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Classification of signatures of Bovine Spongiform Encephalopathy in serum using infrared spectroscopy†

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Signatures of Bovine Spongiform Encephalopathy (BSE) have been identified in serum by means of "Diagnostic Pattern Recognition (DPR)". For DPR-analysis, mid-infrared spectroscopy of dried films of 641 serum samples was performed using disposable silicon sample carriers and a semi-automated DPR research system operating at room temperature. The combination of four mathematical classification approaches (principal component analysis plus linear discriminant analysis, robust linear discriminant analysis, artificial neural network, support vector machine) allowed for a reliable assignment of spectra to the class "BSE-positive" or "BSE-negative". An independent, blinded validation study was carried out on a second DPR research system at the Veterinary Laboratory Agency, Weybridge, UK. Out of 84 serum samples originating from terminally-ill, BSE-positive cattle, 78 were classified correctly. Similarly, 73 out of 76 BSE-negative samples were correctly identified by DPR such that, numerically, an accuracy of 94.4 % can be calculated. At a confidence level of 0.95 ($\alpha = 0.05$) these results correspond to a sensitivity $> 85\%$ and a specificity $> 90\%$. Identical class assignment by all four classifiers occurred in 75% of the cases while ambiguous results were obtained in only 8 of the 160 cases. With an area under the ROC (receiver operating charateristics) curve of 0.991, DPR may potentially supply a valuable surrogate marker for BSE even in cases in which a deliberate bias towards improved sensitivity or specificity is desired. To the best of our knowledge, DPR is the first and—up to now—only method which has demonstrated its capability of detecting BSE-related signatures in serum.

Introduction

Sponge-like modifications of brain tissue are the main pathological characteristics of a family of diseases, collectively known as Transmissible Spongiform Encephalopathies (TSE) (see *e.g.* refs. 1–3). Scrapie was the first example identified within this group of diseases, reported in sheep almost 300 years ago. Besides scrapie, other forms of TSE are known today, such as Kuru, Gerstmann–Sträussler–Scheinker Syndrome, Fatal Familiar Insomnia, and Creutzfeldt–Jakob Disease in humans or Bovine Spongiform Encephalpathy (BSE), Chronic Wasting Disease, Feline Spongiform Encephalopathy and Transmissible Mink Encepahlopathy in cattle, deer, cats and mink, respectively. Since changes of the Prion protein from PrP^{c} (c: cellular) to PrP^{sc} (sc: scrapie) were directly linked to TSE,[4] the disorders are often called Prion diseases.

In 1986 an epidemic broke out within the cattle population of the UK and to date more than 180,000 cases of BSE have been reported worldwide.[1–3] Many countries have implemented BSE screening and testing programs for cattle, in which PrP^{sc} is detected *post-mortem* in brain tissue, *e.g.* by means of the Prionics Check testing.

Despite the success of *post-mortem* testing of BSE-infected brain tissue, a test for the living cattle population remains highly desirable. Such a living animal test would probably be based on fluid samples such as cerebrospinal fluid, blood, serum or urine. Signatures specific to TSE have recently been detected in the serum of artificially infected hamsters[5] and, during a field study, of cattle[6] using mid-infrared spectroscopy and multivariate data analysis methods.

Mid-infrared spectroscopy plays an increasing role in biomedical research.[7–9] It has been shown that the interpretation of mid-infrared spectra of serum in terms of particular diseases allows for the identification of disease-specific signatures *e.g.* for diabetes mellitus,[10,11] the metabolic syndrome[12] or rheumatoid arthritis.[13] Throughout this paper we refer to this combination of the spectroscopy of molecular vibrations and multivariate classification algorithms as "Disease Pattern

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Recognition'' or ''Diagnostic Pattern Recognition'' (DPR).[10–14] The DPR-method yields a number between 1 and 0 (''DPR-score'') which relates to the likelihood of a spectrum resembling typical spectra of serum from donors, who either do or don't suffer from the particular disease under investigation, respectively.

Along the path of the DPR-method from academic research laboratories to routine use, improvements in convenience (*e.g.* room temperature operation, simplified sample and data logistics) and robustness of the DPR-method play a key role. In this paper we present the application of DPR to the detection of BSE-specific signatures in bovine serum with emphasis on the applicability to daily routine. Based on the pioneering work of Lasch *et al.*[6] further progress has to target the following issues:

- The experimental precedure requires simplification and improved convenience (no repeating of measurement, throughput of at least 80 samples per day, single operator only, may be operated by spectroscopically unexperienced users).
- In order to avoid time-consuming washing cycles and due to the infectious nature of BSE, disposable sample carriers need to replace the repetitively cleaned ZnSe sample carrier used in ref. 6.
- The robustness of classification needs to be investigated and classification methods have to be compared in order to choose the optimum method and/or the optimum combination of methods.
- Given the high accuracy of the presently available *post-mortem* tests, further improvement of the overall accuracy of the *ante-mortem* method is required.
- In some application scenarios, sensitivity is more important than specificity. Thus, the possibility to bias the testing towards higher sensitivity at the expense of specificity needs further investigation.

Experimental

A total of 641 serum samples were acquired from confirmed BSE-positive or BSE-negative cattle by the Veterinary Laboratory Agency (VLA), Weybridge, UK and from BSE-negative cattle of a commercial abattoir in southern Germany. All 210 BSE-positive samples were obtained by VLA and originated from cattle in the clinical stage, *i.e.* the animals showed clinical signs of BSE and were subsequently shown to be BSE-positive by histopathological examination. As a reference, 211 BSE-negative samples from the VLA were augmented by 220 BSE-negative samples obtained from a German abbattoir. All of the BSE-negative samples originated from animals which were not suspected to suffer from BSE. However, approximately 1/3 of the confirmed BSE-negative samples originated from farms at which at least one case of BSE-infection had occurred.

After thawing, 3 μl of each sample were pipetted onto each of three disposable silicon sample carriers using a modified COBAS INTEGRA 400 instrument.‖ Each sample carrier carried up to 89 samples. The triplicate pipetting allowed for the improved detection of failures or outliers, which may occur due to variations in the surface properties of the sample carrier, the dispensing probe head, and/or the sample itself. After pipetting, the samples were left to dry in the analyser for 30 min. The drying stage helps to suppress the strong absorption background of water which usually exists in aqueous fluids and which frequently hampers the reproducible spectroscopy in the mid-infrared region. Upon drying, the serum sample forms a homogenous film with a diameter of 6 mm and a thickness of a few micrometers. The film thickness has been optimized to give reasonably large infrared absorbance signals in the spectral regions of interest while simultaneously staying well within the minimum volume capabilities of the liquid handling system.

Spectra were measured using a Matrix HTS/XT spectrometer (Bruker Optics GmbH, Ettlingen). Spectroscopy is performed in transmission using a DLaTGS detector, which in contrast to MCT detectors can be operated without liquid nitrogen cooling. Avoiding the need for liquid nitrogen cooling has indeed been one of the major requirements in terms of convenience and user-friendliness. Each spectrum was recorded in the wavenumber range from 500–4000 cm^{-1} and consisted of 3629 data points. Spectra were acquired at a resolution of 4 cm^{-1} and averaged over 32 scans. Blackmann–Harris 3-term apodization was used and the zero filling factor was 4. The three absorbance spectra of each sample measurement were corrected for the sample carrier background. Automation allowed for routine triplicate measurement of 89 samples per day with one operator.

For teaching, 96 BSE-positive (VLA) and 325 BSE-negative (VLA: 105; German abattoir: 220) samples were measured on a DPR research system in the laboratories of Roche Diagnostics GmbH. A second system was placed at the VLA, and 60 additional serum samples (30 BSE-positive, 30 BSE-negative) of the VLA archive were measured on this system. The corresponding spectral and sample information was also used for teaching the classification algorithms.

Four different approaches were investigated for teaching. Each approach started with a data reduction step, which aimed at the selection of those spectral regions, which are suited for the discrimination between spectra originating from BSE-positive samples *versus* spectra from BSE-negative samples. A classification algorithm was then trained using the reduced data set, namely a linear discriminant analysis (LDA) based on principal components, a robust linear discriminant analysis (R-LDA, see *e.g.* ref. 13), an artificial neural network (ANN, see *e.g.* refs. 5, 6 and 15) and a support vector machine (SVM, see *e.g.* refs. 16–18). To the best of our knowledge this is the first time that an SVM classification algorithm is applied and compared to other classification methods in the context of vibrational spectroscopy.

When training the classifier algorithms, it is important to know that variances between the spectra may be influenced by covariates other than the disease state, such as gender, breed, system-to-system variation, *etc*. Thus, it was helpful to employ a cascade of classification steps within each approach prior to finally performing the discrimination between BSE-positive and BSE-negative. This multilayered/stacked classification has previously been described *e.g.* in the context of microbiology.[15,19]

Finally, the results of the four approaches for differentiating between BSE-positive and BSE-negative serum samples were combined by means of a voting scheme. The combined classifier assigned the sample under investigation to BSE-positive if two or more of the classifiers were positive and assigned a sample to BSE-negative if none of the approaches or only one of them indicated BSE-positive. As an alternative to the voting scheme, the individual DPR scores were arithmetically averaged to give a DPR-score of the combined classifier.

After the teaching was finished, the classifier software was installed at the VLA system and 160 serum samples (released by an independent audit committee, the IAAG, from the VLA data bank) were run through the system in a blinded manner. Note that these samples had not been subject to any DPR investigation before and the individual diagnoses were not known to the operators at the time of the spectral measurement and subsequent analysis. During each measurement, the result of the classification of each validation sample (class assignment based on voting scheme as well as DPR-score of the combined

‖ COBAS INTEGRA is a trademark of a member of the Roche Group.

classifier) was calculated on-line and stored with the sample identification number. After the blinded validation was finished, the data was compared to the true diagnosis (based on a *post-mortem* test of the corresponding brain tissue) and the comparison was reported to Roche Diagnostics.

Results

Analysis of the variation within the triplicate measurement of each sample allowed an evaluation of the reproducibility, which we defined as the (wavenumber dependent) ratio between the mean absorbance and the reproduction error. The results shown in Fig. 1 are based on the comparison of the automated and the manual pipetting of 200 arbitrarily chosen bovine serum samples from this study and are averaged over the wavenumber regions 900–1500 cm^{-1} and 2700–3000 cm^{-1}. Here as well as in the other wavenumber regions, automation of the pipetting step resulted in a significant improvement (*t*-test: $\alpha = 0.05$, $p < 0.001$) of the reproducibility when compared to manual pipetting (see also ref. 20).

The arithmetic mean of the absorbance is shown in Fig. 2 as a function of wavenumber after pre-processing of all the 421 sample spectra measured on the first DPR research system. The relative spectral differences between the spectra of BSE-positive samples and BSE-negative samples are wavenumber-dependent and amount to 2% of the mean absorbance or less for most of the spectrum (see Fig. 2, middle). Since non-disease-specific variations contribute to the spread of spectral shapes around a mean value, the ratio between spread and difference has to be considered on a statistical basis. We have applied the Mann–Whitney (MW) test as a univariate measure of deviation at each wavenumber (see lower curve in Fig. 2). At any given wavenumber, the MW-test gives a value (MW-score) between 0.5 and 1.0 if the BSE-positive absorbance is more frequently above the BSE-negative absorbance and a value between 0 and 0.5 in the opposite case.

The spectral regions selected for classification between BSE-positive and BSE-negative are shown in Fig. 3 for each of the four approaches. Although the different spectral windows vary in number, position and width, some intervals have been selected by all of the classification approaches (1052 cm^{-1}, 1139 cm^{-1}, 1312 cm^{-1}, 1330 cm^{-1}, 1742 cm^{-1}, 2884 cm^{-1}, and 2942 cm^{-1}).

In routine laboratory diagnostics, the quality of a diagnostic marker is frequently measured by the sensitivity (number of correctly classified BSE-positive samples divided by the total number of BSE-positive samples), specificity (number of correctly classified BSE-negative samples divided by the total number of BSE-negative samples) and accuracy (number of correctly classified samples divided by the total number of samples). The results of resampling are summarized in Table 1 in terms of sensitivity and specificity. Combining the four classifiers by means of the voting scheme resulted in the correct classification of 94 out of the 96 BSE-positive and 315 out of the 325 BSE-negative samples. Numerically this corresponds to a sensitivity and specificity of 98% and 97%, respectively, and an acuracy of 97.1%.

After the training of the classifiers had been completed, the classification algorithms together with the voting scheme were implemented into the DPR research system at the VLA. 160 unknown bovine serum samples were measured by the VLA and the DPR-score calculated was displayed on-line and

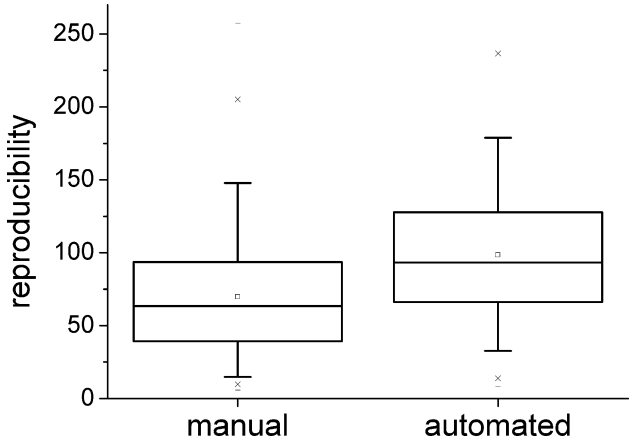

Fig. 1 Ratio between absorbance and reproduction error for 200 bovine serum samples using manual pipetting and automated pipetting. Averages of this ratio in the interval 900–1500 cm^{-1} and 2700–3000 cm^{-1} are given. (lower (upper) symbols of the box-and-whiskers plot: –: 1% (99%), ×: 5% (95%), whiskers: 10% (90%), box: 25% (75%), line: 50%, □: arithmetic mean.)

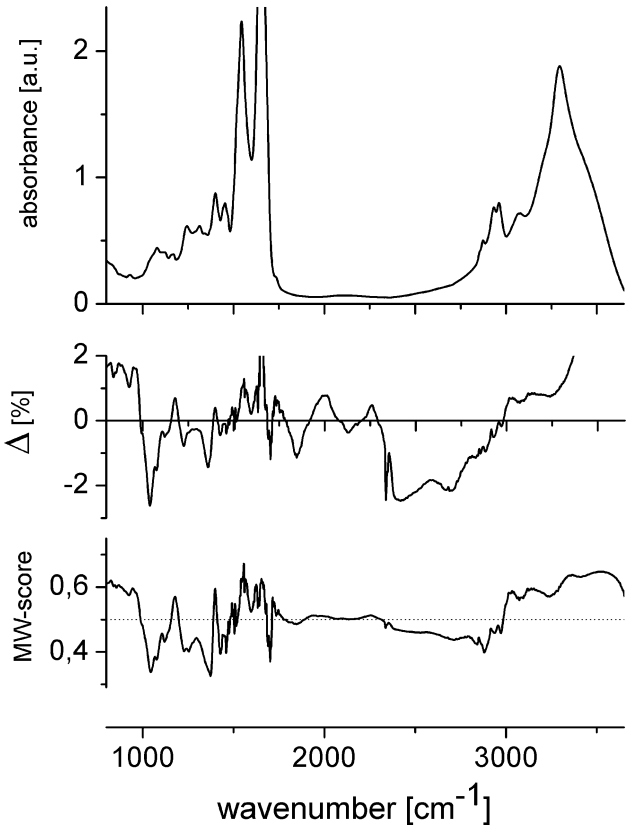

Fig. 2 Mean infrared absorbance spectra of the samples used for teaching the classifiers (top) together with the relative absorbance difference Δ between the BSE-positive and the BSE-negative samples (middle). The lower part of the figure illustrates the statistical significance of the spectral differences by means of the Mann–Whitney score (MW-score). The MW-score equals the area under the ROC curve for univariate analysis, *i.e.* if one were to use a single wavenumber to discriminate between BSE-positive and BSE-negative samples.

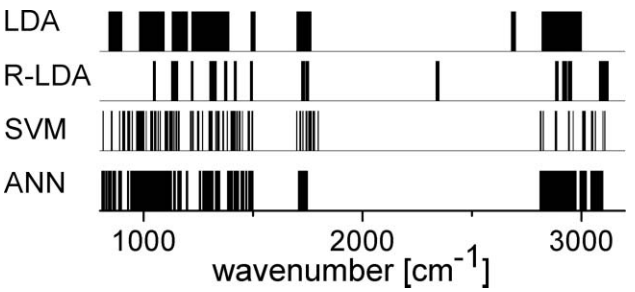

Fig. 3 Spectral regions used for classification by means of the linear discriminant analysis (LDA), the robust linear discriminant analysis (R-LDA), support vector machine (SVM) and artificial neural network (ANN).

Table 1 Sensitivity and specificity of the different classification methods in the teaching set. All values were calculated by resubstituting the teaching spectra. Note that any interpretation of the given numbers needs to take into account the limited number of samples. Furthermore the resubstitution procedure is known to overestimate the accuracy of classification as compared to an independent validation

Method	Sensitivity (%)	Specificity (%)
Linear discriminant analysis	82	93
Robust linear discriminant analysis	85	97
Artificial neural network	99	97
Support vector machine	98	99
Combined classifier	*98*	*97*

recorded for each sample. When all measurements of this blinded validation were finished, the true sample diagnoses (based on the post-mortem BSE-test) were unblinded and compared to the DPR-predictions: The combined classifier correctly classified 78 out of 84 BSE-positive samples, which numerically corresponds to a sensitivity of 93%. Furthermore, 73 out of the 76 BSE-negative samples were correctly classified such that a specificity of 96% can be calculated. Numerically, the accuracy amounts to 94.4%. The individual classifier results are summarized in Table 2. When interpreting these values, however, the low number of samples has to be considered. Requiring a 95% confidence level ($\alpha = 0.05$) results in a sensitivity above 85% and a specificity above 90% for the combined classifier based on the implemented voting scheme.

Analysis of the classification results on the individual sample level showed that all classifiers gave the identical classification result (*i.e.* either BSE-positive or BSE-negative) in 120 cases, out of which 117 were correct with respect to the true diagnosis (Table 3). This finding hints towards a sample-specific origin for misclassification. Only in 8 cases did two classifiers indicate BSE-positive while the other two indicated "BSE-negative". Since the voting scheme was deliberately biased towards higher sensitivity, the samples were assigned BSE-positive by the combined classifier, while it turned out that 6 out of the 8 cases were actually BSE-negative according to the *post-mortem* diagnosis.

In order to quantify the classification result of each sample, a combined DPR-score was calculated as the arithmetic mean of the DPR-scores of those classifiers, which contributed to the majority decision for the voting scheme (see also Fig. 4). The combined DPR-score together with the variation of the threshold value allows for increasing *e.g.* the sensitivity at the expense of specificity or *vice versa*. This interrelation between sensitivity and specificity is frequently illustrated in the form of the Receiver Operating Characteristics[21] (ROC, see Fig. 5). As an example, should a sensitivity of 99% be desired, the threshold value should be lowered to 0.173 (based on the teaching data), which decreases the specificity to 89% in the independent validation. The area under the ROC-curve ranges from 0.5 (worst case) to 1.0 (best case) and may serve as a measure for marker quality regardless of the particular threshold value. The area under the ROC-curve amounts to 0.991 for the validation data shown in Figs. 4 and 5.

Table 2 Sensitivity and specificity of the blinded validation for the different classification methods. The last row gives the results of the combined classification based on the voting scheme, in which—after unblinding—78 out of 84 BSE-positive and 73 out of 76 BSE-negative samples were identified correctly. Note that any interpretation of the given numbers needs to take into account the limited number of samples. Statistical analysis (confidence level: 0.95) shows that the sensitivity and specificity are larger than 85% and 90%, respectively

Method	Sensitivity (%)	Specificity (%)
Linear discriminant analysis	82	93
Robust linear discriminant analysis	80	88
Artificial neural network	93	93
Support vector machine	88	99
Combined classifier	93	96

Table 3 Number of samples (BSE-positive or BSE-negative) of the validation set for which the individual classifiers indicate "BSE-positive"

Number of classifiers	4	3	2	1	0
True BSE positive	61	12	2	0	1
True BSE negative	2	4	6	16	56

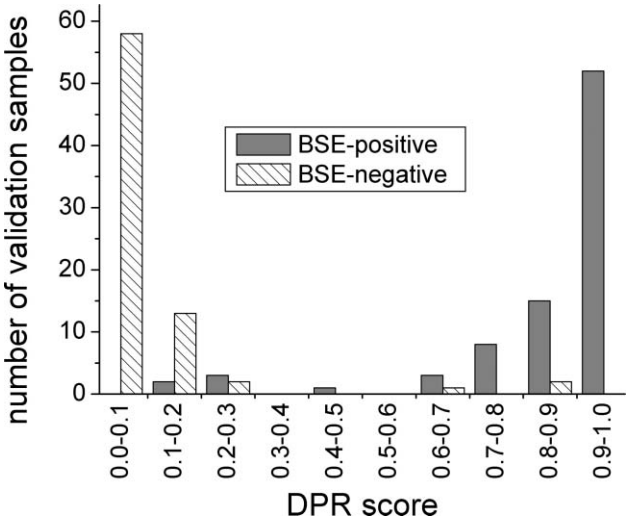

Fig. 4 Results of the blinded validation of 84 BSE-positive and 76 BSE-negative samples using the combined classifier. The *x*-axis shows the combined DPR-score of the different classification approaches (see text). For a DPR-threshold of 0.5 correct classification was obtained for 78 BSE-positive and 73 BSE-negative samples.

Discussion

It is the strength of the DPR method that complete understanding of the biochemical basis for the changes in infrared

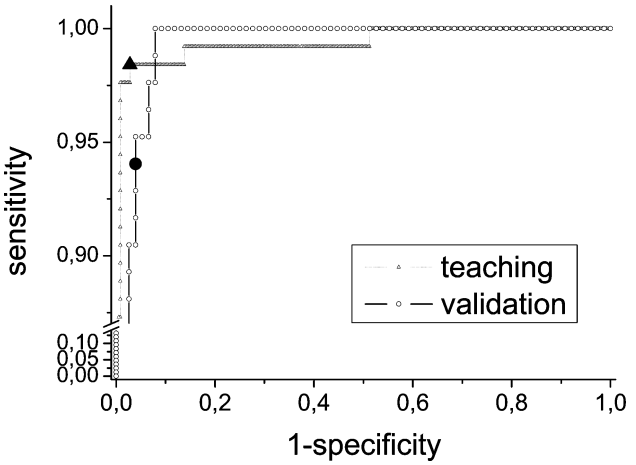

Fig. 5 Receiver Operating Characteristics (ROC) of the combined classifier (area: 0,991) applied to blinded validation data (solid line; 84 BSE-positive and 76 BSE-negative samples) and upon resampling within the teaching set (dotted line; 126 BSE-positive and 355 BSE-negative samples). The filled circle (triangle) marks that combination of sensitivity and specificity within the validation (teaching) set, for which the cut-off in DPR-score is set to 0.5.

spectra is not required for the classification. However, it is legitimate to ask for the biochemical interpretation of the spectral differences observed. Indeed, some of the spectral regions which allowed for discrimination between BSE-positive and BSE-negative were identified independently by all four classifiers. A coarse analysis of the common wavenumbers did not hint towards identification of a specific biochemical group of serum components as the source of the discriminatory signal. Furthermore, the direct detection of conformational changes of the prion protein can be excluded: firstly, the amide I and amide II regions were intentionally excluded from the analysis of the differentiation between BSE-positive and BSE-negative such that the observed discrimination potential is unlikely to be based on specific protein signatures. Secondly, the concentration of prion proteins in serum[22–24] is less than 50 ng ml^{-1} and, hence, well below the mid-infrared detection limit.

In summary, we have shown that the automation of the DPR process was successfully implemented into a DPR research system resulting in added convenience, simplified sample and data logistics and an improvement in reproducibility. The expensive ZnSe plates (which required washing cycles) were successfully replaced with disposable silicon sample carriers, which is particularly useful in an infectious disease environment. Robustness of the analysis method was enhanced by the joint application of four classification approaches. To the best of our knowledge our investigation constitute the first direct comparison of classification methods—and in particular SVMs—in the context of DPR. Given the high measurement reproducibility and the improved stability of analysis, the independent, blinded validation on a second system served as a sound proof of the reliability of the DPR method. Furthermore, with 151 out of 160 samples being correctly identified during the blinded validation, the overall accuracy has substantially improved to 94.4% as compared to the "pure ANN" validation results** (84.8% and 89.2%) reported in ref. 6. Finally, the high value of the area under the ROC curve of 0.991 shows that the method may deliberately be biased towards improved sensitivity by changing the cut-off in DPR-score.

To the best of our knowledge the DPR-method is the only approach which has been able to reliably identify serum samples of cattle with BSE-infection up to now. When aiming to implement the DPR approach into daily routine for BSE-related investigations, further work has to target the impact of covariates such as animal breed, gender, medication and nutrition as well as technical aspects like the system-to-system variation. Furthermore the influence of other diseases (in particular neurological diseases such as listeriosis) onto the classification accuracy has to be clarified. Further experiments should also address the question whether BSE-specific signatures can be identified prior to the onset of clinical symptoms.

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References

1 S. B. Prusiner, *Prion biology and diseases*, Cold Spring Harbor Monograph Series 41, Cold Spring Harbor Laboratory Press, 2004, ISBN 0-87969-693-1.
2 B. K. Nunnally and I. S. Krull, *Prions and mad cow disease*, Marcel Dekker, New York, 2003, ISBN 0-82474-083-1.
3 *Prionen und Prionenkrankheiten*, ed. B. Hörnlimann, D. Riesner and H. A. Kretzschmar, Walter de Gruyter, Berlin, 2001, ISBN 3-11-016361-6 (in German).
4 S. B. Prusiner, *Science*, 1982, **216**, 136; S. B. Prusiner, *Science*, 1991, **252**, 1515; S. B. Prusiner, *Science*, 1997, **278**, 245.
5 J. Schmitt, M. Beekes, A. Brauer, T. Udelhoven, P. Lasch and D. Naumann, *Anal. Chem.*, 2002, **74**, 3865.
6 P. Lasch, J. Schmitt, M. Beekes, T. Udelhoven, M. Eiden, H. Fabian, W. Petrich and D. Naumann, *Anal. Chem.*, 2003, **75**, 6673.
7 W. Petrich, *Appl. Spectrosc. Rev.*, 2001, **36**, 181.
8 D. Naumann, *Appl. Spectrosc. Rev.*, 2001, **36**, 238.
9 *Infrared and Raman spectroscopy of biological materials*, ed. H.-U. Gremlich and B. Yan, Marcel Dekker, Basel, 2001, ISBN 0-8247-0409-6.
10 W. Petrich, A. Staib, M. Otto and R. Somorjai, *Vib. Spectrosc.*, 2002, **28**, 117.
11 W. Petrich, B. Dolenko, J. Früh, M. Ganz, H. Greger, S. Jacob, F. Keller, A. E. Nikulin, M. Otto, O. Quarder, R. L. Somorjai, A. Staib, G. Werner and H. Wielinger, *Appl. Opt.*, 2000, **39**, 3372.
12 J. Früh, S. Jacob, B. Dolenko, H.-U. Häring, R. Mischler, O. Quarder, W. Renn, R. Somorjai, A. Staib, G. Werner and W. Petrich, *Proc. SPIE*, 2002, **4614**, 63.
13 A. Staib, B. Dolenko, D. J. Fink, J. Früh, A. E. Nikulin, M. Otto, M. S. Pessin-Minsley, O. Quarder, R. Somorjai, U. Thienel, G. Werner and W. Petrich, *Clin. Chim. Acta*, 2001, **308**, 79.
14 G. Werner, J. Früh, F. Keller, H. Greger, R. Somorjai, B. Dolenko, M. Otto and D. Böcker, *Proc. SPIE*, 1998, **3257**, 35.
15 J. Schmitt and T. Udelhoven, in ref. 9, pp. 379–420.
16 A. I. Belousov, S. A. Verzakov and J. von Frese, *J. Chemometrics*, 2002, **16**, 482.
17 N. Cristianini and J. Shawe-Taylor, *Support vector machines and other kernel-based learning methods*, Cambrige University Press, 2000, ISBN 0-521-78019-5.
18 B. Schölkopf and A. Smola, *Learning with Kernels*, MIT Press, 2002, ISBN 0-262-19475-9.
19 K. Maquelin, C. Kirschner, L.-P. Choo-Smith, N. A. Ngo-Thi, T. van Vreeswijk, M. Stämmler, H. P. Endtz, H. A. Bruining, D. Naumann and G. J. Puppels, *J. Clin. Microbiol.*, 2003, **41**, 324.
20 J. Moecks, G. Kocherscheidt, W. Köhler and W. Petrich, *Proc. SPIE*, 2004, **5321**, 117.
21 J. A. Swets, *Science*, 1988, **240**, 1285.
22 W. Eberle, private communication.
23 I. MacGregor, J. Hope, G. Barnard, L. Kirby, O. Drummond, D. Pepper, V. Hornsey, R. Barclay, H. Bessos, M. Turner and C. Prowse, *Vox Sang.*, 1999, **77**, 88.
24 I. R. MacGregor and O. Drummond, *Vox Sang.*, 2001, **81**, 236.

** Note that an iterative re-training led to an improved accuracy in ref. 6. We did not yet apply iterative re-training in the present case such that our approach needs to be compared to the "pure ANN" results of ref. 6.