles have been respectively classified as having a terrestrial origin for the samples F4 and F11. Both samples have been analysed by PCR that indicated the presence of trace of terrestrial animal DNA.

Table 3. Results of the NIRM analysis of the raw fraction of 20 fish meal.

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Comparison with classical microscopy and transfer of the method

The correlation between the weight of bones in the sediment obtained with the optical microscopy and NIRM method was higher than 0.85 %. Moreover, the NIRM method developped at CRA-W has been transfered with success to the JRC with an level of false negative inferior to 5 %.

Acknowledgement

Walloon Agricultural Research Centre - Quality department of agrofood products
Chaussée de Namur, 24 - B - 5030 Gembloux - Belgium

Walloon Agricultural Research Centre

P.40.-In-house validation of the near infrared microscopy (NIRM) technique for the detection of animal meal in feedingstuffs

Vincent Baeten[1], Antoine Michotte Renier[1], Christoph von Holst[2], Isabelle Fissiaux[1], Ian Murray[3] & Pierre Dardenne[1]

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Since 1998, near infrared microscopy (NIRM) has been proposed by the Walloon Agricultural Research Centre (CRA-W) as an attractive alternative method for the detection and the quantification of meat and bone meal (MBM)[1,2]. A spectral library including more than 20 000 spectra of particles coming from allowed and forbidden (MBM, Poultry meal, Fish meal and various animal by-products) feed ingredients has been constructed. The samples were well characterised and have been analysed by the reference method (i.e. classical microscopy) and alternative methods (e.g. PCR) in order to check their composition and labelling. This spectral library is being used to construct discriminant equations to detect the presence of animal ingredients in feed ingredients and compound feeds.

In the framework of the STRATFEED project a complete NIRM protocol is being developed and validated to analyse the raw and the sediment fractions of the feedingstuffs[3,4]. The mathematical models generated were tested using several sets of samples including material coming from a wide range of origin. The first set included 21 samples of pure animal meals and compound feeds coming from the industry. The second set included a total of 24 blank (i;e. without MBM) compound feeds and 48 spiked samples adulterated at level ranging from 0.5 to 8 %. The third set was made of pure and spiked fishmeal samples. The analysis of the three sets demonstrated the powerful of the **NIRM method to detect the presence of animal meal in the raw material and in the sediment fraction** as well as the possibility to detect MBM in fishmeal.

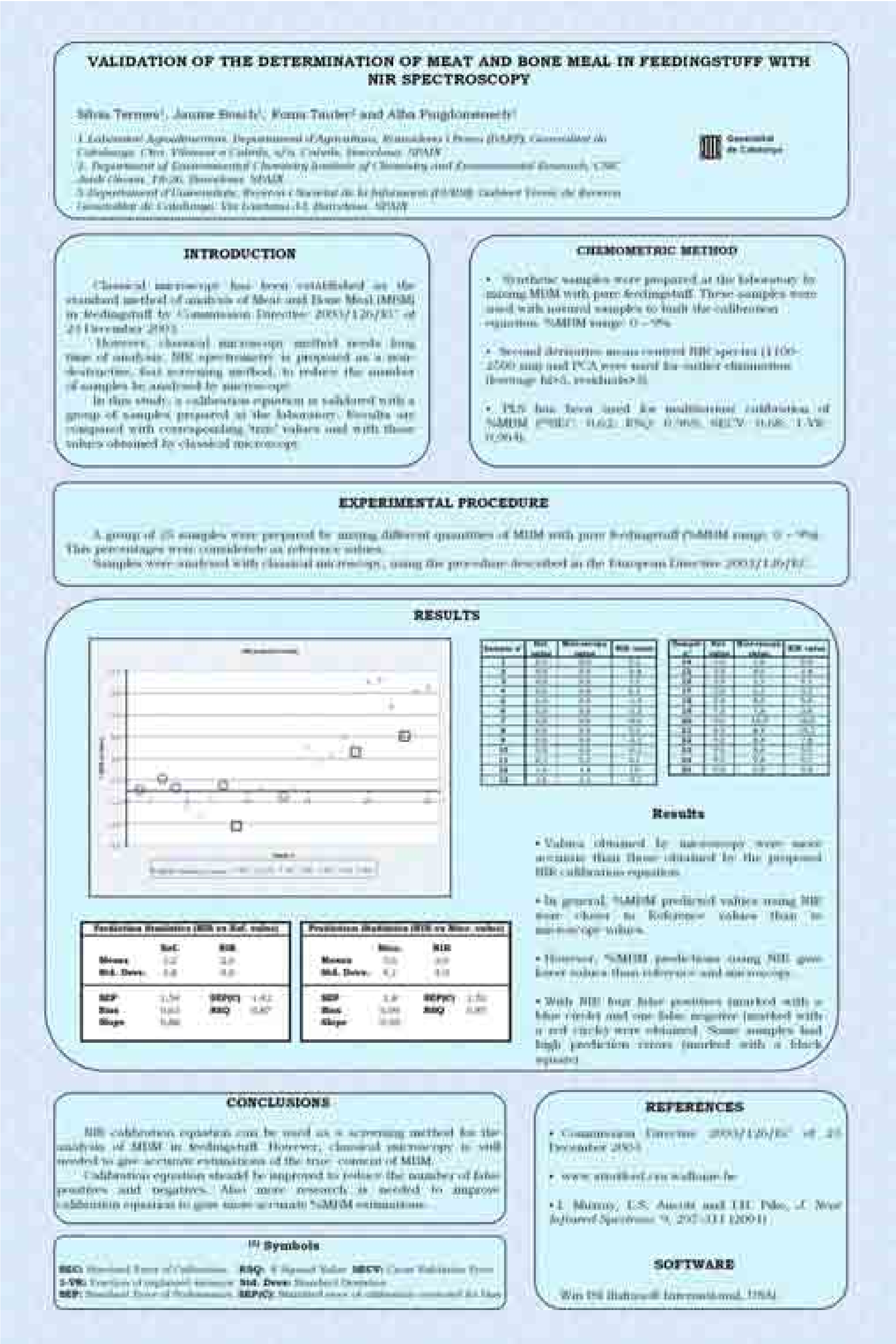
The results of analyses of series of MBM free and spiked samples by NIRM and classical microscopy demonstrate that there is no difference between the outcome of both techniques. Moreover, the method has been successfully transferred to the Joint Research Centre (JRC) and was successfully applied to the homogeneity study of test material that was used in an intercomparison study for the detection of MBM in feed[5].

References

[1]Piraux F. & Dardenne P. (1999). - Feed authentication by near-infrared microscopy. In Proc. of the 9th International Conference on Near-Infrared Spectroscopy (Davies A.M.C. and Giangiacomo R. eds), Verona, Italy, June 1999.
[2]Baeten V., Michotte Renier A., Sinnaeve G. & Dardenne, P. (2001). - Analyse of feedingstuffs by near-infrared microscopy (NIRM): detection and quantification of meat and bone meal (MBM). In Proc. of the sixth International symposium on food authenticity and safety, 28–30 November 2000, Nantes, p. 1-11.
[3]Baeten V. & Dardenne P. (2001). - The contribution of near infrared spectroscopy to the fight against the mad cow epidemic. NIRS news, 12 (6), 12-13.
[4]Baeten V., Michotte Renier A., Sinnaeve G., Garrido Varo A. and Dardenne P. (2004). Analysis of the sediment fraction of feed by Near-Infrared Microscopy (NIRM). In Proc. of the 11th International Conference on Near-Infrared Spectroscopy (Garrido Varo A.), Cordoba, Spain, 6-11 April 2003.
[5] Gizzi G., Baeten V. , Berben G. , van Raamsdonk L. and von Holst C. Intercomparison study for the determination of processed animal proteins including meat and bone meal in animal feed, J AOAC International in print of processed animal proteins including meat and bone meal in animal feed, J AOAC International in print[5]

Keywords

Spectroscopy, microscopy, MBM, fish meal, feedingstuffs, validation


VALIDATION OF THE DETERMINATION OF MEAT AND BONE MEAL IN FEEDINGSTUFF WITH NIR SPECTROSCOPY
INTRODUCTION
CHEMOMETRIC METHOD
EXPERIMENTAL PROCEDURE
RESULTS
Results
CONCLUSIONS
Symbols
REFERENCES
SOFTWARE

P.41.- Validation of the determination of meat and bone meal in feedingstuff with NIR spectroscopy

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Determination of Meat and Bone Meal in feedingstufs is an important topic since their presence has been related with mad cow decease. Several Regulations and Commission Directives have been adapted to avoid the presence of Meat and Bone Meal in feedingstufs and to establish standard methods of analysis. In 2003 the Commission Directive 2003/126/EC of 23 December 2003 established classical microscopy as the preferred method of analysis.

The main inconvenience of microscopy method is that it needs long time of analysis. In this context, NIR spectrometry has been recently proposed as a fast screening method, to reduce analysis time and to reduce the number of samples to be analysed by microscopy. NIR equations developed in the calibration step must be validated before they can be used in routine analysis of feedingstuff. In this work, NIR equations are validated with a group of samples prepared in the laboratory. Values obtained using the proposed NIR spectrometric methods are compared and validated with known 'true' values and with those obtained using microscopy.

Keywords

Meat and Bone Meal, Feedingstuff, NIR Spectrometry, Microscopy, Validation

The use of AGID technique to identify the specie purity in Spray Dried Porcine Plasma

J. Polo, N. Saborido, J. Ródenas and C. Rodríguez

Introduction:

Objectives:

Material and Methods:

Material

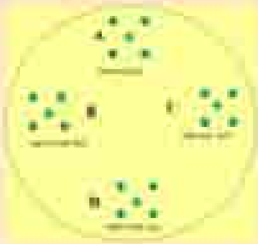
Reagents:

1. Samples:

2. Agar

Method

Interpretation



Results and Discussion:

Bovine inclusion level (% v/v)	Liquid plasma Before control	Liquid plasma After control	SD Plasma Before control	SD Plasma After control
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Conclusions:

Bibliography:

P.42.- The use of AGID technique to identify the specie purity in Spray Dried Porcine Plasma

J. Polo[1]., N. Saborido[1]., J. Ródenas[2]., C. Rodríguez[3]

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There is currently urgent interest in identifying the species of origin of the components of different animal by-products. In Europe, this interest is expected to increase with authorization of the re-introduction of these proteins into animal feed formulations. The number of validated methods to differentiate the species of origin for most of these products is limited. The objective of this study was to develop an easy method, inexpensive, reliable and accurate to detect the presence of bovine protein (IgG) in porcine plasma before and after spray-drying using the Agar Gel Immunodiffusion (AGID) technique.

Bovine blood and porcine blood were directly obtained from a jugular venipuncture of animals and placed in a container with 0.4% (w/v) sodium tri-poly-phosphate as anticoagulant. In the lab, the obtained bovine blood was mixed using calibrated pipettes with porcine blood at different inclusion levels, from 0 to 1% (v/v). These mixed samples were centrifuged to separate the cellular fraction. The liquid plasma obtained was analyzed before and after spray-drying using a lab spray-drier (Buchi 190 Mini Spray Drier) under the same technical conditions used at industrial level. Alternatively, contaminated plasma samples were obtained after the centrifugation of bovine and porcine blood. Pure bovine plasma was mixed with pure porcine plasma with the help of calibrated pipettes at different levels of inclusion, from 0 to 1% (v/v)

The liquid plasma samples were used directly without dilution. The spray dried plasma were diluted to obtain a 7% (w/v) of protein concentration (similar to liquid plasma) in PBS pH 7.2 buffer. Antibodies used against proteins present in the samples**:** anti-IgG bovine (B-8395 Sigma) and anti-IgG porcine (P-0916 Sigma). Positive and negative standards**:** pure bovine plasma (B-8392 Sigma) and pure porcine plasma (P-2891 Sigma). Detailed description of the AGID technique used can be found elsewhere (Polo et al., 2004. JAOAC). The presence of precipitating proteins indicate specific immune-reaction between the antibody and some proteins in the samples.

The technique developed was able to detect the presence of bovine IgG in porcine plasma at inclusion levels above 0.5% v/v in all cases. No differences were found when cross contamination was simulated before or after whole blood centrifugation.

The method described is reliable and inexpensive, the samples for the analyses are easy to prepare, and they require both minimal laboratory equipment and expertise to detect bovine IgG in porcine blood products at inclusion levels of > 0.5% v/v.

Keywords

Species identification, spray dried porcine plasma, blood products, AGID, bovine IgG identification.

Validation study for the detection limit of Enzyme-Linked ImmunoSorbent Assay (ELISA) with heated meat and bone meal as an effective prophylactic method for Bovine Spongiform Encephalopathy (BSE)

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Introduction

It has been widely accepted that the causative agent of Bovine Spongiform Encephalopathy (BSE) is inactivated when the animal materials are treated at 133? with 3 bar under wet sterilization condition for 20 minutes (Anon, 1997). A commercially available ELISA test kit for cooked species identification test kit (Tepnel Co.) and for meat, bone meals and animal feeds (MELISA-TEK, ELISA-TEK) has been used and compared in the current study. Here we report immuno sorbent assay that allows to detect the presence of meat and bone meal (MBM) in a feed at a concentration of about 5% and 1%.

Materials and methods

The whole experiments were carried out according to the method described by the manufacturer for the procedure of the enzyme linked immunosorbent assay (ELISA).
ELISA kit : Tepnel, ELISA-Tek, MELISA-Tek

Results

R-value for the formulated feed containing 1% of heat-treated Korean native cattle meat meal was 1.28 indicating low detection capacity whereas it was 2.69 and positive with 1% of heat-treated dairy cattle meat meal.

R-value for the formulated feed containing 1% of heat-treated pork meat meal was 1.42~1.57 indicating quite low detection level and it was 2.15~2.28 with positive test result with 1% of heat-treated poultry meat meal using ELISA.

When land animal meat was heat-treated without pressure and tested for the contamination using commercially available ELISA kits in beef, pork and poultry was 15.7, 6.84 and 12.95, respectively with A kit and 10.27, 16.51 and 8.22 with B kit, respectively. It appears that detection limit of the assay using known concentration of meat and bone meal for beef, pork and poultry was 0.5, 0.1 and 0.1%, respectively.

Table 1. Results obtained from known amount of samples of Korean Native Cattle Meat Meal treated under the different sterilization condition

Heat treatment Conditions	Additive level of meat meal (R-value) *					
	5.00%			1.0%		
	O. D.	Result #	R-value	O. D.	Result	R-value
20min, T<128? , 2.6bar, Wet sterilization	0.267	P	5.24	0.089	S	1.76
20min, T<135? , 3bar, Wet sterilization	0.257	P	5.04	0.066	S	1.28
20min, T<140? , Dry sterilization	0.384	P	7.53	0.109	S	2.14

* R-value is the optical absorbance of the sample/optical absorbance of negative control
# P : Positive, , S : suspicious

Table 2. Results obtained from known amount of samples of Dairy Cattle Meat Meal treated under the different sterilization condition.

Heat treatment Conditions	Results with additive level of meat meal (R-value) *					
	5.00%			1.0%		
	O.D	Result #	R-value	O.D.	Result	R-value
20min, T<128? , 2.6~2.8bar, Wet sterilization	0.322	P	6.32	0.253	P	4.97
20min, T<135? , 3bar, Wet sterilization	0.145	P	2.88	0.138	P	2.69
20min, T<140? , Dry sterilization	0.699	P	13.72	0.601	P	11.80

* R-value is the optical absorbance of the sample/optical absorbance of negative control
# P: Positive

Table 3. Results obtained from known amount of samples of Pork Meat Meal treated under the different sterilization condition.

Heat treatment Conditions	Results with additive level of meat meal (R-value)					
	5.00%			1.0%		
	O.D.	Result #	R-value	O.D.	Result	R-value
20min, T<128? , 2.6~2.8bar, Wet sterilization	0.115	P	2.03	0.081	N	1.42
20min, T<135? , 3bar, Wet sterilization	0.102	S	1.80	0.089	N	1.57
20min, T<140? , Dry sterilization,	0.134	P	2.36	0.103	S	1.81

* R-value is the optical absorbance of the sample/optical absorbance of negative control.
# P: Positive, N: negative, S: suspicious.

Table 4 Results obtained from known amount of samples of Poultry Meat Meal treated under the different sterilization condition.

Heat treatment Conditions	Results with additive level of meat meal (R-value)					
	5.00%			1.0%		
	O.D.	Result #	R-value	O.D.	Result	R-value
20min, T<128? , 2.6~2.8bar, Wet sterilization	0.223	P	2.59	0.185	S	2.15
20min, T<135? , 3bar, Wet sterilization	0.225	S	2.62	0.190	S	2.22
20min, T<140? , Dry sterilization,	0.250	P	2.90	0.195	S	2.28

Table 5. Results obtained from known amount of samples of Land Animal Meat Meal treated under the different sterilization condition

Heat treatment Conditions	Results with additive level of meat meal (R-value)					
	Beef		Pork		Poultry	
	A	B	A	B	A	B
Raw meats	18.41	13.15	-	-	18.21	-
20min,T<127? , no pressure Dry sterilization	14.80	18.83	8.05	17.70	12.56	12.02
20min,T<135? , no pressure Dry sterilization	15.71	10.27	6.84	16.51	1 2.95	8.22

* R-value : OD sample/OD negative control
# A : according to the ELISA kit manufacturer, B : according to the TEPNEL kit manufacturer

Table 6. Results obtained from known amount of samples of Land Animal Meat Meal treated under the different sterilization condition *.

Heat treatment Conditions	Results with additive level of meat meal (R-value)#		
	Beef	Pork	Poultry
MBM, T<135? , 100%+	2.63	-	-
MBM, T<135? , 50%	2.08	-	-
MBM, T<135? , 10%	1.52	8.80	-
MBM, T<135? , 5%	1.68	5.65	-
MBM, T<135? , 1.0%	1.38	1.40	2.34
MBM, T<135? , 0.5%	1.52	1.44	2.09
MBM, T<135? , 0.2%	-	1.13	2.07
MBM, T<135? , 0.1%	-	1.13	2.01
MBM, T<128? , 1.0%	-	1.86	2.32
MBM, T<128? , 0.5%	-	1.37	2.18
MBM, T<128? , 0.2%	-	1.26	2.07
MBM, T<128? , 0.1%	-	1.16	1.97

* Enzyme linked immunosorbent assay was used (TEPNEL kit).
# R-value; OD sample/OD negative control.
* Additive level of meat and bone meal (MBM) in compound feed-stuffs.

Conclusions

When known concentration of meat and bone meal, heat-treated and pressurized was used for formulated feed and tested for the contamination using commercially available ELISA kit detection limit appeared to be about 1%. However when meat and bone meal, heat-treated without pressure, was added to formulated-feed and tested for the contamination then the limitation of detection for beef, pork and poultry was 0.5, 0.1 and 0.1%, respectively.

References

1. Commission Directive 98/88/EC of 13 November 1998 establishing 'guidelines for the microscopic identification and estimation of constituents of animal origin for the official control of feedingstuffs (O.J.L 318, 27.11.98, p. 45).
2. Council Decision 2000/766/EC of 4 December 2000 concerning certain protection measures with regard to transmissible spongiform encephalopaties and the feeding of animal protein (O. J. L 306, 7.12.2000, p. 32).
3. Commission Directive 76/371/EEC of 1 March 1976 establishing Community method of sampling for the official control of feedingstuffs (O.J. No L102, p. 1, 15.04.1976).

P.43.- Validation study for the detection limit of Enzyme-Linked ImmunoSorbent Assay (ELISA) with heated meat and bone meal as an effective prophylactic method for Bovine Spongiform Encephalopathy (BSE)

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It has been widely accepted that the causative agent of Bovine Spongiform Encephalopathy (BSE) is inactivated when the animal materials are treated at 133° with 3 bar under wet sterilization condition for 20 minutes (Anon, 1997). A commercially available ELISA test kit for the identification of cooked animal species (Tepnel Co.) and for meat, bone meals and animal feeds (MELISA-TEK, ELISA-TEK) has been used and compared in the current study. R-value for the formulated feed containing 1% of heat-treated Korean native cattle meat meal was 1.28 indicating low detection capacity whereas it was 2.69 and positive with 1% of heat-treated dairy cattle meat meal. R-value for the formulated feed containing 1% of heat-treated pork meat meal was 1.42~1.57 indicating quite low detection level and it was 2.15~2.28 with positive test result with 1% of heat-treated poultry meat meal using ELISA. When land animal meat was heat-treated without pressure and tested for the contamination using commercially available ELISA kits in beef, pork and poultry was 15.7, 6.84 and 12.95, respectively with A kit and 10.27, 16.51 and 8.22 with B kit, respectively. It appears that detection limit of the assay using known concentration of meat and bone meal for beef, pork and poultry was 0.5, 0.1 and 0.1%, respectively. In conclusion, when known concentration of meat and bone meal, heat-treated and pressurized was used for formulated feed and tested for the contamination using commercially available ELISA kit detection limit appeared to be about 1%, whereas when meat and bone meal, heat-treated without pressure, was added to formulated-feed and tested for the contamination then the limitation of detection for beef, pork and poultry was 0.5, 0.1 and 0.1%, respectively.

Keywords

Enzyme-Linked ImmunoSorbent Assay, ELISA, heated meat and bone meal, Bovine Spongiform Encephalopathy, BSE, contamination, monitoring

Analysis of products of animal origin in feeds by determination of Carnosine and related dipeptides by High Performance Liquid Chromatography

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As the consequence of the increased appearence of bovine spongioforme encephalopathy the feeding of animal originated products, especially to ruminants was forbidden. The microscope examination is the only official method to prove animal originated adulterations of feeds. It's validated in collaboration studies and manifested in the European law. Unfortunately, the microscopic technique is an estimate method, is time consuming and requires experienced staff. We developed a High Performance Liquid Chromatography (HPLC) method for qualitative determination of animal originated parts in feeds. The evidence is made by the HPLC-determination of the dipeptide carnosine (β-alanyl-L-histidin) which is found in animal tissue exclusively, not in plants. Carnosine and related dipeptides (anserine, balenine) are present in in animal tissues like in heart muscle, kidney and liver but in particularly high concentration especially in muscle tissue. We show the dependence of contents of anserine, balenine and carnosine in compound feeds on content of meat meal in feeds. HPLC analyses will be favorable because it is a simple, less expensive technique and it is essential in most feed science laboratories. Compare to microscopy a much more bigger part of the sample can be investigated. The presented method can complete and confirm the result of the microscopic method for evidence parts of animal origin in feeds. Literatur: SCHÖNHERR,J. *Jour.Agric.Food Chem.* **2002**, *50*, 1945-1950

I. Method description

Extract in with distilled water

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Filtration

↓

Precipitation of proteins by sulfosalicylic acid

↓

Cleaning and concentration of the extract by PRS-SPE cartridge

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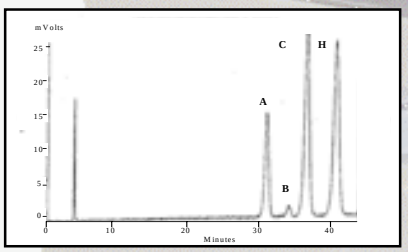
Sample derivatisation by CFC (N-Chloroformylcarazole)

↓

HPLC separation of the derivates by an RP-C18 column with fluorescence- or UV-detection

HPLC separation of standard solution *(Isocratioc elution)*



Anserin (A) 3,23 ng; Balenin (B) 0,37 ng; Carnosin (C) 6,25 ng; Histidin (H) 5 ng

Statistical data of HPLC-method

Detection limit for carnosine (*calibration curve method; calculation by area*)
gradient elution programm; 200 µl injection volume: **25 µg/kg**
isocratic elution; 20 µl injection volume: **540 µg/kg**

Repeatability of the method **3.8%** (n=8)

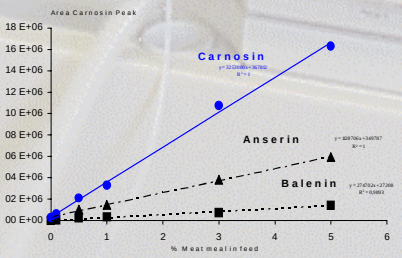
Recovery for carnosine from spiked feed (cattle, pig, poultry) **88.0% – 104.2%**

II. Dependence of parts of animal origin and carnosin content

To prove the relations between contents of anserine, balenine and **carnosine** and contents of products of animal origin 6 compound feeds (made from shredded extracted soya, wheat, barley, mineral feed and increasing parts of meat meal) were mixed. With increasing meat meal quantities the quantity of soya was decreased proportional to get the same protein content in each sample.

	Wheat (g)	Barley (g)	Soya (g)	Mineral (g)	Meat meal (g)
0-Feed	1000	560	380	60	0
0.1%-Feed	1000	560	378	60	2
0.5%-Feed	1000	560	370	60	10
1%-Feed	1000	560	360	60	20
3%-Feed	1000	560	320	60	60
5%-Feed	1000	560	280	60	100

Contents of anserine, balenine and carnosine in depence on content of meat meal in defined mixed feeds



Both the peaks of **carnosine**, **anserine** (β-alanyl-1-methylhistidine) and **balenine** (β-alanyl-3-methylhistidine) correlate very well with the content of parts of animal origin in feeds. The gradient elution HPLC method was optimized for carnosine.

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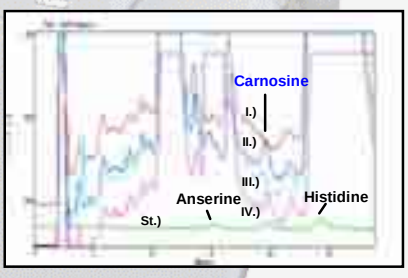
III. Carnosine in feeds

No anserine, balenine and carnosine were found in 15 samples of plant origin analysed. More than 50 compound feeds for cattle, pig and poultry were analysed for its contents of the dipeptides. If there were found parts of animal origin (also in traces) by microscopy a carnosine peak was detected by the HPLC-method in each case. The determination of carnosine in microscopic not recognizable structures such as carcass is possible.
A species determination by the ratio carnosine / anserine / balenine seems possible if only one species is the source of parts of animal origin.

Contents of anserine, balenine and carnosine in feeds of animal origin

(ppm)	n	Anserine	Balenine	**Carnosine**
Meat Meal	14	132-862	29-101	556-1857
Meat Meal ?	1	497	n.d.	9
Meat bone meal	6	88-527	26-34	651-1088
Fish meal	1	35	n.d.	6
Feather meal	1	95	n.d.	21
Blood meal	1	n.d.	n.d.	6

HPLC separation of samples of the STRATFEED Study *(Gradient elution)*



I.) Negative sample
II.) 0.1% Mammalian Meat Bone Meal
III.) 5 % Fish Meal
IV.) 5 % Fish Meal with 0.1% Mammalian Meat Bone Meal
St.) Anserine/Carnosine/Histidine Standard

P.44.- Analysis of products of animal origin in feeds by determination of Carnosine and related dipeptides by High Performance Liquid Chromatography

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As the consequence of the increased appearence of bovine spongioforme encephalopathy the feeding of animal originated products, especially to ruminants was forbidden. The microscope examination is the only official method to prove animal originated adulterations of feeds. It's validated in collaboration studies and manifested in the European law. Unfortunately, the microscopic technique is an estimate method, is time consuming and requires experienced staff. We developed a High Performance Liquid Chromatography (HPLC) method for qualitative determination of animal originated parts in feeds. The evidence is made by the HPLC-determination of the dipeptide carnosine (b-alanyl-L-histidin) which is found in animal tissue exclusively, not in plants. Carnosine and related dipeptides (anserine, balenine) are present in in animal tissues like in heart muscle, kidney and liver but in particularly high concentration especially in muscle tissue. We show the dependence of contents of anserine, balenine and carnosine in compound feeds on content of meat meal in feeds. HPLC analyses will be favorable because it is a simple, less expensive technique and it is essential in most feed science laboratories. Compare to microscopy a much more bigger part of the sample can be investigated. The presented method can complete and confirm the result of the microscopic method for evidence parts of animal origin in feeds.

Keywords

Feeds – Meat meal – High Performance Liquid Chromatography (HPLC) – Solid-phase extraction (SPE) - Dipeptides - Carnosine –Anserine-Balenine

POTENTIAL APPLICATION OF ELECTONIC NOSE IN PAP DETECTION IN FEEDSTUFFS

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Introduction

·The need to develop improved techniques to characterize feed components has become mandatory

·It is essential that new methods proposed can be widely applied in order to enforce the new legislation and limit illegal substitution and fraud

·Obviously to combine these requirements with the need for accurate, fast and objective quality determination of feed value and safety standards could be very difficult

·Electronic nose provide already a powerful modern analytical methods in food industry, addressing many quality, safety and process challenger facing manufactures

·Electronic noses consist of chemical detectors with non-specific responses to simple or complex vaporphase analyte mixtures

Aim

The aim of this study was to evaluate possible application of electronic nose for PAP detection and identification in feed

Materials and Methods

·Samples: six reference compound feeds (Agricultural Research Centre of Gembloux, Belgium, STRATFEED Project) fortified with PAP (meat and bone meal (MBM) and/or fish meal) at different concentration levels were used. Samples schedule was the follow: sample A, MBM 0.5%, sample B, MBM 0.5% + Fish 5%, sample C, Fish 5%, sample D, blank (PAP absent), sample E, MBM 0.5%, sample F MBM 0.5% + Fish meal 5%.

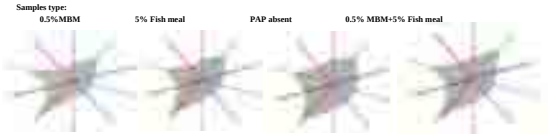
·Instrument: electronic nose pen2 (Airsense Analytics GmbH, Schwerin, Germany)

·Sample treatment: 1.2 g of each feed sample was equilibrated at 40° C temperature for 5 min in glass vials and the odour profile for each sample was determined by the 10 MOS (Metal Oxide Semi-Conductor) sensors of the electronic nose. The sampling time was 3 min and the flush time between sampling was 4 min.

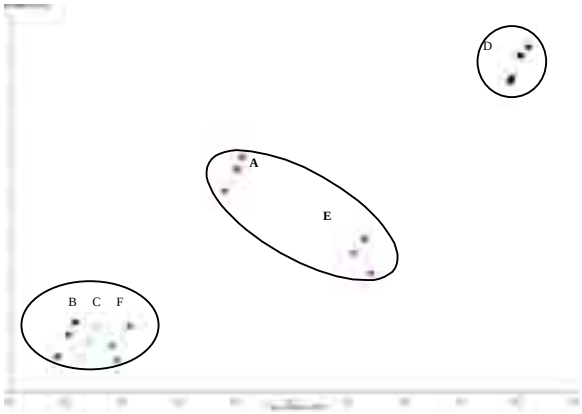
·The flow rate was 400 ml/min. Four replicates were taken for each feed sample. Ten different descriptors, representing each of the 10 sensors of electronic nose (aromatic1, broadrange, aromatic3, hydrogen, arom-aliphatic, broad-methane, sulphur-organic, broad-alcohol, sulph-chlor, methane-aliphatic), were used to characterise the odour of each sample.

·Data were analysed by PRINCOMP (Principal Component Analysis) andCLUSTER (Ward's Minimum Variance Cluster Analysis) procedures of SAS (2001).

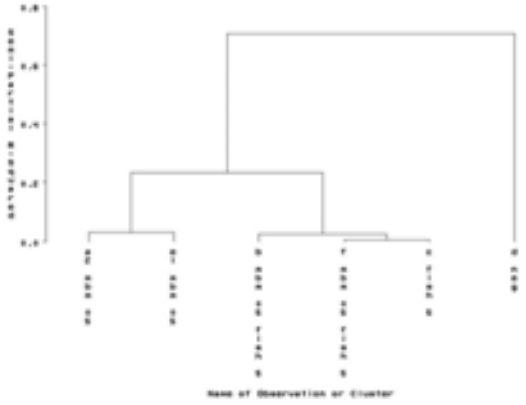
Results & Discussion



•Figure 1. Eletronic nose measurements Radar Plot (175 sec).



·Figure 2. Results of Principal Component Analysis (PCA). 1° PC (X axis): values of sulphur-org sensonsor; 2° PC (Y axis): values of broadrange sensor. Sample A, MBM 0.5%, sample B, MBM 0.5% + Fish 5%, sample C, Fish 5%, sample D, blank (PAP absent), sample E, MBM 0.5%, sample F, MBM 0.5% + Fish meal 5%.



· Figure 3. Results Ward's Minimum Variance Cluster Analysis

·Electronic nose was able to detect PAP in samples containing levels of MBM as low as 0.5%.

·Electronic nose was also able to distinguish between MBM and fish meal.

·Samples containing a combination of MBM 0.5% + Fish meal 5%, were not discriminate from samples fortified with 5% of fish meal solely.

Conclusions

·This study has demonstrated that the electronic nose is a promising analytical approach to PAP detection in feedstuffs, particularly for screening raw materials in the feed industry, although further studies on more and more varied samples are needed.

·However, further study is also necessary to determine the real potential of the technique in this field. For instance, as suggested by Feast (2001), it would be useful carry out "training" tests with representative samples in order to construct a model for the recognition of unknown samples. After training, unknown samples could be matched to the model and classified as either one of the training groups or as unknown.

Keywords: PAP determination, Electronic nose.

P.45.- Potential application of electronic nose in PAP detection in feedstuffs

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Electronic nose and olfactometry techniques have already proven to be a powerful modern analytical method in the food industry, in which they address many quality, safety and process challenger facing manufactures. The aim of this study was to evaluate possible application of electronic nose in PAP detection and recognition in feed. For this purpose 6 reference feedstuffs (Agricultural Research Centre of Gembloux, Belgium, STRATFEED Project) were used. The base of the test samples was a compound feed for bovine fortified with PAP (meat and bone meal (MBM) and/or fish meal) at different concentration. Samples schedule was the follow: containing sample A, MBM 0.5%, sample B, MBM 0.5%+ Fish 5%, sample C, Fish 5%, sample D, blank (PAP absent), sample E, MBM 0.5%, sample F MBM 0.5% + Fish meal 5%. Each feed sample was tested in glass vials and the odour profile was determined by the 10 MOS (metal oxide semi-conductor) sensors of the electronic nose (Airsense Analytics GmbH, Schwerin, Germany). Four replicates were taken for each sample. Ten different descriptors, representing each 10 sensors of electronic nose, were used to characterise the odour of each sample. Obtained data were analysed using the PRINCOMP and CLUSTER procedures of SAS (2001). In the present study electronic nose was able to discriminate the blank sample from all others samples containing PAP (MBM, fish meal or both). Samples containing the 0.5% of MBM and 5% of fish meal were identified, while samples containing higher fish meal (5%) associated with low MBM content (0.5%) were not discriminate from samples containing solely fish meal at the higher level (5%). This latter indicate that probably the high level fish meal, in samples containing both MBM and fish meal, tended to mask MBM odour. It was also evident that two odour sensors (sulphur-organic and broad-alcohol) were enough to explain 72.12% of total variability in odour pattern. In view of these results, it could be suggested that electronic nose and olfactometry techniques can provide an interesting approach for screening raw materials in feed industry, even though further studies using different and larger number of samples are needed.

Keywords

PAP determination, Electronic nose.

Technological Development for Detection of Animal Materials in Feed in Japan

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The methods applied for detection of animal materials in feed, such as microscopic examination, near infrared reflectance spectroscopy (NIRS), ELISA technique and DNA technology (PCR) are being improved time to time and there is no common techniques internationally accepted. In Japan, authorized agencies are conducting an examination of contamination of animal materials in feed by the combination of microscopic examination, PCR and ELISA. While, we have initiated a research project to develop/upgrade detection methods of animal materials in feed as for evaluation of feed safety in relation to BSE, which involves in NIRS, ELISA, PCR and DNA chip. Current outcomes are outlined in this report.

NIRS

Analytical accuracy of NIRS to detect MBM in formula feeds for swine, poultry and cattle was examined using samples containing MBM from 0.15 to 6.0 %. The results indicated that NIRS could be used to distinguish MBM material included in formula feed within 0.5% level (Fig. 1). It was also shown (Fig. 2) that MBM included in fishmeal could be clearly distinguished (Amari et al. 2003).

PCR

The PCR primers for the detection of materials derived from ruminants, pigs and chickens were newly designed on the basis of sequences of the Art2 short interspersed repetitive element (SINE), PRE-1 SINE, and CR1 long interspersed repetitive element, respectively. With the primers, detection of Art2, PRE-1, or CR1 in test feed at concentrations of 0.01% MBM or less was possible (Fig. 3). This method was suitable for the detection of micro-contamination of feed by animal materials (Tajima et al., 2002).

The PCR method using primers for mitochondrial DNA (mtDNA, Kusama et al., 2004), which is applied for practical examination by authorized agencies in Japan, was modified by adding an isolation procedure of bone particles (Toyoda et al., 2004). Bone particles were isolated from feed containing bovine MBM and milk products as precipitates in chloroform solution, treated with sodium hypochlorite solution and EDTA/proteinase K solution, and then subjected to PCR examination. As bovine DNA derived from milk products can be eliminated by this procedure, this method is suitable for the selective detection of MBM in feed (Fig. 4 and Fig. 5). It allowed detection of the presence of bovine mtDNA in feed containing 0.1% of bovine MBM. When the treatment with sodium hypochlorite was excluded, the detection limit was improved up to 0.0001% at the expense of specificity.

ELISA

Sandwitch ELISA system, constructed by the combination of rabbit polyclonal antibody with mouse monoclonal antibody detactable cattle-origin antigen, showed very high reaction to cattle-origin antigen with low reaction to sheep- and goat-origin antigen and without any reaction to swine- and poultry-origin antigen (Hosokowa-Kanai and Tuchiya, unpublished). It would be a promising finding to develop new commercial ELISA system and related research is now underway (Fig. 6).

The achievements of these technological developments will permit to increase the safety of food supply and consequently to recover from the drop of consumer confidence related to BSE incidence. The present study was conducted under the framework of "Research project for utilizing advanced technologies in agriculture, forestry and fisheries", Ministry of Agriculture, Forestry and Fisheries, Japan.

References

Amari, M. et al., 2003, Proc. 11th Int. Conf. on NIRS 5, 116.

Kusama T. et al., 2004, J. Food Prot. vol. 67 (in press).

Tajima, K. et al., 2002, Biosci. Biotechnol. Biochem. 66, 2247-2250.

Toyoda, A. et al., 2004, J. Food Prot. (in press).

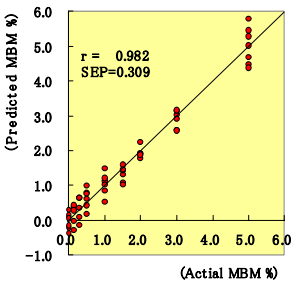

Fig.1 Actual MBM contents vs predicted contents in formula feed by NIRS

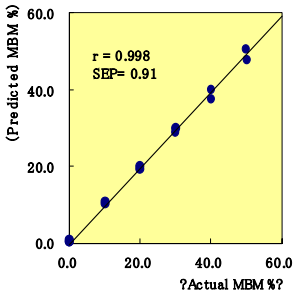

Fig.2 Actual MBM contents vs predicted contents in fish-meal by NIRS

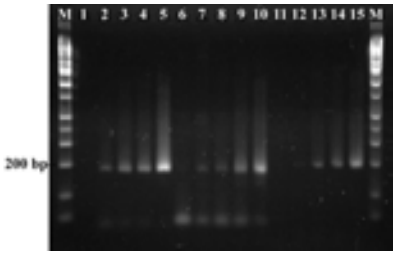

Fig.3. Detection of animal DNAs in test feed
Lane M, DNA size markers. Lanes 1 to 5, PCR products amplified total DNA from the test feed containing zero, 0.001, 0.01, 0.1 and 1.0% MBM with Art2 primer. Lanes 6 to 10, PCR products amplified with PRE-1 primer. Lanes 11 to 15, PCR products amplified with CR1 primer.

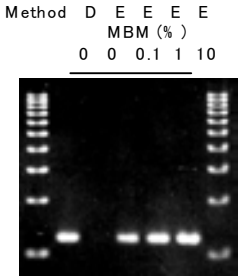

Fig. 4. PCR amplification of bovine mtDNA from compound feed containing bovine MBM and milk replacer. The test feeds were the mixture consisting of 20 % milk replacer and 80% compound feed. D: Direct and conventional extraction method. E: Our extraction method. Lane M: DNA size markers.

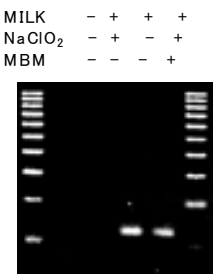

Fig. 5. Effect of the treatment with sodium hypochlorite on detection of bovine mtDNA in compound feed. MILK: milk replacer. $NaClO_2$: sodium-hypochlorite treatment. MBM: 10% MBM. Lane M: DNA size markers.

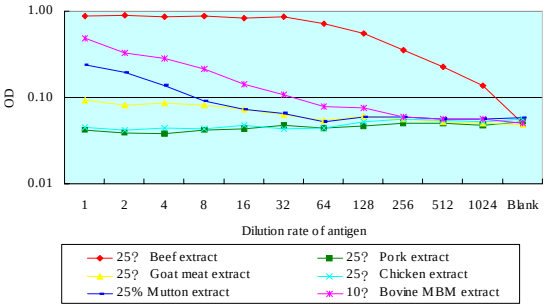

Fig. 6. Development of ELISA System to detect cattle-specific antigen

P.46.- Technological Development for Detection of Animal Materials in Feed in Japan

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We have initiated a research project to develop/upgrade a detection method of animal materials in feed towards an eradication of BSE, which involves in NIRS, ELISA, PCR and DNA chip, while authorized agencies in Japan are conducting examination of contamination of animal materials by the combination of microscopic examination, PCR and ELISA. NIRS: Analytical accuracy of NIRS to detect MBM in formula feeds for swine, poultry and cattle was examined using samples containing MBM from 0.15 to 6.0 %. The results indicated that NIRS could be used to distinguish MBM material included in formula feed within 0.5% level. It was also shown that MBM included in fishmeal could be clearly distinguished (Amari et al. 2003). PCR: The PCR primers for the detection of materials derived from ruminants, pigs and chickens were newly designed on the basis of sequences of the Art2 short interspersed repetitive element (SINE), PRE-1 SINE, and CR1 long interspersed repetitive element, respectively. With the primers, detection of Art2, PRE-1, or CR1 in test feed at concentrations of 0.01% MBM or less was possible. This method was suitable for the detection of micro-contamination of feed by animal materials (Tajima et al., 2002). The PCR method using primers for mitochondrial DNA (mtDNA, Kusama et al., 2004), which is applied for practical examination by authorized agencies in Japan, was modified by adding an isolation procedure of bone particles (Toyoda et al., 2004). Bone particles were isolated from feed containing bovine MBM and milk products as precipitates in chloroform solution, treated with sodium hypochlorite solution and EDTA/proteinase K solution, and then subjected to PCR examination. As bovine DNA derived from milk products can be eliminated by this procedure, this method is suitable for the selective detection of MBM in feed. It allowed detection of the presence of bovine mtDNA in feed containing 0.1% of bovine MBM. When the treatment with sodium hypochlorite was excluded, the detection limit was improved up to 0.0001% at the expense of specificity. ELISA: Sandwitch ELISA system, constructed by the combination of rabbit polyclonal antibody with mouse monoclonal antibody detectable cattle-origin antigen, showed very high reaction to cattle-origin antigen with low reaction to sheep- and goat-origin antigen and without any reaction to swine- and poultry- origin antigen. References: Amari, M. et al., 2003, Proc. 11th Int. Conf. on Near Infrared Spectroscopy 5, 116; Kusama T. et al., 2004, J. Food Prot. vol. 67 (in press); Tajima, K. et al., 2002, Biosci. Biotechnol. Biochem. 66, 2247-2250; Toyoda, A. et al., 2004, J. Food Prot. (in press).

Keywords

NIRS, PCR, ELISA, meat and bone meal, BSE

Determination of animal constituents in feeding stuff: Evaluation of diverse methods and their results

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ABSTRACT

Two methods for the determination of animal ingredients in feeding stuff - classical microscopy and PCR-analysis - were compared in the following study. Aims of the investigations were (i) to determine the capacity and the limits of both methods and (ii) to determine the optimum area of application for each method. For these purposes mixed feed samples were amended with different artificially prepared animal meals and investigated with both methods.

In summary of the results obtained with the artificially prepared animal meals in the mixed feed samples both methods complemented each other.

The different targets that are determined as animal specific characteristics with each method have to be taken into consideration for the choice of the most suitable determination method for animal ingredients in feeding stuff as well as for the evaluation of the results obtained from these investigations in highly processed feed matrizes. While microscopy is a very effective method for the analysis of animal meat and bone meal in feeding stuff it can be useful to combine microscopy and PCR-analysis for special application purposes.

Methods

Microscopical determinations were made according to the EU guideline 98/88/EG that was provided by the IAG feedstuff analysis section feedstuff microscopy.

PCR-analyses were made according to the VDLUFA-method "Molecularbiological determination of animal ingredients - PCR-method". This method consists of different modules. DNA-sequences from the animal specific mitochondrial cytochrome-b-gene were used as universal targets for animal detection, while determinations of animal species were made by RFLP-analyses of the amplified cytochrome-b gene sequences and with species-specific primer pairs.

Tab. 1: Detailed results of microscopic analysis (A) and PCR-analysis (B) of feed samples contaminated with different artificially prepared animal meals

sample No.	animal species	animal content [%]	animal components	microscopical determination of animal ingredients [%] A: qualitative	A: quantitative (details): (determinated from sediment fraction =bone fragments)	A: quantitative (details): (determinated from water fraction =muscle fibers)	A: RESULTS animal ingredients [%]	PCR-analysis B: animal ingredients	PCR-analysis B: animal species
1	animal A	0	-	no animal ingredients	-	-	no animal ingredients	no animal ingredients	--
2	animal A	0,1	intestines	no animal ingredients	-	-	no animal ingredients	animal ingredients	n.d.
3	animal A	0,5	intestines	no animal ingredients	-	-	no animal ingredients	animal ingredients	cattle
4	animal A	2,0	intestines	animal ingredients	<0,1%	-	<0,1% traces of animal	animal ingredients	n.d.
5	animal B	0,1	bone fragments, muscle fibers	warm blooded animal	0,1-0,3%	0,1-0,2%	0,1 - 0,3% warm blooded animal	animal ingredients	n.d.
6	animal B	0,5	bone fragments, muscle fibers	warm blooded animal	0,9-2,9%	1,0-1,5%	1,0 - 1,5% warm blooded animal	animal ingredients	chicken
7	animal C	0,1	intestines	no animal ingredients	-	-	no animal ingredients	animal ingredients	n.d.
8	animal C	0,5	intestines	no animal ingredients	-	-	no animal ingredients	animal ingredients	pig
9	animal D	0,1	cartilage, muscle fibers	animal	-	0,1-0,3%	0,1 - 0,3% animal	animal ingredients	n.d.
10	animal D	0,5	cartilage, muscle fibers	animal	-	0,7-1,3%	0,7 - 1,3% animal	animal ingredients	n.d.
11	animal E	0,1	bone fragments, muscle fibers	fish	< 0,1%	ca. 0,1%	0,05 - 0,15% fish	no animal ingredients	n.d.
12	animal E	0,5	bone fragments, muscle fibers	fish	0,4-1,0%	0,3-0,5%	0,3 - 0,7% fish	no animal ingredients	n.d.
13	mixture of all	0,5	bone fragments, muscle fibers, intestines	warm blooded animal and fish	0,2-1,4%	0,2-0,5%	0,2 - 0,6% fish and warm blooded animal	animal ingredients	cattle, chicken, pig

n.d.= not determined

Preparation of the test samples

Production of animal meals: Material of 5 different animal species (swine, cattle, sheep, chicken and herring) was ground. After autoclaving at 133 °C and 3 bar for 20 minutes the run-down fat was separated and the remaining material was dried at 60°C and ground again.

Production of feed samples: Feeding stuff for dairy cows was mixed with the animal meals. 13 feed samples were prepared differing in

- **the amount of added animal meal (0%, 0,1%, 0,5% und 2%)**
- **different animal species (swine, cattle, sheep, chicken and herring)**
- **the material chosen for animal meal production (meat/ intestines/ whole animals/ butcher residues).**

The feed samples were investigated with both methods. The detailed results are presented in Tab. 1. The findings of the investigations were confirmed by diverse laboratories of the IAG feedstuff microscopy and the PCR-AG of VDLUFA in ring tests.

Tab.2: Overview about the results obtained by microscopy or PCR-analysis in feed samples contaminated with different artificially prepared animal meals

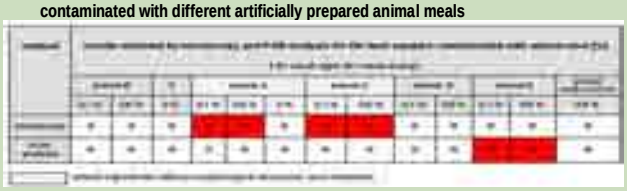
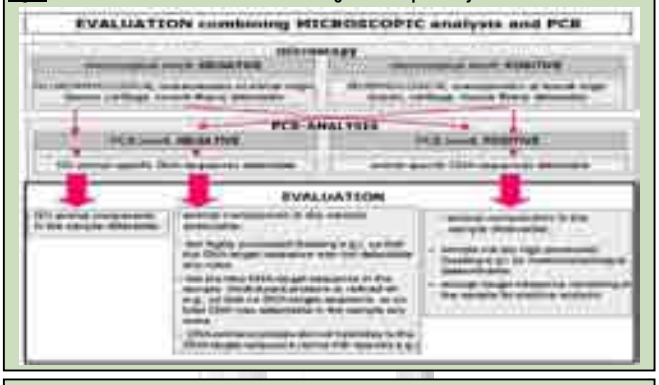


Fig. 1: Evaluation of the results when combining microscopic analysis and PCR



Results

The microscopic method allowed the detection of all added animal ingredients (0,1%, 0,5% and 2%) with morphological structures like bone particles, cartilage and/or muscle fibers (Tab. 1A). The limit of detection was dependent on the content of morphological structures in the animal meals. Besides the presence or absence of animal constituents in the feed sample a differentiation between fish and mammalian animals as well as reliable quantitative results were obtained in presence of morphological structures of animals in the feed samples. These aspects confirm the suitability of the microscopic method in the official control of feeding stuff.

With the PCR-method all samples containing terrestrial animal meal (0,1%, 0,5% and 2%) were determined positively - even samples with animal meal having nearly no morphological detectable structures (Tab. 1B). Two different modules of the PCR-method (species-specific PCR and RFLP-analyses) allowed to distinguish between animal groups and to identify diverse animal species. Herring was not detected sufficiently with the tested screening primer pairs.

In summary the tested methods of classical microscopy and PCR-analysis complemented each other with respect to the determination of different artificially composited animal meals in feeding stuff (Tab. 2).

> The microscopical approach allowed a reproducible, highly sensitive and quantitative determination of animal ingredients with morphological detectable structures like meat and bone meal in feed including a differentiation between fish and terrestrial animals in presence of bone fragments.

> The PCR-analysis allowed the detection of animal ingredients in feed even in absence of morphological detectable structures. This method enabled to differentiate between animal groups and species and to identify diverse species.

CONCLUSIONS

In case of searching for the most suitable method or when combining both methods it is necessary to interprete the obtained data in consideration of the different target structures that are detected by the methods and their quality in processed feed (Fig. 1):
Microscopy provides reliable results even in highly processed feed matrizes with well-preserved morphological animal structures also when the genetic material is highly degraded.
The PCR-method tested provides results in feed samples with preserved genetic animal material even after the separation of morphological structures.

The demonstrated methodic particularities have to be considered with respect to the choice of a suitable method for determination of animal ingredients in feeding stuff as well as for the correct evaluation of the results obtained from these investigations in highly processed feed matrizes.

Acknowledgements: Skilful technical work by M. Parsch, D. Kraus and M. Jayme from LUFA Speyer is gratefully acknowledged.

P.47.- Determination of animal constituents in feedingstuff: Evaluation of diverse methods and their results

D. Hormisch

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Two methods for the determination of animal ingredients in feedingstuff were compared in the following study. Classical microscopy was done according to the EU guideline 98/88/EC that was provided by the IAG feedstuff analysis section feedstuff microscopy. PCR-analysis was done according to the VDLUFA-method „Molecular biological determination of animal ingredients - PCR-method“. A set of 13 feed samples with artificially added animal meals of different composition was investigated to check abilities and limits of the methods. The results presented were confirmed by laboratories of the IAG feedstuff microscopy and the PCR-AG of VDLUFA in ring tests. The microscopical method allowed the detection of all added animal ingredients (0,1%, 0,5% and 2%) with morphological structures (bone particles, cartilage and/or muscle fibres). The limit of detection was dependent on the content of morphological structures in the animal meals. Besides the presence or absence of animal constituents a differentiation between fish and terrestrial animals as well as reliable quantitative results were obtained in presence of morphological structures of animals in the feed samples. These aspects confirm the suitability of the microscopical method in the official control of feedingstuffs. With the PCR-method all samples containing terrestrial animal meal (0,1%, 0,5% und 2%) were determined positively - even samples with animal meal having nearly no morphological detectable structures. Two different modules of the PCR-method (species-specific PCR and RFLP-analyses) allowed to distinguish between animal groups and to identify diverse animal species. Herring was not detected sufficiently with the tested screening primerpairs. The results showed that microscopy and PCR analyses complement each other: The microscopical approach allows a reproducible, high sensitive and quantitative determination of animal ingredients with morphological detectable structures in feed with a differentiation between fish and terrestrial animals in presence of bone fragments. The PCR-analysis allows the detection of animal ingredients in feed even in absence of morphological detectable structures. This method enables to differentiate between animal groups and species and to identify diverse species. When combining both methods it is necessary to interpret the obtained data in consideration of the different targets of both methods: Microscopy provides reliable results also in highly processed feed matrices with well-preserved morphological animal structures even when the genetic material is highly degraded. The PCR-method tested provides results in feed samples with preserved genetic animal material even after the separation of morphological structures. These specialties have to be considered with respect to the choice of a suitable determination method for animal ingredients in feedingstuff as well as for the evaluation of the results obtained from these investigations in highly processed feed matrices.

Keywords

Meat and Bone Meals (MBM), Feedingstuffs, classical microscopy, DNA technology (PCR), comparison of methods and results

Studies on the detection and monitoring for the contamination of land animal protein in domestic and imported fish meals as prophylactic for BSE

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Introduction

There has been a concern about the contamination of causative agents for the Bovine Spongiform Encephalopathy (BSE) from land animal protein sources into fish meal. European Union (EU) and other many countries are monitoring and limiting its use in the animal feed industry and therefore it is becoming an important issue to control the contamination by monitoring. The objective of the current study was to detect the presence and contamination of land animal proteins in the domestic and imported fish meals by using enzyme linked immunosorbent assay (ELISA) and further to be used for monitoring.

Materials and methods

Sample
Grind the material
Chloroform sedimentation
(2g of the sample + 15ml C_2Cl_4, 1~3min)
the fraction with staining reagent

Microscopy
Stereomicroscopical(25X, 40X) analysis

ELISA : Protocol of Tepnel ELISA-Tek
MELISA-Tek

Results

Microscopic test and ELISA were used to monitor the contamination of land animal protein in fish meal. According to the result there was no indication of contamination with ruminant related protein in domestic fish meal. One factory was found to be suspicious with pork related protein contamination. After continuous education and training on the importance of processing, human health and awareness, there have been no cases for the contamination lately. However it appeared that imported fish meals from some other countries were suspicious for or detected to have a contamination with land animal protein sources.

Figure 1. Microscopically identifiable characteristic of animal bone fragments

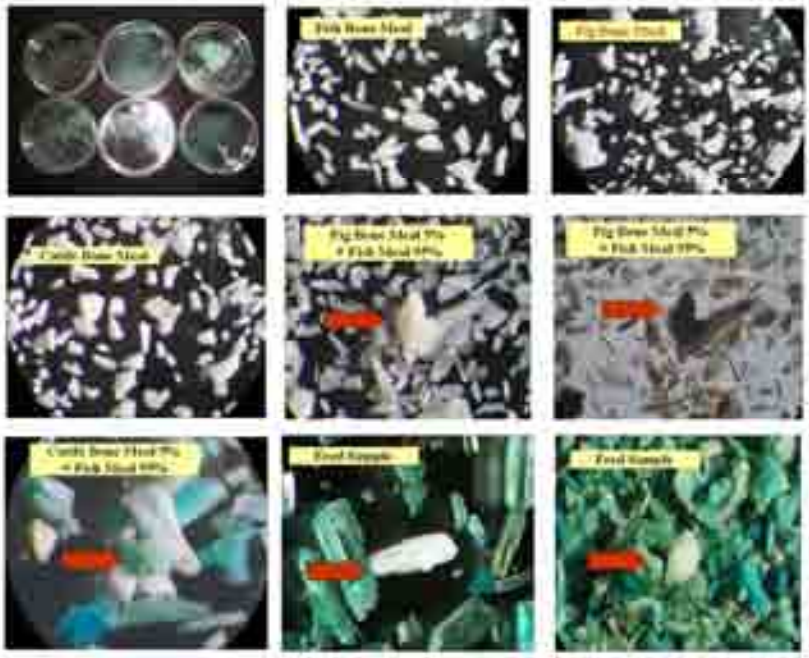

Table 1. Detection of land animal protein in the domestic fish meal using ELISA.

Table 2. Detection of land animal protein in the domestic fish meal by microscopic observation.

	A-1	D	ND	D	ND	ND	ND	D
	A-2	ND	ND	ND	ND	ND	ND	ND
	B	ND	ND	ND	ND	ND	ND	ND
	C	ND	ND	ND	ND	ND	ND	ND

S, suspicious; D, detected; ND, not detected.

Table 3. Monitoring for the land animal protein in fish meal from domestic production line using ELISA.

Material source	Ruminant (R-value)	Pork (R-value)	Poultry (R-value)
Company A	1.14	0.03	1.05
Company B	1.05 1.25	0.09 1.24	1.04 1.02
Company C	1.15 1.03 1.07	3.20 0.44 0.04	1.00 0.99 0.99

Table 4. Microscopic monitoring of land animal protein in fish meal from domestic production line.

Table 5. Monitoring for the land animal protein in imported fish meal using ELISA.

Peru	0.94~1.70	0.90~1.70	0.92~1.07
Chile	0.89~1.10	0.17~1.07	0.93~1.04
Denmark	0.96~1.27	0.99~1.77	0.95~1.55
Australia	6.09~36.72	1.37~2.77	1.02~2.06
U.S.A	1.02~1.04	0.12~0.97	0.93~1.41
Vietnam	1.11~1.15	0.44~1.06	0.99~1.16
China	1.01~1.13	0.99~1.06	0.95~1.00

Table 6. Microscopic observation and monitoring of land animal protein in imported fish meal.

Material source	Imported Years		Detection No. /Analysis No.
	2002	2003	
Peru	D	ND	1/7
Chile	S	S	4/10
Denmark	S	S	2/6
Australia	-	D	2/2
U.S.A	-	S	1/2
Vietnam	-	S	2/2
China	-	ND	0/4

Conclusions

Improved technical education and monitoring allow the factory to produce non-contaminated domestic fish meal with a land animal protein in Korea although it was rather commonly detected in the past because of contamination during the collection of ingredients. However our data suggested that a few imported fish meal from some countries were suspicious for or detected to have the contamination with land animal protein sources. Therefore it seems necessary to have continuous monitoring for all imported fish meal and to make an observation list.

References

1. Commission Directive 98/88/EC of 13 November 1998 establishing guidelines for the microscopic identification and estimation of constituents of animal origin for the official control of feedingstuffs (O.J L 318, 27.11.98, p. 45).
2. Council Decision 2000/766/EC of 4 December 2000 concerning certain protection measures with regard to transmissible spongiform encephalopathies and the feeding of animal protein (O. J. L 306, 7.12.2000, p. 32).
3. Commission Directive 76/371/EEC of 1 March 1976 establishing Community method of sampling for the official control of feedingstuffs (O.J. No L102, p. 1, 15.04.1976).

P.48.- Studies on the detection and monitoring for the contamination of land animal protein in domestic and imported fish meals as prophylactic for BSE

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There has been a concern about the contamination of causative agents for the Bovine Spongiform Encephalopathy (BSE) from land animal protein sources into fish meal. European Union (EU) and other many countries are monitoring and limiting its use in the animal feed industry and therefore it is becoming an important issue to control the contamination. The objective of the current study was to detect the presence and contamination of land animal proteins in the domestic and imported fish meals by using enzyme linked immunosorbent assay (ELISA) and microscopic test further to be used for monitoring.There was no indication of contamination with ruminant related protein in domestic fish meal. One factory was found to be suspicious with pork related protein contamination. After continuous education and training on the importance of processing together with human health and awareness, there have been no cases for the contamination lately. However it appeared that imported fish meals from some other countries were suspicious for or detected to have a contamination with land animal protein sources. In conclusion, improved technical education and monitoring allow the factory to produce non-contaminated domestic fish meal with a land animal protein in Korea although it was rather commonly detected in the past because of contamination during the collection of ingredients. However our data suggested that a few imported fish meal from some countries were suspicious for or detected to have the contamination with land animal protein sources. Therefore it seems necessary to have continuous monitoring for all imported fish meal and to make an observation list.

Keywords

Enzyme-Linked ImmunoSorbent Assay, ELISA, microscopic test, Bovine Spongiform Encephalopathy, BSE, contamination, mornitoring

P.49.- Methods for species identification

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In 2002 the Animal By-Product Regulation (EC) No. 1774/2002 was published, imposing a ban on feeding animals with proteins from the same species. The availability of an analytical technique for species identification would be helpfull to lift the feed ban.
The suitability of immunochemical and PCR techniques is tested with different reference materials prepared in a dedicated 140 Litre steriliser under strict controls and conditions (133 and 159°C, pressure 3 bar and during 20 minutes).
As reference materials are prepared (both pre-pressure and post-pressure cooked):
- porcine soft material (100% large intestines (empty))
- porcine bone material (5% tails and 95% hind-legs)
- chicken soft material (100% digestive system)
- chicken bone material (40% heads and 60% shanks)
- bovine, ovine, porcine and avian bone material and bovine muscle, heated 133, 137, 141 and 145°C, are prepared by PDM Ltd (Doncaster, UK).

The following commercial available kits are tested:
- Food Expert ID, DNA chip technique (BioMérieux)
- SureFood Animal-ID, PCR-ELISA (Congen Biotechnologie GmbH)
- MELISA-TEK, ELISA (ELISA Technologies Inc.)
- AgriScreen, dipstick immunoassay (Neogen Corporation)
- FeedChek, lateral flow test (Strategic Diagnostics Inc.)

Also one ELISA assay and three PCR assays, which are still in development, of four different research institutes are compared.
The results (green when the result is okay and red when the result is wrong / no result) of the assays with the various reference materials are presented in a survey.
Most of the tested assays are more or less able to characterise the species identity of reference materials, even after heating for twenty minutes at the highest temperatures. However, often false positive and sometimes false negative results are found.
Dilemma: the current authorisation of the use of "non prohibited proteins" (including e.g. milk-products, egg-products) in feed will hamper the correct identification of the other tissues of that species which are banned by the intra-species recycling ban.

Keywords

Species identification, anti-cannibalism, PCR, ELISA, DNA chip, reference materials

Methods for species identification

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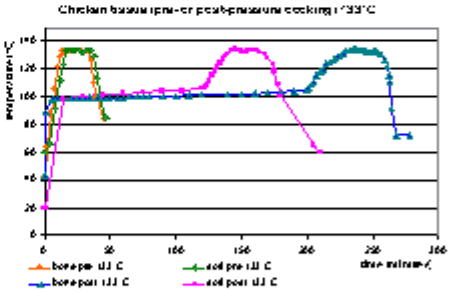
In 2002 the Animal By-Products Regulation (EC) No. 1774/2002 was published, imposing a ban on feeding animals with proteins from the same animals. The availability of an analytical technique for species identification, would facilitate the lift of this ban.

The suitability of immunochemical and PCR techniques is tested with different reference materials.

Materials and methods

Reference samples are prepared in a dedicated 140 litre steriliser under strict controls and conditions (133°C and 159°C during 20 minutes; both pre-pressure and post-pressure cooked):

- Porcine soft material
  (100% large intestines (empty))
- Porcine bone material
  (5% tails and 95% hind-legs)
- Chicken soft material
  (100% digestive system)
- Chicken bone material
  (40% heads and 60% shanks)



- Bovine, ovine, porcine and avian bone material and bovine muscle, heated at 133°C, 137°C, 141°C and 145°C, are prepared by PDM Ltd (Doncaster, UK)

Also some 'pure' commercial available products are used as reference samples.

Results

See table with results of the assays of institutes and commercial available test kits mentioned in the heading of the columns.

Conclusions

Most of the tested assays are more or less suitable for characterisation of the species identity of reference materials, even after heating for twenty minutes at the highest temperatures. However, often false positive and sometimes false negative results are found.

Acknowledgements
This study is a collaboration of the European Fat Processors and Renderers Association (EFPRA). It is funded by the Natural Organic Products group of Sobel (Best, the Netherlands).

Results species identification

DETECTION OF ANIMAL DNA IN FEEDSTUFFS BY POLYMERASE CHAIN REACTION COMPARED WITH THE OFFICIAL MICROSCOPIC METHOD

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INTRODUCTION

The bovine spongiform encephalopathy (BSE) European epidemic has focused the attention on the importance of adopting stringent control measures to avoid the risk of the diffusion of the disease through meat meal-based animal feedstuffs. EU directives 89/535/EC and 2000/1/2/EC establish the official control to evaluate the presence of animal-derived constituents in feedstuffs by **microscopic examination**; however, this method presents some drawbacks, cause it's time consuming and the reliability mainly depends on the evaluation of specialised staff. The microscopic technique is able to discriminate between the classes of animal bone fragments but not among the species and it can not be used to detect the presence of some banned animal by-products, as blood.

Afterwards the need of alternative analytical approaches is largely highlighted. In the last years many studies extensively investigated the application of molecular techniques like **Polymerase Chain Reaction** (PCR) to such analyses. PCR seems to be a specific and sensitive method and it can help even in the detection of animal DNA traces from heat processed products. A one step multiplex PCR for simultaneous detection of ruminant, poultry, fish and pork material was developed (Dalmasso et al. Mol. Cell. Probes 2004; 18: xxx-x).

AIM OF THE STUDY: in this study this multiplex PCR method was compared to microscopic official technique to detect animal materials in feedstuffs from routine official controls.

MATERIALS AND METHODS

201 feed samples from official control were examined for the presence of animal-derived products both with microscopic method and with PCR.

MICROSCOPIC METHOD

The microscopic method was performed in feedstuffs analysis to detect animal-derived constituents according to EU regulation 2000/2/EC and to national ordinance D.M. 22/07/96.

500g of each sample was weighted and grounded, if necessary. The grinded material was divided in two portions: the first for the sieve fractions (< 0.5mm and > 0.5mm), the others for the concentrated sediment (10g extracted with 100ml tetrachloroethylene). The fraction > 0.5mm was examined under stereomicroscope. The fraction < 0.5mm and the concentrated sediment were screened both under stereomicroscope and compound microscope.

PCR

DNA extraction was performed both by CTAB method and by ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems).The extracted DNA samples were first investigated for vertebrate specific DNA (16S rRNA gene) by simplex PCR according to Bottero protocol (Buttero et al., *J of Food Prot.* 2003;66, vol.12). The samples resulted positive for animal DNA where then analyzed with one step multiplex PCR for simultaneous detection of ruminant, poultry, fish and pork material in feeds (Dalmasso et al.Mol. Cell. Probes 2004; 18: xxx).2 µl stumpion PCR was performed in a final volume of 25 µl containing 1.5 U of Platinum Taq DNA polymerase (Invitrogen); 0.1 mg/ml BSA (Promega); 0.2 mM of each dATP, dGTP, dTTP, dCTP (Fisher); 2mM of $MgCl_2$ and 10, 20, 12.5, 40 pmol of ruminant, pork, fish and poultry primers respectively. DNA amplification was performed in a 9700 Thermal Cycler (Applied Biosystems) with the following conditions: denaturation step at 94°C for 2 min, then 35 cycles at 94°C for 30s, 60°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. Amplicons were then resolved by electrophoresis on a 2% agarose gel, stained with Ethidium Bromide-dNuRed and run in a Tris Acetate EDTA for 70 min at 110 V.

RESULTS

Of 201 feed samples were analysed, 156 (77.6%) were negative both for microscopic and PCR methods; 45 (22.4%) were positive for animal DNA by PCR (Fig.1) while only 11 (5.5%) were positive by microscopic method (Tab.1). Of these, only 5 (2.5%) were positive for animal DNA by both methods (Fig.2 lane 7) and 1 for pork DNA by multiplex PCR (Tab.2 and Fig.3 lane 7). None of the 201 samples were positive by official microscopy test.

Tab.1 — Presence of animal-derived constituents

analysed feedstuffs	Positive results (microscopic method)	Positive results (PCR)
201	0	45

Fig.1 — Results with multiplex PCR

Tab.2 — RESULTS

16	5	1	0

Fig.2 — Simplex PCR (16S rRNA gene)

Fig.3 — Multiplex PCR (pork, ruminant, poultry, fish)

CONCLUSIONS

- The PCR analytical method seems to be sensitive and it also seems to overcome the resolution efficiency of the official microscopic method. However, this kind of analysis system, as a qualitative one and as, it could be difficult to discriminate between traces and non intentional contamination of animal origin in feed samples.
- PCR analysis can not discriminate the source of animal constituents (blood, milk, etc.) and so the presence of not banned animal-derived products, as milk (Regulation 2000/1/2/EC), that could mask the presence of not allowed animal materials.
- In conclusion PCR analysis could be considered a useful tool to identify the species of animal constituents in feeds and it could be applied to confirm positive samples. It cannot be used as the only screening method.

P.50.- Detection of animal DNA in feedstuffs by Polymerase Chain Reaction compared with the official microscopic method

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EU regulations (98/88/EC and 2003/126/EC) established the official control to evaluate the presence of animal-derived constituents in feedstuffs by a microscopic examination. However, the need of alternative analytical approaches is largely highlighted.
In the last years many studies extensively investigated the application of molecular techniques (PCR) to such analysis.

The aim of this study is to compare the official microscopic method with a multiplex PCR method to detect animal materials in feedstuffs. 201 feed samples from the routinely official controls resulted negative for the presence of constituents of animal origin by microscopic method.
The same samples were then investigated for vertebrates specific DNA (16S rRNA gene) by PCR (Bottero et al. J. Food Prot. 2003: 66, vol. 12) and the presence of animal-derived constituents was detected in 45 samples. For the simultaneous detection of ruminant, pork, fish and poultry DNA a multiplex PCR (Dalmasso et al. Mol. Cell. Probes 2004, 18 (2) was performed; 16 samples resulted positive for ruminant DNA, 5 for poultry and 1 for pork.

The PCR analytical method seems to be sensitive and it also seems to overcame the resolution efficiency of the official microscopic method. Therefore, because of high sensitivity of PCR in detecting animal DNA in feeds, it would be difficult to discriminate between fraud and non intentional contamination of animal origin. PCR analysis can detect as well the presence of animal constituents, as blood, that are impossible to discriminate by the official microscopic method. On the other hand the presence of not banned animal-derived products (EU Regulation 2003/1234/EC) could mask the presence of not allowed animal materials.

In conclusion PCR analysis could be a useful tool to confirm positive samples and also to identify the species of animal constituents in feeds but it cannot be used as the only screening method.Select the field and type or paste the text.

Keywords

Feedstuffs, microscopic method, PCR

Possibilities of FT-IR and PCR to discriminate species by animal fats

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Introduction

After the first verified case of BSE in 1986, feeding of farmed animals was regulated. These regulations concern TSE in general, removal of risk material, surveillance and testing, feed ban and animal by products.

The safe use of animal by-products (ABPs) such as meat and bone meal (MBM) or fat for feeding farmed animals is regulated by EC Regulation 1774/2002 prohibiting intra-species recycling of ABPs and by EC Regulation 1234/2003 introducing a total ban on MBM and other ABPs. The latter measure will be reconsidered when species specific analytical methods are available. Here we report on species analysis of animal fat.

Infrared spectroscopy

Samples of fish oil (Fi), lard (La), tallow (Ta), lamb (Sh), chicken (Ch), horse (Ho) and rabbit (Ra) fats analysed were taken from various butcheries and market.

The samples were analysed in Attenuated total Reflectance mode (ATR) from a heated drop (50°C) deposited on a ZnSe ATR crystal. Principal Components Analysis (PCA) applied on the full spectra separates the fish oil from other samples. The PCA on 920-980 cm^{-1} MIR region allows the separation of butter and sheep fat from other samples and shows a trend to cluster lard and tallow samples together (Figure 1). The stepwise linear discimiminant analysis (SLDA) (Figure 2) and the use of 2D graph of the values of two signal ratios 966/956 and 990/980 are robust tools to distinguish sample.

Figure 1: PCA on 920-980 cm^{-1} based on raw ZnSe ATR MIR spectra

Figure 2: SLDA on 956, 966 and 990 cm^{-1}

Polymerase Chain Reaction (PCR) is a technique able to detect the presence of animal sources of DNA even in heat treated samples by using short targets but it is unable to distinguish forbidden materials (MBM) from authorised ones (e. g. milk powder or blood).

DNA extractions were attempted on a limited set of three industrial fat samples (lard, tallow and chicken fat). Short mitochondrial targets furthermore used for MBM detection were tested in real time PCR. Significant amplification curves were obtained with each of the three samples.

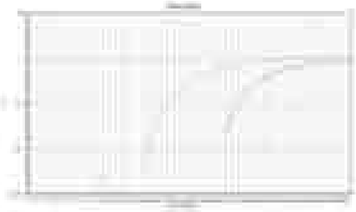

Figure 3: Amplification curves obtained with the bovine target
— Positive control – DNA extracted from blood
— Sample – DNA extracted from tallow

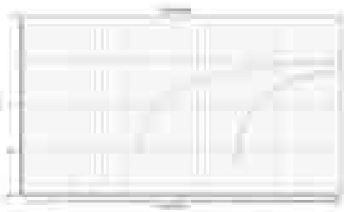

Figure 4: Amplification curves obtained with the pig target
— Positive control – DNA extracted from blood
— Sample – DNA extracted from lard

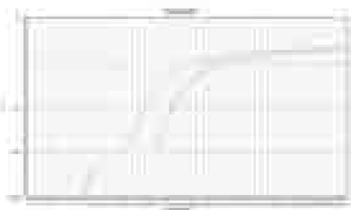

Figure 5: Amplification curves obtained with the chicken target
— Positive control – DNA extracted from blood
— Sample – DNA extracted from chicken fat

Conclusion

Based on these preliminary results, FT-IR and PCR could be used in combination for the control of the species origin of the fat incorporated in feed. The spectroscopic methods could be used for the screening and the PCR for confirmation as a forensic method. The results obtained with the real time PCR show that DNA is still present in such samples and significant amplification is possible with appropriate targets.

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P.51.- Possibilities of FT-IR and PCR to discriminate species by animal fats

Possibilities of FT-IR and PCR to discriminate species by animal fats

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Animal by-products represent yearly in the EU more than 10 million tons of materials derived from healthy animals which have to be incinerated or transformed in a variety of products used among others in human food and animal feed. A significant part of these by-products are made up of fats. **The Regulation (EC) N° 1774/2002**[1] extends the current ruminant intra-species recycling (cannibalism) ban to other species and maintains current EU total ban on the feeding of meat and bone meal to farmed animals. The Regulation establishes clear safety rules for the production of meat and bone meal in case it is ever re-authorised for inclusion in feed for non-ruminant species, e.g. poultry and pigs. Therefore it will be necessary to clearly identify the species origin of by-products included in feed[2] with efficient analytical methods.

Near Infrared (NIR) region can be used to determine the origin of the fat samples, however differences of the spectra in the Middle Infrared (MIR) region are more significant and allow to get better discrimination. The use of multivariate statistical tools (PCA, SLDA) on the full spectra allows to distinguish fish from other samples. The chemometric analysis in the fingerprint region (1000-900 cm^{-1}) allows the classification of some fat samples according to their origin. Butter and sheep are clearly separated from the other samples and a lot of the tallow samples tend to cluster together. The use of 2D graph of the values of the absorbances at two signal ratios 966/956 cm^{-1} and 990/980 cm^{-1} is a robust tool to distinguish samples.

PCR is a species-specific technique which could be a helpful complement of the spectroscopic methods. Successful attempts of DNA extraction and PCR amplification were realised at CRA-W on three industrial fat samples (lard, tallow and chicken fat). Short mitochondrial targets initially developed for MBM detection were tested in real time PCR and gave significant results[3].

Based on these preliminary results, FT-IR and PCR could be used in combination for the control of the species origin of the fat incorporated in feed. The spectroscopic methods could be used for screening and PCR for confirmation as a forensic method. The results obtained with the real time PCR show that DNA is still present in such samples and significant amplification is possible with appropriate targets.

Keywords

Animal fats, animal by-products, species identification, FT-IR, spectroscopy, PCR

[1] Regulation (EC) N°1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. *Off. J. Eur. Comm.* L273, 10/10/2002, 1-95.

[2] EC DG Health and Consumer Protection; MEMO/04/107Brussels, 6 May 2004 Questions and Answers on animal by-products

[3] Dubois M., Fumière O., von Holst C. & Berben G. (2002). - Meat and bone meal detection in feed by search of specific animal DNA segments. *181st meeting of the Belgian Society of Biochemistry and Molecular Biology*, 4th of May, Katholieke Universiteit Leuven (KUL), Heverlee (Belgium), abstract nr. 7

Relationship between rendering process temperatures and DNA degradation in Meat and Bone Meals by Real Time PCR assay

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Several methods for the detection of Processed Animal Proteins (PAPs), based on the Polymerase Chain Reaction (PCR) technique have been described to implement taxon-specific identification, presently not fully supported by microscopic analysis. Most of PCRs report qualitative data, with performances expressed as detection limits. Nevertheless, their performances could depend on the integrity of the target DNA, influenced by the different rendering processes. This has prompted us to set up a quantitative Real Time PCR to evaluate the target DNA degradation, on a defined MBM, processed at 133°, 137°, 141° and 145°C , fulfilling the present legislation (20 min, ≥3 bar).

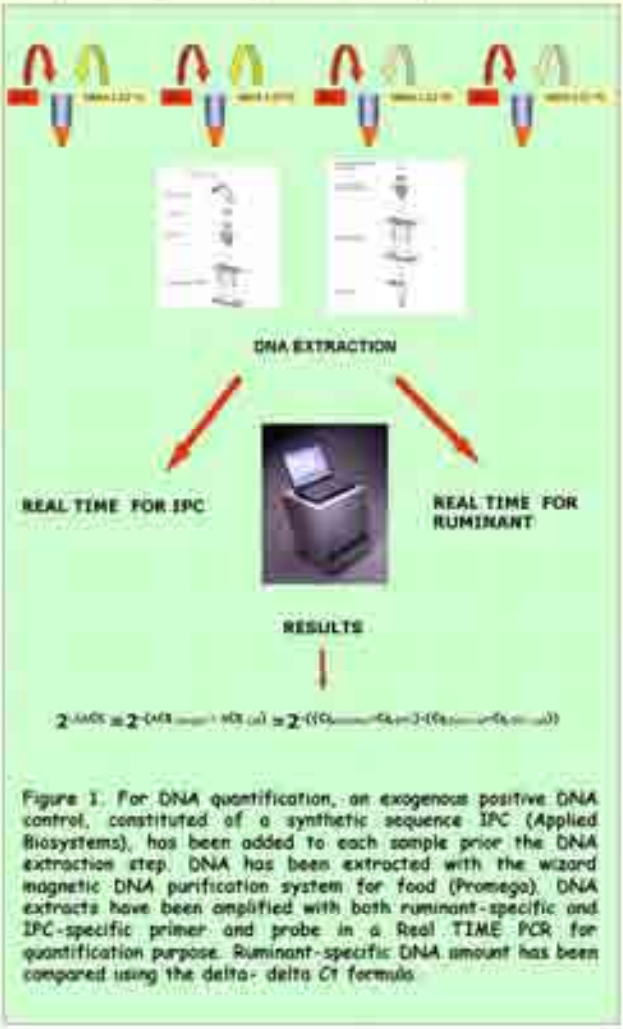

Figure 1. For DNA quantification, an exogenous positive DNA control, constituted of a synthetic sequence IPC (Applied Biosystems), has been added to each sample prior the DNA extraction step. DNA has been extracted with the wizard magnetic DNA purification system for food (Promega). DNA extracts have been amplified with both ruminant-specific and IPC-specific primer and probe in a Real TIME PCR for quantification purpose. Ruminant-specific DNA amount has been compared using the delta- delta Ct formula.

To quantify the target mithocondrial DNA specific for ruminant, we use an exogenous target control. It consists of an exogenous DNA used as internal positive control also for the evaluation of the DNA extraction efficiency. Such approach is mandatory because the absence in MBMs of an endogenous target control. Our results demonstrate that the method could detect ruminant specific DNA also in 145°C treated MBM. The DNA determination indicates that with the increase of the temperatures, the quantity of DNA that can be amplified decreases with an exponential factor. In particular, in the range analysed, we observe a two log decrease of the amplified DNA amount. between 133°C and 145°C treated MBM.

Owing to the above, the assessment of PCR method performances (such as detection limit and quantification) are greatly affected by the kind of MBM tested. Therefore, it must be considered that the quantification of MBM in feeds, by PCR techniques, must be related to the rendering process. For official control purposes, this fact could not allow to use PCR results to discriminate an intentional addition of MBM in feeds from a possible occasional contamination. On the other side, PCR could help within the same rendering plant to monitor the effectiveness of the process, by evaluating the progressive DNA degradation under the present legislative frame

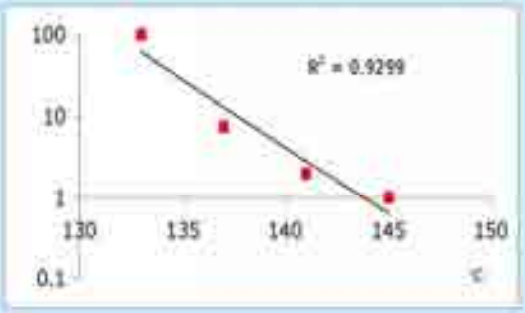

Figure 2. Ruminant-specific DNA amount (Y values) for each MBM produce under different heat treatment (X values) condition has been expressed in comparison to what observed on the MBM treated at 145°C which has been set to a value of 1. DNA decrease 100 fold from the sample treated at 133°C to that at 145°C.

Acknowledgment: work granted by EU project G6RD-2000-CT-00414, STRATFEED, Dr. Pierre Dardenne - Coordinator

P.52.- Relationship between rendering process temperatures and DNA degradation in Meat and Bone Meals by Real Time PCR assay

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Keywords

PCR, DNA degradation,MBM

Glyceroltriheptanoate as marker for category 1 and 2 animal by-products

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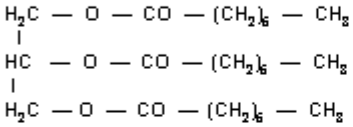
From 1 May 2003 products derived from category 1 and 2 animal by-products should be marked according to Regulation (EC) No. 1774/2002. Glyceroltriheptanoate (GTH) is suitable as marker.

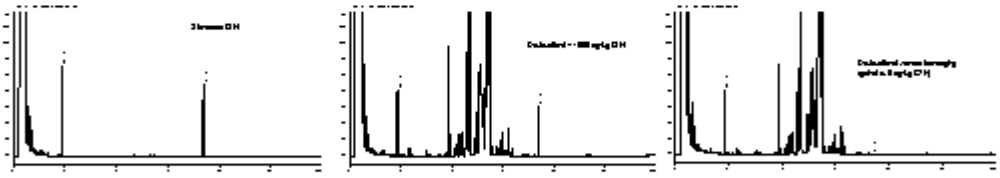


GTH is artificial: it is not found in nature
GTH is safe: it has applications in the food industry
GTH is stable during the rendering process
GTH is not removable from the marked materials

Analysis of GTH in MBM and fat

GTH is extracted with light petroleum 40 – 70
- Clean up of the extract with a disposable silica column
- Analysis of the extract with gas chromatography with flame ionisation detection.

Detection limit: 5 mg/kg (on fat basis)
Analytical recovery: 97 %



Pilot experiment on rendering plant

GTH is homogeneously added to animal slaughter by-products (532 g/ metric tonne on fat basis).
Every 8 hours a sample is taken from the MBM and the fat product.
Nearly 57 % of the added GTH is found in both products.

Conclusion

In practice a dosage of 100 g GTH/ metric tonne raw material (= 500 g/mt on fat basis) is efficient as marker for category 1 and 2 animal by-products.

Acknowledgements
This study is a collaboration of the European Fat Processors and Renderers Association (EFPRA). It is funded by the Natural Organic Products group of Sobel (Best, the Netherlands).

P.53.- Glyceroltriheptanoate as marker for category 1 and 2 animal by-products

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From 1 May 2003 products derived from category 1 and 2 animal by-products should be marked according to Regulation (EC) No. 1774/2002. To establish a suitable marking method for the traceability of MBM and fat, the price and non-removability are important and a reliable detection method with a low detection limit will be required.
Glyceroltriheptanoate (GTH) is suitable as a marker for category 1 and 2 animal by-products.
GTH is a clear transparant liquid with three C7-fatty acids esterified with glycerol. GTH is a safe product: it has applications in the food industry (e.g. marking of butter).
The C7 fatty acids of GTH are normally not found in nature, but heptanoic acid can also be found at low levels (10 - 20 mg/kg) in fat after bacterial deterioration. Therefore, an analytic technique is succesfully developed for analysis of the intact GTH instead of heptanoic acid after saponification.
GTH is extracted from the sample with light petroleum 40 - 70, after this the extract is purified by use of gas chromatrography with a silica column. The elution uses a mixture of light petroleum 40-70 and diethylether. The fraction with GTH is collected, concentrated and after silylation separated with a capillary column using gas chromatography and detected using flame ionisation detection (FID). The concentrations are determined by comparisons with the pure standards and are expressed in mg/kg on fat basis. As the GTH is trapped in the fat fraction, it can be found in the separated fat and in the fat fraction of the meal.

At a rendering company GTH (532 mg/kg added on fat basis) is added to the raw (animal slaughter by-products) material at the transport screws. The GTH dosage pump is automatically switched on when the transport screws are working. Every 8 hours a sample is taken to see if the GTH was homogenious divided within the contents. As a mean 292 mg/kg of the GTH (55% of the added amount) was found in the MBM. 303 mg/kg of the GTH (57% of the added amount) was found in the fat.

The detection limit of the technique used for analysis is set at < 5 mg/kg GTH on fat basis. In practice a dosage of 100 gram GTH/ metric tonne raw animal material (this is about 500 gram GTH/ metric tonne on fat basis) is sufficient.
GTH is recommended as a very suitable marker for all category 1 and 2 materials.

Keywords

Marker; animal by-products; analysis; gas chromatography; MBM; GTH; fat

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